Association of Rare CYP39A1 Variants With Exfoliation Syndrome Involving the Anterior Chamber of the Eye

The Genetics of Exfoliation Syndrome Partnership

**IMPORTANCE** Exfoliation syndrome is a systemic disorder characterized by progressive accumulation of abnormal fibrillar protein aggregates manifesting clinically in the anterior chamber of the eye. This disorder is the most commonly known cause of glaucoma and a major cause of irreversible blindness.

**OBJECTIVE** To determine if exfoliation syndrome is associated with rare, protein-changing variants predicted to impair protein function.

**DESIGN, SETTING, AND PARTICIPANTS** A 2-stage, case-control, whole-exome sequencing association study with a discovery cohort and 2 independently ascertained validation cohorts. Study participants from 14 countries were enrolled between February 1999 and December 2019. The date of last clinical follow-up was December 2019. Affected individuals had exfoliation material on anterior segment structures of at least 1 eye as visualized by slit lamp examination. Unaffected individuals had no signs of exfoliation syndrome.

**EXPOSURES** Rare, coding-sequence genetic variants predicted to be damaging by bioinformatic algorithms trained to recognize alterations that impair protein function.

**MAIN OUTCOMES AND MEASURES** The primary outcome was the presence of exfoliation syndrome. Exome-wide significance for detected variants was defined as \( P < 2.5 \times 10^{-6} \). The secondary outcomes included biochemical enzymatic assays and gene expression analyses.

**RESULTS** The discovery cohort included 4028 participants with exfoliation syndrome (median age, 78 years [interquartile range, 73-83 years]; 2377 [59.0%] women) and 5638 participants without exfoliation syndrome (median age, 72 years [interquartile range, 65-78 years]; 3159 [56.0%] women). In the discovery cohort, persons with exfoliation syndrome, compared with those without exfoliation syndrome, were significantly more likely to carry damaging CYP39A1 variants (1.3% vs 0.30%, respectively; odds ratio, 3.55 [95% CI, 2.07-6.10]; \( P = 6.1 \times 10^{-7} \)). This outcome was validated in 2 independent cohorts. The first validation cohort included 2337 individuals with exfoliation syndrome (median age, 74 years; 1132 women; n = 1934 with demographic data) and 2813 individuals without exfoliation syndrome (median age, 72 years, 2417 women; n = 2421 with demographic data). The second validation cohort included 1663 individuals with exfoliation syndrome (median age, 75 years; 587 women; n = 1064 with demographic data) and 3962 individuals without exfoliation syndrome (median age, 74 years; 951 women; n = 1663 with demographic data). Of the individuals from both validation cohorts, 5.2% with exfoliation syndrome carried CYP39A1 damaging alleles vs 3.1% without exfoliation syndrome (odds ratio, 1.82 [95% CI, 1.47-2.26]; \( P < .001 \)). Biochemical assays classified 34 of 42 damaging CYP39A1 alleles as functionally deficient (median reduction in enzymatic activity compared with wild-type CYP39A1, 94.4% [interquartile range, 78.7%-98.2%] for the 34 deficient variants). CYP39A1 transcript expression was 47% lower (95% CI, 30%-64% lower; \( P < .001 \)) in ciliary body tissues from individuals with exfoliation syndrome compared with individuals without exfoliation syndrome.

**CONCLUSIONS AND RELEVANCE** In this whole-exome sequencing case-control study, presence of exfoliation syndrome was significantly associated with carriage of functionally deficient CYP39A1 sequence variants. Further research is needed to understand the clinical implications of these findings.
Exfoliation syndrome (also known as pseudoxfoliation syndrome) is a systemic disorder characterized by progressive accumulation of abnormal fibrillar protein aggregates manifesting clinically in the anterior chamber of the eye (Figure 1). The fibrillar protein aggregates obstruct drainage of aqueous humor and cause increased intraocular pressure, resulting in glaucoma. Exfoliation syndrome is distinct from true exfoliation, which is a condition linked to heat or environmental exposure–related eye injury. Exfoliation syndrome affected up to 70 million people in 2010 and remains a major cause of irreversible blindness worldwide. An association of exfoliation syndrome with increased risk of cardiovascular diseases has been reported, although this association could be confounded by several factors including age, sex, vascular abnormalities, and serum homocysteine levels.

Although previous genome-wide association studies (GWAS) have identified 7 loci associated with exfoliation syndrome, it has been challenging to ascertain which genes were affected by the noncoding variants discovered through GWAS. The modest effect sizes of GWAS-discovered common variants limit interpretation of their clinical relevance. Conversely, rare, coding-sequence genetic variants often have larger effect sizes and are able to directly implicate causative genes and illuminate disease biology. Genetic studies of rare variants also have facilitated advances in the treatment of other common systemic diseases via the development of potential drug targets. Although more than 95% of coding-sequence variants are rare (defined as an allele frequency <1%) individually, their cumulative prevalence may be sufficient for detection of statistically significant differences when the aggregated rare variant counts within each gene are compared between cases and controls.

The objective of this study was to use whole-exome sequencing to assess whether rare, coding-sequence genetic variants are related to the pathogenesis of exfoliation syndrome by directly altering protein function. Functional assays and gene expression analyses were used to characterize a single gene that surpassed exome-wide significance.

**Key Points**

**Question** Is the presence of exfoliation syndrome, a common cause of glaucoma, associated with rare, protein-changing variants?

**Findings** In this multicenter, case-control, whole-exome sequencing study that included 20,441 participants, persons with exfoliation syndrome, compared with those without exfoliation syndrome, were significantly more likely to be carriers of functionally deficient CYP39A1 alleles (odds ratio, 2.03).

**Meaning** The presence of exfoliation syndrome was significantly associated with carriage of functionally deficient CYP39A1 sequence variants.

**Methods**

**Study Design and Participants**

The sequencing and analytical approaches used to identify genes associated with exfoliation syndrome are depicted in Figure 2. This case-control, whole-exome sequencing study included a discovery stage (discovery cohort) and a validation stage with 2 independently ascertained cohorts to evaluate significant findings from the discovery stage. Study participants from 14 countries were enrolled between February 1999 and December 2019. The date of last clinical follow-up was December 2019. Patients with exfoliation syndrome and those without exfoliation syndrome were enrolled after obtaining written informed consent, adhering to the tenets of the Declaration of Helsinki. All relevant local and hospital institutional review boards approved the studies.

**Discovery Stage**

For the discovery cohort, the enrollment criteria for individuals with exfoliation syndrome were: (1) aged older than 50 years at time of recruitment and (2) presence of exfoliation material visualized by slit lamp examination of the eye along the pupillary margin, anterior lens surface, or other anterior segment structures of at least 1 eye. Individuals who were younger than aged 50 years were excluded as well as those who had other secondary forms of glaucoma (such as neovascular glaucoma) or those with uveitis.

Included individuals without exfoliation syndrome were aged 60 years or older and had a complete eye examination to confirm the absence of exfoliation syndrome and other eye diseases. Because even subtle differences in geographical population structure can confound genetic analysis, individuals without exfoliation syndrome were matched to individuals with exfoliation syndrome by enrollment site and self-reported ancestry.

**Validation Stage**

For the 2 independently enrolled validation cohorts, the inclusion criteria for persons with exfoliation syndrome were:

1. Aged older than 50 years at time of recruitment.
2. Presence of exfoliation material visualized by slit lamp examination of the eye along the pupillary margin, anterior lens surface, or other anterior segment structures of at least 1 eye.
3. Individuals who were younger than aged 50 years were excluded as well as those who had other secondary forms of glaucoma (such as neovascular glaucoma) or those with uveitis.
4. Matched to individuals with exfoliation syndrome by enrollment site and self-reported ancestry.
Figure 2. Sequencing and Analytical Approaches to Identify Genes Associated With Exfoliation Syndrome

- **4028** Participants with exfoliation syndrome underwent whole-exome sequencing during discovery stage
- **5638** Participants without exfoliation syndrome underwent whole-exome sequencing during discovery stage

Identification of qualifying rare variants in all genes (minor allele frequency <1%) predicted to be damaging (by the Polyphen-2 HumDiv software) and that disrupt the protein-coding sequence

Statistical analysis performed using gene-based burden of qualifying rare variants to determine significance (exome-wide significance set at 2-sided \( P < 2.5 \times 10^{-6} \))

- **CYP39A1** was found and further analysis was performed
- **LOXL1** found, no further analysis performed

- **2337** Participants with exfoliation syndrome underwent whole-exome sequencing during first validation stage
- **2813** Participants without exfoliation syndrome underwent whole-exome sequencing during first validation stage

Identification of qualifying rare variants in CYP39A1 (minor allele frequency <1%) predicted to be damaging (by the Polyphen-2 HumDiv software) and that disrupt the protein-coding sequence

Statistical analysis performed using gene-based burden of qualifying rare variants to determine significance (statistical significance set at 2-sided \( P < 0.05 \))

- **1663** Participants with exfoliation syndrome underwent targeted sequencing during second validation stage
- **3962** Participants without exfoliation syndrome underwent targeted sequencing during second validation stage

Identification of qualifying rare variants in CYP39A1 (minor allele frequency <1%) predicted to be damaging (by the Polyphen-2 HumDiv software) and that disrupt the protein-coding sequence

Statistical analysis performed using gene-based burden of qualifying rare variants to determine significance (statistical significance set at 2-sided \( P < 0.05 \))

- **42** CYP39A1 genetic variants predicted to be damaging (by the Polyphen-2 HumDiv software) using biochemical assays
- **Human tissue analysis using differential gene expression and cholesterol deposition**

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*a* Each participant with exfoliation syndrome was matched to at least 1 participant without exfoliation syndrome (for every individual with exfoliation syndrome, \( \geq \)1 individual without exfoliation syndrome could be recruited as a matching control). Matching was by geographical site of recruitment and self-reported ancestry.

*b* Most disease-causing genetic variants are maintained at low frequencies by purifying evolutionary selection.

*c* Polymorphism Phenotyping version 2 (Polyphen-2) is a widely used computer algorithm to identify genetic variants that damage protein function. Such variants are likely to cause disease. Alleles predicted to be benign were excluded because their inclusion could have masked disease associations caused by damaging allelic variants.

*d* Many medical conditions are caused by haploinsufficiency, which is a state where 1 copy of a gene has been damaged by alterations, leaving the remaining normally functioning gene copy insufficient to sustain normal function. In many of these conditions, different alterations within a given gene causes the same damaging consequences to the encoded protein product. In this study, the burden test was designed to compare the number (or burden) of damaging genetic alterations found in each gene among persons with exfoliation syndrome vs those without exfoliation syndrome.

*e* Validation of original discovery stage findings in 2 independently enrolled validation cohorts increases the confidence that the original observations were not false discoveries.

*f* Genes identified to be significantly associated with disease during the discovery and validation stages were characterized further using post hoc experimental assays to garner additional insights into disease pathogenesis.

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Identical to those used for the discovery cohort. Persons without exfoliation syndrome who were aged 60 years or older and who had complete eye examinations to verify absence of exfoliation syndrome were enrolled in Austria, Georgia, Greece,
Japan, Poland, Romania, Russia, Spain, and Turkey. For the US study, the inclusion criteria for individuals without exfoliation syndrome were aged 50 years or older with a complete eye examination. Age-based inclusion criteria for individuals without exfoliation syndrome were not used for Canada and Germany. All individuals without exfoliation syndrome were matched to individuals with exfoliation syndrome by enrollment site (city [e.g., Erlangen, Germany] or province [e.g., Nova Scotia, Canada]) and self-reported ancestry (Appendix and eFigure 1 in Supplement 1). The study details for each sample set (by location) appear in eTable 1 in Supplement 1.

Whole-Exome Sequencing and Targeted Sequencing
Genomic DNA from all participants was extracted from venous blood. Whole-exome sequencing and targeted sequencing (targeting the CYP39A1 [Refseq NM_016593] coding sequence) libraries were prepared using hybridization capture kits (Roche-Nimblegen SeqCap). The DNA samples from individuals with exfoliation syndrome and individuals without exfoliation syndrome were sequenced together using 2 × 151 base-pair, paired-end reads on Illumina instruments.

Postsequencing Bioinformatics Analyses
The bioinformatics procedures were applied uniformly across all samples and were blinded by case vs control status. The DNA sequence reads were mapped using Burrows-Wheeler Aligner software (version 0.7.16a-r1181). Variant calling was performed using the Genome Analysis Tool Kit (version 3.7) and the Picard (version 2.18.11) software packages (Broad Institute for both). Quality control procedures were performed using VCFtools (version 0.1.15), BCFtools (version 1.9), and PLINK (version 1.9). Variant annotation was performed using the Ensembl variant effect predictor (GRCh37).

Due diligence procedures, such as the use of information on synonymous variants (eFigure 2 and eTables 2-3 in Supplement 1), ancestry principal components (eFigures 3-4 in Supplement 1), exome-wide variant count (eFigures 5–6 in Supplement 1), singleton-variant count (eFigure 7 in Supplement 1), and excessive heterozygosity (eFigure 8 in Supplement 1), were used to avoid systematic differences in variant detection between individuals with exfoliation syndrome and those without exfoliation syndrome (eAppendix in Supplement 1).

Identification of Rare Genetic Variants Predicted to Be Damaging
Rare genetic variants predicted to have a damaging effect on protein function were identified using the following criteria: (1) minor allele frequency less than 1% across all ethnic groups studied; (2) variants that disrupt the protein-coding sequence (these refer to stop-gained, start-loss, frameshift, or canonical splice-site alterations); and (3) missense variants predicted to be damaging by the Polymorphism Phenotyping version 2 (Polyphen-2) computer prediction algorithm.

The filter for minor allele frequencies less than 1% was applied because genetic variants with minor allele frequencies greater than 1% were well represented by previously used GWAS genotyping arrays. All bioinformatics filters were pre-specified and applied uniformly across the discovery and validation cohorts using PLINK (version 1.9).

Sanger Capillary Sequencing
Participants carrying CYP39A1 rare alleles were randomly selected for capillary sequencing to confirm the rare allele calls from exome sequencing, which was consistent with previous reports that used capillary sequencing to validate a subset of rare variant calls detected from whole-exome sequencing.

Analysis of Individual CYP39A1 Variant Activity
CYP39A1 metabolizes 24(S)-hydroxycholesterol to 24(S)-7α,24-dihydroxycholesterol. The enzymatic activity of 50 CYP39A1 coding-sequence variants was tested via transfection into human embryonic kidney 293 cells. For transfection, the reference CYP39A1 complementary DNA sequence and the variants were cloned into an expression plasmid. During the transfection process, 6 μg of plasmid was transfected into 1.8 million human embryonic kidney 293 cells in a 10-cm dish using 18 μL of Lipofectamine 2000 (Thermo Fisher Scientific). Twenty-four hours later, 5 μM of 24(S)-hydroxycholesterol was added to the transfected cells, followed by a further 24-hour incubation period before harvesting. Liquid chromatography–tandem mass spectrometry was used to assay harvested cell lysates for the abundance of 24(S)-7α,24-dihydroxycholesterol as a direct readout of enzymatic activity (eAppendix and eTables 4-5 in Supplement 1).

Expression Analysis of CYP39A1 in Eye Tissues
Quantitative real-time polymerase chain reaction was performed on a panel of eye tissues from patients with exfoliation syndrome and from matched individuals without exfoliation syndrome. Disease-specific expression was tested in an independent panel (eAppendix and eTable 6 in Supplement 1). The sample sizes were consistent with previous reports assessing differential gene expression for significant associations with disease genes.

Immunohistochemical analysis was performed on age-matched donor eyes from individuals with exfoliation syndrome and those without exfoliation syndrome using antibodies against CYP39A1 on cryosections and paraffin-embedded sections via heat-induced antigen retrieval. Filipin staining for esterified and free unesterified cholesterol was performed as previously described. Additional staining for β1 integrin (a cell membrane marker) and apolipoprotein E and LOXL1 (both are markers found in abnormal exfoliative material) was performed to assess co-localization. Differences in CYP39A1 protein expression were based solely on visual inspection of stained slides.

To detect unesterified free cholesterol, tissue cryosections were fixed with 4% paraformaldehyde and incubated with 250 μg/mL of filipin for 1 hour. To detect esterified cholesterol in cryosections, free cholesterol was extracted using 70% ethanol for 30 minutes, followed by conversion of esterified to unesterified cholesterol by incubation with 2 U/mL of cholesterol esterase for 3 hours at 37°C before detection with fili-
pin. Sections were counterstained with propidium iodide and examined under a fluorescence microscope. Filipin fluorescence was observed using a UV filter set (λex/λem = 350 nm/455 nm). As a negative control, cholesterol esterase was replaced by phosphate-buffered saline.

**Statistical Analysis**

An assessment of the statistical power was made (statistical power calculations appear in eTable 7 in Supplement 1). All rare genetic variants predicted to impair protein function (ie, damaging) within each gene were aggregated together for an association analysis using the burden test. The burden test evaluated whether any of the approximately 20,000 genes across the human exome bore an excess or a deficit of damaging genetic variants in individuals with exfoliation syndrome vs those without exfoliation syndrome. Because many medical conditions show a haploinsufficiency effect with allelic heterogeneity, the burden test was used to evaluate whether different damaging variants within the same gene are, in aggregate, associated with the presence of disease.

**Discovery Stage**

A stratified Cochran Mantel-Haenszel fixed-effects meta-analysis (without continuity correction) was used to summarize gene-based burden test results across the exomes of the study participants from the 3 countries studied in the discovery cohort. The accuracy of this method was verified by adjusting the primary association test statistics for potential confounders (such as ancestry principal component scores and exome-wide variant count) using Firth penalized logistic regression. Exome-wide significance was specified at 2-sided \( P < 2.5 \times 10^{-6} \) to reflect multiple testing correction for 20,000 genes.

**Validation Stage**

**First Cohort** | The newly identified genes surpassing exome-wide significance during the discovery stage (ie, CYP39A1) were evaluated for validation. A meta-analysis using the same stratified Cochran Mantel-Haenszel fixed-effects method as described for the discovery stage was used for the association between CYP39A1 rare variant burden and the presence of exfoliation syndrome across the participants from 8 countries in the first validation cohort. The association test was adjusted for ancestry principal components and exome-wide variant count. Because only 1 gene (CYP39A1) was tested here, 2-sided \( P < .05 \) was considered statistically significant.

**Second Cohort** | A meta-analysis using the same stratified Cochran Mantel-Haenszel fixed-effects method across the 4 participating countries was performed in the second validation cohort. The Fisher exact test was used to evaluate the individual strata. Because only 1 gene (CYP39A1) was tested here, 2-sided \( P < .05 \) was considered statistically significant.

**First and Second Cohorts** | A meta-analysis using the same stratified Cochran Mantel-Haenszel fixed-effects method was used for the association between CYP39A1 rare variant burden and the presence of exfoliation syndrome across the participants from all 12 countries.

**Post Hoc Analysis of CYP39A1 Biochemical Enzymatic Activity**

Enzymatic activity of 50 tested CYP39A1 variants were compared with enzymatic activity of wild-type CYP39A1 using the absolute range of their experimentally measured enzymatic activity. A variant was classified as increased function if its experimentally measured enzymatic activity range was significantly higher than, and did not overlap with, that of wild-type CYP39A1. Conversely, a variant was classified as deficient if its experimentally measured enzymatic activity range was significantly lower than, and did not overlap with, that of wild-type CYP39A1. For the 34 variants that were classified as functionally deficient, their median reduction in enzymatic activity was determined from all their measured data points (34 variants measured in 3 independent biological replicates, resulting in 102 data points; eFigure 11 in Supplement 1).

**Post Hoc Analysis of Gene Expression in Eye Tissues**

Expression levels of assessed genes were normalized relative to the GAPDH housekeeping gene. Comparison of gene expression levels between tissues from patients with exfoliation syndrome and those without exfoliation syndrome were made using the unpaired 2-tailed \( t \) test. A 2-sided \( P < .05 \) was considered significant. All statistical analyses were performed using R statistical software package version 3.4.3 (R Foundation for Statistical Computing).

**Results**

**Rare Coding Variants Associated With Exfoliation Syndrome**

The discovery cohort included 4028 participants with exfoliation syndrome (median age, 78 years [interquartile range, 73-83 years]; 2377 [59.0%] women) and 5638 participants without exfoliation syndrome (median age, 72 years [interquartile range, 65-78 years]; 3159 [56.0%] women) (Table). A total of 415,671 rare variants predicted to impair protein function were identified across 18,753 genes. An exome-wide significant association with exfoliation syndrome was observed at genes LOXLI (Refseq NM_005576) and CYP39A1 (Figure 12 in Supplement 1 and eTable 8 in Supplement 2). The association at LOXLI was driven by rare variants that conferred protection likely from exfoliation syndrome (eTable 9 in Supplement 1). Persons with exfoliation syndrome were significantly more likely to carry damaging CYP39A1 variants compared with persons without exfoliation syndrome (52 of 4028 [1.3%] vs 17 of 5638 [0.30%], respectively; odds ratio [OR], 3.55 [95% CI, 2.07-6.10], \( P = 6.1 \times 10^{-7} \); Figure 3).

The first validation cohort included 2337 individuals with exfoliation syndrome (median age, 74 years; 1132 women; \( n = 1934 \) with demographic data) and 2813 individuals without exfoliation syndrome (median age, 72 years; 1287 women; \( n = 2421 \) with demographic data). Persons with exfoliation syndrome were again observed to be significantly more likely to carry damaging CYP39A1 variants vs those without...
Table. Baseline Participant Characteristics in the Discovery Cohorta

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>With exfoliation syndrome</th>
<th>Without exfoliation syndrome</th>
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<td>Japanb</td>
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<tr>
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<tr>
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<td>1729 (41.2)</td>
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<tr>
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<td>4197</td>
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<tr>
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<tr>
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<td>67 (63-73)</td>
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<td>Male</td>
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<td>Exfoliation glaucoma, No. (%)</td>
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<td>69 (63-76)</td>
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<tr>
<td>No. of participants</td>
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</table>

Abbreviation: IQR, interquartile range.

a All participants underwent whole-exome sequencing.

b Indicates the country where participants were recruited.

exfoliation syndrome (128 of 2337 [5.5%] vs 84 of 2813 [3.0%], respectively; OR, 1.86 [95% CI, 1.38-2.51]; P < .001). This association was reassessed in a second validation cohort including 1663 individuals with exfoliation syndrome (median age, 75 years; 587 women; n = 1064 with demographic data) and 3962 individuals without exfoliation syndrome (median age, 74 years; 951 women; n = 1555 with demographic data). Persons with exfoliation syndrome were observed to be significantly more likely to carry damaging CYP39A1 variants compared with persons without exfoliation syndrome (79 of 1663 [4.8%] vs 123 of 3962 [3.1%], respectively; OR, 1.78 [95% CI, 1.32-2.41]; P < .001; Figure 3). Of the individuals from both validation cohorts, 5.2% of individuals (207 of 4000) with exfoliation syndrome carried CYP39A1 damaging alleles vs 3.1% of individuals (207 of 6775) without exfoliation syndrome (OR, 1.82 [95% CI, 1.47-2.26]; P < .001).

A meta-analysis of all participants confirmed that patients with exfoliation syndrome were significantly more likely to carry rare, damaging CYP39A1 variants compared with persons without exfoliation syndrome (OR, 2.03 [95% CI, 1.67-2.46]; P < .001; Figure 3 and eTable 10 in Supplement 1). The heritability explained by CYP39A1 rare variant burden was estimated to be 0.2%. Complete concordance between the rare variant calls from exome sequencing and capillary sequencing was observed for 130 CYP39A1 rare variant carriers selected for capillary sequencing (eAppendix and eFigure 13 in Supplement 1).

Post Hoc Evaluation of CYP39A1 Activity of CYP39A1 Rare Coding Alleles

Sequencing of all 20,441 study participants identified 483 individuals carrying 42 unique CYP39A1 rare variants that were predicted to be damaging by Polyphen-2 (eTable II in Supplement 1).

Biochemical assays classified 34 of 42 damaging CYP39A1 alleles as functionally deficient (median reduction in enzymatic activity compared with wild-type CYP39A1, 94.4% [interquartile range, 78.7%-98.2%] for the 34 deficient variants). Seven variants carried by 7 individuals had enzymatic activity ranges that overlapped with that of wild-type CYP39A1. One variant (p.F175L), which appeared to have increased enzymatic activity compared with wild-type CYP39A1, was carried by 2 individuals (Figure 4). The enzymatic assays were highly reproducible when 16 deficient variants were randomly selected and retested (eFigure 14 in Supplement 1). Persons with exfoliation syndrome were significantly more likely to carry 1 of the 34 deficient variants compared with persons without exfoliation syndrome (OR, 2.02 [95% CI, 1.66-2.47]; P < .001).

The enzymatic activity of 3 common CYP39A1 variants and 5 CYP39A1 rare variants predicted by Polyphen-2 to be benign also were measured. All 5 benign variants had enzymatic activity comparable with wild-type CYP39A1. Two of the 3 common variants showed increased function relative to wild-type CYP39A1 (Figure 4). None of the 8 variants were significantly associated with the presence of exfoliation syndrome (eTables 12-13 in Supplement 1).

Post Hoc Differential Gene Expression of CYP39A1 in Eye Tissues

CYP39A1 expression was observed in the liver and in all normal ocular tissues analyzed (eFigure 15 in Supplement 1). CYP39A1 was evaluated with several other functionally related CYP enzymes (eFigure 16 in Supplement 1) for differences in gene expression between eye tissues affected by exfoliation syndrome (n = 23) and unaffected eye tissues (n = 23). CYP39A1 was the only CYP enzyme tested to show consistent and significant downregulation across all affected eye tissues (eg, mean reduction for ciliary body of 47% [95% CI, 30%-64%]; P < .001) compared with unaffected eye tissues (Figure 5), as well as significant downregulation in eye tissues from all disease stages compared with unaffected eye tissues (eFigure 17 in Supplement 1).

Immunohistochemical analyses using anti-CYP39A1 antibodies showed CYP39A1 localized to the ciliary epithelium, iris, choroid, and retina (eFigure 18 in Supplement 1). Visual inspection showed reduced immunostaining for CYP39A1 in the ciliary epithelium (eFigure 19 in Supplement 1) and in the retina (eFigure 20 in Supplement 1) of patients with exfolia-
tions syndrome compared with those without exfoliation syndrome. Immunostaining for CYP39A1 was visually observed to be lower in the ciliary epithelium from an individual with exfoliation syndrome carrying a deficient CYP39A1 sequence variant (p.G410R) compared with individuals with exfoliation syndrome not carrying a deficient CYP39A1 sequence variant (eFigure 19 in Supplement 1).

Because of the role of CYP39A1 in cholesterol metabolism, potential differences in localization of cholesterol in its esterified and unesterified forms were assessed between affected and unaffected eye tissues using immunofluorescence analysis. Abnormal extracellular signals for esterified cholesterol localizing within the exfoliation material deposits on the surface membrane of the ciliary epithelium of affected eye tissues were observed. These signals showed clear co-localization with known markers of exfoliation material (such as apolipoprotein E and LOXL1). Conversely, these abnormal extracellular signals for esterified cholesterol were not observed in unaffected tissues (eFigure 21 in Supplement 1).

Discussion
The study of 20 441 participants found that individuals with exfoliation syndrome were significantly more likely to carry damaging CYP39A1 alleles compared with those without exfoliation syndrome. The full concordance observed between next-generation exome sequencing and capillary sequencing made it unlikely that the CYP39A1 rare alleles identified were sequencing artifacts.

Deficient CYP39A1 function impairs cellular metabolism of 24(S)-hydroxycholesterol to downstream intermediates. Because 24(S)-hydroxycholesterol regulates cellular lipid homeostasis, its dysregulation may lead to abnormalities in
cholesterol homeostasis and transport. This may result in excess cholesterol accumulation in extracellular aggregates of exfoliation material, which represent the hallmark of the disease. It is possible that the association between CYP39A1 deficiency and the presence of exfoliation syndrome was not detected in prior GWAS because GWAS tend to focus on common genetic variants, most of which have modest ORs for...
disease susceptibility. The low heritability fraction explained by CYP39A1 rare variant burden is consistent with similar studies of other complex diseases.9,18,19

The findings raise a possibility that future research efforts aimed at restoring deficient CYP39A1 function and inhibiting the formation of exfoliation material in the eye (thereby preventing complications such as blinding from its accumulation) could be an approach to assess new strategies to treat exfoliation syndrome.

Limitations
This study has several limitations. First, the deficient CYP39A1 variants were present in only a small percentage of persons with exfoliation syndrome. Thus, even if there was a causal link between these variants and exfoliation syndrome, the variants accounted for only a very small proportion of patients with the disorder.

Second, the observational design of this case-control study precluded conclusions about causal relationships.

Third, this study was insufficiently powered to assess potential interactions between CYP39A1 rare alleles and other loci.

Fourth, this study did not explore experimental perturbation of CYP39A1 in an animal model that recapitulates exfoliation syndrome. Such research is complex and could be addressed by future work.

Conclusions
In this whole-exome sequencing case-control study, presence of exfoliation syndrome was significantly associated with carriage of functionally deficient CYP39A1 sequence variants. Further research is needed to understand the clinical implications of these findings.
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Obtained funding: Kazama, Sugimoto, Rolfes, Founti, Haines, Wigs, Aung, Tam, Khor.

Critical revision of the manuscript: Z. Li, Wang, Lee, Zenkel, Pasutto, Schlötzer-Schrehardt, Ho, Aung, Tam, and Khor.


Obtained funding: Kazama, Sugimoto, Rolfes, Founti, Haines, Wigs, Aung, Tam, Khor.


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Conflict of Interest Disclosures: Dr Topouzis reported receiving grants from Pfizer, Thea Pharmaceuticals, Novartis, Novo, Alcon, Omikron, Bayer, and Bausch & Lomb; and receiving personal fees from Novartis and Omikron. Dr Chan reported receiving grants from the Genome Institute of Singapore, for technical support without receiving compensation.

Additional Information: Coauthor Yury S. Astakhov, MD, died on June 10, 2020, and coauthor Robert P. Igo Jr, PhD, died on July 19, 2020.

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Association of Rare CYP39A1 Variants With Exfoliation Syndrome Involving the Anterior Chamber of the Eye


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