

Prevalence of *FOXC1* Variants in Individuals With a Suspected Diagnosis of Primary Congenital Glaucoma

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IMPORTANCE Both primary and secondary forms of childhood glaucoma have many distinct causative mechanisms, and in many cases a cause is not immediately clear. The broad phenotypic spectrum of secondary glaucoma, particularly in individuals with variants in *FOXC1* or *PITX2* genes associated with Axenfeld-Rieger syndrome, makes it more difficult to diagnose patients with milder phenotypes. These cases are occasionally classified and managed as primary congenital glaucoma.

OBJECTIVE To investigate the prevalence of *FOXC1* variants in participants with a suspected diagnosis of primary congenital glaucoma.

DESIGN, SETTING, AND PARTICIPANTS Australian and Italian cohorts were recruited from January 1, 2007, through March 1, 2016. Australian individuals were recruited through the Australian and New Zealand Registry of Advanced Glaucoma and Italian individuals through the Genetic and Ophthalmology Unit of l'Azienda Socio-Sanitaria Territoriale Grande Ospedale Metropolitan Niguarda in Milan, Italy. We performed exome sequencing, in combination with Sanger sequencing and multiplex ligation-dependent probe amplification, to detect variants of *FOXC1* in individuals with a suspected diagnosis of primary congenital glaucoma established by their treating specialist. Data analysis was completed from June 2015 to November 2017.

MAIN OUTCOME AND MEASURES Identification of single-nucleotide and copy number variants in *FOXC1*, along with phenotypic characterization of the individuals who carried them.

RESULTS A total of 131 individuals with a suspected diagnosis of primary congenital glaucoma were included. The mean (SD) age at recruitment in the Australian cohort was 24.3 (18.1) years; 37 of 84 Australian participants (44.0%) were female, and 71 of 84 (84.5%) were of European ancestry. The mean (SD) age at recruitment was 22.5 (18.4) years in the Italian cohort; 21 of 47 Italian participants (44.7%) were female, and 45 of 47 (95.7%) were of European ancestry. We observed rare, predicted deleterious *FOXC1* variants in 8 of 131 participants (6.1%), or 8 of 166 participants (4.8%) when including those explained by variants in *CYP11B1*. On reexamination or reinvestigation, all of these individuals had at least 1 detectable ocular and/or systemic feature associated with Axenfeld-Rieger syndrome.

CONCLUSIONS AND RELEVANCE These data highlight the genetic and phenotypic heterogeneity of childhood glaucoma and support the use of gene panels incorporating *FOXC1* as a diagnostic aid, especially because clinical features of Axenfeld-Rieger syndrome can be subtle. Further replication of these results will be needed to support the future use of such panels.

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Glaucoma is a heterogeneous group of optic neuropathies associated with irreversible visual field loss.¹ Childhood glaucoma (defined here as glaucoma occurring with an age at onset younger than 18 years)² is generally a more severe disease than its adult counterpart, and it often requires multiple surgical interventions to control high intraocular pressure (IOP). Causes of childhood glaucoma are typically classified as primary or secondary²; primary childhood glaucoma includes primary congenital glaucoma (PCG) and juvenile open-angle glaucoma, while secondary causes include Axenfeld-Rieger anomaly (ARA), Peters anomaly, aniridia, and ectopia lentis.

Primary congenital glaucoma is the most common non-syndromic cause of childhood glaucoma and is characterized by elevated IOP and associated sequelae, including optic disc cupping, buphthalmos, Haab striae, enlarged corneal diameter, corneal edema, and/or increased axial length. The disease has an approximate incidence of 1:18 500 in Great Britain³ and 1:30 000 in Australia.⁴ There is an increased incidence of PCG in ethnic groups in which parental consanguinity is more common (such as Pakistani British populations),³ or where there is a strong founder effect (eg, an incidence of 1:1250 among Romani Slovakian individuals),^{5,6} which is consistent with autosomal recessive inheritance.

Axenfeld-Rieger anomaly is a rare condition defined by a collection of 1 or more ocular features, including posterior embryotoxon, iris stromal hypoplasia, peripheral anterior synechiae (PAS), corectopia, or pseudopolycoria.⁷ This condition may also be associated with 1 or more systemic features, in which case it is referred to as Axenfeld-Rieger Syndrome (ARS). Glaucoma is present in approximately 50% of individuals with ARS.⁸

Both primary and secondary childhood glaucoma may be associated with variants in one of several genes. Cases of PCG are most commonly associated with autosomal recessive variants in *CYP11B1*⁹ or less commonly with heterozygous variants of *TEK*¹⁰ or *ANGPT1*.¹¹ Causes of secondary glaucoma include microspherophakia and/or ectopia lentis associated with variants in *LTBP2*,¹²⁻¹⁴ aniridia owing to variants in *PAX6*,¹⁵ Peters anomaly owing to variants in *PAX6*, *CYP11B1*, *PITX2*, *FOXC1*, or other genes,¹⁶ and ARA or ARS owing to variation in *PITX2*¹⁷ or *FOXC1*.¹⁸

Key phenotypic differences exist between ARS associated with *FOXC1* or *PITX2* variants. Cases of ARS associated with *PITX2* typically present with dental and umbilical anomalies,^{7,19,20} while sensorineural hearing loss, intellectual disability, and congenital heart defects are more characteristic of patients with variants in *FOXC1*.²¹ Facial dysmorphism, including maxillary hypoplasia, a broad and flat nasal bridge, telecanthus, or hypertelorism, can be observed in both. *FOXC1*-associated ARS typically presents with glaucoma at a younger age than *PITX2*-associated ARS, with 23% of *FOXC1* carriers diagnosed before the age of 3 years compared with 7% of *PITX2* carriers.²¹

Ocular or systemic features of ARS are not always readily apparent; posterior embryotoxon, for example, may not always be visible on slitlamp examination,²² and systemic features may not be apparent until later in life, if at all. In our pre-

Key Points

Question What is the prevalence of *FOXC1* variants, which are associated with Axenfeld-Rieger syndrome and secondary glaucoma, in individuals with a suspected diagnosis of primary congenital glaucoma?

Findings Among a cohort of 166 Australian and Italian affected individuals, predicted deleterious *FOXC1* variants were observed in approximately 1 in 20 participants. On reexamination or reinvestigation, all of these individuals had at least 1 detectable ocular and/or systemic feature associated with Axenfeld-Rieger syndrome.

Meaning These findings highlight the genetic and phenotypic heterogeneity of childhood glaucoma and suggest that *FOXC1* sequencing may aid its diagnosis.

vious study,²¹ 1 *FOXC1* carrier displayed systemic features in the first few years of life, compared with all of the *PITX2* carriers. These ambiguities, combined with the difficulties associated with thorough pediatric ophthalmic examination (often requiring general anesthesia), can occasionally lead to a diagnosis of PCG. Overall, this suggests that *FOXC1* variants might account for suspected diagnoses of PCG. In this study, we used sequencing and copy-number analysis of the *FOXC1* locus to refine the diagnostic classification of 166 participants with a suspected diagnosis of PCG.

Methods

Participant Recruitment

Australian individuals with PCG and their families were recruited through the Australian and New Zealand Registry of Advanced Glaucoma (ANZRAG),²³ with Italian individuals with PCG and their families recruited at the Genetic and Ophthalmology Unit of l'Azienda Socio-Sanitaria Territoriale Grande Ospedale Metropolitan Niguarda (Milan, Italy). Patients with a suspected diagnosis of PCG made by their treating ophthalmologist were included from the Australian cohort (n = 84) and the Italian cohort (n = 47). Control individuals (n = 106) were confirmed to not have glaucoma or anterior segment dysgenesis on ocular examination.

Informed written consent was obtained from participants or their guardians, and blood or saliva samples collected along with clinical details from the referring ophthalmologist. Ethics approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee (Australia), the Medical Faculty of the University of Erlangen-Nürnberg (Germany), and l'Azienda Socio-Sanitaria Territoriale Grande Metropolitan Niguarda Hospital (Italy).

Sequencing

For exome sequencing, genomic DNA was extracted from blood samples using a QIAamp Blood Maxi Kit (Qiagen) and subjected to exome capture (Agilent SureSelect, version 4). Saliva samples were collected with Oragene DNA Self-Collection Kits (DNA Genotek Inc) and DNA extracted per the

manufacturer's instructions. All paired-end libraries were sequenced on a HiSeq 2000 (Illumina), with the exception of a single Italian trio that was sequenced on the SOLiD 4 System (Life Technologies). Reads were mapped to the human reference genome (hg19) using Burrows-Wheeler Aligner (<http://bio-bwa.sourceforge.net>), and duplicates marked and removed with Picard Tools (Broad Institute). Variants were called using Sequence Alignment/Map (SAM) tools and annotated with ANNOVAR (<http://annovar.openbioinformatics.org/en/latest/>). All variants were annotated using the genome aggregation database (gnomAD) variant database (release 2.0.2; <http://gnomad.broadinstitute.org>), and with Sorting Intolerant From Tolerant (SIFT) and PolyPhen-2 scores. *FOXC1* variants were annotated against the consensus transcript (NM_001453.2) and protein (NP_001444.2) sequences. For capillary sequencing, genomic DNA was prepared from 200 μ L of venous blood collected in EDTA-coated tubes and extracted using an Illustra Blood Genomic Prep MiniSpin kit (GE Healthcare), or Flexi-Gene kit (Qiagen), according to the manufacturer's instructions. Primers for amplification of *FOXC1* genomic DNA are available on request. All variants are publicly available at the ClinVar database (accession numbers SCV000494251, SCV000494255, SCV000494257, SCV000494258, SCV000494261, SCV000494267, SCV000693681, and SCV000693680).

Exome Copy Number Variant Analysis

Coverage depth across the *FOXC1* locus and neighboring loci was extracted from exome Binary Alignment/Map files using SAMtools. For copy number variations analysis using Copy Number Inference From Exome Reads (CoNIFER; version 0.2.2), the same interval was analyzed in 343 contemporaneously sequenced exomes using the parameters singular value decomposition (SVD) version 5 and the *z* score of the reads per kilobase per million mapped reads (ZRPKM) version 1.5.

Multiplex Ligation-Dependent Probe Amplification

FOXC1 copy number was measured by multiplex ligation-dependent probe amplification (MLPA) using the SALSA MLPA P054-B2 *FOXL2-TWIST1* probemix (MRC Holland) according to the manufacturer's instructions. Ligation sites for the *FOXC1* probes (FOXC1-A [02561-LO2029]) and FOXC1-B [02562-LO2030]) were located at 925 base pairs (bp) and 1532 bp, respectively, of NM_001453.2. The copy number present in the original DNA specimen was determined from the relative amplitude of each amplicon product detected using the ABI 3130xl Genetic Analyzer, and data analyzed using Peak Scanner version 2.0 (ThermoFisher Scientific).

Results

Genetic Analysis

In the Australian cohort, mean (SD) age at recruitment was 24.3 (18.1) years; 37 of 84 (44.0%) were female, and 71 of 84 (84.5%) were of European ancestry. In the Italian cohort, the mean (SD) age at recruitment was 22.5 (18.4) years; 21 of 47 (44.7%) were female, and 45 of 47 (95.7%) were of European ancestry.

From a collection of Australian individuals with a suspected diagnosis of PCG, we selected 65 probands whose conditions were not explained by homozygous or compound heterozygous variants of *CYP1B1*. All 65 individuals underwent exome sequencing, and variation at the *FOXC1* locus was examined. Rare *FOXC1* variants predicted to be deleterious were identified in 2 of the 65 individuals, including frameshift deletion c.718_719delCT, p.(Leu240ValfsTer65) in participant PCG128 and missense variant c.269C>A, p.(Ala90Asp) in participant PCG140 (Figure 1A).

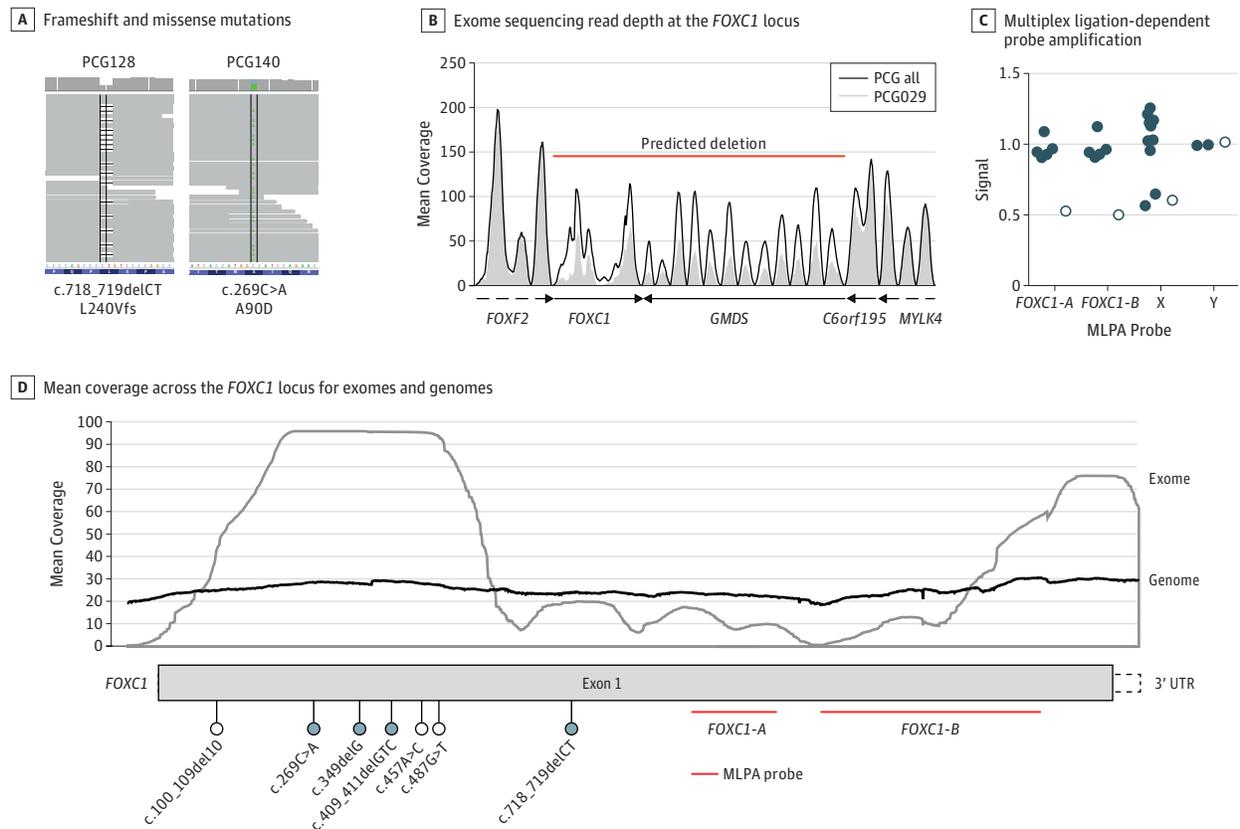
By examining the depth of coverage across the *FOXC1* locus, a large deletion spanning *FOXC1* and the neighboring *GMDS* gene was identified in participant PCG029 (Figure 1B). It also became clear that portions of *FOXC1* were poorly covered by exome capture and sequencing (Figure 1B and C), likely because of a high guanine-cytosine content.²⁴ The single *FOXC1* coding exon was therefore resequenced in all 65 proband individuals by capillary sequencing, alongside an additional 19 PCG probands whose conditions were not explained by *CYP1B1* variants. Multiplex ligation-dependent probe amplification was performed on 73 of these individuals to detect *FOXC1* copy number variations (both gains and losses). An additional 3 rare protein-coding variants were identified by capillary sequencing, including nonsense variant c.487G>T, p.(Glu163Ter) in participant PCG033, missense variant c.457A>C, p.(Thr153Pro) in participant PCG057, and frameshift deletion c.100_109del10, p.(Gly34ThrfsTer8) in participant PCG125 (Figure 1C). All variants and deletions identified by exome sequencing were validated by capillary sequencing or MLPA (Figure 1D).

None of the 6 variants identified in the Australian cohort were present in 106 controls exomes sequenced in parallel, nor were they reported in the gnomAD variant collection. In total, *FOXC1* variants accounted for 6 of 84 participants (7.1%) in the Australian cohort or 6 of 104 participants (5.8%) when including those whose conditions were explained by variants in *CYP1B1*.

In a separate cohort of Italian participants with suspected PCG, 22 family trios whose conditions were not explained by variants in *CYP1B1* underwent exome sequencing. *FOXC1* was also covered by capillary sequencing in all 22 probands plus an additional 25 Italian probands in which *CYP1B1* variants had been excluded by capillary sequencing. Predicted pathogenic *FOXC1* variants were identified in 2 of 47 individuals (4.2%) in the Italian cohort. De novo in-frame deletion c.409_411del, p.(Val137del) in participant PCG402-IT, identified by exome sequencing, has previously been described.²⁵ A second de novo frameshift deletion, c.349delG, p.(Asp117ThrfsTer64), was identified in participant PCG297-IT by capillary sequencing. Neither variant was present in 680 European control participants without glaucoma, and neither was reported in gnomAD. When including patients whose conditions were explained by variants in *CYP1B1*, we identified *FOXC1* variants in 2 of 62 participants (3.2%) of the Italian PCG cohort (Figure 1C and D).

Across both cohorts, we identified predicted pathogenic *FOXC1* variants in 8 of 131 participants (6.1%) who had conditions that were not explained by *CYP1B1* variants, and 8 of 166 participants (4.8%) when including individuals whose conditions were explained by *CYP1B1* variants (Table 1).

Figure 1. *FOXC1* Variants Associated With Suspected Primary Congenital Glaucoma (PCG)



A, Frameshift and missense variants identified by exome sequencing. B, Exome sequencing read depth at the *FOXC1* locus (chr6:1390204-2679449, hg19) for participant PCG029 compared with the mean read depth across all suspected PCG samples. C, Multiplex ligation-dependent probe amplification (MLPA) using probes within *FOXC1* (chr6) and on both sex chromosomes. Signal intensity was measured in duplicate, normalized, and plotted as the mean for participant PCG029 and all other samples processed in the same batch (control

participants). D, Mean coverage across the *FOXC1* locus for exomes (gray) and genomes (black) from the gnomAD release 2.0.2 collection, indicating location of variants identified by exome (blue fill) or capillary (no fill) sequencing. Multiplex ligation-dependent probe amplification probe locations are indicated in red. Most gnomAD exome sequences (approximately 77%) were processed with the same or similar exome capture kits as those used in the current study (Agilent SureSelect).

Table 1. Heterozygous *FOXC1* Variants Identified in Affected Individuals

Proband Identification No.	Cohort	Coordinates (hg19)	Method	<i>FOXC1</i> Complementary DNA ^a	<i>FOXC1</i> Protein	PolyPhen 2, HumVar	SIFT
PCG402-IT	Italian	6:1611089-1611091	Exome	c.409_411del	p.(Val137del)	NA	NA
PCG297-IT	Italian	6:1611029-1611029	Capillary	c.349delG	p.(Asp117ThrfsTer64)	NA	NA
PCG029	Australian	NA	Exome	c.902-?-1560+?del	NA	NA	NA
PCG033	Australian	6:1611167-1611167	Capillary	c.487G>T	p.(Glu163Ter)	NA	NA
PCG057	Australian	6:1611137-1611137	Capillary	c.457A>C	p.(Thr153Pro)	0.978 (Probably damaging)	0 (Damaging)
PCG125	Australian	6:1610780-1610789	Capillary	c.100_109del10	p.(Gly34ThrfsTer8)	NA	NA
PCG128	Australian	6:1611398-1611399	Exome	c.718_719delCT	p.(Leu240ValfsTer65)	NA	NA
PCG140	Australian	6:1610949-1610949	Exome	c.269C>A	p.(Ala90Asp)	0.999 (Probably damaging)	0 (Damaging)

Abbreviations: NA, not available; SIFT, Sorting Intolerant From Tolerant.

^a Coordinates of complementary DNA and protein variants are with respect to consensus complementary DNA and amino acid sequences (NM_001453.2 and NP_001444.2, respectively).

Table 2. Clinical Details of the Probands

Proband Identification No.	Age at Diagnosis	Diagnosis	Ocular Features	Facial Dysmorphism	Systemic Features	Best-Corrected Visual Acuity	Maximum Intraocular Pressure	Cup-Disc Ratio
PCG402-IT	14 y	Congenital glaucoma	Buphthalmos, posterior embryotoxon, peripheral anterior synechiae, megalocornea	Yes	NA	20/30:20/30	50/50	0.6/0.7
PCG297-IT	1 mo	Congenital glaucoma	Buphthalmos, posterior embryotoxon, iris stromal hypoplasia, peripheral anterior synechiae, corectopia, corneal opacity, megalocornea	Yes	NA	20/40:20/30	23/22	0.6/0.3
PCG029	0 y	Primary congenital glaucoma	Corneal opacity, nystagmus	NA	Hearing loss, hydrocephalus, inguinal hernia, heart murmur	Count fingers:20/40	22/27	No view
PCG033	1 y	Congenital glaucoma	Posterior embryotoxon, peripheral anterior synechiae, corneal opacity, megalocornea	NA	Hearing loss, congenital hip dislocation	20/200:20/70	30/30	0.9/0.9
PCG057	0 y	Primary congenital glaucoma	Haab striae, iris stromal hypoplasia, corectopia	Yes	Short stature	20/40:20/30	48/40	0.6/0.8
PCG125	8 y	Primary congenital glaucoma	Buphthalmos, Haab striae, posterior embryotoxon, iris stromal hypoplasia, peripheral anterior synechiae, corneal decompensation	Yes	Developmental delay	20/40:20/25	40/30	0.95/0.6
PCG128	0 y	Primary congenital glaucoma	Buphthalmos, Haab striae, peripheral anterior synechiae	Yes	Developmental delay	NA	50/50	0.75/0.8
PCG140	0 y	Primary congenital glaucoma	Accommodative esotropia	NA	Mitral valve replacement, short stature	20/40:20/40	23/21	0.3/0.6

Abbreviation: NA, not applicable.

Clinical Reinvestigation

Having uncovered potentially pathogenic *FOXC1* variants, all probands were reinvestigated for evidence of ocular and systemic features of ARS (Table 2). Family pedigrees were ascertained, with variant segregation testing performed where possible (Figure 2). Ocular features of the probands and their examined family members are shown in eFigure in the Supplement.

Detailed ophthalmological reexamination of participant PCG402-IT revealed prominent posterior embryotoxon and PAS on gonioscopy.²⁵ Facial dysmorphism included a flat philtrum, a flat nasal bridge, a prominent forehead, lateral bushy eyebrows, hypertelorism, and a thin upper lip. No other systemic features were present. These features all suggested a revised diagnosis of ARS.

Review of the clinical records of participant PCG297-IT led to a revised diagnosis of glaucoma with ARA by 1 year of age. Posterior embryotoxon, PAS, optic disc cupping, and corneal edema were present in both eyes, with corneal opacity and sclerocornea in the right eye. Other systemic features associated with ARS were not present, with the exception of potential midface dysmorphism, including hypertelorism, a thin up-

per lip, a flat philtrum, dysmorphic ears, and lateral bushy eyebrows with medial flare.

Review of participant PCG029 did not reveal any anterior segment anomalies consistent with ARA. A more detailed examination by the individual's current ophthalmologist was complicated by bilateral corneal opacity, making a formal exclusion of ARA difficult. Possible syndromic associations included hydrocephalus and hearing loss. The same *FOXC1* deletion was absent in all family members tested (ie, 1 parent and 5 siblings), and although the other parent could not be tested, this suggested a de novo origin.

For participant PCG033, corneal opacity again complicated detailed anterior segment examination for ARA. Review of previous medical records indicated prominent posterior embryotoxon and PAS, with a possible systemic association of hearing loss. Both 1 parent of participant PCG033 and that parent's parent were affected by glaucoma, although a detailed medical history (including ages at diagnosis) could not be obtained.

The diagnosis for participant PCG057 was revised to ARS with secondary glaucoma after the referral of 2 of the participant's relatives, of whom 1 was diagnosed with ARA or the other

present in a further 2 probands, although this is not typically associated with ARS. All 5 of the probands examined for facial features had some degree of facial dysmorphism consistent with features reported in ARS. Based on their newly ascertained genetic evidence, all 8 probands were therefore given a revised diagnosis of ARS.

Axenveld-Rieger anomaly and Axenveld-Rieger syndrome are rare conditions that can be difficult to diagnose in children, especially without detailed ophthalmic examination under anesthesia. Ocular signs such as posterior embryotoxon or PAS may only be visible by gonioscopy,²² and even then, these may only become apparent with age. Diagnosis may also be complicated in adults, particularly if corneal opacity obscures a thorough anterior segment examination. Both *PITX2* and *FOXC1* show phenotypic variation within families, and only approximately 30% of individuals with *FOXC1* variants have extraocular features.²¹ When compared with *PITX2*, *FOXC1* is less likely to be associated with more striking systemic features, such as dental or umbilical anomalies,²⁰ and is therefore more likely to resemble PCG. Systemic features of ARS, particularly those in *FOXC1*-associated disease, often become apparent only later in life (eg, hearing loss, developmental delay, and short stature), making an accurate diagnosis of *FOXC1*-associated ARA or ARS even more challenging. Furthermore, *FOXC1* variants can be inherited de novo (as was confirmed in 2 families and suspected in a further 3 participants in this study), making clinical diagnosis more difficult than probands with a known family history of ARS (as reported in 3 pedigrees). All of these issues highlight the difficulties in reaching a precise clinical diagnosis of ARA or ARS at an early age. These data show the value of early genetic testing in presentations of suspected PCG cases and the revision of several diagnoses as a direct result.

Accurate diagnosis of PCG has a number of important implications for genetic counseling. Biallelic variants of *CYP11B1* are the most common known genetic cause of PCG, accounting for 22% of affected individuals in the Australian population.²⁶ Therefore families with children affected by PCG are often counseled for autosomal recessive inheritance. Revising this diagnosis to ARA or ARS caused by autosomal dominant *FOXC1* variants therefore requires a revision of counseling: rather than a maximum risk to siblings of 25%, first-degree relatives of individuals with inherited disease-

causing *FOXC1* variants have a risk of 50% for ARA and/or ARS, with approximately 50% of these expected to develop glaucoma. Importantly, in families with de novo inheritance, the risk of recurrence for siblings of affected individuals is low. Glaucoma is also not the only concern for a family with ARA; the presence of multiple associated systemic features should also be closely investigated and managed. Reaching the correct clinical and genetic diagnosis allows all family members the opportunity for genetic testing, appropriate referrals to be made for surveillance and management, and discussion of reproductive options.

Our data support the importance of genetic information in a clinical context, especially for conditions, such as childhood glaucoma, that carry a broad phenotypic spectrum and genetic heterogeneity. Indeed, one might consider sequencing not only *FOXC1*, but all genes associated with childhood glaucoma. This might be achieved with exome or genome sequencing; however, exome sequencing is likely to be less reliable, given the uneven capture and coverage of *FOXC1* and the limited ability to detect copy number variations.²⁷ At the very least, exome and other capture-based sequencing tests should be interpreted cautiously when excluding variants in poorly captured genes such as *FOXC1*.

Limitations

These cohorts were predominantly of European ancestry, and these findings may therefore not be generalizable to other populations, although lower prevalence of *FOXC1* variants has been reported in PCG cohorts from India (0% to 2.4%).^{28,29} Additionally, MLPA failed in 11 of 84 participants from the Australian cohort, which means that the true prevalence of *FOXC1* variants could be higher.

Conclusions

In summary, our data support the use of *FOXC1* sequencing as a diagnostic aid in cases of suspected PCG, although further replication will be needed to support the future use of such panels. Timely sequencing has the potential to accelerate diagnosis, minimize excessive examinations under anesthesia, refine genetic counseling, guide appropriate therapy, and maximize long-term patient benefit.

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