Received: 15 July 2022 | Accepted: 18 July 2022

DOI: 10.1002/mgg3.2023

Molecular Genetics & Genomic Medicine Open Access WILEY

ORIGINAL ARTICLE

Diagnostic yield of candidate genes in an Australian corneal dystrophy cohort

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Funding information
National Health and Medical Research Council

Abstract

Corneal dystrophies describe a clinically and genetically heterogeneous group of inherited disorders. The International Classification of Corneal Dystrophies (IC3D) lists 22 types of corneal dystrophy, 17 of which have been demonstrated to result from pathogenic variants in 19 identified genes. In this study, we investigated the diagnostic yield of genetic testing in a well-characterised cohort of 58 individuals from 44 families with different types of corneal dystrophy. Individuals diagnosed solely with Fuchs endothelial corneal dystrophy were excluded. Clinical details were obtained from the treating ophthalmologist. Participants and their family members were tested using a gene candidate and exome sequencing approach. We identified a likely molecular diagnosis in 70.5% families (31/44). The detection rate was significantly higher among probands with a family history

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of corneal dystrophy (15/16, 93.8%) than those without (16/28, 57.1%, $p = .015$), and among those who had undergone corneal graft surgery (9/9, 100.0%) compared to those who had not (22/35, 62.9%, $p = .041$). We identified eight novel variants in five genes and identified five families with syndromes associated with corneal dystrophies. Our findings highlight the genetic heterogeneity of corneal dystrophies and the clinical utility of genetic testing in reaching an accurate clinical diagnosis.

KEYWORDS

corneal dystrophy, genetic testing, molecular diagnosis, *TGFBI*

1 | INTRODUCTION

Corneal dystrophies are a heterogeneous group of inherited disorders affecting the cornea. With a few exceptions, these clinical entities are usually bilateral, symmetric, slowly progressive and non-syndromic (Weiss et al., 2015). The second edition of the International Classification of Corneal Dystrophies (IC3D) incorporates clinical, histopathological and genetic information and lists 22 corneal dystrophies subdivided into epithelial and subepithelial dystrophies, epithelial-stromal transforming growth factor beta-induced (*TGFBI)* dystrophies, stromal dystrophies and endothelial dystrophies (Table 1) (Weiss et al., 2015). This revised classification system includes (1) a modification of the anatomical classification to reflect the involvement of multiple corneal layers in some dystrophies, and (2) genetic evidence highlighting the phenotypic continuum of a single dystrophy rather than distinct clinical entities.

Genes have been identified for 17 of the corneal dystrophies listed in IC3D (Table 1), with *TGFBI* the most commonly implicated (Munier et al., 1997). The genetics of corneal dystrophy highlight both genetic heterogeneity (e.g., Meesmann corneal dystrophy associated with variants in *KRT3* and *KRT12*) and phenotypic heterogeneity (e.g., variants in *TGFBI* associated with five different epithelial-stromal corneal dystrophies). A clinical diagnosis can be difficult to establish in some patients (e.g., Schnyder corneal dystrophy in the absence of crystals [Weiss, 2009]), and in these cases, genetic testing can assist in reaching a more definitive diagnosis. Furthermore, genetic testing may improve the diagnosis of syndromes associated with corneal dystrophy and facilitate counselling about modes of inheritance and the risk for other family members. In this study, we investigated the underlying genetic cause of a well-characterised corneal dystrophy cohort using candidate gene and exome sequencing and discussed clinical utility of genetic testing.

2 | MATERIALS AND METHODS

2.1 | Participants and ethical considerations

Ethics approval was obtained from the Southern Adelaide Clinical Human Research Ethics Committee and the Royal Victorian Eye and Ear Hospital Human Research Ethics Committee. The study adhered to the revised Declaration of Helsinki and the National Health and Medical Research Council statement of ethical conduct in research involving humans. All participants provided informed consent. This was a retrospective clinical and molecular cohort study. Individuals with a clinical diagnosis of corneal dystrophy, and their family members when available, were recruited between 2007 and 2020. Clinical details were obtained from the treating ophthalmologist and clinical diagnoses were reviewed by a cornea specialist (RAM). Individuals diagnosed solely with Fuchs endothelial corneal dystrophy were excluded from the study. Blood or saliva samples were collected for DNA extraction purposes.

2.2 | Genetic testing

Sanger sequencing of four candidate genes was performed on a subset of individuals: *TGFBI* was sequenced in 28 probands (primers in Supplementary Table S1), *CHST6* in four probands with macular corneal dystrophy at the Casey Eye Institute (Portland, OR, USA), and *UBIAD1* in one proband with Schnyder corneal dystrophy at SA Pathology (Flinders Medical Centre, Adelaide, Australia).

Exome sequencing was performed on 30 probands without a molecular diagnosis from candidate gene sequencing. DNA was prepared from whole blood and subjected to exome capture (Agilent SureSelect v5) as previously described (Siggs et al., 2019). Reads were processed through the GATK 'Best Practice' variant calling workflow using bcbio-nextgen (v.1.2.8), with alignment

TABLE 1 Classification of corneal dystrophies and associated genes and loci

Classification	Type	OMIM	Locus	Gene	OMIM	Inheritance
Epithelial & subepithelial	Epithelial basement membrane dystrophy	121820	5q31.1	*TGFBI*	601692	Sporadic/AD
	Epithelial recurrent erosion dystrophy	122400	10q25.1	*COL17A1*	113811	AD
	Subepithelial mucinous corneal dystrophy	612867	–	–	–	AD?
	Meesmann corneal dystrophy	618787, 122100	12q13.13, 17q21.2	*KRT3*, *KRT12*	148043, 601687	AD
	Lisch epithelial corneal dystrophy	300778	Xp22.3	–	–	XL
	Gelatinous drop-like corneal dystrophy	204870	1p32.1	*TACSTD2*	137290	AR
Epithelial-stromal *TGFBI*	Reis-Bücklers corneal dystrophy	608470	5q31.1	*TGFBI*	601692	AD
	Thiel-Behnke corneal dystrophy	602082	5q31.1	*TGFBI*	601692	AD
	Lattice corneal dystrophy	122200	5q31.1	*TGFBI*	601692	AD
	Granular corneal dystrophy, type 1	121900	5q31.1	*TGFBI*	601692	AD
	Granular corneal dystrophy, type 2	607541	5q31.1	*TGFBI*	601692	AD
Stromal	Macular corneal dystrophy	217800	16q23.1	*CHST6*	605294	AR
	Schnyder corneal dystrophy	121800	1p36.22	*UBIAD1*	611632	AD
	Congenital stromal corneal dystrophy	610048	12q21.33	*DCN*	125255	AD
	Fleck corneal dystrophy	121850	2q34	*PIKFYVE*	609414	AD
	Posterior amorphous corneal dystrophy	612868	12q21.33	*KERA, LUM, DCN, EPYC*[a]	–	AD
	Central cloudy dystrophy of François	217600	–	–	–	–
	Pre-Descemet corneal dystrophy	–	–	–	–	–
Endothelial	Fuchs endothelial corneal dystrophy	136800, 613267, 613268, 613270, 615523, 610158, 613269, 613271	1p34.3, 18q21.2, 20p13, 10p11.22, 15q25.3, 13pter-q12.13, 5q33.1-q35.2, 9p24.1-p22.1	*COL8A2*, *TCF4*, *SLC4A11*, *ZEB1*, *AGBL1*	120252, 602272, 610206, 189909, 615496	AD
	Posterior polymorphous corneal dystrophy	122000, 609140, 609141, 618031	20p11.23, 1p34.3, 10p11.22, 8q22.3	*OVOL2*, *COL8A2*, *ZEB1, GRHL2*	616441, 120252, 189909, 608576	AD
	Congenital hereditary endothelial dystrophy	217700	20p13	*SLC4A11*	610206	AR
	X-linked endothelial corneal dystrophy	300779	Xq25	–		XL

Abbreviations: AD, autosomal dominant; AR, autosomal recessive; XL, X-linked.

[a]Contiguous gene deletion syndrome.

to the GRCh37 human reference genome. We used a candidate gene approach and screened for all genes listed in Table 1 as well as two genes associated with syndromes that can present with corneal dystrophy listed in the IC3D: the gelsolin gene (*GSN*, 9q33.2, MIM 137350), associated with amyloidosis of the Finnish type (MIM 105120), and the steroid sulfatase gene (*STS*, Xp22.31, MIM 300747), associated with X-linked ichthyosis (MIM 308100). Variant were filtered if they were nonsense, frameshift, essential splice or missense; had a gnomAD (v.2.1.1) allele frequency <0.001; and were predicted deleterious by Combined Annotation Dependent Depletion (CADD).

Variants identified through *TGFBI* sequencing or exome sequencing were validated by a National Association of Testing Authorities-accredited laboratory (SA Pathology) using bi-directional direct sequencing of the relevant PCR-amplified regions. Deletions on chromosome X of the *STS* gene were confirmed by single nucleotide polymorphism (SNP) array using the Illumina Infinium CytoSNP-850 K Beadchip. The recommendations from the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (Richards et al., 2015) and the ClinGen Sequence Variant Interpretation working group (https://www.clinicalgenome.org/working-groups/sequence-variant-interpretation/) were used to assess variant pathogenicity. Variants classified as pathogenic, likely pathogenic and of uncertain significance (VUS) are presented. All variants, including novel ones, have been deposited in ClinVar (Accession numbers SCV001981633.1-SCV001981655.1).

2.3 | Histopathology

Histopathological and electron microscopy analysis were performed when available using corneal thickness specimens obtained from participants undergoing full-thickness corneal graft surgery. Formalin-fixed paraffin-embedded specimens were used for histopathology. Diagnostic stains, which were chosen on a case-by-case basis according to clinical findings, included (but were not limited to) haematoxylin and eosin, periodic acid-Schiff, Masson trichrome, and Congo red. Transmission electron microscopy was performed using sections of the same tissues collected in glutaraldehyde. All images were assessed by a pathologist (SK) with expertise in ophthalmic pathology.

2.4 | Statistics

PASW Statistics, Rel. 18.0.1.2009 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Data are presented as mean ± standard deviation and 95%CI. Fisher's exact test was used to assess differences in categorical data whereas Mann–Whitney U test was used to assess differences in mean.

3 | RESULTS

The cohort consisted of 58 individuals from 44 families. Mean age at recruitment was 48.3 ± 20.0 years (95%CI 43.0–53.6 years), and 27 (46.6%) were female. The majority of families (40/44, 90.9%) were of self-reported European ancestry. Clinical details of the cohort are described in Supplementary Table S2. We achieved a probable molecular diagnosis in 31 families (70.5%, Table 2), including 11 families via direct sequencing of the *TGFBI* gene, three families via direct sequencing of the *CHST6* gene, one family via direct sequencing of the *UBIAD1* gene and 16 families using exome sequencing. The detection rate was significantly higher among probands with a family history of corneal dystrophy (15/16, 93.8%) compared with probands without a family history (16/28, 57.1%, $p = .015$), and among probands with one or more corneal grafts (9/9, 100.0%) compared with those without a graft (22/35, 62.9%, $p = .041$). Moreover, individuals with a molecular diagnosis had a younger age at diagnosis (32.5 ± 20.2 years, 95%CI 26.1–38.9 years) compared with individuals with no molecular diagnosis (53.5 ± 21.6 years, 95%CI 40.4–66.5 years, $p = .004$). The genetic results and the clinical associations are discussed below, with pedigrees of the solved families illustrated in Supplementary Figure S1.

3.1 | Epithelial-stromal TGFBI corneal dystrophies

Twenty-three individuals from 16 families had a clinical diagnosis of an epithelial-stromal corneal dystrophy. We achieved a molecular diagnosis in 14 families (87.5%). Exome sequencing did not identify pathogenic variants in the genes investigated in one proband with a clinical presentation of epithelial basement membrane dystrophy but histopathology suggesting Reis-Bücklers corneal dystrophy (CDSA315), and one proband who had anterior stromal opacities with subtle lattice-like appearance in both eyes but not typical of granular corneal dystrophy type 2 (CDSA043). In the whole cohort, six of the 10 individuals (60.0%) who required corneal graft surgery carried *TGFBI* pathogenic variants, with a mean age at surgery of 52.0 ± 18.6 years (95%CI 32.5–71.5 years). In contrast, individuals with epithelial-stromal corneal dystrophy and no corneal graft were younger, with a

TABLE 2 Genetic results of the probands

ID	Gene	gDNA	cDNA	Protein	Status	gnomAD AC	ClinVar ID	ACMG classification
CDSA001	*TGFBI*	g.135382096G>A (NC_000005.9)	c.371G>A (NM_000358.3)	p.Arg124His (NP_000349.1)	heterozygous	1/112,864	7869	P
CDSA028	*KRT12*	g.39019532_39019543del (NC_000017.10)	c.1148_1159del (NM_000223.4)	p.(Gln383_Val387delinsLeu) (NP_000214.1)	heterozygous	Nil	1300203	VUS
CDSA041	*STS*	g.6575925_8173249del (NC_000023.10)	N/A	N/A	heterozygous	Nil	1300212	P
CDSA116	*TGFBI*	g.135392469C>T (NC_000005.9)	c.1663C>T (NM_000358.3)	p.Arg555Trp (NP_000349.1)	heterozygous	Nil	7866	P
CDSA117	*TGFBI*	g.135382096G>A (NC_000005.9)	c.371G>A (NM_000358.3)	p.Arg124His (NP_000349.1)	heterozygous	1/112,864	7869	P
CDSA118	*GSN*	g.124073097G>A (NC_000009.11)	c.640G>A (NM_000177.5)	p.Asp214Asn (NP_000168.1)	heterozygous	0/129,136	16180	P
CDSA128	*TGFBI*	g.135382096G>A (NC_000005.9)	c.371G>A (NM_000358.3)	p.Arg124His (NP_000349.1)	heterozygous	1/112,864	7869	P
CDSA135	*TGFBI*	g.135396583A>C (NC_000005.9)	c.1864A>C (NM_000358.3)	p.(Asn622His) (NP_000349.1)	heterozygous	Nil	1300215	LP
CDSA140	*TGFBI*	g.135382095C>T (NC000005.9)	c.370C>T (NM_000358.3)	p.Arg124Cys (NP_000349.1)	heterozygous	1/128,304	7868	P
CDSA142	*STS*	g.6456940_8135053del (NC_000023.10)	N/A	N/A	heterozygous	Nil	1300213	P
CDSA155	*PIKFYVE*	g.208579356_209357870del (NC_000002.11)	N/A	N/A	Heterozygous	Nil		P
CDSA160	*CHST6*	g.75512730A>G (NC000016.9)	c.997T>C (NM_021615.5)	p.Trp333Arg (NP_067628.1)	homozygous	2/113,120	1300206	LP
CDSA174	*TGFBI*	g.135382096G>A (NC_000005.9)	c.371G>A (NM_000358.3)	p.Arg124His (NP_000349.1)	heterozygous	1/112,864	7869	P
CDSA175	*TGFBI*	g.135392470G>A (NC_000005.9)	c.1664G>A (NM_000358.3)	p.Arg555Gln (NP_000349.1)	heterozygous	Nil	7867	P
CDSA177	*CHST6*	g.75513423A>C (NC_000016.9)	c.304T>G (NM_021615.5)	p.Cys102Gly (NP_067628.1)	compound heterozygous	3/127,712	5076	P
		g.75513128A>C (NC_000016.9)	c.599T>G (NM_021615.5)	p.Leu200Arg (NP_067628.1)	compound heterozygous	59/125,324	5075	P
CDSA197	*TGFBI*	g.135382096G>A (NC_000005.9)	c.371G>A (NM_000358.3)	p.Arg124His (NP_000349.1)	heterozygous	1/112,864	7869	P

(Continues)

TABLE 2 (Continued)

ID	Gene	gDNA	cDNA	Protein	Status	gnomAD AC	ClinVar ID	ACMG classification
CDSA262	*GSN*	g.124083678T>C (NC_000009.11)	c.1477T>C (NM_000177.5)	p.(Trp493Arg) (NP_000168.1)	heterozygous	Nil	984957	VUS
CDSA265	*PIKFYVE*	g.209200915G>A (NC_000002.11)	c.4511G>A (NM_015040.4)	p.(Trp1504Ter) (NP_055855.2)	heterozygous	Nil	1300208	P
CDSA288	*TGFBI*	g.135382096G>A (NC_000005.9)	c.371G>A (NM_000358.3)	p.Arg124His (NP_000349.1)	heterozygous	1/112,864	7869	P
CDSA305	*CHST6*	g.75512879_75512880delinsCA (NC_000016.9)	c.847_848delinsTG (NM_021615.5)	p.Glu283Ter (NP_067628.1)	compound heterozygous	4/127,444	1300205	P
		g.75513423A>C (NC_000016.9)	c.304T>G (NM_021615.5)	p.Cys102Gly (NP_067628.1)	compound heterozygous	3/127,712	5076	P
CDSA314	*PIKFYVE*	g.209189647C>T (NC_000002.11)	c.2344C>T (NM_015040.4)	p.(Arg782Ter) (NP_055855.2)	heterozygous	Nil	1300207	P
CDSA316	*TGFBI*	g.135382095C>T (NC_000005.9)	c.370C>T (NM_000358.3)	p.Arg124Cys (NP_000349.1)	heterozygous	1/128,304	7868	P
CDSA319	*SLC4A11*	g.3214873C>T (NC_000020.10)	c.508G>A (NM_001174090.1)	p.Glu170Lys (NP_001167561.1)	homozygous	1/113,652	1068022	P
CDSA324	*TGFBI*	g.135392418A>C (NC_000005.9)	c.1612A>C (NM_000358.3)	p.(Thr538Pro) (NP_000349.1)	heterozygous	Nil	1300214	LP
CDSA333	*TGFBI*	g.135382096G>A (NC_000005.9)	c.371G>A (NM_000358.3)	p.Arg124His (NP_000349.1)	heterozygous	1/112,864	7869	P
CDSA336	*UBIAD1*	g.11345889G>A (NC_000001.10)	c.718G>A (NM_013319.3)	p.Asp240Asn (NP_037451.1)	heterozygous	Nil	1300209	LP
CDSA344	*TGFBI*	g.135382095C>A (NC000005.9)	c.370C>A (NM_000358.3)	p.Arg124Ser (NP_000349.1)	heterozygous	Nil	7873	P
CDSA360	*KRT12*	g.39019540A>G (NC_000017.10)	c.1151T>C (NM_000223.4)	p.(Leu384Pro) (NP_000214.1)	heterozygous	Nil	1300204	VUS
CDSA361	*ZEB1*	g.31803530G>A (NC_000010.10)	c.688-1G>A (NM_001174096.2)	N/A	heterozygous	Nil	1300211	LP
CDSA368	*ZEB1*	g.31803530G>A (NC_000010.10)	c.688-1G>A (NM_001174096.2)	N/A	heterozygous	Nil	1300211	LP
PPCD003	*ZEB1*	g.31799739dup (NC_000010.10)	c.623dup (NM_001174096.2)	p.(Tyr208Ter) (NP_001167567.1)	heterozygous	Nil	1300210	LP

Notes: Positions are referenced using hg19 (GRCh37). AC, allele counts; AC are reported for the Non-Finnish European population in the gnomAD database (v2.1.1). N/A, not applicable. VUS, variant of uncertain significance; LP, likely pathogenic; P, pathogenic.

mean age at recruitment of 45.1 ± 18.7 years (95%CI 35.5–54.7 years).

The most common type of epithelial-stromal corneal dystrophy was granular corneal dystrophy type 2, present in 14 individuals from seven families. All of these individuals harboured the pathogenic *TGFBI* p.Arg124His variant. Interestingly, in one family (CDSA001, Figures 1a and 2a–d), three individuals were concurrently affected by Fuchs corneal dystrophy.

One individual (CDSA175, Figure 1b) originally diagnosed with Reis-Bücklers corneal dystrophy was later diagnosed, based on electron microspcopy, as having Thiel-Behnke corneal dystrophy (Figure 2e–h). Sequencing of *TGFBI* identified the pathogenic p.Arg555Gln variant, which has been reported in both phenotypes (Munier et al., 1997; Munier et al., 2002). Two known pathogenic *TGFBI* variants were identified in individuals with granular corneal dystrophy type 1 (p.Arg555Trp in CDSA116 and p.Arg124Ser in CDSA344).

We achieved a molecular diagnosis in the four probands with lattice corneal dystrophy. The pathogenic *TGFBI* p.Arg124Cys variant was identified in two probands (CDSA140 & CDSA316, Figure 1c). Additionally, we identified two previously reported *TGFBI* variants associated with clinical variants of lattice corneal dystrophy (p.(Asn622His) and p.(Thr538Pro)) in two other families (CDSA135 (Figures 1d and 2i–l) and CDSA324 (Figure 1e), respectively).

3.2 | Meesmann corneal dystrophy

Two probands (CDSA028 & CDSA360 [Figure 1f]) had a clinical diagnosis of Meesmann corneal dystrophy. A third proband (CDSA274) had Meesmann-like corneal dystrophy with microcystic changes of the corneal epithelium. We identified a novel in-frame deletion/insertion (p.(Gln383_Val387delinsLeu)) and a novel missense variant (p.(Leu384Pro)) classified as VUS in *KRT12* of CDSA028 and CDSA360, respectively. These two variants are located just outside of the helix termination motif but occur in a highly conserved region among KRT12 orthologues, providing evidence toward pathogenicity. The parents were not available for examination or genetic testing. We did not identify pathogenic variants in the third proband (CDSA274).

3.3 | Macular corneal dystrophy

Four individuals from three families had macular corneal dystrophy. One proband (CDSA160) was originally thought to have mucopolysaccharidosis (Figure 2m–p), while another one (CDSA177) was initially diagnosed with granular corneal dystrophy. We identified biallelic pathogenic variants in *CHST6* in all three families. CDSA160 was homozygous for a missense variant predicted likely pathogenic (p.Trp333Arg) while CDSA177 was compound heterozygous for two previously reported pathogenic missense variants (p.Cys102Gly and p.Leu200Arg) (Aldave et al., 2004), each inherited from one parent. In the third family, both affected siblings (CDSA305.1 [Figure 1g] & CDSA305.2) were compound heterozygous for the same pathogenic missense variant as CDSA177 (p.Cys102Gly) and a novel nonsense variant predicted pathogenic (p.Glu283Ter) inherited from one parent each (Shields et al., 2020).

3.4 | Schnyder corneal dystrophy

A single individual with Schnyder corneal dystrophy was recruited (CDSA336, Figure 1h) after presenting at the age of 50 years with bilateral central corneal opacity and corneal arcus without crystalline deposits. We identified a previously reported missense variant classified as likely pathogenic in *UBIAD1* (p.Asp240Asn) (Weiss et al., 2010).

3.5 | Fleck corneal dystrophy

Three probands were diagnosed with fleck corneal dystrophy. Two probands (CDSA265 & CDSA314 [Figure 1i]) had novel nonsense variants classified pathogenic in *PIKFYVE* (p.(Trp1504Ter) and p.(Arg782Ter) respectively). The third proband (CDSA155) had fleck corneal dystrophy as well as coloboma of the iris and choroid and congenital microphthalmia in her right eye. A whole gene deletion of *PIKFYVE* as well as the *CRYG* gene cluster was identified. Interestingly, coloboma and microphthalmia have been reported with crystalline genes before (Sun et al., 2017). However, it is unclear at this stage if these additional ocular features can be caused by the identified deletion. The parents were not available for examination or genetic testing in the three families.

3.6 | Posterior polymorphous corneal dystrophy

Six probands were diagnosed with posterior polymorphous corneal dystrophy. One proband (CDSA361) had phocomelia major and congenital heart disease believed to be due to thalidomide exposure in-utero while four affected individuals from family PPCD003 had abdominal hernias. We identified a novel splice site variant

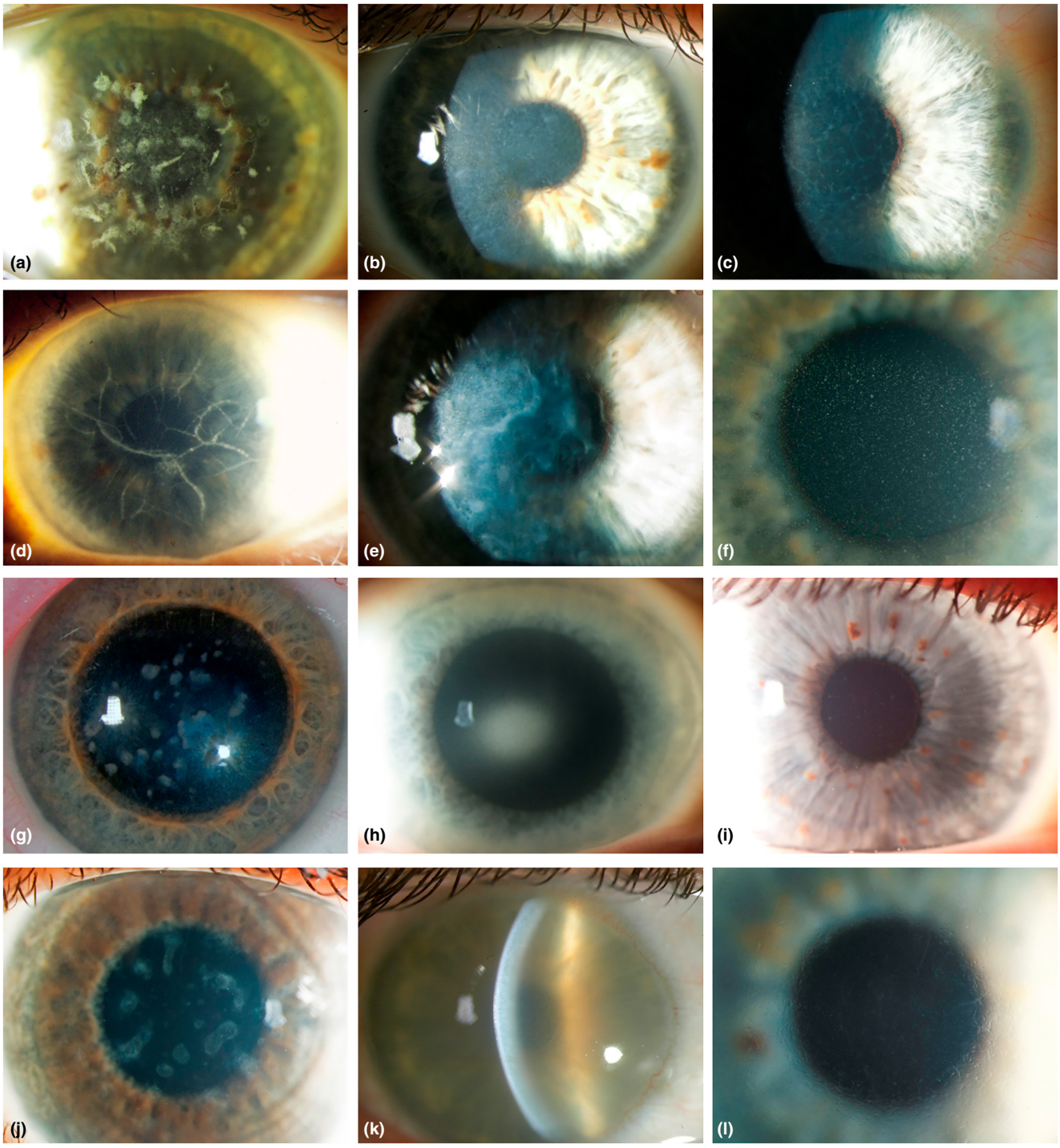

FIGURE 1 Clinical photographs. (a) Star, icicle-shaped, spiny and ring-like opacities (granular corneal dystrophy type 2, CDSA001.2, *TGFBI*:p.Arg124His). (b) Diffuse irregular subepithelial and anterior stroma honeycomb opacities (Thiel-Behnke corneal dystrophy, CDSA175, *TGFBI*:p.Arg555Gln). (c) Thin branching refractile lines and whitish branching stromal opacities (lattice corneal dystrophy, CDSA316, *TGFBI*:p.Arg124Cys). (d) Thick central ropy-appearing lattice lines (lattice corneal dystrophy variant CDSA135, *TGFBI*:p.Asn622His). (e) Diffuse confluent stromal opacities with multiple translucent thin lattice lines (lattice corneal dystrophy variant, CDSA324, *TGFBI*:p.Thr538Pro). (f) Diffuse solitary microcysts of the epithelium (Meesmann corneal dystrophy, CDSA360, *KRT12*:p.Leu384Pro). (g) Central irregular whitish opacities with diffuse stromal haze of the entire cornea (macular corneal dystrophy, CDSA305.1, *CHST6*:p.Glu283Ter/p.Cys102Gly). (h) Disc-shaped central opacity with no crystals and peripheral arcus lipoides (Schnyder corneal dystrophy, CDSA336, *UBIAD1*:p.Asp240Asn). (i) Infrequent small discrete opacities at various levels in the cornea (fleck corneal dystrophy, CDSA314, *PIKFYVE*:p.Arg782Ter). (j) Polymorphic grey opacities in deep stroma just anterior to Descemet membrane (posterior polymorphous corneal dystrophy, CDSA368, *ZEB1*:c.688-1G>A). (k) Diffuse ground-glass milky haze opacities with thickening of the cornea (congenital hereditary corneal dystrophy, CDSA319, *SLC4A11*:p.Glu170Lys/p.Glu170Lys). (l) Linear and punctate stromal deposits that appear opaque under direct illumination but translucent under indirect illumination (lattice corneal dystrophy with familial amyloidosis CDSA262.1, *GSN*:p.Trp493Arg).

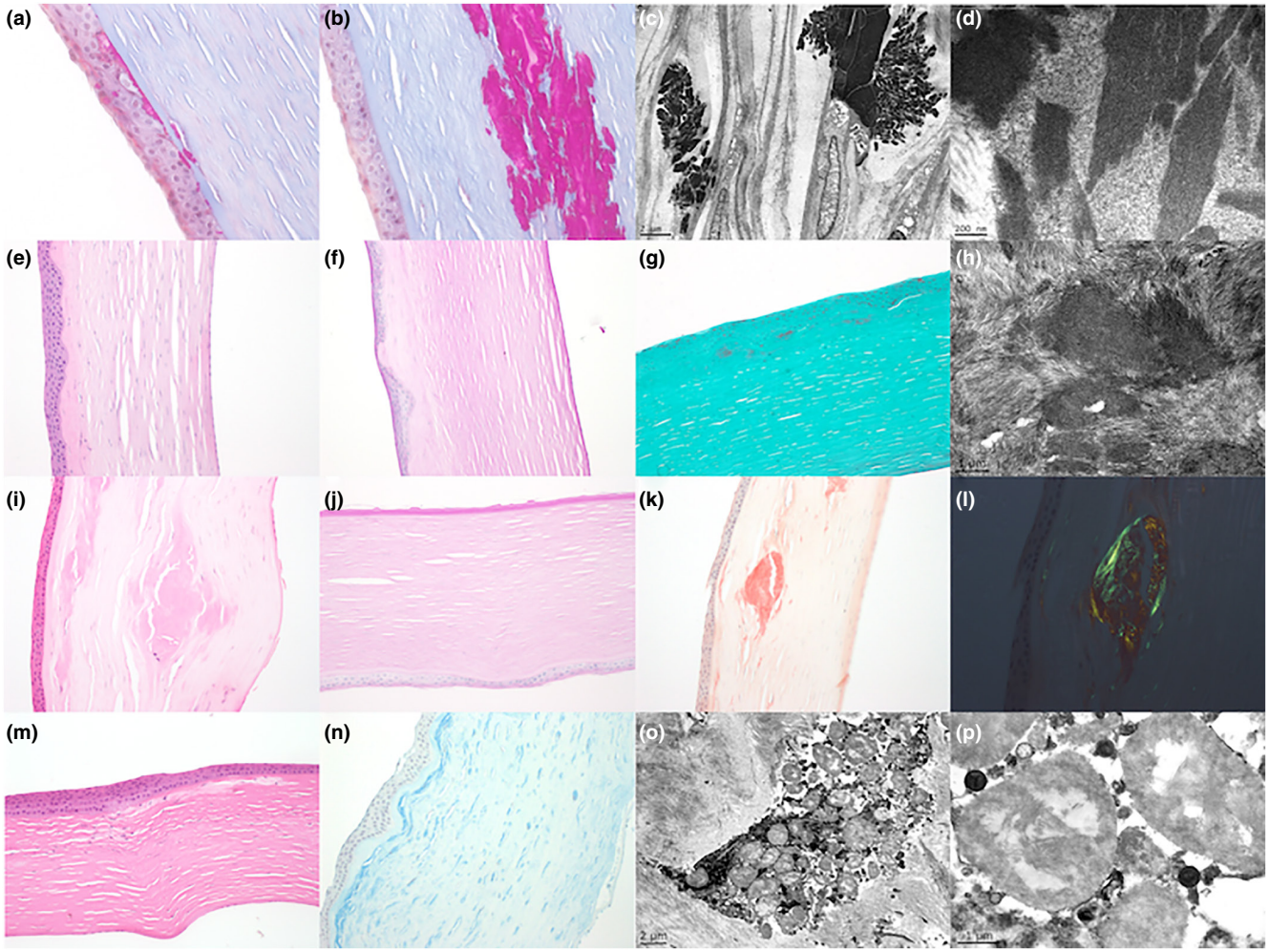

FIGURE 2 Corneal histopathology and electron microscopy. (a–d) Granular corneal dystrophy type 2 (CDSA001.3). (a,b) Trichrome staining demonstrated intense trichrome positive subepithelial deposits, morphologically indistinguishable from the deposits seen in granular corneal dystrophy (a, 40X magnification) and stromal (b, 40X magnification) deposits. (c,d) Electron microscopy revealed these deposits to be abundant with large, electron dense granules. (e–h) Thiel-Behnke corneal dystrophy (CDSA175). (e,f) A thick and irregular subepithelial layer observed in a haematoxylin and eosin (H&E) stained corneal section (e, 20X magnification) demonstrated negative periodic acid-Schiff (PAS) staining (f, 20X magnification). (g) Undifferentiated Mason trichrome staining revealed subepithelial deposit in a pannus-like pattern in the region of Bowman's layer (20X magnification). (h) Electron microscopy demonstrated an abundance of thick (14 nm diameter) curly collagen fibres within these depots, distinguishing the phenotype of Thiel-Behnke from Reis-Bücklers corneal dystrophy. (i–l) Lattice corneal dystrophy variant (CDSA135). (i) Subepithelial thickening and anterior to mid-stromal eosinophilic extracellular deposits were observed in an H&E stained specimen (20X magnification). (j) Loss of PAS staining in the region of Bruch's membrane suggested subepithelial deposition (20X magnification). (k) Congo red staining further highlighted the characteristic features of amyloidosis, including with well-delineated red colouration of amyloid deposits (20X magnification). (l) Apple-green birefringence under polarised light (20X magnification). (m–p) Macular corneal dystrophy (CDSA160). (m) H&E slides demonstrated patchy loss of subepithelial haematoxylin uptake. (n) Alcian blue (pH 2.5) showed these areas corresponded to positive fine granular deposits, typical of acid mucopolysaccharide accumulation within keratocytes throughout the entire thickness of the corneal stroma (20X magnification). (o,p) Electron microscopy revealed corneal collagen with deposition of electron-dense lysosomal granules, affirming the diagnosis of macular corneal dystrophy.

(c.688-1G>A, CDSA361 & CDSA368 [Figure 1j]) and a novel nonsense variant (p.(Tyr208Ter), PPCD003.5) predicted likely pathogenic in *ZEB1* in three probands. The parents of CDSA361 were not available for examination or genetic testing. We did not identify pathogenic variants in the other three probands (CDSA215, CDSA328 and CDSA329).

3.7 | Congenital hereditary endothelial dystrophy

One proband (CDSA319, Figure 1k) was diagnosed with corneal oedema and an undefined corneal dystrophy at the age of 3 years. He received a clinical diagnosis of congenital hereditary endothelial dystrophy when he was

15 years old. A homozygous variant identified in *SLC4A11* (p.Glu170Lys) was classified as pathogenic. There was no family history of corneal dystrophy but two siblings had a history of hearing loss.

3.8 | Unclassified corneal dystrophies

Twelve individuals from 10 families had corneal dystrophies that did not fit the IC3D classification. Two probands (CDSA041 and CDSA142) had deep stromal focal opacities compatible with pre-Descemet corneal dystrophy and ichthyosis. Exome sequencing revealed an absence of coverage on chromosome X and deletions of 1.6 Mb and 1.7 Mb encompassing *STS* were confirmed by SNP array in both probands, associated with a diagnosis of X-linked ichthyosis.

One proband (CDSA261) had anterior stromal opacities that were mainly circular, with some fleck-like in nature and confluent inferiorly. Although there was no history of ocular inflammation, serology was positive for Epstein–Barr virus (EBV) and EBV-associated keratitis could not be excluded as a cause. One proband had bilateral corneal opacities with both superficial and deep components (CDSA131) that did not fit any recognisable pattern. He had central anterior opacities that looked like band keratopathy, peripheral Salzmann-like small subepithelial scars and deeper multifocal translucent stromal lesions that looked like amyloid or were in keeping with polymorphic dystrophy.

One proband (CDSA118.1) and a relative with lattice corneal dystrophy but no *TGFBI* variant had a known pathogenic variant (p.Asp214Asn) in *GSN* that led to a subsequent clinical diagnosis of amyloidosis of the Finnish type.

Finally, five probands had corneal opacities similar to the polymorphic stromal corneal dystrophies described by Thomsitt and Bron (1975) and Mannis et al. (1981). A novel variant classified as VUS in *GSN* (p.(Trp493Arg)) segregated in four affected relatives in one family (CDSA262, Figure 1l). All four individuals had systemic features consistent with a diagnosis of amyloidosis of the Finnish type, including drooping eyelids, dry skin, cutis laxa and eczema. Amyloid deposition was present in the anterior corneal stroma in the excised cornea of the proband and immunohistochemistry reported the presence of the GSN protein within corneal amyloid deposits (Mullany et al., 2021). We did not achieve a molecular diagnosis in the other four probands (CDSA188, CDSA249, CDSA289 & CDSA350).

4 | DISCUSSION

Using a combination of candidate gene and exome sequencing, we reached a probable molecular diagnosis in over two-thirds of the families in our cohort. Moreover, we identified novel variants in several genes, broadening the allelic heterogeneity of associated genes.

4.1 | Epithelial-stromal corneal dystrophies

Heterozygous variants in *TGFBI* cause a number of clinically distinct corneal dystrophies involving the corneal epithelium and/or stroma (Munier et al., 1997). The majority of variants occur at residues 124 and 555, with haplotype analyses suggesting a mutational hotspot rather than a founder effect (Korvatska et al., 1998). Interestingly, different heterozygous variants in *TGFBI* are associated predominantly with different corneal dystrophies: Reis-Bücklers corneal dystrophy is associated with p.Arg124Leu, Thiel-Behnke corneal dystrophy with p.Arg555Gln, lattice corneal dystrophy with p.Arg124Cys, granular corneal dystrophy type 1 with p.Arg555Trp and granular corneal dystrophy type 2 with p.Arg124His (Munier et al., 1997; Munier et al., 2002). In this cohort, all individuals with an epithelial-stromal-*TGFBI* corneal dystrophy had genetic results consistent with their clinical diagnosis. These results support strong correlations between genotype and phenotype for *TGFBI* variants.

Lattice corneal dystrophy is mainly associated with *TGFBI* p.Arg124Cys (Munier et al., 1997) while clinical variants of the condition are usually associated with *TGFBI* variants in downstream exons (Schmitt-Bernard et al., 2000). In the five families with lattice corneal dystrophy in this study, two had sequence variants in downstream exons (p.Thr538Pro in exon 12 and p.Asn622His in exon 14). The first variant (p.Thr538Pro) was previously reported in one Indian and two Chinese families with lattice corneal dystrophy, and was classified as likely pathogenic (Long et al., 2011; Paliwal et al., 2010; Yu et al., 2006). The second variant (p.Asn622His) was identified in an individual with unilateral lattice corneal dystrophy and was classified as a VUS. Interestingly, the same variant was previously reported in an individual with a similar phenotype of unilateral non-progressive lattice corneal dystrophy (Stewart et al., 1999).

4.2 | Epithelial and sub-epithelial corneal dystrophies

Meesmann corneal dystrophy is characterised by multiple fine round intraepithelial microcysts that can appear by 12 months of age which lead to corneal fragility and recurrent painful erosions (Szaflik et al., 2008; Weiss et al., 2015). This condition is caused by variants in *KRT3*

and *KRT12* (Irvine et al., 1997). Keratin proteins are expressed in pairs in a tissue-specific manner and form heterodimers of a type I acidic protein and a type II basic protein. The expression of *KRT3* (type II) and *KRT12* (type I) is specific to corneal epithelial cells and these two genes play an important role in maintaining normal corneal epithelial function (Kao et al., 1996). Reported pathogenic variants have been identified in the helix initiation and termination highly conserved motifs of the central rod domains (Szaflik et al., 2008), which are essential for polymerisation and proper filament formation. These dominant-negative variants result in protein misfolding and aggregation in corneal epithelial cells, which may trigger an unfolded protein response and apoptosis (Allen et al., 2016). In this study, we identified two novel variants in the helix termination motif of *KRT12*, expanding the list of *KRT12* variants potentially associated with Meesmann corneal dystrophy. Although *COL17A1* variants have previously been reported in four families with epithelial recurrent erosion dystrophies (Oliver et al., 2016), we did not identify any *COL17A1* variants in this cohort.

4.3 | Stromal corneal dystrophies

Macular corneal dystrophy is an autosomal recessive condition characterised by superficial, central and irregular grey-white corneal opacities that progressively increase to include the entire corneal stroma and the limbus, with corneal thinning (Weiss et al., 2015). This condition is caused by biallelic variants in *CHST6* (Akama et al., 2000). We identified biallelic variants in *CHST6* in the three families with macular corneal dystrophy, including one novel variant predicted to be pathogenic.

Schnyder corneal dystrophy is an autosomal dominant condition characterised by an accumulation of cholesterol and phospholipids in the corneal stroma. Crystalline corneal deposits are reported in approximately half of affected individuals (Weiss et al., 2015). This condition is caused by heterozygous variants in *UBIAD1* (Orr et al., 2007), with all known pathogenic variants located in the prenyltransferase domain (Nickerson et al., 2013). We identified a previously reported variant (p.Asp240Asn) (Weiss et al., 2010) classified as likely pathogenic in one proband with no family history who was diagnosed at the relatively late age of 50 years. This is likely due to an absence of crystalline deposits, which often results in a more subtle presentation and may contribute to the difficulty of reaching diagnosis (Weiss, 2009).

Fleck corneal dystrophy is a typically asymptomatic autosomal dominant condition caused by variants in *PIKFYVE* (Li et al., 2005), and is characterised by small white-fleck opacities scattered through the corneal stroma (Weiss et al., 2015). Apart from one missense variant (Li et al., 2005), all reported *PIKFYVE* pathogenic variants are nonsense or frameshift. In this study, we identified two novel predicted pathogenic nonsense variants in exons 19 and 27 as well as a whole gene deletion.

4.4 | Endothelial corneal dystrophies

Posterior polymorphous corneal dystrophies display geographic opacities and vesicular lesions of Descemet's membrane and the endothelium, and are phenotypically and genetically heterogeneous. There are four subtypes of posterior polymorphous corneal dystrophies associated with variants in four different genes inherited in an autosomal dominant manner (Table 1). In this cohort, we identified two novel loss-of-function *ZEB1* variants associated with posterior polymorphous corneal dystrophy type 3. Interestingly, missense variants in *ZEB1* are associated with Fuchs endothelial corneal dystrophy whereas loss-of-function variants are associated with posterior polymorphous corneal dystrophy (Riazuddin et al., 2010). Abdominal hernias which have been described with *ZEB1* variants (Krafchak et al., 2005), were recorded in one of the families reported here. The different disease mechanisms leading to different phenotypes are supported by the findings from this study. The diagnostic yield in this study was the lowest (50.0%) for posterior polymorphous corneal dystrophy. The condition can be caused by non-coding variants (promoter region of *OVOL2* or intronic variants in *GRHL2*). Therefore, it is possible that the individuals with no molecular diagnosis have non-coding variants in these genes not covered by exome sequencing.

Congenital hereditary endothelial dystrophy (CHED) is caused by biallelic variants in *SLC4A11* (Vithana et al., 2006), which have also been associated with Harboyan syndrome (MIM 217400), a condition characterised by CHED and sensorineural deafness (Harboyan et al., 1971). Interestingly, the same variants have been reported in individuals with CHED or Harboyan syndrome (Desir et al., 2007; Mehta et al., 2010; Sultana et al., 2007). Sensorineural hearing loss in Harboyan syndrome usually appears in the teenage years, meaning that ocular features are generally the first symptoms identified and reported. The lack of auditory assessment and the young age of reported individuals with CHED make it difficult to assess the true prevalence of hearing loss in CHED. A small study reported subsequent diagnosis of sensorineural hearing loss in four individuals originally diagnosed with CHED (Siddiqui et al., 2014). Harboyan syndrome and CHED are likely to represent a single clinical entity with variable expressivity rather than two distinct clinical entities. In the family reported in this study, the proband did

not have hearing loss but two of his siblings did. Testing of family members would be needed to assess if they have Harboyan syndrome.

4.5 | Corneal grafts

Among the nine probands who had undergone at least one corneal graft, all had a molecular diagnosis. Five of the probands who required corneal grafting had an epithelial-stromal *TGFBI* corneal dystrophy, two had macular corneal dystrophy, one had CHED, and one had amyloidosis of the Finnish type. This was not surprising considering that epithelial-stromal corneal dystrophies are characterised by painful and recurrent corneal erosions that lead to corneal scarring and progressive deterioration of vision (Weiss et al., 2015).

4.6 | Clinical utility of genetic testing

In 2012, the American Academy of Ophthalmology published recommendations supporting routine genetic testing for inherited eye diseases (Stone et al., 2012). Genetic testing has proven clinical utility for inherited eye disorders, including corneal dystrophies (Lenassi et al., 2020). As discussed below, it can assist in establishing a more accurate clinical diagnosis, define inheritance patterns and recurrence risks, inform future reproductive options, and facilitate appropriate referral and management of extraocular features in disorders affecting multiple systems.

Even for experienced cornea specialists, accurately diagnosing corneal dystrophies can remain a challenge due to their rarity, clinical heterogeneity, and the difficulties associated with identifying early and more subtle changes in younger individuals (Weiss et al., 2015). In these cases, genetic testing, in combination with the clinical examination, and family and medical history, can assist with diagnosis. This is illustrated by some of the families in this study who had a reclassification of their clinical diagnosis after review by a cornea specialist and genetic testing. For example, two out of the three families in this cohort with a confirmed molecular diagnosis of macular corneal dystrophies had different initial clinical diagnoses. Similarly, variable expressivity and the absence of crystals in 50% of affected individuals with Schnyder corneal dystrophy can make diagnosis difficult (Weiss, 2009). Our findings support a role for genetic testing for corneal dystrophies to confirm the clinical diagnosis or provide a more accurate diagnosis.

Corneal dystrophies can be isolated or part of multisystemic disorders. In the latter, molecular diagnosis can help identify unrecognised syndromic presentations. In this study, we have identified several occurrences of syndromes associated with corneal dystrophies, including Harboyan syndrome, X-linked ichthyosis, and amyloidosis of the Finnish type. Some had been clinically identified while others were not known to the patient or the clinician. These families may benefit from appropriate referrals to assess the presence of associated systemic features and initiate adequate surveillance or management when necessary.

In families with a single affected individual, the mode of inheritance is often unclear, with counselling to inform on the risk for other relatives or future children depending on the clinical diagnosis. Family history may not be obvious, especially for milder corneal dystrophies that may require eye examination to assess the presence of subtle corneal opacities and whether they are inherited or de novo. However, as discussed above, reaching an accurate clinical diagnosis of corneal dystrophy can be difficult. Genetic testing can assist in establishing a molecular diagnosis and inform on the mode of inheritance in sporadic cases. Patients and their families can then be adequately counselled about the risk to other relatives and benefit from predictive genetic testing to identify presymptomatic individuals. For severe and early-onset corneal dystrophies, couples may choose to access potential reproductive options, such as prenatal diagnosis and preimplantation diagnosis. Finally, corneal dystrophies associated with syndromes may have different modes of inheritance than isolated dystrophies, with a molecular diagnosis allowing more accurate counselling with respect to the risk to relatives and future children.

4.7 | Genetic testing approach to corneal dystrophies

The genetic heterogeneity of corneal dystrophies and the difficulty in establishing an accurate clinical diagnosis supports a role for panel-based genetic testing. In this study, with a combination of candidate gene and exome sequencing, we achieved a diagnostic yield of 70.5%. The epithelial-stromal corneal dystrophies and macular corneal dystrophy had the highest detection rates at 87.5% and 100%, respectively. A significantly higher detection rate was achieved in individuals with a family history of corneal dystrophies (93.8%) than in those with no family history (57.1%). Gene-panel testing has already proved efficient in improving diagnostic yields for several inherited eye diseases including congenital cataracts and inherited retinal dystrophies (Javadiyan et al., 2017; Lenassi et al., 2020; Taylor et al., 2017). Considering the contribution of syndromes associated with corneal dystrophy, testing panels should include their associated genes.

Initiatives such as ClinGen that assess the relevance of gene to disease are key in confirming gene-disease association to develop gene panels for specific conditions (Rehm et al., 2015).

Technologies such as exome sequencing carry some limitations: non-coding variants are not sequenced and copy number variant analysis can be limited. Individuals without a molecular diagnosis may have variants in genes with poor coverage, non-coding regions of the genome, copy number variations not detected by the current methodology or genes yet to be associated with corneal dystrophies. For example, posterior polymorphous corneal dystrophy 1 is caused by variants in the promoter region of *OVOL2* and posterior polymorphous corneal dystrophy 4 has been associated with intronic variants in *GRHL2*, both of which would not be detected by exome sequencing. This might explain the low detection rate for the condition in this study (50.0%). Finally, the detection rate was low in individuals with corneal dystrophies that did not fit the IC3D classification (40.0%). These individuals may carry genetic variation in genes that have not yet been associated with corneal dystrophies or have non-genetic forms of corneal dystrophy.

5 | CONCLUSIONS

A molecular diagnosis was established in over two-thirds of families investigated by candidate gene or exome sequencing, which was comparable to other inherited eye diseases such as congenital cataracts and retinal dystrophies. Our findings highlight the genetic heterogeneity of corneal dystrophies and the clinical utility of genetic testing in reaching an accurate clinical diagnosis. Gene-panel testing is likely to be the most efficient approach to achieve high diagnostic yields for corneal dystrophy due to the genetic heterogeneity of the various entities and the difficulty in establishing accurate clinical diagnoses.

AUTHOR CONTRIBUTIONS

Conceptualization: E.S., O.M.S., J.E.C., R.A.M.; Data curation: E.S., S.M., M.M.H., A.C., H.B., L.S.K., S.E.S., A.W.H., D.A.M., A.G., S.K., J.E.C., R.A.M.; Formal analysis: E.S., O.M.S., A.D., J.B., J.N., J.H., K.P.B., S.K., J.E.C., R.A.M.; Funding acquisition: J.E.C., R.A.M.; Writing – original draft: E.S.; Writing – review & editing: E.S., O.M.S., S.M., M.M.H., A.D., A.C., J.B., J.N., J.H., L.S.K., S.E.S., A.W.H., D.A.M., A.G., K.P.B., S.K., J.E.C, R.A.M.

ACKNOWLEDGMENTS

This project was supported by the Australian National Health and Medical Research Council (NHMRC) Centres of Research Excellence Grant (GNT1116360). Jamie E Craig is an NHMRC Practitioner Fellow (GNT1154824) and Emmanuelle Souzeau has an Early Career Fellowship from the Hospital Research Foundation. The Centre for Eye Research Australia receives Operational Infrastructure Support from the Victorian Government. We thank John Brealey (SA Pathology) for the electron microscopy and Matthew Hussey (SA Pathology) for histology.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Souzeau, E., Siggs, O. M., Mullany, S., Schmidt, J. M., Hassall, M. M., Dubowsky, A., Chappell, A., Breen, J., Bae, H., Nicholl, J., Hadler, J., Kearns, L. S., Staffieri, S. E., Hewitt, A. W., Mackey, D. A., Gupta, A., Burdon, K. P., Klebe, S., Craig, J. E., & Mills, R. A. (2022). Diagnostic yield of candidate genes in an Australian corneal dystrophy cohort. *Molecular Genetics & Genomic Medicine*, *10*, e2023. https://doi.org/10.1002/mgg3.2023