Molecular Vision 2020; 26:613-622 <http://www.molvis.org/molvis/v26/613>
Received 21 October 2019 | Accepted 21 August 2020 | Published 23 August 2020

The impact of the c.5603A>T hypomorphic variant on founder mutation screening of *ABCA4* for Stargardt disease in South Africa

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Purpose: Seven founder mutations in *ABCA4* underlie a large proportion of Stargardt disease in the South African Caucasian population of Afrikaner descent. The Quick 7 assay was locally developed to test for these specific mutations and is available through the National Health Laboratory Service. However, in 2017 it was suggested that one of these mutations, c.2588G>C (p.Gly863Ala), is only pathogenic when present *in cis* with the c.5603A>T (p.Asn1868Ile) hypomorphic variant. Several patients and family members have been screened and have had their results delivered; thus, a retrospective analysis for the presence of c.5603A>T in all resolved *ABCA4* cases was warranted.
Methods: In this study, probands with biallelic mutations in *ABCA4* and all families carrying the c.2588G>C variant were genotyped for c.5603A>T with restriction fragment length polymorphism analysis. Cosegregation analysis was performed to ascertain the phase of causative mutations.
Results: The downgraded c.2588G>C variant was present in 26 families, of whom 24 (92.31%) also carried the hypomorphic variant (*cis* phase confirmation was possible in 12 families). Two families (7.69%) carried the downgraded variant without the hypomorphic variant; however, in these cases the second disease-causing variant had not been identified. These two families remained in research mode; therefore, family follow-up was not immediately required. Additionally, the hypomorphic variant occurred *in cis* with two of the other Quick 7 mutations.
Conclusions: This study adds to the evidence of the pathogenicity downgrade of c.2588G>C, as it results in disease when *in cis* with c.5603A>T in this cohort. This work highlights the value of a close link between research and diagnostic laboratories, in keeping abreast of the functionality of variants. It is recommended that the Quick 7 assay be expanded to include c.5603A>T, and that only the complex c.[2588G>C;5603A>T] allele be reported as pathogenic. Confirmation of *cis* or *trans* configuration of alleles by the inclusion of familial samples is strongly recommended.

Inherited retinal diseases (IRDs) encompass a genetically and clinically heterogeneous group of diseases characterized by vision loss due to the degradation of photoreceptors (rods and cones) and retinal pigment epithelium (RPE) cells. Investigation into IRDs was initiated in South Africa in 1985 through the collaboration of the Division of Human Genetics at the University of Cape Town (UCT) and the national patient support group Retina South Africa [1,2]. Due to this ongoing collaboration, the UCT IRD research project has a large diagnostic aspect, which includes the use of our medical genetic and genetic counseling services for the delivery of results to families upon the identification of disease-causing mutations in affected individuals, and those at risk. Obtaining a molecular diagnosis is highly beneficial as this may confirm or refine a clinical diagnosis, impact family planning, and determine whether a patient is suitable for participation in gene-based clinical trials [2].

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Mutations in *ABCA4* (Gene ID: HGNC ID = HGNC:34; NCBI Gene ID = 24; OMIM 601691) underlie a spectrum of autosomal recessive IRDs exhibiting extensive phenotypic variability which can, in part, be explained by the vast allelic heterogeneity reported for the *ABCA4* gene. To date, more than 1,000 mutations have been identified in the coding regions and splice sites of *ABCA4* [3]. Mutations in *ABCA4* underlie retinitis pigmentosa (RP), cone-rod dystrophy (CRD), and Stargardt disease (STGD) [4]. RP is the most common IRD, affecting one in 3,000 individuals worldwide [5]. This progressive disorder is caused by the degeneration of rod cells, resulting in night blindness and loss of peripheral vision [6]. In contrast, CRD is caused by the initial deterioration of cone cells and subsequently, rod cells, and thus, results in an initial loss of central vision, followed by a progressive loss of peripheral vision [6]. Finally, STGD, the most common macular dystrophy, is caused by cone cell degeneration and results in reduced central vision, while peripheral vision is usually conserved [6,7]. STGD can be characterized as early

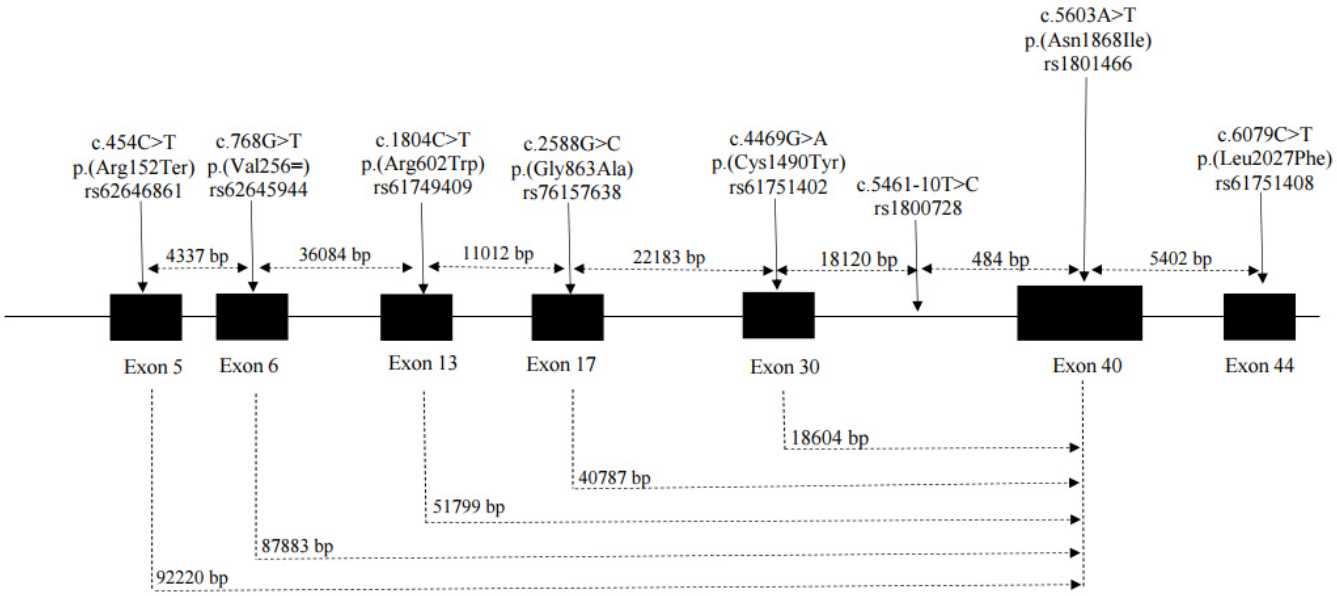

Figure 1. Diagram of the location and base pair distance between the seven common mutations in *ABCA4* (accession sequence NM_000350.3) used in a diagnostic screening test for STGD in South Africa, namely, c.454C>T, c.768G>T, c.1804C>T, c.2588G>C, c.4469G>A, c.5461–10T>C, and c.6079C>T, as well as the variant of interest in this study, c.5603A>T.

onset (≤10 years), classical (11–44 years; sometimes referred to as adult onset), or late onset (>44 years). However, the age cut-off between classical and late onset varies among studies, ranging from 35 to 45 years [8,9]. STGD is fundoscopically characterized by lipofuscin deposits in the RPE appearing as yellow-white flecks in the macula [8,10].

Previous research using single-stranded conformation polymorphism screening [11] and arrayed primer extension (APEX) *ABCA4* microarrays (Asper Biogene, Tartu, Estonia) [6,12,13] identified several founder effects which lead to STGD in the (European derived) South African Caucasian population of Afrikaner ancestry. Seven common mutations were identified, which accounted for about 50% of disease-causing alleles with a carrier frequency of 4.46% (Figure 1) [12]. A locally developed assay, referred to as the Quick 7 assay, was subsequently established to test for these mutations in patients with STGD. This assay is offered for diagnostic screening via the National Health Laboratory Service (NHLS) and resolves about 52% of Afrikaner STGD cases (data not shown). Originally, the seven mutations were genotyped using Snapshot technology [12]. However, the NHLS has transferred to sequencing technology using the Big Dye ® Direct cycle sequencing kit (Thermo Fisher Scientific, Waltham, MA). Although the technology has changed, the basis of the test remains the genotyping of the distinct mutations of interest. In our resource-limited environment, the Quick 7 assay takes advantage of the presence of founder mutations to offer a more affordable genetic testing option, but it is not designed to be comprehensive.

Ideally, a diagnostic test would elucidate the complete genetic landscape of an individual, with coding, non-coding, and structural variant analysis. Understanding of the genetic determinants of *ABCA4*-associated retinopathies has improved in recent years, implicating all of the mentioned variants as causal for disease [3,8,14-24]. Copy number variants (CNVs), which elude detection by sequencing, occur in *ABCA4* but are rare [3,15,17,24,25]. Furthermore, many deep intronic *ABCA4* variants are found to be pathogenic, usually due to altered splicing [14-17,21,22,24-30]. In silico programs cannot always accurately predict the effects on pre-mRNA splicing [23], and the sequence context of intronic variants is vital when assessing splicing. Consequently, midigene assays have been designed for in vitro assessment of suspected splice variants [23]. Finally, certain *ABCA4* variants previously considered benign due to a high minor allele frequency (MAF) in the general population have been reclassified as mild conditional alleles which can cause disease under particular conditions, for example, the c.5603A>T variant [3,29].

The *ABCA4* c.5603A>T variant has a high MAF of about 7% in the non-Finnish European population, and thus, was previously classified as benign [4]. However, in 2017 this variant was found to be associated with STGD and reclassified as a mild conditional allele [3]. This hypomorphic variant is phenotypically expressed only when present *in trans* with a

severe mutation in *ABCA4*. The resulting phenotype is mild, manifesting as classical or late-onset STGD with relative foveal sparing. This variant was found to account for about 80% of late onset cases, and its reclassification as pathogenic resolved >50% of the previously unresolved monoallelic *ABCA4* cases [3].

In addition to the reclassification of c.5603A>T, Zernant et al. proposed that the third most frequent *ABCA4* variant, c.2588G>C, is pathogenic only when present *in cis* with c.5603A>T [3]. This is significant in the South African context as c.2588G>C is one of the common mutations incorporated in the Quick 7 assay. Several patients and family members have already been screened and had results delivered by an ophthalmologist or genetic counselor.

Due to the reclassification of c.5603A>T as pathogenic, all unresolved cases would be screened for c.5603A>T within a large, multicenter collaboration screening the entire *ABCA4* gene [17]. However, due to the recommended reclassification of c.2588G>C to 'no longer pathogenic' as a simplex (i.e., single variant) allele, we set out to investigate the presence of c.5603A>T in all families carrying c.2588G>C. Furthermore, a retrospective analysis for the presence of c.5603A>T in all resolved cases was warranted, to ascertain whether c.5603A>T frequently occurs *in cis* with any of the other Quick 7 mutations. A better understanding of the complex alleles, which occur among the Quick 7 mutations, is vital, as it has diagnostic implications and may be important for additional genotype–phenotype studies [31]. Furthermore, because a lack of familial DNA samples is often a limiting factor in human genetic studies, it is highly beneficial to know the probability of *cis* or *trans* configuration of frequent mutations.

METHODS

Cohort selection: This study was approved by the Human Research Ethics Committee (HREC), UCT (HREC reference number: 226/2010), and informed consent was obtained from each participant during recruitment according to the tenets of the Declaration of Helsinki and ARVO statements of ethical principles for medical research involving human subjects. Previous genotyping results archived in the UCT IRD registry were interrogated to select the cohort for this study. Due to the reclassification of c.5603A>T as pathogenic, a retrospective analysis for the presence of c.5603A>T in all resolved cases (182 families, n = 354 individuals) was warranted. These cases had been previously considered resolved by either SSCP screening [11], Asper Biogene *ABCA4* microarray [6], or the Quick 7 assay [12]. Therefore, the study cohort consisted of one affected proband with two identified mutations in *ABCA4* from each family in diagnostic mode. Furthermore, due to the recommended pathogenicity reclassification of c.2588G>C, the cohort included all families identified as carrying c.2588G>C (26 families, n = 76 individuals), regardless of whether they were unresolved or in diagnostic mode. Control samples for each c.5603A>T genotype (AA, AT and TT) were selected from the IRD registry.

Genomic DNA extractions were performed from blood or saliva samples. Peripheral blood was collected and stored in ethylenediaminetetraacetic acid (EDTA) tubes. DNA extractions were performed immediately, using a salting out method [32]. Alternatively, the buffy coat was removed from the blood sample and frozen, and later processed using the salting out method. Saliva samples were collected using the Oragene® saliva kit (DNA Genotek, Ottawa, Ontario, Canada) and processed according to the manufacturer's instructions. The DNA was re-suspended in 1X Tris-EDTA buffer and stored at -4 ºC or -20 ºC until use. The DNA concentration and relative purity of the samples were assessed using the NanoDrop® ND1000 Spectrophotometer (Thermo Fisher Scientific).

N1868I genotyping: Samples were genotyped for c.5603A>T using either the APEX *ABCA4* microarrays (Asper Biogene; n = 159 individuals) [6] or restriction fragment length polymorphism (RFLP) analysis (n = 271 individuals). WebCutter (accessed 30 April 2018) was used to assess whether c.5603A>T alters a restriction enzyme recognition site. The variant creates an additional recognition site (/aatt), in comparison to the wild-type sequence, recognized by four isoschizomers: *Sse9I*, *Tsp509I*, *TspEI*, and *MluCI*. The following fragments were generated for the various c.5603A>T genotypes: AA: 192 and 24 bp; A/T: 192, 157, 35, and 24 bp; and T/T: 157, 35, and 24 bp.

PCRs were performed in a reaction volume of 25 µl comprised of 1 X GoTaq® flexi buffer (Promega, Madison, WI), 2 mM $MgCl_2$ (Promega), 0.2 mM dNTPs (Kapa Biosystems (Pty) Ltd, Cape Town, South Africa), 10 pmol forward primer for *ABCA4* exon 40 (5′-AGC TGG GGC GGC TGA AG-3′), 10 pmol reverse primer for *ABCA4* exon 40 (5′-TGC CCT GAG CTG CCC AC-3′), 0.5 U GoTaq® G2. DNA Polymerase (Promega), and 100 ng DNA. The thermocycling parameters consisted of an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of successive denaturation, annealing, and extension (i.e., 94 °C for 30 s, 70 °C for 30 s, 72 °C for 40 s), and concluded with a final extension step at 72 °C for 7 min.

Each 15 µl RFLP reaction consisted of 12 µl PCR product, 1 X CutSmart buffer (New England Biolabs, Ipswich, MA), and 5 U Time-Saver™ *MluCI* (New England Biolabs). The

reaction was incubated for 30 min at 37 °C on an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific), according to the manufacturer's instructions. Digest products were visualized on a 2.8% (w/v) agarose gel.

Cosegregation: Cosegregation analysis was performed to assess the inheritance of *ABCA4* variants, i.e., whether variants were inherited *in cis* or *in trans.* All family members of probands identified as carrying c.5603A>T were genotyped with RFLP analysis. Furthermore, due to the high prevalence of c.5603A>T in *ABCA4*-associated retinopathies, it is plausible that family members of individuals not carrying c.5603A>T may carry the variant. Thus, in addition, family members of individuals not carrying c.5603A>T but in whom genotyping may resolve the phase, were genotyped with RFLP analysis. The genotype results for c.5603A>T were overlaid on pedigrees displaying mutation results previously archived in the IRD registry, and the phase of variants was manually assessed. The median age of onset (AOO) and the AOO range were calculated for different genotypes using the values extracted from the registry. However, in cases where the data were not numerical, birth was interpreted as 0 years old, child as 5 years old, and teen as 13 years old.

Sanger sequencing validation: Selected genotyping results of the RFLP analysis were validated with Sanger sequencing (36/247 samples successfully genotyped with RFLP; 14.57%). In this study, 8.9 µl of PCR product was purified with 1 U Thermosensitive Alkaline Phosphatase (FastAp; Thermo Fisher Scientific) and 2 U Exonuclease I (*Exo I*; Thermo Fisher Scientific). The reaction was incubated on an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific) at 37 °C for 60 min, followed by enzyme denaturation at 75 °C for 15 min and finally, at 95 °C for 5 min.

Each sequencing reaction contained 5 µl PCR purified PCR product, 10 pmol of the reverse primer, 1 X BigDye® Terminator v3.1 buffer (Applied Biosystems by Thermo-Fisher Scientific), and 1 X BigDye® Terminator v3.1 Cycle Sequencing mix (Applied Biosystems, Foster City, CA). The sequencing reaction was incubated on an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific) for an initial denaturation step at 96 °C for 5 min, followed by 30 cycles of 96 °C for 30 s, 50 °C for 15 s, and 60 °C for 4 min. The sequencing products were purified via ethanol precipitation.

Following ethanol precipitation, 10 µl of Hi-Di Formamide (Thermo Fisher Scientific) was added to the purified sequencing products which were subsequently denatured at 95 °C for 5 min on an Applied Biosystems 2720 Thermal Cycler (Thermo Fisher Scientific). The sequencing products were separated according to size by capillary electrophoresis using ABI PRISM® 3130xl Genetic Analyzer (Applied Biosystems).

The sequences were captured using Foundation Data Collection software v3.1.1 (Applied Biosystems) and aligned to the *ABCA4* reference sequence (NM_000350.2) obtained from Ensembl Genome Browser (accessed 16 April 2018).

RESULTS

N1868I genotyping: In total, 406 samples (Table 1) were successfully genotyped for c.5603A>T (94.42% of the cohort; 159 individuals were genotyped using the APEX *ABCA4* microarray (Asper Biogene) [6] and 247 individuals genotyped with RFLP analysis). Amplification of the remaining samples failed due to degraded or poor-quality DNA with no alternative sample available.

Cosegregation: Cosegregation analysis was performed to assess the phase of the previously ascertained causal *ABCA4* variants, with the c.5603A>T variant. All c.5603A>T genotyping results were overlaid on pedigrees showing mutation results, as extracted from the registry. Clinical records for members of each family were evaluated, particularly with respect to the AOO of disease, which was the most consistent clinical datum available. The results are summarized in Table 2.

In total, 124 families contained individuals with c.5603A>T. From these individuals, the phase could definitively be determined in 34.68% of the families (n = 43 families). The remaining families had insufficient DNA samples

Table 1. Summary of the c.5603A>T genotype results for families carrying biallelic *ABCA4* mutations, and families carrying c.2588G>C.

c.5603A>T genotype	Probands carrying biallelic *ABCA4* mutations, and family members	Probands carrying c.2588G>C, and family members	Total
AA	146	24	170
AT	147	35	182
TT	43	11	54
n=	336	70	406

from additional family members to reliably determine the phase. In the 43 families, a total of 139 individuals carried c.5603A>T. The simplex variant, i.e., not *in cis* with another variant, occurred in eight unaffected individuals. Of these individuals, six carried the heterozygous simplex allele, one individual was homozygous for c.5603A>T, and one individual carried c.6079C>T *in trans*. In the remaining individuals (n = 131), the variant occurred *in cis* with three of the Quick 7 mutations, specifically c.4469G>A, c.2558G>C, or c.5461–10T>C.

In total, 89 families did not harbor c.5603A>T. The Quick 7 mutations observed in these families included c.454C>T, c.6079C>T, c.768G>T, c.1804C>T, c.4469G>A, and c.2588G>C. However, more than 30 different mutations (other than the Quick 7) were observed in these families. Notably, c.2588G>C occurred without c.5603A>T in two individuals (from two families), and c.4469G>A occurred without c.5603A>T in four individuals (from three families). Examples of selected pedigrees are shown in Figure 2.

DISCUSSION

The recent reclassification of the hypomorphic *ABCA4* variant c.5603A>T from benign to pathogenic has had a knock-on effect. c.2588G>C, one of the seven common mutations

Table 2. A summary of the cosegregation results for individuals carrying c.5603A>T.

Genotypes		Number of unaffected individuals	Number of affected individuals	Median age of onset	Age of onset range
Allele1	Allele2				
c.5603A>T		6	0	N/A	N/A
c.5603A>T	c.5603A>T	1	0	N/A	N/A
c.5603A>T	c.6079C>T	1	0	N/A	N/A
[c.5603A>T; c.4469G>A]		36	4*	12	12
[c.5603A>T; c.4469G>A]	c.5603A>T	4	0	N/A	N/A
[c.5603A>T; c.4469G>A]	[c.5603A>T;c.4469G>A]	0	12	8	7 - 10
[c.5603A>T; c.4469G>A]	c.6079C>T	0	7	11	7 - 15
[c.5603A>T; c.4469G>A]	c.454C>T	0	2	8	7 - 9
[c.5603A>T; c.4469G>A]	c.3304G>T	0	1	13	13
[c.5603A>T;c.4469G>A]	c.1804C>T	0	10	8.5	4 - 12
[c.5603A>T; c.4469G>A]	c.768G>T	0	3	7	5 - 9
[c.5603A>T; c.4469G>A]	c.6107A>G	0	1	18	18
[c.5603A>T; c.4469G>A]	c.4169T>C	0	2	8	8
[c.5603A>T; c.4469G>A]	c.5714+5G>A	0	2	10	9 - 11
[c.5603A>T; c.5461–10T>C]		12	0	N/A	N/A
[c.5603A>T; c.5461–10T>C]	c.5603A>T	1	0	N/A	N/A
[c.5603A>T; c.5461–10T>C]	[c.5603A>T; c.5461–10T>C]	0	4	7	6 - 9
[c.5603A>T; c.5461–10T>C]	c.454C>T	0	2	6.5	5 - 8
[c.5603A>T; c.5461–10T>C]	c.3056C>T	0	2	7.5	6 - 9
[c.5603A>T; c.5461–10T>C]	c.2966T>C	0	1	13	13
[c.5603A>T; c.5461–10T>C]	c.1804C>T	0	1	11	11
[c.2588G>C; c.5603A>T]		7	1*	6	6
[c.2588G>C; c.5603A>T]	c.5603A>T	1	0	N/A	N/A
[c.2588G>C; c.5603A>T]	c.768G>T	0	1	20	20
[c.2588G>C; c.5603A>T]	c.454C>T	0	2	30	30
[c.2588G>C; c.5603A>T]	[c.6282+7G>A>A,c.4319T>C]	0	2	16	5 - 27
[c.5603A>T, c.4469G>A]	[c.5603A>T; c.2588G>C]	0	2	17.5	16 - 19
[c.5603A>T; c.4469G>A]	[c.5603A>T; c.5461–10T>C]	0	8	8	0 - 10

Each individual (n=139) is represented once in the table. * denotes an unresolved case, i.e. second change is unknown.

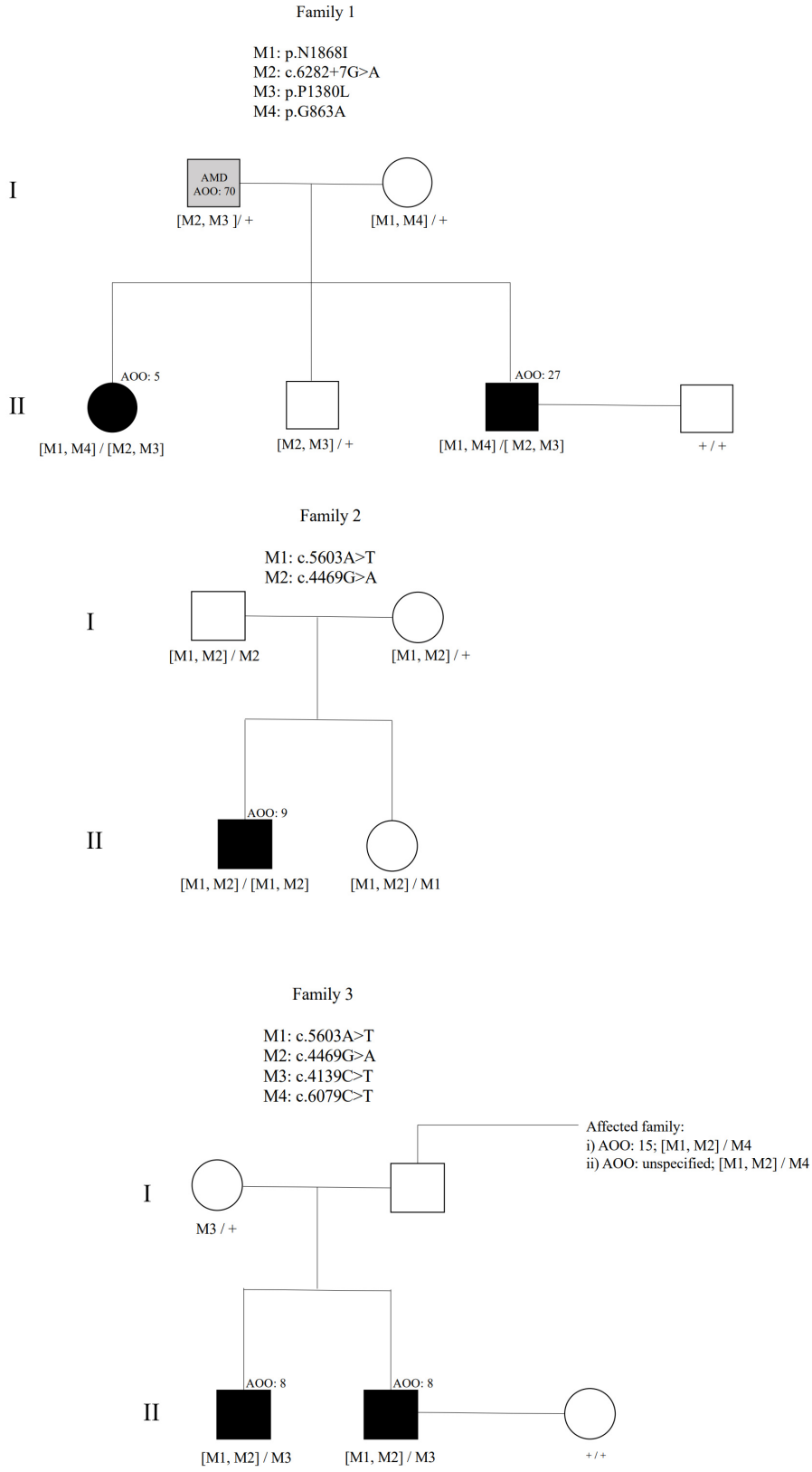

Figure 2. Example pedigrees overlaid with disease, genotype, and AOO information used for cosegregation analysis.

that are targeted for diagnostic screening in South African patients of Afrikaner descent with STGD, is now considered pathogenic only when present *in cis* with c.5603A>T [3]. As c.5603A>T has not been thoroughly investigated in a South African context, it was imperative to assess the presence of c.5603A>T in all families carrying c.2588G>C, and in all resolved cases, for a fuller understanding of the underlying genetic determinants of disease in this population.

The cis and trans configuration of c.5603A>T alleles: Investigation of complex alleles is essential due to the complex genetic landscape of *ABCA4* [17]. To further investigate the complex alleles that occur among the Quick 7 mutations [12], we were particularly interested in the phase of the c.5603A>T variant and the discussed mutations.

Of the 124 families with at least one individual carrying c.5603A>T, cosegregation analysis was possible in 43 families (34.68%), representing 139 individuals. The c.5603A>T variant was present *in cis* with a causal *ABCA4* variant in 131 individuals (94.24%). This high incidence was due to the nature of the study, which focused on families in diagnostic mode, that is, with biallelic mutations identified. The c.5603A>T variant has frequently been reported *in cis* with known mutations in *ABCA4*. This is potentially due to the high prevalence of the variant which occurs in up to 6.6% of the background population and about 20% of *ABCA4*-associated retinopathy cases [3,18]. Seven individuals carried a single allele (c.5603A>T; n = 6) or were homozygous for c.5603A>T (n = 1), and were unaffected family members of resolved STGD cases. This result was expected, as disease was observed only in individuals where c.5603A>T was *in trans* with a severe mutation in *ABCA4*. Finally, one individual carried c.5603A>T *in trans* with c.6079C>T. This individual was unaffected at the time of recruitment. However, further clinical follow-up is recommended as this genotype would be expected to manifest with mild STGD, with a late disease onset.

The c.5603A>T variant occurred *in cis* with c.5461–10T>C, c.4469G>A, and c.2588G>C, which are all Quick 7 mutations. Of the individuals carrying c.5461–10T>C, the c.5603A>T variant was always present *in cis* (100%), that is, never occurring without c.5603A>T. This observation has previously been reported, with no studies to date reporting c.5461–10T>C without c.5603A>T [3,8,17,18]. This is not unexpected, due to the short genomic distance between the two variants (484 bp) resulting in linkage disequilibrium. In the recent *ABCA4* landscape study by Khan et al., this complex allele was the third most common causal allele [17].

Of the individuals carrying c.4469G>A where phase determination was possible, 95% carried the complex allele c.[4469G>A;5603A>T], whereas only 5% carried the simplex allele (c.4469G>A). This particular complex allele has previously been reported by Runhart et al. and Collison et al [8,18]. Khan et al. reported it was the fifth most common causal allele [17], and Zernant et al. reported the complex allele occurring in 100% of c.4469G>A cases [3].

Two founder haplotypes for c.4469G>A in South Africa have previously been reported [11]. In this study, we identified cases of c.4469G>A with and without c.5603A>T, which strongly suggests one ancestral allele carrying c.4469G>A *in cis* with c.5603A>T, and another ancestral allele carrying only c.4469G>A.

The downgraded c.2588G>C variant was present in 26 families, of whom 24 (92.31%) also carried the hypomorphic variant (*cis* phase confirmation was possible in 12 families). In concordance with previous studies, no affected individuals in diagnostic mode in the registry carried the homozygous c.2588G>C allele [3]. The occurrence of the complex allele and the absence of the homozygous c.2588G>C variant in affected individuals were two factors that originally cast doubt on the pathogenicity of c.2588G>C [3]. The downgrade of c.2588G>C to a “conditional allele,” pathogenic only when *in cis* with c.5603A>T [3], was of concern for molecular diagnostic services in South Africa. This variant was included in the targeted screen for common mutations in *ABCA4* locally, and many patients had already received their diagnostic results. As 24 out of 26 families were identified as being likely to carry the complex allele (*cis* phase confirmation possible in only 12 families owing to a lack of familial samples), they still have two biallelic, pathogenic mutations in *ABCA4* identified. The remaining two families, who did not carry c.5603A>T, had not previously been contacted, as their second causative mutation had not yet been discovered; that is, they were still in research mode. For these two families, the simplex (and therefore, benign) nature of c.2588G>C was noted on the UCT IRD registry, to ensure they are fully resolved before they are given the results.

Clinical implications: In the present study, the simplex variant allele (c.5603A>T) was detected, albeit in unaffected members of resolved families. Thus, c.5603A>T can be expected to be observed *in trans* with a severe mutation in *ABCA4* in the South African population. This combination of mutations is reported to result in late-onset disease with relative foveal sparing [3]. All unresolved STGD cases in the IRD registry are currently under investigation, and any cases carrying that particular combination of variants will be assessed. However, due to the nature of the present study, which focused on the resolved cases (with other biallelic mutations), the hallmark c.5603A>T-associated phenotype did not occur. In total, for

the 139 individuals carrying c.5603A>T for which cosegregation was possible, the median AOO was 9 years, ranging from birth to 30 years. The comparatively early median AOO exhibited probably results from the severity of the variants *in cis* with c.5603A>T, and the mutation(s) *in trans* with the complex alleles.

A gender bias has previously been reported for *ABCA4*-associated retinopathies, in which men are often unaffected or display non-penetrance due to the 1.5-fold higher *ABCA4* expression in men compared to women [8,33]. By this hypothesis, men with a highly expressed mild mutation in *ABCA4* may be less susceptible to disease compared to women with the same mutation. Notably, c.5603A>T is reportedly overrepresented in female patients compared to male patients. Furthermore, asymptomatic men have been identified within families carrying c.5603A>T *in trans* with another mutation [8]. These men represent cases of either non-penetrance or delayed age of onset and generate scope for further research into genetic modifiers of disease. A gender bias was not observed in this study between the affected individuals carrying c.5603A>T. Of the 157 affected individuals carrying c.5603A>T, 49.68% were female, and 50.32% were male, with an average AOO of 11.67 years and 10.03 years, respectively. This was to be expected, as the study focused on individuals with moderate-strength causal mutations in complex alleles with the hypomorphic variant. However, an individual example of gender bias was observed. In family 1 of Figure 2, a male family member (II:III) and a female family member (II:I) presented with an AOO of 27 years and 5 years, respectively. These individuals carried identical underlying genetic mutations. Therefore, the varying AOO may be attributed to the higher expression of *ABCA4* in men, or to other undetermined genetic or environmental modifiers.

The complex allele c.[4469G>A;5603A>T] occurred *in trans* with c.5603A>T in four individuals who were unaffected at the time of recruitment. This genotype would be expected to produce a mild form of the disease, resulting in late onset and foveal sparing. The individuals were two women, unaffected at ages 46 and 8 years, and two men, unaffected at ages 38 and 55 years. The unaffected status of these individuals may either be due to their recruitment before the onset of disease, or non-penetrance of c.5603A>T previously reported [8]. Figure 2 shows an example: In family 2, the male family member (I:I) was unaffected at the age of 38 years, and his daughter (II:II) was unaffected at the age of 8 years. Further clinical follow-up of these individuals is recommended.

The severity of missense mutations can be determined by their presence *in trans* with c.5603A>T; that is, it acts as a litmus test [3]. This is observed in family 3 of Figure 2, where a slight delay in the AOO was observed in different mutation branches of this family. All individuals carried the complex allele c.[4469G>A;5603A>T]. However, the one branch was *in trans* with c.6079C>T, resulting in an AOO of 15 years, while the other branch was *in trans* with c.4139C>T, resulting in an AOO of 8 years. From this, it may be inferred that c.4139C>T may be a more severe mutation than c.6079C>T.

Limitations: The lack of familial DNA samples was a limitation in this study, as is often the case in human genetic studies. The phase of mutations could be conclusively determined in only 43 families carrying c.5603A>T (34.68%). In the remaining families, samples from additional family members are required for cosegregation to be performed. Missing clinical data were an additional limitation of the study. The most consistent clinical information captured was the AOO; however, this was self-reported. Subclinical cases can occur in IRDs, where photoreceptor degeneration has commenced, but the patient may not have noticed any change in vision. Thus, the AOO used in this study may be a late estimate.

Conclusions and further work: A clinical reexamination is recommended for all individuals identified in this study (n = 4) carrying the complex allele [c.5603A>T;c.4469G>A] *in trans* with the simplex allele (c.5603A>T; family 2 in Figure 2), and the individual carrying c.5603A>T *in trans* with c.6079C>T (n = 1). Although these individuals were unaffected at the time of recruitment, it is possible that they may have subsequently manifested with late onset, or mild disease, or may be non-penetrant. Thus, there will be a diagnostic impact on these individuals as their status may change from carrier to affected, with consequent implications for other family members.

This study adds to the evidence supporting the downgrade of c.2588G>C from a pathogenic variant to a mild conditional allele, as it results in disease only when present *in cis* with c.5603A>T in the patients in this cohort. From this finding, and due to the high prevalence of the complex allele c.[2588G>C;5603A>T] in the Afrikaner population of South Africa, it is strongly recommended that the Quick 7 assay be expanded to include c.5603A>T, and to report only the complex allele [c.5603A>T;c.4469G>A] as pathogenic. Finally, given the complex genetic landscape of *ABCA4* recently reported [17], we amended the translational protocols so that in the future families will be placed in diagnostic mode only when the phase of the likely causal variants has been confirmed.

ACKNOWLEDGMENTS

The authors thank Retina South Africa and The Medical Research Council of South Africa for their contribution to funding for this research. We are most grateful to the ophthalmologists and families involved in this study.

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Articles are provided courtesy of Emory University and the Zhongshan Ophthalmic Center, Sun Yat-sen University, P.R. China. The print version of this article was created on 23 August 2020. This reflects all typographical corrections and errata to the article through that date. Details of any changes may be found in the online version of the article.