Variants in the *ABCA4* gene in a Brazilian population with Stargardt disease

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Purpose: The aim of this study was to analyze and report pathogenic variants in the *ABCA4* gene in Brazilian patients with a clinical diagnosis of Stargardt disease.
Methods: This retrospective study evaluated variants in the *ABCA4* gene in Brazilian patients with Stargardt disease. The patients' visual acuity and age of symptom onset were obtained from previous medical records. The patients were classified according to the autofluorescence patterns.
Results: Fifty patients aged between 10 and 65 years from 44 families were included in the study. Among these cases, the mean age of symptom onset was 14 years (range, 5–40 years). *ABCA4* gene sequencing was conclusive in 40 patients (80%), negative in two patients (4%), and inconclusive in eight patients (16%). Four families carried homozygous pathogenic variants. Segregation analysis results were available for 23 families. One novel variant was found: p.Ala2084Pro. The most frequent pathogenic variant in this group was p.Arg602Trp (12/100 alleles). Based on the phenotypic characteristics assessed with fundus autofluorescence imaging, 12 patients were classified as having type I phenotype, 16 as having type II, and 18 patients as having type III. The cases classified as type III phenotype included patients who were homozygous for the p.Asn96Asp and p.Arg2030* variants. One patient with a type I phenotype carried the homozygous intronic variant c.3862+1G>A.
Conclusions: Next-generation sequencing was effective for the molecular diagnosis of genetic diseases and specifically allowed a conclusive diagnosis in 80% (40/50) of the patients. As the *ABCA4* gene does not show a preferential region for pathogenic variants, the diagnosis of Stargardt disease depends on broader analysis of the gene. The most common pathogenic variants in the *ABCA4* gene described in the literature were also found in these Brazilian patients. Although some genotype–phenotype correlations were found, more studies regarding the progression of Stargardt disease will help increase our understanding of the pathogenicity of these gene variants.

Stargardt disease (STGD, OMIM 248200) is an autosomal recessive retinal degenerative disease caused by defects in the *ABCA4* gene (OMIM 601691). The disease causes functional and structural retinal damage, and the main characteristic is central vision impairment. Stargardt disease exhibits great clinical variability and may diffusely alter the retina in the most severe cases. Since the first descriptions of this disease, diagnosis has been based on phenotypic characteristics [1,2]. Because the gene responsible for this disease has been identified, clinical diagnosis of these patients may be confirmed genetically with gene sequencing [3].

Decreased central vision is the initial symptom of Stargardt disease; it classically occurs between the first and second decades of life and progressively worsens with age

[1,2]. This progressive decline in bilateral central vision is related to retinal atrophy of the macular region due to loss of the external segments of photoreceptors and retinal pigment epithelium (RPE) cells [4,5]. Lipofuscin deposits in the RPE and photoreceptors (called flecks) are key markers for a diagnosis of Stargardt disease. In addition, these clinical signs, which are clearly identified with a fundus autofluorescence examination, determine the phenotypic classification of patients with this disease [6]. For example, areas of RPE atrophy are hypoautofluorescent on retinal autofluorescence examination, whereas the hyperautofluorescence points identified in this examination are related to lipofuscin accumulation in RPE cells [6]. Lipofuscin accumulation in the RPE layer masks choroidal fluorescence on fluorescein angiography, an effect is known as the dark or silent choroid sign [7].

The *ABCA4* gene is exclusively expressed in photoreceptors, cones and rods [8,9]. The loss or decreased function of the protein encoded by the *ABCA4* gene causes deposition of the main component of lipofuscin

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[N-retinylidene-N-retinylethanolamine (A2-E)] in the RPE and the formation of retinal flecks. This accumulation slows adaptation to darkness [10,11]. The presence of lipofuscin in RPE cells causes oxidative damage to these cells, which become apoptotic. Moreover, loss of the RPE layer leads to secondary photoreceptor death, resulting in reduced vision [11-13].

ABCA4 is a polymorphic gene with low conservation due to the high frequency of allelic variation in the gene. It presents many benign variants, with a frequency higher than 1% in the population, as are hundreds of pathogenic variants [14]. The majority of variants are missense mutations, followed by protein-truncating variants [15]. Complex alleles and intronic variants have also been described for the *ABCA4* gene [14-17]. The presence of pathogenic variants of this gene is the most common cause of retinal diseases among children and young adults and is primarily related to autosomal recessive Stargardt disease [14,18]. Involvement of *ABCA4* has also been reported in cases of cone–rod dystrophy and autosomal recessive retinitis pigmentosa [19,20].

The phenotypic characteristics of each case demonstrate particularities corresponding to the combination of pathogenic variants in the *ABCA4* gene in compound heterozygous and homozygous individuals. The phenotypic characteristics of certain variants, including p.Gly1961Glu and the complex allele p.[Leu541Pro; Ala1038Val], have been established [21,22]. Overall, the clinical outcome depends on the severity of the combined alleles [15].

Little is known to date about the pathogenic variants in the Brazilian population with Stargardt disease. Thus, the aim of this study was to analyze and describe pathogenic variants in the *ABCA4* gene in Brazilian patients with a clinical diagnosis of Stargardt disease.

METHODS

This retrospective study evaluated variants in the *ABCA4* gene in Brazilian patients with a Stargardt disease diagnosis. Cases were selected based on a review of 254 genetic tests for retinal dystrophy performed between January 2009 and January 2017 at the Federal University of São Paulo (UNIFESP) and the Ocular Genetics Institute (IGO), São Paulo, Brazil. All patients signed informed consent. The study was approved by the Research Ethics Committee of UNIFESP (Protocol No. 6159), Brazil, and was conducted in accordance with the ethical standards of the 1964 Declaration of Helsinki and the Association for Research in Vision and Ophthalmology (ARVO) statement on human subjects, and its subsequent amendments.

From a pool of 254 retinal dystrophy tests, patients with Stargardt for whom the *ABCA4* gene was sequenced using next-generation sequencing (NGS) were included in the study. Patients with a clinical diagnosis of Stargardt disease and conclusive molecular testing regarding other Stargardt genes were excluded. Among the study group, there were patients with clinically Stargardt with a conclusive *ABCA4* test, an inconclusive test with one variant, and patients with no variant.

The Ion PGM™ System (Life Technologies, Carlsbad, CA) and MiSeq by Illumina (Illumina, San Diego, CA) platforms were used for the molecular diagnosis of the cases. The *ABCA4* gene was included in gene panels related to Stargardt disease or macular dystrophies. Only coding regions and immediately flanking intron sequences were examined. Changes in the promoter region, deep intronic regions, or other non-coding regions of the gene were not evaluated. Among the patients, only those with an initial clinical diagnosis of Stargardt disease were selected for inclusion in this study.

Segregation results available in medical records were analyzed and included in the study. Sanger sequencing was used to identify in relatives pathogenic variants found in probands.

Gene sequencing data collected from medical records were compared with variants available in the following databases: Human Gene Mutation Database (HGMD) [23], ClinVar [24], 1000 Genomes Project [25], Exome Aggregation Consortium (ExAC) [26], and Leiden Open Variation Database (LOVD) [27]. The following programs were used to predict the pathogenicity of new variants: CADD (deleterious threshold: 20) [28], PolyPhen-2 [29], MutationTaster [30], and Sorting Intolerant from Tolerant (SIFT) [31]. The nucleotide numbering is based on the reference sequence NM_000350, with the A of the initiation codon (ATG) representing number 1.

Data on the patients' visual acuity and age of symptom onset were collected from medical records. Complementary examinations, including retinography (RG), fundus autofluorescence imaging (FAF), and optical coherence tomography (OCT), were reassessed to confirm the clinical diagnosis of Stargardt disease.

The patients were classified according to signs found in the autofluorescence test (Heidelberg Retina Angiograph, HRA 2, Heidelberg Engineering, Heidelberg, Germany), as described by Fujinami et al. [6]. This classification separates patients into the following three types: type I (an area of foveal hypoautofluorescence surrounded by a retina with a

TABLE 1. FUNDUS AUTOFLUORESCENCE TYPE I PATIENTS. PATHOGENIC VARIANTS IN THE ABCA4 GENE AND CLINICAL DATA.

Pt	S	Pathogenic variants				Visual acuity	Age of onset/ data collect/ disease dura-tion (years)
		Allele 1		Allele 2			
		Nucleotide change	Protein change	Nucleotide change	Protein change	RE LE	
1•	F	c.4926C>G c.5044_5058del15‡	p.Ser1642Arg p.Val1682_Val1686del‡	c.5882G>A	p.Gly1961Glu	20/200 20/200	12/30/18
9	M	c.6112C>T	p.Arg2038Trp	c.6320G>A	p.Arg2107His	20/200 20/150	11/35/24
14	M	not detected		not detected		FC 2m 20/40	30/42/12
31•	M	c.1622T>C c.4328G>A	p.Leu541Pro p.Arg1443His	c.3386G>T	p.Arg1129Leu	N/A N/A	N/A/23/ N/A
49•	M	c.5282C>G c.6316C>T	p.Pro1761Arg p.Arg2106Cys	c.2345G>A	p.Trp782*	20/400 20/400	15/25/10
43•	F	c.5882G>A	p.Gly1961Glu	c.66G>T	p.Lys22Asn	20/60 20/60	19/20/1
51•	M	c.1622T>C c.3113C>T	p.Leu541Pro p.Ala1038Val	c.5882G>A	p.Gly1961Glu	20/30 20/30	10/11/1
28	M	c.70C>T	p.Arg24Cys	c.1804C>T	p.Arg602Trp	20/50 20/60	16/26/10
33§•	M	c.3862+1G>A†	p.?	c.3862+1G>A†	p.?	20/200 20/200	10/10/0
35•	M	c.6079C>T	p.Leu2027Phe	**c.6250G>C**	**p.Ala2084Pro**	20/25 20/25	20/20/0
48•	F	c.5882 G>A c.3113 C>T	p.Gly1961Glu p.Ala1038Val	c.1364T>A	p.Leu455Gln	20/60 20/150	32/32/0
36•	F	c.1804C>T	p.Arg602Trp	not detected		20/25 20/25	7/28/21

Pt, Patient; S, Sex; N/A, not available; FC, Finger counting. Novel variant. • Segregation analysis performed †Homozygous variants ‡c.5044_del15bp, nonframeshift deletion (c.5044_5058delGTTGCCATCTGCGTG). § Consanguinity Families: Siblings: 41(Table 4) and 49. Mother 44 (Table 3) and daughter 43. Mother 45 (Table 3) and daughter 36.

homogeneous appearance, with or without foci of high or low autofluorescent signals), type II (an area of foveal hypo-fluorescence surrounded by an area with a heterogeneous appearance and hyperautofluorescent and hypoautofluores-cent points that extend to the temporal arcades, giving the retina a reticulate appearance), and type III (extensive areas of hypoautofluorescence in the posterior pole and a hetero-geneous appearance of the remaining retinal areas, with hyperautofluorescent and hypoautofluorescent points) [6].

RESULTS

A total of 50 patients from 44 families (including four sibling pairs and two mother-daughter pairs) aged between 10 and 65 years were included in the study. Three families had a history of consanguinity (siblings 4 and 6 and patients 33 and 40).

Table 1, Table 2, Table 3, Table 4 shows the molecular analysis results and clinical characteristics of the Stargardt disease cases, including the age of symptom onset (range, 5–40 years; mean, 14 years) and visual acuity, which ranged from 20/40 to counting fingers at 20 cm during the examination.

All patients included showed changes in pigmentation or macular atrophy on retinography examination. Retinal flecks were found in almost all cases. OCT revealed focal areas of photoreceptor layer discontinuity corresponding to macular atrophy (Figure 1). The patients were classified into three types of phenotypic characteristics according to the fundus autofluorescence examination results (Table 1, Table 2, Table 3 and Figure 2) [6]. Twelve patients were classified as type I (Table 1), 16 patients as type II (Table 2), and 18 patients as type III (Table 3). Records of the fundus autofluorescence

examination were lacking for four patients (Table 4), who, therefore, were not classified.

The *ABCA4* gene sequencing results were conclusive for 40 patients (80%) but negative for patients 8 and 14 (4%). An additional eight patients (7, 11, 16, 21, 26, 32, 36, and 39) carried only one pathogenic variant in the *ABCA4* gene, rendering their molecular test inconclusive (16%). Patients 8, 14, 7, 11, 16, 21, and 39 were evaluated using a small Stargardt disease panel that included the *ABCA4* (ID:

24, OMIM: 601691), *ELOVL4* (ID: 6785, OMIM: 605512), and *PROM1* (ID: 8842, OMIM: 604365) genes, although no pathogenic variants in those other genes were found. Patients 26 and 32 were assessed for the *ABCA4, ELOVL4, PROM1, BEST1* (ID: 7439, OMIM: 607854), and *PRPH2* (ID: 5961, OMIM: 179605) genes and patient 36 for the *ABCA4, ELOVL4, PROM1, BEST1, PRPH2, CDH3* (ID: 1001, OMIM: 114021), *EFEMP1* (ID: 2202, OMIM: 601548), *IMPG1* (ID: 3617, OMIM: 602870), *IMPG2* (ID:50939, OMIM: 607056)

TABLE 2. FUNDUS AUTOFLUORESCENCE TYPE II PATIENTS. PATHOGENIC VARIANTS IN THE ABCA4 GENE AND CLINICAL DATA.

Pt	S	Pathogenic variants				Visual acuity	Age of onset/ data collect/ disease dura-tion (years)
		Allele 1		Allele 2		RE LE	
		Nucleotide change	Protein change	Nucleotide change	Protein change		
3•	F	c.4926C>G c.5044_5058del15‡	p.Ser1642Arg p.Val1682_Val1686del‡	c.2791G>A	p.Val931Met	20/150 20/150	14/37/23
5	F	c.3898C>T	p.Arg1300*	c.1804C>T	p.Arg602Trp	20/200 20/200	7/17/10
8	F	not detected		not detected		20/400 20/400	32/44/12
11	M	c.6079C>T	p.Leu2027Phe	not detected		FC1m 20/200	12/24/12
12•	M	c.4328G>A c.1622T>C	p.Leu541Pro p.Arg1443His	c.2743G>A	p.Asp915Asn	20/200 20/200	8/13/5
16	M	c.4720G>T	p.Glu1574*	not detected		20/150 20/200	6/38/32
18	M	c.634C>T	p.Arg212Cys	c.1804C>T	p.Arg602Trp	20/400 20/400	6/31/25
19	M	c.634C>T	p.Arg212Cys	c.1804C>T	p.Arg602Trp	FC1.5m FC1.5m	11/26/15
21	M	c.3386G>T	p.Arg1129Leu	not detected		20/150 20/150	16/32/16
22•	M	c.4926C>G c.5044_5058del15‡	p.Ser1642Arg p.Val1682_Val1686del‡	c.4340A>T	p.Glu1447Val	20/200 FC2m	8/10/2
29	F	c.223T>G	p.Cys75Gly	c.6088C>T	p.Arg2030*	N/A N/A	8/16/8
32	M	c.455G>A	p.Arg152Gln	not detected		20/30 FC10cm	36/39/3
46	F	c.5461–10T>C	p.[Thr1821Valfs*13, Thr1821Aspfs*6]	c.1804C>T	p.Arg602Trp	20/200 20/200	9/10/1
47•	F	c.1622T>C c.4328G>A	p.Leu541Pro p.Arg1443His	c.286A>G	p.Asn96Asp	20/400 20/400	7/26/19
50•	M	c.1804C>T	p.Arg602Trp	c.5381C>A	p.Ala1794Asp	20/400 20/400	15/31/16
26	F	c.5714+5G>A	p.?	not detected		20/80 20/80	9/14/5

Pt, Patient; S, Sex; N/A, not available; FC, Finger counting.• Segregation analysis performed ‡c.5044_del15bp, nonframeshift deletion (c.5044_5058delGTTGCCATCTGCGTG).Families: Siblings: 18 and 19.

TABLE 3. FUNDUS AUTOFLUORESCENCE TYPE III PATIENTS. PATHOGENIC VARIANTS IN THE ABCA4 GENE AND CLINICAL DATA.

| Pt | S | Pathogenic variants | | | | Visual acuity | Age of onset/ data collect/ disease dura-tion (years) |
| | | Allele 1 | | Allele 2 | | | |
		Nucleotide change	Protein change	Nucleotide change	Protein change	RE LE	
4§	F	c.286A>G	p.Asn96Asp[†]	c.286A>G	p.Asn96Asp[†]	20/400 20/400	23/39/16
6§	M	c.286A>G	p.Asn96Asp[†]	c.286A>G	p.Asn96Asp[†]	FC2m FC2m	14/47/33
7	M	c.3862+1G>A	p.?	not detected		FC20cm FC20cm	16/63/47
10•	F	c.4926C>G c.5044_5058del15[‡]	p.Ser1642Arg p.Val1682_Val1686del[‡]	c.3056C>T	p.Thr1019Met	FC2m FC1m	5/26/21
13•	M	c.4926C>G c.5044_5058del15[‡]	p.Ser1642Arg p.Val1682_Val1686del[‡]	c.1804C>T	p.Arg602Trp	FC50cm FC2m	8/26/18
15	F	c.5714+5G>A	p.?	c.6005+1G>T	p.?	FC20cm FC10cm	12/40/28
17•	F	c.1622T>C c.3113C>T	p.Leu541Pro p.Ala1038Val	c.3329–2A>T	p.?	HM FC10cm	7/59/52
20•	M	c.1622T>C c.3113C>T	p.Leu541Pro p.Ala1038Val	c.3329–2A>T	p.?	HM HM	8/65/57
23•	F	c.1622T>C c.4328G>A	p.Leu541Pro p.Arg1443His	c.1804C>T	p.Arg602Trp	FC1.5m FC1.5m	7/31/24
24	M	c.4457C>T	p.Pro1486Leu	c.1804C>T	p.Arg602Trp	FC1.5m FC1.5m	15/46/31
44•	F	c.4926C>G c.5044_5058del15[‡]	p.Ser1642Arg p.Val1682_Val1686del[‡]	c.66G>T	p.Lys22Asn	N/A N/A	N/A
30•	F	c.6088C>T	p.Arg2030* [†]	c.6088C>T	p.Arg2030* [†]	FC1.5m 20/400	7/38/31
37	F	c.4003_4004delCC	p.Pro1335Argfs*86	c.658C>T	p.Arg220Cys	20/30 20/30	28/31/3
39	F	c.1648G>A	p.Gly550Arg	not detected		FC1.5m FC1.5m	8/36/28
42•	M	c.2894 A>G	p.Asn965Ser	c.5381C>A	p.Ala1794Asp	20/400 20/400	17/38/21
25	M	c.5044_5058del15[‡]	p.Val1682_Val1686del[‡]	c.1804C>T	p.Arg602Trp	20/400 20/400	7/26/19
34•	M	c.4926C>G c.5044_5058del15[‡]	p.Ser1642Arg p.Val1682_Val1686del[‡]	c.1622T>C c.4328G>A	p.Leu541Pro p.Arg1443His	N/A N/A	7/33/26
45•	F	c.1804C>T	p.Arg602Trp	c.3386G>T	p.Arg1129Leu	20/400 20/400	18/61/43

Pt, Patient; S, Sex; N/A, not available; HM, Hand movement; FC, Finger counting.• Segregation analysis performed †Homozygous variants. ‡c.5044_del15bp, nonframeshift deletion (c.5044_5058delGTTGCCATCTGCGTG). § Consanguinity Families: Siblings:4 and 6; 17 and 20. Mother 44 and daughter 43 (Table 1). Mother 45 and daughter 36 (Table 1).

TABLE 4. PATHOGENIC VARIANTS IN THE ABCA4 GENE AND CLINICAL DATA OF
PATIENTS WITH NO FUNDUS AUTOFLUORESCENCE CLASSIFICATION.

| | | Pathogenic variants | | | | Visual acuity | Age of onset/ data collect/ |
| | | Allele 1 | | Allele 2 | | | disease duration (years) |
Pt	S	Nucleotide change	Protein change	Nucleotide change	Protein change	RE LE	
40§•	M	c.1622T>C	p.Leu541Pro†	c.1622T>C	p.Leu541Pro†	N/A	N/A/35/N/A
		c.4328G>A	p.Arg1443His†	c.4328G>A	p.Arg1443His†	N/A	
41•	F	c.5282C>G	p.Pro1761Arg	c.2345G>A	p.Trp782*	20/200	N/A/27/N/A
		c.6316C>T	p.Arg2106Cys			20/200	
27Ω	F	c.868C>T	p.Arg290Trp	c.5882G>A	p.Gly1961Glu	20/400	40/61/21
		c.2690C>T	p.Thr897Ile			20/400	
38•	M	c.32T>C	p. Leu11Pro	c.3113C>T	p. Ala1038Val	20/400	20/21/1
						20/400	

Pt, Patient; S, Sex; N/A, not available • Segregation analysis performed †Homozygous variants. § Consanguinity Families: Siblings: 41 and 49 (Table 1). ΩPatient 27 present 3 patogenic variants, but without segregation analisys it was impossible to determinate which of them are combined in cis.

and *TIMP3* (ID: 7078, OMIM: 188826) genes. No pathogenic variants were found in the other genes.

In the group of 40 conclusive patients, five (12.5%) had homozygous pathogenic variants in the *ABCA4* gene, with 35 being compound heterozygous variants (85.5%); segregation analysis data were available for 26 patients (65%) [32]. Segregation analysis confirmed complex alleles in 20 patients (50% of 40 patients), all of whom showed three pathogenic variants; confirmation that the third variation was in trans rendered these cases conclusive for molecular diagnosis. Although patient 27 showed three pathogenic variants, segregation analysis data were not available for this patient.

The following six complex alleles were found: p.[Leu541Pro; Arg1443His]; p.[Leu541Pro; Ala1038Val]; p.[Ser1642Arg; Val1682_Val1686del]; p.[Pro1761Arg; Arg2106Cys] and p.[Ala1038Val; Gly1961Glu] p.[Ala1038Val; Met669Ile]. All of these alleles were confirmed with segregation analysis to be in cis.

One novel variant, p.Ala2084Pro (Table 5), not present in genetic variant (HGMD, ClinVar and LOVD) or population (1000 Genomes and ExAC) databases was found. Pathogenicity prediction software (CADD, SIFT, MutationTaster, and PolyPhen-2) classified the variant p.Ala2084Pro as disease-causing. It was found in one allele of patient 35 and was considered pathogenic to conclude the genotype test. In addition, three variants that had previously been described without pathogenic classification or with conflicting pathogenicity classifications (p.Met669Ile, p.Arg152Gln, and p.Thr897Ile) were analyzed, as outlined in Table 5 [33-36].

Among the four families (patients 4 and 6 (siblings), 30, 33, and 40) with homozygous variants (Table 1, Table 3, Table 4) only patient 30 denied a history of consanguinity. The most frequent variant in this population was p.Arg602Trp (12/100 alleles). It was present in two cases with phenotype type I, five with type II, and five with type III.

Other frequent variants among patients with Stargardt disease, such as p.Gly1961Glu, p.[Leu541Pro; Ala1038Val], and c.5461–10T>C, were also found in the study population [15,37]. The variants identified in this study are distributed throughout the *ABCA4* gene, including intronic and exonic regions (Table 6, Table 7), and no preferential region for the occurrence of the variants was observed.

DISCUSSION

The *ABCA4* gene is highly polymorphic, and there are a large number of benign variants in addition to the more than 900 pathogenic variants that have been described to date [14,15,17]. The allelic heterogeneity and distribution of pathogenic variants along the 50 exons and introns of this gene demonstrate the complexity of the encoded protein and its role in Stargardt disease (Table 6, Table 7) [38]. Moreover, the large number of variants complicates predictions of disease-causing mutations [39]. Another characteristic of the *ABCA4* gene is the presence of pathogenic variants in cis (Table 6) [32,40]. For this reason, segregation analysis is crucial for clarifying the family origin of each pathogenic variant in compound heterozygotes and for reaching a conclusive molecular diagnosis.
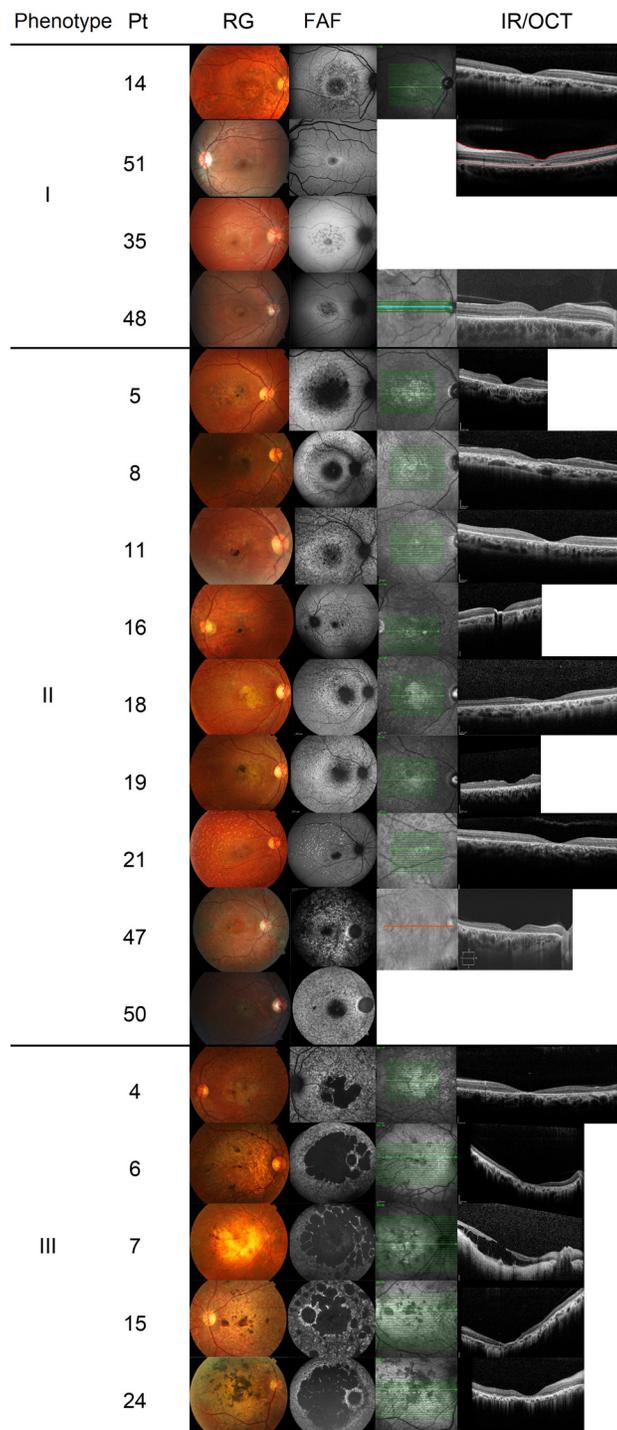
Figure 1. Type I FAF phenotype: patients 14, 51, 35, and 48. Type II FAF phenotype: patients 5, 8, 11, 16, 18, 19, 21, 47, and 50. Type III FAF phenotype: patients 4, 6, 7, 15, and 24. Negative cases at molecular testing: patients 14 and 8. Inconclusive cases at molecular testing: patients 11, 16, 21, and 7. Patient, Pt; retinography, RG; fundus autofluorescence, FAF; infrared imaging, IR; optical coherence tomography, OCT.

Phenotypic severity is directly linked to the protein damage caused by a pathogenic variant [12,15,22], and clinical heterogeneity can be explained by different combinations of pathogenic variants in the *ABCA4* gene [40,41]. Therefore, knowledge of variant pathogenicity may affect disease prognosis and genetic counseling in each case [15]. Some studies have addressed the presence of homozygous mutations in the *ABCA4* gene to assess the effect of a pathogenic variant alone [6,15].

Among the four families with homozygous pathogenic variants in this study, three could be classified based on their phenotypic characteristics. Patients 4 and 6, who were
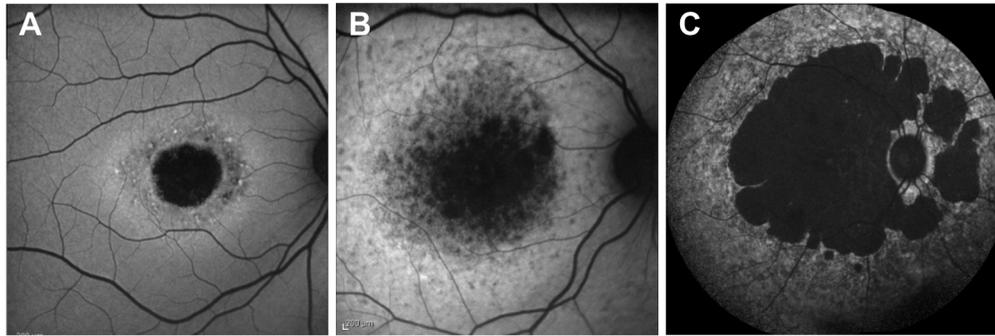
Figure 2. Fundus autofluorescence pictures of Stargardt cases. **A**: Patient 9 with the type I phenotype. **B**: Patient 5 with the type II phenotype. **C**: Patient 6 with the type III phenotype.

siblings, carried the homozygous variant p.Asn96Asp and were classified as having the type III phenotype 16 and 33 years, respectively, after symptom onset. Despite denying family consanguinity, patient 30 carried the homozygous pathogenic variant p.Arg2030* and was also classified with the type III phenotype at 38 years of age. These clinical characteristics suggest that those changes are associated with a worse disease prognosis. Conversely, the phenotype of patient 33, with the homozygous intronic variant c.3862+1G>A, at diagnosis at 10 years of age was type I. The pathogenic variant c.3862+1G>A causes a splicing defect, and this intronic variant has been found in cases of cone–rod degeneration and retinitis pigmentosa in addition to cases of Stargardt disease [42,43]. Follow-up of the phenotypic progression of patient 33 will indicate the prognosis for this variant.

The pathogenic variant most commonly found in this group was p.Arg602Trp, which was present in 12 patients (12 alleles). Wiszniewski et al. described the missense p.Arg602Trp as being responsible for mislocalization of the ABCA4 protein, resulting in the absence of physiologic protein function [21]. These authors further suggested that this missense variant is a severe variant due to the early age of symptom onset when associated with another mislocalization or nonsense variant [21]. Symptoms in patient 5, who presented p.Arg1300* and p.Arg602Trp, started at age 7, with phenotype II showing after 10 years (Table 2). However, the results suggest that the severity of the disease does not depend exclusively on the pathogenic effect of p.Arg602Trp. Patients with this variant exhibited all three phenotypes, demonstrating that the combination of pathogenic variant effects in both alleles is important for determining the phenotype (or

TABLE 5. NOVEL VARIANT AND VARIANTS WITH UNCERTAIN SIGNIFICANCE.				
Patient	**35**	**38**	**32**	**27**
Nucleotide change	c.6250G>C	c.2007G>C	c.455G>A	c.2690C>T
Protein change	p.Ala2084Pro	p.Met669Ile	p.Arg152Gln	p.Thr897Ile
dbSNP	N/A	N/A	rs62646862	rs61749440
ClinVar	N/A	N/A	Conflicting	Conflicting
CADD score/pathogenicity	34/ Deleterious	23.9/ Deleterious	21.5/ Deleterious	23.6/ Deleterious
MutationTaster score/ pathogenicity	1/ Disease-causing	1/ Disease-causing	0.775/ Disease-causing	0.989/ Disease-causing
SIFT score/pathogenicity	0.019/ Deleterious	0.189/ Tolerated	0.124/ Tolerated	0.188/ Tolerated
PolyPhen-2 HumVar score/ pathogenicity	1.000/ Damage	0.413/ Benign	0.015/ Benign	0.994/ Damage
1000 genomes	N/A	N/A	0,00159744	0,00159744
ExAC	N/A	N/A	0,0024	0,0011
References	**Novel variant**	35	33 and 34	32

N/A, Not available

the Stargardt disease severity). This dependence is illustrated in the following cases. Patient 46 presented the first symptoms at age 9, and after only 1 year, her phenotype was type II (Table 2). She carried p.Arg602Trp and c.5461–10T>C variants, and the latter is described as a severe pathogenic variant [37] because it leads to abnormal mRNA and ABCA4 protein truncation. In addition, patients homozygous for this variant have a severe phenotype. In contrast, patient 28 presented two missense variants (p.Arg24Cys and p.Arg602Trp). His first symptoms were at age 16, and after 10 years, his phenotype was type I (Table 1). Although the age of onset of the disease may be related to its severity, the natural history of the disease may better reflect the pathogenicity of variants.

The variant p.Gly1961Glu is associated with milder disease and causes late onset of visual symptoms, with a phenotype that does not evolve to type III [44]. This characteristic was also observed in patients 1, 43, 48, and 51, who carried this variant and showed symptoms after 10 years of age. All patients were classified as type I based on the fundus autofluorescence examinations, and patient 1 maintained these phenotypic characteristics after 18 years of the disease (Table 1). The phenotypic characteristics of these four patients corroborate the hypothesis that variant p.Gly1961Glu causes milder disease.

Variants c.5714+5G>A and c.6005+1G>T were present in patient 15. According to Burke et al., the latter is likely to completely abolish splicing and to cause a frameshift that results in severe protein dysfunction [45]. Conversely, variant c.5714+5G>A is associated with a mild pathogenic effect and a later median age of onset [15,46,47]; nonetheless, Cremers et al. proposed that this variant has a moderately severe effect

[19]. Patient 15 was classified as the type III phenotype at 28 years after symptom onset (Table 3), suggesting a severe effect of those variants.

Analysis of the relationship between the genetic variants and the phenotype of each patient showed that sibling patients had the same mutations and phenotypic classifications. Conversely, the two mother-and-daughter families showed an autosomal dominant inheritance pattern, which suggests that another gene is responsible for their Stargardt disease, even though molecular testing confirmed Stargardt disease linked to the *ABCA4* gene. Because of different mutation combinations, the mother and daughter exhibited different phenotypes. The high frequency of variants in *ABCA4* may have led to the identification of three different pathogenic variants in the same family.

This study reports pathogenic variants in the *ABCA4* gene in Brazilian patients with a clinical diagnosis of Stargardt disease. Thus far, little is known about the genetic characteristics of the Brazilian population with retinal dystrophy. The presence of variants previously described and with a founder effect known for ethnic group-specific populations [48] confirms the miscegenation of Brazilians, including p.Arg1129Leu of Spanish origin [42,49] and p.[Leu541Pro; Ala1038Val] and p.Gly1961Glu of German origin [47,50].

Six complex alleles were found in this study (Table 6). These complex alleles were present in 20 patients distributed among all three phenotypes (Table 1, Table 2, Table 3) and no phenotype–genotype correlation was observed. Although the complex allele p.[Leu541Pro; Ala1038Val] has reportedly high frequency, the allele was present in only three patients in this study [50]. Conversely, the variant p.Leu541Pro formed

TABLE 6. DISTRIBUTION OF PATHOGENIC VARIANTS IN THE *ABCA4* GENE.

Complex alleles

Nucleotide Change	Protein Change	Location	Allele†
c.4926C>G	p.Ser1642Arg	35	7
c.5044_5058del15‡	p.Val1682_Val1686del	36	
c.1622T>C	p.Leu541Pro	12	7
c.4328G>A	p.Arg1443His	29	
c.5282C>G	p.Pro1761Arg	37	2
c.6316C>T	p.Arg2106Cys	46	
c.5882 G>A	p.Gly1961Glu	42	1
c.3113 C>T	p.Ala1038Val	21	
c.1622T>C	p.Leu541Pro	12	3
c.3113C>T	p.Ala1038Val	21	
c.3113C>T	p.Ala1038Val	21	1

† Number of alleles that each complex allele has apear.

TABLE 7. DISTRIBUTION OF PATHOGENIC VARIANTS IN THE *ABCA4* GENE.			
Single alleles			
Nucleotide Change	**Protein Change**	**Location**	**Allele†**
c.32T>C	p.Leu11Pro	1	1
c.66G>T	p.Lys22Asn	1	2
c.70C>T	p.Arg24Cys	2	1
c.223T>G	p.Cys75Gly	3	1
c.286A>G	p.Asn96Asp	3	5
c.455G>A	p.Arg152Gln	5	1
c.634C>T	p.Arg212Cys	6	2
c.658C>T	p.Arg220Cys	6	1
c.1364 T>A	p.Leu455Gln	11	1
c.1648G>A	p.Gly550Arg	12	1
c.1804C>T	p.Arg602Trp	13	12
c.2345G>A	p.Trp782*	15	2
c.2743G>A	p.Asp915Asn	19	1
c.2791G>A	p.Val931Met	19	1
c.2894 A>G	p.Asn965Ser	19	1
c.3056C>T	p.Thr1019Met	21	1
c.3329–2A>T	p.?	Intron 22	2
c.3386G>T	p.Arg1129Leu	23	3
c.3862+1G>A	p.?	Intron 26	3
c.3898C>T	p.Arg1300*	27	1
c.4003_4004delCC	p.Pro1335Argfs*86	27	1
c.4340A>T	p.Glu1447Val	29	1
c.4457C>T	p.Pro1486Leu	30	1
c.4720G>T	p.Glu1574*	33	1
c.5044_5058del15	p.Val1682_Val1686del	36	1
c.5381C>A	p.Ala1794Asp	38	2
c.5461–10T>C	p.[Thr1821Valfs*13, Thr1821Aspfs*6]	Intron 38	1
c.5714+5G>A	p.?	Intron 40	2
c.6005+1G>T	p.?	Intron 43	1
c.6079C>T	p.Leu2027Phe	44	2
c.6088C>T	p.Arg2030*	44	3
c.6112C>T	p.Arg2038Trp	44	1
c.6250G>C	**p.Ala2084Pro**	45	1
c.6320G>A	p.Arg2107His	46	1

Novel vatiant.† Number of alleles that each variant has apear.Patient 27 was excluded of this table due to the absence of segregation analysis. FAF, Fundus autofluorescence; Pt, Patient; N/A, not available; HM, Hand movement; FC, Finger counting. Novel variant. †Homozygous variants. ‡c.5044_del15bp, non-frameshift deletion (c.5044_5058delGTTGCCATCTGCGTG). Families: Siblings: 4 and 6; 17 and 20; 18 and 19; 41 and 49. Mother 44 and daughter 43. Mother 45 and daughter 36. § Consanguinity •Patient 27 presented with 3 pathogenic variants, but without segregation analysis it was impossible to determinate which of them are combined in cis.

a complex allele with p.Arg1443His in patients 12, 23, 31, 34, 40, and 47 [6]. This high frequency of complex alleles (20 of 40 conclusive cases) and the possibility of different variant combinations in cis emphasize the importance of segregation analysis for a correct molecular diagnosis [32].

NGS has been found to be effective for the molecular diagnosis of patients with Stargardt disease [51] and was used for molecular diagnosis in all cases reported in this study. These patients were assessed using a gene panel for Stargardt disease or macular dystrophies. In this study, the rate of conclusive molecular diagnosis was 80% (40/50), higher than the rate reported in other studies with patients with Stargardt disease using NGS (ranging from 50% to 60%) [51-54].

The negative and inconclusive cases may be explained by mutations in non-sequenced regions, such as intronic, untranslated, and regulatory regions, and/or a lack of knowledge of the pathogenicity of new variants in the *ABCA4* gene. In addition, other genes not analyzed or not yet related to the disease might cause these phenotypes [14]. New studies are evaluating other regions of the *ABCA4* gene that were previously considered irrelevant for protein formation, including deep-intronic regions [52,53], in an effort to validate variants as disease causing and to improve molecular diagnosis [14,55,56]. Genes other than *ABCA4* have been evaluated to clarify inconclusive cases [35,57-59].

The importance of a conclusive molecular diagnosis is directly related to new treatments for retinal dystrophies [15]. Identifying pathogenic variants responsible for disease enables the inclusion of patients in clinical trials and renders them eligible for future commercial therapies [14,60].

CONCLUSION: NGS is effective for the molecular diagnosis of genetic diseases, and this approach specifically allowed a conclusive diagnosis in 80% (40/50) of the patients. The *ABCA4* gene does not show a preferential region for pathogenic variants; thus, the diagnosis of Stargardt disease depends on broader analysis of the gene. The most common pathogenic variants in *ABCA4* described in the literature were also found in the Brazilian patients in this study. Some genotype–phenotype correlations were found, but more studies on the progression of Stargardt disease will help us understand the pathogenicity of variants in genes.

ACKNOWLEDGMENTS

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