

# Novel Complex *ABCA4* Alleles in Brazilian Patients With Stargardt Disease: Genotype–Phenotype Correlation

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**PURPOSE.** To analyze the presence of complex alleles of the *ABCA4* gene in Brazilian patients with Stargardt disease and to assess the correlation with clinical features.

**METHODS.** This was an observational cross-sectional study. Patients with a diagnosis of Stargardt disease who presented three pathogenic variants of the *ABCA4* gene or who had variants previously described as complex alleles were included. The relatives of these probands were evaluated in the segregation analysis. The patients were evaluated based on age at symptom onset and visual acuity, and the clinical characteristics were classified according to the findings observed on autofluorescence examination.

**RESULTS.** Among the 47 families analyzed, approximately 30% (14/47) presented complex alleles. The segregation analysis in 14 families with cases of Stargardt disease identified three novel complex alleles and one previously described complex allele. The known complex allele p.[Leu541Pro; Ala1038Val] was identified in two families. The novel complex alleles identified were p.[Leu541Pro; Arg1443His] in five families, p.[Ser1642Arg; Val1682\_Val1686-del] in seven families, and p.[Pro1761Arg; Arg2106Cys] in one family. Furthermore, four new variants (p.Lys22Asn, p.Asp915Asn, p.Glu1447Val, and p.Pro1761Arg) were identified in the second allele of the *ABCA4* gene.

**CONCLUSIONS.** Segregation analysis is important in order to confirm the molecular diagnosis of patients with Stargardt disease, given the frequency of complex alleles in the *ABCA4* gene. The various pathogenic variation combinations observed in this study were associated with different phenotypes.

**Keywords:** Stargardt disease, macular degeneration/genetics, retinal dystrophy, eye diseases, hereditary, *ABCA4* protein, human, complex allele

The autosomal recessive form of Stargardt disease (STGD, Online Mendelian Inheritance in Man [OMIM] 248200) is mainly associated with pathogenic variants of the *ABCA4* gene (OMIM \*601691). This gene is expressed in the outer segments of the retinal photoreceptors, cones, and rods. *ABCA4* encodes a transport protein of the disc membrane in these cells.<sup>1–3</sup> A functional deficiency of this protein leads to the development of several retinal degenerative diseases. The autosomal recessive form manifests as Stargardt disease, fundus flavimaculatus, cone and rod dystrophy, and retinitis pigmentosa.<sup>3–5</sup> This variety of clinical presentation is probably related to the innumerable possible combinations of pathogenic variants of this gene.<sup>5–7</sup>

The *ABCA4* gene has 50 exons, and a large number of variants of this gene have been described.<sup>8</sup> Because *ABCA4* is a polymorphic gene, benign missense variations are often identified in molecular tests,<sup>9–11</sup> hindering the identification of true disease-causing variants.<sup>5,6,9</sup> Cases of homozygous pathogenic variants, “compound heterozygotes,” and complex alleles have been described in studies on the molecular

diagnosis of Stargardt disease.<sup>4,7,12–14</sup> The combined inheritance of two pathogenic variants in the same allele constitutes a so-called complex allele. Complex alleles are common in the *ABCA4* gene and may suggest a common ancestor within an ethnic subgroup in a given population.<sup>4,6</sup>

Stargardt disease manifests mainly during childhood with progressive central vision loss. The presence of flecks on retinal examination is an important diagnostic finding.<sup>15</sup> As the disease progresses, the retinal pigment epithelium and the photoreceptors in the macular region atrophy. In advanced stages, the disease may also affect the periphery of the retina.<sup>14,16</sup> Autofluorescence examination may help in the diagnosis of this condition by revealing the presence of flecks and atrophic areas.<sup>16</sup>

The objective of this study was to clarify the existing haplotypes in Brazilian patients with Stargardt disease who present more than two pathogenic variants on genetic sequencing of the *ABCA4* gene. The clinical features were analysed and correlated with the genetic results.



## MATERIALS AND METHODS

This observational cross-sectional study analysed genetic variants of the *ABCA4* gene in patients with a diagnosis of Stargardt disease who were enrolled between January 2009 and January 2017. All patients signed an informed consent form. The study was approved by the Research Ethics Committee of the Federal University of São Paulo–UNIFESP (No. 6159), Brazil, and was conducted in accordance with the ethical standards of the 1964 Declaration of Helsinki and its subsequent amendments.

The patients were evaluated based on age at symptom onset and visual acuity, and the clinical characteristics were classified according to the findings observed on autofluorescence examination (Heidelberg Retina Angiograph, HRA 2; Heidelberg Engineering, Heidelberg, Germany). Based on autofluorescence findings, patients were classified into one of three subtypes: type I, characterized by an area of foveal hypofluorescence surrounded by a homogeneous-appearing retina; type II, characterized by an area of foveal hypofluorescence surrounded by an area with a more heterogeneous appearance and with foci of hyperautofluorescence and hypoautofluorescence that extend to the temporal arches, giving the retina a reticulate appearance; and type III, characterized by extensive areas of hypoautofluorescence in the posterior pole and a heterogeneous appearance of the remaining retinal areas with foci of hyperautofluorescence and hypoautofluorescence.<sup>14</sup> Relatives of the probands who also had a diagnosis of Stargardt disease underwent the same clinical evaluation whenever possible.

Next-generation sequencing was used to sequence the *ABCA4* gene in the probands. An Ion Torrent barcoded library was set up following the manufacturer's protocol for the Ion AmpliSeq Library Kit 2.0 V (Life Technologies, Carlsbad, CA, USA). Specific primers were designed using the AmpliSeq Designer software (Life Technologies). Amplified libraries were subjected to emulsion PCR using the Ion OneTouch system (Life Technologies). Sequencing was performed using the Ion 314 Chip v2 system and an Ion PGM Sequencer as described by Veronez et al.<sup>17</sup>

Analysis was performed using the Ion Torrent Suite 5.0 platform with CoverageAnalysis (5.0.4.0) and VariantCaller (5.0.5) plug-ins and optimized parameters for a custom panel. The reference genome used was hg19. VCF files were annotated using Annovar<sup>18</sup> in order to filter the variants. The variant filtering criteria included population distribution (variants with more than 1% frequency in the ExAC<sup>19</sup> or 1000 Genomes Project<sup>20</sup> databases were discarded), function (synonymous variants were discarded), and previous non-pathogenic description (variants previously described as nonpathogenic in the ClinVar and HGMD databases 1.35 2016July06 and 2016.2 versions, respectively).<sup>21,22</sup>

Probands who presented three pathogenic variants in the *ABCA4* gene or variants previously described as complex alleles were included in this study. The relatives of these probands were evaluated in a segregation analysis.

For the segregation analysis of the relatives, only regions containing pathogenic variants identified in the probands were sequenced using Sanger technology. Genomic DNA was amplified by PCR with the TopTaq Master Mix Kit (Qiagen, Melbourne, Vic, Australia) and sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) according to the manufacturer's recommendations, using an ABI Prism 3500xl Genetic Analyzer sequencer (Life Technologies) as described by Turaca et al.<sup>23</sup>

The databases used as references for the analysis of new genetic variations were the Human Gene Mutation Database (HGMD),<sup>22</sup> the population analysis of 1000 Genomes Project,<sup>20</sup>

and ExAC.<sup>19</sup> The software programs used for predictive analysis of pathogenicity were CADD (available in the public domain at <http://cadd.gs.washington.edu/>)<sup>24</sup> PolyPhen-2 (available in the public domain at <http://genetics.bwh.harvard.edu/pph2/>),<sup>25</sup> MutationTaster (available in the public domain at <http://www.mutationtaster.org/>),<sup>26</sup> and SIFT (available in the public domain at <http://sift.jcvi.org/>).<sup>27</sup>

## RESULTS

Fourteen families suspected of having complex alleles were identified in a cohort of 47 families who previously underwent sequencing of the *ABCA4* gene (approximately 30%). The probands from these 14 families presented three genetic variants, including pathogenic or unknown variants, or presented variants previously reported as segregating in cis. A total of 55 individuals from these 14 families were included in the segregation analysis.

From the initial group of 47 families, 5 families were identified as carrying the complex allele p.[Leu541Pro; Arg1443His] (5/47, 10.6%). The known complex allele p.[Leu541Pro; Ala1038Val] was identified in two families (2/47, 4.2%). The complex allele p.[Ser1642Arg; Val1682\_Val1686del] was found in seven families (7/47, 14.9%), and p.[Pro1761Arg; Arg2106Cys] was identified in one family (1/47, 2.1%). The molecular analysis results and the clinical characteristics of the Stargardt patients are presented in Table 1 and Figure 1.

### Segregation Analysis

The segregation analysis results are shown in the pedigrees in Figure 2. Individuals BII2, DI1, DII2, and JII1 were described by the probands as having Stargardt disease but were not clinically evaluated.

Family D had a history of consanguinity. The matriarch (DI1) and the brother (DII2), who were also reported to have Stargardt disease, were tested only for segregation. For this reason, the pathogenic variant in the second allele was not found.

Individual JII1 was tested only for segregation of the variants found in proband JII2 in this family; therefore, a second variant was not found. The complex allele p.[Ser1624Arg; Val1682\_Val1686del] was also found in these individuals (JII1 and JII2) as well as in JI3.

Individuals KII2 and NII2, who have Stargardt disease, underwent only segregation analysis, and the molecular diagnosis concluded that they carry the same pathogenic variants as their siblings.

### Pathogenicity of Novel Variants

Four novel variants were identified in this study (p.Lys22Asn, p.Asp915Asn, p.Glu1447Val, and p.Pro1761Arg), and their effects were previously unknown (Table 2). Only the p.Asp915Asn variant had been previously described as a rare variant in the ExAC database. None of the other three novel variants were found in the population databases used as references.<sup>19,20</sup>

The p.Lys22Asn variant (family L) was classified as deleterious by three predictive software programs: CADD (score 24), MutationTaster (score 1.000), and PolyPhen-2 (score 0.701). In contrast, it was classified as tolerated by SIFT (score 0.124).

The pathogenicity of variant p.Asp915Asn (family A) was rated as tolerated/benign by SIFT (score 0.273) and PolyPhen-2 (score 0.001). However, it was classified as probably deleteri-

TABLE 1. Genotyping of ABCA4 Gene and Clinical Characteristics of Patients With Stargardt Disease

Family	Patient	Sex	Allele 1	Allele 2	Age at Onset, Years	Age at Examination, Years	Duration of Disease, Years	RE Visual Acuity	LE Visual Acuity	Classification in Autofluorescence
Fam A	II 1	M	p.[Leu541Pro; Arg1443His]	<b>p.Asp915Asn</b>	8	13	20/200	20/200+2	II	
Fam B	II 1	F	p.[Leu541Pro; Arg1443His]	p.Arg602Trp	7	31	CF 1.5m	CF 1.5m	III	
Fam C	III 1	M	p.[Leu541Pro; Arg1443His]	p.Arg1129Leu	N/A	23	N/A	N/A	I	
Fam D	II 1	M	p.[Leu541Pro; Arg1443His]	p.[Leu541Pro; Arg1443His]	N/A	35	N/A	N/A	N/A	
Fam E	II 1	F	p.[Leu541Pro; Ala1038Val]	c.3329-2A>T	7	59	HM	CF 10cm	III	
Fam E	II 3	M	p.[Leu541Pro; Ala1038Val]	c.3329-2A>T	8	65	HM	HM	III	
Fam F	II 1	M	p.[Leu541Pro; Ala1038Val]	p.Gly1961Glu	10	11	20/30	20/30	I	
Fam G	II 1	F	p.[Ser1642Arg; Val1682_Val1686del]	p.Val931Met	14	37	20/150+2	20/150+2	II	
Fam H	II 1	M	p.[Ser1642Arg; Val1682_Val1686del]	<b>p.Glu1447Val</b>	8	10	20/200	CF 2m	II	
Fam I	II 1	M	p.[Ser1642Arg; Val1682_Val1686del]	p.Arg602Trp	8	26	CF 50cm	CF 2m	III	
Fam J	II 2	F	p.[Ser1642Arg; Val1682_Val1686del]	p.Thr1019Met	5	26	CF 2m	CF 1m	III	
Fam K	II 2	M	p.[Ser1642Arg; Val1682_Val1686del]	p.Gly1961Glu	32	32	20/80	20/80	I	
Fam K	II 3	F	p.[Ser1642Arg; Val1682_Val1686del]	p.Gly1961Glu	12	30	20/200	20/200	I	
Fam L	I 1	F	p.[Ser1642Arg; Val1682_Val1686del]	<b>p.Lys22Ans</b>	N/A	N/A	N/A	N/A	III	
Fam L	II 2	F	p.Gly1901Glu	<b>p.Lys22Ans</b>	19	20	20/60	20/60	I	
Fam M	II 1	M	p.[Ser1642Arg; Val1682_Val1686del]	p.[Leu541Pro; Arg1443His]	N/A	33	N/A	N/A	N/A	
Fam N	II 1	M	p.[Pro1761Arg; Arg2106Cys]	p.Trp782*	15	25	20/400	20/400	I	
Fam N	II 2	F	p.[Pro1761Arg; Arg2106Cys]	p.Trp782*	N/A	26	20/200	20/200	N/A	

RE, right eye; LE, left eye; CF, count fingers; HM, hand movement; N/A, not available. Bold indicates novel variants.



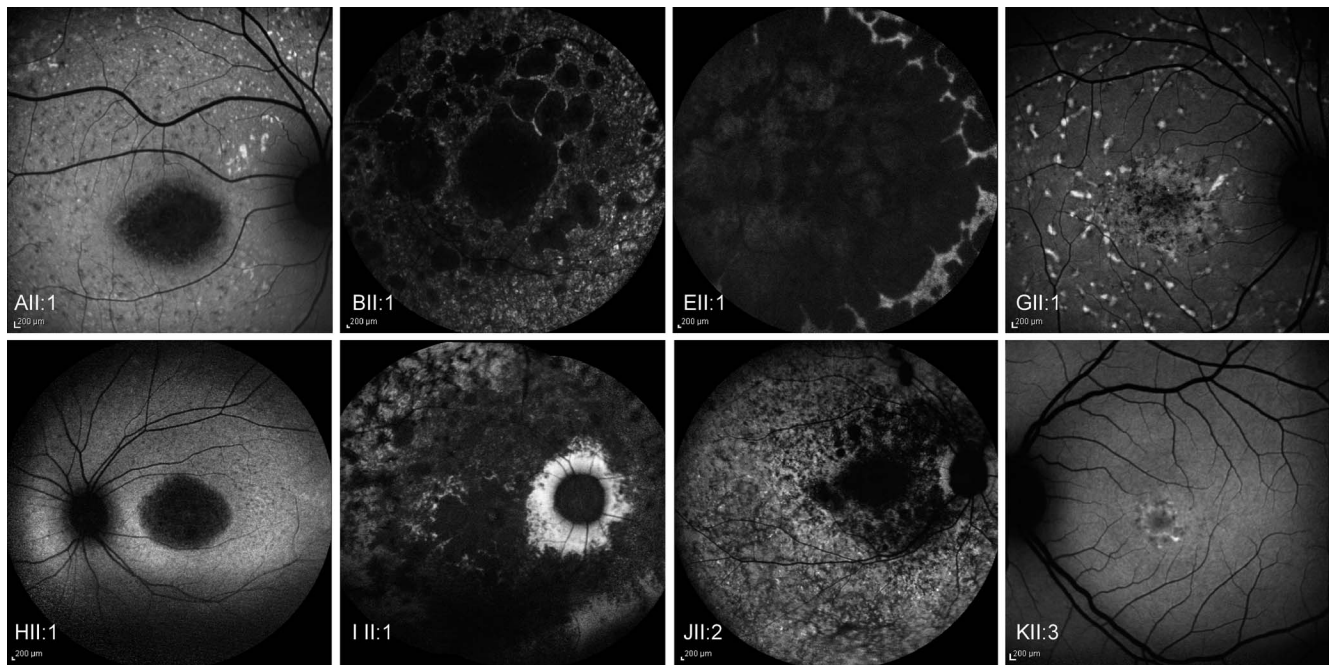


FIGURE 1. Autofluorescence images of probands with Stargardt disease. Type I: KII3; Type II: AII1, GII1, and HII1; Type III: BII1, EII1, I II1, and JII2.

ous by CADD (score 19.92) and as a disease-causing variant by MutationTaster (score 0.914063).

Likewise, because the pathogenicity of the p.Glu1447Val variant (family H) was previously unknown, this variant was evaluated in silico using the same four software tools. It was identified as benign by PolyPhen-2 (score 0.021), SIFT (score 0.062), and MutationTaster (score 0.949215) but as a deleterious variant by CADD (score 23.1).

The p.Pro1761Arg variant (family N) was consistently identified as a disease-causing variant, with scores of 27 in CADD (deleterious), 1.000 in MutationTaster (disease-causing), 0.001 in SIFT (deleterious), and 0.926 in Polyphen-2 (probably damaging).

## DISCUSSION

Since the identification of the *ABCA4* gene, several variants (both pathogenic and benign) have been described and correlated with Stargardt disease.<sup>8</sup> Because this gene is polymorphic and not highly conserved, categorizing new *ABCA4* variants as pathogenic is likely to be important for molecular diagnosis in some patients.<sup>10,11,28,29</sup> Currently, the identification of complex alleles in *ABCA4* gene and their correlation with specific populations have enabled changes in the interpretation of molecular analyses of this gene.<sup>30</sup> In this study of Brazilian patients with Stargardt disease, four pairs of pathogenic variants were observed to cosegregate in the *ABCA4* gene.

### p.Leu541Pro Associated With p.Arg1443His

The pathogenic variants p.Leu541Pro and p.Arg1443His were found in families A, B, C, D, and M and were inherited as complex alleles. Variant p.Arg1443His was described as pathogenic by Rivera et al.<sup>13</sup> In the same study, Rivera et al.<sup>13</sup> described variant p.Arg1443Val in a patient who also presented the pathogenic variants p.Leu541Pro and p.Ala1038Val but who did not undergo segregation analysis.

This case shows the importance of segregation in the presence of three pathogenic variants in the same gene. Cosegregation of the pathogenic variants p.Leu541Pro and p.Arg1443His was identified for the first time in the present study and was present in five families.

### p.Leu541Pro Associated With p.Ala1038Val

The complex allele p.[Leu541Pro; Ala1038Val] was previously identified in several families and had a founder effect associated with German ancestry.<sup>4,7,12,13,31–34</sup> Szczyńska et al.<sup>30</sup> also found a high frequency of this complex allele in the Polish population. This combination was found in families E and F in this study.

The paternal grandmother in family E was of Polish origin. The segregation analysis of this family suggested a paternal origin for this set of pathogenic variants (Fig. 2). The segregation analysis of family F identified complex allele p.[Leu541Pro; Ala1038Val] in the maternal lineage of Italian descent.

These variants previously described as disease-causing may present separately.<sup>4,31,35</sup> Variant p.Ala1038Val is frequent among patients with Stargardt disease and may be the only pathogenic variant in one allele.<sup>13</sup>

### p.Ser1642Arg Associated With p.Val1682\_Val1686del

The probands of families G, H, I, J, K, and M and case II of family L were heterozygous for the pathogenic variant p.Ser1642Arg and the p.Val1682\_Val1686del (c.5044\_5058del15) deletion. The repetition of these two variants in these patients, who were not related, raised the possibility of their cotransmission. This suspicion was confirmed by the results of the segregation analysis. Bertelsen et al.<sup>36</sup> found the pathogenic variants p.Ser1642Arg and p.Val1682\_Val1686del in the same patient, but they did not describe the segregation pattern. Riveiro-Alvarez et al.<sup>37</sup> described cases in which the p.Val1682\_Val1686del deletion was associated with other genetic variants.

TABLE 2. Novel Variants

Novel Variants	HGMD	CADD Score/ Pathogenicity	MutationTaster Score/ Pathogenicity	SIFT Score/ Pathogenicity	PolyPhen-2 HumVar Score/ Pathogenicity	1000 Genomes	ExAC
c.66G>T	N/A	24/deleterious	1.000/disease-causing	0.124/tolerated	0.701/possibly damaging	N/A	N/A
c.2743G>A	N/A	19.92/probably deleterious	0.914063/disease-causing	0.273/tolerated	0.001/benign	N/A	0.00000826
c.4340A>T	N/A	23.1/deleterious	0.949215/polymorphism	0.062/tolerated	0.021/benign	N/A	N/A
c.5282C>G	N/A	27/deleterious	1.000/disease-causing	0.001/deleterious	0.926/probably damaging	N/A	N/A

N/A, not available.

### p.Pro1761Arg Associated With p.Arg2106Cys

The novel variant p.Pro1761Arg, which was predicted to cause disease by CADD, SIFT, Polyphen-2, and MutationTaster,<sup>24-27</sup> was found in family N. However, its frequency in the population has not been described.<sup>19,20</sup> This variant was found to cosegregate with p.Arg2106Cys in family N. The combination of the cosegregating variants p.Pro1761Arg and p.Arg2106Cys was confirmed in the segregation analysis for this family. Variant p.Arg2106Cys is known to be pathogenic and is described in the reference databases.<sup>8,38</sup>

Family N was the only family carrying the complex allele p.[Pro1761Arg; Arg2106Cys] in this study. Nevertheless, the identification of this variant may encourage investigators to search for this potential combination in other families.

### Challenges in the Interpretation of Molecular Diagnosis

The identification of the complex allele p.[Arg1443His; Leu541Pro] in family A required a determination of pathogenicity in the other allele to make conclusions about the molecular diagnosis. The novel variant p.Asp915Asn was identified in the other allele in this family. As this is a novel variant with a frequency that has not been described in the population and is considered pathogenic by the CADD and MutationTaster prediction software programs, its presence may be related to the presentation of Stargardt disease in this case.

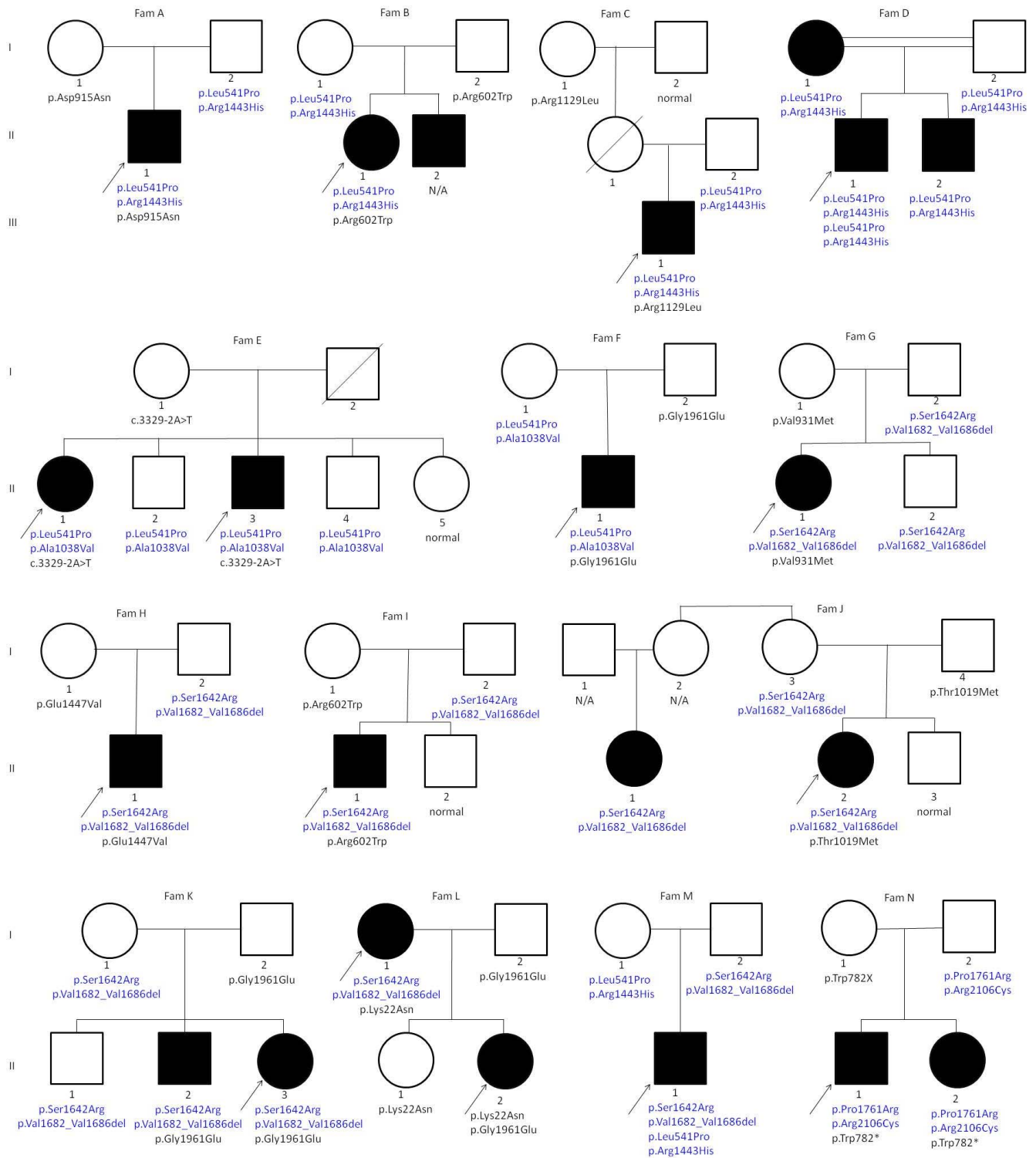
Similarly, in family H, the presence of the complex allele p.[Ser1642Arg; Val1682\_Val1686del] does not allow for a conclusive molecular diagnosis. The novel variant p.Glu1447Val was found in the second allele. However, this variant was predicted to be deleterious only by CADD software. Thus, to infer the molecular diagnosis of this family, it is important to either confirm the pathogenicity of variant p.Glu1447Val or identify another pathogenic variant in this allele.

In family L, the novel variant p.Lys22Asn was predicted to be pathogenic by both CADD and MutationTaster, and its frequency in the population is unknown. This variant may be related to the presentation of Stargardt disease in two individual cases. In individual II, this variant was associated with the complex allele p.[Ser1642Arg; Val1682\_Val1686del], whereas in individual II2, this variant was associated with the p.Gly1901Glu variant. This repetition may support the hypothesis that the variant is indeed pathogenic.

### Genotype–Phenotype Correlation

Cases in which the onset of Stargardt disease occurs during the first decade of life tend to present with a more rapid loss of central vision and more extensive retinal pigment epithelium atrophy.<sup>4</sup> The pathogenicity of the variant in terms of protein function and/or structure may be directly related to the early onset of disease symptoms. Wiszniewski et al.<sup>7</sup> suggested that pathogenic variants with more deleterious effects on protein function resulted in earlier symptoms of Stargardt disease. Westeneng-van Haften et al.<sup>39</sup> also suggested that more deleterious pathogenic variants caused more severe phenotypes. In this study, six cases with symptom onset before 10 years of age were classified as type III by autofluorescence. These individuals currently have had the disease for 18 to 57 years. In contrast, in six patients in whom symptoms of decreased visual acuity presented after the age of 10, the autofluorescence classification was type I.

The observed phenotypic classification in this study was the same among siblings, indicating the same pathogenic effects for each variant. This concordance of autofluorescence results among siblings was also described by Fujinami et al.<sup>14</sup>



**FIGURE 2.** Pedigrees of the 14 families with Stargardt disease and segregation analysis results. The blue text highlights the following complex alleles: p.[Leu541Pro; Arg1443His] in five families (A, B, C, D, M); p.[Leu541Pro; Ala1038Val] in two families (E, F); p.[Ser1642Arg; Val1682\_Val1686del] in seven families (G, H, I, J, K, L, M); and p.[Pro1761Arg; Arg2106Cys] in one family (N). The arrows indicate the probands, whose genomes were fully sequenced. Relatives of the probands underwent only segregation analysis. Individuals BII2, DI1, DII2, and III1 were not formally examined. Circles: females; squares: males. Black symbols: affected. N/A, not available.

However, in a study evaluating the intrafamilial characteristics of the phenotypic manifestations of Stargardt disease, Lois et al.<sup>40</sup> described different ages of symptom onset, different visual acuity, and different autofluorescence findings among siblings.

No concordance of phenotypic manifestations was observed for the four complex alleles described herein. This may be due to different combinations of pathogenic variants that can alter the phenotype of Stargardt patients. Additional



studies of ABCA4 protein activity may provide insights into the phenotypic characteristics of each case.<sup>5,6</sup>

Segregation analysis is important in order to confirm the molecular diagnosis of patients with Stargardt disease, given the high frequency of complex alleles of the ABCA4 gene. The identification of four novel variants and three novel complex alleles in the Brazilian population draws attention to the importance of analyzing molecular diagnoses in different populations. A better understanding of complex alleles may change approaches to sequence analysis and therefore affect genetic counselling practices.

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