The Common ABCA4 Variant p.Asn1868Ile Shows Nonpenetration and Variable Expression of Stargardt Disease When Present in trans With Severe Variants

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utoosomal recessive Stargardt disease (STGD1; Mendelian Inheritance in Man [MIM] 248200) represents the most common hereditary macular dystrophy (estimated prevalence, 1:10,000) and is funduscopically characterized by lipofuscin deposits in the retinal pigment epithelium (RPE), presenting as yellow-white flecks and progressive atrophy of the macular RPE.2,3 STGD1 is caused by sequence variants in the transmembrane ATP-binding cassette transporter type A4 (ABCA4; MIM 601691).4 Different combinations of ABCA4 variants give rise to several phenotypes, which can partly be...
attributed to differences in the residual activity of the ABCA4 protein. A combination of disease-causing ABCA4 alleles may, therefore, result in a severe, early-onset STGD1 phenotype (i.e., onset < 10 years of age), which is often observed as rapidly progressive cystoid dystrophy, in classical STGD1, or as late-onset STGD1 in about half of the patients with late-onset STGD1 (onset > 44 years of age), only one disease-causing allele was identified, despite the sequence analysis of all coding variants.

In carriers of monoallelic ABCA4 variants, the second variant can reside in introns, outside of splice sites, not detected by standard sequencing methods. A very small fraction of these unidentified defects comprise copy number variants that elude detection by PCR-based sequencing techniques. Identification of disease-causing alleles and insight into their associations with differences in disease presentation and severity are essential for the counseling of patients and become increasingly important, as research on therapeutic approaches is rapidly evolving.

Recently, a frequent ABCA4 coding variant, c.5603A>T (p.Asn1868Ile), which thus far was considered benign due to its high minor allele frequency (AF) of 6.6% in the non-Finnish European population, was associated with STGD1. This variant was found to be phenotypically expressed only when it is trans with a deleterious mutation (i.e., presence of the variants on different alleles) and explained >50% of all monoallelic cases and ~80% of late-onset cases in a US cohort. In addition, the c.2588G>C (p.[Gly863Ala, Gly863del]) variant was proposed to act as a mild pathogenic variant only when in cis with p.Asn1868Ile (i.e., presence of the variants on the same allele).

In this study, we investigated STGD1 probands with a single disease-causing ABCA4 allele for the presence of the p.Asn1868Ile variant, performed segregation analysis through the inclusion of unaffected family members to assess the configuration of the identified ABCA4 variants (i.e., in cis or in trans), and performed ophthalmologic studies to evaluate the full spectrum of phenotypes associated with the single-variant allele p.Asn1868Ile (i.e., no ABCA4 variants identified in cis). In addition, we calculated the penetrance for the p.Asn1868Ile variant by using allele frequencies of severe ABCA4 variants.

METHODS

Study Population and Genetic Analysis

We ascertained 67 clinically suspected STGD1 patients in whom only one disease-causing variant in the ABCA4 gene had been identified, from eight ophthalmic centers in The Netherlands (n = 64) and one center in Germany (n = 3). Between 1998 and 2017, these subjects were screened for disease-causing variants in the coding regions and flanking splice sites of ABCA4, and, recently, for 63 subjects, ~95% of the entire ABCA4 gene (exons and introns) was further sequenced by using a custom Haloplex Target Enrichment kit (Agilent, Santa Clara, CA, USA) as described previously. All variants identified by Haloplex-based ABCA4 sequencing were confirmed using Sanger sequencing. We investigated probands harboring monoallelic variants for the presence of the p.Asn1868Ile variant and rare (minor AF < 0.005) deep-intronic variants. Four DNA samples were analyzed using non-Haloplex-based alternative techniques (Supplementary Methods S1).

The segregation of the sequence variants identified in the probands was studied by sequencing the corresponding genomic DNA fragments in relatives (Supplementary Table S1). Participants gave informed consent after an explanation of the nature of the study. The study procedures were performed according to approval of the medical ethics committee of the Radboud University Medical Center and the tenets of the Declaration of Helsinki.

Clinical Evaluation

Probands and affected siblings carrying the single-variant allele p.Asn1868Ile were clinically evaluated. Medical records were reviewed and available clinical data were collected, comprising age at onset and initial symptoms, age at diagnosis, best-corrected visual acuity (BCVA), and findings on ophthalmoscopy. Age of onset was defined as the age at which the initial symptoms were noted by the patient. In asymptomatic subjects and in cases where age at onset was not noted in the patient file, we used the age at which macular abnormalities were first diagnosed by an ophthalmologist.

Furthermore, we examined available data acquired by fundus photography, fundus autofluorescence (FAF) imaging using a confocal scanning laser ophthalmoscope (Spectralis; Heidelberg Engineering, Heidelberg, Germany), spectral domain-optical coherence tomography (OCT; Spectralis), or time-domain OCT (Stratus; Carl Zeiss Meditec, Dublin, CA, USA), fluorescein angiography, and full-field electroretinography, according to the International Society for Clinical Electrophysiology of Vision standards. We assessed presence of foveal sparing, which we defined as RPE atrophy encircling a structurally and functionally (BCVA, ≥20/200) preserved fovea by ≥180° by evaluation of FAF and/or OCT.

Two asymptomatic siblings who carried the same combination of ABCA4 variants as the respective probands were clinically examined by measurement of BCVA and performance of fundus photography, FAF, and OCT.

ABCA4 Allele Frequency Calculations and p.Asn1868Ile Penetrance Estimates

To assess the penetrance of the common variant p.Asn1868Ile when present in trans with a severe ABCA4 variant, we used AF data in ~35,000 non-Finnish European control subjects in Exome Aggregation Consortium (available at: http://exac.broadinstitute.org; accessed September 01, 2017), as at least 24/27 patients belong to this population. First, because we did not know all ABCA4 variants that are in linkage disequilibrium with p.Asn1868Ile, we estimated the frequency of the single-variant allele p.Asn1868Ile. Zernant et al. indicated that 10% of c.2588G>C alleles carry c.5603A>T (p.Asn1868Ile) in cis and that this allele, containing both variants, probably acts as a fully penetrant mild allele if present in a compound heterozygous state with a severe ABCA4 variant. In the same study, consistent with our data, the frequent noncanonical splice site variant c.5461-10T>C was always found in cis with c.5603A>T (p.Asn1868Ile). Therefore, the AF of the single-variant allele p.Asn1868Ile was calculated based on its total AF minus the AF of c.5461-10T>C and 10% of the AF of c.2588G>C. Other variants have been found in cis with p.Asn1868Ile, but their cumulative non-Finnish European AF is likely negligible and was not taken into account.

Second, we calculated a cumulative AF of severe ABCA4 variants. We determined the sum AF of all protein-truncating variants (i.e., stop mutations, frameshift mutations) and canonical splice site variants that, in the majority of cases, result in exon skipping and likely in the absence of ABCA4 activity, and added the AF of noncanonical splice site variants (i.e., RNA splice variants outside the conserved intronic
dinucleotides at the ends of introns) that resulted in less than 25% of normally spliced ABCA4.

Finally, given an estimated prevalence of STGD1 of 1 in 10,000 individuals and a population in The Netherlands of 17,150,000 people, the expected and observed number of STGD1 patients due to the combination of the single-variant allele p.Asn1868Ile and a severe variant in The Netherlands were compared.

**RESULTS**

**Genetic Analysis**

In 14/67 probands, we found p.Asn1868Ile to be in cis with causal protein-coding or splice site variants, that is c.5461-10T>C (n = 9), c.2588G>C (n = 3), c.4469G>A (n = 1), and c.818G>A (n = 1). We excluded these patients from clinical evaluation.
<table>
<thead>
<tr>
<th>Subject / Sex / Age</th>
<th>Latest Visual Acuity</th>
<th>Fundus and Imaging Features</th>
<th>Full-Field ERG†</th>
<th>Genotype: ABCA4 Variant(s) in Trans</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-II:4 / F / 75</td>
<td>20/25 20/20</td>
<td>Yellow-white pisciform flecks throughout the posterior pole, no atrophy</td>
<td>Yes</td>
<td>c.768G&gt;T p.(Leu257Valfs*17)§ S</td>
</tr>
<tr>
<td>B-II:1 / M / 65</td>
<td>20/200 20/200</td>
<td>Yellow-white pisciform flecks throughout the posterior pole and midperiphery with chorioretinal atrophy in macula</td>
<td>Yes</td>
<td>c.768G&gt;T p.(Leu257Valfs*17)§ S</td>
</tr>
<tr>
<td>B-II:3 / F / 60</td>
<td>CF 20/400</td>
<td>Extensive chorioretinal atrophy and pigmentary changes in macula with yellow-gray pisciform flecks throughout posterior pole and midperiphery. Additionally, temporal and inferior peripheral in RE chorioretinal scars of Coats-like lesions and laser therapy.</td>
<td>Yes</td>
<td>c.768G&gt;T p.(Leu257Valfs*17)§ S</td>
</tr>
<tr>
<td>C-II:1 / M / 49</td>
<td>20/20 20/20</td>
<td>Yellow-white pisciform flecks throughout the posterior pole and midperiphery with parafoveal chorioretinal atrophy in the left eye with foveal sparing</td>
<td>NP</td>
<td>c.768G&gt;T p.(Leu257Valfs*17)§ S</td>
</tr>
<tr>
<td>C-II:2 / F / 55</td>
<td>20/50 20/20</td>
<td>Yellow-white pisciform flecks throughout the posterior pole and extensive chorioretinal atrophy with foveal sparing</td>
<td>NP</td>
<td>c.768G&gt;T p.(Leu257Valfs*17)§ S</td>
</tr>
<tr>
<td>D-II:1 / M / 68</td>
<td>CF 20/130</td>
<td>62 y: yellow-white pisciform flecks throughout the posterior pole and parafoveal chorioretinal atrophy with foveal sparing. 68 y: chorioretinal atrophy in macula with a few surrounding pisciform yellow-white flecks</td>
<td>Yes</td>
<td>c.768G&gt;T p.(Leu257Valfs*17)§ S</td>
</tr>
<tr>
<td>D-II:3 / M / 58</td>
<td>20/20 20/22</td>
<td>Predominant perimacular pisciform yellow-white flecks and mottled decreased FAF in macula with foveal sparing</td>
<td>NP</td>
<td>c.768G&gt;T p.(Leu257Valfs*17)§ S</td>
</tr>
<tr>
<td>E-II:3 / F / 64</td>
<td>20/400 20/400</td>
<td>Foveal changes with perimacular and peripapillary irregular yellow-white flecks and mottled decreased FAF in macula (Fig 3D-F)</td>
<td>Yes</td>
<td>c.4462T&gt;C p.(Cys1488Arg) NA</td>
</tr>
<tr>
<td>F-II:2 / M / 41</td>
<td>20/66 20/66</td>
<td>Foveal changes with perimacular and peripapillary irregular yellow-white flecks and mottled decreased FAF in macula (Figs. 3J-L)</td>
<td>Yes</td>
<td>c.1822T&gt;A p.(Phe608Ile) S</td>
</tr>
<tr>
<td>Subject / Sex / Age Latest Visit, y</td>
<td>Age at Onset, y</td>
<td>Latest Visual Acuity</td>
<td>Fundus and Imaging Features</td>
<td>Dark Choroid on Fluorescein Angiography</td>
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<tr>
<td>G-II:1 / F / 76 72</td>
<td>20/25</td>
<td>20/52</td>
<td>Parafoveal pigmentary changes RE, and chorioretinal atrophy with foveal sparing and hyper- and hypoautofluorescent flecks throughout posterior pole on FAF</td>
<td>NP</td>
</tr>
<tr>
<td>G-II:2 / M / 75 69</td>
<td>20/25</td>
<td>20/40</td>
<td>Chorioretinal atrophy with foveal sparing and hyper- and hypoautofluorescent flecks throughout posterior pole and midperiphery on FAF (Figs. 2A–C)</td>
<td>NP</td>
</tr>
<tr>
<td>G-II:4 / F / 70 37</td>
<td>CF</td>
<td>CF</td>
<td>Extensive chorioretinal atrophy and pigmentary changes in posterior pole and hyper- and hypoautofluorescent flecks throughout posterior pole and midperiphery on FAF</td>
<td>NP</td>
</tr>
<tr>
<td>H-II:3 / M / 38 30</td>
<td>20/125</td>
<td>HM¶ 20/125</td>
<td>Perimacular irregular yellow-white flecks and beaten bronze atrophy in macula (no OCT/FAF)</td>
<td>Yes</td>
</tr>
<tr>
<td>I-II:3 / F / 29 18</td>
<td>20/50</td>
<td>20/33</td>
<td>Parafoveal pigmentary changes with yellow-white pisciform flecks throughout the posterior pole and mild atrophy in macula (no OCT/FAF)</td>
<td>No</td>
</tr>
<tr>
<td>J-II:2 / M / 57 56</td>
<td>20/32</td>
<td>20/330</td>
<td>Pigmentary changes in macula, perimacular yellow-white flecks, and chorioretinal atrophy with in RE foveal sparing, in both eyes surrounded by an area of increased background FAF</td>
<td>NP</td>
</tr>
<tr>
<td>K-II:1 / F / 29 23</td>
<td>20/200</td>
<td>20/125</td>
<td>Foveal changes and perimacular yellow-white flecks (no OCT/FAF)</td>
<td>Yes</td>
</tr>
<tr>
<td>L-II:1 / F / 46 31</td>
<td></td>
<td></td>
<td>20/200</td>
<td>Foveal changes with perimacular yellow-white irregular flecks and mottled decreased FAF in macula</td>
</tr>
<tr>
<td>M-II:1 / F / 69 53</td>
<td>20/50</td>
<td>20/100</td>
<td>Foveal changes with yellow-white pisciform flecks throughout the posterior pole and midperiphery and chorioretinal atrophy with foveal sparing; Attenuated arterioles</td>
<td>Yes</td>
</tr>
<tr>
<td>Subject / Sex / Age at Onset, y</td>
<td>Latest Visual Acuity</td>
<td>Fundus and Imaging Features</td>
<td>Dark Choroid on Fluorescein Angiography</td>
<td>Light Adapted (RE/LE)</td>
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<tr>
<td>N-II:1 / F / 71&lt;sup&gt;abc&lt;/sup&gt;</td>
<td>45</td>
<td></td>
<td>CF 20/20</td>
<td>45 y: yellow-white pisciform flecks throughout the posterior pole. 71 y: extensive chorioretinal atrophy in macula of RE after years of foveal sparing, chorioretinal atrophy with foveal sparing in LE.</td>
</tr>
<tr>
<td>O-II:1 / F / 39</td>
<td>32</td>
<td>20/22</td>
<td>20/20</td>
<td>Foveal changes with perimacular yellow-white irregular flecks and mottled FAF in macula with foveal sparing.</td>
</tr>
<tr>
<td>P-II:1 / F / 35&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32</td>
<td>20/50</td>
<td>20/100</td>
<td>Foveal changes with perimacular yellow-white irregular flecks and mottled decreased FAF in macula surrounded by a zone of increased background FAF.</td>
</tr>
<tr>
<td>Q-II:1 / F / 46</td>
<td>42</td>
<td></td>
<td>20/30</td>
<td>20/32</td>
</tr>
<tr>
<td>R-II:1 / M / 30</td>
<td>30</td>
<td></td>
<td>20/28</td>
<td>20/22</td>
</tr>
<tr>
<td>S-II:1 / F / 30</td>
<td>26</td>
<td>20/200</td>
<td>20/50</td>
<td>Foveal changes with perimacular yellow-white irregular flecks and mottled decreased FAF in macula.</td>
</tr>
<tr>
<td>T-II:1 / M / 45&lt;sup&gt;c&lt;/sup&gt;</td>
<td>36</td>
<td>20/66</td>
<td>CF</td>
<td>Foveal pigmentary changes and yellow-white pisciform flecks throughout the posterior pole and midperiphery with chorioretinal atrophy in macula.</td>
</tr>
<tr>
<td>U-II:1 / F / 40</td>
<td>37</td>
<td></td>
<td>20/40</td>
<td>20/66</td>
</tr>
<tr>
<td>V-II:1 / F / 31</td>
<td>27</td>
<td></td>
<td>CF 20/100</td>
<td>Perimacular yellow-white pisciform flecks and mottled decreased FAF in macula, surrounded by a zone of increased background FAF, initially with foveal sparing.</td>
</tr>
<tr>
<td>Subject / Sex / Age at Latest Visit, y‡</td>
<td>Latest Visual Acuity</td>
<td>Fundus and Imaging Features</td>
<td>Dark Choroid on Fluorescein Angiography</td>
<td>Full-Field ERG†</td>
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<tr>
<td>W-II:1 / F / 55</td>
<td>42</td>
<td>20/130</td>
<td>20/200</td>
<td>NP</td>
</tr>
<tr>
<td>X-II:1 / F / 32</td>
<td>20</td>
<td>20/200</td>
<td>20/125</td>
<td>No</td>
</tr>
<tr>
<td>Y-II:1 / F / 53</td>
<td>47</td>
<td>20/25</td>
<td>20/20</td>
<td>Yes</td>
</tr>
<tr>
<td>Z-II:1 / F / 44</td>
<td>21</td>
<td>CF</td>
<td>CF</td>
<td>NP</td>
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<tr>
<td>AA-II:1 / M / 58</td>
<td>57</td>
<td>20/20</td>
<td>20/20</td>
<td>NP</td>
</tr>
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</table>

** CF, counting fingers; ERG, electroretinography; fs, frameshift; HM, hand motion; LE, left eye; M, mild effect; Mo, moderately severe effect; NA, not applicable; NP, not performed; RE, right eye; S, severe effect; N, normal amplitude (equal to or above the lower 5% of the range for a normal population); MR, moderately reduced amplitude (1%–5% of normal range); SR, severely reduced amplitude (<1% of normal range).

† Stop codon.
‡ ERG results at latest visit, unless stated otherwise.
§ Previously mentioned cases: (a) Ref. 16, (b) Ref. 33, (c) Ref. 34, (d) Refs. 13, 20, (e) Ref. 35, and (f) Ref. 15.
§§ Based on in vitro splice assays.27,28
|| Age at diagnosis.
¶ Amblyopic eye.
# trans configuration could not be established by segregation analysis or homozygosity.
** Also CNGB3 c.1148delC heterozygous.
In 27/67 probands, we detected the single-variant allele p.Asn1868Ile; in 23 patients, we could establish the trans configuration of the identified variants (Figs. 1A, 1B; Supplementary Figs. S1, S2), either due to segregation analysis (n = 21) or because the p.Asn1868Ile variant was found in a homozygous state (Y-II:1 and AA-II:1). In the remaining four probands (K-II:1, N-II:1, R-II:1, and Z-II:1), segregation analysis could not be completed due to the absence of DNA of relatives (Table, footnote #; Supplementary Fig. S2). Additionally, in probands H-II:3, J-II:2, Y-II:1, and AA-II:1, we could not exclude the presence of intronic variants in cis with p.Asn1868Ile, as the noncoding regions of ABCA4 in these individuals were not sequenced.

Among the 27 probands harboring the single-variant allele p.Asn1868Ile, 19 carried p.Asn1868Ile together with a protein-truncating mutation due to a stop (n = 4), a frameshift (n = 4), a canonical splice site variant (n = 1), or because of noncanonical splice site variants elsewhere shown to result in protein truncations (n = 10) (Table).27,28 The remaining eight probands harbored seven unique variants: six missense variants and one noncanonical splice site variant (c.5714+5G>A) that has a moderately severe effect on splicing.28 Among these missense variants, p.Gly1961Glu in the vast majority of STGD1 cases acts as a mild variant,26,29,30 whereas p.Phe608Ile is considered a severe variant.31 For the remaining missense variants (p.Glu1087Lys, p.Cys1488Arg, p.Gly1972Arg, and p.Met2143Arg), the effect on protein function is unclear.

Segregation analysis in 10 siblings affected by STGD1 identified 7 individuals who harbored the same causal variants as the respective probands (Fig. 1A). In two families, we found affected siblings carrying different combinations of disease-causing ABCA4 alleles (Supplementary Fig. S1).

Clinical Evaluation

Thirty-four STGD1 patients (27 probands and 7 siblings) that harbor p.Asn1868Ile in combination with a known causal variant were identified, 12 of whom were male and 22 were female. Detailed clinical characteristics of 32/34 subjects carrying p.Asn1868Ile are depicted in the Table. For two individuals, detailed clinical data were not available (A-II:2 and, besides age at onset, A-II:1; Figure 1A).

None of the subjects manifested early-onset STGD1 (age at onset, ≤10 years). Fourteen patients showed late-onset STGD1 (age at onset, >44 years), and 19 subjects manifested classical STGD1 (age at onset, 11–44 years). The mean age of onset was 42 years (SD, 16 years), with the majority of patients presenting with decreased visual acuity. Other initial symptoms included scotoma and metamorphopsia. Patients D-II:5, R-II:1, and U-II:1 did not experience visual symptoms at the time of diagnosis at the age of 52, 30, and 37 years, respectively; all three manifested few flecks, largely confined to the macular region, and a perifoveal ring of RPE atrophy with a relatively spared fovea.

Classical STGD1, in the majority of patients (14/19), was characterized by early involvement of the macula featuring flecks, beaten bronze atrophy, and/or mottled decreased FAF, all largely confined to the macular and perimacular region. In the minority of patients (5/19), flecks throughout the posterior pole were observed, accompanied by pronounced atrophy in the posterior pole and midperiphery in 4/5 patients. The latter combination of fundus characteristics might represent a later disease stage, because available data of the first decade(s) after disease onset for the four patients manifesting atrophy (B-II:3, I-II:3, G-II:4, and T-II:1) were insufficient to assess fundus characteristics early in the disease. However, in 1/5 patients (C-II:1, with an age at onset of 41 years), flecks throughout the posterior pole and midperiphery with parafoveal atrophy were observed in the year after he first experienced symptoms, suggesting either slowly progressive disease that the patient himself had long been unaware of, or a different subphenotype. Foveal sparing in the classical STGD1 group was observed in 5/19 patients (age at onset, 27–41 years) (Table).

Late-onset STGD1, in the majority of clinically evaluated patients (8/13), was characterized by flecks throughout the posterior pole and midperiphery, often followed by sharply demarcated atrophy. Yet, three patients (E-II:3, J-II:2, and Y-II:1, with an age at onset of 56, 56, and 47, respectively) showed early exclusive involvement of the macula with perimacular flecks and macular beaten bronze atrophy and/or macular mottled decreased FAF, which are characteristics that were observed in the majority of the classical STGD1 patients. The remaining two patients showed both flecks throughout the posterior pole and midperiphery as well as mottled, decreased FAF in the macula at first presentation. RPE atrophy in a foveal sparing pattern was observed in 9/13 subjects, whereas 2/13 patients did not show macular atrophy yet.

In two late-onset STGD1 patients (G-II:1 and G-II:2), who were initially diagnosed with age-related macular degeneration (AMD) at the age of 72 and 69, respectively, flecks appeared absent, as assessed by ophthalmoscopy and fundus photography. Yet, they did exhibit widespread hypo- and hyperautofluorescent flecks on FAF (Fig. 2), highlighting the clinical value of FAF in the differentiation between AMD and late-onset STGD1.

Variable Age at Onset and Incomplete Penetrance

When comparing affected siblings who harbor p.Asn1868Ile (Fig. 1A), we observed fairly constant ages at onset in families A, C, and D; whereas in family B, the age at onset between
affected siblings varied tremendously (19 and 54 years of age). In addition to STGD1, B-II:3 suffered from Coats-like exudative vasculopathy, but this was first observed at the age of 57 and affected only the right eye.

Surprisingly, we found three asymptomatic male siblings (E-II:1, F-II:1, and H-II:1) in three STGD1 families that carried the same compound heterozygous variants as their probands (Fig. 1B). Also, unaffected person H-II:2 carried an ABCA4 allele with a severe variant (c.[5461-10T>C; 5603A>T]) and c.5603A>T (p.Asn1868Ile) on the other allele. For asymptomatic siblings E-II:1 and H-II:1, in view of the large gap between their current age and the age at onset of the proband, it is plausible that they remain unaffected and can be considered nonpenetrant. Moreover, current manifestation of STGD1 in E-II:1 was excluded during ophthalmologic examination: fundus photography, FAF, and OCT at the age of 64, FAF reveals hyperautofluorescent lesions corresponding with the flecks and mottled decreased autofluorescence in the macula. OCT shows macular RPE atrophy. (G-I) Asymptomatic sibling F-II:1 shows no abnormalities on fundus photography, FAF, and OCT at the age of 47 years. (J-L) Patient F-II:2 shows foveal changes with perimacular and peripapillary irregular yellow-white flecks, mottled decreased FAF in the macula, and central RPE atrophy at the age of 41 years, 9 years after disease onset.

FIGURE 3. Retinal imaging of two sibling pairs in which an affected and an unaffected sibling harbor the same combination of ABCA4 variants. (A-C) Asymptomatic sibling E-II:1 does not manifest STGD1 as assessed by fundus photography, FAF, and OCT at the age of 70 years. Fundus photography shows one small yellow-white, round lesion, which appears hyperautofluorescent on FAF and is visible as a single subretinal deposit on OCT. (D-F) Patient E-II:3 shows perimacular and peripapillary irregular yellow-white flecks on fundus photography at the age of 64. FAF reveals hyperautofluorescent lesions corresponding with the flecks and mottled decreased autofluorescence in the macula. OCT shows macular RPE atrophy. (G-I) Asymptomatic sibling F-II:1 shows no abnormalities on fundus photography, FAF, and OCT at the age of 47 years. (J-L) Patient F-II:2 shows foveal changes with perimacular and peripapillary irregular yellow-white flecks, mottled decreased FAF in the macula, and central RPE atrophy at the age of 41 years, 9 years after disease onset.
probands, he also underwent ophthalmologic examination, which excluded current manifestations of STGD1. His fundus photography, FAF, and OCT showed no abnormalities at the age of 47, and BCVA was 20/20 in both eyes (Figs. 3G–I). His sibling F-II:2 reported initial symptoms at the age of 32 and was diagnosed with STGD1 at the age of 38 (Figs. 3J–L). The asymptomatic cases in family H were not clinically examined, so current manifestations of mild STGD1 phenotypes could not be excluded.

Individuals harboring an ABCA4 variant that had been designated as severe did not show STGD1 phenotypes more often or more severe compared to individuals who harbored a variant with mild, moderately severe, or unknown effect (Table), with regard to age at onset or occurrence of foveal sparing.

p.Asn1868Ile Shows a Very Low Penetrance in the Caucasian Population

Based on previous findings and the results of this study, we can conclude that p.Asn1868Ile represents a mild ABCA4 variant that in the majority of cases only results in STGD1 when present in trans with a severe ABCA4 variant.

First, the maximum AF of the single-variant allele p.Asn1868Ile among ~66,000 non-Finnish European control alleles, based on its total AF minus the AF of c.5603A>T and ~10% of the AF of c.2588G>C, was 0.06453 (Fig. 4).

Second, the total AF of severe ABCA4 variants (protein-truncating variants and noncanonical splice site variants) was 0.00083, which yielded an estimated prevalence of individuals with one severe ABCA4 variant and p.Asn1868Ile of 0.000349 (Fig. 4C). Given a total population in the Netherlands of 17,150,000 persons, the theoretical number of STGD1 cases with a severe ABCA4 variant and p.Asn1868Ile thereby is 5991.

Third, the prevalence of STGD1 is estimated at 1 in 10,000 individuals, and in the Nijmegen STGD1 cohort, STGD1 due to a severe ABCA4 variant and p.Asn1868Ile was observed in 25/300 (8.3%) probands (Fig. 4D). Given a total population in The Netherlands of 17,150,000 people, STGD1 due to this combination of variants was expected in ~143 persons. If the non-Finnish European AF are comparable to those in The Netherlands, we concluded that the penetrance of the single-variant allele p.Asn1868Ile, when present in combination with a severe ABCA4 variant, was 2.4% (Fig. 4E).

As these calculations were based on a few assumptions, we also calculated the penetrance based on alternative assumptions regarding the prevalence of STGD1 and the frequency of severe ABCA4 variants (Supplementary Results S2). When uncertainties in these factors of the calculation were taken into account and in the unlikely situation that the alternative assumptions would act in concert, the penetrance of p.Asn1868Ile ranged between 0.24% and 9.54%.

DISCUSSION

By combining ABCA4 sequence analysis and segregation analysis of the identified variants, we detected the common variant p.Asn1868Ile as the second allele in 40% of cases carrying monoallelic variants in our genetically unexplained...
Reduced Penetrance in Stargardt Disease

STGD1 cohort. In the majority of the cases, p.Asn1868Ile was found in trans with severe ABCA4 variants, thus pointing toward the mild nature of this exonic variant in STGD1. In agreement with a previous study, we found that a high proportion of these p.Asn1868Ile carriers manifest the late-onset STGD1 phenotype. However, among 414 probands, age at onset was highly variable. Strikingly, we identified one asymptomatic sibling and two unaffected siblings, all of whom carried the same combination of ABCA4 variants as the respective probands. The very low calculated penetrance of p.Asn1868Ile of 2.4% in addition to the observed variability in age at onset and phenotypes among individuals carrying the same combinations of ABCA4 variants, lead us to hypothesize that cis- or trans-acting genetic modifiers or environmental factors greatly influence ABCA4 gene expression and, thereby, the clinical presentation of STGD1.

As yet, very little is known about the potential nature, role, and mechanisms of modifiers in STGD1. One group studied gene expression variation in the human retina and found that ABCA4 showed 1.5-fold higher expression in males compared to females. Males carrying a high-expressed mild ABCA4 allele, thereby, may be less prone to develop STGD1. In our study, we also observed an overrepresentation of females (22/34, 65%) among patients carrying p.Asn1868Ile (one-sample one-sided binomial test, \( P = 0.0615 \)). Furthermore, all three putative nonpenetrant siblings are male. Further research is needed to identify possible modifiers, explain the mechanisms whereby they modify gene expression, and, most interestingly, explore their potential as therapeutic targets.

Clinical overlap with other macular diseases, along with inconclusive results of genetic analyses, can make the diagnosis of late-onset STGD1 particularly challenging, as illustrated by cases G-II:1 and G-II:2 who first experienced symptoms at the age of 72 and 69, respectively. FAP and genetic analysis in these cases were valuable in distinguishing late-onset STGD1 from AMD. The identification of the second disease-causing allele, whereby a significant fraction of late-onset STGD1 cases is now genetically explained, strengthens the assumption that late-onset STGD1 represents a distinct disease entity that should not be confused with AMD. An accurate diagnosis is essential for appropriate counseling of patients regarding prognosis and inheritability, and is relevant for putative therapies, for example either recommendation or dissuading of vitamin A supplement use in AMD and STGD1, respectively. Moreover, several therapeutic trials in STGD1 patients are ongoing (http://www.clinicaltrials.gov, last accessed 21-03-2018), requiring identification of both disease-causing ABCA4 alleles.

The assessment of nonpenetrance has a few limitations. First, this study showed that age at onset, which constitutes a rather subjective measure, can vary tremendously even among siblings carrying the same ABCA4 variants. Therefore, nonpenetration cannot easily be confirmed by clinical examination of unaffected persons carrying bi-allelic disease-causing ABCA4 variants. Regardless of whether the currently unaffected subjects develop STGD1 later in life, a highly variable disease expression was observed that raises new questions about the disease mechanisms.

Second, although we have experimentally proven the veracity of a nonpenetrance phenomenon, we are conscious of the fact that nonpenetration estimates cannot be considered as definitive. Most importantly, late-onset STGD1 currently may be underdiagnosed or misdiagnosed due to the clinical overlap with AMD. In an AMD cohort (\( n = 698 \)) from the Radboudumc, The Netherlands, however we did not observe persons with a combination of a severe ABCA4 variant and p.Asn1868Ile, suggesting that misdiagnosis cannot be a major factor in centers of clinical expertise on STGD1. Moreover, because several nontruncating ABCA4 variants might also behave like severe variants, our estimate of the cumulative AF of severe variants in the population may in fact be higher, which would make the resultant penetrance estimate (2.4%) even lower.

Conclusions

Identification of genetic variants underlying STGD1 is essential to provide accurate counseling to patients and relatives and to drive therapeutic approaches. A significant proportion of STGD1 patients carries p.Asn1868Ile, which is associated with late-onset STGD1 and a variable disease expression. In line with these findings, we calculated an exceptionally low penetrance of the p.Asn1868Ile variant, which may be explained by the action of cis- or trans-acting genetic modifiers or nongenetic factors in STGD1.

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