Late-Onset Stargardt Disease Due to Mild, Deep-Intronic ABCA4 Alleles

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PURPOSE. To investigate the role of two deep-intronic ABCA4 variants, that showed a mild splice defect in vitro and can occur on the same allele as the low penetrant c.5603A>T, in Stargardt disease (STGD1).

METHODS. Ophthalmic data were assessed of 18 STGD1 patients who harbored c.769-784C>T or c.4253+43G>A in combination with a severe ABCA4 variant. Subjects carrying c.[769-784C>T; 5603A>T] were clinically compared with a STGD1 cohort previously published carrying c.5603A>T noncomplex. We calculated the penetrances of the intronic variants using ABCA4 allele frequency data of the general population and investigated the effect of c.769-784C>T on splicing in photoreceptor progenitor cells (PPCs).

RESULTS. Mostly, late-onset, foveal-sparing STGD1 was observed among subjects harboring c.769-784C>T or c.4253+43G>A (median age of onset, 54.5 and 52.0 years, respectively). However, ages of onset, phenotypes in fundus, and visual acuity courses varied widely. No significant clinical differences were observed between the c.[769-784C>T; 5603A>T] cohort and the c.4253+43G>A or the c.5603A>T cohort. The penetrances of c.769-784C>T (20.5%–39.6%) and c.4253+43G>A (35.8%–43.1%) were reduced, when not considering the effect of yet unidentified or known factors in cis, such as c.5603A>T (identified in 7/7 probands with c.769-784C>T; 1/8 probands with c.4253+43G>A). Variant c.769-784C>T resulted in a pseudo-exon insertion in 15% of the total mRNA (i.e., ~30% of the c.769-784C>T allele alone).

CONCLUSIONS. Two mild intronic ABCA4 variants could further explain missing heritability in late-onset STGD1, distinguishing it from AMD. The observed clinical variability and calculated reduced penetrance urges research into modifiers within and outside of the ABCA4 gene.

Keywords: Stargardt disease, ABCA4, disease expression, penetrance, differential diagnosis

Stargardt disease (STGD1 [Mendelian Inheritance in Man: 248200]) is typically characterized by rapid visual acuity (VA) decline in childhood or early adulthood, and by yellow-white pisciform flecks throughout the posterior pole and macular atrophy on fundoscopy.1,2 We previously described a cohort of patients with late-onset STGD1, which was characterized by slowly progressive sharply demarcated retinal pigment epithelium (RPE) atrophy in a foveal-sparing pattern.3,5 However, the diagnosis of late-onset STGD1 remained questionable due to unidentified disease-causing ABCA4 alleles, and phenotypic overlap with other macular diseases, the most frequent of which is AMD.6 Recently, a large part of the missing heritability of late-onset STGD1 was elucidated by the frequent ABCA4 coding variant c.5603A>T (p.Asn1868Ile), which was found to cause STGD1 only when present in combination with a deleterious mutation.7 Interestingly, this mild variant was associated with an exceptional clinical variability.8 Moreover, this variant clearly showed reduced penetrance in the Dutch population (~5%), that is, the vast majority of individuals carrying c.5603A>T in trans with (i.e., positioned on the other allele as) a severe variant do not manifest STGD1.8,9 Reduced penetrance has rarely been reported in autosomal-recessive retinal diseases.10–13 Occurrence of this phenomenon has major implications for genetic counseling and future directions for research into modifying factors, which could result in targets for therapeutics.

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4249

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Intronic ABCA4 Alleles in Late-Onset Stargardt Disease

Very recently, we identified eight deep-intronic variants among 67% of genetically unsolved STGD1 patients.14 The two most frequently identified variants, c.769-784C>T and c.4253+43G>A, only showed a partial splice defect in vitro. Variant c.769-784C>T strengthened a cryptic splice site at a noncanonical nucleotide position resulting in pseudo-exon inclusion. As this c.769-784C>T variant was consistently found in cis with (i.e., positioned on the same allele as) the mild c.5603A>T variant, its causal role could not be fully established yet. Variant c.4253+43G>A disrupted predicted splice silencers and created an exonic splicing enhancer leading to partial skipping of exon 28. Like c.5603A>T, variant c.4253+43G>A was associated with late-onset STGD1 and found to be phenotypically expressed only when in trans with a severe variant.15

In this study, we investigate the phenotypic characteristics associated with deep-intronic ABCA4 variants c.769-784C>T and c.4253+43G>A, and clinically compared these patients with our previously described patient cohort carrying c.5603A>T without variants in cis. Furthermore, we aim to strengthen the observation that c.769-784C>T has an effect on splicing, and to assess the penetrance of both intronic variants. This information is paramount for the differential diagnostics of late-onset STGD1 and to discern this disease from AMD.

METHODS

Subjects
Participants originated from the population of STGD1 patients in ophthalmic centers in The Netherlands participating in the RD5000 study group.16 Fifteen genetically unexplained probands and three affected siblings in whom the c.769-784C>T variant or the c.4253+43G>A variant was found as the second allele, after HaloPlex-based (Agilent, Santa Clara, CA, USA) sequencing and segregation analysis by Sangermano et al.14 were identified. All records were reviewed retrospectively. Additionally, skin biopsies were collected from a STGD1 patient harboring c.769-784C>T and a control individual, and splice assays were performed in photoreceptor progenitor cells (PPCs). Ultimately, we performed penetrance calculations employing ABCA4 allele frequency data of one European and two Dutch population databases.

All procedures were approved by the Medical Ethics Committee of Erasmus Medical Center (MEC-2010-359). Informed consent was obtained after explanation of the study, and the study adhered to the tenets of the Declaration of Helsinki.

Clinical Evaluation
Clinical data collected from medical records included age of onset and initial symptoms, age at diagnosis, best-corrected VA, findings on ophthalmoscopy, retinal imaging, and full-field (ff) ERG. We examined available 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 Cirrus; Carl Zeiss Meditec, Inc., Dublin, CA, USA), and ffERG acquired according to the International Society for Clinical Electrophysiology of Vision standards.7 We assessed the presence of foveal sparing, which we defined as RPE atrophy encircling a structurally and functionally (VA ≥20/200 Snellen) preserved fovea by 180° or more, by evaluation of FAF and/or OCT.4 The previously described definition ‘definitely decreased autofluorescence’ was used in the assessment of FAF.18

Age of onset was defined as the age at which the patient first experienced visual complaints. In asymptomatic subjects or in case age at onset was not reported, we used the age at which decrease in VA was first documented or the age at which macular abnormalities were first diagnosed. Age of onset was compared across patient groups with different ABCA4 genotypes, using Mann-Whitney U tests for independent samples. A P value < 0.05 was considered statistically significant.

We performed Kaplan-Meier survival analysis to determine the median survival time to develop severe visual impairment, defined as a VA of less than 20/200 Snellen (decimal, 0.10) in accordance with the International Classification of Diseases, 11th revision.19 Patients were censored if the time interval between the first measurement of severe visual impairment and the prior visit was more than 5 years, and patients were excluded if VA was available at only one time point and already was less than 20/200 Snellen. Additionally, we investigated whether c.769-784C>T has an additive effect on disease severity compared with the c.5603A>T variant alone by including age at onset and VA data of a previously described cohort of STGD1 patients carrying the noncomplex c.5603A>T variant.8

Assessment of RNA Splice Defects in Photoreceptor Progenitor Cells
To determine whether deep-intronic variant c.769-784C>T results in aberrant splicing of ABCA4 pre-mRNA, fibroblasts of a patient and a control were reprogrammed into induced pluripotent stem cells and differentiated into PPCs as previously described.20 All differentiation were performed in duplicate. RTPCR was performed using ABCA4 exonic primers located in ABCA4 exon 6 and 8. Gel resolution, excision, and purification of the bands of RT-PCR products, and Sanger sequencing were performed as described elsewhere.20 Details of RT-PCR primers are given in Supplementary Table S1. To determine the fraction of correctly spliced product, densitometric analysis was performed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).21 The analysis was performed in duplicate, and values were normalized for the size of the different fragments present in each RT-PCR product mixture.

Penetrance Calculations
We assessed the penetrance of c.769-784C>T and c.4253+43G>A, when present in trans with a severe ABCA4 variant, by comparing the observed number of patients carrying this combination of variants with the expected number of patients.

The expected number of patients was calculated based on ABCA4 allele frequency data of the general population, as previously described.8,10 The cumulative frequency of severe ABCA4 alleles was 0.00195, determined in our in-house whole-exome sequencing dataset, containing data of 21,559 individuals.10 We investigated the minor allele frequency (MAF) of the intronic variants of interest in the non-Finnish European (nFE) population in the Genome Aggregation Database (gnomAD),22,23 and the Dutch population in the Genome of The Netherlands database (GoNL).24,25 The median age at diagnosis in the study cohort of interest (i.e., c.769-784C>T or c.4253+43G>A carriers) served as a cut-off value to determine the number of individuals in The Netherlands at risk for disease due to both genotype and age.26

The observed number of patients carrying the intronic variant of interest in The Netherlands could be extrapolated...
given the proportion of patients harboring the intronic variant of interest at Radboud University Medical Center, the estimated STGD1 prevalence of 1 in 10,000 individuals, and the total Dutch population.

RESULTS

Genetic Characteristics

A total of 15 probands was included in this study, and in 13 probands, the trans configuration of the identified ABCA4 variants was confirmed (i.e., variants positioned on different alleles; Fig. 1). In families S and R, DNA of relatives was not available. Seven probands and one affected sibling harbored c.769-784C>T; 5603A>T in trans with a severe ABCA4 variant. Eight probands and two affected siblings harbored c.4253+43G>A in trans with a severe ABCA4 variant. One proband (M-II:1) also carried c.5603A>T on the same allele as c.4253+43G>A.

Clinical Characteristics

Detailed clinical characteristics of all subjects are displayed in Supplementary Table S2. Patients presented with decreased VA, central scotoma, and/or distorted vision, and one patient was asymptomatic. Patients harboring c.769-784C>T; 5603A>T reported a median age at onset of 54.5 (range, 20–69) years. This did not significantly differ from the age of onset among 33 patients carrying the noncomplex c.5603A>T variant previously reported, of whom the median was 41 (range, 18–72) years (Mann-Whitney U: P = 0.146, Supplementary Fig. S1). Patients harboring c.4253+43G>A had a median age at onset of 52.0 (range, 20–68) years, while the single patient who carried c.4253+43G>A; 5603A>T had the youngest age at onset of 18 years.

Patients harboring c.769-784C>T; 5603A>T mainly manifested late-onset STGD1 characterized by pisciform yellow-white flecks throughout the posterior pole and midperiphery on fundoscopy, appearing hyperautofluorescent on FAF, and sharply demarcated RPE atrophy in the macula on FAF, corresponding to loss of the outer retinal layers from the outer plexiform layer to the RPE on OCT, in a foveal-sparing pattern (Fig. 2A).

One patient, sibling Q-II:2, had a discordant onset: Q-II:2 already reported initial symptoms at the age of 20 years. Fundoscopy showed an abnormal foveal reflex with surrounding hypopigmented lesions and yellow flecks at the age of 23, with a VA of 20/100 in the right eye and 20/200 in the left eye (Supplementary Fig. S2). Over the years, macular lesions of RPE atrophy extended, surrounded by yellow-white flecks throughout the posterior pole and midperiphery. During triennial follow-up, VA gradually declined and reached 20/200 in the left eye at the age of 26 years but not in the right eye until the age of 39 years. In striking contrast, the brother, Q-II:1, had an age at onset of 69 years and fundus features in accordance with the characteristic findings of the group. The VA first reached 20/200 in the right eye at the age of 75, while
VA in the left eye still was 20/100 at the last visit, at age 78 years (Supplementary Fig. S2).

Patients harboring c.4253+43G>A also mainly manifested the characteristic late-onset STGD1 features. Six patients (H-II:2, J-II:2, J-II:3, N-II:3, and R-II:3; age of onset 53, 68, 56, 61, 31, and 52 years, respectively) showed foveal sparing up to the latest visit at age 63, 71, 75, 71, 42, and 68 years, respectively (Fig. 2B). Yet, three patients (D-I:1, F-II:2, G-I:2) showed fewer and smaller flecks, mostly confined to the macula, and did not exhibit the typical foveal sparing atrophy. Instead, FAF of patient D-I:1 and G-I:2 showed mottled decreased autofluorescence in the macula accompanied by one parafoveal, over the years expanding, sharply demarcated atrophic lesion. Interestingly, patient D-I:1 showed distinct intereye discordance in

![Figure 2](image1.png)

**Figure 2.** Fundus imaging follow-up and visual acuity decline of three subjects who harbor different ABCA4 variants. The vertical lines represent the moments that the FAF images of the right eyes were acquired. (A) Fundus photography (A1) and FAF of patient L-II:1 at the age of 63 (A2) and 69 years (A3). VA remained stable at 20/25 Snellen (0.8 decimal) (A4). This patient harbored c.[769-784C>T; 5603A>T] in trans with c.768G>T. (B) Fundus photography (B1) and FAF of patient N-II:3 at the age of 38 (B2) and 42 years (B3), when VA was 20/25 (0.8) and 20/28 (0.7), respectively (B4). This patient harbored c.4253+43G>A in trans with c.4773+1G>A. (C) Fundus photography (C1) and FAF of patient M-II:1 at the age of 21 (C2) and 24 years (C3), when VA was 20/50 (0.4) and 20/60 (0.3), respectively (C4). The patient harbored c.[4253+43G>A; 5603A>T] in trans with c.4539+2001G>A.

![Figure 3](image2.png)

**Figure 3.** Visual acuity survival. Kaplan-Meier survival plots for the time to reach severe visual impairment of patients carrying either of the two intronic variants or the noncomplex c.5603A>T.
Penetrance of a severe variant with the intronic variant (observed/expected number of patients)

<table>
<thead>
<tr>
<th></th>
<th>c.769-784C&gt;T</th>
<th>c.4253+43G&gt;A</th>
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<tbody>
<tr>
<td>Observed number of patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proportion of STGD1 cases carrying severe variant and intronic variant of interest</td>
<td>0.0167</td>
<td>0.0267</td>
</tr>
<tr>
<td>Number of STGD1 cases with severe variant and intronic variant of interest in the NL*</td>
<td>29</td>
<td>46</td>
</tr>
<tr>
<td>Expected number of patients</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cumulative allele frequency of ABCA4 protein-truncating, canonical splice-site variants, and severe noncanonical splice site variants in the NL† (p)</td>
<td>0.001953</td>
<td>0.001953</td>
</tr>
<tr>
<td>Allele frequencies of intronic variant of interest (q)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nFE gnomAD</td>
<td>0.004147</td>
<td>0.006050</td>
</tr>
<tr>
<td>GoNL</td>
<td>0.008016</td>
<td>0.005010</td>
</tr>
<tr>
<td>Frequencies of individuals with severe variant and intronic variant of interest (2pq)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Based on q nFE gnomAD</td>
<td>0.00001620</td>
<td>0.00002335</td>
</tr>
<tr>
<td>Based on q GoNL</td>
<td>0.00003151</td>
<td>0.00001957</td>
</tr>
<tr>
<td>Number of STGD1 cases with severe variant and the intronic variant of interest in the NL older than the median age at diagnosis‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Based on q nFE gnomAD</td>
<td>72</td>
<td>128</td>
</tr>
<tr>
<td>Based on q GoNL</td>
<td>139</td>
<td>106</td>
</tr>
</tbody>
</table>

Penetrance

Penetrance of a severe variant with the intronic variant (observed/expected number of patients)

<table>
<thead>
<tr>
<th></th>
<th>c.769-784C&gt;T</th>
<th>c.4253+43G&gt;A</th>
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<tbody>
<tr>
<td>Based on q nFE gnomAD</td>
<td>39.6%</td>
<td>35.8%</td>
</tr>
<tr>
<td>Based on q GoNL</td>
<td>20.5%</td>
<td>43.1%</td>
</tr>
</tbody>
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* Conservatively assuming STGD1 prevalence in The Netherlands to be 1:10,000. Total Dutch population: 17,150,000.
† Based on in vitro splice assays of noncanonical splice-site variants.35
‡ Median age at diagnosis in the c.769-784C>T cohort and the c.4253+43G>A cohort was 59 and 55 years, respectively.
The ‘expected’ number of patients carrying c.769-784C>T in combination with a severe variant was 72, based on the nFE MAF of c.769-784C>T of 0.00415. Based on the 2-fold higher Dutch MAF of c.769-784C>T (0.00802), 139 individuals were expected to be at risk for disease due to the ABCA4 variants they harbored and their age (higher than median age at diagnosis in patient cohort).

For c.4253+43G>A, the ‘expected’ number of patients was 128, based on a nFE MAF of c.4253+43G>A of 0.00603. Based on a slightly different Dutch MAF of c.4253+43G>A of 0.00501, 106 individuals were expected to be at risk.

For c.769-784C>T and c.4253+43G>A in trans with a severe ABCA4 variant, comparison of the expected numbers of patients with the observed number of patients in The Netherlands resulted in an estimated penetrance of 20.5% (Dutch MAF) to 39.6% (nFE MAF) and 35.8% (nFE MAF) to 43.1% (Dutch MAF), respectively.

**DISCUSSION**

Following the common c.5603A>T coding variant, two recently discovered frequent deep-intronic ABCA4 variants could further explain the missing heritability in late-onset STGD1. The majority of the subjects in this study manifested late-onset STGD1 (72%) characterized by pisciform flecks throughout the posterior pole and midperiphery, and macular sharply demarcated RPE atrophy—if present at all—in a foveal-sparing pattern. No significant differences with respect to phototopic features and VA course were observed between the c.769-784C>T and the c.4253+43G>A cohort.

The identification of the second disease-causing ABCA4 allele in an increasing number of patients with late-onset STGD1 undeniably anchors the described phenotype in the spectrum of ABCA4 disease. Still, patients are initially misdiagnosed with AMD on a regular basis. Important phototopic differential diagnostic findings in late-onset STGD1 are the flecks, which, compared with drusen, are more irregularly shaped, often more intensely hyperautofluorescent on FAF images, often but not always more diffusely spread across the posterior pole and midperiphery and correspond to hyperreflective depositions traversing photoreceptor-attributable bands on OCT. Also, in contrast to AMD, STGD1 is only very rarely complicated by a neovascularization. The correct clinical diagnosis and the identification of underlying genetic variants have become paramount with emerging therapies for both STGD1 and geographic atrophy in AMD, not only for proper patient selection for trials, but also for the identification of therapeutic targets. In fact, both c.769-784C>T and c.4253+43G>A ABCA4 variants were already shown to be targets for partial splice correction by antisense oligonucleotides.

Although most subjects exhibited characteristic late-onset STGD1 features, we observed notable clinical variability among subjects carrying the same combination of an intronic and a severe ABCA4 variant, even among siblings (family Q) and within subjects (D-I:1). Both the c.769-784C>T; 5603A>T and c.4253+43G>A cohort had a large range in age of onset, and VA courses varied widely. No comorbidities nor other factors were perceived that might explain the clinical variability. These results suggest that factors outside ABCA4 may modify the disease course. A few studies indicated that the activation of the complement cascade could play a role in the pathogenesis of STGD1. Parallel to the complement cascade, other overlapping disease-modifying factors might act in late-onset STGD1 and AMD.

RNA analysis in patient-derived PPCs showed that c.769-784C>T results in a pseudo-exon insertion in 15% of total mRNA, compared with 8.6% in HEK293T cells assessed previously. As the other allele of this patient harbored a missense variant (p.Phe680Ile), which is not expected to affect mRNA stability, the 15% defective total mRNA is considered to result from a 30% defective mRNA transcript from the c.769-784C>T allele alone, assuming that the missense allele in trans is equally expressed. The RNA analysis in PPCs corroborated a mild effect of c.769-784C>T on splicing. A similar observation was made for variant c.4539+2028C>T, which led to a 345 nt pseudo-exon insertion in patient-derived PPCs in 15% of total mRNA and approximately 30% of mRNA derived from the allele carrying c.4539+2028C>T. In contrast, no pseudo-exon insertion was found in fibroblasts of the same patient. These results strongly suggest that retina-specific splice factors play a role in the defects observed for both c.769-784C>T and c.4539+2028C>T.

Despite the enrichment of the complex c.769-784C>T; 5603A>T allele among patients versus controls and our splice assays that indicate that variant c.769-784C>T has an additive pathogenic effect, significant clinical differences between patients who harbor c.769-784C>T; 5603A>T versus patients only harboring c.5603A>T were not observed. In the genetic study, the age of onset among patients carrying c.769-784C>T; 5603A>T was higher than the age of onset of patients carrying the single c.5603A>T variant, but the other cohort was smaller and included patients from abroad. Variant c.4253+43G>A was found in cis with c.5603A>T in only one patient. Interestingly, this patient had a strikingly early age of onset (18 years), discordant phenotype in fundo and a relatively fast VA decline. These results suggest that variant c.5603A>T and other, yet unidentified, cis variants might influence the expression, and possibly the penetrance, of the ABCA4 allele.

We calculated the penetrance of c.769-784C>T and c.4253+43G>A, when present in trans with a severe variant, and found that, respectively, 20.5% to 39.6% and 35.8% to 43.1% of the individuals carrying these ABCA4 variants actually manifest STGD1. Other ABCA4 variants on the same allele might well influence the penetrance. In fact, in this study cohort, variant c.769-784C>T was consistently found in cis with c.5603A>T, the mild variant for which we previously calculated an extremely low penetrance (~5%), and for which we consequently argued that yet unidentified factors explain its penetrance in patients. It is possible that c.5603A>T acts as a modifier that results in full penetrance of the complex c.769-784C>T: 5603A>T allele. As expected, we recently identified variant c.769-784C>T without c.5603A>T in two late-onset STGD1 cases in a large follow-up study (Khan M, Cremers FPM, unpublished observations, 2019). Further studies are needed to corroborate current findings and provide undeniable evidence of the pathogenicity of the c.769-784C>T variant.

This study has a few limitations, mainly due to the complexity of penetrance calculations discussed previously. Population differences in the prevalence of the variants of interest and severe variants influence the calculated penetrance. To minimize the risk of this sampling bias, we used and compared allele frequencies of the European and the Dutch population. Missense variants that might have a severe effect on ABCA4 protein function were not included in the calculation of severe ABCA4 variant frequency, which has most likely resulted in overestimation of the calculated penetrance. Second, age of onset was defined as the age at which the patient first experienced visual complaints. Alternatively, if complaints were absent or not reported (in only 2 subjects), we used age at which decrease in VA was first documented or the age at which macular abnormalities were first diagnosed. To a larger degree, age of onset might be
Influenced by recall bias and other patient-specific factors, such as daily activities, comorbidities, and location of lesions.

In conclusion, this study showed that intronic ABCA4 variants resulting in a mild splice defect, in addition to the coding c.5603A>T variant, could genetically explain a large proportion of the late-onset STGD1 cases, which aids in the differential diagnosis of late-onset STGD1 and AMD. At the same time, these mild variants were associated with a notable clinical variability, which needs to be considered in the design of upcoming clinical trials. The results of the present study and our previous studies on c.5603A>T,8,10 indicating that cis variants, such as c.5603A>G, as well as factors outside ABCA4 could modify the disease course, should be considered in family counseling and warrant further studies on STGD1 modifiers.

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