Author Response: Penetrance of the *ABCA4* p.Asn1868Ile Allele in Stargardt Disease

We read with interest the commentary[1] on our recent manuscript,[2] in which we argue that the *ABCA4* variant c.5603A>T (p.Asn1868Ile) in *trans* with a loss-of-function mutation leads to Stargardt disease (STGD1) in less than 5% of individuals. Below, we strengthened our previous findings by employing control population-matched *ABCA4* variant frequencies from the Netherlands instead of the European population. We calculated the penetrance assuming our best-estimated prevalence of STGD1 in the Netherlands, but also based on a 2-fold higher prevalence, considering the possibility that late-onset STGD1 may be underdiagnosed.

Allikmets and colleagues[1] state that we only provide evidence for non-penetrance in 2 of 27 pedigrees. This was in fact proposed for asymptomatic males in three pedigrees (E-II:1, F-II:1, H-II:1).[2] Moreover, a fourth asymptomatic biallelic person (H-II:2 in Fig. 1),[2] who is the spouse of H-II:1, was identified. Clinical evidence of an absent STGD1 phenotype was provided for two out of the four cases (E-II:1, F-II:1). The other two individuals (H-II:1, H-II:2) were not clinically examined so we cannot formally rule out current mild manifestation of late-onset STGD1. There is, however, a substantial difference in age at onset (>37 years) between H-II:1 and his younger affected sibling H-II:3, even if H-II:1 would have STGD1 symptoms in the future. Reduced penetrance in a disease with a highly variable age of onset arguably is difficult to substantiate as disease may develop later in life. However, the age of onset of recently described cohorts of patients harboring p.Asn1868Ile[2–4] (Fig.) shows a normal distribution with a peak incidence between 30 and 50 years, and 49/57 (86%) of p.Asn1868Ile cases show STGD1 symptoms before the age of 60 years. Although this distribution does not rule out the possibility that some late-onset STGD1 cases remain undiagnosed to date, it does indicate that the vast majority of p.Asn1868Ile patients has developed disease before the age of 60 years.

In our cohort of 27 families, we found eight p.Asn1868Ile-carrying families in which more than one person carries p.Asn1868Ile in *trans* with a loss-of-function allele. If we only consider the non-probands—to partly overcome the selection bias toward the inherent full penetrance in probands—7/11 biallelic persons (63.6%) show STGD1. This penetrance is much higher than our theoretically calculated penetrance of STGD1 (2.4%) due to a single p.Asn1868Ile variant in *trans* with a loss-of-function allele for the entire population.[2] We hypothesize that this difference is due to shared genetic and environmental factors that increase the penetrance of the c.5603A>T variant. Therefore, siblings of probands are more likely affected than random individuals in the population that carry c.5603A>T in *trans* with a loss-of-function variant.

Our previous calculation of reduced penetrance was indeed, as suggested by Allikmets et al.,[1] and explicitly and repeatedly indicated in our manuscript,[2] based on several assumptions. The points raised are addressed below.

First, we agree that there are large differences between European subpopulations, both in nature and frequencies of *ABCA4* alleles. Initially, we employed ExAC allele frequency data of non-Finnish Europeans (nFE), as these most closely matched our population. To address this concern, we additionally collected *ABCA4* allele frequency data from whole exome sequencing performed in 21,559 persons in the Radboud University Medical Center. This included individuals who were clinically suspected to suffer from any inherited disease, and excluded all patients with ocular disease as the medical indication sent in by the ophthalmologists or clinical geneticists. Hereby, the frequency of *ABCA4* variants among these patients should match the frequency in the general population, not enriched for *ABCA4* variants. More than 90% of this cohort, coined "SE-NL" (South-East Netherlands), consists of persons with ancestry in the Netherlands (Astuti GDN, Gilissen C, unpublished data, 2018), which mirrors the STGD1 cohort published before.[2] We performed similar calculations as described earlier, i.e., we summed up the allele frequencies of *ABCA4* protein-truncating alleles (nonsense, frameshift, canonical splice site variants), and we added the sum allele frequency of the non-canonical splice site variants, which previously were shown to result in severe splice defects[5] (Supplementary Table S1). There is one difference compared to our previous calculations. Assuming that the frequent non-canonical splice site variant c.5714+5G>A (p.[=, Glu1863Leufs*33])[5] displays a severe effect in only a small proportion of alleles,[6] we included 20% of the observed alleles in our calculation (see Supplementary Table S1). The total frequency of 'loss-of-function' alleles in SE-NL thereby is 0.00195, which is somewhat lower than the sum frequency of these alleles in nFE ExAC (0.00265; Supplementary Table S2).

The allele frequency for the 'single-variant' allele c.5603A>T (p.Asn1868Ile), minus the allele frequencies of c.[2588G>C;5603A>T] and c.[5461-10T>C; 5603A>T] was 0.0667, which is a bit higher than its frequency based on nFE ExAC numbers (0.0645).[2]

Second, Allikmets et al.[1] argued that STGD1 prevalence could be much higher than the very crude estimate made by Blacharski,[7] (i.e., 1:10,000). This also may be the case in the Netherlands, which would influence our calculations significantly. There are no accurate prevalence number for STGD1 in the Netherlands. With a population of 17.15 million, 1:10,000 equals ~1715 STGD1 cases in the Netherlands. The Department of Ophthalmology in the RadboudUMC in Nijmegen is the main referral center for STGD1 in the Netherlands and has knowledge of 300 well-genotyped STGD1 cases (in 250 families; Runhart EH, Lambertus S, Bax NM, Valkenburg D, Hoyng CB, unpublished observations, 2018). Based on RD5000 database[8] entries, and according to our best estimate, there could be a maximum of 1200 STGD1 cases in the Netherlands, which points to a prevalence of less than 1:10,000. Late-onset STGD1 may be underdiagnosed as its clinical appearance overlaps with age-related macular degeneration. We consider this a small effect as we could not identify bi-allelic *ABCA4* cases in 960 genotyped AMD cases (Geerlings M, den Hollander AI, unpublished observations, 2018). Still, to address the uncertainty in STGD1 prevalence, we made two new calculations of the penetrance of a single p.Asn1868Ile variant in *trans* with a loss-of-function allele, one based on our best estimate of STGD1 prevalence in the Netherlands (1:14,290) and one that is two times higher (1:7143). For the calculated number of STGD1 patients, we considered the part of the population older than the median age at diagnosis (42 years), counting 8,6 million persons. Assuming a STGD1 prevalence of 1:14,290, the penetrance is 4.5%; assuming a prevalence of 1:7145, it is 8.9% (Table). When we consider the *ABCA4* variant allele frequencies in the nFE of ExAC, the penetrances are 3.4% and 6.8%, respectively (Supplementary Table S2).

Moreover, the most frequent loss-of-function variant in SE-NL was c.768G>T (p.Leu257Valfs*17)[5] which was found in 25/21,559 (0.12%) of Dutch control persons, in 7/27 families with p.Asn1868Ile in *trans* with a loss-of-function variant, and in 56/250 of all genotyped Nijmegen STGD1 families. Based on the above calculations, we theoretically expect [0.00058 × 0.06668

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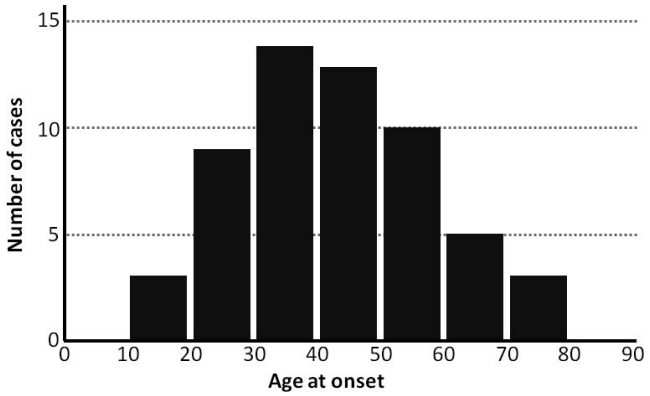

Figure. Age at onset histogram for 57 STGD1 cases carrying c.5603A>T (p.Asn1868Ile) in *trans* with a severe *ABCA4* variant.[2,4] The mean age at onset is 41.9 years (standard deviation 15.0).

× 2 × 8,600,000] 665 persons to carry c.768G>T and c.5603A>T in a compound heterozygous manner. Assuming that the Nijmegen STGD1 cohort, in which we found 10 cases,[2] encompasses one quarter of STGD1 cases in the Netherlands, the total number of observed cases in the Netherlands would be ~40, illustrating a huge (16-fold) discrepancy between expected and observed numbers of cases, which is in line with the calculations above for all loss-of-function mutations in *ABCA4*.

Third, Allikmets et al.[1] state that nonsense mutations may not result in a complete loss-of-function. Most of the known examples of nonsense mutation-mediated splicing defects are single-exon skipping events as shown for nonsense mutations found in *OAT* and *CEP290* in gyrate atrophy and early-onset retinal dystrophy cases, respectively.[9–11] Skipping of the majority of single *ABCA4* exons (33/50) would result in a shift of the open reading frame. The resulting mRNA would then either be degraded through nonsense-mediated decay or the translated protein would be truncated and non-functional. Skipping of one of the remaining 17 exons would not lead to a frameshift but to the absence of transmembrane or nucleotide binding domains and thus to a non-functional protein.

As stated by Allikmets et al.,[1] the alleles in *trans* to p.Asn1868Ile found in STGD1 cases can be considered loss-of-function or highly deleterious alleles. We, however, observed exceptions in our series. We found c.5714+5G>A (p.[=, Glu1863Leufs*33])[5] and c.5882G>A (p.Gly1961Glu), which, based on previous studies, were considered mild alleles, to behave as severe variants in a small percentage of alleles.[6] Nevertheless, assuming that the cases with p.Asn1868Ile are a "litmus test" for severity of the allele in *trans*, we performed an additional calculation in which we included the sum allele frequency of 29 *trans* alleles in SE-NL data set, but excluded the presumed mild alleles (Supplementary Table S1). This sum (0.00063) was added to 0.00195, yielding a total 'loss-of-function' allele frequency of 0.00258. The resulting penetrance scores then are 3.4% and 6.8%, depending on the higher or lower prevalence estimates of STGD1, respectively (Supplementary Table S3). We also calculated the penetrances employing nFE-ExAC data, which are 2.6% or 5.2%, depending on a higher or lower prevalence of STGD1, respectively (Supplementary Table S4). We consider that the actual penetrances in the Netherlands and in Europe are even lower as there are additional 'loss-of-function' missense and deep-intronic variants (Cremers FPM, unpublished observations, 2018),[12] which have not been taken into consideration. The most frequent (moderately) severe 'missense mutation' observed in STGD1 patients is the complex allele p.[Leu541Pro; Ala1038Val] (256 alleles in *ABCA4*-LOVD vs. 140 alleles for

Table. Penetrance Calculations for c.5603A>T in *trans* With Severe *ABCA4* Variants in the Netherlands as Based on a Prevalence of STGD1 in the Netherlands of Either 1:14,290 (I) or 1:7145 (II)

A. Allele Frequencies c.5603A>T in the Netherlands	
c.5603A>T	0.0676945
c.[5641-10T>C; 5603A>T]*	0.0002783
c.[2588G>C; 5603A>T]†	0.0007398
Single variant allele c.5603A>T (p)	0.0666764
B. Allele Frequencies of Severe *ABCA4* Variants in the Netherlands	
Protein-truncating and canonical splice site variants	0.0015539
Severe non-canonical splice site variants‡	0.0003989
Severe *ABCA4* variants (q)	0.0019528
C. Calculated Number of STGD1 Cases With Severe Variant and c.5603A>T in the Netherlands	
Frequency of STGD1 cases with severe variant and c.5603A>T (2pq)	0.000260
Calculated number of STGD1 cases in the NL with severe variant and c.5603A>T (2pq x §)	2240
D. Observed Number of STGD1 Cases With Severe Variant and c.5603A>T in the Netherlands	
Proportion of STGD1 cases in Nijmegen with severe variant and c.5603A>T (a)	0.0833
I. Assuming STGD1 prevalence in the Netherlands to be 0.00007 (b^{I}):	
Prevalence STGD1 cases with severe variant and c.5603A>T (ab^{I})	0.000005831
Observed number of cases with severe variant and c.5603A>T (ab^{I} x ||)	100
II. Assuming STGD1 prevalence in the Netherlands to be 0.00014 (b^{II}):	
Prevalence STGD1 cases with severe variant and c.5603A>T (ab^{II})	0.000011662
Observed number of cases with severe variant and c.5603A>T (ab^{II} x ||)	200
E. Penetrance of c.5603A>T With Severe Variant (observed/calculated) Assuming I.	0.045
Penetrance of c.5603A>T With Severe Variant (observed/calculated) Assuming II.	0.089

* Based on the observation that in an AMD cohort, 10% of individuals with c.2588G>C also carry c.5603A>T.[4]

† Assuming complete linkage disequilibrium between c.5603A>T and c.5641–10T>C (Cremers FPM, unpublished observations, 2018).[2,4]

‡ Based on in vitro splice assays of noncanonical splice site variants.[5]

§ Considering the number of persons in the Netherlands of ≥42 years (8,600,000 persons), which is the median age at diagnosis for STGD1 due to c.5603A>T and a severe *ABCA4* variant.

|| Total Dutch population: 17,150,000 persons in 2017.

c.768G>T),[6] which was found as a *trans* allele in three p.Asn1868Ile cases.[3] As the allele frequency of this complex allele is not present in SE-NL nor in nFE ExAC, we did not include it in our calculations.

Fourth, in the commentary it was stated that modifiers should only be mentioned if these have actually been identified, such as *cis*-modifiers for c.5603A>T. Although we cannot exclude the possibility that as yet unrecognized variants in *cis* with c.5603A>T partly explain the reduced penetrance mentioned above, we consider our findings in STGD1 families, a likely sex imbalance of the cases, and the calculations above, strongly indicate that modifiers do play a big role. To find these modifiers nevertheless will represent a formidable challenge in the next years.

In conclusion, based on the above calculations using actual *ABCA4* allele frequency numbers in the Netherlands, we estimate the penetrance of STGD1 due to p.Asn1868Ile and a loss-of-function allele in *trans*, to be ∼5%. We hypothesize that other genetic or non-genetic factors play a major role in the expression of disease in this STGD1 cases carrying p.Asn1868Ile and a loss-of-function allele. If so, the same modifiers may also explain phenotypic differences between other STGD1 cases that carry the same combination of *ABCA4* alleles.

Frans P. M. Cremers[1,2]
Stéphanie S. Cornelis[1,2]
Esmee H. Runhart[2,3]
Galuh D. N. Astuti[1,4]

[1]Department of Human Genetics, Radboud University Medical Center, Nijmegen, The Netherlands; [2]Donders Institute for Brain, Cognition and Behaviour, Radboud University Medical Center, Nijmegen, The Netherlands; [3]Department of Ophthalmology, Radboud University Medical Center, Nijmegen, The Netherlands; and the [4]Division of Human Genetics, Center for Biomedical Research, Faculty of Medicine, Diponegoro University, Semarang, Indonesia.
E-mail: Frans.Cremers@radboudumc.nl.

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