Quantitative Fundus Autofluorescence in *ABCA4*-Related Retinopathy -Functional Relevance and Genotype-Phenotype Correlation

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• PURPOSE: To investigate lipofuscin-related quantitative autofluorescence measures and their association with demographic characteristics, retinal structure, retinal function and genotype in *ABCA4*-related retinopathy (Stargardt disease 1).

• DESIGN: Cross-sectional study with age-matched healthy control subjects.

• METHODS: A total of 77 patients with *ABCA4*-related retinopathy and 110 control subjects underwent quantitative fundus autofluorescence (qAF) imaging using a confocal scanning laser ophthalmoscope equipped with an internal fluorescent reference to measure qAF as surrogate for lipofuscin accumulation. Measures of qAF were correlated with demographic characteristics, structural alterations on optical coherence tomography and fundus autofluorescence imaging, retinal function assessed by full-field electroretinography (ERG) and fundus-controlled perimetry, and genotype.

• RESULTS: Most patients (76.6%) had qAF levels >95% prediction interval of the age-related control group, with best discrimination between cases and control subjects in younger patients. Reduced discrimination based on qAF measures was associated with mild disease, more advanced disease with dark flecks, or older age because of the physiological age-related increase in qAF and a ceiling effect in patients. Nullizygous patients presented with high qAF levels earlier in life compared with those with at least 1 milder *ABCA4* variant. Within the sectors of qAF measurements, at approximately 7-9° eccentricity, increased qAF without flecks or with only bright flecks was associated with topographically related preserved retinal thickness and fundus-controlled perimetry results, and with normal full-field ERG recordings. All 3 parameters were increasingly abnormal with the development of dark flecks and decreasing qAF.

• CONCLUSIONS: The accumulation of lipofuscin depends on the severity of *ABCA4* variants, precedes other structural changes, and may remain without clinically relevant effect on retinal function. (Am J Ophthalmol 2021;222:340–350. © 2020 Elsevier Inc. All rights reserved.)

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ONE OF THE MOST COMMON FORMS OF INHERITED retinal diseases is caused by biallelic mutations in the adenosine triphosphate (ATP)—binding cassette A4 (*ABCA4*) gene (Online Mendelian Inheritance in Man # 601691).[1–3] The severity of *ABCA4*-related retinopathy may vary considerably, and clinical phenotypes include Stargardt disease, cone-rod dystrophy, and bull's-eye maculopathy.[4] A rather consistent finding in patients and in the *Abca4*$^{-/-}$ mouse model is an increased accumulation of lipofuscin in the retinal pigment epithelium (RPE)[5,6] that results in an increased fundus autofluorescence (AF) intensity upon excitation with blue light.[6–12] The increased AF signal may be evident before the development of pattern-like fundus changes[6,9] and can be measured using quantitative fundus AF (qAF).[10,13] Hence, qAF has been shown to allow discrimination of *ABCA4*-related retinopathy from different retinal diseases that appear similar on clinical examination and other retinal imaging modalities.[12,14,15]

With the prospect of gene therapy and pharmacological approaches that include attempts to lower lipofuscin accumulation,[16–19] outcome measures for clinical trials and features for identifying the therapeutic window are gaining importance in *ABCA4*-related retinopathy.[20–22] In this context, understanding of the functional relevance of morphological alterations is essential. However, detailed studies on the relation of retinal function with AF intensity levels as a surrogate marker for lipofuscin accumulation are currently scarce.[9]

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Herein, we performed an in-depth genotype-phenotype analysis in patients with *ABCA4*-related retinopathy who underwent qAF imaging. Moreover, we assessed potential associations of qAF levels with retina-wide and focal functional measures, using electroretinography (ERG) and fundus-controlled perimetry (microperimetry), respectively.

METHODS

THIS MONOCENTER CASE-CONTROL STUDY WAS performed at the Department of Ophthalmology, University of Bonn, Germany. The study was in adherence with the Declaration of Helsinki. The institutional review board approved the study (Ethikkommission, Medizinische Fakultät, Rheinische Friedrich-Wilhelms-Universität Bonn, #316/11 and #288/17). Written patients' informed consent was obtained after the explanation of the nature and possible consequences of the study.

• **PATIENT CHARACTERISTICS:** The study included 77 unrelated Caucasian patients with *ABCA4*-related retinopathy with a mean age ± standard deviation (SD) of 34.0 ± 14.8 years (range: 9-63 years) from a previously genotyped cohort.[1] The qAF data of a subset of the included patients was published previously in a general overview on qAF imaging in retinal dystrophies,[12] but without detailed correlation of the genotype and functional data. The diagnosis of *ABCA4*-related retinopathy was based on a compatible phenotype and the presence of at least 1 variant in *ABCA4*.[21] Nullizigosity was assumed in the presence of 2 variants with a presumed null effect, including truncating mutations (ie, stop, frameshift), exon deletions, and missense or splice variants that were recently suggested to have a null-like effect.[23,24] To minimize an influence of factors known to alter qAF measurements,[13] exclusion criteria included an age 65 years or older, race other than Caucasian, significant lens opacities, dilated pupil diameter <7 mm, unstable fixation, refractive error larger than ±6 diopters (spherical equivalent), and any other additional known ocular pathology or previous ophthalmic surgery, including cataract surgery.

All patients underwent a complete ophthalmologic examination, including best corrected visual acuity (BCVA) testing using Early Treatment Diabetic Retinopathy Study charts and the protocol of the Comparison of AMD Treatments Trials (CATT) study,[25] slit lamp examination, full-field ERG (Toennies Multiliner Vision 1.70, Hochberg, Germany), indirect ophthalmoscopy, spectral domain optical coherence tomography, and fundus AF imaging (both Spectralis HRA+optical coherence tomography, Heidelberg Engineering, Heidelberg, Germany). The BCVA letter score was converted to logarithm of minimum angle of resolution (logMAR). Before imaging, pupils were dilated using 0.5% tropicamide and 2.5% phenylephrine.

To account for the variable disease manifestation, patients were stratified into 3 different groups based full-field ERG testing according to the classification by Lois and associates.[26] Briefly, group 1 included patients with normal responses on scotopic and photopic full-field ERG testing. Patients with reduced (>2 SDs) photopic B-wave and 30-Hz flicker amplitudes but who had normal scotopic responses were assigned to group 2. Patients who had reduced rod- and cone-driven responses were classified to group 3. This disease grading was previously shown to be associated with disease progression.[27]

• **QAF IMAGING:** qAF imaging was performed in all 77 patients and 110 age-matched healthy control subjects (mean age ± SD: 39.6 ± 14.6 years; range: 8-65 years)[11] without ocular diseases (Supplemental Table 1), as described by Delori and associates.[13] Herein, an internal fluorescence reference was placed in the intermediate retinal plane of a Spectralis HRA (Heidelberg Engineering) to control for fluctuations in detector sensitivity and laser power. Details regarding reference data, emission spectrum, and calibration were published previously by our group.[11] The right eye was used for analysis using a custom-made image analysis program written in IGOR (WaveMetrics, Lake Oswego, OR, USA). In the case of unreliable recordings, for instance, due to poor image quality or anatomical abnormalities, the left eye was used instead (19 patients and 16 control subjects) (Supplemental Table 1). The qAF value of 8 segments that formed a ring with an eccentricity of approximately 7-9° centered to the fovea (0.58-0.78 times the horizontal distance to the temporal border of the optic disc) was calculated from the gray values of the respective segment, and the reference, the offset of the laser, the axial length, and corneal curvature (measured using the IOL-Master 500, Carl Zeiss Meditec, Jena, Germany) assumed lens opacity (based on age-matched normative data)[28] as well as a device-specific calibration factor.[13] Vessels and areas of atrophy were excluded based on histographic analysis.[10] The individual qAF_8 value was then computed as the mean of the qAF values of the 8 segments (Supplemental Figure 1).

• **FUNDUS-CONTROLLED PERIMETRY:** For point-wise examination of retinal sensitivity, 73 of the 77 patients (age range: 9-63 years) and 37 age-matched control subjects (not included in the qAF comparison group; age range: 13-64 years) underwent fundus-controlled mesopic perimetry (Macular Integrity Assessment, MAIA, CenterVue, Padova, Italy) (Supplemental Table 1). The acquisition protocol was similar to that previously published.[29] Briefly, after pupil dilatation and 20 minutes of adaptation to the red test background luminance at 1.27 cd/m^2, a custom perimetric test pattern was used (adapted from the foveo-papillary profile of Cideciyan and colleagues[30]) that examined 50 loci (Goldmann III, white, 200 ms in duration, 4-2 staircase strategy, 3.6 log units measurement range), mostly

placed along the horizontal line through the foveal center, covering 30 degrees of the central visual field (Supplemental Figure 1). The patient was asked to maintain fixation on a red central circle of 1° diameter at a preferred retinal locus. On the same day, 2 full perimetric tests were performed with each eye; only the results of the second test were analyzed to minimize learning effects. The in-built confocal scanning laser ophthalmoscope (830 nm, 36 × 36° field) enabled a real-time eye tracking (25 Hz) by retinal landmarks to assure precise topographic light stimuli projection.

For analysis, the microperimetry results were aligned to the qAF images using custom-made software (Multi-Modal-Mapper).[31] The resulting images of each patient were overlaid using Photoshop CS 6.0 (Adobe, San Jose, California). Retinal sensitivity of the nasal and temporal segment of the qAF_8 ring was calculated and compared with qAF values in the respective segment to examine possible structure−function correlations (Supplemental Figure 1).

• **OPTICAL COHERENCE TOMOGRAPHY:** Volume optical coherence tomography scans were recorded with 61 scans covering an area of 25 × 30° (~120 μm distance between the scans), centered on the fovea. Sixteen images per scan were averaged. Segmentation of layers was controlled manually and corrected, if necessary, using the Heidelberg Eye Explorer (Heidelberg Engineering) software. Mean retinal thickness of the eccentric ring of the Early Treatment Diabetic Retinopathy Study grid, which covers the area of the qAF measurement ring, was measured from the internal limiting membrane to Bruch's membrane, and association with qAF was examined. In addition, the outer retinal thickness was measured at 8° eccentricity nasal and temporal to the fovea from the inner border of the outer nuclear layer to the Bruch's membrane to evaluate possible correlations with retinal sensitivity and qAF in the respective segments.

• **STATISTICAL ANALYSIS:** For control subjects, qAF_8 was modeled using age-adjusted linear regression, with log-transformations of qAF_8 and age. The 95% prediction interval was calculated using the standard error of the forecast. The standardized residuals from these models for the nasal and temporal retinal segments, as well as qAF_8, were calculated to form standardized scores for all subjects as described previously.[15] Comparisons between men and women, between cases and control subjects, between genotypes, between ERG-based groups, and according to BCVA were then performed using univariable linear regression of the standardized qAF_8 scores, as were analyses of the associations between qAF and both retinal thickness and retinal sensitivity in specific sectors. An interaction term between quartiles of the age of disease onset (defined the age at which symptoms were first noticed) and age of test was added to the regression model to assess the association between duration of symptoms and qAF_8.

Diagnostic accuracy (sensitivity, specificity and area under the receiver-operating characteristic curve) of qAF_8 for *ABCA4*-related retinopathy was investigated using a standardized qAF_8 score of greater than +2 (representing 2 SDs from the mean of control subjects at each age) as the cut-off.[15] All analyses were conducted using Stata 13.1 (StataCorp, College Station, Texas, USA).

RESULTS

TWENTY-EIGHT (36.4 %) OF THE 77 INCLUDED PATIENTS WERE men and 49 (63.6 %) were women (Supplemental Table 1). Biallelic *ABCA4* variants were present in 74 patients (96.1%), and only monoallelic variants were identified in 3 patients (3.9%). The most common variants were c.[3113C>T; 1622T>C]/p.(Leu541Pro-Ala1038Val) in 16 alleles, c.5882G>A/p.(Gly1961Glu) in 21 alleles, and c.5603A>T/p.(Asn1868Ile) in 23 alleles. The latter was observed in *cis* with the variant c.2588G>C/p.(Gly863Ala) in 8 instances. The group of patients with nullizygous mutations (n = 46) included 4 patients with biallelic nullizygosity.

When disease severity was determined according to retina-wide functional impairment on full-field ERG testing, 36 patients were classified as group 1 (normal scotopic and photopic responses), 26 as group 2 (normal scotopic responses, reduced photopic responses), and 15 as group 3 (reduced scotopic and photopic responses). Accordingly, morphologic changes ranged from circumscribed foveal and/or macular alterations to panretinal disease manifestation. Individual demographic, genetic and clinical data of all patients are provided in Supplemental Table 2.

• **GENERAL OBSERVATIONS USING QAF:** The control population showed the physiological age-dependent increase of qAF_8 levels (Figure 1A), which was comparable to previously published data of an independent cohort.[32] In 59 (76.6 %) of the 77 patients, qAF_8 levels were above the age-related 95% prediction interval of the control group (Figure 1A). In 16 (20.8%) or 2 (2.6 %) patients, qAF_8 levels were within or below the 95% prediction interval, respectively. The distribution of qAF_8 values among patients was fairly similar across age groups, with an apparent ceiling effect of qAF_8 values at approximately 800 AU. Despite the differences in the qAF_8 levels, spatial distribution of qAF levels along the circle at 7-9° eccentricity was comparable between patients and control subjects (Supplemental Figure 2). The qAF_8 scores were similar between men and women for both patients ($P = .12$) and in control subjects ($P = .31$). Discriminatory efficiency was better at a younger age (Figure 1).

The relative difference compared with age-related normal qAF_8 levels could be demonstrated after normalization of individual measures to the regression curve shown in

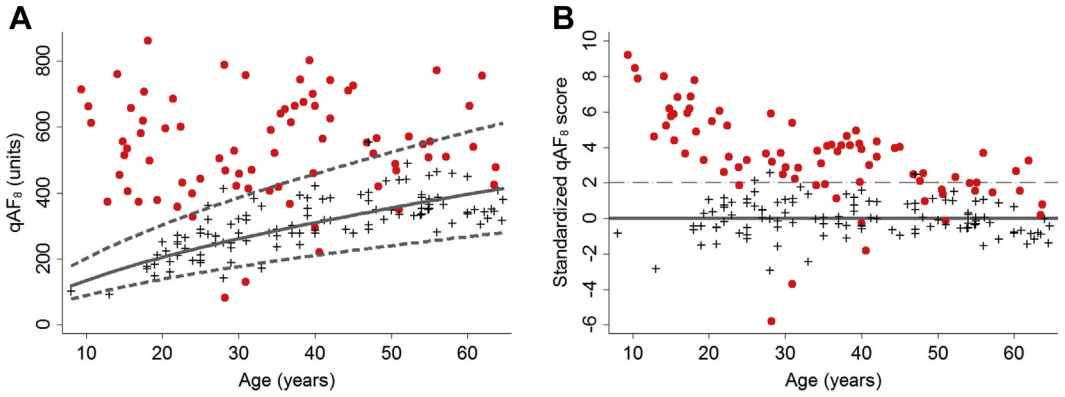

FIGURE 1. Quantitative fundus autofluorescence (qAF_8) in *ABCA4*-related retinopathy. (A) QAF_8 values and standardized (B) qAF_8-scores revealed that patients (red dots) had significantly elevated AF intensity compared with control subjects (black crosses) with highest discrimination in the younger age group. Dashed lines show the (A) 95% prediction interval and (B) 2 SD of control subjects, respectively.

Figure 1A. The resulting standardized qAF_8 score illustrated the –fold difference in SD from the mean of control subjects (Figure 1B).[15] In this sample, a cutoff of 2 SDs allowed to discriminate patients from control subjects with a specificity of 97.3% (95% confidence interval [CI]: 92.2-99.4), a sensitivity of 76.6% (95% CI: 65.6-85.5), and an area under the curve of 0.87 (95% CI: 0.82-0.92). The mean standardized qAF_8 score was greater among patients (mean ± SD: 3.45 ± 2.50; range: −5.79 to 9.21) compared to control subjects (0.00 ± 1.00; range: −2.93 to 2.58) with a mean difference of 3.45 (95% CI: 2.93-3.97).

In patients older than 35 years of age and with qAF_8 scores >2, a late disease onset (30 years or older) was common (11 of 24 patients [45.8%]). None of the patients with early onset of disease (younger than 10 years) and older than 25 years had elevated qAF levels (qAF_8 score >2) (Supplemental Figure 3).

• **MORPHOLOGIC PHENOTYPE AND QAF:** Within the circular area assessed for determining the qAF values, 93.2% of eyes with qAF_8 score >2 (ie, >2 SDs from the mean of control subjects; n = 59) revealed hyper-autofluorescent flecks or an increased background AF without flecks. In contrast, most patients (55.6 %) with a qAF_8 score of <2 (n = 18) showed hypo-autofluorescent (ie, dark) flecks within the region of interest, which represented more advanced fundus changes (Table 1; Supplemental Figure 4). Accordingly, outer retinal thickness was preserved in eyes with elevated qAF in the associated segment, whereas qAF values within and/or below the normal range of control subjects were often associated with thinning of the retinal layers (Supplemental Figure 5).

Six of 8 patients with the less frequent combination of mild phenotype and a qAF_8 score of <2 were older than 50 years of age, when the predictive interval of control subjects distinctly diverged. They had normal scotopic function (4/6) or normal scotopic and photopic retinal function (2/6) on ERG testing, as well as preserved (≤0.2 logMAR; 3/6) or only moderately impaired visual acuity (0.3-0.6 logMAR; 3/6). The remaining 2 patients with this combination were younger (age: 34 and 35 years). Their qAF_8 scores were only slightly below the threshold of 2. All of the exceptional cases with severe phenotype and a standardized qAF_8 score of >2 (n = 4) showed the consistent features of young age (younger than 35 years) and grade 3 ERG-based disease stage. No older patient with a severe phenotype in combination with a standardized qAF_8 score of >2 was observed.

TABLE 1. Relation Between Standardized qAF_8 Scores and Phenotypes Observed on AF Images Within the Eccentric Area of qAF_8 Measurements

	Standardized qAF_8 Score	
AF Phenotype	>2 (n = 59)	≤2 (n = 18)
No AF pattern	30 (51)	5 (28)
Hyper-AF flecks, no hypo-AF flecks	25 (42)	3 (17)
Hypo-AF flecks	4 (7)	10 (56)

qAF = quantitative fundus autofluorescence.
Values are n (%).

• ***ABCA4* GENOTYPE AND QAF:** Nullizygosity of *ABCA4* resulted in high qAF_8 in the first decade of life, ceiling levels at approximately 800 AU in the second decade, and thereafter decreased qAF_8 associated with dark flecks and/or atrophic changes (Figure 2A, red squares).

In contrast, patients with the Gly1961Glu variant in combination with a null mutation *in-trans* seemed to reach qAF_8 ceiling levels at much older age (between 30 and 60 years), or possibly even later (Figure 2B, blue squares). The

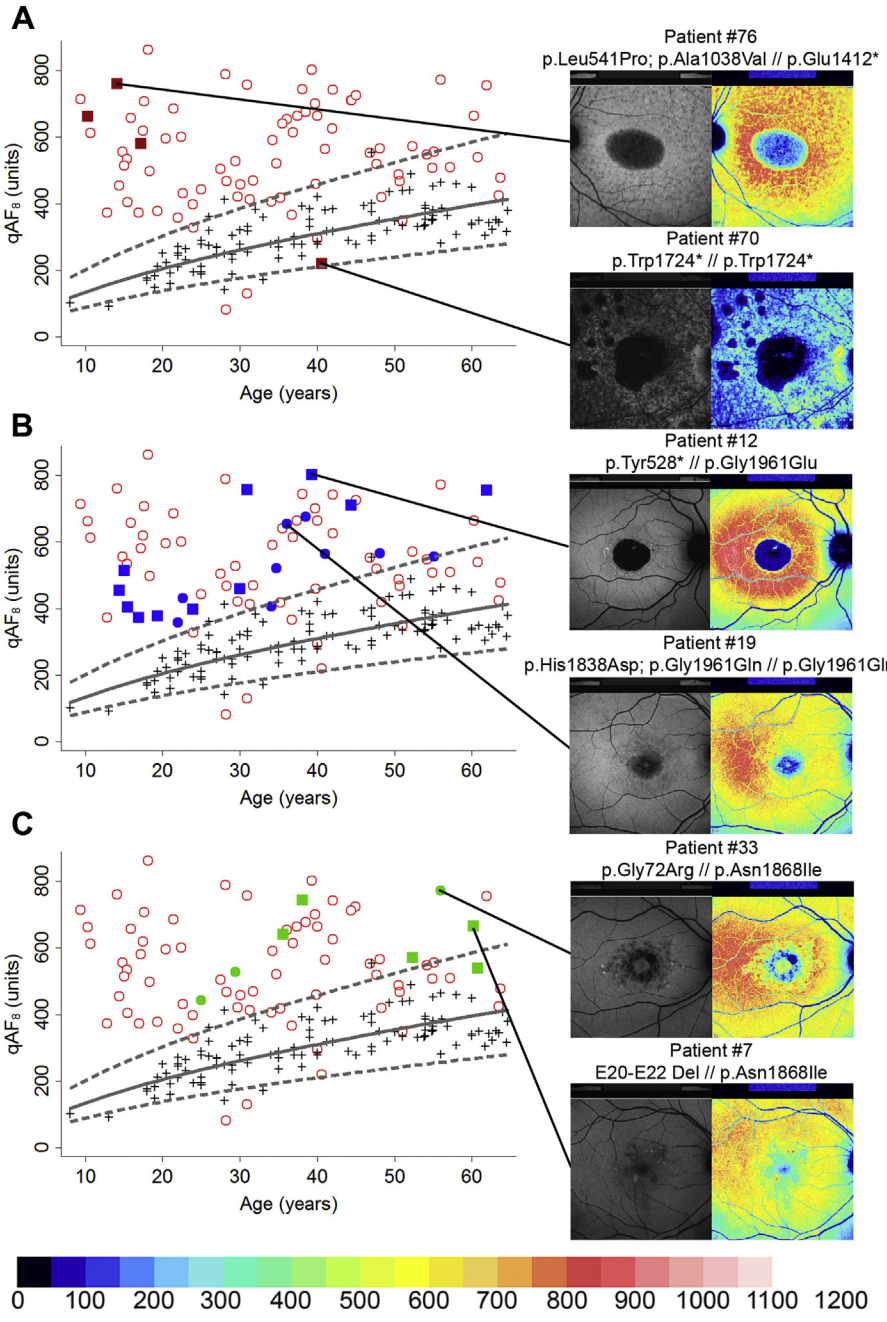

FIGURE 2. Quantitative fundus autofluorescence (qAF_8) and *ABCA4* variants. QAF_8 values as a function of age (left) and exemplary cases (right) for common variants in *ABCA4*. (A) Patients with biallelic null mutations (dark red squares) lead to high qAF levels early in life followed by a fast decline. (B) The Gly1961Glu variant with missense mutation *in trans* (blue dots) results in a slower increase in qAF_8 compared with those with null mutations in trans (blue squares). (C) The mild Asn1868Ile variant is associated with only mild effects on qAF levels (null mutation *in trans*, green squares; missense mutation *in trans*, green dots). Dashed lines show 95% prediction interval of control subjects (black crosses).

combination of the Gly1961Glu variant with another milder mutation resulted in even less but still elevated qAF_8 levels and less macular atrophy at a similar age (Figure 2B, blue dots). Bright flecks were usually only observed within areas of high background AF that, if present, surrounded macular atrophy.

Even milder elevations of qAF_8 levels were observed if 1 allele exclusively harbored the Asn1868Ile variant in combination with a severe mutation *in-trans* (Figure 2C). These patients usually showed no or visually nonsignificant atrophic changes up to the sixth decade. The retinal disease, therefore, was an incidental finding in 63% (5/8 patients).

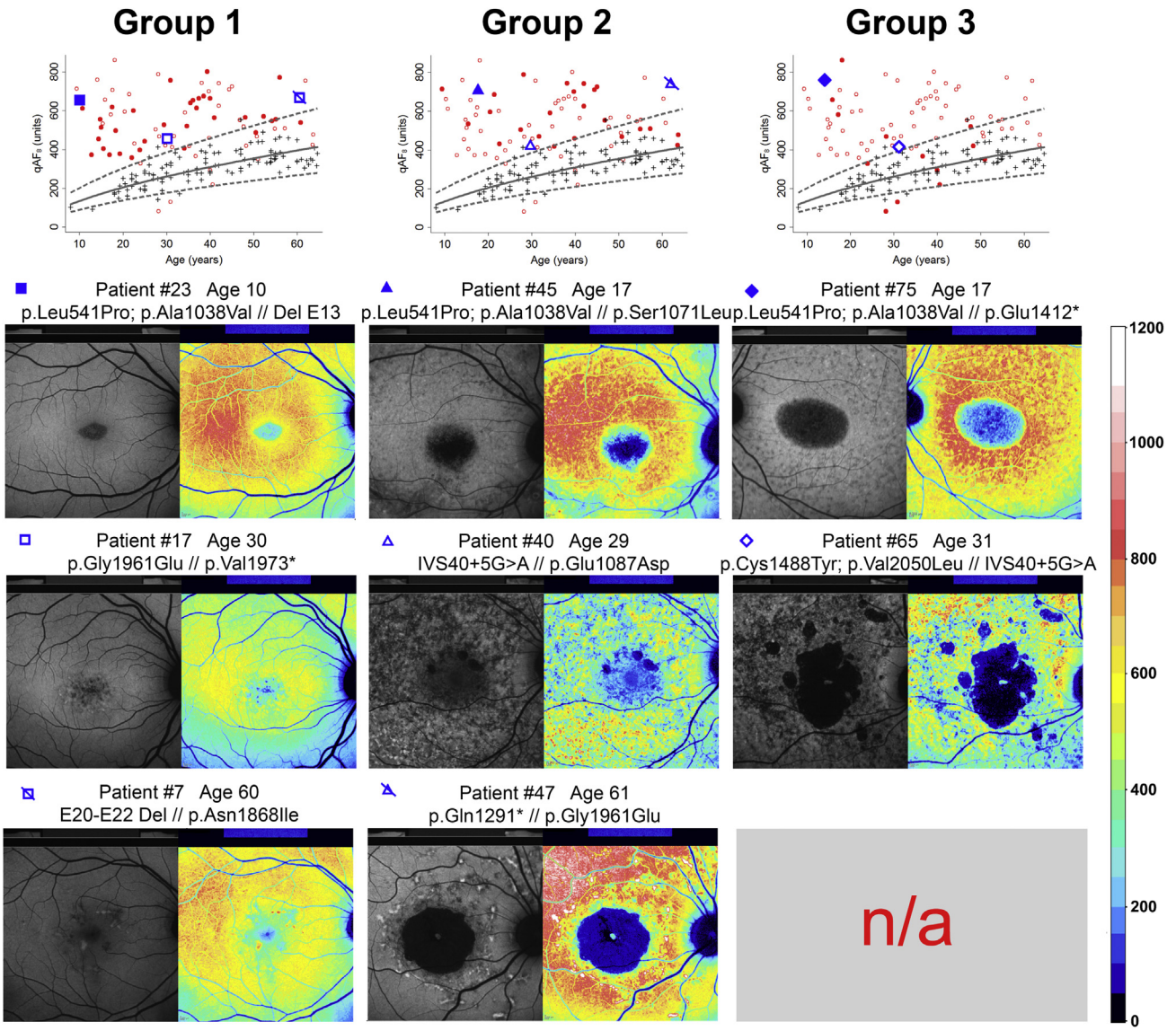

FIGURE 3. Quantitative fundus autofluorescence (qAF_8) and retina-wide function. QAF_8 values (first row) are highlighted as red dots for each group based on findings on full-field electroretinopathy testing. In group 1 (left), qAF_8 values seem to slowly increase with age. Group 2 eyes (middle) show a rather constant distribution of qAF_8 values across age groups. A marked age-dependent decrease of qAF_8 was observed in group 3 (right). Dashed lines mark the 95% prediction interval of control subjects (black crosses). In the second to last row, AF images and corresponding color-coded quantitative qAF images of exemplary patients are shown at similar age and with similar qAF_8 measures. High qAF_8 levels in young patients (second row, blue full symbols) of group 1 were commonly associated with milder foveal involvement and a surrounding homogeneous background with or without flecks of increased AF. In groups 2 and 3, there was an increasingly pronounced macular atrophy with surrounding flecks of increased and mildly decreased AF. In middle-aged patients (age 25-40 years) with a minor increase in qAF_8 (third row, blue open symbols), group 1 eyes usually showed only central retinal irregularities. In contrast, similar qAF_8 levels in groups 2 and 3 were associated with widespread dark flecks and areas of severely reduced AF in this age group (group 2 < group 3), indicating atrophy of the outer retina and the retinal pigment epithelium. High qAF_8 levels in older patients (last row, crossed blue symbols) were only observed in patients with relatively mild or absent retina-wide dysfunction (groups 1 and 2) and at least 1 *ABCA4* variant with mild effect.

Although the exemplary cases in Figure 2 are representative of mutational effects in most patients with similar genetic constellation, few cases were notably different. In 1 patient with the Gly1961Glu variant and 1 case with Asn1868Ile variant, macular atrophy, and paracentral hypo- or hyper-autofluorescent flecks were surrounded by qAF_8 levels in the upper range of normal control subjects. Effects of other, less frequent *ABCA4* variants against the background of a null mutation are shown in Supplemental Figure 6.

• **RETINA-WIDE FUNCTION AND QAF:** Each ERG-based group revealed significantly higher overall standardized qAF_8 scores compared with the control groups ($P < .001$ for each) (Supplemental Table 3), despite different age-dependent distribution and phenotypes (Figure 3 and Supplemental Figures 7-9). Group 1 eyes usually featured a phenotype without qualitative AF alteration within the area assessed for determining the qAF_8 values, group 2 eyes showed more widespread RPE atrophy and flecks, and group 3 eyes typically had advanced atrophic changes and dark flecks. Group 1 and 2 eyes revealed elevated qAF_8 levels across the whole age range, whereas group 3 eyes only featured elevated qAF_8 levels in young patients. Therefore, different ERG-based phenotypes, despite similar elevated qAF_8, could especially be observed at a young age (Figure 3).

A comprehensive genotype-phenotype analysis (Supplemental Figures 7-9) revealed that variants commonly considered to have a mild effect[19] (eg, Asn1868Ile, Gly1961Glu) were typically found in group 1 eyes with moderately and highly elevated qAF_8 in young and old patients, respectively. All but 1 patient older than 23 years had at least 1 allele with such a mild variant. In group 2, these variants were rare and only present in eyes of older patients (older than 35 years) that showed qAF_8 measures in the ceiling range and typically carried a nullizygous variant *in-trans*. In group 3, none of the patients carried these mild *ABCA4* variants. Higher qAF_8 levels at a young age were generally found in eyes of patients with more severe variants on both alleles. In group 1, this genotype was only found in young patients, whereas it was more common in groups 2 and 3. In group 3, biallelic nullizygosity was associated with qAF_8 in the ceiling range early in life (Supplemental Figure 10).

• **FUNDUS-CONTROLLED PERIMETRY AND QAF:** Despite elevated qAF_8 levels, retinal sensitivity in the nasal and temporal qAF segment (based on fundus-controlled perimetry) was preserved in eyes with no qualitative AF alterations or only scattered bright flecks in the respective area. These eyes showed ERG findings assigned to group 1 or less frequently to group 2. Other group 2 eyes with dark flecks, and hence, more advanced structural alterations within the area of qAF_8 measurements showed impaired retinal sensitivity in the respective region. However, the AF intensity was consistently high in these cases. Group 3 eyes showed the most severe disease manifestation (ie, widespread dark flecks and atrophy) associated with the lowest retinal sensitivity. Here, the functional impairment was highly correlated with lower qAF_8 levels (nasal, $P < .001$; temporal, $P = .013$) (Figure 4A; Supplemental Table 4). When qAF, microperimetry, and ERG data were plotted together, a relationship unfolded in which high qAF was present in eyes with good retinal function. Although qAF was still in the upper range, retinal function deteriorated, and a significant reduction of qAF was consistently associated with loss of function (Figure 4B).

• **VISUAL ACUITY AND QAF:** There was no consistent correlation between BCVA and qAF_8 score (0.05 decrease for each 0.1 logMAR unit increase in BCVA; 95% CI: −0.08 to −0.18) in patients with *ABCA4*-related retinopathy because most patients of each group lost central vision early in disease progression, regardless of qAF value. Preserved visual acuity (≤0.2 logMAR; n = 16) was associated with either foveal sparing at higher age (n = 13; all but 2 of those showed late age of onset) or early disease manifestation at young age (n = 3; each of them revealed a disease duration of ≤4 years and was assigned to group 1). All of these patients without significant loss of visual acuity had qAF_8 levels above the age-related 95% perception interval of control subjects (qAF_8-scores >2; $P < .001$), except for patient #50. The latter was the overall second oldest included patient and already had advanced fundus changes, including dark flecks, in the measurement area despite foveal sparing.

DISCUSSION

THIS STUDY CONFIRMS IN A LARGE PATIENT COHORT THAT increased qAF_8 is a consistent feature in *ABCA4*-related retinopathy, as indicated by previous studies and the *Abca4*$^{-/-}$ mouse model.[6,10,11] The ceiling of qAF_8 levels was observed at different ages, and the timepoint seemed to be related to the individual genotype. Patients with 2 truncating *ABCA4* variants appeared to reach ceiling qAF_8 levels in the second decade of life, whereas hemizygosity for the milder Gly1961Glu variant led to similar qAF_8 levels not before the fourth or fifth decade of life.[10] Hemizygosity for the hypomorphic variant Asn1868Ile was associated with only mildly increased qAF_8 levels that usually did not reach ceiling levels even at older age. In accordance with this data, genotype effects on lipofuscin-associated qAF levels could similarly be investigated for other *ABCA4* variants, but larger patient cohorts representing individual variants would be required to obtain robust data. In advanced disease stages with prevailing dark flecks and/or atrophy, qAF decreases again.

In healthy control subjects, qAF_8 levels and variability increased with age.[32] Thus, a score that indicated qAF levels relative to age-matched control subjects (qAF_8 score) allowed to better illustrate relative abnormalities. QAF_8 scores were particularly high in young patients with *ABCA4*-related retinopathy, and only slightly elevated in older patients. Late-onset Stargardt disease could clinically be confused with age-related macular degeneration (AMD).[33,34] However, even slightly increased qAF_8 scores would be uncommon in patients with AMD, and hence, might allow to distinguish these diseases.[35]

Higher qAF levels were not necessarily associated with functional decline. In some patients, qAF_8 was observed to be in the range of ceiling levels, whereas full-field ERG

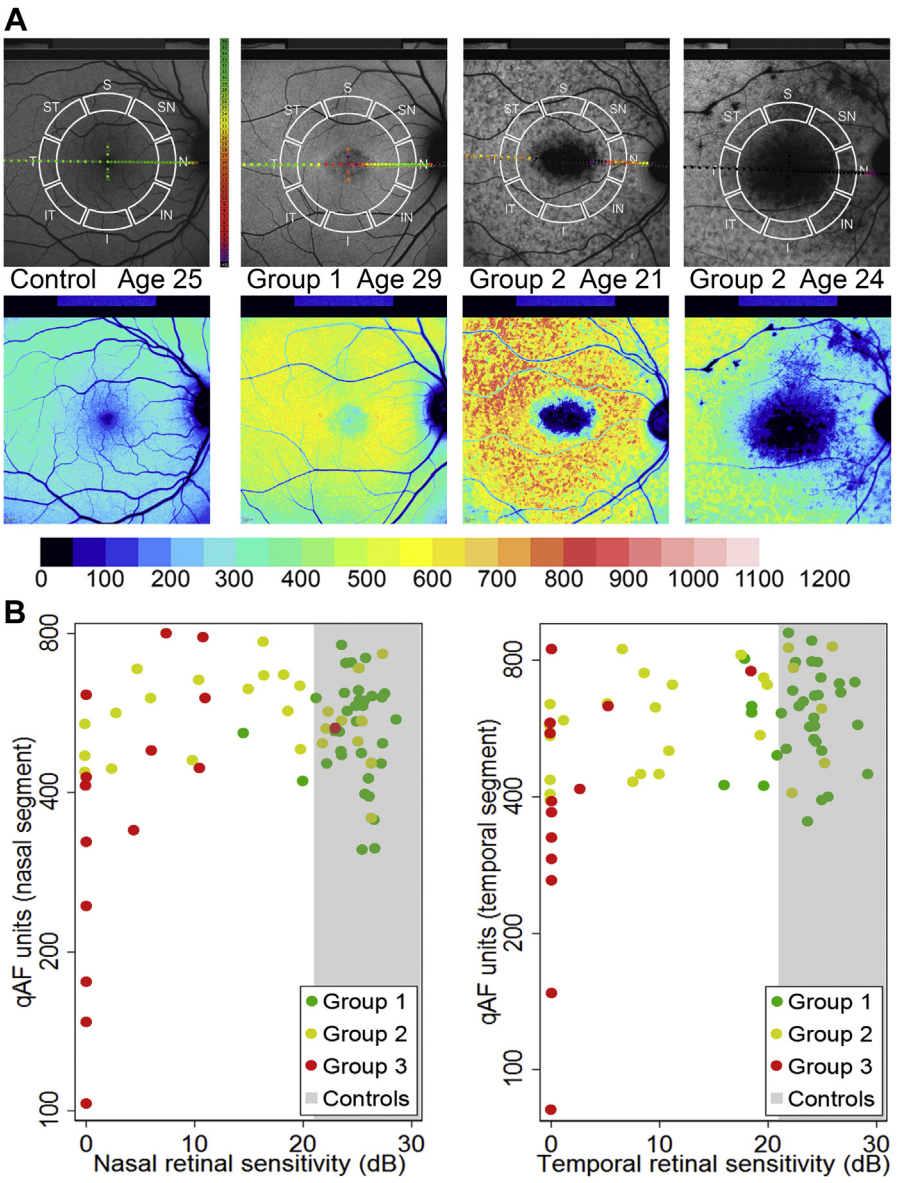

FIGURE 4. Quantitative fundus autofluorescence (qAF) and focal retinal sensitivity. (A) AF images (top row) and corresponding color-coded qAF images (bottom row) of an exemplary control subject (first column) as well as patients assigned to full-field electroretinography (ERG)-based groups (second to last column). AF images are overlaid with the qAF_8 measurement ring segments (white) and the respective functional results of localized retinal sensitivity acquired by fundus controlled perimetry, where green indicates preserved, yellow slightly impaired, red distinctly impaired, and black severe loss of retinal sensitivity. The age (years) of each individual at examination is also provided. (B) The scatter plots demonstrate the association of each individual qAF value and the respective retinal sensitivity in the nasal (left) and temporal (right) segment, color-coded across the 3 ERG-based groups. The gray area represents the range of the fundus-controlled perimetry results of healthy control subjects. Most group 1 (green dots) and some group 2 (yellow dots) eyes showed normal retinal sensitivity despite significantly elevated qAF. Eyes assigned to group 3 revealed severe functional impairment with loss of AF intensity. I = inferior; IN = inferonasal; IT = inferotemporal; N = nasal; S = superior; SN = superonasal; ST = superotemporal; T = temporal.

responses and fundus-controlled perimetry measures were still in the normal range. This observation supports the conclusion by Cideciyan and associates that increased AF intensity would be "the only abnormality that occurred with normal values for all other parameters,"[9] a finding based on semi-quantitative AF assessment and visual field data. Hence, multimodal phenotyping, including qAF imaging, fundus-controlled perimetry, and ERG recordings indicated that *ABCA4*-related retinopathy was characterized by elevated RPE lipofuscin early in the disease course. This concept is recapitulated in the *Abca4*$^{-/-}$ mouse, in which an extensive increase in lipofuscin-related AF precedes photoreceptor loss and functional decline.[6] Overall, functional loss was usually observed with the occurrence of

dark flecks and when qAF_8 declined, although longitudinal data in individual eyes would be required for confirmation.

A commonly used endpoint in current clinical trials for Stargardt disease is the growth of RPE atrophy.[36,37] However, there is the possibility that this functionally relevant parameter assesses the natural disease course at a time point when cell death cannot be prevented or deferred anymore ("point of no return").[38–41] Although qAF is not highly informative with regard to vision loss, qAF measures appear to be a suitable outcome parameter for therapies aimed at reducing lipofuscin accumulation. A sophisticated patient selection would include demographic, genetic, phenotypic, and functional parameters before trial inclusion to predict the expected short-term change in AF intensity. Eyes of young patients, eyes of patients with mild mutation, eyes assigned to ERG-based group 1, as well as eyes with no or mild AF pattern in the area assessed for qAF_8 measurements, are expected to show an increase in future qAF_8 assessment. In contrast, eyes assigned to ERG-based group 3, eyes with qAF levels in the ceiling range, and eyes with severe widespread AF alterations and dark flecks are expected to lose their AF intensity based on our cross-sectional data.

Even if longitudinal data are still pending, a model may summarize the relationship between qAF, retinal function, and genotype (see Supplemental Figure 10 for the most common *ABCA4* variants in this cohort). Retinal function may be well preserved while lipofuscin-related qAF increases up to ceiling levels, which may be reached at different time points depending on disease severity and disease progression. Subsequently, cell death with concurrent loss of function may occur. Assuming qAF_8 levels are close to zero at birth, the average increase of qAF would be about 50 units/year (although this is likely nonlinear) if the ceiling range of approximately 800 AU is reached in the mid-teenage years, which would be expected in a patient with biallelic null variants. In a patient with the Gly1961Glu *in-trans* with a null variant who would reach a similar maximum qAF_8 level at approximately the age of 40 years, the rate would be abound 20 units/year.

A drawback to use qAF as a trial outcome is the variability of recordings. Test-retest variability of ±9.4 % has been observed among healthy eyes in controlled settings.[32] However, real-life, test-retest variability of qAF measurements using current technology might be even higher due to the demanding imaging protocol (eg, optimal photopigment bleaching, camera position, and focus) as well as patient characteristics (eg, media opacities). The consequence of increased variance is decreased statistical power, which necessitates large samples size to show treatment effects. In this context, the inclusion of a lens meter in future study protocols might allow to correct for individual media opacity and to calculate qAF measures more accurately than with currently used corrections based on normative data. Moreover, reliable photopigment bleaching is needed for consistent qAF measurements using 488-m excitation light, which requires extensive light exposure associated with patient discomfort and theoretical negative effects on *ABCA4*-related retinopathy.[42,43] Such limitations might be overcome in the future using longer wavelength excitation light and improved testing protocols.[6,44]

CONCLUSIONS

IN CONCLUSION, WE CONFIRM A CONSISTENT PATTERN of lipofuscin-related qAF-intensity measures in patients with *ABCA4*-related retinopathy that depends on the severity of the genotype. High qAF was associated with no or minimal structural and functional alterations, indicating that the accumulation of lipofuscin had no clinically relevant direct effect on retinal function. The AF intensity may be in the normal range in mild disease, and may be normal or low in advanced cases with degenerative changes and functional decline. Findings on qAF imaging provide an additional diagnostic dimension, but its usefulness as potential outcome measure may require further optimization.

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