Quantitative Fundus Autofluorescence Distinguishes ABCA4-Associated and Non–ABCA4-Associated Bull’s-Eye Maculopathy

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Purpose: Quantitative fundus autofluorescence (qAF) and spectral-domain optical coherence tomography (SD OCT) were performed in patients with bull’s-eye maculopathy (BEM) to identify phenotypic markers that can aid in the differentiation of ABCA4-associated and non–ABCA4-associated disease.

Design: Prospective cross-sectional study at an academic referral center.

Subjects: Thirty-seven BEM patients (age range, 8–60 years) were studied. All patients exhibited a localized macular lesion exhibiting a smooth contour and qualitatively normal-appearing surrounding retina without flecks. Control values consisted of previously published data from 277 healthy subjects (374 eyes; age range, 5–60 years) without a family history of retinal dystrophy.

Methods: Autofluorescence (AF) images (30°, 488-nm excitation) were acquired with a confocal scanning laser ophthalmoscope equipped with an internal fluorescent reference to account for variable laser power and detector sensitivity. The grey levels (GLs) from 8 circularly arranged segments positioned at an eccentricity of approximately 7° to 9° in each image were calibrated to the reference (0 GL), magnification, and normative optical media density to yield qAF. In addition, horizontal SD OCT images through the fovea were obtained. All patients were screened for ABCA4 mutations using the ABCR600 microarray, next-generation sequencing, or both.

Main Outcome Measures: Quantitative AF, correlations between AF and SD OCT, and genotyping for ABCA4 variants.

Results: ABCA4 mutations were identified in 22 patients, who tended to be younger (mean age, 21.9±8.3 years) than patients without ABCA4 mutations (mean age, 42.1±14.9 years). Whereas phenotypic differences were not obvious on the basis of qualitative fundus AF and SD OCT imaging, with qAF, the 2 groups of patients were clearly distinguishable. In the ABCA4-positive group, 37 of 41 eyes (19 of 22 patients) had qAF8 of more than the 95% confidence interval for age. Conversely, in the ABCA4-negative group, 22 of 26 eyes (13 of 15 patients) had qAF8 within the normal range.


Varying definitions of bull’s-eye maculopathy (BEM) have been reported1–4 since the term was introduced in 1966 to describe the fundus appearance of chloroquine retinopathy.5 The classical BEM phenotype presents as concentric parafoveal rings of increased and decreased fundus autofluorescence (AF).6–8 However, the rings in BEM can be less obvious because of mottling, bright or reduced foveal AF, diffuse hyperautofluorescence, or a combination thereof, and the lesion can be round or elliptical.6,8,9 The BEM phenotype also can progress to extensive macular atrophy.9 Some patients who are homozygous for disease-causing mutations in the ABCA4 gene can present with the BEM phenotype.2,4 However, BEM is not specific to ABCA4 and also can be caused by mutations in phototransduction-related genes (e.g., GUCA1A, RPGR),10–11 mutations in a gene (PROM1)12 that encodes a protein involved in outer segment morphogenesis, and mutations in a gene (RIM1)13 that encodes a photoreceptor synaptic protein. Thus, pinpointing the underlying cause of BEM in an individual patient can be challenging.

Advances in noninvasive imaging have facilitated the diagnosis and differentiation of retinal dystrophies. Two imaging methods that have proven especially valuable in this regard are spectral-domain optical coherence tomography...
(SD OCT) and fundus AF. By SD OCT, structural changes of the retina can be studied almost at a cellular level. Fundus AF emerges from retinal pigment epithelium (RPE) lipofuscin,12 a mixture of fluorophores with spectral emission features that reflect the bisretinoid constituents that have been characterized.13 Retinal pigment epithelium lipofuscin accumulates with age in healthy eyes,14,15 and its formation is accentuated in recessive Stargardt disease (STGD1).16–19 Disease-related processes also can alter the distribution of the AF signal. Through image registration, SD OCT and AF can be correlated.

Using quantitative fundus AF (qAF), an approach we developed to measure fundus AF intensities, we recently reported increased qAF levels in STGD1 patients with confirmed ABCA4 mutations.20 In these patients, qAF levels were elevated even at young ages and in qualitatively unaffected-appearing fundus areas. Whether qAF levels also are increased in retinal dystrophies related to mutations other than ABCA4 and to what extent lipofuscin is involved in the pathogenesis of these conditions remains to be determined.

The objective of this study was to investigate whether qAF, a noninvasive approach to measuring RPE lipofuscin in vivo, could aid in distinguishing ABCA4-associated from non–ABCA4-associated disease in BEM, a phenotype that is common to some retinal dystrophies. In addition, we were interested to determine whether, based on qualitative assessment of AF and SD OCT images, a distinction between ABCA4-positive and ABCA4-negative patients is feasible.

Methods

Patients and Genetic Testing

Thirty-seven BEM patients (age range, 8–60 years) from 35 families were recruited prospectively at the Department of Ophthalmology, Columbia University. All subjects were examined by a retinal specialist (S.H.T.) and had clear media except for some floaters. Patients exhibiting the BEM phenotype were selected on the basis of fundus AF images. All patients exhibited a localized macular lesion exhibiting a smooth contour; outside the macula, the retina was qualitatively normal and without flecks. Of the ABCA4-positive patients who have undergone qAF imaging at our institute, approximately 35% exhibited a BEM phenotype without flecks. The criteria of BEM without flecks may have developed at a later stage. Demographic, clinical, and genetic information on the study cohort is presented in Table 1.

The ABCA4 microarray was used for initial screening of most study subjects, followed by direct Sanger sequencing to confirm identified changes, as described previously.21 Because the array screening had been performed over many years, different versions of the ABCA4 chip had been used, from the least representative (approximately 300 mutations) to the recent version of the array (>600 variants). More recently recruited patients and patients in whom the array screening identified only 1 mutated ABCA4 allele or no ABCA4 mutations at all were subjected to next-generation sequencing.

All 50 exons and exon—intron boundaries of the ABCA4 gene were amplified using TruSeq Custom Amplicon protocol (Illumina, San Diego, CA), followed by sequencing on the Illumina MiSeq platform. The next-generation sequencing reads were analyzed and compared with the reference genome GRCh37/hg19 using the variant discovery software NextGENe (SoftGenetics LLC, State College, PA). All detected variants possibly associated with disease were confirmed by Sanger sequencing and analyzed with Alamut software (http://www.interactive-biosoftware.com). Segregation of the new variants with the disease was analyzed in families if family members were available.

All procedures adhered to the tenets of the Declaration of Helsinki, and written informed consent was obtained from all subjects after a full explanation of the study procedures was provided. The study was approved by the Institutional Review Board of Columbia University and complied with the Health Insurance Portability and Accountability Act of 1996.

Image Acquisition

Protocols for the acquisition of AF images that meet the quality standards necessary for quantification have been described previously.14,20,22,23 Fundus AF images (30°; 488-nm excitation) were acquired using a confocal scanning laser ophthalmoscope (Spectralis HRA+OCT; Heidelberg Engineering, Heidelberg, Germany) modified by the insertion of an internal fluorescent reference to account for variations in laser power and detector gain.22 The barrier filter in the device transmitted light from 500 to 680 nm. Before image acquisition, pupils were dilated to at least 7 mm with topical 1% tropicamide and 2.5% phenylephrine. With room lights turned off, a near-infrared reflectance image was recorded first. After switching to fundus AF mode (488-nm excitation; beam power, <260 μW), the camera was moved slowly toward the patient to allow the patient to adapt to the blue light. Patients were asked to focus on the central fixation light of the device. The fundus was exposed for 20 to 30 seconds to bleach rhodopsin,22 whereas focus and alignment were refined to produce a maximum and uniform signal over the entire field. The detector sensitivity was adjusted so that the grey levels (GLs) did not exceed the linear range of the detector (GL, <175).22 Two or more images then were recorded (each of 9 frames, in video format) in the high-speed mode (8.9 frames/second) within a 30°×30° field (768×768 pixels). To assess the repeatability of qAF measurements, a second session of AF images was recorded after repositioning the patient and camera. After imaging, all videos were inspected for image quality and consistency in GLs. For an imaging session, 2 videos were selected to generate the AF images for analysis. At least 4 of 9 frames without localized or generalized decreased AF signal (because of eyelid interference or iris obstruction) and no large misalignment of frames (causing double images after alignment) were considered for each image. The frames then were aligned and averaged with the system software and saved in nonnormalized mode (no histogram stretching). In total, AF imaging was performed for 73 eyes, and for 52 of those eyes, a second AF imaging session was completed. After reviewing the frames of all AF videos, 18 imaging sessions were excluded because image quality was not considered sufficient enough for quantification. Hence, qAF images of 67 eyes, 40 of which had second imaging sessions were included in this study. Quantitative AF images from all eyes included in this study are presented in the Supplemental Appendix (available at www.aaojournal.org).

In addition, a horizontal 9-mm SD OCT image through the fovea registered to a simultaneously acquired AF or near-infrared reflectance (NIR-R) image was recorded in high-resolution mode as an average of 100 individual images for each eye. The optical depth resolution in the Spectralis is currently approximately 7 μm. In cases where the SD OCT image had been registered to an NIR-R image, i2kRetina software (DualAlign LLC, Clifton Park, NY) was used for...
alignment of the AF image of the same field. The assignment of reflectivity bands was based on the study by Spaide and Curcio.24

Image Analysis

Fundus AF images were analyzed under the control of an experienced operator (T.D.) with dedicated image analysis software written in IGOR (WaveMetrics, Lake Oswego, OR) to determine qAF.22 The software recorded the mean GLs of the internal reference and of 8 circularly arranged segments positioned at an eccentricity of approximately 7° to 9° (Fig 1). Segments were scaled to the horizontal distance between the fovea and the temporal edge of the optic disc. If the fovea could not be identified, then its position was estimated based on an SD OCT scan that was registered to an AF or NIR-R image. When a myopic crescent obscured the true edge of the optic disc on AF, an NIR-R image aided in identifying the disc edge (crescent has high reflectance). The software accounted for the presence of vessels and marked atrophy in the segments and automatically excluded segments that were positioned, even partially, at a distance farther than 15° from the center of the image. The mean GLs of each segment then were measured, and qAF for that segment was calculated, taking into account the reference calibration factor, the internal reference GL (0 GL), magnification, and optical media density from normative data on lens transmission spectra.22,24 For each eye, the average qAF from the 8 segments (qAF<sub>8</sub>) from all available images per eye were calculated, as shown in Table 1.

Table 1. Summary of Demographic, Clinical, and Genetic Data

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age (yrs)</th>
<th>Race or Ethnicity</th>
<th>Best-Corrected Visual Acuity (Logarithm of the Minimum Angle of Resolution Units)</th>
<th>Genetic Data</th>
<th>Average of Quantitative Fundus Autofluorescence Values of the 8 Segments</th>
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<tr>
<td></td>
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<td>Right Eye</td>
<td>Left Eye</td>
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<tr>
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</tr>
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<tr>
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<tr>
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<td>1.00</td>
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<tr>
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<td>0.00</td>
<td>0.00</td>
<td>p.G1961E</td>
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</tbody>
</table>

F = female; M = male; ND = not determined; WES = whole-exome sequencing.

<sup>*</sup>Indicates stop codon.

<sup>1</sup>Foveal sparing.

<sup>2</sup>Optical empty lesion.

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Values of the 8 segments is determined using a master image (and from 8 circularly arranged segments (outlined in white). The horizontal distance, FD, between the temporal edge of the optic disc (white vertical line) and the center of the fovea (white cross) was used to define inner and outer radii of the ring of segments (0.58 × FD and 0.78 × FD, respectively). The average of quantitative fundus autofluorescence values of the 8 segments is defined as qAF₈.

(2 or 4, depending on whether a second imaging session was performed) was generated for comparison with other patients. Control values used in this study consisted of previously published data from 277 healthy subjects (374 eyes; age range, 5–60 years) without a family history of retinal dystrophy.14 During the course of the study, the detection aperture of the Spectralis was changed from 6 to 5 mm in diameter to minimize pupil interference. This modification had no effect on qAF measurements because the AF signals of both fundus and reference were similarly affected by the smaller aperture size. The measurements were obtained with different Spectralis devices and internal references; in each case, the reference calibration factor12 was determined using a master fluorescent reference. Correlations between AF and SD OCT images were made by 2 of the authors (T.D., J.R.S.), who reviewed and discussed the images.

Statistical Analyses

Analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA). The qAF₈ values were compared with the 95% confidence intervals (CIs) of healthy subjects of the same age and race or ethnicity.14 To evaluate the repeatability of the measurements between sessions, we used the method described by Bland and Altman13 to compute the coefficient of repeatability (CR, 95% CI) for the differences [log(qAF₈) - log(qAF₈)], where qAF₈ and qAFₛ are the qAF₈ in the 2 sessions. The CR expressed as a percentage of the mean qAF₈ was calculated as CR = 100 × (10CR - 1). The coefficient of agreement between the qAF₈ of the right and left eyes was computed similarly. Sensitivity and specificity of qAF as a test to differentiate between ABCA4-associated and non-ABCA4-associated BEM were calculated using MedCalc (http://www.medcalc.org/calc/diagnostic_test.php).

Results

ABCA4 mutations were identified in 22 patients, including 21 patients (95%) with both disease-causing ABCA4 variants (Table 1). One patient was homozygous and 13 patients were compound heterozygous for the p.G1961E variant. ABCA4 was excluded as the causal gene in 15 patients because no mutations were detected after complete sequencing of the ABCA4 exons and adjacent intronic sequences. ABCA4-positive patients tended to be younger (mean age, 21.9±8.3 years) than ABCA4-negative patients (mean age, 42.1±14.9 years; P<0.0001, unpaired t test). There was no difference in visual acuities between the 2 groups (right eyes, P = 0.88, and left eyes, P = 0.13, unpaired t test). International Society for Clinical Electrophysiology of Vision standardized full-field electroretinography was performed in 16 of 22 ABCA4-positive patients. All of these patients were classified as having isolated maculopathy (normal full-field electroretinography).3,26-29 Of the 15 ABCA4-negative patients, 10 manifested generalized cone dysfunction, 3 had normal electroretinography results, and 2 did not participate in electroretinography testing.

ABCA4-positive and ABCA4-negative patients shared similar AF features (Figs 2 and 3; AF images of all patients are available at www.aaojournal.org). For instance, we found that in both groups of patients, the BEM lesion could be distinctly circular (e.g., ABCA4-positive patients 8 and 9; ABCA4-negative patients 26, 30, 31, 33, and 37) or elliptical (e.g., ABCA4-positive patients 3, 7, and 16; ABCA4-negative patients 27 and 32). The parafovea could be mottled (e.g., ABCA4-positive patients 3, 4, 9, 15, and 17; ABCA4-negative patients 25, 26, 30, and 33), and the hyper autofluorescent ring could be bright with well-defined borders (e.g., ABCA4-positive patients 5 and 7; ABCA4-negative patients 25, 26, and 28), relatively faint (e.g., ABCA4-positive patients 11, 13, 19, and 21; ABCA4-negative patients 27, 29, 30, 31, and 37), or diffuse (e.g., ABCA4-positive patients 18 and 22; ABCA4-negative patients 23 and 34). The fovea also could appear bright (e.g., ABCA4-positive patients 2, 6, 9, and 16; ABCA4-negative patients 30, 32, 33, and 35) or dark (e.g., ABCA4-positive patients 14 and 18; ABCA4-negative patients 25, 27, and 28). Lesion size also varied among the patients, whereas there was a remarkable similarity between fellow eyes with respect to lesion size, shape, and AF pattern.

Quantitative Fundus Autofluorescence

The difficulties inherent in distinguishing patients with and without ABCA4 mutations based on conventional fundus AF images are illustrated in Figure 4, where age-similar ABCA4-negative patients (left) and ABCA4-positive patients (right) are presented side by side. When comparing the corresponding color-coded qAF maps of these patients, however, it becomes immediately notable that ABCA4-positive BEM eyes exhibit higher qAF levels than ABCA4-negative BEM eyes.

This difference in qAF levels also is shown in Figure 5, where the qAF₈ of each eye is plotted versus age and compared with the 95% CIs of healthy subjects.14 ABCA4-positive patients tend to cluster at young ages, whereas ABCA4-negative patients are represented at all ages, with a tendency to cluster at older ages. In the ABCA4-positive group, 37 of 41 eyes (19 of 22 patients) had qAF₈ of more than the 95% CI for age. Conversely, in the ABCA4-negative group, 22 of 26 eyes (13 of 15 patients) had qAF₈ within the normal range. This difference between the ABCA4-positive and ABCA4-negative groups was significant (chi-square [2, n = 67], 37.5; P<0.0001). Of the 2 ABCA4-positive patients who had qAF₈ within the normal range for age, one was homozygous and the other was heterozygous for p.G1916E. Considering only white ABCA4-positive patients.
younger than 30 years, we found that mean \( qAF_8 \) was 366±72 qAF units for patients carrying the p.G1961E mutation and 416±89 for patients carrying variants other than p.G1961E, but the difference was not significant (\( P = 0.2, 2\text{-tailed } t\text{ test} \)). There was no difference in age between \( ABCA4 \)-positive patients who carried the p.G1961E variant and those who did not (\( P = 0.1, \text{unpaired } t\text{ test} \)).

The sensitivity of qAF measurements in this study was calculated as the percentage of \( ABCA4 \)-positive BEM patients with a qAF value of more than the 95% CI for age divided by the total number of BEM patients; sensitivity was 86.4% (95% CI, 65.1%–96.94%). The specificity was 86.67 (95% CI, 59.5%–97.9%), calculated as the probability that a qAF value will be within the 95% CI for age when the patient is \( ABCA4 \) negative.

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**Figure 2.** Fundus autofluorescence images of \( ABCA4 \)-positive patients: (A) patient 12, (B) patient 7, (C) patient 8, (D) patient 4, (E) patient 17, (F) patient 16, (G) patient 21, and (H) patient 1. The internal autofluorescence reference is visible in the top of the image.

**Figure 3.** Fundus autofluorescence images of \( ABCA4 \)-negative patients: (A) patient 36, (B) patient 29, (C) patient 28, (D) patient 27, (E) patient 24, (F) patient 35, (G) patient 26, and (H) patient 34. The internal autofluorescence reference is visible in the top of the image.
For $qAF_e$ of right and left eyes ($n = 30$), the Bland-Altman coefficient of agreement was $15.6\%$. The between-session Bland-Altman CR was $\pm 8.8\%$ (40 eyes of 24 patients). The study cohort had a coefficient of agreement between eyes and a between-session CR that were similar to those of healthy subjects ($\pm 15.3\%$ and $\pm 9.4\%$, respectively), $^{15}$ STGD1 patients ($\pm 13\%$ and $\pm 10.3\%$, respectively), $^{20}$ and Best vitelliform macular dystrophy patients ($\pm 19.2\%$ and $\pm 7.0\%$, respectively). $^{23}$

**Spectral-Domain Optical Coherence Tomography**

Correlations between AF and SD OCT images were made for 73 eyes. Severe nystagmus did not permit acquisition of an AF image for the right eye of patient 25. Fellow eyes of each patient generally had very similar SD OCT findings. In Figure 6, representative SD OCT images of (A) a healthy subject, (B) patient 10, (C) patient 15, (D) patient 14, and (E) patient 25 are shown. The high AF ring surrounding the low AF lesion area correlated in all eyes with the outer limits of a central area of inner segment ellipsoid loss or break-up. The SD OCT features of the low AF lesion area included obvious RPE thinning in 53 eyes (27 patients, 15 of whom were ABCA4 positive), increased choroidal reflectivity in 61 eyes (32 patients, 19 of whom were ABCA4 positive), and variable amounts of hyperreflective debris above the RPE–Bruch’s membrane complex in 53 eyes (28 patients, 15 of whom were ABCA4 positive). An optical empty lesion (OEL) with localized inner segment...
ellipsoid loss at the fovea and an intact external limiting membrane (ELM) throughout the SD OCT scan were present in 10 eyes (5 patients, all ABCA4 positive; Fig 6B). Relative foveal sparing, as shown in Figure 6D, E, was present in 9 eyes (7 patients, 3 of whom were ABCA4 positive). In all eyes except for those of patient 36, the outer nuclear layer (ONL) was thinned in the central part of the lesion and in an adjacent transition zone. In 32 eyes (16 patients, 14 of whom were ABCA4 positive), the ONL was disrupted by hyperreflective dots in the transition zone bordering the central lesion. Interestingly, this feature was most pronounced in patients with an OEL (Fig 6B). In some patients, the ELM appeared more pronounced than in healthy subjects (Fig 6C). At the eccentricities where qAF measurements were obtained (double-headed arrows in Fig 6), abnormalities were observed in at least some eyes in the form of hyperreflective dots at the level of the ONL (Fig 6B), ELM thickening (Fig 6C), or ONL thinning (Fig 6E).

Discussion

In this study, we assessed whether qAF, an indirect measure of RPE lipofuscin, could aid in differentiating ABCA4-positive from ABCA4-negative cases of BEM. Quantitative AF clearly distinguished the 2 groups. Specifically, qAF analysis indicated that ABCA4-positive BEM patients have increased lipofuscin levels throughout the posterior pole, whereas patients with BEM resulting from mutations in other genes have qAF levels within normal limits for age. These data reinforce the clinical usefulness of qAF. Because of the association between ABCA4-related disease and a dark choroid in fluorescein angiography, the latter imaging method also can benefit diagnosis. However, qAF has the advantage of being noninvasive. It is also worth noting that in the case of G1961E mutations in ABCA4, neither fluorescein angiography nor qAF may be helpful imaging methods; a dark choroid is absent in these individuals, and in the presence of the G1961E mutation, qAF values can be within the normal range for age (Fig 5).

The ABCA4 disease pathway is known. Functioning ABCA4 protein is needed to remove N-retinylidene-phosphatidylethanolamine, formed by the binding of retinaldehyde to phosphatidylethanolamine, from the outer segment disc membranes of rods and cones. Failure of this process, such as in STGD1, permits N-retinylidene-phosphatidylethanolamine to accumulate in excess such that a second molecule of retinaldehyde can react with N-retinylidene-phosphatidylethanolamine, leading to the formation of A2E and related bisretinoids. These molecules are phagocytosed and stored in RPE cells as lipofuscin and have potential to cause cellular damage. The disease pathways in ABCA4-negative BEM patients are less clear, and the phenotypic similarities between ABCA4-positive and ABCA4-negative patients currently are difficult to explain. Moreover, although lipofuscin accumulation likely is involved in the primary pathogenesis of ABCA4-related disease, whether this is the case for non–ABCA4-disease is not known.

Although retinal changes were most obvious in the central macula, we also observed ONL thinning in a transition zone at the lesion border in all but 1 patient. In a subgroup of patients, the ONL in the transition zone was altered by the presence of hyperreflective material. The ELM also was thinned outside the central part of the lesion in some ABCA4-positive patients. A case of ELM thickening in a young STGD1 patient without any obvious RPE changes has been reported before, and it was suggested that ELM thickening could be an early disease marker. Although in our study cohort all patients with an OEL were ABCA4 positive, other studies have shown that OEL also can be associated with mutations in other genes, for example, in achromatopsia. We identified several quantifiable features by SD
OCT; however, none of these clearly distinguished \textit{ABCA4}-positive and \textit{ABCA4}-negative cases.

Because all photoreceptors of \textit{ABCA4}-positive cases have a defective visual cycle, it is perhaps not surprising that retinal changes also extended into areas that appeared unaffected on AF imaging. It is interesting to consider, however, why the central macula seems to be more susceptible to the damaging effects of the \textit{ABCA4} mutations than the rest of the retina. One possibility is that certain \textit{ABCA4} mutations, for instance, the p.G1961E variant, may elicit a stronger effect on cones than rods. Although p.G1961E is the most frequent \textit{ABCA4} variant in STGD1 (allele frequency, approximately 10\%),\textsuperscript{2,33} it is still remarkable that 14 of 22 \textit{ABCA4}-positive BEM patients (64\%) carried this variant. It has been noted before, however, that the p.G1961E variant seems to be associated with the BEM phenotype and a milder disease spectrum.\textsuperscript{2,20,32,34} The notion that STGD1 patients with a BEM phenotype have less widespread disease is supported further by Figure 7, which shows the \textit{qAF}\textsubscript{8} levels of the white \textit{ABCA4}-positive BEM patients in comparison with previously published\textsuperscript{20} results from white STGD1 patients with phenotypes other than BEM, including patients with extramacular fundus changes. \textit{ABCA4}-positive BEM patients have higher \textit{qAF} levels than normal subjects (and \textit{ABCA4}-negative BEM patients) but can have relatively low \textit{qAF} levels when compared with other STGD1 patients.

It is perhaps not a coincidence that most \textit{ABCA4}-positive patients in our study clustered at young ages. In STGD1, the BEM phenotype in some may cases be a transient stage, and
eventually more extensive fundus changes will develop in patients. This is illustrated in Figure 8, in which disease progression over 2.5 years is shown for the brother of patient 15, who also carries the complex allele L541P/A1038V. At the initial visit (Fig 8A), this patient readily could have been classified as having BEM. However, we decided not to include the patient in this study because some peripheral flecks were visible already. At subsequent visits 1.2 (Fig 8B) and 2.5 (Fig 8C) years later, the flecks were more pronounced and numerous.

A question that is not only of interest for the interpretation of the AF signal in BEM, but also for other retinal dystrophies, is what may be the basis for the high AF ring that surrounds the low AF part of the central lesion. We found that, similar to retinitis pigmentosa, the inner segment ellipsoid was discontinued at the outer border of or within the high AF ring. Thus, the high AF ring may reflect an accelerated production of bisretinoids within degenerating photoreceptors. As an alternative explanation, Freund et al recently suggested that the high AF ring may result from what they termed a window defect. They proposed that loss of the outer retina may cause less absorption of the AF signal originating from the RPE. However, photopigment, the major absorber of the fundus AF exciting and emitted light, was bleached before image acquisition in our protocol.

A limitation of this study is that retinal layer thicknesses on SD OCT were not analyzed quantitatively. By segmenting retinal layers, one could confirm whether the ONL was thinned throughout the retina or whether the thinning was limited to the central part of the lesion and the adjacent transition zone.

Although finding the definitive causal genes in the ABCA4-negative patients is beyond the scope of this study, we investigated the genetic causality in most ABCA4-negative cases by whole-exome sequencing (7/15 patients) or targeted candidate gene sequencing (4/15 patients). Although these studies have not yielded the causal gene in most cases, we found that (1) the genetic causality varies widely in the ABCA4-negative group; (2) except for one patient with an RPGR mutation, in all cases we excluded known genes that previously have been associated with the BEM phenotype (PROM1, GUCA1A, etc.); and (3) in some cases, we unexpectedly found disease-causing mutations in

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**Figure 7.** Scatterplot showing quantitative fundus autofluorescence in patients homozygous or compound heterozygous for disease-causing mutations in ABCA4. The average of quantitative fundus autofluorescence values of the 8 segments (qAF8) of both eyes of white recessive Stargardt disease (STGD1) patients with a phenotype other than bull’s-eye maculopathy (BEM; white circles)13 are shown together with qAF8 of white ABCA4-positive BEM patients (black circles). For comparison, qAF8 from white healthy subjects14 (grey circles) together with their 95% confidence intervals (CIs; dashed lines) and means (solid line) are shown.

**Figure 8.** Fundus autofluorescence images from 3 visits (A–C) are shown for the brother of patient 15 at the indicated ages, illustrating that patients with a bull’s-eye maculopathy (BEM) phenotype can develop extramacular disease and flecks. This ABCA4-positive patient was not included in the study (because of presence of peripheral flecks), but he also carries the complex allele L541P/A1038V.
known retinal disease genes such as *CRX* and *CNGA3*, which have not been previously associated with the BEM phenotype, suggesting phenotypic expansion (BEM phenotype in genes known to cause a very different disease phenotype). Because almost all of our patients exhibited sporadic disease with no available family members, we were not able to identify the causal gene even by whole-exome sequencing, in most cases because unless one finds clearly pathogenic variants in known disease genes it is impossible to distinguish between many candidate genes that result from whole-exome sequencing studies of a single patient. In summary, we found a very likely causal gene in 5 of 15 cases, excluded known BEM-associated genes in most cases, and identified multiple possible new causal genes in 4 of 15 cases that need detailed follow-up analyses.

A common challenge in the diagnosis of retinal dystrophies is that mutations in different genes can result in very similar phenotypes. Identifying the causal gene has implications with respect to the patient’s prognosis and will be critical when personalized treatment options such as gene therapy become available. Quantitative AF cannot circumvent the need for genetic testing of causal genes such as *ABCA4*. However, clinical criteria such as qAF that provide some indication of how likely a specific gene is the cause of a patient’s phenotype can be a valuable clinical tool. For instance, in patients with a clinical diagnosis of STGD1, complete sequencing of exons and adjacent intronic sequences in the *ABCA4* gene may identify only 1 disease-causing mutation in 15% to 20% of individuals and no mutations in 10% to 15% of individuals. In these groups of patients, qAF analysis can be particularly valuable. Although qAF levels in the normal range do not completely exclude the possibility of *ABCA4* mutations, they substantially increase the likelihood of finding causal mutations in genes other than *ABCA4*.

Quantitative AF is clinically useful to recognize BEM patients with qAF levels higher than the normal limits for age; in these patients, one may expect to confirm the presence of *ABCA4* mutations with genotyping. Thus, the qAF method may guide genetic testing and help to counsel patients at a stage where genetic results are not yet available. Ongoing longitudinal qAF studies involving STGD1 patients hold promise in providing valuable information regarding the natural course of the disease. Quantitative AF also may help to ascertain the role of lipofuscin in different disorders. Whether the qAF approach will be useful as an outcome measure in clinical trials remains to be determined.

**References**

Footnotes and Financial Disclosures

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Abbreviations and Acronyms:
AF = autofluorescence; BEM = bull's-eye maculopathy; CI = confidence interval; CR = coefficient of repeatability; ELM = external limiting membrane; GL = grey level; NIR-R = near-infrared reflectance; qAF = quantitative fundus autofluorescence; OEL = optical empty lesion; qAFs = mean quantitative fundus autofluorescence from the 8 segments; ONL = outer nuclear layer; RPE = retinal pigment epithelium; SD OCT = spectral-domain optical coherence tomography; STGD1 = recessive Stargardt disease.

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