Mutations in GPR143/OA1 and ABCA4 Inform Interpretations of Short-Wavelength and Near-Infrared Fundus Autofluorescence

Maarjaliis Paavo,1 Jin Zhao,1 Hye Jin Kim,1 Winston Lee,1 Jana Zernant,1 Carolyn Cai,1 Rando Allikmets,1,2 Stephen H. Tsang,1,2 and Janet R. Sparrow1,2

1Department of Ophthalmology, Columbia University Medical Center, New York, New York, United States
2Department of Pathology and Cell Biology, Columbia University Medical Center, New York, New York, United States

PURPOSE. We sought to advance interpretations and quantification of short-wavelength fundus autofluorescence (SW-AF) emitted from bisretinoid lipofuscin and near-infrared autofluorescence (NIR-AF) originating from melanin.

METHODS. Carriers of mutations in X-linked GPR143/OA1, a common form of ocular albinism; patients with confirmed mutations in ABCA4 conferring increased SW-AF; and subjects with healthy eyes were studied. SW-AF (488 nm excitation, 500–680 nm emission) and NIR-AF (excitation 787 nm, emission >850 nm) images were acquired with a confocal scanning laser ophthalmoscope. SW-AF images were analyzed for quantitative autofluorescence (qAF). Analogous methods of image acquisition and analysis were performed in albino and pigmented Abca4<sup>−/−</sup> mice and wild-type mice.

RESULTS. Quantititation of SW-AF (qAF), construction of qAF color-coded maps, and examination of NIR-AF images from GPR143/OA1 carriers revealed mosaics in which patches of fundus exhibiting NIR-AF signal had qAF levels within normal limits whereas the hypopigmented areas in the NIR-AF image corresponded to foci of elevated qAF. In patients diagnosed with ABCA4-associated disease, NIR-AF increased in tandem with increased qAF originating in bisretinoid lipofuscin. Similarly in Abca4<sup>−/−</sup> mice having increased SW-AF, NIR-AF was more pronounced than in wild-type mice.

CONCLUSIONS. These studies corroborate RPE melanin as the major source of NIR-AF but also indicate that bisretinoid lipofuscin, when present at sufficient concentrations, contributes to the NIR-AF signal. Ocular melanin attenuates the SW-AF signal.

Keywords: fundus autofluorescence, near-infrared autofluorescence, short-wavelength autofluorescence, GPR143/OA1, X-linked albinism, recessive Stargardt disease, ABCA4

The clinical diagnosis and monitoring of retinal disease often relies on changes in the patterns and intensities of retinal autofluorescence (AF). This autofluorescence is commonly imaged at the fundus by confocal scanning laser ophthalmoscopy (cSLO) with excitation by short-wavelength (SW-AF; 488 nm) light. The cellular source, spectral features, and age relationship of SW-AF are indicative of an origin from visual cycle products that accumulate in RPE as lipofuscin. These bisretinoid fluorophores are a family of retinaldehyde-adducts that form nonenzymatically in photoreceptor cells and are transferred within phagocytosed outer segment membrane to RPE cells. Bisretinoids form in all healthy eyes, but are particularly abundant in recessive Stargardt disease (STGD1) due to mutations in the ABCA4 (ATP-binding cassette transporter 4) gene. A second AF originating in large part from RPE melanin is emitted with near-infrared (787 nm) excitation (NIR-AF).

In the macula of a healthy subject, the distributions of the NIR-AF and SW-AF signals exhibit different patterns. In particular, the NIR-AF emission is high centrally due to increased optical density of melanin in an area approximately 8° in diameter. On the other hand, the SW-AF signal in central macula and especially the fovea is reduced due to absorption of the excitation light by macular pigment. Peripheral to the macula, the NIR-AF and SW-AF signals are relatively uniform.

In addition to the high foveal NIR-AF signal that corresponds to elevated melanin optical density in RPE, the assertion that NIR-AF originates primarily from RPE melanin is based on research showing that the NIR-AF signal is bright within the window-defect created by a full-thickness macular hole. In addition, melanocytic choroidal nevi fluoresce under NIR-AF excitation, as do melanosomes isolated from RPE, cutaneous melanin, and the pigmented epithelium of the iris. Conversely, in the presence of RPE atrophy such as occurs in STGD1 due to mutations in the ABCA4 gene, central lesions present as areas of reduced SW- and NIR-AF due to a loss of RPE.

Nevertheless, the origin of NIR-AF is not fully understood. Bone spicule–like pigmentation in the peripheral fundus of
retinitis pigmentosa (RP) patients can be readily apparent in color fundus photographs while being hypofluorescent in NIR-AF images and nonreflective in infrared reflectance (IR-R) images. Moreover, the prevailing assumption that NIR-AF originates only from melamin is a barrier to an explanation for why bright AF rings in SW-AF images of RP patients are also visible in NIR-AF images (787 nm). Other observations also have been puzzling. For instance, although both modalities are thought to originate from RPE, discrepancies can occur as to the boundaries of atrophy in recessive STGD1, with zones of RPE atrophy being larger in NIR-AF images than in SW-AF images. Why bright AF rings in SW-AF images of RP patients are also origins only from melamin is a barrier to an explanation for the difference was not detectable in healthy control eyes.

As a component of our efforts to advance clinical interpretations of SW-AF and NIR-AF images, we studied NIR-AF and SW-AF images in patients carrying mutations in ABCA4 that confer increased SW-AF intensity. We also examined fundus images acquired from female carriers of GPR143/OA1 mutations that are the most common cause of ocular albinism. Random X-inactivation of the OA1/GPR143 gene in RPE cells of carriers of OA1/GPR143 mutations causes melanin pigmentation in retina to acquire a mosaic pattern. Several mouse lines differing in their content of RPE melanin and lipofuscin also were studied. While confirming that melatin is the major source of NIR-AF, we demonstrated that retinal lipofuscin imaged as SW-AF can contribute to NIR-AF intensities. Conversely, ocular pigmentation modulates SW-AF intensities. To the best of our knowledge, this study is the first to present NIR-AF images and quantitative AF (qAF) of GPR143/OA1 mutation carriers.

METHODS

Patients, Clinical Evaluation, and Genetic Testing

A retrospective analysis was performed of fundus AF images from five carriers of mutations in the X-linked ocular albinism gene GPR143/OA1 (Table 1). Three of the carriers (patient P1, P2, P3) were the daughters of a GPR143/OA1 male proband (P6) who presented to the clinic. The fourth and the fifth carriers (P4, P5) were not related to the aforementioned family. All subjects had a comprehensive ophthalmological examination by a retina specialist and assessment of best-corrected visual acuity. For genetic testing, blood was collected from all patients and genomic DNA was extracted from blood lymphocytes using a standard protocol.

Direct Sanger sequencing revealed that the three sisters from the same family (P1, P2, P3) were all heterozygous carriers of a novel mutation c.461delT (p.Ile154fs) in GPR143/OA1. All nine exons were amplified and directly sequenced, and no other variants were found. Sequence analysis of GPR143/OA1 in P4 disclosed heterozygosity for the c.455+3A>G mutation. Results of genetic testing were not available for P5; the clinical diagnosis was based on iris transillumination and typical RPE mosaicism in the fundus.

P6 (father of P1, P2, P3) was hemizygous for the mutation c.461delT (p.Ile154fs) in GPR143/OA1 and is described in the Supplement.

A prospective analysis of fundus AF images acquired from 25 patients (age range 8.3-51.5 years) with clinically and genetically confirmed diagnosis of STGD1 was carried out. Images of patients with advanced disease (appreciable loss of the NIR-AF signal throughout the 30° field) were not included. Clinical, demographic, and genetic data are presented in Table 1. At least one known mutation in the ABCA4 gene was detected in all patients by direct sequencing. Two (expected) ABCA4 mutations were detected in 22 (88%) of 25 patients. The control group consisted of 15 individuals without a history of eye disease. The mean age was 34.9 years (range 12.7-52.7 years) and 10 subjects identified themselves as Caucasian, 3 as Hispanic, 1 Asian, and 1 African American. Both groups underwent a comprehensive eye examination.

All procedures adhered to the tenets of the Declaration of Helsinki and written informed consent was obtained from all patients after full explanation of the procedures. The study was carried out with the approval of the Institutional Review Board of Columbia University, and all patients were enrolled in accordance with the tenets set out in the Declaration of Helsinki. Informed consent was obtained before enrollment.

Image Acquisition and Analysis in Patients and Subjects

SW-AF (blue AF; 488 nm excitation, 500-680 nm emission) images (55° × 55° and 30° × 30° fields) were acquired with cSLO (Spectralis HRA-OCT; Heidelberg Engineering, Heidelberg, Germany) and saved in normalized mode. SW-AF images for qAF analysis were obtained with a Spectralis modified by the insertion of an internal fluorescent reference. Videos of either 9 or 12 frames were captured in the high-speed mode (8.9 frames/s) and saved in non-normalized mode (i.e., without histogram stretching). Protocols used for qAF have been published.

To calculate qAF and generate qAF maps, gray levels (GLs) in predetermined segments centered on the fovea (7°-9° eccentricity) were normalized to GLs in the internal fluorescent reference while standardizing for the zero GL, the magnification (refraction), and ocular media absorption. Values in the eight segments were averaged to obtain qAFs. The normative database of healthy eyes (574 eyes; age range, 5-60 years) has been described.

NIR-AF images (55° × 55° and 30° × 30° field) were obtained with an HRA2-SLO (Heidelberg Engineering; excitation 787 nm, emission >830 nm; without injection of dye) and a sensitivity of 96. The NIR-AF signal acquired with the HRA2-SLO is more robust than with the Spectralis because in the latter the detection channel is divided between the SD-OCT and NIR-AF modalities. The eye-tracking function was used and 100 single frames were averaged. Images were saved in both normalized and non-normalized mode. The image of the right eye was chosen for analysis, but if that image was not usable, the left eye was analyzed. Images were selected based on sufficient image quality and STGD1 images with extensive flecks or atrophy were not included.

To quantify NIR-AF in 30° × 30° fields, non-normalized images of STGD1 patients (25) and healthy control eyes (15) were imported into an open source image analysis software (Fiji; National Institutes of Health, Bethesda, MD, USA). The straight-line analysis tool was used to draw a horizontal line through the fovea for the full width of the image and GL values were acquired along this horizontal axis. The points on the horizontal axis first presented distance from fovea in pixels and were later converted into micrometers from fovea using the scaling factor (µm/pixel) provided by Heidelberg software. The GL values were then adjusted by subtracting the NIR-AF image GL offset-value generated by the Heidelberg software. GL profiles of STGD1 patients and age-matched healthy eyes were then plotted as a function of distance along the x-axis in the temporal (0 to −4 mm) and nasal (0 to +4 mm) direction relative to the fovea. The profiles were aligned so that the fovea was always at position zero (0).

SD-OCT images were acquired in high-resolution mode (9 mm foveal and volume scans) and registered to a simulta-
neously acquired near-infrared reflectance (IR-R; 820 nm) image using the Spectralis HRA+OCT. The Heidelberg Eye Explorer software was used to determine mean foveal thickness using the central subfield (diameter 1 mm) of the Early Treatment Diabetic Retinopathy Study grid. The control group consisted of 51 females without evidence of eye disease (aged 7–55, mean age 29) of whom 10 identified as Asian, 7 African American, 7 Hispanic, 4 Indian, and 23 white.

Swept-source widefield OCT (12 mm, 9 mm, and 6 mm cube and angiography scans) images were obtained with the Cirrus PLEX Elite 9000 (Carl Zeiss Meditec, Jena, Germany) and en face images adjusted to the level of RPE were created with Cirrus PLEX Elite 9000 software. Color fundus photographs were obtained with a FF 450plus Fundus Camera (Carl Zeiss Meditec). Ultra-widefield high-resolution optomap images were also captured (Optos Daytona; Optos, Inc., Marlborough, MA, USA) in the composite color and AF (excitation 532 nm) mode.

### Mice

Albino *Abca4/Abcr* null mutant mice (*Abca4<sup>−/−</sup>”), homozygous for Rpe65-Leu450, were reared and genotyped<sup>24</sup> (Table 2). Agouti *Abca4<sup>−/−</sup>*/ (*129S-Abca4tm1Ght/J; Rpe65-Leu450) and agouti *Abca4<sup>−/−</sup>* (*129S1/SvImJ; Rpe65-Leu450) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred in-house. Agouti *Rdh8<sup>−/−</sup>Abca4<sup>−/−</sup>* mice (Rpe65-Leu450) were acquired as a gift from Krzysztof Palczewski, Case Western Reserve University (Cleveland, OH, USA). Black *C57BL/6j* and albino *C57BL/6jf<sup>−/−</sup>* wild-type mice (Rpe65-Met<sup>450</sup>) were purchased from The Jackson Laboratory. The research was approved by the Institutional Animal Care and Use Committee and adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

### Table 1. Clinical, Demographic, and Genetic Data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at Visit</th>
<th>Eye Analyzed</th>
<th>Ethnicity</th>
<th>OD</th>
<th>OS</th>
<th>BCVA*</th>
<th>Genetic Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>OA1/GPR143</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>13.5</td>
<td>OA</td>
<td>Hispanic</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>Heterozygous mutation GPR143/OA1 gene, c.461del_T (p.Ile154fs)</td>
</tr>
<tr>
<td>2</td>
<td>17.3</td>
<td>OA</td>
<td>Hispanic</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>Heterozygous mutation GPR143/OA1 gene, c.461del_T (p.Ile154fs)</td>
</tr>
<tr>
<td>3</td>
<td>21.7</td>
<td>OA</td>
<td>Hispanic</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>Heterozygous mutation GPR143/OA1 gene, c.461del_T (p.Ile154fs)</td>
</tr>
<tr>
<td>4</td>
<td>34.6</td>
<td>OA</td>
<td>Asian</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>Heterozygous mutation GPR143/OA1 gene, c.455+3A&gt;G</td>
</tr>
<tr>
<td>5</td>
<td>20.1</td>
<td>OA</td>
<td>Caucasian</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>Not available</td>
</tr>
<tr>
<td>6</td>
<td>43.1</td>
<td>OA</td>
<td>Hispanic</td>
<td>1</td>
<td>1</td>
<td>0.3</td>
<td>Hemizygous mutation GPR143/OA1 gene, c.461del_T (p.Ile154fs)</td>
</tr>
</tbody>
</table>

**STGD1**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at Visit</th>
<th>Eye Analyzed</th>
<th>Ethnicity</th>
<th>OD</th>
<th>OS</th>
<th>BCVA*</th>
<th>Genetic Variants</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14.3</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.7</td>
<td>1</td>
<td>p.G1961E</td>
<td>p.P1380L</td>
</tr>
<tr>
<td>2</td>
<td>8.3</td>
<td>OD</td>
<td>Indian</td>
<td>0.5</td>
<td>0.6</td>
<td>p.R653C</td>
<td>p.G1961E</td>
</tr>
<tr>
<td>3</td>
<td>12.9</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.9</td>
<td>0.9</td>
<td>p.[L541PA1038V]</td>
<td>p.L2027F</td>
</tr>
<tr>
<td>4</td>
<td>17.2</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.1</td>
<td>0.1</td>
<td>p.R1108C</td>
<td>p.Q1412</td>
</tr>
<tr>
<td>5</td>
<td>17.7</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.7</td>
<td>0.7</td>
<td>p.G1961E</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>18.5</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.8</td>
<td>0.9</td>
<td>p.A1773V</td>
<td>p.G1961E</td>
</tr>
<tr>
<td>7</td>
<td>21.6</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.7</td>
<td>0.7</td>
<td>p.G1961E</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>22.6</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.5</td>
<td>0.2</td>
<td>p.R602W</td>
<td>p.N1868I</td>
</tr>
<tr>
<td>9</td>
<td>24.5</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.9</td>
<td>0.4</td>
<td>p.G1961E</td>
<td>p.N96D</td>
</tr>
<tr>
<td>10</td>
<td>25.9</td>
<td>OS</td>
<td>Caucasian</td>
<td>0.7</td>
<td>0.7</td>
<td>p.G1961E</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>27.8</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.7</td>
<td>0.6</td>
<td>p.G1961E</td>
<td>p.C2150R</td>
</tr>
<tr>
<td>13</td>
<td>29.9</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.9</td>
<td>0.9</td>
<td>c.3050+5G&gt;A</td>
<td>p.G1961E</td>
</tr>
<tr>
<td>14</td>
<td>31.6</td>
<td>OS</td>
<td>African</td>
<td>0.1</td>
<td>0.1</td>
<td>p.R1300</td>
<td>p.R2106C</td>
</tr>
<tr>
<td>15</td>
<td>36.3</td>
<td>OS</td>
<td>Caucasian</td>
<td>0.7</td>
<td>0.7</td>
<td>p.N1799D</td>
<td>p.N1868I</td>
</tr>
<tr>
<td>16</td>
<td>45</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.4</td>
<td>0.4</td>
<td>p.R2106C</td>
<td>p.[L541PA1038V]</td>
</tr>
<tr>
<td>17</td>
<td>46.6</td>
<td>OD</td>
<td>Indian</td>
<td>0.3</td>
<td>0.6</td>
<td>c.1938–822_1938–808del15</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>50.7</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.4</td>
<td>0.4</td>
<td>p.R2077G</td>
<td>p.R2107H</td>
</tr>
<tr>
<td>19</td>
<td>51.5</td>
<td>OS</td>
<td>Caucasian</td>
<td>0.1</td>
<td>0.1</td>
<td>p.N1868I</td>
<td>p.P1380L</td>
</tr>
<tr>
<td>20</td>
<td>45.8</td>
<td>OS</td>
<td>Caucasian</td>
<td>0.17</td>
<td>0.17</td>
<td>p.G1961E</td>
<td>p.G1961E</td>
</tr>
<tr>
<td>21</td>
<td>21.9</td>
<td>OD</td>
<td>Caucasian</td>
<td>0.1</td>
<td>0.1</td>
<td>p.G1961E</td>
<td>p.N96D</td>
</tr>
<tr>
<td>22</td>
<td>25.4</td>
<td>OS</td>
<td>Caucasian</td>
<td>0.7</td>
<td>0.7</td>
<td>p.G1748E</td>
<td>p.R2106H</td>
</tr>
<tr>
<td>23</td>
<td>15.2</td>
<td>OS</td>
<td>Caucasian</td>
<td>0.1</td>
<td>0.1</td>
<td>p.G1961E</td>
<td>p.V615A</td>
</tr>
<tr>
<td>24</td>
<td>11.4</td>
<td>OD</td>
<td>Caucasian</td>
<td>1</td>
<td>1</td>
<td>p.[L541PA1038V]</td>
<td>c.768+5G&gt;A</td>
</tr>
<tr>
<td>25</td>
<td>13.5</td>
<td>OD</td>
<td>Caucasian</td>
<td>1</td>
<td>0.5</td>
<td>p.V989A</td>
<td>c. 2918+5G&gt;A</td>
</tr>
</tbody>
</table>

* Best-corrected visual acuity logMAR equivalent.

### Table 2. Mouse Strains Used in These Studies

<table>
<thead>
<tr>
<th>Mouse Line</th>
<th>Melanin Status</th>
<th>Bisretinoid Status</th>
<th>Rpe65–450 Variant</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Abca4&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Albino</td>
<td>Elevated relative to albino <em>Abca4&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Leu-450&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Abca4&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Albino</td>
<td>Elevated relative to agouti <em>Abca4&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Leu-450&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Abca4&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Agouti</td>
<td>Elevated relative to agouti <em>Abca4&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Leu-450&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>Abca4&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Agouti</td>
<td>Elevated relative to agouti <em>Abca4&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Leu-450&lt;sup&gt;+&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C57BL/6j&lt;sup&gt;−/−&lt;/sup&gt;</em></td>
<td>Albino</td>
<td>Reduced relative to black <em>C57BL/6j</em></td>
<td>Met-450&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td><em>C57BL/6j</em></td>
<td>Black</td>
<td></td>
<td>Met-450&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Leucine at position 450.
† Methionine at position 450.
Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Protocols for positioning, pupillary dilation, temperature monitoring, and placement of contact lens have been described. After visual pigment bleaching, fundus images (55° lens) were acquired with a cSLO (Spectralis HRA+OCT) equipped with an incident laser beam of 0.98 mm and an internal fluorescence reference. Nine successive frames were acquired in high-speed mode (8.9 images/s), using a sensitivity of 95 to 100; the frames were averaged and saved in the non-normalized mode. Fundus SW-AF images were analyzed as described and qAF was calculated by calibrating GLs, in predetermined fundus segments, to the GL of the fluorescent reference (GLR). All mice were exposed to only a single occasion of imaging.

High-resolution NIR-AF (787 nm excitation, >830 nm emission) images were acquired at a sensitivity of 105 and after averaging 100 frames with background subtraction, the images were saved in non-normalized mode. GLs were measured using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MA, USA). Between-session coefficient of repeatability (Bland-Altman) of NIR-AF GL values was 3.5%.

**Image Acquisition and Analysis in Mice**

AF images were acquired as described and qAF was calculated by calibrating GLs, in predetermined fundus segments, to the GL of the fluorescent reference (GLR). All mice were exposed to only a single occasion of imaging.

High-resolution NIR-AF (787 nm excitation, >830 nm emission) images were acquired at a sensitivity of 105 and after averaging 100 frames with background subtraction, the images were saved in non-normalized mode. GLs were measured using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MA, USA). Between-session coefficient of repeatability (Bland-Altman) of NIR-AF GL values was 3.5%.

**Image Acquisition and Analysis in Mice**

Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Protocols for positioning, pupillary dilation, temperature monitoring, and placement of contact lens have been described. After visual pigment bleaching, fundus images (55° lens) were acquired with a cSLO (Spectralis HRA+OCT) equipped with an incident laser beam of 0.98 mm and an internal fluorescence reference. Nine successive frames were acquired in high-speed mode (8.9 images/s), using a sensitivity of 95 to 100; the frames were averaged and saved in the non-normalized mode. Fundus SW-AF images were analyzed as described and qAF was calculated by calibrating GLs, in predetermined fundus segments, to the GL of the fluorescent reference (GLR). All mice were exposed to only a single occasion of imaging.

High-resolution NIR-AF (787 nm excitation, >830 nm emission) images were acquired at a sensitivity of 105 and after averaging 100 frames with background subtraction, the images were saved in non-normalized mode. GLs were measured using ImageJ (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MA, USA). Between-session coefficient of repeatability (Bland-Altman) of NIR-AF GL values was 3.5%.

**Figure 1.** GPR143/OA1 carrier (Patient 3). (A) Pseudocolor (Optos System). (B) SW-AF (488 nm) (Heidelberg Spectralis), composite image. Pigmented (blue arrow) and hypopigmented (white arrow) areas are indicated in pseudocolor and SW-AF images.

**Figure 2.** NIR-AF (787 nm) and SW-AF (488 nm) images of GPR143/OA1 carriers. P3 (A, B) and P4 (C, D). (Spectralis, Heidelberg Engineering). Areas in the rectangles in (A-D) are magnified in images in 1–4.
Mouse eyecups (4–6 per sample) were homogenized and extracted for quantitation by HPLC (Alliance system; Waters, Corp, Milford, MA, USA) as previously described.26

Statistics
Analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA, USA) and the statistical tests as indicated.

RESULTS

Carriers of GPR143/OA1 Mutations
The five female carriers of GPR143/OA1 mutations (P1–P5; mean age 21.7, age range 13.5–34.6) were asymptomatic, with the exception of P3 who complained of difficulty seeing in the dark. All carriers had best-corrected visual acuity of 20/20. The three sisters (P1, P2, P3) showed no iris transillumination defects on anterior segment examination; for P4 those data were not available. Patient 5 exhibited iris transillumination on slit-lamp examination.

The five GPR143/OA1 carriers also exhibited nonuniform fundus pigmentation with radially directed hypopigmented streaks stretching from the macular area to the peripheral fundus in SW-AF images (Figs. 1A, 1B) as previously observed.27 The elevated NIR-AF emission typically observed in central fundus of healthy eyes, was less obvious in these individuals (Figs. 2A, 2C). Additionally, in OA1 carriers, subtle patterns in fundus NIR-AF images were visible (Fig. 2). Specifically, the units of the mosaic exhibiting NIR-AF signal (Figs. 2A, 2C) were associated with lower SW-AF (Figs. 2B, 2D), whereas the hypopigmented areas in the NIR-AF image (Figs. 2A, 2C) corresponded to foci of readily detectable SW-AF (Figs. 2B, 2D).

GPR143/OA1 Carriers: Quantitative Fundus AF
To further understand patterns of AF, we quantified SW-AF intensities using qAF protocols. Levels of qAF (7°–9° eccentricity) in GPR143/OA1 carriers fell within the 95% confidence intervals for age-matched healthy eyes (Fig. 3B). Comparison of GPR143/OA1 carriers and age-matched healthy subjects using color-coded qAF maps scaled to a qAF range of 0 to 1200, revealed that in carriers, the pigmented units of the mosaic corresponded to the level of normal AF, whereas the hypopigmented areas exhibited significantly higher focal areas of SW-AF (Fig. 3A).

Carriers of GPR143/OA1 Mutations: SD-OCT and En Face OCT Images
All of the carriers exhibited normal foveal depression with no signs of foveal hypoplasia (Fig. 4). The carriers had a moderately reduced central subfield foveal thickness (mean 253.0 μm ± 18.42 SD, 7.519 SEM) as compared with the control group (mean 265.9 μm ± 18.57 SD, 2.600 SEM), but the difference was not statistically significant (P > 0.05, unpaired two-tailed t-test).

En face fundus images extracted at the level of RPE for the four carriers (P1, P2, P3, P5) revealed reflectivity that was homogeneous (Fig. 4). Melanin is a major reflective element in the RPE; nevertheless, we did not observe transmission into the choroid in association with melanin-deficient patches in the eyes of GPR143/OA1 carriers (Fig. 4). En face images for P4 were not available.

STGD1/ABCA4 Disease: NIR-AF Signal
We have previously reported that SW-AF intensities measured as qAF are considerably elevated in retinal disease caused by...
FIGURE 4. Images obtained from GPR143/OA1 carriers (P1, 2, 3, 4, 5) and a healthy eye (age 29.6 years) by IR-R and horizontal SD-OCT (Spectralis). En face OCT images at levels of RPE are shown (swept-source widefield OCT), and at the bottom an SS-OCT scan showing the segmentation for RPE en face slab.
ABCA4 mutations, and we have speculated as to an accompanying upturn in NIR-AF in these patients. To more fully examine this issue, GL NIR-AF intensities were measured in STGD1 and healthy retina at 1-mm intervals (nine positions) along a horizontal axis beginning at the fovea and proceeding temporally (0 to −4) and nasally (0 to +4) (Fig. 5). As expected, healthy eyes exhibited an increase in NIR-AF signal in a zone approximately 8° (2.3 mm from fovea) in diameter centered on the fovea. Within the same area, NIR-AF signal was reduced in the STGD1 patients because of central atrophy. Outside the fovea, GL values were consistently elevated in the STGD1 cohort, with the differences in GL values between the STGD1 and healthy eyes being statistically significant at all positions (P < 0.05, ANOVA and Sidak’s multiple comparison test) (except at the 4-mm nasal position due to absent NIR-AF signal at the optic disc). The difference was as much as 3-fold (2 mm nasal to fovea).

**Fundus AF in Mice**

Using mice varying in melanin and RPE lipofuscin levels, together with instrumentation (Spectralis HRA+OCT) used in humans, we sought information supporting our observations in the human patients (Fig. 6). We first assessed for relationships among age (2–9 months) and genotype-related increases in RPE lipofuscin (measured as qAF) and increases in NIR-AF. Agouti Abca4−/− mice serve as a model of accelerated formation of bisretinoid lipofuscin; the inadequate clearance of retinaldehyde in these mice results in bisretinoid accumulation that is approximately 3-fold higher in Abca4−/− mice than wild-type (Fig. 6A). As shown in Figure 6, the NIR-AF signal in the agouti Abca4−/− mice having elevated RPE lipofuscin (increased SW-AF) was greater than in agouti Abca4+/+ mice on the same genetic background (Fig. 6A). In those fundus images in which the SW-AF signal in Abca4−/− mice appeared just as bright or brighter than in the Abca4+/+ mouse (Fig. 6A), it is noted that the internal fluorescent reference, visible in the image, was also brighter, indicating that the image was acquired using a greater sensitivity setting. Plots of NIR-AF versus SW-AF (age 2–9 months) in agouti Abca4−/− (72.4 ± 8, SE) versus Abca4+/+ (156 ± 63, SE) mice revealed different slopes (t-test, P < 0.05). Moreover, for the same age interval, the greater change in SW-AF (x-axis) in the agouti Abca4−/−
versus SW-AF for albino wild-type mice (C57BL/6Jc2j, (Abca4
elevated. (C57BL/6Jc2j mice (age 8 months). (NIR-AF signal in the albino
vessions in the agouti carriers, SW-AF intensities were increased, we
also studied albino
Abca4
mice.34,35 The stepwise increase in SW-AF (qAF) in the agouti
escalation as compared with the single knockout
Abca4+/− mice.25 Thus, the more intense SW-AF in the albino would
be due to the greater irradiance received by the RPE cell fluorophores in the eyes lacking melanin and due to light reflected by the sclera.29 By plotting NIR-AF values as a function of SW-AF (age 4 and 6 months) for albino wild-type
Abca4+/− mice, it was apparent that NIR-AF levels did not change when SW-AF intensity was limited to levels less than 1 qAF-unit. It is also notable that NIR-AF was more intense in mice having a black (i.e., C57BL/6J) versus agouti (Abca4+/−) coat color (Fig. 6A versus 6F).

FIGURE 6. Fundus AF and HPLC analysis of mouse eyes. (A) SW-AF (488 nm) and NIR-AF (787 nm) images of mouse fundus. Abca4+/− and wild-type (Abca4+/+) albino mice and mice with an agouti coat color were imaged as indicated. The internal AF reference is visible in the top of the image. Note that in the case of the Abca4+/+ mouse, the fundus has relatively high GLs, but the internal fundus AF reference is also brighter, reflecting lower qAF levels. (B) Analysis of SW-AF and NIR-AF images acquired in mice at indicated ages, SW-AF is quantified as qAF units and NIR-AF as GLs. (B) SW-AF and NIR-AF plotted as a function of age in agouti
Abca4+/− and
Abca4+/+ mice reveal different slopes (t-test, P < 0.05), indicating a greater increase in NIR-AF in mice exhibiting increased SW-AF (Abca4+/−). (C) SW-AF and NIR-AF in agouti
Abca4+/−, Abca4−/−, and
Rdh8+/−/Abca4−/− mice, indicating increases in NIR-AF intensity as SW-AF is elevated. (E) SW-AF and NIR-AF plotted as a function of age in albino
Abca4−/− mice. (F) NIR-AF plotted versus SW-AF for black C57BL/6J and albino C57BL/6J/c2j mice (age 8 months). (G) HPLC quantitation of the bisretinoid A2E in black C57BL/6J and C57BL/6J/c2j mice. (H) Plotting of NIR-AF versus SW-AF for albino wild-type mice (C57BL/6J/c2j, Abca4+/−) and Abca4−/− mice (age 4 and 6 months). Means ± SEM are based on 2 to 11 mice.

mice resulted in a greater change in NIR-AF (y-axis) than in the
gouti
Abca4+/+ mice (Fig. 6B).

We also recorded AF levels in agouti
Rdh8−/−/Abca4−/− double knockout mice. Absence of Rdh8 (retinol dehydrogenase 8) causes bisretinoid levels to undergo additional escalation as compared with the single knockout
Abca4−/− mice.36–38 Consequently, melanin is
organized as it is synthesized.36–38 Moreover, the organization of melanin in the melanosomes is altered.37,38 Consequently, melanin is
synthesized but melanosomes are grossly enlarged and reduced in number in the presence of
GPR143/OA1 mutations39 and the organization of melanin in the melanosomes is altered.57,58

DISCUSSION

The most common form of ocular albinism is caused by mutations in the heterotrimeric G protein–coupled receptor, GPR143/OA1. OA1 loss of function does not impair the activity of tyrosinase, the enzyme controlling the production of melanin, but reduces the expression of premelanosomal protein (PMEL), a major melanosomal structural protein that provides the melanosome fibrillar matrix for melanin polymerization as it is synthesized.56–58 Consequently, melanin is
synthesized but melanosomes are grossly enlarged and reduced in number in the presence of
GPR143/OA1 mutations39 and the organization of melanin in the melanosomes is altered.57,58
This condition contrasts with that in the albino mice that lack melanin synthesis.

Melanin Is the Major Contributor to the NIR-AF of the Fundus

In this study, we have added NIR-AF imaging to the examination of the fundus in GPR143/OA1 carriers. In these individuals, small-scale patches of NIR-AF darkness alternated with similar-sized foci of brightness in the NIR-AF images. The signal for NIR-AF originates from melanin, at least in part, and thus the darkness in the NIR-AF images of GPR143/OA1 carriers corresponded to nonpigmented areas of the fundus, while brightness corresponded to the presence of melanin pigment, with the signal primarily originating in the RPE melanin (as opposed to choroidal melanin). This pattern of AF pigmentation, with the signal primarily originating in the RPE and brightness corresponding to the presence of melanin, supports previous reports indicating that NIR-AF originates from melanin.

NIR-AF Signal Can Undergo Increases in Tandem With SW-AF

In STGD1/ABCA4 patients, we confirmed that the increase in SW-AF intensity was paralleled by a generalized increase in NIR-AF signal. Similarly, the NIR-AF signal in agouti Abca4−/− mice was elevated relative to the NIR-AF signal in agouti Abca4+/+ mice. By way of explaining the enhanced NIR-AF, it is conceivable that NIR-AF may be increased if lipofuscin-containing organelles in the RPE modulate the NIR-AF signal originating from melanin. For instance, the broadband absorbance spectrum of melanin leads to density-dependent self-absorbance of the fluorescence emitted by neighboring melanin molecules. Accordingly, quenching of the NIR fluorescence emission by secondary absorbance could be reduced if the packing density of melanosomes was altered by the interspersion of melanosomes with lipofuscin storage bodies. Under these circumstances, more pronounced release of the fluorescence emission of melanin could occur. Nevertheless, this mechanism does not account for the observation that in albino Abca4−/− mice, fundus NIR-AF intensities were elevated in tandem with increases in SW-AF even in the absence of melanin. The NIR-AF difference in albino Abca4−/− versus albino wild-type mice, both of which are deficient in the tyrosinase gene required for melanin synthesis, indicates that bisretinoid is not stimulating increased melanin synthesis, as has been postulated previously.

Similarly, an increase in numbers of melanosomes paralleling a rise in NIR-AF intensity was not observed in pigmented Abca4−/− mice. An alternative explanation is that bisretinoid can contribute to the NIR-AF signal.

An age-related increase in the perifoveal NIR-AF due to a contribution from increasing levels of lipofuscin could explain the age-related decline in the foveal-to-perifoveal NIR-AF ratio. An increase in NIR-AF intensity also occurs in conjunction with the SW-AF rings that are often observed in the fundus of retinitis pigmentosa patients. The hyperautofluorescent rings in NIR-AF images are unlikely to be due to a change in transmission in the retinal layers that are anterior to the RPE (unmasking), because tissue absorption at wavelengths between 600 and 1300 nm is relatively low. Pigmentary changes corresponding to the distribution of the hyperautofluorescent NIR-AF ring are not observed in color fundus photographs and changes in the thickness of RPE/Bruch’s membrane-attributable hyperreflective band is not visible by SD-OCT as has been reported at the borders of geographic atrophy in AMD.

We note, however, that melanin is the major source of NIR-AF. Indeed, lower levels of RPE lipofuscin, such as those responsible for SW-AF in albino wild-type mice, do not make a detectable difference to NIR-AF intensities probably because the contribution to the NIR-AF signal in the wild-type is minor in comparison with melanin (Fig. 6). This observation is likely applicable to the healthy human eye.

IR-R and Melanin

Melanin is also responsible for IR-R by the RPE monolayer. In fundus images. Although in NIR-AF images of GPR143/OA1 carriers the fundus presented as a mosaic of variable AF, a mosaic of differing reflectivity was not observed in en face OCT images at the level of RPE. Also, in horizontal SD-OCT scans, zones of increased transmission into the choroid were not observed. Thus, the impact of GPR143/OA1 deficiency on the NIR-AF and reflectivity properties of the melanosomes are not the same.

SW-AF Signal Is Attenuated by the Presence of RPE Melanin

With examination of the SW-AF images acquired from GPR143/OA1 carriers, we found that the foci that were less intense in SW-AF images (dark) were associated with bright focal areas in NIR-AF images, indicating that the SW-AF signal is attenuated by the presence of RPE melanin. Conversely, abnormal SW-AF appeared brighter in areas characterized by an absence of melanin. This is consistent with the observation that qAF was elevated in the melanin-free albino mice. Because the absence of pigment allows more light to be transmitted through the iris and through the eye wall posterior to the iris, one can expect intraocular light to be increased. It is also for this reason that subjects having lighter iris pigmentation have reduced contrast sensitivity and larger b-wave amplitudes at all illumination levels. As demonstrated by increased intraocular light in the presence of hypopigmentation (carriers of GPR143/OA1 mutations) or albinism (albino Abca4−/−, C57BL/6j), the SW-AF signal is modulated by variations in the levels of ocular pigment.

This study has clinical implications. The influence of ocular pigmentation on SW-AF intensities is important to an understanding of ethnicity-related differences in qAF values. A limitation of this study was that the cohort of GPR143/OA1 carriers was relatively small. The difference in NIR-AF intensity in agouti- versus black-coated mice may call into question the assumption that RPE cell melanin content does not vary in concert with the melanin concentration in other tissues (e.g., iris, choroid, skin). If this is the case, we will require further study. Nevertheless, NIR-AF imaging was shown to be of practical value for the identification of female GPR143/OA1 carriers and affected patients. SW-AF imaging has become a part of standard care in retinal clinics, whereas NIR-AF imaging is rarely used. Nevertheless, the acquisition of NIR-AF images (HRA2; Heidelberg Engineering) is comfortable for patients because the NIR light is invisible. Diseased versus nondiseased areas of retina can be distinguished in NIR-AF images because of good contrast, and we found that a decline in NIR-AF can be predictive of ellipsoid zone loss in SD-OCT scans. Thus efforts to understand the NIR-AF signal, as shown here, are important.

Acknowledgments

Supported by the National Eye Institute grants EY12951, EY024091, and P30EY019007, and a grant from Research to
Prevent Blindness to the Department of Ophthalmology, Columbia University.

Disclosure: M. Paavo, None; J. Zhao, None; H.J. Kim, None; W. Lee, None; J. Zernant, None; C. Cai, None; R. Allikmets, None; S.H. Tsang, None; J.R. Sparrow, None

References


