RESEARCH ARTICLE

Fundus autofluorescence, spectral-domain optical coherence tomography, and histology correlations in a Stargardt disease mouse model

Yuan Fang1 | Alexander Tschulakow1,2 | Tatjana Taubitz1 | Barbara Illing1 | Antje Biesemeier1 | Sylvie Julien-Schraermeyer1,2 | Roxana A. Radu3 | Zhichun Jiang3 | Ulrich Schraermeyer1,2

1Division of Experimental Vitreoretinal Surgery, Center for Ophthalmology, Institute of Ophthalmic Research, University of Tuebingen, Tuebingen, Germany
2Preclinical Drug Assessment, STZ OcuTox, Hechingen, Germany
3UCLA Stein Eye Institute, Department of Ophthalmology, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

Abstract

Stargardt disease (STGD1), known as inherited retinal dystrophy, is caused by ABCA4 mutations. The pigmented Abca4−/− mouse strain only reflects the early stage of STGD1 since it is devoid of retinal degeneration. This blue light-illuminated pigmented Abca4−/− mouse model presented retinal pigment epithelium (RPE) and photoreceptor degeneration which was similar to the advanced STGD1 phenotype. In contrast, wild-type mice showed no RPE degeneration after blue light illumination. In Abca4−/− mice, the acute blue light diminished the mean autofluorescence (AF) intensity in both fundus short-wavelength autofluorescence (SW-AF) and near-infrared autofluorescence (NIR-AF) modalities correlating with reduced levels of bisretinoid-fluorophores. Blue light-induced RPE cellular damage preceded the photoreceptors loss. In late-stage STGD1-like patient and blue light-illuminated Abca4−/− mice, lipofuscin and melanolipofuscin granules were found to contribute to NIR-AF, indicated by the colocalization of lipofuscin-AF and NIR-AF under the
INTRODUCTION

Stargardt disease with an autosomal recessive inheritance is the most prevalent form of hereditary macular dystrophy. The onset age of Stargardt disease and the rate of vision deterioration vary widely among individuals. Nevertheless, in most patients, Stargardt disease initially manifests with central vision impairment, including dyschromatopsia and central scotomata, in the first or second decades of their life and develops into a poor vision outcome. Stargardt disease is characterized by central (macular) retinal pigment epithelium (RPE) atrophy and the presence of macular and/or peripheral yellowish-white flecks on color fundus photography. About 80% of the cases shows black choroids in fluorescein angiography. A comprehensive diagnosis of Stargardt disease is established by undertaking fluorescein angiography, fundus autofluorescence (FAF), spectral-domain optical coherence tomography (SD-OCT), and electrophysiological assessment (including pattern, full-field, and multifocal electroretinography (ERG)).

Mutations in the ABCA4 gene, a member of the ATP-binding cassette (ABC) transporter gene superfamily A, are responsible for the Stargardt disease 1 (STGD1, OMIM #248200) phenotype. ABCA4 protein, encoded by the ABCA4 gene, is a membrane-associated protein localized in the outer segments (OS) of rod and cone photoreceptors and RPE cells. Biochemical evidence has illustrated that ABCA4 protein translocates N-ret-PE (conjugate of all-trans-retinal and phosphatidylethanolamine [PE]) and 11c-N-ret-PE (conjugate of 11-cis-retinal and PE) to the cytoplasmic side of the disk membrane and RPE endolysosomes, thereby acting in the clearance of all-trans-retinal and 11-cis-retinal.

Lack of functional ABCA4 protein induces the accumulation of N-ret-PE, further resulting in the formation of di-retinoid-pyridinium-phosphatidylethanolamine (A2PE) in the disk, N-retinylidene-N-retinylethanolamine (A2E) and other bisretinoids, the main constituents of lipofuscin in RPE cells.

A2E was proven to be an initiator in the process of blue light-induced apoptosis and necroptosis of RPE cells. Upon exposure to blue light, A2E and iso-A2E generate reactive oxygen species (ROS), which directly damage cells. Photo-oxidation of bisretinoids in RPE cells is known to damage DNA of RPE cells in the presence of A2E epoxides and oxiranes, activating the complement system and altering the transcription of genes for the stress response, apoptosis, and immune response. Recent studies have suggested that the toxicity of 415- to 455-nm blue-violet light generates the highest ROS levels and produces the highest level of mitochondrial dysfunction in A2E-loaded RPE cells. In real life, one source of blue light is digital screens. Studies have suggested that periodic exposure to smartphone-like low-luminance blue light induces photoreceptor atrophy, decreases retinal thickness, and injures neuron transduction in the retina. Additionally, the RPE cells of STGD1 patients with higher oxygen uptake and light absorption by lipofuscin granules are more susceptible to photo-oxidative stress thanagematched controls.

The pigmented Abca4−/− mouse, the first animal model for Stargardt disease, is characterized by delayed dark adaptation, delayed clearance of all-trans-retinal after partial photobleaching using white light, and marked accumulation of A2E and lipofuscin granules in RPE cells. However, with the absence of retinal degeneration, this mouse model manifests rather the pathology of the early stage of Stargardt disease. Considering the deleterious effect of excessive blue light and lipofuscin in the retina, studying clinical ophthalmic photography, retinal function, and histology in blue light-irradiated pigmented Abca4−/− mice may provide insights into developing a phenotype of the advanced stage of Stargardt disease and age-related macular degeneration (AMD).

Here, we investigated retinal function, fundus alterations, and retinal histology in pigmented Abca4−/− mice after blue light illumination (BLI). The results of fundus AF were correlated with the findings from optical coherence tomography (OCT), light and fluorescence microscopy. Mouse data were correlated with the fluorescence microscopy analysis of the peri-macular tissue of a STGD1-like patient.
2 | MATERIALS AND METHODS

2.1 | Mice

Pigmented Abca4−/− mice (129S4/SvJae-Abca4tm1Ght) were purchased from The Jackson Laboratory (Bar Harbor, Maine, USA). Pigmented wild-type (WT) mice (129S2/SvPas) were purchased from Janvier Labs (Le Genest-Saint-Isle, France). In each group, the male-to-female ratio was 1:1. The mice were bred and housed in a 12:12-h light (approximately 50 lux in cages)-dark cycle with food and water ad libitum. All experiments involving animals were designed to conform to German laws governing the use of experimental animals. All procedures were conducted with the approval of the local agency for animal welfare (Einrichtung für Tierschutz, Tierärztlichen Dienst und Labor tierkunde der Eberhard Karls Universität Tübingen, Tuebingen, Germany) and local authorities (Regierungspräsidium Tübingen, Tuebingen, Germany).

2.2 | Blue-light illumination (BLI)

Age-matched pigmented Abca4−/− and WT mice (9-month-old) were intraperitoneally anesthetized with three-component narcosis comprising 0.05 mg/kg of fentanyl, 5 mg/kg of midazolam, and 0.5 mg/kg of medetomidine. The pupils were dilated with a mixture of 0.5% tropicamide and 2.5% phenylephrine hydrochloride. METHOCEL (Omni Vision, Puchheim, Germany) was applied to moisten the cornea. During light illumination, a glass slip was placed on the cornea of the exposed eye. The illuminated eye of each mouse was exposed to blue light (wavelength: 430-nm) at an intensity of 50 mW/cm² for 15 minutes. The nonilluminated eye as a control was covered carefully to shield from stray light. After light illumination, the mice were hypodermically injected with the antidote, a mixture of 1.2 mg/kg of naloxone, 0.5 mg/kg of flumazenil and 2.5 mg/kg of atipamezole and then kept in the dark room before the further investigations.

2.3 | Electroretinography (ERG)

Full-field ERG was performed before and seven days after BLI. Abca4−/− mice were dark-adapted for more than 16 hours before ERG investigation. All procedures were completed in a dark room under the dim red light. Mice were intraperitoneally injected with ketamine (100 mg/kg) and xylazine (5 mg/kg). The pupils were fully dilated, and a pair of gold wire rings serving as positive electrodes were lightly placed onto the cornea of both eyes. The reference electrode was inserted into the skin of the forehead, and the ground electrode was placed subcutaneously near the tail. To evaluate rod photoreceptor function (scotopic ERG), seven strobe flash stimuli were presented in a Ganzfeld with flash intensities from 0.0003 to 25 cds/m² (0.0003, 0.00095, 0.0095, 0.095, 3, 10, 25 cds/m²). After 10 minutes of light adaptation (30 cds/m² white-light), the function of cone photoreceptors (photopic ERG) was investigated using 3 and 10 cds/m² white-flash stimuli. The amplitude of the a-wave was measured from the baseline to the maximum a-wave peak and the b-wave was measured from the trough of the a-wave to the peak of the b-wave. The a-wave shows the function of the photoreceptors, while the b-wave reflects the function of bipolar cells and Müller cells.

2.4 | Fundus autofluorescence image acquisition

Before BLI and seven days after BLI, confocal scanning laser ophthalmoscopy (cSLO: Spectralis HRA+OCT, Heidelberg Engineering, Heidelberg, Germany) was used for fundus AF image acquisition as reported previously.25 The Abca4−/− mice were anesthetized using the three-component narcosis as described earlier. METHOCEL was applied on the surface of the cornea after full dilation of pupils (diameters >2 mm). To apply the Spectralis to the analysis of mouse retina, we fixed a 78-dpt noncontact slit lamp lens (Volk Optical, Inc., Mentor, OH 44060, USA) directly in front of the device. Additionally, a custom-made contact lens (100 dpt) was positioned onto the cornea. The mouse was placed on a three-dimensional platform that is adjustable to acquire clear scanning images.

The near-infrared reflectance (NIR-R) mode was performed first to align the camera and acquire well-focused images centered on the optic nerve head (ONH). Short-wavelength autofluorescence (SW-AF) images and near-infrared autofluorescence images (NIR-AF) were recorded simultaneously with excitation of the 488-nm laser and 788-nm laser, respectively. All the fundus fluorescence images were recorded with 55 and 30°angles of view, a 768 × 768 pixel image size, and a detector sensitivity setting at 100 after the fundus was exposed to the blue laser for 20 seconds. The automatic real-time function was activated for image capture. Fifteen consecutive frames were captured in the video format and then the averaged images were also saved in the “non-normalization” mode for the quantitation of fundus AF. Normalized images as the average of 100 successive frames were also captured. For correlative analysis, the normalized images with different excitations were coded with different colors and merged using Image J software.

2.5 | Image analysis

The quantitation of fundus AF was analyzed by Image J software according to the published method.25 Nonnormalized
images recorded with a 55° angle of view were used for quantitative analysis. Briefly, the mean grey value of each raw nonnormalized image was measured within a circle (200 pixels-radius) concentric to the optic disc center subtracted from the area of the optic nerve head. The grey value of the zero signal affected by ambient light was recorded automatically for each image and was shown as “offset” in the “information panel.” To calculate the corrected grey value of fundus AF images, the “grey value offset” was subtracted from the grey value measured from the area of interest. The corrected grey value was used to represent the AF intensity of the fundus AF image.

2.6 | Optical Coherence Tomography (OCT)

Spectalis HRA+OCT was also used for OCT scans. All OCT images were recorded in the 30° field of view. To measure the retinal thickness in vivo, we performed line scans averaging 100 B-scans vertically through the optic nerve head. Volume scans centered at the optic nerve head were recorded in both the horizontal and vertical directions. At least 32 B-scans were acquired for the volume scan and were registered to NIR-R images or SW-AF images, which were simultaneously recorded to correlate the SW-AF images, NIR-AF images, and OCT scans.

2.7 | Light Microscopy and Transmission Electron Microscopy (TEM)

For histological analysis, the pigmented Abca4+/− and WT mice were sacrificed seven days after BLI between 4 PM and 5 PM. The eyes were marked with a stitch at the 12 o’clock position before enucleation, followed by fixation in 5% glutaraldehyde overnight at 4°C. For embedding the eyes, the eyecups were hemisected vertically through the optic disc after removal of the cornea and lens. One half was embedded for ultra-thin sectioning and was postfixed with 1% osmium tetroxide (OsO4) in 0.1 M cacodylate buffer (pH 7.4), followed by staining with uranyl acetate (UAC). The other half for fluorescence microscopy was only washed with 0.1 M cacodylate buffer. Both halves were dehydrated with a graded series of ethanol and propylene oxide. The specimens were then embedded in Epon. Reagents were purchased from Serva (Heidelberg, Germany), AppliChem (Darmstadt, Germany) and Merck (Darmstadt, Germany). Semi-thin sections (700 nm) stained with toluidine blue were examined by light microscopy (Zeiss Axiosplan2 imaging system; Zeiss, Jena, Germany). Ultra-thin sections (70 nm) were examined by electron microscopy (Zeiss EM 900; Jena, Germany).

2.8 | Quantification of RPE and photoreceptor nuclei

The images of semi-thin sections through the optic nerve head were recorded using a 63× oil objective for quantification of RPE and photoreceptor nuclei. The number of nuclei was quantified within each 200-µm interval starting next to the optic nerve head and continuing superiorly and inferiorly along the vertical meridian. The area from 2-mm superior to 2-mm inferior was included for the analysis. The average of the data from three sections of each eye was obtained as the mean value of each interval.

2.9 | Fluorescence microscopy

The hemisphere without OsO4 fixation or UAC staining was used to examine retinal AF. The semi-thin sections (500 nm) were photographed using a 63× oil objective and a Zeiss Axioplan2 imaging microscope (Zeiss, Jena, Germany). The images were obtained using a lipofuscin filter set (excitation: 370/36 nm; emission: 575/15 nm, 400 nm beam splitter) for lipofuscin-AF23 and a Cy7 filter set (excitation: 708/75 nm; emission: 809/81 nm, 757 nm beam splitter) for NIR-AF.26 Fluorescence images were merged using Image J software.

2.10 | Immunofluorescence staining

The eyeballs were fixed in 4% paraformaldehyde (Carl Roth, Karlsruhe, Germany). Next, they were immersed in 70, 96, and 99% (v/v) ethanol for 1 hours successively for dehydration. Subsequently, the eyecups were cleared in xylol before being embedded in paraffin. Sections (4-µM thick) of the entire retina, with the optic nerve head, were deparaffinized, dehydrated and heated for 2 minutes in citrate buffer at pH 6 for antigen retrieval. The sections were then incubated with the primary antibody goat anti-RPE65 (1:100; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and rabbit anti-Iba1 (1:1000; FUJIFILM Wako, Japan) at 4°C overnight. Cy3 rabbit anti-goat IgG (H+L) and Cy3 goat anti-rabbit IgG (H+L) (Jackson ImmunoResearch, Ely, UK) were applied as secondary antibodies for anti-RPE65 and anti-Iba1, respectively. 4′,6-Diamidino-2-phenylindole (DAPI) was used to counterstain cell nuclei. The slides were cover-slipped with fluorescence mounting medium (Dako Omnis, Denmark) and analyzed using a Zeiss Axioplan2 imaging microscope (Zeiss, Jena, Germany). Contiguous sections were stained with hematoxylin and eosin.
2.11 | Quantification of bisretinoids by High-Performance Liquid Chromatography (HPLC)

Seven days after BLI, the eyes from the Abca4−/− mice were enucleated, immediately snap-frozen and stored at −80°C for further processing. All the tissue preparation was made under dim red light and bisretinoids were extracted from the whole eyes and analyzed by HPLC following published methods.27 Data were presented as picomoles per eye for A2E and milli-absorbance (mAU) per eye for the other bisretinoids: A2PE, dihydro-A2PE (A2PE-H2), and all-trans-RAL-dimer PE conjugates (at-RAL dimer-PE).

2.12 | Human Tissue

The peri-macular tissue of a 72-year-old human donor with a clinical diagnosis of Stargardt disease was obtained from the Foundation Fighting Blindness (Columbia, MD, USA), as previously described.24 In the meantime, a mutation in the peripherin-2 (PRPH2) gene (c.629>G, p. (Pro210Arg)) was identified in the donor. Therefore, the patient has now been characterized in this study as an STGD1-like patient. The tissue was prepared into semithin sections (without OsO4 fixation or UAC staining). To investigate lipofuscin AF and NIR-AF, the sections were examined under a fluorescence microscope. The use of the peri-macular tissue of the human donor in medical research was approved by the Institutional Review Board of the University of Tuebingen (Ethik-Kommission an der Medizinischen Fakultät der Eberhard-KarlsUniversity und am Universitätsklinikum Tübingen, Tuebingen, Germany, approval number 462/2009BO2), and written informed consent was obtained from the donor prior to the study. All experiments were conducted according to the Declaration of Helsinki.

2.13 | Statistics

All results are presented as the means ± SD (standard deviation). The grey value of fundus AF, the retinal thickness, and the amplitudes of the ERG test were analyzed by the paired t test. The number of nuclei from each group was compared using the unpaired t test. All the statistical analyses were performed using two-tailed tests. Statistical significance was set at P < .05. Statistical analysis was performed using SPSS25.0 software (SPSS, Chicago, USA) and GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA).

3 | RESULTS

3.1 | Retinal dysfunction after BLI in Abca4−/− mice

Scotopic and photopic ERG were performed to show the effect of blue light on retinal function before and seven days after BLI (Figure S1A-B). The results showed that blue light decreased the a-wave amplitude by 53.3% (n = 10 eyes; P < .01; paired t test) and 56.3% (n = 10 eyes; P < .001; paired t test) at the intensity of 9.49 cds/m2 and 25 cds/m2 of scotopic ERG, respectively (Figure S1C). Similarly, b-wave amplitudes of scotopic ERG at intensities from 0.095 cds/m2 to 25 cds/m2 were reduced by 46.9% (n = 10 eyes; P < .001; paired t test), 51.8% (n = 10 eyes; P < .001; paired t test), 57.4% (n = 10 eyes; P < .001; paired t test), and 58.4% (n = 10 eyes; P < .01; paired t test) after BLI, respectively (Figure S1D). In photopic ERG, compared with the data before BLI, the b-wave amplitude was diminished by 63.9% at the intensity of 3 cds/m2 (n = 10 eyes; P < .001; paired t test) and by 53.3% at the intensity of 9.49 cds/m2 (n = 10 eyes; P < .01; paired t test) after BLI (Figure S1F). However, the difference in the photopic a-wave amplitude was not statistically significant (Figure S1E). The above results indicated that the current blue light model affects not only the outer retina but also the inner retina.

3.2 | Findings in SW-AF and NIR-AF images

To investigate blue light damage to the retina in Abca4−/− mice, we examined the fundus AF using SW-AF (488 nm) and NIR-AF (788 nm) modalities concurrently. SW-AF was introduced as a noninvasive technique to visualize the accumulation of lipofuscin.28 NIR-AF is thought to originate predominantly from melanin in the RPE.29 Before BLI, fundus AF was homogeneous in the SW-AF images of pigmented Abca4−/− mice. In NIR-AF images, a mottling AF pattern was distributed uniformly in the fundus of all mice (Figure 1A). Seven days after illumination, SW-AF images showed that the optic nerve head (ONH) was surrounded by a demarcated area with the simultaneous presence of hyper-autofluorescence (hyper-AF, relative to background) and hypo-autofluorescence (hypo-AF, relative to background). Additionally, the area was discovered to incline to the superior part of the fundus in all mice. Meanwhile, a corresponding area exhibiting irregular flecks with hyper-AF or hypo-AF was observed in the fundus in all mice (Figure 1B). For analysis of the correlation of AF alterations in SW-AF and NIR-AF imaging after BLI, green-coded SW-AF images and the corresponding red-coded NIR-AF images were
FIGURE 1  Effect of blue light on fundus AF in both SW-AF and NIR-AF modalities in 9-month-old pigmented Abca4−/− mice. A, Representative nonnormalized fundus SW-AF (left) and NIR-AF (right) images were recorded from pigmented Abca4−/− mice before BLI. B, Representative fundus SW-AF (left) and NIR-AF (right) nonnormalized images were recorded from pigmented Abca4−/− mice seven days after BLI. C–E, Correlation analysis of SW-AF and NIR-AF imaging from three pigmented Abca4−/− mice seven days after BLI. The combination of SW-AF (green-color coded) and NIR-AF (red-color coded) images created color composite images (rightmost). Some AF alterations were markedly hyperautofluorescent on SW-AF but were hypoautofluorescent on NIR-AF, appearing green in composite images (boxes labeled 1). Some AF abnormalities were hyperautofluorescent patches in both modalities and appeared orange or yellow on image composition (boxes labeled 2). Dark patches were found in both SW-AF and NIR-AF images, indicating GA (boxes labeled 3). No area was observed with dark patches in SW-AF, but hyper-AF was observed in NIR-AF. F, Measurement of the fundus AF intensity of SW-AF and NIR-AF images from pigmented Abca4−/− mice before and seven days after BLI. Data are expressed as the mean ± SD, n = 8 eyes. Significant differences were analyzed using the paired t test. ***P < .001 and ****P < .0001, before BLI vs after BLI.
It was revealed that a greater part of AF alterations was hyperautofluorescent in SW-AF and hypoautofluorescent (near to background) in NIR-AF and appeared green in merged images. Nevertheless, some AF abnormalities were found as hyperautofluorescent patches in both modalities and appeared orange or yellow in image composition. Furthermore, dark patches of absent AF in SW-AF images corresponded to dark patches in NIR-AF images, indicating geographic atrophy (GA), as typically seen in patients with AMD and Stargardt disease. No area was observed with hypo SW-AF but with hyper NIR-AF (Figure 1C-E). In Figure 1C,E, GA is surrounded by hyper SW-AF. These observations suggest that blue light-induced abnormalities in fundus AF manifest with varying AF intensities in different modalities of fundus AF imaging.

### 3.3 Quantitative analysis of fundus AF

To evaluate the effect of blue light on the distribution of retinal pigments, we measured and corrected the mean grey value of nonnormalized SW-AF and NIR-AF images for quantitative analysis of fundus AF. As shown in Figure 1F, the quantified AF intensity in SW-AF images was decreased dramatically (n = 8 eyes, $P < .0001$, paired $t$ test) after BLI. Similarly, blue light reduced the AF intensity on NIR-AF distinctly (n = 8 eyes; $P < .001$; paired $t$ test).

### 3.4 Retinal thickness measurement using OCT

Total retinal thickness (the distance from the retinal nerve fiber layer (RNFL) to the inner segment/outer segment of photoreceptors (IS/OS)) was measured and plotted every 200-µm intervals beginning from the ONH to superior and inferior positions along the vertical axis in OCT images (Figure 2A). The plot showed that the lesion area caused by blue light was from the 400-µm inferior position to ONH and 700-µm superior position to ONH along the vertical meridian (n = 9 eyes; $P < .05$; paired $t$ test) (Figure 2B). Acute blue light reduced the thickness of the superior retina more dramatically. The decrease in retinal thickness was 14.9-21.3% inferiorly and 17.1-34.8% superiorly (n = 9 eyes; $P < .05$; paired $t$ test).

**FIGURE 2** Comparison of the retinal thickness among 9-month-old pigmented Abca4<sup>−/−</sup> mice before and seven days after BLI. A, Representative cross-sectional OCT images were acquired through the ONH along the vertical axis from a normal 9-month-old pigmented Abca4<sup>−/−</sup> mouse. RNFL: retinal nerve fiber layer; GC: ganglion cell; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; ELM: external limiting membrane; EZ: ellipsoid zone; IS: inner segment; OS: outer segment; IZ: interdigitation zone; RPE: retinal pigment epithelium; BM: Bruch’s membrane. B, The total retinal thicknesses were measured (from RNFL to IS/OS, including both layers) and plotted in pigmented Abca4<sup>−/−</sup> mice before and seven days after BLI. Data are expressed as the mean ± SD, n = 9 eyes. Significant differences were analyzed using the paired $t$ test. *$P < .05$, **$P < .01$, and ***$P < .001$, before BLI vs after BLI.
3.5 | Findings in Optical Coherence Tomography (OCT)

OCT images recorded on volume scans were used to analyze alterations in the retinal structure after BLI. Five categories of lesions invading different retinal layers were highlighted by OCT imaging.

As revealed in Figure 3A, hyperreflective material accumulated within the area of IS/OS from RPE to the external limiting membrane (ELM), which was classified as Category 1. Category 1 lesions corresponded to hyper-AF in both AF modalities and appeared as yellow flecks in merged AF images (Figure 3B,C). Analysis of OCT images also revealed that Category 2 lesions, which can be described as the disruption of IS/OS with loss of the ellipsoid zone (EZ) and ELM, corresponded to hyper-AF in SW-AF (green-colored in merged AF images) (Figure 3D-F). Category 3 lesions were observed as the disruption of IS/OS and the accumulation of hyperreflective material from the RPE layer and extending to the outer nuclear layer (ONL), with the thinning of the ONL (Figure 3G). Importantly, some lesions in Category 3 correlated with green patches on AF image composition and others appeared yellow (Figure 3H-I). Category 4 lesions appeared with the disruption of IS/OS, hyperreflective material accumulating around the RPE layer and distributing into the ONL, and infoldings of the OPL (outer plexiform layer) expanding to the inner nuclear layer (INL) (Figure 3J). Furthermore, lesions in Category 4 appeared as hyper-AF in both SW-AF and NIR-AF modalities, resulting in orange-colored patches in merged AF images (Figure 3K-L). We observed lesions with loss of the ONL, loss of IS/OS, and disruption of the RPE in OCT images, defined as Category 5 lesions (Figure 3M). Loss of the RPE and photoreceptors resulted in the loss of fundus AF and appeared as dark patches in SW-AF and NIR-AF images. Some Category 5 lesions with a high amount of hyperreflective material accumulating around the RPE layer exhibited increased fundus AF in both modalities evidenced by orange-colored patches in merged AF images. Additionally, Category 5 lesions with a lower amount of hyperreflective material showed hyper-AF in SW-AF and appeared as green in AF composite images (Figure 3N,O).

3.6 | Quantification of RPE and photoreceptor nuclei in the pigmented WT and Abca4−/− mice

In WT mice, nuclei quantification revealed no significant decrease in the number of RPE nuclei induced by blue light (n = 5 eyes, P > .05, unpaired t test) (Figure 4A). Blue light induced the marked loss of photoreceptor in the area from 600-µm superiorly to the ONH (P < .05), but not in the inferior retina in WT mice (n = 5 eyes, P > .05, unpaired t test) (Figure 4B). In Abca4−/− mice, blue light decreased the RPE nuclei number pronouncedly in the area from 400-µm inferiorly to the ONH and 800-µm superiorly to the ONH (n = 8 eyes; P < .05; unpaired t test) (Figure 4C), which was contrary to its effect on RPE in WT mice. The decrease in the RPE nuclei number was 66.0-69.0% inferiorly and 50.0-87.5% superiorly. Additionally, the number of photoreceptor nuclei in ONL was significantly reduced in light-illuminated eyes from the 200-µm inferior position to the 600-µm superior position (n = 8 eyes; P < .05; unpaired t test) (Figure 4D). The reduction in the photoreceptor nuclei number reached 25.5-48.7% in the superior hemiretina and 36.4% in the inferior hemiretina.

3.7 | Histological features in pigmented WT and Abca4−/− mice seven days after BLI

Compared with the nonilluminated eyes of WT mice (Figure 5A), the light-illuminated eyes showed the loss of OS/IS and progressive thinning of ONL, which were confined to the superior retina (Figure 5B). After BLI, subretinal nonpigmented cells were observed in the area of photoreceptor loss (Figure 5C), whereas no change was observed in RPE cells after BLI under the light microscope (Figure 5B,C).

The nonilluminated eyes of Abca4−/− mice showed no degeneration of photoreceptors or RPE (thickness: 6-8 µm), as previously described by Charbel Issa et al.23 and shared the similar structure of the retina with the nonilluminated eyes of WT mice (data not shown). In the light-illuminated eyes of the Abca4−/− mice, subretinal pigmented cells were found in the margin of the light-induced lesion where the retina was relatively intact (Figure 5D). As opposed to WT mice, RPE cells were damaged by blue light in Abca4−/− mice and appeared as thinning of the RPE layer (thickness < 3 µm) with vacuole-like structures and irregular shaped nuclei (Figure 5D,E), increased pigmentation in attenuated RPE cells (Figure 5C), multilayers of RPE (Figure 5F), and loss of the RPE layer (Figure 5G-I). Blue light damaged photoreceptors and caused progressive thinning of the retina in Abca4−/− mice as in WT mice. Differently, in Abca4−/− mice, the photoreceptor degeneration was always accompanied by RPE damage caused by blue light (Figure 5E-I). Additionally, in Abca4−/− mice but not in WT mice, the retinal laminar architecture was deformed after blue light exposure, appearing as a rosette-like structure comprising the ONL and residual IS (Figure 5F). The rosette-like structure extended to the OPL (Figure 5C) and even intruded into the INL (Figure 5F) with the presence of different types of RPE damage. After BLI, in the areas with RPE and photoreceptor degeneration in Abca4−/− mice, subretinal pigmented cells with a spherical shape in various amounts were observed to attach to Bruch’s membrane (Figures 5G-I,
FIGURE 3  Correlation between OCT and fundus AF imaging among 9-month-old pigmented Abca4<sup>−/−</sup> mice seven days after BLI. A, Cross-sectional OCT image of Category 1 lesions (hyperreflective material located in the IS/OS). B, Category 1 lesions appear as yellow flecks in the color composite AF image. D, Cross-sectional OCT image of Category 2 lesions (disruption of IS/OS with loss of EZ and ELM). E, Category 2 lesions correspond to the green-colored area in the color composite AF image. G, Category 3 lesions (disruption of IS/OS and hyperreflective material located from the RPE layer and extending to the ONL and thinning of the ONL) were recorded in the OCT image. H, Category 3 lesions correlate with a green patch (red box 1) or yellow-color patch (red box 2) in the AF image composite. J, Category 4 lesions (disruption of IS/OS, hyperreflective material accumulating around the RPE layer and distributing into the ONL, and infoldings of the OPL expanding to the INL) in the OCT image. K, Category 4 lesions appear as an orange-colored area in the color composite AF image. M, Category 5 lesions (loss of the ONL, disruption of the IS/OS, and disruption of the RPE) in the OCT image. N, Category 2 lesions appear as an orange-colored patch (red box 1), a green-colored patch (red box 2) or a dark patch (red box 3) in the color composite AF image. C, F, I, L, O, Higher magnifications of the white boxed areas in B, E, H, K, and N, respectively.
3.8 Ex vivo retinal AF in pigmented Abca4<sup>−/−</sup> mice after BLI and in a donor eye with late-stage STGD1-like phenotype

In the semi-thin sections of the nonilluminated retina in pigmented Abca4<sup>−/−</sup> mice, the lipofuscin-AF in RPE cells was visualized at an excitation of 360 nm under the fluorescence microscope. Melanosomes were observed with the pigmentation of melanin in the bright-field images. With NIR excitation, AF was located in the RPE layer and choroid and mainly corresponded to melanin pigmentation as shown in the bright-field image (Figure 6B). The subretinal pigmented cells accumulated after BLI and showed a higher density of melanin pigmentation, hyper lipofuscin-AF, and hyper NIR-AF. The melanin in these pigmented cells exhibited higher NIR-AF intensity than that in normal RPE cells. In the merged image, more pigment granules (in red/white circles) without melanin pigmentation were autofluorescent at both excitation wavelengths. Adjacent multi-layered RPE cells also contained pigment granules, showing hyper-AF in both modalities (Figure 6C). Additionally, photoreceptors were not observed with lipofuscin-AF or NIR-AF.

The peri-macular tissue from a 72-year-old patient with late-stage STGD1-like phenotype was investigated for ex vivo retinal AF. RPE cells of the peri-macular tissue were packed

**FIGURE 4** Effect of blue light on the RPE and photoreceptor cells in 9-month-old pigmented WT (A-B) and Abca4<sup>−/−</sup> mice (C-D). In the pigmented WT mice, (A) the number of RPE nuclei and (B) the number of nuclei in the ONL were measured in the nonilluminated retina (control) and the light-illuminated retina (after BLI) and were plotted as functions of the distance from the ONH along the vertical axis. Data are expressed as the mean ± SD, n = 5 eyes. Significant differences were analyzed using the unpaired t-test. ***P < .001, control vs after BLI. In the pigmented Abca4<sup>−/−</sup> mice, (C) The number of RPE nuclei and (D) number of nuclei in the ONL were measured in nontreated (control) pigmented Abca4<sup>−/−</sup> mice and blue light-illuminated pigmented Abca4<sup>−/−</sup> mice (after BLI) and were plotted as functions of the distance from the ONH along the vertical axis. Data are expressed as the mean ± SD, n = 8 eyes. Significant differences were analyzed using the unpaired t-test. *P < .05, **P < .01, and ***P < .001, control vs after BLI.
with pigment granules, including lipofuscin and melanolipofuscin stained by toluidine blue but devoid of melanosomes (Figure 6D), as previously shown by Taubitz et al.²⁴ Figure 6E,F show hyper lipofuscin-AF and hyper NIR-AF of pigment granules in RPE cells, respectively. The overlay yellow color in the merged fluorescence image demonstrated that most pigment granules were hyperautofluorescent at both excitation wavelengths (white arrows). Additionally, few pigment granules presented only NIR-AF (red arrowheads) and only some exhibited lipofuscin-AF (white arrowheads) (Figure 6G).
FIGURE 6 Ex vivo retinal AF in both modalities from nontreated (control) and blue light-illuminated 9-month-old pigmented Abca4−/− mice (A-C) and a 72-year-old human donor eye with late-stage STGD1-like phenotype (D-G). (A) Bright-field image, fluorescence image showing lipofuscin AF (green), NIR-AF image (red), and merged fluorescence image of an unstained semi-thin section from nontreated (control) pigmented Abca4−/− mice. Scale bar: 5 µm. (B) Seven days after BLI, in blue light-damaged RPE cells, some pigment granules (white/red arrows in white/red circle) without melanin pigmentation in the bright-field image, exhibiting lipofuscin AF (green) and NIR-AF (red), appear yellow in the merged image. The areas in the red and white boxes are shown on higher magnification (rightmost). Scale bar: 5 µm. (C) After BLI, in the subretinal pigmented cells and multi-layered RPE cells, pigment granules (white/red arrows in the white/red circle) without melanin pigmentation, exhibiting lipofuscin AF (green) and NIR-AF (red), were observed with a yellow color in the merged image. The areas in the red and white boxes were shown in the higher magnification (rightmost). Scale bar: 5 µm. (D) Overview of the RPE layer from the perimacular tissue of the donor eye with late-stage STGD1-like phenotype in toluidine blue-stained semi-thin sections. Scale bar: 50 µm. (E) The fluorescence image shows the distribution of lipofuscin-AF in RPE cells from the donor eye. Scale bar: 50 µm. (F) The corresponding image of E shows NIR-AF in RPE cells. (G) Merged image of E and F. Overlay areas appeared as yellow (white arrows). The pigment granules only exhibited lipofuscin AF (white arrowheads) and some only exhibited NIR-AF (red arrowheads).
3.9 Correlation among the findings of fundus AF, OCT, light microscopy, and fluorescence microscopy in pigmented Abca4<sup>−/−</sup> mice after BLI

To associate in vivo imaging with ex vivo imaging, OCT and histologic images were captured at the same location and were resized to the same scale for alignment. As shown in Figure 7 (red box 1), GA was recognized as the loss of RPE and photoreceptors in histologic images with dark patches in fundus AF images. However, the adjacent area (red box 2) appearing as GA but with the accumulation of hyperreflective material on OCT corresponded to hyper-AF in both modalities (yellow in the composite image). In the microscopic image, abundant subretinal pigmented cells accumulated above Bruch’s membrane at the corresponding location.

Fluorescence microscopy analysis was employed to further investigate these findings. In Figure 8A, the orange-colored area in the composite AF image (red box 1) indicated hyper-AF in both SW-AF and NIR-AF. The corresponding change showed as substantial hyperreflective material adhering to the thicker RPE layer, loss of EZ, and infolding of the inner plexiform layer (IPL) evidenced by the OCT analysis. The histologic image showed an increased number of pigmented cells accumulated above Bruch’s membrane at the corresponding location.

Fluorescence microscopy analysis was employed to further investigate these findings. In Figure 8A, the orange-colored area in the composite AF image (red box 1) indicated hyper-AF in both SW-AF and NIR-AF. The corresponding change showed as substantial hyperreflective material adhering to the thicker RPE layer, loss of EZ, and infolding of the inner plexiform layer (IPL) evidenced by the OCT analysis. The histologic image showed an increased number of pigmented cells accumulated above Bruch’s membrane at the corresponding location.

3.10 Ultrastructural changes after BLI in pigmented Abca4<sup>−/−</sup> and WT mice

In pigmented WT mice, the nonilluminated eyes contained barely any lipofuscin in the RPE cells (Figure 9A). In the light-illuminated eyes, there was no gross evidence of RPE degeneration. However, the elongation of microvilli and increased phagosomes with partially degraded photoreceptor discs were observed in the RPE cells after BLI (Figure 9B). Cells containing abundant phagosomes were found in the subretinal space of WT mice after BLI, which were likely to be macrophages (Figure 9C).

As reported previously, normal monolayered RPE cells of 9-month-old pigmented Abca4<sup>−/−</sup> mice possessed regularly shaped basal infoldings, with numerous mitochondria located in this area, and well-developed apical microvilli. The features of pigmented Abca4<sup>−/−</sup> RPE cells also included extensive lipofuscin, melanin, and melanolipofuscin in the cytoplasm (Figure 9D). After BLI, unlike the observations in WT mice, many more phagosomes...
with undegraded photoreceptor discs accumulated and extensively occupied the cytoplasm of damaged RPE cells, accompanied by hypopigmentation and fewer basal infoldings in Abca4<sup>−/−</sup> mice (Figure 9E). The damaged RPE cells appeared thinning and contained flattened RPE nuclei (Figure 9F,G). The reductions of the microvilli number and basal infolding expansion (Figure 9F,G) along with vacuole-like structures in the RPE cytoplasm (Figure 9G) were evident as well. Furthermore, blue light in Abca4<sup>−/−</sup> mice led to geographic atrophic lesions as
FIGURE 9  Ultrastructural changes in 9-month-old pigmented WT (A-C) and Abca4$^{-/-}$ (D-I) mice seven days after BLI. A, The RPE cells in a nonilluminated eye of WT mice under the electron microscope. Scale bar: 2 µm. B, Increased phagosomes (black arrows) show in the RPE cells of the light-illuminated eye of WT mice. The microvilli are elongated. Scale bar: 2 µm. C, In the light-illuminated eye, the subretinal cell which may be a macrophage contains abundant phagosomes. Scale bar: 2 µm. D, RPE cells in the nonilluminated eye of Abca4$^{-/-}$ mice under the electron microscope. Scale bar: 2 µm. E-H, Light-illuminated eye of Abca4$^{-/-}$ mice presents the following structural changes: (E) numerous undigested phagosomes (black arrows) accumulate and extensively occupy the cytoplasm of atrophic RPE cells, accompanied by hypopigmentation and fewer basal infoldings (white arrowheads). Scale bar: 2 µm. F, An isolated spherical-shaped pigmented cell is located in the subretinal space. Scale bar: 5 µm. G, Damaged RPE cells appear as thinning and contained flattened RPE nuclei, less pigment, and reduction of basal infolding expansion; vacuole-like structure (white arrow) was also observed. Scale bar: 2 µm. H, Geographic atrophy due to the loss of RPE cells. Scale bar: 5 µm. I, Multi-layered RPE cells contained extensive undigested phagosomes (black arrows) and decreased melanin and lipofuscin in the cytoplasm. Smaller lipofuscin-like granules (red arrowheads) were observed at the basal side of RPE cells next to Bruch’s membrane. Scale bar: 10 µm. BM: Bruch’s membrane; L: lipofuscin; M: melanin; ML: melanolipofuscin; nu: nucleus; PE: retinal pigment epithelium; ld: lipid droplet
shown by the absence of RPE and IS/OS of the photoreceptor cells (Figure 9H). Unlike the observed subretinal cells in WT mice (Figure 9C), in the light-illuminated Abca4−/− mice, the subretinal spherical-shape pigmented cells were densely packed with melanin, lipofuscin, and melanolipofuscin granules (Figure 9F).

It appears that the blue light exposure induced the formation of multi-layered RPE cells in Abca4−/− mice. As shown in Figure 9I, the RPE cells displayed multilayers containing extensive undigested phagosomes and decreased melanin and lipofuscin in the cytoplasm. Basal infoldings were limited, even absent, and apical microvilli were not evident. Thus, RPE cells lost apical-basal polarity. Smaller lipofuscin-like granules (<0.3 µm in diameter) were observed on the basal side of RPE cells next to Bruch’s membrane accompanied by the absence of mitochondria (Figure 9I).

### 3.11 Immunofluorescence findings

To identify the cell type of the subretinal pigmented cells, the blue light illuminated eyes of Abca4−/− mice were immunostained with anti-RPE65 (used as RPE marker) and anti-Iba1 (a macrophage/microglia marker) antibodies. Loss of photoreceptors and loss of RPE in the hematoxylin and eosin (HE)-stained sections (Figure 10A) corresponded to the loss of RPE65 expression, as revealed by immunofluorescence staining (Figure 10B). The subretinal pigmented cells with spherical shape were positive for Iba1, both in the area with GA (Figure 10C) and the margin of the light-induced lesion (Figure 10D). Combined with the TEM results, the data suggest that blue light causes the accumulation of macrophages phagocytosing melanin and lipofuscin in the subretinal space in Abca4−/− mice.

### 3.12 Bisretinoids analysis in Abca4−/− mice after BLI

Levels of bisretinoids A2E, A2PE, A2PE-H2, and at-RAL dimmer-PE were quantified by HPLC in the nonilluminated eyes and the light-illuminated eyes of Abca4−/− mice. As shown in Figure 11, blue light induced a decrease in the levels of A2E and other bisretinoids dramatically in the pigmented Abca4−/− mice (n = 6 eyes, P < .05; unpaired t test), suggesting the loss of the RPE cells.

### 4 DISCUSSION

A correlation was found between the foveal NIR-AF pattern and vision acuity in Stargardt patients. Cideciyan et al. suggested the importance of melanin-dependent NIR-AF to follow the progression of the early-stage of ABCA4-associated retinal disease. RPE melanin plays a great role in the pathology and diagnosis of Stargardt disease. Thus, we used pigmented Abca4−/− mice to establish a blue light-induced retinal damage model. This mouse model manifested retinal dysfunction, abnormalities in fundus AF, accumulation of hyperreflective material on OCT, and histological evidence of photoreceptor and RPE degeneration. The correlation of in vivo and ex vivo assessments helped us to interpret the histological features of abnormal fundus AF induced by blue light.

In contrast to WT mice, Abca4−/− mice showed the accumulation of lipofuscin in RPE cells. Lipofuscin containing bisretinoids is known to disrupt lysosomal and antioxidant systems in RPE and be phototoxic to RPE cells as a photoinducible generator of ROS. In this study, we postulated that the pathological changes in Abca4−/− mice after BLI were attributed to lipofuscin accumulation. Consistently, RPE loss induced by blue light was only present in Abca4−/− mice (Figures 4 and 9). Other structural differences between WT and Abca4−/− mice exposed to blue light include the number of phagosomes in RPE cells and the degree of degradation of ingested photoreceptor discs within the phagosomes (Figure 9). These structural changes likely resulted from the dysfunction of RPE lysosomes secondary to lipofuscin accumulation. Blue light induced the loss of photoreceptor nuclei in the superior area, known to be highly susceptible to light damage, was evidenced in both WT and Abca4−/− mice (Figure 4). However, the inferior retina of Abca4−/− mice was more susceptible to blue light than that of WT mice (Figure 4), which might be secondary to RPE damage. Retinaldehyde released by photobleaching visual pigments is involved in acute light-induced retinal degeneration and cannot explain the severe RPE degeneration observed in Abca4−/− mice after BLI. Recently, it was shown that bisretinoids formation can be initiated endogenously in the RPE, independently of the photoreceptor pathway, from the free retinaldehydes released after rhodopsin proteolysis. Lenis et al. showed that the absence of ABCA4 in the RPE internal membranes led to the enhanced deposition of bisretinoids. In rodents, RPE cells in the central retina contain a higher level of lipofuscin and a lower level of melanin than in the peripheral retina. The RPE degeneration induced by blue light was mainly located in the central area of eyes in pigmented Abca4−/− mice, consistent with the spatial distribution of phototoxic RPE lipofuscin and RPE melanin that is known to have antioxidative properties. Based on our research, we conclude that lipofuscin accumulated in the RPE is the main mediator of photodamage in Abca4−/− mice. The photoreceptor and RPE degeneration in blue light-exposed Abca4−/− mice were dependent on mutation in ABCA4.
Repeated blue light exposure (high intensity and short term) has been suggested to cause mild RPE degeneration, which is transient and preceding photoreceptor degeneration in pigmented Abca4−/− mice. In our study, RPE in WT mice might be transiently damaged by blue light but then recovered quickly due to the protective effect of melanin. Consequently, RPE degeneration was not observed in WT mice seven days after BLI. In rhesus, the initial lesion in the paramacular induced by blue light exposure (1000 seconds) was localized predominantly in the RPE. Wu et al suggested that bisretinoids of lipofuscin increase with age, so the age-related increase in retinal damage induced by blue light in albino Abca4−/− may point to injury that is initiated in RPE cells. Figure 4C,D revealed a greater area of the RPE loss compared to the photoreceptor loss. These implied that RPE loss may precede photoreceptor loss after short-duration blue light injury in the current study.

FIGURE 10 Blue light induced the reduction of RPE65 expression and accumulation of Iba1 positive cells in the subretinal space in pigmented Abca4−/− mice seven days after BLI. A, Representative image of hematoxylin- and eosin-stained paraffin section recorded from a blue light-illuminated eye. Scale bar: 200 µm. B, RPE65 immunostaining (red) and DAPI staining (blue) in the adjacent section showed no evidence of RPE65 expression in geographic atrophy. Additionally, no expression of RPE65 was observed in the subretinal pigmented cells (white arrows in A). Scale bar: 200 µm. Artificial retinal detachment in A and B. C, The isolated subretinal pigmented cells in areas of GA were found with Iba1-positivity. Scale bar: 20 µm. D, The subretinal pigmented cells in the peripheral zone of the lesion were Iba1-positive. Scale bar: 10 µm. E, Representative images of negative controls with no primary antibody. Scale bar: 20 µm. arrows: the subretinal pigmented cell
A longitudinal study of AF in Stargardt patients demonstrated that fleck lesions were increased in SW-AF over time to a peak of hyperAF, and then decreased in SW-AF subsequently and eventually became hypoAF. NIR-AF of lesions also changed similarly over time, but the rate of decline in NIR-AF to hypoAF occurred more quickly. Aligned with the AF changes in the late stage of Stargardt disease, both the mean intensities of SW-AF and NIR-AF were reduced in blue light-illuminated pigmented Abca4−/− mice (Figure 1). Consistently, the level of bisretinoid fluorophores decreased by blue light in Abca4−/− mice (Figure 11). This might be attributed to the loss of the RPE cells in the blue-light exposed Abca4−/− mice. Additionally, photo-oxidation is known to cause photodegradation of bisretinoids and melanin. Correlative analysis between SW-AF and NIR-AF (Figure 1) revealed three features of fundus AF abnormalities in blue light-illuminated pigmented Abca4−/− mice: (i) most of the lesions showed hyper SW-AF but not hypo NIR-AF (near to background); (ii) some lesions appeared as dark patches in SW-AF and NIR-AF; (iii) some lesions showed hyper-AF in both modalities. Similar AF features were previously reported in patients with ABCA4-associated retinal dystrophies.

Based on the OCT images analysis, lesions were classified into five categories (Figure 3). Lesions under categories 1, 3, 4, and 5 showed hyperreflective material deposited at the IS/OS and RPE/Bruch’s membrane complex. These hyperreflective subretinal deposits in blue-light exposed Abca4−/− mice resemble the appearance of advanced Stargardt disease. In patients affected by Stargardt disease, the degree of hyper-reflective material penetration through the retinal layers was directly correlated with the SW-AF intensity of the retinal flecks. Similarly, in our mouse model, higher SW-AF and NIR-AF signals correlated with hyperreflective material protruding into the outer retinal layers in SD-OCT scans.

In human ABCA4-associated retinal degeneration, the disease sequence was defined as six stages based on the retinal function and structure. Stage I is proposed as a normal retinal structure and function. Stage II, increased AF intensity with normal retinal function, shares similar characteristics with the pigmented Abca4−/− mouse model. Stage III is defined as an increase in the mean fundus AF intensity and AF texture. The advanced stages include increased fundus AF texture with partial degeneration of photoreceptors (Stage IV) and reduction of the mean fundus AF intensity with a
further decline in retinal function (Stage V). Stage VI as the end-stage is characterized as a complete loss of the RPE and photoreceptors. As shown by in vivo assessments, our mouse model mainly reflects the features of advanced stages including Stage IV and V. It also implied that excessive blue light accelerated the progression of ABCA4-associated retinal degeneration.

The histological features of Stargardt disease are not available. Here, using an experimental model, we correlated the findings of light and fluorescence microscopy with fundus AF imaging and SD-OCT scans in Abca4<sup>−/−</sup> mice after BLI (Figure 8). This approach allowed us to interpret the underlying retinal histology of AF abnormalities. First, the common histological characteristics of the sites with hyper SW-AF and hypo NIR-AF (near to background) were associated with changes in the RPE layer highlighted in the semi- and ultra-thin sections. Decreased NIR-AF after blue light can be interpreted by a reduction and loss of melanin in the damaged RPE cells evidenced by electron microscopy (Figure 9E,G). The damaged RPE cells, which contained few melanin but abundant undigested phagosomes in the cytoplasm, reflected the hyper SW-AF and hypo NIR-AF profile (Figure 9E). Undegraded phagosomes in RPE cells have been reported to exhibit lipofuscin-like fluorescence. Release of retinaldehyde following rhodopsin proteolysis in the RPE leads to the formation of bisretinoids with subsequent chemical and oxidative modifications further contributing to the SW-AF. The erythrocytes present in the choroid were autofluorescent under the fluorescence microscope yet no fluorescence was evidenced by SLO modality. Second, the site with hypo AF in SW-AF and NIR-AF (box 2 in Figure 8A) was mainly attributed to the loss of RPE cells. In the STGD1 patients, hypoAF patches associated with poorer visual acuity were prominent in the advanced stage. Third, hyperreflective material (box 1, box 3 in Figure 8B) was evident in the sites with hyperAF in both SW-AF and NIR-AF (box 1 and box 3 in Figure 8A), which corresponded to the accumulation of subretinal macrophages (box 1, box 3 in Figure 8C-E). The presence of hyperreflective lesions in OCT images is a common pathological feature of Stargardt patients. However, the cellular components that generate these lesions are controversial. Battaglia Parodi M. et al. suggested that outer retinal hyperreflective foci result from the transdifferentiation of RPE cells after the epithelial-mesenchymal transition process. Paavo et al. suggested that the origin of hyperreflective foci is bisretinoid residing in photoreceptors. However, in our work, photoreceptors were not observed with AF in pigmented Abca4<sup>−/−</sup> mice before or after BLI under the fluorescence microscope. We demonstrated that the origin of hyperreflective lesions in the illuminated Abca4<sup>−/−</sup> mice is the accumulation of subretinal macrophages with abundant melanin and (melano)lipofuscin from degenerated RPE cells. Additionally, multi-layered RPE cells exhibited much weaker lipofuscin-derived AF and NIR-AF than the subretinal macrophages. In contrast to a previous study, our results do not support that the superimposition of RPE cells causes an increased AF in both modalities.

The presence of macrophages engorged with melanolipofuscin and lipofuscin granules in the outer retina was reported in the donor eyes of a Stargardt patient. However, the isolated pigmented cells in the subretinal space were recognized as migrated RPE cells in Stargardt patients by other studies. The macrophages are known to infiltrate into the subretinal space, clean cellular debris such as OS and accumulate autofluorescent lipofuscin in light-related and all-trans retinal-associated retinal degeneration. The photoreceptor debris-laden macrophages were observed in the WT mice and occasionally in the Abca4<sup>−/−</sup> mice after BLI. These observations indicate that the subretinal macrophages contributed to the clearance of disintegrated RPE and photoreceptor cellular debris. The origin of subretinal macrophages in acute blue light-induced retinal injury has been reported as blood-borne macrophages migrating into the lesion area via the optic nerve and ciliary body and activating resident microglia that possess macrophage characteristics in the injured region. Furthermore, the debris-laden macrophages can leave the retina into the general circulation.

The donor eye with PRPH2 mutation was analyzed in our study. Pathogenic variants in PRPH2 have been suggested to be significantly associated with a broad retinal dystrophy phenotype including STGD1 in patients without ABCA4 variants. Compared with STGD1 patients, PRPH2-positive patients showed similar clinical features including increased fundus autofluorescence and geographic atrophy for cases with late-onset. Although the increase in lipofuscin occurs less rapidly in the PRPH2-positive patients, the deleterious effects of increased RPE lipofuscin are the same as in the STGD1 patients. Correlation of the two modalities under the fluorescence microscope revealed the pigmented granules exhibiting both lipofuscin-derived AF and NIR-AF in the blue light-illuminated mice and donor eye with the late-stage STGD1-like phenotype (Figure 6). We further demonstrated that these granules were lipofuscin and melanolipofuscin formed by the fusion of lipofuscin and melanin with aging and oxidative stress. Previously, we have suggested that the NIR-AF emitted by lipofuscin granules and the lipofuscin moiety of melanolipofuscin granules may be mainly due to the accumulation of melanin degradation products. Bisretinoids contained in lipofuscin granules in degenerating RPE cells can also undergo photo-oxidation leading to additional toxic products. These oxidized and fragmented bisretinoids might contribute to NIR-AF as well. Photobleaching is a known contributor to age-related changes in the melanosome morphology. Also, blue light triggers photobleaching which enhances the fluorescence intensity of melanosomes. This was clearly evidenced by an enhanced NIR-AF in the melanosomes in the subretinal pigmented cells.
of blue light-exposed Abca4/−/− mice. Likewise, the increase in melanin-dependent NIR-AF was demonstrated in most ABCA4-STGD1 patients.70 These findings together indicate that photo-oxidation linked to the changes in fluorescence properties of pigment granules (melanin, lipofuscin, and melanolipofuscin), is a common mechanism of the STGD1 phenotype and blue light damage in Abca4/−/− mice. Loss of the RPE cells in the blue-light exposed mice was consistent with the reduction in the level of quantified bisretinoids by HPLC.

Further analysis of the chemical alteration of pigment granules (lipofuscin, melanolipofuscin, and melanin) in RPE cells after blue light is needed. Wu et al. suggested that bisretinoids of retinal lipofuscin are contributors to retinal light damage by studying blue light-exposed albino Abca4/−/− mice.45 In the current study, we applied a similar blue light source and a shorter exposure time, but in the pigmented Abca4/−/− mice, which resembled similar phenotypic features of advanced Stargardt disease. Our work also indicates that the fundus AF examination in both SW-AF and NIR-AF provides more information for the diagnosis and evaluation of disease progression.32,71

Our mouse model reflects the clinical manifestations of advanced Stargardt disease and AMD. Quantitative analysis of RPE nuclei and photoreceptor nuclei indicated that blue light-induced RPE loss preceded the photoreceptor loss in Abca4/−/− mice. The correlation of the findings among fundus autofluorescence, SD-OCT, fluorescence microscope, and histology can be used to interpret the underlying histological characteristics of fundus AF abnormalities and structural changes observed by OCT. Subretinal macrophages phagocytizing RPE debris were proven to be sources of both hyper SW-AF and hyper NIR-AF. Melanin in macrophages with higher NIR-AF, lipofuscin and melanolipofuscin granules expressing SW-AF and NIR-AF indicated chemical alterations of pigment granules caused by photo-oxidation. We suggest that this model is appropriate to investigate the correlation of fundus AF and histology and the role of melanin granules. Since the photodamage was mainly mediated by lipofuscin in this model, removal of existing lipofuscin granules might be a potential therapy for Stargardt disease. This model could also be applied to study preclinical experimental therapy strategies for the advanced stage of Stargardt disease and further elucidate its mechanism.

ACKNOWLEDGMENTS

The authors thank the Foundation Fighting Blindness, Vera Bonilha and Joe Hollyfield for collecting and providing the human tissue. The authors thank Monika Rittgarn and Antonina Burda for excellent technical assistance. The authors also thank Judith Birch for language improvement. This work was supported by the Bundesministerium für Bildung und Forschung (grant number 01GQ1422B) and China Scholarship Council (CSC201608080087; to Fang, Y) and by the National Eye Institute (grant R01 EY025002 to Radu R.A.). The funders played no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

AUTHOR CONTRIBUTIONS

Y. Fang wrote the manuscript, conducted the experiments, acquired data, and analyzed the data. A. Tschulakow conducted the experiments, contributed to data acquisition and data analysis. T. Taubitz conducted the experiments. B. Illing provided the electron microscopy data. A. Biesemeier provided the sections of peri-macular tissue of the human donor. Z. Jiang and R. A. Radu analyzed the bisretinoids data. U. Schraermeyer and S. Julien-Schraermeyer contributed to study design, supervised the work and corrected the manuscript. Y. Fang, T. Taubitz, Z. Jiang, R. A. Radu, U. Schraermeyer, and S. Julien-Schraermeyer reviewed and edited the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors have declared that no conflict of interest exists.

[Correction added on February 13, 2020, after first online publication: Reference 3 has been corrected.]

REFERENCES

1. Blacharski PA. Fundus flavimaculatus. In: Newsome DA, eds. Retinal dystrophies and degenerations. New York: Raven Press; 1988:135-139.
2. Rotenstreich Y, Fishman GA, Anderson RJ. Visual acuity loss and clinical observations in a large series of patients with stargardt disease. Ophthalmology. 2003;110(6):1151-1158.
3. Gelisken O, De Laey JJ. A clinical review of Stargardt’s disease and/or fundus flavimaculatus with follow-up. Int Ophthalmol. 1985;8:225-235.
4. Lambertus S, van Huet RAC, Bax NM, et al. Early-onset stargardt disease: phenotypic and genotypic characteristics. Ophthalmology. 2015;122(2):335-344.
5. Fujinami K, Zernant J, Chana RK, et al. Clinical and molecular characteristics of childhood-onset Stargardt disease. Ophthalmology. 2015;122(2):326-334.
6. Allikmets R, Singh N, Sun H, et al. A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. Nat Genet. 1997;15:236-246.
7. Molday LL, Rabin AR, Molday RS. ABCR expression in foveal cone photoreceptors and its role in Stargardt macular dystrophy. Nat Genet. 2000;25:257-258.
8. Lenis TL, Hu J, Ng SY, et al. Expression of ABCA4 in the retinal pigment epithelium and its implications for Stargardt macular degeneration. Proc Natl Acad Sci USA. 2018;115(47):E11120-E11127.
9. Weng J, Mata NL, Azarian SM, Tzekov RT, Birch DG, Travis GH. Insights into the function of rim protein in photoreceptors and etiology of Stargardt’s disease from the phenotype in abcr knockout mice. Cell. 1999;98(1):13-23.
10. Quazi F, Molday RS. ATP-binding cassette transporter ABCA4 and chemical isomerization protect photoreceptor cells from the toxic accumulation of excess 11-cis-retinal. Proc Natl Acad Sci USA. 2014;111(13):5024-5029.
11. Mata NL, Weng J, Travis GH. Biosynthesis of a major lipofuscin fluorophore in mice and humans with ABCR-mediated retinal and macular degeneration. Proc Natl Acad Sci USA. 2000;97:7154-7159.

12. Sparrow JR, Gregory-Roberts E, Yamamoto K, et al. The bis-retinoids of retinal pigment epithelium. Prog Retin Eye Res. 2012;31(2):121-135.

13. Sparrow JR, Nakanshi K, Parish CA. The Lipofuscin Fluorophore A2E mediates blue light- induced damage to retinal pigmented epithelial cells. Invest Ophthalmol Vis Sci. 2000;41:1981-1989.

14. Lu B, Zhang P, Zhou M, et al. Involvement of XBP1s in blue light-induced A2E-containing retinal pigment epithelium cell death. Ophthalmic Res. 2017;57(4):252-262.

15. Sparrow JR, Zhou J, Ben-Shahat S, Vollmer H, Itagaki Y, Nakanshi K. Involvement of oxidative mechanisms in blue-light-induced damage to A2E-laden RPE. Invest Ophthalmol Vis Sci. 2002;43:1222-1227.

16. Sparrow JR, Vollmer-Snarr HR, Zhou J, et al. A2E-epoxides damage DNA in retinal pigment epithelial cells. Vitamin E and other antioxidants inhibit A2E-epoxide formation. J Biol Chem. 2003;278(20):18207-18217.

17. Radu RA, Mata NL, Bagla A, Travis GH. Light action spectrum on the biosynthesis of a major lipofuscin fluorophore in mice and humans. Proc Natl Acad Sci USA. 2015;112(24):7530-7535.

18. Zhou J, Jang YP, Kim SR, Sparrow JR. Complement activation by photooxidation products of A2E, a lipofuscin constituent of the retinal pigment epithelium. Proc Natl Acad Sci USA. 2006;103(44):16182-16187.

19. van der Burght BW, Hansen M, Olsen J, et al. Early changes in gene expression induced by blue light irradiation of A2E-laden retinal pigment epithelial cells. Acta Ophthalmol. 2013;91(7):e537-e545.

20. Marie M, Bigot K, Angebault C, et al. Light action spectrum on oxidative stress and mitochondrial damage in A2E-loaded retinal pigment epithelial cells. Cell Death Dis. 2018;9(3):287.

21. Lin C-H, Wu M-R, Li C-H, et al. Editor’s highlight: periodic exposure to smartphone-mimic low-luminance blue light induces retinal damage through Bcl-2/BAX-dependent apoptosis. Toxicol Sci. 2017;157(1):196-210.

22. Teusssink MM, Lamberts S, de Mul FF, et al. Lipofuscin-associated photo-oxidative stress during fundus autofluorescence imaging. PLoS ONE. 2017;12(2):e0172635.

23. Charbel Issa P, Barnard AR, Singh MS, et al. Fundus autofluorescence in the Abca4(-/-) mouse model of Stargardt disease: correlation with accumulation of A2E, retinal function, and histology. Invest Ophthalmol Vis Sci. 2013;54(8):5602-5612.

24. Taubitz T, Tschulakow AV, Tikhonovich M, et al. Ultrastructural alterations in the retinal pigment epithelium and photoreceptors of a Stargardt patient and three Stargardt mouse models: indication for the central role of RPE melanin in oxidative stress. PeerJ. 2018;6:e5215.

25. Charbel Issa P, Singh MS, Lipinski DM, et al. Optimization of in vivo confocal autofluorescence imaging of the ocular fundus in mice and its application to models of human retinal degeneration. Invest Ophthalmol Vis Sci. 2012;53(2):1066-1075.

26. Oguchi Y, Sekiryu T, Takasumi M, Hashimoto Y, Furuta M. Near-infrared and short-wave autofluorescence in ocular specimens. Jpn J Ophthalmol. 2018;62(5):605-613.

27. Lenis TL, Sarfare S, Jiang Z, Lloyd MB, Bok D, Radu RA. Complement modulation in the retinal pigment epithelium rescues photoreceptor degeneration in a mouse model of Stargardt disease. Proc Natl Acad Sci USA. 2017;114(15):3987-3992.

28. Delori FC, Dorey CK, Staurenghi G, Arend O, Goger DG, Weiner JJ. In vivo fluorescence of the ocular fundus exhibits retinal pigment epithelium lipofuscin characteristics. Invest Ophthalmol Vis Sci. 1995;36:719-729.

29. Keilhauer CN, Delori FC. Near-infrared autofluorescence imaging of the fundus: visualization of ocular melanin. Invest Ophthalmol Vis Sci. 2006;47(8):3556-3564.

30. Cukras CA, Wong WT, Caruso R, Cunningham D, Zein W, Sieving PA. Centrifugal expansion of fundus autofluorescence patterns in Stargardt disease over time. Arch Ophthalmol. 2012;130(2):171-179.

31. Parodi MB, Iacono P, Triolo G, et al. Morpho-functional correlation of fundus autofluorescence in Stargardt disease. Br J Ophthalmol. 2015;99(10):1354-1359.

32. Cideciyan AV, Swider M, Schwartz SB, Stone EM, Jacobson SG. Predicting progression of ABCA4-associated retinal degenerations based on longitudinal measurements of the leading disease front. Invest Ophthalmol Vis Sci. 2015;56(10):5946-5955.

33. Shamji FA, Boulton M. Inhibition of RPE lysosomal and antioxidant activity by the age pigment lipofuscin. Invest Ophthalmol Vis Sci. 2001;42:3041-3046.

34. Wassell J, Davies S, Bardsley W, Boulton M. The photoreactivity of the retinal age pigment lipofuscin. J Biol Chem. 1999;274:23828-23832.

35. Rózanowska M, Jarvis-Evans J, Korytowski W, Boulton ME, Burke JM, Sarna T. Blue light-induced reactivity of retinal age pigment. In vitro generation of oxygen-reactive species. J Biol Chem. 1995;32:18825-18830.

36. Finnemann SC, Leung LW, Rodriguez-Boulan E. The lipofuscin component A2E selectively inhibits phagolysosomal degradation of photoreceptor phospholipid by the retinal pigment epithelium. Proc Natl Acad Sci USA. 2002;99:3842-3847.

37. Holz FG, Schütt F, Kopitz J, et al. Inhibition of lysosomal degradative functions in RPE cells by a retinoid component of lipofuscin. Invest Ophthalmol Vis Sci. 1999;40:737-743.

38. Rapp LM, Williams TP. A parametric study of retinal light damage in albino and pigmented rats. In: Williams TP, Baker BN, eds. The effects of constant light on visual processes. New York: Plenum Press; 1980:133-159.

39. Maeda A, Maeda T, Goliczak M, et al. Involvement of all-trans-retinal in acute light-induced retinopathy of mice. J Biol Chem. 2009;284(22):15173-15183.

40. Grey AC, Crouch RK, Koutalos Y, Schey KL, Ablonczy Z. Spatial distribution of A2E mediates blue light-induced damage to retinal pigmented epithelial cells. Invest Ophthalmol Vis Sci. 2009;284(22):15173-15183.

41. Polosa A, Bassaklia H, Lachapelle P. Light-induced retinopathy: young age protects more than ocular pigmentation. Curr Eye Res. 2017;42(6):924-935.

42. Wang Z, Dillon J, Gaillard ER. Antioxidant properties of melanin in retinal pigment epithelial cells. Photochem Photobiol. 2006;82(2):474-479.

43. Nakamura M, Yako T, Kuse Y, et al. Exposure to excessive blue LED light damages retinal pigment epithelium and photoreceptors of pigmented mice. Exp Eye Res. 2018;177:1-11.

44. Ham WT Jr, Ruffolo JJ Jr, Mueller HA, Clarke AM, Moon ME. Histologic analysis of photochemical lesions produced in rhesus retina by short-wave-length light. Invest Ophthalmol Vis Sci. 1978;17:1029-1035.
45. Wu L, Ueda K, Nagasaki T, Sparrow JR. Light damage in Abca4 and Rpe65d12 mice. Invest Ophthalmol Vis Sci. 2014;55(3):1910-1918.
46. Ueda K, Zhao J, Kim HJ, Sparrow JR. Photodegradation of retinal bisretinoids in mouse models and implications for macular degeneration. Proc Natl Acad Sci USA. 2016;113:6904-6909.
47. Sarna T, Burke JM, Korytowski W, et al. Loss of melanin from human RPE with aging: possible role of melanin photooxidation. Exp Eye Res. 2003;76(1):89-98.
48. Dontsov AE, Sakina NL, Ostrovsky MA. Loss of melanin by eye melanocytes initiate microglial activation via Toll-like receptor 4 in flavimaculatus. Ophthalmology. 1994;101:1211-1219.
49. Voigt M, Querques G, Atmani K, et al. Analysis of retinal flecks in fundus flavimaculatus using high-definition spectral-domain optical coherence tomography. Am J Ophthalmol. 2010;150(3):330-337.
50. Ciccone L, Lee W, Zernant J, et al. Hyperreflective deposit in the background of advanced stargardt disease. Retina. 2018;38:2214-2219.
51. Katz ML, Wendt KD, Sanders DN. RPE65 gene mutation prevents progression of Stargardt disease caused by compound heterozygous ABCA4 mutations. Invest Ophthalmol Vis Sci. 2003;44:3631-3639.
52. Battaglia Parodi M, Sacconi R, Romano F, Bandello F. Hyperreflective foci in Stargardt disease: 1-year follow-up. Graefes Arch Clin Exp Ophthalmol. 2017;255(10):1917-1922.
53. Battaglia Parodi M, Sacconi R, Romano F, Bandello F. Hyperreflective foci in Stargardt disease: 1-year follow-up. Graefes Arch Clin Exp Ophthalmol. 2017;255(10):1917-1922.
54. Wang J, Wang J, Wang J, et al. Age-related changes in the morphology, absorption and fluorescence of melanosomes and lipofuscin granules of the retinal pigment epithelium. Vis Res. 1990;30(9):1291-1303.
55. Sears AE, Bernstein PS, Cideciyan AV, et al. Towards treatment of Stargardt disease: workshop organized and sponsored by the foundation fighting blindness. Transl Vis Sci Technol. 2017;6(5):6.
56. Strauss RW, Muñoz B, Ho A, et al. Progression of Stargardt disease as determined by fundus autofluorescence in the retrospective progression of Stargardt disease study (ProgStar. Report No. 9). JAMA Ophthalmol. 2017;135(11):1232-1241.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Fang Y, Tschulakow A, Taubitz T, et al. Fundus autofluorescence, spectral-domain optical coherence tomography, and histology correlations in a Stargardt disease mouse model. The FASEB Journal. 2020;34:3693–3714. https://doi.org/10.1096/fj.201901784RR