Published in final edited form as:

J Neurosci Res. 2019 January ; 97(1): 98–106. doi:10.1002/jnr.24252.

Photoreceptor Cells as a Source of Fundus Autofluorescence in Recessive Stargardt Disease

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Abstract

Bisretinoid fluorophores form in photoreceptor outer segments from non-enzymatic reactions of vitamin A aldehyde. The short wavelength autofluorescence (SW-AF) of fundus flecks in recessive Stargardt disease (STGD1) suggests a connection to these fluorophores. Through multimodal imaging we sought to elucidate this link. Flecks observed in SW-AF images often colocalized with foci exhibiting reduced or absent near infrared autofluorescence signal, the source of which is melanin in retinal pigment epithelial (RPE) cells. With serial imaging, changes in NIR-AF preceded the onset of fleck hyperautofluorescence in SW-AF images and fleck profiles in NIR-AF images tended to be larger. Flecks in SW-AF and NIR-AF images also corresponded to hyperreflective lesions traversing photoreceptor-attributable bands in horizontal SD-OCT scans. The hyperreflective lesions interrupted adjacent OCT reflectivity bands and were associated with thinning of the outer nuclear layer. These SD-OCT findings are attributable to photoreceptor cell degeneration. Progressive increases and decreases in the SW-AF intensity of flecks were evident in color-coded quantitative fundus autofluorescence (qAF) maps. In some cases flecks appeared to spread radially from the fovea to approximately 8° of eccentricity, beyond which a circumferential spread characterized the distribution. Since the NIR-AF signal is derived from melanin and loss of this autofluorescence is indicative of RPE atrophy, the SW-AF of flecks cannot be accounted for by bisretinoid lipofuscin in RPE. Instead, we suggest that the bisretinoid serving as the source of the SW-AF signal, resides in photoreceptors, the cell that is also the site of bisretinoid synthesis.

Graphical abstract

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Associate Editor: Royce Mohan

CONFLICT OF INTEREST STATEMENT

None of the authors has conflicts of interest to disclose.

AUTHOR CONTRIBUTIONS

All authors had full access to all of the data in the study and take responsibility for the integrity of the data and accuracy of the data analysis. Study concept and design: JRS. Acquisition of data: MP, JRS, RA, SHT, LW. Analysis and interpretation of the data: JRS, MP. Drafting of the manuscript: JRS. Critical revision of the manuscript for important intellectual content: JRS, MP. Obtained funding: JRS.

In recessive Stargardt disease, a early onset form of macular degeneration, hyperautofluorescent foci are often features of short wavelength fundus autofluorescence. In color-coded quantitative autofluorescence maps the distributions and intensity changes of these flecks are apparent. We provide evidence that flecks are a manifestation of groups of degenerating photoreceptor cells.

Keywords

recessive Stargardt disease; ABCA4; retinal pigment epithelium; fundus autofluorescence; spectral domain optical coherence tomography; SD-OCT; bisretinoid

Photoreceptor cells are highly specialized neurons of retina that mediate phototransduction. Within the outer segments of rod and cone photoreceptor cells, opsins densely concentrated in the disc and plasma membrane, bind the 11-*cis*-retinal chromophore. Lesser amounts of rhodopsin are also present in the plasma membrane of rod inner segments (Hicks et al. 1989). After absorbing a photon of light, the 11-*cis*-retinal chromophore of rhodopsin and cone pigment undergoes isomerization to all-*trans*-retinaldehyde. The latter retinaldehyde when released from photoactivated rhodopsin reacts with phosphatidylethanolamine (PE) in the disc membrane to form the reversible Schiff base adduct, *N*-retinylidene-PE (NRPE) (Ahn et al. 2000; Anderson and Maude 1970; Beharry et al. 2004; Molday et al. 2000; Quazi and Molday 2014; Sun et al. 1999; Sun and Nathans 1997; Sun and Nathans 2001). The ATP binding cassette (ABC) transporter ABCA4 (Illing et al. 1997; Papermaster et al. 1978; Sun et al. 1999; Sun and Nathans 1997) that is expressed by photoreceptor cells, aids in delivery of retinaldehyde in the form of NRPE to the cytoplasmic retinol dehydrogenase (Rdh) that reduces all-*trans*-retinal to the less reactive alcohol (all-*trans*-retinol). Nevertheless, some retinaldehyde avoids reduction, thus allowing two retinaldehydes to condense with PE non-enzymatically so as to irreversibly form bisretinoid fluorophores in the outer segment compartments of the photoreceptor cells. If these light-absorbing fluorophores were to collect in outer segments, they could, in the very least, interfere with photon absorbance by cone and rod visual pigments. Thus to keep them to a minimum in healthy photoreceptor cells, bisretinoids are transferred to RPE cells within the outer segment debris that is shed by

photoreceptor cells and phagocytosed by RPE on a daily basis. Consequently these bisretinoid fluorophores accumulate in RPE cells as lipofuscin.

Deficiency of ABCA4 activity as occurs in recessive Stargardt disease (Allikmets et al. 1997) greatly accelerates the formation of these bisretinoid molecules and while the gene mutation is expressed by photoreceptor cells, the impact of the mutation is expressed first in RPE cells where toxic bisretinoids accumulate. Pronounced bisretinoid accumulation is replicated not only in *Abca4* null mutant mice (Kim et al. 2004; Weng et al. 1999) but also in mice deficient in retinol dehydrogenase-8 and 12 (*Rdh8*; *Rdh12*) (Chrispell et al. 2009; Maeda et al. 2009; Maeda et al. 2008). It is likely that both all-*trans*-retinal and 11-*cis*-retinal can contribute to bisretinoid formation (Boyer et al. 2012; Ueda et al. 2016). Various bisretinoids of RPE lipofuscin, including A2-GPE (A2-glycero-phosphoethanolamine), A2-DHP-PE (A2-dihydropyridine-phosphatidylethanolamine), all-*trans*-retinal dimer-PE (all-*trans*-retinal dimer phosphatidylethanolamine) and A2E (adduct of A2 and ethanolamine) (Parish et al. 1998; Sparrow et al. 2012a; Wu et al. 2009; Yamamoto et al. 2011) have been isolated and structurally characterized.

Bisretinoids are also the primary source of the natural autofluorescence of the retina that can be elicited non-invasively with excitation by blue light (488 nm) (short-wavelength fundus autofluorescence, SW-AF) and captured in fundus images by confocal scanning laser ophthalmoscopy (cSLO). Alterations in the patterns of SW-AF can occur due to autofluorescence intensity loss, spatial heterogeneities and focal or widespread increases in fluorescence. These patterns can be integral to the clinical diagnosis and monitoring of several retinal diseases including recessive Stargardt disease (STGD1) and retinitis pigmentosa (RP). Changes in fundus autofluorescence can be defined further by measuring fluorescence intensities such as in the quantitative fundus autofluorescence (qAF) method in current use. With this approach, image grey levels are calibrated to an internal fluorescent reference without histogram stretching so as to enable comparisons amongst images (Duncker et al. 2014a; Duncker et al. 2015). In ABCA4-associated disease, the increase in bisretinoid lipofuscin in RPE was evidenced by elevated qAF in 36 of 42 patients having genetically confirmed recessive Stargardt disease; the remaining 6 patients were homozygous or heterozygous for the G1961E mutation in *ABCA4* (Burke et al. 2014). The adverse effects of augmented bisretinoid deposition leads to RPE cell atrophy as demonstrated in the fundus by decreased SW-AF and near-infrared autofluorescence (Cideciyan et al. 2004; Duncker et al. 2014b; Kellner et al. 2009).

In ABCA4-associated disease, one of the most noticeable changes in the SW-AF signal is imposed by autofluorescent foci (fundus flecks). Since flecks are dynamic features of the fundus, we have analyzed flecks in SW-AF, near-infrared autofluorescence (NIR-AF) and infrared reflectance (IR-R) images and by qAF. We have related these multi-modal findings to structural information acquired by spectral domain optical coherence tomography (SD-OCT).

MATERIALS AND METHODS

Patients

Images from 12 patients (6 female, 6 male; age range 9 to 61 years) who had been recruited between 6/2012 and 11/2016 were studied retrospectively after obtaining a clinical diagnosis of STGD1 and subsequent genetic screening. Disease-causing variants were detected by direct sequencing of the *ABCA4* locus as previously described (Zernant et al. 2011; Zernant et al. 2014). Patients were further selected on the basis of the presence of flecks visible in fundus SW-AF images and the availability of NIR-AF and spectral domain optical coherence tomography (SD-OCT) images. Exclusion criteria were poor quality images and extensive atrophy. Demographic, clinical and genetic information is presented in Table 1. The study was carried out with the approval of the Institutional Review Board of Columbia University and complied with the Health Insurance Portability and Accountability Act of 1996. All patients were enrolled in accordance with the tenets set out in the Declaration of Helsinki. Informed consent was obtained prior to enrollment.

Short-wavelength fundus autofluorescence (SW-AF): image acquisition and qAF analysis

SW-AF images were acquired using a Spectralis HRA+OCT (Heidelberg Engineering, Heidelberg, Germany) (488 nm excitation, 500 nm barrier filter) and a previously described protocol that was followed to acquire high-quality SW-AF images that can be used for qAF (Burke et al. 2014). Photopigments were bleached for 20-30 seconds while focus and alignment were adjusted (Delori et al. 2011). Images were obtained in video format (9-12 frames), videos were examined for image quality and the frames were aligned, averaged and saved in non-normalized mode (no histogram stretching). To generate qAF maps, gray levels (GL) were normalized to GL in the internal fluorescent reference while standardizing for zero GL, magnification (refraction), and ocular media absorption (Duncker et al. 2015).

Near-infrared autofluorescence (NIR-AF)

Near-infrared autofluorescence (NIR-AF) images (30° X 30° field, 787 nm excitation, >830 nm emission) were acquired with the HRA2-SLO (Heidelberg Engineering) using the indocyanine-green angiography mode (787 nm excitation) and a sensitivity of 96. To obtain high-quality images the eye-tracking function was utilized, and 100 single frames were averaged and saved in normalized mode.

Spectral domain optical coherence tomography (SD-OCT)

Horizontal 9 mm scans through the macula were acquired in high-resolution mode (minimum of 9, maximum of 100 single scans were averaged) with the Spectralis HRA+OCT. The scans were automatically registered to a simultaneously acquired near infrared reflectance (IR-R) fundus image. The nomenclature used for identification of reflectivity bands in SD-OCT was previously published (Staurengi et al. 2014).

Fleck analysis

For analysis, all images were registered and aligned using 12kRetina software (DualAlign LLC, Clifton Park, NY USA). For illustrative purposes some non-aligned images were used

in figures. To delineate an area for fleck analysis, a square was drawn in superior hemiretina such that all sides were equal to the optic disc-fovea distance in the corresponding image. In SW-AF images 5 hyperautofluorescent flecks were chosen (total of 95 flecks; 19 eyes, 10 patients) and then scored according to appearance in NIR-AF images.

RESULTS

Fundus Flecks in SW-AF, NIR-AF and IR-R images

In ABCA4-related disease, bisretinoids undergo accelerated formation due to reduced or absent activity of ABCA4 (Burke et al. 2014). In addition to this generalized increase in SW-AF, intensely fluorescent foci (flecks) are readily recognizable in the fundus in many cases of ABCA4-associated disease. We undertook a study of flecks using SW-AF that originates primarily in bisretinoid lipofuscin and NIR-AF that is generated primarily from RPE melanin with less pronounced input from choroidal melanin. In SW-AF images flecks were often distinctly hyperautofluorescent while being hypoautofluorescent (12/12 patients) in NIR-AF images (Kellner et al. 2009; Sparrow et al. 2015) (Fig. 1, green rectangles; Fig. 2). Other flecks were found to be dark in NIR-AF images while being barely visible in SW-AF images (Fig. 1, yellow arrows). The fleck profiles in the NIR-AF images also tended to be larger (Figs. 1, 2) while in SW-AF images flecks were sometimes encircled by a halo of weaker autofluorescence (12/12 patients) (Figs. 2, 3). In NIR-AF images, some flecks exhibited a bright center and dark surround; these flecks could also be bright in SW-AF and often were at more peripheral positions in the fundus (Fig. 1, turquoise rectangle). We selected hyperautofluorescent flecks in a pre-determined area of superior hemiretina in SW-AF images (total of 95 flecks; 19 eyes, 10 patients) and scored them according to appearance in NIR-AF images. We found that 7.3% were hyperautofluorescent in NIR-AF images, 7% appeared to be not different than the neighbouring intensities and 85% were hypoautofluorescent. The latter included flecks having a bright center with a dark-surround. A *chi*-square test of independence revealed that the differences in percentages were significant ($X^2(2, N=100) = 122.9, p < 0.01$).

The SW-autofluorescence of flecks declines with time (Cukras et al. 2012) and in central locations, flecks could be darkened in both SW-AF and NIR-AF modalities (Fig. 1, red rectangles). Accordingly, fleck profiles in SW-AF and NIR-AF images were often contiguous with central areas of atrophy (17/21 eyes); these flecks were typically hypoautofluorescent in SW-AF and NIR-AF (Fig. 1) but were occasionally of increased intensity in SW-AF images (Fig. 2). Flecks were also visible in infrared reflectance images (Fig. 1C) (12/12 patients); with this modality flecks could be hyper- or hyporeflective (Fig. 1C; 2B, 3C, E, G, I).

Tracking the distribution of flecks in both short wavelength and near infrared fundus autofluorescence revealed that under both modalities flecks often first became visible in central retina and then with disease progression become increasingly distributed toward more eccentric locations (8/11 patients) (Figs. 4, 5). The spread of flecks in NIR-AF was marked by an increase in darkened profiles while the advancement of flecks in SW-AF images was led by hyperautofluorescent foci (9/9 patients) (Fig. 4). In some cases flecks appeared to spread radially from the fovea to approximately 8° of eccentricity, beyond which

a circumferential spread characterized the distribution (8/12 patients) (Figs. 4B, 5F). In clinical settings, flecks are typically visualized within the 30° field, but as seen in wide-field images, flecks form well beyond this range (12/12 patients) (Fig. 4). In patients (7/10) in which serial images were obtained after a lapse of 1 year, some flecks were observed to be hypoautofluorescent in NIR-AF images (Fig. 4 B) before any evidence of hyperautofluorescence in SW-AF images (Fig. 4 A versus C).

Flecks in SD-OCT scans

Flecks in SW-AF, NIR and IR-R (Fig. 3A,3B) also co-localized with hyperreflective lesions in SD-OCT images; these lesions traversed photoreceptor-attributable OCT bands (12/12 patients) (Fig. 3D, F, H, J). In the case of some flecks the hyperreflectivity appeared as a thickening of the interdigitation zone (IZ) while other flecks extended through the ellipsoid zone (EZ) and external limiting membrane (ELM) and up into the outer nuclear layer (ONL). As fleck height increased anteriorly, ONL thinning was evident (12/12 patients) (Fig. 3 D, H, blue and red arrows). Fleck profiles in SD-OCT scans were often associated with decreased transmission into the choroid although other flecks that in SW-AF images exhibited fluorescence extinction were accompanied in SD-OCT images by increased transmission into the choroid (4/12 patients) (Fig. 3 A, B, H, purple arrow).

Quantitative autofluorescence and color coded images

The autofluorescence of flecks was also examined in color-coded qAF images in which colors were assigned to each pixel based on qAF values scaled to a range of 0-1200. In qAF maps acquired serially, changes in the brightness of flecks could be appreciated. Shown in Figure 6, is a patient (P1) imaged at 15-month (Fig. 6, A–B) and 18- month (Fig. 6, B–C) intervals. In this patient flecks were initially more dense and of greater intensity in superotemporal retina. Progression of these features proceeded superiorly before being visible in inferior macula. This pattern of fleck evolution was observed in 5/10 patients (9/16 eyes). Flecks that transitioned to foci of intense autofluorescence (red/white foci) (Fig. 6A left panel) were later reduced in brightness. (8/10 patients) (Fig. 6A, right panel, yellow and green foci). The peripapillary area was notable for being spared from flecks and atrophy. The area of central atrophy was typically marked by gradations of color (black to green).

DISCUSSION

For many years it had been assumed that flecks represent lipofuscin-laden RPE cells (Eagle et al. 1980; Lopez et al. 1990). However, the observations reported here indicate that fundus flecks are a manifestation of degenerating photoreceptor cells in STGD1. This conclusion is based on the finding that the flecks visualized in SW-AF images corresponded to hyperreflective aberrations extending radially within photoreceptor-attributable bands in SD-OCT scans. Moreover, as flecks expanded anterior they gradually interrupted, rather than displaced, the EZ and ELM bands and ONL width was progressively reduced over the hyperreflective fleck. This outer to inner expansion of flecks in SD-OCT scans likely represents stages in the degeneration of groups of photoreceptor cells within individual flecks.

Importantly, the hyperreflectivity of flecks in SD-OCT scans also co-localized with hypoautofluorescent foci in NIR-AF images. Since the NIR-AF signal is derived from melanin the loss of this autofluorescence is indicative of RPE atrophy. Taken together these observations support our previously reported evidence (Sparrow et al. 2015) that the anomalous autofluorescence of flecks in SW-AF images originates in photoreceptor cells degenerating secondary to RPE atrophy. The latter interpretation is supported by the instances in which flecks become hyperautofluorescent in SW-AF images after darkening in NIR-AF images and by the observation that hypoautofluorescent flecks in NIR-AF images were larger than bright flecks in SW-AF images. Since RPE melanin is a major reflector of light it is also significant that IR-R is reduced in association with reduced NIR-AF signal at positions of flecks. The conclusion that the aberrant SW-AF of flecks is generated from outer segments of degenerating photoreceptor cells is buttressed by abundant evidence showing that the bisretinoid lipofuscin that is the source of SW-AF is known to be produced in photoreceptor cell outer segments before phagocytosis by RPE (Sparrow et al. 2012b). We previously proposed that SW-AF fundus autofluorescence does not just signal the status of RPE but, under some circumstances, can be indicative of the health of photoreceptor cells (Sparrow et al. 2015). For instance, we have noted abrupt elevations in SW-AF that co-localized with photoreceptor cell degeneration in acute macular neuroretinopathy (Gelman et al. 2012; Sparrow et al. 2015) and elevated qAF that corresponded to EZ loss and ONL thinning at the lesion border in acute zonal occult outer retinopathy (AZOOR) (Boudreault et al. 2017). Within autofluorescence rings that are often a feature of the fundus in retinitis pigmentosa (RP) (Aizawa et al. 2010; Duncker et al. 2013; Popovic et al. 2005; Robson et al. 2003; von Ruckmann et al. 1999) we also found that qAF intensity was higher than at equivalent fundus positions in healthy eyes (Schuerch et al. 2017). Abnormally increased SW-AF is also associated with outer segments that form the core of photoreceptor cell rosettes in degenerating retina (Flynn et al. 2014).

As compared to histological analysis, multimodal *in vivo* imaging allows identified flecks to be linked to changes in the fluorescence of bisretinoid lipofuscin (SW-AF) and melanin (NIR-AF) and to be correlated spatially with structural information available in SD-OCT images. The aberrant SW-AF associated with flecks cannot be accounted for by accelerated phagocytosis of photoreceptor outer segments (Aizawa et al. 2010; Lima et al. 2012; Robson et al. 2006), since bisretinoid fluorophore formation occurs in photoreceptor cells prior to outer segment shedding and subsequent phagocytosis. RPE autofluorescence unmasking consequent to outer segment degeneration and photopigment loss cannot account for the hyperautofluorescence of flecks in SW-AF images since in the current work, image acquisition was preceded by bleaching of photoreceptor visual pigment. A window defect associated with retinal thinning is unlikely to explain the foci of elevated SW-AF since one would expect the NIR-AF of flecks to also be consistently higher under these circumstances, yet the NIR-AF was more often reduced when SW-AF was increased. The reduced or absent NIR-AF (787 nm excitation) at positions of flecks is also unlikely to be due to reduced light transmission since tissue absorption at wavelengths between 600-1300 nm is minimal (Tsai et al. 2001). With time, SW-AF bleaching (Hunter et al. 2012) and resolution of degenerating debris would likely explain the reduction in SW-AF intensity observed. An

alternative interpretation of the findings is that during RPE atrophy, hyperAF contents are extruded into the subretinal space and become the source of SW-AF intensity.

Since bisretinoid initiates phototoxicity (Sparrow et al. 2012b) these findings indicate a role for excess bisretinoid formation in the natural history of fleck formation in STGD1. Aberrant bisretinoid production could also serve as a potential target for therapy. For instance, since flecks track photoreceptor cell degeneration, therapeutic interventions that limit bisretinoid production (Sparrow 2016) may be gauged by evaluating fleck numbers and progression.

Reticular pseudodrusen also present as hyperreflective lesions in photoreceptor-attributable OCT bands (Paavo et al. 2017). Indeed the appearance of RPD in OCT images is quite similar to flecks and in both cases the hyperreflective foci can extend through the EZ and ELM and into a thinning ONL. In other cases of elevated SW-AF, for instance the AF rings in RP, corresponding hyperreflectivity is not observed in OCT scans. Instead, in RP the reflectivity band attributable to ONL is thinned and disruptions in the EZ band are observed (Lima et al. 2009). Perhaps foci of outer retina hyperreflectivity are a feature of photoreceptor cells degenerating secondary to RPE changes.

Acknowledgments

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

References

- Ahn J, Wong JT, Molday RS. 2000; The effect of lipid environment and retinoids on the ATPase activity of ABCR, the photoreceptor ABC transporter responsible for Stargardt macular dystrophy. *J Biol Chem.* 275(27):20399–20405. [PubMed: 10767284]
- Aizawa S, Mitamura Y, Hagiwara A, Sugawara T, Yamamoto S. 2010; Changes of fundus autofluorescence, photoreceptor inner and outer segment junction line, and visual function in patients with retinitis pigmentosa. *Clin Experiment Ophthalmol.* 38(6):597–604. [PubMed: 20456441]
- Allikmets R, Singh N, Sun H, Shroyer NF, Hutchinson A, Chidambaram A, Gerrard B, Baird L, Stauffer D, Peiffer A, Rattner A, Smallwood P, Li Y, Anderson KL, Lewis RA, Nathans J, Leppert M, Dean M, Lupski JR. 1997; A photoreceptor cell-specific ATP-binding transporter gene (ABCR) is mutated in recessive Stargardt macular dystrophy. *Nat Genet.* 15(3):236–246. [PubMed: 9054934]
- Anderson RE, Maude MB. 1970; Phospholipids of bovine outer segments. *Biochemistry.* 9(18):3624–3628. [PubMed: 5509846]
- Beharry S, Zhong M, Molday RS. 2004; N-retinylidene-phosphatidylethanolamine is the preferred retinoid substrate for the photoreceptor-specific ABC transporter ABCA4 (ABCR). *J Biol Chem.* 279:53972–53979. [PubMed: 15471866]
- Boudreault K, Schuerch K, Zhao J, Lee W, Cabral T, Yannuzzi LA, Tsang SH, Sparrow JR. 2017 Quantitative autofluorescence intensities in acute zonal occult outer retinopathy vs healthy eyes. *JAMA ophthalmology.*
- Boyer NP, Higbee D, Currin MB, Blakeley LR, Chen C, Ablonczy Z, Crouch RK, Koutalos Y. 2012; Lipofuscin and N-retinylidene-N-retinylethanolamine (A2E) accumulate in the retinal pigment epithelium in the absence of light exposure: their origin is 11-cis retinal. *J Biol Chem.* 287:22276–22286. [PubMed: 22570475]

- Burke TR, Duncker T, Woods RL, Greenberg JP, Zernant J, Tsang SH, Smith RT, Allikmets R, Sparrow JR, Delori FC. 2014; Quantitative fundus autofluorescence in recessive stargardt disease. *Invest Ophthalmol Vis Sci.* 55(5):2841–2852. [PubMed: 24677105]
- Chrispell JD, Feathers KL, Kane MA, Kim CY, Brooks M, Khanna R, Kurth I, Huebner CA, Gal A, Mears AJ, Swaroop A, Napoli JL, Sparrow JR, Thompson DA. 2009; Rdh12 activity and effects on retinoid processing in the murine retina. *J Biol Chem.* 284:21468–21477. [PubMed: 19506076]
- Cideciyan AV, Aleman TS, Swider M, Schwartz SB, Steinberg JD, Brucker AJ, Maguire AM, Bennett J, Stone EM, Jacobson SG. 2004; Mutations in ABCA4 result in accumulation of lipofuscin before slowing of the retinoid cycle: a reappraisal of the human disease sequence. *Hum Mol Genet.* 13(5): 525–534. [PubMed: 14709597]
- Cukras CA, Wong WT, Caruso R, Cunningham D, Zein W, Sieving PA. 2012; Centrifugal expansion of fundus autofluorescence patterns in Stargardt disease over time. *Arch Ophthalmol.* 130:171–179. [PubMed: 21987580]
- Delori F, Greenberg JP, Woods RL, Fischer J, Duncker T, Sparrow J, Smith RT. 2011; Quantitative measurements of autofluorescence with the scanning laser ophthalmoscope. *Invest Ophthalmol Vis Sci.* 52(13):9379–9390. [PubMed: 22016060]
- Duncker T, Greenberg JP, Ramachandran R, Hood DC, Smith RT, Hirose T, Woods RL, Tsang SH, Delori FC, Sparrow JR. 2014a; Quantitative fundus autofluorescence and optical coherence tomography in Best vitelliform macular dystrophy. *Invest Ophthalmol Vis Sci.* 55(3):1471–1482. [PubMed: 24526438]
- Duncker T, Marsiglia M, Lee W, Zernant J, Tsang SH, Allikmets R, Greenstein VC, Sparrow JR. 2014b; Correlations Amongst Near-Infrared and Short-Wavelength Autofluorescence and Spectral Domain Optical Coherence Tomography in Recessive Stargardt Disease. *Invest Ophthalmol Vis Sci.* 55:8134–8143. [PubMed: 25342616]
- Duncker T, Tabacaru MR, Lee W, Tsang SH, Sparrow JR, Greenstein VC. 2013; Comparison of near-infrared and short-wavelength autofluorescence in retinitis pigmentosa. *Invest Ophthalmol Vis Sci.* 54:585–591. [PubMed: 23287793]
- Duncker T, Tsang SH, Lee W, Zernant J, Allikmets R, Delori FC, Sparrow JR. 2015; Quantitative fundus autofluorescence distinguishes ABCA4-associated and non-ABCA4-associated Bull's-Eye Maculopathy. *Ophthalmology.* 122:345–355. [PubMed: 25283059]
- Eagle RC, Lucier AC, Bernardino VB, Yanoff M. 1980; Retinal pigment epithelial abnormalities in fundus flavimaculatus. *Ophthalmol.* 87:1189–1200.
- Flynn E, Ueda K, Auran E, Sullivan JM, Sparrow JR. 2014; Fundus autofluorescence and photoreceptor cell rosettes in mouse models. *Invest Ophthalmol Vis Sci.* 55(9):5643–5652. [PubMed: 25015357]
- Gelman R, Chen R, Blonska A, Barile G, Sparrow JR. 2012; Fundus autofluorescence imaging in a patient with rapidly developing scotoma. *Retinal Cases & Brief Reports.* 6:345–348. [PubMed: 23293707]
- Hicks D, Sparrow JR, Barnstable CJ. 1989; Immunoelectron microscopical examination of the surface distribution of opsin in rat rod photoreceptor cells. *Exp Eye Res.* 49(1):13–29. [PubMed: 2527162]
- Hunter JJ, Morgan JJ, Merigan WH, Sliney DH, Sparrow JR, Williams DR. 2012; The susceptibility of the retina to photochemical damage from visible light. *Prog Retin Eye Res.* 31:28–42. [PubMed: 22085795]
- Illing M, Molday LL, Molday RS. 1997; The 220-kDa rim protein of retinal rod outer segments is a member of the ABC transporter superfamily. *J Biol Chem.* 272(15):10303–10310. [PubMed: 9092582]
- Kellner S, Kellner U, Weber BH, Fiebig B, Weinitz S, Ruether K. 2009; Lipofuscin- and melanin-related fundus autofluorescence in patients with ABCA4-associated retinal dystrophies. *Am J Ophthalmol.* 147:895–902. [PubMed: 19243736]
- Kim SR, Fishkin N, Kong J, Nakanishi K, Allikmets R, Sparrow JR. 2004; The Rpe65 Leu450Met variant is associated with reduced levels of the RPE lipofuscin fluorophores A2E and iso-A2E. *Proc Natl Acad Sci U S A.* 101(32):11668–11672. [PubMed: 15277666]

- Lima LH, Burke T, Greenstein VC, Chou CL, Cella W, Yannuzzi LA, Tsang SH. 2012; Progressive constriction of the hyperautofluorescent ring in retinitis pigmentosa. *Am J Ophthalmol.* 153:718–727. [PubMed: 22137208]
- Lima LH, Cella W, Greenstein VC, Wang NK, Busuioc M, Smith RT, Yannuzzi LA, Tsang SH. 2009; Structural assessment of hyperautofluorescent ring in patients with retinitis pigmentosa. *Retina.* 29:1025–1031. [PubMed: 19584660]
- Lopez PF, Maumenee IH, de la Cruz Z, Green WR. 1990; Autosomal-dominant fundus favimaculatus. Clinicopathologic correlation. *Ophthalmol.* 97:798–809.
- Maeda A, Golczak M, Maeda T, Palczewski K. 2009; Limited roles of Rdh8, Rdh12, and Abca4 in all-trans-retinal clearance in mouse retina. *Invest Ophthalmol Vis Sci.* 50:5435–5443. [PubMed: 19553623]
- Maeda A, Maeda T, Golczak M, Palczewski K. 2008; Retinopathy in mice induced by disrupted all-trans-retinal clearance. *J Biol Chem.* 283:26684–26693. [PubMed: 18658157]
- Molday LL, Rabin AR, Molday RS. 2000; ABCR expression in foveal cone photoreceptors and its role in Stargardt macular dystrophy. *Nat Genet.* 25(3):257–258. [PubMed: 10888868]
- Paavo M, Lee W, Merriam J, Bearely S, Tsang S, Chang S, Sparrow JR. 2017; Intraretinal Correlates of Reticular Pseudodrusen Revealed by Autofluorescence and En Face OCT. *Invest Ophthalmol Vis Sci.* 58(11):4769–4777. [PubMed: 28973322]
- Papermaster DS, Schneider BG, Zorn MA, Kraehenbuhl JP. 1978; Immunocytochemical localization of a large intrinsic membrane protein to the incisures and margins of frog rod outer segment disks. *J Cell Biol.* 78:415–425. [PubMed: 690173]
- Parish CA, Hashimoto M, Nakanishi K, Dillon J, Sparrow JR. 1998; Isolation and one-step preparation of A2E and iso-A2E, fluorophores from human retinal pigment epithelium. *Proc Natl Acad Sci U S A.* 95(25):14609–14613. [PubMed: 9843937]
- Popovic P, Jarc-Vidmar M, Hawlina M. 2005; Abnormal fundus autofluorescence in relation to retinal function in patients with retinitis pigmentosa. *Graefes Arch Clin Exp Ophthalmol.* 243:1018–1027. [PubMed: 15906064]
- Quazi F, Molday RS. 2014; ATP-binding cassette transporter ABCA4 and chemical isomerization protect photoreceptor cells from the toxic accumulation of excess 11-cis-retinal. *Proc Natl Acad Sci U S A.* 111(13):5024–5029. [PubMed: 24707049]
- Robson AG, El-Amir A, Bailey C, Egan CA, Fitzke FW, Webster AR, Bird AC, Holder GE. 2003; Pattern ERG correlates of abnormal fundus autofluorescence in patients with retinitis pigmentosa and normal visual acuity. 44:3544–3450.
- Robson AG, Saihan Z, Jenkins SA, Fitzke FW, Bird AC, Webster AR, Holder GE. 2006; Functional characterisation and serial imaging of abnormal fundus autofluorescence in patients with retinitis pigmentosa and normal visual acuity. *Br J Ophthalmol.* 90:472–479. [PubMed: 16547330]
- Schuerch K, Woods RL, Lee W, Duncker T, Delori FC, Allikmets R, Tsang SH, Sparrow JR. 2017; Quantifying fundus autofluorescence in patients with retinitis pigmentosa. *Invest Ophthalmol Vis Sci.* 58(3):1843–1855. [PubMed: 28358950]
- Sparrow JR. 2016; Vitamin A-aldehyde adducts: AMD risk and targeted therapeutics. *Proc Natl Acad Sci U S A.* 113(17):4564–4569. [PubMed: 27071115]
- Sparrow JR, Gregory-Roberts E, Yamamoto K, Blonska A, Ghosh SK, Ueda K, Zhou J. 2012a; The bisretinoids of retinal pigment epithelium. *Prog Retin Eye Res.* 31:121–135. [PubMed: 22209824]
- Sparrow JR, Gregory-Roberts E, Yamamoto K, Blonska A, Ghosh SK, Ueda K, Zhou J. 2012b; The bisretinoids of retinal pigment epithelium. *Prog Retin Eye Res.* 31(2):121–135. [PubMed: 22209824]
- Sparrow JR, Marsiglia M, Allikmets R, Tsang S, Lee W, Duncker T, Zernant J. 2015; Flecks in Recessive Stargardt Disease: Short-Wavelength Autofluorescence, Near-Infrared Autofluorescence, and Optical Coherence Tomography. *Invest Ophthalmol Vis Sci.* 56(8):5029–5039. [PubMed: 26230768]
- Staurengi G, Sadda S, Chakravarthy U, Spaide RF. 2014; Proposed lexicon for atomic landmarks in normal posterior segment spectral-domain optical coherence tomography: The IN^{*} OCT consensus. *Ophthalmology.* 121:1572–1578. [PubMed: 24755005]

- Sun H, Molday RS, Nathans J. 1999; Retinal stimulates ATP hydrolysis by purified and reconstituted ABCR, the photoreceptor-specific ATP-binding cassette transporter responsible for Stargardt disease. *J Biol Chem.* 274(12):8269–8281. [PubMed: 10075733]
- Sun H, Nathans J. 1997; Stargardt's ABCR is localized to the disc membrane of retinal rod outer segments. *Nat Genet.* 17:15–16. [PubMed: 9288089]
- Sun H, Nathans J. 2001; Mechanistic studies of ABCR, the ABC transporter in photoreceptor outer segments responsible for autosomal recessive Stargardt disease. *J Bioenerg Biomembrane.* 33(6): 523–530. [PubMed: 11804194]
- Tsai CL, Chen JC, Wang WJ. 2001; Near-infrared absorption property of biological soft tissue constituents. *J Med Biol Engineering.* 21:7–14.
- Ueda K, Zhao J, Kim HJ, Sparrow JR. 2016; Photodegradation of retinal bisretinoids in mouse models and implications for macular degeneration. *Proc Natl Acad Sci.* 113:6904–6909. [PubMed: 27274068]
- von Ruckmann A, Fitzke FW, Bird AC. 1999; Distribution of pigment epithelium autofluorescence in retinal disease state recorded in vivo and its change over time. *Graefe's Arch Clin Exp Ophthalmol.* 237:1–9. [PubMed: 9951634]
- Weng J, Mata NL, Azarian SM, Tzekov RT, Birch DG, Travis GH. 1999; Insights into the function of Rim protein in photoreceptors and etiology of Stargardt's disease from the phenotype in abcr knockout mice. *Cell.* 98(1):13–23. [PubMed: 10412977]
- Wu Y, Fishkin NE, Pande A, Pande J, Sparrow JR. 2009; Novel lipofuscin bisretinoids prominent in human retina and in a model of recessive Stargardt disease. *J Biol Chem.* 284:20155–20166. [PubMed: 19478335]
- Yamamoto K, Yoon KD, Ueda K, Hashimoto M, Sparrow JR. 2011; A novel bisretinoid of retina is an adduct on glycerophosphoethanolamine. *Invest Ophthalmol Vis Sci.* 52:9084–9090. [PubMed: 22039245]
- Zernant J, Schubert C, Im KM, Burke T, Brown CM, Fishman GA, Tsang SH, Gouras P, Dean M, Allikmets R. 2011; Analysis of the *ABCA4* gene by next-generation sequencing. *Invest Ophthalmol Vis Sci.* 52:8479–8487. [PubMed: 21911583]
- Zernant J, Xie YA, Ayuso C, Riveiro-Alvarez R, Lopez-Martinez MA, Simonelli F, Testa F, Gorin MB, Strom SP, Bertelsen M, Rosenberg T, Boone PM, Yuan B, Ayyagari R, Nagy PL, Tsang SH, Gouras P, Collison FT, Lupski JR, Fishman GA, Allikmets R. 2014; Analysis of the *ABCA4* genomic locus in Stargardt disease. *Hum Mol Genet.* 23(25):6797–6806. [PubMed: 25082829]

SIGNIFICANCE

Fundus flecks are conspicuous features of short wavelength fundus autofluorescence images in many patients presenting with recessive Stargardt disease (STGD1). Here, we examined flecks across multiple imaging modalities. In optical coherence tomography scans, flecks were associated with photoreceptor cell-attributable lesions and in near infrared autofluorescence images, the signal from retinal pigment epithelial (RPE) melanin was greatly reduced. These findings indicate that flecks in STGD1 are a manifestation of groups of photoreceptor cells degenerating after the loss of RPE. The elevated production of bisretinoid reflected in increased short wavelength fundus autofluorescence signal could accelerate photoreceptor cell degeneration and contribute to the natural history of STGD1.

Fig. 1.

Fundus flecks in an STGD1 patient are viewed in short-wavelength autofluorescence (SW-AF) (A), near infrared autofluorescence (NIR-AF) (B) and infrared reflectance (IR-R) (C) images (Patient 5). Flecks can be hyperautofluorescent in SW-AF, hypoautofluorescent in NIR-AF and bright in IR-R images (green rectangles); bright in SW-AF, NIR-AF and IR-R images (turquoise rectangles); barely detectable in SW-AF, dark in NIR-AF and bright or not detectable in IR-R (yellow arrows). Flecks can be dark in all modalities (red rectangles);

Fig. 2.

Flecks surround a central area of atrophy (Patient 9). A. Short-wavelength autofluorescence (SW-AF) (A), infrared reflectance (IR-R) (B), near infrared autofluorescence (NIR-AF) (C). Isolated flecks in SW-AF images are often situated within hypoautofluorescent zones in NIR-AF images.

Fig. 3.

Retinal flecks in STGD1 imaged by short-wavelength autofluorescence (SW-AF) (A), near infrared autofluorescence (NIR-AF) (B) and IR-R (C, E, G, I) registered to images obtained by spectral domain optical coherence tomography (D, F, H, J) (Patient 4). The horizontal axis and extent of SD-OCT image are indicated by horizontal green lines ending in arrows. Flecks are visible in SD-OCT images as hyperreflective radial lesions that occupy photoreceptor-attributable bands. Flecks are color-coded to indicate correspondence in successive imaging modalities.

Fig. 4. Spatial distribution of fundus flecks in serial images (Patient 3). Short-wavelength (SW-AF) (A, C) and near infrared (B) wide-field images. The SW-AF image in C was acquired 12 months after the SW-AF and NIR-AF images in A and B. Flecks (arrows) are hypoautofluorescent in NIR-AF images (B) while invisible or faintly visible in SW-AF images (A) before appearing hyperautofluorescent in SW-AF images after 12 months (C).

Fig. 5. Progression of fleck formation in short wavelength (A, B, C, D) and near infrared (D, F, G) autofluorescence images (Patient 1). Serial images acquired at intervals of 13 months (A to B, C); 12 months (B,C to D, E); 6 months (D,E to F, G).

Fig. 6.

Flecks visualized in color-coded quantitative fundus autofluorescence (qAF) images (upper panel) shown together with the corresponding fundus autofluorescence (AF; 488 nm) images (lower panel) of Patient 1. Serial images were acquired at a 15-month (A –B) and 18- month (B-C) interval. Lower qAF levels are coded in blue and higher qAF levels as red or white.

Table 1

Demographic, clinical and genetic summary of the Stargardt disease patient cohort.

Patient	Age	Age of onset	Race/Ethnicity	Iris color	BCVA		ABCA4 Mutations	
					OD	OS	Allele 1	Allele 2
1	14	10	White	Green	20/70	20/80	p.[L541P/A1038V]	p.N1868I; c.768+358C>T [‡]
2	9	9	White	Hazel	20/200	20/200	p.W855*	p.T1526M
3	18	14	White	Blue	20/100	20/125	p.T972N	p.L2027F
4	23	18	White	Blue	20/30	20/30	p.C54Y	c.5196+1137G>A; [‡] c.1555:2745A>G [‡]
5	14	10	White	Blue	20/150	20/40	c.4773+3A>G	p.[L541P/A1038V]
6	12	8	White	Blue	20/300	20/200	c.5312+1G>A	p.R2030*
7	16	10	White	Blue	20/40	20/60	p.[L541P/A1038V]	p.L2027F
8	30	23	Indian	Brown	20/60	20/70	p.R653C	p.G1961E
9	61	52	White	Green	20/125	20/20	c.250_251insCAAA	p.N1868I
10	16	8	White	Brown	20/25	20/25	p.R1108C	p.Q1412*
11	41	18	White	Blue	20/200	20/150	p.G1961E	p.N1868I; c.2160+584A>G [‡]
12	55	50	White	Hazel	20/50	20/40	p.R2077W	p.N1868I

BCVA, best-corrected visual acuity; OD, right eye; OS, left eye;

[‡] possibly pathogenic (pending functional validation studies)