Acta Ophthalmologica

Spectral fundus autofluorescence peak emission wavelength in ageing and AMD

Rowena Schultz,[1] Linda Schwanengel,[1] Matthias Klemm,[2] Daniel Meller[1] and Martin Hammer[1,3]

[1]Department of Ophthalmology, University Hospital Jena, Jena, Germany
[2]Institute of Biomedical Engineering and Informatics, Technical Univ. Ilmenau, Ilmenau, Germany
[3]Center for Medical Optics and Photonics, Univ. of Jena, Jena, Germany

ABSTRACT.

Purpose: **To investigate the spectral characteristics of fundus autofluorescence (FAF) in AMD patients and controls.**

Methods: **Fundus autofluorescence spectral characteristics was described by the peak emission wavelength (PEW) of the spectra. Peak emission wavelength (PEW) was derived from the ratio of FAF recordings in two spectral channels at 500–560 nm and 560–720 nm by fluorescence lifetime imaging ophthalmoscopy. The ratio of FAF intensity in both channels was related to PEW by a calibration procedure. Peak emission wavelength (PEW) measurements were done in 44 young (mean age: 24.0 ± 3.8 years) and 18 elderly (mean age: 67.5 ± 10.2 years) healthy subjects as well as 63 patients with AMD (mean age: 74.0 ± 7.3 years) in each pixel of a 30° imaging field. The values were averaged over the central area, the inner and the outer ring of the ETDRS grid.**

Results: **There was no significant difference between PEW in young and elderly controls. However, PEW was significantly shorter in AMD patients (ETDRS grid centre: 571 ± 26 nm versus 599 ± 17 nm for elderly controls, inner ring: 596 ± 17 nm versus 611 ± 11 nm, outer ring: 602 ± 16 nm versus 614 ± 11 nm). After a mean follow-up time of 50.8 ± 10.8 months, the PEW in the patients decreased significantly by 9 ± 19 nm in the inner ring of the grid. Patients, showing progression to atrophic AMD in the follow up, had significantly ($p \leq 0.018$) shorter PEW at baseline than non-progressing patients.**

Conclusions: **Peak emission wavelength (PEW) is related to AMD pathology and might be a diagnostic marker in AMD. Possibly, a short PEW can predict progression to retinal and/or pigment epithelium atrophy.**

Key words: age-related macular degeneration – fluorescence spectra – fundus autofluorescence – retinal pigment epithelium

[Correction added on 03 February 2022, after first online publication: The author Matthias Klemm has been added to the authors' list.]

Acta Ophthalmol. 2022: 100: e1223–e1231

doi: 10.1111/aos.15070

Introduction

Although fundus autofluorescence (FAF) imaging is part of routine clinical diagnostics now, there is still limited knowledge on the involved fluorophores and their role in the pathophysiology of retinal diseases. Most of our knowledge comes from post mortem isolation and analysis of fluorophores (Marmorstein et al. 2002; Han et al. 2006; Warburton et al. 2007; Sparrow et al. 2010; Feldman et al. 2015, 2018; Ben Ami et al. 2016; Tong et al. 2016; Guan et al. 2020; Yakovleva et al. 2020), from investigations in transgenic mice with specific alterations in the visual cycle (Boyer et al. 2012, 2021; Stremplewski et al. 2015; Meleppat et al. 2020; Palczewska et al. 2020), and from cell culture experiments using bisretinoids and their oxidation products as known fluorophores of lipofuscin of the retinal pigment epithelium (RPE) (Ben-Shabat et al. 2002; Sparrow et al. 2002; Hammer et al. 2006, 2008a, 2008b). In these investigations, the molecule N-retinylidene-N-retinyl-ethanolamine (A2E) got the most attention as it was regarded the most fluorescent component of lipofuscin. But also the possibility to synthesise A2E (Parish et al. 1998) for the use in cell culture experiments might have contributed to its popularity. In fact, the molecular composition of lipofuscin is much more complex. Ten different compounds have been isolated by thin-layer chromatography (Eldred & Katz 1988) and taking precursor and oxidation products into account, about 25 molecules are reported (Sparrow et al. 2012).

Differentiation of fluorophores *in vivo* needs a description of the fluorescence beyond fluorescence intensities. Two parameters of the fluorescence have been investigated so far, the fluorescence lifetime and the emission spectrum. Whereas several recent reports show alterations of fluorescence lifetimes in various retinal diseases

(Dysli et al. 2015, 2016, 2016, 2017, 2017; Sauer et al. 2016, 2018a, 2018b, 2018c, 2019; Solberg et al. 2019; Hammer et al. 2020; Schultz et al. 2020a, 2020), there are relatively few publications on *in vivo* measurements of FAF spectra. Delori et al. (1995) reported a fundus camera based instrument measuring FAF spectra at single locations of the retina in 1994 (Delori 1994) and measured spectra of a cohort of healthy subjects. This technique was applied to a series of seven patients suffering from age-related macular degeneration (AMD; Arend et al. 1995). However, the patient group was divers, no conclusion on spectral changes by AMD could be drawn with statistical evidence. Investigations in AMD and diabetic patients, using two spectral channels only, revealed general long wavelength fluorescence, resulting from Lipofuscin, and additional short wavelength emission, which could be addressed to collagen in disciform scars secondary to AMD, drusen, and advanced glycation end products in the diabetic patients (Hammer et al. 2008a, 2008b). Borrrelli et al. used a slit-scanning camera to record colour FAF images showing that the assessment of green-emitting fluorophores is repeatable and in areas of macular atrophy, shortwave fluorophores are present and appear to correspond to residual debris or drusenoid materials (Borrelli et al. 2018, 2020; Borrelli et al. 2018).

In this study, we used the fluorescence lifetime imaging ophthalmoscopy (FLIO) device; however, the temporal resolution was neglected. Photons from all time channels were binned and only their distribution over the two spectral channels was considered. This way, the peak emission wavelength (PEW) per pixel was determined for healthy subjects of different age and AMD-patients.

Methods

First, we tested the test re-test reliability of the PEW measurement. We performed FLIO measurements of FAF in 22 eyes of 11 young healthy subjects (age: 28.7 ± 3.6 years). Within two weeks, all subjects were measured twice on two days by the same operator using the same device with identical settings. Peak emission wavelength (PEW) were averaged over standardised areas at the fundus by centering the standard ETDRS grid at the fovea manually and calculating the mean values in the centre, the inner, and the outer ring of the grid.

Forty-four young (<40 years, mean age: 24.0 ± 3.8 years) and 18 elderly (>40 years, mean age: 67.5 ± 10.2 years) healthy subjects as well as 63 patients with AMD (mean age: 74.0 ± 7.3 years) were included. Patients were seen repeatedly and assigned to two follow-up intervals (12–36 and 37–72 months); demographic data is given in Table 1. Mean ages of all groups were significantly different. The study was approved by the ethics committee of the University Hospital Jena and adhered to the tenets of the declaration of Helsinki. All participants gave written informed consent prior to study inclusion and underwent a full ophthalmologic examination including best corrected visual acuity, OCT (Cirrus-OCT, Carl-Zeiss Meditec AG, Jena, Germany) and CFP (Visucam, Carl-Zeiss Meditec AG, Jena, Germany). Pupils were dilated using tropicamide (Mydriaticum Stulln, Pharma Stulln GmbH, Nabburg, Germany) and phenylephrine-hydrochloride (Neosynephrin-POS 5%, Ursapharm GmbH, Saarbrucken, Germany). After pupil dilation, patients underwent FLIO imaging, OCT, and CFP. No sodium fluorescein was administered topically or intravenously prior to FLIO investigation.

Fluorescence lifetime imaging ophthalmoscopy (FLIO) image capture is based on a picosecond laser diode coupled with a laser scanning ophthalmoscope (Spectralis, Heidelberg Engineering, Heidelberg, Germany), exciting retinal autofluorescence at 473 nm with a repetition rate of 80 Mhz. Fluorescence photons were detected by time-correlated single photon counting (SPC-150, Becker&Hickl GmbH, Berlin, Germany) in short-wavelength (SSC: 498–560 nm) and long-wavelength (LSC: 560–720 nm) spectral channel. Fluorescence lifetime imaging ophthalmoscopy (FLIO) provides 30° field images with a frame rate of nine frames per second and a resolution of 256 × 256 pixels. In this study, we neglected the time resolution of the measurement and only counted all photons per spectral channel and pixel. The ratio of the photons in SSC and LSC was calculated and calibrated to the PEW as described previously (Schultz et al. 2021). Briefly, the calibration used the known fluorescence emission spectra of healthy RPE (Delori et al. 1995) in vivo and the human lens (Zuclich et al. 2005), compared that with the SSC/LSC ratio from FLIO measurements at the fundus and lens in a cohort of healthy young subjects, and established a linear relationship between the ratio and the PEW.

Table 1. Survey of patient demographics

Patient information	
Patients at baseline	63 (100%)
Female	35 (56%)
Male	28 (44%)
Age (years, mean±SD)	74.0±7.3
Patients with drusen	50 (79%)
Patients with SDD	18 (29%)
Patients at follow up 1	39 (62%)
Patients at follow up 2	25 (40%)
Eyes progressed to atrophy	10 (16%)

SPSS 27.0 (IBM, SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Mann-Whitney *U*-test was used for the comparison of group median values if data in the groups was not normally distributed (tested by Kolmogorov-Smirnov test). Otherwise, mean values were compared by *t*-test or ANOVA with *post-hoc* tests using Bonferroni correction for multiple comparisons. Wilcoxon test was used for the paired testing of median values of the test re-test PEW measurements, the intraclass correlation coefficient was determined and a Bland-Altman plot was produced.

Results

In the repeatability investigation, a significant correlation between the first and the second measurement was found in the inner and the outer ring as well as the centre of the ETDRS grid (Fig. 1, $p = 0.003$, $p < 0.001$, and $p < 0.001$, respectively). In a test re-test analysis (see Bland-Altman plots in Fig. 1), the intraclass correlation coefficient was 0.577 (95% CI 0.219–0.799) for the centre of the ETDRS grid, 0.800 (95% CI 0.575–0.912) for the inner, and 0.826 (95% CI 0.627–0.924) for the outer ring. The median values of the first and the second measurement did not differ significantly ($p = 0.154$, 0.765, and 0.527 for the respective grid areas in Wilcoxon test).

The PEW tends to shift towards shorter wavelengths with ageing. A significant difference for subjects of <40 and >40 years of age, however, was only found in the outer ring of the ETDRS grid (*t*-test, $p = 0.017$). In an

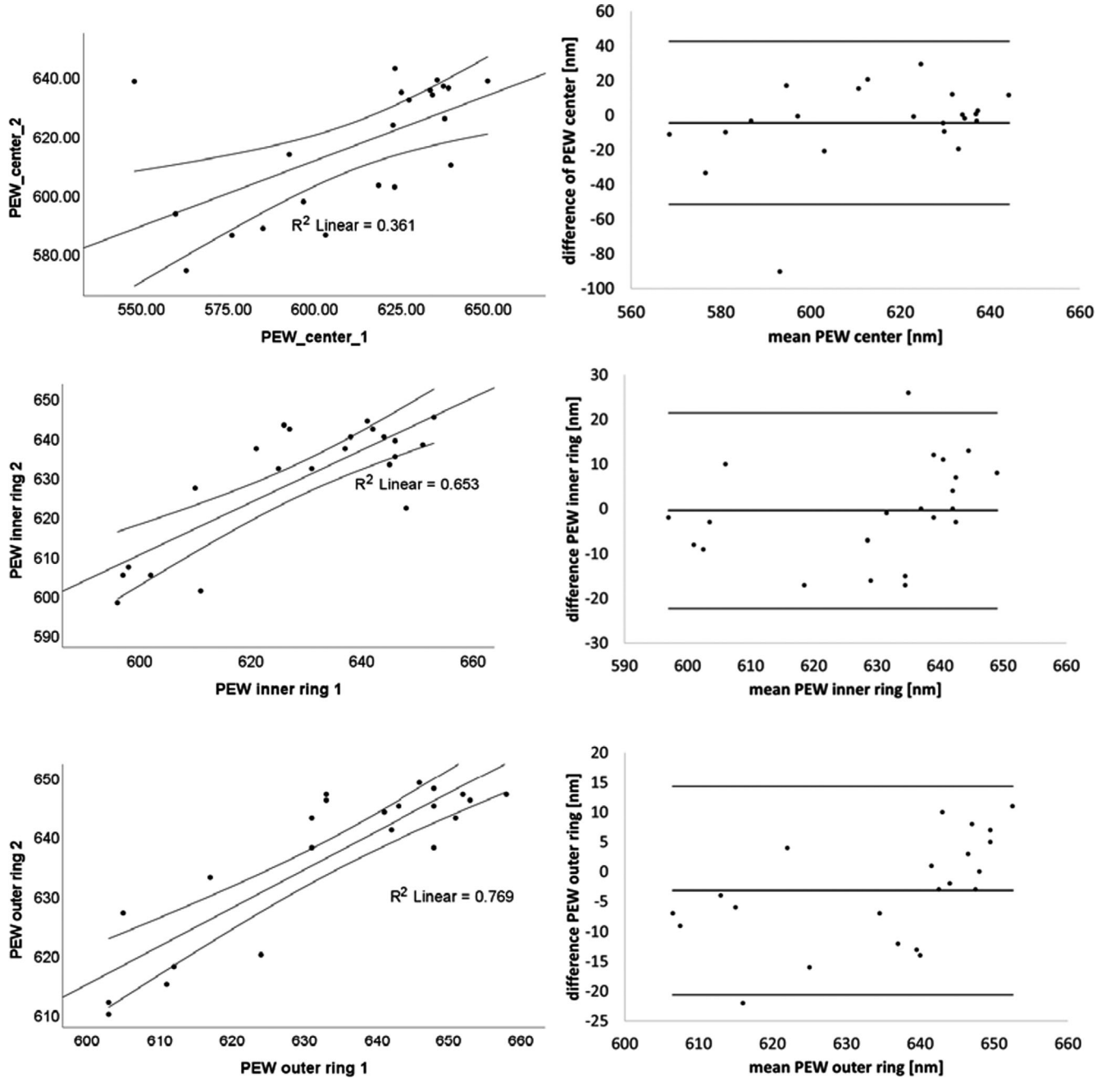

Fig. 1. PEW measures in the second versus the first imaging session (left) and Bland-Altman plots (right) in the centre (top), the inner ring (middle), and the outer ring (bottom) of the ETDRS grid.

ANOVA, accounting for multiple comparisons, there was no significant difference found for healthy subjects of both age groups, however the AMD-patients had significantly shorter PEW in all areas of the ETDRS grid (see Table 2 and Fig. 2). As a typical example, FAF and PEW-images (colour-coded from 560 to 650 nm according to the scale bar) of a 68-year-old AMD patient and a 65-year-old control are shown in Fig. 3. The difference of the PEW is clearly seen. Both subjects were phacic. In our AMD-cohort, 27 subjects were pseudophacic (clear lenses only, yellow lenses were excluded). There was no difference in the median PEW between phacic and pseudophacic patients ($p = 0.738$ in Mann-Whitney *U*-test).

Thirty-nine patients had follow-up investigations, 30 within 12–36 months (mean follow-up time: 24.0 ± 8.7 months), 25 within 37–72 months (mean follow-up time: 50.8 ± 10.8 months), and 16 had repeated visits in both follow-up intervals. Ten cases were excluded from the follow-up since they progressed to retinal or RPE atrophy. A further shortening of the PEW was seen for both follow up intervals in all areas of the ETDRS grid (Table 2 and Fig. 4) which, however, was significant for the centre of the ETDRS grid and inner ring at the last follow-up (37–72 months) only.

A hypsochromic shift of the PEW may precede the advent of ORA or cRORA. Figure 5 shows a 78-year-old patient who developed cRORA in the follow up. Already at baseline, shorter PEW was found superior to the fovea

Table 2. Mean values and standard deviations of PEW for healthy subjects <40 years and >40 years of age as well as AMD patients.

	Healthy < 40 years	Healthy < 40 years	AMD
ETDRS centre	602 ± 16 nm	599 ± 17 nm	571 ± 26 nm
ETDRS inner ring	614 ± 12 nm	611 ± 11 nm	596 ± 17 nm
ETDRS outer ring	621 ± 11 nm	614 ± 11 nm	602 ± 16 nm

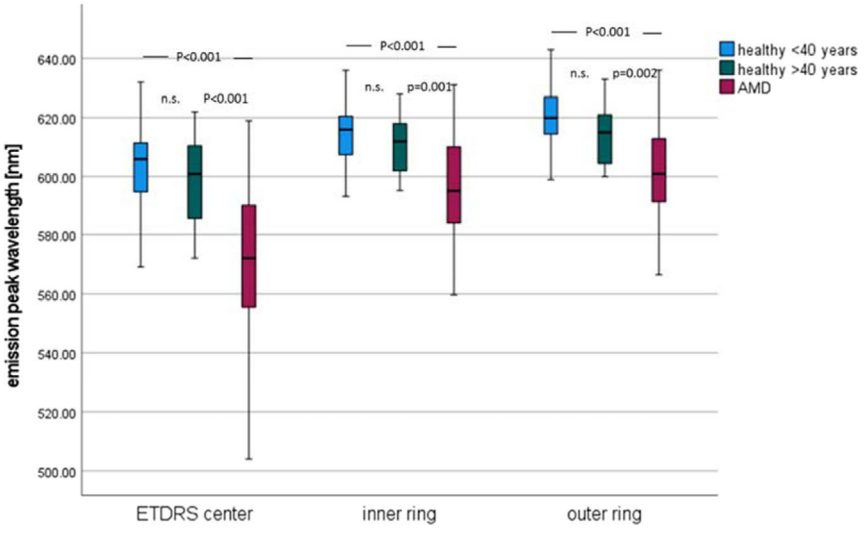

Fig. 2. Boxplots of emission peak wavelengths for healthy controls younger and older than 40 years as well as AMD patients in the center, inner, and outer ring of the ETDRS grid. *p*-values from ANOVA and post-hoc test with Bonferroni correction for multiple testing are given for significant differences.

where atrophy developed later. The 10 subjects, showing AMD progression to ORA or cRORA, showed significantly shorter PEW in all areas of the ETDRS-grid at baseline than the 39 subjects who did not progress (Table 3 and Fig. 6).

Eighteen eyes (29% of all eyes) had subretinal drusenoid deposits (SDD). The PEW tended to be shorter in the SDD cases than in the drusen-dominated eyes (centre: 564 ± 27 nm versus 574 ± 25 nm, inner ring: 591 ± 15 nm versus 598 ± 18 nm, outer ring: 597 ± 12 nm versus 604 ± 17 nm) but none of these differences were significant. The rate of progression to ORA or cRORA was higher in eyes with SDD (5 out of 16 eyes in follow up, 31%) than in eyes without SDD (5 out of 33 eyes, 15%).

Discussion

Good repeatability of the PEW measurement was shown for the inner and outer ring of the ETDRS grid. In the centre, the inter-individual variance was higher. This can be accounted to differences in the Xanthophyll concentration between subjects (Delori et al. 2001). However, also the Pearson correlation coefficient and the intra-class correlation coefficient were lower here than in the other areas, the PEW measurement has to be considered less repeatable. The reason might be the lower fluorescence intensity due to Xanthophyll absorption of the excitation light.

This is the first report of PEW measurements from FLIO in healthy eyes and that of AMD patients. Our findings indicate a slight hypsochromic emission shift with ageing and significantly shorter emission wavelengths in AMD versus controls. Patients with progression to ORA or cRORA at follow up show shorter PEW in the baseline measurement already. Furthermore, in agreement with the literature (Smith et al. 2009; Heesterbeek et al. 2020), the rate of disease progression was higher in the patients showing SDD and these patients had insignificantly lower PWE. It remains to be determined in further prospective studies, adjusting for disease stage and duration, whether PEW is indicative for AMD progression.

Our measurements should be referenced to such of full spectra as described by Delori (1994). His instrument uses a sophisticated procedure for the compensation of the spectral transmission of all optical media and the sensitivity of the detector. Thus, these spectra are considered the gold standard; however, the spectral resolution is achieved on cost of spatial resolution. Due to the limited number of fluorescence photons, it provides spectra at single spots (minimal size 1.3°) but no two-dimensional images. Using this technique, Delori et al. (1995) determined lipofuscin as the dominant retinal fluorophore, but also found a minor fluorophore with peak emission at 520–540 nm, possibly related to flavin adenine dinucleotide (FAD). This fluorophore was seen most clearly where lipofuscin fluorescence was low, e.g. in the macula. This is in agreement with our findings of shorter PEW in the macula in healthy subjects. These authors reported an increase of the fluorescence intensity with age, but no spectral shift. Arend et al. performed spectral measurements in seven AMD patients and two controls (Arend et al. 1995). Unfortunately, the sample size and the diversity of AMD stages did not allow conclusions on spectral differences between patients and controls. However, they reported shorter emission wavelengths (maximum emission at 560 nm) in cases with drusen at the location of the measurement. Although in the current investigation, we sought for global differences rather than local ones, Figs 3 and 5 show shortwave emission at drusen as well. This is in agreement with our previous findings (Hammer et al. 2020). Shortest PEW were found in the central subfield of the ETDRS grid (Fig. 2, Table 4). As this holds especially for AMD patients and soft drusen have highest abundance in the central macula, they might contribute to these short PEW.

Colour-CCD based measurements of FAF in two spectral channels are reported previously. We investigated pseudophacic AMD and diabetic retinopathy patients as well as healthy controls (Hammer et al. 2008a, 2008b). In agreement with the current investigation, we found a slight, non-significant green-shift of the emission with age. Generally, the emission

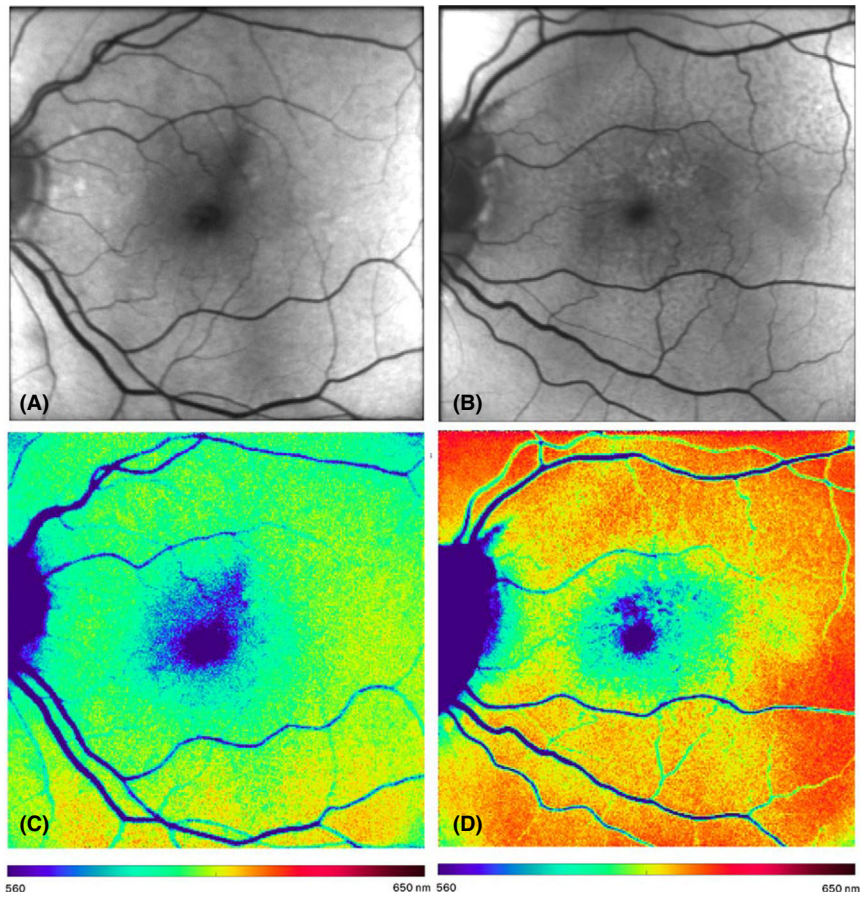

Fig. 3. (A) FAF images of a 65-year-old control and (B) a 68-year-old AMD patient. (B) and (C): colour-coded PEW for both subjects. The patient shows shorter FAF emission wavelength than the control.

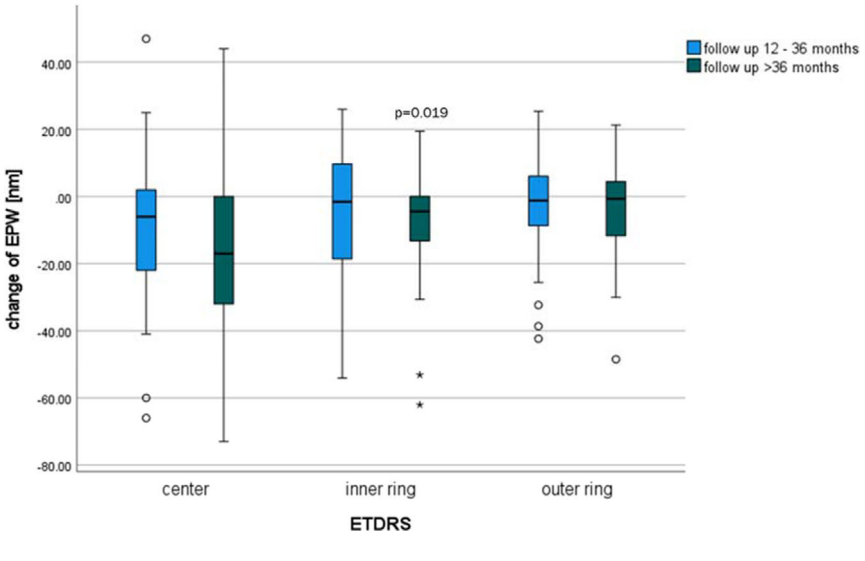

Fig. 4. Change of the EPW during follow up of 12–36 or >36 months compares to baseline. A hypsochromic shift of the EPW was observed. This was significant for the follow up >36 months in the inner ring of the ETDRS grid.

wavelength was shortest in the diabetics, intermediate in AMD, and longest in the controls; however, the differences were not significant. A reason could be that this fundus camera based approach lacks confocality and all structures anterior to the retina contributed to the fluorescence as well. Using a slit-scanning device with fluorescence detection in a green and a red camera channel upon excitation at 450 nm, Borrelli et al. (2018) reported a good visibility of areas of geographic atrophy in a red-filtered image of FAF and a remaining green fluorescence in areas of macular atrophy, associated with hyperreflective material atop Bruch's membrane seen in OCT (Borrelli et al. 2018). All studies so far indicate a weak, short-wave emission in RPE atrophy (Arend et al. 1995; Hammer et al. 2008a, 2008b; Borrelli et al. 2018). When lacking lipofuscin fluorescence, FAF is dominated by contributions of Bruch's membrane and remaining basal linear and laminar deposits, eventually also from the retina and deeper layers such as choroid and sclera. For this reason, we excluded patients with ORA and cRORA from the comparison with healthy controls as well as from the analysis of spectral changes of FAF in the follow up of AMD.

As FAF emission wavelengths shorten in AMD (eventually in normal ageing as well) and short wavelength emission seems to be predictive for AMD progression, reasons for short-wave FAF would be of utmost interest. Related fluorophores were studied in in vitro experiments or from post mortem donor eyes. However, wavelengths are not entirely comparable. Already Delori et al. found a hypsochromic shift of FAF spectra post mortem versus *in vivo* (Delori et al. 1995). An effect of tissue fixation was not found (Delori et al. 1995; Schultz et al. 2020a, 2020), however, altered FAD concentrations post mortem as well as oxidation of fluorophores upon oxygen exposure of tissue during the preparation process may account for this shift (Hammer et al. 2018). Marmorstein et al. (2002) reported a maximal emission at 555 nm for RPE and 545 nm for sub-RPE deposits from histologic sections upon excitation at 488 nm. There was no difference between AMD- and control donor eyes. In contrast, we found a considerable difference in RPE and sub-RPE emission (610 versus 570 nm) upon 2-photon excitation (960 nm), but also only minor differences between AMD patients and controls (Schultz et al. 2020a, 2020). From slices of mouse eyes, broad emission spectra upon 3-photon

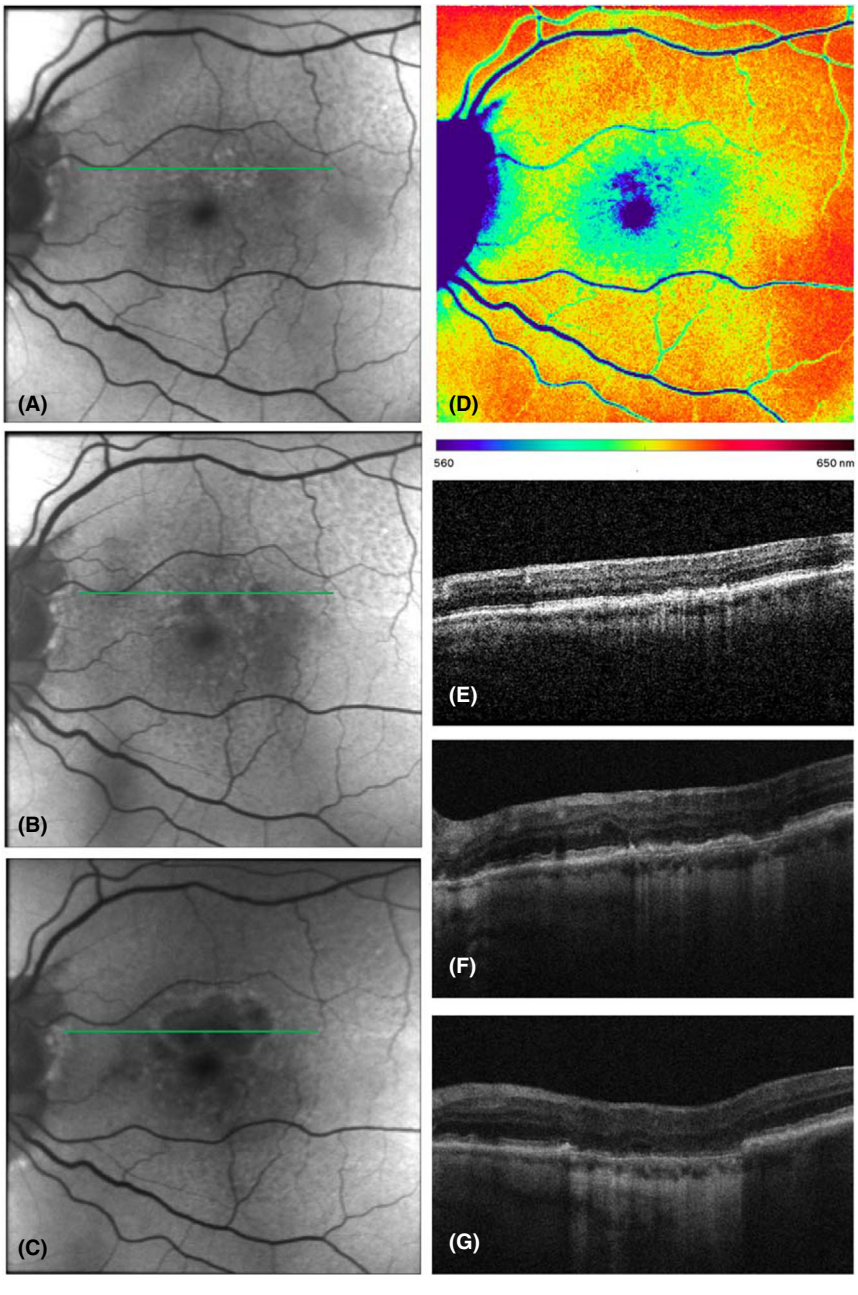

Fig. 5. Fundus autofluorescence (FAF) of a 78-year-old AMD patient at (A) baseline and follow up of (B) 19 and (C) 52 months. The development of geographic atrophy is seen. (E) and (F): OCT at green lines in A and B revealing cRORA. (D): EPW image at baseline. Areas of later atrophy show shorter FAF emission wavelengths at baseline already.

Table 3. Difference of PEW at follow-up investigations versus baseline.

	Follow-up 12–36 months	Follow-up 37–72 months
ETDRS centre	**−10 ± 23 nm**	**−17 ± 26 nm**
ETDRS inner ring	−6 ± 26 nm	**−9 ± 19 nm**
ETDRS outer ring	−3 ± 18 nm	−4 ± 16 nm

Note: Mean values and standard deviations, bold values show significant differences.

excitation at 1.07 µm with very short pulses (40 ps) are reported with a strong contribution of retinal layers, which, however, is not well explained (Murashova et al. 2017). Han et al. (2006) found RPE cells with "abnormal lipofuscin granules" showing short-wavelength autofluorescence at 520 nm versus 556 nm in other cells upon 436 nm excitation in RPE flat-mounts. This corresponds to the finding of lipofuscin granules of different density and fluorescence properties (Guan et al. 2020) as well as to the finding of large granules with short-wavelength emission in RPE cells of an elderly donor (Han et al. 2007). However, it could not be stated whether cells containing larger granules with shortwave emission are associated with pathology.

Several authors report measurements in mouse strains overexpressing or lacking lipofuscin. Abca4$^{-/-}$ mice, a model for Stargardt's disease, show a massive accumulation of lipofuscin with same emission spectra as wild type animals in RPE flat mounts (emission peak at 610 nm upon 488 nm excitation; Boyer et al. 2012; Meleppat et al. 2020). The major source of fluorescence is regarded to be A2E generated from 11-cis-retinal rather than all-trans-retinal (Boyer et al. 2021). However, as the fluorescence intensity in different mouse strains is not proportional to the A2E level (Boyer et al. 2012), other fluorophores have to be considered as well. RPE65$^{-/-}$ mice, in which the visual cycle is blocked due to lack of the enzyme RPE65, which converts all-trans-retinal to 11-cis-retinal, do not accumulate bisretinoids but retinyl esters in the retinosomes of RPE cells. For these mice, a different fluorescence emission peaking at 524 nm upon 2-photon excitation at 730 nm *in vivo*, was found (Stremplewski et al. 2015). Two-photon fluorescence (excitation at 730 nm) was able to distinguish bisretinoids and retinyl esters by their emission spectra in Rdh8$^{-/-}$Abca4$^{PV/PV}$ mice (Palczewska et al. 2020).

Several studies report spectral measurements on Chloroform/Methanol extracts of lipid compounds from human donor eyes. The separation of compounds from the extracts by thin layer chromatography revealed ten fluorescent species (Eldred & Katz 1988). Three of them could be excited at wavelengths above 400 nm and showed yellow-orange fluorescence. High Performance Liquid Chromatography (HPLC) analysis revealed one of them, A2E, as a strong fluorophore which, subsequently, was widely studied in cell culture experiments as it could be synthesised (Parish et al. 1998). A2E solution in PBS showed

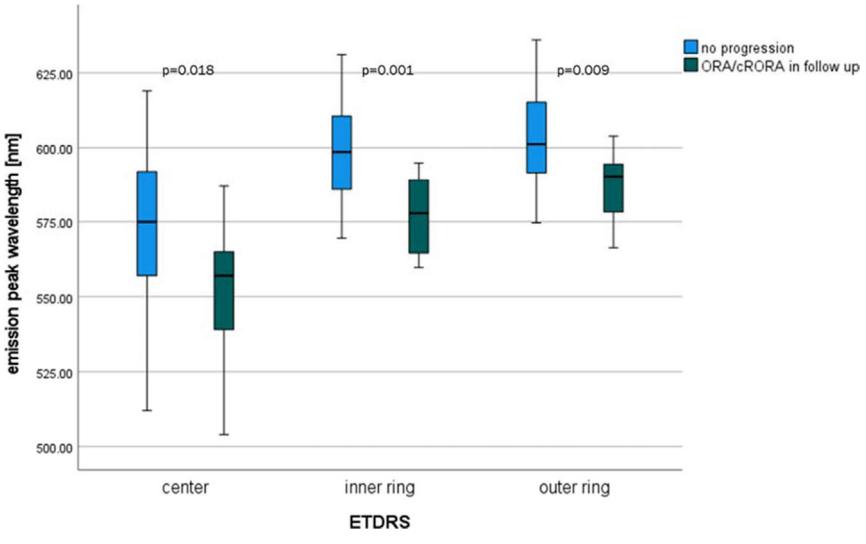

Fig. 6. Baseline EPWs for patients who progressed to ORA or cRORA in the follow up versus patients who did not. p-Values from the Mann-Whitney U-Test show significant differences between the groups.

Table 4. Median and interquartile ranges of PEW at baseline for patients with progression or no progression to ORA or cRORA.

	No AMD progression	ORA/cRORA in follow-up	p-Value
ETDRS centre	575 [39] nm	557 [32] nm	0.018
ETDRS inner ring	599 [25] nm	578 [25] nm	0.001
ETDRS outer ring	601 [25] nm	590 [18] nm	0.009

Note: p-Values from Mann-Whitney *U*-test show significance between the groups.

an emission maximum at 610 nm (excitation at 380 nm; Sparrow et al. 1999). However, the emission is sensitive to the chemical environment (Ragauskaite et al. 2001) and can be shifted to shorter wavelengths by photo-oxidation (Sparrow & Duncker 2014). Later studies, using HPLC for compound separation, found A2E to be only minor fluorescent but fluorescence of photo-degradation products of bisretinoids was highest with fluorescence from photo-oxidised A2E (Feldman et al. 2015; Yakovleva et al. 2020). Furthermore, these authors found a hypsochromic shift of the fluorescence of RPE cells from AMD donors compared to healthy controls (Feldman et al. 2018; Yakovleva et al. 2020). Warburton et al. observed shorter emission wavelength for melano-lipofuscin, not related to photoreceptor phagocytosis, than for lipofuscin (Warburton et al. 2007).

Thus, the shorter PEW of AMD patients versus controls, observed here, could be attributed to changes in the RPE fluorophore composition, specifically a photo-oxidation of bisretinoids or an increase of the ratio of melano-lipofuscin to lipofuscin. However, other explanations should be considered as well: As lipofuscin is known to decrease in AMD (Ach et al. 2015; Reiter et al. 2019), the relative contribution of other fluorophores can increase. These can be located in sub-RPE drusen and basal-linear deposits, known to have shortwave emission (Ben Ami et al. 2016; Tong et al. 2016; Hammer et al. 2020; Schultz et al. 2020a, 2020) or in the retina (Murashova et al. 2017; Hammer et al. 2018). Furthermore, we consistently observed shorter PEW in the macula in all subjects. This might be related to the fluorescence of the macular pigment (Sauer et al. 2016, 2018a, 2018b, 2018c) or to a lack of A2E in the fovea (Ablonczy et al. 2013).

This study has several limitations: First of all, it has to be emphasised that the reported PEW are subject to a calibration procedure relying on assumptions (Schultz et al. 2021). Although these assumptions are reasonable and well documented in the literature, they might not be absolutely correct. This could result in small deviations of the absolute values of PEW. The reported differences between groups, however, will not be affected as the calibration was used for all PEW values. Another point, which can corrupt the PEW, is background light in the measurement. All measurements were taken in a completely dark room and background, usually, is very low. However, as the detectors have single photon sensitivity; few background photons might have been recorded. If their number, relative to that of the fluorescence photons, is different in both spectral channels, this will influence the PEW. Finally, the AMD patients were on average 6.5 years older than the elderly control subjects. Considering the inter-individual variance of the PEW values, we do not think that this age difference of the subjects contribute much to the PEW-difference between the groups, yet we cannot exclude a certain age effect.

In conclusion, PEW might be a diagnostic marker in AMD. It was not only shorter in the patients than in the controls but also shorter in patients, who later progressed to ORA or cRORA than in those, who did not. Thus, short PEW might be an indicator for AMD progression.

References

Ablonczy Z, Higbee D, Anderson DM et al. (2013): Lack of correlation between the spatial distribution of A2E and lipofuscin fluorescence in the human retinal pigment epithelium. Invest Ophthalmol vis Sci **54**: 5535–5542.

Ach T, Tolstik E, Messinger JD, Zarubina AV, Heintzmann R & Curcio CA (2015): Lipofuscin redistribution and loss accompanied by cytoskeletal stress in retinal pigment epithelium of eyes with age-related macular degeneration. Invest Ophthalmol vis Sci **56**: 3242–3252.

Arend O, Weiter JJ, Goger DG & Delori FC (1995): In vivo Fundus Fluoreszenzmessungen bei Patienten mit altersbedingtrer Makuladegeneration. Der Ophthalmologe **92**: 647–653.

Ben Ami T, Tong Y, Bhuiyan A, Huisingh C, Ablonczy Z, Ach T, Curcio CA & Smith RT (2016): Spatial and spectral characterization of human retinal pigment epithelium fluorophore families by ex vivo hyperspectral autofluorescence imaging. Transl vis Sci Technol **5**: 5.

Ben-Shabat S, Itagaki Y, Jockusch S, Sparrow JR, Turro NJ & Nakanishi K (2002): Formation of a nonaoxirane from A2E, a lipofuscin fluorophore related to macular degeneration, and evidence of singlet oxygen involvement. Angew Chem Int Ed Engl **41**: 814–818.

Borrelli E, Battista M, Zuccaro B et al. (2020): Spectrally resolved fundus autofluorescence in healthy eyes: repeatability and topographical analysis of the green-emitting fluorophores. J Clin Med **9**: 2388.

Borrelli E, Lei J, Balasubramanian S et al. (2018): Green emission fluorophores in eyes with atrophic age-related macular degeneration: a colour fundus autofluorescence pilot study. Br J Ophthalmol **102**: 827–832.

Borrelli E, Nittala MG, Abdelfattah NS et al. (2018): Comparison of short-wavelength blue-light autofluorescence and conventional blue-light autofluorescence in geographic atrophy. Br J Ophthalmol. **103**: 610–616.

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 retinal pigment epithelium in absence of light exposure: their origin is 11-cis-retinal. J Biol Chem **287**: 22276–22286.

Boyer NP, Thompson DA & Koutalos Y (2021): Relative contributions of all-trans and 11-cis retinal to formation of lipofuscin and A2E accumulating in mouse retinal pigment epithelium. Invest Ophthalmol vis Sci **62**: 1.

Delori FC (1994): Spectrometer for noninvasive measurement of intrinsic fluorescence and reflectance of ocular fundus. Appl Optics **33**: 7439–7452.

Delori FC, Dorey KC, Staurenghi G, Arend O, Goger DC & Weiter JJ (1995): In vivo fluorescence of the ocular fundus exhibits retinal pigment epithelium lipofuscin characteristics. Invest Ophthalmol **36**: 718–729.

Delori FC, Goger DG, Hammond BR, Snodderly DM & Burns SA (2001): Macular pigment density measured by autofluorescence spectrometry: comparison with reflectometry and heterochromatic flicker photometry. J Opt Soc Am A Opt Image Sci vis **18**: 1212–1230.

Dysli C, Berger L, Wolf S & Zinkernagel MS (2017): Fundus autofluorescence lifetimes and central serous chorioretinopathy. Retina **37**: 2151–2161.

Dysli C, Fink R, Wolf S & Zinkernagel MS (2017): Fluorescence lifetimes of drusen in age-related macular degeneration. Invest Ophthalmol vis Sci **58**: 4856–4862.

Dysli C, Wolf S, Hatz K & Zinkernagel MS (2016): Fluorescence lifetime imaging in stargardt disease: potential marker for disease progression. Invest Ophthalmol vis Sci **57**: 832–841.

Dysli C, Wolf S & Zinkernagel MS (2015): Fluorescence lifetime imaging in retinal artery occlusion. Invest Ophthalmol vis Sci **56**: 3329–3336.

Dysli C, Wolf S & Zinkernagel MS (2016): Autofluorescence lifetimes in geographic atrophy in patients with age-related macular degeneration. Invest Ophthalmol vis Sci **57**: 2479–2487.

Eldred GE & Katz ML (1988): Fluorophores of the human retinal pigment epithelium: Separation and spectral characterization. Exp Eye Res **47**: 71–86.

Feldman TB, Yakovleva MA, Arbukhanova PM, Borzenok SA, Kononikhin AS, Popov IA, Nikolaev EN & Ostrovsky MA (2015): Changes in spectral properties and composition of lipofuscin fluorophores from human-retinal-pigment epithelium with age and pathology. Anal Bioanal Chem **407**: 1075–1088.

Feldman TB, Yakovleva MA, Larichev AV, Arbukhanova PM, Radchenko AS, Borzenok SA, Kuzmin VA & Ostrovsky MA (2018): Spectral analysis of fundus autofluorescence pattern as a tool to detect early stages of degeneration in the retina and retinal pigment epithelium. Eye (Lond) **32**: 1440–1448.

Guan Z, Li Y, Jiao S, Yeasmin N, Rosenfeld PJ, Dubovy SR, Lam BL & Wen R (2020): A2E distribution in RPE granules in human eyes. Molecules **25**: 1413.

Hammer M, Konigsdorffer E, Liebermann C, Framme C, Schuch G, Schweitzer D & Strobel J (2008): Ocular fundus autofluorescence observations at different wavelengths in patients with age-related macular degeneration and diabetic retinopathy. Graefes Arch Clin Exp Ophthalmol **246**: 105–114.

Hammer M, Richter S, Guehrs K-H & Schweitzer D (2006): Retinal pigment epithelium cell damage by A2-E and its photoderivatives. Mol vis **12**: 1348–1354.

Hammer M, Richter S, Kobuch K, Mata N & Schweitzer D (2008): Intrinsic tissue fluorescence in an organotypic perfusion culture of the porcine ocular fundus exposed to blue light and free radicals. Graefes Arch Clin Exp Ophthalmol **246**: 979–988.

Hammer M, Sauer L, Klemm M, Peters S, Schultz R & Haueisen J (2018): Fundus autofluorescence beyond lipofuscin: lesson learned from ex vivo fluorescence lifetime imaging in porcine eyes. Biomed Opt Express **9**: 3078–3091.

Hammer M, Schultz R, Hasan S et al. (2020): Fundus autofluorescence lifetimes and spectral features of soft drusen and hyperpigmentation in age-related macular degeneration. Transl vis Sci Technol **9**: 20.

Han M, Bindewald-Wittich A, Holz FG et al. (2006): Two-photon excited autofluorescence imaging of human retinal pigment epithelial cells. J Biomed Opt **11**: 010501.

Han M, Giese G, Schmitz-Valckenberg S, Bindewald-Wittich A, Holz FG, Yu J, Bille JF & Niemz MH (2007): Age-related structural abnormalities in the human retina-choroid complex revealed by two-photon excited autofluorescence imaging. J Biomed Opt **12**: 024012.

Heesterbeek TJ, Lores-Motta L, Hoyng CB, Lechanteur YTE & den Hollander AI (2020): Risk factors for progression of age-related macular degeneration. Ophthalmic Physiol Opt **40**: 140–170.

Marmorstein AD, Marmorstein LY, Sakaguchi H & Hollyfield JG (2002): Spectral profiling of autofluorescence associated with lipofuscin, Bruch's Membrane, and sub-RPE deposits in normal and AMD eyes. Invest Ophthalmol vis Sci **43**: 2435–2441.

Meleppat RK, Ronning KE, Karlen SJ, Kothandath KK, Burns ME, Pugh EN Jr & Zawadzki RJ (2020): In situ morphologic and spectral characterization of retinal pigment epithelium organelles in mice using multicolor confocal fluorescence imaging. Invest Ophthalmol vis Sci **61**: 1.

Murashova GA, Mancuso CA, Canfield JL, Sakami S, Palczewski K, Palczewska G & Dantus M (2017): Multimodal nonlinear optical imaging of unstained retinas in the epi-direction with a sub-40 fs Yb-fiber laser. Biomed Opt Express **8**: 5228–5242.

Palczewska G, Boguslawski J, Stremplewski P et al. (2020): Noninvasive two-photon optical biopsy of retinal fluorophores. Proc Natl Acad Sci USA **117**: 22532–22543.

Parish CA, Hashimoto M, Nakanishi K, Dillon J & Sparrow J (1998): Isolation and one step preparation of A2-E and iso-A2-E fluorophores from human retinal pigment epithelium. Proc Natl Acad Sci USA **95**: 14609–14613.

Ragauskaite L, Heckathorn RC & Gaillard ER (2001): Environmental effects on the photochemistry of A2-E, a component of human retinal lipofuscin. Photochem Photobiol **74**: 483–488.

Reiter GS, Told R, Schlanitz FG, Bogunovic H, Baumann L, Sacu S, Schmidt-Erfurth U & Pollreisz A (2019): Impact of drusen volume on quantitative fundus autofluorescence in early and intermediate age-related macular degeneration. Invest Ophthalmol vis Sci **60**: 1937–1942.

Sauer L, Andersen KM, Li B, Gensure RH, Hammer M & Bernstein PS (2018): Fluorescence lifetime imaging ophthalmoscopy (FLIO) of macular pigment. Invest Ophthalmol vis Sci **59**: 3094–3103.

Sauer L, Gensure RH, Andersen KM, Kreilkamp L, Hageman GS, Hammer M & Bernstein PS (2018): Patterns of fundus autofluorescence lifetimes in eyes of individuals with nonexudative age-related macular degeneration. Invest Ophthalmol vis Sci **59**: AMD65–AMD77.

Sauer L, Gensure RH, Hammer M & Bernstein PS (2018): Fluorescence lifetime imaging ophthalmoscopy: a novel way to assess macular telangiectasia type 2. Ophthalmol Retina **2**: 587–598.

Sauer L, Komanski CB, Vitale AS, Hansen ED & Bernstein PS (2019): Fluorescence lifetime imaging ophthalmoscopy (FLIO) in eyes with pigment epithelial detachments due to age-related macular degeneration. Invest Ophthalmol vis Sci **60**: 3054–3063.

Sauer L, Peters S, Schmidt J, Schweitzer D, Klemm M, Ramm L, Augsten R & Hammer M (2016): Monitoring macular pigment changes in macular holes using fluorescence lifetime imaging ophthalmoscopy. Acta Ophthalmol. **95**: 481–492.

Schultz R, Gamage KCLK, Messinger JD, Curcio CA & Hammer M (2020): Fluorescence lifetimes and spectra of RPE and sub-RPE deposits in histology of control and AMD eyes. Invest Ophthalmol vis Sci **61**: 9.

Schultz R, Hasan S, Schwanengel LS & Hammer M (2020): Fluorescence lifetimes increase over time in age-related macular degeneration. Acta Ophthalmol. **99**: e970–e972.

Schultz R, Klemm M, Meller D & Hammer M (2021): Spectral calibration of fluorescence lifetime imaging ophthalmoscopy. Acta Ophthalmol. [Epub ahead of print]. DOI: 10.1111/aos.14950

Smith RT, Sohrab MA, Busuioc M & Barile G (2009): Reticular macular disease. Am J Ophthalmol **148**: 733–743 e732.

Solberg Y, Dysli C, Moller B, Wolf S & Zinkernagel MS (2019): Fluorescence lifetimes in patients with hydroxychloroquine retinopathy. Invest Ophthalmol vis Sci **60**: 2165–2172.

Sparrow JR & Duncker T (2014): Fundus autofluorescence and RPE lipofuscin in age-related macular degeneration. J Clin Med **3**: 1302–1321.

Sparrow JR, Gregory-Roberts E, Yamamoto K, Blonska A, Ghosh SK, Ueda K & Zhou J (2012): The bisretinoids of retinal pigment epithelium. Prog Retin Eye Res **31**: 121–135.

Sparrow JR, Parish CA, Hashimoto M & Nakanishi K (1999): A2E, a lipofuscin fluorophore, in human retinal pigmented epithelial cells in culture. Invest Ophthalmol vis Sci **40**: 2988–2995.

Sparrow JR, Wu Y, Nagasaki T, Yoon KD, Yamamoto K & Zhou J (2010): Fundus autofluorescence and the bisretinoids of retina. Photochem Photobiol Sci **9**: 1480–1489.

Sparrow JR, Zhou J, Ben-Shabat S, Vollmer H, Itagaki Y & Nakanishi K (2002): Involvement of oxidative mechanisms in blue-light-induced damage to A2E-laden RPE. Invest Ophthalmol vis Sci **43**: 1222–1227.

Stremplewski P, Komar K, Palczewski K, Wojtkowski M & Palczewska G (2015): Periscope for noninvasive two-photon imaging of murine retina in vivo. Biomed Opt Express **6**: 3352–3361.

Tong Y, Ben Ami T, Hong S et al. (2016): Hyperspectral autofluorescence imaging of drusen and retinal pigment epithelium in donor eyes with age-related macular degeneration. Retina **36**(Suppl 1): S127–S136.

Warburton S, Davis WE, Southwick K, Xin H, Woolley AT, Burton GF & Thulin CD (2007): Proteomic and phototoxic characterization of melanolipofuscin: correlation to disease and model for its origin. Mol vis **13**: 318–329.

Yakovleva MA, Radchenko AS, Feldman TB, Kostyukov AA, Arbukhanova PM, Borzenok SA, Kuzmin VA & Ostrovsky MA (2020): Fluorescence characteristics of lipofuscin fluorophores from human retinal pigment epithelium. Photochem Photobiol Sci **19**: 920–930.

Zuclich JA, Previc FH, Novar BJ & Edsall PR (2005): Near-UV/blue light-induced fluorescence in the human lens: potential interference with visual function. J Biomed Opt **10**: 44021.

Received on June 30th, 2021.
Accepted on November 19th, 2021.

Correspondence:
Martin Hammer
University Hospital Jena
Department of Ophthalmology
Am Klinikum 1
07747 Jena
Germany
Telephone: +49-3641-9390860
Fax: +49-3641-9390867
Email: martin.hammer@med.uni-jena.de