

Structural Changes in Optical Coherence Tomography Underlying Spots of Increased Autofluorescence in the Perilesional Zone of Geographic Atrophy

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PURPOSE. To investigate structural correlates corresponding to the appearance of increased fundus autofluorescence (FAF) in the perilesional area of geographic atrophy (GA) secondary to age-related macular degeneration.

METHODS. Serial FAF images of 181 eyes with GA of 134 patients participating in the Directional Spread in Geographic Atrophy study (NCT02051998) were screened for increased FAF spots that had developed during the review period. Thickness and reflectivity of the retinal pigment epithelium (RPE)-basal lamina complex, as well as the integrity of the external limiting membrane (ELM) and the ellipsoid zone (EZ), respectively, in corresponding optical coherence tomography (OCT) scans were compared between the time points before and after the appearance of increased FAF. Adjacent areas without development of abnormal FAF were assessed as internal control.

RESULTS. A total of 36 areas (15 eyes) with de novo developed increased FAF spots and 54 control areas were included. Analysis of the corresponding OCT images revealed an increase in RPE-basal lamina complex thickness (31.8 ± 7.3 to $42.1 \pm 11.9 \mu\text{m}$ [$P < 0.001$]) and reflectivity (reflectivity ratio: 1.42 ± 0.11 to 1.54 ± 0.27 [$P = 0.009$]) corresponding to an increased FAF signal while there was no significant change in control areas. Development of increased FAF spots was associated with disruption of the ELM and the EZ.

CONCLUSIONS. Increase of RPE-basal lamina complex thickness and reflectivity was spatially and temporally associated with the development of increased FAF spots in eyes with GA. In addition, outer retinal disruption may contribute to the corresponding increased FAF signal.

Keywords: geographic atrophy, fundus autofluorescence, age-related macular degeneration, optical coherence tomography, retinal pigment epithelium

Geographic atrophy (GA) represents a morphologic end stage in various retinal diseases including advanced age-related macular degeneration (AMD). The exact pathophysiology of GA is yet to be elucidated. In multifactorial, complex AMD, accumulation of lipofuscin has been considered as a contributing pathogenetic factor. Lipofuscin has photoreactive properties and mediates oxidative stress, thus leading to inflammasome activation.^{1,2}

Fundus autofluorescence (FAF) visualizes intrinsic fluorophores including lipofuscin in the retina.³ Increased FAF in the border zone of GA has been associated with a faster atrophy progression.^{4,5} Furthermore, specific patterns of increased FAF in eyes with GA have been classified to be associated with different disease progression rates.^{6–9} Thus, increase of FAF may represent an important biomarker for GA progression over time. However, the underlying structural correlate and cellular and molecular mechanisms responsible for localized increases in FAF signal intensity are not well understood. Histopathologic study indicates that vertically superimposed cells and cellular fragments containing fluorophore are the source of increased FAF in AMD.^{10,11} However, these studies were based on postmortem eyes, and it is unclear as to whether these appear clinically in vivo.

Previous studies investigating the border zone of GA used optical coherence tomography (OCT) for the in vivo sectional analysis of retinal layers. These studies reported presence of hyperreflective material, irregularity of the outer retina, and thickening of the retinal pigment epithelium (RPE) at the perilesional zone of GA, including increased FAF area.^{12–15} However, the observations were qualitative or cross-sectional. In addition, alterations of the RPE are not the only possible cause for increased FAF signals. Reduction of optical pigment density due to disruption of the outer retina can also be associated with increased FAF.^{11,16} Investigating the disruption of the outer retina in combination with the state of the RPE would provide more detailed information about the changes underlying increased FAF in association with GA.

In this longitudinal observation study we focused on the development of new spots with increased FAF in order to investigate the structural changes underlying the increased FAF.

METHODS

Patients were recruited from the Directional Spread in Geographic Atrophy (DSGA) study (NCT02051998) encom-



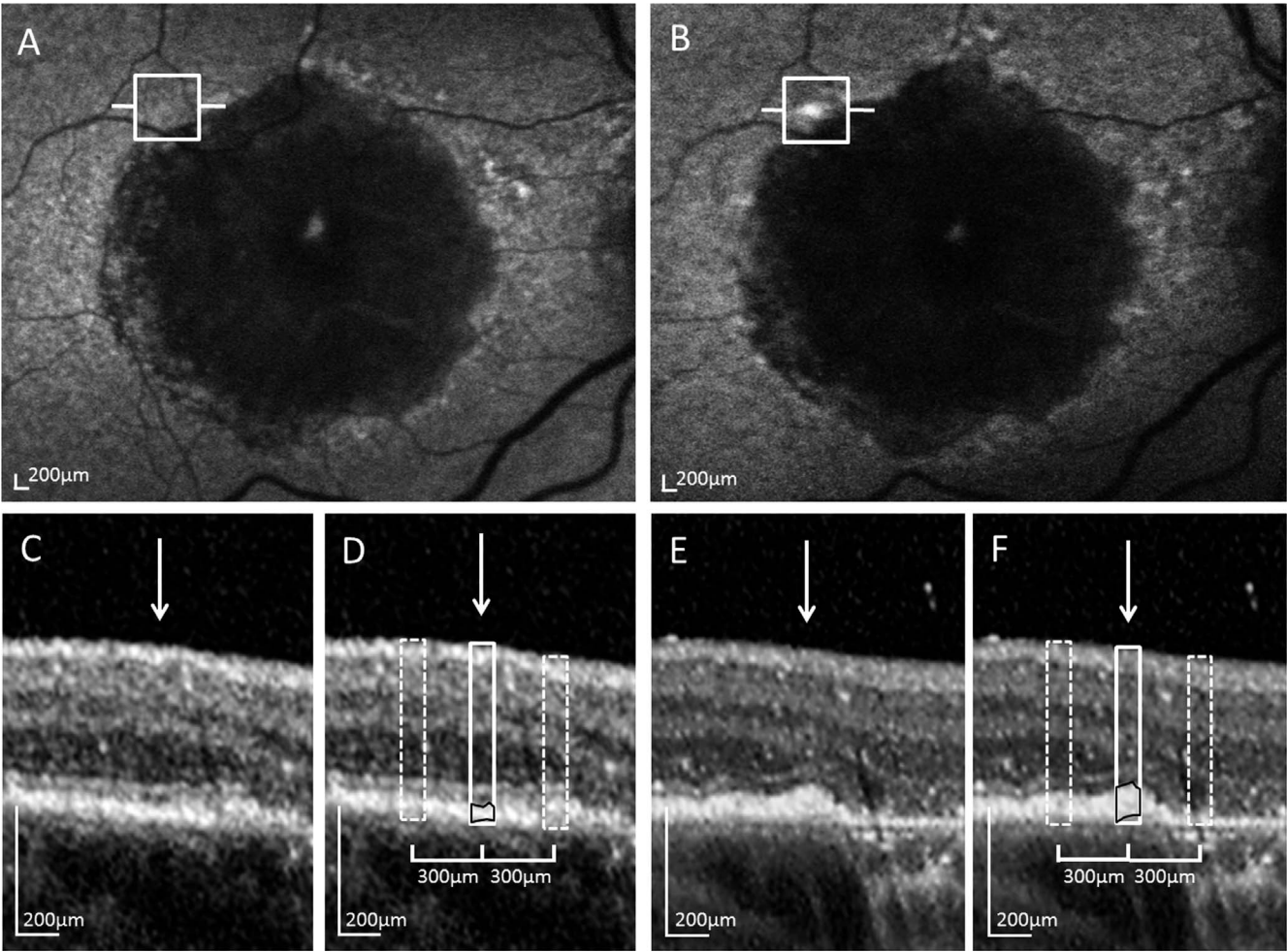


FIGURE 1. Measurement of the RPE-basal lamina complex at increased FAF spots. An increased FAF spot highlighted in (B) and the corresponding area at baseline (A) were identified on FAF images. The corresponding location of the OCT image was precisely determined using Region Finder, ImageJ software, and Heidelberg Eye Explorer (C, E). White lines in FAF images indicate the range of OCT images. We demarcated the hyperreflective structure continuous to the RPE-basal lamina complex (black polygon in [D, F]). The measurement was done in a 100-μm-width region. The mean reflectivity of the whole retina was measured as a reference (solid white rectangle in [D, F]). As a control, the same measurement was done in 100-μm-wide regions 300 μm temporal and nasal to the spot (dashed white rectangles in [D, F]). *Measurement of nasal dashed rectangle in this case was not used for the analysis since the area was within GA.

passing 181 eyes of 134 patients. This noninterventional, prospective natural history study followed the ethical principles of the Declaration of Helsinki, and the design was approved by the Ethics Committee of the Medical Faculty, University of Bonn, Germany. Informed consent was obtained from each participant after explanation of the study's nature and possible consequences of participation.

As part of the study procedure, all patients underwent FAF imaging and OCT on the same day. Only eyes presenting with (1) good-quality FAF, near-infrared reflectance (NIR), and OCT images (2) for a period of at least 6 months were included. Eyes with neovascular AMD or other retinal diseases such as diabetic

retinopathy and retinal detachment were excluded. If both eyes of a patient met the criteria, both eyes were included.

Image Acquisition and Processing

FAF images were obtained with HRA2 or Spectralis (Heidelberg Engineering, Heidelberg, Germany). OCT and confocal scanning laser ophthalmoscope (cSLO) images were simultaneously obtained using Spectralis with a volume scan mode. Automatic fundus tracking and follow-up mode were used to enable a longitudinal comparison. Region Finder software version 2.5.8.0 (Heidelberg Engineering) and ImageJ software ([**TABLE.** SD-OCT Image Measurements and the States of the Outer Retina Before and After the Appearance of Increased FAF Spots](http://</p></div><div data-bbox=)

	Control Area		P Value	Increased FAF Spots		P Value
	Before	After		Before	After	
Reflectivity ratio of the RPE-basal lamina complex, mean ± SD	1.40 ± 0.14	1.42 ± 0.16	0.37	1.42 ± 0.11	1.54 ± 0.27	0.009
Thickness of the RPE-basal lamina complex, μm, mean ± SD	31.9 ± 8.7	31.0 ± 7.4	0.52	31.8 ± 7.3	42.1 ± 11.9	<0.001
Ellipsoid zone, continuous/disrupted	45/9	43/11	0.63	30/6	3/33	<0.001
External limiting membrane, continuous/disrupted	53/1	52/2	1.00	36/0	27/9	0.03

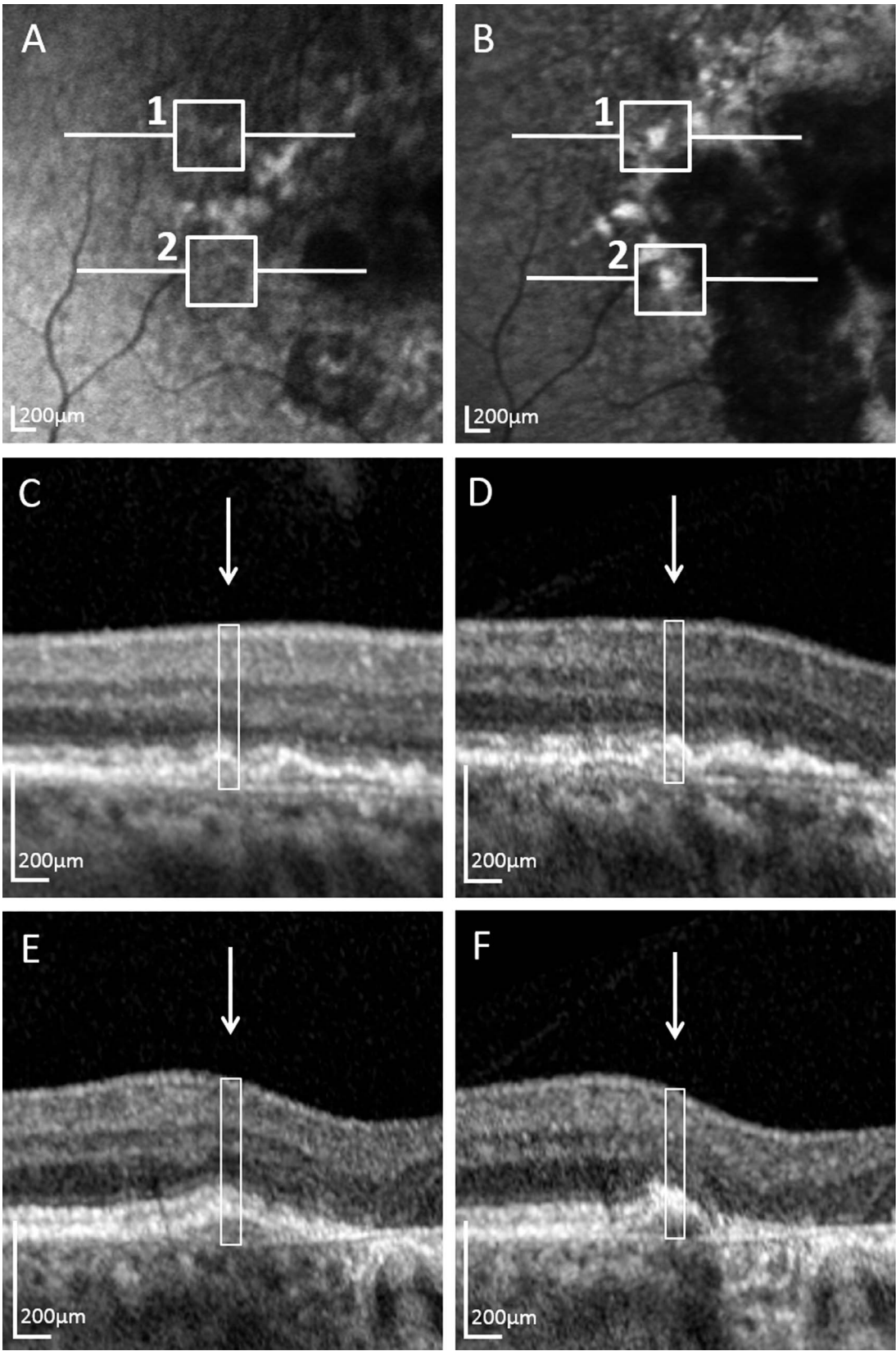


FIGURE 2. A representative case with appearance of increased FAF spots. Spot 1 in FAF images (**A**, **B**) corresponds to an *arrow* in the OCT images (**C**, **D**), spot 2 to (**E**, **F**). At spot 1, the RPE-basal lamina complex shows focal multilayered appearance (**D**), which was not found before the spot showed increased FAF signal (**C**). At spot 2, focal thickening was observed corresponding with increased FAF spot (**F**).

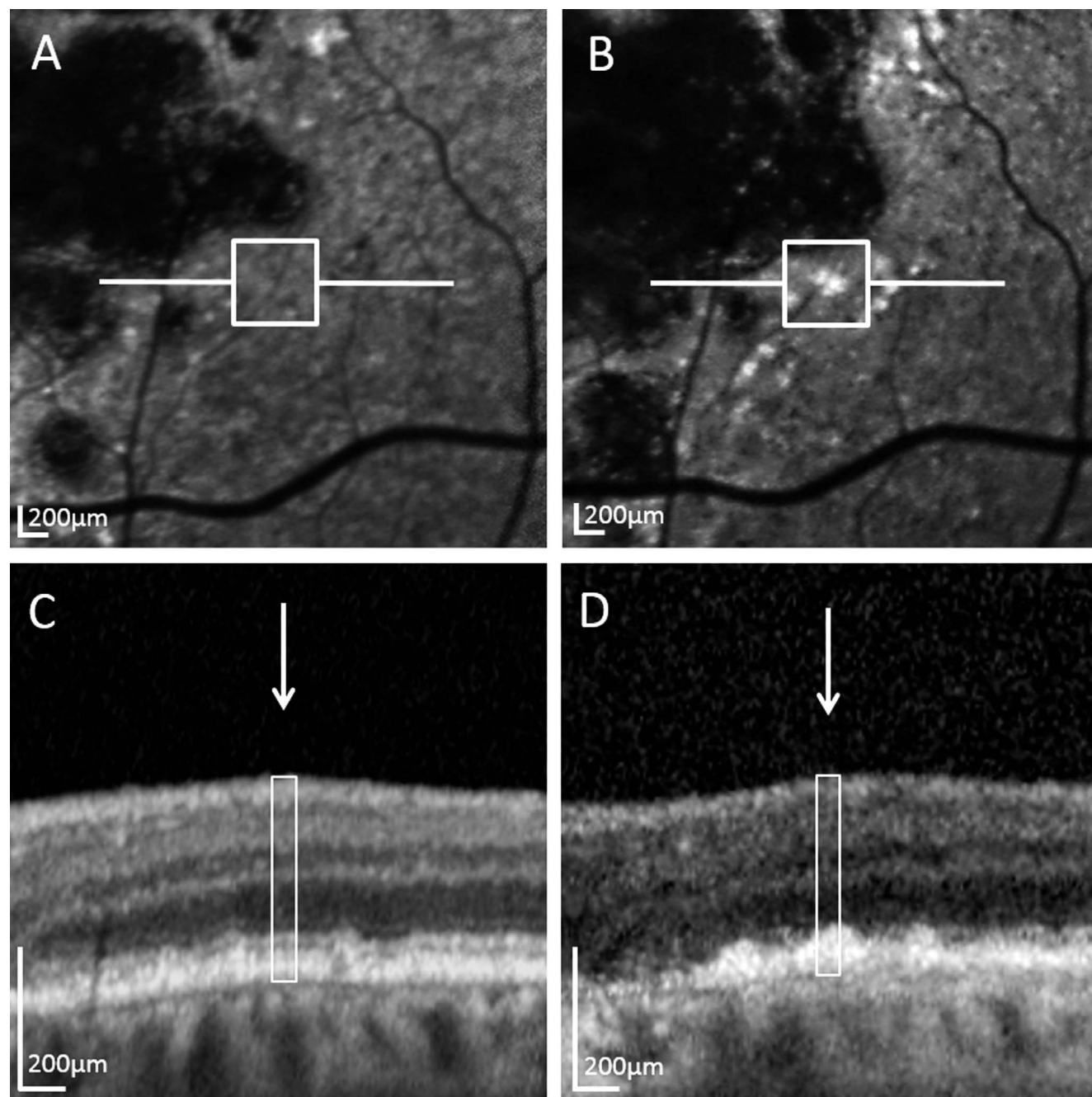


FIGURE 3. Interval between before (A) and after the occurrence of an increased FAF spot (B) is 20 months. The spot shows an almost normal outer retina on OCT before the appearance of the increased FAF spot (C). After the appearance of the increased FAF spot, the RPE-basal lamina complex shows thicker, mass-like appearance extending from Bruch's membrane toward the ellipsoid zone (D). The ellipsoid zone is disrupted. Reflectivity ratio is also increased in the region.

imagej.net/Fiji; in the public domain) were used to confirm correct registration of FAF images and NIR images. Loci in OCT images corresponding to a specific point of interest in NIR images were checked using Heidelberg Eye Explorer version 1.9.10.0 (Heidelberg Engineering).

Definition of an Increased FAF Spot Developing During the Review Period

In the current study, an increased FAF spot was defined as a well-demarcated area $\geq 100 \mu\text{m}$ but $< 500 \mu\text{m}$ in diameter with clearly increased FAF compared to the background signal. FAF

images were screened by two graders independently, and only the spots identified by both of the graders were further analyzed. Included were only spots (1) that had not shown increased FAF signals in the corresponding area on at least one previous examination and (2) for which serial OCT scans of the exact area were available.

Measurements and Grading of Spectral-Domain (SD)-OCT Images

In the present study, we use the term RPE-basal lamina complex, which was modified from a previously used term,

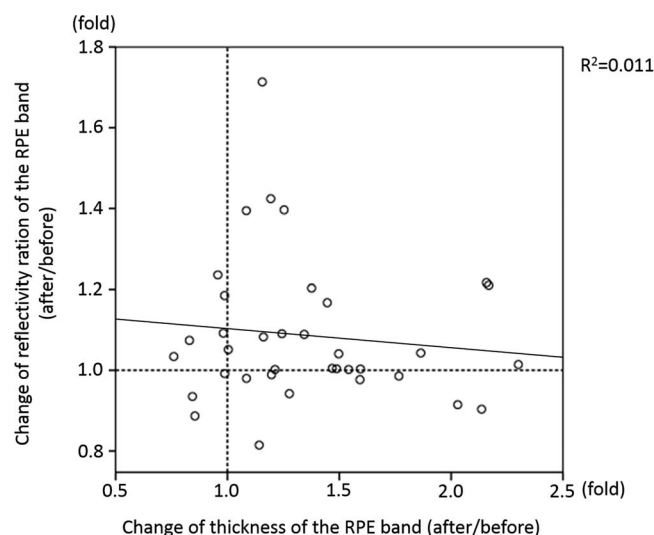


FIGURE 4. Scatter plot showing the correlation between changes of reflectivity ratio and thickness of the RPE-basal lamina complex after the appearance of increased FAF spots. There was no significant correlation between the parameters ($R^2 = 0.011$). Of note, some cases showed an increase of reflectivity ratio without an increase of thickness (*upper left quadrant*).

RPE-basal lamina-Bruch's complex,¹⁷ to describe the fourth outer retinal hyperreflective band, since the standard nomenclature¹⁸ is not applicable to such diseased eyes. To investigate whether the reflectivity of the RPE on SD-OCT differed before and after the defined area showed hyperautofluorescence, the mean reflectivity of the RPE-basal lamina complex corresponding to increased FAF spots was measured in the width of 100 μm using the ImageJ software. Hyperreflective structure continuous to the RPE-basal lamina complex was demarcated. The mean reflectivity of the entire retina (between Bruch's membrane and the internal limiting membrane) was measured as internal reference, and the ratio of the mean reflectivity of the RPE-basal lamina complex to that of entire retina was used as a "reflectivity ratio" for each spot (modified method from a previous report¹⁹; Fig. 1). The area of the RPE-basal lamina complex within the same 100- μm -width region was also measured. The mean thickness of the RPE-basal lamina complex was calculated by dividing the area by 100 μm . All spots were measured independently by two graders (MO and AO), and the mean values of the two graders were used for statistical analysis. The graders were masked to FAF images during the SD-OCT measurements.

External limiting membrane (ELM) and ellipsoid zone (EZ) states were categorized in two groups: "continuous" when they appeared as a line without disruption throughout the 100- μm width and "disrupted" when the structure showed discontinuity. Again grading was performed by two graders (MO and AO) independently, and in cases of discrepancies these were discussed and arbitrated.

In order to confirm whether the changes of these parameters are specific to de novo development of increased FAF spots, we investigated control areas of 100- μm width located 300 μm nasal and temporal to the center of each spot. Thickness and reflectivity of the RPE-basal lamina complex were measured in these areas with the same methods (Fig. 1). Since the maximal size of increased FAF spots was 500 μm , the area in 300- μm distance to the center of the lesion did not overlap with the spot under investigation. If the area was within GA or outside of the corresponding OCT scan, it was

excluded from the analysis. The integrity of the ELM and the EZ was examined in the control areas as well.

Statistical Analysis

Data were analyzed by using the SPSS software version 19.0 (IBM SPSS Statistics, Chicago, IL, USA). Interobserver agreement was assessed using the interclass correlation coefficient (ICC) for the value of reflectivity ratio and thickness of the RPE-basal lamina complex. In order to assess the differences between before and after the appearance of increased FAF spots, paired *t*-tests were applied for reflectivity ratio or thickness of the RPE-basal lamina complex, and the McNemar test was applied for the states of ELM or EZ. Correlation between changes of reflectivity ratio and changes of thickness of the RPE-basal lamina complex was analyzed with the Pearson's correlation coefficient test.

RESULTS

A total of 36 spots with increased FAF had developed de novo in 15 eyes of 15 patients during the review period and were scanned by SD-OCT. Out of 72 candidate control areas, 54 areas were included. (Seventeen areas were located in GA and one area was outside of the corresponding OCT scan.) Mean age of the participants was 78.9 ± 5.6 years, and mean interval between the time points before and after the occurrence of increased FAF spots was 17.4 ± 8.0 months (range, 6–36 months).

Assessment of the interobserver agreement of the measurement revealed an ICC of 0.944 (0.925–0.959, 95% confidence interval [CI]) for reflectivity and of 0.834 (0.778–0.876, 95% CI) for thickness of the RPE-basal lamina complex. The results confirmed the reproducibility of the method.

The results of the measurements are summarized in the Table. No significant change was observed in control areas. On the other hand, in de novo developed FAF spots, the mean reflectivity ratio of the RPE-basal lamina complex was 1.42 ± 0.11 before the appearance of increased FAF spots and 1.54 ± 0.27 after the appearance of increased FAF spots; the difference was statistically significant ($P = 0.009$). The mean thickness of the RPE-basal lamina complex increased from 31.8 ± 7.3 to 42.1 ± 11.9 μm after hyperautofluorescence appeared ($P < 0.001$). Representative images are shown in Figures 2 and 3. Overall, there was no linear relationship between the change of reflectivity ratio and the change of thickness of the RPE-basal lamina complex ($R^2 = 0.011$, Fig. 4). Both reflectivity ratio and thickness increased in most cases; however, some cases showed increased reflectivity ratio without an increase of RPE-basal lamina complex thickness being evident. To further assess possible reasons for the increase in FAF in these cases, an explorative assessment was performed. As shown in a representative example in Figure 5, the RPE-basal lamina complex shows increased reflectivity but no evident increase of thickness. Disruption of the outer retina was also observed in the case.

The results for ELM and EZ assessment are shown in the Table. There was no significant change of either of these parameters in control areas. As for increased FAF spots, all cases showed continuous appearance of ELM before the spots showed increased FAF, and most of them (27/36 cases, 75.0%) were still classified as continuous after the spots showed increased FAF. EZ was found to be continuous in most cases (30/36 cases, 83.3%) prior to the spots showing increased FAF. On FAF increase in the aforementioned spots, however, 33 cases were classified as disrupted and only 3 out of 36 cases (8.3%) presented a continuous appearance. Both of these differences were statistically significant ($P = 0.03$ and $P < 0.001$, respectively).

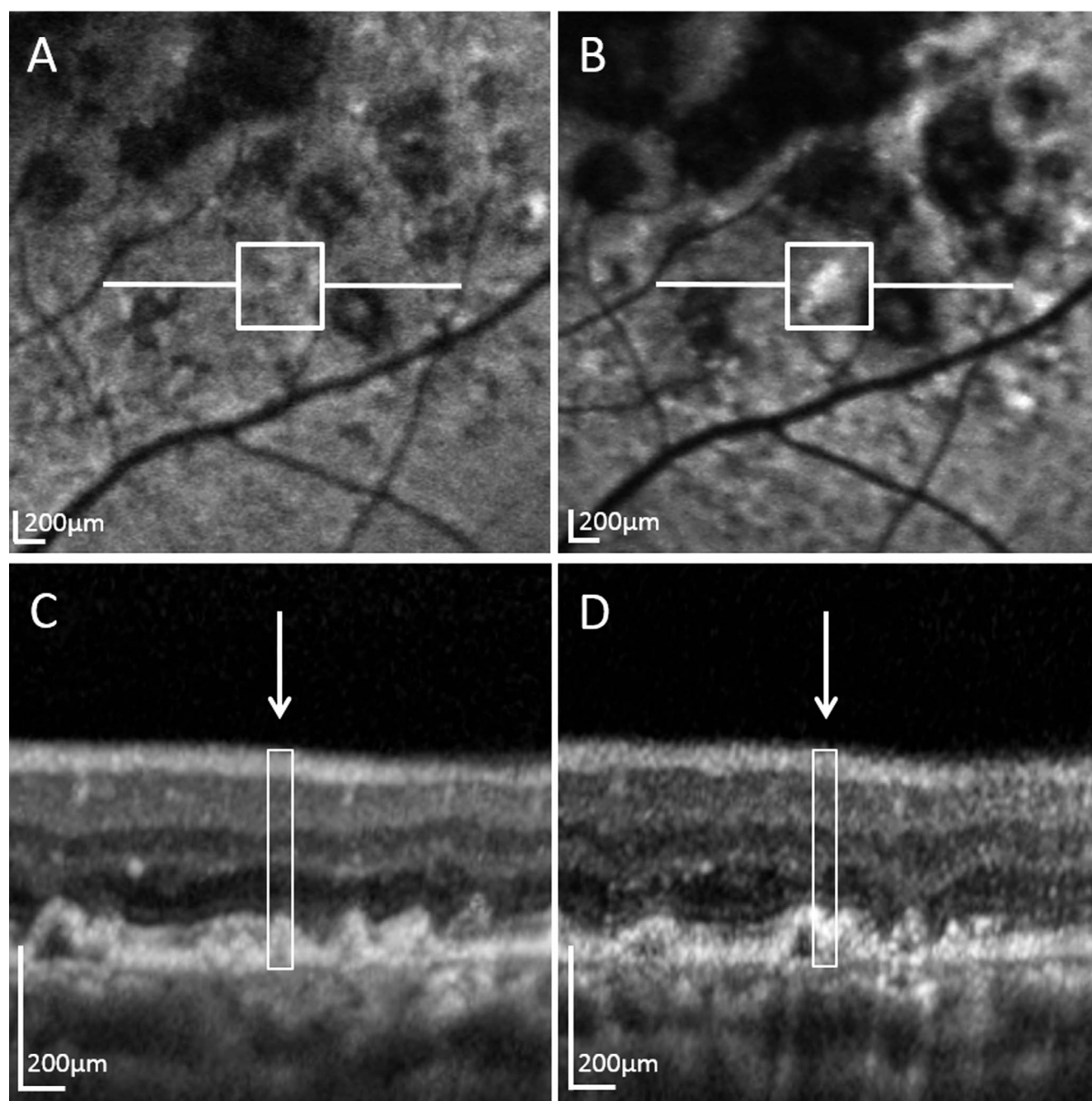


FIGURE 5. A representative case without thickening of RPE-basal lamina complex after the appearance of increased FAF spots. The RPE-basal lamina complex looks more reflective after the appearance of an increased FAF spot (**A**, **B**) while the thickness does not show remarkable changes (**D**) compared to that before the development of the increased FAF spot (**C**).

DISCUSSION

The findings of this study indicate that a de novo occurrence of increased FAF spots in eyes with GA due to AMD correlates with both increase in reflectivity and thickness of the RPE-basal lamina complex. Thus, structural changes underlie focal enhancements of FAF. Coregistration of both imaging modalities, that is, FAF and SD-OCT imaging with exact topographic alignment, adds to our understanding on the phenotypic variety in FAF signals in the context of dry AMD.

Increased thickness of the RPE-basal lamina complex in SD-OCT may correlate with vertically superimposed RPE cells and cell fragments as previously reported in histopathologic studies

of GA eyes.^{10,20} The reported phenotype of RPE cells such as sloughed, heaped, or multilayered cells in the perilesional zone of GA would be expected to present as increased thickness of the RPE-basal lamina complex on OCT images. Furthermore, enlargement of the RPE cells could also add to the increase of thickness.^{10,21} In addition, accumulation of reflective material in the basal laminar deposit observed in these studies may also partly account for an increase of thickness.

Increased reflectivity of the RPE-basal lamina complex may indicate accumulation of optically reflective material in the cells. Previous histopathologic studies of GA reported hyperpigmented RPE, that is, accumulation of melanin, melanolipofuscin, and other optically reflective materials.^{20–26} Thickening

of F-actin bundles of RPE cells was also reported in the course of AMD and may cause increased reflectivity of the RPE band on OCT images.²⁶ Among potential substrates, melanolipofuscin would increase both reflectivity and FAF and may be an explanation for the present findings of increased reflectivity in association with the appearance of increased FAF spots. The role of intracellular lipofuscin accumulation in the pathogenesis of GA is currently under debate.¹¹

Reduction of optical pigment, which can cause increased FAF,¹⁵ might also contribute to the increased reflectivity of the RPE-basal lamina complex on OCT.²⁷ In a previous study,¹³ the disruption of outer retinal layers in areas with increased FAF was also observed at the border zone of GA. With a reduction of optical pigment, more light reaches the RPE and thus may increase the signal originating from the RPE. In the current study, a disruption of the EZ occurred in 75% of cases concurrently with the development of increased FAF spots. The results of our longitudinal observation represent further evidence that changes in the outer retina may be associated with changes in the acquired FAF signal. The outer retinal damage at the increased FAF spots may also account for a previously reported spatial reduction of retinal sensitivity in areas with an increased FAF signal.^{28,29}

As the thickness of RPE cells may vary depending on their distance from the fovea as well as individual characteristics,³⁰ case-control studies have to take such variations into account. The present study, however, provides a longitudinal analysis of exactly the same topographic locations of a given patient and thus does not need to consider these variations.

Registration of the different imaging modalities was performed by automated software and was manually confirmed by the graders, which represents a particular strength of this study. Thus, we can safely conclude that the observed changes appeared concomitantly with the increased FAF spots.

Limitations of this study include the relatively small number of cases and lack of histopathologic correlation as the phenomena were studied in vivo. Although the comparison before and after provides longitudinal information, the inclusion criteria limited the number of cases. We believe the exclusion of small lesions and scans, which are not certain whether they accurately show corresponding spot, is necessary considering the residual colocalization error between IR and the corresponding OCT image.³¹ While the identification of increased FAF spots was subjective, independent grading by two separate readers was performed to substantiate the grading. Lack of evidence about the exact source of the increased FAF is another limitation. Future analysis taking more detailed emission spectra into account might enhance our understanding of the origin of FAF.³²

In summary, we demonstrate that increases in RPE thickness and reflectivity along with disintegration of outer retinal structures on OCT images correlate with newly occurring spots of increased FAF. Additional longitudinal studies are warranted to further elucidate the natural history of the observed FAF changes.

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