Original Investigation

Quantitative Fundus Autofluorescence in Early and Intermediate Age-Related Macular Degeneration

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IMPORTANCE Increased lipofuscin accumulation is assumed to be an important factor in the pathogenesis of age-related macular degeneration (AMD), although direct evidence for this hypothesis is missing.

OBJECTIVE To quantitatively investigate lipofuscin-associated fundus autofluorescence (AF) in patients with early and intermediate AMD.

DESIGN, SETTING, AND PARTICIPANTS A prospective, single-center, case-control study was conducted from August 1, 2014, to October 31, 2015, at a university referral center. Participants included 40 patients aged 65 years or younger and 108 individuals without eye disease serving as controls. All participants underwent quantitative fundus AF (qAF) imaging with a modified scanning laser ophthalmoscope equipped with an internal fluorescent reference. Mean qAF values of an 8-segment circular ring centered on the fovea (qAF_8) were measured and compared between patients and controls. For subgroup analysis, drusen were categorized as soft drusen, cuticular drusen, and/or reticular pseudodrusen (RPD).

MAIN OUTCOMES AND MEASURES The qAF_8 levels.

RESULTS In the 40 patients with AMD, mean (SD) age was 54.8 (5.6) years, and 32 (80%) were women. None of the investigated patients had qAF_8 values above the 95% prediction interval (PI) of the 108 controls. In the soft drusen (28 [70%]) and cuticular drusen (8 [20%]) groups, qAF_8 levels within the 95% PI were noted in 22 patients (79%; 95% CI, 60% to 90%) and 7 patients (88%; 95% CI, 51% to 99%) respectively. The qAF_8 values in the RPD group (4 [10%]) were below the 95% PI in 3 patients (75%; 95% CI, 29% to 97%). Compared with the controls, statistical analysis revealed lower qAF_8 values in the overall AMD cohort after adjusting for age (difference, −19.9% [95% CI, −25.6% to −12.7%], $P < .001$) as well as in all subgroups (soft drusen, −17.1% [95% CI, −24.1% to −9.5%], $P < .001$; cuticular drusen, −19.6% [95% CI, −30.3% to −7.2%], $P = .003$; and RPD, −34.5% [95% CI, −47.1% to −21.3%]; $P < .001$).

CONCLUSIONS AND RELEVANCE The qAF_8 measurements in this sample showed no increased lipofuscin-related fundus AF in patients with early and intermediate AMD. Lower qAF levels in certain subgroups may point to subnormal lipofuscin levels in the retinal pigment epithelium or, alternatively, limitations to detection of true retinal pigment epithelial lipofuscin content. The results of this study might expand the understanding of the pathogenesis of AMD and may have an effect on upcoming treatment trials that aim to modify lipofuscin accumulation.

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Lipofuscin accumulates throughout life within the lysosomal compartment of the postmitotic retinal pigment epithelium (RPE) as a byproduct mainly of the visual cycle.[1] Although this accumulation may represent an aging process without pathologic significance of its own, abnormally high lipofuscin accumulation as observed in *ABCA4*-related retinopathy is associated with development of atrophy of the RPE and photoreceptor layer.[2-4]

Lipofuscin accumulation has also been discussed[5] as a possible factor contributing to the development of multifactorial, complex age-related macular degeneration (AMD), which is the main cause of irreversible severe visual loss. This assumption is based on various observations: (1) age-dependent lipofuscin accumulation parallels the increased prevalence of AMD in older patients,[6-8] (2) lipofuscin concentration is highest within the macular region,[6-8] (3) enhanced autofluorescence (AF) surrounding atrophy of the RPE and photoreceptor layer suggests elevated lipofuscin levels,[9] and (4) lipofuscin may have toxic effects on the RPE[10] and induce complement activation, a process that plays an eminent role in the development of AMD.[11,12] However, direct evidence is missing for an abnormally increased lipofuscin load in patients with AMD.

Lipofuscin has autofluorescent properties that allow visualizing its accumulation in vivo using fundus AF imaging.[13] Recent modifications of this imaging technique resulted in the possibility to quantify AF intensities at the ocular fundus as a surrogate for lipofuscin accumulation in the RPE.[14,15] The aim of this study was to evaluate whether early and intermediate AMD are associated with increased lipofuscin-related quantitative AF (qAF) levels.

Key Points

Question Is lipofuscin-related quantitative fundus autofluorescence (qAF) increased in patients with early or intermediate age-related macular degeneration (AMD)?

Findings In this case-control study, none of 40 patients with early or intermediate AMD had qAF values above the 95% predictive interval of healthy control individuals. Overall, statistical analysis revealed significantly lower qAF values compared with controls.

Meaning The results provide evidence against an abnormally high lipofuscin accumulation in patients with the early or intermediate stage of AMD, a finding that may influence the understanding of the pathogenesis of this multifactorial disease.

Methods

Patients

This prospective, cross-sectional, case-control study was conducted from August 1, 2014, to October 31, 2015, at the Department of Ophthalmology of the University of Bonn, Bonn, Germany. All participants were recruited from the retina clinic of the Department of Ophthalmology, University of Bonn.

The inclusion criteria for patients were the clinical diagnosis of drusen (≥63 µm) in white individuals aged 45 to 65 years. Exclusion criteria were any signs of choroidal neovascularization, pigment epithelial detachments, polypoidal chroidal vasculopathy or geographic atrophy, drusen or reticular pseudodrusen (RPD) associated with a known monogenic disease, any other disease within the macular region, any abnormality affecting the ocular media (eg, corneal opacities, cataract unusual for age, or vitreous opacities), unstable fixation, refractive error greater than ±6 diopters (spherical equivalent), and dilated pupil diameter less than 7 mm. All patients underwent a complete ophthalmologic examination including best-corrected visual acuity testing, slitlamp examination, and ophthalmoscopy with dilated pupils.

The study was in adherence with the Declaration of Helsinki.[16] Institutional review board approval was provided by the Ethikkommission der Medizinischen Fakultät at the Rheinische Friedrich-Wilhelms-Universität Bonn, and written informed consent was obtained from each participant. There was no financial compensation.

Image Acquisition and Analysis

After dilation of the pupils by instillation of tropicamide, 0.5%, and phenylephrine, 2.5%, all probands underwent a standardized imaging protocol. The protocol consisted of fundus photography (Visucam; Carl Zeiss Meditec), spectral-domain optical coherence tomography, and fundus AF imaging with a confocal scanning laser ophthalmoscope with 488-nm excitation (Spectralis HRA+OCT; Heidelberg Engineering).

The qAF imaging was performed according to the method developed by Delori et al[14] and established in our center by Müller et al.[17] An internal fluorescent reference was integrated into the scanning laser ophthalmoscope to account for fluctuations in laser power and detector sensitivity. The material of the reference was identical to that characterized by Delori et al.[4,17] The qAF scale was calibrated with a master reference provided by Heidelberg Engineering. The calibration procedure was routinely repeated and yielded comparable results.

For qAF imaging, the camera was positioned centered on the fovea of the proband by using the near-infrared reflectance mode and the internal fixation light. After switching to the qAF mode (488-nm excitation and 500- to 680-nm detection), focus and alignment were readjusted to obtain a maximum and uniform signal. Overexposure was avoided by reducing the detector sensitivity. For bleaching of the visual pigment, the retina was exposed to the blue excitation light for at least 20 to 30 seconds. The optimal camera position was rechecked and the patient was asked to blink a few times to provide optimal imaging conditions. A series of 12 successive images was then recorded using customized software for recording qAF images developed by Heidelberg Engineering (30° field of view and 768 × 768 pixels).

After the recording of each series, the quality of the acquired images was evaluated. In cases of insufficient quality, images were excluded from further analysis. Exclusion criteria included inhomogeneous illumination, sectorial opacities (eg, eyelashes or floaters), or unstable fixation. The minimal number of remaining images required for further analysis of an image series was set to 9. The mean of the images was then

Table. Demographic Data and Analysis of Quantitative Fundus Autofluorescence Measures

Characteristic	AMD	Soft Drusen	Cuticular Drusen	RPD
Patients, No. (%)	40 (100)	28 (70)	8 (20)	4 (10)
Age, mean (SD) [range], y	54.8 (5.6) [45-65]	54.5 (5.4) [45-64]	52.5 (3.8) [46-60]	61.2 (4.8) [53-65]
Sex, No. (%)				
Male	8 (20)	4 (14)	2 (25)	2 (50)
Female	32 (80)	24 (86)	6 (75)	2 (50)
qAF_8 difference from control, %[a]				
95% CI	−19.9 (−25.6 to −12.7)	−17.1 (−24.1 to −9.5)	−19.6 (−30.3 to −7.2)	−34.5 (−47.1 to −21.3)
P value	<.001	<.001	.003	<.001

Abbreviations: AMD, age-related macular degeneration; qAF_8, quantitative fundus autofluorescence of an 8-segment circle centered on the fovea; RPD, reticular pesudodrusen.

[a] Based on linear regression of the natural log of qAF_8 adjusted for the natural log of age.

determined and the images were saved without normalization in the Heidelberg Eye Explorer (HEYEX; Heidelberg Engineering) database. The right eye was used for analysis. The left eye was used instead only in cases of poor image quality or anatomical abnormalities.

For further processing, images were exported from the HEYEX software to a customized image analysis program written in IGOR (WaveMetrics Inc). The mean gray values of the reference and a circular region with 8 subsegments and an eccentricity of approximately 7° to 9° centered on the fovea were measured (eFigure 1A in the Supplement). Retinal vessels were excluded from analysis by automated histogram analysis. The gray values were exported to a spreadsheet analysis program (Excel; Microsoft). The qAF values were calculated according to the formula described by Delori et al[14] and accounted for the gray value of the reference and the 8 segments, the offset of the laser, the magnification, the age-adjusted lens opacity (based on normative data), and a device-specific calibration factor. The overall qAF value was computed as the mean of the qAF values of the 8 segments (qAF_8). The qAF_8 values were compared with those of 108 healthy control individuals of white ethnicity (age range, 18-64 years) without eye disease. Color-coded qAF maps were computed based on pixelwise transformation of the qAF values into colors by using a custom-made extension of the HEYEX software (eFigure 1B in the Supplement).

The qAF_8 values represent the mean qAF of an 8-segment circular region centered on the fovea. Pathologic changes, such as drusen, within this circular region may affect qAF_8 measurements, potentially resulting in incorrect estimations of background qAF levels. Furthermore, qAF_8 measures do not allow evaluation of qAF levels associated with individual lesions such as drusen. To overcome these limitations, horizontal qAF profiles were investigated. For this purpose, gray-level histograms along a 3-pixel-wide horizontal band through the fovea were extracted and qAF values were calculated as described above. For this analysis, qAF values of the patients were compared with those of 45 age-matched (40-65 years) controls without eye disease.

Definitions

Patients with early or intermediate AMD were identified based on the presence of drusen 63 µm or larger in color fundus photography and spectral-domain optical coherence tomographic images. Patients with drusen of 63 µm or larger but smaller than 125 µm were classified as having early AMD. Those with drusen 125 µm or larger, or patients with drusen of 63 µm or larger but smaller than 125 µm and pigmentary abnormalities, were classified as having intermediate AMD according to the Beckmann classification.[18] Drusen were further subclassified as soft drusen, cuticular drusen, and RPD. Detailed definitions are reported in the eTable in the Supplement. Selected patients with cuticular drusen additionally underwent fluorescein angiography to identify the characteristic "stars in the sky" appearance.[19]

Statistical Analysis

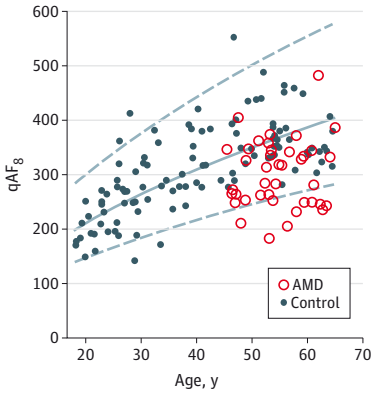
Prediction intervals (PIs) were calculated assuming a linear association between the log of age and the log of qAF_8 units for all control participants. Linear regression of the log of qAF_8 was used to compare patients with controls after adjusting for the log of age. Topographic distribution of qAF values along the qAF_8 circle was compared between groups by 1 factorial analysis of variance followed by the Tukey test for the pairwise comparison of groups. Categorical variables were assessed using a modified Wald method and are reported as 95% CIs. Statistical analyses were performed using Stata/IC, version 13.1 (StataCorp LP) and SPSS, version 22 (IBM Corp).

Results

The 40 patients included in this study had a mean (SD) age of 54.8 (5.6) years, and 32 (80%) were women (**Table**). Four patients had early AMD and 36 had intermediate AMD. None of the patients had a qAF_8 level above the 95% PI of healthy controls (**Figure 1**). The qAF values were within the 95% PI in most patients (30 [75%]; 95% CI, 60%-86%) and were mainly distributed in the lower normal range. The remaining patients had qAF values below the normal 95% PI (10 [25%]; 95% CI, 14%-40%). Quantitative analysis showed significantly lower qAF_8 values of patients with AMD compared with controls after adjusting for age ($P < .001$) (Table).

Phenotypic subclassification revealed 28 patients (70%) with soft drusen, 8 patients (20%) with cuticular drusen, and 4 patients (10%) with RPD (Table). Eight patients of the soft drusen group had concomitant cuticular drusen (n = 6) or RPD (n = 2).

Figure 1. Quantitative Fundus Autofluorescence (qAF) in Age-Related Macular Degeneration (AMD)



Mean qAF values of an 8-segment circular ring centered on the fovea (qAF_8) of all individual patients with early or intermediate AMD compared with normative data. The blue line represents the regression curve and the dotted blue lines, the 95% prediction interval of the control individuals.

The qAF_8 levels were within the 95% PI of controls in most patients with soft (22 of 28 [79%]; 95% CI, 60%-90%) and cuticular (7 of 8 [88%]; 95% CI, 51%-99%) drusen. In both groups, individual measures were again distributed in the lower normal range. The remaining patients as well as most with RPD (3 of 4 [75%]; 95% CI, 29%-97%) had qAF_8 levels below the 95% PI (**Figure 2**). Compared with controls, statistical analysis in the patients revealed significantly lower qAF_8 values for each subgroup (soft drusen, −17.1% [95% CI, −24.1% to −9.5%], $P < .001$; cuticular drusen, −19.6% [95% CI, −30.3% to −7.2%], $P = .003$; and RPD, −34.5% [95% CI, −47.1% to −21.3%]; $P < .001$) (Table). Color-coded qAF maps of representative patients with different lesion subtypes compared with an age-matched representative control are shown in **Figure 3**.

The topographic qAF distribution along the circular segments investigated for qAF_8 analysis showed no differences compared with controls for the overall AMD cohort as well as for the different drusen subgroups except for the superotemporal segment in patients with soft drusen (difference from control: 4.5%; 95% CI, 0.2%-8.7%; $P = .03$). The highest qAF values were located in the superotemporal segment except for the RPD subgroup, in which the highest qAF values were determined in the temporal segment (eFigure 2 in the Supplement).

Pathologic changes such as drusen may affect qAF_8 measurements within the analyzed circular region. To investigate qAF levels in areas with and without drusen, qAF profiles through the fovea were computed and compared with normative data of age-matched controls. As illustrated by representative examples in **Figure 4**, the background qAF level (ie, between drusen) was within the normal range of the controls (mean ± 1 SD) in most individuals with soft drusen (24 of 28, [86%]; 95% CI, 68%-95%) and in all patients with cuticular drusen (8 [100%]; 95% CI, 63%-100%). The background qAF level was slightly above and below this range in 1 (4%; 95% CI, 0%-19%) and 3 (11%; 95% CI, 3%-28%) patients with soft drusen, respectively. Background qAF in the 4 patients with RPD was below (n = 2), within (n = 1), or above (n = 1) this normal range. The qAF signal of drusen compared with the background qAF level depended on the drusen subtype and was slightly increased in soft drusen (definable on AF images in 19 of 28 patients [68%]; 95% CI, 49%-82%) and decreased in cuticular drusen (definable in 6 of 8 patients [75%]; 95% CI, 40%-94%) and RPD (definable in 4 of 4 patients [100%]; 95% CI, 45%-100%).

Discussion

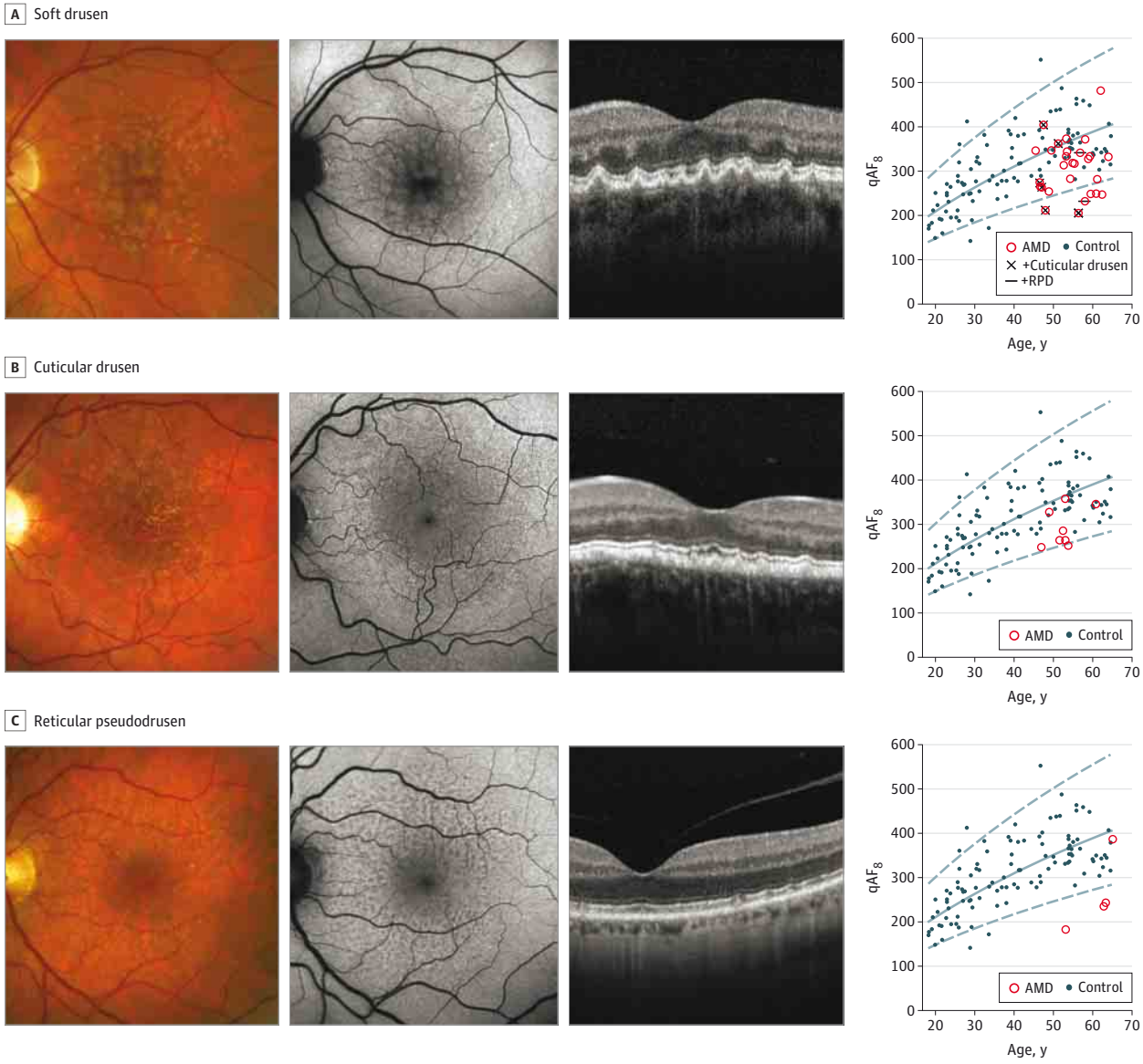
Based on our sample of patients, lipofuscin-related fundus AF levels were not increased in early or intermediate AMD. The qAF measures were either normal or slightly reduced compared with the measures in controls. Two other studies revealed comparable results in eyes with AMD: one[20] reported quantitative fluorescence measures in flat mounts of human postmortem eyes and the other[21] reported in vivo fluorophotometric AF measurements. Thus, 3 independent studies using 3 different methods for assessing the lipofuscin content at the ocular fundus in AMD are in accordance.

Because the AF signal analyzed in the present study largely derives from lipofuscin,[13] the results are indicative of normal to slightly subnormal lipofuscin levels in patients with early and intermediate AMD. In eyes with low qAF measures, the lipofuscin content of single RPE cells or the number of RPE cells could be reduced, as suggested in a histopathologic report on AMD eyes.[22] Reduced lipofuscin levels within single RPE cells might, for instance, be explained by decreased lipofuscin accumulation (eg, due to a reduced rod/cone density or a general slowing of the visual cycle in patients with AMD) or by increased degradation, although direct evidence for such hypothesis is currently missing.

When interpreting the results of this study, one must consider that qAF is not a direct measure of lipofuscin concentration. The measurement of qAF may be affected by several additional factors leading to low qAF measures despite a normal lipofuscin load. First, different packing and distribution of lipofuscin within RPE cells could affect measurement, as shown by Ach and coworkers[22] in a histopathologic study. Second, the composition of lipofuscin fluorophores and the resulting autofluorescent properties could differ in patients with AMD compared with individuals with healthy eyes. For instance, interactions of lipofuscin (granules) with other components of the phagolysosomal complex and/or melanin granules may result in the formation of other fluorophores, such as melanolipofuscin.[23] Therefore, the composition and distribution of fluorophores need to be addressed in future studies. Third, qAF measures may be reduced by absorbers of the excitation or fluorescence light within (eg, melanin granules[23]) or anterior to (eg, subretinal deposits[24]) the RPE.

Despite these limitations, the findings in AMD clearly contrast with results in *ABCA4*-related retinopathy, in which increased lipofuscin accumulation[25] and elevated qAF values are well documented.[4,17] Furthermore, the normal, age-dependent lipofuscin accumulation in healthy human eyes was not found to be associated with RPE cell loss, which raises ques-

Figure 2. Quantitative Fundus Autofluorescence (qAF) Associated With Drusen Subtypes

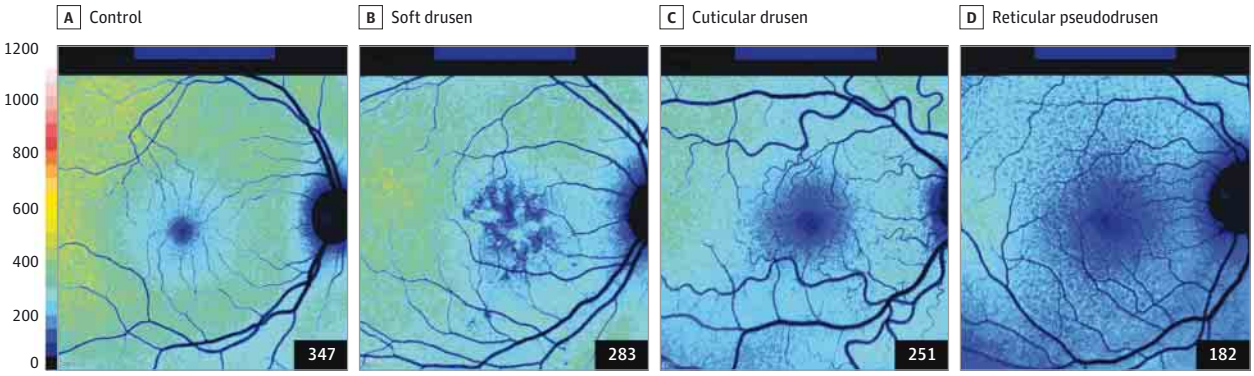


From left to right, fundus color, fundus autofluorescence, and optical coherence tomographic images as well as mean qAF values of the 8-segment circular ring centered on the fovea (qAF_8) of the investigated subgroups compared with the control individuals are shown. AMD indicates age-related macular degeneration; RPD, reticular pseudodrusen. In the graphs, the blue line indicates the regression curve; dotted lines, the 95% prediction interval of controls.

tions regarding the cytotoxic effects of lipofuscin at these mildly elevated levels.[26] Thus, direct lipofuscin-related cytotoxic effects as suggested[1] for *ABCA4*-related retinopathy are unlikely to be a major pathogenic factor in AMD. However, even normal to slightly subnormal lipofuscin accumulation might still affect other disease processes that play an important role in the multifactorial pathogenesis of AMD, such as complement activation. For example, the dysregulation of the complement system in AMD might result in increased lipofuscin-dependent complement activation even at normal or low lipofuscin levels.

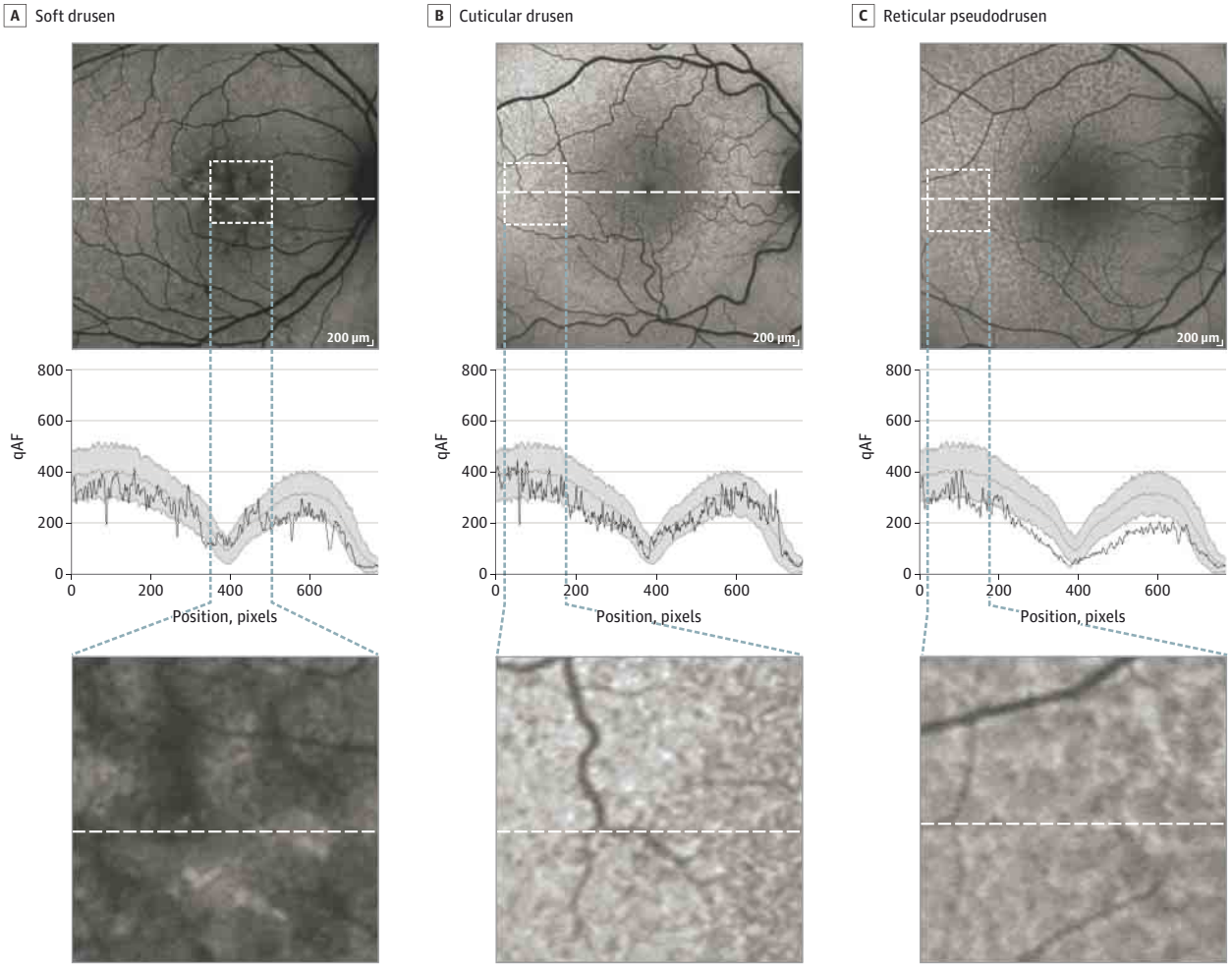
Based on the assumed pathogenicity of lipofuscin accumulation in the RPE, several treatments aiming at lowering lipofuscin are under development or in clinical trials.[27] The lack of direct evidence for increased lipofuscin accumulation in AMD suggests that such compounds may have limited effect, although vulnerability to, for example, complement activation might be reduced. In addition, rather low qAF levels in AMD result in larger effects of measurement noise and a low range to monitor potential effects of lipofuscin reduction. Thus, qAF imaging may be more appropriate to assess lipofuscin-lowering therapies in *ABCA4*-related retinopathy.

Figure 3. Color-Coded Quantitative Fundus Autofluorescence Maps in Age-Related Macular Degeneration



Images of an age-matched healthy control individual and characteristic patients with soft drusen, cuticular drusen, and reticular pseudodrusen. Quantitative values of the 8-segment circular ring centered on the fovea are shown in the lower right corner of each image.

Figure 4. Horizontal Quantitative Fundus Autofluorescence (qAF) Profiles of Representative Patients With Age-Related Macular Degeneration



The upper row shows the fundus autofluorescence (AF) image. In the middle row, the black line illustrates the qAF measures along the horizontal dotted white line in the AF image above, and the gray area indicates the mean (±1 SD) qAF profile of 45 age-matched controls. Lower row shows 5-fold magnification of areas of interest highlighted by the square superimposed on the AF image in the row above.

Based on the lipofuscin toxicity hypothesis, qAF could be considered a possible screening tool to identify patients at risk for developing late AMD stages. This tool would allow early diagnosis and initiation of therapy before the onset of functionally relevant retinal damage. However, since qAF was not increased in early or intermediate AMD in this study, the results challenge this notion and suggest that qAF testing would not be effective to evaluate risk for progression in patients with AMD.

Investigation of qAF patterns of different drusen subtypes revealed slightly increased qAF measurements over most larger soft drusen. Descriptions of AF patterns of soft drusen in the literature[28-30] are inconsistent and range from hypoautofluorescent to hyperautofluorescent patterns. These discrepancies might be explained by less sensitive imaging devices or less detailed phenotyping used in these older studies. Possible explanations for an increased drusen-associated AF compared with background found in the present study encompass greater accumulation of fluorophores within the overlying RPE, intrinsic fluorescence of drusen, and, at least for foveal drusen, decreased macular pigment.

In contrast, reduced qAF patterns of cuticular drusen and RPD were in line with previous reports[31,32] using conventional fundus AF imaging and compatible with a proposed thinning of the overlying RPE in cuticular drusen and a masking of the autofluorescence owing to the assumed subretinal localization of RPD.[33] Reduced qAF values might be a characteristic feature of patients with RPD since qAF was low even in areas without this lesion subtype. It has been suggested[34,35] that RPD may result from dysfunction of the choroid-Bruch membrane-RPE complex, which might be associated with a slowing of the visual cycle, reduced rod and cone densities, or different composition of lipofuscin, resulting in reduced lipofuscin accumulation and/or lower qAF measures.

This study has several limitations. First, measurements were not performed in patients older than 65 years. This cutoff was set because of the increasing variability of lens opacities with age, which makes estimation of lens opacities and the resulting qAF values more inaccurate. Second, we report on a relatively small number of patients, mainly because of strict inclusion criteria aiming to include only patients with high image quality and distinct phenotypes. Therefore, the results should be generalized with caution, although the uniformity of the findings renders random effects unlikely. Third, patients with late AMD were not investigated because such patients are frequently older than 65 years and present with more progressed fundus changes requiring different examination strategies to acquire reliable data. We therefore cannot rule out that certain subgroups (eg, older patients or those with late AMD) may have different qAF levels. For instance, relatively increased AF signals have been described[36] in the junctional zone of geographic atrophy based on conventional AF imaging, although background qAF levels were not assessed.

Conclusions

Patients with early and intermediate AMD analyzed in this study demonstrated normal to reduced lipofuscin levels. This finding has implications for understanding the pathogenesis of AMD and for upcoming treatment trials. Because of the limitations of qAF imaging to directly measure lipofuscin or analyze its components, further studies to substantiate these findings are required.

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Study concept and design: Gliem, Holz, Charbel Issa.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: Gliem, Charbel Issa.
Critical revision of the manuscript for important intellectual content: Müller, Finger, McGuinness, Holz.
Statistical analysis: Gliem, Müller, Finger, McGuinness.
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Invited Commentary

New Understanding of Age-Related Macular Degeneration Through Quantitative Autofluorescence

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The study of Gliem et al[1] in this issue of *JAMA Ophthalmology* appears to be the first application of the powerful quantitative autofluorescence (qAF) technology to age-related macular degeneration (AMD) and warrants our full attention. To appreciate the significance of this study, recall that the retinal pigment epithelium (RPE) has been clinically imaged for nearly 2 decades by AF scans that record the AF of its lipofuscin granules. The value of clinical AF imaging for phenotype description and as a qualitative disease marker has continued to grow exponentially, not only in retinal degenerations such as AMD, Stargardt disease, and Best disease, but also in the wide spectrum of inflammatory disorders and choroidal tumors. Quantitative AF, introduced in 2011, is performed by calibrating the AF image to an embedded reference of known fluorescence efficiency, making it possible to reproducibly quantify and compare the AF intensity of the RPE, a surrogate for its lipofuscin content, between patients and across time. For example, a broad increase in qAF with age in individuals with healthy eyes was confirmed, documenting how lipofuscin accumulates. Interesting and unexplained sex and ethnic variations in these trends were found. The addition of qAF to the study of genotype/phenotype correlation in Stargardt disease has begun, with milder disease pheno-

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