

# Impact of Drusen Volume on Quantitative Fundus Autofluorescence in Early and Intermediate Age-Related Macular Degeneration

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Submitted: January 4, 2019

Accepted: April 4, 2019

Citation: Reiter GS, Told R, Schlanitz FG, et al. Impact of drusen volume on quantitative fundus autofluorescence in early and intermediate age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 2019;60:1937-1942. <https://doi.org/10.1167/iovs.19-26566>

**PURPOSE.** Drusen volume (DV) and quantitative autofluorescence (qAF) are potential indicators of progression in age-related macular degeneration (AMD). This prospective and observational study examined the association between DV and qAF of the retinal pigment epithelium.

**METHODS.** Eighty-eight eyes of 52 patients with early and intermediate AMD were included. The mean follow-up was  $9.2 \pm 5.6$  months, resulting in 312 examinations. DV was extracted from  $6 \times 6$ -mm spectral-domain optical coherence tomography scans. qAF was measured using a rotated Delori pattern. The associations between qAF and DV, age, sex, and lens status were investigated using linear mixed models.

**RESULTS.** Patients' mean age was  $75.6 \pm 5.0$  years (range, 61.0–83.4 years). Sixty-eight eyes (77.3%) were from females. No significant association between DV and qAF was found, neither within the total outer Early Treatment Diabetic Retinopathy Study (ETDRS) grid nor for ETDRS segments six to nine (all  $P > 0.05$ ). Sex and lens status were not associated with qAF ( $P = 0.429$  and  $P = 0.213$ , respectively). A significant association between age and qAF ( $P = 0.025$ ) was found, indicating a decrease of qAF with age.

**CONCLUSIONS.** Quantification of DV and fundus autofluorescence did not reveal any correlation in early and intermediate AMD. qAF decreased with age, whereas DV increased in about half of the patients. This finding is a quantitative corroboration that fundus autofluorescence and the buildup of drusen are not correlated processes in AMD.

**Keywords:** age-related macular degeneration, drusen, lipofuscin, fundus autofluorescence, retinal pigment epithelium

Drusen and pigmentary alterations are well-known hallmark signs of age-related macular degeneration (AMD) and are used in clinical classifications to calculate individual risk factors.<sup>1,2</sup> Drusen have been studied extensively in relation to disease progression, as for example by algorithms automatically quantifying drusen on fundus color photos,<sup>3,4</sup> spectral-domain optical coherence tomography (SD-OCT),<sup>5-10</sup> and polarization-sensitive OCT (PS-OCT) images.<sup>11</sup> Changes in drusen volume have been examined as a possible marker for disease progression to create new end points for clinical studies.<sup>11-13</sup> Consequently, automated and manual drusen volume segmentations have been validated with proper repeatability and reproducibility.<sup>14-16</sup> Drusen and drusenoid pigment epithelium detachments have a characteristic life cycle before disease progression.<sup>13,17</sup> Drusen volume has been shown to regress preceding the onset of geographic atrophy or choroidal neovascularization (CNV) in AMD.<sup>11</sup>

A healthy retinal pigment epithelium (RPE) cell contains hundreds of long-lasting inclusion bodies, subdivided into lipofuscin, melanolipofuscin, and melanosomes.<sup>18</sup> Lipofuscin accumulates in healthy RPE cells until the age of 70 years and

declines thereafter.<sup>19,20</sup> In AMD, lipofuscin is redistributed by degranulation, which has been described as the earliest subcellular alteration seen in affected RPE cell bodies.<sup>21</sup> Lipofuscin accumulation in RPE cell bodies has been discussed for decades as a disease indicator in AMD. This accumulation was first described in histopathology studies<sup>22-25</sup> and later in studies using short-wave fundus autofluorescence (FAF).<sup>26-28</sup> A disintegration of the RPE above drusen may result in a subsequent decline of FAF emitted from lipofuscin granules in the RPE cell bodies, which can be imaged using a confocal scanning laser ophthalmoscope (cSLO).<sup>26,27</sup> Regarding changes over drusen, autofluorescence intensity was directly shown to be lower in eyes in which drusen changed internal composition (i.e., calcification).<sup>29-31</sup> In vivo excitation of lipofuscin ranges between 400 nm and 590 nm and presents a peak at 490 to 510 nm, whereas emission of FAF ranges between 520 and 800 nm, culminating at 590 to 630 nm.<sup>32</sup>

Quantitative autofluorescence (qAF), a method recently presented by Delori et al.,<sup>33</sup> quantifies FAF signals by using a cSLO modified by an internal reference and shows good reliability and repeatability in patients with AMD.<sup>34</sup> The FAF



signal distribution shows the signal is at its minimum at the fovea due to absorption by macular pigment and maximum between 7° and 15° from the fovea, with a peak in the superotemporal region.<sup>20,35</sup> Patients with AMD have been found to have overall lower levels of qAF than healthy age-matched individuals.<sup>36,37</sup> An assessment of the intraindividual in vivo course of lipofuscin concentration in AMD progression only became available with the development of qAF. The present study was conducted to quantify autofluorescence values and investigate their possible association with drusen volume in patients with early and intermediate AMD.

## METHODS

This study adhered to the Declaration of Helsinki including current revisions and the Good Clinical Practice guidelines. The study protocol was approved by the Ethics Committee of the Medical University of Vienna. After giving written informed consent, 52 patients with early and intermediate AMD were included based on the classification published by Ferris et al.<sup>1</sup> Patients were excluded if they had late AMD or had received anti-VEGF treatment or intraocular surgery, except uncomplicated cataract surgery. Lens opacities in phakic eyes were graded using the lens opacities classification system III,<sup>38</sup> and patients were excluded when they exceeded nuclear subcategories of 3.0 and cortical or subcapsular posterior subcategories of 2.0. The study was conducted from August 2015 until June 2017. Each patient visit included a best-corrected visual acuity (BCVA) test and a complete ophthalmologic examination including funduscopy, infrared images, qAF images, and SD-OCT (all Spectralis HRA+OCT; Heidelberg Engineering, Heidelberg, Germany). After assessing BCVA, eye drops containing 0.5% tropicamide and 2.5% phenylephrine were administered to dilate pupils to at least 7 mm. Patients were asked to return to our clinic every 3 months for repeat examinations.

### qAF Image Acquisition

qAF images were acquired by a cSLO (Spectralis HRA+OCT, Heidelberg Engineering) by using 488 nm excitation and a barrier filter, which transmits light between 500 and 680 nm, modified with an internal fluorescence reference provided and calibrated by Heidelberg Engineering, which accounts for laser power and detector sensitivity.<sup>33</sup> Details of the acquisition procedures have been published.<sup>33,39</sup> In short, the infrared mode was used to align the camera on the fundus. The room light was switched off to avoid influence of room light on image quality and to reduce distraction of the patients. After warning the patients about the bright blue light, the qAF mode was activated and the camera was aligned to reach the maximum signal and sharpness. After a minimum of 20 seconds of bleaching using the 488-nm light to minimize light absorption by rhodopsin, qAF images with 12 frames covering a field of 30 by 30° (768 × 768 pixels) were acquired in the high-speed mode while paying attention to camera alignment and ensuring the maximum signal level without oversaturation. Patients were asked to blink before each acquisition and avoid eye movements. Two qAF videos were acquired on each visit with a short break of 1 minute in between. The videos were checked for adequate quality immediately after recording and, if insufficient (less than nine useable frames per video), qAF video was reacquired.

### qAF Image Evaluation

Images were analyzed using the manufacturer's software HEYEX (Heidelberg Engineering). Single frames were checked

and consistently removed if of poor quality or if artefacts, such as motion artefacts or emerging lids or lashes, were seen. A total of nine frames had to be available to process an average image. For this study, we used the Delori pattern, which consists of one center circle and four concentric rings around the fovea.<sup>33,35</sup> The inner four-segment ring, together with the outer eight-segment ring was omitted for this study. The eight segments of both the inner and middle ring were used for analysis. The Delori pattern was centered on the fovea and rotated by 22.5 degrees (see Figure) for alignment with the Early Treatment Diabetic Retinopathy Study (ETDRS) grid of the drusen volume. Vessels were automatically excluded from analysis by the software. The threshold setting was manually adjusted if necessary. Phakic eyes were subsequently corrected for normative age-related optical media density.<sup>35,40</sup> The mean qAF of all segments of the inner and middle eight-segment rings of the rotated Delori pattern (qAF<sub>IMS</sub>) were selected for further evaluation, and values were exported in tabular form. Because qAF results were displayed in an anatomic description and ETDRS segment results were exported in an anticlockwise description, care was taken to correlate segments seven and nine to the corresponding temporal and nasal values exported after qAF analysis. We used the segment numbers of the ETDRS grid for statistical analysis to enable easier description (see Figure). qAF<sub>IMS</sub> was used representatively for the mean qAF of each eye, instead of qAF of the middle eight segment ring (qAF<sub>MS</sub>).<sup>36,41</sup>

### SD-OCT Imaging Protocol

SD-OCT imaging was performed with Spectralis HRA+OCT (Heidelberg Engineering) by using a 6 × 6-mm grid with a resolution of 1024 × 97 (A-scans × B-scans) centered on the fovea. The follow-up mode, provided by the system's software, was used to acquire images thereafter. Volume scans were exported for automated drusen segmentation.

### Drusen Segmentation

Three hundred twelve OCT volume scans were segmented using a published algorithm<sup>10</sup> and manually corrected using the IOWA OCT Explorer 3.0 (The University of Iowa, Iowa City, IA, USA). Drusen volume was computed from an overlying 6-mm-diameter ETDRS grid showing results for each individual segment and the total results within the ETDRS grid.

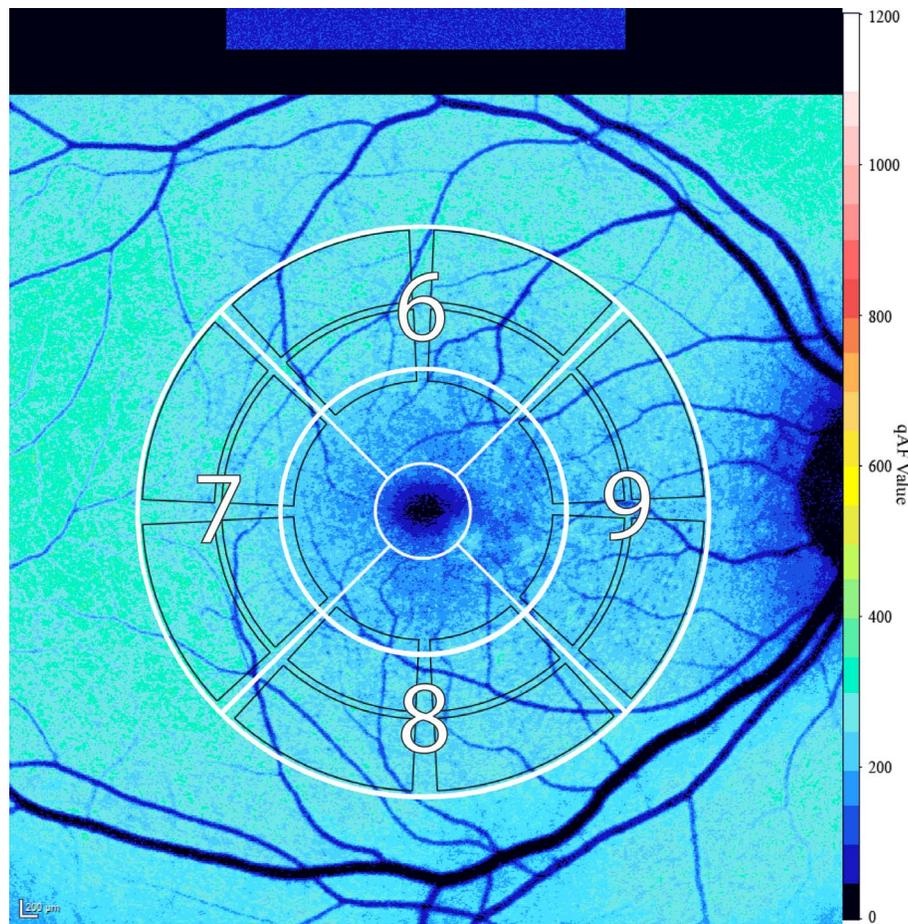
### Statistics

A linear mixed model was used to assess whether there was an association between qAF and drusen volume. qAF was used as the dependent variable and drusen volume, age, sex, and lens status as independent variables. Furthermore, random intercepts were specified for each patient and each eye. The covariance structure was specified as an autoregressive model of the first order (AR1). This model was computed once for the averaged qAF<sub>IMS</sub> and drusen volume within the 6-mm-diameter ETDRS grid. The same model was applied for segments six to nine of the ETDRS grid. A *P* value <0.05 was considered statistically significant. Due to exploratory reasons, no correction for multiple testing was performed in this study.

## RESULTS

### Study Participants

Eighty-eight eyes of 52 patients were included in the study. Forty-four eyes (50%) were right eyes and 44 were left eyes. Out of 52 patients, 40 (76.92%) were female and 68 eyes



**FIGURE.** Quantitative autofluorescence with the inner and middle eight-segment ring of a rotated Delori pattern (black) and an overlying ETDRS grid (white). The outer eight-segment ring of the Delori pattern is not shown in the figure. Numbers 6 to 9 corresponding to the ETDRS segments were used in this study.

(77.3%) were from females. The mean age at baseline was  $75.58 \pm 4.96$  years (range, 61.0–83.4 years). Forty-two eyes (48%) were pseudophakic and 46 eyes (52%) were phakic. The mean BCVA at baseline was  $0.08 \pm 0.12$  logarithm of the minimum angle of resolution (logMAR) (Snellen equivalent approximately 20/24). The monitoring period ranged from 0 to 21 months, resulting in a total of 312 baseline and follow-up visits. The mean drusen volume per eye within the 6-mm-diameter ETDRS grid at baseline was  $0.21 \pm 0.17 \text{ mm}^3$  and the mean  $\text{qAF}_{\text{IMS}}$  was  $165.3 \pm 49$  qAF units. A complete listing of the descriptive characteristics for each ETDRS segment is given in Table 1.

Of the 88 eyes included in the study, the total drusen volume continuously increased during the observational period in 49 eyes (55.7%), whereas 23 eyes (26.1%) remained stable and drusen regressed in 16 (18.2%) with a rapid decline in volume. Five eyes of four patients developed CNV. Patients were excluded from analysis starting with one visit previous to CNV detection.

### Linear Mixed Model

The results of the primary model ( $\text{qAF}_{\text{IMS}}$ , total drusen volume) are shown in Table 2. We found no significant association between drusen volume and  $\text{qAF}_{\text{IMS}}$  ( $P = 0.904$ ). Likewise, no association between drusen volume and qAF was found in the other single segments of the ETDRS grid ( $P = 0.736$ ,  $P = 0.451$ ,  $P = 0.141$ , and  $P = 0.697$ ).

No significant association was found between sex and qAF ( $P = 0.429$  in the primary model;  $P = 0.301$ ,  $P = 0.333$ ,  $P = 0.679$ , and  $P = 0.557$  for the ETDRS segments six to nine) or between lens status and qAF after correction for normative age-related optical media density ( $P = 0.213$  in the primary model;  $P = 0.224$ ,  $P = 0.528$ ,  $P = 0.156$ , and  $P = 0.208$  for the ETDRS segments six to nine). However, we found a significant association between age and qAF in the primary model ( $P = 0.025$ ) and in two out of four ETDRS segments ( $P = 0.067$ ,  $P = 0.008$ ,  $P = 0.015$ , and  $P = 0.068$ ). In the primary model, age and qAF revealed a negative slope coefficient of  $-2.7$ , indicating a decrease of qAF by 2.7 qAF units with an increase of each year in age. The qAF decrease per age ranged between  $-2.4$  to  $-3.2$  for the ETDRS segments six to nine.

### DISCUSSION

We found no statistically significant association between drusen volume and qAF in individuals with early and intermediate AMD. As AMD progresses, drusen volume may increase before eventually regressing and converting into geographic atrophy or CNV.<sup>11</sup> The RPE overlaying the druse thins, disintegrates, and segregates from the RPE during the life cycle with hyperreflective retinal foci appearing in OCT imaging.<sup>13,17,42</sup> Lipofuscin redistribution by degranulation was the earliest subcellular alteration described in AMD.<sup>21</sup> Following degranulation, one would expect FAF to decline in

TABLE 1. Descriptive Statistics at Baseline for Each ETDRS Segment and the Total Area

Variables	Mean	SD	Min	Max
Drusen volume, mm <sup>3</sup> (ETDRS segment 6)	0.02	0.02	0.00	0.13
Drusen volume, mm <sup>3</sup> (ETDRS segment 7)	0.02	0.02	0.00	0.14
Drusen volume, mm <sup>3</sup> (ETDRS segment 8)	0.02	0.02	0.00	0.10
Drusen volume, mm <sup>3</sup> (ETDRS segment 9)	0.02	0.02	0.00	0.15
Drusen volume, mm <sup>3</sup> (total outer ETDRS)	0.21	0.17	0.01	0.95
qAF, qAF units (ETDRS segment 6)	166.36	51.30	58.64	349.14
qAF, qAF units (ETDRS segment 7)	167.35	48.88	64.77	318.80
qAF, qAF units (ETDRS segment 8)	154.64	49.27	61.56	296.19
qAF, qAF units (ETDRS segment 9)	169.99	53.90	61.21	352.66
qAF, qAF units (total inner + middle 8)	164.58	48.82	61.54	324.96

areas with a higher drusen volume due to a loss of lipofuscin in RPE areas above the drusen where thinning and disintegration of the RPE is perceivable during the drusen life cycle.

Specific detection of melanin-enriched cells is possible by using PS-OCT,<sup>43</sup> which has shown that the RPE above drusen disintegrates over time with increasing drusen volume.<sup>44</sup> In fact, for drusenoid pigment epithelium detachments, these changes may even precede changes in volume during the lifecycle of this lesion, demonstrating a degree of overlap with the natural history of drusen. As major druse components are RPE derived, it is plausible that dysfunction or loss of RPE cells actually drives the decline in drusen volume, rather than increasing drusen volume causing RPE loss.<sup>13</sup> For healthy individuals, qAF ex vivo applied to flatmounts of RPE from young and aged donors showed no net cell loss or abnormal cellular morphology in aging despite progressive lipofuscin accumulation, the opposite of what would be expected if lipofuscin were toxic.<sup>45</sup> There is long-standing confusion in the literature about the relationship of drusen and FAF. Lipofuscin is an organelle of the RPE that sequesters FAF byproducts of the visual cycle. However, it once was also thought that lipofuscin and its precursors, incompletely digested photoreceptor membranes, could accumulate in Bruch's membrane in the form of drusen.<sup>46</sup> In fact, modern concepts of drusen biogenesis do not involve lipofuscin or its precursors. The formation of drusen and its precursor basal linear deposit appear to be driven by lipid deposition from the RPE on Bruch's membrane, also known as the "oil spill hypothesis." The RPE secretes apolipoprotein B particles of unusual composition into Bruch's membrane, where they accumulate with age, eventually forming a lipid wall, a precursor of basal linear deposit, and may interact with reactive oxygen species to form proinflammatory peroxidized lipids.<sup>47</sup> Our findings indicate drusen volume and FAF within the surrounding RPE may take different pathophysiologic paths during the progression of AMD. Although drusen volume seems to increase to a critical value until it rapidly regresses, the intensity of FAF reaches a critical threshold, even before AMD develops, and does not change along with drusen volume, as proven in this study.

Autofluorescent particles with the same autofluorescent properties as lipofuscin can also be found within drusen.<sup>48</sup>

Apart from the lipofuscin within the RPE, drusen themselves have a distinct, diffuse autofluorescence that is excited by violet light (436 nm), that emits at 520 nm, and is more weakly excited by 480 nm light with an emission near 550 nm.<sup>49</sup> In particular, this spectral signature does not match the lipofuscin signatures of the RPE found by hyperspectral imaging but could contribute to FAF at 488 nm excitation. This finding is quite different from the few, small, and isolated FAF particles found within drusen that do have the same autofluorescent properties as lipofuscin organelles, are of similar size, and are unlikely to add to FAF.<sup>48</sup> Therefore, autofluorescent particles within drusen may not have an impact that is detectable with qAF.

Age correlates significantly with qAF ( $P = 0.025$ ) with an estimated slope coefficient of  $-2.7$ , which predicts a decline of qAF of 2.7 qAF units per year. This could be interpreted in two different ways. First, it may be argued that patients with phakic eyes manipulate these results because the lens absorbs more light and qAF declines, although this effect should be mostly avoided applying the normative age-related optical media density correction.<sup>40</sup> On the other hand, the autofluorescence signal has been shown to reach a peak at the age of 70 and decline thereafter.<sup>19</sup> Considering our cohort had a mean age of  $75.58 \pm 4.96$  years, the more likely interpretation is a slowly declining FAF intensity, which can be shown by qAF. In addition, this study showed no association between qAF and lens status after correcting for normative age-related optical media density and excluding patients with considerable cataract.

However, some limitations have to be considered. Most importantly, as is inevitable when investigating AMD, we included patients aged 65 and above. Previous studies of qAF excluded this age group due to the fact that the different light absorption of a lens with cataract influences qAF. However, we did exclude patients with considerable cataract and believe by proper evaluation, age correction, as well as statistical model we were able to avoid the effect of lens status on qAF. Another limitation from using qAF is that it shows same session repeatability of approximately  $\pm 8.0\%$  in patients with AMD,<sup>34</sup> which may potentially affect the short-term association due to minor changes in drusen volume. Although drusen volume has been described as a monitoring marker in early and interme-

TABLE 2. Association Between Drusen Volume, Age, Sex, Lens Status, and qAF

Variables	Estimate	Lower CL	Upper CL	SE	P Value
Drusen volume, mm <sup>3</sup>	2.636	-40.012	45.283	21.759	0.904
Age, y	-2.728	-5.097	-0.36	1.208	0.025
Sex (male)	-10.674	-36.93	15.582	13.396	0.429
IOL (yes)	-13.783	-35.058	7.493	10.855	0.213

Estimates are given for the change of qAF unit per one unit of the investigated parameter. CL, confidence level; IOL, intraocular lens.

diate AMD with adequate repeatability and reproducibility, qAF changes might be better detectable in long-term studies. Further long-term intraindividual results are needed to show the course of qAF in early and intermediate AMD.

Use of the standard software provided by the manufacturer was a drawback because it can measure qAF mainly by using the Delori pattern. Therefore, qAF detected the autofluorescence signal not only in the regions above drusen but also in the regions between drusen. A completely accurate description of the drusen overlying RPE may, therefore, not be possible using the standard software. An individual analysis of the overlying RPE based on drusen maps might reveal detailed alterations in its autofluorescence properties. In a similar way, subretinal drusenoid deposits, which were not excluded from this study, could have an effect on the association between qAF and drusen volume. In a subgroup comparison, patients with reticular pseudodrusen showed overall the lowest qAF values.<sup>36</sup> Individually custom-built maps of subretinal drusenoid deposits might also show specific alterations for these patients.

In conclusion, we found no association between qAF and drusen volume, sex, or lens status but did find a significant association between qAF and age. These results indicate different pathomechanisms in the progression of drusen volume and loss in FAF intensity in the aging RPE during AMD progression, with decrease of qAF likely representing physiologic aging together with the overall lower level of autofluorescence in AMD and drusen being a pathognomonic AMD feature.

### Acknowledgments

Presented in part at the ARVO 2018 annual meeting in Honolulu, Hawaii, United States, on May 2, 2018.

Disclosure: **G.S. Reiter**, None; **R. Told**, None; **F.G. Schlanitz**, None; **H. Bogunovic**, None; **L. Baumann**, None; **S. Sacu**, None; **U. Schmidt-Erfurth**, None; **A. Pollreis**, None

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