Dynamic Changes of Retinal Microaneurysms in Diabetes Imaged With In Vivo Adaptive Optics Optical Coherence Tomography

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PURPOSE. To prospectively monitor microaneurysms (MAs) in three dimensions using adaptive optics optical coherence tomography (AOOCT).

METHODS. Patients with diabetes mellitus and parafoveal MAs were included in this longitudinal study. At baseline, MAs were identified in standard fluorescein angiography (FA) and subsequently imaged with an AOOCT prototype, incorporated into an AO fundus camera (RTX1, Imagine Eyes) device. Imaging was repeated every 3 months in each patient to explore the potential structural change of MAs over time including size, shape, intraretinal position, (intra-) luminal reflectivity, and other qualitative morphologic characteristics.

RESULTS. We imaged 18 MAs in seven eyes (two left eyes) of five patients (mean age: 69 ± 7 years) over 18 months. All MAs appeared as saccular in the en face imaging plane at baseline, and no change in shape was observed in any of the MAs during follow-up. Evaluation of the AOOCT volumes revealed dynamic changes of MAs during follow-up including intermittent growth (n = 2), progressive involution (n = 3), total disappearance (n = 2), and MA division (n = 1). Intraluminal hyperreflective material was visualized in 11 out of 18 MAs, which remained stable (n = 3), increased (n = 2), regressed (n = 1), or fluctuated (n = 5). Three MAs without intraluminal spots at baseline progressively developed distinct hyperreflectivities.

CONCLUSIONS. AOOCT illustrates the structurally dynamic evolution of MAs in vivo in three dimensions. Despite a consistent saccular shape in the en face view, AOOCT volumes revealed a heterogeneous behavior in regard to size and reflective status of MAs over time.

Keywords: microaneurysm, diabetes, adaptive optics, optical coherence tomography, imaging
coherence tomography (SD-OCT), thereby offering the great potential to unravel retinal microvascular details in all three dimensions.\textsuperscript{14} A recent cross-sectional study compared details of more than 500 diabetes MAs visualized in AOOCT with those identified with commercially available retinal imaging techniques including FA, SD-OCT, and OCT angiography. AOOCT successfully delineated distinct characteristics of MAs including MA intraluminal and wall reflectivity, as well as MA feeding and draining vessels and their origin from the individual retinal capillary plexuses. Though intraluminal clots were found in some MAs, most of the strong intraluminal reflections delineated with AO fundus camera imaging merely correlated to a thickening of the MA wall in the corresponding AOOCT volumes.\textsuperscript{15}

The purpose of the present study was to use AOOCT to prospectively observe retinal MAs in order to investigate changes of three-dimensional morphologic details in the natural history of their dynamic turnover.

**METHODS**

All study investigations were conducted in accordance with the tenets of the Declaration of Helsinki. The study was approved by the Ethics Committee of the Medical University of Vienna (EK 1244/2014) and the board of governmental authorities. All patients were recruited in the outpatient clinic for DR at the Department of Ophthalmology, Medical University of Vienna, and gave written informed consent to all procedures prior to study inclusion.

In this prospective study, we included adult patients with a confirmed diagnosis of diabetes mellitus type 1 or 2 and any level of DR (Early Treatment of Diabetic Retinopathy Study, ETDRS level 20-75) who showed at least one parafoveal MA and good image quality in OCT and FA. Exclusion criteria were any inflammatory eye disease, any retinal disease other than DR, or any condition that might preclude pupil dilation.

Routine clinical examination included best-corrected visual acuity testing (Snellen), a slit-lamp examination of the anterior eye, intraocular pressure measurement, SD-OCT (macula cube 512 × 128, Cirrus SD-OCT; Carl Zeiss Meditec, Jena, Germany), and dilated fundus biomicroscopy. Axial eye length was measured using optical biometry (IOL Master 500, Carl Zeiss Meditec). Standard FA (Spectralis OCT+HRA; Heidelberg Engineering, Heidelberg, Germany) was performed as part of clinical routine examinations, where MAs of interest could precisely be localized. All patients then underwent adaptive optics imaging. Patients were elective for follow-up only if AOOCT image quality was sufficient at baseline. Every patient was followed at 3-monthly intervals for at least 18 months. Each follow-up comprised a routine clinical eye exam and adaptive optics imaging.

**AOOCT System**

The AOOCT system used in this study was a prototype designed at the Center for Medical Physics and Biomedical Engineering of the Medical University Vienna.\textsuperscript{16,17} In brief, it combines two different imaging modalities in a single compact instrument: an AO fundus camera and AOOCT. The AO fundus camera (RTX1; Imagine Eyes, Orsay, France) records en face fundus reflectance images with a field of view of 4° × 4° and a transverse resolution of ~4 μm. The AOOCT system is based on SD-OCT and is operated with a super luminescent diode with a center wavelength of 841 nm (bandwidth of ~50 nm). Assuming a refractive index of 1.4 of the retina, a theoretical axial resolution of ~3 μm is achieved in the retina. The acquisition of one volume (at an A-scan rate of 200 kHz) takes 0.8 seconds and covers a field of view of 1.8° × 2° consisting of 565 (x) times 400 (y) pixels. Patient alignment is facilitated by the use of an anterior segment camera and the possibility to move the compact instrument in three dimensions. The light power of the OCT imaging beam at 841 nm was 500 μW; the guide star at 750 nm was 50 μW; and the AO fundus camera at 850 nm was 1.1 mW. There was simultaneous light exposure for the patient of 50 μW and 500 μW when the OCT was on, as well as 50 μW and 1.1 mW when the AO fundus camera was on. The combined light power used for the system was below the limits for safe exposure according to the European standards.\textsuperscript{18}

**AAOCT Imaging Protocol and Postprocessing**

Imaging was conducted in mydriasis and cycloplegia by applying one drop of tropicamide 1% (Mydriaticum Agepha, Vienna, Austria) and phenylephrine 2.5% (Pharmacy General Hospital Vienna, Vienna, Austria) to the study eye prior to imaging. MAs of interest were defined in the early phase FA images. The individual patient was aligned at the AO system and instructed to follow an internal fixation target, which was moved accordingly to localize the MA at the center of the AO fundus camera image. The internal fixation target can move vertically from −8° to +8° and horizontally from −10° to +10° from the center of the fovea, which defined the borders of the parafoveal imaging area and the cutoffs for eccentricity in this study. The focus was then set to the inner retinal layers, and multiple AO fundus images were acquired. The depth of focus of the system is ~120 μm and therefore sufficiently large to be able to set the focus either to the anterior layers or to the photoreceptor layers without any specific nuances. However, loss of signal may occur if the focus is set off the anterior layers of the retina. The device was switched to the AOOCT mode to record at least four perpendicular volumes at the same focus settings. The acquired AO fundus camera images were registered to each other and averaged with software provided by the manufacturer. Details of AOOCT data post processing were published previously.\textsuperscript{16,17} B-scans of sufficiently high quality were correlated and aligned by the software to eliminate axial eye motion artifacts.

**Image Analyses**

All analyses were performed by the same grader trained on advanced retinal image analyses (J.H.) using ImageJ (software version 2.0.0; National Institutes of Health, Bethesda, MD, USA). First, each MA was identified in the early phase FA image and assigned to the corresponding location in the AO fundus image. AOOCT volumes of the best quality were chosen for further analysis. All AOOCT volumes were rotated to align the retinal pigment epithelium in the B-scan horizontally before assessing the en face view in order to ensure even levels of retinal capillary plexuses. Image analysis was based on a complete review of each AOOCT volume stack in all three dimensions for each MA. Single, preselected AOOCT images were chosen as figures for publication to illustrate MA morphologic criteria of interest. At baseline, all AOOCT volumes were evaluated for the intraretinal position of each MA, which was defined as the retinal layer where the center of the MA was located. At baseline, the number of feeding and/or draining vessels of the MA and their origin from the individual retinal capillary plexuses (superficial, intermediate, and deep capillary plexus) were recorded. The feeding and/or draining vessels included in the MA were classified in the en face plane of the AOOCT volumes as being either saccular, fusiform, or a focal bulge according to the

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TABLE 1. Baseline Characteristics of Study Patients (n = 5)

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<th>Interval Between Study Visits in Days, Mean ± SD</th>
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<td>11</td>
<td>2</td>
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<td>93 ± 14‡</td>
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</table>

HbA1c, glycated hemoglobin.
* One MA location could not be relocated at one follow-up visit.
† Lost to follow-up at month 15.
‡ Month 3 missing.

classification scheme proposed by Moore et al.19 MA intraluminal reflectivity was graded by comparing it to the MA wall reflectivity. It was considered hyperreflective if the intensity of the reflected signal was similar or stronger compared to that of the MA wall or hyporeflective otherwise. In addition, the homogeneity of the hyperreflective signal as well as the presence of well-circumscribed hyperreflective material in an otherwise hyporeflective MA lumen was assessed.

In a second step, the stacks of the follow-up visits were compared to those at baseline in order to investigate specifications regarding a morphologic change of the MA including MA shape, MA luminal appearance, MA division, and MA size.

Due to the lack of a validated three-dimensional segmentation algorithm for AOOCT volume stacks, precise MA volume measurements cannot be performed at present. For the purpose of this explorative longitudinal study, change in size was measured in the B-scan view of the stacks with respect to the extension of the MA over the individual retinal layers, as well as in the en face view with respect to the largest visible diameter of the MA. In both views, the outer MA wall borders were considered for all measurements. First, the scan displaying the maximum size of the MA was identified in both views in the full AOOCT volume. In order to assess the variability in respect to the chosen B-scan location, the size of the MAs was measured in seven adjacent scans (three adjacent scans in both directions from the “maximum size scan,” respectively). The size of the MA was then defined as the mean of these measurements, and the variability was defined as the standard deviation of this measurement. Microaneurysm size is presented in micrometers (m) for all results. Images were scaled using the previously calibrated axial pixel size of the instrument (1 pixel corresponds to 3 m, assuming a refractive index of 1.41 of the retina) and the axial eye length (AL) information of all study patients. For lateral scaling the scanning angle was calibrated using a model eye and a calibration target as retina. With a scanning angle of 2°, the conversion from angle to distance on the retina (x) was performed using following equation: x = 2° (AL – AD)/(1.33) tan (1), where AL is the axial eye length, AD is the anterior chamber depth, and 1.33 is the refractive index of water. As an example, for an eye length of 24 mm, AD = 5.5 mm, two degrees of scanning angle correspond to 540 μm. This distance is sampled with 400 pixels, which results in 1 pixel corresponding to 1.35 μm. All these two-dimensional measurements were performed by the same grader, and the evaluation of intergrader variability was forgone at this stage, considering the lack of a robust three-dimensional AOOCT segmentation algorithm.

RESULTS

Five out of eight study patients met the image quality criteria at baseline to be included in this follow-up study. Seventeen MAs from six eyes (two left eyes) of four patients were followed for 18 months; one patient (patient 3, see Table 1) was lost to follow-up after 15 months. Three patients underwent an additional AOOCT imaging session at month 21. One of these patients, however, missed the scheduled visit at month 3 (patient 5, see Table 1). In another two patients with complete follow-up (18 months), one MA location of interest was not correctly identified with the AO fundus camera at one visit (month 3 for patient 1, and month 9 for patient 4, see Table 1).

The baseline characteristics of all study patients, study eyes, and followed MAs are presented in Table 1. Figure 1 shows a direct comparison of each followed MA location in the early phase FA (top row), the AO fundus camera (middle row), and the AOOCT en face view (bottom row) image at baseline.

Shape of MAs

All MAs included in this AOOCT analysis were graded as being saccular in shape in the en face imaging plane at baseline, which implied the visualization of an asymmetric dilation with respect to the long axis of the associated vessel(s). We observed no change of this saccular morphology in any of the MAs during follow-up (Figs. 2–6).

Stability in Size and Growth of MAs

Besides stability in size over the observation period of 18 months as exemplified over a period of 6 months in Figure 2, we observed intermittent growth in two MAs, which is clearly visualized in MA “a” of Figure 3, which is part of a cluster of four MAs in the superior parafoveal region. This MA grows from baseline to month 12. In the same Figure, MA “d” grows from baseline to month 6, remaining stable to month 12.

Division and Fusion of a Single MA

Microaneurysm division was observed in one MA. Figure 3 shows MA division in MA “b,” which transforms from a single MA at baseline into two small “daughter” MA bulges at month 6. From month 9 on, only one MA can again be visualized, which remains stable in size until month 18. Figure 4 shows an example of suspected MA fusion from months 6 to 15 in the right eye of patient 4 (also shown in Supplementary Video S1).

Regression and Disappearance of MAs

Three of the followed MAs progressively regressed during follow-up. MA “c” in Figure 3 progressively decreases in size until month 21. MA “d” totally disappeared in the time span from month 12 to month 15. Another example of MA involution is seen in the 3-month interval in Figure 5. The MA, which is centered in the inner nuclear layer (INL), decreases in size from month 12 on. Regression of a single MA
is also demonstrated in Figure 6 from baseline to month 18 (the MA could not be relocalized at month 9) in patient 4 (see also Supplementary Video S2).

Figures 7A and 7B illustrate the course of change in size for the individual MAs that have grown, regressed, or disappeared during follow-up as determined in the en face and the cross-sectional AOOCT imaging planes, respectively.

**MA Vessel Wall Reflectivity**

The majority of MAs featured heterogeneous reflective signal intensity along the MA vessel wall with increased reflectivity where the wall was bulging outward from the originating capillary plexus. MA wall thickening with enhanced reflectivity at the bulge can be visualized in MA “c” of Figure 3 throughout the visits. More dynamic changes in MA wall reflectivity can be observed in MA “d” of Figure 5. At baseline, the MA vessel wall cannot be delineated from the surrounding retinal tissue. During follow-up the MA can be well delineated, showing a typical enhanced reflectivity at the outward-bulging wall, which again diminishes at month 12 before the MA disappears.

A granular wall appearance with several hyperreflective spots within or adjacent to the MA vessel wall is presented in Figure 5. The hyperreflective spots move over 3 months, but are still visible in the en face view, even if not visible in the B-scan. The en face image at month 18 reveals persistence of these hyperreflective spots at the same vessel wall location.

**MA Lumen Reflectivity**

At baseline, in 11 out of 18 MAs (61%), hyperreflective intraluminal spots were identified in either the en face or the cross-sectional AOOCT imaging plane, which remained stable \( (n = 5) \), increased \( (n = 2) \), regressed \( (n = 1) \), or fluctuated \( (n = 5) \) during follow-up.

Figure 5 illustrates an MA with moderate intraluminal hyperreflectivity at months 12 and 15, which increases to an intense hyperreflective intraluminal signal suggesting full clotting of the MA at month 18. Figure 2 highlights the benefit of three-dimensional AOOCT imaging in regard to the assessment of luminal reflective appearance of MAs: MA “a” appears fully hyperreflective in the en face view at month 18, but analysis of this MA in the B-scan imaging planes reveals a stable moderate hyperreflective luminal appearance comparable to that of months 15 and 21.

Besides a gradual increase, we also recorded a regression of intraluminal hyperreflectivity. MA “d” of Figure 5 displays only some tiny hyperreflective spots in the otherwise hyporeflective lumen at baseline, which regress to leave this MA totally hyporeflective (M12) before it disappears (M15).

In the majority of MAs graded as hyperreflective at baseline \( (n = 5) \), the homogeneity and magnitude of intraluminal hyperreflectivity fluctuated in the course of follow-up. In Figure 5, this fluctuation of intraluminal appearance between the individual imaging sessions can be observed in MAs “a” to “c.” All three MAs display changes in intraluminal and wall reflectivity.
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FIGURE 3. Dynamic natural history of four MAs during observation. Images refer to MAs identified in the right eye of patient 5. The four MAs of interest are highlighted by the white square in the early phase fluorescein angiography image. MAs, marked as “a,” “b,” “c,” and “d” at baseline, are shown in the en face AOOCT intensity images at the level of the superficial capillary plexus at each visit. The white horizontal lines feature the level of the en face imaging plane in the corresponding B-scan and vice versa. Vertical and horizontal scale bars: 100 μm across for AOOCT images. Scale bar for FA image: 400 μm.

reflectivity over the course of the observation period. Fluctuating intraluminal hyperreflectivity can also be observed in Figure 8: At month 6, the intraluminal hyperreflective spot is clearly attached to the vessel wall as visualized in both imaging planes. At month 9, it has grown, leaving only a small residual lumen in both views. However, at month 12, the hyperreflective intraluminal material has almost totally disappeared. The hyperreflective status of the MA lumen remained stable in the remaining 3 of these 10 hyperreflective MAs during follow-up. As visualized in the representative images, the internal reflective signal intensity appeared heterogeneous in all these MAs with a hyperreflective content, and an attachment of the material to the MA vessel wall was clearly visualized in at least one AOOCT imaging plane.

The remaining eight MAs were considered hyperreflective at baseline, three of which developed distinct intraluminal hyperreflective spots during the observational period.

Supplementary Video S3 shows the full AOOCT stack in the en face view of the four MAs presented in Figure 3 at follow-up visit month 6. The data are shown in logarithmic representation to improve visualization of the individual retinal layers.

DISCUSSION

The present study constitutes the first approach to monitor retinal MAs prospectively in three dimensions using in vivo AOOCT. Reliable imaging and analysis of MA structural change is critical considering that their absolute count and turnover reflect retinopathy severity and represent a valuable biomarker for disease progression. The characterization of MA natural history in vivo remains challenging to date, but single pioneering observations on their structural change over time have already been made: Chui et al. used AOSLO structural maps to analyze geometric changes of four MAs in one retinal region after 4, 14, and 20 weeks in a patient with proliferative DR. In another approach, one MA disappearance and one enlargement were shown in a total of four AOSLO imaging sessions in a patient with severe NPDR. Besides AOSLO structural imaging, single MAs have recently been followed over 4 months in two patients with hypertensive retinopathy and central retinal vein occlusion to reveal MA formation and regression using AOSLO FA. However, we could recently demonstrate that AOOCT can reveal further details of MA.

FIGURE 4. Microaneurysm fusion over a period of 9 months. Images refer to MAs identified in the right eye of patient 4. Imaging sessions at months 6, 9, 12, and 15 are shown in the respective columns (M6, M9, M12, M15). AOOCT B-scans are demonstrated on top of the corresponding en face AOOCT intensity images at the level of the intermediate capillary plexus at each visit. The white arrow is pointing at two MAs (M6), which fuse to form one MA at month 15. Vertical and horizontal scale bar: 100 μm across for AOOCT images.
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As precise three-dimensional volume measurements of MAs will first require the development of a robust three-dimensional AOOCT segmentation algorithm, we performed manual MA diameter measurements in the transverse and axial dimensions of AOOCT images separately, thus accepting a certain level measurement variability in this preliminary approach (see Figs. 7A, 7B). Besides structural stability over 21 months, we observed intermittent growth \((n = 2)\) and progressive involution \((n = 3)\), as well as total resolution \((n = 2)\) of MAs in the context of the neural retina within single follow-up visits. Beyond these changes in MA size, AOOCT imaging illustrated the process of MA division in one MA. At this point it should be emphasized that caution has to be taken in the process of analysis in order to detect hidden motion artifacts resulting in a “doubling” of MAs, which may simulate division of a former single MA. However, this phenomenon was observed only for a single MA in this study.

Though all of the MAs in this study were graded as saccular and no change of shape in the en face view was observed, their natural course and behavior were highly heterogeneous during follow-up. Classifying MAs based on their two-dimensional geometry may therefore not be sufficiently predictive for the longitudinal course of MA pathology.

Most MAs \((56\%)\) featured an intraluminal hyperreflectivity at baseline and either showed a stable hyperreflective appearance or a fluctuation in its extent during follow-up. Only a minority of MAs showed a constant intraluminal hyporeflective signal over 21 months. These findings support earlier reports of an association between focal hypofluorescence and the larger, pedunculated or irregular MA type, as we did not include any MAs graded as pedunculated or irregular in this study.\(^{13}\) As presented in Figure 5, progressive increase of intraluminal hyperreflective material to the stage of “total clotting” was associated with regression of MAs and might therefore signify thrombogenesis. However, it has to be emphasized that we refer to the term MA “clotting” as a morphologic description, as the actual histologic MA lumen composition was not investigated in this study. Our structural findings are well in line with the results of former histologic studies, demonstrating that hyperreflective material in the internal lumen of MAs is likely to represent extensive fibrosis and infiltration by a variety of cells ranging from acute inflammatory cells, such as polymorphonuclear cells and monocytes, to red blood cell breakdown products and cellular debris.\(^{22}\) Figure 5 demonstrates hyperreflective spots within or adjacent to the MA vessel wall. Bolz et al.\(^{23}\) also found hyperreflective deposits adjacent to and within the vessel wall of MAs with SD-OCT and, based on histologic findings, suggested an association to the presence of dense lipoprotein concentration related to macrophage infiltration.\(^{24}\) Similarly, Vujosevic et al.\(^{25}\) classified the appearance of this organized

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**Figure 5.** Microaneurysm regression over 6 months. Images refer to MAs identified in the right eye of patient 1. Imaging sessions at months 12, 15, and 18 are shown in the respective columns (M12, M15, M18). AOOCT B-scans are demonstrated on top of the corresponding en face AOOCT intensity images at the level of the intermediate capillary plexus at each visit. Vertical and horizontal scale bar: 100 µm across for AOOCT images.

**Figure 6.** Microaneurysm regression over 18 months. Images refer to a MA identified in the right eye of patient 4. Imaging sessions at baseline and months 3, 6, 12, 15, and 18 are shown in the respective columns (BL, M3, M6, M12, M15, M18). The MA could not be relocalized at month 9, but a significant change in size is shown from BL to M18, especially in the period between M6 and M12. AOOCT B-scans are demonstrated on top of the corresponding en face AOOCT intensity images at the level of the intermediate capillary plexus at each visit. Vertical and horizontal scale bar: 100 µm across for AOOCT images.
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FIGURE 7. Microaneurysm change in size during follow-up. The course of change in MA size is shown as measured in the en face (A) and cross-sectional (B) AOOCT imaging planes. The table below the graph presents MA size at the specific visits (BL, baseline; M, months 3, 6, 9, 12, 15, 18, 21) in micrometers, and indicates the patient identification number and study eye referring to Tables 1–3; asterisk indicates missing visit, triangle indicates inability to relocalize MA with AOOCT, rectangle indicates disappearance of MA. MA 1 grew and corresponds to MA “a” in Figure 3. MA 2 exemplifies growth as well as disappearance and refers to MA “d” in Figure 3. Disappearance is also illustrated in the graph of MA 3. MA 4 to 6 exemplify MA regression. MA 4 is also shown in Figure 4, MA 5 represents MA “c” of Figure 3, and MA 6 is shown in Supplementary Video S2.
hyperreflective material as activated microglial cells representing reactive immune defense. We hypothesize this recruitment of immunologic cells including microglia and macrophages as the initiating event for MA regression as observed in Figure 5. The thickened wall bulge appearance observed in some MAs (see Figs. 3, 8) might correspond to a hyalinized basement membrane or a multilayering of endothelial cells.

Further longitudinal studies are required in order to relate our preliminary findings to functional outcome, including risk of MA leakiness or response to treatments such as anti-VEGF interventions. In this study, however, we observed highly dynamic changes of MAs irrespective of whether or not the patients received further intraocular injections during follow-up.

In conclusion, in this study AOOCT illustrated the structurally dynamic evolution of MAs in vivo in three dimensions with incomparable resolution to reveal a consistent saccular shape, but a heterogeneous behavior in regard to MA

<table>
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<th>TABLE 2.</th>
<th>Baseline and Follow-Up Characteristics of Study Eyes (n = 7)</th>
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BCVA, best corrected visual acuity; NPDR, nonproliferative diabetic retinopathy; CSME, clinically significant macular edema; PDR, proliferative diabetic retinopathy; VEGF, vascular endothelial growth factor; PRP, panretinal photocoagulation; n, no; y, yes.

* According to the International Clinical Classification System, American Academy of Ophthalmology.

<table>
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<th>TABLE 3.</th>
<th>Baseline Characteristics of Microaneurysms (n = 18)</th>
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No., number; SCP, superficial capillary plexus; ICP, intermediate capillary plexus; DCP, deep capillary plexus; N, nasal; V, vertical; T, temporal; IPL, inner plexiform layer; GCL, ganglion cell layer; OPL, outer plexiform layer.
size and reflective status of the lumen and vessel wall over time. AOQCT may help to establish new MA categorization schemes allowing a more efficient stratification of patients according to their risk for DR progression in the future.

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