Association of Microaneurysms on Adaptive Optics Scanning Laser Ophthalmoscopy With Surrounding Neuroretinal Pathology and Visual Function in Diabetes

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PURPOSE. We evaluate diabetic microaneurysm (MA) features on high-resolution adaptive optics scanning laser ophthalmoscopy (AOSLO) and their correlations with visual acuity (VA) and local retinal pathology on spectral domain optical coherence tomography (SDOCT).

METHODS. Diabetic participants underwent VA testing and AOSLO and SDOCT imaging of MAs. AOSLO images were graded for MA dimension, wall hyperreflectivity (WH), intraluminal hyperreflectivity (IH), and perfusion pattern. SDOCTs centered on each MA were graded for disorganization of the retinal inner layers (DRIL) and other neuroretinal pathology.

RESULTS. We imaged 109 MAs (30 eyes). Multivariate modeling, including statistically significant covariates from bivariate analyses, associated WH with greater MA size (P = 0.001) and DRIL (P = 0.04). IH was associated with perfusion (P = 0.003) and MA visibility on photographs (P = 0.0001), and larger MA size with partial perfusion (P = 0.05), MA ring signs (P = 0.0002), and photographic visibility (P = 0.01). Multivariate modeling revealed an association of WH and VA with DRIL.

CONCLUSIONS. AOSLO imaging demonstrates associations of hyperreflective MA walls with MA size and adjacent DRIL, as well as the presence of DRIL with lower VA. This study identifies a correlation between vascular and neural pathology associated with VA decline. Further studies of MA structure and neuroretinal disorganization may enable novel approaches to assess anatomic and functional outcomes in the diabetic eye.

Keywords: diabetic retinopathy, DR, microaneurysms, MA, retina, optical coherence tomography, OCT, adaptive optics scanning laser ophthalmoscope, AOSLO

The onset of clinically visible microaneurysms (MA) is an early hallmark of diabetic retinopathy (DR). Indeed, extent and severity of MAs in an individual eye are used to determine DR severity and strongly correlate with subsequent disease progression.1–5

Ex vivo histopathologic studies from animal models and human specimens have shown that selective loss of pericytes in retinal capillaries precedes the appearance of MAs. The weakened vessel wall support in areas of profound pericyte loss may lead to MAs of the capillary wall, which commonly are associated with endothelial cell alterations, basement membrane thickening, and lumen clotting.6–8 In addition, leakage of fluid from capillaries and MAs is a primary mechanism for the onset and progression of diabetic macular edema (DME), one of the most common causes of vision loss in diabetic individuals. However, neither histopathologic studies nor current standard in vivo imaging modalities, such as color fundus photography and fluorescein angiography, allow the dynamic characterization of MAs at the cellular level in humans.

The advent of adaptive optics scanning laser ophthalmoscopy (AOSLO), which provides noninvasive, high-resolution retinal imaging, enables evaluation of the living human retinal vasculature at the cellular level. Adaptive optics systems correct more than 90% of the wavefront aberrations from an individual eye through the coupling of a wavefront sensor with a control system that achieves aberration compensation through deformable mirror technology, enabling a transverse resolution limit of approximately 2 μm.9–11 Using AOSLO, dynamic and static imaging of the human retinal vasculature became feasible, providing insight into the vascular wall microstructure as well as flow dynamics within the vascular tree.12–15

AOSLO imaging combined with commercially available spectral domain optical coherence tomography (SDOCT) enables an added understanding of the retinal neurovascular unit as a whole, since SDOCT provides axial resolution of approximately 5 μm and can resolve individual retinal neural layers in the healthy and diseased states.16 Thus, SDOCT imaging provides complementary characterization of the neural tissue surrounding specific MAs, including the presence of adjacent intraretinal cysts, disorganization of the retinal layers, or changes to overall retinal thickness adjacent to the MA.17–20

We performed AOSLO and SDOCT imaging of individual retinal MAs and the local surrounding neural retina in persons with diabetes to determine if structural or physiologic AOSLO
MA & VA Associations Imaged by AOSLO & OCT in DR

MA characteristics correlate with local retinal neural pathology on SD OCT. We also assessed if MA parameters evaluated on AOSLO are associated with altered local retinal visual function within the central macula.

**Materials and Methods**

The study was approved by the Joslin Diabetes Center institutional review board and adhered to the tenets of the Declaration of Helsinki. Before enrollment and the performance of any study procedures, written informed consent was obtained from each participant.

**Patients and Examination**

Patients were recruited for this study from regularly scheduled retina clinics at the Beetham Eye Institute of Joslin Diabetes Center, a tertiary care referral center specializing in diabetic eye disease. Patients were eligible to participate if they met the following inclusion criteria: minimum age 18 years, diagnosis of type 1 or type 2 diabetes, clear optical media (cornea, lens, vitreous), and stable central fixation. Patients with substantial media opacities precluding good quality imaging or retinal pathology due to nondiabetic diseases were excluded from study participation.

During a single study visit, all participants underwent a comprehensive, dilated ophthalmologic evaluation, including Early Treatment Diabetic Retinopathy Study (ETDRS)—protocol procedures of both eyes: refraction, best-corrected visual acuity (BCVA), and modified 7-field color fundus photography (PP4 Fundus Camera; Carl Zeiss Meditec, Inc., Dublin, CA, USA). Additionally, AOSLO and SD OCT imaging (Spectralis OCT; Heidelberg Engineering, Heidelberg, Germany) was performed. DR severity based on the ETDRS DR severity scale for each eye was determined by grading of color fundus photographs. The SD OCT imaging protocol consisted of 49 B-scans (spacing between scans, 120 µm; high resolution mode, 16 frames Automated Real-Time Averaging [ART]), covering a field of 20° × 20° centered at the fovea.

**Adaptive Optics Imaging and Image Post-Processing**

The AOSLO (Boston Micromachines, Cambridge, MA, USA) used for this study was a subtype of the Indiana system that has been described previously. In brief, the confocal SLO subsystem operates with a 13 nm imaging beam bandwidth centered at 830 nm. The AO subsystem uses a deformable mirror (Boston Micromachines) and a Shack-Hartman wavefront sensor (180 samples within a nominal pupil of 6.4 mm on the eye) controlled by custom software. The field size is approximately 1° × 1.2° with a lateral resolution limit of 2.5 µm on the retina.

The retinal posterior pole was imaged by moving the scanning field stepwise across the 10° × 10° retinal area centered at the fovea. MAs within this area were identified for AOSLO imaging based on fundus photos and SD OCT images. Additional outpouchings of the capillary walls not initially visible on fundus photos or SD OCT that were detected during AOSLO imaging sessions also were defined as MAs and included in the analysis. Imaging of MAs was performed by shifting focus between the retinal nerve fiber layer and RPE. Audio-video interleave (AVI) files of up to 10 blocks were recorded on each MA at a rate of 30 frames per second. Each block consisted of 50 frames, and blocks were acquired at differing levels of focus that ranged through the anterior block consisted of 50 frames, and blocks were acquired at a rate of 30 frames per second. Each study eye’s axial length was assessed using an IOL master (Carl Zeiss Meditec, Inc.) to reliably measure and quantitate MA size (in µm) on the AOSLO images.

**Image Analysis**

AOSLO images were registered to corresponding fundus photographs based on vascular landmarks and known imaging coordinates. For all MAs, visibility on fundus photography and infrared images was assessed and MAs were graded as visible or not visible on these imaging modalities. Eccentricity from the foveal center point also was calculated for each MA (Fig. 1). AOSLO and SD OCT images were exported for further offline processing and analysis in Fiji (Fiji is Just ImageJ; National Institutes of Health [NIH], Bethesda, MD, USA). The following variables were graded in a binary fashion on the high-resolution AOSLO videos: MA perfusion status (completely perfused versus partially perfused), presence of intraluminal hyperreflectivity (IH, present versus absent) and presence of MA wall hyperreflectivity (WH, present versus absent). WH was defined as any portion of increased pixel intensity along the MA wall compared to the remaining MA wall. Longest diameter of each MA was measured in µm. Perfusion was defined as visible flow of blood cells within the graded MA on AOSLO videos. IH was defined as hyperreflectivity within the MA lumen with a rough appearance and/or connection to the MA wall that also had adjacent blood cell flow near the IH. Smooth, centrally located hyperreflectivity with no local disturbances in blood flow was ascribed to lensing artifact due to the dome-shaped architecture of MAs, and was not included in the analysis (see Fig. 2).

For assessment of morphologic alterations of the neural retinal layers, SD OCT substacks of 5 B-scans centered on each MA were generated. Within each B scan, a 500 µm box centered on each MA was assessed for retinal volume, outer layer disruption, disorganization of retinal inner layers (DRIL), intraretinal cysts, hyperreflective retinal spots, and MA ring signs. All SD OCT variables were graded in binary fashion as present versus absent on each B scan.

**Statistical Analysis**

Associations between AOSLO parameters and SD OCT parameters were first assessed using nonparametric Wilcoxon rank sum or Fisher’s exact test analyses treating results from each MA as an independent observation. To adjust for possible confounding parameters that were statistically significant in these analyses then were included in mixed effects logistic regression models to determine the strength of the relationship between variables while adjusting for correlations between MAs from the same eye within 500 µm of each other. Statistical analyses were run in SAS (version 9.4; SAS Institute, Inc., Cary, NC, USA). The level of statistical significance was set at $P < 0.05$ for these exploratory analyses.

**Results**

We studied 30 eyes of 29 individuals. Median (interquartile range [IQR]) age was 46 (37–57) years, and 11 (38%) were female. Of the participants, 22 (76%) had type 1 diabetes with...
a median (IQR) diabetes duration of 26 (18–32) years and hemoglobin A1c (HbA1c) of 7.7% (6.5%–8.4%). Median logMAR visual acuity (VA) in the study eyes was 0.04 (0.10–0; Snellen equivalent of 20/20 +2 letters [20/25–20/20]). A detailed description of the study population characteristics is reported in Table 1.

AOSLO MA Parameters Associations With Morphologic Alterations

A total of 109 MAs were imaged. In unadjusted, bivariate analyses, associations were found between the presence of WH using AOSLO imaging and longer MA dimension (WH present, 104.6 [75.9–129.1] μm versus no WH present, 50.0 [38.3–65.2] μm; P < 0.0001), the presence of intraluminal MA hyperreflectivity (WH present, 82.4% versus no WH, 33.3%; P < 0.0001), visible perfusion within MA (WH present, 76.5% versus no WH, 33.3%; P < 0.0001), DRIL (WH present, 14.7% versus no WH, 2.7%, P = 0.03), and visibility on fundus photography (WH present, 93.2% versus no WH, 59.1%; P = 0.001). No significant relationship with WH was found for sex, age, duration of diabetes, thickness, or volume of the ETDRS subfield of the respective MA, presence of intraretinal cysts ≥250 μm, MA ring signs, epiretinal membranes, hyperreflective retinal spots, or outer retinal layer disruption as seen on SDOCT, or MA wall deformability as seen on AOSLO (Table 2).

The presence of IH found in AOSLO imaging was associated with shorter duration of diabetes (IH present, 26 [15–35] years versus no IH, 29 [17–42] years; P = 0.047), longer MA dimension (IH, 6.5 [59.2–119.0] μm versus no IH, 48.5 [35.1–65.6] μm; P < 0.0001), visible perfusion within each MA (IH, 79.3% [n = 42] versus no IH, 16.1% [n = 9]; P < 0.0001) and visibility on fundus photography (IH present, 95.9% [n = 47] versus no IH, 42.6% [n = 20]; P < 0.0001). No significant relationship was found with sex, age, thickness, or volume of the ETDRS subfield of the respective MA, presence of MA ring signs, intraretinal cysts, epiretinal membranes, hyperreflective retinal spots, outer retinal layer disruption or DRIL as seen on SDOCT, or MA wall deformability as seen on AOSLO (Table 2).

Multivariable modeling was performed adjusting for all characteristics that were statistically significant in unadjusted analyses as well for correlations between MAs from the same eye within 500 μm of each other. For WH in AOSLO imaging, only MA dimension (point estimate [95% confidence limits], 0.04 [0.02, 0.07]; P = 0.001) and DRIL (1.29 [0.04, 2.53]; P = 0.04) remained associated. IH remained related to visible
perfusion (2.33 [0.79, 3.87]; P = 0.003), and visibility on fundus photography (3.12 [1.66, 4.56]; P < 0.0001). For MA size, associations with visible perfusion (2.58 [0.32, 4.84]; P = 0.03), presence of MA ring signs on SDOCT (3.55 [1.71, 5.40]; P = 0.0002), and visibility on fundus photography (2.98 [0.76, 5.21]; P = 0.01) remained statistically significant (Fig. 3).

AOSLO MA Characteristics and VA
To assess the relationship of AOSLO MA parameters with retinal function, the relationship between VA and AOSLO characteristics of MAs located within 500 μm of the foveal center (n = 41) was analyzed. In a bivariate analysis, the absence of WH was associated with better logMAR VA (−0.11, −0.01; P = 0.02). Worse logMAR VA also was associated with larger MA diameter (0.001 [0.0005, 0.001]; P = 0.001) and presence of DRIL (0.17 [0.13, 0.19]; P < 0.0001). A multivariable model assessing the relationship between VA and WH, MA size, IH, and presence of DRIL found that only DRIL (0.13 [0.06, 0.19]; P = 0.004) remained associated with VA (Fig. 4).

DISCUSSION
In this cross-sectional exploratory study, we demonstrated that the combination of high resolution AOSLO imaging with SDOCT can provide detailed assessment of the in vivo interplay between vascular and neural pathology in the diabetic retina. Using these state-of-the-art imaging modalities, we were able to identify an association between specific AOSLO MA wall characteristics and neural disorganization and worse visual outcomes. Hyperreflectivity of MA walls on AOSLO is related to greater likelihood of local presence of neural disorganization as assessed by DRIL on SDOCT. This is important, since DRIL extent is associated with concurrent and predictive of future visual outcomes in eyes with DME.33-35 Indeed, in this study, the presence of DRIL within 500 μm of the foveal center was significantly associated with worse VA.

The cause of wall hyperreflectivity on AOSLO has yet to be determined, although possible correlates are suggested by histopathology. Stitt et al.5 proposed a classification of MA based on the integrity of pericytes, endothelial cells and the basement membrane, as well as the accumulation of cells within the MA lumen. Four different types were described ranging from loss of pericytes with preservation of endothelial cells (type I) to loss of pericytes and endothelial cells (type II and III), to late stages with loss of wall cells accompanied by an increase in basement membrane thickness (type III and IV). The significant association of MA WH on AOSLO with MA size in our data suggests that these MAs might represent later stage MAs that have grown in size and possibly progressed to increased basement membrane thickening. In this study, MA WH on AOSLO did not correlate with the presence of hyperreflective MA ring signs on SDOCT, suggesting that the two different imaging modalities are sensitive to different structural aspects of MA walls.

The hypothesis that MAs with WH are more mature and/or older is consistent with our results demonstrating that MA WH is significantly associated, not only with larger MA size, but also with presence of local neural retinal pathology. Foveal DRIL is a biomarker that is strongly correlated with current and future visual outcomes in eyes with DME. Greater extent of central retinal DRIL is associated with worse current VA in eyes with existing or resolved center-involved DME.55 Furthermore, early 4-month change in DRIL extent is highly correlated with longer-term change in VA over 1 year, independent of change in SDOCT central subfield thickness.55 The developmental relationship between WH on AOSLO and DRIL on SDOCT has

**TABLE 1. Baseline Characteristics of the Study Population n = 29**

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Median (IQR); No. (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>46 (37–57)</td>
</tr>
<tr>
<td>Women</td>
<td>11 (38%)</td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>22 (76%)</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>7.9 (6.5–8.4)</td>
</tr>
<tr>
<td>Type II</td>
<td>7 (24%)</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>6.4 (6.3–8.1)</td>
</tr>
<tr>
<td>Duration, y</td>
<td>26 (18–32)</td>
</tr>
<tr>
<td>VA, logMAR</td>
<td>0.04 (–0.10 to 0.00)</td>
</tr>
<tr>
<td>VA, Snellen equivalent</td>
<td>20/20 +2 letters (20/25–20/20)</td>
</tr>
<tr>
<td>Axial length, mm</td>
<td>25.54 (23.30–24.27)</td>
</tr>
<tr>
<td>Severity of DR</td>
<td></td>
</tr>
<tr>
<td>Mild NPDR</td>
<td>5 (10.3%)</td>
</tr>
<tr>
<td>Moderate NPDR</td>
<td>8 (27.6%)</td>
</tr>
<tr>
<td>Severe NPDR</td>
<td>7 (24.1%)</td>
</tr>
<tr>
<td>PDR</td>
<td>10 (34.5%)</td>
</tr>
<tr>
<td>QPDR</td>
<td>1 (3.5%)</td>
</tr>
</tbody>
</table>

* Percentages may not sum up to 100.00% because of rounding.

**FIGURE 2. Examples of microaneurysms in AOSLO (A1–D1) and SDOCT imaging (A2–4–D2). Arrowheads indicate examples of hyper-reflective walls, arrows indicate examples of IH at the center and wall. Note the rough structure of the IH in all examples (arrows), with IH connected to the MA wall in (A1), (B1), and (C1). The central, bright IHs in A1 are examples of lensing effects. See Discussion for details. (A2–4–D2) Corresponding SDOCT B-scans to respective AOSLO images. The *white box* indicates the 500 μm surrounding the microaneurysm. Note the proximity of intraretinal cysts (*asterisk in (A2–4)) to the MA and the disorganization of retinal inner layers adjacent to/or caused by the respective MA (A2–4–C2). Scale bars in AOSLO images (A1–D1) = 100 μm, in SDOCT images (A–4–D2) = 200 μm.**
Table 2. Unadjusted Analyses of Associations Between MA AOSLO Variables and Demographic and SD-OCT Parameters, n = 109 MAs

<table>
<thead>
<tr>
<th>Variable</th>
<th>MA Size ≥ 14 μm</th>
<th>Wall Hyperreflectivity</th>
<th>Intraluminal Hyperreflectivity</th>
<th>MAs in Relation to Retinal Capillary Plexuses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variable</td>
<td>P Value</td>
<td>P Value</td>
<td>P Value</td>
<td>P Value</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.77</td>
<td>0.14</td>
<td>0.59</td>
<td>0.73</td>
</tr>
<tr>
<td>Age (y)</td>
<td>0.69</td>
<td>0.60</td>
<td>0.08</td>
<td>0.60</td>
</tr>
<tr>
<td>Diabetes duration (y)</td>
<td>1.00</td>
<td>0.10</td>
<td>0.78</td>
<td>0.58</td>
</tr>
<tr>
<td>Longest dimension of MA (μm)</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Retinal subfield volume (mm³)</td>
<td>0.18</td>
<td>0.18</td>
<td>0.17</td>
<td>0.17</td>
</tr>
<tr>
<td>MA perfusion*</td>
<td>0.0013</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
</tr>
<tr>
<td>Interaluminal flow</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
</tr>
<tr>
<td>Presence of intraretinal cysts</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
</tr>
<tr>
<td>Hyperreflective foci*</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
</tr>
<tr>
<td>Wall deformability*</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
</tr>
<tr>
<td>Visibility on fundus photo</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

* Assessed on AOSLO.† Assessed on SD-OCT.‡ Wilcoxon 2-sample / Kruskal-Wallis test.§ Fisher’s exact test.
Figure 3. Forest plots reporting the point estimates and 95% confidence intervals of multivariable models for wall hyperreflectivity of MA (top), IH of MA (middle), and MA size (bottom). All variables significantly associated in the respective unadjusted analyses were included in creating each of the models. Analyses and P values were adjusted for correlations between MAs from the same eye within 500 µm of each other.

Figure 4. Forest plots reporting the point estimates and 95% confidence intervals of multivariable models for visual acuity in the subgroup of MA located within 500 µm of the foveal center. Variables significantly associated in the respective unadjusted analyses were included in creating each of the models. Analyses and P values were adjusted for correlations between MAs from the same eye within 500 µm of each other.
outcomes. Wall hyperreflectivity of MAs within the foveal center together with DRIL on SD-OCT appear to be new markers associated with VA in patients with DR. Future studies will explore and elucidate longitudinal associations of AOSLO MA characteristics and onset, worsening, or improvement of local neural retinal pathology and visual function over time.

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