Retinal Oximetry and Vessel Diameter Measurements With a Commercially Available Scanning Laser Ophthalmoscope in Diabetic Retinopathy

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PURPOSE. To test the hypothesis that retinal vascular diameter and hemoglobin oxygen saturation alterations, according to stages of diabetic retinopathy (DR), are discernible with a commercially available scanning laser ophthalmoscope (SLO).

METHODS. One hundred eighty-one subjects with no diabetes (No DM), diabetes with no DR (No DR), nonproliferative DR (NPDR), or proliferative DR (PDR, all had photocoagulation) underwent imaging with an SLO with dual lasers (532 nm and 633 nm). Customized image analysis software determined the diameters of retinal arteries and veins (DA and DV) and central retinal artery and vein equivalents (CRAE and CRVE). Oxygen saturations of hemoglobin in arteries and veins (SO2A and SO2V) were estimated from optical densities of vessels on images at two wavelengths. Statistical models were generated by adjusting for effects of sex, race, age, eye, and fundus pigmentation.

RESULTS. DA, CRAE, and CRVE were reduced in PDR compared to No DM (P ≤ 0.03). DV and CRVE were similar between No DM and No DR, but they were higher in NPDR than No DR (P ≤ 0.01). Effect of stage of disease on SO2A differed by race, being increased relative to No DM in NPDR and PDR in Hispanic participants only (P ≤ 0.02). Relative to No DM, SO2V was increased in NPDR and PDR (P ≤ 0.05).

CONCLUSIONS. Alterations in retinal vascular diameters and SO2 by diabetic retinopathy stage can be detected with a widely available SLO, and covariates such as race can influence the results.

Keywords: diabetic retinopathy, retinal oximetry, retinal vessel diameter, scanning laser ophthalmoscope

Diabetic retinopathy (DR) is a major cause of visual loss. DR is usually categorized clinically by the presence of retinal lesions such as microaneurysms, hemorrhages, exudates, venous beading, and other lesions on inspection by ophthalmoscopy and biomicroscopy. This information is qualitative, or at best semiquantitative, and does not reveal the underlying pathophysiology of the disease. It is reasonable that evaluation of DR in terms of quantitative biomarkers that are closely related to the underlying physiology of the vasculature might allow finer quantitative assessment of disease severity and prognosis.

A major component of the pathophysiology of DR is abnormal retinal oxygenation, especially in the advanced, sight-threatening stages. This is supported by the presence of nonperfusion on fluorescein angiography and by the efficacy of treating neovascularization with inhibitors of vascular endothelial growth factor, which is induced by hypoxia. Retinal oxygenation is related to the rate of retinal blood flow and the concentrations of oxygen in the arterial and venous blood. The blood flow, in turn, is dependent on the diameters of the retinal arteries (DA) and veins (DV). Accordingly, measurement of DA and DV has attracted considerable attention, and changes related to diabetes have been identified.

The concentrations of oxygen in the arterial and venous blood are proportional to the oxygen saturations of hemoglobin (SO2). SO2 can be measured by oximetry on the basis of the differences in absorption of light by oxyhemoglobin and deoxyhemoglobin at two or more wavelengths. Studies using oximetry in individuals with diabetes have reported elevation of retinal venous SO2 (SO2V), but changes in arterial SO2 (SO2A) and the saturation difference between artery and vein (SO2AV) are not consistent. It might be expected that a variety of variables might influence SO2. A few of the above reports on diabetes have investigated several covariates, but only one has related them to the stages of retinopathy and included race, but its emphasis is on the effects of light flicker stimulation.

Most of these studies have been done with one of the two commercially available instruments for oximetry (Oxymap ehf., Reykjavik, Iceland, or Imedos, Jena, Germany). Recently, oximetry results in retinal vein occlusion subjects and infants have been presented, using a commercially available scanning laser ophthalmoscope (SLO) (Optos200TX; Optos, Dunfermi...
wide availability in clinical centers. Furthermore, retinal vessel diameters also have been measured with an SLO. There are several advantages to using the SLO compared to fundus camera–based systems, including the use of dual monochromatic lasers, less light exposure for the eye, data acquisition through an undilated pupil, a large field of view up to 200°, and wide availability in clinical centers.

To date, D_A, D_V, SO_2A, and SO_2V have not been evaluated with an SLO in subjects with DM. We tested the hypothesis that retinal vascular diameter and oxygen saturation alterations according to the progressive stages of DR are discernible with a commercially available dual wavelength SLO. We also tested the hypothesis that age, race, sex, and pigmentation index influence these retinal vascular variables by constructing a statistical model to account for their effects.

Methods

Subjects

The study was approved by an Institutional Review Board of the University of Illinois at Chicago. Before enrollment, the research study was explained to subjects and informed consent was obtained from each according to the tenets of the Declaration of Helsinki. The study was part of a broader research project to identify biomarkers of diabetic retinopathy. Therefore, for analysis, it represents a convenience sample. The results from 181 subjects are reported. The subjects indicated their race, and there were too few Asians to be included. Only one eye of each individual was included, with the right eye was selected unless only the left eye was eligible. The eyes of each subject were classified by one of four participating retinal specialists (NPB, JIL, FYC, or YL) on clinical examination as normal in control subjects without DM (No DM; n = 46), or in subjects with DM as no visible DR (No DR; n = 41), nonproliferative DR (NPDR; n = 59), or proliferative DR (PDR; n = 35). All of the eyes with PDR had been treated with panretinal photoagulation. In this report we will use the term “stage of disease” rather than “stage of DM” when referring to the previous four groups together, since the individuals without DM cannot be said to have any stage of retinopathy. Exclusion criteria were unwillingness or inability to cooperate with the experimental protocol; opacities of the media precluding clear imaging; diseases that could affect the retina or optic nerve (aside from DM), such as retinal vascular occlusions, sickle cell disease, age-related macular degeneration, glaucoma, or high myopia (spherical equivalent > -6.00 diopters); and intraocular surgery performed within 9 months of participation. In 10 subjects without DM, imaging was performed at two visits to determine the repeatability of measurements.

Imaging

Images were acquired with the SLO (Optos200TX) at laser wavelengths of 532 nm and 633 nm (images532 and image633) with a 60° field of view centered at the optic disc. Images at the two wavelengths appeared in good focus and registration, thus no correction for chromatic aberrations was performed. Our previously developed software program was used for segmentation of retinal vessels and measurement of DO, SO_2A, and SO_2V. This last study uses a customized oximetry instrument, and some subjects were included in both studies. Briefly, SLO images were cropped to 30° × 30° (1536 × 1536 pixels), with the optic nerve head centered within the field of view. Cropping eliminated regions of the SLO images not used for vascular measurements and increased computational efficiency of the program by reducing image sizes. A circumpapillary region of interest was defined, extending between one and two optic disc radii from the optic disc edge, as shown in Figure, left panel.

Vascular Diameter Measurements. Vessel boundaries and diameters were determined from the full width at half maximum of the perpendicular intensity profiles generated from images532 by using a previously published method. [59,40] Diameter measurements were then averaged along each individual blood vessel segment to derive a mean arterial (D_Aind) and venous (D_Vind) diameter (Fig., left panel). These diameter measurements in units of pixels were converted to micrometers by using a constant calibration factor (5.8 μm/pixel), which was derived from the known field of view and pixel dimensions of the SLO images. Central retinal artery (CRAE) and vein equivalents (CRVE) were determined with previously defined equations that included the six largest D_Aind and D_Vind measurements. [41-44] In addition, D_Aind and D_Vind measurements were averaged in all vessels to derive D_A and D_V for each eye.

Vascular Hemoglobin Oxygen Saturation Measurements. Retinal vascular SO_2 was calculated by using optical density (OD) and optical density ratio (ODR) measurements. From images obtained at each wavelength, OD along each perpendicular intensity profile was calculated as log(I_{outside}/I_{inside}), where I_{inside} and I_{outside} represent the average pixel intensity inside and outside the vessel, respectively. I_{outside} was measured by averaging the lowest 50% of pixel values within the vessel boundaries, which minimized reflectance contribution from the bright central reflex of the vessel. I_{outside} was determined by averaging a percentage of background pixel values (based on the vessel diameter) at locations corresponding to the maximum negative curvatures of perpendicular intensity profile. These locations were determined from the minima of a second-order derivative of the perpendicular intensity profile, as previously described. [59,40] ODRs were calculated as OD_{633}/OD_{532}, where OD_{633} and OD_{532} were the retinal vascular optical densities calculated from image633 and image532, respectively. ODR measurements along vessel segments were averaged to derive a mean ODR value in individual arteries (ODR_{Aind}) and veins (ODR_{Vind}). ODR measurements have been previously shown to have a linear relationship to hemoglobin oxygen saturation. [45] Accordingly, ODR_{Vind} values were adjusted for diameter and converted.
OEF is the fraction of the total oxygen entering the eye. The oxygen extraction fraction (OEF) was calculated as the difference between SO2A and SO2V (SO2AV) divided by SO2V.

Table 1: Demographic Characteristics of Subjects by Stage of Disease

<table>
<thead>
<tr>
<th>Stage of Disease</th>
<th>Total, N = 181</th>
<th>No DM, n = 46</th>
<th>No DR, n = 41</th>
<th>NPDR, n = 59</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>68</td>
<td>37.6%</td>
<td>28.3%</td>
<td>39.0%</td>
<td>45.7%</td>
</tr>
<tr>
<td>Female</td>
<td>113</td>
<td>62.4%</td>
<td>71.7%</td>
<td>61.0%</td>
<td>54.3%</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>74</td>
<td>40.9%</td>
<td>10.9%</td>
<td>63.4%</td>
<td>47.5%</td>
</tr>
<tr>
<td>White</td>
<td>61</td>
<td>33.7%</td>
<td>76.1%</td>
<td>22.0%</td>
<td>16.9%</td>
</tr>
<tr>
<td>Hispanic</td>
<td>46</td>
<td>25.4%</td>
<td>13.0%</td>
<td>14.6%</td>
<td>35.6%</td>
</tr>
<tr>
<td>Age, mean (SD), y</td>
<td>57.3 (11.8)</td>
<td>59.1 (12.2)</td>
<td>56.2 (12.5)</td>
<td>59.5 (10.9)</td>
<td>52.5 (10.7)</td>
</tr>
<tr>
<td>&lt;30</td>
<td>5</td>
<td>1.7%</td>
<td>0.0%</td>
<td>2.4%</td>
<td>3.5%</td>
</tr>
<tr>
<td>30-39</td>
<td>13</td>
<td>7.2%</td>
<td>6.5%</td>
<td>9.8%</td>
<td>3.4%</td>
</tr>
<tr>
<td>40-49</td>
<td>26</td>
<td>14.3%</td>
<td>17.4%</td>
<td>12.2%</td>
<td>8.5%</td>
</tr>
<tr>
<td>50-59</td>
<td>55</td>
<td>30.4%</td>
<td>23.9%</td>
<td>31.7%</td>
<td>33.9%</td>
</tr>
<tr>
<td>60-69</td>
<td>57</td>
<td>31.5%</td>
<td>32.6%</td>
<td>29.3%</td>
<td>33.9%</td>
</tr>
<tr>
<td>70+</td>
<td>27</td>
<td>14.9%</td>
<td>19.6%</td>
<td>14.6%</td>
<td>18.6%</td>
</tr>
<tr>
<td>Eye</td>
<td>166</td>
<td>91.7%</td>
<td>100.0%</td>
<td>92.7%</td>
<td>89.8%</td>
</tr>
<tr>
<td>Pigmentation index, mean (SD)</td>
<td>3.4 (2.1)</td>
<td>3.6 (1.8)</td>
<td>3.3 (2.3)</td>
<td>3.5 (1.9)</td>
<td>3.6 (2.4)</td>
</tr>
<tr>
<td>&lt;2.5</td>
<td>62</td>
<td>34.3%</td>
<td>28.3%</td>
<td>41.5%</td>
<td>33.9%</td>
</tr>
<tr>
<td>2.5-3.8</td>
<td>61</td>
<td>33.7%</td>
<td>37.0%</td>
<td>34.1%</td>
<td>33.9%</td>
</tr>
<tr>
<td>3.8+</td>
<td>58</td>
<td>32.0%</td>
<td>34.7%</td>
<td>24.4%</td>
<td>32.2%</td>
</tr>
</tbody>
</table>

Significant P values in bold. One eye was included per individual. AA, African American; No DM, individuals without diabetes mellitus; No DR, individuals with diabetes, but with no visible retinopathy; NPDR, individuals with nonproliferative diabetic retinopathy; PDR, individuals with proliferative diabetic retinopathy.

† P value from analysis of variance test of means across stages of disease.

Data Analysis

Outcome Biomarker Variables. Eight continuous biomarker variables (Dn, CRAE, DRVE, SO2A, SO2V, SO2AV, and OEF) were evaluated to assess the relationship between each and the stage of disease (No DM, No DR, NPDR, and PDR).

Statistical Analysis. Repeatability was determined by percentage change, which was calculated as the absolute value of the difference between measurements divided by the average of the two measurements.

The distributions of the biomarker data were evaluated in 189 individuals for data normalcy and to identify outliers. Regression diagnostics including Cook’s distance were performed to assess the linear relationship between the stage of disease and each biomarker to identify data points that were outliers, had leverage, or were influential. Eight outliers were identified, which were removed from further analyses, leaving data from 181 participants.

Descriptive statistics were compared for demographic and clinical variables by using the χ² test and t-tests. Linear regression was used to assess the independent effect of stage of disease on each biomarker. Multivariable linear regression models were constructed by using a priori–selected covariates from univariate models to compute the parameter estimates (β) and 95% confidence intervals. The covariates chosen were sex, race, eye examined (categorical variables), and age and pigmentation index (continuous variables). Effect modification between race and stage of disease was assessed by including the pairwise interaction term and the two corresponding main effects into the final model. The interaction was significant for one biomarker; as such, this model was stratified by race with race-specific results presented. All analyses were performed in Stata (version 12; StataCorp LP, College Station, TX, USA). Statistical significance was set to P ≤ 0.05, and all statistical tests were two-sided.

Results

Demographic Characteristics

The demographic characteristics of participants are presented in Table 1. No differences in the distribution of sex among the stages of disease were found. The distribution of races among the stages of disease differed significantly with a disproportionately high percentage of whites in the No DM stage and a disproportionately low number of Hispanics in the No DR stage. The mean age was lower in the subjects with PDR, but the distributions of ages were similar among the stages of disease. There were relatively fewer right eyes in the subjects...
with PDR than in the other stages of disease. The pigmentation indices were evenly distributed among the stages of disease.

**Repeatability**

Repeatability was calculated as the mean percentage change averaged over data in 10 subjects without DM. The repeatability of DA, Dv, SO2A, and SO2V measurements were 5%, 5%, 7%, and 13%, respectively. These results are comparable to those reported by O’Connell et al.50

**Vascular Biomarkers**

**Vascular Diameter.** Mean and SD values of the unadjusted retinal vascular diameters according to stage of disease are presented in Table 2. Significant differences were present among stages of disease on DA and CRAE. The highest values were seen in No DR and NPDR, and the lowest were seen in PDR. Significant differences were also observed on Dv and CRVE. The highest values were present in NPDR and the lowest in No DM.

Estimates of retinal vascular biomarker differences between No DM and the stages of DR from the statistical model adjusting for age, race, sex, eye examined, and pigmentation index are displayed in Table 3. The P value for DA in PDR became significant and that of CRAE became more significant when compared to the value in No DM in the adjusted model as compared to the unadjusted model. After adjusting for the covariates, CRVE in PDR was revealed as being lower than in No DM. The probabilities of the pairwise comparisons of the vascular diameter biomarkers among the stages of DR in the statistical model are presented in Table 4.

**Vascular Hemoglobin Oxygen Saturation.** Mean and SD values of the unadjusted retinal hemoglobin oxygen saturations according to stage of disease are presented in Table 2. Significant differences were present among stages of disease on SO2A and SO2V. The highest values were found in NPDR and PDR and the lowest in No DR. SO2AV and OEF did not differ among stages of disease.

Table 2 indicates the results of estimated vascular hemoglobin oxygen saturation differences between No DM and the stages of DR in the model adjusting for age, race, sex, eye examined, and pigmentation index. The main effect of stage of disease on SO2A was that it was higher in PDR than in No DM. However, the interaction between race and stage of disease was significant. Thus, the evidence indicates that the effect of the stage of disease on SO2A differs by race, so that interpretation of the main effects is difficult. Accordingly, we report these interactions separately.

Table 3. Statistical Model With Adjusted Estimates of Mean Differences of Retinal Vascular Diameter Biomarkers by Stage of Disease*

<table>
<thead>
<tr>
<th>Stage of Disease</th>
<th>DA, µm</th>
<th>CRAE, µm</th>
<th>DA, µm</th>
<th>CRVE, µm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>β</td>
<td>P</td>
<td>β</td>
<td>P</td>
</tr>
<tr>
<td>Intercept</td>
<td>86</td>
<td>&lt;0.01</td>
<td>176</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Stage of disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No DM, n = 46</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>No DR, n = 41</td>
<td>1</td>
<td>0.52</td>
<td>1</td>
<td>0.72</td>
</tr>
<tr>
<td>NPDR, n = 59</td>
<td>3</td>
<td>0.16</td>
<td>-1</td>
<td>0.85</td>
</tr>
<tr>
<td>PDR, n = 35</td>
<td>-5</td>
<td>0.01</td>
<td>-18</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Interactions†</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Race*stage</td>
<td>0.06</td>
<td>0.17</td>
<td>0.57</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Significant P values in bold. Ref, reference; β, parameter estimate.
* Estimates of differences in retinal diameter with No DM as the reference group, obtained with multivariable linear regression analysis, adjusted for race, sex, eye examined (categorical variables), age and pigmentation index (continuous variables).
† Interaction P values determined by likelihood ratio test for addition of interaction term into the model.
simple main effects were calculated in each racial group and are shown in Table 6. The only significant differences on SO2A by race were elevations in the NPDR and PDR stages of disease as compared to No DM among Hispanics. Table 5 also shows that, as in the unadjusted analysis, SO2V was greater in both NPDR and PDR than in No DM. The pairwise comparisons of the vascular hemoglobin oxygen saturation biomarkers among the stages of DR in the statistical model are presented in Table 4. The pairwise comparisons on SO2A are difficult to interpret because of the significant interaction between race and stage of disease, but OEF was revealed to be lower in NPDR than in No DR.

**DISCUSSION**

This study demonstrated that retinal vascular diameter and oxygen saturation alterations according to stage of diabetic retinopathy are discernible with the dual wavelength SLO. The main results were that in NPDR retinal venous oxygen saturation was increased, while in PDR retinal arterial diameter was decreased and venous oxygen saturation was increased. Most of the statistically significant differences in the unadjusted analysis were also significant with the model that accounted for the effects of covariates. However, some significant differences not present in the unadjusted comparisons were revealed when the model was applied. Importantly, the model revealed significant interactions by race and stage of disease on how SO2A varies, such that it was higher in NPDR and PDR among Hispanic subjects. These results suggest the importance of identifying and adjusting for covariates to fully account for the natural population variance in these biomarkers and thereby improve their sensitivity to report development and progression associated with DR.

**Vascular Diameter**

Considerable information has been published on retinal vessel diameters in DM.12–27 There have been some discrepancies that may be related to variations in study design and participant populations. The most consistent findings of retinal vascular diameter measurements in DM to date are near-normal values in No DR, increasing values of venous biomarkers in NPDR, and reduced values in treated PDR. Overall, our results are consistent with those previously reported.

**Vascular Hemoglobin Oxygen Saturation**

Several groups have reported values of SO4 in DM. We found SO2A to be elevated in NPDR and PDR as compared to No DM and No DR in Hispanics, but not in whites or African Americans. Several investigators31,32,34 have found SO2A to be greater in PDR than in No DM, while one study33 has reported lower values in PDR than in the other stages of disease. Increases in SO2A as compared to No DM have been reported in the combined stages of DR,30 in NPDR,34 and in the more severe stages of NPDR.32 On the other hand, one group31 has found no difference in SO2A between No DM and the stages of NPDR that were studied. As in the present study, two reports with a No DR stage have found SO2A in this stage not to differ from No DM31,32 but one has found SO2A to be elevated in No DR as compared to No DM.34 Two studies28,29 have reported no difference in SO2A between No DM and the stages of DR that were studied. In our adjusted model, SO2A was found to depend on race. Since the statistical model adjusted for pigmentation, some other as yet unknown factor or factors were responsible for this racial difference. Racial effects on oximetry results have not been discussed previously.

We found SO2V to be increased in NPDR and PDR as compared to No DM and No DR. These findings are consistent with those previously reported.

### Table 4. Probability Values of Pairwise Comparisons in Statistical Model With Adjusted Mean Values of Retinal Vascular Diameter and Oxygen Biomarkers by Stage of Disease

<table>
<thead>
<tr>
<th>Stage comparison</th>
<th>DAV, P</th>
<th>CRAE, P</th>
<th>DAV, P</th>
<th>CRVE, P</th>
<th>SO2A, P</th>
<th>SO2V, P</th>
<th>SO2AV, P</th>
<th>OEF, P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No DR vs. NPDR</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>0.49</td>
<td>0.02</td>
<td>0.15</td>
<td>0.03</td>
</tr>
<tr>
<td>No DR vs. PDR</td>
<td>&lt;0.01</td>
<td>0.27</td>
<td>0.38</td>
<td>0.02</td>
<td>0.04</td>
<td>0.08</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>NPDR vs. PDR</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>0.05</td>
<td>0.94</td>
<td>0.10</td>
<td>0.25</td>
<td></td>
</tr>
</tbody>
</table>

Significant P values in bold.

* Estimates of stage of disease comparisons obtained with multivariable linear regression outcomes, adjusted for race, sex, eye examined (categorical variables), age, and pigmentation index (continuous variables).

### Table 5. Statistical Model With Adjusted Estimates of Mean Differences of Retinal Vascular Oxygen Biomarkers by Stage of Disease

<table>
<thead>
<tr>
<th>Stage of disease</th>
<th>SO2A, %</th>
<th>SO2V, %</th>
<th>SO2AV, %</th>
<th>OEF, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>94</td>
<td>&lt;0.01</td>
<td>59</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Stage of disease</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No DM, n = 46</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>No DR, n = 41</td>
<td>2</td>
<td>0.64</td>
<td>0</td>
<td>0.99</td>
</tr>
<tr>
<td>NPDR, n = 59</td>
<td>4</td>
<td>0.26</td>
<td>7</td>
<td>0.03</td>
</tr>
<tr>
<td>PDR, n = 35</td>
<td>10</td>
<td>0.01</td>
<td>8</td>
<td>0.05</td>
</tr>
<tr>
<td>Interactions†</td>
<td>0.02</td>
<td>0.28</td>
<td>0.13</td>
<td>0.43</td>
</tr>
</tbody>
</table>

Significant P values in bold.

* Estimates of differences in retinal oxygen biomarkers with No DM as the reference group, obtained with multivariable linear regression analysis, adjusted for race, sex, eye examined (categorical variables), age, and pigmentation index (continuous variables).

† Interaction P values determined by likelihood ratio test for addition of interaction term into the model.
with all other studies that have found elevated values of SO\textsubscript{2AV} in PDR\textsuperscript{29,31–34} some stages of NPDR\textsuperscript{29,31,32,34} or the combined stages of DR as compared to No DM\textsuperscript{30,35} The three reports with a No DR stage have found no difference in SO\textsubscript{2AV} from No DM\textsuperscript{31,32,34} One group\textsuperscript{28} investigated early NPDR and found an increase in SO\textsubscript{2AV} with age.

We found no difference on SO\textsubscript{2AV} in any of the stages of disease that we studied, similar to other published studies\textsuperscript{30–32} However, some studies\textsuperscript{29,31,33,35} have reported a decrease in SO\textsubscript{2AV} in various stages of DR. In addition, one group\textsuperscript{28} has found a decrease in SO\textsubscript{2AV} with age. We found a reduction in OEF in NPDR as compared to No DR, as also was found in another report\textsuperscript{34}.

The differences between our oximetry results and those previously published, as well as discrepancies among those previously published, may be related to variations in study populations as well as to instrumentation. Another factor highlighted in the current report is that previous research has not adjusted for race or other important covariates. The oximetry biomarkers that we investigated may prove useful in monitoring DR and its treatment, since we have shown differences in some of them by stage of disease. To date, no longitudinal studies are available on SO\textsubscript{2} in DM. Longitudinal studies may reveal more consistent and informative prognostic information about individuals than are possible with cross-sectional studies such as the current one. Overall, the most significant and consistent findings of oximetry in DM so far are no abnormalities in eyes with No DR and abnormally high values of SO\textsubscript{2} in the more advanced stages of NPDR and in PDR.

### Retinal Oxygenation and the Biomarkers

It would be of particular interest if the biomarkers we studied could be used to indicate the oxygenation of the retinal tissue in DR in which hypoxia is often present. The first factor that determines retinal oxygenation is the retinal blood flow. Vessel diameter is a major determinant of blood flow. In fact, according to Poiseuille’s equation, flow is proportional to the fourth power of the diameter\textsuperscript{11} The presence of increased diameters in some stages of disease in the current and in other studies suggests that blood flow was increased. However, no conclusion can be made since we did not measure the blood velocity.

The second factor that determines the retinal oxygenation is the arteriovenous difference in oxygen content of the blood, which is proportional to SO\textsubscript{2AV}. We, and others, have found increases in both SO\textsubscript{2A} and SO\textsubscript{2V} in both NPDR and PDR. SO\textsubscript{2A} is dominated by factors acting before the blood arrives at the arterial measurement site near the optic nerve, whereas SO\textsubscript{2V} is the result of removal of oxygen by the retinal tissue as blood passes from the arterial to the venous measurement site. Hence, SO\textsubscript{2AV} is related to the rate that oxygen is withdrawn by the tissue. However, even though we found no difference from normal in DR on SO\textsubscript{2AV} we cannot conclude that the rate oxygen was withdrawn from the blood was unaltered in DM, since we did not measure the blood flow. Because the reported measurements of retinal blood flow in diabetic patients have not been consistent\textsuperscript{51,52} we cannot be certain of blood flow alterations in the current study. OEF has the advantage of being independent of blood flow.\textsuperscript{53–57} OEF also equals the ratio of inner retinal oxygen metabolism to retinal vascular oxygen delivery.\textsuperscript{53–57} We did find a reduction of OEF in NPDR as compared with No DR. This may have been due to a relatively high oxygen delivery, since DA and SO\textsubscript{2A} each had the highest value in NPDR, though neither was significantly different from their values in No DM. A decrease in inner retinal oxygen metabolism may also have been present.

There were several limitations of the current study. First, the wavelengths of the SLO were not optimized for oximetry. However, despite this we found statistically significant differences among the stages of disease. Second, we used only one image for analysis, but in the future more images can be acquired to reduce measurement variability. In fact, the standard deviations in our study usually were larger than those in previous reports (though similar to those using standard deviations in our study usually were larger than those in previous reports (though similar to those using SLO\textsuperscript{56}), which may have reduced the power to discern differences in biomarker variables among the stages of disease. Third, a fixed calibration factor was used to calculate vessel diameters and, thus, did not account for variations in refractive error among subjects. However, subjects with high refractive error (>6 diopters) were excluded from the study. Fourth, we did not account for measurement variations as a function of the cardiac cycle. While Knudtson et al.\textsuperscript{53} have found that image quality is more important than the cardiac cycle as a source of measurement variability, Chen et al.\textsuperscript{54} have reported changes in vessel diameter during the heart cycle as 3.46% and 4.82% in arteries and veins, respectively. This suggests that the heart cycle may have been a major source of variability in our data over and above the error of measurement. In the future, taking this into account may reduce the variability substantially. Fifth, aging has been shown to affect retinal SO\textsubscript{2} measurements in healthy and diabetic subjects.\textsuperscript{28,55} Although control and diabetic subjects were age matched, adjustments for age were taken into account in the models. While changes in the optical properties of the eye due to disease were minimized by the calculation of optical density ratios, future studies are needed for rigorous determination of the effects of alterations in lenticular light transmission on SO\textsubscript{2} measurements. Sixth, our method of calibrating values of SO\textsubscript{2} from values of ODR sometimes led to values exceeding 100%. However, we used the same method other investigators have used and they also obtained SO\textsubscript{2} values above 100%. Finally, the distribution of
participants by race and diabetic group was not even (e.g., an underrepresentation of African Americans and Hispanics among No DR and also an underrepresentation of whites among NPDR and PDR). Nevertheless, the observational study design and multivariate linear regression models allow for estimates that appropriately adjust for race.

In summary, we demonstrated that alterations in retinal vascular diameters and hemoglobin oxygen saturations according to the stages of diabetic retinopathy can be detected with a widely available SLO and that statistical modeling can reveal the influences of covariates such as race on the results.

Acknowledgments

The authors thank Andrew Cross and Ruth Zelkha for subject recruitment.

Supported by Research Grants DK104393 and EY001792 from National Institutes of Health, Bethesda, Maryland, United States; senior scientific investigator award (MS) and a departmental award to Research to Prevent Blindness, New York, New York, United States.

Disclosure: N.P. Blair, None; J. Wanek, None; A.E. Felder, None; C.E. Joslin, None; J.K. Kresovich, None; J.I. Lim, None; F.Y. Chau, None; Y. Leiderman, None; M. Shahidi, None

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