Oxygen Saturation of Retinal Vessels in All Stages of Diabetic Retinopathy and Correlation to Ultra-Wide Field Fluorescein Angiography

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Purpose. The purpose of this study was to determine retinal hemoglobin oxygen saturation (SO_2) in patients with diabetic retinopathy (DR) using retinal oximetry (RO) and to correlate the degree of retinal ischemia using intravenous fluorescein angiography (IVFA).

Methods. This is a single-center cross-sectional cohort study. Twenty-seven controls and 60 adult patients with diabetes mellitus (16 without DR and 44 with DR) were enrolled. Patients were stratified according to DR severity. Using RO, SO_2 was measured in major retinal arterioles (SaO_2) and venules (SvO_2). Using IVFA, the percentage of retinal ischemia in 31 patients with DR was calculated and correlated with RO.

Results. Pairwise one-way analysis of variance (ANOVA) showed a significant increase in SaO_2 and SvO_2 in patients with proliferative DR (PDR) compared with controls (SaO_2: PDR, 100 ± 7% vs. controls, 91 ± 4% [$P = 0.003$]; SvO_2: PDR, 66 ± 11% vs. controls, 53 ± 6% [$P < 0.00001$]). The percentage of retinal ischemia also increased with DR severity: ANOVA showed a significant difference in retinal ischemia between all categories of nonproliferative DR vs. PDR: 2.31 ± 2% vs. 7.92 ± 9% ($P = 0.017$), respectively. Pearson two-tailed correlation showed significant correlation between SaO_2 and ischemia ($R = 0.467$, $P = 0.011$).

Conclusions. Hemoglobin oxygen saturation of retinal arterioles and venules increases with DR severity; SaO_2 correlates with increasing ischemia measured by IVFA. Retinal oximetry may complement current imaging strategies to noninvasively augment the diagnosis and risk stratification of patients with diabetes.

Keywords: retinal oximetry, diabetic retinopathy, fluorescein angiography

Ischemia plays a central role in the pathophysiology of diabetic retinopathy (DR). As ischemia increases, untreated DR generally progresses through four stages in an orderly fashion: mild nonproliferative DR (NPDR), moderate NPDR, severe NPDR, and proliferative DR (PDR). Intravenous fluorescein angiography (IVFA) is the gold standard to assess retinal ischemia and perfusion and is also helpful when determining treatment for DR. However, IVFA is relatively invasive as it requires the intravenous injection of contrast dye. Furthermore, IVFA provides only the anatomic state of retinal vessels and does not give any metabolic information, such as oxygenation.

Recently, quantitative measurement of oxygen saturation (SO_2) has been possible using retinal oximetry (RO), a noninvasive imaging modality used to estimate hemoglobin oxygen saturation in retinal arterioles and venules. The technical aspects of RO have been previously described in the literature.[1–3] Briefly, RO is based on similar principles as standard pulse oximetry, utilizing the light absorbance of oxyhemoglobin and deoxyhemoglobin at the carefully selected wavelengths of 570 and 600 nm. Studies have shown that the retinal oximeter produces repeatable and reliable measurements when detecting differences in SO_2 levels in controls, as well as in patients with retinal pathology.[3–7] Although normative values for RO have been developed,[8–12] less is known about the measurement of RO in all stages of DR. Furthermore, prior to our work, the correlation of oxygen saturation measured by RO (metabolic state) with the percentage of ischemia assessed by IVFA (anatomic state) was unknown.

Hence, in this study, we report the retinal vessel oxygenation and perfusion status of patients with diabetes mellitus (DM) with all stages of DR with two imaging modalities: RO and IVFA. We also examined differences in arteriolar and venular hemoglobin oxygenation saturation between controls and patients with DM.

Methods

Patients

The study was a single-center cross-sectional cohort. Twenty-seven controls were enrolled from the University of North Carolina (UNC) comprehensive ophthalmology clinic, and 60 patients with DM were enrolled from the UNC Retina Clinic

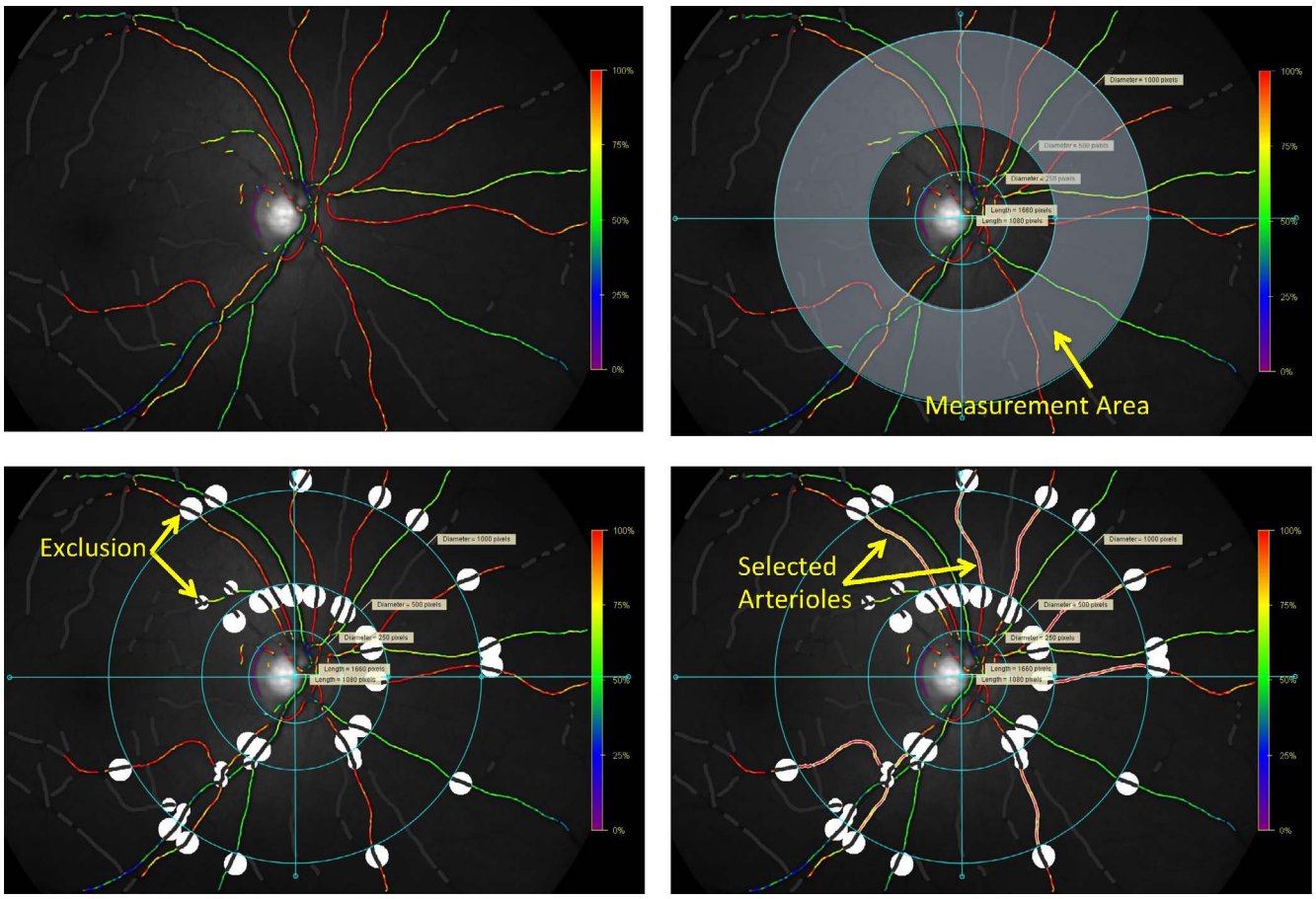

FIGURE 1. Ring method analysis of retinal oximetry images. Image when opened with Oxymap software (*top left*). Quadrant delineation and circle outlines in place (*top right*). Exclusionary areas mapped (*bottom left*). Overall selection of arterioles (*bottom right*).

with the following clinical diagnoses: DM without DR ($n = 16$), mild NPDR ($n = 6$), moderate NPDR ($n = 14$), severe NPDR ($n = 7$), and PDR ($n = 17$). The patients were classified according to the international clinical DR severity scale.[13] Inclusion criteria were at least 18 years of age with type I or type II DM. Exclusion criteria were patients with a history of retinal vascular occlusion, glaucoma, and AMD (i.e., ischemic ocular conditions that could potentially confound the results) and patients with a media opacity such as dense cataracts or severe vitreous hemorrhage (i.e., conditions that could obscure RO images or retinal photographs). Patients with systemic conditions that could confound RO results were also excluded, such as severe respiratory disease (e.g., chronic obstructive pulmonary disease), severe anemia, or sickle cell disease. The protocol was approved by the UNC Institutional Review Board. All patients enrolled provided written informed consent, the study was conducted in accordance to the tenets of the Declaration of Helsinki, and all work was HIPAA compliant.

Retinal Oximetry

The retinal oximeter consists of a fundus camera with an attached image splitter, as well as a digital camera (Oxymap T1 device connected to Topcon TRC50-DX fundus camera; Oxymap ehf., Reykjavik, Iceland). The device captures images at two wavelengths, one sensitive to oxyhemoglobin (600 nm) and one isosbestic (570 nm), where the absorption spectra of oxyhemoglobin and hemoglobin cross. Computer software detects retinal vessels and uses relative light intensities inside and outside a vessel to calculate the optical density (light absorbance) of a vessel at both wavelengths. The hemoglobin oxygen saturation (SO_2) of a vessel can therefore be calculated, because the optical density ratio at these wavelengths has been shown to have an approximately inverse linear relationship with SO_2.[1]

Prior to image capture, patients' eyes were dilated with one drop each of 1% tropicamide and 2.5% phenylephrine. Using the oximeter, trained retinal photographers obtained images of both retinas, centered on the optic disc. The images were 1200 $\times$ 1600 pixels and covered a 50° field of the central retina.

For each patient, one eye was randomly selected for analysis. Images were analyzed in a masked fashion by two independent observers with the Oxymap Analyzer software (version 2.3.2; Oxymap ehf.), which automatically detects vessels greater than eight pixels in diameter, using the ring-method protocol previously described by this research group (Fig. 1).[8] The analysis area was selected by centering quadrant lines on the optic disc. An initial central circle was used to delineate the optic disc. Two additional measurement circles, an inner and outer circle, two and four times the diameter of the central circle, respectively, were then demarcated. To exclude the peripheral retina beyond the outer circle and the optic nerve, only the area between the circles centered on the optic disc were analyzed. The width of this ring was two disc diameters. Vessels beyond the area of analysis, certain areas where vessel detection would prove inaccurate (branching, overlapping, or intersecting), and segments of vessels less than 19 pixels in length were excluded. Vessels to be measured were selected manually. Measurements were first taken to yield arteriolar SO_2 (SaO_2) by quadrant. This was

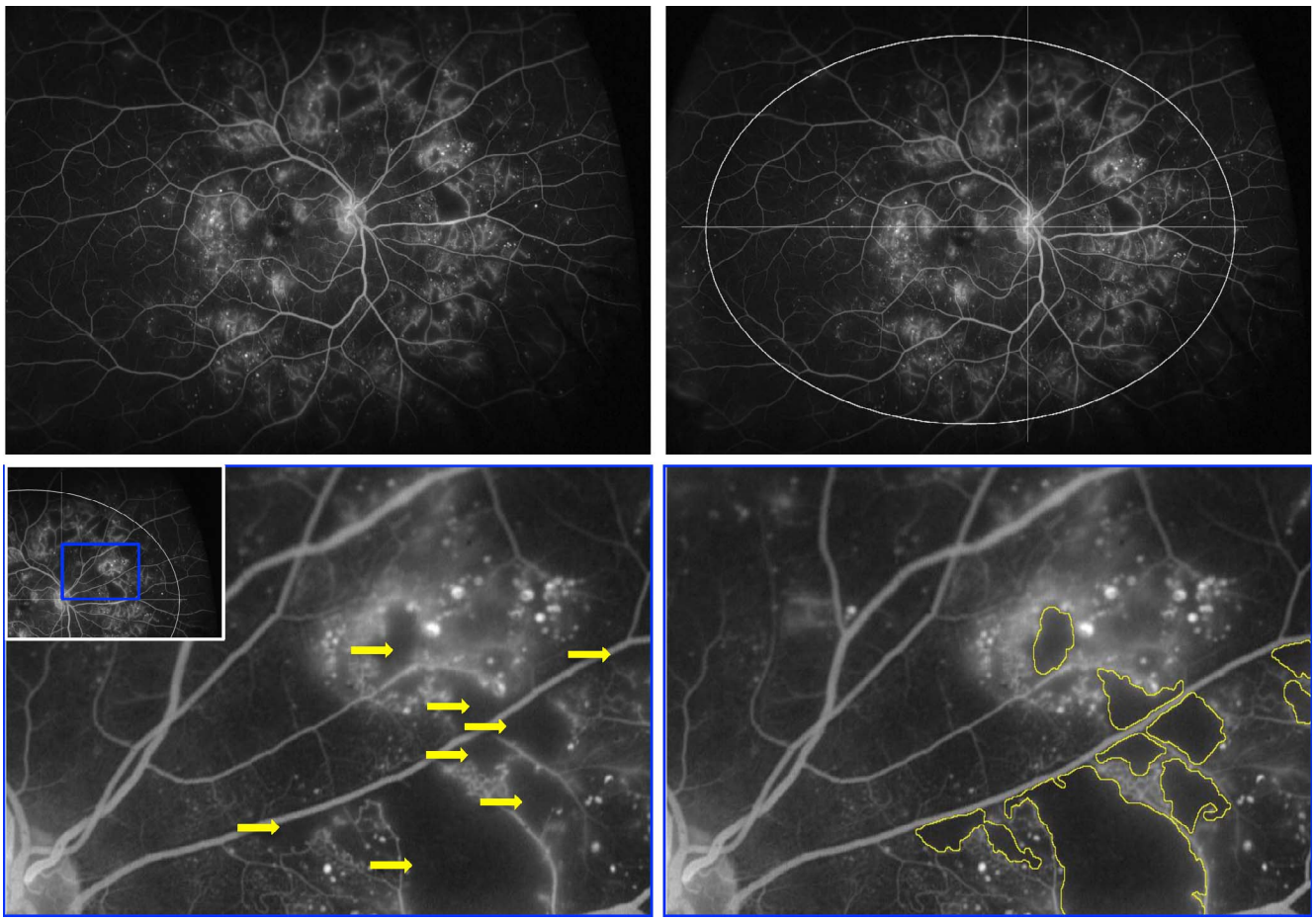

FIGURE 2. Fluorescein angiogram image opened in ImageJ software (*top left*). Quadrants and ellipse in place (*top right*). Ischemic areas indicated with *yellow arrows* (*bottom left*). Selections of ischemic areas outlined in yellow for pixel measurement (*bottom right*).

repeated for venules to yield venular SO_2 (SvO_2) by quadrant. Overall saturation was computed by averaging saturation values of each quadrant. Because Oxymap software measurements are calibrated to nondiabetic young individuals, results are relative to that calibration, occasionally resulting in SO_2 measurements greater than 100%. These values were not truncated to 100%, per established oximetry protocol.[5,8,14]

Statistical analyses were implemented with Statistical Package for the Social Sciences (SPSS) software (version 20.0; SPSS, Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) with Tukey's post hoc test was used to compare means, with DR stage as the independent variable and SO_2 as the dependent variable.

Fluorescein Angiography

Ultra-wide field intravenous fluorescein angiography (UWF-IVFA) is a relatively new diagnostic imaging modality used to assess retinal perfusion simultaneously in 200° of the retina. The photographic equipment consists of a scanning laser ophthalmoscope that captures rapid sequential images, as well as separate laser for angiography (Optos 200 Tx; Optos plc., Queensferry House, Scotland, United Kingdom). First, approximately 5 mL sodium fluorescein dye that becomes luminescent when excited by certain wavelengths of light (between 465 and 490 nm) is injected intravenously, typically in the antecubital vein. Fundus images are captured in rapid sequence to allow the visualization of blood flow through the retina and choroid.[15] In this study, patients with DR were imaged with UWF- IVFA after RO imaging. The image with optimal perfusion of dye (approximately 40–50 seconds after injection) was used for analysis. Because IVFA is relatively invasive, controls and patients with DM without DR did not undergo this imaging study.

After accounting for DR patients who were not able to obtain an IVFA, and patients with poor image quality, 31 patient UWF-IVFA images were analyzed according to the following protocol. For each patient, the same eye that was randomly selected for RO analysis was used for IVFA analysis, using ImageJ Software (Version 1.48v; US National Institutes of Health, Bethesda, MD, USA) (Fig. 2). The IVFA images were analyzed by two independent observers who were masked to patient identity, DR stage, and RO results. Quadrant lines were transferred from the corresponding RO image analysis to the IVFA image. An ellipse was delineated to capture the retinal area (approximately a 4.2-megapixel area) in the image with the optimal dye perfusion. Nonperfused areas on the image were manually outlined and measured. The percentage of retinal ischemia was then calculated by dividing the area of ischemia by the total delineated elliptical area and multiplying by 100.

Statistical analyses were performed on the 31 images using SPSS (version 20.0; SPSS, Inc.). One-way ANOVA with Tukey's post hoc test was used to compare means, with DR stage as the independent variable and percentage of ischemia as the dependent variable.

Table 1. Demographic Data for Study Patients

Demographic Data	Control ($n = 27$)	DM w/o DR ($n = 16$)	Mild NPDR ($n = 6$)	Moderate NPDR ($n = 14$)	Severe NPDR ($n = 7$)	PDR ($n = 17$)
Age, y*	59 ± 9	56 ± 10	58 ± 19	53 ± 10	53 ± 11	52 ± 12
Sex, no. of male (%)†	9 (33)	6 (38)	5 (83)	9 (94)	0 (0)	6 (35)
Race, no. of patients (%)*						
Caucasian	11 (41)	9 (56)	1 (17)	6 (43)	2 (29)	6 (35)
African American	9 (33)	6 (38)	4 (67)	8 (57)	3 (43)	8 (47)
Hispanic	5 (18)	1 (6)	–	–	–	1 (6)
Other	3 (7)	–	1 (17)	–	2 (29)	2 (12)
HTN, no. of patients (%)‡	8 (30)	15 (94)	4 (67)	13 (93)	5 (83)	11 (78)
Smoking history, no. of patients (%)*	8 (30)	6 (38)	2 (33)	6 (43)	0 (0)	1 (4)
Hemoglobin A1c (%)*	–	7.7 ± 2.4	7.6 ± 1.2	8.5 ± 2.2	8.1 ± 1.3	8.3 ± 1.6

* No significant difference among groups.
† χ^2: $P = 0.019$.
‡ χ^2: $P < 0.00001$.

Results

Patient demographics are summarized in Table 1. A difference was considered to be significant if the P value was less than 0.05. Overall, ANOVA and χ^2 tests revealed no significant difference among the groups regarding age, race, smoking history, and hemoglobin (Hb)A1c. There was a higher percentage of patients with hypertension in the diabetes group than the control group, and the difference was significant among the groups ($P < 0.00001$). Statistically, there was a significant difference among the groups in terms of sex ($P = 0.019$); however, this is only because there were no males in the severe NPDR group.

Mean SaO_2, SvO2, and arteriovenous differences are detailed in Table 2. Distribution of the individual patient values can be visualized in the box and whisker plot of Figure 3. A significant increase in SaO_2 was found in patients with PDR compared with controls ($P = 0.003$) and patients with DM without DR ($P = 0.001$). A significant increase in SvO_2 was found in patients with moderate NPDR compared with controls ($P = 0.038$), as well as in PDR patients compared with controls ($P < 0.00001$) and DM without DR ($P = 0.004$). No significant difference was found in SaO_2 or SvO_2 measurements between any other subgroups. A significant increase in the arteriovenous difference was also found in moderate NPDR patients compared with controls ($P = 0.027$). However, there was not a noticeable trend in arteriovenous difference with increasing DR severity.

Percentage of retinal ischemia by DR stage were as follows: mild NPDR, 0.76 ± 0.6%; moderate NPDR, 2.21 ± 2%; severe NPDR, 3.50 ± 2%; PDR, 7.92 ± 9% (Fig. 4). Significance was found in the percentage of ischemia between all NPDR patients (2.31 ± 2%) and PDR patients (7.92 ± 9%; $P = 0.017$).

Pearson two-tailed correlation analysis demonstrated significance in the positive correlation between SaO_2 and percentage of ischemia ($R = 0.46$, $P = 0.011$; Fig. 5). However, there was only a small positive correlation between SvO_2 and percentage of ischemia ($R = 0.254$, $P = 0.18$; Fig. 5).

Discussion

In this single-center study of 87 participants, we observed the following. First, RO measurements showed a trend of increasing arteriolar and venular oxygen saturation with increasing severity of DR. Retinal arteriolar oxygen saturation (SaO_2) was significantly higher in patients with PDR than in patients with DM without DR and in controls. Retinal venular oxygen saturation (SvO_2) was significantly higher in patients with moderate NPDR and PDR than in patients with DM without DR and in controls. Second, the retinal vessel oxygenation and perfusion status of patients with DM with all stages of DR were evaluated with the two imaging modalities RO and IVFA. We found a trend of increasing retinal tissue ischemia with increasing severity of DR. We demonstrated a significant increase in ischemia in patients with PDR compared with patients with NPDR. Moreover, SaO_2 and SvO_2 correlated with percentage of ischemia measured by IVFA, with SaO_2 showing a significant correlation.

With respect to RO findings in various stages of DR, this study adds to the existing literature. Two studies have demonstrated significantly increased SvO_2 and SaO_2 in patients with PDR compared with controls, but did not find trends of progressively increasing SO_2 with severity of disease.[16,17] Two other studies demonstrated that only SvO_2 (and not SaO_2) increased with DR severity.[18,19] Khoobehi et al.[14] reported increasing SvO_2 and SaO_2 with increasing severity of DR and only found significance in the comparison of controls with severe NPDR and PDR groups. We observed a similar trend with increasing disease severity but only determined significance in the comparison of controls and DM without DR with

Table 2. Global Oxygen Saturation Values (%) for Retinal Arterioles, Venules, and the Arteriovenous Difference in Oxygen Saturation for All Patients, Given as Mean ± SD (min to max)

Stage	Arterioles (SaO_2)	Venules (SvO_2)	Arteriovenous (A-V) difference
Normal ($n = 27$)	91 ± 4 (82.5–100.7)	53 ± 6 (40.9–64.9)	38 ± 5 (29.9–49.1)
DM w/o DR ($n = 16$)	89 ± 8 (66.8–102.0)	53 ± 10 (23.4–65.0)	36 ± 6 (24.4–43.6)
Mild NPDR ($n = 6$)	92 ± 11 (71.5–102.4)	53 ± 14 (34.6–69.1)	39 ± 8 (30.6–52.3)
Moderate NPDR ($n = 14$)	93 ± 5 (87.1–99.7)	62 ± 6 (53.1–74.4)	31 ± 4 (25.3–38.1)
Severe NPDR ($n = 7$)	96 ± 14 (77.0–124.5)	63 ± 13 (40.9–76.5)	33 ± 11 (21.3–48.4)
PDR ($n = 17$)	100 ± 7 (85.3–112.5)	66 ± 11 (40.0–83.4)	34 ± 11 (20.2–58.2)

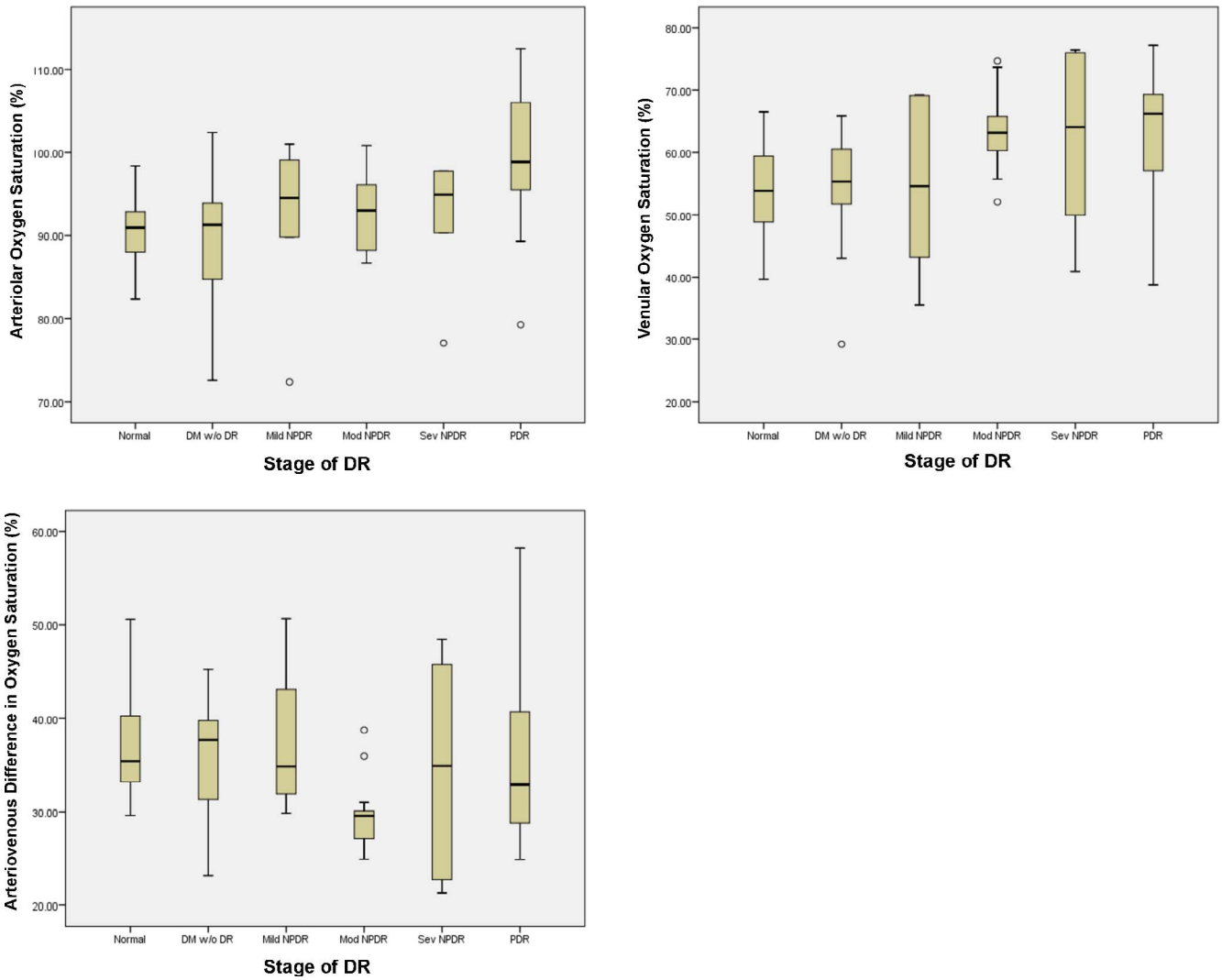

FIGURE 3. Assessment of arteriolar oxygen saturation (%) (*top left*), venular oxygen saturation (%) (*top right*), and arteriovenous difference in oxygen saturation (%) (*bottom left*) for normal, diabetic, and DR patients with increasing severity of disease. *Circles* indicate outliers.

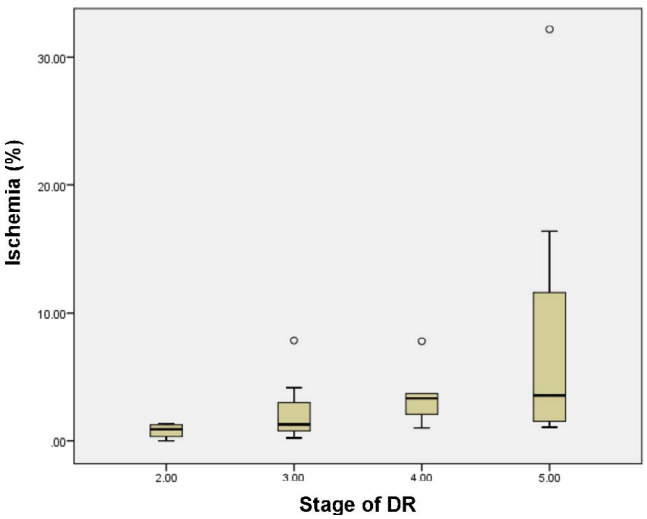

FIGURE 4. Assessment of tissue ischemia (%) for DR patients with increasing severity of disease. *Circles* indicate outliers.

PDR, as well as the comparison of controls with moderate NPDR for venular oxygenation.

Additionally, our study is the first to demonstrate correlation of IVFA with RO in various stages of DR. Our findings are robust, as we had strict exclusion criteria to exclude those with either ocular or systemic hypoxia from any cause to reduce the chances of confounding our RO and IVFA results. Our observers were masked to patient identity and DR stage to avoid selection bias. We included all the stages of DR, including those with DM without DR. Although several studies cited above included homogeneous populations, we recruited a multiethnic cohort, thereby increasing the generalizability of our results. Finally, the novel comparison of RO results was to the latest generation of ultra-wide field IVFA.

There are two major mechanisms that may explain the increase in retinal vessel oxygenation and increase in retinal tissue ischemia in DR: (1) capillary nonperfusion and shunting and (2) thickening of capillary vessel walls. In capillary shunting, some vessels dilate and others constrict, leading to blood flow bypassing parts of the capillary network.[20,21] This leads to capillary nonperfusion, in which blood is quickly transported through dilated capillaries, reducing the amount of oxygen provided to retinal tissues. Second, capillary walls thicken with DR, increasing the distance oxygen must diffuse

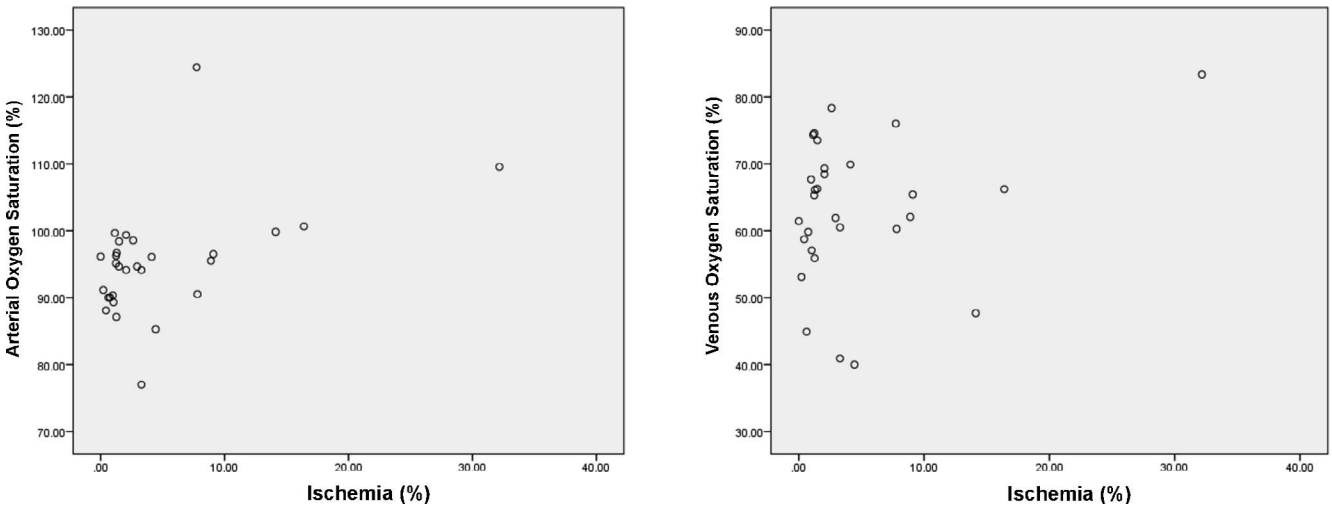

FIGURE 5. Scatterplot depicting the relationship of patients' tissue ischemia (%) with (*left*) arteriolar oxygen saturation (%) ($R = 0.467$, $P = 0.011$) and (*right*) venular oxygen saturation (%) ($R = 0.254$, $P = 0.184$; $n = 31$).

from the bloodstream to reach retinal tissue.[22,23] Blood flow maldistribution and changes in oxygen extraction could explain retinal vessel hyperoxia and retinal tissue hypoxia in later stages of retinopathy. The increase in arteriolar oxygenation may be attributed to an increase in oxygen demand.[14] As retinal tissue becomes ischemic, more blood is directed to the tissue; therefore, the increase in the oxygenation in the arterioles. In patients with DM, the decreased extraction of oxygen results in hyperoxia of the venules as well. In the central region around the optic nerve, where vessels are in close apposition, this pathophysiology must also be considered in the context of counter current exchange, in which oxygen diffuses directly from arterioles to venules. With increased blood flow in this region, as in DR, counter current exchange may become less effective and lead to increased SaO_2.[24]

The findings of the study should be interpreted in the context of the following limitations. The study sample size was small, given the strict exclusion criteria to minimize confounders. This may explain the lack of a significant positive correlation for ischemia and SvO_2; nonetheless, the size and direction of the correlation coefficient were generally similar for SvO_2 and SaO_2. Another limitation is that the that intravascular O_2 saturation approximates tissue O_2 saturation rather than directly measuring the partial pressure of oxygen; however, at this time, there is no other imaging technology that gives tissue metabolic information. Other imaging modalities such as IVFA, optical coherence tomography, and even the most recent optical coherence tomography angiography provide only anatomic information.

The combined use of RO and IVFA to analyze DR severity has clinical implications. Intravenous fluorescein angiography is the current standard for qualitative classification of DR.[25] Quantitative IVFA could provide standardization for existing qualitative IVFA studies and could be used in conjunction with RO to provide not only anatomic staging of DR but also metabolic staging. Moreover, RO may be used as a tool to help assess and risk stratify patients with DR noninvasively. The ability to detect changes in retinal oxygenation, metabolism, and perfusion could be important for early detection of DR, as well as potentially monitoring response to therapy in later stages of DR.

Management of mild DR is typically systemic glycemic and blood pressure control until a patient presents with advanced disease, such as PDR. Quantifying the ischemia in IVFA imaging, we found that ischemia significantly increased in PDR compared with all stages of NPDR. Similarly, we found that SaO_2 and SvO_2 significantly increased from controls to patients with PDR. The study showed the range of RO values that are concerning for a patient with severity of disease that calls for targeted management and has correlated these values with an increase in tissue ischemia. Thus, significant findings for both imaging methods were established for PDR, the severity of DR that requires active treatment.

In conclusion, this study found a significant correlation between stage of DR and RO and was the first to correlate RO with IVFA. Further longitudinal studies are needed to determine the role of RO in risk-stratifying patients for progression of DR and investigate the effect of DR treatments on retinal oxygenation. Although larger studies are needed to confirm our findings, we hope that in the future, RO may ultimately become not only a scientific tool to evaluate the role of oxygen in human retinal pathology but also a clinical tool to diagnose DR and monitor retinal disease progression, as well as response to treatments.

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