

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

Norman P. Blair,¹ Justin Wanek,¹ Anthony E. Felder,¹ Charlotte E. Joslin,¹⁻³ Jacob K. Kresovich,² Jennifer I. Lim,¹ Felix Y. Chau,¹ Yannek Leiderman,¹ and Mahnaz Shahidi⁴

¹Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, Chicago, Illinois, United States

²Division of Epidemiology and Biostatistics, School of Public Health, University of Illinois at Chicago, Chicago, Illinois, United States

³University of Illinois Cancer Center, Population Health, Behavior, and Outcomes Program, Chicago, Illinois, United States

⁴Department of Ophthalmology, University of Southern California, Los Angeles, California, United States

Correspondence: Norman P. Blair, Department of Ophthalmology and Visual Sciences, University of Illinois at Chicago, 1855 West Taylor Street, Chicago, IL 60612, USA; npblair@uic.edu.

Submitted: March 25, 2017

Accepted: September 7, 2017

Citation: Blair NP, Wanek J, Felder AE, et al. Retinal oximetry and vessel diameter measurements with a commercially available scanning laser ophthalmoscope in diabetic retinopathy. *Invest Ophthalmol Vis Sci*. 2017;58:5556-5563. DOI:10.1167/iops.17-21934

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 (D_A and D_V) and central retinal artery and vein equivalents (CRAE and CRVE). Oxygen saturations of hemoglobin in arteries and veins (SO_{2A} and SO_{2V}) were estimated from optical densities of vessels on images at the two wavelengths. Statistical models were generated by adjusting for effects of sex, race, age, eye, and fundus pigmentation.

RESULTS. D_A , CRAE, and CRVE were reduced in PDR compared to No DM ($P \leq 0.03$). D_V and CRVE were similar between No DM and No DR, but they were higher in NPDR than No DR ($P \leq 0.01$). Effect of stage of disease on SO_{2A} differed by race, being increased relative to No DM in NPDR and PDR in Hispanic participants only ($P \leq 0.02$). Relative to No DM, SO_{2V} was increased in NPDR and PDR ($P \leq 0.05$).

CONCLUSIONS. Alterations in retinal vascular diameters and SO_2 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 ophthalmoscopy

Diabetic retinopathy (DR) is a major cause of visual loss.^{1,2} 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,³⁻⁷ which is induced by hypoxia.⁸ Retinal oxygenation is related to the rate of retinal blood flow and the concentrations of oxygen in the arterial and venous blood.^{9,10} The blood flow, in turn, is dependent on the diameters of the retinal arteries (D_A) and veins (D_V).¹¹ Accordingly, measure-

ment of D_A and D_V has attracted considerable attention, and changes related to diabetes have been identified.¹²⁻²⁷

The concentrations of oxygen in the arterial and venous blood are proportional to the oxygen saturations of hemoglobin (SO_2). SO_2 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 SO_2 (SO_{2V}), but changes in arterial SO_2 (SO_{2A}) and the saturation difference between artery and vein (SO_{2AV}) are not consistent.²⁸⁻³⁵ It might be expected that a variety of variables might influence SO_2 . 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,³⁴ 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, Dunfer-



line, Scotland) in combination with modified commercial software from Oxymap.^{36,37} Furthermore, retinal vessel diameters also have been measured with an SLO.³⁸ 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, and 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 photocoagulation. 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 (image₅₃₂ 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 D_A , D_V , SO_{2A} , and SO_{2V} .⁹ 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

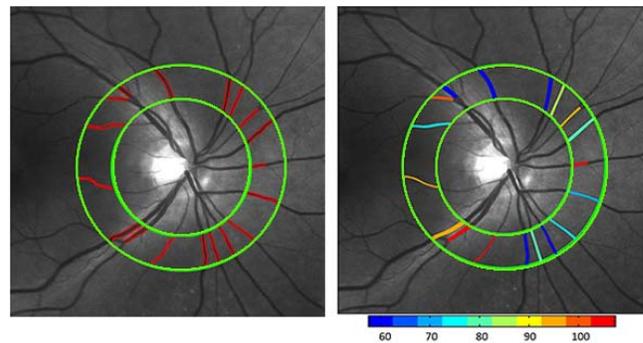


FIGURE. Fundus images in a subject without diabetes acquired at 532 nm with a commercially available dual wavelength scanning laser ophthalmoscope. (*Left panel*) Vessel boundaries for diameter measurements between one and two optic disc radii from the optic disc (indicated by the *green lines*) are shown in *red*. (*Right panel*) Vascular hemoglobin oxygen saturation values (%) between one and two optic disc radii from the optic disc (indicated by the *green lines*) are displayed in mm Hg according to the *color bar*.

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 image₅₃₂ by using a previously published method.^{39,40} 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 $\mu\text{m}/\text{pixel}$), 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_{inside} 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.^{39,40}

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_{ind} values were adjusted for diameter⁹ and converted

TABLE 1. Demographic Characteristics of Subjects by Stage of Disease

	Stage of Disease					<i>P</i> [*]	
	Total, <i>N</i> = 181		No DM, <i>n</i> = 46	No DR, <i>n</i> = 41	NPDR, <i>n</i> = 59		PDR, <i>n</i> = 35
	<i>n</i>	%	%	%	%		
Sex						0.43	
Male	68	37.6	28.3	39.0	39.0	45.7	
Female	113	62.4	71.7	61.0	61.0	54.3	
Race						<0.0001	
AA	74	40.9	10.9	63.4	47.5	42.9	
White	61	33.7	76.1	22.0	16.9	20.0	
Hispanic	46	25.4	13.0	14.6	35.6	37.1	
Age, mean (SD), y	57.3 (11.8)		59.1 (12.2)	56.2 (12.5)	59.5 (10.9)	52.5 (10.7)	0.02†
<30	3	1.7	0.0	2.4	1.7	2.9	0.59
30-39	13	7.2	6.5	9.8	3.4	11.4	
40-49	26	14.3	17.4	12.2	8.5	22.9	
50-59	55	30.4	23.9	31.7	33.9	31.4	
60-69	57	31.5	32.6	29.3	33.9	28.6	
70+	27	14.9	19.6	14.6	18.6	2.9	
Eye							0.04
Right	166	91.7	100.0	92.7	89.8	82.7	
Left	15	8.3	0.0	7.3	10.2	17.1	
Pigmentation index, mean (SD)	3.4 (2.1)		3.6 (1.8)	3.3 (2.3)	3.5 (1.9)	3.6 (2.4)	0.91†
<2.5	62	34.3	28.3	41.5	33.9	34.3	0.85
2.5-3.8	61	33.7	37.0	34.1	33.9	28.6	
3.8+	58	32.0	34.7	24.4	32.2	37.1	

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 χ^2 test of proportions.

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

to SO_2 in individual arteries (SO_{2Aind}) and veins (SO_{2Vind}) by linear regression in 20 No DM subjects, using previously published retinal arterial and venous SO_2 values.⁴⁶ Figure (right panel) illustrates an example of SO_2 values obtained in major retinal arteries and veins in a No DM subject. SO_{2Aind} and SO_{2Vind} measurements were then averaged to derive mean arterial and venous SO_2 values, SO_{2A} and SO_{2V} , for each eye. The difference between SO_{2A} and SO_{2V} (SO_{2AV}) was calculated. Also, the oxygen extraction fraction (OEF) was calculated as SO_{2AV}/SO_{2A} .⁹ OEF is the fraction of the total oxygen entering the retinal arterial system that is removed by the tissue as blood flows from artery to vein.^{9,47} The pigmentation index for each eye was calculated as $\log(I_{outside633}/I_{outside532})$ where $I_{outside633}$ and $I_{outside532}$ equal the average pixel intensities outside the vessels on image₆₃₃ and image₅₃₂, respectively.⁴⁸

Data Analysis

Outcome Biomarker Variables. Eight continuous biomarker variables (D_A , CRAE, D_V , CRVE, SO_{2A} , SO_{2V} , SO_{2AV} , 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 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 χ^2 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.⁴⁹ 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 $P \leq 0.05$, 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

TABLE 2. Unadjusted Mean and SD Values of Retinal Vascular Diameter and SO₂ by Stage of Disease

	Stage of Disease					<i>P</i> *
	Total, <i>N</i> = 181	No DM, <i>n</i> = 46	No DR, <i>n</i> = 41	NPDR, <i>n</i> = 59	PDR, <i>n</i> = 35	
<i>D</i> _A , μm						
Mean	73	70	75	75	69	0.0004
SD	9	7	9	9	10	
CRAE, μm						
Mean	144	142	151	148	132	<0.0001
SD	18	13	17	17	21	
<i>D</i> _V , μm						
Mean	91	86	89	96	91	0.0007
SD	13	11	10	14	13	
CRVE, μm						
Mean	227	216	227	238	221	0.002
SD	30	26	24	32	32	
SO _{2A} , %						
Mean	100	97	97	100	107	0.02
SD	15	9	12	17	19	
SO _{2V} , %						
Mean	66	64	61	69	70	0.03
SD	16	15	16	15	15	
SO _{2AV} , %						
Mean	34	33	36	31	37	0.38
SD	17	12	18	19	21	
OEF						
Mean	0.33	0.34	0.36	0.30	0.33	0.22
SD	0.15	0.14	0.17	0.15	0.16	

Significant *P* values in bold. CRAE, central retinal artery diameter equivalent; CRVE, central retinal vein diameter equivalent; *D*_A, retinal arterial diameter; *D*_V, retinal venous diameter; OEF, retinal vascular oxygen extraction fraction; SO_{2A}, retinal arterial hemoglobin oxygen saturation; SO_{2AV}, retinal arteriovenous hemoglobin oxygen saturation difference; SO_{2V}, retinal venous hemoglobin oxygen saturation.

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

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 *D*_A, *D*_V, SO_{2A}, and SO_{2V} 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 *D*_A 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 *D*_V 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 *D*_A 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 SO_{2A} and SO_{2V}. The highest values were found in NPDR and PDR and the lowest in No DR. SO_{2AV} and OEF did not differ among stages of disease.

Table 5 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 SO_{2A} 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 SO_{2A} differs by race, so that interpretation of the main effects is difficult. Accordingly,

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

	<i>D</i> _A , μm		CRAE, μm		<i>D</i> _V , μm		CRVE, μm	
	<i>β</i>	<i>P</i>	<i>β</i>	<i>P</i>	<i>β</i>	<i>P</i>	<i>β</i>	<i>P</i>
Intercept	86	<0.01	176	<0.01	113	<0.01	279	<0.01
Stage of disease								
No DM, <i>n</i> = 46	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
No DR, <i>n</i> = 41	1	0.52	1	0.72	-4	0.09	-9	0.16
NPDR, <i>n</i> = 59	3	0.16	-1	0.85	4	0.08	5	0.36
PDR, <i>n</i> = 35	-5	0.01	-18	<0.01	-2	0.57	-14	0.03
Interactions†								
Race*stage		0.06		0.17		0.57		0.30

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.

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*

	D_A, P	CRAE, P	D_V, P	CRVE, P	SO_{2A}, P	SO_{2V}, P	SO_{2AV}, P	OEF, P
Stage comparison*								
No DR vs. NPDR	0.42	0.54	<0.01	0.01	0.49	0.02	0.15	0.03
No DR vs. PDR	0.01	<0.01	0.27	0.38	0.02	0.03	0.82	0.45
NPDR vs. PDR	<0.01	<0.01	0.01	<0.01	0.05	0.94	0.10	0.23

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), and age and pigmentation index (continuous variables).

simple main effects were calculated in each racial group and are shown in Table 6. The only significant differences on SO_{2A} 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, SO_{2V} 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 SO_{2A} 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 SO_{2A} 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 SO_2 in DM. We found SO_{2A} 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 investigators^{31,32,34} have found SO_{2A} 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 SO_{2A} 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 SO_{2A} 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 SO_{2A} in this stage not to differ from No DM,^{31,32} but one has found SO_{2A} to be elevated in No DR as compared to No DM.³⁴ Two studies^{28,29} have reported no difference in SO_{2A} between No DM and the stages of DR that were studied. In our adjusted model, SO_{2A} 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 SO_{2V} to be increased in NPDR and PDR as compared to No DM and No DR. These findings are consistent

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

	$SO_{2A}, \%$		$SO_{2V}, \%$		$SO_{2AV}, \%$		OEF	
	β	P	β	P	β	P	β	P
Intercept	94	<0.01	59	<0.01	35	<0.01	0.36	<0.01
Stage of disease								
No DM, $n = 46$	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
No DR, $n = 41$	2	0.64	0	0.99	2	0.69	0.01	0.88
NPDR, $n = 59$	4	0.26	7	0.03	-4	0.37	-0.06	0.08
PDR, $n = 35$	10	0.01	8	0.05	3	0.55	-0.02	0.58
Interactions†								
Race*stage		0.02		0.28		0.13		0.43

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.

TABLE 6. Statistical Model With Adjusted Mean Values of Retinal Vascular SO_{2A} by Stage of Disease Within Racial Groups

	AA, <i>n</i> = 74			White, <i>n</i> = 61			Hispanic, <i>n</i> = 46		
	<i>n</i>	β	<i>P</i>	<i>n</i>	β	<i>P</i>	<i>n</i>	β	<i>P</i>
Intercept		93	<0.01		99	<0.01		94	<0.01
Stage of disease*									
No DM, <i>n</i> = 46	5	Ref	Ref	35	Ref	Ref	6	Ref	Ref
No DR, <i>n</i> = 41	26	-4	0.59	9	3	0.53	6	2	0.83
NPDR, <i>n</i> = 59	28	-6	0.45	10	-3	0.50	21	21	0.02
PDR, <i>n</i> = 35	15	-1	0.92	7	9	0.08	13	25	0.01

Significant *P* values in bold.

* Estimates of stage of disease differences obtained with multivariable linear regression outcomes.

with all other studies that have found elevated values of SO_{2V} in PDR,^{29,31-34} some stages of NPDR,^{29,31,32,34} or the combined stages of DR as compared to No DM.^{30,35} The three reports with a No DR stage have found no difference in SO_{2V} from No DM.^{31,32,34} One group²⁸ investigated early NPDR and found an increase in SO_{2V} with age.

We found no difference on SO_{2AV} in any of the stages of disease that we studied, similar to other published studies.³⁰⁻³² However, some studies^{29,31,33,35} have reported a decrease in SO_{2AV} in various stages of DR. In addition, one group²⁸ has found a decrease in SO_{2AV} with age. We found a reduction in OEF in NPDR as compared to No DR, as also was found in another report.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_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_{2V} 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.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_{2AV} . We, and others, have found increases in both SO_{2A} and SO_{2V} in both NPDR and PDR. SO_{2A} is dominated by factors acting before the blood arrives at the arterial measurement site near the optic nerve, whereas SO_{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_{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_{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,^{51,52} we cannot be certain of blood flow alterations in the current study. OEF has the advantage of being independent of blood flow.^{9,47} OEF also equals the ratio of inner retinal oxygen metabolism to retinal vascular oxygen delivery.^{9,47} 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 D_A and SO_{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 SLO³⁶), 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.⁵³ have found that image quality is more important than the cardiac cycle as a source of measurement variability, Chen et al.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_2 measurements in healthy and diabetic subjects.^{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_2 measurements. Sixth, our method of calibrating values of SO_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_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 from 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

References

1. Kempen JH, O'Colmain BJ, Leske MC, et al. The prevalence of diabetic retinopathy among adults in the United States. *Arch Ophthalmol*. 2004;122:552-563.
2. Roy MS, Klein R, O'Colmain BJ, Klein BE, Moss SE, Kempen JH. The prevalence of diabetic retinopathy among adult type 1 diabetic persons in the United States. *Arch Ophthalmol*. 2004;122:546-551.
3. Ernst BJ, Garcia-Aguirre G, Oliver SC, Olson JL, Mandava N, Quiroz-Mercado H. Intravitreal bevacizumab versus panretinal photocoagulation for treatment-naive proliferative and severe nonproliferative diabetic retinopathy. *Acta Ophthalmol*. 2012;90:e573-e574.
4. Grisanti S, Biester S, Peters S, et al. Intracameral bevacizumab for iris rubeosis. *Am J Ophthalmol*. 2006;142:158-160.
5. Martinez-Zapata MJ, Marti-Carvajal AJ, Sola I, et al. Anti-vascular endothelial growth factor for proliferative diabetic retinopathy. *Cochrane Database Syst Rev*. 2014;11:CD008721.
6. Shimizu K, Kobayashi Y, Muraoka K. Midperipheral fundus involvement in diabetic retinopathy. *Ophthalmology*. 1981; 88:601-612.
7. Silva PS, Dela Cruz AJ, Ledesma MG, et al. Diabetic retinopathy severity and peripheral lesions are associated with nonperfusion on ultrawide field angiography. *Ophthalmology*. 2015;122:2465-2472.
8. Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev*. 2004;25:581-611.
9. Felder AE, Wanek J, Blair NP, Shahidi M. Inner retinal oxygen extraction fraction in response to light flicker stimulation in humans. *Invest Ophthalmol Vis Sci*. 2015;56:6633-6637.
10. Wanek J, Teng PY, Albers J, Blair NP, Shahidi M. Inner retinal metabolic rate of oxygen by oxygen tension and blood flow imaging in rat. *Biomed Opt Express*. 2011;2:2562-2568.
11. Feke GT, Tagawa H, Deupree DM, Goger DG, Sebag J, Weiter JJ. Blood flow in the normal human retina. *Invest Ophthalmol Vis Sci*. 1989;30:58-65.
12. Grunwald JE, Riva CE, Sinclair SH, Brucker AJ, Petrig BL. Laser Doppler velocimetry study of retinal circulation in diabetes mellitus. *Arch Ophthalmol*. 1986;104:991-996.
13. Kifley A, Wang JJ, Cugati S, Wong TY, Mitchell P. Retinal vascular caliber, diabetes, and retinopathy. *Am J Ophthalmol*. 2007;143:1024-1026.
14. Klein R, Klein BE, Moss SE, et al. Retinal vascular abnormalities in persons with type 1 diabetes: the Wisconsin Epidemiologic Study of Diabetic Retinopathy: XVIII. *Ophthalmology*. 2003;110:2118-2125.
15. Klein R, Klein BE, Moss SE, Wong TY, Sharrett AR. Retinal vascular caliber in persons with type 2 diabetes: the Wisconsin Epidemiological Study of Diabetic Retinopathy: XX. *Ophthalmology*. 2006;113:1488-1498.
16. Nguyen TT, Wang JJ, Sharrett AR, et al. Relationship of retinal vascular caliber with diabetes and retinopathy: the Multi-Ethnic Study of Atherosclerosis (MESA). *Diabetes Care*. 2008; 31:544-549.
17. Tsai AS, Wong TY, Lavanya R, et al. Differential association of retinal arteriolar and venular caliber with diabetes and retinopathy. *Diabetes Res Clin Pract*. 2011;94:291-298.
18. Klein R, Klein BE, Moss SE, et al. The relation of retinal vessel caliber to the incidence and progression of diabetic retinopathy: XIX: the Wisconsin Epidemiologic Study of Diabetic Retinopathy. *Arch Ophthalmol*. 2004;122:76-83.
19. Klein R, Myers CE, Lee KE, Gangnon R, Klein BE. Changes in retinal vessel diameter and incidence and progression of diabetic retinopathy. *Arch Ophthalmol*. 2012;130:749-755.
20. Alibrahim E, Donaghue KC, Rogers S, et al. Retinal vascular caliber and risk of retinopathy in young patients with type 1 diabetes. *Ophthalmology*. 2006;113:1499-1503.
21. Cheung N, Rogers SL, Donaghue KC, Jenkins AJ, Tikellis G, Wong TY. Retinal arteriolar dilation predicts retinopathy in adolescents with type 1 diabetes. *Diabetes Care*. 2008;31: 1842-1846.
22. Falck A, Laatikainen L. Retinal vasodilation and hyperglycaemia in diabetic children and adolescents. *Acta Ophthalmol Scand*. 1995;73:119-124.
23. Kifley A, Wang JJ, Cugati S, Wong TY, Mitchell P. Retinal vascular caliber and the long-term risk of diabetes and impaired fasting glucose: the Blue Mountains Eye Study. *Microcirculation*. 2008;15:373-377.
24. Klein R, Klein BE, Moss SE, Wong TY. Retinal vessel caliber and microvascular and macrovascular disease in type 2 diabetes: XXI: the Wisconsin Epidemiologic Study of Diabetic Retinopathy. *Ophthalmology*. 2007;114:1884-1892.
25. Rogers SL, Tikellis G, Cheung N, et al. Retinal arteriolar caliber predicts incident retinopathy: the Australian Diabetes, Obesity and Lifestyle (AusDiab) study. *Diabetes Care*. 2008;31: 761-763.
26. Roy MS, Klein R, Janal MN. Retinal venular diameter as an early indicator of progression to proliferative diabetic retinopathy with and without high-risk characteristics in African Americans with type 1 diabetes mellitus. *Arch Ophthalmol*. 2011;129:8-15.
27. Yau JW, Xie J, Lamoureux E, et al. Retinal microvascular calibre and risk of incident diabetes: the multi-ethnic study of atherosclerosis. *Diabetes Res Clin Pract*. 2012;95:265-274.
28. Schweitzer D, Lasch A, van der Vorst S, et al. Change of retinal oxygen saturation in healthy subjects and in early stages of diabetic retinopathy during breathing of 100% oxygen [in German]. *Klin Monbl Augenbeilk*. 2007;224:402-410.
29. Hammer M, Vilser W, Riemer T, et al. Diabetic patients with retinopathy show increased retinal venous oxygen saturation. *Graefes Arch Clin Exp Ophthalmol*. 2009;247:1025-1030.
30. Hardarson SH, Stefansson E. Retinal oxygen saturation is altered in diabetic retinopathy. *Br J Ophthalmol*. 2012;96: 560-563.
31. Jorgensen CM, Hardarson SH, Bek T. The oxygen saturation in retinal vessels from diabetic patients depends on the severity

- and type of vision-threatening retinopathy. *Acta Ophthalmol.* 2014;92:34-39.
32. Khoobehi B, Firn K, Thompson H, Reinoso M, Beach J. Retinal arterial and venous oxygen saturation is altered in diabetic patients. *Invest Ophthalmol Vis Sci.* 2013;54:7103-7106.
 33. Kashani AH, Lopez Jaime GR, Saati S, Martin G, Varma R, Humayun MS. Noninvasive assessment of retinal vascular oxygen content among normal and diabetic human subjects: a study using hyperspectral computed tomographic imaging spectroscopy. *Retina.* 2014;34:1854-1860.
 34. Felder AE, Wanek J, Blair NP, et al. The effects of diabetic retinopathy stage and light flicker on inner retinal oxygen extraction fraction. *Invest Ophthalmol Vis Sci.* 2016;57:5586-5592.
 35. Man RE, Sasongko MB, Xie J, et al. Associations of retinal oximetry in persons with diabetes. *Clin Exp Ophthalmol.* 2015;43:124-131.
 36. Kristjansdottir JV, Hardarson SH, Halldorsson GH, Karlsson RA, Eliasdottir TS, Stefansson E. Retinal oximetry with a scanning laser ophthalmoscope. *Invest Ophthalmol Vis Sci.* 2014;55:3120-3126.
 37. Vehmeijer WB, Magnusdottir V, Eliasdottir TS, Hardarson SH, Schalijs-Delfos NE, Stefansson E. Retinal oximetry with scanning laser ophthalmoscope in infants. *PLoS One.* 2016; 11:e0148077.
 38. Pellegrini E, Robertson G, Trucco E, et al. Blood vessel segmentation and width estimation in ultra-wide field scanning laser ophthalmoscopy. *Biomed Opt Express.* 2014; 5:4329-4337.
 39. Moss HE, Treadwell G, Wanek J, DeLeon S, Shahidi M. Retinal vessel diameter assessment in papilledema by semi-automated analysis of SLO images: feasibility and reliability. *Invest Ophthalmol Vis Sci.* 2014;55:2049-2054.
 40. Patton N, Aslam TM, MacGillivray T, et al. Retinal image analysis: concepts, applications and potential. *Prog Retin Eye Res.* 2006;25:99-127.
 41. Hubbard LD, Brothers RJ, King WN, et al. Methods for evaluation of retinal microvascular abnormalities associated with hypertension/sclerosis in the Atherosclerosis Risk in Communities Study. *Ophthalmology.* 1999;106:2269-2280.
 42. Knudtson MD, Lee KE, Hubbard LD, Wong TY, Klein R, Klein BE. Revised formulas for summarizing retinal vessel diameters. *Curr Eye Res.* 2003;27:143-149.
 43. Parr JC, Spears GE. Mathematic relationships between the width of a retinal artery and the widths of its branches. *Am J Ophthalmol.* 1974;77:478-483.
 44. Parr JC, Spears GE. General caliber of the retinal arteries expressed as the equivalent width of the central retinal artery. *Am J Ophthalmol.* 1974;77:472-477.
 45. Beach JM, Schwenzler KJ, Srinivas S, Kim D, Tiedeman JS. Oximetry of retinal vessels by dual-wavelength imaging: calibration and influence of pigmentation. *J Appl Physiol.* 1999;86:748-758.
 46. Schweitzer D, Hammer M, Kraft J, Thamm E, Konigsdorffer E, Strobel J. In vivo measurement of the oxygen saturation of retinal vessels in healthy volunteers. *IEEE Trans Biomed Eng.* 1999;46:1454-1465.
 47. Teng PY, Wanek J, Blair NP, Shahidi M. Inner retinal oxygen extraction fraction in rat. *Invest Ophthalmol Vis Sci.* 2013;54: 647-651.
 48. Hammer M, Vilser W, Riemer T, Schweitzer D. Retinal vessel oximetry-calibration, compensation for vessel diameter and fundus pigmentation, and reproducibility. *J Biomed Optics.* 2008;13:054015.
 49. Hidalgo B, Goodman M. Multivariate or multivariable regression? *Am J Public Health.* 2013;103:39-40.
 50. O'Connell RA, Anderson AJ, Hosking SL, Batcha AH, Bui BV. Test-retest reliability of retinal oxygen saturation measurement. *Optom Vis Sci.* 2014;91:608-614.
 51. Pemp B, Schmetterer L. Ocular blood flow in diabetes and age-related macular degeneration. *Can J Ophthalmol.* 2008; 43:295-301.
 52. Pournaras CJ, Rungger-Brandle E, Riva CE, Hardarson SH, Stefansson E. Regulation of retinal blood flow in health and disease. *Prog Retin Eye Res.* 2008;27:284-330.
 53. Knudtson MD, Klein BE, Klein R, et al. Variation associated with measurement of retinal vessel diameters at different points in the pulse cycle. *Br J Ophthalmol.* 2004;88:57-61.
 54. Chen HC, Patel V, Wiek J, Rassam SM, Kohner EM. Vessel diameter changes during the cardiac cycle. *Eye.* 1994;8(pt 1): 97-103.
 55. Geirsdottir A, Palsson O, Hardarson SH, Olafsdottir OB, Kristjansdottir JV, Stefansson E. Retinal vessel oxygen saturation in healthy individuals. *Invest Ophthalmol Vis Sci.* 2012;53:5433-5442.