Hemorheologic Abnormalities Associated with HIV Infection: In Vivo Assessment of Retinal Microvascular Blood Flow

Hajir Dadgostar,1,2 Gary N. Holland,1,2,3 Xin Huang,4 Adnan Tufail,1,2 Alisa Kim,1,2 Timothy C. Fisher,5 William G. Cumberland,3,4 Herbert J. Meiselman,5 Arthur Benjamin,1,2 and Dirk-Uwe Bartsch6

PURPOSE. To evaluate retinal microvascular blood flow in human immunodeficiency virus (HIV)-infected individuals using scanning laser Doppler flowmetry (SLDF) and to seek correlations between flow and various laboratory measures that may predict alterations in flow.

METHODS. The Heidelberg Retina Flowmeter and SLDF software were used to acquire in vivo retinal blood flow data from 24 HIV-infected individuals and 16 HIV-negative control subjects. In each subject, separate scans were performed in each of six retinal regions: nasal parapapillary retina; macula; and the superior, nasal, inferior, and temporal periphery. Erythrocyte aggregation (assessed in vitro by a fully automatic erythrocyte aggregometer and by zeta sedimentation ratio (ZSR, a hematocrit-independent sedimentation rate)), serum fibrinogen level, plasma viscosity, and leukocyte rigidity (assessed in vitro with a cell transit analyzer) were compared with flow in selected regions.

RESULTS. Flow was significantly higher in the periphery (superior, nasal, inferior, temporal) than in the posterior retina (nasal parapapillary retina, macula). Flow was highest in the temporal periphery for both HIV-infected subjects and control subjects. Flow in the posterior retina was significantly lower in HIV-infected individuals than in control subjects (P < 0.0001). Among HIV-infected individuals, flow in the macula correlated negatively with ZSR (r = −0.397, P = 0.0547) and leukocyte rigidity (r = −0.505, P = 0.0119).

CONCLUSIONS. Microvascular blood flow in the posterior retina is reduced in HIV-infected individuals. Both increased erythrocyte aggregation and increased leukocyte rigidity contribute to this hemorheologic abnormality. (Invest Ophthalmol Vis Sci. 2006;47:3933–3938) DOI:10.1167/iovs.06-0138

We have shown that erythrocyte aggregation is increased in human immunodeficiency virus (HIV)-infected individuals.1 Erythrocyte aggregation is a determinant of blood flow, and our finding therefore suggests the possibility of reduced flow in the retinal microvasculature of people with HIV disease. Our previous study included indirect indicators of such reduced flow, including (1) an association between increased erythrocyte aggregation and cotton-wool spots, which are signs of retinal ischemia, and (2) the presence of sludging of blood flow in conjunctival vessels by slit lamp biomicroscopy.2 Subsequently, reduced blood flow has been shown by use of the blue-light entoptic phenomenon3 and by scanning laser ophthalmoscopy combined with fluorescein angiography.4 A direct relationship between erythrocyte aggregation and in vivo measures of reduced retinal blood flow has not been confirmed, however.

Kim et al.5 have shown that increased erythrocyte aggregation persists in HIV-infected individuals despite immune reconstitution attributable to potent antiretroviral drugs, emphasizing the continued importance of HIV-related hemorheologic abnormalities. To investigate the relationship between erythrocyte aggregation and retinal microvascular blood flow, we returned to a previously unpublished 1996 database, in which measures of erythrocyte aggregation and in vivo measures of blood flow were collected from the same subjects, at the same visit. We also investigated topographic variations in retinal microvascular blood flow and investigated a possible effect of other determinants, including leukocyte rigidity, on blood flow.

METHODS

During the period September to December 1996, adult HIV-infected subjects were recruited from the Jules Stein Eye Institute and the UCLA Center for AIDS Research and Education (CARE) Clinic, without regard to specific age, gender, or race and ethnicity. Adult HIV-negative control subjects were recruited from the Jules Stein Eye Institute during the same period, without regard to specific age, gender, or race and ethnicity. Excluded were individuals with diabetes mellitus, hypertension, any form of systemic vasculitis, rheumatologic disease, tobacco use, blood transfusion within the prior 3 months, or evidence of glaucoma or ocular hypertension. The study adhered to the tenets of the Declaration of Helsinki and was approved by the UCLA and USC...
Institutional Review Boards. All subjects provided written informed consent.

For each HIV-infected subject, the presence or absence of cytomegalovirus (CMV) retinitis was determined by indirect ophthalmoscopy. The use of antiretroviral drugs was not recorded. On the day of in vivo blood flow determination, blood pressure was determined in both HIV-infected subjects and control subjects.

**Scanning Laser Doppler Flowmetry**

Confocal scanning laser Doppler flowmetry (SLDF) with the Heidelberg Retina Flowmeter (HRF; Heidelberg Engineering, Heidelberg, Germany) was used to measure retinal microvascular blood flow, as previously described.5,6 Briefly, SLDF measures the Doppler frequency shift in each of 16,384 retinal image points with a size of 10 × 10 µm per pixel in a retinal area of approximately 2.7 × 0.7 mm within 2 seconds. After Fourier transformation of the time-domain reflectance data into the spectral domain, it becomes possible to determine two independent variables: number of moving particles (volume) and number of moving particles times frequency shift (flow). The flow data describe an index for the distance traveled by all moving particles inside the sample volume per unit time.7 For a valid estimation of retinal blood flow, some assumptions must be made regarding brightness, absence of movement, and Doppler shift < 2000 Hz. To meet these requirements, the perfusion images were processed by the SLDF software, as previously described.7 The laser was targeted using confocal optics to bring the real-time retinal image, displayed on a monitor, into sharp focus. A 10° by 2.5° area of the retina displayed on the screen was then scanned, and the data were stored. Scans from both eyes were obtained for most subjects, and in most cases, the right eye was scanned first.

For the purposes of this study, the retina was divided into six regions. The nasal parapapillary retina was bound by the superior and inferior nasal vascular arcades and was within one disc diameter of the nasal margin of the optic disc. The macular region was defined as the area bound by the superior and inferior vascular arcades, excluding the area within 1 disc diameter of the temporal margin of the optic disc.

The four peripheral retinal regions (superior, nasal, inferior, and temporal) were defined as being outside the temporal vascular arcades in the midperiphery, more than 1 disc diameter from the nasal margin of the optic disc, and more than 2 disc diameters temporal to the fovea. The four peripheral areas to be scanned were located by instructing the subject to fixate with the nonexamine eye on four predetermined targets in the examination room. The superior peripheral area was located directly superior to the optic disc, and the inferior peripheral area was located directly inferior to the optic disc. Temporal and nasal peripheral areas were located on the horizontal meridian just beyond the macular and nasal parapapillary regions, respectively.

For most, but not all subjects, scans of the nasal parapapillary retina and macula were obtained from both eyes, and for many subjects, scans of all regions were obtained from both eyes. To evaluate the reproducibility of measurements, three replicate scans of the posterior retinal regions (nasal parapapillary retina and macula) were performed. Single scans of the four peripheral regions were obtained. Scans were separated in time by 2 to 3 minutes, while the next area to be scanned was located and brought into focus. For those HIV-infected subjects with CMV retinitis, scans were taken only from areas of the retina with no clinical lesions.

Original 1996 data files were transformed for analysis by software for automated full-field analysis of perfusion images (SLDF software, ver. 3.11; Heidelberg Engineering, Heidelberg, Germany)7 that was not yet available when data were collected. Before data extraction, individual scans were reviewed in detail, and portions containing motion artifact or large blood vessels were blocked electronically; scans that retained less than 20% of the area after this blocking procedure were excluded from analysis. Using the data analysis function of the SLDF software, mean flow was calculated for each region and placed in a spreadsheet for statistical analysis. Flow data are expressed in terms of arbitrary units, according to the manufacturer.

To identify topographic variations in flow, we determined differences between regions in multiple pair-wise comparisons for both HIV-infected subjects and HIV-negative control subjects. To determine whether retinal microvascular blood flow is affected by HIV status, we performed a region-by-region comparison of flow between HIV-infected subjects and HIV-negative control subjects.

**Laboratory Studies**

We also measured erythrocyte aggregation, zeta sedimentation ratio (ZSR), plasma fibrinogen level, plasma viscosity, and leukocyte rigidity on peripheral blood specimens of HIV-infected subjects. Specimens were obtained by venipuncture, using a 19-gauge needle and vacuum tubes containing standard concentrations of the appropriate anticoagulant for each test: EDTA for erythrocyte aggregation and ZSR; sodium citrate for fibrinogen level; and sodium heparin for plasma viscosity and leukocyte rigidity. All laboratory tests were performed at the time of specimen collection in 1996.

Leukocyte rigidity was determined with a cell transit analyzer (CTA; ABX Hematologie, Montpellier, France), as described previously.8 Erythrocyte aggregation was quantified with a photometric rheoscope (Myrenne Aggregometer; Myrenne GmbH, Rötgen, Germany), as described previously.7 ZSR, a hematocrit-independent measure of erythrocyte aggregation, was performed as described previously.10 Plasma viscosity relative to water at 37°C was determined on a cone-plate viscometer as described previously.11 Plasma fibrinogen level was determined on frozen citrated plasma by Specialty Laboratories (Santa Monica, CA).

We investigated the relationships between flow and each of the laboratory parameters described earlier, using flow data from the macula (chosen as a representative posterior region because of its clinical significance) and from the temporal periphery (chosen as a representative peripheral region, because it had the highest blood flow, and greatest difference from the posterior retina; see the Results section).

**Statistical Analysis**

All comparisons of flow (left eye versus right eye, topographic pair-wise comparisons between retinal regions, and comparisons between subject groups) were performed by using a multilevel mixed model analysis with random effects for person and eye or region, as needed, to account for the different correlations among measurements, taking the natural log of the mean flow as the response variable. For confirmatory purposes, paired t-tests and Wilcoxon signed rank analyses were also performed. Spearman correlation coefficients were calculated to determine relationships between continuous variables for mean flow (macula or temporal periphery) and laboratory data. For the above comparisons and correlations, primary analyses were performed with data from both eyes. Repeat analyses were performed using data from the right eye only, and from the first eye scanned for each subject only.

**RESULTS**

We evaluated 24 HIV-infected subjects, of whom 12 had CMV retinitis affecting one or both eyes, and 16 HIV-negative control subjects. Two of the HIV-negative control subjects were excluded from analyses because retinal scan quality was inadequate for data acquisition.

Table 1 shows demographic and laboratory data. There was no significant difference in mean systolic blood pressure or diastolic blood pressure between HIV-infected individuals and HIV-negative control subjects. Both mean hematocrit and leukocyte count were significantly lower in HIV-infected subjects. Mean age was significantly higher in HIV-infected subjects.

There was close clustering of the three replicate flow values for the nasal parapapillary retina and for the macula, when
compared with the variability of flow between subjects (Fig. 1).

Using a multilevel mixed-model analysis of log-transformed data to accommodate correlations among observations, we found that blood flow in the nasal parapapillary retina was significantly higher in the left eyes than in the right eyes of all subjects (P < 0.0001), and in HIV-infected subjects only (P < 0.0001) and in HIV-negative control subjects only (P = 0.0386). Similar data were obtained when comparing blood flow between the left and right eyes by using paired t-tests (P = 0.00413 for all subjects) or the Wilcoxon signed rank test (P = 0.00016 for all subjects). A similar difference was observed for the superior periphery, although the relationship was less strong (P = 0.0159 for all subjects). In all other regions, there was no statistically significant difference in flow between left and right eyes.

Figure 1. Scatterplots showing clustering of results from repeated scans of flow in the nasal parapapillary retina (A) and macula (B) of the right eye in all subjects. Subjects for whom no data points are shown had no scans of the given region for the right eye or had scans of poor quality that were excluded from analysis. AU, arbitrary units.

Table 1. Demographic and Laboratory Data

<table>
<thead>
<tr>
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<th>HIV-Infected Subjects</th>
<th>HIV-Negative Control Subjects</th>
<th>P*</th>
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<tbody>
<tr>
<td>Subjects (n)†</td>
<td>24</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>Age (y)</td>
<td>42.9 ± 8.2</td>
<td>36.4 ± 10.2</td>
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</tr>
<tr>
<td>CD4⁺ T-lymphocyte count (cells/μL)</td>
<td>111.32 ± 150.46 (n = 19)</td>
<td>36.4 ± 10.2</td>
<td></td>
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<tr>
<td>HIV blood level (copies/mL)</td>
<td>125,150.46 ± 223,552.69 (n = 13)</td>
<td>NE</td>
<td></td>
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<tr>
<td>Blood pressure (mm Hg)</td>
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<tr>
<td>Systolic</td>
<td>130.47 ± 8.16 (n = 17)</td>
<td>132.75 ± 11.98 (n = 4)</td>
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</tr>
<tr>
<td>Diastolic</td>
<td>79.82 ± 7.37 (n = 17)</td>
<td>70.75 ± 15.22 (n = 4)</td>
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<tr>
<td>Hematocrit (%)</td>
<td>37.67 ± 8.50 (n = 19)</td>
<td>42.86 ± 2.73 (n = 7)</td>
<td>0.028</td>
</tr>
<tr>
<td>Leukocyte count (×10³ cells/μL)</td>
<td>3.99 ± 1.90 (n = 10)</td>
<td>5.63 ± 0.23 (n = 3)</td>
<td>0.024</td>
</tr>
</tbody>
</table>

* Two-sample t-test.
† Laboratory data were not available for every subject. The number of subjects for whom specific results were available is shown after each mean value.

Table 2 and Figure 2 show flow data for each region. There was no significant difference in flow between the two posterior regions (nasal parapapillary retina versus macula), but flow was consistently slower in the posterior regions than in each of the peripheral regions (Fig. 2). Comparison of flow between different peripheral regions showed that the temporal periphery had the greatest flow for both HIV-infected and HIV-negative control subjects. Peripheral regions in the horizontal meridian (temporal versus nasal) were not significantly different from each other, and those in the vertical meridian (superior versus inferior) were not significantly different from each other. Flow in the temporal peripheral region was significantly greater than that in either the superior or inferior peripheral regions (P = 0.0008 and 0.0001 respectively). Flow in the nasal peripheral region was also greater than in the superior and inferior peripheral regions, but the relationships were weak (P = 0.0868 and 0.0285, respectively).

Similar results were obtained whether flow was analyzed from both eyes of all subjects (Fig. 2) or the right eye only or left eye only of either HIV-infected subjects alone or HIV-negative control subjects alone (data not shown).

Relationships to HIV Infection

For both the nasal parapapillary retina and macula, blood flow was lower in HIV-infected subjects than in HIV-negative control subjects (P < 0.0001). For the superior periphery, blood flow was also lower in HIV-infected subjects than in HIV-negative control subjects, although this relationship was less strong (P = 0.0374). For all other peripheral regions, there was no significant difference in blood flow between HIV-infected subjects and HIV-negative control subjects.

Relationships to Host Factors

No correlation was found between CD4⁺ T-lymphocyte count and flow for either the macula (r = 0.045, P = 0.86) or temporal periphery (r = 0.21, P = 0.41). Likewise, no correlation was found between HIV blood level and flow for either the macula (r = 0.33, P = 0.28) or temporal periphery (r = 0.24, P = 0.45).

Flow was consistently lower for those HIV-infected subjects who had CMV retinitis when compared with those without CMV retinitis, but the difference was not statistically significant in either the nasal parapapillary retina (P = 0.146) or macula (P = 0.511). There was, however, a greater and statistically stronger difference in flow between HIV-infected subjects with CMV retinitis (nasal parapapillary retina, 4.63; macula, 4.71) and HIV-negative control subjects (nasal parapapillary retina, 5.25, P = 0.0002; macula, 5.15, P = 0.0012) than between...
HIV-infected subjects without CMV retinitis (nasal parapapillary retina, 4.91; macula, 4.90) and HIV-negative control subjects (nasal parapapillary retina, \( P = 0.0445 \); macula, \( P = 0.0354 \)), indirectly suggesting a relationship between reduced flow and CMV retinitis.

Results similar to each of those described were obtained when comparing right eyes only and first-scanned eyes only (data not shown).

Table 3 shows correlation coefficients for comparisons between flow data and laboratory test results. We found a marginally significant negative correlation between flow in the macula and ZSR (\( r = -0.40, P = 0.0547 \) for both eyes). This correlation was stronger when analyzing flow from the right eye only (\( r = -0.48, P = 0.0209 \)) or from the first-scanned eye only (\( r = -0.44, P = 0.0314 \)). We also found a significant negative correlation between flow in the macula and leukocyte rigidity (\( r = -0.50, P = 0.0119 \)). Similar results were obtained when analyzing data from the right eye only (\( r = -0.56, P = 0.0057 \)) and from the first-scanned eye only (\( r = -0.59, P = 0.0024 \)). None of the other three laboratory parameters (erythrocyte aggregation, plasma viscosity, or fibrinogen level) correlated significantly with flow in the macula. There was no consistent correlation between flow in the temporal periphery and any of the five laboratory parameters.

**DISCUSSION**

The HRF is a reliable and accurate tool for quantifying retinal microvascular blood flow and has been used to document changes in several disease conditions.\(^{12-16}\) Using this instrument, we found that blood flow in the posterior retina (nasal parapapillary retina and macula) was reduced in HIV-infected individuals when compared with HIV-negative control subjects. Our results complement those reported by Lim et al.,\(^2\) who used the subjective blue field entoptic phenomenon to estimate macular leukocyte velocity. Erythrocytes and leukocytes have different flow characteristics, and the HRF is believed to reflect total microvascular blood flow rather than the behavior of a single component of blood. Furthermore, it provides an objective measure that is less dependent on subject participation and is able to assess blood flow in the peripheral as well as the posterior retina.

Although the HIV-infected subgroup was statistically older, past studies of healthy subjects have found no correlation between age and HRF flow measurements in the macula\(^{17,18}\) or optic disc.\(^{19}\) Also, we have found that age differences of this magnitude have no meaningful effect on erythrocyte aggregation,\(^9\) and in a PubMed search of the literature, we found no documentation that age has an effect on leukocyte rigidity.

Our results support the hypothesized relationship between erythrocyte aggregation and microvascular blood flow in HIV-infected individuals. Engstrom et al.\(^1\) found that sludging of blood and vascular structural abnormalities in the conjunctiva were related to increased ZSR, erythrocyte sedimentation rate (ESR), and fibrinogen levels, all of which are indicators of increased erythrocyte aggregation. We found a weak, negative correlation between flow and ZSR, but no correlation with direct measurement of erythrocyte aggregation or fibrinogen level. A definite conclusion about the relationship between erythrocyte aggregation and retinal microvascular blood flow in this setting is therefore difficult. The lack of a significant correlation between blood flow and other indicators of erythrocyte aggregation may be due to the small sample sizes, but a relationship may have been obscured by other factors that influence blood flow, such as cumulative structural damage to the microvasculature. A relationship between blood flow and erythrocyte aggregation may be easier to identify by using the ZSR than with other tests because the ZSR is a hematocrit-independent measure of erythrocyte aggregation.\(^{19}\) As in the
study by Engstrom et al., we found no apparent relationship between blood flow and plasma viscosity.

Leukocytes may be important determinants of blood flow in small vessels because of their large size. Altered leukocyte dynamics may be an especially important mechanism for decreased microvascular blood flow in HIV-infected individuals, who have been shown to have increased leukocyte rigidity.

We found a significant negative correlation between leukocyte rigidity and flow in the macula, in support of that hypothesis. The strength of the relationship suggests that it may be a more important contribution than erythrocyte aggregation.

Of interest, the correlations between flow and either erythrocyte aggregation or leukocyte rigidity were observed in the macula, but not in the temporal periphery, a finding that may be related to differences in blood flow regulation between the posterior and peripheral retina. Variable results with regard to regional differences in retinal blood flow have been reported in previous studies in which the HRF was used to examine healthy subjects. They include reports of higher blood flow in the superotemporal arteriole than the inferotemporal arteriole; lack of a significant difference between superior and inferior retinal flow; significantly greater flow in the inferotemporal arteriole than in the superotemporal arteriole; decreased blood flow in the temporal neuroretinal rim; and lack of a difference in capillary flow among various regions within the macula. This variability in findings may, in part, be a result of differences in protocol, as well as the retinal regions and vessel diameters under study. Our findings indicate that microvascular blood flow is significantly higher in all peripheral quadrants than it is in the posterior retina. Furthermore, of the four peripheral regions, the temporal periphery had the highest flow. This regional difference was observed whether or not subjects were HIV infected. The finding of greater flow in the temporal periphery is in agreement with previous findings in healthy subjects.

Studies of the topographic distribution of retinal damage in HIV-infected individuals have also produced variable results. Sample et al. suggest that nerve fiber layer loss may be more severe in the inferior retina, whereas Mansour et al. report that the frequency of cotton-wool spots is greatest in the temporal periphery.

Studies of AIDS-related CMV retinitis also have revealed a nonuniform distribution of lesions, with relatively fewer lesions in the peripheral retina. One might speculate that more rapid microvascular blood flow in the periphery decreases the likelihood that CMV-infected leukocytes or free virus particles will interact with vascular walls and establish foci of retinal infection. If this hypothesis is correct, one might expect to find significantly slower blood flow in HIV-infected subjects with CMV retinitis that in those without CMV retinitis. Although our results suggested a trend in that direction, the difference did not reach statistical significance. The small number of subjects in these subgroups may have resulted in a failure to detect real differences. In addition, however, blood flow is a dynamic process, and flow at the time of the scans may have changed since the development of CMV retinitis in these subjects. The risk of CMV retinitis in people with reduced flow would best be investigated in a longitudinal study.

A surprising finding was the difference in parapapillary flow between left and right eyes of the same subject. In the macula and in most of the periphery, no such difference existed, consistent with previous reports indicating no left–right difference in macular blood flow of healthy subjects. Anatomically, parapapillary capillaries are most proximal to the ophthalmic artery, and thus may be more likely to reflect tiny differences in flow dynamics related to left–right differences in vascular branching at the aortic arch. Another possibility, however, is that this finding represents an artifact related to the sequential order in which the eyes were scanned. In all but three cases, the right eye was scanned first. In two HIV-infected subjects, the left eye was scanned first, and one had scans of the left eye only. We found no information in the literature as to whether scanning order influences blood flow measurements, but one could speculate that there may be subtle alterations in flow between scans of the two eyes, attributable to vascular autoregulation, as subjects remained seated with minimal physical activity. We accounted for these possibilities by repeating comparisons using data from the right eye only and using data from the first-scanned eye only. In each of these secondary analyses, relationships remained unchanged.

Previous studies suggest that immune reconstitution by potent antiretroviral drugs may not influence abnormalities in macular leukocyte velocity, leukocyte rigidity, and erythrocyte aggregation. The present study did not address a possible influence of antiretroviral drug use on hemorheologic factors; data were collected when these drugs were just becoming available, and their profound effect on HIV disease was not yet known. We found no correlation between flow and either CD4+ T-lymphocyte count or HIV blood levels, however.

Our study has several other limitations. As mentioned, it is possible that there was insufficient power to detect real but subtle differences because of small sample sizes. We found that leukocyte counts were significantly lower in HIV-infected individuals than in HIV-negative control subjects, which may have influenced the observed correlation between flow and leukocyte rigidity, but we could not adjust for leukocyte count in our analysis because of the small number of subjects with leukocyte counts. We did not adjust for multiple comparisons, and thus, weak associations should be interpreted with caution.

There may be unknown factors that have altered the magnitude of hemorheologic changes in HIV-infected individuals since 1996, but there is no reason to suspect that the relationships between flow and erythrocyte aggregation or leukocyte rigidity have been altered by those factors. Because both erythrocyte aggregation and leukocyte rigidity are known to be significantly increased, even among HIV-infected individuals.
taking potent antiretroviral drugs, it is reasonable to conclude that reduced blood flow continues to be a problem in the current era. It has been hypothesized that microvascular disease and hemorheologic abnormalities contribute to visual disturbances that can occur in HIV-infected individuals, even after immune reconstitution.

In summary, retinal blood flow varies topographically, with flow in the peripheral retina being greater than in the posterior retina. Furthermore, our data suggest that microvascular blood flow in the posterior retina is reduced in HIV-infected individuals. This reduction is related in part to increased leukocyte rigidity and possibly to increased erythrocyte aggregation. These changes may have implications for development of HIV-related retinal disease, even in the setting of immune reconstitution.

Acknowledgments
The authors thank Heidelberg Engineering (Heidelberg, Germany) for providing the Heidelberg Retina Flowmeter used in the study; John Hawley (Heidelberg Engineering, Carlsbad, CA) and Thomas Tomasso (Heidelberg Engineering, Smithfield, RI) for technical advice and assistance in the transformation and analysis of data; Alexander N. Fleshman, MS, and Sonia Minassian, DrPH (Department of Biostatistics, UCLA School of Public Health, Los Angeles, CA) for assisting with the statistical analyses; and Ingwald Gangsaas, MD, and Michael Mosher, MD (David Geffen School of Medicine of UCLA, Los Angeles, CA) for assistance with collection of data.

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