Rapid, Noninvasive Detection of Diabetes-Induced Retinal Metabolic Stress

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Objective: To test whether subjects with diabetes mellitus (DM) have enhanced retinal flavoprotein autofluorescence compared with age-matched control subjects using a rapid, noninvasive clinical imaging method.

Methods: Twenty-one subjects with DM and 21 healthy age-matched control volunteers were subjected to retinal imaging using 1-ms flashes of 467-nm light. Flavoprotein autofluorescence for each flash at 535 nm was recorded using an electron-multiplying charged-coupled device camera with a 512 × 512-pixel chip. The average intensity and the average curve width of retinal flavoprotein autofluorescence were determined by analyzing histograms of pixel intensities plotted for each eye.

Results: When stratified by age, the mean average intensity and average curve width levels in subjects with DM were significantly greater than those in controls across all 3 consecutive decades of life studied (P ≤ .004 and P ≤ .006, respectively). An overall comparison of the mean average intensity and average curve width levels in all subjects with DM vs all controls, with adjustment for age, was consistent with the results found in each age category (P = .001 and P < .001, respectively). Subjects having DM with retinopathy in at least 1 eye had significantly greater average intensity and average curve width than subjects having DM without retinopathy in either eye (P = .002 and P = .005, respectively).

Conclusions: Flavoprotein autofluorescence measurements may be clinically useful to rapidly and noninvasively identify diabetic metabolic tissue stress and disease severity. Development of flavoprotein autofluorescence technology is likely to result in a tool that will improve DM screening and disease management.

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HYPERGLYCEMIA INDUCES mitochondrial stress and apoptotic cell death in diabetic tissues soon after disease onset and before involvement can be detected by any current clinical diagnostic method.1-6 This suggests that measurement of mitochondrial metabolic activity can serve as an early indicator of the onset of disease.7 Before apoptosis, mitochondria exhibit impaired electron transport by energy-generating enzymes in the respiratory chain,1,8 causing increased percentages of flavoproteins in the chain to be oxidized and rendered capable of absorbing blue light and emitting green autofluorescence.9-11 This phenomenon leads to the hypothesis that increased flavoprotein autofluorescence (FA) may be an early indicator of diabetic metabolic tissue stress.

The standard criterion diagnostic method for diabetes mellitus (DM) is the oral glucose tolerance test. However, this method is cumbersome and is often avoided by patients.12 Thus, many subjects with DM may remain undiagnosed until they develop diabetic microvascular and macrovascular complications.12-13

A noninvasive method of measuring FA to detect early ocular dysfunction due to disease has been previously described.14 In this study, we compared retinal FA levels in subjects with DM regardless of disease severity or duration with those of age-matched healthy control subjects.

METHODS

To measure retinal FA, a modified fundus camera containing 467-nm excitation and 533-nm emission filters (Omega Optical, Brattleboro, Vermont), 2 back-illuminated electron-multiplying charge-coupled device (EMCCD) cameras (Photometrics 512B; Roper Scientific, Tucson, Arizona), and customized computer hardware and software were used. The equipment has been previously described.14

Twenty-one subjects, aged 30 to 59 years, with established type 1 or type 2 DM and without ophthalmic disease other than retinopathy (hereafter referred to as “cases”) were enrolled consecutively between June 11, 2007, and September 17, 2007, at the University of Michigan, Ann Arbor.
Michigan, Ann Arbor, during routine funduscopic examinations (Figure 1). Plasma glucose levels (obtained at the examination) were assessed by the glucose oxidase method, and hemoglobin A1c (HbA1c) levels were measured by high-performance liquid chromatography. Twenty-one age-matched healthy controls with normal glucose tolerance,15 normal blood pressure,16 and normal lipid profile17 according to recognized guidelines and standards were recruited as the control population.

This study was approved by the institutional review board at the University of Michigan; all subjects gave written informed consent. The study was organized and was performed according to the Standards for Reporting of Diagnostic Accuracy Initiative.18,19

After pupillary dilation, an EMCCD camera was used to visualize the macula using commercially available software (RSImage, Roper Scientific). For each eye, a second EMCCD camera with interfaced software (MetaVue; MDS Analytical Technologies, Toronto, Ontario, Canada) was used to capture 3 to 5 FA 535-nm readings, each induced by a 1-ms flash of 467-nm light. Imaging required 5 minutes per patient. The depth of instrument focus results in the capture of FA from all retinal layers.

The FA images, stored as 512×512-pixel files, were analyzed to produce histograms using available software (MetaVue; Adobe Photoshop CS2; Adobe Systems, San Jose, California; and Lispix; National Institute of Standards and Technology, Gaithersburg, Maryland). Histograms of pixel intensities (Figure 2), ranging from 0 to 256 U gray scale, were plotted for each eye to yield the average intensity (AI) and the average curve width (ACW) of retinal FA. All images were independently interpreted by 2 research associates (M.G.F. and J.M.F.) trained in FA image evaluation. If disagreement was encountered, a consensus reading was performed. At the time of imaging and statistical analysis, the research associates knew if the patient had DM, but test review bias was minimized by not excluding any subject’s data and by relying on objective results of FA testing.1 Test and analysis of variance were used to compare the AI and the ACW in cases vs controls. Comparisons of eye-specific AI and ACW in cases vs controls were made using mixed linear regression analysis to adjust for intereye dependency and age (where appropriate). Commercially available software (SAS 9.0; SAS Institute Inc, Cary, North Carolina) was used for all statistical analyses. P < .05 was considered significant.

RESULTS

Twenty-one of 33 consecutive subjects with DM referred for imaging met the inclusion criteria (Figure 1) and were imaged to determine the AI and the ACW for each eye. Among cases, the mean (SE) age was 44.8 (10.0) years (age range, 30-59 years); the mean (SE) duration of DM was 10.5 (9.8) years, and the mean

![Figure 1. Flow of participants in the study. Subjects were classified as having diabetes mellitus by plasma testing, which served as the reference standard. The index test was retinal flavoprotein autofluorescence (FA) of each subject’s eyes. Results of the index test were then compared with the reference standard.](image-url1)

![Figure 2. Retinal flavoprotein autofluorescence (FA) histograms of pixel intensities collected from 3 age-matched subjects. Each panel displays 4 histograms of the right (black line) and left (blue line) eyes of each subject. Right shift of histograms designates increased retinal FA intensity. ACW indicates average curve width; AI, average intensity; L, left; and R, right. Numerals are given as mean (SE) gray scale units.](image-url2)
(SE) HbA1c level was 8.5% (1.9%) (to convert to proportion of the total hemoglobin, multiply by 0.01). There were 6 subjects with type 1 DM and 15 subjects with type 2 DM. Diabetic retinopathy was present in 12 subjects. The mean (SE) age among controls was 44.7 (9.4) years (age range, 30-59 years).

As shown in Figure 3 and summarized in the Table, for all 3 age strata (30-39, 40-49, and 50-59 years), the mean AI in cases was significantly greater than that in controls (P < .004). An overall comparison of the mean AI in all cases vs all controls, with adjustment for age, was consistent with the results found in each age category (P < .001). Similar findings are seen for mean ACW levels, which were significantly greater in cases than in controls within each age strata (P ≤ .006) and in an overall comparison with adjustment for age (P < .001).

The AI and the ACW in cases vs controls were compared to determine the age dependence of FA because other endogenous autofluorescent molecules (such as lipofuscin) accumulate with age and may affect FA intensity. In each age group, the AI and the ACW in cases were greater than those in controls, with the controls showing gradual steady increases of FA with age. However, the relative elevations of FA in cases vs controls seemed to be independent of patient age, suggesting that elevated FA in subjects with DM is not caused by lipofuscin or other similar fluorophores.

Differences in FA values between cases with type 1 vs type 2 DM were considered. The FA in 15 cases with type 2 DM (mean AI, 59.5 [23.6] U gray scale) and in 6 cases with type 1 DM (mean AI, 55.8 [25.9] U gray scale) did not differ (P = .65).

Elevated AI and ACW were detected in cases regardless of whether retinopathy was detected on fundus examination by an ophthalmologist specializing in diabetic retinopathy (D.G.P.). In fact, 9 of 21 cases had no visible

Figure 3. Average intensity and average curve width of retinal flavoprotein autofluorescence from 21 cases with (blue squares) or without (white squares) retinopathy and from 21 age-matched controls across 3 consecutive decades of life. For each subject, values for the right and left eyes are shown paired. * Indicates the only case whose average intensity and average curve width in each eye overlapped with those of controls.
retinopathy (Figure 3), indicating that retinal metabolic stress due to DM is present before any visible retinopathy.

We studied the associations between FA, HbA1c level, and the presence of retinopathy (Figure 4). The mean HbA1c level among cases with retinopathy in at least 1 eye (8.9% [2.1%]) was not significantly different from those among cases without retinopathy in either eye (7.9% [1.5%]) (P=.23). However, the mean AI and ACW among cases with retinopathy in at least 1 eye (69.7 [18.3] and 63.2 [14.5] U gray scale, respectively) were significantly different from those among cases without retinopathy in either eye (43.5 [12.2] and 44.8 [10.5] U gray scale, respectively) (P=.002 and P=.005, respectively).

To consider the possibility that FA imaging might measure acute fluctuations in plasma glucose levels rather than metabolic effects of chronic hyperglycemia, the FA of 4 volunteers was measured in a fasting state and at 1 hour after 75-g oral glucose challenge. No significant differences were observed in FA values, indicating that acute elevations in plasma glucose levels do not affect retinal FA.

The results of FA imaging in subjects with DM differ significantly from those in age-matched controls. Only 1 case (indicated by an asterisk in Figure 3), an intensively treated subject within 1 year of diagnosis and with an HbA1c level of 7%, had AI and ACW in each eye that overlapped with those of age-matched controls. Thus, even our proof-of-concept prototype measures significant differences between groups of controls and cases regardless of disease duration or severity. In several subjects (controls and cases), there is a statistical difference in AI and ACW values between their 2 eyes, suggesting that FA measures could be detecting increased retinal stress in one eye as opposed to the other. In fact, a high degree of retinal FA asymmetry between eyes of the same individual is a strong indicator of disease.

For cases, FA levels seem to be associated with the severity of retinal damage (Figure 3). In addition, our limited data suggest that FA may be more strongly associated with retinopathy than HbA1c levels, which are considered the most reliable measure of metabolic control. The value of FA imaging is supported by 2 cases with retinopathy (Figure 4) who had elevated FA but low HbA1c levels. Future studies may show FA to be useful in monitoring disease progression and its mitigation by treatment. Unlike glucose monitoring, elevations in FA reflect ongoing diabetic tissue damage and may provide patient and caregiver motivation for intensifying disease management.

### Table. Average Intensity and Average Curve Width in Cases and in Controls by Age Category

<table>
<thead>
<tr>
<th>Age Strata, y</th>
<th>Mean (SE)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cases</td>
<td>Controls</td>
</tr>
<tr>
<td>Average intensity, U gray scale</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td>52.4 (8.4)</td>
<td>18.3 (1.8)</td>
</tr>
<tr>
<td>40-49</td>
<td>54.2 (6.3)</td>
<td>30.4 (2.1)</td>
</tr>
<tr>
<td>50-59</td>
<td>68.9 (8.0)</td>
<td>35.4 (1.4)</td>
</tr>
<tr>
<td>Overall</td>
<td>58.4 (4.2)</td>
<td>28.2 (1.0)</td>
</tr>
<tr>
<td>Average curve width, U gray scale</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-39</td>
<td>49.0 (6.3)</td>
<td>21.3 (2.6)</td>
</tr>
<tr>
<td>40-49</td>
<td>52.8 (6.0)</td>
<td>31.8 (1.8)</td>
</tr>
<tr>
<td>50-59</td>
<td>64.1 (4.6)</td>
<td>34.2 (1.3)</td>
</tr>
<tr>
<td>Overall</td>
<td>55.2 (3.1)</td>
<td>29.1 (1.2)</td>
</tr>
</tbody>
</table>

*There were 7 cases and 7 controls in each age category.
*From least squares method.
*From mixed linear regression analysis. The overall comparison includes adjustment for age.

Until the recent development of a noninvasive method for clinical use, FA had not been used in human clinical studies of disease, to our knowledge. Ocular emission spectrophotometry has shown that mitochondrial FA constitutes a shoulder of a broad emission spectrum of other fluorescent species, especially lipofuscin. For maximal metabolic contrast, only a narrow emission band at the FA maximum is acquired, effectively excluding most of the emission of lipofuscin. To account for the residual portion of the FA signal derived from age-dependent accumulation of lipofuscin, age-matched FA comparisons were used to correct for this variable. Our approach in obtaining metabolic contrast seems to satisfy the requirements of a disease-sensitive tool. Nevertheless, careful quantification of the effects of potentially confounding ocular fluorophores on FA measurements is warranted in future studies.

Because neuronal loss and microangiopathy occur early in human and animal DM, tissue damage begins at the earliest stages of the disease, before it is clinically evident or detected by fasting blood glucose screening. Early diagnosis and treatment are likely to prevent this...
Our data suggest that development of FA imaging for DM, including its evaluation in longitudinal clinical trials, is likely to result in a tool that will become increasingly important in DM detection and management.

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Author Contributions: Messrs Field and Feuerman and Dr Musch had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Financial Disclosure: Drs Elner and Petty have a financial interest in the presented material by having founded OcuSciences, Inc, to commercialize the technology.

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