Multimodal functional and anatomic imaging identifies preclinical microvascular abnormalities in type 1 diabetes mellitus


1Centre for Experimental Medicine, Queen’s University Belfast, Belfast, Northern Ireland; and 2Regional Medical Physics Service, Northern Ireland, Royal Victoria Hospital, Belfast, Northern Ireland

Submitted 30 May 2014; accepted in final form 28 September 2014

Lockhart CJ, McCann AJ, Pinnock RA, Hamilton PK, Harbinson MT, McVeigh GE. Multimodal functional and anatomic imaging identifies preclinical microvascular abnormalities in type 1 diabetes mellitus. Am J Physiol Heart Circ Physiol 307: H1729–H1736, 2014.—Structural and functional changes in the microcirculation in type 1 diabetes mellitus predict future end-organ damage and macrovascular events. We explored the utility of novel signal processing techniques to detect and track changes in ocular hemodynamics in patients with this disease. Twenty-four patients with uncomplicated type 1 diabetes mellitus and eighteen age- and sex-matched control subjects were studied. Doppler ultrasound was used to interrogate the carotid and ophthalmic arteries, and digital photography was used to image the retinal vasculature. Frequency analysis algorithms were applied to quantify velocity waveform structure and retinal photographic data at baseline and after inhalation of 100% O2. Frequency data were compared between groups. No significant differences were found in the resistive index between groups at baseline or after inhaled O2. Frequency analysis of Doppler flow velocity waveforms identified significant differences in bands 3–7 between patients and control subjects in data captured from the ophthalmic artery (P < 0.01 for each band). In response to inhaled O2, changes in frequency band amplitudes were significantly greater in control subjects compared with patients (P < 0.05). Only control subjects demonstrated a positive correlation (R = 0.61) between changes in retinal vessel diameter and frequency band amplitudes derived from ophthalmic artery waveform data. The use of multimodal signal processing techniques applied to Doppler flow velocity waveforms and retinal photographic data identified preclinical changes in the ocular microcirculation in patients with uncomplicated diabetes mellitus. An impaired autoregulatory response of the retinal microvasculature may contribute to the future development of retinopathy in such patients.

diabetes; microvascular; doppler; retina; wavelet transform

CARDIOVASCULAR COMPLICATIONS represent the major cause of morbidity and mortality in type 1 diabetes mellitus (24). Clinically apparent diabetic retinopathy is recognized as a major cause of blindness and additionally stratifies a patient with diabetes into a high risk group for future cardiovascular events (20). Recent work using computer-based image analysis techniques to measure and quantify subtle abnormalities of structure and function in the retinal vasculature have documented associations with systemic vascular disease (25, 43). These changes in vessel wall properties cannot be detected by routine visual inspection or retinal photography that identifies overt disease at a later stage in the disease process after leakage of blood and lipid from the microvessels into the retina. It is known that abnormalities in wall properties of the retinal microvasculature have also been shown to mirror similar changes in cerebral and coronary microcirculations (8), suggesting that local changes in retinal vascular structure may be representative of generalized structural alterations in remote microvascular beds. The clinical rationale for identifying abnormalities in the structure and reactivity of retinal vessels is that early detection may improve risk stratification beyond traditional risk factor assessment and provide a valid surrogate to guide the use of therapeutic interventions to delay or prevent future cardiovascular complications.

For type 1 diabetes, there is a window between the initial diagnosis and detection of overt diabetic eye disease that can be sight threatening. In contrast to the delayed development of clinical macrovascular events, it is in vulnerable microvascular beds where early structural and functional abnormalities are identified that can predate or accompany the earliest stages of the cardiovascular disease process (10, 23). Maladaptive remodeling of the microvasculature is a characteristic feature and the primary driver promoting target organ damage in diabetes mellitus (9, 45). Deranged blood flow, autoregulation, and structure of the microvasculature influence organ perfusion and alter both pulsatile and steady-state pressure and flow characteristics of the vascular bed (27). Remodeling of the microcirculation represents one of the first manifestations of target organ damage and is a dynamic process that is reversible and thus a target for interventions that hold therapeutic and prognostic significance (27, 49). Therefore, the ability to accurately assess and monitor this section of the circulation could have value.

The aim of the present study was to investigate the clinical utility of applying novel signal processing algorithms to Doppler flow velocity waveforms perfusing the ocular circulation combined with simultaneously recorded digital retinal photography data in patients with type 1 diabetes mellitus and healthy control subjects. This multimodal approach provides a comprehensive functional and anatomic assessment linking arteriolar and microvascular function and permitted exploration of the dynamic association between changes in retinal microvascular geometry and the functional response of the flow velocity input in response to O2 inhalation.

MATERIALS AND METHODS

Participants

This parallel group case-control study included 24 patients with uncomplicated type 1 diabetes mellitus and 18 age- and sex-matched control subjects. Patients were eligible for the study if they had glycated hemoglobin (hemoglobin A1C) between 6.5% (48 mmol/mol) and 10% (86 mmol/mol) and were excluded if they had a history of overt cardiovascular disease, retinopathy, microalbuminuria, or
hypertension. No subjects were taking any prescribed medications other than insulin at the time of study. Glucose and plasma insulin levels were not measured on the day of the study. Written informed consent was obtained from all subjects. This study was performed in accordance with the Declaration of Helsinki (2000) and was approved by the local research ethics committee. The registered clinical trial number is NCT01045005.

**Procedures**

Experiments were performed in the morning after an overnight fast. Subjects attended on one occasion. Subjects refrained from consuming alcohol, tobacco, or caffeine for 12 h before testing. Subjects with diabetes withheld their usual morning insulin dose until the study was complete. We used B-mode and Doppler ultrasound to interrogate the right ophthalmic and carotid arteries using a Philips HDI-3500 ultrasound system equipped with a 12.5-MHz linear array probe. All subjects underwent baseline retinal photography using a Canon CR-DGi Non-Mydriatic Retinal Camera.

All subjects had O2 administered using a gas delivery system configured for the administration of 100% O2. The sequential rebreathing system comprised a fresh gas reservoir and an expiratory gas reservoir each connected to the patient by one-way valves. The system was assembled by adding a gas reservoir to the expiratory port of a commercial three-valve O2 delivery system. Flow from the gas tanks was controlled using standard rotometers as flow meters. O2 (100%) was then delivered for 5 min [sufficient time to produce a maximal vasoconstrictor response (12, 18)] via a tightly fitting face mask. After administration of 100% O2, retinal photography and ultrasound assessments were then repeated.

**Ophthalmic Artery Doppler Waveform Analysis**

Subjects were studied in the supine position with their head comfortably supported on a pillow and maintained fixation with their nonexamined eye on a point marked on the ceiling directly above their head. The operator sat behind the subject’s head and lightly placed the ultrasound probe, coupled with gel, on the closed eyelid of the subject. Ultrasound image quality was optimized, and the machine settings were kept constant for the remainder of the entire examination. Color imaging mode was used to locate the ophthalmic artery as it coursed along the medial side of the optic nerve. Pulsed Doppler recordings of flow velocity were made with the Doppler angle maintained under 60°, as previously described (32). A 30-s train of blood flow velocity signals were digitized at 200 Hz and exported to a personal computer using HDI lab software (Advanced Technologies Laboratory, Bothell, WA). Peak velocity envelopes of 10 consecutive flow velocity waveform signals were stored for offline analysis using customized software developed in our department.

**Carotid Artery Doppler Waveform Analysis**

The patient was in a supine position with their head supported on a single pillow. The head was extended by 10° and rotated by 45° to the opposite side. Color Doppler was used to locate the carotid artery, and a point 2 cm distal to the carotid bulb was insonated. A 15-s train of pulsed Doppler recordings of flow velocity was performed with the Doppler angle maintained at <60°.

**Spectral Analysis of Flow Velocity Waveforms**

The resistive index was calculated along standard lines using the maximum and minimum velocities only (1). We used the discrete wavelet transform, a time-frequency signal analysis method, to quantify changes in the Doppler flow waveform structure. This mathematical tool decomposes signals into discrete frequency bands and is superior to the traditional Fourier approach in resolving the range of frequencies contained in the Doppler flow velocity waveforms, as shown in Fig. 1. A detailed mathematical description of the discrete wavelet transform is beyond the scope of this paper (1).

Waveform data were characterised using six frequency bands. Lower frequency information was available for each signal, but since such data incorporate frequencies less than the heart rate and therefore are unrelated to the pulsatile component of the velocity waveform, they were omitted. In addition, very high frequency information, which we considered to not represent physiological phenomena, were ignored as these data relate to electronic noise of a nonphysiological origin. Measures of reproducibility of this technique have been previously published as percent quartile coefficients of variation in waveform outputs by frequency band and vascular location. They are in the order of 6–15% (28).

**Retinal Image Capture and Retinal Gating**

Centered between the optic disc and macula, retinal photographs encompassed the optic disc, the macula, substantial portions of the temporal vascular arcades, and approximately two disk diameters of retina nasal to the optic disk. This follows previously published protocols for retinal image capture (17).

**Retinal Image Analysis**

Images were captured as full color jpeg files, and analysis was carried out using the green channel since this gives the best contrast between the fundus and blood vessels (17, 44, 46). Before vessel diameter calculation, image data were preprocessed to improve vessel identification and registered to facilitate comparison between images (Fig. 2, A–C). Preprocessing was carried out using software developed by the authors using Borland Delphi 7.0 professional. To enhance contrast and reduce the effect of uneven illumination, a high-pass filter was used (46). Pre- and post-O2 inhalation images were registered using the Generalized Dual Bootstrap Iterative Closest Point method (52) to allow for comparisons between corresponding vessels. The resulting data were then segmented using a threshold value to generate a mask of the vessel network. The stages in generating this network are shown in Fig. 2. After segmentation, the vessel network was skeletonized to leave the vessel centerlines and vessel bifurcations. Crossings were removed, leaving isolated vessel network bifurcations.

---

**Fig. 1.** Note the discrete wavelet transform (DWT) reproduces the synthesized signal more accurately than the short-time Fourier transform (STFT).
segments. Short vessel segments were then removed, the orientation for each remaining centerline pixel was calculated, and the full width at half-maximum of the image profile perpendicular to the vessel segment was calculated for each centerline pixel. Finally, these values were averaged to give a single diameter measurement for each vessel segment (Fig. 3).

Tortuosity was defined as the ratio of the straight line distance between the end points of the vessel segment and the distance measured along the vessel’s centerline. Vessel parameters (including percent changes) were averaged over all vessels to give a single value for each subject. An augmented version of software created by Bankhead et al. (4) was used for the analysis (4).

A repeatability analysis was carried out for the measurement of vessel diameters. Vessel diameters were calculated for 10 subjects each seen on 2 different occasions. For each subject, the data set used consisted of three images: two images captured at the first visit and the third image captured at the second visit. This analysis indicated that in the absence of any change in vessel diameter, 95% of vessel diameter measurements would change by <2.2 pixels within a single visit.

Statistical Analysis

Statistical analysis was performed using the Statistical Package for the Social Sciences software (SPSS version 15). Descriptive variables are presented as means ± SD and were compared using the independent-samples t-test when data were normally distributed. The robustness of multivariate normality assumptions was assessed via Hotelling’s T² permutation test. Nonparametric tests were used as appropriate to perform statistical comparisons between groups for retinal vessel parameters and waveform data using the Mann-Whitney test. Comparisons were carried out separately for retinal venules and arterioles. Statistical significance was set at $P < 0.05$. Covariate analysis was applied to potential confounding outcome measures at baseline, each chosen because of established status as global cardiovascular risk factors (age, sex, smoking status, blood pressure, and dyslipidemia) in turn via multivariate models of covariance in SPSS.

RESULTS

Study Sample Characteristics

Study groups were well matched with respect to age, sex, and body mass index (Table 1). The mean age of type 1 diabetic subjects was $37 ± 10$ yr. The mean duration from diagnosis of diabetes was $16 ± 10$ yr. All patients with diabetes were receiving insulin therapy in the form of an evening dose long-acting preparation and a short-acting preparation with meals. The average total daily insulin dose was $53 ± 15$ units. There were no significant differences between groups with respect to prior cardiovascular events or family history of overt cardiovascular disease. The mean hemoglobin A1C in patients was $8.2\%$ ($66$ mmol/mol). Patients exhibited a higher heart rate and albumin-to-creatinine ratio than control subjects ($P < 0.01$ for both), although the values were within normal range for each measure.

Vascular and Imaging Data at Baseline and After Inhaled O₂ Administration

Baseline carotid and ophthalmic Doppler flow waveform analysis. No differences were found in the calculated resistive index derived from flow velocity waveforms captured from any

Fig. 2. A: the green channel was extracted from the full-color image, providing the best contrast between the vessel and fundus. The image was then processed to remove the effects of uneven illumination and reduce the prominence of the optic disk. B: all images were then registered to a single baseline image. C: the wavelet transform was applied at different scales to identify vessel segments.

Fig. 3. Profiles perpendicular to vessel centerline along a single vessel segment. Profiles shown in blue are outliers and were not included in the calculation of vessel segment diameter. For each vessel segment identified in the baseline image, the corresponding segments in other images were identified based on their location. Corresponding vessel segments were analyzed only if they meet criteria for minimum overlap between images and minimum vessel segment length.
arterial site at baseline (0.74 vs. 0.74, \( P = 0.68 \) for the ophthalmic artery, and 0.78 vs. 0.80, \( P = 0.65 \) for the carotid artery).

Frequency analysis of the ophthalmic artery Doppler flow velocity waveform data, used to quantify waveform structure over the duration of the cardiac cycle, revealed significant differences in frequency bands 3–6 (\( P < 0.01 \)) between groups (Table 2). Analysis of flow waveform data recorded in the carotid artery also revealed differences in the majority of frequency bands, although the differences between patients and control subjects were less prominent than those found in recordings made in the ophthalmic artery (Table 2). After adjustment for various potential confounding influences, the observed differences in the frequency analysis data were preserved between groups, thus suggesting that the differences reflect the disease state as opposed to other established cardiovascular risk factors.

**Baseline retinal imaging data analysis.** Comparison of a number of vascular parameters from retinal images (Figs. 2 and 3) captured at baseline in patients and control subjects were performed. No differences in length-to-diameter ratios or vessel tortuosity measures were apparent between groups (\( P > 0.05 \) for all measures).

**Effect of administration of inhaled \( O_2 \) on systemic hemodynamics and Doppler flow waveform structure.** No significant changes in blood pressure or heart rate occurred in response to the administration of inhaled \( O_2 \) in either group. No significant differences were apparent in the resistive index calculated from flow velocity waveform data obtained from ophthalmic and carotid arteries in response to the administration of \( O_2 \) in patients or control subjects (\( P = 0.68 \) for each site). The administration of 100% inhaled \( O_2 \) was associated with a change in the frequency content of the Doppler flow velocity waveform structure (Table 3). The change in frequency band amplitudes from baseline was significantly greater in control subjects compared with patients (\( P < 0.05 \) for all frequency bands). The change in frequency band amplitude from baseline was more evident and found in all frequency bands captured from the ophthalmic artery compared with data captured from the carotid artery.

**Relationship between change in Doppler flow waveform structure and retinal diameters in response to inhaled \( O_2 \).** For unclassified vessels, a significant difference was found between control subjects and patients in terms of the magnitude of the vasoconstrictor response observed with inhaled \( O_2 \) (\( P < 0.05 \)), with control subjects demonstrating a greater vasoconstrictor response for both vessel classifications. A significant difference was found between control subjects and patients in terms of the degree of vasoconstriction observed in response to inhaled \( O_2 \) for veins (\( P = 0.07 \)) and arterioles (\( P < 0.05 \)), with control subjects demonstrating a greater vasoconstrictor response for each vessel classification (Fig. 4). Furthermore, only control subjects exhibited a positive correlation between the percent change in retinal vessel diameter and change in ophthalmic artery Doppler flow velocity input wavelet amplitude for the different frequency bands (Table 4 and Fig. 5) in data captured from the ophthalmic artery in response to inhaled \( O_2 \). No relationship was identified between the change in Doppler flow velocity waveform structure captured from the carotid artery and change retinal vessel diameters in response to inhaled \( O_2 \).

**DISCUSSION**

Using novel frequency-based approaches applied to the Doppler flow velocity input perfusing the ocular microcirculation, we identified significant differences in the flow waveform morphology in patients with type 1 diabetes mellitus compared with age- and sex-matched healthy control subjects. Changes in the Doppler flow velocity input perfusing the ocular circulation were identified in patients without clinical retinopathy that could be detected by retinal photography. In addition, a

### Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td><strong>Age, yr</strong></td>
<td>41</td>
<td>11.0</td>
</tr>
<tr>
<td><strong>Height, m</strong></td>
<td>1.74</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Weight, kg</strong></td>
<td>76.5</td>
<td>15.7</td>
</tr>
<tr>
<td><strong>Body mass index</strong></td>
<td>25.2</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Hemoglobin A1C, %</strong></td>
<td>5.3</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Total cholesterol, mmol/l</strong></td>
<td>5.1</td>
<td>0.9</td>
</tr>
<tr>
<td><strong>High-density lipoprotein-cholesterol, mmol/l</strong></td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td><strong>Low-density lipoprotein-cholesterol, mmol/l</strong></td>
<td>2.9</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Triglycerides, mmol/l</strong></td>
<td>1.5</td>
<td>0.8</td>
</tr>
<tr>
<td><strong>Creatinine, ( \mu )mol/l</strong></td>
<td>82.8</td>
<td>12.0</td>
</tr>
<tr>
<td><strong>Albumin-to-creatinine ratio, mg/mmol</strong></td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Systolic blood pressure, mmHg</strong></td>
<td>118</td>
<td>17</td>
</tr>
<tr>
<td><strong>Diastolic blood pressure, mmHg</strong></td>
<td>68</td>
<td>12</td>
</tr>
<tr>
<td><strong>Mean arterial pressure, mmHg</strong></td>
<td>85</td>
<td>13</td>
</tr>
<tr>
<td><strong>Hemoglobin A1C, %</strong></td>
<td>5.3</td>
<td>0.2</td>
</tr>
<tr>
<td><strong>Body mass index</strong></td>
<td>25.2</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Height, m</strong></td>
<td>1.74</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Weight, kg</strong></td>
<td>76.5</td>
<td>15.7</td>
</tr>
</tbody>
</table>

\( n = 18 \) control subjects and 24 patients. NS, not significant.

### Table 2. Baseline time domain and frequency domain measures in control subjects and patients

<table>
<thead>
<tr>
<th></th>
<th>Control Subjects</th>
<th>Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Interquartile range</td>
</tr>
<tr>
<td><strong>Ophthalmic artery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resitive index</td>
<td>0.74</td>
<td>0.74</td>
</tr>
<tr>
<td>Frequency band amplitude 2</td>
<td>0.35</td>
<td>0.07</td>
</tr>
<tr>
<td>Frequency band amplitude 3</td>
<td>0.92</td>
<td>0.17</td>
</tr>
<tr>
<td>Frequency band amplitude 4</td>
<td>1.56</td>
<td>0.47</td>
</tr>
<tr>
<td>Frequency band amplitude 5</td>
<td>2.0</td>
<td>0.82</td>
</tr>
<tr>
<td>Frequency band amplitude 6</td>
<td>2.71</td>
<td>1.40</td>
</tr>
<tr>
<td><strong>Carotid artery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resitive index</td>
<td>0.78</td>
<td>0.80</td>
</tr>
<tr>
<td>Frequency band amplitude 2</td>
<td>0.81</td>
<td>0.29</td>
</tr>
<tr>
<td>Frequency band amplitude 3</td>
<td>2.54</td>
<td>1.01</td>
</tr>
<tr>
<td>Frequency band amplitude 4</td>
<td>4.59</td>
<td>3.0</td>
</tr>
<tr>
<td>Frequency band amplitude 5</td>
<td>4.79</td>
<td>2.9</td>
</tr>
<tr>
<td>Frequency band amplitude 6</td>
<td>6.26</td>
<td>3.0</td>
</tr>
</tbody>
</table>

\( P = 0.01 \) for all frequency bands.
positive correlation was found between the magnitude of the diameter change of retinal arterioles in response to inhaled O2 and the amplitude of specific frequency bands used to quantify the change in the Doppler flow velocity input perfusing the ocular microcirculation. The response of retinal vessels to inhaled O2 was significantly greater in control subjects, with a significant positive association between the change in Doppler flow velocity waveform structure and retinal vessel diameter found in response to inhaled O2, which was evident only in recordings obtained from the ophthalmic artery. These data represent the first description in patients with diabetes linking impaired reactivity of a downstream microvascular bed with diameter changes in arteriolar vessels perfusing the microvasculature. The novel findings indicate that simultaneous analysis techniques applied to retinal arteriolar vessel measurement and Doppler flow velocity waveform data can identify static and dynamic abnormalities in the physical properties of the ocular microcirculation in patients with type 1 diabetes mellitus, before the identification of overt retinal disease.

Existing data now convincingly show links between a range of visible microvascular signs, altered morphology of Doppler flow velocity waveforms perfusing vulnerable microvascular beds, and the development of both clinical and subclinical cerebrovascular, cardiovascular, and metabolic consequences (3, 5, 15, 27). The eye and kidney in particular have been recognized as vulnerable target end-organs in diabetes where subtle pathophysiological changes in the microcirculation predates and predict future cardiovascular events (2, 27). Of the risk factors known to be associated with the development of microvascular complications, the duration of diabetes and diabetes control are recognized as important contributors.

However, in the diabetes control and complications trial, hemoglobin A1c and the duration of diabetes explained only 11% of the variation in retinopathy risk, suggesting the need for additional markers of risk for diabetic microvascular complications (16, 21). The patients in this study were not in the earliest stages of the disease process in terms of the duration of diabetes but nevertheless were free from complications of arterial vascular disease that could be detected by standard screening tests.

Microvascular beds have been recognized as sites where the earliest manifestations of inflammatory processes occur, and endothelial activation initiated in this section of the vasculature may play an important role in driving the development of atherosclerotic disease in large arteries (37, 42, 47). The microcirculation in diabetes displays a change in phenotype and function of the endothelium and smooth muscle and altered structure and composition of the extracellular matrix. As a result, the diabetic microvasculature exhibits altered wall-to-lumen ratios and stiffer vessel walls than vessels from subjects without diabetes (13, 23, 33, 40). Furthermore, in addition to altering wall properties, the complex diabetic milieu influences vascular reactivity that varies with vessel size, region, and function (11, 40). Abnormalities in function and geometry of the retinal microvascular network have recently been described in patients with type 1 diabetes without clinical retinopathy (36, 41), indicating that subtle structural and functional changes can be detected at an early preclinical stage in the disease process.

Global changes in the geometry, wall characteristics, and caliber of vessels manifest throughout the microvascular network will alter the opposition to both steady-state and pulsatile components of the blood flow input into, and the pattern of wave reflection emanating from, the microcirculation (27, 31, 50). The microvasculature has been recognized as the most

### Table 3. Percent changes in vessel diameter and changes in wavelet amplitude for different frequency bands in the ophthalmic artery in response to inhaled O2.

<table>
<thead>
<tr>
<th>Subject Group</th>
<th>Average Change in Vessel Diameter, %</th>
<th>Mean Change in Frequency Band 2</th>
<th>Mean Change in Frequency Band 3</th>
<th>Mean Change in Frequency Band 4</th>
<th>Mean Change in Frequency Band 5</th>
<th>Mean Change in Frequency Band 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control subjects</td>
<td>-10.75</td>
<td>-0.001</td>
<td>-0.065</td>
<td>0.017</td>
<td>-0.11</td>
<td>-0.23</td>
</tr>
<tr>
<td>Patients</td>
<td>-8.40</td>
<td>-0.085</td>
<td>-0.195</td>
<td>-0.10</td>
<td>-0.48</td>
<td>-0.83</td>
</tr>
</tbody>
</table>

\( n = 18 \) control subjects and 24 patients.

### Table 4. Correlation between the percent change in retinal vessel diameter and changes in the frequency band amplitude in response to inhaled O2.

<table>
<thead>
<tr>
<th>Frequency Band</th>
<th>Correlation Coefficient</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frequency band 2</td>
<td>0.573</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Frequency band 3</td>
<td>0.534</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Frequency band 4</td>
<td>0.519</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Frequency band 5</td>
<td>0.479</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Frequency band 6</td>
<td>0.612</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Frequency band 2</td>
<td>-0.045</td>
<td>NS</td>
</tr>
<tr>
<td>Frequency band 3</td>
<td>-0.016</td>
<td>NS</td>
</tr>
<tr>
<td>Frequency band 4</td>
<td>-0.133</td>
<td>NS</td>
</tr>
<tr>
<td>Frequency band 5</td>
<td>0.026</td>
<td>NS</td>
</tr>
<tr>
<td>Frequency band 6</td>
<td>-0.033</td>
<td>NS</td>
</tr>
</tbody>
</table>

\( n = 18 \) control subjects and 24 patients.
important site for wave reflection in the arterial system, and
alterations in the pattern of wave reflection, indicative of altered physical properties of microvessels downstream to the site of measurement, distort the shape of incident waveform, which can be quantified to provide important information that may be predictive of adverse clinical outcomes (49). For many years, the resistive index, derived from Doppler flow velocity waveforms recorded from larger arteries supplying organ beds, has been used to provide an estimate of the resistance to blood flow presented by the downstream microcirculation. In some studies, this index has been shown to represent a marker for future microvascular dysfunction in organ beds and adverse clinical outcomes (14). However, it has now been recognized that the resistive index has significant limitations and is a poor discriminator for identifying early vascular disease and the effects of altered hemodynamics in influencing the pattern of wave reflection and changes in flow velocity waveform (7, 26, 38).

In the present study, we investigated the clinical utility and novel application of signal processing algorithms applied to the structure of Doppler flow velocity waveforms and retinal photography data of the retinal vasculature to determine the association between physical properties of the retinal network and static and dynamic functional responses of the flow velocity input perfusing the ocular microvascular bed. The use of multimodal technologies provides a more comprehensive functional and anatomic assessment of a regional microcirculation in humans. The time-frequency based analysis applied to Doppler flow velocity waveforms identified and quantified changes in the maximal flow velocity envelope in patients compared with control subjects. In contrast, the resistive index failed to discriminate between groups. Quantifiable differences in the flow velocity waveform were most evident in waveforms recorded in the immediate proximity of the ophthalmic artery microvascular bed compared with recordings made in the more proximal carotid conduit artery, a finding we have previously reported in patients with systemic lupus erythematosus (51). Propagating waves can add destructively as well as constructively, and the effects of wave reflection in altering the Doppler flow velocity waveshape depend on the distance between recording and reflecting sites, the magnitude of wave reflection, the speed of the pulse wave, and the degree of attenuation and energy dissipation produced by blood viscosity and damping by the vessel walls (6, 22, 39). As forward and reflected flow waves add negatively, it is plausible that reflected energy emanating from distant microvascular beds will have largely dissipated in recordings made in more proximal conduit arteries.

Abnormalities in the retinal circulation may not be evident in patients with type 1 diabetes with good glycemic control even after several years of disease, and a dynamic response to physiological and pharmacological challenges may be required to uncover subtle microvascular dysfunction (29). Inhalation of 100% O2 is associated with retinal arteriolar vasoconstriction with minimal effect on systemic hemodynamics, and O2 administration has been previously used as a test of retinal vascular reactivity by a number of investigators (19, 35, 48). We found that inhalation of 100% O2 was accompanied by vasoconstriction of the retinal arterioles and that the magnitude of the change was greater in control subjects compared with patients with type 1 diabetes. It has been proposed that a defective myogenic response in this low-impedance network exposes the retinal microvasculature to large pulsatile pressure and flow fluctuations that may predispose to the future development of clinical retinopathy (30, 34). For the first time in humans, we have documented a significant correlation between the magnitude of vasoconstriction in the microvascular bed of the retina in response to O2 inhalation, identified by changes in the frequency components that quantify the structure of the Doppler flow velocity waveshape, and changes in the diameter of arteriolar feeding vessels supplying the retinal circulation. As inhaled O2 did not alter the Doppler flow velocity profile in the proximal carotid vessel that perfuses the ophthalmic artery, these data provide compelling evidence that suggests dynamic alterations of flow waveform structure can identify and track subtle changes in tone of the downstream retinal microvascular bed.

We have shown the application of novel signal processing algorithms applied to multimodal technologies that identifies the presence of static and dynamic microvascular abnormalities at an early preclinical stage in the disease process in the ocular vasculature of patients with type 1 diabetes mellitus. The ability to measure and quantify subtle structural and functional abnormalities in microvascular beds may have clinical utility and help facilitate understanding of the role of the microcirculation in the development of vascular complications. The information may also improve risk stratification beyond conventional risk assessment and function as a potential valid surrogate to gauge the efficacy of new therapeutic agents.

**Limitations**

There was a relatively small sample size in this study, which is a clear limitation. However, we believe that these data lay the basis for future larger cohort studies. In addition, plasma glucose and insulin levels were not measured on the day of the study, which again is a limitation; future studies will incorporate these additional measures.
ACKNOWLEDGMENTS

The authors acknowledge the contributions of Dr. Peter Banckhead, Dr. Canice McGivern, and Dr. Christina Agnew to this work.

GRANTS

This work was supported by a grant from the Research and Development office (Northern Ireland).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: C.J.L. and G.E.M. conception and design of research; C.J.L. performed experiments; C.J.L., A.M., and R.P. analyzed data; C.J.L. and P.H. interpreted results of experiments; C.J.L. prepared figures; C.J.L. drafted manuscript; C.J.L., P.H., M.H., and G.E.M. edited and revised manuscript; C.J.L., A.M., P.H., M.H., and G.E.M. approved final version of manuscript.

REFERENCES