Small Nerve Fiber Quantification in the Diagnosis of Diabetic Sensorimotor Polyneuropathy: Comparing Corneal Confocal Microscopy With Intraepidermal Nerve Fiber Density

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OBJECTIVE
Quantitative assessment of small fiber damage is key to the early diagnosis and assessment of progression or regression of diabetic sensorimotor polyneuropathy (DSPN). Intraepidermal nerve fiber density (IENFD) is the current gold standard, but corneal confocal microscopy (CCM), an in vivo ophthalmic imaging modality, has the potential to be a noninvasive and objective image biomarker for identifying small fiber damage. The purpose of this study was to determine the diagnostic performance of CCM and IENFD by using the current guidelines as the reference standard.

RESEARCH DESIGN AND METHODS
Eighty-nine subjects (26 control subjects and 63 patients with type 1 diabetes), with and without DSPN, underwent a detailed assessment of neuropathy, including CCM and skin biopsy.

RESULTS
Manual and automated corneal nerve fiber density (CNFD) ($P < 0.0001$), branch density (CNBD) ($P < 0.0001$) and length (CNFL) ($P < 0.0001$), and IENFD ($P < 0.001$) were significantly reduced in patients with diabetes with DSPN compared with control subjects. The area under the receiver operating characteristic curve for identifying DSPN was 0.82 for manual CNFD, 0.80 for automated CNFD, and 0.66 for IENFD, which did not differ significantly ($P = 0.14$).

CONCLUSIONS
This study shows comparable diagnostic efficiency between CCM and IENFD, providing further support for the clinical utility of CCM as a surrogate end point for DSPN.
Methods to quantify neuropathy include clinical scores based on symptoms and neurological tests, quantitative sensory testing (QST), electrophysiological measurements, in the form of nerve conduction studies (NCS), and intraepidermal nerve fiber density (IENFD) in skin biopsy specimens (5). The neurological examination involves an assessment, such as the modified Neuropathy Disability Score (NDS) (6), a composite score that assesses touch, temperature, and vibration perception and reflexes, which requires expert clinical judgment, a strong element of subjectivity, and hence, poor reproducibility (7). Neurophysiology is objective and reproducible and is currently considered to be the most reliable measurement for confirming the diagnosis of diabetic neuropathy and indeed represents an essential part of the Toronto Criteria (TC) to identify those with “Confirmed DSPN: the presence of an abnormality of NCS and a symptom or symptoms or a sign or signs of neuropathy” (8). However, these measures mainly assess large nerve fibers, making them less sensitive to early DSPN, which is more likely to involve small fibers (9,10).

Small fibers can be assessed by quantifying thermal thresholds (11) and IENFD in skin biopsy specimens (12). Although QST assessment has been shown to have good repeatability (11), IENFD is considered to be the most objective and quantitative for the diagnosis of small fiber neuropathy (13,14). However, its invasive nature makes it unsuitable for repeated investigations (12). Furthermore, the reliability of IENFD for the diagnosis of DSPN has never been thoroughly validated in a large cohort of patients with diabetes (15). Thus diabetic neuropathy currently lacks a noninvasive surrogate for accurately detecting small nerve fiber damage and repair. Several studies (16–20) have shown that corneal confocal microscopy (CCM) is capable of making a quantitative assessment of small fiber damage and has the potential to be a surrogate end point for DSPN (9). Quantitative analysis using manual annotation of CCM images to identify fibers and branches is labor-intensive and subjective. However, a fully automated nerve fiber quantification method has been shown to have high correlation with the manually obtained measurements (21,22), and our recent study (23) compared manual and automated image analysis in a large cohort of patients with diabetes. We previously assessed CCM and IENFD in the same patients and showed that the measures were related (17). However, to date there has been no attempt to directly compare the ability of CCM and IENFD in the diagnosis of DSPN. In this report, we comprehensively evaluate manually and automatically quantified CCM-derived measures of nerve fiber morphology and compare their diagnostic performance with IENFD measurements according to the presence or absence of DSPN using the TC.

RESEARCH DESIGN AND METHODS

Study Subjects
The study recruited 63 patients with type 1 diabetes from clinics of the Manchester Diabetes Center, Manchester Royal Infirm, and age-matched control subjects from the community. The updated TC was used to assess all subjects for the presence and severity of DSPN between 2010 and 2011 (8). This research adhered to the tenets of the Declaration of Helsinki and was approved by the North Manchester Research Ethics Committee. Informed written consent was obtained from all participants before their enrollment in the study. All assessments were performed by trained staff in a purpose-designed clinical research facility in central Manchester. Inclusion criteria were age between 14 and 85 years and a history of type 1 diabetes. Exclusion criteria were a positive history of malignancy, connective tissue or infectious disease, deficiency of vitamin B_{12} or folate, chronic renal failure, liver failure, active diabetic foot ulceration, family history of peripheral neuropathy, active ocular disease, systemic disease known to affect the cornea other than diabetes, or chronic corneal pathologies. All participants underwent assessment of glycated hemoglobin (HbA_{1c}), HDL and LDL cholesterol, triglycerides, BMI, and renal status (estimated glomerular filtration rate and albumin-to-creatinine ratio). Participants in this study represent a subcohort of participants with type 1 diabetes (n = 110) and control subjects (n = 97) who agreed to undergo skin biopsy in addition to routine neurological testing.

Peripheral Neuropathy Assessment
All study participants underwent an assessment of neurological deficits (NDS) (6) and symptoms (Diabetic Neuropathy Symptom [DNS] score) (24). Vibration perception threshold (VPT) was tested using a Horwell Neurothesiometer (Scientific Laboratory Supplies, Nottingham, U.K.). Cold thresholds (CT) and warm thresholds (WT) were established on the dorsolateral aspect of the left foot (51) using the TSA-II NeuroSensory Analyzer (Medoc Ltd., Ramat-Yishai, Israel). Electrodiagnostic studies were undertaken using a Dantec Keypoint system (Dantec Dynamics Ltd., Bristol, U.K.) equipped with a DISA temperature regulator to keep limb temperature constantly between 32° and 35°C. Sural sensory nerve amplitude (SSNamp), sural sensory nerve conduction velocity (SSNCV), peroneal motor nerve amplitude (PMNamp), and peroneal motor nerve conduction velocity (PMNVC) were assessed by a consultant neurophysiologist.

The Toronto Diabetic Neuropathy Expert Group (8) recommendation was followed to define an individual to have neuropathy if he or she met both of the following criteria: 1) abnormal nerve conduction—a PMNVC of <42 m/s; and 2) a symptom or sign of neuropathy, defined as one of the following: a) DNS of 1 or more of 4, or b) NDS of 3 or more of 10.

For the IENFD assessment, a 3-mm punch skin biopsy specimen was obtained from the dorsum of the foot, and a bright-field immunohistochemistry protocol was used according to published guidelines (12). Linear IENFD (number of fibers/mm) was established in at least four sections of 50-μm thickness according to published counting rules (IENFD have to cross or originate at the dermal–epidermal junction, and secondary branches and fragments are not counted) (14). The assessments were performed by two experts (M.J. and R.M.) who were masked to the neuropathic/diabetes status of participants and were cross-validated.

Manual and Automated Quantification of Corneal Nerves
CCM images (Fig. 1A) were captured from all participants using the Heidelberg Retina Tomograph Rostock Cornea Module (HRT-III), as described (23,25), by two purpose-trained optometrists (I.N.P. and M.T.). Their dimensions are 384 × 384 pixels with the pixel size of 1.0417 μm. During a bilateral CCM scan, more than 100 images per patient were
Figure 1—A: Original CCM image. B: Manually quantified CCM image. C: Automatically quantified CCM image. The red lines represent main nerve fibers, blue lines are branches, and green spots indicate branch points on the main nerve trunks. CCM images of the subbasal nerve plexus from a control subject (D), a DSPN(−) patient with type 1 diabetes (E), and a DSPN(+) patient with type 1 diabetes (F) show the reduction in corneal nerves in the DSPN(+) patient. The red arrows indicate main nerve fibers (to calculate CNFD), and yellow arrows indicate branch fibers (to calculate CNBD). Box plots of IENFD (G) manual CNFD values (H), automated CNFD (I), and automated CNFL (J) values in controls and in DSPN(−) and DSPN(+) patients with type 1 diabetes based on the TC. K: ROC curves for manual CNFD (MCNFD), automated CNFD (ACNFD), and IENFD to discriminate DSPN(+) and DSPN(−) patients with diabetes. G–J: Red lines represent median, the box borders 25th and 75th percentile. Whiskers represent the range of the data (without outliers). Red plus symbols represent outliers.
typically captured from all corneal layers, and 6 subbasal images from the right and left eyes were selected for analysis. Criteria for image selection were depth, focus position, and contrast. One experienced examiner (I.N.P.), masked from the outcome of the medical and peripheral neuropathy assessment, manually quantified 1,506 images of all study participants using purpose-written, proprietary software (CCMetrics, M.A. Dabbah, Imaging Science, University of Manchester) (Fig. 1B). The specific parameters measured per frame were corneal nerve fiber density (CNFD) (number of main fibers per mm²), corneal nerve fiber length (CNFL) (total length of main fibers and branches per mm²), and corneal nerve branch density (CNBD) (number of branches per mm²) in accordance with our previously published protocol (23,25).

Automated corneal nerve fiber quantification consists of two steps: 1) CCM image enhancement and nerve fiber detection and 2) quantification of the three morphometric parameters. As described in our earlier work (21), a dual-model feature descriptor combined with a neural network classifier was used to train the detection software to distinguish nerve fibers from the background (noise and underlying connective tissue). In the nerve fiber quantification process, all of the end points and branch points of the detected nerve fibers are extracted and used to construct a connectivity map. Each segment in the connectivity map is then connected and classified as a main nerve fiber or branch (Fig. 1C). The software for automated CCM image quantification (ACCMetrics) is available via http://www.click2go.umip.com/i/software/Biomedical_Software/accmetrics_v2.html.

To evaluate the effectiveness of using IENFD and manually and automatically generated CCM features to diagnose DSPN, we used the TC as ground truth to categorize the subjects with diabetes into those with DSPN [DSPN+] and without DSPN [DSPN[−]].

Statistical Analysis
Statistical analysis and the receiver operating characteristic (ROC) curves were performed and generated using MATLAB R2012a software (The MathWorks Inc.). One-way ANOVA (nonparametric Kruskal-Wallis) was used to evaluate within- and between-group differences (control group, the DSPN[+] group, and the DSPN[−] group). A $P < 0.05$ was considered significant. The area under the ROC curve (AUC) values, 95% CIs, and sensitivity and specificity at the equal error-rate point and at the threshold of 2 standard deviations below the mean of the control group were calculated for comparison. MedCalc 14.12.0 software (MedCalc Software bvba) was used to compare the difference between two ROC curves. The power analysis was performed using Software G*Power 3.1.9.2 software. The power analysis was performed based on the Wilcoxon-Mann-Whitney test comparing the group with type 1 diabetes and the control group. For PMNVC, the power was 0.999 (assuming an error rate $\alpha = 0.01$), indicating that a sample size of 46 DSPN[−] and 17 DSPN[+] was sufficient to find a statistically significant difference.

RESULTS
Demographics, Metabolic, and Anthropometric Assessment
The demographics and metabolic and anthropometric measurements in patients with diabetes and control subjects are summarized in Table 1. In the patients with type 1 diabetes, 57% were on a multiple daily insulin injection regimen, and 43% were on continuous subcutaneous insulin infusion. Other medications included an ACE inhibitor or angiotensin receptor blocker in 36%

| Table 1—Clinical demographic results and neuropathy assessment in control subjects and in DSPN(−) and DSPN(+) patients with type 1 diabetes |
|---|---|---|---|
| Variable | Control subjects | DSPN(−) | DSPN(+) |
| | (n = 26) | (n = 46) | (n = 17) |
| Age | 44 ± 15 | 44 ± 13 | 59 ± 11 |
| Duration of diabetes | N/A | 23 ± 15 | 39 ± 14 |
| HbA1c (%)‡ | 5.5 ± 0.3 | 8.2 ± 1.4 | 8.5 ± 1.3 |
| Cholesterol (mmol/L) | 37.1 ± 3.5 | 62.2 ± 24.1 | 69.3 ± 14.3 |
| BMI (kg/m²)† | 26.8 ± 4.0 | 26.4 ± 4.5 | 27.5 ± 3.5 |
| HDL | 5.0 ± 0.8 | 4.4 ± 0.9 | 4.3 ± 0.9 |
| Triglycerides (mmol/L) | 1.4 ± 0.7 | 1.2 ± 0.7 | 1.3 ± 0.6 |
| Blood pressure (mmHg) | 126.7 ± 16.3 | 130.3 ± 17.8 | 141.1 ± 25.2‡§ |
| Systolic† | 70.2 ± 9.1 | 71.6 ± 9.6 | 73.0 ± 9.8 |
| Diastolic‡ | 6.0 ± 5.5 | 7.6 ± 5.5 | 25.2 ± 13.4¶§ |
| VPT (V)¶ | 36.4 ± 2.0 | 38.7 ± 3.6§ | 43.5 ± 4.9§ |
| CT (°C)† | 28.8 ± 1.6 | 27.1 ± 2.7§ | 16.8 ± 10.7†§ |
| PMNVC (ms)§ | 49.1 ± 3.4 | 43.9 ± 3.1¶ | 31.0 ± 9.5§§ |
| SSNVC (ms)‡ | 50.9 ± 3.9 | 45.3 ± 5.2¶ | 37.8 ± 6.8§§ |
| PMNamp (µV)¶ | 6.0 ± 2.4 | 6.0 ± 8.3 | 1.6 ± 1.6¶§ |
| SSNamp (µV)‡ | 19.7 ± 8.3 | 12.5 ± 6.9§ | 4.3 ± 3.5§ |
| CCM and IENFD for Nerve Fiber Quantification | 36.8 ± 5.3 | 28.3 ± 7.2¶ | 16.9 ± 10.1¶ |
| CNBD* | 92.8 ± 36.4 | 56.1 ± 30.3¶ | 48.2 ± 32.9¶ |
| CNFL† | 26.7 ± 3.7 | 20.2 ± 5.1¶ | 14.8 ± 8.3§ |

Results are expressed as mean ± SD. N/A, not applicable for this group. Statistically significant differences using ANOVA/Kruskal-Wallis: *P < 0.05; †P < 0.01; ‡P < 0.001; §P < 0.0001. Post hoc results for DSPN(+) significantly different from †control subjects; and §DSPN(−).
of subjects and statins in 71%. Age was comparable between control subjects and patients with diabetes. HbA1c was significantly higher in patients with diabetes than in control subjects, with no difference between DSPN(+) and DSPN(−) patients. BMI was significantly higher in DSPN(+) patients with diabetes compared with control subjects. Total cholesterol was significantly lower in DSPN(+) and DSPN(−) patients with diabetes, whereas HDL and triglycerides did not differ between the groups. Systolic blood pressure was significantly higher in DSPN(+) patients with diabetes compared with control subjects, whereas diastolic blood pressure did not differ between groups.

Neurological Assessment
The NDS differed significantly between DSPN(+) patients and control subjects (Table 1).

Electrophysiology
PMNCV, SSNCV, and SSNamp were significantly reduced in DSPN(−) patients with diabetes compared with control subjects (Table 1). PMNCV, SSNCV, PMNamp, and SSNamp were all reduced in DSPN(+) patients with diabetes compared with control subjects and DSPN(−) patients with diabetes.

IENFD
IEFND was significantly reduced in DSPN(+) patients (P = 0.002) and in DSPN(−) patients (P = 0.001), and was further reduced in DSPN(+) compared with DSPN(−) patients (P = 0.05) (Table 1 and Fig. 1G and Fig. 2). The median value of the control group was 9.35 and the 0.05 quantile was 4.31, which is consistent with previously published IENFD measurements (12).

CCM
Manual CNFD was significantly reduced in DSPN(+) patients (P < 0.0001) and in DSPN(−) patients (P < 0.0001) compared with control subjects and was further reduced in DSPN(+) patients compared with DSPN(−) patients (P < 0.0001) (Table 1 and Fig. 1H). Manual CNBD was significantly reduced in DSPN(+) patients (P < 0.0001) but not in DSPN(−) patients (P = 0.09) compared with control subjects. Manual CNFL was significantly reduced in DSPN(+) patients (P < 0.0001) and in DSPN(−) patients (P < 0.0001) compared with control subjects and was further reduced in DSPN(+) patients compared with DSPN(−) patients (P = 0.001). Automated CNFD was significantly reduced in DSPN(+) patients (P < 0.0001) and DSPN(−) patients (P < 0.0001) compared with control subjects and was further reduced in DSPN(+) patients compared with DSPN(−) patients (P < 0.0001) (Fig. 1J).

ROC Analysis
The patients with diabetes were categorized into DSPN(−) (n = 46) and DSPN(+) (n = 17). Table 2 reports the AUC values, 95% CIs, and sensitivity/specificity at the equal error-rate point on the ROC curve for manual and automated CCM features as well as IENFD values. The highest AUC values among the manual and automated CCM measures were obtained for CNFD, with AUC values of 0.82 and 0.80, respectively. Almost all individual CCM measurements resulted in higher AUC values than IENFD (0.66). Furthermore, sensitivity and specificity values were calculated at the equal error-rate point for the purpose of consistency. For this measure of diagnostic performance also, CNFD provided the best discrimination (76% for manual measurement and 70% for automated measurement), which exceeded the 65% achieved by IENFD.

In using IENFD to identify DSPN, a decision threshold for neuropathy is commonly set at 2 standard deviations below the mean of the control group. Table 2 also reports the sensitivity/specificity values obtained by applying this

| Table 2—AUC, 95% CI values, and sensitivity-specificity for manual and automated CCM and IENFD for the diagnosis of DSPN |
|-------------------------------|-----------------|-------------------|------------------|
|                             | CCM and IENFD   | 95% CI            | Sensitivity-specificity at error-rate | Sensitivity-specificity at mean ± 2 SD (threshold) |
| Manual                      |                 |                   | equal-error rate |                                                                 |
| CNFD                        | 0.82            | 0.68–0.95         | 0.76             | 0.82/0.71 (24.0) |
| CNFL                        | 0.70            | 0.54–0.885        | 0.71             | 0.59/0.74 (16.5) |
| CNBND                       | 0.59            | 0.43–0.75         | 0.53             | 0.17/0.96 (15.0) |
| Automated                   |                 |                   |                  |                                                                 |
| CNFD                        | 0.80            | 0.66–0.93         | 0.70             | 0.60/0.83 (15.5) |
| CNFL                        | 0.77            | 0.63–0.91         | 0.70             | 0.59/0.80 (15.5) |
| CNBND                       | 0.70            | 0.55–0.86         | 0.59             | 0.29/0.98 (4.0)  |
| IENFD                       | 0.66            | 0.50–0.82         | 0.65             | 0.53/0.76 (3.3)  |
threshold. When this threshold was used, manual CNFD and automated CNFD result in better sensitivity/specificity than IENFD: 0.82/0.71, 0.60/0.83, and 0.53/0.76, respectively. There were no statistically significant differences between the ROC curves for manual CNFD and IENFD (P = 0.14) and for automated CNFD and IENFD (P = 0.19) (26). However, CCM measurements show considerably less variability within the subject groups than IENFD measurements (Fig. 1G) and larger AUC values (Fig. 1K).

CONCLUSIONS
There is a need for surrogate end points of diabetic neuropathy that accurately detect early disease, quantify disease progression, and measure therapeutic response (2). The current gold standard for the diagnosis of neuropathy, neurophysiology, is a robust measure but has poor reproducibility (27). Other measures of neuropathy, such as symptoms and signs, are also poorly reproducible (7), and although QST is reproducible, it and signs, are also poorly reproducible for the individual diagnosis of DSPN has not been reported to date. In this report, we present a comparison of nerve fiber features, quantified manually or automatically from CCM images (CNFL, CNFD, and CNBD) with IENFD measurement in identifying DSPN in individuals. CCM and IENFD are comparable in their diagnostic performance for detecting patients with diabetic neuropathy. Neither technique appears to have an optimal diagnostic performance. However, there were relatively small numbers of patients in the study because a significant proportion were not willing to undergo biopsy. Furthermore, the diagnosis of DSPN does not incorporate a measure of small fiber damage, which limits the assessment of the diagnostic performance of these small fiber tests. The added advantage of CCM compared with IENFD assessment is the more rapid and noninvasive acquisition of images and automated corneal nerve image analysis allowing rapid and consistent quantification (22,23,35). The exception is the manually measured CNBD, which has been found previously (25) to be unreliable due to the subjective judgment required in identifying branches. The algorithmic definition of branches in the automated measurement results in greater consistency, although this is the least useful individual automated CCM measurement. CCM and IENFD both seek to measure small fibers, but IENFD showed a poorer discrimination between DSPN(+) and DSPN(−) patients. Furthermore, CCM measurements show considerably less variability within the subject groups than IENFD measurements. Interestingly, very low IENFD values were observed, even in control subjects.

This study has strengths and limitations. Strengths include the study design and techniques used to assess neuropathy. This is the first study to report the clinical utility of two highly sensitive techniques, CCM and skin biopsy, in the same group of patients with type 1 diabetes and control subjects. Thus, CCM appears to be an emerging surrogate end point of diabetic neuropathy that shows comparable performance to the current gold standard of IENFD.

The limitations of the current study are the relatively small number of patients with established neuropathy and the use of the more distal site for the biopsy, which makes comparison of the IEFND results with other studies difficult. Furthermore, these data are only applicable to Caucasian patients with type 1 diabetes and need to be confirmed in nondiabetic neuropathies.

In conclusion, we show that the diagnostic efficiency of CCM is comparable to IENFD. However, CCM may be preferred due to its rapid, noninvasive, automated, and hence, unbiased means of quantifying small nerve fiber damage and repair in DSPN(+) patients.

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