Diabetic sensorimotor polyneuropathy (DSPN) is a frequent complication of diabetes affecting up to 53% of people with diabetes. Diagnosis of the condition is important to define at-risk patients, anticipate deterioration, and assess new therapies. Neuropathic symptoms and signs, together with electrodiagnostic studies are the endpoints of choice to diagnose DSPN and assess therapeutic outcomes. Although these tests offer a robust means of assessing neuropathy, they predominantly focus on large fiber deficits, yet the earliest alterations occur in the small unmyelinated C- and thinly myelinated Aδ-nerve fibers. Small fiber neuropathy can be evaluated using quantitative sensory testing of thermal thresholds or skin biopsy to quantify intra-epidermal nerve fiber density (IENFD). However, the assessment of thermal thresholds using IVCCM in DSPN.

Conclusions. Diabetic peripheral neuropathy is associated with significant corneal nerve loss detected with IVCCM. Fully automated corneal nerve quantification provides an objective and reproducible means to detect human diabetic neuropathy.

Keywords: corneal confocal microscopy, diabetic neuropathy, diabetes
we described an algorithm that concurrently uses a dual-model feature descriptor and a neural network classifier to distinguish nerve fibers from the background and presented an evaluation of its performance against other available detection methods. The aim of the present study was to assess the diagnostic validity of a fully automated image analysis algorithm of in vivo confocal microscopy images in quantifying corneal subbasal nerves to diagnose diabetic neuropathy.

**METHODS**

**Study Subjects**

One hundred eighty-six patients with diabetes mellitus (108 male/78 female) and 55 age-matched control subjects (28 male/27 female) (50.4 ± 14.1 vs. 51.7 ± 11.4 years) were assessed for the presence and severity of DSPN between 2010 and 2011 based on the updated Toronto consensus criteria.² Informed written consent was obtained from all participants prior to their enrolment to the study. This research adhered to the tenets of the Declaration of Helsinki and was approved by the North Manchester Research Ethics Committee. Subjects were excluded if they had a history of malignancy, connective tissue or infectious disease, deficiency of vitamin B12 or folate, chronic renal failure, liver failure, active diabetic foot ulceration, and/or family history of peripheral neuropathy. Control subjects were excluded from the study if they had evidence of neuropathy or risk factors likely to cause neuropathy. All subjects were also assessed for the presence of corneal lesions by means of relevant history and slit-lamp biomicroscopy. Subjects were excluded if they had active ocular disease (e.g., severe dryness), systemic disease known to affect the corneal subbasal innervation, other than diabetes or chronic corneal pathologies (cystic corneal disorders, epithelial basement membrane dystrophies).

**Medical Status Assessment**

All participants underwent assessment of their cardiometabolic [glycated hemoglobin (HbA1c), total cholesterol (TC), triglycerides and body mass index (BMI)] and renal status [estimated glomerular filtration rate (eGFR) and albumin to creatinine ratio (ACR)].

**Peripheral Neuropathy Assessment**

The neuropathy disability score (NDS), a scale of 0 to 10, was used to stratify the neuropathic severity of the study participants into none (0–2), mild (3–5), moderate (6–8), and severe (9–10) as described elsewhere³¹ (Tables 1, 2). The neuropathy symptom profile (NSP) was employed to assess symptoms of neuropathy. Vibration perception threshold (VPT) was evaluated on the hallux of both feet with a Neurothesiometer (Horwell Scientific Laboratory Suppliers, Wilford, UK). Cool and warm thermal (CT/WT) thresholds and cold- and heat-induced pain (CIP/HIP) were established on the non-dominant hand. The neuropathy disability score (NDS), a scale of 0 to 10, was used to stratify the neuropathic severity of the study participants into none (0–2), mild (3–5), moderate (6–8), and severe (9–10) as described elsewhere³¹ (Tables 1, 2). The neuropathy symptom profile (NSP) was employed to assess symptoms of neuropathy. Vibration perception threshold (VPT) was evaluated on the hallux of both feet with a Neurothesiometer (Horwell Scientific Laboratory Suppliers, Wilford, UK). Cool and warm thermal (CT/WT) thresholds and cold- and heat-induced pain (CIP/HIP) were established on the non-dominant hand.

**Table 1. Medical and Peripheral Neuropathy Status**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls, n = 55, NDS = 0</th>
<th>DSPN (−), n = 86, NDS ≤ 2</th>
<th>DSPN (+), n = 100, NDS &gt; 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of diabetes</td>
<td>N/A</td>
<td>24.2 ± 21.2</td>
<td>34.4 ± 17.3</td>
</tr>
<tr>
<td>HbA1c, %/mmol/mol‡</td>
<td>5.5 ± 0.3/34 ± 3.3</td>
<td>7.7 ± 1.6/61 ± 17.5‡</td>
<td>7.9 ± 1.6/63 ± 17.5‡</td>
</tr>
<tr>
<td>BMI, Kg/m²</td>
<td>25.6 ± 4.6</td>
<td>27.2 ± 5.2</td>
<td>27.6 ± 5.8‡</td>
</tr>
<tr>
<td>TC, mM‡</td>
<td>5.1 ± 0.9</td>
<td>4.3 ± 1.2§</td>
<td>4.4 ± 0.9§</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>1.5 ± 0.8</td>
<td>1.5 ± 0.9</td>
<td>1.4 ± 0.9</td>
</tr>
<tr>
<td>eGFR, ml/min/L‡</td>
<td>85.8 ± 7.8</td>
<td>81.8 ± 18.2</td>
<td>70.0 ± 24.5§</td>
</tr>
<tr>
<td>ACR, mg/mmol‡</td>
<td>1.0 ± 1.4</td>
<td>2.9 ± 1.3</td>
<td>18.8 ± 11.3§</td>
</tr>
<tr>
<td>BP, systolic/diastolic, mm Hg</td>
<td>122 ± 16/70 ± 8.8</td>
<td>130 ± 18/71 ± 9</td>
<td>138 ± 23/72 ± 8</td>
</tr>
<tr>
<td>NSP</td>
<td>0</td>
<td>1.9 ± 3.0</td>
<td>5.6 ± 6.2</td>
</tr>
<tr>
<td>VPT, V‡</td>
<td>5.8 ± 4.6</td>
<td>9.2 ± 6.5§</td>
<td>22.3 ± 12.6§</td>
</tr>
<tr>
<td>WT¹/ACT; °C</td>
<td>37.0 ± 3.0/28.2 ± 2.2</td>
<td>39.6 ± 3.9/27.0 ± 9.2</td>
<td>42.7 ± 4.6/20.8 ± 9.2</td>
</tr>
<tr>
<td>HIP/CIP; °C</td>
<td>44.8 ± 2.9/11.9 ± 9.2</td>
<td>45.5 ± 6.6/9.8 ± 10.7</td>
<td>46.9 ± 7.3/4.1 ± 6.2</td>
</tr>
<tr>
<td>PMNCV, m/s‡</td>
<td>48.8 ± 3.3</td>
<td>43.7 ± 4.7§</td>
<td>39.2 ± 6.1§</td>
</tr>
<tr>
<td>SSNCV, m/s¹</td>
<td>51.0 ± 4.8</td>
<td>46.4 ± 5.8§</td>
<td>42.2 ± 6.4§</td>
</tr>
<tr>
<td>PMNamp, µV²</td>
<td>5.2 ± 1.8</td>
<td>4.5 ± 3.2</td>
<td>2.4 ± 2.1</td>
</tr>
<tr>
<td>SSNamp, µV²</td>
<td>20.0 ± 9.7</td>
<td>12.5 ± 7.8§</td>
<td>6.5 ± 6.6§</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD, statistically significant differences using ANOVA/Kruskal-Wallis. N.A, not applicable for this group.

* P < 0.05.
† P < 0.001.
‡ P < 0.0001; post hoc results for DSPN (+) significantly different from § control subjects and || DSPN (−).

**Table 2. IVCCM Assessment of DSPN Status**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls, NDS = 0</th>
<th>DSPN (−), NDS ≤ 2</th>
<th>DSPN (+), NDS &gt; 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual IVCCM quantification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNFDm, no./mm²²</td>
<td>37.2 ± 6.7</td>
<td>26.7 ± 8.5</td>
<td></td>
</tr>
<tr>
<td>CNBsm, no./mm²²</td>
<td>92.7 ± 38.6</td>
<td>54.9 ± 35.7</td>
<td></td>
</tr>
<tr>
<td>CNFsm, mm²/mm²²</td>
<td>26.4 ± 5.6</td>
<td>20.3 ± 6.7</td>
<td></td>
</tr>
<tr>
<td>Automated IVCCM quantification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNFDm, no./mm²²</td>
<td>50.0 ± 6.9</td>
<td>20.1 ± 8.7</td>
<td></td>
</tr>
<tr>
<td>CNBsm, no./mm²²</td>
<td>50.4 ± 24.7</td>
<td>31.4 ± 25.6</td>
<td></td>
</tr>
<tr>
<td>CNFsm, mm²/mm²²</td>
<td>21.2 ± 5.5</td>
<td>17.1 ± 4.5</td>
<td></td>
</tr>
<tr>
<td>Corneal sensation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCCA, mbar¹</td>
<td>0.7 ± 0.5</td>
<td>0.9 ± 0.8</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD, statistically significant differences using ANOVA/Kruskal-Wallis. no., number; mbar, millibar.

* P < 0.05.
† P < 0.01.
‡ P < 0.00.
§ P < 0.0001; post hoc results for diabetes DSPN (+) significantly different from || control subjects and ¶ DSPN (−).
Automated Detection of Diabetic Neuropathy

Nerve conduction studies (NCS) were undertaken by a consultant neurophysiologist (AM) as previously described.\textsuperscript{24} Peroneal motor nerve amplitude (PMNamp) and conduction velocity (PMNCV) and sural sensory nerve amplitude (SSNmap) and conduction velocity (SSNCV) were assessed. The diabetes cohort included 11 patients that did not agree or were unable to undergo NCS. These patients were not excluded from the study, but were not considered when NCS results were assessed.

Study Definition of Peripheral Neuropathy

The Toronto Diabetic Neuropathy Expert Group\textsuperscript{2} recommendation was followed to define "Confirmed DSPN: the presence of an abnormality of NCS and a symptom or symptoms or a sign or signs of neuropathy. In the absence of an abnormal NCS, a validated measure of small fiber neuropathy should be used" and "Subclinical DSPN: the presence of no signs or symptoms of neuropathy confirmed with an abnormal NCS or a validated measure of small fiber neuropathy." To define an abnormal result for NCS and QST we have used a mean $\pm$ 2 SD cutoff based on our control population.

In Vivo Corneal Confocal Microscopy

All study subjects were scanned with a laser IVCCM (Heidelberg Retinal Tomograph III Rostock Cornea Module [HRT III RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany) as described elsewhere.\textsuperscript{20} The overall examination took approximately 5 minutes for both eyes of each subject, and in this study two experienced optometrists performed all IVCCM scans. All images were captured using the "section" mode and prior to scanning corneal sensation was assessed using noncontact corneal aesthesiometry (NCA) as described elsewhere.\textsuperscript{25}

Manual Image Analysis

During a bilateral IVCCM scan more than 100 images per patient were typically captured from all corneal layers. Six subbasal images from right and left eyes were selected for analysis. Criteria for image selection were depth, focus position, and contrast. A single experienced examiner (INP), masked from the outcome of the medical and peripheral neuropathy assessment, quantified 1506 images of all study participants using purpose-written, proprietary software (CCMetrics, MA Dabbah; Imaging Science and Biomedical Engineering, University of Manchester, Manchester, UK). The specific parameters measured per frame were: CNFD (no./mm$^2$), CNFL (mm/mm$^2$), and CNBD (no./mm$^2$) in accord with our previously published protocol.\textsuperscript{20}

Automated Image Analysis

Automated corneal nerve fiber quantification consists of two steps: (1) IVCCM image enhancement and nerve fiber detection, and (2) quantification of the three morphometric parameters. As described in our earlier work,\textsuperscript{22,23} a dual-model feature descriptor combined with a neural network classifier was used to train the computer to distinguish nerve fibers from the background (noise and underlying connective tissue). In the nerve fiber quantification process, all 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 was then connected and classified as main nerve fibers or branches.

Statistical Analysis

Statistical analysis was performed using StatsDirect for Windows (version 2.7.9; StatsDirect Ltd., Cheshire, UK) and STATA 12 for Windows (Stata Corporation, College Station, TX, USA) was used to generate the receiver operating characteristic curves (ROC). Correlation analysis was performed to assess the strength of the relationship between automated and manually generated variables. Linear regression analysis was used to assess the consistency of the responses from the fully automated algorithm for a given manual estimate. The intraclass correlation coefficient (ICC) was calculated as a measure of reliability of the automated image analysis algorithm over repeated assessment of the dataset. One-way ANOVA (nonparametric Kruskal-Wallis) were used to evaluate within and between group differences. P value was maintained at 0.05 for multiple comparisons (Bonferroni adjustment or Conover-Imman pairwise comparisons) and a $P < 0.05$ was considered significant.

Receiver operating characteristic curves analysis was performed for all corneal nerve parameters to identify the point closest to the upper left corner of the ROC graph, which concurrently optimized sensitivity and specificity and the AUC, OR, and positive (+LR) and negative likelihood ratios (−LR) associated with the point were calculated. The diagnostic validity of IVCCM was assessed in relation to four established measures of DSPN (PMNamp, SSNamp, PMNCV, and WT). A $χ^2$ test was used to compare the AUCs generated for all IVCCM parameters.

Results

Medical Status and DSPN Assessment

Detailed medical and DSPN assessment results for subjects with diabetes and controls are presented in Table 1. Diabetic sensorimotor polyneuropathy(+) compared with DSPN(−) and controls had a lower eGFR ($P < 0.0001$), higher ACR ($P < 0.0001$), systolic blood pressure (BP) ($P < 0.0001$), VPT ($P < 0.0001$), WT ($P = 0.0005$), and lower CT ($P = 0.0004$), CIP ($P < 0.0001$), PMNCV ($P < 0.0001$), SSNCV ($P < 0.0001$), PMNamp ($P < 0.0001$), and SSNamp ($P < 0.0001$). Diabetic sensorimotor polyneuropathy(+) subjects had a longer duration of diabetes ($34.4\pm17.3$ vs. $24.2\pm21.2$, $P = 0.01$) and were older compared with DSPN(−) ($55.3\pm12.4$ vs. $47.3\pm15.6$, $P = 0.001$). Metabolic control and BMI were significantly different between controls (HbA$1c$, $P < 0.0001$; BMI, $P < 0.05$) and patients with diabetes, but comparable between DSPN(+) and DSPN(−). Total cholesterol (TC) was similar between the two groups with diabetes, and lower compared with controls ($P < 0.0001$), which is likely due to statin used in the diabetes cohort.

Manual and Automated Assessment of DSPN With IVCCM

Diabetic sensorimotor polyneuropathy(+) compared with DSPN(−) and controls had significantly lower manually quantified CNFD$_A$ ($P < 0.0001$), CNBD$_A$ ($P = 0.0005$), CNFL$_A$ ($P = 0.0002$), and automatically quantified CNFD$_A$ ($P < 0.0001$), CNBD$_A$ ($P = 0.0002$), and CNFL$_A$ ($P < 0.0001$) parameters. A significant reduction was also detectable between DSPN(−) and controls in CNFD$_M$ ($P < 0.0001$), CNBD$_M$ ($P = 0.0006$), CNFL$_M$ ($P = 0.0003$), and CNFD$_A$ ($P < 0.0001$), CNBD$_A$ ($P = 0.0003$), and CNFL$_A$ ($P < 0.0001$). Changes detected using automated image quantification were associated with a stronger significance level. Noncontact corneal aesthesiometry showed a significant elevation in the
There were 53 (30%) diabetic patients who had neuropathy based on abnormal PMNamp. A CNFDa less than 18.7 no./mm² was the point where sensitivity (0.79) and specificity (0.78) were concurrently optimized and associated with the highest AUC = 0.84, OR = 16.5, +LR = 4.6 (95% confidence interval [CI] 5.0–6.9), and −LR = 0.3 (95% CI 0.2–0.4). The corresponding point for automated analysis was CNFDa less than 14.7 no./mm² with sensitivity (0.76) and specificity (0.72) and AUC = 0.80, OR = 11.0, +LR = 3.4 (95% CI 2.4–4.9), and −LR = 0.3 (95% CI 0.2–0.5) (Fig. 2A). Similarly, CNFDa and CNFDa were associated with an AUC of 0.82 and 0.84 respectively, +LR = 3.25 (95% CI 2.5–4.6) and −LR = 0.35 (95% CI 0.2–0.5) (Fig. 2).

SSNamp Less Than 5.5 μV. When an abnormal SSNamp result was used as an indicator of neuropathy, the number of abnormal cases increased to 72 (40%). Automatically quantified CNFLa was associated with the highest AUC (0.77) and the highest OR = 5.1. A CNFLa less than 16.1 mm/mm² optimized sensitivity (0.72) and specificity (0.66) with +LR = 2.1 (95% CI 1.6–2.9) and −LR = 0.4 (95% CI 0.3–0.6). A CNFLa less than 19.1 mm/mm² optimized sensitivity (0.68) and specificity (0.67), but was associated with a lower AUC (0.70) and OR = 4.6 and comparable +LR = 2.1 (95% CI 1.5–3.0) and −LR = 0.5 (95% CI 0.3–0.7). Both CNFDM and CNFDa were equally capable in ruling out neuropathy. Both CNBDa and CNBDa showed limited ability to differentiate between cases with and without neuropathy.

PMNCV Less Than 42 M/S. There were 96 (54%) diabetic patients who had an abnormal PMNCV result. Automatically quantified CNFLa was associated with the highest AUC (0.79) and a CNFLa less than 16.0 mm/mm² optimized sensitivity (0.74) and specificity (0.71) with +LR = 2.6 (95% CI 1.9–3.8) and −LR = 0.3 (95% CI 0.2–0.5). A CNFLa less than 19.7 mm/mm² was associated with 0.74 sensitivity and 0.65 specificity, AUC = 0.75, OR = 4.8, +LR = 2.0 (95% CI 1.6–2.6), and −LR = 0.4 (95% CI 0.3–0.6). Both CNFDM and CNFDa had comparable AUC, OR, LR, and sensitivity/specificity to rule out neuropathy.

WT Greater Than 42°C. There were 95 (51%) patients with diabetes who had an abnormal WT greater than 42°C. Both CNFDM and CNFDa were associated with the highest AUC and modest OR. Specifically, a CNFDM less than 24.0/ mm² optimized sensitivity (0.65) and specificity (0.62) and was associated with AUC = 0.69, OR = 2.9, +LR = 1.6 (95% CI 1.2–2.1) and −LR = 0.7 (95% CI 0.5–0.8). The number of patients with an abnormal CNFDM and a WT was 61 (64%), while 35 (37%) had reduced CNFDM without a normal WT result. All CNFDM, CNFDM, and CNFDM values were comparable, but were associated with slightly lower AUC and OR while sensitivity and specificity remained modest (Table 3).

DISCUSSION

Diabetic peripheral neuropathy is the main initiating factor for foot ulceration and amputation and is associated with heavy morbidity, reduced quality of life, and poor healthcare.
outcomes. The prevalence of DSPN, in the diabetic population varies from 10% to 53%. However, only a few studies have used objective endpoints to estimate the rates of neuropathy and this may explain the reported variability. Dyck and colleagues found that when NCS was used in combination with a functional abnormality to diagnose DSPN, there was an overestimation with less experience has been described. The detection of nerve structures in IVCCM images is a challenging task: Nerve fibers often show poor contrast on a relatively noisy background due to image analysis is a labor-intensive task, where a human investigator applies subjective criteria to define a nerve and distinguish a nerve structure from noise. In contrast, manual axon reflectivity, to construct a connectivity map and quantify method.

<table>
<thead>
<tr>
<th>Definition of DSPN</th>
<th>IVCCM Value (Sensitivity/Specificity)</th>
<th>AUC</th>
<th>Odds Ratio (95% CI)</th>
<th>+LR (95% CI)</th>
<th>–LR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PMN&lt;sub&gt;amp&lt;/sub&gt;, &lt;1.4 μV</strong></td>
<td>CNFD&lt;sub&gt;d&lt;/sub&gt; 18.7 (0.79/0.78) 0.84 16.5 (7.0–39.9) 4.6 (3.0–7.0) 0.3 (0.2–0.4) &lt;br&gt; CNFD&lt;sub&gt;A&lt;/sub&gt; 14.7 (0.76/0.72) 0.80 11.0 (4.8–24.8) 3.4 (2.4–4.9) 0.3 (0.2–0.5) &lt;br&gt; CNBD&lt;sub&gt;d&lt;/sub&gt; 41.7 (0.75/0.68) 0.75 5.9 (2.7–13.1) 2.3 (1.7–3.1) 0.4 (0.2–0.6) &lt;br&gt; CNBD&lt;sub&gt;A&lt;/sub&gt; 14.9 (0.74/0.73) 0.79 9.2 (4.1–21.4) 2.9 (2.1–4.7) 0.3 (0.2–0.5) &lt;br&gt; CNFL&lt;sub&gt;d&lt;/sub&gt; 15.8 (0.77/0.76) 0.82 9.8 (4.4–22.0) 3.2 (2.3–4.6) 0.3 (0.2–0.5) &lt;br&gt; CNFL&lt;sub&gt;A&lt;/sub&gt; 14.6 (0.77/0.74) 0.84 12.9 (5.5–31.8) 3.3 (2.4–4.6) 0.2 (0.1–0.4)</td>
<td>&lt;br&gt; <strong>SSN&lt;sub&gt;amp&lt;/sub&gt;, &lt;5.5 μV</strong></td>
<td>CNFD&lt;sub&gt;d&lt;/sub&gt; 23.1 (0.72/0.67) 0.74 4.7 (2.3–10.0) 1.9 (1.5–2.6) 0.4 (0.3–0.6) &lt;br&gt; CNFD&lt;sub&gt;A&lt;/sub&gt; 18.9 (0.73/0.56) 0.72 5.1 (2.4–11.1) 1.9 (1.5–2.5) 0.4 (0.2–0.6) &lt;br&gt; CNBD&lt;sub&gt;d&lt;/sub&gt; 47.1 (0.61/0.56) 0.65 2.1 (1.1–4.9) 1.4 (1.0–1.9) 0.7 (0.5–1.0) &lt;br&gt; CNBD&lt;sub&gt;A&lt;/sub&gt; 25.4 (0.63/0.54) 0.70 2.1 (1.1–4.2) 1.4 (1.0–1.9) 0.7 (0.5–0.9)</td>
<td>&lt;br&gt; <strong>CNFL&lt;sub&gt;d&lt;/sub&gt;, &lt;0.6 μl</strong></td>
<td>CNFD&lt;sub&gt;d&lt;/sub&gt; 19.4 (0.68/0.67) 0.70 4.6 (2.3–9.3) 2.1 (1.5–3.0) 0.5 (0.3–0.7) &lt;br&gt; CNFD&lt;sub&gt;A&lt;/sub&gt; 16.1 (0.72/0.66) 0.77 5.1 (2.5–10.4) 2.1 (1.6–2.9) 0.4 (0.3–0.6)</td>
</tr>
</tbody>
</table>
In this study, automated analysis of CNBD was more capable in staging neuropathy than manually quantified CNBD, likely due to less variability compared with manual human analysis.

Recently, two studies have assessed the validity of IVCCM in diagnosing DSPN. Tavakoli et al. reported a CNFD less than or equal to 27.8 no./mm² and less than or equal to 20.8 no./mm² as the values with the highest validity to define disease status among patients with mild and more severe neuropathy respectively. Ahmed et al. found that a CNFL less than or equal to 14.0 mm/mm² was the value with the highest validity to rule in DSPN. We assessed the performance of manual and automated IVCCM quantification to identify patients “with” or “without” neuropathy based on gold standard measures of peripheral nerve damage. We found that CNFDₐ, CNFDₐ, CNFLₐ, and CNFLₐ were associated with the highest sensitivity and specificity to diagnose DSPN when PMNamp was used as the primary measure of neuropathy. Corneal nerve branch density showed less but acceptable validity in diagnosing DSPN and CNBDₐ had a significantly higher AUC and OR compared with CNBDₐ. When other endpoints of DSPN were used, such as SSNamp and PMNCV, the diagnostic validity of IVCCM remained high and CNFLₐ was consistently associated with the highest AUC and OR among all parameters. We observed a significant decline in sensitivity and specificity when an abnormality in WT was used as the primary marker of neuropathy. One would expect the opposite since warm detection is mainly mediated by small nerve fibers, and previously we have shown an association between IENFD and corneal nerve morphology. More recently CNFL has been related to three different measures of small fiber neuropathy. This is likely for two main reasons: NCS offer a robust and objective means of assessing neuropathy, while WT is a subjective measurement of small fiber function. Cassanova et al. in their study found that even patients with no IENFs had consistent responses in WT and concluded that it is possible for partially damaged nerve endings to still generate a propagated action potential. We speculate that a similar association may exist for the corneal subbasal nerves.

The validity of fully automated corneal nerve quantification was comparable and in several cases exceeded the performance of human expert assessment in ruling out DSPN. A CNFLₐ between 14.6 mm/mm² and 16.1 mm/mm² was the value consistently associated with the highest AUC and OR given the case definition employed. Both CNFDₐ (18.7–25.4 no./mm²) and CNFDₐ (14.7–19.7 no./mm²) also showed excellent performance with high OR, but were slightly more variable.

This study has several strengths and limitations. The strengths of this study are the detailed clinical assessment by gold standard clinical techniques of a relatively large number of participants with diabetes, representing a wide range of disease duration and neuropathic severity. Moreover, the same highly trained individuals performed all examinations for the 241 participants of this study ensuring consistency of the results. Our findings and cutoff points selected for the diagnosis of DSPN by IVCCM are comparable with the previous studies of Ahmed et al. and Tavakoli et al.; slight differences could be due to the case definition of neuropathy employed in each study, the number of patients investigated, and the disease severity in each group. We have compared IVCCM with several objective and subjective markers of DSPN with significant findings for the validity of the technique. There are no directly comparable published results for the fully automated algorithm employed in this study, therefore we cannot exclude the possibility that another system may be superior to the one presented here. This is to date the only available purpose-built, automated corneal nerve quantification system that has been validated in a large cohort of patients.
with diabetes and varying degrees of DSPN. Our results are cross-sectional and ongoing longitudinal studies will determine the ability of IVCCM to predict the development and progression or regression of DSPN. Recent data generated from wide-field assessment of the subbasal plexus have suggested that both central and inferior whorl nerve density may be reduced early and therefore future studies should explore this further.

In conclusion, we show that diabetic peripheral neuropathy is paralleled by a significant and progressive reduction in central CNFD and CNFL. We have validated a rapid fully automated analysis system to quantify alterations to replace human manual quantification. The use of this system will clearly enhance reproducibility, eliminate inconsistencies, and make the technique suitable to clinical practice and research centers worldwide.

Acknowledgments
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