Rapid Automated Diagnosis of Diabetic Peripheral Neuropathy With In Vivo Corneal Confocal Microscopy

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Purpose. 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. One hundred eighty-six patients with type 1 and type 2 diabetes mellitus (T1/T2DM) and 55 age-matched controls underwent assessment of neuropathy and bilateral in vivo corneal confocal microscopy (IVCCM). Corneal nerve fiber density (CNFD), branch density (CNBD), and length (CNFL) were quantified with expert, manual, and fully-automated analysis. The areas under the curve (AUC), odds ratios (OR), and optimal thresholds to rule out neuropathy were estimated for both analysis methods.

Results. Neuropathy was detected in 53% of patients with diabetes. A significant reduction in manual and automated CNBD ($P < 0.001$) and CNFD ($P < 0.0001$), and CNFL ($P < 0.0001$) occurred with increasing neuropathic severity. Manual and automated analysis methods were highly correlated for CNFD ($r = 0.9$, $P < 0.0001$), CNFL ($r = 0.89$, $P < 0.0001$), and CNBD ($r = 0.75$, $P < 0.0001$). Manual CNFD and automated CNFL were associated with the highest AUC, sensitivity/specificity and OR to rule out neuropathy.

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

Diabetic sensorimotor polyneuropathy (DSPN) is a frequent complication of diabetes affecting up to 53% of people with diabetes.[1] 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.[2] 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.[3] 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 is subjective and therefore liable to variability,[4] while skin biopsy is an invasive and costly technique, which is not routinely available across healthcare systems.[5]

We have pioneered the use of IVCCM and shown that this rapid, noninvasive ophthalmic technique can accurately quantify changes in the human subbasal nerve plexus of patients with diabetes.[6] Alterations in the subbasal corneal nerves occur early, increase with neuropathic severity,[7] and are paralleled by significant IENF loss.[8] Recent studies have shown that chronic glycemic exposure,[9] even in subjects without overt diabetes,[10] hypertension,[9] and elevated serum triglycerides,[11] are strong risk factors for corneal subbasal nerve loss. Furthermore, early reinnervation of the cornea has been shown in recipients of simultaneous pancreas and kidney transplantation (SPK).[12,13] It is important to note that other ocular diseases, such as dry eyes,[14] atopic keratoconjunctivitis,[15] epithelial membrane basement dystrophies,[16] cystic corneal disorders,[17] and other conditions[18] may also affect corneal innervation, and should therefore be excluded in any study using IVCCM in DSPN.

Concerns regarding the use of IVCCM have focused on the reproducibility[19,20] of the technique, its ability to prospectively assess neuropathy, and the absence of an automated image analysis system to allow objective corneal nerve quantification. The latter is essential to eliminate inconsistencies, produce comparable outcomes across centers, and enable the deployment of IVCCM for diagnosis, and as a surrogate endpoint in clinical trials of diabetic neuropathy. Previous studies[21–23] have proposed a variety of quantification algorithms, which differ by methodology and detection properties. In our recent work,[23]

Table 1. Medical and Peripheral Neuropathy Status

Variable	Controls, $n = 55$, NDS = 0	DSPN (−), $n = 86$, NDS ≤ 2	DSPN (+), $n = 100$, NDS > 2
Duration of diabetes	N/A	24.2 ± 21.2	34.4 ± 17.3
HbA_{1c}, %/mmol/mol‡	5.5 ± 0.3/34 ± 3.3	7.7 ± 1.6/61 ± 17.5§	7.9 ± 1.6/63 ± 17.5§
BMI, Kg/m*	25.6 ± 4.6	27.2 ± 5.2	27.6 ± 5.8‖
TC, mM‡	5.1 ± 0.9	4.3 ± 1.2§	4.4 ± 0.9§
Triglycerides, mM	1.5 ± 0.8	1.5 ± 0.9	1.4 ± 0.9
eGFR, mL/min/L‡	85.8 ± 7.8	81.8 ± 18.2	70.0 ± 24.5§‖
ACR, mg/mmol‡	1.0 ± 1.4	2.9 ± 1.3	18.8 ± 11.3§‖
BP, systolic†/diastolic, mm Hg	122 ± 16/70 ± 8.8	130 ± 18§/71 ± 9	138 ± 23§‖/72 ± 8
NSP	0	1.9 ± 3.0	5.6 ± 6.2
VPT, V‡	5.8 ± 4.6	9.2 ± 6.5§	22.3 ± 12.6§‖
WT†/CT†, °C	37.0 ± 3.0/28.2 ± 2.2	39.6 ± 3.9§/27.0 ± 9.2§	42.7 ± 4.6‖/20.8 ± 9.2§‖
HIP/CIP‡, °C	44.8 ± 2.9/11.9 ± 9.2	45.5 ± 6.6/9.8 ± 10.7	46.9 ± 7.3/4.1 ± 6.2§‖
PMNCV, m/s‡	48.8 ± 3.3	43.7 ± 4.7§	39.2 ± 6.1§‖
SSNCV, m/s‡	51.0 ± 4.8	46.4 ± 5.8§	42.2 ± 6.4§‖
PMNamp, μV‡	5.2 ± 1.8	4.5 ± 3.2	2.4 ± 2.1§‖
SSNamp, μV‡	20.0 ± 9.7	12.5 ± 7.8§	6.5 ± 6.6§‖

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 (−).

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 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, 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 elsewhere[21] (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 dorsolateral aspect of the left foot (S1) with a TSA-II

Table 2. IVCCM Assessment of DSPN Status

Variable	Controls, NDS = 0	DSPN (−), NDS ≤ 2	DSPN (+), NDS > 2
Manual IVCCM quantification			
$CNFD_M$, no./mm^2§	37.2 ± 6.7	26.7 ± 8.5‖	20.5 ± 9.5‖¶
$CNBD_M$, no./mm^2‡	92.7 ± 38.6	54.9 ± 35.7‖	48.7 ± 33.2‖
$CNFL_M$, mm/mm^2‡	26.4 ± 5.6	20.3 ± 6.7‖	16.7 ± 7.6‖¶
Automated IVCCM quantification			
$CNFD_A$, no./mm^2§	30.0 ± 6.9	20.1 ± 8.7‖	14.4 ± 8.9‖¶
$CNBD_A$, no./mm^2‡	50.4 ± 24.7	31.4 ± 25.6‖	20.1 ± 18.7‖¶
$CNFL_A$, mm/mm^2§	21.2 ± 3.5	17.1 ± 4.5‖	13.7 ± 5.2 ‖¶
Corneal sensation			
NCCA, mbar†	0.7 ± 0.5	0.9 ± 0.8‖	1.5 ± 2.1‖

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 (−).

NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel) using the method of limits.

Nerve conduction studies (NCS) were undertaken by a consultant neurophysiologist (AM) as previously described.[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[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 ± 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.[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 (NCCA) as described elsewhere.[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.[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,[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-Inman pairwise comparisons) and a P less than 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) ($P = 0.0003$), 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 ± 17.3 vs. 24.2 ± 21.2, $P = 0.01$) and were older compared with DSPN(–) (55.3 ± 12.4 vs. 47.3 ± 15.6, $P = 0.001$). 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 $CNFD_M$ ($P < 0.0001$), $CNBD_M$ ($P = 0.0005$), $CNFL_M$ ($P = 0.0002$), 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

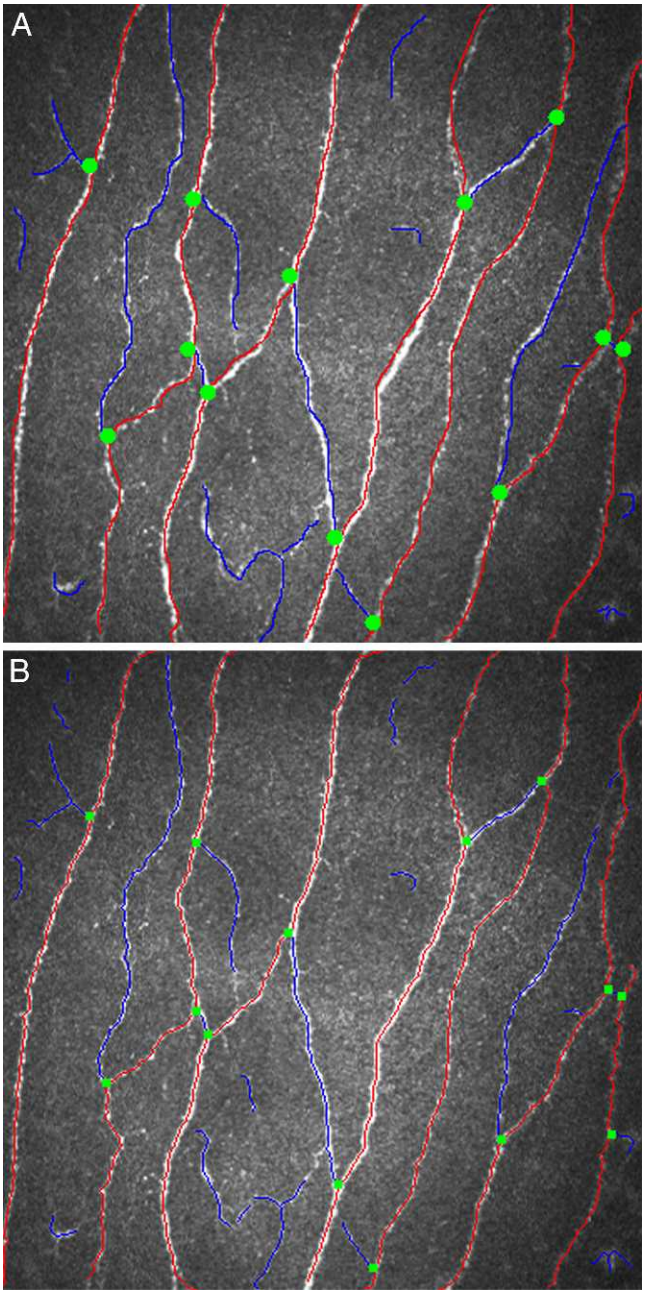

FIGURE 1. An IVCCM image of a control subject analyzed using (**A**) manual expert and (**B**) fully-automated image analysis to quantify corneal subbasal nerve morphology in DSPN. Use of either quantification method results in the detection of comparable structures in the image.

corneal sensation threshold in diabetic subjects and control subjects ($P = 0.004$). All results are presented in Table 2.

Manual Versus Automated Image Analysis

Manual and automated results were strongly correlated for CNFD (adjusted $R^2 = 0.81$, $r = 0.90$, $P < 0.0001$), CNBD (adjusted $R^2 = 0.58$, $r = 0.75$, $P < 0.0001$), and CNFL (adjusted $R^2 = 0.79$, $r = 0.89$. $P < 0.0001$) (Figs. 1A–C). Upon revaluation of the same dataset the reproducibility of the automated algorithm was excellent (ICC = 1.0) across all IVCCM parameters. Automated quantification significantly reduced image analysis time. Each image required 10 to 22 seconds to be processed automatically, while manual analysis took 2 to 7 minutes per image depending on the density of the nerves. Examples of analyzed images using the two methods are presented in Figure 1.

Validity of IVCCM Image Quantification for Diagnosis of DSPN. Receiver operating characteristic curves were inspected for concurrent optimization of sensitivity and specificity and the associated AUCs were calculated for manual and automated IVCCM parameters with respect to the study definition of "neuropathy" (Table 3).

PMNamp Less Than 1.4 μv. There were 53 (30%) diabetic patients who had neuropathy based on abnormal PMNamp. A $CNFD_M$ less than 18.7 no./mm^2 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] 3.0–6.9), and –LR = 0.3 (95% CI 0.2–0.4). The corresponding point for automated analysis was $CNFD_A$ less than 14.7 no./mm^2 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, $CNFL_M$ and $CNFL_A$ were associated with an AUC of 0.82 and 0.84 respectively, +LR = 3.23 (95% CI 2.3–4.6) and –LR = 0.33 (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 $CNFL_A$ was associated with the highest AUC (0.77) and the highest OR = 5.1. A $CNFL_A$ less than 16.1 mm/mm^2 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 $CNFL_M$ less than 19.1 mm/mm^2 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 $CNFD_M$ and $CNFD_A$ were equally capable in ruling out neuropathy. Both $CNBD_A$ and $CNBD_M$ 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 $CNFL_A$ was associated with the highest AUC (0.79) and a $CNFL_A$ less than 16.0 mm/mm^2 optimized sensitivity (0.74) and specificity (0.71) with OR = 7.2, +LR = 2.6 (95% CI 1.9–3.8), and –LR = 0.3 (95% CI 0.2–0.5). A $CNFL_M$ less than 19.7 mm/mm^2 was associated with 0.74 sensitivity and 0.63 sensitivity, AUC = 0.73, OR = 4.8, +LR = 2.0 (95% CI 1.6–2.6), and –LR = 0.4 (95% CI 0.3–0.6). Both $CNFD_A$ and $CNFD_M$ 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 $CNFD_M$ and $CNFD_A$ were associated with the highest AUC and modest OR. Specifically, a $CNFD_M$ less than 24.0/mm^2 optimized sensitivity (0.63) 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 $CNFD_M$ and a WT was 61 (64%), while 35 (37%) had reduced $CNFD_M$ with a normal WT result. All $CNFD_A$, $CNFL_M$, and $CNFL_A$ 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

Table 3. Validity and Associated Probabilities of DSPN Detection Using Manual and Automated IVCCM Parameters Quantification

Definition of DSPN	IVCCM Value (Sensitivity/Specificity)	AUC	Odds Ratio (95% CI)	+LR (95% CI)	−LR (95% CI)
PMNamp, <1.4 μV					
$CNFD_M$	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)
$CNFD_A$	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)
$CNBD_M$	41.7 (0.73/0.68)	0.75	5.9 (2.7–13.1)	2.3 (1.7–3.1)	0.4 (0.2–0.6)
$CNBD_A$	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)
$CNFL_M$	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)
$CNFL_A$	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)
SSNamp, <5.5 μV					
$CNFD_M$	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)
$CNFD_A$	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)
$CNBD_M$	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)
$CNBD_A$	23.4 (0.63/0.54)	0.70	2.1 (1.1–4.2)	1.4 (1.0–1.9)	0.7 (0.5–0.9)
$CNFL_M$	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)
$CNFL_A$	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)
PMNCV, <42.0 m/s					
$CNFD_M$	25.4 (0.78/0.70)	0.74	8.2 (4.1–17.3)	2.6 (1.9–3.7)	0.3 (0.2–0.5)
$CNFD_A$	19.7 (0.80/0.61)	0.74	7.8 (3.7–16.7)	2.2 (1.7–3.0)	0.3 (0.2–0.4)
$CNBD_M$	49.0 (0.69/0.61)	0.68	3.7 (1.9–7.2)	1.8 (1.3–2.5)	0.5 (0.4–0.7)
$CNBD_A$	24.9 (0.68/0.52)	0.67	2.4 (1.2–4.6)	1.4 (1.1–1.9)	0.6 (0.4–0.9)
$CNFL_M$	19.7 (0.74/0.63)	0.73	4.9 (2.4–9.7)	2.0 (1.5–2.8)	0.4 (0.3–0.6)
$CNFL_A$	16.0 (0.74/0.71)	0.79	7.2 (3.5–14.7)	2.6 (1.8–3.8)	0.4 (0.3–0.5)
WT, >41°C					
$CNFD_M$	24.0 (0.63/0.62)	0.69	2.9 (1.5–5.3)	1.7 (1.3–2.3)	0.6 (0.4–0.8)
$CNFD_A$	17.3 (0.63/0.60)	0.67	2.5 (1.4–4.6)	1.5 (1.2–2.1)	0.6 (0.5–0.8)
$CNBD_M$	47.2 (0.65/0.55)	0.65	2.1 (1.2–3.8)	1.4 (1.1–1.9)	0.7 (0.5–0.9)
$CNBD_A$	22.9 (0.60/0.58)	0.64	2.1 (1.1–3.9)	1.4 (1.1–2.0)	0.7 (0.5–0.9)
$CNFL_M$	19.2 (0.63/0.61)	0.67	2.7 (1.5–5.0)	1.6 (1.2–2.2)	0.6 (0.4–0.8)
$CNFL_A$	15.9 (0.61/0.61)	0.68	2.3 (1.3–4.2)	1.5 (1.1–2.1)	0.7 (0.5–0.9)

outcomes.[26] The prevalence of DSPN, in the diabetic population varies from 10% to 53%.[1,27–29] 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[30] found that when NCS was used in combination with a functional abnormality to diagnose DSPN as opposed to conventional clinical examination, twice as many patients were detected. Electrodiagnostic studies are the gold standard to diagnose neuropathy, but they are limited to large fibers and previous research has shown that small nerve fibers are affected first.[3] An objective, noninvasive surrogate of small fiber damage, such as IVCCM,[7] is therefore desirable to diagnose neuropathy early and define patients at risk.

Previous studies have identified age, duration of diabetes, renal status, BP, cardiometabolic control, and anthropometric parameters as risk factors for the onset and severity of DSPN.[29,31–33] Recent studies using IVCCM, have reported an association between levels of HbA_{1c}, BP, and triglycerides with the density of corneal innervation.[9–11] This study assessed 188 subjects with diabetes, but no other identifiable cause of neuropathy, and found that a significant decline in eGFR, increased ACR, and systolic BP were associated with neuropathy. Both diabetes groups [DSPN (+), DSPN (−)] had modest to poor metabolic control.

Corneal confocal microscopy provides the unique opportunity to repeatedly and reliably visualize the corneal nerves adjacent to Bowman's membrane. An increasing body of literature supports the use of IVCCM in the diagnosis and severity stratification of DSPN.[6,7,9,34] At present, a major drawback is the absence of an automated analysis system, which would eliminate inconsistencies and make the technique suitable to a clinical setting. This study assessed, for the first time, the performance and validity of a novel fully-automated image analysis algorithm compared with manual human expert analysis in relation to multiple gold standard clinical endpoints used to define neuropathy.

We found that both methods of image quantification were highly correlated primarily for CNFD and CNFL but also CNBD. We detected a slight underestimation of corneal nerve density and length when automated analysis was used, which was however consistent. 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 microscope properties and underlying structures. As described in our earlier work,[23] the algorithm operates through a combination of detection methods and predefined criteria, mainly nerve-specific characteristics such as orientation and axon reflectivity, to construct a connectivity map and distinguish a nerve structure from noise. In contrast, manual image analysis is a labor-intensive task, where a human investigator applies subjective criteria to define a nerve and an overestimation with less experience has been described.[20] In this study, we found a significant and progressive reduction in nerve density, branching, length between diabetic patients with and even without DSPN, and controls using either quantification method.

Corneal nerve branch density showed a significant positive correlation between manual and automated assessment, but this was not as high as for CNFD and CNFL. Corneal nerve branch density, a measurement of nerve branches directly connected to nerve fibers, has been reported to be highly variable and appears to have modest validity in diagnosing

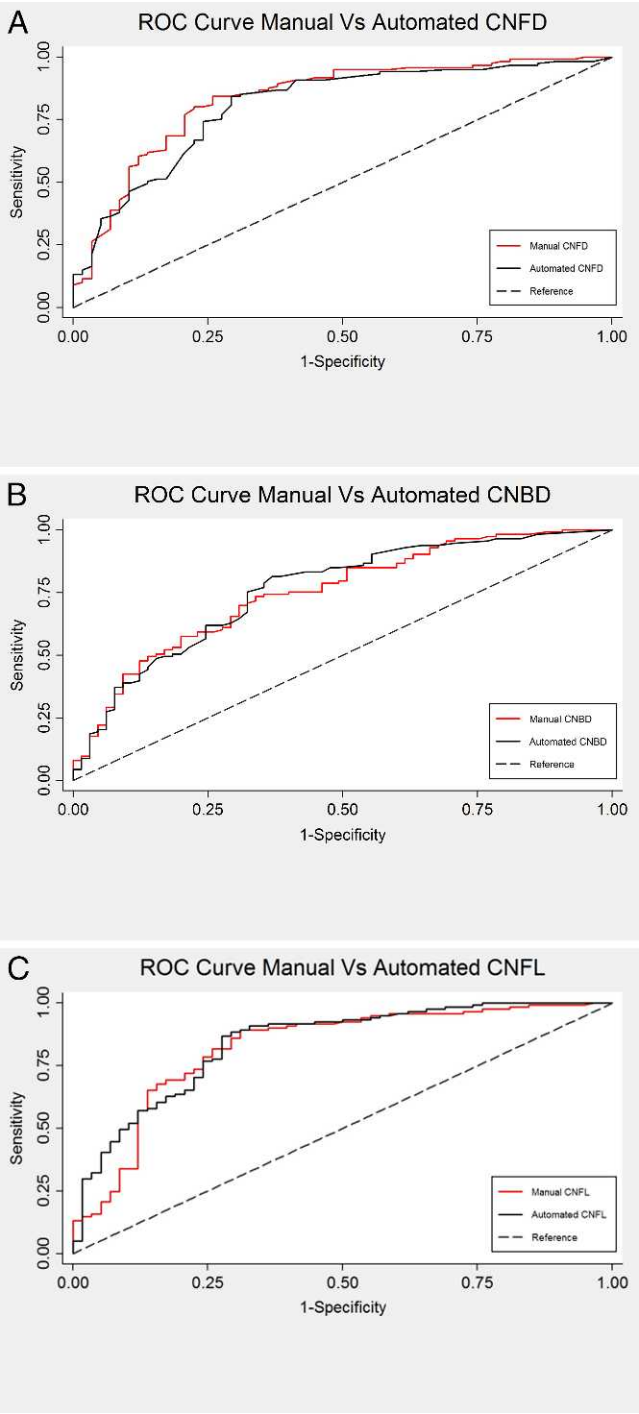

Figure 2. Receiver operating characteristic curves for manual (*solid black*) and automated (*red*) CNFD (**A**), CNBD (**B**), and CNFL (**C**). Corneal nerve fiber density and CNFL showed the highest validity to diagnose DSPN with comparable AUCs (no significant difference). Manual CNFD and automated CNFL were associated with the highest OR.

neuropathy in this and other studies.[13,34] Moreover, inter- and intraobserver estimation of the parameter in highly innervated corneas has shown moderate reproducibility.[20] The relevance of corneal nerve branching to DSPN is not clear. In our recent study,[35] of the 1-year effects of SPK transplantation in type 1 DM recipients, we found a significant and stable increase before an improvement in any other measure of regeneration. 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.[7] has reported a CNFD less than or equal to 27.8 no./mm^2 and less than or equal to 20.8 no./mm^2 as the values with the highest validity to define disease status among patients with mild and more severe neuropathy respectively. Ahmed et al.[34] found that a CNFL less than or equal to 14.0 mm/mm^2 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 $CNFD_M$, $CNFD_A$, $CNFL_M$, and $CNFL_A$ 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 $CNBD_A$ had a significantly higher AUC and OR compared with $CNBD_M$. When other endpoints of DSPN were used, such as SSNamp and PMNCV, the diagnostic validity of IVCCM remained high and $CNFL_A$ 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.[8] More recently CNFL has been related to three different measures of small fiber neuropathy.[36] 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.[37] 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 $CNFL_A$ between 14.6 mm/mm^2 and 16.1 mm/mm^2 was the value consistently associated with the highest AUC and OR given the case definition employed. Both $CNFD_M$ (18.7–25.4 no./mm^2) and $CNFD_A$ (14.7–19.7 no./mm^2) 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.[34] and Tavakoli et al.[7]; 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

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with diabetes and varying degrees of DSPN. Our results are cross-sectional and ongoing longitudinal studies[38] 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.[39]

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.

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References

1. Dyck PJ, Kratz KM, Karnes JL, et al. The prevalence by staged severity of various types of diabetic neuropathy, retinopathy, and nephropathy in a population based cohort. *Neurology*. 1993;43:817–824.
2. Tesfaye S, Boulton AJM, Dyck PJ, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. *Diabetes Care*. 2010;33:2285–2293.
3. Dyck PJ, Giannini C. Pathologic alterations in the diabetic neuropathies of humans: a review. *J Neuropathol Exp Neurol*. 1996;55:1181–1193.
4. Freeman R, Chase KP, Risk MR. Quantitative sensory testing cannot differentiate simulated sensory loss from sensory neuropathy. *Neurology*. 2003;60:465–470.
5. Lauria G, Lombardi R, Camozzi F, Devigili G. Skin biopsy for the diagnosis of peripheral neuropathy. *Histopathology*. 2009; 54:273–285.
6. Malik RA, Kallinikos P, Abbott CA, et al. Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. *Diabetologia*. 2003;46:683–688.
7. Tavakoli M, Quattrini C, Abbott C, et al. Corneal confocal microscopy: a novel non invasive test to diagnose and stratify the severity of human diabetic neuropathy. *Diabetes Care*. 2010;33:1792–1797.
8. Quattrini C, Tavakoli M, Jeziorska M, et al. Surrogate markers of small fiber damage in human diabetic neuropathy. *Diabetes*. 2007;56:2148–2154.
9. Ishibashi F, Okino M, Ishibashi M, et al. Corneal nerve fiber pathology in Japanese type 1 diabetic patients and its correlation with antecedent glycemic control and blood pressure. *J Diabetes Investig*. 2012;3:191–198.
10. Wu T, Ahmed A, Bril V, et al. Variables associated with corneal confocal microscopy parameters in healthy volunteers: implications for diabetic neuropathy screening. *Diabet Med*. 2012;29:e297–e303.
11. Tavakoli M, Marshall A, Pitceathly R, et al. Corneal confocal microscopy: a novel means to detect nerve fibre damage in idiopathic small fibre neuropathy. *Exp Neurol*. 2010;223:245–250.
12. Mehra S, Tavakoli M, Kallinikos PA, et al. Corneal confocal microscopy detects early nerve regeneration after pancreas transplantation in patients with type 1 diabetes. *Diabetes Care*. 2007;30:2608–2612.
13. Hertz P, Bril V, Orszag A, et al. Reproducibility of in vivo corneal confocal microscopy as a novel screening test for early diabetic sensorimotor polyneuropathy. *Diabet Med*. 2011;28: 1253–1260.
14. Benítez-del-Castillo JM, Acosta MC, Wassfi MA, et al. Relation between corneal innervation with confocal microscopy and corneal sensitivity with noncontact esthesiometry in patients with dry eye. *Invest Ophthalmol Vis Sci*. 2007;48:173–181.
15. Hu Y, Matsumoto Y, Adan ES, et al. Corneal in vivo confocal scanning laser microscopy in patients with atopic keratoconjunctivitis. *Ophthalmology*. 2008;115:2004–2012.
16. Rosenberg ME, Tervo TMT, Petroll WM, Vesaluoma MH. In vivo confocal microscopy of patients with corneal recurrent erosion syndrome or epithelial basement membrane dystrophy. *Ophthalmology*. 2000;107:565–573.
17. Chiou AG-Y, Kaufman SC, Beuerman RW, Ohta T, Soliman H, Kaufman HE. Confocal microscopy in cornea guttata and Fuchs' endothelial dystrophy. *Br J Ophthalmol*. 1999;83:185–189.
18. Kaufman SC, Musch DC, Belin MW, et al. Confocal microscopy: a report by the American Academy of Ophthalmology. *Ophthalmology*. 2004;111:396–406.
19. Efron N, Edwards K, Roper N, et al. Repeatability of measuring corneal subbasal nerve fiber length in individuals with type 2 diabetes. *Eye Contact Lens*. 2010;36:245–248.
20. Petropoulos IN, Manzoor T, Morgan P, et al. Repeatability of in vivo corneal confocal microscopy to quantify corneal nerve morphology. *Cornea*. 2013;32:e83–e89.
21. Scarpa F, Grisan E, Ruggeri A. Automatic recognition of corneal nerve structures in images from confocal microscopy. *Invest Ophthalmol Vis Sci*. 2008;49:4801–4807.
22. Dabbah M, Graham J, Petropoulos I, Tavakoli M, Malik R. Dual-model automatic detection of nerve-fibres in corneal confocal microscopy images. In: *Medical Image Computing and Computer-Assisted Intervention - MICCAI 2010*. Berlin: Springer Berlin Heidelberg; 2010. Vol. 6361:300–307.
23. Dabbah MA, Graham J, Petropoulos IN, Tavakoli M, Malik RA. Automatic analysis of diabetic peripheral neuropathy using multi-scale quantitative morphology of nerve fibres in corneal confocal microscopy imaging. *Med Image Anal*. 2011;15:738–747.
24. Petropoulos IN, Alam U, Fadavi H, et al. Corneal nerve loss detected with corneal confocal microscopy is symmetrical and related to the severity of diabetic polyneuropathy. *Diabetes Care*. 2013;36:3646–3651.
25. Tavakoli M, Kallinikos PA, Efron N, Boulton AJ, Malik RA. Corneal sensitivity is reduced and relates to the severity of neuropathy in patients with diabetes. *Diabetes Care*. 2007;30: 1895–1897.
26. Boulton AJM, Vileikyte L, Ragnarson-Tennvall G, Apelqvist J. The global burden of diabetic foot disease. *Lancet*. 2005;366: 1719–1724.
27. Tesfaye S, Stevens LK, Stephenson JM, et al. Prevalence of diabetic peripheral neuropathy and its relation to glycaemic control and potential risk factors: the EURODIAB IDDM Complications Study. *Diabetologia*. 1996;39:1377–1384.
28. Young M, Boulton A, Macleod A, Williams D, Sonksen P. A multicentre study of the prevalence of diabetic peripheral neuropathy in the United Kingdom hospital clinic population. *Diabetologia*. 1993;36:150–154.

29. Adler AI, Boyko EJ, Ahroni JH, Stensel V, Forsberg RC, Smith DG. Risk factors for diabetic peripheral sensory neuropathy: results of the Seattle Prospective Diabetic Foot Study. *Diabetes Care*. 1997;20:1162–1167.
30. Dyck PJ, Davies JL, Litchy WJ, O'Brien PC. Longitudinal assessment of diabetic polyneuropathy using a composite score in the Rochester Diabetic Neuropathy Study cohort. *Neurology*. 1997;49:229–239.
31. Turner R, Holman R, Cull C, et al. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). *Lancet*. 1998;352:837–853.
32. Tesfaye S, Chaturvedi N, Eaton SE, et al. Vascular risk factors and diabetic neuropathy. *N Engl J Med*. 2005;352:341–350.
33. Reichard P, Nilsson B-Y, Rosenqvist U. The effect of long-term intensified insulin treatment on the development of microvascular complications of diabetes mellitus. *N Engl J Med*. 1993; 329:304–309.
34. Ahmed A, Bril V, Orszag A, et al. Detection of diabetic sensorimotor polyneuropathy by corneal confocal microscopy in type 1 diabetes: a concurrent validity study. *Diabetes Care*. 2012;35:821–828.
35. Tavakoli M, Mitu-Pretorian M, Petropoulos IN, et al. Corneal confocal microscopy detects early nerve regeneration in diabetic neuropathy after simultaneous pancreas and kidney transplantation. *Diabetes*. 2013;62:254–260.
36. Sivaskandarajah GA, Halpern EM, Lovblom LE, et al. Structure-function relationship between corneal nerves and conventional small-fiber tests in type 1 diabetes. *Diabetes Care*. 2013; 36:2748–2755.
37. Casanova-Molla J, Grau-Junyent JM, Morales M, Valls-Sole J. On the relationship between nociceptive evoked potentials and intraepidermal nerve fiber density in painful sensory polyneuropathies. *PAIN*. 2011;152:410–418.
38. Edwards K, Pritchard N, Vagenas D, Russell A, Malik RA, Efron N. Utility of corneal confocal microscopy for assessing mild diabetic neuropathy: baseline findings of the LANDMark study. *Clin Exp Opt*. 2012;95:348–354.
39. Edwards K, Pritchard N, Gosschalk K, et al. Wide-field assessment of the human corneal subbasal nerve plexus in diabetic neuropathy using a novel mapping technique. *Cornea*. 2012;31:1078–1082.

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