

The Inferior Whorl For Detecting Diabetic Peripheral Neuropathy Using Corneal Confocal Microscopy

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PURPOSE. In vivo corneal confocal microscopy (CCM) is increasingly used as a surrogate endpoint in studies of diabetic polyneuropathy (DPN). However, it is not clear whether imaging the central cornea provides optimal diagnostic utility for DPN. Therefore, we compared nerve morphology in the central cornea and the inferior whorl, a more distal and densely innervated area located inferior and nasal to the central cornea.

METHODS. A total of 53 subjects with type 1/type 2 diabetes and 15 age-matched control subjects underwent detailed assessment of neuropathic symptoms (NPS), deficits (neuropathy disability score [NDS]), quantitative sensory testing (vibration perception threshold [VPT], cold and warm threshold [CT/WT]), and cold- and heat-induced pain [CIP/HIP]), and electrophysiology (sural and peroneal nerve conduction velocity [SSNCV/PMNCV], and sural and peroneal nerve amplitude [SSNA/PMNA]) to diagnose patients with (DPN+) and without (DPN−) neuropathy. Corneal nerve fiber density (CNFD) and length (CNFL) in the central cornea, and inferior whorl length (IWL) were quantified.

RESULTS. Comparing control subjects to DPN− and DPN+ patients, there was a significant increase in NDS (0 vs. 2.6 ± 2.3 vs. 3.3 ± 2.7 , $P < 0.01$), VPT (V; 5.4 ± 3.0 vs. 10.6 ± 10.3 vs. 17.7 ± 11.8 , $P < 0.01$), WT (°C; 37.7 ± 3.5 vs. 39.1 ± 5.1 vs. 41.7 ± 4.7 , $P < 0.05$), and a significant decrease in SSNCV (m/s; 50.2 ± 5.4 vs. 48.4 ± 5.0 vs. 39.5 ± 10.6 , $P < 0.05$), CNFD (fibers/mm²; 37.8 ± 4.9 vs. 29.7 ± 7.7 vs. 27.1 ± 9.9 , $P < 0.01$), CNFL (mm/mm²; 27.5 ± 3.6 vs. 24.4 ± 7.8 vs. 20.7 ± 7.1 , $P < 0.01$), and IWL (mm/mm²; 35.1 ± 6.5 vs. 26.2 ± 10.5 vs. 23.6 ± 11.4 , $P < 0.05$). For the diagnosis of DPN, CNFD, CNFL, and IWL achieved an area under the curve (AUC) of 0.75, 0.74, and 0.70, respectively, and a combination of IWL-CNFD achieved an AUC of 0.76.

CONCLUSIONS. The parameters of CNFD, CNFL, and IWL have a comparable ability to diagnose patients with DPN. However, IWL detects an abnormality even in patients without DPN. Combining IWL with CNFD may improve the diagnostic performance of CCM.

Keywords: corneal confocal microscopy, diabetic neuropathy, image analysis

Neuropathy affects at least 50% of patients with diabetes,¹ and is associated with increased morbidity in the form of painful neuropathy² and foot ulceration, but also increased mortality.³ Quantifying the corneal subbasal innervation using corneal confocal microscopy (CCM) has provided important diagnostic⁴ and prognostic⁵ information in diabetic patients. A perceived limitation of CCM is the relatively small area of central corneal tissue scanned in the majority of studies. A variety of enhanced imaging techniques, which include central in-depth point scanning,⁴ wide-field assessment,⁶ image montage, and reconstruction techniques,^{7,8} as well as central and pericentral scanning,⁹ have been used to quantify a range of corneal nerve parameters.^{4,10,11} The estimation of corneal nerve fiber length (CNFL) and density (CNFD) via a central scan has been associated with the highest validity to diagnose

diabetic polyneuropathy (DPN),^{4,12} and recently a semiquantitative grading scale has been shown to be reliable, precise, and accurate compared to CNFL.¹³ Furthermore, automated, semi-automated, and manual quantification of CNFL has been found to have a comparable ability to diagnose DPN.¹⁴ While the tortuosity-standardized CNFL has been shown to be better than CNFL in differentiating diabetic patients with and without neuropathy, both metrics were derived from the same central images.¹⁵

Recently, a pilot study using a novel mapping technique, revealed a patchy pattern for corneal nerve loss in a patient with severe neuropathy, mostly affecting the central and inferior cornea.⁶ In a study of experimental diabetes, patchy corneal nerve degradation with focal loss has been demonstrated in the inferior whorl (IW).¹⁶ The IW has been

identified previously as an area with a vortex-like pattern, located inferior and slightly nasal to the corneal apex.¹⁷ Its characteristic appearance makes it an ideal anatomical landmark for consistent scanning in the cornea and a previous study has used this area to measure nerve migration over time.¹⁸ It is unknown whether defects in these different areas occur concomitantly, one precedes the other, or whether an alternative imaging strategy that combines more than one area could improve the diagnostic performance of CCM, while keeping image acquisition time within acceptable limits. The aim of this cross-sectional, observational study was to compare the ability of CNFD, CNFL, and inferior whorl length (IWL) alone and in combination for the diagnosis of DPN.

METHODS

Study Subjects

A total of 53 patients with type 1/type 2 diabetes and 15 healthy, age-matched controls underwent a comprehensive assessment of peripheral neuropathy and in vivo CCM. 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 subjects before participation in the study. Participants were excluded if they had a positive history of malignancy, connective tissue or infectious disease, deficiency of vitamin B12 or folate, chronic renal failure, liver failure, active diabetic foot ulceration, or a family history of peripheral neuropathy. Participants also were excluded if they had active ocular disease, systemic disease known to affect the cornea other than diabetes, or cystic corneal disorders.

Clinical and Peripheral Neuropathy Assessment

All study participants underwent assessment of body mass index (BMI), glycated hemoglobin (HbA_{1c}), lipid fractions (high [HDL]C, low density lipoprotein cholesterol [LDL]C, total cholesterol [TC] and triglycerides), and estimated glomerular filtration rate (eGFR). Evaluation of DPN was based on an assessment of neuropathic symptoms (NSP) and deficits (neuropathy disability score [NDS])¹⁹; vibration perception threshold (VPT) on the hallux using a Neurothesiometer (Horwell; Scientific Laboratory Supplies, Wilford, Nottingham, UK), cold (CT) and warm (WT) threshold, cold-induced (CIP) and heat-induced (HIP) pain on the dorsolateral aspect of the left foot (S1) with a TSA-II NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel) using the method of limits. Electrodiagnostic studies were undertaken using a Dantec “Keypoint” system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32°C and 35°C. Sural nerve amplitude (SSNA), sural nerve conduction velocity (SSNCV), peripheral nerve amplitude (PMNA), and peripheral nerve conduction velocity (PMNCV) were assessed by a consultant neurophysiologist.²⁰ Presence of DPN was defined in accord with the Toronto consensus,²¹ which requires presence of signs of neuropathy (NDS ≥ 2) and at least one abnormality among two nerve electrophysiology parameters (PMNCV < 40 m/s, PMNA < 2.0).

Corneal Confocal Microscopy

Both eyes of all participants were scanned with a CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module; Heidelberg Engineering GmbH, Heidelberg, Germany) using our established procedure.²⁰ The overall examination took a

maximum of 10 minutes/subject, and images were obtained from the central and inferior corneal locations at the level of the subbasal nerve plexus using the “section” mode. A charge couple device camera attached to the microscope and anatomical landmarks were used to identify the areas of interest. Initially, the subject was asked to fixate with the fellow eye on an outer fixation target and the central area, that is, the corneal apex, was scanned. After a sufficient number of images were captured ($n \approx 50$), the fixation target was moved slowly upwards and the subject was asked to follow the target until the IW became visible. As the IW is not always vertical to the apex, the examiner had to move the target to left/right directions occasionally. Gentle forward and backwards movements of the tip of the microscope optimized image contrast and visibility of the subbasal nerves in both areas. Based on a previous immunohistochemistry study of the human cornea,¹⁷ the whole subbasal nerve plexus occupies an area approximately 6 mm in diameter and the anatomical center of the IW ranges between 2.18 and 2.92 mm, from the corneal apex. Images ($n \approx 20$) were taken from the center of the whorl and more proximal areas. The same scanning protocol then was applied to the contralateral eye.

Image Selection and Quantification

Six central and 8 IW images, equally divided, from both eyes of each participant were selected for quantification. There is no consensus on image selection for assessment of DPN-related nerve damage with CCM. Common criteria in previous studies^{12,20,22} have been image quality and location. Vagenas et al.⁹ have reported that five to eight images provide adequate accuracy to quantify corneal innervation, but this study focused on central and pericentral corneal locations and did not include the IW. The number of IW images selected for analysis was chosen based on image quality and the variability observed in the area, and included one image centered on the whorl and three images of the surrounding areas, in which the IW still was visible in varying locations (upper right/left corner and bottom right corner, Fig. 1). Three parameters were quantified by two experienced examiners as described in our previous work²³: CNFD (fibers/mm²) and CNFL (mm/mm²) in central CCM images. Due to the highly complex pattern of nerves in the IW it is not possible to distinguish major nerves from branches. Therefore, the IWL (mm/mm²), which assesses the length of all nerve structures in the whorl region, was quantified.

Inter- and Intraobserver Agreement of IWL

To ensure that the IWL can be assessed consistently between different observers, we conducted an experiment whereby two different observers assessed a subset of patients with diabetes ($n = 11$) on the same day on two consecutive examinations. To assess interobserver agreement, both observers (masked from one another) were asked to determine from their set of captured images which image best represents the whorl center and then estimate IWL. Subsequently, to assess intraobserver agreement, one of the two observers (observer 1), after a washout period of 1 day, repeated the exact same procedure and re-estimated the IWL.

Statistical Analysis

We used SPSS (Version 19 for Macintosh; IBM Statistics, Yorktown Heights, NY, USA) and OriginPro (Version 8.5 for Windows; Origin Lab, Northampton, MA, USA) for data analysis and plotting. The 1-way ANOVA or nonparametric Kruskal

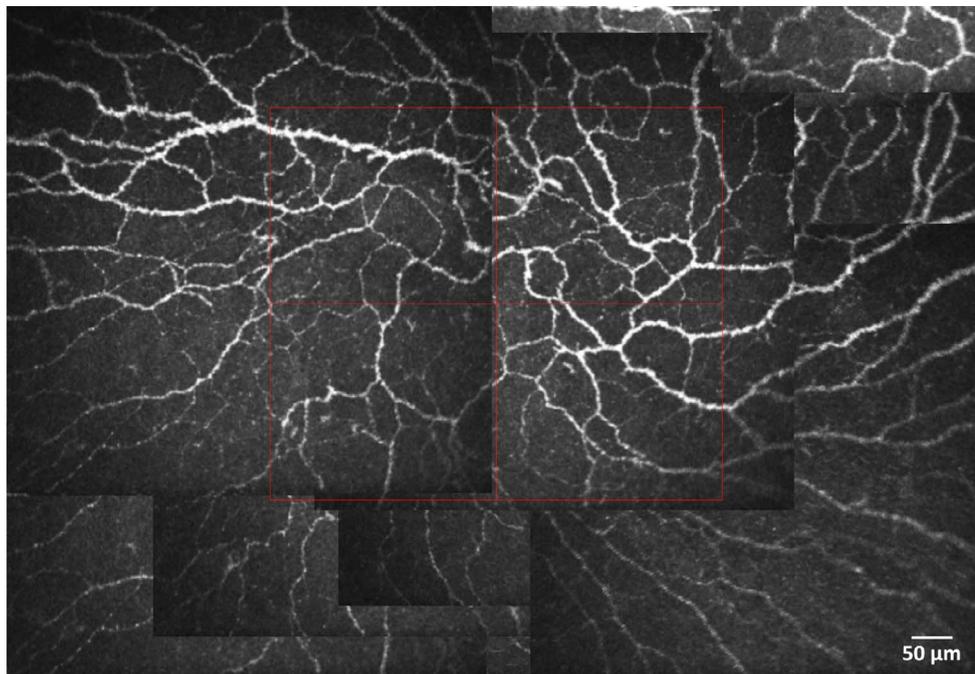


FIGURE 1. A montage of CCM images from the IW region demonstrating the highly complex pattern of nerves. The *grid* indicates the area of images captured for the estimation of IWL.

Wallis (eGFR, triglycerides, LDLC, HbA_{1c}, BMI, NPS, VPT, CT, WT, CIP, HIP, SSNA) were used for group comparisons and a $P < 0.05$ was considered significant (post hoc Tukey or nonparametric Conover-Inman). Spearman's correlation coefficient (r_s) was calculated to assess the relationship between the corneal nerve parameters per subject. Receiver operating characteristic (ROC) curve analysis was performed for each of the corneal nerve parameters or combinations of these parameters, for example, CNFD + CNFL, CNFD + IWL, CNFL + IWL, or CNFD + CNFL + IWL. The area under the curve (AUC) and the ability of each parameter to diagnose DPN was determined by selecting the point that concurrently optimized sensitivity and specificity at a ratio of 1:1. For the assessment of inter- and intraobserver agreement Spearman's r ; the Bland-Altman plots and a t -test were used to assess differences between measurements.

RESULTS

Clinical Assessment

Based on the case definition of neuropathy used in this study, patients were classified into those without (DPN⁻, $n = 28$) or with (DPN⁺, $n = 25$) neuropathy. There was no significant difference in age between controls and subjects with diabetes; however, subjects with DPN⁺ were older than DPN⁻ (60.1 ± 10.2 vs. 42.4 ± 14.7 , $P < 0.0001$). Systolic blood pressure (134 ± 16 vs. 120 ± 15 mm Hg, $P = 0.03$) and HbA_{1c} (%) (7.6 ± 1.5 vs. 5.4 ± 0.5 , $P < 0.0001$) were significantly higher, and TC (4.2 ± 1.2 vs. 5.0 ± 0.7 mmol/L, $P = 0.0007$) and LDLC (2.1 ± 1.0 vs. 2.7 ± 0.7 mmol/L, $P = 0.004$) were significantly lower in DPN⁺ compared to controls, due to statin use in patients with diabetes. There was no significant difference in diastolic blood pressure (69 ± 6 vs. 65 ± 9 mm Hg, $P > 0.05$), BMI (28.6 ± 6.0 vs. 25.9 ± 5.6 kg/m², $P > 0.05$), eGFR (72.8 ± 19.8 vs. 86.5 ± 5.2 mL/min/1.73m², $P > 0.05$), HDLC (1.5 ± 0.4 vs. 1.5 ± 0.4 mmol/L, $P > 0.05$), and triglycerides (1.4 ± 0.6 vs. 1.7 ± 1.1 mmol/L, $P > 0.05$) between DPN⁺ and

controls. There were no significant differences in clinical parameters between DPN⁺ and DPN⁻ patients.

Neuropathy Assessment

All neuropathy assessment results for controls and subjects with diabetes are presented in Table 1. The VPT (V; 16.9 ± 9.3 vs. 5.8 ± 3.8 , $P < 0.0001$) and HIP (°C; 47.9 ± 2.4 vs. 45.7 ± 3.5 , $P = 0.03$) were significantly higher, and CT (°C; 23.5 ± 6.9 vs. 27.5 ± 4.2 , $P = 0.001$), PMNA (mV; 1.6 ± 1.1 vs. 5.4 ± 1.4 , $P < 0.0001$), PMNCV (m/s; 39.0 ± 5.9 vs. 47.7 ± 5.5 , $P < 0.0001$), SSNA (μV; 7.7 ± 5.6 vs. 18.5 ± 7.1 , $P < 0.0001$), and SSNCV (m/s; 40.0 ± 10.2 vs. 49.6 ± 5.7 , $P < 0.0001$) were significantly lower in DPN⁺ compared to controls. Only SSNCV (46.5 ± 4.8 vs. 49.6 ± 5.7 m/s, $P = 0.03$) also was significantly lower in DPN⁻ compared to controls.

Corneal Confocal Microscopy

A significant reduction was found in CNFD (fibers/mm²; 22.4 ± 9.6 vs. 37.2 ± 5.9 , $P < 0.0001$), CNFL (mm/mm²; 17.0 ± 7.5 vs. 27.5 ± 4.6 , $P < 0.0001$), and IWL (mm/mm²; 18.2 ± 8.0 vs. 31.5 ± 7.7 , $P < 0.0001$) in DPN⁺ compared to controls (Table 1). While CNFD and CNFL did not differ, IWL also was significantly lower in DPN⁻ compared to controls (25.0 ± 9.0 vs. 31.5 ± 7.7 , $P = 0.03$, Fig. 2). The CNFD and CNFL parameters were highly significantly correlated with each other ($r_s = 0.8$, $P < 0.0001$), and IWL correlated with CNFD ($r_s = 0.51$, $P = 0.002$) and CNFL ($r_s = 0.68$, $P < 0.0001$).

For the diagnosis of DPN, ROC curve analysis (Table 2) showed that a CNFL = 21.9 mm/mm² had the highest AUC = 0.75 and sensitivity/specificity = 0.76/0.65, followed by CNFD = 28.4 fibers/mm² with an AUC = 0.74 and sensitivity/specificity = 0.68/0.61, and IWL = 20.0 mm/mm² with an AUC = 0.70 and sensitivity/specificity = 0.68/0.67. A combination of CNFL and IWL outperformed all other combinations of parameters as it was associated with the highest AUC = 0.75 and a sensitivity/specificity = 0.80/0.71 for DPN (Fig. 3).

TABLE 1. Clinical and Peripheral Neuropathy Status

Variable	Controls, <i>n</i> = 15 No Neuropathy	DPN-, <i>n</i> = 28	DPN+, <i>n</i> = 25
Duration of diabetes	N/A	16.2 ± 9.3	24.8 ± 19.5
NPS*	0	2.3 ± 5.1†‡	2.5 ± 2.2‡§
NDS*	0	1.7 ± 1.5†‡	3.9 ± 2.5‡§
VPT, V*	5.8 ± 3.8	6.6 ± 4.3	16.9 ± 9.3¶
CT/WT, °C*	27.5 ± 4.2/38.5 ± 4.3	26.0 ± 5.7/39.5 ± 4.0	23.5 ± 6.9¶§/41.3 ± 3.9
CIP/HIP, °C*	12.6 ± 10.5/45.7 ± 3.5	12.5 ± 9.0/46.1 ± 2.4	7.4 ± 8.0/47.9 ± 2.4†**
PMNCV, m/s	47.7 ± 5.5	45.5 ± 3.8	39.0 ± 5.9¶
SSNCV, m/s	49.6 ± 5.7	46.5 ± 4.8†**	40.0 ± 10.2¶
PMNA, mV	5.4 ± 1.4	5.8 ± 3.2	1.6 ± 1.1¶
SSNA, μV*	18.5 ± 7.1	16.2 ± 7.7	7.7 ± 5.6¶
CNFD, no./mm ²	37.2 ± 5.9	31.2 ± 8.2	22.4 ± 9.6¶
CNFL, no./mm ²	27.5 ± 4.6	23.4 ± 7.0	17.0 ± 7.5¶
IWL, no./mm ²	31.5 ± 7.7	25.0 ± 9.0†**	18.2 ± 8.0¶

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

* Nonparametric test was used for comparison.

† Post hoc results for DPN+ significantly different from control subjects.

‡ Post hoc results for DPN+ significantly different from DPN-.

§ *P* < 0.05.

¶ *P* < 0.01.

|| *P* < 0.001.

** *P* < 0.0001.

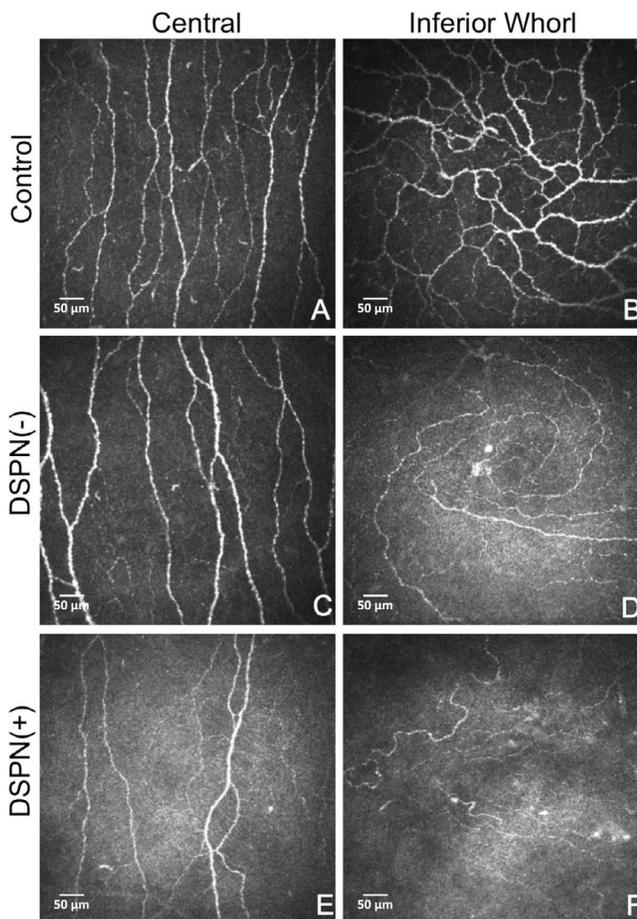


FIGURE 2. Central corneal and corresponding IW CCM images for a control subject (A, B) and a DPN- (B, C) and DPN+ (C, D) patient demonstrating nerve depletion affecting both areas and the IW particularly in DPN-.

Inter- and Intraobserver Agreement

There was excellent inter- and intraobserver agreement for the estimation of IWL (Figs. 4A, 4B). There was no significant difference (17.8 ± 8.5 vs. 17.7 ± 9.1 mm/mm², *P* > 0.05) between observers 1 and 2 with a strong correlation (Spearman's *r* = 0.96, *P* < 0.0001; Fig. 4C). There was no significant difference (17.8 ± 8.5 vs. 17.2 ± 8.0 mm/mm², *P* > 0.05) when the same observer assessed and reassessed images of the IW center selected from the same dataset on two separate occasions. The measurements by the same observer on two subsequent days also were strongly correlated (Spearman's *r* = 0.99, *P* < 0.0001, Fig. 4C).

DISCUSSION

Over the past decade, in vivo CCM has emerged as a powerful endpoint to quantify and stage the severity of human DPN, due to its rapid noninvasive ability to acquire and quantify corneal nerve damage. Several groups have shown significant decreases in corneal subbasal innervation in patients with minimal and more advanced neuropathy,^{5,20,22,24-27} and more recently, in experimental models of diabetes.²⁸⁻³⁰ The majority of studies in humans have visualized the central and pericentral cornea to quantify subbasal nerve density and length. A limitation of CCM is the relatively small field of view, which allows only a proportion of the total subbasal nerve plexus to be scanned at any given time. To overcome this, studies have proposed alternative image acquisition methods,⁶ including fully-automated montaging of CCM images.⁸ However, the clinical use of these techniques remains to be established given the much longer image acquisition times and a lack of validation in larger cohorts of patients.

Edwards et al.⁶ have described a novel, wide-field assessment technique that allows real time mapping of the subbasal nerves and visualization of the entire nerve plexus. Their study revealed patchy nerve loss in a patient with severe neuropathy, mostly affecting the central and inferior areas, including the IW, compared to a patient without neuropathy and minimal corneal nerve alterations. This patchy pattern of corneal nerve loss with focal reduction in the IW region also has been

TABLE 2. Performance of CCM Parameters to Diagnose Neuropathy

	AUC	Sensitivity	Specificity
Single parameters			
CNFD	0.74 (28.4 fibers/mm ²)	0.68	0.61
CNFL	0.75 (21.9 mm/mm ²)	0.76	0.65
IWL	0.70 (20.0 mm/mm ²)	0.68	0.68
Combined parameters			
CNFD + CNFL	0.74	0.72	0.71
CNFD + IWL	0.74	0.80	0.71
CNFL + IWL	0.75	0.80	0.71
CNFD+ CNFL + IWL	0.74	0.80	0.70

described in experimental DPN, and was associated with epithelial thinning and reduced basal cell density.¹⁶ Furthermore, Davidson et al.³¹ showed that corneal nerve loss occurred predominantly in the IW region and before loss of nerves in the central cornea, leading the authors to suggest that if this could be translated to patients then it may allow the detection of earlier nerve damage, thereby improving the diagnostic ability of CCM. They also showed that only a fraction of the subbasal nerves were visible on IVCCM, compared to in vitro imaging, and suggested that further enhancement of visualization with CCM would likely improve our ability to detect early neuropathic changes. Recently, Marfurt et al.¹⁷ have undertaken detailed immunohistochemical staining of the entire subbasal nerve plexus in 16 donor corneas and showed that a gentle, spiral-like structure of long, curvilinear nerve fibers converges on a vortex, located inferior

and slightly nasal to the apex. Given that DPN is a length-dependent, symmetrical neuropathy with initial involvement of the most distal sensory nerves,³² one may expect nerves in the IW, the most distal part of the subbasal plexus, to be depleted before the more proximal central nerves. Indeed, in this study we showed that patients with DPN had a significantly reduced CNFD, CNFL, and IWL compared to patients without DPN and controls. However, in addition, we showed that patients without DPN showed a significant reduction in IWL, but no difference in CNFD or CNFL when compared to controls. This suggests that a reduction in IWL may well be the earliest defect in corneal nerve pathology as previously observed in experimental diabetes.³¹

We also have shown that that the diagnostic performance of IWL is not as good as CNFD and CNFL in diagnosing diabetic patients with neuropathy. This is not surprising given that IWL is already abnormal in diabetic patients without neuropathy. However, combining IWL with CNFD and CNFL, indeed, all three metrics increased the sensitivity of CCM for the diagnosis of DPN. Hence, a scanning protocol that incorporates imaging of the central and IW regions may offer diagnostic advantage over central scanning alone. Perhaps a real-time mapping technique, such as that described by Edwards et al.,⁶ may be superior to any point scanning methodology. However, the scanning time of 15 to 20 minutes per cornea can be uncomfortable to the patient and suboptimal for a healthcare system. Furthermore, if IVCCM is to be deployed for neuropathy screening and, indeed, in children,²⁶ then scanning should be rapid and well tolerated.

This study is cross-sectional and, therefore, does not address the question of how the IWL may perform prospectively. Identification of the IW center may be challenging, especially in patients with a loss of corneal nerve fibers (Fig. 2F). To

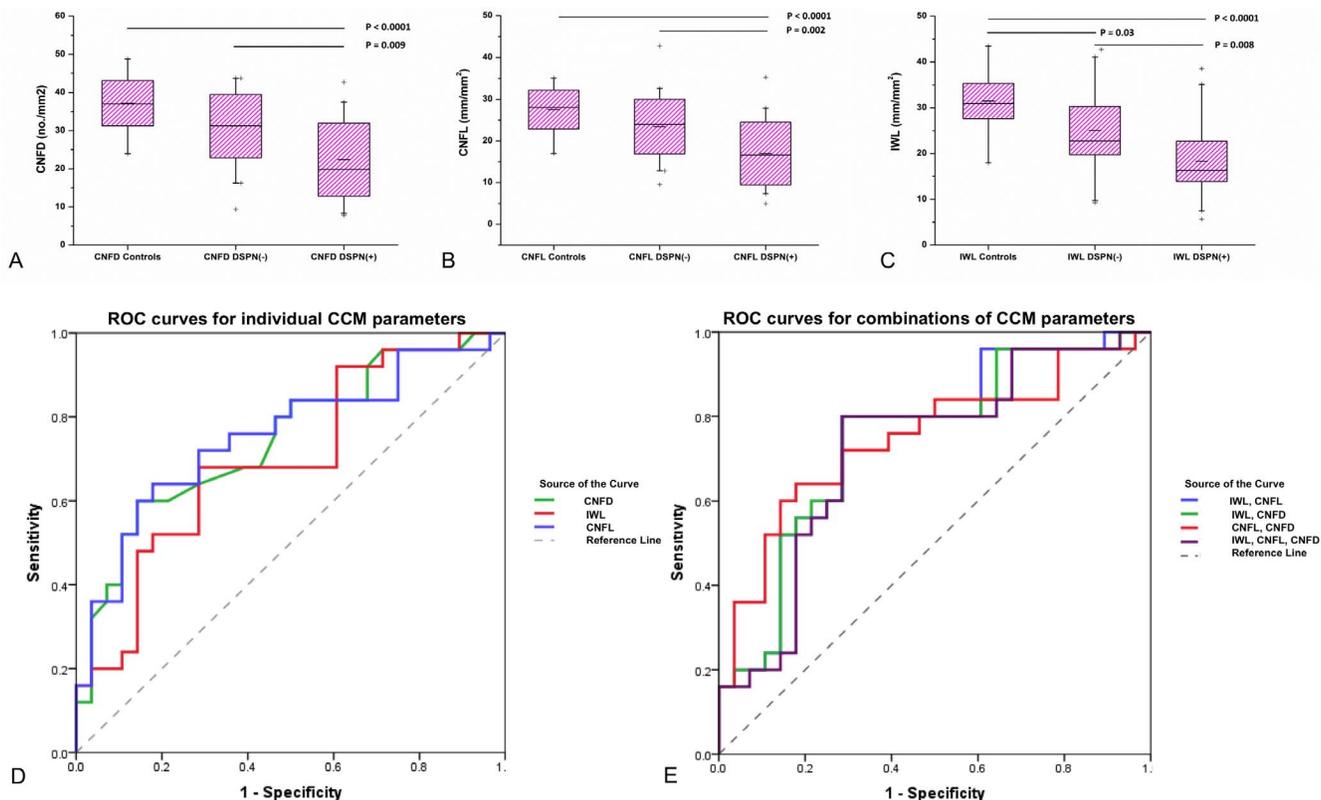


FIGURE 3. Box plots for CNFD (A), CNFL (B), and IWL (C) in controls, DPN- and DPN+. The ROC curves demonstrating the efficacy of individual parameters (D) and various combinations (E) for detecting DSPN.

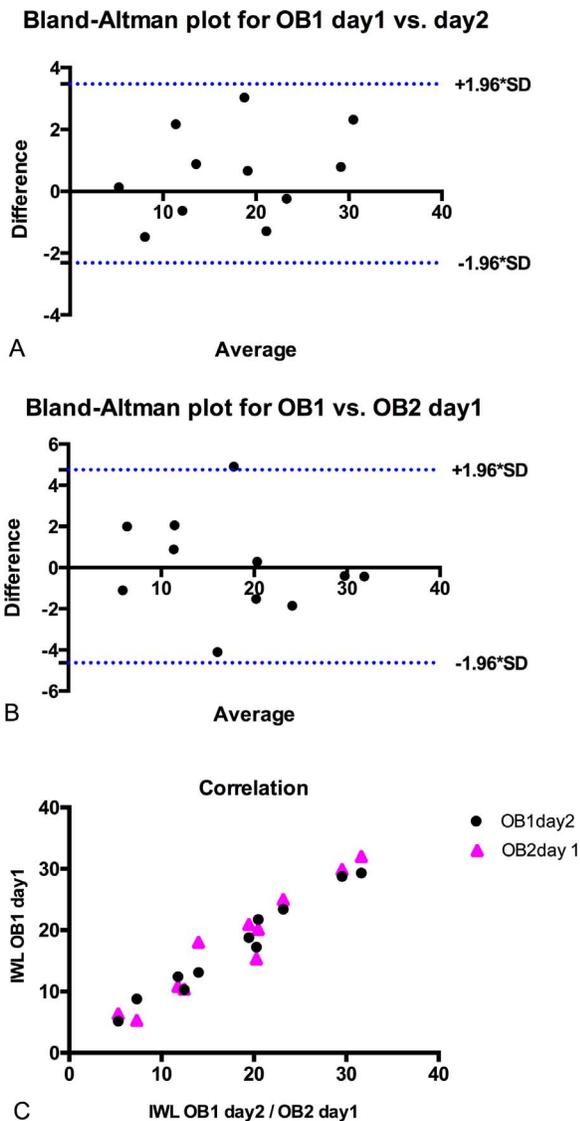


FIGURE 4. Bland-Altman plots with upper and lower limits of agreement ($\pm 1.96 \times SD$, blue dashed line) for intraobserver (A) and interobserver (B) agreement, and a correlation plot (C) for intraobserver (OB1, black circle) and interobserver (OB2, pink triangle) measurements.

address this limitation we have undertaken an analysis, which demonstrates excellent inter- and intraobserver agreement in identifying the IW and assessing the IWL. However, a larger study should evaluate whether this finding is influenced by neuropathic severity.

In conclusion, to our knowledge this study is the first to compare nerve pathology in central and inferior regions of the cornea in patients with and without diabetic neuropathy. We showed that a reduction in corneal nerve length at the IW occurs earlier than in the central cornea and combining this abnormality with changes in the central cornea may improve the diagnostic performance of in vivo CCM.

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