



Comparison of Novel Wide-Field In Vivo Corneal Confocal Microscopy With Skin Biopsy for Assessing Peripheral Neuropathy in Type 2 Diabetes

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Diabetic peripheral neuropathy (DPN) is a serious complication of diabetes, where skin biopsy assessing intraepidermal nerve fiber density (IENFD) plays an important diagnostic role. In vivo confocal microscopy (IVCM) of the corneal subbasal nerve plexus has been proposed as a noninvasive diagnostic modality for DPN. Direct comparisons of skin biopsy and IVCM in controlled cohorts are lacking, as IVCM relies on subjective selection of images depicting only 0.2% of the nerve plexus. We compared these diagnostic modalities in a fixed-age cohort of 41 participants with type 2 diabetes and 36 healthy participants using machine algorithms to create wide-field image mosaics and quantify nerves in an area 37 times the size of prior studies to avoid human bias. In the same participants, and at the same time point, no correlation between IENFD and corneal nerve density was found. Corneal nerve density did not correlate with clinical measures of DPN, including neuropathy symptom and disability scores, nerve conduction studies, or quantitative sensory tests. Our findings indicate that corneal and intraepidermal nerves likely mirror different aspects of nerve degeneration, where only intraepidermal nerves appear to reflect the clinical status of DPN, suggesting that scrutiny is warranted concerning methodologies of studies using corneal nerves to assess DPN.

Diabetic peripheral neuropathy (DPN) is characterized by a symmetrical and predominantly sensory neuropathy,

ARTICLE HIGHLIGHTS

- Comparison of intraepidermal nerve fiber density with automated wide-field corneal nerve fiber density in participants with type 2 diabetes revealed no correlation between these parameters.
- Intraepidermal and corneal nerve fibers both detected neurodegeneration in type 2 diabetes, but only intraepidermal nerve fibers were associated with clinical measures of diabetic peripheral neuropathy.
- A lack of association of corneal nerves with peripheral neuropathy measures suggests that corneal nerve fibers may be a poor biomarker for diabetic peripheral neuropathy.

with loss of sensory function initially affecting the distal parts of the lower extremities. DPN is the most common type of neuropathy globally, and >50% of patients with type 2 diabetes will develop a neuropathy that affects the peripheral nerves during their lifetime (1–5). Moreover, DPN is considered a risk factor for mortality in type 2 diabetes and has a prevalence that increases with age and duration of diabetes. Moreover, the level of glycosylated hemoglobin (HbA_{1c}) and hyperlipidemia, obesity, and smoking have all been found to have an association with DPN (6–8).

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In small fiber neuropathy (SFN), the thinly myelinated A δ and unmyelinated C fibers are mainly affected (9,10). SFN is a neuropathy subtype that can be diagnosed from skin biopsy samples (dermal punch biopsy at calf or thigh), where the intraepidermal nerve fiber density (IENFD) is determined by immunostaining of histologic skin sections. Skin biopsy is an established method to quantify SFN (11), consisting of morphologic quantification of nociceptor axons at the level of the basement membrane of the epidermis (12–14). IENFD has been reported to have high diagnostic efficacy (88%), positive predictive value (75%), and negative predictive value (90%) for neuropathy (15). Reduced IENFD has been associated with the risk of neuropathic pain development but not with its intensity (16–18).

Diagnosis of DPN is often based on several diagnostic criteria complementary to IENFD, including neuropathic symptoms, neuropathic deficits, pathological nerve conduction studies, pathological quantitative sensory testing, and pathological quantitative autonomic testing (19,20). Studies have shown temporal deterioration in IENFD in healthy participants as well as in participants with type 2 diabetes (15,17,18,21,22). IENFD findings can be impacted by the staining method used, as IENFD values have been shown to be greater with immunofluorescence relative to the brightfield staining method (23). Additionally, skin biopsy is invasive, and the results are not immediately available. As a complementary or alternative means to diagnose DPN, noninvasive *in vivo* confocal microscopy (IVCM) of the cornea has gained increasing attention (24–26).

The cornea contains nerve fibers (called subbasal nerves) of both A δ and C subtypes that are arranged in a dense nerve plexus that can be noninvasively imaged and quantified, owing to the cornea's transparency and accessibility of the tissue for *in vivo* examination. Quantitative measurement of corneal subbasal nerve fiber length density (CNFL) is a parameter often evaluated as a surrogate biomarker in the diagnosis of SFN (7,24,27–30). The relationship of CNFL with IENFD and the ability of corneal nerve fiber changes to serve as a noninvasive marker for DPN, and thus, as an alternative to invasive IENFD, is presently unclear. Therefore, in this study, we aimed to objectively compare these methods.

To overcome the limitations of IVCM in imaging only a small area of the corneal subbasal nerve plexus and potential bias in manual selection of corneal nerve images, we used a specialized imaging protocol and software to automatically stitch together wide-field images (mosaics) of the corneal subbasal nerve plexus, constituting a corneal area of 6 mm² in size, which is 37 times larger than the area represented by single-nerve images in prior studies (31). We also applied fully automated computer algorithms to detect and quantify nerve parameters without human involvement to further reduce bias. We present results in terms of a new parameter, the mosaic CNFL (mCNFL), that reflects the corneal subbasal nerve density in a wide-field area as opposed to small images (32).

IVCM has been suggested to be advantageous relative to invasive methods, such as skin biopsy and other clinical methods of assessing DPN (27,28,30,33). However, methodological limitations of quantifying corneal nerves have led to conflicting results regarding the utility of IVCM versus skin biopsy and clinical measures of DPN (34,35). For this reason, we aimed to evaluate the diagnostic utility and efficacy of IENFD relative to corneal nerve density using the largest area of the subbasal plexus examined to date in a diabetes cohort, using the mCNFL parameter to avoid human image selection bias. We further investigated the relationship of mCNFL with other clinical measures of DPN in a population with type 2 diabetes. The findings could have important implications for the future detection, monitoring, and management of DPN.

RESEARCH DESIGN AND METHODS

Study Design and Subjects

Participants were included in the study as part of a prospective, longitudinal, population-based study in Sweden. The participants, who were matched for sex and age, were initially recruited to the cohort and examined from 2004 to 2007 at the baseline examination and thereafter underwent follow-up examinations in 2014, as described in detail elsewhere (32,36). The present inclusion criterion was being a participant in the baseline study, wherein the initial baseline study, participants were recruited with normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and type 2 diabetes; the mean age was 60 $\pm$ 1 years. The NGT and IGT groups underwent two oral glucose tolerance tests, 1 week apart, to establish their glucose status, where both results should be within the cutoff values for fasting plasma glucose (fPG) and 2-h plasma glucose (2hPG) as follows (NGT: fPG <7.0 mmol/L and 2hPG <8.9 mmol/L; IGT: fPG <7.0 and 2hPG $\geq$ 8.9 to <12.2 mmol/L based on the 1999 World Health Organization definition of diabetes) (37). Exclusion criteria consisted of nutritional deficiencies (four individuals were vitamin B12 and folate deficient) and neuropathy asymmetry due to sciatica and stroke (three individuals). The study was conducted according to the tenets of the Declaration of Helsinki; participants provided informed consent. The study protocol was approved by the local ethics committee in Umeå, Sweden (Ethical Application no. 2013-21-31 M) (32).

Clinical Examinations

Study participants underwent a wide range of examinations, including laboratory tests, peripheral neurological examinations, a clinical signs and symptoms evaluation (Dyck Neuropathy Disability Score [NDS] and Neuropathy Symptom Score [NSS], and skin biopsy for assessment of IENFD). The diagnostic criteria for peripheral neuropathy in participants were absent ankle reflexes, and/or reduced sensory perception, and/or neuropathic symptoms in toes or feet (20,38).

Neuropathy Assessment

Clinical examinations included peripheral neurologic examination of nerve function, which was evaluated using NDS and NSS to assess the severity and incidence of neuropathy, respectively. NSS and NDS were performed using the modified Dyck scale (20). NSS addresses the presence or absence of symptoms in the feet, including paresthesia (numbness), abnormal sensation for heat and cold, touch (dysesthesia), pins-and-needles sensation, and various types of pain sensation (burning, dull, or stabbing). NDS assesses the severity of symptoms, which is then scored from 0 to 3 (0 indicating lack of symptoms; 1, sometimes; 2, often; and 3, during most nights), resulting in a range of scores from 0 to 21 (39,40).

Sensory and Nerve Conduction Tests

A single neurophysiologist, masked to the diabetes status of participants, performed nerve conduction measurements at the Clinical Neurophysiology Laboratory, Umeå University. Measurements included the amplitude and conduction velocity of the sural nerve and conduction velocity of the peroneal nerve, all measured on the right leg. Quantitative sensory testing consisted of measurement of heat and cold perception thresholds measured at the dorsum of the right and left foot in each participant using ThermoTest equipment (Somedic AB, Hörby, Sweden).

Skin Biopsy and Immunohistochemistry

The skin biopsy for the assessment of IENFD was taken by only one examiner using a 3-mm disposable punch biopsy instrument and taking dermal samples from a location 10 cm above the lateral malleolus of the right leg of all study participants. The wound was allowed to heal spontaneously without sutures. The skin biopsy samples for the assessment of IENFD were taken at baseline (2004–2007) and follow-up (2014), and specimens were processed for immunohistochemistry as previously detailed (38).

IENFD Assessment

Light microscopy was used to perform the counting of nerve fibers and measurement of epidermal length on two consecutive central sections of the prepared biopsy, with magnification of $\times 200$ and $\times 400$, respectively. IENFD, which is defined as the identifiable number of nerve fibers with the length of at least one-half the epidermal thickness in the area (recorded as a whole number) per unit of epidermal length (1 mm), was calculated and recorded as the number of fibers per mm. The criteria for quantification of positively stained nerves (PGP 9.5 antibody positive) was based on modified recommendations according to the European Federation of Neurological Societies and earlier studies (40,41). An experienced observer conducted the IENFD analysis blinded to the group from which each sample originated, and results have been reported previously in a separate study (38).

Corneal IVCN

IVCM of the cornea was used to acquire images of the sub-basal nerve plexus at follow-up only, as the microscope was not available at baseline. A Heidelberg Retinal Tomograph 3 with Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany) was used for image acquisition. One examiner conducted the examinations. Image acquisition was performed based on an adaptive method of image acquisition by which three-dimensional confocal images stacks were acquired. The method consisted of manual raster scanning of the microscope field of view across the corneal sub-basal layer parallel to the corneal surface (along x - to y -axes) and simultaneous adaptive correction of the depth along the z -axis to keep the nerve layer in focus. The acquired images were then used to produce mosaic images by an automated algorithm described elsewhere (31,42). Further automated algorithms for nerve tracing and quantification as described elsewhere were used to quantify the CNFL density in the entire mCNFL, and nerve length density in a 400- μ m-diameter circle centered on the inferocentral whorl region of the sub-basal nerve plexus, representing the densest region of corneal nerves (wCNFL) (43). The entire raw image data set and full set of mosaics have been published to share with the wider scientific community and are freely accessible (42).

Statistical Analyses

Data are presented as mean $\pm$ SD or number of participants. Differences between groups in the cohort characteristics were assessed with the independent sample t test or χ^2 test for continuous or categorical data, respectively. The association between outcome and exposure variables was analyzed with linear regression and adjusted for other relevant variables by including them as additional independent variables in the statistical models. $P < 0.05$ was regarded as statistically significant. Analyses were performed using Stata/SE 17.0 for Windows (64-bit) statistical software (StataCorp LLC, College Station, TX).

Data and Resource Availability

Raw IVCN image data, processed mosaic images, and clinical parameter data upon which this study is based are freely accessible (44). The detailed description of the data and its reuse is considered as a resource to interested researchers (31).

RESULTS

The characteristics of cohorts, including demographic, physiologic, and neuropathy parameters, are detailed in Table 1. At the baseline examination, 82 participants were included in the cohort. At follow-up, 77 participants (mean age 69.1 ± 1.2 years), consisting of 36 females (mean age 69.2 ± 1.1 years) and 44 males (69.0 ± 1.2 years), were available for examination and agreed to participate in the study. Data on both IENFD and corneal nerve fiber density parameters of wCNFL and mCNFL were available at the follow-up, whereas the corneal nerve parameters were not investigated at baseline.

Table 1—Cohort characteristics in terms of demographics, physiologic variables, nerve fiber characteristics, and neuropathy parameters

	Nondiabetes	Diabetes	P
Baseline			
Participants, <i>n</i>	43	39	
Age, years	61 ± 0.8	61 ± 1.3	0.44
Female/male sex (ratio)	19/23 (0.82)	17/22 (0.77)	0.88
HbA _{1c} , mmol/mol	25.5 ± 7.0	43.1 ± 15.2	<0.001
BMI, kg/m ²	26.3 ± 4.6	28.9 ± 4.1	0.007
IENFD, fibers/mm	2.9 ± 1.5	1.9 ± 1.2	0.002
Follow-up			
Participants, <i>n</i>	41	36	
Age, years	69 ± 1.5	69 ± 0.7	0.90
Female/male sex (ratio)	19/23 (0.82)	17/22 (0.77)	0.88
HbA _{1c} , % (mmol/mol)	5.7 (38.4 ± 2.9)	7.2 (54.9 ± 11.8)	<0.001
BMI, kg/m ²	26.0 ± 4.3	29.0 ± 4.1	0.002
IENFD, fibers/mm	0.8 ± 0.7	0.9 ± 1.0	0.71
NDS	6.6 ± 5.7	8.1 ± 6.7	0.27
NSS	1.3 ± 2.6	2.0 ± 3.1	0.30
mCNFL, mm/mm ²	15.0 ± 3.2	13.2 ± 4.1	0.025
wCNFL, mm/mm ²	18.7 ± 5.1	18.8 ± 4.7	0.97
Amplitude, sural nerve, μV	7.1 ± 4.1	6.1 ± 4.1	0.33
Conduction velocity, m/s			
Sural nerve	45.7 ± 4.6	44.5 ± 5.9	0.32
Peroneal nerve	45.9 ± 5.4	45.4 ± 9.7	0.79
Heat threshold, °C			
Right foot	40.9 ± 3.9	42.7 ± 3.7	0.043
Left foot	41.1 ± 4.1	41.6 ± 3.8	0.59
Cold threshold, °C			
Right foot	26.6 ± 4.3	25.9 ± 4.8	0.56
Left foot	27.6 ± 3.0	25.5 ± 5.0	0.034

Data are mean ± SD unless otherwise indicated. Significant values are indicated in boldface.

The mean time between baseline and follow-up examinations was 7.9 ± 0.75 years. Participants were grouped based on the diagnosis of type 2 diabetes into either diabetes or nondiabetes groups. IEFND and IVCN parameters (wCNFL, mCNFL) were compared between the diabetes and nondiabetes groups at baseline and follow-up where data were available.

At follow-up, HbA_{1c} and BMI differences noted at baseline remained, but IENFD did not differ between the groups. Of the IVCN parameters, mCNFL was reduced in the diabetes group ($P = 0.025$), whereas wCNFL was not. Only the heat threshold in the right foot and the cold threshold in the left foot were reduced in the diabetes group.

Association of IENFD With Corneal Nerve Density by IVCN

There was no association between mCNFL at the follow-up and IENFD at baseline both before adjustment ($P = 0.094$) and after adjusting for age, sex, and HbA_{1c} ($P = 0.323$). Considering only measurements at follow-up, no association between mCNFL and IENFD was found either before adjustment ($P = 0.195$) or after adjusting for age, sex, and HbA_{1c} ($P = 0.178$). The regression lines at follow-up are shown in Fig. 1 and stratified by group, indicating the absence of an association between IENFD and mCNFL in the diabetes and nondiabetes groups (Fig. 1). For corneal nerves

in the densest whorl region of the plexus, no association was found between wCNFL at follow-up and IENFD at baseline either before ($P = 0.926$) or after adjustment for age, sex, and HbA_{1c} ($P = 0.755$). Considering only data obtained at follow-up (Fig. 2), there was no association of wCNFL with IENFD before ($P = 0.121$) or after adjustment for age, sex, and HbA_{1c} ($P = 0.093$) (Fig. 2).

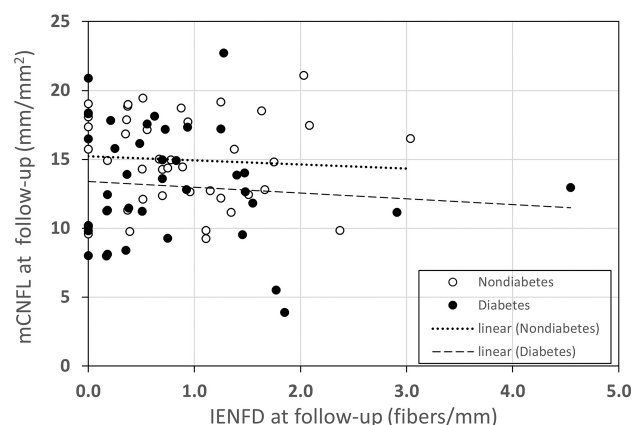


Figure 1—Scatterplot indicating the relationship between mCNFL and IENFD in the same participants with and without diabetes at follow-up. The linear regression is indicated by the dashed lines.

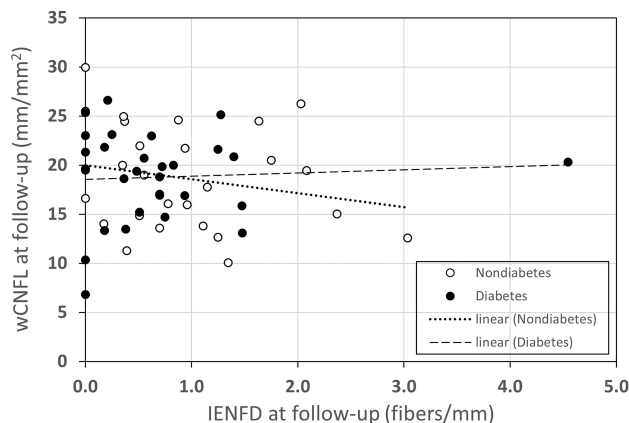


Figure 2—Scatterplot indicating the relationship between wCNFL and IENFD in the same participants with and without diabetes at follow-up. The linear regression is indicated by the dashed lines.

Association of Temporal Change in IENFD With Corneal Nerve Density by IVCN

An association of mCNFL was found with the change in IENFD (Δ IENFD) defined as the follow-up minus the baseline value (Fig. 3). The association was present before adjustment ($P = 0.014$, coefficient -0.788) and was slightly attenuated after adjusting for age, sex, and HbA_{1c} ($P = 0.040$, coefficient -0.722). The negative association of Δ IENFD with the mCNFL at follow-up indicates that participants who lost the most intraepidermal nerves during the 8-year follow-up had the highest corneal nerve density at final follow-up and tended to be those without diabetes, while those who lost the fewest epidermal nerves during the 8-year period had the lowest corneal nerve density at final follow-up and tended to have diabetes (Fig. 3). To better understand this association,

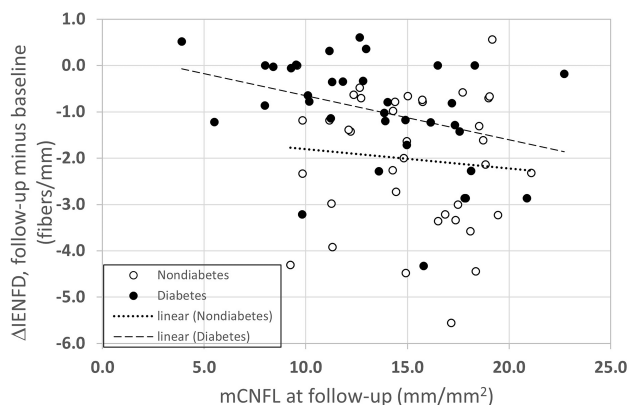


Figure 3—The relationship between Δ IENFD and mCNFL at follow-up, indicating subgroups with and without type 2 diabetes, and corresponding regression lines. The diabetes group lost the fewest intraepidermal nerves during follow-up and had the lowest density of corneal nerves at the final follow-up, while the nondiabetes group lost the most intraepidermal nerves during follow-up and had the highest density of corneal nerves at the final follow-up.

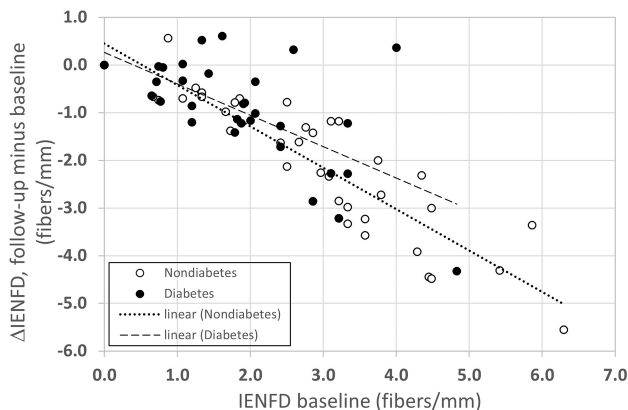


Figure 4—The relationship between Δ IENFD during an 8-year follow-up period and IENFD at baseline in groups with and without type 2 diabetes. Those with diabetes had the lowest IENFD at baseline and lost fewer intraepidermal nerves during follow-up, while those without diabetes had the greatest IENFD at baseline and lost relatively more intraepidermal nerves during follow-up.

the relationship of Δ IENFD with baseline IENFD was examined (Fig. 4). Participants who lost the fewest epidermal nerves during the follow-up tended to be those with diabetes who had the fewest nerves at baseline and thus could only lose a limited number of nerves during follow-up. Conversely, participants who lost the most intraepidermal nerves during follow-up tended to be those without diabetes who had more nerves at baseline and could thus lose more nerves during follow-up. The reduction in mCNFL in type 2 diabetes as noted in Table 1 and Fig. 3 was also discernible by visual inspection of the subbasal nerve plexus. In the same participants, and at the same time point (follow-up), the corresponding IENFD did not yield a noticeable difference (Fig. 5). In contrast to mCNFL, the nerve density in the whorl region of the nerve plexus, wCNFL, was not associated with Δ IENFD either before ($P = 0.303$) or after ($P = 0.138$) adjustment for age, sex, and HbA_{1c} .

Association of IVCN Parameters With Measures of Diabetic Neuropathy

Linear regression was performed to examine the association of IVCN parameters, mCNFL and wCNFL, with the clinical neuropathy measures obtained from symptom scoring, nerve conduction studies (NCS), and quantitative sensory testing (QST) in the entire cohort. Regression was first performed without adjustment and then repeated after adjustment for age, sex, and HbA_{1c} as covariates (Table 2). Regression analyses were initially performed without multiple testing correction; however, after Benjamini-Hochberg correction, none of the clinical neuropathy parameters were significantly associated with the corneal nerve parameters. This result did not change even when only heat and cold thresholds from one foot were considered. Thus, there was no association between the total subbasal plexus or whorl (mCNFL or wCNFL, respectively) with diabetic neuropathy parameters.

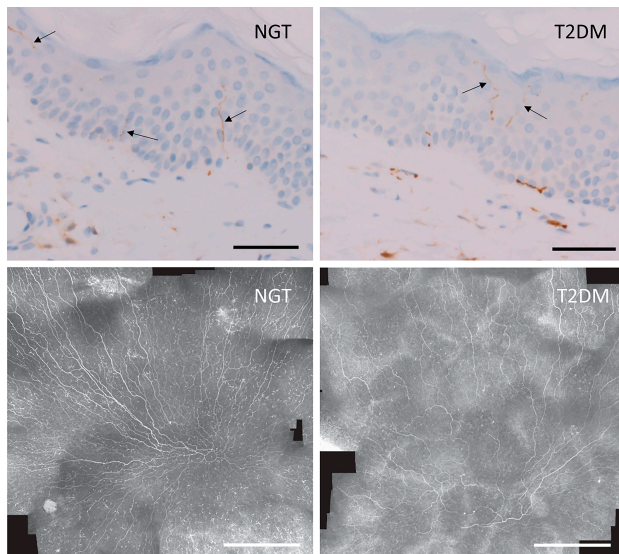


Figure 5—Representative images of skin biopsy sections stained with PGP 9.5 antibody (top row, brown color) to highlight intraepidermal nerve fibers (top row, arrows) and corneal subbasal nerve plexus mosaics obtained by IVCN (bottom row), both at final follow-up. In a 69-year-old female participant with NGT (left column), intraepidermal nerve fibers were detected, while in the cornea of the same participant, a dense distribution of subbasal nerves and inferocentral circular whorl were apparent. In a 68-year-old female participant with type 2 diabetes mellitus (T2DM) diagnosed >25 years prior to examination (right column), intraepidermal nerve fibers were identified, whereas the corneal subbasal nerve plexus appeared less densely innervated. Scale bars: 50 μ m (top row), 500 μ m (bottom row).

Grading DPN

The degree of DPN was determined by grouping participants from the entire cohort into those without neuropathy symptoms (NSS = 0) and those with symptomatic DPN (NSS > 0) at the final follow-up. Neuropathy was also separately assessed considering the NDS by grouping participants into

three tertiles consisting of low, medium, and high score categories. The relationship of IENFD, nerve conduction, quantitative sensory tests, and the IVCN parameters in these subgroupings of participants is given in Table 3. While HbA_{1c}, NSS, NDS, and IENFD, as well as many of the nerve conduction and quantitative sensory test values, significantly differed at follow-up across groups with varying degrees of neuropathy symptoms and disability, Δ IENFD and IVCN parameters did not vary across these groupings.

DISCUSSION

The main findings in this study were a lack of association between skin biopsy and corneal IVCN findings in the same participants taken at the same time point as well as a lack of association between IVCN parameters and clinical measures of peripheral neuropathy, including clinical sensory testing, nerve conduction studies, and NSS or NDS. Interestingly, IENFD was associated with both NSS and NDS, whereas IVCN parameters were not. Moreover, while asymptomatic participants were well differentiated from those with neuropathy symptoms and disability at follow-up according to HbA_{1c}, IENFD, nerve conduction studies, and quantitative sensory tests, IVCN parameters failed to discriminate between groups with or without neuropathy symptoms or groups with low or high neuropathy disability levels. Although reports have described a moderate to strong association between clinical DPN parameters with corneal nerve parameters (25–28,45), prior studies quantified nerves in a very small area of the cornea (0.2% of the plexus area in a single image) and relied on subjective, manual methods of image selection that are prone to human bias. All subsequent quantitative analyses are ultimately limited by the subjective choice of images. These methodological deficiencies can have a large impact on the results obtained (31,35), and this may be limiting corneal nerve assessment in achieving the level of maturity and widespread acceptance as clinical measures of DPN.

Table 2—Overview of correlation between clinical neuropathy parameters with IVCN parameters mCNFL and wCNFL before and after adjustment for age, sex, and HbA_{1c} as covariates and after multiple testing correction for neuropathy parameters

Parameter	mCNFL			wCNFL		
	Unadjusted	Adjusted	B-H correlation	Unadjusted	Adjusted	B-H correlation
NDS	0.59	0.89	NS	0.73	0.55	NS
NSS	0.89	0.86	NS	0.28	0.42	NS
Amplitude, sural nerve, μ V	0.58	0.83	NS	0.82	0.60	NS
Conduction velocity, m/s						
Sural nerve	0.12	0.20	NS	0.43	0.60	NS
Peroneal nerve	0.02	0.03	NS	0.32	0.42	NS
Heat threshold, $^{\circ}$ C						
Right foot	0.39	0.61	NS	0.91	0.45	NS
Left foot	0.80	0.48	NS	0.78	0.43	NS
Cold threshold, $^{\circ}$ C						
Right foot	0.31	0.41	NS	0.11	0.41	NS
Left foot	0.90	0.67	NS	0.36	0.51	NS

All correlations based on final follow-up examination data and linear regression analysis. B-H, Benjamini-Hochberg. Significant values indicated in boldface.

Table 3—Relationship of DPN symptom and disability scores with IVCN and skin biopsy parameters

	NSS			NDS			
	Asymptomatic (0)	Symptomatic (>0)	P (t test)	Tertile 1 (low)	Tertile 2 (medium)	Tertile 3 (high)	P (ANOVA)
Participants, <i>n</i>	46	34		27	26	29	
NSS	0 ± 0	3.9 ± 3.2	<0.001	0.7 ± 2.0	1.4 ± 2.1	2.8 ± 3.7	0.006^a
NDS	5.4 ± 5.8	9.7 ± 5.9	0.002	1.2 ± 1.1	5.9 ± 2.5	14.6 ± 3.5	<0.001^b
IENFD at follow-up, fibers/mm	1.0 ± 0.9	0.6 ± 0.6	0.025	1.3 ± 1.0	0.6 ± 0.6	0.6 ± 0.6	0.009^c
ΔIENFD, fibers/mm	−1.5 ± 1.3	−1.5 ± 1.6	0.995	−1.7 ± 1.3	−1.8 ± 1.3	−1.0 ± 1.5	0.094
HbA _{1c} , % (mmol/mol)	6.2 (43.8 ± 8.6)	6.7 (49.8 ± 14.4)	0.023	6.0 (42.6 ± 6.6)	6.3 (45.2 ± 12.8)	6.8 (51.3 ± 13.4)	0.030^d
BMI, kg/m ²	27.1 ± 3.5	27.9 ± 5.5	0.476	26.8 ± 4.0	26.2 ± 3.6	29.2 ± 5.1	0.029^e
Amplitude, sural, μV	7.3 ± 4.2	5.6 ± 3.8	0.071	8.9 ± 4.0	5.8 ± 3.8	4.8 ± 3.3	<0.001^c
Conduction velocity, m/s							
Sural	45.1 ± 5.4	45.1 ± 5.1	0.960	45.8 ± 5.4	45.2 ± 4.5	44.2 ± 6.1	0.607
Peroneal	47.4 ± 9.0	43.2 ± 5.2	0.014	49.5 ± 9.7	46.1 ± 5.8	41.4 ± 5.2	<0.001^d
Heat threshold, °C							
Right foot	40.6 ± 3.7	43.5 ± 3.5	<0.001	40.6 ± 3.7	42.5 ± 4.2	42.4 ± 3.5	0.122
Left foot	40.0 ± 3.7	43.2 ± 3.4	<0.001	39.8 ± 3.6	41.6 ± 3.9	42.7 ± 3.8	0.037^a
Cold threshold, °C							
Right foot	27.1 ± 3.8	25.0 ± 5.2	0.058	28.7 ± 2.7	25.0 ± 5.0	25.0 ± 4.7	0.003^c
Left foot	27.8 ± 3.1	24.7 ± 5.0	0.003	28.7 ± 2.1	26.3 ± 4.0	24.4 ± 5.1	0.002^c
mCNFL, mm/mm ²	14.2 ± 3.8	14.0 ± 3.9	0.833	14.2 ± 4.1	14.2 ± 3.2	13.8 ± 4.1	0.893
wCNFL, mm/mm ²	18.5 ± 5.0	19.2 ± 4.5	0.566	18.2 ± 4.6	19.4 ± 5.8	18.9 ± 4.3	0.728

Significant values are indicated in boldface. ^aKruskal-Wallis one-way ANOVA, Dunn post hoc test: significance between tertiles 1 and 3. ^bKruskal-Wallis one-way ANOVA, Dunn post hoc test: significance among all pairwise tertiles. ^cKruskal-Wallis one-way ANOVA, Dunn post hoc test: significance between tertiles 1 and 2 and tertiles 1 and 3. ^dKruskal-Wallis one-way ANOVA, Dunn post hoc test: significance between tertiles 1 and 3 and tertiles 2 and 3. ^eOne-way ANOVA, Tukey post hoc test: significance between tertiles 2 and 3.

Furthermore, IVCN is additionally challenging to apply in a clinical setting, as the experience of the operator is essential for acquiring good-quality images. Notably, most prior studies fail to make available the raw IVCN image data sets upon which the analyses were based; therefore, it is impossible for others to assess image quality or to reproduce the results based on the same set of raw images. Thus, it is exceedingly difficult to ascertain how human selection bias influences the results. We previously showed that a tendency toward selection of images depicting many sub-basal nerves results in an overestimation of CNFL by 15–20% (46) and that the use of multiple single-IVCN images for analysis leads to large deviations from the true value of CNFL (31). Here, we reduced the element of human bias by automated construction of wide-field mosaic images of the corneal subbasal nerve plexus combined with fully automated nerve tracing and quantification. The raw data sets and wide-field mosaics used in this study are published and openly accessible (31).

We could not find a direct relationship of corneal nerve density to IENFD at a single time point, but instead, found that corneal nerve density was related to the degree of IENFD loss during the prior 8 years, from 61 to 69 years of age. Counterintuitively, a greater IENFD loss during this

8-year period corresponded to higher mCNFL at age 69. This greater loss of intraepidermal nerve fibers was observed mainly among participants without type 2 diabetes. Conversely, and also potentially counterintuitively, those who lost fewer intraepidermal nerve fibers (lower value of ΔIENFD) during the 8-year period had lower mCNFL at age 69 and tended to be those with type 2 diabetes. These results could be explained by considering that participants who lost the most intraepidermal nerves during follow-up had higher numbers of these nerves at baseline (those without diabetes), while those starting with only a few nerves at baseline (those with diabetes) did not have many nerves left to lose during the follow-up period.

Although IENFD was lower in the diabetes group at baseline, at the final follow-up 8 years later, IENFD was no longer different between groups, with the participants without diabetes having fully caught up with those with diabetes by age 69. This finding confirms the known decline in IENFD with age, even in healthy individuals (15,17,21,38,47), and indicates that the intraepidermal nerve fiber loss in type 2 diabetes occurs at an earlier age than in those without diabetes. However, there is a need for further development of the IENFD quantitative method to reach a greater sensitivity for mild changes and better discrimination between individuals.

Interestingly, whereas IENFD at follow-up did not mirror the presence of type 2 diabetes (Table 1), mCNFL was reduced in participants with diabetes relative to those without diabetes at follow-up. This reduction in corneal nerves was discernible by visual inspection of the distribution of mCNFL (Fig. 3), where values $<10 \text{ mm/mm}^2$ were almost exclusively seen in participants with type 2 diabetes, and a clear loss of nerves was visible in wide-field images of the subbasal nerve plexus (Fig. 5). The greater amount of nerve information present in the corneal subbasal plexus relative to intraepidermal nerve fibers viewed in a particular histologic section of tissue can also be appreciated from Fig. 5. Corneal subbasal nerve density as a parameter was also found to be more sensitive than IENFD, with mCNFL representing a range of nonzero values and where IENFD was zero in both the diabetes and nondiabetes groups. Despite this, the lack of relationship of mCNFL in this study with clinical measures of DPN and the lack of discriminatory power of IVCN for neuropathy signs and symptoms suggest that the corneal nerve fibers may reflect a different aspect of nerve degeneration than clinical neuropathy measures, thus bringing into question the utility of IVCN in the assessment and monitoring of DPN. This result contrasts many prior studies indicating the utility of IVCN for neuropathy assessment (26,27,48,49). Our automated imaging and quantification methods and strict inclusion of participants of the same age and only with type 2 diabetes may account for these differences; however, more studies without requiring selection of single IVCN images are warranted to verify our findings. Two earlier studies with substantially smaller imaged areas than in the current study also reported poor correlation of IVCN with IENFD or neuropathy disability (34,35). Use of corneal nerves as a surrogate marker for detecting neuropathy in diabetes should therefore be considered with caution, and studies should be assessed based on the area of the subbasal nerve plexus imaged and the potential for bias in selection of nerve images for analysis.

It is of note that a potential relationship between mCNFL and peroneal nerve conduction velocity may have existed, given the significant association detected by linear regression in this study. This association, however, disappeared after multiple testing correction was applied. This result could be hypothesis generating, requiring detailed investigation in further studies and also highlights the pitfalls of testing for associations between multiple parameters without applying a priori physiologically or biologically relevant hypotheses. Many prior studies reporting a strong association between IVCN parameters and diabetic neuropathy tested a number of IVCN parameters, including nerve density, branching, number of nerves, nerve tortuosity, beadings, and other related parameters, without appropriate statistical adjustment (24–26,29,50). The chance of detecting spurious associations is therefore greater in such cases. Additionally, no standardized definition of DPN has emerged from prior studies using corneal nerve parameters, with different

studies reporting DPN severity based on different clinical tests.

We did not detect any relationship of corneal nerves with DPN or IENFD when considering only the densest region of corneal nerves at the inferocentral corneal apex, represented by the anatomic spiral pattern of nerves in this whorl region. The wCNFL was not sensitive to any neuropathy parameter, which differs from prior reports indicating the whorl region to be sensitive to pathology (25,51,52). This discrepancy may again be a result of our use of the full whorl region for analysis, as opposed to single-observer, manually selected images considered to be within the whorl region. The wCNFL parameter was also not sensitive to the presence of type 2 diabetes in this study, being roughly equal between the diabetes and nondiabetes groups. Our findings suggest that contrary to prior reports, the whorl region tends to be preserved in type 2 diabetes and with the development of DPN.

A limitation of the current study was the lack of IVCN data from participants at baseline. Baseline examinations commenced in 2004, predating the commercial availability of the laser scanning IVCN system. Furthermore, algorithms making wide-field mosaics of the subbasal nerve plexus practical in a clinical setting only became a reality a decade later, making it impossible to obtain a comparable data set at baseline. A further limitation was the lack of specific measures to assess painful diabetic neuropathy in the cohort, a subgroup of clinical importance. Future studies should assess this subgroup in relation to mosaic-based IVCN parameters.

In conclusion, in our cohort of age- and sex-matched participants with and without type 2 diabetes, we did not find an association of corneal subbasal nerve density with IENFD or clinical DPN parameters. The total corneal nerve fiber density at follow-up was, however, negatively associated with Δ IENFD during the 8-year follow-up period, indicating that IVCN may be a sensitive technique for assessing peripheral nerve loss in type 2 diabetes but not in DPN.

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Authors Contributions. R.A.B., L.E., A.H.P., E.E., and N.L. performed the data analyses and interpretation. R.A.B., L.E., E.E., L.B.D., O.R., and N.L. conducted the experiments and data acquisition. R.A.B., L.B.D., O.R., and N.L. were responsible for the conception and design of the study. R.A.B. and N.L. drafted the manuscript. L.E., A.H.P., T.P.U., E.E., L.B.D., and O.R. contributed to the critical revision of the manuscript. T.P.U., L.B.D., O.R., and N.L. supervised the experiments and data collection. R.A.B., L.B.D., O.R., and N.L. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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