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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Comparison of novel wide-field in vivo corneal confocal microscopy with skin biopsy for assessing peripheral neuropathy in type 2 diabetes mellitus

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Tweet: Comparison of intraepidermal and corneal nerve fibers in type 2 diabetes reveals both are uncorrelated and mirror nerve degeneration but only intraepidermal nerve density is associated with peripheral diabetic neuropathy. #diabetes #diabeticneuropathy #cornea @liu_universitet (Figure 1).
ABSTRACT

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 non-invasive diagnostic modality for DPN. Direct comparisons of skin biopsy and IVCM in controlled cohorts are lacking, while 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 type 2 diabetes and 36 healthy subjects, 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 subjects, 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 scrutiny is warranted concerning methodologies of studies using corneal nerves to assess DPN.
Introduction

Diabetic peripheral neuropathy (DPN) is characterized by a symmetrical and predominantly sensory 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 over 50% of those with type 2 diabetes mellitus (T2DM) will develop neuropathy affecting the peripheral nerves during their lifetime (1-5). Moreover, DPN is considered a risk factor for mortality in T2DM and has a prevalence that increases with age and duration of diabetes. Moreover, the level of glycosylated hemoglobin (HbA1c) and hyperlipidemia, obesity and smoking have all been found to have an association with DPN (6-8).

In small-fiber neuropathy (SFN), the thinly myelinated Aδ 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 immune staining 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 subjects as well as in subjects with T2DM (15, 17, 18, 21, 22). IENFD 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, non-invasive in vivo confocal microscopy (IVCM) of the cornea has gained increasing attention (24-26).

The cornea contains nerve fibers (called subbasal nerves) of both Aδ and C subtypes, arranged in a dense nerve plexus that can be non-invasively 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 small fiber neuropathy (7, 24, 27-30). The relationship of CNFL to IENFD and the ability of corneal nerve fiber changes to serve as a non-invasive marker for DPN and, thus, as an alternative to invasive IENFD, is at present unclear. In this study, we therefore 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 corneal subbasal nerve fiber length density (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 to other clinical measures of DPN in a T2DM population. The findings could have important implications for the future detection, monitoring and management of DPN.

**Methods**

**Study design and subjects**
Participants were included in the study as part of a prospective longitudinal population-based study in Sweden. Subjects, 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 criteria consisted of being a participant in the baseline study; in the initial baseline study participants were recruited with normal glucose tolerance (NGT), impaired glucose tolerance (IGT), and type 2 diabetes, aged 60 ± 1 years. The NGT and IGT groups underwent two oral glucose tolerance tests (OGTT), one week apart, to establish their glucose status, where both results should be within the cut-off values for fasting plasma glucose (fPG) and 2-hour plasma glucose (2hPG) as follows (NGT: fPG < 7.0 mmol/l and 2hPG < 8.9 mmol/l, IGT: fPG < 7.0 and 2hPG ≥ 8.9 to < 12.2 mmol/l based on WHO definition of diabetes, 1999).

Exclusion criteria consisted of nutritional deficiencies (four individuals due to vitamin B12 and folate deficiency), and neuropathy asymmetry due to sciatica and stroke (three individuals). The study was conducted according to the tenets of the Declaration of Helsinki, receiving informed consent from participants. The study protocol was approved (Ethical Application no. 2013-21-31 M) in Umeå, Sweden (32).

**Clinical examinations**
Study participants underwent a wide range of examinations, including laboratory test, peripheral neurological examinations, clinical signs and symptoms evaluation (Dyck’s Neuropathy Disability Score (NDS), Neuropathy Symptom Score (NSS), and skin biopsy for assessment of intra epidermal nerve fiber density (IENFD). The diagnostic criteria for peripheral neuropathy in patients were absent ankle reflexes, and/or reduced sensory perception, and/or neuropathic symptoms in toes or feet (20, 37).

**Neuropathy assessment**
Clinical examinations included peripheral neurological examination of nerve function, which was evaluated using Neuropathy Disability Score (NDS) and Neuropathy Symptom Score (NSS) to assess the severity and incidence of neuropathy, respectively. NSS and NDS were performed using the modified Dyck’s scale (20). NSS addresses presence or absence of symptoms in the feet, including paresthesia (numbness), abnormal sensation for heat and cold, touch (dysesthesia), pins and needles sensation, and different types of pain sensation (burning, dull or stabbing pain). NDS assesses the severity in case of presence of a symptom; severity of a symptom was then scored from 0 to 3; 0 for lack of symptoms, 1 for some times, 3 for often and for during most nights, resulting in a range of scores from 0 to 21 (38, 39).

Sensory and nerve conduction tests.
A single neurophysiologist masked to diabetes status of participants performed nerve conduction measurements at the clinical neurophysiology laboratory, Umeå University, Sweden. 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 subject 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, by application of a 3-mm disposable punch biopsy instrument, taking dermal samples from a location 10 cm above the lateral malleolus of the right leg of all study subjects. The wound was not sutured but was allowed to heal spontaneously. The skin biopsy samples for the assessment of the IENFD were taken at baseline (2004 to 2007) and at the follow-up (2014), and specimens were processed for immunohistochemistry as previously detailed (37).

Intraepidermal nerve fiber density assessment
Light microscopy was used to perform counting of nerve fibers and measurement of epidermal length on two consecutive central sections of the prepared biopsy, with magnification of 200x and 400x, respectively. Intraepidermal nerve fiber density (IENFD), which is defined as the identifiable number of nerve fibers with the length of at least half the epidermal thickness in the area (recorded as whole number) per unit of epidermal length (one millimeter) was calculated and recorded as the number of fibers/mm. The criteria for quantification of positively stained nerves (PGP 9.5 positive) was based on the modified recommendations according to the European Federation of Neurological Societies (EFNS) and earlier studies (39, 40). 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 (37).

Corneal in vivo confocal microscopy
In vivo confocal microscopy (IVCM) of the cornea was used to acquire images of the subbasal 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,
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 3D confocal images stacks was acquired. The method consisted of manual raster scanning of the microscope field of view across the corneal subbasal layer parallel to the corneal surface (along X-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, 41). Further automated algorithms for nerve tracing and quantification as described elsewhere were used to quantify the corneal subbasal nerve fiber length density in the whole mosaic (mCNFL), and nerve length density in a 400µm diameter circle centered on the inferocentral whorl region of the subbasal nerve plexus, representing the densest region of corneal nerves (wCNFL) (42). The entire raw image dataset and full set of mosaics have been published to share with the wider scientific community, and are freely accessible (41).

**Statistical analyses**

Data was described with mean and standard deviation or number of subjects. Difference between groups in the cohort characteristics were assessed with the independent samples t-test or chi-square 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. A p-value less than 0.05 was regarded as statistically significant. Analyses were performed using the software Stata (StataCorp LLC, USA, Stata/SE 17.0 for Windows (64-bit)).

**Data and resource**

Raw IVCM image data, processed mosaic images, and clinical parameter data upon which this study is based are freely accessible at (43). The detailed description of the data and its reuse are considered as a resource to interested researchers (31).

**Results**

The characteristics of cohorts including demographic, physiological and neuropathy parameters are detailed in Table 1. At the baseline examination, 82 subjects were included in the cohort. At follow-up, a total of 77 subjects (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.

The mean time between baseline and follow-up examinations was 7.9 ± 0.75 years. Study subjects were grouped based on the diagnosis of type 2 diabetes to either DM or non-DM groups; IEFND and IVCM parameters (wCNFL, mCNFL) were compared between these two groups of diabetes vs. nondiabetes at baseline and follow-up, where data was available.

At follow-up, HbA1c and BMI differences noted at baseline remained, but IENFD did not differ between the groups. Of the IVCM parameters, mCNFL was reduced in the diabetes
(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 IVCM**

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 HbA1c (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 HbA1c (p = 0.178). The regression lines at follow-up are shown in Figure 1 and stratified by group, indicating absence of association between IENFD and mCNFL in diabetes and nondiabetes groups. (Figure 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 HbA1c (p = 0.755). Considering only data obtained at follow-up (Figure 2), there was no association of wCNFL with IENFD before (p = 0.121) or after adjustment for age, sex, and HbA1c (p = 0.093) (Figure 2).

**Association of temporal change in IENFD with corneal nerve density by IVCM**

An association of mCNFL was found with the change in IENFD (ΔIENFD) defined as the follow-up minus the baseline value (Figure 3). The association was present both before adjustment (p = 0.014, coeff -0.788) and was slightly attenuated after adjusting for age, sex, and HbA1c (p = 0.040, coeff = -0.722). The negative association of ΔIENFD with the mCNFL at follow-up indicated that subjects who lost the most intraepidermal nerves during the 8-year follow-up period had highest corneal nerve density at final follow-up, and tended to be those without diabetes, while those who lost the least epidermal nerves during the 8-year period had the lowest corneal nerve density at final follow-up and tended to have diabetes (Figure 3).

To better understand this association, the relationship of ΔIENFD to baseline IENFD was examined (Figure 4). Those who lost the fewest epidermal nerves during the follow-up period tended to be subjects with diabetes who had the fewest nerves at baseline, and thus could only lose a limited amount of nerves during follow-up. Conversely, those who lost the most intraepidermal nerves during follow-up tended to be subjects without diabetes who had more nerves at baseline and could thus lose more nerves during follow-up.

The reduction in mCNFL in T2DM as noted in Table 1 and Figure 3 was also discernible by visual inspection of the subbasal nerve plexus. In the same subjects, and at the same time point (follow-up), the corresponding IENFD did not yield a noticeable difference (Figure 5). In contrast to mCNFL, the nerve density in the whorl region of the nerve plexus, wCNFL, was not associated with ΔIENFD either before (p = 0.303) or after (p = 0.138) adjustment for age, sex and HbA1c.
Association of IVCM parameters with measures of diabetic neuropathy

Linear regression was performed to examine the association of the IVCM parameters mCNFL and wCNFL with the clinical neuropathy measures obtained from symptom scoring, NCS and QST in the entire cohort. Regression was first performed without adjustment and then repeated after adjustment for age, sex, and HbA1c as covariates, with results given in 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 (Table 2). This result did not change even where only heat/cold thresholds from one foot were considered. Thus, there was no association between the total subbasal plexus or whorl (mCNFL or wCNFL, respectively) to diabetic neuropathy parameters (Table 2).

Grading of diabetic peripheral neuropathy (DPN)

The degree of DPN was determined by grouping subjects 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 subjects into three tertiles consisting of low, medium and high NDS score categories. The relationship of IENFD, nerve conduction, quantitative sensory tests and the IVCM parameters in these subgroupings of participants is given in Table 3. While HbA1c, 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, ΔIENFD and IVCM parameters did not vary across these groupings.

Discussion

The main findings in this study were a lack of association between skin biopsy and corneal in vivo confocal microscopy (IVCM) findings in the same patients taken at the same time point, and a lack of association between IVCM parameters and clinical measures of peripheral neuropathy, including clinical sensory testing, nerve conduction studies, and scoring of neuropathy symptoms or disability. Interestingly, IENFD was associated with both NSS and NDS, whereas IVCM parameters were not. Moreover, while asymptomatic patients were well differentiated from those with neuropathy symptoms and disability at follow-up according to HbA1c, IENFD, nerve conduction studies and quantitative sensory tests, IVCM 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, 44), prior studies quantify nerves in a very small area of the cornea (0.2% of the plexus area in a single image) and rely 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.
IVCM is furthermore 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 IVCM image datasets upon which the analyses are based; therefore, it is impossible for others to assess image quality or to reproduce the results based on the same set of raw images. It is thus exceedingly difficult to ascertain how human selection bias influences the results. We previously showed that a tendency towards selection of images depicting many subbasal nerves results in an overestimation of CNFL by 15-20% (45) and that the use of multiple single IVCM 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-area mosaic images of the corneal subbasal nerve plexus, combined with fully automated nerve tracing and quantification. The raw datasets and wide-area 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 corneal nerve density was related to the degree of IENFD loss during the prior 8 years, from 61 – 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 subjects without T2DM. 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 T2DM. These results could be explained by considering that those 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 subjects without diabetes having fully ‘caught up’ with those having diabetes by age 69. This confirms the known decline in IENFD with age even in healthy subjects (15, 17, 21, 37, 46), and indicates that the intraepidermal nerve fiber loss in T2DM 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 higher sensitivity for mild changes and better discrimination between individuals.

Interestingly, whereas IENFD at follow-up did not mirror the presence of T2DM (Table 1), mCNFL was reduced in T2DM subjects relative to those without diabetes at follow-up. This reduction in corneal nerves was discernible by visual inspection of the distribution of mCNFL (Figure 3), where values below 10 mm/mm² were almost exclusively seen in T2DM subjects, and a clear loss of nerves was visible in wide-area images of the subbasal nerve plexus (Figure 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 Figure 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, where IENFD was zero in both 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 IVCM for neuropathy signs and symptoms suggests that the corneal nerve fibers may reflect a different aspect of nerve
degeneration than clinical neuropathy measures, thus bringing into question the utility of IVCM in the assessment and monitoring of DPN. This result is in contrast to many prior studies indicating the utility of IVCM for neuropathy assessment (26, 27, 47, 48). Our automated imaging and quantification methods and strict inclusion of subjects of the same age and only with T2DM may account for these differences; however, more studies without requiring selection of single IVCM images are warranted to verify our findings. Two earlier studies with substantially smaller imaged areas than in the present study, also reported poor correlation of IVCM 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 of mCNFL with 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. This also highlights the pitfalls of testing for associations between multiple parameters without applying a priori physiologically or biologically relevant hypotheses. Many prior studies reporting strong association between IVCM parameters and diabetic neuropathy test a number of IVCM parameters, including nerve density, branching, number of nerves, nerve tortuosity, beadings, and other related parameters, without appropriate statistical adjustment for testing these multiple parameters (24-26, 29, 49). 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 to DPN or IENFD when considering only the densest region of corneal nerves at the infero-central corneal apex, represented by the anatomic spiral pattern of nerves in this ‘whorl’ region. The wCNFL was not sensitive to any neuropathy parameter. This differs from prior reports indicating the whorl region to be sensitive to pathology (25, 50, 51). 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 not either sensitive to the presence of T2DM in this study, being roughly equal between T2DM and nondiabetes groups. Our findings suggest that contrary to prior reports, the whorl region tends to be preserved in T2DM and with the development of DPN.

A limitation of the present study was the lack of IVCM data from subjects at baseline. Baseline examinations commenced in 2004, predating the commercial availability of the laser-scanning IVCM system. Furthermore, algorithms making wide-area 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 dataset 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 IVCM parameters.

In conclusion, in our cohort of T2DM and age- and sex-matched nondiabetes subjects, 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 the change in intraepidermal nerve fiber density, ΔIENFD, during the 8-year follow-up period, indicating that IVCM may be a sensitive technique for assessing peripheral nerve loss in T2DM but not in DPN.

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**Duality of Interest.**
Authors declare no potential conflicts of interest related to the material presented herein.

**Authors Contributions.**
N.L., R.A.B., L.B.D. and O.R. were responsible for the conception and design of the study. N.L., L.B.D., T.P.U. and O.R. supervised the experiments and data collection. R.A.B., N.L., L.E., E.E., L.B.D. and O.R. conducted the experiments and data acquisition. R.A.B., N.L., L.E., E.E. and A.H.P. performed the data analyses and interpretation. R.A.B and N.L. drafted the manuscript. R.A.B, N.L., L.B.D. and O.R. are the guarantors of this study 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. L.E., T.P.U., E.E., A.H.P., L.B.D. and O.R. contributed to the critical revision of the drafted manuscript.

**References**

## Table 1

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|                      |             |          |         |
| **Follow-up**        |             |          |         |
| N                    | 41          | 36       |         |
| Age                  | 69 ± 1.5    | 69 ± 0.7 | 0.90    |
| Female/male (ratio)  | 19/23 (0.82)| 17/22 (0.77) | 0.88    |
| HbA1c, % (mmol/mol)  | 5.7 (38.4 ± 2.9) | 7.2 (54.9 ± 11.8) | < 0.001 |
| BMI (kg/m²)          | 26.0 ± 4.3  | 29.0 ± 4.1 | 0.002   |
| IENFD                | 0.8 ± 0.7   | 0.9 ± 1.0 | 0.71    |
| Neuropathy disability score (NDS) | 6.6 ± 5.7 | 8.1 ± 6.7 | 0.27    |
| Neuropathy symptom score (NSS) | 1.3 ± 2.6 | 2.0 ± 3.1 | 0.30    |
| mCNFL                | 15.0 ± 3.2  | 13.2 ± 4.1 | 0.025   |
| wCNFL                | 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, sural nerve (m/s) | 45.7 ± 4.6 | 44.5 ± 5.9 | 0.32    |
| Conduction velocity, peroneal nerve (m/s) | 45.9 ± 5.4 | 45.4 ± 9.7 | 0.79    |
| Heat threshold (right foot) (°C) | 40.9 ± 3.9 | 42.7 ± 3.7 | 0.043   |
| Heat threshold (left foot) (°C)   | 41.1 ± 4.1 | 41.6 ± 3.8 | 0.59    |
| Cold threshold (right foot) (°C)   | 26.6 ± 4.3 | 25.9 ± 4.8 | 0.56    |
| Cold threshold (left foot) (°C)    | 27.6 ± 3.0 | 25.5 ± 5.0 | 0.034   |

Cohort characteristics in terms of demographics, physiological variables, nerve fiber characteristics and neuropathy parameters. N: number, HbA1c: glycated hemoglobin, BMI: body mass index, IENFD: intraepidermal nerve fiber density (fibers/mm), mCNFL: corneal subbasal nerve fiber length density in the whole mosaic (mm/mm²), wCNFL: nerve length density (within the inferocentral whorl region, mm/mm²). Significant values indicated in bold.
Table 2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>mCNFL</th>
<th>wCNFL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no adj</td>
<td>adjusted</td>
</tr>
<tr>
<td>Neuropathy disability score (NDS)</td>
<td>0.59</td>
<td>0.89</td>
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<tr>
<td>Neuropathy symptom score (NSS)</td>
<td>0.89</td>
<td>0.86</td>
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<tr>
<td>Amplitude, sural nerve (µV)</td>
<td>0.58</td>
<td>0.83</td>
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<tr>
<td>Conduction velocity, sural nerve (m/s)</td>
<td>0.12</td>
<td>0.20</td>
</tr>
<tr>
<td>Conduction velocity, peroneal nerve (m/s)</td>
<td>0.02</td>
<td>0.03</td>
</tr>
<tr>
<td>Heat threshold (right foot) (°C)</td>
<td>0.39</td>
<td>0.61</td>
</tr>
<tr>
<td>Heat threshold (left foot) (°C)</td>
<td>0.80</td>
<td>0.48</td>
</tr>
<tr>
<td>Cold threshold (right foot) (°C)</td>
<td>0.31</td>
<td>0.41</td>
</tr>
<tr>
<td>Cold threshold (left foot) (°C)</td>
<td>0.90</td>
<td>0.67</td>
</tr>
</tbody>
</table>

Overview of correlation between clinical neuropathy parameters with IVCM parameters mCNFL and wCNFL before and after adjustment for age, sex, and HbA1c as covariates, and after multiple testing correction for neuropathy parameters. All correlations based on final follow-up examination data and linear regression analysis. Adj: adjusted; no adj: not adjusted; B-H corr: Benjamini-Hochberg multiple testing correction; ns: non-significant.
Table 3

<table>
<thead>
<tr>
<th></th>
<th>NSS</th>
<th>NDS</th>
<th>P-value</th>
<th>Tertile 1</th>
<th>Tertile 2</th>
<th>Tertile 3</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Asymptotic</td>
<td>Symptomatic</td>
<td>(t-test)</td>
<td>Low NDS</td>
<td>Medium NDS</td>
<td>High NDS</td>
<td></td>
</tr>
<tr>
<td>No of subjects</td>
<td>(NSS = 0)</td>
<td>(NSS &gt; 0)</td>
<td></td>
<td>27</td>
<td>26</td>
<td>29</td>
<td></td>
</tr>
<tr>
<td>NSS</td>
<td>46</td>
<td>34</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDS</td>
<td>0 ± 0</td>
<td>3.9 ± 3.2</td>
<td>&lt;0.001</td>
<td>0.7 ± 2.0</td>
<td>1.4 ± 2.1</td>
<td>2.8 ± 3.7</td>
<td>0.006</td>
</tr>
<tr>
<td>IENFD at follow-up (fibers/mm)</td>
<td>5.4 ± 5.8</td>
<td>9.7 ± 5.9</td>
<td>0.002</td>
<td>1.2 ± 1.1</td>
<td>5.9 ± 2.5</td>
<td>14.6 ± 3.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ΔIENFD (fibers/mm)</td>
<td>1.0 ± 0.9</td>
<td>0.6 ± 0.6</td>
<td>0.025</td>
<td>1.3 ± 1.0</td>
<td>0.6 ± 0.6</td>
<td>0.6 ± 0.6</td>
<td>0.009</td>
</tr>
<tr>
<td>HbA1c, % (mmol/mol)</td>
<td>6.2 (43.8 ± 8.6)</td>
<td>6.7 (49.8 ± 14.4)</td>
<td>0.023</td>
<td>6.0 (42.6 ± 6.6)</td>
<td>6.3 (45.2 ± 12.8)</td>
<td>6.8 (51.3 ± 13.4)</td>
<td>0.030</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.1 ± 3.5</td>
<td>27.9 ± 5.5</td>
<td>0.476</td>
<td>26.8 ± 4.0</td>
<td>26.2 ± 3.6</td>
<td>29.2 ± 5.1</td>
<td>0.029</td>
</tr>
<tr>
<td>Amplitude, sural (µV)</td>
<td>7.3 ± 4.2</td>
<td>5.6 ± 3.8</td>
<td>0.071</td>
<td>8.9 ± 4.0</td>
<td>5.8 ± 3.8</td>
<td>4.8 ± 3.3</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>CV, sural (m/s)</td>
<td>45.1 ± 5.4</td>
<td>45.1 ± 5.1</td>
<td>0.960</td>
<td>45.8 ± 5.4</td>
<td>45.2 ± 4.5</td>
<td>44.2 ± 6.1</td>
<td>0.607</td>
</tr>
<tr>
<td>CV, peroneal (m/s)</td>
<td>47.4 ± 9.0</td>
<td>43.2 ± 5.2</td>
<td>0.014</td>
<td>49.5 ± 9.7</td>
<td>46.1 ± 5.8</td>
<td>41.4 ± 5.2</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Heat threshold (right foot) (°C)</td>
<td>40.6 ± 3.7</td>
<td>43.5 ± 3.5</td>
<td>&lt; 0.001</td>
<td>40.6 ± 3.7</td>
<td>42.5 ± 4.2</td>
<td>42.4 ± 3.5</td>
<td>0.122</td>
</tr>
<tr>
<td>Heat threshold (left foot) (°C)</td>
<td>40.0 ± 3.7</td>
<td>43.2 ± 3.4</td>
<td>&lt; 0.001</td>
<td>39.8 ± 3.6</td>
<td>41.6 ± 3.9</td>
<td>42.7 ± 3.8</td>
<td>0.037</td>
</tr>
<tr>
<td>Cold threshold (right foot) (°C)</td>
<td>27.1 ± 3.8</td>
<td>25.0 ± 5.2</td>
<td>0.058</td>
<td>28.7 ± 2.7</td>
<td>25.0 ± 5.0</td>
<td>25.0 ± 4.7</td>
<td>0.003</td>
</tr>
<tr>
<td>Cold threshold (left foot) (°C)</td>
<td>27.8 ± 3.1</td>
<td>24.7 ± 5.0</td>
<td>0.003</td>
<td>28.7 ± 2.1</td>
<td>26.3 ± 4.0</td>
<td>24.4 ± 5.1</td>
<td>0.002</td>
</tr>
<tr>
<td>mCNFL (mm/mm²)</td>
<td>14.2 ± 3.8</td>
<td>14.0 ± 3.9</td>
<td>0.833</td>
<td>14.2 ± 4.1</td>
<td>14.2 ± 3.2</td>
<td>13.8 ± 4.1</td>
<td>0.893</td>
</tr>
<tr>
<td>wCNFL (mm/mm²)</td>
<td>18.5 ± 5.0</td>
<td>19.2 ± 4.5</td>
<td>0.566</td>
<td>18.2 ± 4.6</td>
<td>19.4 ± 5.8</td>
<td>18.9 ± 4.3</td>
<td>0.728</td>
</tr>
</tbody>
</table>

Relationship of diabetic peripheral neuropathy symptom and disability scores with in vivo confocal microscopy and skin biopsy parameters.

CV = conduction velocity

1Kruskal-Wallis one-way ANOVA, Dunn's post-hoc test: significance between tertiles 1 and 3
2Kruskal-Wallis one-way ANOVA, Dunn's post-hoc test: significance between all pairwise tertiles
3Kruskal-Wallis one-way ANOVA, Dunn's post-hoc test: significance between tertiles 1 and 2 and tertiles 1 and 3
4Kruskal-Wallis one-way ANOVA, Dunn's post-hoc test: significance between tertiles 1 and 3 and tertiles 2 and 3
5One-way ANOVA, Tukey’s post-hoc test: significance between tertiles 2 and 3
6Kruskal-Wallis one-way ANOVA, Dunn's post-hoc test: significance between tertiles 1 and 2 and tertiles 1 and 3
7Kruskal-Wallis one-way ANOVA, Dunn's post-hoc test: significance between tertiles 1 and 3 and tertiles 2 and 3
8Kruskal-Wallis one-way ANOVA, Dunn's post-hoc test: significance between tertiles 1 and 3
9Kruskal-Wallis one-way ANOVA, Dunn's post-hoc test: significance between tertiles 1 and 2, and tertiles 1 and 3
Kruskal-Wallis one-way ANOVA, Dunn's post-hoc test: significance between tertiles 1 and 2, and tertiles 1 and 3

Significant values indicated in bold.
Figure legends

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

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

Figure 3. The relationship between ∆IENFD and mCNFL at follow-up, indicating subgroups with and without type 2 diabetes, and corresponding regression lines. The group with diabetes 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 the change in intraepidermal nerve fiber density (∆IENFD) during an 8-year period and IENFD at baseline in groups with and without type 2 diabetes. Those with diabetes had 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.

Figure 5. Representative images of skin biopsy sections stained with PGP9.5 antibody (top row, brown color) to highlight intraepidermal nerve fibers (top row, arrows) and corneal subbasal nerve plexus mosaics obtained by IVCM (bottom row), both at final follow-up. In a 69-year-old female subject with normal glucose tolerance (NGT, left column), intraepidermal nerve fibers were detected while in the cornea of the same subject a dense distribution of subbasal nerves and inferocentral circular whorl were apparent. In a 68-year-old female with T2DM diagnosed over 25 years prior to examination (right column), intraepidermal nerve fibers were identified whereas the corneal subbasal nerve plexus appeared less densely innervated. Scale bars: 50µm (top row), 500µm (bottom row).