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Original article

Assessment and comparison of the morphology and function of the corneal sub-basal nerve plexus in type-1 diabetes mellitus patients and in healthy subjects

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Abstract

Aim/purpose: It is believed that small nerve bundles are damaged in the earliest stages of neuropathy caused by diabetes mellitus (DM). Our goal was to evaluate and compare anatomical characteristics of corneal nerve fibers and corneal sensitivity in type-1 DM patients and in healthy control subjects.
Design: A prospective, masked, controlled cross-sectional clinical study.
Method: Thirty patients with type-1 DM and ten non-diabetic healthy subjects underwent a corneal confocal microscopy to evaluate the corneal sub-basal nerve fibers (density, number of nerves and branches, total nerve length) and contact corneal esthesiometry.
Results: Diabetic patients had significantly lower corneal nerve fiber density density (14.32 ± 5.87 vs. 19.71 ± 5.59 mm/mm2; p = 0.023) nerve branches number (4.57 ± 3,91 vs. 9.90 ± 5.8 n°/image; p = 0.006) , nerve fiber length (2.28 ± 0.94 vs. 3.13 ± 0.89 mm; p = 0.032) and corneal sensitivity (1.13 ± 0.29 vs. 0.98 ± 0.058 gr/mm2 p = 0.02), as compared with controls. A negative correlation was found between corneal nerve fiber length, corneal nerve number, corneal nerve fiber density and disease duration ($p < 0.05$).
Conclusion: Corneal confocal microscopy and corneal sensitivity evaluation are

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noninvasive techniques helping to detect early changes in the sub-basal nerve plexus characteristic for diabetic neuropathy (DN) in patients with type-1 DM. Further studies are required to investigate the role of corneal neuropathy assessment using these novel techniques as a toll to detect early DN.

Key words: contact corneal esthesiometry, corneal confocal microscopy, corneal sensitivity, diabetes mellitus, diabetic neuropathy.

1. Introduction

Diabetic neuropathy (DN) is a significant and prevalent complication of diabetes mellitus (DM), which has no effective treatment once established and can ultimately result in foot ulceration and lower extremity amputation.[1] Up to 50% of patients with DM will develop distal symmetric polyneuropathy at some point during their illness.[2] The natural history of nerve damage in patients with type-1 DM is not entirely clear but we do know that the development of diabetic neuropathy has been related not only to glycemic control but also to conventional cardiovascular risk factors such as hypertension and dyslipidemia.[3] Unfortunately, demonstrating an improvement in neuropathy over time has been much more difficult to achieve than preventing progression.[4] Recent studies demonstrated significant abnormalities in the small fibers in subjects with impaired glucose tolerance and DM, despite normal electrophysiology, suggesting that the earliest nerve fiber damage is to the small fibers. Clinical assessments and scales have the advantage of taking into account the patients' symptoms and a neurologic examination, but a recent work has indicated that this approach may have a poor reproducibility.[5]

Traditional neuropathy diagnostic tools do not objectively and accurately assess small nerve fibers, which are often the first to be injured and perhaps the first to be repaired[6], and which has a very important value in clinical trials when investigating interventions for the prevention and treatment of DN. Although electrophysiology correlates with large fiber's damage, it does not assess small fibers (Aδ and C fibers) and the test for preclinical small nerve damage, the skin punch biopsy, which can detect intra-epidermal nerve fiber density and is generally regarded as the gold standard of small nerve fiber degeneration is an invasive procedure.[7,8] Several groups have reported the use of corneal confocal microscopy (CCM) evaluation of corneal nerve structures and corneal sensitivity measurement as a reliable assessments of DN[9,10] and has been shown to be effective as a rapid, noninvasive, repeatable tool that allows detection of neuropathy in patients with DM.[11] In this study we assessed the corneal sensitivity and corneal nerve morphology using contact corneal esthesiometry and CCM in DM patients and healthy control subjects aiming to detect and compare small corneal nerves alterations that might possibly predict development of neuropathy and stratify diabetic patients with increasing neuropathic severity.

2. Materials and methods

2.1. Subjects

This prospective, masked, controlled cross-sectional clinical study, conducted in accordance with the Declaration of Helsinki with the approval of the Human Research Committee with applicable regulations pertaining to Good Clinical Practice was conducted in the Eye Clinic and Endocrinology Clinic of the Lithuanian University of Health Sciences' Hospital.

All subjects were 18 and older and all signed an informed consent form. The patients underwent a single medical examination during which several factors were assessed (age, gender, diabetes mellitus duration, glycated hemoglobin A1c (HbA1c), detailed medical history, family history, lifestyle factors, comorbidities, etc.) and the ophthalmological examination performed (best corrected visual acuity, refraction, intraocular pressure, biomicroscopy, ophthalmoscopy, corneal confocal microscopy, contact corneal esthesiometry).

The study group included type-1 DM patients whereas the control group included healthy subjects.

Exclusion criteria: neuropathy attributable to causes other than diabetes, diseases known to affect the corneal sub-basal innervation (*i.e.*, Herpes zoster), contact-lens wear, concomitant active or past corneal or ocular surface diseases (*i.e.*, active or a history of ocular herpes simplex infection), systemic disease known to affect the corneal sub-basal innervation, (*i.e.*, dry eye in Sjögren syndrome), corneal dystrophy, previous corneal surgery, corneal opacification or visible corneal edema, severe movement disorders (strabismus, nystagmus, poor compliance, or fixation issues),[12-15] refractive laser procedure (LASIK) in the past five years,[16] known allergy to proparacaine .

DM type 1 was defined based on the following criteria for the diagnosis of diabetes: symptoms of diabetes (polydipsia, polyuria, weight loss, fatigue, dizziness, nausea etc.) plus casual plasma glucose concentration ≥ 11.1 mmol/l or Fasting Plasma Glucose (FPG) ≥ 7.0 mmol/l twice on two separate days. Diabetic neuropathy was defined using a modified Neuropathy Disability Score (NDS), which is based on the assessment of ankle reflexes and sensory modalities on the big toes of both feet, and scored: (i) ankle reflexes (0, normal; 1, present with reinforcement; 2, absent); (ii) vibration with a 128-Hz tuning fork; (iii) pinprick; and (iv) temperature (warm and cool) (0, normal; 1, abnormal). Final scores of 3-5, 6-8 and 9-10 were considered to be evidence of mild, moderate and severe clinical neuropathy, respectively.[17]

2.2. Corneal confocal microscopy

All participants underwent in-vivo corneal confocal microscopy (IVCCM) examination of the sub-basal nerve fiber plexus (SNP), comprised between the basal epithelium and the Bowman's layer of the cornea scanned with a laser IVCCM (Heidelberg Engineering GmbH, Heidelberg, Germany) to produce a 0.4 mm x 0.4 mm (384 pixel

x 384 pixel) applying an established methodology.[18] The device is a laser-scanning confocal microscope that uses a visible 670 nm red wavelength diode laser source. The subject's eyes were topically anesthetized using a drop of 0.5% proparacaine hydrochloride, and an ocular 2% carbomer containing gel was applied on the surface of the eye for lubrication. A drop of gel was placed on the tip of the objective lens, which was covered by a sterile disposable TomoCap® to allow the optical coupling of the objective lens to the cornea after another drop of gel had been applied on the surface of the TomoCap® Subjects fixed their gaze on a target positioned behind the corneal confocal microscope device and the examiner used a side-view digital video camera to ensure the apex of the central cornea was scanned. Five to ten high-quality images with the best resolution of the SNP were acquired from the center of the cornea.

The investigator who examined the cornea and undertook morphometric measurements of the images was masked with respect to the identity of the patients as well as medical and neurological results of the subjects. From the images showing well-focused nerves from the central cornea, one image was randomly selected. The priority was given to the picture with the highest quality. The SNP density was calculated, defined by the length of nerves per square millimeters of image area.[9] The following variables were quantified: (1) corneal nerve fiber density (CNFD) – the total number of all nerve fibers per mm^2 (in mm/mm^2); (2) corneal nerve fiber length (CNFL) – the total length of nerve fiber in mm; (3) corneal nerve branches number (CNBN) – the number of branches per image; (4) corneal nerve number per image (CNN) – the total number of major nerves per image. Measures 1 and 2 were calculated using the NeuronJ plug-in of the computer software ImageJ (ImageJ 1.49d, the Java-based image processing environment developed at the National Institutes of Health, Bethesda, MD, USA), which allows a manual semi-automatic nerve tracing and further calculation of the nerve plexus. The pictures on a single plane were mandatory, no oblique pictures were selected, and only the visible parts of the nerve were marked with the ImageJ software.[18]

2.3. Corneal sensitivity

Corneal sensitivity examination is performed to assess the sensory function of the cornea. The examination was carried out using a handheld Cochet-Bonnet esthesiometer (Luneau Ophtalmologie, France), which allows to measure the sensation level and record its numerical value. The device relies on the principle of contact esthesiometry. It contains a thin (0.12 mm in diameter), retractable, nylon monofilament, which has a length that can be regulated in order to increase or reduce the amount of pressure applied. The fully extended length of nylon filament was 60 mm, at which length the cornea was firstly tested. If a positive answer was not detected the filament length was shortened by steps of five mm and the procedure was repeated until a positive response was obtained and each of the pressure value obtained was written down.[9,19]

Five positions were tested in each eye using the esthesiometer, the first one being the central cornea. The cornea was then virtually divided horizontally and vertically into four quadrants, which were tested one after the other. After all five zones had been tested, the average pressure needed to elicit a positive answer was calculated.

2.4. Statistics

SPSS 17.0 for Windows was used to compute the results. The analysis included descriptive and frequency statistics. All data are expressed as means (x(SD)). P value less than 0.05 was considered as significant. The Mann-Whitney test was used to compare patients with type-1 diabetes and controls.

3. Results

Thirty type-1 diabetic patients aged 34 ± 10.4 years and ten control subjects aged 29 ± 11.2 years were included in the study (p = 0.13). There were statistically more female patients in the diabetic group than in control (p = 0.03) (participants characteristics are shown in Table 1).

Table 1. Clinical demographics and corneal sensitivity in control subjects and diabetic patients**.**

	Healthy volunteers (N = 10)	**Diabetic patients (N = 30)**	***p value***
Female sex, N (%)	6 (60%)	27 (90%)	**0.03**
Age (years)	29 ± 1.2	34 ± 10.4	0.13
Diabetes duration (years)	-	13 ± 9.8	-
HbA1c (%)	-	8,5 ± 1,4	-
CCM parameters:			
CNFL (mm)	3.14 ± 0.9 [3.13 (1.74; 4.59)]	2.29 ± 0.9 [2.27 (0.36; 3.87)]	**0.032**
CNFD (mm/mm^2)	19.72 ± 5.6 [20.06 (10.88; 28.71)]	14.32 ± 5.9 [14.19 (2.25;24.18)]	**0.023**
CNBN (number/image)	9.9 ± 5.8 [9 (2;22)]	4.6 ± 3.9 [3 (0;16)]	**0.006**
CNN (number/image)	5.7 ± 1.9 [6.0 (3;8)]	5.3 ± 1.9 [6 (1;8)]	0.67
Corneal sensitivity (gr/mm^2)	0.99 ± 0.06 [0.96 (0.96;1.14)]	1.13 ± 0.29 [1.01 (0.96; 2.24)]	**0.02**

Mean ± SD [median (min.; max.)]. p values were calculated with non-parametric Mann-Withey Test, significance level $p < 0.05$. HbA1c = Glycated hemoglobin A1c; CNFL = corneal nerve fiber length; CNFD = corneal nerve fiber density; CNBN = corneal nerve branches number; CNN = corneal nerves number.

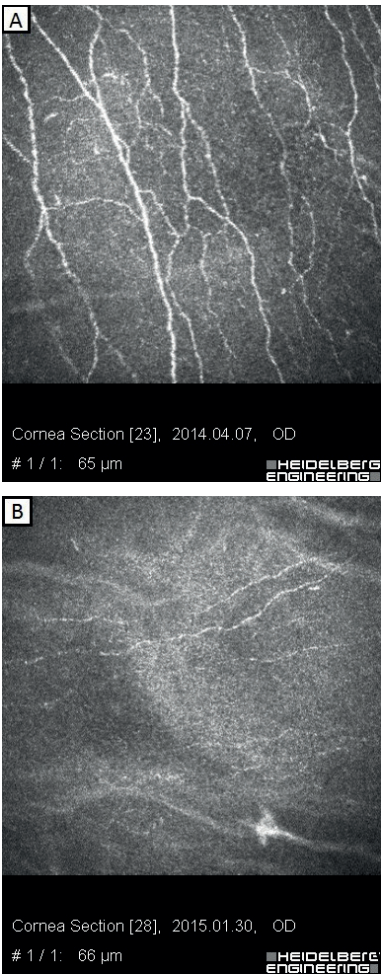

Fig. 1. CCM images from Bowman's layer of cornea: **A.** A 23-years-old healthy patient, CNFD 21.81 mm/mm^2, corneal sensitivity 1.14 gr/mm^2. **B.** A 35-years-old patient, diabetes duration 23 years, CNFD 14.15 mm/mm^2, corneal sensitivity 1.43 gr/mm^2.

Safety: none of the participants experienced any visual or corneal sequelae as a result of the examinations.

Figure 1 displays a sample of the pictures obtained using the CCM. The interconnected sub-basal nerve fiber plexus is represented as elongated hyperreflective structures in both a DM and a healthy person at the sub-basal level, located between the basal layer of the epithelium and the Bowman's membrane (Fig. 1).

Diabetic patients compared to controls had a significantly lower CNFL (2.28 ± 0.94 vs. 3.13 ± 0.89 mm; p = 0.032), CNFD (14.32 ± 5.87 vs. 19.71 ± 5.59 mm/mm^2; p

= 0.023), CNBN (4.57 ± 3.91 vs. 9.90 ± 5.8 no/image; p = 0.006), while the CNN values were not statistically significantly different between the two groups (5.3 ± 1.9 vs. 5.7 ± 1.9 n°/image; p = 0.67). Estimated corneal sensitivity was also lower in diabetic patients group compared to the healthy controls (0.99 ± 0.06 vs. 1.13 ± 0.29 gr/mm^2; p = 0.02). A negative correlation was found between CNFL, CNN, CNFD and diabetes duration (r = -0.366, r = - 0.464, r = -0.363; p < 0.05, respectively) and no statistical significance was found between diabetes duration and corneal sensitivity (p > 0.05) (Figs. 2, 3, 4). There were no statistically significant correlations between HbA1c, corneal sensitivity and sub-basal nerve parameters in the diabetic subjects group. We found no correlations between age and SNP morphological changes or corneal sensitivity in both diabetic and healthy control groups (p > 0,05).

4. Discussion

DN development is the main initiating factor for foot ulceration and lower extremity amputation. The lack of early biomarkers for nerve injury hinders the process of drug development in clinical research, which highlights the urgent need for a valid screening test in clinical practice that overcomes the limitations in their specificity as predictive markers for the future onset of neuropathy.[20] Over the past decade there has been increasing research interest in modeling the relationship between corneal nerve fiber loss and diabetes. There is evidence suggesting that CCM can detect early small fiber changes in patients with type-1 diabetes without neuropathy and accurately quantify the severity of DN.[21]

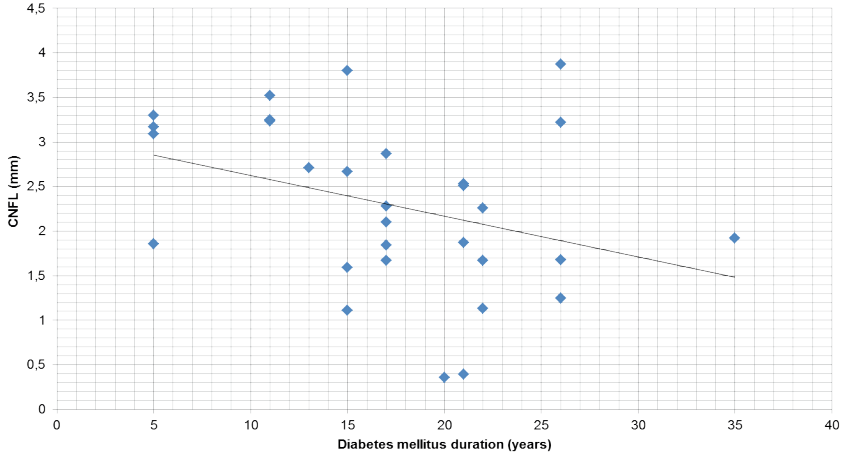

Fig. 2. Correlation between changes in nerve fiber density and duration of diabetes mellitus (r = -0.363, p = 0.049).

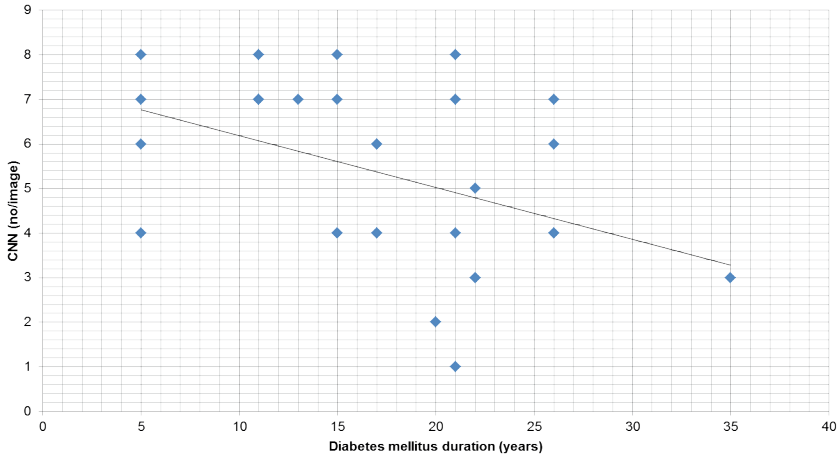

Fig. 3. Correlations between changes in nerve fiber length and duration of diabetes mellitus (r = -0.366, p = 0.046)

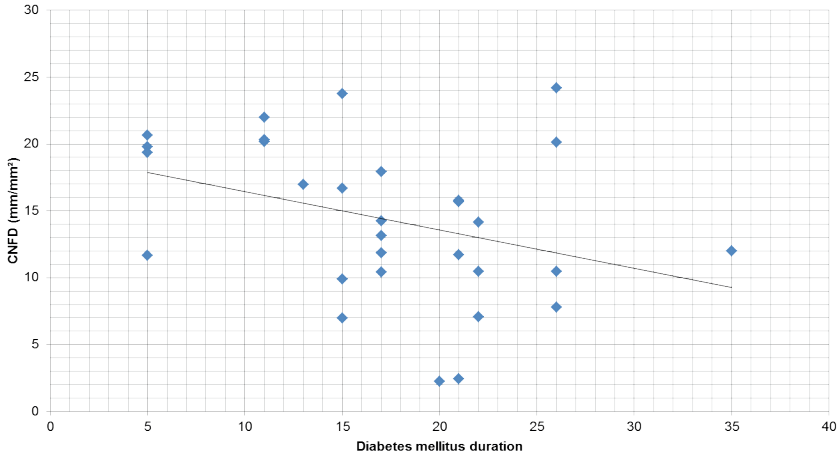

Fig. 4. Correlations between changes in nerve fiber number and duration of diabetes mellitus (r = - 0.464, p = 0.010)

The purpose of this cross-sectional study was to evaluate the differences of corneal sub-basal nerve plexus and corneal sensitivity in subjects with type-1 DM and healthy controls. We hypothesized that values between diabetic and healthy control groups indicate incipient nerve injury that represents those individuals with future neuropathy risk. The study was conducted using two novel noninvasive measures of neuropathy, namely contact corneal esthesiometry and corneal

confocal microscopy.

Our study estimated that corneal sub-basal nerve plexus parameters and corneal sensitivity are significantly reduced in diabetic patients compared with healthy controls and these results are in agreement with several other studies.[9-11,22,23] Rosenberg *et al.* demonstrated that patients with type-1 DM have a reduction in corneal sensitivity and in the number of corneal nerve fiber bundles, and these correlate statistically significantly with the severity of neuropathy.[9] We did not stratify the severity of somatic nerve neuropathy in diabetic patients because of a small number of participants, who have been diagnosed with DN. In Tavakoli *et al.* study, 101 diabetic patients underwent neurological evaluation, the neuropathy deficit score (NDS) was established and the severity of neuropathy was determined. They demonstrated a progressive reduction in corneal sensitivity and increasing corneal nerve degeneration with increasing severity of diabetic neuropathy.[10] In another study conducted by Ishibashi *et al.*, 38 controls and 38 diabetic patients were recruited. DN was not stratified into severity levels, but diabetic patients were divided into two groups based on the presence or absence of DN and was named as 'probable DN'. The authors also established that CCM parameters' alterations were found in patients without neuropathy compared with healthy subjects.[22]

We found no statistically significant correlations between HbA1c, corneal sensitivity and sub-basal nerve parameters in the diabetic patient's group. This could be due to a relatively small sample size. Ishibashi *et al.* found an inversed correlation between CNFD and CNFL and the mean annual HbA1c levels for a period of seven to ten years prior to the examination, suggesting that the mean HbA1c level during this period was an independent predictor of reduced CNFD and CNFL in type-1 DM, but, in their study, this significant correlation disappeared abruptly beyond ten years, probably because of a decrease in the number of subjects.[22]

Although another study conducted by Tavakoli *et al.*, including 25 patients with DM, showed that improvement in nerve fiber density correlated significantly with the improvement in HbA1c ($r = -0.51$; $p = 0.008$) and confirmed the negative association between HbA1c and nerve fiber density ($P = 0.02$), they did not find significant correlations between HbA1c and other corneal sub-basal nerve parameters (*i.e.* corneal nerve branches number, nerve branch density, nerve fiber length, corneal nerves number).[24]

We did not find any statistically significant correlation between gender, corneal sensitivity and sub-basal nerve parameters. In our study, there were more female than male patients (40 participants, 33 of which were females), however, in a 2013 study conducted by Parissi *et al.* including 106 patients, 59 of which were females and 47 males, no differences in the mean sub-basal nerve density between genders were identified.[18]

Interestingly, our study showed that diabetes duration is negatively correlated to CNFL, CNN, CNFD ($p < 0.05$) and that the disease duration has no significant correlation with corneal sensitivity ($p = 0.295$), while Ishibashi *et al.* found no statis-

tically significant correlation, despite a very similar duration of diabetes in studies (13 ± 9.8 vs.15.4 ± 1.5 years). These discrepancies might become significant because of the different number of diabetic participants. Also Ishibashi *et al.* found no difference in nerve branches between controls and diabetic patients,[22] whereas our study showed that the number of nerve branches was statistically significantly lower in the diabetic group compared to healthy patients ($p < 0.05$). The branches, when analyzed by CCM, possess a smaller diameter than the main nerve trunks. They were significantly less numerous in diabetic patients than in our control group, which may confirm that smaller diameter nerve fibers, corresponding to sensory nerve fibers, are affected primarily in the course of DN, and they are probably responsible for the increased threshold required to elicit a corneal sensation.[7] In contrast with another study conducted by Rosenberg *et al.*,[9] we did not find statistically significant changes in CNN between both groups ($p = 0.67$), while they found that patients with diabetes had less nerve fiber bundles than healthy control subjects ($p = 0.035$).[9] This might be due to differences in estimation methods, in the evaluation of the branches and nerves per image. Also different from our study, where we did not find any significant correlation, Rosenberg *et al.* estimated that corneal sensitivity was inversely correlated with the duration of diabetes ($r = -0.630$; $p = 0.001$) and this might be caused by discrepancies in diabetes duration mean between both studies (13 ± 9.8 vs. 25.9 ± 8.1 years).[9]

Despite the results, showing similarities between different studies, there are differences not only in the number of participants, but also in the methodologies and in the technique of images capture, selection, and analysis. First of all, some investigations that quantified CCM parameters in diabetic and healthy subjects used slit-scanning confocal microscopy,[9] and not laser scanning microscopy. Different software was used to measure CCM parameters, which makes it more difficult to compare the parameters between healthy controls and diabetic patients. Secondly, although diminished, corneal sensitivity in diabetes with symmetrical involvement was first described by Schwartz using a Cochet and Bonnet esthesiometer (and later by others),[25] there were differences in corneal sensitivity assessment methods in some recent studies. Tavakoli *et al.* also found significant differences of corneal sensitivity between diabetic and healthy persons ($p < 0.001$), but the study was conducted using a noncontact corneal esthesiometer (NCCA) and assessing just the center of the cornea,[10] which differed from our study.

We acknowledge limitations to the interpretation of our results. Firstly, the data were cross-sectional and correlated with clinical factors retrospectively. Although we define diagnostic thresholds for CNFL, we acknowledge that the small number of participants, differences in gender sample and measurement error could limit the precision of these specific threshold values. Secondly, although we evaluated the corneal sensitivity, it was performed by contact corneal esthesiometry, and not using newer non-contact gas-esthesiomer, which is due to the very limited availability of such devices.[1] Thirdly, we acknowledge that protocols using fully automated

image analysis will likely be needed for this diagnostic test to be generalized into clinical practice.[26-27]

Because of such limitations, a longitudinal study, including more participants, a similar number of different genders and more data concerning the relationship between the morphological anomalies on CCM and the contributing clinical factors is needed.

Many studies use a cross-sectional methodology and it is still unclear how the early nerve regeneration seen in the cornea could be related to the functional improvements of peripheral neuropathy. The possibility remains that corneal nerves and sensory/motor nerves in the feet are unrelated. At present, underdiagnoses impede the benefits of early identification, thus delaying early management and prevention of neuropathy. Although CCM and corneal esthesiometry have the potential to be a game changer in the neuropathy outcome assessment, additional researches[28] as well as a longitudinal study are needed to provide more robust data regarding the ability of CCM to identify patients at risk of developing neuropathy.

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