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CORNEAL CONFOCAL MICROSCOPY – A NOVEL, NONINVASIVE METHOD TO ASSESS DIABETIC PERIPHERAL NEUROPATHY

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Abstract

Background and aims. *This article aims to compare corneal confocal microscopy (CCM) with acknowledged tests of diabetic peripheral neuropathy (DPN), to assess corneal nerve morphology using CCM in diabetic patients, and to underline possible correlations between clinical and biological parameters, diabetes duration and DPN severity.* ***Material and methods.*** *A total of 90 patients with type 2 diabetes were included in the study for whom we measured anthropometric parameters and we performed laboratory measurements (tests). The patients were assessed for diabetic peripheral neuropathy using Semmes-Weinstein Monofilament Testing (SWMT), Rapid-Current Perception Threshold (R-CPT) measurements using the Neurometer®, and CCM. We stratified the patients according to DPN severity, based on four parameters extracted after image analysis.* ***Results.*** *A higher percentage of patients were diagnosed with DPN using CCM (88.8%), compared with SWMT and R-CPT measurement (17.8% and 40% respectively). The incidence of DPN detected with CCM was considerable in patients with normal protective sensation and with normal R-CPT values.* ***Conclusions.*** *Our study showed that corneal confocal microscopy is a useful noninvasive method for diabetic neuropathy assessement in early stages. It was proven to directly quantify small fiber pathology, and to stratify neuropathic severity, and therefore can be used as a new, reliable tool in the diagnosis, clinical evaluation, and follow-up of peripheral diabetic neuropathy.*

key words: *diabetic neuropathy, corneal confocal microscopy*

Background and aims

Diabetic peripheral neuropathy (DPN) is one of the most common complications of diabetes [1]. A total of 60-70% of diabetic patients will develop peripheral neuropathy during life, or lose sensation in their feet [2]. DPN may be present at the diagnosis of type 2 diabetes in up to 10% of patients, and more than 90% of people with diabetic peripheral neuropathy are unaware they have it [3]. Chronic sensorimotor diabetic peripheral neuropathy is by far the most common form of DPN and it is proven to be one of the major risk factors for foot ulcers development. Up to 25% of diabetic patients will develop a foot ulcer [4]. The yearly incidence of diabetic foot ulcers is known to range from 2% to 32%, depending on ADA risk classification

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[5,6]. Diabetic foot ulcers double mortality and heart attack risk while increasing risk for stroke by 40%, preceding in most of the cases a lower limb amputation [7]. Up to 70% of all leg amputations are performed in people with diabetes, so it is important to detect peripheral neuropathy early in the natural history of the disease so that diabetic foot care education can be provided, and protective measures can be used to avoid devastating complications associated with the diabetic foot. There is evidence that instituting a structured diabetic foot program can yield a 75% reduction in amputation rates and a near fourfold reduction in inpatient mortality [8].

Although electrophysiology, quantitative sensory testing (QST), and assessment of neurological disability are advocated to define neuropathy severity, they have limitations when they are used to define therapeutic efficacy in clinical intervention trials [9,10]. Only biopsy of the sural nerve and skin biopsy allows a direct examination of nerve fiber damage and repair [11]. However, both are invasive procedures, and assessment of therapeutic efficacy in clinical trials requires repeated biopsies [12].

Recent studies suggested that small unmyelinated C-fibers are damaged early in diabetic neuropathy [13]. Diagnosing a DPN that preferentially involves small nerve fibers (small-fiber neuropathy, SFN) can be challenging. Emerging markers of SFN include nerve biopsy (invasive and highly specialized), skin biopsy measurement of intraepidermal nerve fiber density (IENFD) (minimally invasive and good sensitivity and specificity), corneal confocal microscopy (CCM) (potential surrogate for SFN), and nerve axon reflex/flare response (requires further validation) [14]. The 2009 Toronto Consensus Panel proposed three categories to define SFN: possible (length-dependent symptoms and/or signs of small-fiber damage), probable (length-dependent symptoms, clinical signs, and normal sural NCS findings), and definite (fulfilling the definition of probable, plus abnormal IENFD at the ankle or abnormal quantitative thermal threshold at the foot) [14,15].

Corneal confocal microscopy is a novel clinical technique for the study of corneal cellular structure. Because corneal confocal microscopy is a noninvasive technique for in vivo imaging of the living cornea, it has huge clinical potential to investigate numerous corneal diseases. Most recently, it has been used as a surrogate for peripheral nerve damage in a variety of peripheral neuropathies (including diabetic neuropathy) and may have potential in acting as a surrogate marker for endothelial abnormalities [16].

Recent studies showed that CCM identifies early small nerve fiber damage and accurately quantifies the severity of diabetic neuropathy [17,18]. It was also proven that CCM relates to intraepidermal nerve fiber loss and detects nerve fiber damage when the results of electrophysiology tests and quantitative sensory testing (QST) are normal [12].

The aim of our study was to assess corneal nerve morphology using CCM in diabetic patients, to establish possible correlations between clinical and biological parameters and diabetes duration with DPN severity, and also to compare CCM with established tests of diabetic neuropathy in diagnosing DPN.

Material and methods

A total of 90 patients with type 2 diabetes from Cluj-Napoca Diabetes Clinical Center were included in the study, between September 2012-November 2012. We measured the following anthropometric parameters: weight, height, waist circumference and we calculated body mass index (BMI). The laboratory measurements

included: glycated hemoglobin, total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerides. The patients were assessed for the presence of DPN using Semmes-Weinstein Monofilament Testing (SWMT), Rapid-Current Perception Threshold (R-CPT) measurements using the Neurometer®, and CCM. All patients signed an informed consent prior to inclusion in the study and the protocol was approve by the local review board.

Semmes-Weinstein Monofilament Testing

We used the technique recommended by the American College of Physicians in Diabetic Foot Ulcers (Clinical Skills Module), to assess protective sensation [19]. A 5.07 Semmes-Weinstein nylon monofilament was employed to apply a consistent 10 g force on 4 different sites on the plantar surface of the foot (plantar surface of the great toe, base of the first metatarsal head, base of the third metatarsal head, base of the fifth metatarsal head). With the patients unable to see their feet, the monofilament was placed on the plantar surface of the foot at 90 degree angles to the skin and the pressure increased until the filament buckled. The patients were asked to say when they felt something. Protective sensation is considered to be present if the patient correctly answers two or more of the three applications, one of which was a sham. If the patient correctly answers only one or none of the three applications, the sites will be retested. The patient is considered to have insensate feet if they fail on retesting at just one more site on either foot [19].

Rapid current perception threshold measurement using the Neurometer®

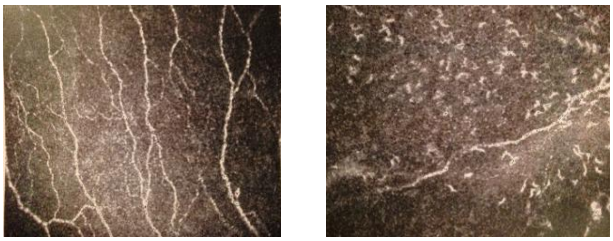
All the patients underwent rapid current perception threshold (R-CPT) measurements using the Neurometer®. The Neurometer® generates R-CPT readings based on the minimal strength of alternating current (AC) stimulus that the patient can detect. AC stimulus was applied to the hallux. At each test site, three different frequencies of 2000, 250 and 5 Hz of AC current were applied to stimulate large myelinated A-beta fibers, small myelinated A-delta fibers and small unmyelinated C fibers, respectively [20]. A-beta fibers detect cutaneous pressure; A-delta fibers detect fast pain and temperature, whereas small diameter C fibers detect slow pain and temperature. At each frequency (2000, 250 and 5 Hz), an R-CPT value was generated and ranged from 1 to 25. A value ranging from 6 to 13 was classified as normal, while a value ranging from 1 to 5 showed hyperesthesia (increased sensation). A value between 14 and 25 showed hypoesthesia (decreased sensation). Both hyperesthesia and hypoesthesia indicated the presence of sensory neuropathy [21]. In the present paper we analyzed only R-CPT obtained by applying 5Hz frequency, known to be the most sensitive in detecting small fiber neuropathy.

Corneal confocal microscopy

The cornea is the most densely innervated part of the human body containing myelinated A-delta and unmyelinated C fibers derived from the ophthalmic division of the trigeminal nerve. A rich network of nerves known as the sub-basal nerve plexus lies between the basal epithelium of the cornea and Bowman's membrane. This layer appears with the confocal microscopy as a dense neural plexus characterized by tortuous and thin, beaded nerve fibers, with a homogeneous reflectivity that is distinct from the background.

We used a Heidelberg Retina Tomograph for CCM assessment. This is a confocal retinal microscope but has attached a Rostock module that allows it to shrink focal length and so, can make cornea and no retinal section. The images (Figure 1) were analyzed by an ophthalmologist and four parameters were quantified: the total

number of nerves per image (TNN); the number of main branches (NMN), the number of branches connections (NBC), and the branches/connections ratios (BCR).



A **B**

Figure 1. *Sub-basal nerve plexus of a subject without DPN (A) and of a patient with severe DPN (B).*

Depending on the parameters mentioned above, DNP was stratified into three severity classes as presented in Table 1. This is an original protocol (conceived by the partner ophthalmologist who analyzed corneal images) for quantifying the severity of lesions in diabetic neuropathy, designed to quickly assess, studying only one or a few representative images, whether or not the patient has diabetic neuropathy and its severity.

Table 1. DPN severity according to confocal microscopically analysis.

	DPN severity			
Parameters	Without DPN	Mild	Moderate	Severe
TNN	>25	20	15	10
NMB	>8	5-7	2-4	≤1
NBC	>20	<15	<10	<5
BCR	<1.25	1.3	1.5	>2

Statistical analysis

For statistical analysis we used SPSS 15. All the numeric variables were expressed as mean value ± standard deviation. A value of $p < 0.05$ was considered statistically significant. The statistical significance of frequencies distribution was analized with Chi square test.

Results

The baseline characteristics of the study group are shown in Table 2.

Table 2. Baseline characteristics of the study patients.

No. of patients	90
Mean age (years)	61.58±10.092
Sex (male)	43.3% (n=39)
Mean diabetes duration (years)	8.31±6.61
Smoking	16.7% (n=15)
Alcohol	28.9% (n=26)
Hypertension	82.2% (n=74)
Total cholesterol (mg/dl)	179.3±40.6
HDL-cholesterol (mg/dl)	45.3±13.3
LDL-cholesterol (mg/dl)	101.3± 35.7
Triglycerides (mg/dl)	163.4±69.1
Mean HbA1c (%)	7.5±1.1
BMI (kg/m^2)	31.6±5.14
Retinopathy	23.3% (n=21)

SWMT detected DPN in only 17.8% of all patients. When applying 5Hz frequency to assess R-CPT of small unmyelinated nerve fiber, 40% of all subjects were diagnosed with DPN, whereas CCM identified 88.8% of patients with DPN, as shown in Figure 2. Thus, a higher percentage of patients were diagnosed with DPN using CCM, compared with SMWT ($p=0.119$) and with R-CPT ($p=0.171$), even if we do not notice a statistical significance.

Of the 74 patients without DPN after protective sensation assessment using SWMT, 29 (39.1%) were diagnosed with mild DPN after performing CCM, 27 (36.4%) with moderate DPN and 8 (10.8%) with severe DPN, whereas only 10 (13.51%) of them had no pathological findings on corneal analisys.

We also analyzed the 54 patients without DPN according to R-CPT measurement and we observed that 22 (40.7%) of them had mild DPN after performing CCM, 19 (35.1%) had moderate DPN, 5 (9.25%) had severe DPN and only 8 (14.81%) of them had normal parameters after performing CCM as shown in Figure 3.

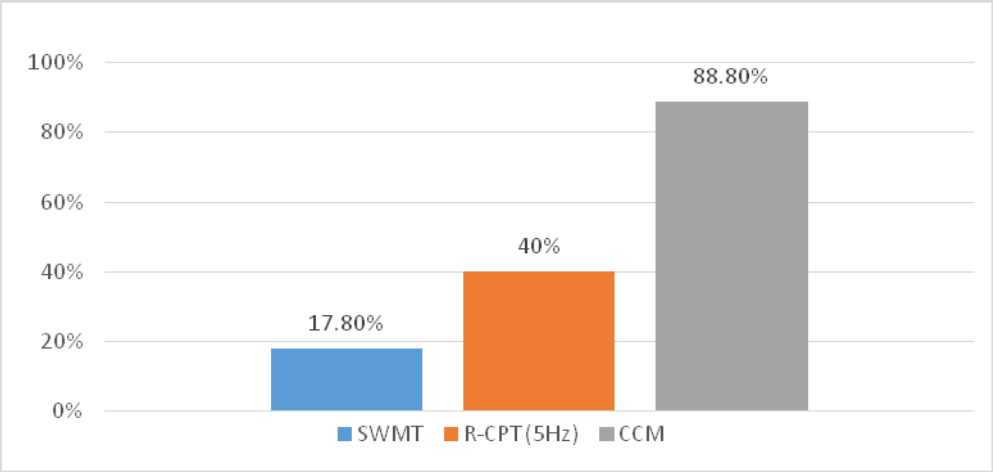


Figure 2. *Incidence of DPN with different modalities of assessment.*

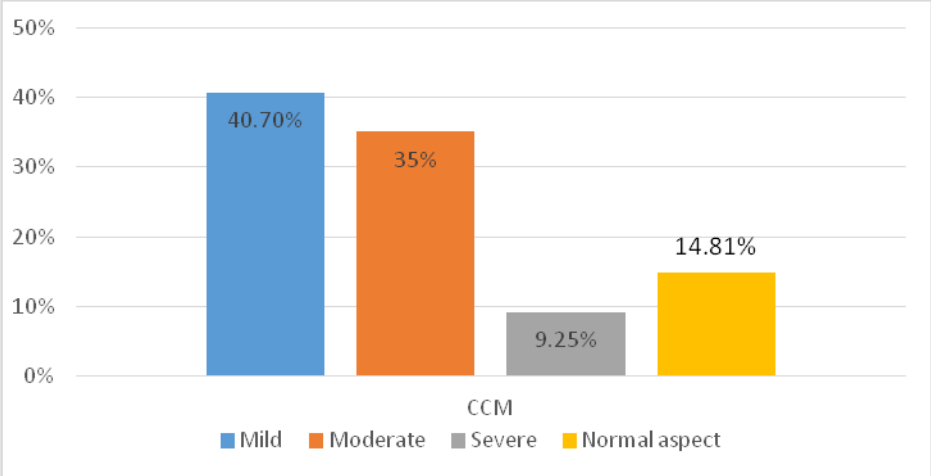


Figure 3. *Incidence of DPN detected by CCM in patients with normal R-CPT values after applying 5Hz AC frequency.*

When we analyzed the incidence of DPN detected by CCM according to diabetes duration, the results showed an ascending trend of DPN incidence with increasing duration of diabetes, more obvious in the moderate intensity DPN group. Surprisingly, an important percentage of patients with a diabetes duration of less than five years, was found with severe neuropathy. The results are presented in Table 3.

Table 3. Incidence of DPN detected by CCM according to diabetes duration

	DPN severity (assessed by CCM)		
Diabetes duration	Mild	Moderate	Severe
<5 years	41.9%	22.5%	19.3%
5-10 years	32.3%	44.1%	8.8%
>10 years	40%	52%	8%

The patients were divided into two groups based on their baseline HbA1c: <7% (28 patients) and ≥7% (59 patients). For three of the patients included in this study the HbA1c values were not available. In the HbA1c <7% group, a smaller proportion of patients were diagnosed with mild DPN compared with the HbA1c ≥7% group (28.5% vs. 42.3%, p=0.035), as shown in Figure 4. There were no significant differences between the two groups for the other forms of DPN, moderate and severe (p=0.888).

We also evaluated the incidence and severity of DPN in the study group using CCM as diagnosis method, after dividing the patients in 2 arms, based on their treatment regimen: insulin treated patients and subjects on oral antidiabetic medication. We observed that a higher

percentage of patients treated with oral agents had mild neuropathy (p = 0.072), while a higher proportion of insulin treated patients were diagnosed with moderate neuropathy (p = 0.072). The results are shown in Figure 5.

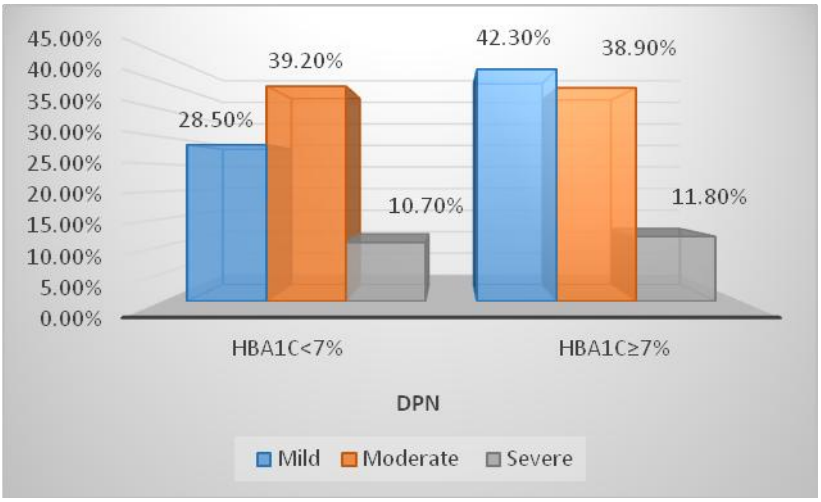


Figure 4. *Incidence of DPN detected by CCM according to HbA1c value.*

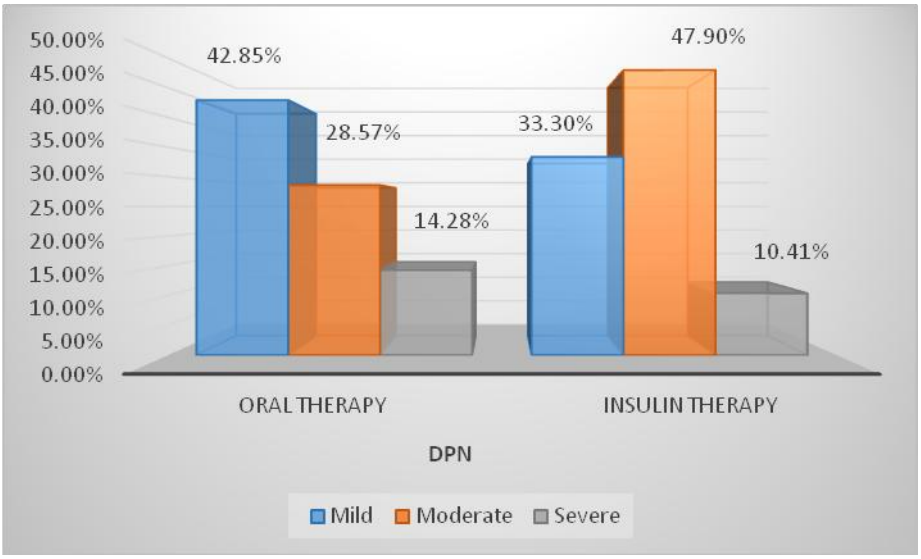


Figure 5. *Incidence and severity of DPN detected by CCM according to type of treatment.*

Discussions

In this paper we documented that corneal confocal microscopy as a noninvasive method to assess the presence and severity of diabetic neuropathy. Our data showed that CCM could identify corneal nerve fiber damage in patients considered to have no evidence of neuropathy based on neurological evaluation and QST, consistent with the existing evidence. In our research, CCM proved to be superior in identifying patients with DPN, compared with monofilament testing, and also with another important method in assessing small fiber neuropathy, rapid current perception threshold measurement using the-Neurometer®.

A recent study showed that CCM may be used to detect early nerve damage and to stratify diabetic patients with increasing neuropathic severity [22]. Another study conducted on 42 patients and 27 age-matched controls used CCM to quantify the following parameters: the number of fibers, the tortuosity of fibers, the number of

beadings, and the branching pattern of the fibers. This study found that diabetes damages corneal nerves, particularly the corneal sub-basal nerve plexus, and this damage may be easily and accurately documented using corneal confocal microscopy [23].

It was also shown that CCM correlate with the intraepidermal nerve fiber (IENF) loss in skin biopsies from the dorsum of the foot in diabetic patients [12] and may also show nerve repair after pancreas transplantation [24].

Results of our study showed that an important number of patients with a diabetes duration of less than 5 years had specific morphological changes of corneal nerve fibres suggestive of neuropathy, so it is important to conduct a chronic complications screening as early as possible after diabetes diagnosis.

The present study is, to our knowledge, the first of its kind held in our country, and can open great opportunities for research in this area, in a first stage. But, hopfully, in the near future, we will be able to apply routinely in our daily practice this early and noninvasive assessment method of DPN.

Our study has its limitations. The analysis of the images obtained with CCM, using interactive image analysis is highly labor intensive and requires considerable expertise to quantify nerve-fiber pathology. We were unable to compare CCM with the golden standard method for DPN diagnosis, nerve conduction studies. Continuing this research will be our goal, focusing on deepening statistical analysis to confirm the results obtained in the present paper.

Conclusions

Our study showed that corneal confocal microscopy is a noninvasive method and may be used to detect diabetic neuropathy in its early stages. It was proven to directly quantify small fiber pathology, and to stratify neuropathic severity, and therefore can be used as a new, reliable tool in the diagnosis, clinical evaluation, and follow-up of peripheral diabetic neuropathy.

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REFERENCES

1. Cornblath DR. Diabetic neuropathy: diagnostic methods. *Adv Stud Med* 4(8A): S650-S661, 2004. Accessed at: http://www.wellworksservices.com/wp-content/uploads/diabetic-neuropathy.pdf

2. Dyck PJ, Davies JL, Wilson DM, Service FJ, Melton LJ 3rd, O'Brien PC. Risk factors for severity of diabetic polyneuropathy: intensive longitudinal assessment of the Rochester Diabetic Neuropathy Study cohort. *Diabetes Care* 22: 1479-1486, 1999.

3. Bongaerts BWC, Rathmann W, Heier M et al. Older subjects with diabetes and prediabetes are frequently unaware of having distal sensorimotor polyneuropathy: the KORA F4 study. *Diabetes Care* 36: 1141-1146, 2013.

4. Singh N, Armstrong DG, Lipsky BA. Preventing foot ulcers in patients with diabetes. *JAMA* 293: 217–28, 2005.

5. Boulton AJ, Armstrong DG, Albert SF et al. Comprehensive foot examination and risk assessment: a report of the task force of the foot care interest group of the American Diabetes Association, with endorsement by the American Association of Clinical Endocrinologists. *Diabetes Care* 31: 1679-1685, 2008.

6. Sibbald G, Goodman L, Norton L, Krasner DL, Ayello EA. Prevention and treatment of pressure ulcers**.** *Skin Therapy Lett* 17: 4-7, 2012.

7. Brownrigg JR, Davey J, Holt PJ et al. The association of ulceration of the foot with cardiovascular

and all-cause mortality in patients with diabetes: a meta-analysis. *Diabetologia* 55: 2906–2912, 2012.

8. Weck M, Slesaczeck T, Paetzold H et al. Structured health care for subjects with diabetic foot ulcers results in a reduction of major amputation rates. *Cardiovasc Diabetol* 12: 45, 2013.

9. Boulton AJ, Malik RA, Arezzo JC, Sosenko JM. Diabetic somatic neuropathies. *Diabetes Care* 27: 1458 –1486, 2004.

10. Mojaddidi M, Quattrini C, Tavakoli M, Malik RA. Recent developments in the assessment of efficacy in clinical trials of diabetic neuropathy. *Curr Diab Rep* 5: 417–422, 2005.

11. Malik RA, Tesfaye S, Newrick PG et al. Sural nerve pathology in diabetic patients with minimal but progressive neuropathy. *Diabetologia* 48: 578 –585, 2005.

12. Quattrini C, Tavakoli M, Jeziorska M et al. Surrogate markers of small fiber damage in human diabetic neuropathy. Diabetes 56: 2148-2154, 2007.

13. Umapathi T, Tan WL, Loke SC, Soon PC, Tavintharan S, Chan YH. Intraepidermal nerve fiber density as a marker of early diabetic neuropathy. *Muscle Nerve* 35: 591–598, 2007.

14. Albers JW, Pop-Busui R. Diabetic neuropathy: mechanisms, emerging treatments, and subtypes. *Curr Neurol Neurosci Rep* 14: 473, 2014.

15. Tesfaye S, Boulton AJ, Dyck PJ et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. *Diabetes Care* 33: 2285–2293, 2010.

16. Tavakoli M, Hossain P, Malik RA. Clinical applications of corneal confocal microscopy. *Clin Ophthalmol* 2: 435–445, 2008.

17. Kallinikos P, Berhanu M, O'Donnell C, Boulton AJ, Efron N, Malik RA. Corneal nerve tortuosity in diabetic patients with neuropathy. *Invest Ophthalmol Vis Sci* 45: 418–422, 2004.

18. Malik RA, Kallinikos P, Abbott CA et al. Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. *Diabetologia* 46: 683–688, 2003.

19. American College of Physicians. Clinical Skills Module "Diabetic Foot Ulcers", 2007. Accessed at: https://store.acponline.org/ebizatpro/ProductsandServices/Products/ProductDetail/tabid/202/Default.aspx?ProductId=16334

20. Masson EA, Veves A, Fernando D, Boulton AJ. Current perception thresholds: a new, quick and reproducible method for the assessment of peripheral neuropathy in diabetes mellitus. *Diabetologia* 32: 724–728, 1989.

21. www.neurotron.com

22. Tavakoli M, Quattrini C, Abbott C et al. Corneal confocal microscopy: a novel noninvasive test to diagnose and stratify the severity of human diabetic neuropathy. *Diabetes Care* 33: 1792–1797, 2010.

23. Midena E, Brugin E, Ghirlando A, Sommavilla M, Avogaro A. Corneal diabetic neuropathy: a confocal microscopy study. J Refract Surg 22(9 Suppl): S1047-S1052, 2006.

24. Mehra S, Tavakoli M, Kallinikos PA et al. Corneal confocal microscopy detects early nerve regeneration after pancreas transplantation in patients with type 1 diabetes. *Diabetes Care* 30: 2608–2612, 2007.