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Corneal confocal microscopy in patients with distal symmetric polyneuropathy compared to controls

Microscopia confocal de córnea em pacientes com polineuropatia simétrica distal comparados a controles

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Abstract

Background Diabetic neuropathy (DN) is a very common clinical condition throughout the world. The diagnostic tests currently recommended have low sensitivity, such as electromyography, or are invasive, such as skin biopsy. New techniques have been developed to identify the early involvement of the peripheral nerve. With the advent of corneal confocal microscopy (CCM), a reduction in corneal innervation in patients with DN has been observed.

Objective To compare, through CCM, diabetic patients with symptomatic distal symmetric polyneuropathy (DSP) and controls.

Methods In the present study, through CCM, we compared the morphological changes in the sub-basal epithelial corneal plexus of 35 diabetic patients with symptomatic DSP with 55 controls. Moreover, we sought to determine a pattern of change regarding the severity stages of DSP, comparing the clinical, laboratory, and nerve-conduction (NC) variables.

Results Differences between the control and diabetic groups were observed for the following variables, respectively: age (44.9 ± 13.24 years versus 57.02 ± 10.4 years; $p < 0.001$); fiber density (29.7 ± 10.2 versus 16.6 ± 10.2; $p < 0.001$); number of fibers (4.76 ± 1.30 versus 3.14 ± 1.63; $p < 0.001$); number of Langerhans cells (4.64 ± 8.05 versus 7.49 ± 10.3; $p = 0.035$); tortuosity ($p < 0.05$); and thickness ($p < 0.05$). Furthermore, inverse relationships were found regarding fiber density and age ($p < 0.01$) and fiber density and the severity of the disease ($p < 0.05$). A positive relationship between the conduction velocity of the fibular nerve and fiber density ($p < 0.05$) was also observed.

Keywords
- Diabetes Mellitus
- Peripheral Nervous System Diseases
- Confocal Microscopy

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Conclusion Corneal confocal microscopy proved to be a fast, noninvasive and reproducible method for the diagnosis, staging, and monitoring of diabetic DSP.

Resumo

Antecedentes A neuropatia diabética (ND) é condição clínica muito frequente no mundo inteiro. Os testes diagnósticos atualmente preconizados são pouco sensíveis, como a eletroneuromiografia, ou invasivos, como a biópsia de pele. Novas técnicas de investigação complementares têm sido desenvolvidas a fim de identificar o acometimento precoce do nervo periférico. Com o advento da microscopia confocal de córnea (MCC), observou-se redução da inervação da córnea em pacientes com ND.

Objetivo Comparar, por meio da MCC, pacientes diabéticos com polineuropatia simétrica distal (PSD) sintomática e controles.

Métodos Neste estudo, por meio da MCC, comparamos as alterações morfológicas do plexo sub-basal epitelial da córnea de 35 pacientes diabéticos com PSD sintomática com 55 indivíduos controles. Além disso, buscamos determinar um padrão de alteração entre os estágios de gravidade da PSD, comparando variáveis clínicas, laboratoriais e de neurocondução.

Resultados Diferenças entre os grupos controle e diabéticos foram verificadas com relação às seguintes variáveis, respectivamente: idade ($44{,}9 \pm 13{,}24$ anos *versus* $57{,}02 \pm 10{,}4$ anos; $p < 0{,}001$); densidade das fibras ($29{,}7 \pm 10{,}2$ *versus* $16{,}6 \pm 10{,}2$; $p < 0{,}001$); número de fibras ($4{,}76 \pm 1{,}30$ *versus* $3{,}14 \pm 1{,}63$; $p < 0{,}001$); número de células de Langerhans ($4{,}64 \pm 8{,}05$ *versus* $7{,}49 \pm 10{,}3$; $p = 0{,}035$); tortuosidade ($p < 0{,}05$), e espessura ($p < 0{,}05$). Além disso, relações inversamente proporcionais foram verificadas entre a densidade das fibras e a idade ($p < 0{,}01$), e entre a densidade das fibras e a gravidade da doença ($p < 0{,}05$). Observou-se ainda uma relação positiva entre a velocidade de condução do nervo fibular e a densidade das fibras ($p < 0{,}05$).

Conclusão A MCC constitui um método rápido, não invasivo e reprodutível para o diagnóstico, o estadiamento, e o acompanhamento da PSD diabética.

Palavras-chave
- Diabetes Mellitus
- Doenças do Sistema Nervoso Periférico
- Microscopia Confocal

INTRODUCTION

Diabetic neuropathy (DN) is a very common, clinical condition worldwide, and it has many different clinical forms, such as distal symmetric polyneuropathy (DSP), the most frequent presentation and the main mechanism for the development of diabetic foot.[1] It predominantly presents with positive, sensory (burning, tingling) and negative symptoms (numbness, loss of sensation); however, it may develop asymptomatically. It is usually associated with autonomic signs and symptoms and is rarely observed with motor manifestations.[2] Approximately 20% of patients with DSP present neuropathic pain, which sometimes becomes chronic and disabling.[3]

If the diagnosis is made early and correctly, it enables the proper treatment and hinders the progression of the neuropathy, preventing serious complications.

Thus, obtaining the clinical history and performing a detailed neurological examination are necessary to identify the signs of impaired nerve fibers.

Importantly, even in the absence of signs on the physical examination and the nerve conduction study (NCS), small fiber neuropathy may still be present. Therefore, new techniques have been developed to more accurately identify the early involvement of these small-diameter myelinated (Aδ) and unmyelinated (C) fibers. Among these new techniques, we highlight neurophysiologic, morphological, and autonomic testing methods which, though currently recommended for the diagnosis of DSP, are not sensitive, such as the NCS, and are invasive, like skin biopsy.

Recently, the sub-basal plexus of the human cornea, which is composed of small fibers, was mapped through confocal microscopy, which enabled the characterization of its pattern and the of distribution of nerve fibers in healthy subjects of both sexes and different ages.[4]

In a series of 18 cases of diabetic patients compared with controls, the authors[5] demonstrated for the first time a significant reduction in the fiber density of the sub-basal plexus by CCM, and highlighted that this examination may be used to establish a morphological diagnosis, and it is rapid, noninvasive and reproducible for the identification of DSP.

It is possible to estimate several aspects of the sub-basal plexus of the cornea, such as the density of the fibers and nerve trunks, as well as their size, degree of tortuosity and thickness, and, finally, the number of Langerhans cells per field. These measurements are used in the qualitative and quantitative assessment of the innervation of the cornea, enabling its comparison with various interest groups of study.[6]

In the present study, we compared the morphological changes in the sub-basal corneal epithelial plexus through in vivo CCM (IVCCM) in 35 diabetic patients with symptomatic

DSP to 55 control subjects in a Brazilian sample taken from a previous study.[7] In addition, we sought to determine a pattern of change regarding the stages of severity of the DSP, comparing clinical, laboratory, and NCS variables.

METHODS

Patients

We selected 35 consecutive outpatient subjects with symptomatic DSP in neurology and endocrinology and 55 healthy individuals in Hospital Universitário Antônio Pedro (HUAP) from Universidade Federal Fluminense (UFF), Rio de Janeiro, Brazil, between November 2011 to March 2013. The present study was analyzed and approved by the HUAP Review Board on June 17, 2011 (CAAE: 0144.0.258.000–11). The patients were informed about all the research steps, and they read and signed two copies of the consent form before their inclusion in the study.

Study design

Subjects who met the inclusion criteria for the group of diabetic patients underwent clinical, laboratory, and neurophysiologic studies, as well as the performance of CCM. The 55 control subjects were submitted to a clinical assessment, and those who did not present relevant changes in the neurological examination underwent the CCM.

The study included patients between 21 and 70 years of age. Patients in the diabetic group had to fulfill the current criteria of the American Diabetes Academy for the diagnosis of type-2 diabetes mellitus, present neuropathic symptoms such as paresthesia, pain, or imbalance, and have a Neuropathy Disability Score (NDS) >3 for the establishment of the clinical diagnosis of DSP. We excluded patients with a history of trauma or corneal diseases, previous eye surgeries, those who had used contact lenses less than 24 hours before the CCM, those who had a history of conditions that could compromise the cornea and/or peripheral nerve, patients who, for fewer than 6 months, had used drugs that are potentially toxic to the nerves and/or cornea, and/or had cognitive and/or sensory deficits that impair the scores on the scales or the exam.

The clinical evaluation was based on data from the history, the physical and neurological examinations, and specific scales for neuropathy, neuropathic pain, and disability. The NCS was performed using an electromyograph (Nihon Kohden Corp., Shinjuku, Tokyo, Japan).

We applied the NDS to assess the severity of the neuropathy,[8] the Leeds Assessment of Neuropathic Symptoms and Signs (LANSS)[9] to evaluate the neuropathic pain, and the modified Rankin scale for disability,[10] all validated for the Portuguese language.

We used the Heidelberg Retina Tomograph III Rostock Corneal Module (HRT3 RCM, Heidelberg Engineering GmbH, Heidelberg, Germany) confocal microscope to capture images of the sub-basal corneal epithelial plexus. These tests were performed by an ophthalmologist who was an expert in the field.

The patients' corneas were anesthetized using a drop of benoxinate hydrochloride at 0.4% and lubricated each eye with carbomer 0.2% eye gel, as well as the surface of the TOMOCAP (Heidelberg Engineering GmbH). Soon after, the patient was positioned comfortably and appropriately for the beginning of the study (►**Figure 1**).

Figure 1 The HRT3 RCM confocal microscope.

Images from the five layers of the corneas were obtained. After identification of the epithelium, the objective lenses were positioned at 0 mm in depth, always exploring a fixed field of 0.16 mm^2. From this point, the lenses were deepened to the subepithelial layer, where multiple images of the sub-basal plexus were obtained. The distance between each image was of $\sim 1\,\mu m$.[11]

The five best photos of the sub-basal plexus of the cornea of each eye were selected. The images of individuals from both groups were subjected to a blinding process. Each image was analyzed manually and separately by three observers for the following previously-established parameters: the number of fibers and of the Langerhans cells per field, the degree of tortuosity, and the thickness of the fibers.

The degrees of tortuosity established were: 1 for linear and parallel fibers; 2 for tortuous and non-parallel fibers, but in the same direction; and 3 for tortuous fibers not in the same direction. The degrees of thickness were classified as: 1 for thick fibers; 2 for fibers of medium thickness; and 3 for thin fibers.

Statistical analysis

Comparison of the findings of the CCM in both groups

The results of the CCM in the diabetic and control patients were compared by means and proportion tests. The Kolmogorov-Smirnov and the Shapiro tests were used to verify the normality of the continuous or discrete variables, and the mean differences were evaluated through the *t*-test or Mann-Whitney U test.

The percentage differences noted regarding the qualitative variables were evaluated using the Chi-squared statistic.

All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS for Windows, SPSS Inc., Chicago, IL, US) software, version 16.0.

RESULTS

Description of the study population

We evaluated 90 individuals, 35 diabetic patients with symptomatic DSP (39%) and 55 control subjects (61%). The differences between the control and diabetic groups were observed in the following variables, respectively: age (44.9 ± 13.24 years versus 57.02 ± 10.4 years; $p < 0.001$); fiber density (29.7 ± 10.2 versus 16.6 ± 10.2; $p < 0.001$); mean number of fibers (4.76 ± 1.30 versus 3.14 ± 1.63; $p < 0.001$); mean number of Langerhans cells (4.64 ± 8.05 versus 7.49 ± 10.3; $p = 0.035$); tortuosity ($p < 0.05$); and thickness (Chi-squared $p < 0.05$) (►**Table 1**).

Table 1 Demographic features, CCM and NCS results, laboratory and clinical findings

Variables		Diabetics ($n = 35$)	Controls ($n = 55$)	*p*-value
Male sex (%)		25.7%	45.5%	
Age (mean ± SD)		57.2 ± 10.4	44.9 ± 13.24	< 0.001[1]
Number of fibers (mean ± SD)		3.14 ± 1.63	4.76 ± 1.30	< 0.001[2]
Fiber density (mean ± SD)		16.6 ± 10.2	29.7 ± 10.2	< 0.001[2]
Number of Langerhans cells (mean ± SD)		7.49 ± 10.3	4.64 ± 8.05	0.035[1]
Tortuosity (%)	Normal	–	3.6%	< 0.05
	Paralell	5.7%	45.5%	
	Non-parallel	82.9%	45.5%	
	Not in the same direction	11.4%	5.5%	
Thickness (%)	Normal	–	3.6%	< 0.05
	Thin	74.3%	29.1%	
	Medium	25.7%	63.3%	
	Large	–	3.6%	
Time since the onset of diabetes (mean ± SD)		9.89 ± 6.87	–	–
Time since the onset of symptoms (mean ± SD)		3.49 ± 2.56	–	–
Metabolic syndrome (yes: %)		65.7%	–	–
Diabetic foot (yes: %)		14.3%	–	–
Macrovascular complication (yes: %)		25.7%	–	–
Microvascular complication (yes: %)		57.1%	–	–
Retinopathy (yes: %)		45.7%	–	–
Nephropathy (yes: %)		37.1%	–	–
Pain (yes: %)		37.1%	–	–
Treatment: insulinotherapy (%)		48.6%	–	–
Glycated hemoglobin (%)		7.54 ± 2.14		
Hypertension (yes: %)		85.7%	–	–
Dyslipidemia (yes: %)		45.7%	–	–
BMI Kg/m^2(mean ± SD)		29.1 ± 5.49	–	–
Mild or moderate disability (%)		34.3%	–	–
Neuropathy Disability Score	Mild	31.4%	–	–
	Moderate	31.4%	–	–
	Severe	37.1%	–	–

Abbreviations: BMI, Body Mass Index; CCM, corneal confocal microscopy; NCS, nerve conduction study; SD, standard deviation.

Table 2 Summary and dispersion measurements of CCM variables according to study groups for the entire population

		N	Mean	SD	95%CI		Min.	Max.
Number of fibers	Healthy controls	55	4.76*	1.305	4.41	5.12	2	7
	Diabetics	35	3.14*	1.630	2.58	3.70	0	7
	Total	90	4.13	1.637	3.79	4.48	0	7
Density	Healthy controls	55	29.7*	10.201	27.56	31.97	12.50	43.75
	Diabetics	35	16.6*	10.184	16.14	23.14	0.00	43.75
	Total	90	25.83	10.229	23.69	27.97	0.00	43.75
Langerhans cells	Healthy controls	55	4.64*	8.054	2.46	6.81	0	45
	Diabetics	35	7.49*	10.305	3.95	11.03	0	44
	Total	90	5.74	9.049	3.85	7.64	0	45

Abbreviations: 95%CI, 95% confidence interval; CCM, corneal confocal microscopy; Max., maximum; Min., minimum; SD, standard deviation.
Note: *Differences in means with $p < 0.05$.

In the diabetic group, 31.4% of the patients had mild DSP (NDS: 3 to 5), 31.4% had moderate (NDS: 6 to 8), and 37.1% had severe DSP (NDS: 9 to 10) (►**Table 1**).

Comparison of the CCM findings between the groups

For the mean number of fibers and density, we found a statistically significant difference between the control and diabetic groups ($p < 0.001$). A statistically significant difference was also noted when the means of the Langerhans cells ($p = 0.035$) were compared. The equality of the variances was tested for several fibers with the Levene test ($p = 0.367$) (►**Table 2**, ►**Figure 2**).

The variables were reclassified in tortuosity (fibers with no tortuosity or tortuousness) and thickness (absent or thin fibers, medium or large fibers). We observed statistically significant differences in the percentage categories of tortuosity and the thickness between the groups (Chi-squared $p < 0.001$) (►**Table 1**).

Pattern of the morphological changes

The pattern of the morphological changes was determined in three stages by comparing the clinical, laboratory, and NCS findings of the CCM. The following variables were statistically different from the mean in the categories of mild and moderate neuropathy / severe: the duration of the diabetes and the SNAP of the sural nerve ($p < 0.05$). The mean differences in symptom duration, glycated hemoglobin (HbA1C), CV, and compound muscle action potential (CMAP) of the fibular nerve were observed between mild and severe neuropathy (►**Table 3**, ►**Figure 3**).

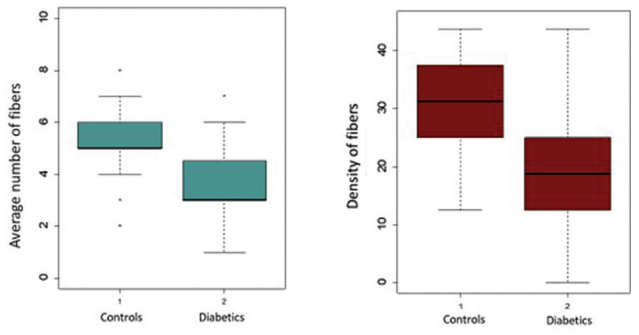

Figure 2 Comparison of the findings of IVCCM between the diabetic and control study groups.

Regarding the NCS, we observed statistically significant differences regarding the mean SNAP of the sural nerve and the three stages of severity. In the conduction velocity of the fibular nerve, differences in the means were noted between patients with mild and severe conditions ($p < 0.05$). And, in relation to the fibular CMAP, these differences were significant between patients with mild and severe neuropathy (►**Table 3**).

In the subgroup of the diabetic patients with mild neuropathy, or small-fiber neuropathy, ~ 90% were female, aged between 27 and 70 years, ~ 80% had had the diagnosis of DM and the symptoms for fewer than 5 years, and all were using oral hypoglycemic agents, without need for insulin therapy. The mean body mass index (BMI) was of 30.4 kg/m^2, 36% reported pain as a symptom, and 100% had an NCS within the normal range. Of these, 27% had other microvascular complications and a mean of HbA1c of 6.2%. The mean number of fibers was 3.8 ± 1.6, and the mean density was of 23.8 ± 10. As for the Langerhans cells, the mean number was 7 ± 9.8. About 90% had no parallel fibers, and 64% had thin fibers (►**Table 3**).

In the subgroup of patients with moderate neuropathy, 100% were female, aged between 49 and 69 years, with a diabetes duration of 7 to 20 years, a mean BMI of 29.2 kg/m^2, and 54% had pain. Furthermore, 64% were dependent on insulin therapy, 90% had pure sensory neuropathy on the NCS, 45% exhibited at least 1 microvascular complication, and their mean HbA1C was of 7.4%. About 30% had a mild disability. The mean number of fibers was 3.2 ± 1.8, and the mean density was of 20 ± 11.3. The Langerhans cells had a mean number of 10.0 ± 14.1. About 90% had no parallel fibers, and 85% had thin fibers (►**Table 3**).

Among the patients with severe neuropathy, 61% were male, aged between 40 and 70 years. The time since their diagnosis of diabetes ranged from 24 to 30 years, their mean BMI was of 28 kg/m^2, and 23% had neuropathic pain. Approximately 80% were using insulin, 38% had diabetic foot complications, and 100% experienced sensorimotor DSP

Table 3 Demographic, clinical, laboratory, CCM, and NCS variables according to severity stages

Neuropathy				
		Mild ($n = 11$)	Moderate ($n = 11$)	Severe ($n = 13$)
Age (years)[1]		54.3 ± 13.6	60.7 ± 7.97	53.3 ± 15.0
BMI[2] (Kg/m^2)		30.4 ± 5.9	29.2 ± 5.28	28 ± 2.0
Time since the onset of diabetes (years)[1]		4.18 ± 4.2*§	11.7 ± 5.7*§	17.3 ± 7.1*§
Time since the onset of symptoms (years)[1]		1.91 ± 14.5**§	3.30 ± 2.9	6.67 ± 2.9**§
HbA1C[1] (%)		6.27 ± 1.35	7.40 ± 1.57	7.0 ± 1.0
CCM	Numberoffibers[2]	3.82 ± 1.6	3.20 ± 1.8	3.00 ± 2.0
	Langerhanscells[1]	7.0 ± 9.8	10.0 ± 14.1	2.33 ± 4.0
	Density[2]	23.8 ± 10.0	20 ± 11.3	18.75 ± 12.5
NCS	Sural amplitude uV(d^1/e^2)	11.3 ± 4.1*§/11.64 ± 3.2§	4.0 ± 1.3*§/6.5 ± 4.7§	3.0 ± 1.7*§/4.0 ± 3.6§
	Fibular conductionvelocity in m/s(d^2/e^2)	47.1 ± 4.1§/46.3 ± 3.1§	43.9 ± 4.5§/44.2 ± 4.8§	41.3 ± 4.9§/41.6 ± 3.5§
	Fibular CMAP mV(d^2/e^1)	4.45 ± 1.7/4.5 ± 2.0**§	4.7 ± 1.89/4.1 ± 1.4**§	5.0 ± 1.7/3.6 ± 0.6**§

Abbreviations: BMI, Body Mass Index; CCM, corneal confocal microscopy; CMAP; d; e; HbA1C, glycated hemoglobin; mV; NCS, nerve conduction study; uV.
Notes:
[1]One-way anaçysis of variance (Tukey).
[2]Kruskal-Wallis test.
*Difference between mild and moderate/severe.
**Difference between mild and severe.
§$p < 0.05$.

according to the NCS. The mean HbA1c was of 7.0%. About 90% had other microvascular complications and 61.5% were classified as having mild or moderate degrees of disability. Regarding the CCM, the mean number of fibers was 3.0 ± 2.0, with a mean density of 18.75 ± 12.5. The mean number for the Langerhans cells was 2.3 ± 4.0. None these patients had parallel fibers and 85% had thin fibers (►**Table 3**).

Positive relationships were found between the duration of the diabetes and: the diabetes symptoms (r = 0.791; $p < 0.01$), the NDS (r = 0.580; $p < 0.01$), the duration of the symptoms (r = 0.487; $p < 0.01$), and the NDS (r = 0.531; $p < 0.01$) (►**Table 4**).

Regarding the results of the NCS, negative correlations were observed: inversely proportional relationships between the

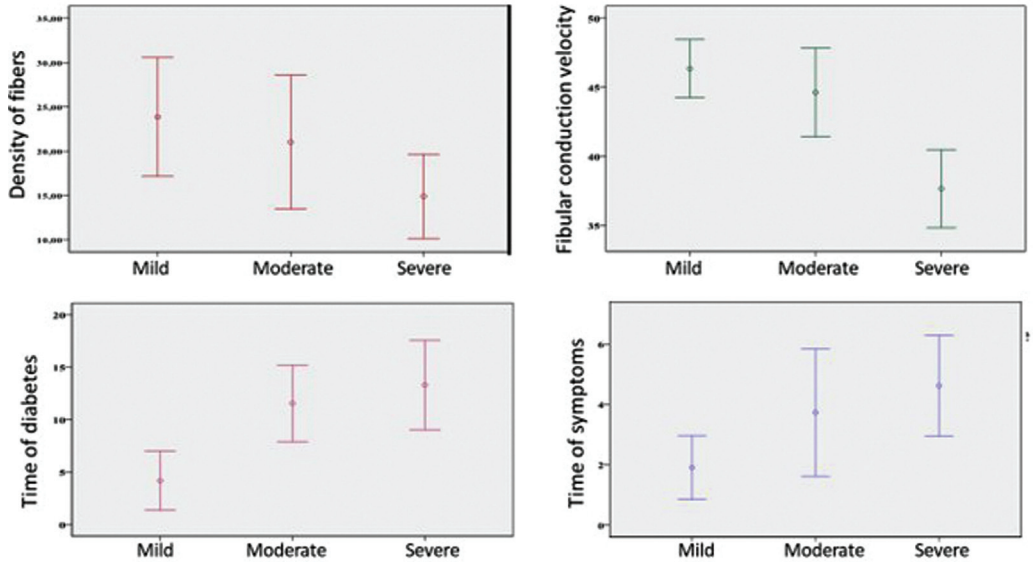

Figure 3 Comparison of the morphological changes in three stages of severity by clinical, laboratory, and NCS variables and the findings of IVCCM.

Table 4 Pearson and Spearman correlation regarding the clinical variables, laboratory tests, CCM, and NCS

		Clinical and laboratory						CCM		NCS					
		Age	TD	TS	BMI	HbA1C	NDS	Dens	LC	ARS	ALS	CVRF	CVLF	RFA	LFA
Clínical and laboratory	Age[2]	1													
	TD[2]	0.162	1												
	TS[2]	0.101	0.791**	1											
	BMI[2]	-0.158	-0.340*	-0.075	1										
	HbA1C[2]	-0.206	0.325	0.167	-0.220	1									
	NDS[2]	0.011	0.580**	0.487**	-0.113	0.537**	1								
CCM	Dens[1]	-0.432**	-0.085	0.043	0.047	-0.174	-0.405*	1							
	LC[2]	0.315	0.042	-0.033	0.183	0.017	-0.038	-.318	1						
NCS	ARS[2]	-0.303	-0.677**	-0.437*	0.023	-0.316	-0.863**	.200	-.086	1					
	ALS[1]	-0.293	-0.538**	-0.247	0.173	-0.145	-0.581**	.074	.084	.567**	1				
	CVRF[1]	-0.305	-0.437*	-0.261	0.057	-0.278	-0.637**	0.386*	0.139	0.584**	0.485*	1			
	CVLF[1]	-0.366*	-0.260	-0.262	-0.137	-0.309	-0.632**	0.446*	-0.153	0.556**	0.424*	0.860**	1		
	RFA[1]	-0.173	-0.172	-0.010	0.156	-0.224	-0.274	0.304	0.149	0.066	-0.047	0.499**	0.364*	1	
	LFA[2]	0.031	-0.165	-0.201	0.192	-0.623**	-0.405*	0.185	-0.063	0.199	-0.162	0.406*	0.349	0.362*	1

Abbreviations: ALS, amplitude of the left sural nerve; ARS, amplitude of the right sural nerve; BMI, Body Mass Index; CCM, corneal confocal microscopy; CVLF, conduction velocity of the left fibular nerve; CVRF, conduction velocity of the right fibular nerve; Dens, density; HbA1C, glycated hemoglobin; LC, Langerhans cells; LFA, left fibular amplitude; NCS, nerve conduction study; NDS, Neuropathy Disability Score; RFA, right fibular amplitude; TD, time since the onset of diabetes; TS, time since the onset of symptoms.

Notes:

[1]Pearson correlation.

[2]Spearman correlation.

*$p < 0.05$.

**$p < 0.01$.

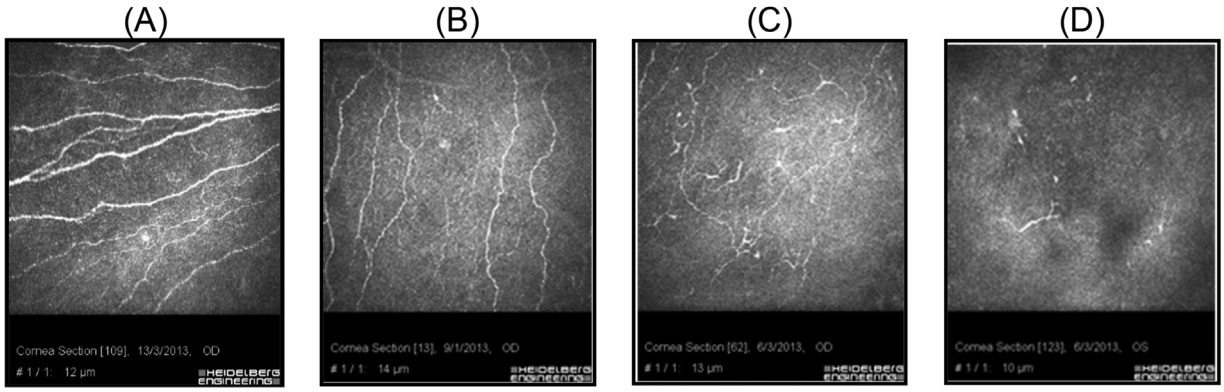

Figure 4 (**A**) Example of thick (thickness: 3), linear, and parallel (tortuosity: 1) fibers, with 7 fibers and 1 Langerhans cell. (**B**) Example of medium fibers (thickness: 2), somewhat tortuous and not parallel, but in the same direction (tortuosity: 2), and with 5 fibers and 1 Langerhans cell. (**C**) Example of thin fibers (thickness: 1), very tortuous and without direction (tortuosity: 3), with 2 fibers and 14 Langerhans cells. (**D**) Example of absence of fibers and 9 Langerhans cells. Images extracted from the database of the present study.

NDS and the SNAP of the sural nerve ($r = -0.863$ and $r = -0.581$ respectively; $p < 0.01$), the NDS and the fibular nerve CV ($r = -0.637$ and $r = -0.632$ respectively; $p < 0.01$), and the NDS and the CMAP of the fibular nerve ($r = -0.405$; $p < 0.05$).

Considering the results of the CCM, inverse relationships were found between the fiber density and age ($r = -0.432$; $p < 0.01$) and the fiber density and the NDS ($r = -0.405$; $p < 0.05$). Another highlight is a positive relationship between the CV of the fibular nerve and the fiber density ($r = 0.386$ and $r = 0.446$ respectively; $p < 0.05$) (►**Table 4**).

DISCUSSION

The involvement of the cornea in diabetic patients has been reported over the years by several authors.[12] The advent of CCM enabled the direct visualization, in the layers of the cornea, of the nerve fibers and the impairment in patients with DN.[5]

Considering the techniques available for the diagnosis of DSP, this method has attracted great interest from the scientific community because it is fast and non-invasive. Since then, several studies have been conducted to evaluate the best technique, the best parameters, and, primarily, a standardization of the method. To this end, some researchers[13] have endeavored to characterize the distribution of the sub-basal plexus of the cornea in healthy individuals, their morphology, and differences between genders and age groups.

We found statistically significant differences ($p < 0.05$) in all the CCM variables studied. In the diabetic group, there was a considerable reduction in the number of fibers, fiber density, as well as increased number of Langerhans cells. In addition, a higher tortuosity index and a lower degree of thickness were observed when compared with controls. This same pattern of changes is mentioned in studies performed with the same purposes as the present one.[5,6,11,14–19]

These data are similar to those of several studies[20] conducted to identify the clinical variables as risk factors for the development and progression of DSP.

We found a positive relationship between the CCM parameters and the modified Rankin Scale, which demonstrates the correlation between the progression of the DSP and disability. Corroborating this finding was the worsening of functionality to evaluate the quality of life and depression, according to the severity of DSP in diabetics previously highlighted.[8]

The results of the NCS, represented by the SNAP of the sural nerve, CV, and CMAP of the fibular nerve, corresponded to the findings already widely demonstrated[21–24] in the progression of diabetic DSP. There was an inverse relationship between these parameters and the NDS, the values of HbA1c, and the duration of diabetes and symptoms.[21–24]

Regarding the CCM parameters, inverse relationships between age and fiber density, and fiber density and the NDS were observed (►**Figure 4**). Moreover, a positive correlation was found between the CV of the fibular nerve and fiber density.

There is no consensus in the literature about the effect of aging on the density of the fibers of the sub-basal corneal epithelial plexus.[25] However, a study[26] showed a yearly linear decrease of 0.9% in fiber density, corroborating the results of the present study.

There seems to be a proportional reduction in the density of the fibers of the sub-basal plexus of the cornea along the natural history of diabetic DSP. Furthermore, it is believed that the fibers become thinner due to the process of axonal degeneration in the course of the disease. The increased tortuosity index and the decrease in thickness were attributed to axonal degeneration and regeneration in DSP, as in the pattern of the histopathologic findings in the peripheral nerve (sprouting), suggesting a chronic, axonal damage.[27]

A significant increase in the number of Langerhans cells was found in the diabetic group compared with the controls, with greater distribution among patients with mild and moderate forms of DSP, corroborating a previous study,[11] which may indicate the contribution of the immune mechanism in the pathogenesis and a perpetuation of the axonal impairment.

In the literature, few papers have been published demonstrating the efficacy of CCM in identifying the regeneration of nerve fibers in the sub-basal plexus of the cornea after pancreas transplantation in patients with type-1 diabetes mellitus and after improvement of the risk factors for DSP in

patients with type-2 diabetes mellitus, whereas functional methods of assessing the peripheral nerve, such as NCS and the Quantitative Sensory Test (QST), were not able to detect such improvement.[28]

Several comparisons with other diagnostic methods, such as NCS, skin biopsy, and the QST, have shown that CCM had greater sensitivity and specificity in identifying diabetic DSP in the early stage, when there is only the involvement of small fibers. Moreover, it is rapid, noninvasive, reproducible, and enables multiple assessments throughout the evolution of the patient. The reduced fiber density in the epithelial sub-basal corneal plexus has a strong correlation with the degree of involvement of the peripheral nerve, which shows this method as a possible diagnosis for testing in the evolution of the severity of the neuropathy. These features make this method very promising for use not only in the clinical practice, but also in longitudinal studies and clinical trials, to evaluate the therapeutic efficacy of certain drugs in diabetic DSP.[6]

An increasing number of articles on the use of IVCM to quantify diabetic neuropathy has demonstrated a reduction in corneal sub-basal nerve fiber density and an increase in nerve fiber tortuosity in diabetes, correlated with the stage or severity of the peripheral neuropathy.[29]

In summary, to date, several groups[25] have employed CCM of the corneal sub basal nerves in diabetic subjects to identify patients with minimal neuropathy, quantify the severity of the neuropathy, and follow the progression or assess the therapeutic response in DN. In vivo CCM has shown moderate-to-high specificity in the diagnosis of DN[16] and the usefulness of the corneal nerves as DN biomarkers.[12,16,19]

In conclusion, CCM is a fast, non-invasive method, with good reproducibility, which contributes to the identification of changes in corneal innervation in patients with diabetic DSP. In addition, the reduction in fiber density of the epithelial sub-basal plexus of the cornea has a high correlation with the degree of involvement of the peripheral nerve, that is, the severity of the neuropathy, standing out as a possible diagnostic and evolutionary test of the disease. These characteristics of the method favor its applicability both in the clinical practice and in longitudinal studies, such as clinical trials.

The method's limitations include the high cost of the device, the need for a qualified professional, and the fact that there are still few studies on standardization.

Future research with larger samples is necessary to standardize and consolidate the method as a diagnostic test for diabetic DSP and also for other small-fiber neuropathies.

Authors' Contributions

CP: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, and project administration; GD, RD: data curation, review; and ON: review.

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Conflict of Interest

The authors have no conflict of interests to declare.

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