Corneal Subbasal Nerve Plexus Evaluation By In Vivo Confocal Microscopy In Multiple Sclerosis

Diogo Hipólito Fernandes[1]; Maria Elisa Luís[1]; Joana Cardigos[1]; Miguel Vieira[1]; Marta Alves[2,4]; Ana Luísa Papoila[2,3,4]; João Paulo Cunha[1,3]; Joana Tavares Ferreira[1,3]

[1] Ophthalmology Department, Lisbon Central Hospital Centre, Lisbon, Portugal

[2] Epidemiology and Statistics Unit, Research Centre, Lisbon Central Hospital Centre, Lisbon, Portugal

[3] NOVA Medical School/Faculdade de Ciências Médicas, Nova Lisbon University, Lisbon, Portugal

[4] CEAUL (Centre of Statistics and its Applications), Lisbon University, Lisbon, Portugal

ABSTRACT

Purpose: Multiple Sclerosis (MS) is the most frequent cause of neurologic disability in young adults, characterized by demyelination and axonal degeneration. Monitoring this last component remains an important challenge. Our study aims to assess corneal subbasal nerve plexus morphology by *in vivo* confocal microscopy (CCM) and explore the possibility of using this noninvasive technology to obtain a biomarker of axonal degeneration.

Methods: In this cross-sectional study 30 patients with MS and 22 healthy controls were included. All participants underwent full ophthalmology standard evaluation, CCM and optical coherence tomography (OCT). The following corneal subbasal nerve plexus morphology parameters were analysed: corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD), corneal nerve fibre length (CNFL) and corneal nerve fibre tortuosity (CNFT). Neurological disability of MS patients was accessed using Expanded Disability Status Scale (EDSS) and MS Severity Score (MSSS).

Results: Compared to controls, MS patients have lower CNFD, CNBD and CNFL ($p<0.001$) but no significant difference was found related to CNFT ($p=0.108$). No significant differences were found related to corneal subbasal plexus parameters between MS patients with or without optic neuritis (MSON *vs* MSNON). CNFD and temporal-inferior peripapillary retinal nerve fibre layer (ppRNFL) showed inverse association both with EDSS ($r_S=-0.62$, $p<0.001$ for CNFD; $r_S=-0.53$, $p=0.003$ for temporal-inferior ppRNFL) and MSSS ($r_S=-0.44$, $p=0.018$ for CNFD; $r_S=-0.49$, $p=0.009$ for temporal-inferior ppRNFL) scores.

Conclusions: CNFD, CNBD and CNFL are decreased in MS patients, suggesting axonal degeneration. Further longitudinal studies are needed to confirm whether CNFD could be a promising imaging parameter in MS severity evaluation.

Keywords: multiple sclerosis, axonal degeneration, optical coherence tomography, corneal confocal microscopy, expanded disability status scale.

RESUMO

Objetivos: A Esclerose Múltipla (EM) representa a causa mais frequente de morbilidade neurológica em adultos jovens, sendo caracterizada pela desmielinização e degeneração axonal. A monitorização deste último componente mantém-se um desafio relevante. Este estudo pretende avaliar a morfologia do plexo nervoso sub-basal da córnea por microscopia confocal *in vivo* (MCC) e explorar a possibilidade de utilizar esta tecnologia não invasiva como biomarcador dessa mesma degeneração axonal.

Métodos: Estudo transversal envolvendo 30 doentes com EM e 22 controlos saudáveis, submetidos a uma avaliação oftalmológica completa, MCC e tomografia de coerência ótica (TCO). Os seguintes parâmetros da morfologia do plexo nervoso sub-basal da córnea foram analisados: densidade das fibras nervosas corneanas (DFNC), densidade de ramificações (DRNC), comprimento total de nervos corneanos (CFNC) e tortuosidade dos nervos corneanos (TFNC). A avaliação da incapacidade neurológica dos doentes com EM foi obtida através da utilização do *Expanded Disability Status Scale* (EDSS) e do *Multiple Sclerosis Severity Score* (MSSS).

Resultados: Em comparação com o grupo controlo, os doentes com EM apresentaram uma DFNC, DRNC e um CFNC inferior ($p<0.001$) mas não houve diferença estatisticamente significativa relativamente à CNFT ($p=0.108$). Não houve diferenças significativas relativamente aos parâmetros do plexo sub-basal da córnea medidos entre os doentes com EM com e sem história pregressa de nevrite ótica. A DFNC e a espessura temporal-inferior da camada de fibras nervosas peripapilar (CFNpp) mostraram uma associação inversa tanto com o *score* EDSS ($r_S=-0.62$, $p<0.001$ para a CNBD; $r_S=-0.53$, $p=0.003$ para a CFNpp temporal-inferior) como com o MSSS ($r_S=-0.44$, $p=0.018$ para a CNFD; $r_S=-0.49$, $p=0.009$ para a CFNpp temporal-inferior)

Conclusões: A DFNC, a DRNC e o CFNC estão diminuídos nos doentes com EM, sugerindo degeneração axonal. São necessários estudos longitudinais para confirmar que a DFNC poderá ser um promissor biomarcador imagiológico para aferir a gravidade da EM.

Palavras-chave: esclerose múltipla, degeneração axonal, tomografia de coerência ótica, microscopia confocal da córnea, *expanded disability status scale*

INTRODUCTION

Multiple Sclerosis (MS) is a chronic inflammatory, auto-immune and degenerative central nervous system (CNS) disease, characterized by demyelination and axonal degeneration. Affecting mainly patients between 20-40 years old, MS interferes both with social and professional activities. In Portugal, the disease affects 56.2 per 100 000 individuals, with an estimated direct plus indirect costs between 16 500€ to 34 400€ per patient, according to disease severity.[1,2]

Axonal degeneration, a crucial component of MS is a major determinant of permanent neurological impairment,[3] being correlated with a poor prognosis.[4] However, one of the biggest challenges is how to accurately monitor axonal degeneration and potential regeneration with treatment, which has been a major obstacle for the development of new disease-modifying therapies.[5]

Magnetic resonance spectroscopic imaging (MRSI) detects changes in metabolites such as N-acetyl aspartate (NAA), a marker of axonal integrity, being able to distinguish MS from healthy subjects.[6,7] Those changes potentially identify the progressive stage of the disease in different clinical groups. However, being an expensive and time-consuming methodology, its clinical application is limited.

The eye is also affected by MS, with optic neuritis being the initial presentation in 20% of patients, while others will develop this inflammatory condition during disease course. Optical coherence tomography (OCT) studies reported a decreased peripapillary retinal nerve fibre layer (ppRNFL) thickness in MS patients, even without previous optic neuritis.[8–12] Furthermore, RNFL thickness appears to correlate with visual function, clinical disability and magnetic resonance imaging indices.[13,14] However, conflicting findings were found regarding the relationship between RNFL thickness and Expanded Disability Status Scale (EDSS),[15–18] the worldwide used method to quantify disability in MS and to monitor it over time. The occurrence of optic neuritis causes additional damage to optic nerve which limits OCT usage as disease progression biomarker in those patients.

The cornea is the most densely innervated tissue in the human body, with approximately 7000 free epithelial nerve endings by millimetre square,[19,20] receiving its somatic supply from the ophthalmic division of the trigeminal nerve. Autonomic corneal innervation also exists but it is believed to be scarce (sympathetic) or unknown (parasympathetic). Corneal confocal microscopy (CCM) is a noninvasive technique of *in vivo* cornea visualization with high quality resolution, allowing the evaluation of corneal structures, as subbasal nerve plexus. The growing interest about this technology gave rise to several studies not only in corneal diseases,[21] but also in some systemic ones, such as diabetes,[22–24] peripheral and central neurodegenerative diseases.[25–32]

The high frequency of optic neuritis in MS patients, as stated before, limits the utility of OCT to evaluate progressive axonal degeneration. However, trigeminal involvement was reported in just 2.9% of patients,[33] which increased the interest in the study of corneal subbasal nerve plexus in MS patients.

Our study aims to evaluate corneal subbasal nerve plexus morphology by *in vivo* CCM in MS patients and explore the possibility of using this noninvasive technology as a methodology to access axonal degeneration. Besides, we want to evaluate the relationship between corneal subbasal nerve plexus parameters, the occurrence of optic neuritis and patient's neurologic disability status.

METHODS

Patients and Controls

In this cross-sectional study 30 patients with MS and 22 healthy controls were recruited between May 2018 and July 2018. The study adhered to the principles of the Declaration of Helsinki and was approved by our health institution ethics committee. Informed written consent was obtained from both patients and controls before study participation.

Patients between 18-65 years old, with MS diagnosis (relapsing-remitting or secondary-progressive) according to the McDonald criteria[34] were considered eligible. In MS patients, a complete neurologic evaluation was performed by a neurologist and disability classification was accessed using EDSS (score range 0-10 – higher score represents greater disability). After that, Multiple Sclerosis Severity Score (MSSS) was calculated from EDSS and disease duration[35] (range 0.01-9.99 – higher score represents greater severity). The occurrence of previous optic neuritis and the duration of the disease were also recorded. Before CCM and OCT, full standard ophthalmologic evaluation was performed.

For patients with bilateral or no optic neuritis, by convention, only the right eye was accessed. In those with unilateral optic neuritis only the affected eye was evaluated.

Exclusion criteria were a history of optic neuritis within 6 months prior to recruitment, diabetes mellitus, any other known neurologic disease, any possible cause of peripheral neuropathy, previous ophthalmologic surgery, ocular trauma, glaucoma or other optic neuropathy unrelated with MS, any corneal disease, contact lens use and high refractive error (spherical equivalent ± 6.0D).

Optical Coherence Tomography

Peripapillary retinal nerve fibre layer (RNFL) thickness measurements were obtained using spectral-domain OCT (Spectralis®, Heidelberg Engineering, Germany). The light source of SD-OCT was a superluminescent diode with 850nm wavelenght, getting 40 000 A-scans/second. Circular B-scans (3.4 mm diameter, 12º scanning angle) centred at the optic disc were obtained, with eye movement tracker activated to reduce motion artefacts. The high-speed resolution mode (768 pixels, 1536 A-scans/second for peripapillary 360º) was used to collect the images. OCT images were obtained by a trained technician (R.L.) and accessed by two ophthalmologists (J.F, M.E.L.), independently from each other, masked from the disability status of the patients. Low quality images (Q<20) were not accepted. Spectralis® own-algorithm determines the inner and outer limits of ppRNFL and estimates its thickness. The peripapillary region was segmented into 6 sectors: nasal (N; 135-225º), nasal-superior (NS; 225-270º), nasal-inferior (NI; 90-135º), temporal (T; 315-45º), temporal-superior (TS; 45-90º) and temporal-inferior (TI; 270-315º) – an average global thickness is automatically measured by the software. RNFL thickness measurements were recorded in micrometres (μm).

In Vivo Corneal Confocal Microscopy

To access corneal subbasal nerve plexus, CCM was performed using Heidelberg Retinal Tomograph III, Rostock Cornea Module (Heidelberg Engineering, Germany), a device that uses a helium neon diode laser with 670-nm wavelength. The combination of a small aperture with a 63x objective lens allows 800-fold magnification. Each two-dimensional image has 340x340 μm (15ºx15º field of view; 10 μm per pixel transversal resolution). Before examination, one drop of topical anaesthetic (oxybuprocaine hydrochloride 0.4%) was used to anaesthetize each eye and Vidisic gel® (Bausch & Lomb, Germany) as coupling agent between cornea and applanation cap (TomoCap; Heldelberg Engineering, Germany). During the exam, an outer fixation light was used to correctly position the eye. The total duration of CCM was 5 minutes per eye and all images were obtained by the same trained ophthalmologist (D.H.), masked from patient's disability status. Three to 5-high quality corneal subbasal nerve plexus images per patient were considered, and the results are the mean values of those measurements.

Validated automated CCMetrics software, version 2.0 (University of Manchester, UK) was used to analyse the images[36–40] (figure 1). The following measurements were quantified: corneal nerve fibre density (CNFD – total number of major nerves per square millimetre), corneal nerve branch density (CNBD – number of branches emanating from major nerve trunks per square millimetre), corneal nerve fibre length (CNFL – total length of all nerve fibres and branches, in millimetres per square millimetre). To measure corneal nerve fibre tortuosity (CNFT – tortuosity coefficient, which represents the degree of tortuosity from a straight line joining the ends of each main nerve fibre) manual CCMetrics software, version 1.1. (University of Manchester, UK) was used. Images analysis was performed by a single observer (D.H.).

Statistical Analysis

Demographics and clinical characteristics of patients were described with frequencies (percentages) and with mean (SD: standard deviation) or with median and interquartile range (IQR: 25th percentile-75th percentile), as appropriate. Nonparametric Fisher's exact test, Mann-Whitney test and Kruskal-Wallis test were applied, as appropriate. To study the association between CCM parameters and RNFL thickness measurements, and disability classification, Spearman correlation coefficients were estimated (r_S).

A level of significance α=0.05 was considered. Data were analysed using the Statistical Package for the Social Science for Windows, version 22.0 (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.).

RESULTS

Demographics and Clinical Characteristics

A total of 30 patients with MS diagnosis (7 men and 23 women) and 22 healthy controls (6 men and 16 women) were included in this study. The mean age of the patients with MS was 41.50 (8.73) years and in healthy subjects was 38.68 (8.60) years. The median duration of MS was 8.50 (3.75-14.25) years. Twenty-nine patients (96.7%) were receiving disease modifying drugs, with dimethyl fumarate being the most frequent drug used (31.0%), followed by

natalizumab (24.1%). The median EDSS and MSSS score was 2.50 (2.00-4.25) and 3.58 (2.37-6.75), respectively. Between MS patients, 27 (90%) had relapsing-remitting sub-type and three (10%) had primary-progressive sub-type. Fourteen MS patients (46.7%) had a history of at least one episode of optic neuritis (ON). No significant differences were found between healthy control group, MS patients with ON (MSON) and MS patients without ON (MSWON) regarding demographic data (Table 1). Comparing MSON and MSWON patients there were not significant differences between them regarding EDSS (p=0.087) and MSS scores (p=0.105). Clinical characteristics are also summarized in Table 1.

Table 1 – Demographic and clinical characteristics of the patients by group

Characteristics	Healthy Controls (n=22)	Patients with MS		
		MSON (n=14)	MSWON (n=16)	*p*-value
Age, years	38.68 (8.60)	38.57 (8.61)	44.06 (8.25)	0.112*
Male gender, n (%)	6 (27.27)	4 (28.57)	3 (18.75)	0.781†
Disease Duration, years	-	9.5 (2.75-19.25)	7.5 (4.25-11.75)	0.544‡
EDSS score	-	3.00 (2.50-6.00)	2.00 (1.63-3.38)	0.087‡
MSSS	-	4.95 (2.80-7.04)	2.93 (1.98-3.90)	0.105‡

Results are expressed as mean (standard deviation) or median (P_{25}-P_{75}). EDSS – Expanded Disability Status Scale; MS – multiple sclerosis; MSON – multiple sclerosis with optic neuritis; MSSS – Multiple Sclerosis Severity Score; MSWON – multiple sclerosis without optic neuritis. *Kruskal-Wallis test; †Fisher's exact test; ‡Mann-Whitney test.

Optical Coherence Tomography

There was a significant reduction in global ppRNFL thickness mean values in MS patients compared with healthy controls (84.73 *vs.* 99.82; p<0.001). This significant reduction was also found in all sectors except for NI (p=0.247). Comparing the MSON with MSWON, MSON patients had a significant thinner ppRNFL in all sectors except in N sector (T and TI p<0.001; G and TS p=0.001; NS p=0.010; NI p=0.046). Between healthy controls and MSON there was a significant reduction in all sectors of ppRNFL (p<0.001 to 0.031). No significant difference was found between healthy controls and MSWON patients regarding OCT measurements. Detailed description of ppRNFL thickness in all groups is depicted in Table 2.

Table 2 – Peripapillary RNFL thickness measurements by OCT, by group.

Measurements	Healthy Controls (n=22)	Patients with MS			*p*-value			
		Total (n=30)	MSON (n=14)	MSWON (n=16)	Patients with MS *vs.* Healthy Controls	MSON *vs.* MSWON	MSON *vs.* Healthy Controls	MSWON *vs.* Healthy Controls
RNFL G, µm	99.82 (6.59)	84.73 (14.49)	73.21 (9.73)	94.81 (9.57)	<0.001‡	0.001*	<0.001*	0.530*
RNFL T, µm	71.14 (12.19)	54.07 (16.02)	41.00 (9.81)	65.50 (10.68)	<0.001‡	<0.001*	<0.001*	0.584*
RNFL TS, µm	137.86 (16.20)	119.50 (22.33)	104.07 (16.49)	133.00 (17.67)	0.003‡	0.001*	<0.001*	1.000*
RNFL TI, µm	139.64 (16.65)	122.83 (22.04)	107.43 (11.39)	136.31 (20.27)	0.005‡	<0.001*	<0.001*	1.000*
RNFL N, µm	77.77 (10.67)	67.63 (15.14)	63.29 (15.56)	71.44 (14.15)	0.009‡	0.372*	0.008*	0.470*
RNFL NS, µm	121.14 (19.39)	101.07 (22.29)	87.71 (14.53)	112.75 (21.59)	0.001‡	0.010*	<0.001*	0.647*
RNFL NI, µm	108.55 (26.01)	99.63 (24.03)	88.29 (19.36)	109.56 (23.79)	0.247‡	0.046*	0.031*	1.000*

Results are expressed as mean (standard deviation). G – global; MS – multiple sclerosis; MSON – multiple sclerosis with optic neuritis; MSWON – multiple sclerosis without optic neuritis; N – nasal; NS – nasal-superior; NI – nasal-inferior; OCT – Optical coherence tomography; RNFL – retinal nerve fibre layer; T – temporal; TS – temporal-superior; TI – temporal-inferior. ‡Mann-Whitney test; *Kruskal-Wallis test.

In Vivo Corneal Confocal Microscopy

MS patients showed significant reduction in CNFD (32.02 *vs.* 39.40; p<0.001), CNBD (47.76 *vs.* 63.12; p<0.001), and CNFL (18.29 *vs.* 21.25; p=0.001) compared to healthy controls. No difference was found in CNFT (10.98 *vs.* 9.73; p=0.152) between these two groups (Table 3 and Figure 2).

Subgroup analysis (healthy controls, MSON and MSWON) revealed that there is a statistically significant decrease in CNFD and CNBD between the MSON patients and controls (p<0.001 and 0.003, respectively), and between the MSWON patients and controls in CNFD, CNBD and CNFL measurements (p=0.002 to 0.010). However, in comparison of the MSWON and the MSON patients there are no significant differences in all CCM measurements (p=0.632 to 1.000) (Table 3).

Table 3 – Corneal Confocal Microscopy parameters measurements, by group

Measurements	Healthy Controls (n=22)	Patients with MS			p-value			
		Total (n=30)	MSON (n=14)	MSWON (n=16)	Patients with MS *vs.* Healthy Controls	MSON *vs.* MSWON	MSON *vs.* Healthy Controls	MSWON *vs.* Healthy Controls
CNFD fibers/mm^2	39.40 (5.16)	32.02 (6.66)	30.43 (5.91)	33.41 (7.14)	<0.001‡	0.632*	<0.001*	0.010*
CNBD branches/mm^2	63.12 (11.11)	47.76 (12.76)	47.52 (12.65)	47.96 (13.28)	<0.001‡	1.000*	0.003*	0.002*
CNFL mm/mm^2	21.25 (2.05)	18.29 (3.28)	18.89 (3.41)	17.77 (3.18)	0.001‡	1.000*	0.079*	0.003*
CNFT tortuosity coefficient	9.73 (2.16)	10.98 (3.07)	10.43 (2.39)	11.46 (3.57)	0.152‡	1.000*	1.000*	0.385*

Results are expressed as mean (standard deviation). CNBD – corneal nerve branch density; CNFD – corneal nerve fibre density; CNFL – corneal nerve fiber length; CNFT – corneal nerve fibre tortuosity; MS – multiple sclerosis; MSON – multiple sclerosis with optic neuritis; MSWON – multiple sclerosis without optic neuritis. ‡Mann-Whitney test; *Kruskal-Wallis test.

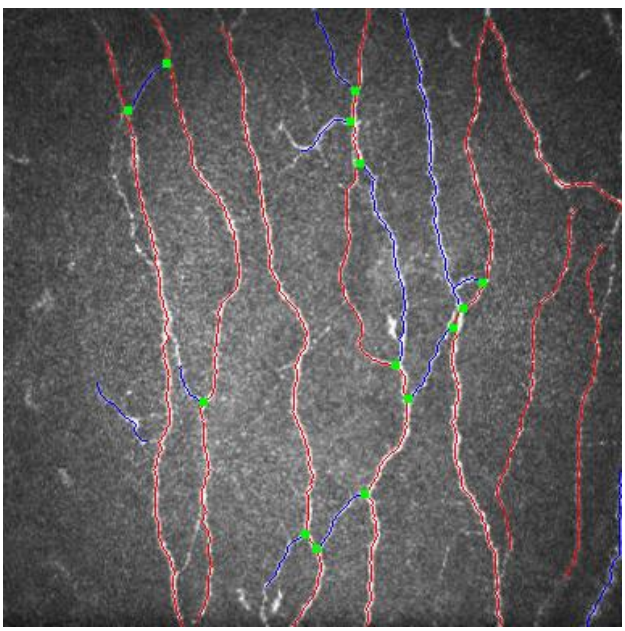

Figure 1 – Automatic CC Metrics layout: red lines represent main nerve fibers, blue lines represent accessory nerve fibers; green spots represent main nerve fiber's branches.

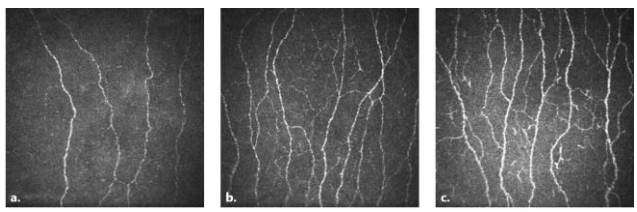

Figure 2 – CCM images corresponding to a MS patient with EDSS = 6.5 (a.), a MS patient with EDSS = 1 (b.) and a control subject (c.)

Clinical Disability Status and Correlations

Spearman's correlation coefficient showed that CNFD and temporal-inferior ppRNFL were negatively correlated, both with EDSS (r_S=-0.62, p<0.001 for CNFD; r_S=-0.53, p=0.003 for temporal-inferior ppRNFL) and MSSS (r_S=-0.44, p=0.018 for CNFD; r_S=-0.49, p=0.009 for temporal-inferior ppRNFL) scores. In the MSON group, CNFD was also negatively correlated with EDSS (r_S=-0.63, p=0.021) and with MSSS (r_S=-0.61, p=0.028). Regarding the MSWON group, CNFD, CNBD and temporal-inferior ppRNFL were negatively correlated with EDSS (r_S=-0.59,

p=0.016; r_S=-0.56, p=0.023; r_S=-0.51, p=0.045, respectively).

DISCUSSION

In this study we used CCM to evaluate corneal subbasal nerve plexus in MS patients. Overall, we found a decrease in all CCM parameters except CNFT, compared to control subjects. Between those measurements, CNFD had a significant association with EDSS score.

Permanent neurological deficits in MS patients have axonal degeneration as a major determinant[41] being partially independent of primary demyelination.[42] However, to date we still do not have any accurate way to access it.

Measurement of RNFL thickness to evaluate retinal axonal loss using OCT has been widely studied. Our results revealed thinner ppRNFL in MS patients, compared to healthy controls, which corroborate several other studies.[8–14,43–49] Regarding neurologic clinical status, EDSS was inversely associated with TI ppRNFL thickness only in MSWON patients which agrees with recent data.[10,11] Moreover, Graham *et al*[50] and Martinez-Lapiscina *et al*[51] reported RNFL measurements by OCT as being a useful aid to evaluate axonal loss but focusing only in this subset of patients. In fact, on the other hand, ON, as an inflammatory event, provokes initial increase in ppRNFL thickness followed by further thinning[52] which may limit the usage of OCT as a long-term analysis to evaluate axonal loss in MSON patients.

Corneal nerves approach the cornea radially around the limbus, losing their perineurium and myelin sheath, running centripetally towards the centre, creating a dense network of unmyelinated axons – subbasal nerve plexus. CCM, being noninvasive and allowing corneal subassal nerve plexus evaluation, gained significant interest between scientific community to study several peripheral neuropathies and even central neurodegenerative diseases,[25–30,32] including MS. The present study revealed significant lower CNFB, CNBD and CNFL between MS patients, compared to healthy controls. These results were independent of previous ON development. Our data agree with literature available to date.[44–46] Unlike RNFL thickness, CCM measurements do not seem to be affected by inflammation, and eventually by transsynaptic retragade axonal degeneration. These findings suggest that corneal subbasal nerve plexus evaluation may be a promising imaging modality to evaluate more objectively axonal loss in MS patients.

Furthermore, our study also revealed that CNFD was inversely correlated with both EDSS and MSSS scores. There are some conflicting data regarding the relationship between CCM measurements and clinical disability scores. Mikolajzak *et al*[46] revealed an inverse correlation between CNFL and EDSS and Petropoulos *et al*[45] found an inverse correlation between CNBD and the same clinical disability status score, but no correlation was found with CNFD and CNFL. On the other hand, Bitirgen *et al*[44] results agree with our findings, revealing a correlation between CNFD and both EDSS and MSSS.

Some factors may explain these different results. First, all four studies (ours and the referred ones) included a limited number of patients (the highest sample was 57 patients in Betirgen *et al*[44] study, followed by ours) and second, Mikolajzak *et al*[46] did not use the same methodology used by the others to quantify corneal subbasal nerve plexus.

Regardless these differences, the truth is that all studies revealed a relationship between any CCM parameter and neurologic disability scores which suggests this technology as promising to evaluate MS progress.

We still want to highlight our results about CNFT. A higher value of tortuosity has been found in a variety of diseases,[53–58] but the truly meaning of this parameter is still debatable with some authors advocating it as a marker of nerve regeneration.[59] Our study was the first one evaluating CNFT in MS patients, and we didn't find any difference compared to healthy subjects.

Evidently, we need to be aware of our limitations. This was a pilot cross-sectional study with a small sample size which did not allow us to draw solid conclusions but gave rise to a larger prospective investigation to corroborate these primary results. Besides, in our study a significant number of patients (96.7%) were receiving disease-modifying drugs. This is the reason why in our future larger study a higher number of untreated patients will be enrolled which is needed to allow a multivariable analysis, to be sure that CCM results are not only related to possible medication's effect.

Concluding, this study represents the second largest CCM study in MS to date, revealing a decrease in three of the CCM parameters (CNFD, CNBD and CNFL) and ppRNFL in MS patients. Furthermore, it also demonstrates a relationship between TI ppRNFL and EDSS score in MSWON patients. Finally, the association between CNFD and both EDSS and MSSS scores really encourage further investigation to access CCM ability as a diagnosis and prognosis image modality in MS.

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CONTACT

Diogo Hipólito Fernandes
Ophthalmology Department
Lisbon Central Hospital Centre
Alameda Santo António dos Capuchos
1169-050 Lisboa
E-mail: cdiogo777@gmail.com

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