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Corneal Subbasal Nerve Plexus Evaluation by *in Vivo* Confocal Microscopy in Multiple Sclerosis: A Potential New Biomarker

Diogo Fernandes[a], Maria Luís[a], Joana Cardigos[a], Catarina Xavier[a], Marta Alves[b,c,d], Ana Luísa Papoila[b,c,d], João Paulo Cunha[e,f], and Joana Tavares Ferreira[e,f,g,h]

[a]Ophthalmology Department, Centro Hospitalar Universitário de Lisboa Central, Lisbon, Portugal; [b]Epidemiology and Statistics Unit, Research Centre, Centro Hospitalar Universitário de Lisboa Central, Lisbon, Portugal; [c]Faculdade de Ciências Médicas, NOVA Medical School, Lisbon, Portugal; [d]Centro de Estatística e Aplicações da Universidade de Lisboa, Lisbon, Portugal; [e]Department of Ophthalmology, Hospital CUF Cascais, Cascais, Portugal; [f]Department of Orthoptics and Vision Sciences, Escola Superior de Tecnologia da Saúde de Lisboa, Lisbon, Portugal; [g]Department of Ophthalmology, Hospital CUF Descobertas, Lisbon, Portugal; [h]Neuro-Ophthalmology Department, Centro Hospitalar Universitário de Lisboa Norte, Lisbon, Portugal

ABSTRACT

Purpose/Aim: Our study aims to evaluate corneal subbasal nerve plexus morphology by *in vivo* corneal confocal microscopy (CCM) in Multiple Sclerosis (MS) patients and to explore its potential ability to distinguish between MS patients and healthy subjects.

Materials and methods: Cross-sectional study, including 60 MS patients and 22 healthy subjects. Expanded Disability Status Scale (EDSS) was used to assess neurological disability. All participants underwent full ophthalmology evaluation, CCM and optical coherence tomography (OCT). Corneal nerve fibre density (CNFD), branch density (CNBD), fibre length (CNFL) and fibre tortuosity (CNFT) were analysed. Generalized additive regression models were used to analyse the data.

Results: Compared to controls, MS patients had lower CNFD, CNBD and CNFL ($p < .001$) and higher CNFT ($p = .002$). The area under the ROC curve to distinguish MS patients from healthy controls with CNFD and CNBD was 0.84 (95%CI: 0.75 to 0.93; 95%CI: 0.75 to 0.92, respectively). A nonlinear association between EDSS and CNFD was found, with an initial density increase followed by a significant decrease until more severe disability status. EDSS was associated with CNFL and CNBD, with values being significantly lower for patients with an EDSS > 2.5 (−2.06 mm/mm2; 95%CI: −3.84 to −0.28; $p = .027$ and −8.70 branches/mm2; 95%CI: −14.69 to −2.71; $p = .006$, respectively). An optic neuritis (ON) history did not influence CCM parameters.

Conclusions: Our results confirm CCM parameters' potential to differentiate MS patients from healthy subjects, not being influenced by a previous ON history. A significant relationship between patient's disability and corneal nerve morphology was also found.

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Introduction

Multiple Sclerosis (MS) is a chronic inflammatory, autoimmune and degenerative central nervous system (CNS) disease, affecting mainly active patients between 20 and 40 years old, with severe social and professional consequences.[1] Currently, there is not any clinical, laboratory, pathological or imaging pathognomonic determinants of MS, posing the development of new diagnostic and prognostic biomarkers as crucial.

Along with the acute inflammatory episodes of demyelination, axonal degeneration is a fundamental component of MS and a major determinant of permanent neurological impairment.[2] However, we still lack an accurate methodology to monitor axonal damage and possible nerve regeneration with treatment, which represents a major obstacle for new disease-modifying therapies development.[3] Magnetic resonance spectroscopic imaging (MRSI) detects changes in metabolites such as N-acetyl aspartate, a marker of axonal integrity, being able to distinguish MS from healthy subjects.[4,5] However, this methodology does not allow direct axon imaging, being also expensive and a time-consuming methodology, which limits its clinical application. Optic neuritis (ON) is the initial presentation in 20% of MS 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.[6–8] However, conflicting findings were found regarding the relationship between retinal nerve fibre layer (RNFL) thickness and Expanded Disability Status Scale (EDSS),[9–11] the worldwide used method to quantify disability in MS and to monitor it over time. The occurrence of optic neuritis causes additional damage tothe optic nerve, which limits OCT usage as disease monitoring and progression biomarker.

Corneal nerve fibres are particularly vulnerable to degeneration caused by metabolic, inflammatory, or toxic changes, due to lack of direct vascular supply. In fact, several studies in peripheral and central neurodegenerative diseases[12–16] support the potential role of corneal subbasal nerve plexus study in such diseases. Regarding MS, trigeminal involvement was reported in just 2.9% of patients,[17] which increases the interest in corneal nerves investigation.

Our study aims to evaluate corneal subbasal nerve plexus morphology by *in vivo* corneal confocal microscopy (CCM) in MS patients and to identify possible associated factors (such as

CONTACT Diogo Fernandes cdiogo777@gmail.com Ophthalmology Department, Centro Hospitalar Universitário de Lisboa Central, Alameda Santo António dos Capuchos, Lisboa 1169-050, Portugal.

patient's age, EDSS and ppRNF thickness). Exploring the possibility of using this noninvasive technology as a methodology to assess axonal degeneration by studying their potential ability to distinguish patients from healthy subjects was also an objective of this study.

Methods

Patients and controls

This cross-sectional study was conducted at the Ophthalmology and Neurology Departments of the Centro Hospitalar Universitário de Lisboa Central, between May 2018 and February 2019. Two groups were recruited: 60 MS patients and 22 healthy controls. 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 and 65 years old, with MS diagnosis (relapsing-remitting or secondary-progressive) according to the McDonald criteria[18] were considered eligible. MS patients were submitted to a complete neurologic evaluation, performed by a neurologist, and disability classification was assessed using EDSS (score range 0–10, higher score represents greater disability). Multiple Sclerosis Severity Score (MSSS) was then calculated from EDSS and disease duration[19] (range 0.01–9.99, higher score represents greater severity). Previous optic neuritis history and disease duration were also recorded. Before CCM and OCT, full standard ophthalmologic evaluation (best-corrected visual acuity evaluation, biomicroscopy, Goldmann applanation tonometry and fundoscopy) was performed in both healthy controls and MS patients.

We only included patients with an optic neuritis episode occurred more than 6 months prior to the study. For patients with bilateral or no optic neuritis, by convention, only the right eye was assessed. In those with unilateral optic neuritis, only the affected eye was evaluated.

Exclusion criteria for patients group include: optic neuritis history within 6 months prior to recruitment; for both patients and controls: history of trigeminal neuralgia, 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, dry eye disease, contact lens use and high refractive error (spherical equivalent ± 6.0D).

Optical coherence tomography

Peripapillary RNFL thickness measurements were obtained through spectral-domain OCT (Spectralis®, Heidelberg Engineering, Germany). The light source of SD-OCT was a superluminescent diode with 850 nm wavelength, 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 well-trained technician (R.L.) – three scans per patient, from which the one with the best quality was selected for analysis – and assessed by two ophthalmologists (J.F, M.E.L.), independently from each other, masked from to the participant's group. Only good-quality scans with well-focused images, without overt misalignment, continuous scan patterns without missing or blank areas, without artefacts, and a signal strength better than 20 (40¼ maximum) were included in the analyses. 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 assess 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 400 × 400 μm (15º × 15º field of view; 1 μm per pixel lateral digital 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 examination, an outer fixation light was used to correctly position the eye. The total duration of CCM was 5 min per eye and all images were obtained by the same trained ophthalmologist (D.H.), masked from to the participant's group. Three to five high-quality corneal subbasal nerve plexus images per patient were considered, and the results are the means of those measurements.

Validated CCMetrics software, version 2.0 (University of Manchester, UK) was used to analyse the images.[15,20–23] 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. Image analysis was performed by a single observer (D.H.), from to the participant's group.

Statistical analysis

Demographics and clinical characteristics of patients were described with frequencies (percentages) and with mean (SD:

standard deviation) for symmetric distributions or with median and interquartile range (25th percentile-75th percentile) for skewed distributions. Nonparametric Chi-square, Mann–Whitney and Kruskal–Wallis tests were applied, as appropriate. CCM parameters' ability to discriminate MS patients from healthy controls was assessed by the area under the receiving operating characteristic curve – AUC (interpretation: excellent ≥0.90, adequate 0.70 to 0.89, poor <0.70). Generalized additive regression models were used to identify the variables that explain each CCM parameter (CNFD, CNBD, CNFL, and CNFT) variability of MS patients. The effect of age, sex, EDSS, disease duration, ON patients' group (MSON and MSWON), and ppRNFL thickness measurements were considered in these analyses. Those variables nonlinearly associated with the outcomes were modelled with splines. For variable selection purposes, a univariable analysis was performed and all the variables with a p-value < 0.25 were candidates for the multivariable models. Normality assumption of the residuals was verified using Shapiro–Wilk goodness-of-fit test.

A level of significance α = 0.05 was considered. Data were analysed using R (R: A Language and Environment for Statistical Computing, R Core Team, R Foundation for Statistical Computing, Vienna, Austria, year = 2019, http://www.R-project.org.).

Results

Patient demographics and clinical characteristics

A total of 60 patients with MS diagnosis (14 males) and 22 healthy controls (6 males) were included. The mean age of MS patients was 43.88 (10.71) years and of healthy subjects was 38.68 (8.60) years. The median MS duration was 10.0 (5.00–-14.50) years. Fifty-eight patients (96.7%) were receiving disease modifying drugs, with dimethyl fumarate being the most frequent drug used (25.9%), followed by fingolimod (17.2%). The median EDSS and MSSS scores were 2.00 (1.00–3.50) and 2.71 (0.93–4.92), respectively. Fifty-five (91.7%) patients had relapsing-remitting MS, four (6.7%) primary-progressive and one (1.7%) secondary-progressive MS. A history of at least one episode of optic neuritis was found in 24 (40%) patients.

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 no significant differences considering disease duration (p = .860), EDSS (p = .297) and MSS scores (p = .220) (Table 1).

Optical coherence tomography

There was a significant reduction in global ppRNFL thickness means in MS patients compared with healthy controls (84.65 μm vs. 99.82 μm; p < .001). This significant reduction was also found in all sectors except for NI (p = .179). MSON patients had a significant thinner ppRNFL in all sectors compared to MSWON patients (G, T, TI, and TS p < .001; N p = .013; NS p = .004; NI p = .003). There was a significant reduction in ppRNFL thickness in MSON patients compared to healthy controls (p < .001 to 0.011), unlike MSWON patients. A detailed description of ppRNFL thickness in all groups is depicted in Table 2.

In vivo corneal confocal microscopy

MS patients showed a significant reduction in means of CNFD (31.81 fibers/mm^2 vs. 39.40; p < .001), of CNBD (47.62 branches/mm^2 vs. 63.12; p < .001), and of CNFL (18.47 mm/mm^2 vs. 21.25; p < .001) compared to healthy controls. CNFT mean was higher in MS patients compared with healthy controls (12.14 of tortuosity coefficient vs 9.73, p = .002) (Table 3 and Figure 1).

Considering MSON and MSWON patients, no significant differences in any of the CCM measurements between MS patients with and without optic neuritis (p = .596 to 1.000) were found (Table 3). There was a statistically significant decrease in CNFD and CNBD between the MSON patients and healthy controls (p < .001 and 0.002, respectively). Every CCM parameter was significantly different between MSWON patients and healthy controls (p < .001 to 0.005) (Table 3).

Corneal subbasal nerve plexus parameters discriminative ability

ROC curves were built and corresponding AUCs were estimated in order to evaluate CCM parameter capacity to discriminate MS patients from healthy controls. The AUC for CNFD and CNBD was 0.84 (95% CI: 0.75 to 0.93 for CNFD; 95% CI: 0.75 to 0.92 for CNBD). The AUCs for CNFL and CNFT were lower (0.74; 95% CI: 0.64 to 0.85, and 0.72; 95% CI: 0.61 to 0.84, respectively). ROC curves are represented in Figure 2.

Table 1. Demographic and clinical characteristics of the patients, by group.

		Patients with MS		
Characteristics	Healthy Controls (n = 22)	MSON (n = 24)	MSWON (n = 36)	p-value
Age, years	38.68 (8.60)	42.46 (10.04)	44.83 (8.25)	.091[†]
Male sex n (%)	6 (27.3)	8 (33.3)	6 (16.7)	.316[‡]
Disease Duration, years	-	9.50 (5.00–14.50)	10.50 (5.00–14.50)	.860[§]
EDSS score	-	2.50 (1.00–3.50)	2.00 (1.00–3.88)	.297[§]
MSSS	-	3.90 (0.94–6.43)	2.52 (0.91–3.89)	.220[§]

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.
[†]ANOVA.
[‡]Chi-square test.
[§]Mann–Whitney test.

Table 2. Peripapillary RNFL thickness measurements, by group.

Measurements	Healthy Controls (n = 22)	Patients with MS			*p*-value			
		Total (n = 60)	MSON (n = 24)	MSWON (n = 36)	Patients with MS vs. Healthy Controls	MSON vs. MSWON	MSON vs. Healthy Controls	MSWON vs. Healthy Controls
RNFL G, µm	99.82 (6.59)	84.65 (14.65)	73.21 (9.73)	94.81 (9.57)	<.001†	<.001‡	<.001‡	.179‡
RNFL T, µm	71.14 (12.19)	55.44 (15.46)	41.00 (9.81)	65.50 (10.68)	<.001†	<.001‡	<.001‡	.340‡
RNFL TS, µm	137.86 (16.20)	117.65 (20.46)	104.07 (16.49)	133.00 (17.67)	<.001†	<.001‡	<.001‡	.245‡
RNFL TI, µm	139.64 (16.65)	123.23 (24.43)	107.43 (11.39)	136.31 (20.27)	.004†	<.001‡	<.001‡	1.000‡
RNFL N, µm	77.77 (10.67)	66.00 (16.54)	63.29 (15.56)	71.44 (14.15)	<.001†	.013‡	<.001‡	.319‡
RNFL NS, µm	121.14 (19.39)	99.25 (21.37)	87.71 (14.53)	112.75 (21.59)	<.001†	.004‡	<.001‡	.074‡
RNFL NI, µm	108.55 (26.01)	99.11 (24.63)	88.29 (19.36)	109.56 (23.79)	.179†	.003‡	.011‡	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.

Table 3. Corneal confocal microscopy parameter measurements, by group.

Measurements	Healthy Controls (n = 22)	Patients with MS			*p*-value			
		Totalv (n = 60)	MSON (n = 24)	MSWON (n = 36)	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)	31.81 (6.32)	31.44 (6.00)	32.06 (6.60)	<.001†	1.000‡	<.00‡	<.001‡
CNBD branches/mm^2	63.12 (11.11)	47.62 (11.95)	49.67 (12.49)	46.26 (11.55)	<.001†	.956‡	.002‡	<.001‡
CNFL mm/mm^2	21.25 (2.05)	18.47 (3.44)	19.17 (3.56)	18.00 (3.33)	<.001†	.596‡	.102‡	.001‡
CNFT tortuosity coefficient	9.73 (2.16)	12.14 (3.26)	11.49 (2.59)	12.57 (3.61)	.002†	1.000‡	.099‡	.005‡

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.

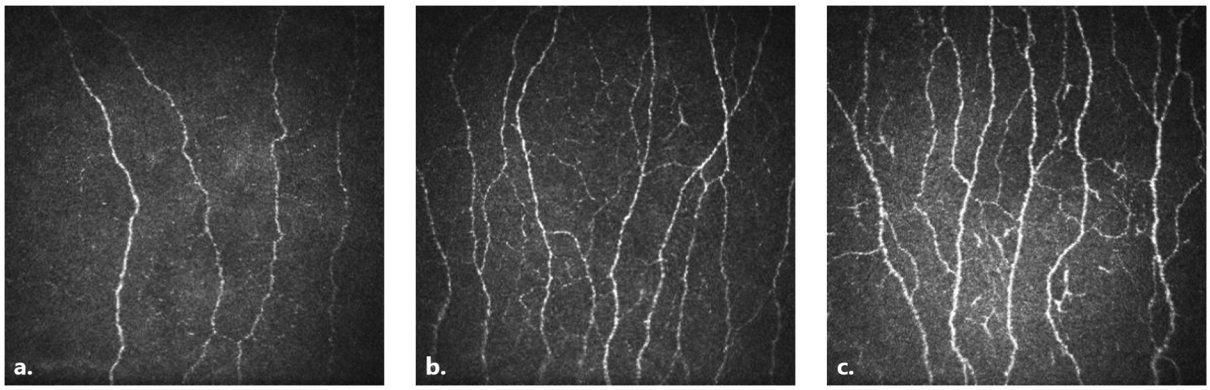

Figure 1. Corneal confocal microscopy images corresponding to a Multiple Sclerosis (MS) patient with EDSS = 6.5 (a) a MS patient with EDSS = 1 (b) and a control subject (c). Each image has 400 x 400µm.

Corneal subbasal nerve plexus parameter associations in MS patients

Variable selection for the multivariable models that will identify the variables that may influence CCM parameters is based on the univariable study whose results are depicted in Table S1. Table 4 presents the multivariate analysis.

The multivariable model for CNFD showed a mean reduction of 0.14 fibers/mm^2 for each year increase of age (95% CI: −0.27 to −0.01; p = .042), and a mean increase of 0.07 fibers/mm^2 for each ppRNFL NI unit increase (95% CI: 0.01 to 0.13; p = .027), independently of previous history of ON that revealed no statistical association with both outcomes (p = .704). Due to the nonlinear association between EDSS and CNFD (Figure 3a), this variable was modelled with splines (p < .001). An initial moderate density increase until around an EDSS value of 1.5 may be observed in this figure, followed by a more significant decrease until more severe disability status.

For CNBD and CNFL multivariable models (Figure 3b,c, respectively), EDSS was reclassified into two categories, no-to-mild disability (EDSS ≤ 2.5) and moderate-to-severe disability (EDSS > 2.5). Results showed that independently of a previous history of ON that revealed no statistical association with both outcomes (p = .120 for CNFL and p = .167 for CNBD), EDSS was associated with CNFL and CNBD, with values being significantly lower for patients with EDSS > 2.5 when compared with patients with EDSS≤ 2.5 (on average −2.06 mm/mm^2; 95% CI: −3.84 to −0.28; p = .027, and on average −8.70 branches/mm^2; 95% CI: −14.69 to −2.71; p = .006, respectively).

For CNFT, no multivariable model was achieved.

Figure 3 corresponding scatter plots are presented in Figure S1.

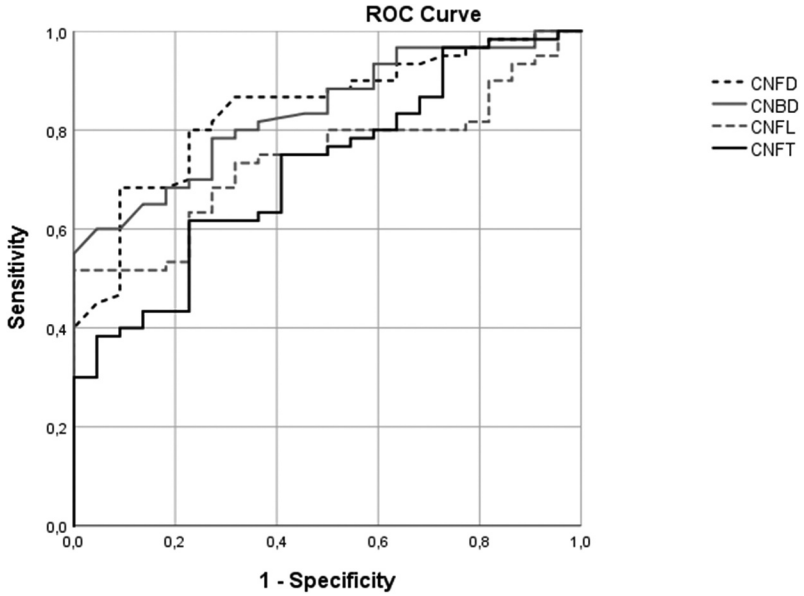

Figure 2. Receiver operating characteristic curves corresponding to corneal confocal microscopy. CNFD – corneal nerve fibre density; CNBD – corneal nerve branch density; CNFL – corneal nerve fibre length; CNFT – corneal nerve fibre tortuosity.

Table 4. Results of the multivariable regression analysis – dependent variable: CCM parameters (CNFD, CNBD, and CNFL) (fibers/mm^2).

Model	Coefficient Estimate	95% Confidence Interval	*p*-value
Dependent variable: CNFD[a]			
Age (years)	−0.14	−0.27 to −0.01	.042
Optic neuritis	−0.58	−3.57 to 2.41	.704
ppRNFL Nasal Inferior (µm)	0.07	0.01 to 0.13	.027
Dependent variable: CNBD			
EDSS > 2.5	−8.70	−14.69 to −2.71	.006
Optic neuritis	4.25	−1.69 to 10.18	.167
Dependent variable: CNFL			
EDSS > 2.5	−2.06	−3.84 to −0.28	.027
Optic neuritis	1.42	−0.34 to 3.19	.120

[a]Due to the nonlinear association between CNFD and EDSS, this variable was modelled with splines ($p < .001$); *p*-values were obtained by generalized additive regression models.

Abbreviations: CCM, corneal confocal microscopy; CNBD, corneal nerve branch density; CNFD, corneal nerve fibre density; CNFL, corneal nerve fibre length; EDSS, Expanded Disability Status Scale; MSSS, ppRNFL, peripapillary retinal nerve fiber layer.

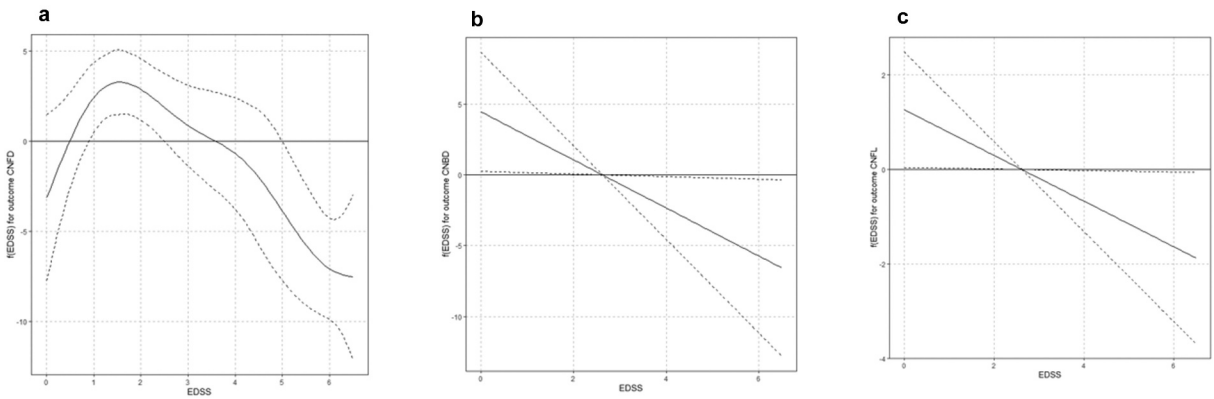

Figure 3. Fitted smooth effects (f(EDSS): black curve) of EDSS on outcomes CNFD (a), CNBD (b), and CNFL (c) with corresponding 95% confidence intervals (dashed lines) obtained by generalized additive models. These smooth effects reflect a nonlinear change of the expected mean value of CNFD considering each value of EDSS, whereas for outcomes CNBD and CNFL that change is linear. At a certain observed point of EDSS, a negative value of f(EDSS) means that, at that point, the expected mean of the outcome suffers a decrease in an amount corresponding to that value. At the contrary, if f(EDSS) is positive, the expected mean of the outcome increases by that amount; f(EDSS) = 0 means no effect of EDSS on the outcome.

Discussion

This cross-sectional study evaluated corneal subbasal nerve plexus in MS patients through CCM. Overall, we found a significant decrease in CNFD, CNBD and CNFL and a significant increase in CNFT in MS patients when compared to healthy controls, which agrees with recent literature.[24–26]

Axonal loss may be the most significant pathophysiological factor in MS and its monitorizationmight be extremely helpful to assess disease progression.[27] Corneal nerves approach the cornea radially around the limbus, losing their perineurium and myelin sheath, running centripetally towards the centre, creating a network of unmyelinated axons – subbasal nerve plexus. CCM, allowing a non-invasive evaluation of such plexus, is gaining popularity in the scientific community as there is increasing evidence of small unmyelinated fibre loss in central neurodegenerative diseases. In fact, considering the great potential of corneal nerve plexus evaluation, as well described in a recent revision by Petropoulos et al.[28] could it be a biomarker of MS-related neurodegeneration, disease progression and treatment response?

The first step to consider CCM parameters as potential biomarkers is to confirm if they really differ between MS patients and healthy subjects. Our results support that hypothesis in two ways. First, we clearly found a difference between both groups in all parameters, reflecting higher corneal subbasal nerve plexus neurodegeneration in MS patients. Second, their discriminative power to distinguish MS patients from controls seems very relevant, particularly CNFD and CNBD, both with an AUC of 0.84. However, considering the exploratory nature of our research, we consider prudent not to determine any cut-off value yet. This may be significant considering MS diagnosis, as the 2017 revised McDonald criteria, even with an improved sensitivity due to oligoclonal band inclusion, still lack specificity.[29,30] Moreover, an alternative less technically demanding and lower costing biomarker than oligoclonal bands might be useful.

Another important fact is that when we compared CCM parameters between patients with and without optic neuritis, there were no differences between them, unlike ppRNFL thickness, in which significant differences were found between those groups, in agreement with some publications.[26,31] These results reinforce corneal subbasal nerve plexus potential relatively to ppRNFL since an history of optic neuritis does not seem to influence the differences relatively to healthy subjects. This looks particularly true for CNFD and CNBD, in which both groups (MSON and MSWON) maintained their differences from controls even after subgroup analysis.

Regarding MS patient's disability status, measured by EDSS, our analysis revealed a significant association with CNFD, CNFL and CNBD, and a non-linear association between CNFD and EDSS was identified. According to our model, it seems that an initial increase in CNFD (from EDSS 0 to 1.5) is followed by a more marked, continuous decrease until more severe disability status. Furthermore, EDSS was associated with CNFL and CNBD, with values being significantly lower for patients with EDSS > 2.5. Regardless of EDSS association with CCM parameters, our models also showed that, unlike for ppRNFL, a history of ON did not have an impact on corneal subbasal nerve parameters.

These findings raise questions about MS patients with low EDSS scores and the relationship between continuous background axonal loss and measurable patient's disability.

EDSS, despite being the most widely used outcome measure for disability progression in MS, has some well-known limitations, such as subjectivity and poor reliability within and between ratters; it is heavily dependent on the motor system; it does not represent patient's disability as a whole, being more insensitive to cognitive impairment and sensory system dysfunction.[32] Also, the true relationship between EDSS and axonal loss is still not well established since both MRI and OCT studies found some controversial results so far.[2,33]

Corneal subbasal nerve plexus, despite its vulnerability, also developed several different mechanisms to regenerate, which was demonstrated after refractive surgery or after neurotrophic keratopathy treatment.[34,35] Considering the above-mentioned, we believe that our findings regarding the relationship between EDSS and CCM parameters may be explained by some factors. Despite their immunosuppressive and immunomodulatory role, different disease modifying drugs (DMD) may have different impacts on neuronal regeneration. Fingolimod, for example, recently showed promising capacity to promote nerve regeneration.[36] In our cohort, none of the patients with EDSS equal 0 was taking fingolimod, unlike patients with EDSS between 1 and 2. This example does not explain the initial rise of CNFD but may reflect some of our study limitations, particularly MS duration heterogeneity and, even more, different therapeutics. We do consider the hypothesis that in patients with none-to-mild disability being treated with DMD, drug's effect on the inflammatory environment may contribute to some corneal nerve regeneration, even without returning to normal values. In fact, we are able to find in literature some clinical trials on diabetes revealing an improvement in corneal nerve morphology after treatment.[37–40] Our study also revealed that EDSS was associated with CNFL and CNBD, with values being significantly lower for patients with moderate-to-severe disability, compared to patients with EDSS ≤2.5. We believe that when patients' disability score rises above a certain threshold, the background axonal loss may not be overcome by corneal regenerative capacity, resulting in a significant progressive decrease in CCM parameters according to EDSS severity.

To date, we still lack strong evidence regarding corneal subbasal nerve plexus evaluation in MS.[24–26] However, regardless of the limited number of patients included so far, all studies revealed a significant difference between MS patients and healthy controls, and a relationship between at least one CCM parameter and neurologic disability scores. These results reinforce this technology as promising to be used in MS.

We want to emphasise that until now, these are all hypotheses. This was a pilot cross-sectional study with a small sample size which did not allow us to draw solid conclusions. We strongly encourage further investigation regarding the potential role of corneal subbasal nerve plexus evaluation in MS, particularly prospective studies to evaluate its progression over time, its ability to distinguish patients at risk of disability progression and to find the DMD effects on corneal nerve regeneration. Another limitation that should be considered is that despite using 3–5 high-quality CCM images per patient (as recommended by Cruzat A, Qazi Y and Hamrah P[41] and

generally used by other authors,[22,24–26,42]) we did not use the optimal number of non-overlapping images (eight) defined by Vagenas et al.[43] In conclusion, our research represents the largest CCM study in MS to date, revealing a significant difference between MS patients and healthy controls regarding corneal subbasal nerve plexus parameters, a difference that is independent of an ON history. Finally, we found a significant relationship between patient disability and CCM parameters that needs further validation, particularly the influence of other factors not evaluated in this study, such as different treatment regimens.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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