Corneal Confocal Microscopy: An Imaging Endpoint for Axonal Degeneration in Multiple Sclerosis

Ioannis N. Petropoulos,[1] Saadat Kamran,[2] Yi Li,[1] Adnan Khan,[1] Georgios Ponirakis,[1] Naveed Akhtar,[2] Dirk Deleu,[2] Ashfaq Shuaib,[2] and Rayaz A. Malik[1]

[1]Weill Cornell Medicine–Qatar, Qatar Foundation, Education City, Doha, Qatar
[2]Institute of Neurosciences, Hamad General Hospital, Doha, Qatar

Purpose. To evaluate whether corneal confocal microscopy (CCM) detects axonal degeneration and whether this is associated with retinal nerve fiber degeneration and clinical disability in patients with multiple sclerosis (MS).

Methods. Twenty-five patients with MS and 25 healthy control subjects underwent CCM, optical coherence tomography (OCT), and assessment of neurological disability using the expanded disability status scale (EDSS) and MS severity score (MSSS).

Results. In patients with MS compared with controls, there was a significant reduction in corneal nerve fiber density (CNFD), branch density (CNBD), and length (CNFL). There was no significant difference in CCM parameters between patients with optic neuritis (MS-ON) and without (MS-NON), or between relapsing-remitting (RRMS) and secondary-progressive MS (SPMS). There was significant thinning of the retinal nerve fiber layer (RNFL) in the global, temporal, temporal superior, and temporal inferior quadrants, with no difference between MS-ON and MS-NON. Patients with SPMS compared with RRMS had a significantly lower global, temporal superior, temporal inferior, nasal, and nasal superior RNFL. The EDSS and MSSS correlated significantly with CNBD, nasal, nasal superior, and nasal inferior RNFL and with CNBD and nasal inferior RNFL, respectively.

Conclusions. CCM and OCT detect significant corneal and retinal nerve degeneration which relates to the severity of neurological deficits in patients with mild MS.

Keywords: axonal loss, cornea, corneal confocal microscopy, multiple sclerosis

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Multiple sclerosis (MS) is a chronic inflammatory, neurodegenerative disease of the central nervous system and is the leading cause of neurological disability in young adults. The hallmarks of MS are inflammation, demyelination, and axonal degeneration. The latter is associated with significant disability and poor prognosis.[1] In a postmortem study of patients with MS, there was evidence of significant small fiber axonal loss in both corticospinal and sensory tracts.[2] Indeed, neuropathic pain is a feature of MS and may occur in approximately 50% of patients in association with altered thermal thresholds.[3] Furthermore, the transition to progressive disease is believed to occur when cumulative axonal degeneration surpasses the capacity for regeneration.[4] A major challenge in MS is how to accurately monitor axonal degeneration with disease progression and potential regeneration with treatment.

Quantification of neurological deficits and brain imaging form the mainstay for evaluating patients in the clinic and for assessing therapeutic response in clinical trials of MS. The expanded disability status scale (EDSS) has low sensitivity and reproducibility and does not capture the full spectrum of deficits encountered by patients with MS.[5] Magnetic resonance imaging is the principal imaging tool to detect and monitor pathological changes underpinning MS. Serial T2-weighted images are used to assess and follow-up the number of new hyperintense brain lesions, as opposed to T1-weighted images, which are used to identify hypointense lesions, which represent areas of permanent demyelination and axonal loss. Gadolinium-enhanced T1-weighted images are used to detect areas of active inflammation. However, magnetic resonance imaging has limited histopathological specificity, as T2-weighted images identify inflammation, edema, and demyelination, as well as axonal loss with gliosis. Quantifying global brain atrophy can provide an index of axonal degeneration, but suffers from the "clinical-radiological paradox," with only a modest correlation to clinical signs and symptoms of MS.[6] Indeed, to date it has not been accepted by the US Food and Drug Administration as a surrogate endpoint in clinical trials of MS.

Parisi and colleagues[7] have previously reported that the retinal nerve fiber layer (RNFL) is decreased in patients with MS and this has been confirmed in subsequent studies,[8] particularly in the affected eyes of patients with optic neuritis (ON). Indeed, RNFL is now increasingly used as a co-endpoint in trials of MS. Although, in the recent RENEW trial,[9] opicinumab following a first episode of ON showed an improvement in optic nerve conduction latency but no improvement in RNFL thickness or visual function. Additionally, in the SYNERGY study of opicinumab in patients with relapsing-remitting MS (RRMS) there was no significant improvement in a multicomponent endpoint evaluating physical disability.[10] The results of these trials highlight the shortcomings of currently advocated Food and Drugs Administration endpoints in clinical trials of MS.

We have pioneered corneal confocal microscopy (CCM), a rapid noninvasive ophthalmic imaging modality that objectively quantifies axonal degeneration and regeneration in diabetic neuropathy,[11] Fabry's disease,[12] hereditary neuropathy,[13] and chemotherapy-induced neuropathy.[14] We and others have shown that CCM quantifies early axonal loss[15] reliably with high sensitivity and specificity,[16] correlates to the severity of intraepidermal nerve fiber loss,[17] and predicts incident diabetic neuropathy.[18] It detects nerve regeneration after simultaneous pancreas and kidney transplantation[19] and in patients with diabetes[20] and sarcoid neuropathy[21] after administration of the erythropoietic peptide ARA290. Recent studies have demonstrated corneal nerve fiber loss in relation to motor deficits[22] and affective touch,[23] and corneal nerve loss with preservation of intraepidermal nerve fibers in patients with Parkinson's disease.[24] Corneal nerve fiber loss also correlates with reduced bulbar function in patients with amyotrophic lateral sclerosis measured with the amyotrophic lateral sclerosis severity score, a tool designed to measure speech, swallowing, and upper and lower extremity disability.[25] A recent study also has demonstrated a significant reduction in corneal nerve fiber density (CNFD) using CCM in patients with MS.[26]

The aim of the present study was to determine whether CCM can detect axonal degeneration and whether this is associated with ON or clinical disability status in patients with MS.

METHODS

Study Subjects

A total of 25 patients with MS attending the neurology outpatient clinic at Hamad General Hospital in Doha, Qatar, and 25 healthy age-matched controls were studied. This study adhered to the tenets of the declaration of Helsinki and was approved by the Institutional Review Board of Weill Cornell Medicine–Qatar of Cornell University (approval 15-00064). Informed written consent was obtained from all subjects before participation in the study. Patients with a diagnosis of MS by a board-certified neurologist (SK) who were 18 to 75 years old were included in the study. Patients with MS and healthy controls who were contact lens users, or had been diagnosed with ophthalmic disease (e.g., glaucoma, retinal or corneal disorders) or had an incident of ON within 6 months from assessment were excluded. Before CCM, patients with MS and healthy controls underwent slit lamp biomicroscopy. Due to the well-established relationship between corneal nerve density and peripheral neuropathy, other causes of neuropathy were excluded based on HbA1c, anti-nuclear antibody (ANA), serum B12/folate, and immunoglobulins (IgG). The neurological status of patients with MS was assessed using the EDSS (SK) and the multiple sclerosis severity score (MSSS) was calculated from the EDSS and disease duration.[27] MS duration was based on the date of diagnosis and secondary-progressive MS (SPMS) was defined based on EDSS and accumulation of neurological disability over time.

Optical Coherence Tomography

Peripapillary RNFL thickness measurements were performed with a spectral-domain optical coherence tomography (OCT) system (Spectralis OCT; Heidelberg Engineering GmbH, Heidelberg, Germany) in 50 MS eyes and 50 healthy control eyes by a single trained examiner (INP) who was masked from the disability status of the subject. RNFL measurements were performed by using circular scans with a scanning angle of 12°, which equates to a retinal diameter of 3.5 mm when assuming a standard corneal curvature of 7.7 mm and with the eye tracker activated. This OCT system uses a superluminescent diode with a center wavelength of 840 nm and obtains up to 40,000 A-scans per second with a depth resolution of 7 μm and transverse optical resolution of 14 μm. All RNFL scans in this study were performed in high-speed mode, which contains 768 A-scans along a peripapillary circle of 360°. A built-in algorithm determines the inner and outer boundaries of the RNFL and estimates the RNFL thickness along the circular scan. Within one session, three scans were taken per eye for both eyes with the eye tracker on, ensuring a minimum signal strength of 20 as per default settings (of 30) and optimal centering on the optic nerve head performed manually by a trained examiner. The average value per area of the optic nerve from three scans was calculated for estimation of regional RNFL thickness. For interpretation of the RNFL scan, the optic disk is segmented as follows: temporal (315–45°), temporal superior (45–90°), temporal inferior (270–315°), nasal (135–225°), nasal inferior (90–135°), nasal superior (225–270°), and an averaged global classification. RNFL data are presented as an average of left and right eyes for patients with MS and healthy controls.

Corneal Confocal Microscopy

All study subjects underwent CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module; Heidelberg Engineering GmbH). This device uses a 670-nm wavelength helium neon diode laser, which is a class I laser and therefore does not pose any ocular safety hazard. A ×63 objective lens with a numerical aperture of 0.9, and a working distance, relative to the applanating cap (TomoCap; Heidelberg Engineering GmbH, Heidelberg, Germany), of 0.0 to 3.0 mm is used. The size of each two-dimensional image produced is 384 × 384 μm with a 15° × 15° field of view and 10 μm per pixel transverse optical resolution. To perform the CCM examination, two drops of local anesthetic (0.4% benoxinate hydrochloride; Chauvin Pharmaceuticals, Chefaro, UK) was used to anaesthetize each eye and Viscotears (Carbomer 980, 0.2%; Novartis UK, Surrey, UK) were used as the coupling agent between the cornea and the applanating cap. All subjects were asked to fixate on an outer fixation light throughout the CCM scan and a charge-coupled device camera was used to correctly position the applanating cap onto the central cornea. The examination took approximately 10 minutes for both eyes and a single experienced examiner (INP), masked from the subject's disability status, performed CCM and acquired images using the "section" mode. Based on depth, contrast, and focus position, six images per subject (three per eye) were selected and analyzed from the central subbasal nerve plexus.[16] In total, 308 CCM images were analyzed using validated, purpose-written software (CCMetrics; M. A. Dabbah, ISBE, University of Manchester, Manchester, UK). CNFD, corneal nerve branch density (CNBD), and length (CNFL) were quantified according to a previously established protocol.[16] Data are presented as an average of left and right eyes for patients with MS and healthy controls.

Statistical Analysis

All statistical analyses and graphic illustrations were generated using Prism 6 (version 6.0g for Mac; GraphPad Software, Inc., San Diego, CA, USA) and Stats Direct (StatsDirect Ltd., Altrincham, Cheshire, UK). A Shapiro-Wilk test was used to assess data for normality ($P < 0.05$). Data were confirmed to follow a normal distribution and an unpaired t-test was used for comparisons between the MS and healthy control groups. Bonferroni adjustment was used for multiple comparisons; type I error was maintained at 0.05 and a $P < 0.05$ was considered significant. To determine the effect of ON history

TABLE 1. Clinical and Demographic Characteristics for Healthy Controls and Patients With MS

	Controls, $n = 25$	MS Patients, $n = 25$	P
Age	35.1 ± 9.1	34.4 ± 8.9	NS
MS duration, y	–	7.2 ± 5.5	–
Sex, male:female	10:15	7:18	–
No. of relapses	–	2.6 ± 1.9	–
EDSS	–	2.3 ± 2.1	–
MSSS	–	3.3 ± 2.9	
ON (R/L/R&L)	–	12/25 (9/1/2)	–
HbA1c, mmol/mol	35.9 ± 2.8	31.5 ± 3.1	NS
Total cholesterol, mM	4.8 ± 0.9	4.5 ± 0.4	NS
HDL cholesterol, mM	1.5 ± 0.4	1.2 ± 0.2	NS
LDL cholesterol, mM	2.7 ± 0.7	1.8 ± 0.6	NS
IgG	Normal	Normal	–
ANA	Negative	Negative	–

R, ON right eye; L, ON left eye; R&L, ON right and left eye.

and MS stage, patients with MS were divided into two subgroups: MS with ON (MS-ON) and MS without ON (MS-NON) and RRMS and SPMS. The Pearson correlation coefficient was used to estimate the strength of the relationship between clinical disability (EDSS, MSSS) and CCM and OCT parameters. A multiple linear regression model was used to estimate the effects of age, MS duration, and EDSS on CCM-generated parameters and global RNFL and a logistic regression model to estimate the effects of previous ON history and stage of MS. Based on the variability seen in our previously published studies, we estimated by means of a paired *t*-test that a sample of 25 patients with MS and 25 healthy controls will give us 90%

power to demonstrate a meaningful difference in CCM measures of axonal loss.

RESULTS

Demographic and Clinical Status

No healthy control or MS eyes were excluded from analysis of the results. There was no significant difference in age between control subjects and patients with MS. Patients with MS had a disease duration of 7.2 ± 5.5 years, based on the date of diagnosis, an EDSS of 2.3 ± 2.1, and MSSS of 3.3 ± 2.9 (Table 1). The male-to-female ratio was 7:18 for the MS patient group and 10:15 for the healthy control group. Twelve patients with MS had at least one previous episode of ON. There was no significant difference in any of the demographic or clinical parameters between patients with and without ON. Sixteen patients with MS had RRMS and nine had SPMS. Patients with SPMS compared with RRMS were older (39.11 ± 9.56 vs. 31.69 ± 9.54 years, $P = 0.05$), had longer duration of MS (11.75 ± 6.03 years vs. 4.63 ± 3.12 years, $P = 0.003$), significantly more relapses (3.9 ± 1.45 vs. 1.8 ± 1.64, $P = 0.001$), higher EDSS (4.11 ± 1.62 vs. 1.31 ± 1.71, $P = 0.001$), and MSSS (5.01 ± 2.47 vs. 2.25 ± 2.72, $P = 0.01$).

Corneal Confocal Microscopy

In patients with MS, there was a significant reduction in CNFD (26.6 ± 9.04 vs. 38.11 ± 6.25 fibers/mm², $P < 0.0001$), CNFL (18.19 ± 6.06 vs. 27.96 ± 5.53 mm/mm², $P < 0.0001$), and CNBD (56.08 ± 29.46 vs. 94.81 ± 34.01 branches/mm², $P = 0.0003$) (Fig. A, B, C; Table 2) compared with controls. There was no significant difference for any CCM parameter between
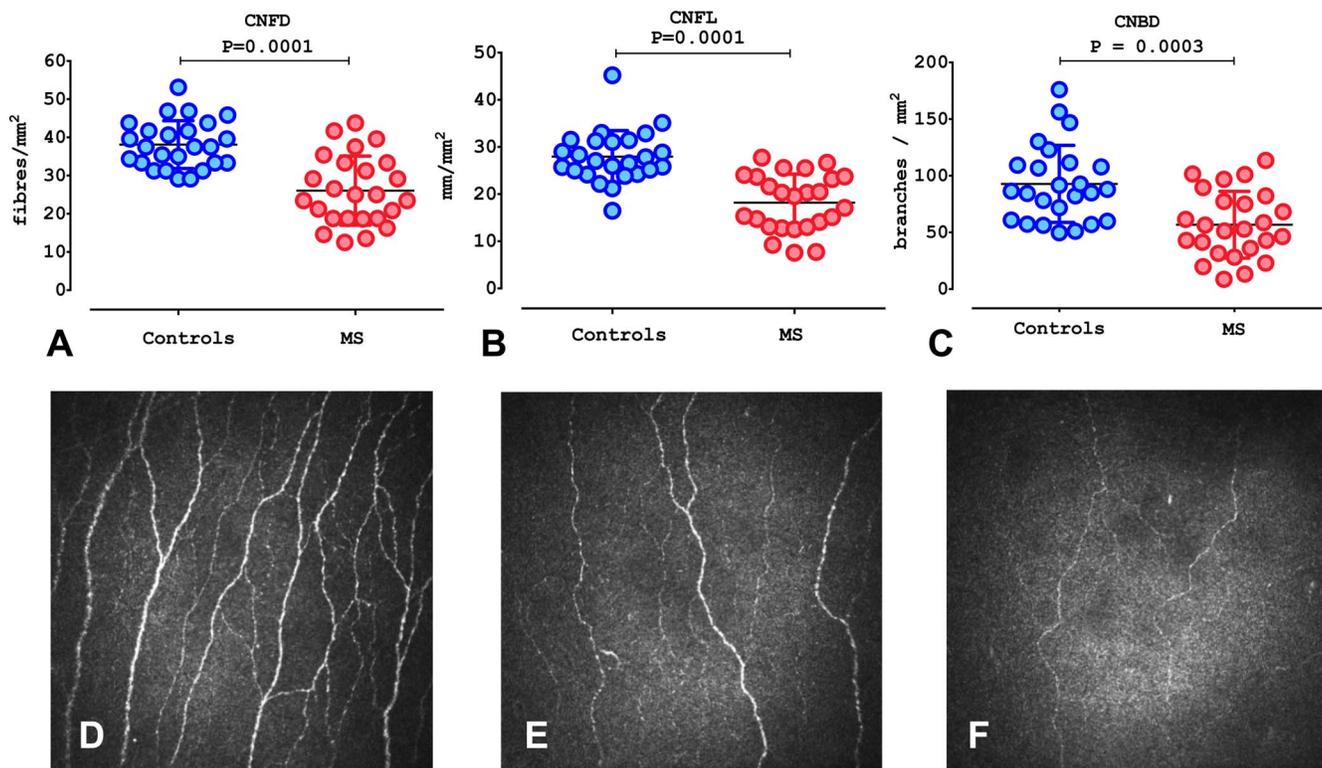
FIGURE. Graphs indicating mean ± range (1%–99%) for CNFD (**A**), CNFL (**B**), and CNBD (**C**) in controls (*blue*) compared with MS patients (*red*). CCM images correspond to a healthy control patient (**D**); an MS patient with EDSS = 0, disease duration of 2 years, and history of previous ON (**E**); and an MS patient with EDSS = 6, disease duration of 4 years, and history of previous ON (**F**).

TABLE 2. Ophthalmic Results for Healthy Controls and Patients With MS

	Controls	MS Patients	P
CNFD, fibers/mm²	38.1 ± 5.0	26.7 ± 8.5	0.0001
CNFL, mm/mm²	27.9 ± 5.5	18.2 ± 6.1	0.0001
CNBD, branches/mm²	91.9 ± 34.1	56.8 ± 29.5	0.0003
RNFL G, µm	98.43 ± 7.68	88.66 ± 15.66	0.01
RNFL T, µm	73.34 ± 12.85	58.96 ± 12.79	0.0004
RNFL TS, µm	138.57 ± 11.28	124.96 ± 23.85	0.01
RNFL TI, µm	139.91 ± 9.81	124.62 ± 22.38	0.004
RNFL N, µm	72.0 ± 13.57	67.21 ± 15.39	NS
RNFL NS, µm	104.51 ± 15.82	104.11 ± 21.54	NS
RNFL NI, µm	109.29 ± 23.61	98.17 ± 28.64	NS

G, global; T, temporal; TS, Temporal superior; TI, temporal inferior; N, nasal; NS, nasal superior; NI, nasal inferior.

patients with and without ON or RRMS and SPMS. There was no significant effect of age, MS duration, EDSS, ON history, and MS stage on CNFD and CNFL. There was a significant effect of EDSS on CNBD ($P = 0.01$) with no effect of age, MS duration, ON history, and MS stage.

Optical Coherence Tomography

In patients with MS, there was a significant reduction in global (88.66 ± 15.66 vs. 98.43 ± 7.68 µm, $P = 0.01$), temporal (58.96 ± 12.79 vs. 73.34 ± 12.85 µm, $P = 0.0004$), temporal superior (124.96 ± 23.85 vs. 138.57 ± 11.28 µm, $P = 0.01$), and temporal inferior (124.62 ± 22.38 vs. 139.91 ± 9.81 µm, $P = 0.004$) RNFL thickness compared with healthy controls (Table 2). There was no difference in RNFL in patients with MS-ON compared with MS-NON. Patients with SPMS compared with RRMS had significantly lower global (78.77 ± 19.34 vs. 93.6 ± 11.09 µm, $P = 0.02$), temporal superior (108.76 ± 26.39 vs. 134.07 ± 17.1 µm, $P = 0.007$), temporal inferior (112.5 ± 27.77 vs. 131.44 ± 15.89 µm, $P = 0.04$), nasal (56.33 ± 14.96 vs. 73.32 ± 12.19 µm, $P = 0.005$), and nasal superior (92.11 ± 16.83 vs. 110.86 ± 21.37 µm, $P = 0.03$) RNFL thickness. There was a significant effect of MS duration ($P = 0.001$) and MS stage ($P = 0.04$) on global RNFL thickness with no effect of age, EDSS, and ON history.

Clinical Disability and Axonal Loss

EDSS showed a significant inverse correlation with CNBD ($r = -0.45$, $P = 0.02$), nasal ($r = -0.56$, $P = 0.004$), nasal superior ($r = -0.46$, $P = 0.01$). and nasal inferior ($r = -0.50$, $P = 0.01$) RNFL thickness. MSSS showed a significant inverse correlation with CNBD ($r = -0.5$, $P = 0.01$) and nasal inferior RNFL thickness ($r = -0.39$, $P = 0.05$). There was no significant relationship between any of the CCM parameters and RNFL thickness.

DISCUSSION

In the present study, we demonstrate a reduction in CNFD, and branch density and length in patients with mild MS-related disability. This reduction appears to be independent of age, ON history, MS duration and stage, and RNFL loss. However, there was a significant effect of EDSS, a well-established clinical measure of MS severity on CNBD. These findings agree with findings in another recent report by Mikolajczak and colleagues,[26] which showed that reduction in corneal nerve fiber length was associated with clinical severity of MS. We also show a significant reduction in RNFL thickness between patients with MS and healthy controls, which was more marked in patients with SPMS and was associated with MS duration and severity, which is consistent with earlier studies.[28,29]

Axonal loss occurring in MS[4] is partially independent of primary demyelination,[30] and is predictive of irreversible neurological disability.[31] A major challenge is how to monitor axonal loss in patients with MS. Estimation of RNFL thickness by OCT in previous studies[7,8] has shown promise as an imaging biomarker of axonal loss and correlates with visual function, clinical disability, and magnetic resonance imaging indices of demyelination. However, ON, a recurrent inflammatory event in MS, can cause an initial increase in RNFL followed by a reduction.[32] The cornea receives sensory innervation from the ophthalmic branch of the trigeminal ganglion, which in turn receives its input from the brainstem nuclei. These nerves penetrate the cornea at the posterior stroma where they lose their myelin sheath, run forward, and terminate in the anterior cornea as a dense network (19,000–44,000 axons within 90 mm²) of unmyelinated axons termed the subbasal nerve plexus.[33] CCM is an ophthalmic imaging device that has emerged as a surrogate endpoint of axonal loss in diabetic[16] and a range of peripheral and recently central neurodegenerative diseases.[13-15,17,18,22-25,34,35] Furthermore, CCM with the addition of wide field montaging capabilities can be used to track nerve migration overtime, a normal physiological process that may be reduced significantly in the presence of axonal degeneration.[36] We also have shown that CCM detects early nerve regeneration in trials of the nonerythropoietic peptide ARA290 in diabetic[20] and sarcoid neuropathy[21] and following kidney and pancreas transplantation in patients with diabetes.[19]

Our findings in this study suggest that corneal subbasal innervation may be a useful biomarker for the detection of neuroaxonal injury in MS. We hypothesize that the lack of difference in CNFD, CNBD, and CNFL between the RRMS and SPMS groups and with MS duration indicates that subclinical axonal loss may occur early before the development of significant neurological disability. Reduced corneal nerve density was present regardless of ON history and was not associated with RNFL thickness. This suggests that quantification of corneal nerve density may act as a more objective measure of axonal loss in MS independent of inflammation and transsynaptic retrograde axonal degeneration, which can affect RNFL. This is a pilot study of a small cohort of patients with mild MS and therefore has limitations in relation to the interpretation of the utility of CCM in MS. However, reassuringly, it recapitulates the findings of two recent studies.[26,37] Therefore, these two small but independent studies emphasize the need for a larger prospective study to confirm these observations and to assess the diagnostic and prognostic ability of CCM, especially in relation to clinical disability and brain imaging in patients with different subtypes and a wider range of severity of MS.

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