In Vivo Corneal Confocal Microscopy in Multiple Sclerosis: Can it Differentiate Disease Relapse in Multiple Sclerosis?

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• PURPOSE: This study aims to investigate the role of in vivo corneal confocal microscopy (IVCCM) in the detection of corneal inflammatory activity and subbasal nerve alterations in patients with multiple sclerosis (MS) and to further determine whether IVCCM can be used to detect (acute) disease relapse.

• DESIGN: Prospective cross-sectional study, with a subgroup follow-up.

• METHODS: This single-center study included 58 patients with MS (MS-Relapse group [n = 27] and MS-Remission group [n = 31]), and 30 age- and sex-matched healthy control subjects. Patients with a history of optic neuritis or trigeminal symptoms were excluded. Corneal nerve fiber density (CNFD), corneal nerve branch density (CNBD), corneal nerve fiber length (CNFL), and dendritic cell (DC) density were evaluated in all patients with MS and control subjects by IVCCM. Patients in the MS-Relapse group who were in remission for ≥6 months after the MS incident underwent a repeat IVCCM.

• RESULTS: No statistical difference was observed between the MS-Relapse and MS-Remission groups regarding age, sex, MS duration, and the number of relapses ($P > .05$). Compared with healthy control subjects, all subbasal nerve parameters were significantly lower (CNFD: $P < .001$, CNFL: $P < .001$, CNBD: $P < .001$), and the DC density was significantly higher ($P = .023$) in patients with MS. However, no significant difference was observed between MS-Relapse and MS-Remission groups in terms of CNFD (mean [SE] difference −2.05 [1.69] fibers/mm^2 [95% confidence interval {CI} −1.32 to 5.43]; $P < .227$), CNFL (mean [SE] difference −1.10 [0.83] mm/mm^2 [95% CI −0.56 to 2.75]; $P < .190$), CNBD (mean [SE] difference −3.91 [2.48] branches/mm^2 [95% CI −1.05 to 8.87]; $P < .120$), and DC density (median [IQR], 59.38 [43.75-85.0] vs 75.0 [31.25-128.75]; $P = .596$). The repeat IVCCM in relapse patients (n = 16 [59.3%]) showed a significant increase in CNFD ($P = .036$) and CNBD ($P = .018$), but no change was observed in CNFL ($P = .075$) and DC density ($P = .469$).

• CONCLUSION: Although increased inflammation and neurodegeneration can be demonstrated in patients with MS compared with healthy control subjects, a single time point evaluation of IVCCM does not seem to be sufficient to confirm the occurrence of relapse in patients with MS. However, IVCCM holds promise for demonstrating early neuroregeneration in patients with MS. (Am J Ophthalmol 2023;250: 138–148. © 2023 Elsevier Inc. All rights reserved.)

Multiple sclerosis (MS) is a chronic persistent inflammatory, demyelinating, and neurodegenerative disease of the central nervous system (CNS), and is the most frequent nontraumatic disabling disease affecting young adults.[1,2] Relapsing/remitting MS (RRMS) is the most common form, seen in 80% of patients with MS, and is characterized by periods of exacerbation followed by substantial remission.[3] The underlying pathophysiology of relapse is increased inflammation and demyelination of the CNS, resulting in a monophasic clinical episode with patient-reported symptoms and objective findings typical of MS for a duration of ≥24 hours.[4,5]

The diagnosis of MS is made according to the revised 2017 McDonald criteria by the clinical evidence and the additional data gathered with magnetic resonance imaging (MRI) and cerebrospinal fluid.[4] However, the diagnosis of a new relapse is mainly based on clinical evaluation because MRI has low specificity with high false positivity in differentiating the ongoing inflammation and the clinical relapse.[6,7] Yet symptoms that patients report as an MS relapse can also be misleading, and MS clinicians often

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Abbreviations: IVCCM, in-vivo corneal confocal microscopy; MS, multiple sclerosis; RRMS, relapsing/remitting multiple sclerosis; CNS, central nervous system; MRI, magnetic resonance imaging; SNP, subbasal nerve plexus; DC, dendritic cell; EDSS, Kurtzke expanded disability status score; CNFD, corneal nerve fiber density; CNBD, corneal nerve branch density; CNFL, corneal nerve fiber length.

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find it challenging to discriminate between an acute relapse and disease progression or fluctuations of the chronic symptoms.[8] Given that untreated relapses can cause permanent disability, the accurate diagnosis and timely treatment of a new relapse are crucial.[9,10] Moreover, identifying relapse is critical because it indicates the efficacy of disease-modifying treatments on disease progression, and the implementation, escalation, withdrawal, or long-term usage of disease-modifying treatments is decided according to the occurrence of relapse.[6,8,11] For these reasons, besides the patient-reported symptoms, developing an objective imaging method that can detect exacerbation of inflammation and neurodegeneration in MS relapses is pivotal for the documentation, follow-up, and timely treatment of patients.

The cornea is the most densely innervated tissue in the human body, and its transparency makes it unique for in vivo neural and immune evaluations at the cellular level.[12] In vivo corneal confocal microscopy (IVCCM) is a rapid, noninvasive technique with good interobserver repeatability allowing high-resolution imaging of corneal layers, including the subbasal nerve plexus (SNP).[13] Therefore, IVCCM is widely used to investigate the presence of neuropathy in many ophthalmologic, systemic, and neurologic diseases.[14] Previous studies have shown that nerve fiber damage could be demonstrated in patients with MS by IVCCM evaluation of corneal SNP.[15–18] In addition, IVCCM enables the in vivo investigation of the immunologic status of the corneal epithelium with its ability to demonstrate the dendritic cells (DCs), which are the main antigen-presenting cells in the cornea, with comparable results to in vitro immunohistochemical staining.[19,20] The close association that has been demonstrated between corneal DCs and nerves suggests an immune–nervous interplay.[21] Recurrent bouts of inflammation are the primary cause of CNS damage in the course of MS. It has been shown that the number of plasmacytoid DCs increases in the cerebrospinal fluid of patients with untreated MS during a relapse compared with patients in remission, and the number of DCs correlates with the parameters of CNS inflammation.[22,23] However, in MS studies with IVCCM, conflicting results have been reported about DC density that appears to be related to different MS disease characteristics of the patients and the differences in the methodology used.[15,16,24,25] For these reasons, IVCCM could be an excellent candidate to show disease activity in MS disease as it allows assessment of corneal nerve damage as well as the immune status of the cornea in vivo. To the best of our knowledge, we are unaware of any published studies in our literature review investigating SNP parameters and the inflammatory status of the cornea by DC analysis during active relapse and remission phases of MS.

The aim of this study was to investigate the inflammatory and neurodegenerative changes in the cornea of patients with MS during acute relapse or remission phases of disease using IVCCM to determine whether IVCCM can be used as a tool to detect disease activity. Furthermore, longitudinal changes in the studied IVCCM parameters were examined in acute relapse patients who attained a remission ≥6 months after initial treatment.

METHODS

This prospective, nonrandomized, controlled, cross-sectional study was conducted between February 2021 and January 2022 at Marmara University School of Medicine Hospital, Istanbul, Turkey. The study was designed with the approval of the Ethics Committee on Human Research of Marmara University 09.2021.240 protocol number and was conducted following the tenets of the Declaration of Helsinki. Written informed consent was obtained from all patients and healthy controls. This study was registered at ClinicalTrials.gov (identifier NCT05218317).

• **SUBJECTS AND EXAMINATION:** Fifty-eight patients with RRMS and 30 healthy control subjects were included in the study. The RRMS patients were divided into 2 subgroups: the MS-Remission group (n = 31) and the MS-Relapse group (n = 27), in whom the administration of intravenous corticosteroids was decided due to a diagnosis of acute relapse. The diagnosis of RRMS and acute relapse was confirmed and decided by a senior neurologist (K.A.) of Marmara University Department of Neurology according to the revised McDonald criteria based on clinical and radiologic findings.[4] The relapse is defined as a monophasic clinical episode with typical objective findings of MS, such as a focal or multifocal inflammatory demyelinating event in the CNS and acutely or subacutely developed patient-reported symptoms in the absence of fever or infection, lasting ≥24 hours.[4] Exclusion criteria were patients <18 years of age, having any other neurologic, metabolic, or endocrine diseases, having a history of optic neuritis and trigeminal symptoms, having previous ocular trauma or surgery, and a history of ophthalmologic diseases, including uveitis, dry eye disease, contact lens, and artificial tears use. The patients who had a relapse <6 months before the study were also excluded.

All patients underwent complete neurologic and ophthalmologic examinations. The patients diagnosed with new relapses were referred to the ophthalmology department before the administration of intravenous steroids. Age, sex, MS duration, the number of total MS relapses, the site and laterality affected by the last relapse, and the type of relapses were recorded for each patient. The type of relapses was divided into 2 groups; sensory, and motor or motor plus sensory groups for statistical evaluation. The neurologic status of the patients was evaluated by the Kurtzke Expanded Disability Status Score (EDSS).[26] In addition, the plaques on MRI were assessed and located as juxtacortical, infratentorial, periventricular, and spinal lesions in ac-

cordance with the previous study conducted by Filippi and associates[27] and the lesion counts were recorded. Disease-modifying treatments were divided into 2 categories according to their immunosuppressive effects as follows: first-line treatment: interferon-β, teriflunomide, dimethyl fumarate, and glatiramer acetate; second-line treatment: fingolimod, ocrelizumab, cladribine, natalizumab, and rituximab.[28]

• **IN VIVO CORNEAL CONFOCAL MICROSCOPY:** All study subjects underwent IVCCM (Rostock Cornea Module, Heidelberg Retinal Tomograph III). Before the examination, 2 drops of topical anesthetic containing 0.5% proparacaine hydrochloride (Alcaine) were applied to both eyes. A 0.2% carbomer–containing gel (Viscotears) was used as the coupling agent between the cornea and the applanation cap of IVCCM. For proper positioning of the central cornea, all subjects were asked to fixate a determined fixation point, and the fixation was controlled with the device camera. All scans were performed in approximately 5 min for each eye by a single experienced ophthalmologist (S.A.T.) masked from the subject's disease status.

Five high-quality, centered images were collected and analyzed from all subjects. Data acquired from these images were averaged for each eye. Automated tracing of nerve fibers program (ACCMetrics) was used to analyze corneal nerve fiber density (CNFD, total number of major nerves per mm^2), corneal nerve branch density (CNBD, number of branches emanating from major nerve per mm^2), and corneal nerve fiber length (CNFL, total length of all nerve fibers and branches per mm^2). To quantify the density of DCs, the same nonoverlapping images in the central cornea were analyzed with ImageJ software (National Institutes of Health). All image collection and nerve measurements were performed by the same masked researcher (S.A.T.).

The patients in the MS-Relapse group were followed up for 6 months. IVCCM was repeated in patients who did not have any acute exacerbation within this period, and the parameters mentioned above were re-evaluated.

• **STATISTICAL ANALYSIS:** The sample size of the study was calculated based on the study conducted by Petropoulos and associates.[21] This study showed a statistically significant difference in CNBD values between patients with MS (26.76 $\pm$ 8.5 branches/mm^2) and healthy control subjects (38.1 $\pm$ 5.0 branches/mm^2), with 25 patients for each group. Considering these values and the alpha value set to 0.05 with a power of 0.95, 19 patients were required. In addition, a previous nerve regeneration study[29] showed a statistically significant difference in CNFD values of 15 diabetic patients before (9.25 $\pm$ 1.87 fibers/mm^2) and 6 months after pancreas transplantation (18.04 $\pm$ 10.48 fibers/mm^2). Considering these values and the alpha value set to 0.05 with a power of 0.95, 18 patients were required. Assuming the drop rate of the participants as 30%, the sample size was calculated as a minimum of 26 patients.

SPSS software (version 24; IBM) was used for statistical analysis. Categorical variables were analyzed with the Pearson chi-square test. The normality of the distribution of the variables was confirmed with the Kolmogorov-Smirnov test. The normally distributed variables were given as mean and standard deviation (SD) or mean and 95% confidence intervals (CIs), and the nonnormal variables were presented as the median and interquartile range (IQR) as quartile 1 to quartile 3. For unpaired group analysis, an independent sample *t* test and Mann-Whitney *U* test were used, and for paired group analysis, a paired-sample *t* test and Wilcoxon test were used in accordance with the normality of the distribution. Three-group comparisons were performed with a one-way analysis of variance (ANOVA) test followed by the Tukey test or Kruskal-Wallis followed by a pairwise comparison with Bonferroni adjustment. Pearson and Spearman correlation coefficient tests were used to determine associations between parametric and nonparametric variables, respectively. The arithmetic means of the right and left eyes' SNP and DC parameters were used for statistical analysis for all patients with MS since no statistical difference, and a moderate correlation was observed between eyes (statistical differences between right and left eyes were $P = .144$ for CNFD, $P = .456$ for CNBD, $P = .112$ for CNFL, and $P = .118$ for DCs; the correlation between right and left eyes were $r = 0.446$, $P < .001$ for CNFD, $r = 0.549$, $P < .001$ for CNFL, $r = 0.376$, $P = .004$ for CNBD, and $r = 0.742$, $P < .001$ for DCs). $P < .05$ was considered statistically significant.

RESULTS

The study included 58 patients with RRMS and 30 healthy control subjects. The mean (95% CI) age of the RRMS patients was 37.7 (35.3-40.1) years and of the healthy control subjects was 38.3 (35.1-41.6) years ($P = .74$). The female ratio was 65.5% (38:20) in the MS group and 60% (18:12) in the healthy control group ($P = .61$). There was no significant difference among the 3 groups regarding age ($P = .213$) or sex ($P = .403$). In addition, no statistical difference was observed between the MS-Relapse and MS-Remission groups regarding age, sex, MS duration, and the number of previous relapses. The characteristics of the patients and healthy control subjects are summarized in Table 1.

In patients with RRMS compared with healthy control subjects, CNFD (17.68 [95% CI 15.99-19.37] vs 25.62 [95% CI 22.98-28.26] fibers per mm^2, $P < .001$; respectively), CNFL (11.52, [95% CI 10.69-12.35] vs 15.39 [95% CI 14.18-16.61] mm/mm^2, $P < .001$; respectively), and CNBD (18.40 [95% CI 15.89-20.90] vs 36.46 [95% CI 28.67-44.24] branches per mm^2, $P < .001$; respectively) were observed to be significantly lower. In addition, the DC density was significantly higher in patients with RRMS compared with healthy control subjects (median [IQR] 63.75 [34.4-97.65]

TABLE 1. Demographic and Clinical Characteristics of Patients in the Multiple Sclerosis Remission and Multiple Sclerosis Relapse Groups

	MS-Remission, n = 31	MS-Relapse, n = 27	*P* Value
Age (y), mean ± SD	39.6 ± 8.7	35.5 ± 9.3	.092[a]
Sex, n (%)			.702[b]
Female	21 (67.7)	17 (62.9)	
Male	10 (36.3)	10 (37.1)	
MS duration (months), mean ± SD	98.9 ± 57.2	107.2 ± 91.5	.677[a]
No. of relapses, median (IQR)	3 (2-10)	3 (2-7)	.361[c]
No. of juxtacortical lesions, median (IQR)	1 (0-2)	1 (1-2)	.177[c]
No. of infratentorial lesions, median (IQR)	0 (0-1)	1 (0-2)	.285[c]
No. of periventricular lesions, median (IQR)	3 (2-4)	3 (2-3)	.627[c]
No. of spinal lesions, median (IQR)	2 (1-2)	2 (1-3)	.759[c]
EDSS, mean ± SD	1.37 ± 1.2	1.46 ± 1.4	.795[a]

EDSS = Expanded Disability Status Scale; IQR = interquartile range; MS = multiple sclerosis; SD = standard deviation.
[a] Independent-samples *t* test.
[b] Pearson chi-square test.
[c] Mann-Whitney *U* test.

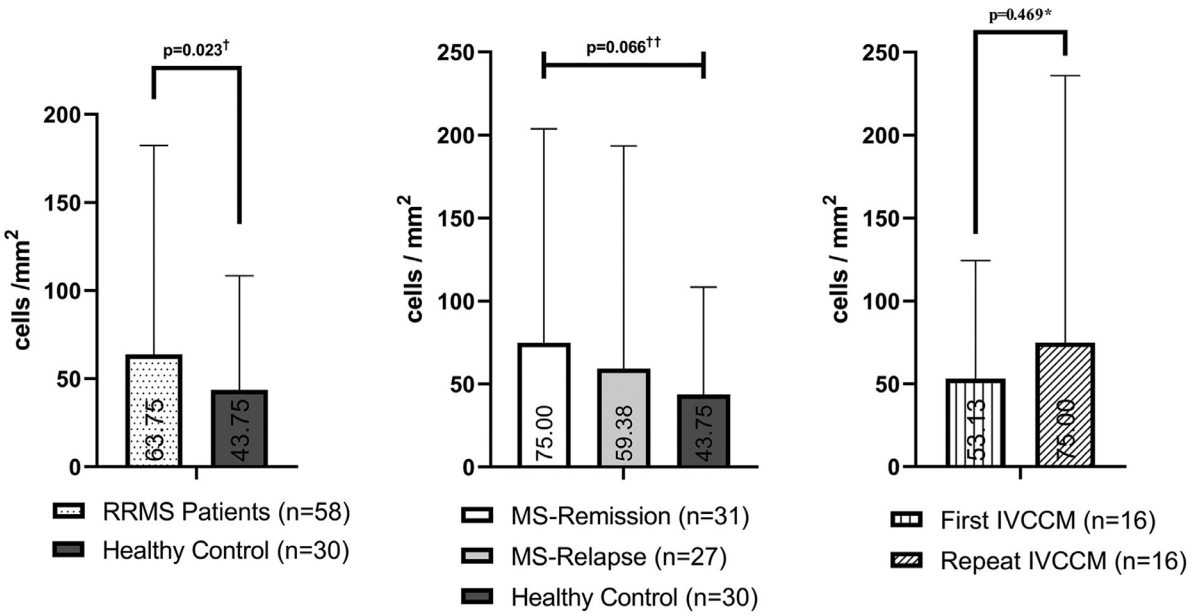

FIGURE 1. Distribution and statistical comparisons of dendritic cell density in different groups and in repeat in vivo corneal confocal microscopy. Median values and 95% confidence intervals are shown. *Wilcoxon test. †Mann-Whitney U test. ††Kruskal-Wallis test. IVCCM = in vivo corneal confocal microscopy; RRMS = relapsing/remitting multiple sclerosis.

vs 43.75 [17.2-78.12] cells/mm^2, $P = .023$, respectively) (Figure 1).

In subgroup analysis, IVCCM parameters were lower in the MS-Relapse group than in the MS-Remission group {CNFD (mean [SE] difference −2.05 [1.69] fibers/mm^2; 95% CI −1.32 to 5.43; $P = .227$), CNFL (mean [SE] difference −1.10 [0.83] mm/mm^2; 95% CI −0.56 to 2.75; $P = .190$), and CNBD (mean [SE] difference −3.91 [2.48] branches/mm^2; 95% CI −1.05 to 8.87; $P = .120$}. However, these differences were not statistically significant (Figure 2). Furthermore, no significant difference was observed in DC density between the MS-Relapse and MS-Remission groups (median [IQR] 59.38 [43.75-85.0] vs 75.0 [31.25-128.75], $P = .596$, respectively). When the MS-Relapse patients

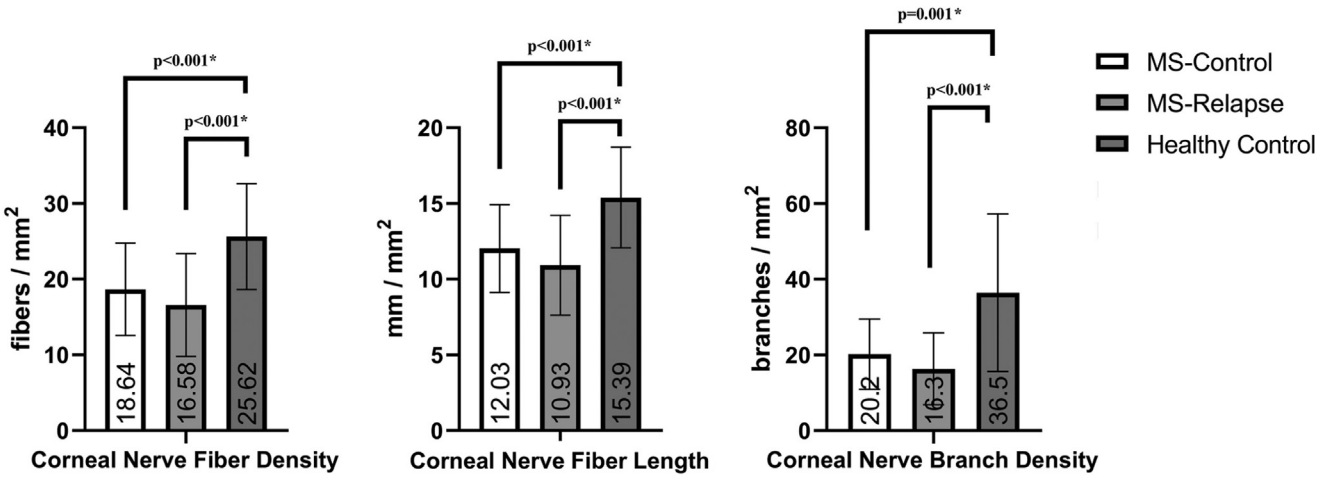

FIGURE 2. Distribution of subbasal nerve layer nerve parameters and statistical comparisons of the 3 groups. Mean values and standard deviations are shown. *One-way analysis of variance. MS = multiple sclerosis.

were grouped regarding the sensory (n = 17) and motor or motor plus sensory (n = 10) symptoms, no difference was observed in IVCCM parameters (CNFD: $P = .401$; CNFL: $P = .103$; CNBD: $P = .136$; DC density: $P = .209$).

A repeat IVCCM was performed in 16 (59.3%) patients in the MS-Relapse group who did not have a relapse for 6 months. In the second IVCCM, a significant increase was observed in CNFD (mean [SE] difference 5.26 [2.78] fibers/mm^2; 95% CI 0.40-10.11; $P = .036$) and CNBD (mean [SE] difference 7.28 [2.73] branches/mm^2; 95% CI 1.46-13.10; $P = .018$) parameters compared with the first IVCCM; however, there were no differences in CNFL (mean [SE] difference 1.89 [0.99] mm/mm^2; 95% CI 0.21-4.01; $P = .075$) and DC density (median [IQR] 75.00 [27.34-126.56] vs 53.13 [32.03.65-77.34], $P = .469$, respectively) (Figures 1, 3, and 4).

Regarding the line of treatment among patients with RRMS, 28 patients (48.3%) were using first-line treatment: 10 patients (17.2%) teriflunomide, 6 patients (10.3%) interferon-β, 6 patients (10.3%) dimethyl fumarate, and 6 patients (10.3%) glatiramer acetate; 27 patients (46.6%) were using second-line treatment: 14 patients (24.1%) fingolimod, 6 patients (10.3%) ocrelizumab, 4 patients (6.8%) natalizumab, 2 patients (3.4%) rituximab, and 1 patient (1.7%) cladribine. Three patients (5.2%) did not use any disease-modifying treatment. No significant difference was found in the mean values of CNFD ($P = .613$), CNBD ($P = .629$), CNFL ($P = .135$), or DC density ($P = .866$) in patients receiving first-line or second-line immunosuppressive treatments. Moreover, no correlation was observed between CNFD, CNBD, CNFL, or DC density parameters and EDSS or the location and number of lesions on MRI. EDSS only correlated with the number of relapses ($r = 0.429$, $P = .001$) and the number of infratentorial lesions ($r = 0.487$, $P < .001$).

DISCUSSION

This study investigated whether IVCCM assessment of corneal nerve fiber degeneration and dendritic cell density at SNP can be a useful tool in diagnosing acute relapse in patients with MS. Three main results are as follows: first, subbasal corneal nerve loss and increased inflammatory cells in patients with MS compared with healthy control subjects were demonstrated by IVCCM. Second, assessment of these morphologic changes at a single time point was not helpful to differentiate relapse and remission in RMMS. Finally, the longitudinal follow-up of relapse patients revealed significant neuroregeneration in SNP parameters of CNFD and CNBD.

Compatible with the results of previous studies,[15–18] this study showed that all SNP parameters were significantly lower in patients with RRMS compared with those in healthy control subjects (Table 2). Previously, the impact of having a history of optic nerve[20,21] or trigeminal nerve involvement[19] as a confounding variable on corneal nerve impairment in patients with MS was also studied. Bitirgen and associates[16] and Petropoulos and associates[17] subgrouped patients with MS with regard to having a history of optic neuritis; however, no significant differences were found in SNP parameters between the patients with and without a history of optic neuritis in both studies. Mikolajczak and associates,[15] on the other hand, investigated the effect of having a history of mild trigeminal symptoms on the corneal nerve density in their small MS cohort (n = 11/25) without a definitive diagnosis of trigeminal neuralgia. Similarly, they did not observe an association between neural degeneration and a previous history of trigeminal symptoms. Nevertheless, patients having a history of

TABLE 2. In Vivo Corneal Confocal Microscopy Findings and Demographic Data of the Published Literature in Patients With Multiple Sclerosis

	Mikolajczak and Associates,[15] n = 26 RRMS Patients	Bitirgen and Associates,[16] n = 57 RRMS Patients	Petropoulos and Associates,[17] n = 16 RRMS, 9 SPMS Patients	Testa and Associates,[24a] n = 14 RRMS, 5 PPMS, 4 SPMS Patients	Petropoulos and Associates,[18a] n = 9 CIS, 20 RRMS, 22 SPMS Patients	Khan and Associates,[25a] n = 9 CIS, 43 RRMS, 22 SPMS Patients	Dericioğlu and Associates,[b] n = 58 RRMS Patients
Age (y), mean ± SD	42.8 ± 9.5	35.4 ±8.9	34.3 ± 8.9	47.9 ± 7.2	34.03 ± 8.20	34.77 ± 8.80	37.7 ± 9.2
MS duration (y), mean ± SD	10.1 ± 5.3	7.5 ± 4.6	7.2 ± 5.5	17.3 ± 10.2	8.15 ± 3.71	7.47 ± 3.43	8.6 ± 6.2
No. of relapses	—	—	Mean ± SD 2.6 ± 1.9	—	Mean ± SD 1.70 ± 1.42	Mean ± SD 1.26 ± 1.00	Median (IQR) 3 (2-7.50)
EDSS	Median (range) 2.5 (1-6.5)	Mean ± SD 2.8 ± 1.2	Mean ± SD 2.3 ± 2.1	Median (IQR) 3 (2.5)	Mean ± SD 0.88 ± 0.98	Mean ± SD 0.93 ± 1.26	Mean ± SD 1.4 ± 1.3
CNFD (fibers/mm^2), mean ± SD	16,531.7 ± 4426.6[c]	26.7 ± 10.2[d]	26.7 ± 8.5[d]	—	19.48 ± 6.15[e]	—	17.7 ± 6.4[e]
CNBD (branches/mm^2), mean ± SD	—	37.1 ± 20.3[d]	56.8 ± 29.5[d]	—	—	—	18.4 ± 9.5[e]
CNFL (mm/mm^2), mean ± SD	—	16.1 ± 4.1[d]	18.2 ± 6.1[d]	—	12.14 ± 2.85[e]	—	11.5 ± 3.2[e]
DC density (cells/mm^2)	Mean ± SD[f] 28.6 ± 24.5	Median (IQR)[g] 27.7 (12.4–66.8)	—	Mean ± SD[g] 33.06 ± 19.03	—	Mean ± SD[d] 58.51 ± 53.18	Median (IQR)[f] 63.8 (34.4 – 97.7)
Correlations	T25FW ∝ CNFD PASAT ∝ CNFD	EDSS ∝ 1/CNFD MSSS ∝ 1/CNFD	EDSS ∝ 1/CNBD	DC density ∝ ongoing DMTs	EDSS ∝ 1/CNFD EDSS ∝ 1/CNFL	EDSS ∝ relapses SDMT ∝ 1/immature DC and 1/total DC	EDSS ∝ relapses EDSS ∝ infratentorial lesions

Note: Studies are sorted by publication date from left to right.

CNBD = corneal nerve branch density; CNFD = corneal nerve fiber density; CNFL = corneal nerve fiber length; CIS = clinically isolated syndrome; DC = dendritic cell; DMT = disease modifying treatment; EDSS = expanded disability status scale; IQR = interquartile range; MS = multiple sclerosis; MSSS = multiple sclerosis severity scores; PASAT = paced auditory serial addition test; PPMS = primer progressive multiple sclerosis; RRMS = relapsing/remitting multiple sclerosis; SD = standard deviation; SDMT = symbol digit modality test; SPMS = seconder progressive multiple sclerosis; T25FW = timed 25-foot walk test; ∝ = direct correlation; ∝1/x = inverse correlation.

[a] Data given for only patients with RRMS.

[b] Present study.

[c] Analyzed with Neuron J software.

[d] Analyzed with CCMetrics.

[e] Analyzed with ACCMetrics.

[f] ImageJ Cell Counter plug-in.

[g] Manually counted.

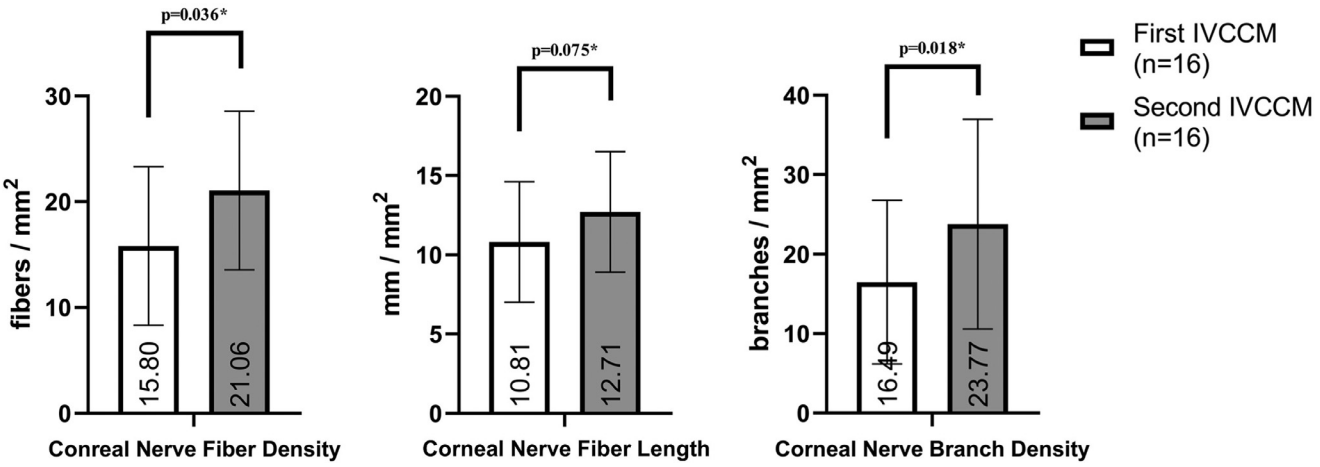

FIGURE 3. Distribution and statistical comparison of subbasal nerve parameters in relapse patients evaluated with the repeat in vivo corneal confocal microscopy. Mean values and standard deviation plots were given, median values were given for dendritic cell. *Paired-samples t test. IVCCM = in vivo corneal confocal microscopy.

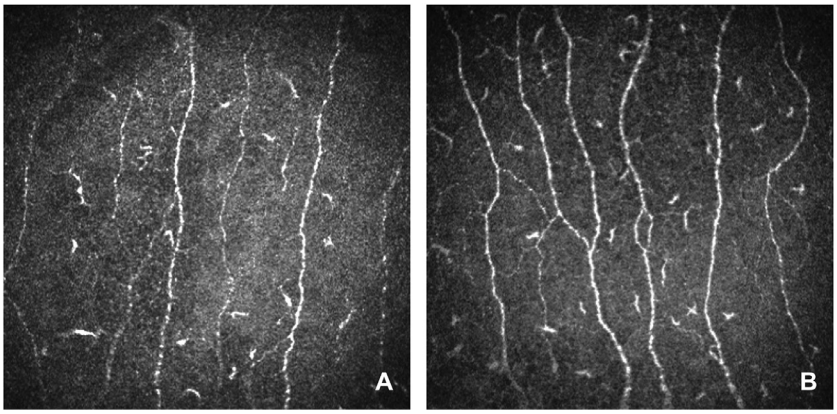

FIGURE 4. In vivo corneal confocal microscopy of a patient with relapsed multiple sclerosis. A. In vivo corneal confocal microscopy of the patient in relapse phase. B. Six months after the relapse phase, improvement is seen in the subbasal nerve plexus.

trigeminal or optic nerve involvement were excluded from this study to ensure a homogenous study group in an attempt to minimize the impact of potential effectors. However, due to a high likelihood of nasociliary nerve involvement, future studies evaluating IVCCM findings in patients with active relapse presenting with trigeminal symptoms may provide valuable information.

DCs are classified into different subtypes by the molecules that they express as conventional, plasmacytoid, Langerhans, and monocyte-derived DCs.[30] Conventional, plasmacytoid, and Langerhans DCs play an essential role in immune surveillance and stimulation of the antigen-specific immune response, as well as in the innate immunity of the cornea.[31] A growing number of studies report that corneal DCs evaluated by IVCCM are increased in systemic inflammatory and neuropathic diseases.[32–36] DCs also play a crucial role in the immunopathogenesis of MS.[37] It has been shown that patients with MS have an increased number of plasmacytoid DCs in the cerebrospinal fluid compared with noninflammatory neurologic diseases.[23] Beyond the immunologic classification, DCs can be found in 2 different functional states as mature or immature which are thought to represent the active and inactive states, respectively.[38]

Morphologically, large cells bearing long processes and smaller cells lacking cell dendrites are classified as mature and immature DCs, respectively.[39] Central corneal DCs are predominantly major histocompatibility complex class-II negative immature cells that upregulate the expression of inhibitory molecules, while peripheral corneal DCs comprehend both immature and mature (major histocompatibility complex II+) phenotypes which can induce specific CD8+ and CD4+ T cell responses.[40] In recent years,

researchers have started to evaluate these 2 phenotypical types of DCs separately in IVCCM studies.[41]

In the current study, the total DC density analysis without maturity discrimination was used, and DC density was found to be higher in patients with RRMS compared with healthy control subjects, supporting the role of chronic inflammation in the pathogenesis of the disease. However, contradictory results have been reported in the literature regarding the density of DCs in the cornea of patients with MS. Bitirgen and associates[16] reported a higher DC density in patients with MS with a history of optic neuritis compared with healthy control subjects; however, they did not observe a significant difference in patients with MS without optic neuritis. Mikolajczak and associates[15] found no significant difference from healthy control subjects in their cohort of 26 patients with RRMS. Khan and associates[25] investigated the density of mature and immature DCs in different MS types, and while reporting an increase in DC density (total and immature immune cells) in patients with RRMS, similar to our study, no significant difference was found in mature DCs. On the contrary, a recent study by Testa and associates[24] found a lower DC density in patients with MS than healthy subjects.[24] They attributed this finding to their study population's longer disease duration (16.6 ± 10.5 years), the heterogeneity of MS subtypes (progressive MS comprising 39% of their MS cohort), and the effect of ongoing disease-modifying treatments.[24] Their findings may also support the natural course of MS because longer disease duration and progressive disease type are both associated with reduced inflammation.[5] In addition, it is noteworthy that the DC density in cerebrospinal fluid has been shown to be associated with disease duration as the number of DC was found to be highest in early MS, and decreased with time.[23] Other possible reasons for these variations include differences in patient demographic characteristics and methodologic approaches used in different studies (Table 2). More numerous DCs are found in the peripheral cornea, and DCs were observed to migrate rapidly toward the central cornea in cases of increased inflammation.[42] Bitirgen and associates[16] and Khan and associates[25] used central cornea for DC evaluation, similar to our study; however, Mikolajczak and associates[15] used composite wide-field images (3.2 mm × 3.2 mm), and Testa and associates[24] used the mean value from the central and 4 peripheral sectors. Moreover, sunlight has immunomodulatory effects on MS severity,[43] and the corneal epithelium and the Bowman layer have high ultraviolet absorption properties.[44] Therefore, the nonidentical exposure to sunlight among patients in studies conducted in different geographic areas might be another confounding factor for corneal DC density measurements. As a result, an increase in the immune cells associated with subbasal corneal nerve damage found in the current study supports a potential relation between the corneal immune and nervous systems as suggested in previous studies.[21,45] Novel immunotherapeutic approaches are being developed to regulate the differentiation and development of DCs, and to use their tolerogenic capacity to limit disease activity and progression in MS. In this respect, DC analysis with IVCCM may hold promise for monitoring treatment efficacy of tolerogenic therapies in the future.[46]

Relapses are the defining determinants of the phenotype of RRMS. In addition to determining disease activation and prognosis, the decision to choose and adjust immunosuppressant and immunomodulatory treatments is made according to the frequency of relapses in patients with RRMS. It has been suggested that to prevent MS progression, new imaging approaches are required to monitor disease activity for this challenging diagnosis.[47] The critical question primarily addressed in this study is whether IVCCM can be used to detect disease activation in RRMS by visualizing the neurodegenerative and inflammatory changes in the cornea. To the best of our knowledge, this is the first study in the literature evaluating the acute relapse in RRMS by IVCCM. In this study, SNP parameters and DC density were lower in relapsed patients with MS compared with those in remission; however, the difference did not reach statistical significance. These findings may indicate a comparable amount of inflammation and neurodegeneration in remission and relapse, which goes in parallel with the natural course of MS. In RRMS, it is known that there is an underlying persistent inflammation and ongoing neurodegeneration.[48] In an MRI study, 7 patients with RRMS were followed up monthly with sequential contrast-enhanced MR imaging. During the study, a total of 51 new enhancing or re-enhancing lesions were observed in 6 of 7 patients on 26 occasions, and only 19% (5/26) of them were associated with a clinical relapse. This could imply an ongoing inflammatory disease activity in clinically silent disease.[49] Another confounding factor is the ongoing immunosuppressive treatment, which may potentially impact inflammatory cell infiltration in the cornea. Therefore, to eliminate the immunosuppressive effect, investigating SNP alterations and corneal immune cells in treatment-naïve patients with RRMS in further studies may better determine the role of IVCCM in detecting relapse in RRMS.

One of the most important findings of the present study is that CNFD and CNBD showed a significant increase in repeat IVCCM in the follow up of relapsed patients with MS who were in remission for ≥6 months after the acute attack. This result can be interpreted as a sign of neural repair at SNP during the remission phase of the disease after an acute attack. The improvement in corneal nerve fiber density has already been demonstrated to occur between 3 and 24 months with treatment in different conditions that affect the corneal nerves.[14] However, significant regeneration of corneal nerves at SNP early after an MS attack has not been shown in the published literature. In a recent study, Bitirgen and associates[50] studied corneal SNP and DC density by IVCCM in 31 patients with RRMS at baseline and after 2 years of follow-up. Con-

trary to the present study population, 29% (9/31) of the patients had a history of relapse during the 2-year follow-up, and nearly half of the patients (48%) had a worsening EDSS in their study cohort. They reported a progressive loss of corneal nerves and an increase in DC density at SNP only in patients with RRMS with worsening neurologic disability, while no significant change was found in patients without progression in EDSS. Moreover, they observed an increase in CNFD, CNBD, and CNFL in 42%, 29%, and 23% of the patients, respectively. It has been shown that myelin repair occurs in acute inflammatory lesions beginning 1 to 2 months after an acute attack in postmortem studies, and remyelination is associated with functional recovery and clinical remittances.[51,52] Therefore, it may be speculated that recovery in corneal innervation early after an acute attack may further deteriorate with subsequent relapses over the long term in patients with RRMS with progression of disease severity. Previous studies have demonstrated a mutual interplay between DCs and SNP nerves in nerve degeneration and repair processes in the cornea.[45] While nerves have a modulatory influence on immune cells via secretion of some neuropeptides, growth factors, and cytokines, recently DCs have also been shown to mediate sensory nerve innervation and regeneration through expression of a major neurotrophic factor: ciliary neurotrophic growth factor.[53] Although not reaching statistical significance, an increasing trend in the density of dendritic cells at the level of SNP in the remission phase after an acute relapse, observed in this study, may have a role in mediating the regeneration of subbasal nerves.

Some limitations should be considered when evaluating our results. The remission patients and healthy control subjects did not undergo repeat IVCCM; therefore, the alterations in these groups could not be assessed and compared with patients with relapses. Although several studies pointed out that the IVCCM has high reproducibility and reliability with the use of automatized methods and adequate image sampling,[54,55] the small field of view (400×400 μm) and its poor ability to collect consecutive images from the exact same area in longitudinal studies are some of the technical challenges. Recently, the whorl-like complex with a distinctive spiraled nerve fiber pattern (inferior whorl), typically located 1 to 2 mm inferior to the corneal apex, has been proposed as a consistent landmark for longitudinal assessment of SNP.[56] However, reduced repeatability of inferior whorl parameters compared with the central cornea has recently been reported by Chiang and associates[57] in chemotherapy-induced neuropathy and healthy eyes. The authors proposed the possible reason for this as the difficulty in identifying the center point of convergence of nerves, which has a highly complex and variable pattern even in healthy eyes, which is further complicated by the loss of nerves in disease states. The fact that DCs were not differentiated as morphologically mature or immature can be considered a limitation in this study. However, many different cutoff values for size, morphologic descriptions, and measurement methods have been used, and no consensus has been reached to make this distinction in the literature.[41] Since these DCs have extensions in XYZ directions, it has been suggested that a volumetric or 3-dimensional IVCCM image analysis will provide more accurate evaluations to reach ideal quantitative and morphologic results.[41]

In addition, immature DCs have centripetally migration capacities, and it has been shown that the migration rate in healthy people is 1.12 ± 0.21 μm/min.[42] Alzahrani and associates[58] showed a significant increase in DC density even after a short time (2 hours) in eyes with contact lens compared with control eyes. The centripetal migration speed of corneal nerves in healthy people is slower than DCs, but it has been shown that SNP has a dynamic structure with dramatic pattern changes in 6 weeks.[59] Al Rashah and associates[60] found this velocity to be 41.1 ± 13.5 μm per week (0.004 μm/min) in healthy eyes. However, it has been shown that this migration rate is significantly reduced in the case of diabetic neuropathy.[61] Nevertheless, the current study does not have sufficient evidence on how DCs and SNP nerves in the central cornea behave after an MS relapse because the IVCCM evaluations were performed only once during the acute phase of relapse, shortly after patients became symptomatic, and at 6 months after remission as a second time. Therefore, it cannot be commented on how relapse affects the migration of DCs and subbasal nerves to the central cornea. For future perspectives, following newly diagnosed patients with MS with frequent IVCCM monitoring can provide critical information for better understanding the in vivo MS pathophysiology and analyzing relapse objectively. In addition, the use of IVCCM for evaluating the course of neural recovery in remission is promising, and needs to be supported by future longitudinal studies in larger cohorts with repeated monitoring.

In conclusion, this study confirms the findings in the literature and shows that IVCCM can demonstrate neurodegeneration and increased inflammation in patients with RRMS. However, because of the chronic inflammatory and neurodegenerative nature of the disease, IVCCM alone may not be sufficient in diagnosing an acute relapse. Moreover, it has been shown that subbasal nerve damage tends to recover after an acute relapse. With the increasing use of technology and artificial intelligence, we think that the ability of IVCCM to demonstrate nerves and inflammatory cells in vivo will gain more importance in the diagnosis and monitoring of treatment efficacy in MS in the future.

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Multiple sclerosis is an inflammatory and neurodegenerative disease characterized by phases of relapse and remission. This study investigated the role of in vivo

corneal confocal microscopy in detecting disease activity and subsequent changes, with its ability to demonstrate corneal nerve degeneration and inflammatory status in vivo. Consequently, this study showed that a single time point corneal confocal evaluation is insufficient to differentiate relapse and remission phases; however, the longitudinal analysis holds promise for demonstrating early neuronal regeneration.

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