Serum MMP-2, MMP-9, TIMP-1 and TIMP-2 levels in multiple sclerosis clinical subtypes and their diagnostic value in the progressive disease course.

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Abstract

In multiple sclerosis (MS), a group of matrix metalloproteinases (MMPs) and their natural tissue inhibitors of metalloproteinases (TIMPs) may play a role in preventing tissue damage. The aim of this study was to determine the correlation between the MMP-9, MMP-2, TIMP-1, and TIMP-2 serum levels and the possible relationship to the type, severity, and progressive course of MS. Eighty-nine patients and 22 healthy volunteers were enrolled in the study. The MS patients were grouped as follows: 35 relapsing-remitting patients with short-disease-duration (RRMS-SDD) (≤5 years), 23 patients with longer-disease-duration (RRMS-LDD) (≥10 years), 21 secondary progressive patients (SPMS), 6 progressive relapsing patients (PRMS) and 4 primary progressive patients (PPMS). Serum levels were analysed by ELISA. Compared with the Control Group, the TIMP-1 serum levels (ng/ml) increased [Group I (RRMS-SDD), 2242.2; Group II (RRMS-LDD), 3108.3; Group III (SPMS), 3108.3; Group IV (PRMS), 3552.3; Group V (PPMS), 2068.6; and Group VI (control group), 1554.9] (p<0.01), and the MMP-9/TIMP-1 ratios decreased [Group I, 0.074; Group II, 0.055; Group III, 0.043; Group IV, 0.028; Group V, 0.116; and Group VI, 0.107] (p<0.05) in all patient groups except the PPMS group. We re-classified the patients to reflect the progressive disease course as non-progressive (all RRMS) and progressive (PPMS, SPMS, and PRMS) groups. Compared with the control group, we again found an increase in TIMP-1 serum levels (ng/ml) [Groups I-II (RRMS=SDD and LDD), 2726.6; Groups III-IV-V (SPMS, PRMS, and PPMS), 3100.9; and Group VI (control group), 1554.9] (p<0.001) and a decrease in the MMP-9/TIMP-1 ratios [Groups I-II, 0.064; Groups III-IV-V, 0.046; and Group VI, 0.107] (p<0.01). In our study, the MMP-2, MMP-9, TIMP-2 serum levels and MMP-2/TIMP-2 ratio were not significantly different between the groups altough reclassification. These results mirror the clinical stability that is related to the disease remission status of our patient sample. Further clinical trials will be necessary to examine potential drug targets to prevent patient attacks involving this dynamic pathophysiologic process of MS.

Keywords: Multiple sclerosis subtypes, Progressive Course, MMP-2, MMP-9, TIMP-1, TIMP-2.

Background and purpose

Multiple sclerosis (MS) is a chronic inflammatory, demyelinating and neurodegenerative disease of the central nervous system (CNS). The pathophysiology of multiple sclerosis is a dynamic process involving concomitant damage and repair mechanisms. It is believed that autoimmune mechanisms, triggered by environmental factors, contribute to MS in genetically susceptible individuals. The pathogenesis of MS is known to be comprised of blood-brain barrier (BBB) degradation and trans-migration of T-lymphocytes into the CNS [1-3].

The entrance of leukocytes into the CNS is dependent on a number of factors, and the expression of matrix metalloproteinases (MMPs) is most likely among these factors [4-6]. Th1 cells have been reported to possess enhanced migration over Th2 cells, and this enhanced migration is associated with higher MMP-2 and MMP-9 expression levels in Th1 cells when relative the migratory capabilities of the pro-inflammatory CD4+ (Cluster of differentiation 4 positive) T cells (Th1), which are involved in the pathogenesis of MS, are compared with regulatory Th2 cells [7]. Migration of leukocytes across the BBB into the CNS depends on a series of events. Selectins and integrins, the ligands that interact with endothelial cell adhesion molecules, chemokines and their receptors, and matrix metalloproteinases (MMPs) enable leukocyte migration into the CNS [8]. The degradation of the subendothelial basal membrane is required during this migration, and the MMPs are responsible for the basal membrane degradation [9].

The integrity of the extracellular matrix is maintained through the balance of the synthesis and proteolysis of its components, and this process is primarily carried out by MMPs and their tissue inhibitors (TIMP). MMPs are considered to be physiological mediators that enable cell migration through various barriers. Astrocytes, oligodendrocytes, microglia, endothelial cells, neurons and lymphocytes provide the main source of MMPs in the CNS [10].

In this study, the serum levels of MMP-2, MMP-9, TIMP-1, TIMP-2 and the ratio of MMP-9/TIMP-1 and MMP-2/TIMP-2 were characterised as biomarkers and correlated with their activity and MS subtype.

Patients and Methods

This study, conducted in the neurology unit of the Ankara Dışkapı Yıldırım Beyazıt Training and Research Hospital between January 2008 and February 2013, consisted of 89 MS patients and 22 healthy control subjects. The inclusion criteria were: age between 15 and 60 years old, confirmed MS according to McDonald's diagnostic criteria (2010 revision), and a remission period of at least two months. The study's exclusion criteria were: MS coexisting with any other systemic or CNS disease and receiving medical treatment for any infectious disease within the two months prior to study enrolment.

The serum of 89 MS patients (30 men and 59 women) and 22 healthy control subjects were examined. The youngest patient was 17, and the oldest patient was 58 years old. Patients with MS were divided into subgroups according to their MS types as RRMS, SPMS, PRMS and PPMS. Patients with RRMS were separated into two groups: patients with a disease duration of 5 years or less [RRMS-Short Disease Duration (RRMS-SDD)] and patients with

a disease duration of 10 years or longer [RRMS-Long Disease Duration (RRMS-LDD)]. The control group consisted of 22 healthy individuals (aged 17 to 48 years old) and gender-matched volunteers (6 men and 16 women), who did not have a previous history of systemic or CNS disease. Subjects with an active infection or who received any medical treatment were excluded from the analyses to avoid any interference from medical treatments and biological variables.

Clinical evaluation of the patients was performed using the Kurtzke Expanded Disability Status Scale (EDSS). Serum samples were obtained from patients, centrifuged and stored at -80°C. Venous blood samples obtained from the 22 healthy control subjects were stored at -80°C immediately after centrifugation. MS patient and control samples were brought to room temperature along with all reagents, and the samples were analysed using an Enzyme Linked-Immuno-Sorbent Assay (ELISA).

The study protocol was approved by the Ethics Committee of the Ankara Dışkapı Yıldırım Beyazıt Training and Research Hospital, and written informed consent was obtained from each participant.

Analysis of the MMP-9, TIMP-1, and TIMP-2 expression levels
The following expression levels were analysed according to the manufacturers' instructions: human MMP-9, TIMP-1, and TIMP-2 ELISA Kits (RayBiotech, Inc., Norcross, GA, USA) ; an MMP-9 ELISA Kit, which is a sandwich ELISA based kit designed specifically to measure MMP-9 in suspended or adherent human cells; a human TIMP-1 sandwich ELISA; a non-isotopic human TIMP-1 colorimetric in vitro assay for tissue culture media or serum; a human TIMP-2 sandwich ELISA based kit; and a non-isotopic in vitro TIMP-2 immunoassay in tissue culture medium, serum, plasma and tissue lysates. Reagents and patient samples were warmed to room temperature. After adding standards and 100 μl of sample into all micro-wells and incubating at room temperature for 2.5 hours, 100 μl of a biotin antibody were added to all wells and incubated at room temperature for 1 hour. After the addition of 100 μl of streptavidin solution, the samples were incubated at room temperature for 45 minutes. 100 μl of TMB One-Step Substrate solution was added, and the samples were incubated at room temperature for 30 minutes. Stop solution (50 μl) was added, and the absorbance values were acquired with an automated ELISA micro-plate reader at a wavelength of 450 nm.

Analysis of MMP-2
The MMP-2 expression levels were analysed according to the following manufacturers' instructions: total MMP-2 (R&D Systems Quantikine® Human/Mouse/Rat MMP-2, MN, USA) and a non-isotopic colorimetric human MMP-2 ELISA kit for tissue culture media, serum, or plasma

(Catalog Number QIA63, Calbiochem, San Diego, USA). The reagents and samples obtained from patients were warmed to room temperature and prepared in polypropylene test tubes. Assay Diluent RD1-74 (100 μl) was added to all wells followed by 50 μl of standard. The microwells were covered with adhesive strips, incubated for 2 hours at room temperature using a horizontal micro-plate shaker, and then washed 4 times by aspiration. After washing, 200 μl of conjugate were added to all microwells, incubated in a shaker for 2 hours, and then washed 4 times by aspiration. Next, 200 μl of substrate solution were added to each well and incubated for 30 minutes in the dark. Finally, 50 μl of stop solution were added to all wells, and within 30 minutes, the absorbance values of the micro-wells were obtained at a wavelength of 450 nm.

Statistics

Data were analysed using the Statistical Package for the Social Sciences (SPSS) 11.5 program for Windows. Descriptive statistics were expressed as follows: for age, mean ± standard deviation; for disease duration, median (minimum-maximum); and for the EDSS, MMP-2, MMP-9, TIMP-1, TIMP-2, MMP-2/TIMP-2 and MMP-9/TIMP-1 levels, median (25-75) percentile. The average age among the groups was evaluated with a One-Way Analysis of Variance and a Tukey post hoc test, and the median expression levels of EDSS, MMP-2, MMP-9, TIMP-1, TIMP-2, MMP-2/TIMP-2 and MMP-9/TIMP-1 for disease duration were analysed with the Kruskal-Wallis test followed by a Conover's multiple comparison post hoc test. The median disease duration and the EDSS of Groups I-II and Groups III-IV-V were compared with

the Mann Whitney U test. To determine whether the gender distribution of the groups was homogeneous, the groups were compared with the Pearson's chi-square test. Statistical significance was set at $p<0.05$.

Results

The patients were separated into the following groups: Group I (RRMS-SDD), Group II (RRMS-LDD), Group III (SPMS), Group IV (PRMS), Group V (PPMS), and Group VI (healthy controls).

There were 35 patients in Group I, 23 patients in Group II, 21 patients in Group III, 6 patients in Group IV, 4 patients in Group V and 22 healthy controls in Group VI.

The age, gender, disease duration and EDSS levels of each group are presented in **Table 1**.

The MMP-2, MMP-9, TIMP-1, TIMP-2 serum levels and the MMP-2/TIMP-2, MMP-9/TIMP-1 ratios of each group are presented in **Table 2**. **Figures 1**, **2**, and **3** show the TIMP-1 serum levels, TIMP-2 serum levels, and MMP9/TIMP-1 ratios of each group, respectively.

No statistically significant differences were found between Groups I and II except for the disease duration (Conover's Multiple Comparison test, $p<0.001$). Statistically significant differences were observed between the EDSS scores of Groups I and II when compared with the other patient groups (Conover's Multiple Comparison test, $p<0.05$).

Table 1. The age, sex, disease duration and EDSS levels of each group.

Groups\Variables	Age (years)	Gender (M/F)	Disease Duration (years)	EDSS
Group I RRMS-SDD (n=35)	32.5 ± 8.6^a	11/24	$3 (1-5)^{a,b,c,d}$	$2 (0-4)^{a,c,d}$
Group II RRMS-LDD (n=23)	37.5 ± 9.3	7/16	$12 (10-18)^b$	$2 (0-4)^{e,f,g}$
Group III SPMS (n=21)	40.3 ± 7.2^a	10/11	$14 (4-28)^a$	$9 (2-24)^c$
Group IV PRMS (n=6)	42.3 ± 6.6	0/6	$9 (2-24)^c$	$6 (4-8)^{c,f}$
Group V PPMS (n=4)	38.0 ± 9.9	2/2	$11 (5-13)^d$	$5 (4-8)^{d,g}$
Group VI Control Group (n=22)	34.7 ± 8.7	6/16		
p-value	0.010†	0,177‡	<0.001¶	<0.001¶

†, One-Way ANOVA; ‡, Pearson's Chi-Square test; ¶, Kruskal Wallis test; a, Group I vs Group III (p<0.05); b, Group I vs Group II (p<0.001); c, Group I vs Group IV (p<0.01); d, Group I vs Group V (p<0.01); e, Group II vs Group III (p<0.001); f, Group II vs Group IV (p<0.001); g, Group II vs Group V (p<0.001).

Table 2. *The MMP-2, MMP-9, TIMP-1, and TIMP-2 serum levels and MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios of each group.*

Groups\Variables	Age (years)	Gender (M/F)	Disease Duration (years)	EDSS
Group I RRMS-SDD (n=35)	32.5 ± 8.6^a	11/24	$3 (1-5)^{a,b,c,d}$	$2 (0-4)^{a,c,d}$
Group II RRMS-LDD (n=23)	37.5 ± 9.3	7/16	$12 (10-18)^b$	$2 (0-4)^{e,f,g}$
Group III SPMS (n=21)	40.3 ± 7.2^a	10/11	$14 (4-28)^a$	$9 (2-24)^c$
Group IV PRMS (n=6)	42.3 ± 6.6	0/6	$9 (2-24)^c$	$6 (4-8)^{c,f}$
Group V PPMS (n=4)	38.0 ± 9.9	2/2	$11 (5-13)^d$	$5 (4-8)^{d,g}$
Group VI Control Group (n=22)	34.7 ± 8.7	6/16		
p-value	0.010†	0,177‡	<0.001¶	<0.001¶

†, Kruskal Wallis test; a, Group I vs Group VI (p<0.05); b, Group II vs Group VI (p<0.001); c, Group III vs Group VI (p<0.001); d, Group IV vs Group VI (p<0.01); e, Group I vs Group III (p<0.001); f, Group III vs Group V (p<0.05); g, Group IV vs Group V (p<0.05); h, Group V vs Group VI (p<0.05); i, Group I vs Group IV (p<0.05).
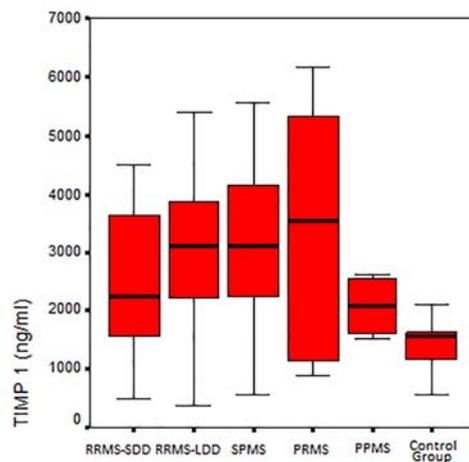

Figure 1. *MS patient group median TIMP-1 serum levels and interquartile ranges. The whiskers above and below the box indicate the maximum and minimum TIMP-1 levels, respectively.*
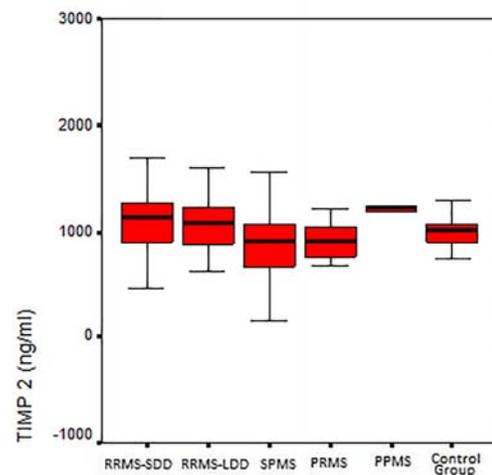

Figure 2. *MS patient group median TIMP-2 serum levels and interquartile ranges. The whiskers above and below the box indicate the maximum and minimum TIMP-2 levels, respectively.*

The average age was 34.5 ± 9.2 in Groups I-II, 40.4 ± 7.3 in Groups III-V and 34.7 ± 8.7 in Group VI. The age differences between Groups I-II and Groups III-V and between Groups III-V and Group VI were statistically significant (p<0.05). The gender distribution (male/female ratio) was 18/40 in Groups I-II, 12/19 in Groups III-V and 6/16 in Group VI, and no statistically significant differences were detected between the groups (p=0.645). The median disease duration was 5 (1 to 18) years in Groups I-II and 12 (2 to 28) years in Groups III-V, and the difference in disease the median disease duration between the groups was statistically significant (p<0.001).

The age, gender, disease duration and EDSS levels of each group, re-classified according to disease progression, are presented in **Table 3**. The median EDSS values were 2 (0 to 4) in Groups I-II and 4 (3 to 9) in Groups III-V, and the difference between the groups was statistically significant (p<0.001).

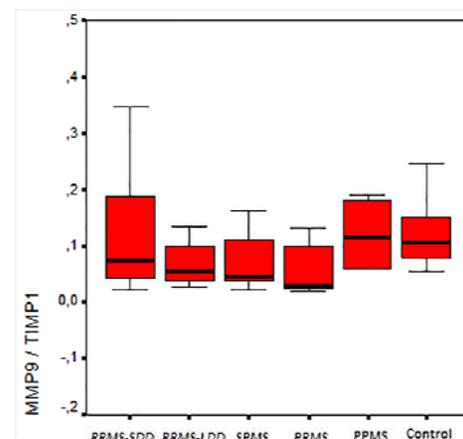***Figure 3.*** *MS patient median MMP9/TIMP-1 ratios and interquartile ranges. The whiskers above and below the box indicate the maximum and minimum MMP9/TIMP-1 ratios, respectively.*

Table 3. *The age, gender, disease duration and EDSS levels of the groups re-classified according to disease progression.*

Groups\Variables	Age (years)	Gender (M/F)	Disease duration (years)	EDSS
Groups I-II (RRMS=SDD + LDD) (n=58)	34.5 ± 9.2^a	18/40	5 (1-18)	2 (0-4)
Groups III-V (SPMS + PRMS + PPMS) (n=31)	$40.4 \pm 7.3^{a,b}$	12/19	12 (2-28)	4 (3-9)
Group VI (Control Group) (n=22)	34.7 ± 8.7^b	6/16		
p-value	0.007†	0.645‡	<0.001¶	<0.001¶

†, One-Way ANOVA; ‡, Pearson's Chi-Square test; ¶, Mann Whitney U test; a, Groups I-II vs Groups III-IV-V (p=0.007); b, Groups III-IV-V vs Group VI (p<0.05).

Table 4. *The MMP-2, MMP-9, TIMP-1, and TIMP-2 serum levels and the MMP-2/TIMP-2 and MMP-9/TIMP-1 ratios of the groups re-classified according to disease progression.*

Groups\Variables	Age (years)	Gender (M/F)	Disease duration (years)	EDSS
Groups I-II (RRMS=SDD + LDD) (n=58)	34.5 ± 9.2^a	18/40	5 (1-18)	2 (0-4)
Groups III-V (SPMS + PRMS + PPMS) (n=31)	$40.4 \pm 7.3^{a,b}$	12/19	12 (2-28)	4 (3-9)
Group VI (Control Group) (n=22)	34.7 ± 8.7^b	6/16		
p-value	0.007†	0.645‡	<0.001¶	<0.001¶

†, Kruskal Wallis test; a, Groups I-II vs Group VI (p<0.01); b, Groups III-IV-V vs Group VI (p<0.001).
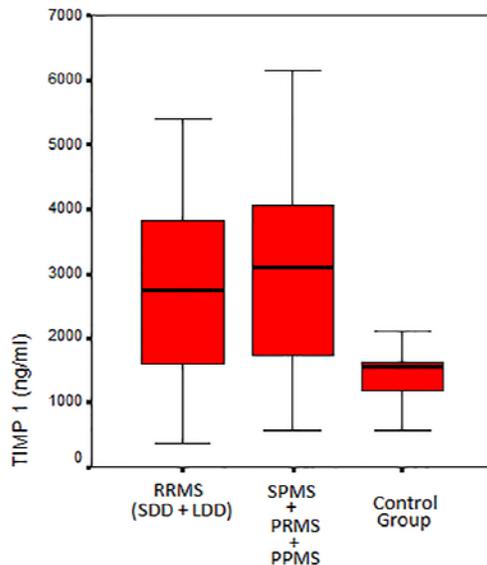

Figure 4. *The median TIMP-1 serum levels and inter-quartile ranges of the re-classified MS patient groups. The groups of MS patients were re-classified according to disease progression. The whiskers above and below the box indicate the maximum and minimum TIMP-1 expression levels, respectively.*
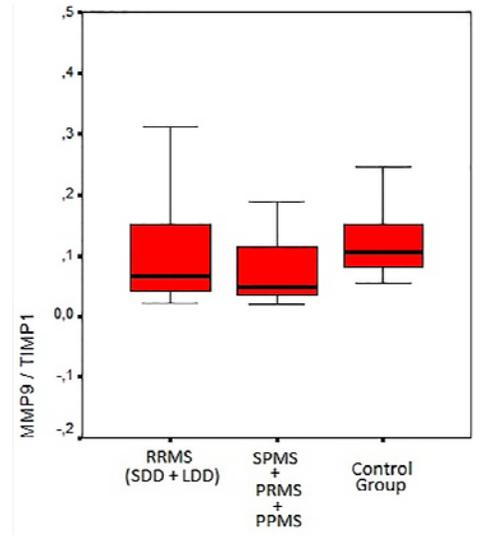
Figure 5. *The median MMP9/TIMP-1 ratios and inter-quartile ranges of the re-classified MS patient groups. The groups of MS patients were re-classified according to disease progression. The whiskers above and below the box indicate the maximum and minimum MMP9/TIMP-1 ratios, respectively.*

The MMP-2, MMP-9, TIMP-1, TIMP-2 serum levels and the MMP-2/TIMP-2, MMP-9/TIMP-1 ratios of each group, re-classified according to disease progression, are shown in **Table 4**. **Figure 4** shows the TIMP-1 serum levels the re-classified groups, and **Figure 5** shows the MMP9/TIMP-1 ratios of the re-classified groups.

Discussion

Reliable biomarkers to evaluate the activity and MS sub-type or to provide information about the clinical prognosis and pathophysiological disease process are still lacking [11-14]. Although MMPs are not the only factors responsible for the pathophysiology of MS, they may be important in evaluating the clinical subtype and disease activity. This study hypothesised that MMP-9 and MMP-2 and their tissue inhibitors TIMP-1 and TIMP-2 are involved in MS pathogenesis and set out to investigate if these factors differ in the clinical disease subtypes and *indisease* progression. The study design was based on previously published work [11].

MMPs have been shown to play a role in active disease exacerbation [12], and MMP-9 expression was observed in acute MS lesions, macrophages, astrocytes, and in white matter perivascular mononuclear cells [15,16]. Various studies of serum and cerebrospinal fluid MMP levels have also been conducted. The majority of these studies have focused on MMP-9, which can easily be detected and measured using gelatin zymography and ELISA from several commercial sources [17]. The increased MMP-9 levels in the cerebrospinal fluid (CSF) of MS patients were associated with BBB damage and gadolinium involvement in contrast-enhanced MRI. Methylprednisolone therapy has been reported to reduce gadolinium involvement as well as the MMP-9 levels in CSF by preventing MMP transcription [18]. However, while increased MMP-9 levels were detected in all patients with RRMS, a ratio of 57% in the CSF was reported in patients with PPMS [19]. Furthermore, several studies have shown that interferon-β therapy caused a decrease in MMP-9 serum levels and MMP-9 mRNA in MS patients [13,20].

Conflicting results from a limited number of studies suggest that MMP-2 can serve as a biomarker for the different MS subtypes. High levels of MMP-2 in progressive MS have been reported to be responsible for the inflammation and disease suppressive effects. However, the role of MMP-2 in the chronic disease process, tissue repair [21], neuronal death [22] and axonal death [23] has already been demonstrated.

In our study, the MMP-9 and MMP-2 serum levels were not significantly different between the groups. Our admission criteria of current remission and no active infection or medical treatment could be a possible cause for the lack of increased MMP-9, as previously discussed in the literature, during the formation of MS lesions [24,25].

While MMP-9 is normally up-regulated during inflammatory conditions, MMP-2 is primarily expressed in the brain [5]. The overexpression of MMP-2 may produce a protective effect in MS but can also be damaging. In contrast, MMP-2, which causes myelin and/or axonal damage through the penetration of CD4+ Th1 cells and macrophages across the BBB, can also increase tissue repair [17,26]. Previous studies have produced conflicting results when examining the MMP-2 levels in CSF, serum and neuropathological conditions in acute and chronic demyelinating lesions [27]. Compared with the control group, the MMP-2 expression levels in MS patients were found to increase, decrease or remain unchanged in CSF [19,27-30], serum [29,31] and peripheral blood mononuclear cells [7,32,33]. Because the "active-MMP-2" possesses catalytic activity and is responsible for its effects, the inability of the previously used methods (immuno-histochemistry, zymography, in situ hybridisation, polymerase chain reaction, flow cytometry and ELISA) to detect active enzyme may explain the observed variability across different studies. Our study used an ELISA method to measure the MMP-2, MMP-9, TIMP-1 and TIMP-2 serum levels.

In another study, the brain tissues of MS patients were examined immuno-histopathologically, and TIMP-1 was detected in regions with MMP-9 reactivity; however, no comparison with the control group was performed [15]. While the serum TIMP-1 levels were not statistically different between the MS groups in our study, the TIMP-1 serum levels of the RRMS-SDD, RRMS-LDD, SPMS and PRMS groups were increased compared with the healthy control group. The absence of this difference in PPMS patients may be due to the small sample size (n=4).

Moreover, in a recent study [14], a significant decrease in the serum TIMP-2 levels and an increase in the MMP-2/TIMP-2 ratio were detected when PPMS patients were compared with RRMS patients. The decrease in serum TIMP levels caused the MMP/TIMP ratio to increase and the subsequent increase in MMP activity. In our study, the TIMP-2 serum levels of the RRMS-SDD group were increased compared with the SPMS and control groups. In the SPMS group, these TIMP-2 serum levels were significantly lower than the PRMS, PPMS and control group levels. After activation, MMPs can be regulated by establishing tight, 1:1 non-covalent complexes with TIMPs [34]. The MMP/TIMP balance reflects the net proteolytic activity present in several physiological processes.

Lichtinghagen et al. [35] reported a higher MMP-9/TIMP-1 ratio in the active MS group than in the control group,

which is consistent with higher proteolytic activity in MS patients. Avolio et al. [11] showed that the serum MMP-9/TIMP-1 ratio in RRMS patients was higher than the ratio in PPMS patients; however, the inverse was observed for the MMP-2/TIMP-2 ratio. The increased MMP-2/TIMP-2 ratio was interpreted as characteristic of chronic disease progression and the higher MMP-9/TIMP-1 ratio as progressing relapsing/remitting disease. The MMP-9 and TIMP-1 expression levels in MS are still controversial, but the relationship between a higher MMP-9/TIMP-1 ratio and lesion formation with new Gd involvement has been emphasised in various studies [36].

Galboitz et al. [13] reported increased MMP-2 expression in RRMS patients when comparing 16 RRMS patients to 12 SPMS patients. In our study, the MMP-2/TIMP-2 ratios were similar in all groups examined. The MMP-9/TIMP-1 ratio of the RRMS-SDD group was higher than the ratio of the PRMS group. Additionally, in the PRMS group, the MMP-9/TIMP-1 ratio was lower compared with the PPMS group. Furthermore, when compared with the control group, this ratio was lower in all the RRMS-SDD, RRMS-LDD, SPMS and PRMS groups, which particularly attracted our attention. The small sample size of the PPMS patients examined can explain the lack of significant differences when compared to the control group.

In this study, the MS patient groups were re-classified by the progressive disease course. With this new classification scheme, no statistically significant differences were observed in the MMP-2, MMP-9 and TIMP-2 serum levels. Although the TIMP-1 serum levels of the RRMS and SPMS-PRMS-PPMS groups were similar, the serum levels of these groups were increased compared with the control group.

In summary, the TIMP-1 serum levels were increased in all MS patients compared with the control group. This observation was remarkable and in contrast to previous reports. While the MMP-2/TIMP-2 ratios were similar between the groups, the MMP-9/TIMP-1 ratios were similar between the groups but decreased in the RRMS and SPMS-PRMS-PPMS patient groups compared with the control group. The decrease of the MMP-9/TIMP-1 ratio was a result of the increased TIMP-1 serum levels.

Conclusions

In all MS subgroups compared with the control group, the TIMP-1 levels were increased, and the MMP-9/TIMP-1 ratios were decreased, except for the PPMS group due to a small sample size (n=4). The increase of MMP-9 expression levels during the active formation of MS lesions has been emphasised in numerous studies. However, when the repair process is initiated, TIMP production may increase to create a normal MMP/TIMP ratio and to regain BBB integrity by decreasing activated T cell migration. Our findings correlate with the clinical stability of our patients. To investigate potential drug targets to prevent patient attacks, further clinical trials should be initiated to examine this dynamic pathophysiologic process of MS.

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