
# TIMP-1 resistant matrix metalloproteinase-9 is the predominant serum active isoform associated to MRI activity in patients with multiple sclerosis



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| | active MMP-9 to the MS pathogenesis.<br>Methods: Serum levels of 82 kDa and TIMP-1 resistant active MMP-9 isoforms were measured by activity assay systems in 86 MS relapsing-remitting MS (RRMS) patients, categorized according to clinical and magnetic resonance imaging (MRI) evidence of disease activity, and in 70 inflammatory (OIND) and 69 non-inflammatory (NIND) controls.<br>Results: Serum levels of TIMP-1 resistant MMP-9 were more elevated in MS patients than in OIND and NIND ($p<0.05$, $p<0.02$, respectively). Conversely, 82 kDa active MMP-9 was higher in NIND than in OIND and MS patients ($p<0.01$ and $p<0.00001$, respectively). MRI active had higher levels of TIMP-1 resistant MMP-9 and 82 kDa active MMP-9 than MRI inactive MS ($p<0.01$ and $p<0.05$, respectively).<br>Conclusion: Our findings suggest that the TIMP-1 resistant MMP-9 would seem to be the predominant active isoform contributing to MS disease activity. |
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# TIMP-1 resistant matrix metalloproteinase-9 is the predominant serum active isoform associated to MRI activity in patients with multiple sclerosis

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**Keywords:** MMP-9; active forms; TIMP-1 resistant MMP-9; serum levels; Multiple Sclerosis; MRI activity

## ABSTRACT

**Background:** The activity of matrix metalloproteinase-9 (MMP-9) depends on to two isoforms, an 82 kDa active MMP-9 modulated by its specific tissue inhibitor (TIMP-1), and a 65 kDa TIMP-1 resistant active MMP-9. The relevance of these two enzymatic isoforms in multiple sclerosis (MS) is still unknown.

**Objective:** To investigate the contribution of 82 kDa and TIMP-1 resistant active MMP-9 to the MS pathogenesis.

**Methods:** Serum levels of 82 kDa and TIMP-1 resistant active MMP-9 isoforms were measured by activity assay systems in 86 MS relapsing-remitting MS (RRMS) patients, categorized according to clinical and magnetic resonance imaging (MRI) evidence of disease activity, and in 70 inflammatory (OIND) and 69 non-inflammatory (NIND) controls.

**Results:** Serum levels of TIMP-1 resistant MMP-9 were more elevated in MS patients than in OIND and NIND ($p<0.05$, $p<0.02$, respectively). Conversely, 82 kDa active MMP-9 was higher in NIND than in OIND and MS patients ($p<0.01$ and $p<0.00001$, respectively). MRI active had higher levels of TIMP-1 resistant MMP-9 and 82 kDa active MMP-9 than MRI inactive MS ($p<0.01$ and $p<0.05$, respectively).

**Conclusion:** Our findings suggest that the TIMP-1 resistant MMP-9 would seem to be the predominant active isoform contributing to MS disease activity.




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## INTRODUCTION

Multiple sclerosis (MS) is an inflammatory disease in which demyelination and neurodegeneration coexist, with supposed autoimmune origin but unknown etiology.[1] Infiltration of activated myelin basic protein (MBP)-reactive CD4+ T-cell in the subarachnoid space is thought to be the first event, with the consequent reactivation in the central nervous system (CNS) by local antigen presenting cells.[2,3] The following inflammation triggered by all these cells leads to the activation of brain vessels ensuring the crossing of circulating activated CD4+ T-cells through the blood-brain barrier (BBB).[1] As a consequence of inflammation, the BBB disrupts, favoring the further passage of active components of the immune system into the brain parenchyma.[4,5] A growing body of evidence suggests that Matrix Metalloproteinases (MMPs), $Zn^{2+}$-dependent and $Ca^{2+}$-requiring endopeptidases, which remodel the extracellular matrix, are involved in various steps of MS pathogenesis, including BBB damage, leukocytes trafficking across the BBB and demyelination.[4,6] In general, MMPs are secreted from cells as inactive pro-enzymes and activated at the extracellular space level, where they exert their catalytic activity.[7] Therefore, under normal conditions they are tightly regulated at both transcription and activation levels, and their activity can be modulated by specific tissue inhibitors (TIMPs).[8] Among all MMPs, a predominant role in MS pathology is devoted to MMP-9. Indeed, it has long been observed that MMP-9 up-regulation is a key factor for the injury to the BBB as well as for the formation of MS lesions.[7,9,10] Previous studies, which mostly evaluated only the total MMP-9 without any distinction between active or latent enzyme, have shown that CSF and serum concentrations of MMP-9 are more elevated in MS patients compared to non-inflammatory neurological disorders (NIND) and healthy controls, but not to other inflammatory neurological disorders (OIND).[11-17] Moreover, high serum levels of total MMP-9 have been related to Magnetic Resonance Imaging (MRI) disease activity,[12-15] and its levels decrease in response to disease modifying therapies (e.g. interferon-β) correlating with a reduction in the number of gadolinium-enhanced (Gd+) lesions.[18] In a recent publication,[19] we found higher levels of active MMP-9, the only enzymatic form which exerts catalytic activity, solely in CSF but

not in serum of MS patients as compared to NIND. More importantly, patients with MRI evidence of disease activity experienced higher levels of CSF and serum active MMP-9 than those with MRI inactivity, suggesting that this enzyme could be implicated in the opening of the BBB, promoting the development of MS inflammatory response.[19] Recently, different forms of circulating active MMP-9 have been described in the serum: 82 kDa active MMP-9, which is inhibited by its specific inhibitor TIMP-1, and a 65 kDa active MMP-9 which is no longer inhibited, called TIMP-1 resistant MMP-9,[20] both produced by a MMP-3-dependent activation.[21] *In vitro* experiments show that MMP-3 at first induces the formation of a N-truncated active enzyme of 82 kDa after the removal of the N-terminal propeptide domain from proMMP-9[22.] Subsequently, it promotes the elimination of the C-terminal hemopexin-like domain interacting with high affinity with TIMP-1 generating a N- and C-truncated active enzyme of 65 kDa[21] that becomes resistant to inhibitory effects of TIMP-1.[23]This study provided the first demonstration that TIMP-1 resistant active MMP-9 can be detected in vivo with gelatin zymography, after separation of MMP-9 from MMP-2 by affinity chromatography and western blot analysis, and measured by an activity assay in presence of TIMP-1.[20] However, the actual biological role of this isoform is currently unknown.[20] In addition, in a more recent work Rossano and colleagues[24] were able to identify the TIMP-1 resistant MMP-9 in the serum of relapsing-remitting (RR) MS patients, without, however, any specific quantification. Therefore, in the present study the aim was to measure the levels of 82 kDa and TIMP-1 resistant MMP-9 in the serum of RRMS patients categorized on the basis of disease activity, and NIND and OIND controls in order to investigate its actual contribution to the MS pathogenesis. Moreover, we related the amount of TIMP-1 resistant MMP-9 to disease severity and duration.

## MATERIALS AND METHODS

### *Patient characteristics*

Eighty-six consecutive patients affected by definite MS according to McDonald criteria[25] and followed at the MS Centre of the Department of Neurology, University of Ferrara, were recruited (Table 1). Patients were classified as having relapsing-remitting (RRMS) according to the criteria of Lublin[26] and evidence of a relapse at admission was considered as clinical disease activity.[27] Accordingly, 66 patients were clinically active whereas 20 were clinically stable. Disease severity was measured by the Kurtzke's Expanded Disability Status Scale (EDSS).[27] Disease duration was scored and expressed in months. At the time of sample collection none of the patients had fever or other signs of acute infections, nor had they been receiving any disease-modifying therapies during the 6 months before the study. Seventy patients with other inflammatory neurological disorders (OIND) and 69 subjects with other non-inflammatory neurological disorders (NIND), who were age and sex matched to RRMS, were recruited as neurological controls (Table 2). All OIND and NIND patients were free of immunosuppressant drugs, including steroids, at the time of sample collection. As further controls we used 40 age and sex matched healthy donors (HD) (28 women and 12 men; mean age ± SD = 37.0 ± 7.5 years). Informed consent was given by all patients before inclusion and the study design was approved by the Local Committee for Medical Ethics in Research.

***Serum sampling***

Serum samples were obtained from centrifugation of blood specimens withdrawn by puncture of an anterocubital vein, collected under sterile conditions, stored in aliquots at -80°C until assay and measured under exactly the same conditions. Serum analysis were performed within 2 weeks of the onset of clinical symptoms in relapsing MS patients, and at least 2 months after the end of clinical exacerbation in clinically stable MS patients.

***MRI examination***

Brain Magnetic Resonance Imaging (MRI) scans were performed at entry using a standard head coil in all patients with a 1.5-Tesla MRI unit (GE Signa Horizon, General Electric Medical Systems, Milwaukee, Wisconsin). Routinely used T1-weighted axial spin echo images were obtained

approximately 10 minutes after intravenous injection of 0.1 mmol/Kg of Gadolinium (Gd)-DTPA in each patient. Lesions showing Gd-enhancement on T1-weighted scans were defined as indicative of MRI activity. Accordingly, 38 MS patients (31 with clinically active and 7 with clinically stable disease; disease duration = 25.1±38.0 months; EDSS = 2.2±1.4) were classified as MRI active and 48 (35 with clinically active and 13 with clinically stable disease; disease duration = 34.6±50.0 months; EDSS = 2.0±1.3) were considered as MRI inactive.

*MMP-9 activity assays*

Active forms of MMP-9 were measured using a commercially available activity assay system (MMP-9 Activity Assay System, GE Healthcare, UK, Cat. No. RPN 2634) following the manufacturer's instructions. Briefly, each sample was analyzed in duplicate into 96-microwell microtiter plates pre-coated with anti-MMP-9 antibodies. Seven dilutions of standard, recombinant human proMMP-9, in the range of 0.125-4 ng/ml were dispensed onto each plate in duplicate. After an overnight incubation at 4°C and four washing cycles, only the enzyme was bound to the wells. The standards were activated by adding 50 µl/well of p-Aminophenylmercuric acetate (APMA). Conversely, to detect endogenous levels of active MMP-9, 50 µl/well of assay buffer was dispensed into each well of serum sample. The plate was then incubated at 37°C for 1.5 hours. Afterwards, fifty microliters of detection reagent, a modified pro-urokinase and urokinase substrate, were pipetted into each well. The amount of active MMP-9 in all samples was determined by interpolation from the standard curve. The MMP-9 activity assay was not able to distinguish between 82 kDa active MMP-9 and 65 kDa TIMP-1 resistant MMP-9. To determine the amount of the last enzymatic isoform, a TIMP-1 inhibition step was added to the aforementioned assay, as published elsewhere.[20] Briefly, after the overnight incubation of the samples in microtiter plates and 4 washing cycles, 100 µl/well of purified TIMP-1 (100 ng/ml; Sigma-Aldrich, Italy, Cat. No. T8947) were added to all samples in order to inhibit the 82 kDa MMP-9 active form. After 1.5 hours of incubation at 37°C and three washing cycles, 50 µl of assay buffer and 50 µl of detection

reagent were applied, and the residual MMP-9 activity was assigned to the TIMP-1 resistant MMP-9. The amount of 82 kDa active MMP-9 was determined by the difference between the total endogenous active MMP-9 and the TIMP-1 resistant MMP-9.

***Gelatin Zymography***

To confirm the presence of TIMP-1 resistant MMP-9, MMP-9 and MMP-2 were separated by chromatography on ConA-Sepharose in serum from 3 patients with RRMS, OIND and NIND as previously described.20 The eluate, containing MMP-9, was tested for the presence of MMP-2 and found negative (data not shown).

***Statistical analysis***

The normality of distribution of the variables was checked by the Kolmogorov-Smirnov test. When normality of data distribution was found for all groups, statistical analysis was performed by using a parametric approach. On the contrary, when normality was rejected a non-parametric approach was used. Accordingly, continuous variables were compared using Kruskal-Wallis, followed by Mann-Whitney U test with Bonferroni correction for multiple comparisons, and by means of Mann-Whitney U test or independent-samples t test. The Spearman rank correlation coefficient test or Linear Regression Analysis were used to identify possible relationships among the different variables. A value of $p<0.05$ was considered statistically significant.

**RESULTS**

***Serum levels of active MMP-9 isoforms in MS patients and controls***

Serum concentrations of total active, 65 kDa TIMP-1 resistant and 82 kDa active MMP-9 were detectable in 100% of cases in RRMS, OIND and NIND. Three illustrative cases demonstrating the zymographic detection of 65 kDa TIMP-1 resistant active MMP-9 in serum samples from RRMS, OIND and NIND are reported in Figure 1. As reported in Figure 2, serum levels of total active MMP-9, TIMP-1 resistant MMP-9 and 82 kDa active MMP-9 were statistically different among the groups ($p<0.00001$). In particular, serum levels of total active MMP-9 were similar in MS, OIND

and NIND patients, but greater in MS, OIND and NIND compared to HD ($p<0.00001$). On the other hand, TIMP-1 resistant MMP-9 was higher in RRMS than in OIND and NIND ($p<0.05$, and $p<0.02$, respectively), and more increased in MS, OIND and NIND compared to HD ($p<0.00001$). Conversely, serum concentrations 82 kDa active MMP-9 were more elevated in NIND than in RRMS and OIND ($p<0.00001$ and $p<0.01$, respectively) and more prominent in MS, OIND and NIND compared to HD ($p<0.001$, $p<0.01$ and $p<0.05$, respectively).

***Serum levels of active MMP-9 isoforms in MS patients categorized according to clinical and MRI activity***

When MS were grouped according to clinical activity, there were no statistical differences between RRMS patients with and without clinical evidence of disease activity for serum levels of total active MMP-9, TIMP-1 resistant MMP-9 and 82 kDa active MMP-9 (Figure 3, panel A). On the contrary, when the patients were categorized according to MRI activity, serum titers of total active MMP-9 and TIMP-1 resistant MMP-9 were more increased in MRI active ($p<0.01$ and $p<0.01$, respectively) than in MRI inactive RRMS, whereas serum values of 82 kDa active MMP-9 were only slightly greater in RRMS patients with MRI active ($p=0.0414$) than in those with MRI stable disease (Figure 3, panel B).

***Correlation between serum levels of active MMP-9 isoforms and MS clinical features***

There were no significant correlations between disease severity measured by EDSS and serum levels of total active MMP-9 (Linear regression; $r^2=0.0025$, beta=-0.12; $p=0.275$), TIMP-1 resistant MMP-9 (Linear regression; $r^2=-0.0094$, beta=-0.05; $p=0.648$) and 82 kDa active MMP-9 (Spearman; $r=-0.0095$; $p=0.931$). No definite association was found between disease duration and serum concentrations of total active MMP-9 (Spearman; $r=0.1766$; $p=0.104$), TIMP-1 resistant MMP-9 (Spearman; $r=0.0095$; $p=0.931$) and 82 kDa active MMP-9 (Spearman; $r=0.1190$; $p=0.275$). Age did not affect any of the variables considered (data not shown).

## DISCUSSION

Prior studies have revealed that MMP-9 may be an important determinant for MS pathogenesis since its CSF and serum levels are increased in MS patients compared to controls,[11-17] as well as during clinical and MRI evidence of disease activity.[12-15] In addition, we previously showed that CSF and serum concentrations of active MMP-9, the only enzyme which exerts catalytic activity, is elevated in MRI active MS patients which indicates that they could be used as a surrogate biomarker for monitoring disease activity.[19] These findings suggest that MMP-9 may lead to BBB impairment which is the essential prerequisite for the formation of Gd enhancing lesions and the occurrence of relapse in MS.[4] On the other hand, we have recently observed[20] that MMP-9 activity in body fluids is due to the presence of two enzymatic forms: an 82 kDa active MMP-9, modulated by its specific inhibitor TIMP-1, and a 65 kDa TIMP-1 resistant active MMP-9 which is no longer inhibited by TIMP-1, escaping physiological regulation.[23] Nevertheless, the actual contribution of the different active MMP-9 isoforms to MS inflammatory response is currently unknown. Thus, in the present study we measured serum levels of 82 kDa and TIMP-1 resistant active MMP-9 in RRMS patients categorized according to disease activity and in controls. In agreement with our previous publication,[19] we here show that serum levels of total active MMP-9 were higher in RRMS, OIND and NIND than in healthy donors, confirming that they were equivalent between RRMS patients and neurological controls, did not correlate with disease duration and severity, but were higher in MRI active than in MRI inactive MS. These findings were only partially concordant with previous studies in which, in particular, serum concentrations of MMP-9 were found to be higher in MS than in controls.[12,13,15-17] Although divergences in patient selection may at least in part explain these conflicting results, it is likely that they are mainly due to methodological differences in determination techniques. Indeed, while we measured serum levels of active MMP-9 by a sensitive activity assay system, the other groups quantified serum concentrations of both active and latent forms of MMP-9 by enzyme-linked immunosorbent assay (ELISA). Intriguingly, although serum amounts of 82 kDa and TIMP-1 resistant active MMP-9 were more increased in RRMS and

neurological controls than in healthy volunteers, the two enzymatic active MMP-9 forms were differentially represented in the examined cohorts, showing reciprocal fluctuations. In fact, while serum levels of TIMP-1 resistant active MMP-9 were more elevated in RRMS than in OIND and NIND, serum concentrations of 82 kDa active MMP-9 were more prominent in NIND than in MS and OIND. However, our main result was the demonstration that serum levels of TIMP-1 resistant active MMP-9 were higher in RRMS patients with evidence of MRI activity than in those without MRI appearance of disease activity. Indeed, although 82 kDa active MMP-9 serum values were also greater in RRMS patients with Gd enhancing lesions than in those without, this difference reached a low level of statistical significance. The well-known superiority of MRI Gd enhancement on clinical examination in measuring MS disease activity[28] can explain why serum concentrations of 82 kDa and TIMP-1 resistant active MMP-9 did not differ between RRMS patients with and without clinical attacks. In addition, we did not recognize any statistical correlations between serum levels of total active MMP-9 and its isoforms and severity or duration of the disease. These findings support and extend data coming from a recent study[24] that describes the presence of TIMP-1 resistant MMP-9 in the serum of RRMS patients by using a 2D-gelatin zymography, a sensitive but semi-quantitative technique.[29] In fact, to the best of our knowledge, this is the first study to quantify the two enzymatic forms of active MMP-9 in the serum of MS patients and controls by employing a modified activity assay.[20] Our data further support the notion that serum MMP-9 activity is not restricted to MS, but is shared by several inflammatory neurological conditions,[9,10,19] and is associated to MRI disease activity.[19] More important, these results indicate that in neurological inflammatory disorders, serum MMP-9 activity seems to be predominantly dependent on the levels of TIMP-1 resistant active MMP-9, and suggest a potential role of this MMP-9 isoform in the development of Gd enhancing lesions in MS patients. Therefore, an overproduction of active MMP-9 not counterbalanced by the inhibitory effect of TIMP-1 could promote the BBB impairment leading to the initiation of MS intrathecal inflammation.[7,19,30] The occurrence of increased levels of MMP-3 during active phase of disease[31] may contribute to enhancing MMP-9

activity in MS since MMP-9 activation is MMP-3-mediated.[21] This study was not without its limitations. Firstly, the small sample size may have weakened the consistency of our data. Secondly, the lack of spinal cord MRI examinations in our study could have had an effect on our findings since some small active lesions may have been missed, leading to potentially inappropriate allocation of patients to the MRI inactive group. Thirdly, as we did not measure CSF levels of 82 kDa and TIMP-1 resistant active MMP-9, whether these MMP-9 isoforms are intrathecally synthesized remains an open issue. However, a local production of active MMP-9 within the CNS was detected only in a subset of MS patients, suggesting that brain-derived 82 kDa and TIMP-1 resistant active MMP-9 concentrations could be detected in a restricted MS population.[19] Fourthly, as peripheral blood cells were not available in our study population, we were unable to perform biochemical experiments useful for the identification of the cellular sources of TIMP-1 resistant active MMP-9 and for a better understanding of the biological significance of this isoform in MS. Finally, a bias in the results due to some experimental variables (e.g. displacement of TIMP-1 from the 82 kDa active MMP-9 during incubation, cross reactivity of capturing antibodies with untested proteases) cannot be excluded. In addition, our results could be weakened by the currently unknown effects of the prolonged storage in frozen conditions on the stability of MMP-9 isoforms. In conclusion, this study strengthens the hypothesis that the proteolytic activity of MMP-9 may be relevant in inducing the formation of Gd enhancing lesions in MS.[9,32] In this context, TIMP-1 resistant active MMP-9 seems to be the major enzymatic form contributing to disease activity as detected by MRI because this pro-inflammatory isoform is able to escape the inhibitory control of its specific inhibitor.[20,23] Further studies in a larger number of patients are needed to verify the actual significance of TIMP-1 resistant active MMP-9 in the modulation of inflammatory response operating in MS. For this purpose, a longitudinal evaluation of changes in serum levels of TIMP-1 resistant active MMP-9 in relation to MRI disease activity would be particularly pertinent.

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## Conflict of Interest Statement

The Authors declare that there is no conflict of interest.

## APPENDIX

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**Table 1.** Demographic, clinical and radiological characteristics in 86 patients with relapsing-remitting multiple sclerosis (RRMS).

| | |
|---|---|
| Sex: Female/Male | 59/27 |
| Age, years: mean ± SD | 37.9 ± 10.9 |
| Disease duration, months: mean ± SD | 30.5 ± 45.1 |
| Disease severity, EDSS: mean ± SD | 2.1 ± 1.4 |
| Clinically Active MS: n/total (%) | 66/86 (77%) |
| Clinically Stable MS: n/total (%) | 20/86 (23%) |
| Gd+ MS: n/total (%) | 38/86 (44%) |
| Gd- MS: n/total (%) | 48/86 (56%) |

EDSS = Expanded disability status scale; Gd+ = MRI appearance of gadolium enhancing lesions; Gd- = no MRI evidence of gadolium enhancing.

**Table 2.** Demographic and clinical features of OIND and NIND patients.

| Patients | n | Female:Male | Age (years) | Type of disease |
|---|---|---|---|---|
| **OIND** | 70 | 49/21 | 38.0 ± 10.7 | |
| | 25 | | | Chronic inflammatory demyelinating polyneuropathy |
| | 15 | | | Acute inflammatory demyelinating polyneuropathy |
| | 5 | | | Herpes simplex virus-1 encephalitis |
| | 5 | | | Bacterial meningitis |
| | 4 | | | Viral meningitis |
| | 3 | | | Varicella-zoster virus encephalitis |
| | 3 | | | Acute disseminated encephalomyelitis |
| | 2 | | | Inflammatory myelitis |
| | 2 | | | Neurolupus |
| | 1 | | | HIV-related leukoencephalopathy |
| | 1 | | | neuroSjogren |
| | 1 | | | neuroBechet |
| | 1 | | | Intracerebral abscess |
| | 1 | | | Post-infectious myelitis |
| | 1 | | | Post-infectious posterior reversible encephalopathy syndrome |
| **NIND** | 69 | 47/22 | 38.5 ± 11.0 | |
| | 13 | | | Headache |
| | 9 | | | transient ischemic attack |
| | 6 | | | amyotrophic lateral sclerosis |
| | 6 | | | Migraine |
| | 6 | | | Epilepsy |
| | 5 | | | mild cognitive impairment |
| | 3 | | | low grade glioma |
| | 3 | | | vascular dementia |
| | 3 | | | Parkinson disease |
| | 3 | | | hereditary neuropathy |
| | 2 | | | Alzheimer disease |
| | 2 | | | compression neuropathy |
| | 2 | | | cervical spondylosis |
| | 2 | | | intracerebral haemorrhage |
| | 1 | | | venous thrombosis |
| | 1 | | | multiple system atrophy |
| | 1 | | | hereditary ataxia |
| | 1 | | | hydrocephalus |

OIND = Other Inflammatory Neurological Diseases; NIND = Non-Inflammatory Neurological Diseases.

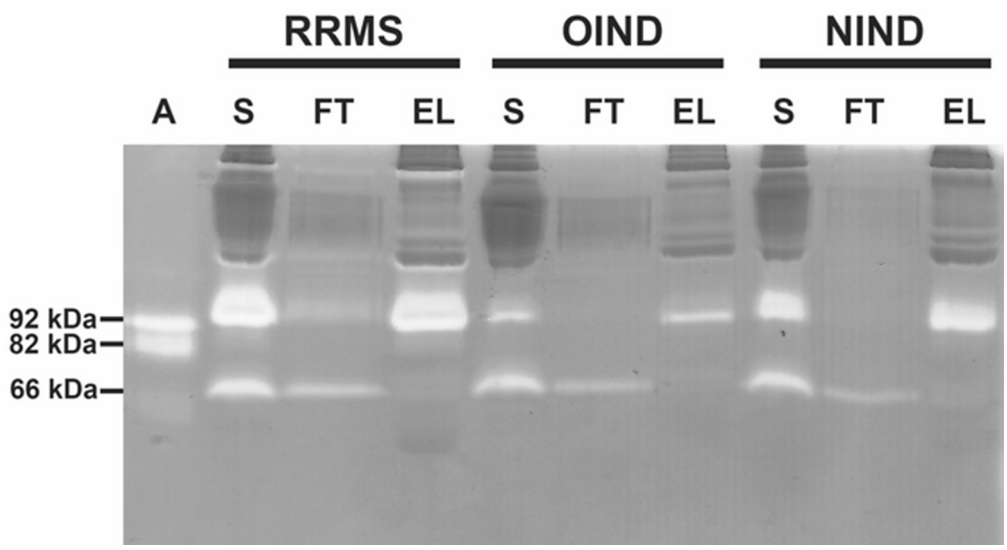
**Figure 1.** Presence of 65 kDa TIMP-1 resistant active MMP-9 in serum samples from 3 patients with relapsing-remitting multiple sclerosis (RRMS), other inflammatory neurological disorders (OIND) and non inflammatory neurological disorders (NIND) demonstrated by gelatin zymography after carbohydrate affinity chromatography. Note the bands in the 66 kDa range of the elution (EL) fractions.

A column with 500 µl of concanavalin-A-Sepharose was equilibrated with 10 volumes of phosphate buffered saline (PBS). After the application of 300 µl of serum diluted 3 times in PBS, the column was first washed with 20 volumes of a 0.3M NaCl buffer and then with 2 volumes of a 0.3 M NaCl and 50 mM methyl-α-D-mannopyranoside buffer. A fraction of the flow through was kept. Finally, the column was eluted with a buffer containing 0.5 M methyl-α-D-mannopyranoside. The fractions were analysed by gelatin zymography. Moreover, the elution fractions were tested for the presence of MMP-2 and found negative. *A*: recombinant human 4 ng/ml MMP-9 partially activated; *S*: serum sample diluted 20 times; *FT*: flow through fraction diluted 2 times; *EL*: elution fraction diluted 2 times. For all the samples the same amount (20 µl) was loaded on the gel.

**Figure 2.** Median and interquartile range (IQR) of serum total active MMP-9, TIMP-1 resistant active MMP-9 and serum 82 kDa active MMP-9 levels in 86 patients with relapsing-remitting multiple sclerosis (RRMS), in 70 patients with other inflammatory neurological disorders (OIND), in 69 patients with non inflammatory neurological disorders (NIND) and in 40 healthy donors (HD).Serum levels of total active MMP-9, TIMP-1 resistant MMP-9 and 82 kDa active MMP-9 were statistically different among the groups (Kruskal-Wallis; $p<0.00001$). Serum concentrations of total active MMP-9 were no different for among RRMS (8.4 ± 4.0 ng/ml), OIND (8.1 ± 4.7 ng/ml) and NIND (8.8 ± 4.6 ng/ml), whereas they were higher (Mann Whitney; $p<0.00001$) in RRMS, OIND and NIND compared to HD (4.0 ± 1.4 ng/ml) **(***Panel A*). Serum levels of TIMP-1 resistant MMP-9 were greater in RRMS (5.3 ± 2.9 ng/ml) than in OIND (4.2 ± 3.2 ng/ml) and NIND (4.0 ± 2.9 ng/ml) (Mann Whitney; $p<0.05$ and $p<0.02$, respectively) and more elevated (Mann Whitney;

p<0.00001) in RRMS, OIND and NIND compared to HD (2.4 ± 1.3 ng/ml) (*Panel B*). Serum amounts of 82 kDa active MMP-9 were more increased in NIND (4.7 ± 3.8) than in RRMS (3.0 ± 2.7) and OIND (3.9 ± 4.2) (Mann Whitney; p<0.00001 and p<0.01, respectively), but more pronounced in RRMS, OIND and NIND compared to HD (1.6 ± 0.6 ng/ml) (*Panel C*). The line between the data represents the median.

**Figure 3.** Median of serum total active MMP-9, TIMP-1 resistant active MMP-9 and serum 82 kDa active MMP-9 levels in 66 clinically active (CA) and 20 clinically stable (CS) RRMS patients (*Panel A*) and in 38 MRI active (Gd+) and 48 MRI inactive (Gd-) RRMS (*Panel B*). No differences were found between clinically active or clinically stable MS patients for serum levels of total active MMP-9 (t test; CA = 8.4 ± 3.7 ng/ml; CS = 8.4 ± 5.0 ng/ml), TIMP-1 resistant MMP-9 (t test; CA = 5.2 ± 2.6 ng/ml; CS = 5.9 ± 3.6 ng/ml) and 82 kDa active MMP-9 (t test; CA = 3.2 ± 2.9 ng/ml; CS = 2.5 ± 1.9 ng/ml) (*Panel A*). Serum concentrations were greater in patients with MRI active than in those with MRI inactive disease for total active MMP-9 (t test; Gd+ = 9.8 ± 4.8 ng/ml; Gd- = 7.3 ± 2.9 ng/ml; p<0.01), TIMP-1 resistant MMP-9 (t test; Gd+ = 6.2 ± 3.5 ng/ml; Gd- = 4.6 ± 2.0 ng/ml; p<0.01) and 82 kDa active MMP-9 (Mann-Whitney; 3.5 ± 3.0 ng/ml vs. 2.7 ± 2.5 ng/ml; p=0.041) (*Panel B*). In *Panel A* and *Panel B*, the line between the data represents the median.

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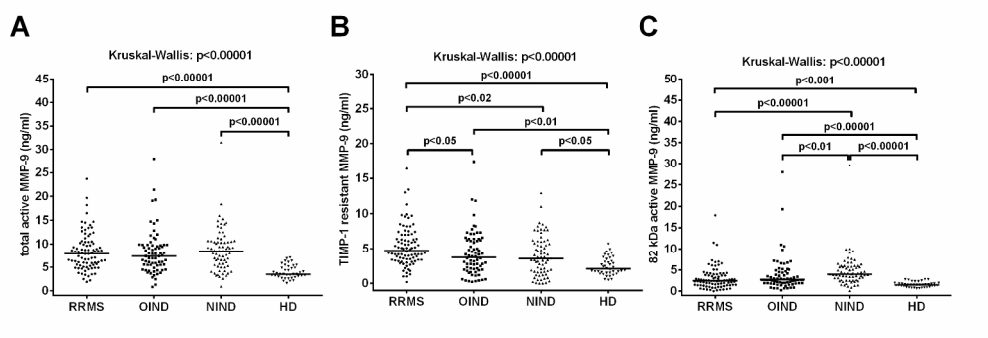


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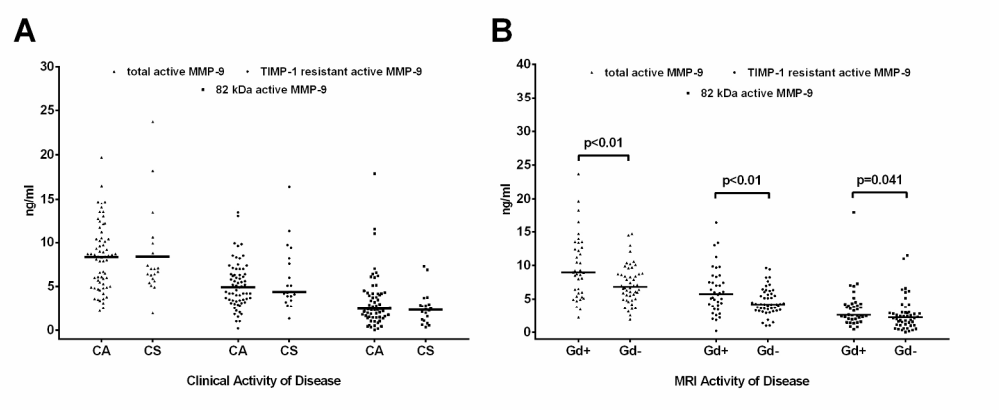


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