

## PAPER

## Serum matrix metalloproteinase-3 levels correlate with disease activity in relapsing-remitting multiple sclerosis

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**Background:** Adhesion molecules and matrix metalloproteinases (MMPs) are known to be relevant to the ongoing development and disappearance of areas of demyelination in the white matter of the CNS of multiple sclerosis (MS) patients. This study examined whether serum matrix metalloproteinase-3 (MMP-3) levels correlate with disease activity in MS.

**Methods:** Serum MMP-3 levels in 47 consecutive patients with relapsing-remitting MS were measured by immunoassay every 4 weeks over a 15 month period.

**Results:** During the study period, 48 clinical relapses occurred. Serum MMP-3 levels within 1 month of relapse were significantly higher than during the remission phase. Sequential analysis showed that serum MMP-3 levels had increased transiently at the time of clinical relapse but returned to the normal range within a month.

**Conclusions:** Circulatory MMP-3 levels are correlated with disease activity in relapsing-remitting MS. This may contribute to the breakdown of the blood-brain barrier at the time of relapse.

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS). A characteristic feature of its disease pathology is the ongoing development and disappearance of areas of demyelination in the white matter of the CNS.<sup>1</sup> Initial events leading to the formation of lesions include an inflammatory reaction, in which lymphocytes, macrophages, and glial cells play a major role,<sup>2</sup> and damage to the blood-brain barrier (BBB) which allows mononuclear cells to enter the CNS. Adhesion molecules and matrix metalloproteinases (MMPs) are known to be relevant to this process.<sup>3-4</sup>

MMPs degrade various extracellular matrix (ECM) components that participate in tissue remodelling during development, wound healing, tumour growth and metastasis, and inflammation. In fact, MMP-7 and MMP-9 are expressed in inflammatory cells in MS plaques.<sup>5-7</sup> That increased CSF MMP-9 levels in MS patients are associated with BBB damage<sup>8</sup> is suggested by gadolinium enhancement on MRI. Moreover, elevated MMP-9 mRNA levels in peripheral blood mononuclear cells and raised MMP-9 levels in serum and CSF have been found in MS patients in association with clinical or MRI disease activity.<sup>9-11</sup> A previous report showed that MMP-2 and MMP-3 were not upregulated and that MMP-7 was not detectable in the CSF of MS patients.<sup>10</sup> No study of changes in MMP-3 levels associated with disease activity, however, has been reported. In the expectation that serum MMP-3 levels are correlated with disease activity, we conducted a longitudinal study of consecutive patients with relapsing-remitting MS.

## METHODS

## Patients

Forty seven Japanese patients with relapsing-remitting MS (40 women, 7 men; mean age  $\pm$  standard deviation (SD)  $37 \pm 12$  years), seen at Chiba University Hospital between September 2002 and December 2003, were enrolled in the study. Their conditions fulfilled McDonald's criteria for MS.<sup>12</sup> Informed consent was obtained from each patient before beginning the study.

Every 4 weeks the clinical condition of these patients was monitored with reference to the Expanded Disability Status

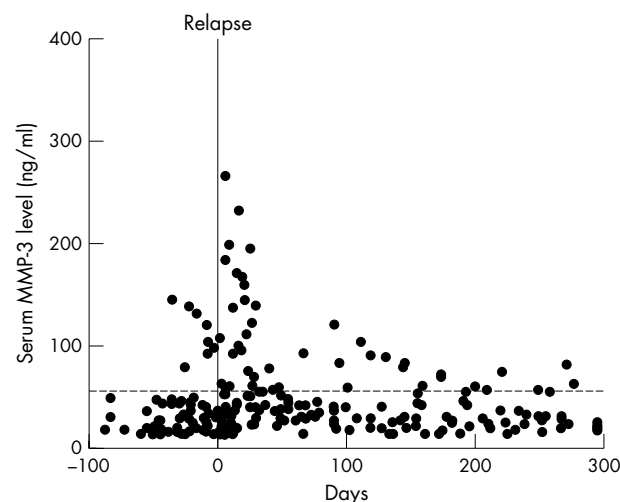
Scale (EDSS). The criteria proposed by McDonald *et al*<sup>12</sup> define clinical relapse as an episode of neurological disturbance of the kind seen in MS, lasting at least 24 h. During the study period, serum or plasma samples were taken every 4 weeks, or at the time of relapse, and stored.

## MMP assays

Patients' sera and plasma were separated by centrifugation for 10 min in dry and EDTA-2Na tubes, then pipetted to dry tubes and frozen at  $-80^{\circ}\text{C}$ . Serum MMP-3 was assayed by a one step sandwich enzyme immunoassay (EIA; Fuji Chemical Industries, Toyama, Japan), as described elsewhere.<sup>13-16</sup> MMP-3 EIA recognises both active and latent forms of the free enzymes or complexes with tissue inhibitors of matrix metalloproteinases (TIMPs). The detection limit for serum was 12.5 ng/ml. Assays were performed sequentially, by researchers blinded to clinical information, every 4 weeks during the 15 month study period. Normal control data for the MMP-3 assay were obtained from 623 healthy subjects. Sera collected from 32 Guillain-Barré syndrome patients within 30 days of neurological onset were used as MMP-3 disease controls.

Serum MMP-1 and MMP-2, and plasma MMP-9 levels were measured by EIAs. For these assays, patients' sera and plasma samples were obtained at the time of clinical relapse and 2-3 months before and after clinical relapse. MMP-1 EIA recognises free MMP-1 and MMP-1 complexes with inhibitors such as TIMP-1, but not  $\alpha_2$  macroglobulin-MMP-1 complexes. Moreover, it recognises the precursor forms of the free enzymes or tissue inhibitors of metalloproteinase-TIMP-2 complexes, but not active forms of the enzymes. MMP-9 EIA recognises the precursor forms of the free enzymes, TIMP-1 complexes, or the 83 kDa active forms, but not the 67 kDa active forms of the enzymes. Plasma MMP-9 levels were measured rather than serum levels because the latter were irregularly elevated during serum separation. The

**Abbreviations:** BBB, blood-brain barrier; CNS, central nervous system; ECM, extracellular matrix; EDSS, Expanded Disability Status Scale; EIA, enzyme immunoassay; MMP, matrix metalloproteinase; MMP-3, matrix metalloproteinase-3; MS, multiple sclerosis; TIMPs, tissue inhibitors of matrix metalloproteinases



**Figure 1** Serum MMP-3 levels based on days from the nearest relapse. Dashed line shows cut off values (2 SD above the mean normal control value).

detection limit for serum was 1.0 ng/ml for MMP-1 and 70.7 ng/ml for MMP-2. In plasma, it was 3.0 ng/ml for MMP-9. Control data from 36 normal subjects were obtained for the MMP-1 assay, from 213 normal subjects for the MMP-2 assay, and from 15 normal subjects for the MMP-9 assay.

MRI was performed at 6 month intervals and at the time of relapse. All MRI data were acquired with a General Electric 1.5 T Signa system (Milwaukee, WI). Scanning sessions included T2 weighted and T1 weighted images. The latter were acquired 30 min after intravenous injection of gadolinium-diethylenetriamine pentaacetic acid (0.1 mmol/kg).

The MMP concentrations (ng/ml) were expressed as means  $\pm$  standard error of the mean (SEM). In the statistical analysis, median differences were tested with the Wilcoxon signed ranks or Mann-Whitney's U test. *p* values lower than 0.05 were considered significant.

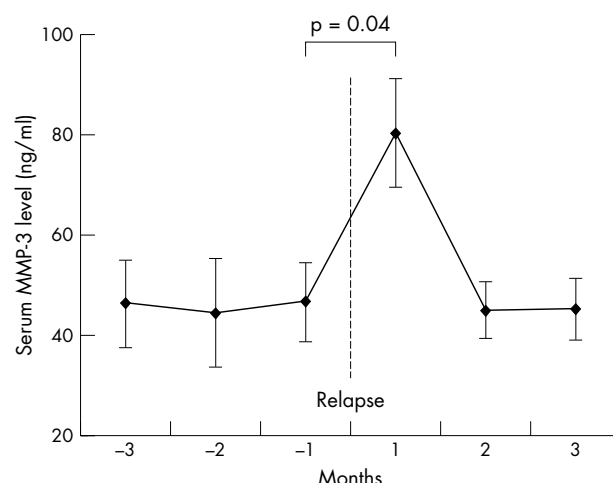
## RESULTS

### Clinical evaluation

During the study period, 48 definite clinical relapses occurred in 22 of the 47 MS patients (mean 1.0 per patient; range 1–4). Of these, 27 (56%) involved the spinal cord and 21 (44%) the brain. Forty of the 48 relapses were preceded by a clinical remittance phase lasting more than 2 months. To eliminate effects of prior relapse, we focused only on those 40 episodes. In 40 of the 48 relapse cases, patients were given oral prednisolone (40–60 mg per day for 7 days) or a high dose of methylprednisolone intravenously (1000 mg a day for 5 consecutive days).

### Serum MMP-3 levels

Figure 1 shows the serum MMP-3 levels of all the samples (313 sera in total) by day from the time of nearest relapse. At the time of clinical relapse, elevated serum MMP-3 levels were present in some sera, but the levels rarely had increased more than 30 days before or after relapse. Figure 2 shows the results of serial analysis of serum MMP-3 levels around relapse preceded by more than 2 months of remission. MMP-3 levels were elevated transiently within 1 month after relapse and had increased significantly within that period ( $p = 0.04$ ). None of the 32 patients with Guillain-Barré syndrome had elevated serum MMP-3 levels. Figure 3 shows a representative case of elevated serum MMP-3 at the time of relapse. During the study period, this 41 year old Japanese woman experienced two relapses involving the spinal cord.



**Figure 2** Serial analysis of mean serum MMP-3 levels at the time of relapse. Error bars indicate SEM. Serum MMP-3 shows a transient increase within 1 month after relapse.

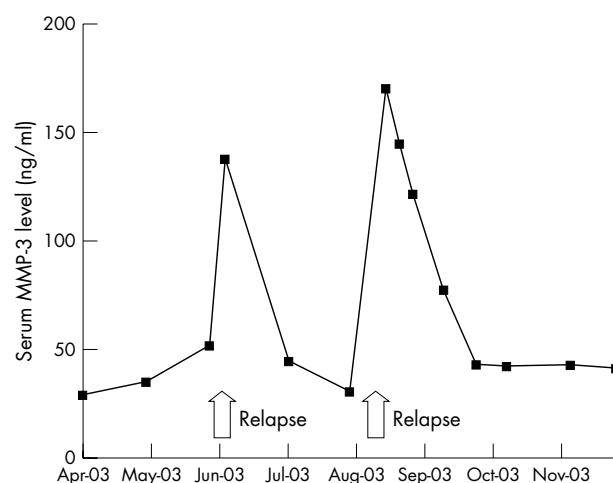
As shown in this case, the increase in MMP-3 levels was transient, returning to normal within 1 month after relapse. Fourteen of the 48 relapses (29%) had increased levels of MMP-3.

### Serum or plasma MMP-1, -2, and -9 levels

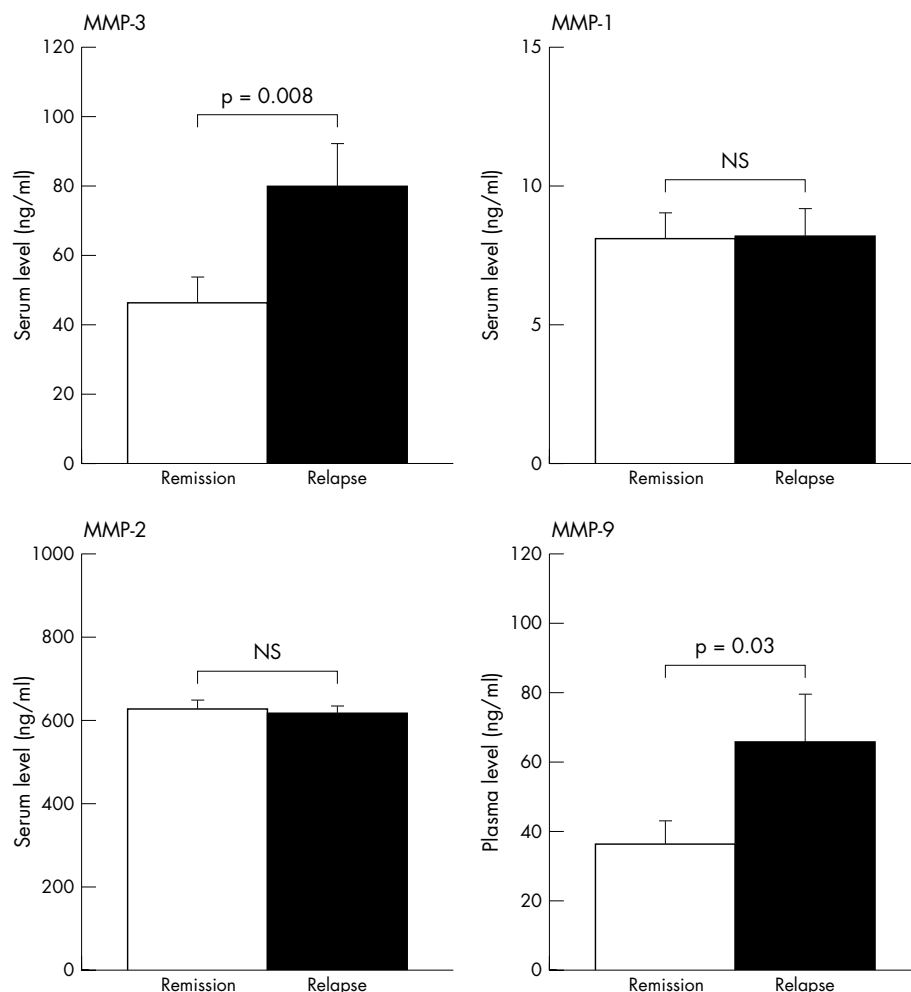
Figure 4 shows serum MMP-1, MMP-2, MMP-3, and plasma MMP-9 levels at the time of clinical relapse and during the remission phase (2 months before relapse). Serum MMP-3 and plasma MMP-9 levels were higher at relapse than during remission ( $p = 0.008$  for MMP-3;  $p = 0.03$  for MMP-9). Serum MMP-1 and MMP-2 levels were similar at the time of relapse and during remission.

### Active MRI lesions and serum MMP-3 levels

Twenty nine MRI scans were performed on the day serum samples were collected for MMP analysis. Fourteen of the 29 MRI scans showed new gadolinium enhanced lesions. MMP-3 measured on the day when MRI showed an active lesion(s) did not differ significantly from values obtained on other days. We compared the MMP-3 levels on the day when MRI scan showed active brain lesion(s) ( $n = 8$ ) with the MMP-3 levels on the day when MRI scan showed active spinal



**Figure 3** A representative case showing elevated serum MMP-3 levels at the time of relapse.



**Figure 4** Differences in serum MMP-1, MMP-2, MMP-3, and plasma MMP-9 levels during the relapse (black columns) and remission (white columns) phases. Serum MMP-3 and plasma MMP-9 levels are increased significantly in the relapse phase.

lesion(s) ( $n = 6$ ). The mean  $\pm$  SD MMP-3 level with active brain MRI lesion(s) was  $54.0 \pm 65.6$ , while that with active spinal MRI lesion(s) was  $54.7 \pm 64.1$ . There was no significant difference in MMP-3 levels between the sera from patients with brain and spinal lesions.

## DISCUSSION

This study shows that serum MMP-3 levels in patients with remitting-relapsing MS were increased at the time of relapse but returned to normal range within a month. This is the first investigation of serum MMP-3 levels and their sequential changes. Levels were significantly higher at relapse than during the remission phase.

Of the various members of the MMP family, stromelysins have special importance as they have broad substrate specificity and degrade such components of the ECM as fibronectin, laminin, elastin, collagen IV, and proteoglycans.<sup>17–19</sup> Laminin, fibronectin, and collagen IV are reported to be major ECMs of the brain capillaries.<sup>20</sup> The elevated MMP-3 in sera of the MS patients in this study, therefore, would degrade ECMs, major components of the BBB. Moreover, MMP-3 activates other pro-MMPs, including pro-MMP-1 and pro-MMP-9.<sup>21–22</sup> As stated in the introduction, several lines of evidence point to the importance of MMP-9 in the pathophysiology of MS. MMP-3 may contribute to the pathogenesis of MS via its effects on MMP-9 in patients with MS. We recently reported the increased ability of

peripheral blood lymphocytes to degrade laminin in MS, in particular in the acute phase.<sup>23</sup> Laminin is degraded by such MMPs as MMP-7 and MMP-3. The increased degradation ability of blood lymphocytes in MS, therefore, may reflect degradation of laminin by MMP-3.

Increased MMP-3 serum levels have been found in patients with active rheumatoid arthritis, lupus erythematoses, and other connective tissue diseases, as well as glomerulonephritis. Moreover, in rheumatoid arthritis the serum level is correlated with disease activity.<sup>24–26</sup> There are several lines of evidence regarding the relationship between MMP-3 and MS. Elevated MMP-3 expression has been found in the CNS lesions,<sup>27</sup> and elevated mRNA expression by in situ hybridisation in peripheral blood mononuclear cells of patients with MS<sup>28–29</sup> and by flow cytometry in mature dendritic cells which are antigen presenting together with high MMP-3 secretion in culture supernatants.<sup>30</sup> Moreover, compared to pretreatment levels, IFN- $\beta$  treatment decreased expression of the mRNA of MMP-3.<sup>31</sup> In the transgenic mouse model of spontaneous demyelinating disease, in which DM20, the major myelin proteolipid protein in early development, is overexpressed, mRNA and protein levels of MMP-3 were elevated before disease onset.<sup>32</sup> We found elevated MMP-3 in sera from patients with MS obtained around relapse, indicative of a correlation with disease activity, whereas corticosteroid treatment might affect serum MMP levels during recovery from a relapse. This is consistent with

published findings described above on the relationship between MMP-3 and MS and supports MMP-3 activity having an important pathogenic role in MS.

There have been studies on the relationship between MMPs and MS, but few provide longitudinal data on MMPs before and after relapse. Waubant *et al* reported that serum MMP-9 and TIMP-1 levels are related to MRI activity in MS,<sup>11</sup> but only showed longitudinal MMP-9 data before and after relapse for one patient.

We were unable to demonstrate a correlation between serum MMP-3 levels from patients with MS and MRI activity. There are two possible explanations: (i) in this study only 29 MRI scans were performed on the day when sera were collected for MMP-3 assays, so our numbers may be too small to show significant correlation; and (ii) our MMP-3 assay may not be sensitive enough to reflect changes in activity in small subclinical relapse.

This is the first report that serial change in peripheral blood MMP levels affects clinical MS relapse and that there is a transient increase in serum MMP-3 at the time of relapse. Serum MMP-3 levels are correlated with clinical disease activity in relapsing-remitting MS and can serve as a marker of relapse.

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