Elevated levels of MMP-9 and TIMP-1 in the cerebrospinal fluid of neuro-Behçet's disease

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This project was supported by the "Ministère de l'Ensenseignement supérieur de la Recherche et de la Technologie" of Tunisia.

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Received on February 17, 2009; accepted in revised form on June 25, 2009.

Clin Exp Rheumatol 2009: 27 (Suppl. 53): S52-S57.

Key words: Inflammatory sites, neuro-Behçet's disease, matrix metalloproteinase, cerebrospinal fluid.

Competing interests: none declared.

ABSTRACT

Matrix metalloproteinases (MMP-) are involved in leukocyte invasion into the central nervous system (CNS) during inflammation. In a retrospective cohort study of 18 neuro-BD patients, CSF samples were studied for MMP-9, TIMP-1 and cell characteristics in neuro-BD patients compared to 12 Headache attributed to BD (HaBD) patients, 15 multiple sclerosis (MS) and 20 Non-inflammatory Neurological Disease (NIND) patients. Concentrations of MMP-9 and TIMP-1 were measured in CSF by using an enzyme-linked immunosorbent assay (ELISA). The MMP-9/TIMP-1 ratio was significantly increased in neuro-BD group (mean ± SD: 0.145±0.045) compared to (HaBD) (0.065±0.029; p=0.0001) and NIND patients (0.070±0.031; p=0.0001). No significant differences were observed between neuro-BD and MS patients. A significant correlation was observed between CSF-PMN cells and MMP-9 in neuro-BD patients (r=0.714; p=0.0009), indicating probably that PMN cells were in part the source of MMP-9. A significantly positive correlation was also observed between MMP-9 and CSF-mononuclear cells in neuro-BD patients (r=0.623; p=0.0012).

This is the first study to evaluate the expression of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinase-1 in cerebrospinal fluid of neuro-BD patients. It demonstrates increased matrix metalloproteinase-9/tissue inhibitors of metalloproteinase-1 ratio. The results suggested that MMPs released in the CSF may be involved in the pathogenesis of neuro-BD by promoting local damage, similarly as suspected in other inflammatory diseases.

Introduction

Behçet's disease (BD) is a chronic, recurrent and inflammatory disorder characterized with recurrent attacks of oral and genital aphtous ulcerations, uveitis, skin lesions and pathergic skin reaction. While numerous tissue types such as blood vessels, eye, skin, mucosa, joint, or lungs may be affected during the course of BD, central nervous system (CNS) involvement, usually named as neuro-Behçet's disease (neuro-BD), remains one of the most serious complications of the disease (1-2).

Pathological examination of the CNS of neuro-BD patients shows non-specific inflammatory reaction with chronic lymphocytic and neutrophilic infiltration and multifocal necrotic foci, predominantly in the brainstem and basal ganglia (1).

A number of factors play a role in the central nervous system (CNS) leukocyte invasion, namely chemokines, cytokines, adhesion molecules and also matrix metalloproteinases (MMP), which are zinc-containing endopeptidases that degrade extracellular matrix. Recently this growing family of proteolytic enzymes (3) could be categorized by substrate specificity into functional groups. Gelatinases (MMP-2 and MMP-9) seem to be involved in leukocyte trafficking within the CNS (4). They are secreted as zymogens (pro-MMP) that are activated by a variety of factors (5). Matrix metalloproteinases are involved in many physiological processes that take place in the CNS, some of them potentially harmful. The activity of the metalloproteinases is regulated at several different levels (6): gene expression, proenzyme activation, and by the activity of the tissue inhibitors of matrix metalloproteinases (TIMPs). Recently Pay *et al*. reported that matrix metalloproteinases (MMP) and particularly MMP-9 can be used as an activity indicator for Behçet's patients (7).

In the current study, we evaluated CSF concentrations of active and pro-matrix metalloproteinase 9 (total MMP-9) and its binding tissue inhibitor, TIMP-1, in active BD patients with neurological manifestations.

Materials and methods

Patients

The 18 Behçet's disease (BD) patients with neurological disorders included in our study fulfilled the diagnostic criteria of the International Study Group for BD (8). Parenchymal neurological diagnosis was made according to previously published clinical parameters (9-11). Cranial magnetic resonance imaging (MRI) and magnetic resonance venography (MRV) were systematically realized in case of neuro-BD. Patients characteristics are presented in Table I. CSF samples were obtained after the patients had given their written informed consent, as approved by the Medicine University Institutional Review Board. The duration of BD ranged from 8 months to 10 years. Patients with BD who have headache without other neurological symptoms and signs or abnormalities on neuro-imaging or in the CSF are not defined as having neuro-BD (11).

A first control group studied was composed of 12 BD patients with only headache, and not considered as neuro-BD "Headache attributed to BD: (HaBD)" (mean age: 30.4±8.2 years; range: 27-39). This type of headache should neither be accompanied by any focal or diffuse neurological signs or magnetic resonance imaging (MRI) findings nor fulfilling the HIS criteria for primary headaches (12). Two other control groups consisted of age- and sex-matched individuals: 15 multiple sclerosis patients (MS) (mean age: 32.7±7.8 years; range: 22-41) with clinically definite relapsing-remitting multiple sclerosis according to Poser criteria (13). Twenty patients with non-inflammatory neurologic disease (NIND) composed of 14 patients with dementia, and 6 patients with stroke (mean age: 40.6±6.5 years; range: 28-44).

CSF analysis

Total CSF leukocyte numbers were done manually using Fuchs-Rosenthal counting chamber and crystal violet dye, followed by cytological staining to look for polymorphonuclear leukocyte (PMN) predominance. Protein concentration was measured by precipitating the proteins with sulphosalicylic acid and comparing the absorbance of the turbidity at 640 nm with that of protein standards. CSF samples were centrifuged, frozen, and stored at –80°C for further measurements.

Enzyme-linked immunosorbent assay (ELISA)

Concentrations of MMP-9 and TIMP-1 in previously frozen CSF samples were determined by using commercially available enzyme-linked immunoassays (Quantikine human MMP-9 (total) and Quantikine human TIMP-1; R&D Systems, Minnneapolis, MN) with the use of an ELx 800 Automated Microplate Reader, Bio-Tek Instruments (Winooski, VT). The assays were performed according to the manufacturer's instruction, and CSF MMP-9/TIMP-1 concentrations were estimated from standard curves made for each assay. The intra- and inter assay precision coefficients of variation (in percent)

Table I. Clinical features in patients with Behçet's disease.

	Age/Sex	Oral ulcers	Genital aphthosis	Ocular involvement	Erythema nodosum	Arthritis/ arthralgia	Positive pathergy test	Hemisphere involvement	Brainstem involvement	Spinal cord involvement
1	22 /M	+	+	+	-	-	+	+	+	-
2*	35 /M	+	+	+	-	+	+	-	-	+
3*	34 /M	+	-	+	+	+	+	+	+	-
4	32 / M	+	+	-	+	+	+	+	+	-
5*	36 /M	+	-	+	+	+	+	-	+	+
6	38 / F	+	+	-	+	+	+	-	+	-
7	36 / M	+	-	+	+	+	-	+	-	-
8*	39 / M	+	+	+	-	+	+	+	-	-
9	38 / M	+	-	+	+	+	+	+	-	-
10*	38 /M	+	+	+	-	+	+	-	+	-
11*	37 / M	+	+	-	+	+	-	+	-	-
12	39 / F	+	+	+	+	-	-	+	-	-
13*	36 / M	+	+	+	-	+	+	+	-	-
14	42 / M	+	-	+	+	+	-	+	-	-
15*	34 / F	+	+	-	+	+	+	+	-	-
16*	22 / M	+	+	+	+	+	+	+	-	-
17*	30 / M	+	+	-	+	+	+	-	+	-
18*	23 / M	+	+	+	+	+	+	+	-	-

All the patients have neurological involvement. They were selected according to the recent report of Houman *et al*. (10) and the report of Al-Araji & Kidd (11). Firstly we have studied 32 patients with neuro-BD, but after revision only 18 patients responded to the criteria of parenchymal neuro-BD (as special caution needs to be applied in diagnosing neuro-BD above the age of 50 years; exclusion of more common neurological disorders, particularly stroke and non-specific changes in white matter on cranial MRI), is very important as reported by Al-Ariji & Kidd [11]. CSF constituents of these patients are altered. CSF protein is raised (median: 0.51 mg/mm^3; range: 0.37-3.79) and oligoclonal bands are usually absent. The CSF cell count is often prominently raised (Table II) and there is usually a CSF neutrophilia replaced sometimes by a lymphocytosis in 75% of the cases. MRI commonly showed hyperintense T2 weighted lesions (plaques) while brain MRV showed no abnormalities as recently reported. Patients without any poor prognostic factor, azathioprine or methotrexate and corticosteroids are recommended as the first-line treatment. For high-risk patients (*), intravenous cyclophosphamide and corticosteroids was used.

were as follows: MMP-9 <2.9/<7.9; TIMP-1 <5.0/<4.9. The minimum detectable dose is 0.156 ng/mL for MMP-9 and 0.08 ng/mL for TIMP-1.

Statistical analysis

Data were analyzed using non-parametric statistical tests. Correlations of levels of MMPs with CSF cellularity and protein concentration were calculated with the Spearman rank test. The concentrations of MMP-9 and the concentrations between different disease groups were compared using the Mann-Whitney U-test and the Kruskal-Wallis test. The paired sign test was used to compare individual levels of PMN-MMP-9. *P*-values <0.05 were considered significant.

Results

The CSF cell count on neuro-BD patients and control groups are displayed in Table II. Neuro-BD patients and MS patients exhibited increased cell count, increased percentage of mononuclear cells and polymorphonuclear cells compared to NIND. Significant differences were also observed between neuro-BD and HaBD patients.

MMP-9 and TIMP-1 expression in neuro-Behçet's disease

MMP-9 and TIMP-1 were detected in 18 neuro-BD patient, in 15 MS patients, in 12 patients with HaBD and in 20 patients with NIND patients (Fig. 1A; Fig. 1B). Concentrations of MMP-9 were significantly elevated in neuro-BD (mean±SD: 3.68±1.47ng/ml; *p=0.0001*) compared to HaBD patients (0.64±0.21 ng/ml) and NIND patients (0.71±0.26 ng/ml). Patients with MS exhibited slightly higher levels of MMP-9 (4.07±0.97 ng/ml) compared to neuro-BD patients (*p=0.028*). No significant differences were observed between NIND and HaBD patients (*p=0.394*). Concentrations of TIMP-1 greatly exceeded concentrations of MMP-9 and were elevated in neuro-BD (mean ± SD: 24.72±8.45 ng/ml) and MS patients (33.0±9.56 ng/ml) compared to HaBD (11.08±4.66 ng/ml) and NIND patients (10.77±3.01 ng/ml) (*p=0.0001*). There was no difference between NIND and HaBD patients (*p=0.821*).

Table II. CSF laboratory findings in neuro-Behçet's disease (neuro-BD). Cerebrospinal fluid (CSF) cell count, percentage of mononuclear cells and CSF total protein in patients with neuro-BD, in Behçet's disease patients without neurological involvement (headache attributed to BD (HaBD)], in multiple sclerosis (MS) and in non-inflammatory neurological disease (NIND) patients. (Values are expressed as mean ± SD). †significantly different from HaBD and NIND patients, $p<0.005$; ††values not significantly different from values observed in neuro-BD patients.

	Neuro-BD (n=18)	HaBD (n=12)	MS (n=15)	NIND (n=20)
CSF cell count (no. per mm^3)	279 ± 153†	50.7 ± 12.6	254 ± 72††	1.2 ± 0.5
CSF mononuclear cells (%)	58.7 ± 13.4†	12.6 ± 3.8	62.4 ± 34.8††	8.6 ± 4.2
CSF polymorphonuclear cells (%)	5.4 ± 1.8†	0.4 ± 0.23	5.7 ± 2.7††	0.23 ± 0.02
CSF total protein (mg/dl)	44 ± 32†	21 ± 4.5	53.9 ± 18.6††	22 ± 14

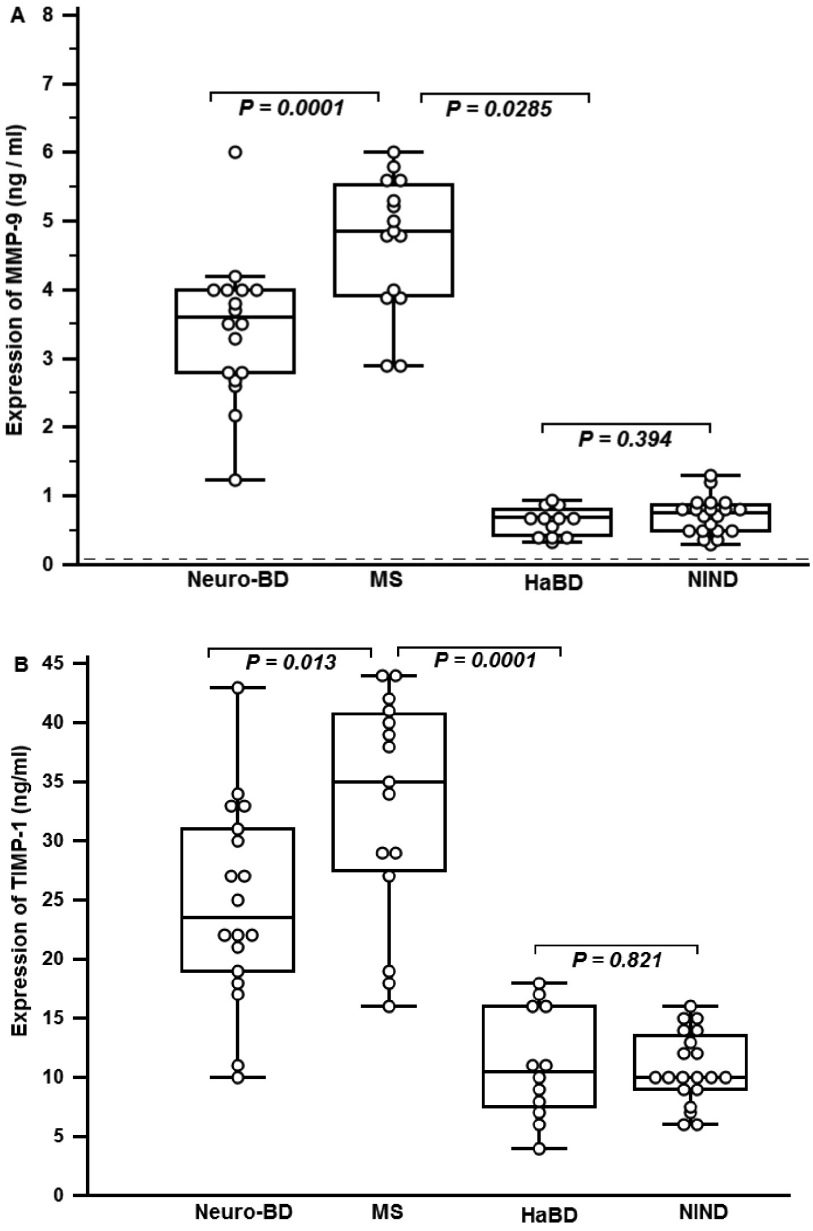

Fig. 1. Concentrations of MMP-9 (**A**) and TIMP-1 (**B**) in cerebrospinal fluid (CSF) in neuro-Behçet's (Neuro-BD) patients, in multiple sclerosis (MS) patients, in headache attributed to BD (HaBD) and in Non-Inflammatory Neurological disease (NIND) patients as measured by using ELISA. Dotted lines indicate the detection limits of the tests used.

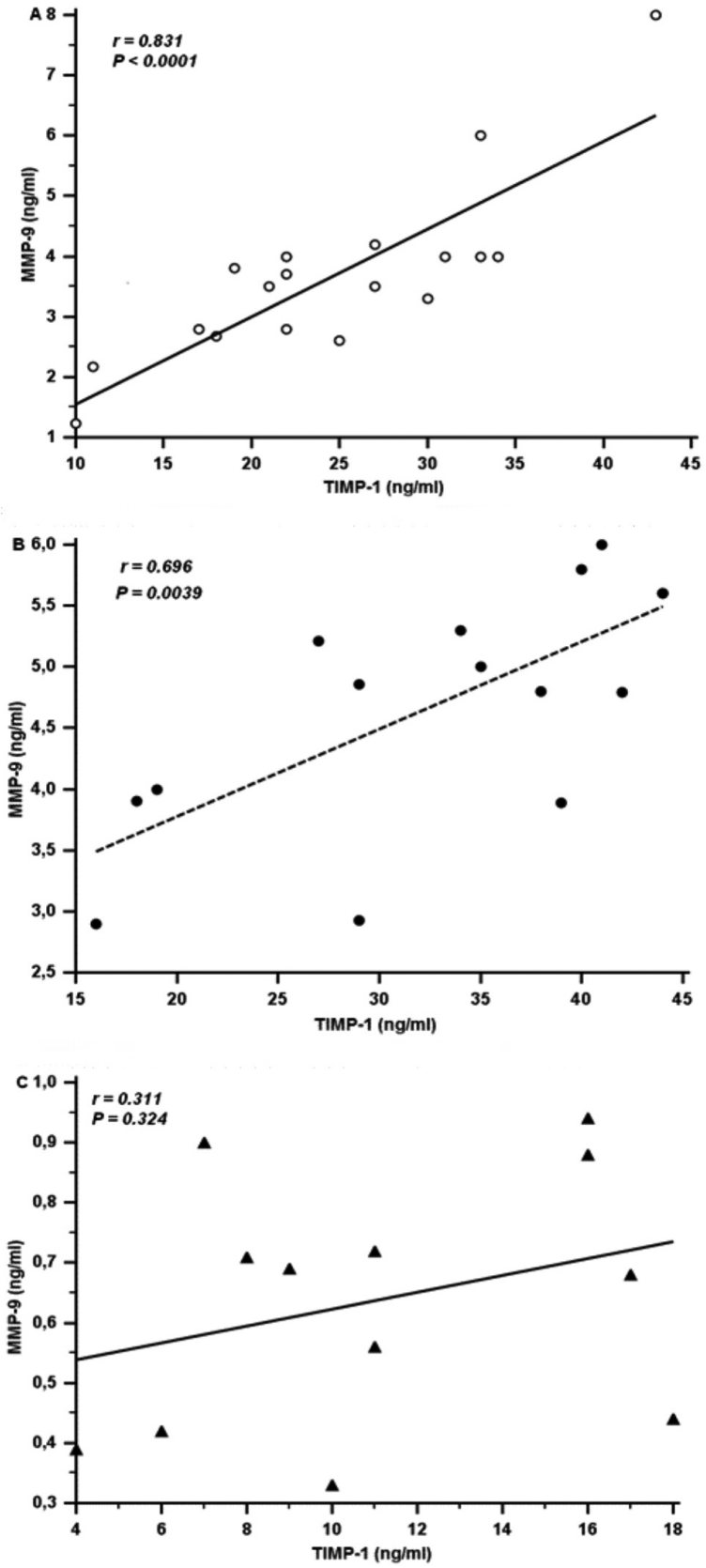

Fig. 2. Correlation between concentrations of MMP-9 and TIMP-1 cerebrospinal fluid in 18 patients with neuro-Behçet's (**A**), in 15 multiple sclerosis patients (MS) (**B**) and in 12 patients with headache attributed to BD (HaBD) (**C**). MMP-9 was significantly correlated with TIMP-1 in cerebrospinal fluid cells (CSF) from neuro-BD and in MS patients. Spearman correlation coefficient (Neuro-BD: 0.831; MS: 0.696; HaBD: 0.311).

Significant differences were observed in the MMP-9 and TIMP-1 expression between neuro-BD and HaBD patients (*p=0.0001*). Increased values of MMP-9 and TIMP-1 were specific to inflammatory lesions.

Correlations and MMP-9/TIMP-1 ratio

Significant positive correlations were observed between MMP-9 and TIMP-1 in neuro-BD patients (r=0.831, *p=0.0001*) and MS patients (r=0.696, *p=0.0039*) (Figs. 2A, 2B). By contrast no correlation was observed in HaBD (r=0.311; *p=0.324*) and NIND patients (r=0.311; *p=0.324*) (Fig. 2C).

MMP-9/TIMP-1 ratio was increased in neuro-BD patients (mean±SD: 0.145±0.045) and in MS patients (0.150±0.040) compared to NIND patients (0.070±0.031; *p=0.0001*) (Fig. 3). Patients with neuro-BD showed a higher ratio than the HaBD ones (0.065±0.029; *p=0.0001*).

MMP-9 in patients with neuro-BD patients appeared to be associated with CSF PMN and mononuclear cells as we observed a significant correlation between MMP-9 and PMN (r=0.714; p=0.0009) (Fig. 4) and mononuclear cells percentages (r=0.623; p=0.0012). TIMP-1 was not correlated with any CSF cellular compound (PMN cells: *r=0.205; p=0.414*; cell count: *r=0.227; p=0.486*; mononuclear cells: *r = 0.361; p=0.387*) in neuro-BD patients.

The level of MMP-9 correlate with total protein concentration in neuro-BD patients (r=0.490; p=0.023) and in MS patients (r=0.582; p=0.016). It was not the case in NIND (r=0.162; p=0.743) and HaBD patients (r=0.239; p=0.493).

Discussion

This study found a significant increase of MMP-9 in CNS of patients with neuro-BD compared to HaBD and to NIND patients. Similar values were observed between neuro-BD and MS patients. Previous work has shown that MMP-9 levels are elevated in the CNS of infectious diseases of viral, bacterial or fungal origin (14-15). The etiology of Behçet's disease does not exclude an infective trigger, which could operate

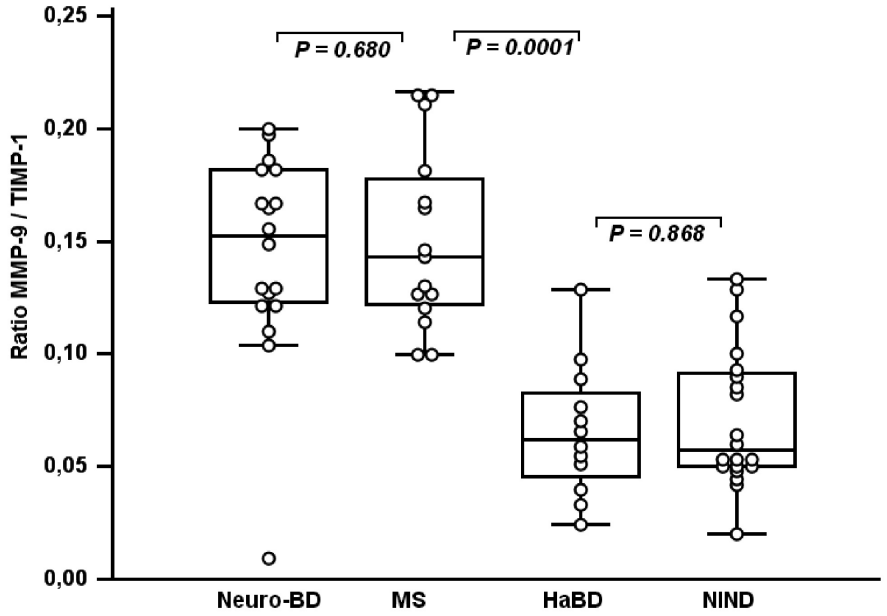

Fig. 3. Ratio of cerebrospinal fluid MMP-9 and TIMP-1 (MMP-9 / TIMP-1) in neuro-Behçet's (Neuro-BD) patients, in multiple sclerosis (MS) patients, in headache attributed to BD (HaBD) and in Non-Inflammatory Neurological disease (NIND) patients. Significant difference was observed between Neuro-BD patients and HaBD patients (*p=0.0001*).

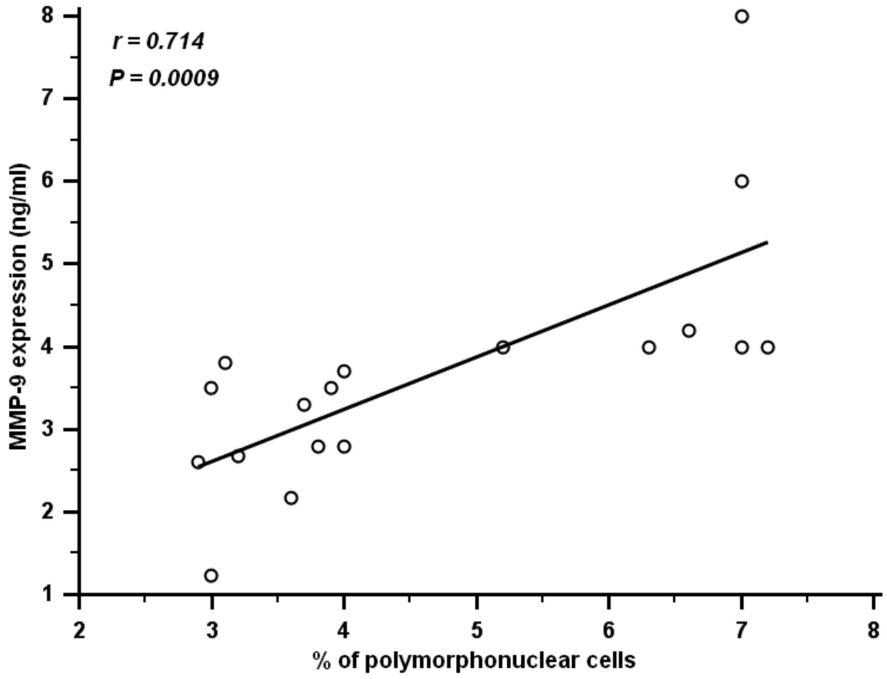

Fig. 4. Correlation of matrix metalloproteinase (MMP-9) and the percentage of PMNL in CSF of patients with neuro-BD patients (*r=0.714; p=0.0009*).

through molecular mimicry (16-18) or some other mechanism, but implies that the disease is perpetuated by an abnormal immune response to an autoantigen in the absence of ongoing infection (17). In Neuro-BD, proinflammatory cytokines (IL-1, IL-6, and TNF-alpha) were found increased both in serum and in inflammatory sites. These studies indicate that the continued hypothetic presence of HSV-DNA contributes to angiogenesis and inflammation in BD, as HSV-DNA elicits the production of angiogenic factor VEGF and MMP-9 (19).

The increased levels of MMP-9 in the CSF of neuro-BD are correlated with inflammatory cells number. On one hand, this fact can be due to MMP-9 facilitating action on leukocyte migration through the basement membrane into the inflammatory site. A recent *in vitro* study has demonstrated that neutrophils use MMP-9 and elastase to migrate across the basement membrane (20). On the other hand, high levels of several cytokines: interleukin 6, 8, 10, interferon-γ and TNF-α, a well-recognized stimulator of MMP-9 production (21-24) have been described in neuro-BD patients CSF, suggesting an association (25). Mononuclear cells and particularly PMN cells, present in the CSF of neuro-BD patients during inflammation, seemed to be the main source of MMP-9. CNS-resident cells (astrocytes, oligodendrocytes and microglia) (26) may also serve as sources of MMP-9, leading to elevation of CSF levels regardless of pleocytosis. Obeying the law of protein filtration at the BBB (6), BBB impairment influences MMP-9 concentration in neuro-BD. More investigations are needed to confirm that MMP-9 elevation is predominantly dependent on cell accumulation in CSF. It might also be possible that other MMPs (more than 15 are known) than MMP-9 are involved in cell extravasation and BBB disturbance.

Imbalance of the MMP/TIMP ratio may contribute to CNS pathology conditions during inflammation. A significant increase in MMP-9/TIMP-1 was seen in neuro-BD and MS patients, in contrast to non-inflammatory control groups. In a mouse model of CNS infection, the imbalance of MMP and TIMP was observed mostly in the brain regions affected by the viral agent (27). We found a positive correlation between MMP-9 and TIMP-1 levels in neuro-BD patients but not in NIND or BD patients free from any CNS inflammation. The ratio of matrix metalloproteinase-9 and tissue inhibitors of metalloproteinase-1 may play a role as a diagnostic tool of inflammation.

A converging body of evidence supports the idea that MMP are crucially involved in a variety of neuropathologies, including CNS infection. Matrix metalloproteinases, especially MMP-9,

have been shown to degrade components of the basal lamina and disrupt the blood-brain barrier (BBB) and thus, contribute to neuroinflammation (28). These observations could be integrated into the inflammatory schema of Behçet's disease, involving Th1 activation and Treg hyper stimulation in the CNS (29). Our present results are in accordance with the reported results from Pay *et al*. (6). Still, much is to be learned about the function of these enzymes. Their impact on the understanding of the pathophysiology of BD and on the practical management of BD patients remains to be established.

Acknowledgments

The authors wish to thank Professor Fayçal Hentati (Department of Neurology, La Rabta Neurology Institute, Tunis University) for his help in the collection of CSF samples from neuro-Behçet's disease and the control diseases. The authors also want to thank the patients for their support.

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