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Serum matrix metalloproteinase-9 (MMP-9) as a biomarker in paediatric and adult tick-borne encephalitis patients

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ABSTRACT

Matrix metalloproteinases (MMPs) play an important role in central nervous system infections. We analysed the levels of 8 different MMPs in the cerebrospinal fluid (CSF) of 89 adult patients infected with tick-borne encephalitis (TBE) virus and compared them with the levels in a control group. MMP-9 was the only MMP that showed significantly increased CSF levels in TBE patients. Serum MMP-9 levels were subsequently measured in 101 adult TBE patients at various time points during the neurological phase of TBE and at follow-up. In addition, serum MMP-9 was analysed in 37 paediatric TBE patients. Compared with control levels, both paediatric and adult TBE patients had significantly elevated serum MMP-9 levels. In most adult patients, serum MMP-9 levels peaked at hospital admission, with higher serum MMP-9 levels observed in patients with encephalitis than in patients with meningitis. Elevated serum MMP-9 levels were observed throughout hospitalisation but decreased to normal levels at follow-up. Serum MMP-9 levels correlated with clinical course, especially in patients heterozygous for the single-nucleotide polymorphism rs17576 (A/G; Gln279Arg) in the *MMP9* gene. The results highlight the importance of MMP-9 in the pathogenesis of TBE and suggest that serum MMP-9 may serve as a promising bioindicator of TBE in both paediatric and adult TBE patients.

1. Introduction

Each year, more than 10,000 people living mainly in Central, Northern, and Eastern Europe and Northeast Asia become infected with tick-borne encephalitis virus (TBEV). TBEV is a flavivirus (genus *Flavivirus*, family *Flaviviridae*) that causes tick-borne encephalitis (TBE), a major tick-borne viral disease of humans that is potentially fatal (Ruzek et al., 2019). Global climatic changes, human-induced environmental changes, and socioeconomic factors are thought to contribute to the increase in TBE cases in endemic regions during recent decades. In Europe, TBEV is predominantly transmitted by *Ixodes ricinus* ticks, while in Asia *I. persulcatus* ticks are its dominant vector (Korenberg, 2009). Next to transmission by ticks, humans can also be infected with TBEV after consuming unpasteurized milk or dairy products from infected ruminants (Kríz et al., 2009; Salat and Ruzek, 2020). TBE can have various clinical manifestations, from a mild flu-like disease to life-threatening encephalitis or encephalomyelitis (Kohlmaier et al., n. d.). In general, TBE has a more favourable course and prognosis in children than in adults (Bogdanavičienė et al., 2022; Krbková et al., 2015; Rostasy, 2012), although severe cases can also be rarely observed in children (Krbková et al., 2021). However, long-term outcomes in childhood after TBE may be unfavourable due to cognitive problems

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associated with impaired brain activity (Palyga-Bysiecka et al., 2022; Schmolck et al., 2005). Typically, TBE has two phases. After the incubation period, which in most cases lasts between 7 and 14 days, the first phase occurs, characterized by nonspecific, mild febrile illness. More than half of patients develop the second phase with neurological symptoms, it is approximately 65% in adults (Kohlmaier et al., n.d.; Ruzek et al., 2019), and 58% to 70% in children (Krbková et al., 2015; Lesnicar et al., 2003). Detection of TBEV-specific IgM and IgG antibodies in serum and/or CSF is crucial for TBE diagnosis (Ergunay et al., 2016; Taba et al., 2017). Detection of TBEV RNA in blood is also possible in the first phase of infection (Saksida et al., 2005). Several biomarkers of TBE have been identified (Gudowska-Sawczuk and Mroczko, 2021). For example, we recently found that serum and CSF phosphorylated neurofilament heavy chain subunit could serve as a marker of neuroaxonal damage in the later stages of TBE (Fortova et al., 2022). However, biomarkers for the early stage of the neurological phase of TBE are needed for prognostic and therapeutic purposes (Gudowska-Sawczuk and Mroczko, 2021). In addition to various cytokines or chemokines, metalloproteinases also appear to play an important role in TBE pathogenesis and could therefore serve as promising biomarkers for the early neurological phase of TBE (Gudowska-Sawczuk and Mroczko, 2021).

Matrix metalloproteinases (MMPs) are a family of zinc-dependent enzymes capable of degrading extracellular matrix proteins. Depending on their substrate specificity, they can be divided into collagenases (MMP-1, MMP-8, and MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, and MMP-11), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MMP-14, MMP-15, MMP-16, MMP-17, MMP-24, and MMP-25), enamelysin (MMP-20), and metalloelastase (MMP-12), or have other functions (MMP-19, MMP-21, MMP-23A, MMP-23B, MMP-27, MMP-28) (Behl et al., 2021; Goetzl et al., 1996; Leppert et al., 2001). MMPs play several important roles in normal and pathological processes, including inflammation, CNS recovery and regeneration, regulation of blood–brain barrier (BBB) function, promotion of influx of immunocompetent cells into the CNS, etc. (Behl et al., 2021; Goetzl et al., 1996; Leppert et al., 2001; Niranjan et al., 2021). Almost every human inflammatory disease is thought to be characterized by upregulation of MMPs (Niranjan et al., 2021). We have previously found that TBE patients have elevated serum levels of MMP-9 (Palus et al., 2014b), and Chinese scientists have found elevated MMP-9 levels in the CSF of patients infected with Far Eastern TBEV and that CSF MMP-9 levels correlate with disease severity (Kang et al., 2013). MMP-9 is largely involved in brain physiology and pathology (Vafadari et al., 2016). There, it is released from various types of cells, including neurons, glia, and leukocytes (Vafadari et al., 2016). Under physiological conditions, the production of MMP-9 is very low in the brain, but it is markedly increased following various pathological insults, including inflammation in response to a CNS infection (Vafadari et al., 2016). The importance of MMP-9 in the pathophysiology of TBE was further emphasised in a genetic study of Russian TBE patients. It was found that a single-nucleotide polymorphism (SNP) in the *MMP9* gene may influence the genetic predisposition to severe forms of this disease (Barkhash et al., 2018). However, the roles of other MMPs in TBE pathophysiology are unknown. It remains to be investigated whether MMP-9 is the only MMP upregulated during TBE and to what extent serum or CSF MMP-9 can be used as a biomarker for severe TBE.

The aim of this study was to measure the concentrations of various MMPs (namely MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13) in the CSF of TBE patients. In addition, serum MMP-9 levels were investigated and the kinetics of serum MMP-9 levels during the neurological phase of TBE and the convalescent phase were analysed, as was the correlation of serum MMP-9 levels with the different clinical manifestations of TBE. Serum MMP-9 levels were compared between adult and paediatric TBE patients. Finally, associations between the *MMP9* SNP and predisposition to severe forms of TBE, and between the SNP and different MMP-9 levels in serum and CSF in Czech TBE patients, were investigated.

2. Patients and methods

2.1. Ethics statement

The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the University Hospital in Brno (date of approval: 27 June 2018). All patients or their parents agreed to participation in the study and signed informed consent.

2.2. Patients

Patients and the control group were prospectively recruited in 2018–2021. The diagnosis of TBE was made according to the case definition and based on the following criteria: (i) the presence of clinical signs of meningitis, meningoencephalitis, or meningoencephalomyelitis; (ii) CSF pleocytosis (>5 cells/μl); and (iii) the presence of TBEV-specific IgM and IgG antibodies in serum or TBE-specific IgM antibodies in CSF (Taba et al., 2017). None of the patients had been vaccinated against TBE. All patients lived in a TBE-endemic region of South Moravia, Czech Republic.

2.3. CSF sampling

CSF samples were obtained from 89 adult patients with TBE (aged 18–84 years, median 49 years; 50 male) on admission to the hospital (or within 24 h from admission). Patients were divided into two subgroups according to the severity of TBE (final diagnosis): patients with meningitis (TBE-mening; $n = 44$, 23 male; age 18–77 years, mean 44.6 years) and patients with CNS involvement (TBE-enc, i.e., meningoencephalitis, meningoencephalomyelitis; $n = 45$, 27 male; age 21–84 years, mean 52.0 years). CNS involvement was diagnosed on the basis of disturbances of consciousness and/or focal neurological signs noted on neurological examination. Data were collected prospectively. Control groups consisted of 28 age-matched patients with acute aseptic meningitis of other etiologies (matched positive controls; non-TBE meningitis, mostly cases of neuroborreliosis or varicella-zoster meningitis) and 71 age-matched controls with initial suspicion of CNS infection later excluded on the basis of CSF analysis for intrathecal synthesis of specific IgM and IgG anti-*Borrelia* antibodies, polymerase chain reaction (PCR) examination of CSF for other relevant viruses, and serum C-reactive protein analysis (unaffected controls). CSF samples were collected by lumbar puncture, centrifuged, and frozen at −80 °C until analysis. Concentrations of MMP-3, MMP-12, and MMP-13 in CSF were measured using Milliplex MAP Human MMP Magnetic Bead Panel 1 (Cat. No. HMMP1MAG-55 K; Millipore), and MMP-1, MMP-2, MMP-7, MMP-9, and MMP-10 were measured using Milliplex MAP Human MMP Magnetic Bead Panel 2 (Cat. No. HMMP2MAG-55 K; Millipore). Assays were performed using a Magpix instrument (Luminex, Austin, TX, USA) according to the manufacturer's instructions.

2.4. Serum sampling

Serum samples were obtained from 101 adult patients (aged 19–86 years, median 47 years), who were also divided into two subgroups (final diagnosis): patients with meningitis (TBE-mening; $n = 50$, 24 male; aged 21–76 years, mean 47 years) and patients with CNS involvement (TBE-enc; $n = 51$, 30 male; aged 19–86 years, mean 51 years). The first serum sample was collected at hospital admission (day 0), the second sample 2 days later (+2 days), the third sample at hospital discharge (4–22 days, mean 11.2 days), and the fourth sample at the first follow-up (26–120 days, mean 56.6 days). Not all samples were available from all patients; there were 78 samples at admission, 70 samples at +2 days, 78 samples at discharge, and 75 samples at follow-up. In 35 patients, all 4 samples were available. Serum samples from 75 healthy blood donors served as controls.

Serum samples were also collected from 37 children (aged 3–17) at hospital admission; the control group consisted of 46 children with acute aseptic meningitis of other aetiology. Serum samples from 35 healthy children served as negative controls. The MMP-9 concentration was measured with the Human MMP9 ELISA kit (Cat. No. BMS2016-2, Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) according to the manufacturer's instructions.

2.5. SNP genotyping

DNA was extracted from whole blood using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The TaqMan SNP Genotyping Assay C_11655953_10 (Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for genotyping the rs17576 SNP (A/G; Gln279Arg) in the *MMP9* gene. Amplification mixtures contained 10 ng of DNA, 1x TaqMan Genotyping Master Mix (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 1x TaqMan SNP Genotyping Assay (Thermo Fisher Scientific, Waltham, Massachusetts, USA) including allele-specific probe and primers labelled with the two allele-specific fluorescent reporter dyes VIC (A allele) and FAM (G allele), and Milli-Q water in a 10 μL reaction. PCR was performed using the LightCycler 480 System (Roche, Hague Rd., Indianapolis, USA) in 96-well plates. The PCR profile was as follows: 10 min at 95 °C and 40 cycles of 15 s at 95 °C followed by 60 s at 60 °C. There were two negative controls on each plate. After amplification, postread allele discrimination analysis was performed.

2.6. Statistical analyses

The data from the CSF samples were log-transformed and analysed using multiple-comparison testing with a two-stage step-up false discovery rate (Benjamini-Krieger-Yekutieli); $Q = 1\%$. The analysis was performed with GraphPad Prism 9 version 9.3.0 (GraphPad Software, La Jolla, CA, USA).

Statistical analysis of data from serum samples was performed with the Mann–Whitney *U* test using GraphPad Prism 7.04 (GraphPad Software, La Jolla, CA, USA). All differences with $p < 0.05$ were considered significant.

Hardy-Weinberg equilibrium was assessed by the χ^2 test. SNP genotypic and allelic frequencies were compared between the studied TBE patient and control groups by χ^2 test using GraphPad Prism 9 version 9.3.0 (GraphPad Software, La Jolla, CA, USA). The difference between two groups was considered significant when the *p*-value was less than 0.05.

3. Results

3.1. Concentrations of MMP-1, 2, 3, 7, 9, 10, 12, and 13 in CSF

CSF samples from 89 adult TBE patients collected on hospital

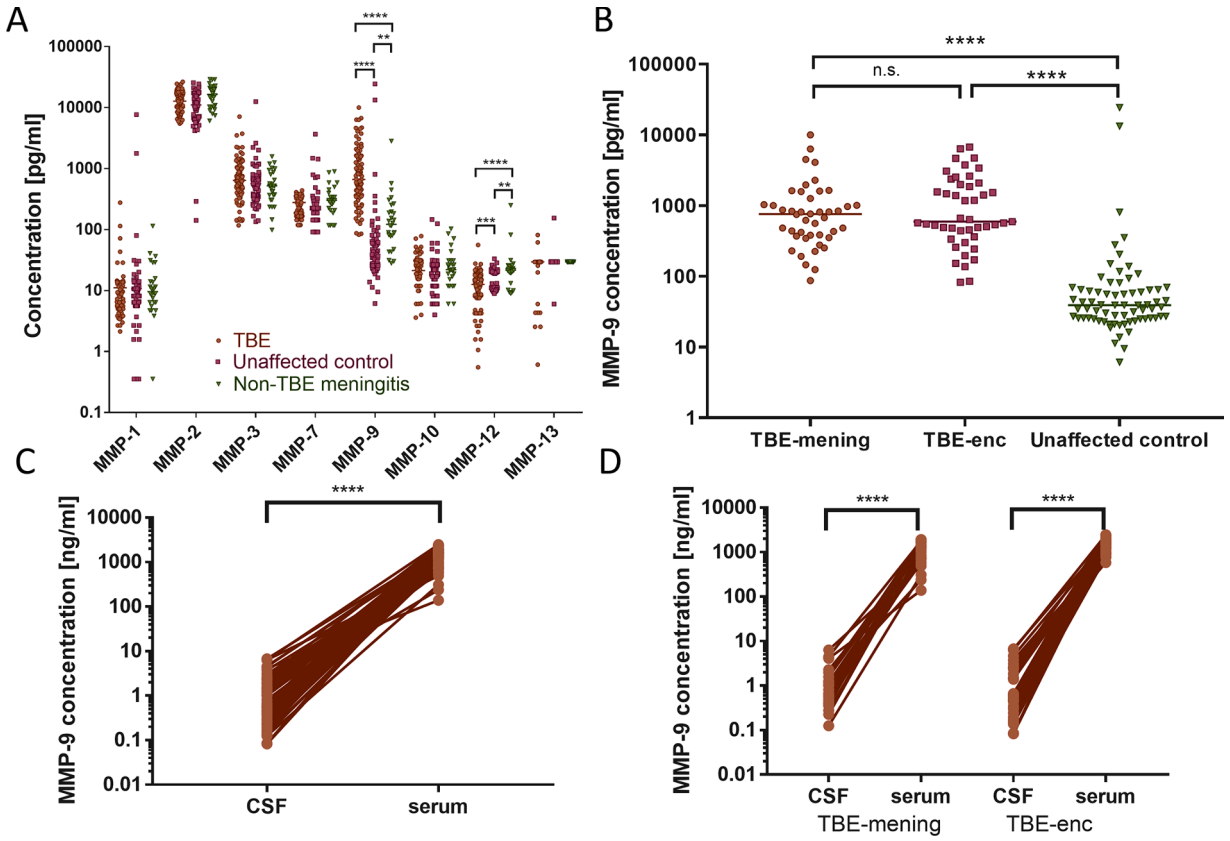

Fig. 1. (A) Levels of MMPs in CSF from adult patients with TBE. CSF samples were collected from adult TBE patients by lumbar puncture at hospital admission. Control groups consisted of patients with acute aseptic meningitis of other aetiology (non-TBE meningitis) and those with initially suspected CNS infection that was excluded on CSF analysis (unaffected controls). The data were log-transformed and compared between the groups (TBE, unaffected control, non-TBE meningitis) using a multiple-comparisons test with a two-stage step-up false discovery rate (Benjamini-Krieger-Yekutieli), $Q = 1\%$. The horizontal lines indicate median values. **(B)** Based on the severity of their TBE, the patients were classified into two subgroups: (i) patients with meningitis (TBE-mening) and (ii) patients with CNS involvement (TBE-enc). The CNS MMP-9 levels were compared amongst TBE-mening, TBE-enc, and non-TBE meningitis and unaffected controls. The data were log-transformed to improve normality and the differences between the groups were statistically tested using an unpaired *t*-test. The horizontal lines indicate median values. **(C)** Comparison of the MMP-9 levels in paired CSF and serum of TBE patients at hospital admission. Data were analysed by Mann-Whitney *U* test. **(D)** Comparison of the MMP-9 levels in paired CSF and serum of TBE-mening and TBE-enc patients at hospital admission. Data were analysed by Mann-Whitney *U* test. n. s., not significant; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$.

admission were analysed for the concentrations of MMP-1, 2, 3, 7, 9, 10, 12, and 13, which were compared with MMP concentrations in samples from 28 patients with acute aseptic meningitis of other aetiology (non-TBE meningitis) and 71 unaffected controls. No statistical differences in the levels of MMP-1, 2, 3, 7, 10, and 13 were observed between TBE patients and non-TBE meningitis patients or unaffected controls ($p > 0.05$) (Fig. 1A). CSF MMP-9 levels were significantly higher in TBE patients than in non-TBE meningitis patients ($p < 0.0001$) and unaffected controls ($p < 0.0001$) (Fig. 1A). CSF levels of MMP-9 were higher in patients with non-TBE meningitis than in unaffected controls ($p < 0.01$) (Fig. 1A). No significant differences in CSF MMP-9 values were found between TBE patients with meningitis and patients with CNS involvement ($p > 0.05$), but MMP-9 levels in each of these two groups differed significantly from the level in unaffected controls ($p < 0.0001$) (Fig. 1B). CSF MMP-12 concentrations were significantly lower in TBE patients than in patients with non-TBE meningitis ($p < 0.0001$) and unaffected controls ($p < 0.001$); however, CSF MMP-12 levels were higher in patients with non-TBE meningitis than in unaffected controls ($p < 0.01$) (Fig. 1A).

3.2. Serum levels of MMP-9 in adult TBE patients

Serum samples were collected from 101 adult TBE patients, and their MMP-9 levels were compared with those of samples from 75 healthy blood donors who served as controls. Samples from TBE patients collected at hospital admission and 2 days later or at hospital discharge all had significantly higher MMP-9 concentrations than did sera from healthy controls ($p < 0.0001$) (Fig. 2A). However, MMP-9 levels in sera

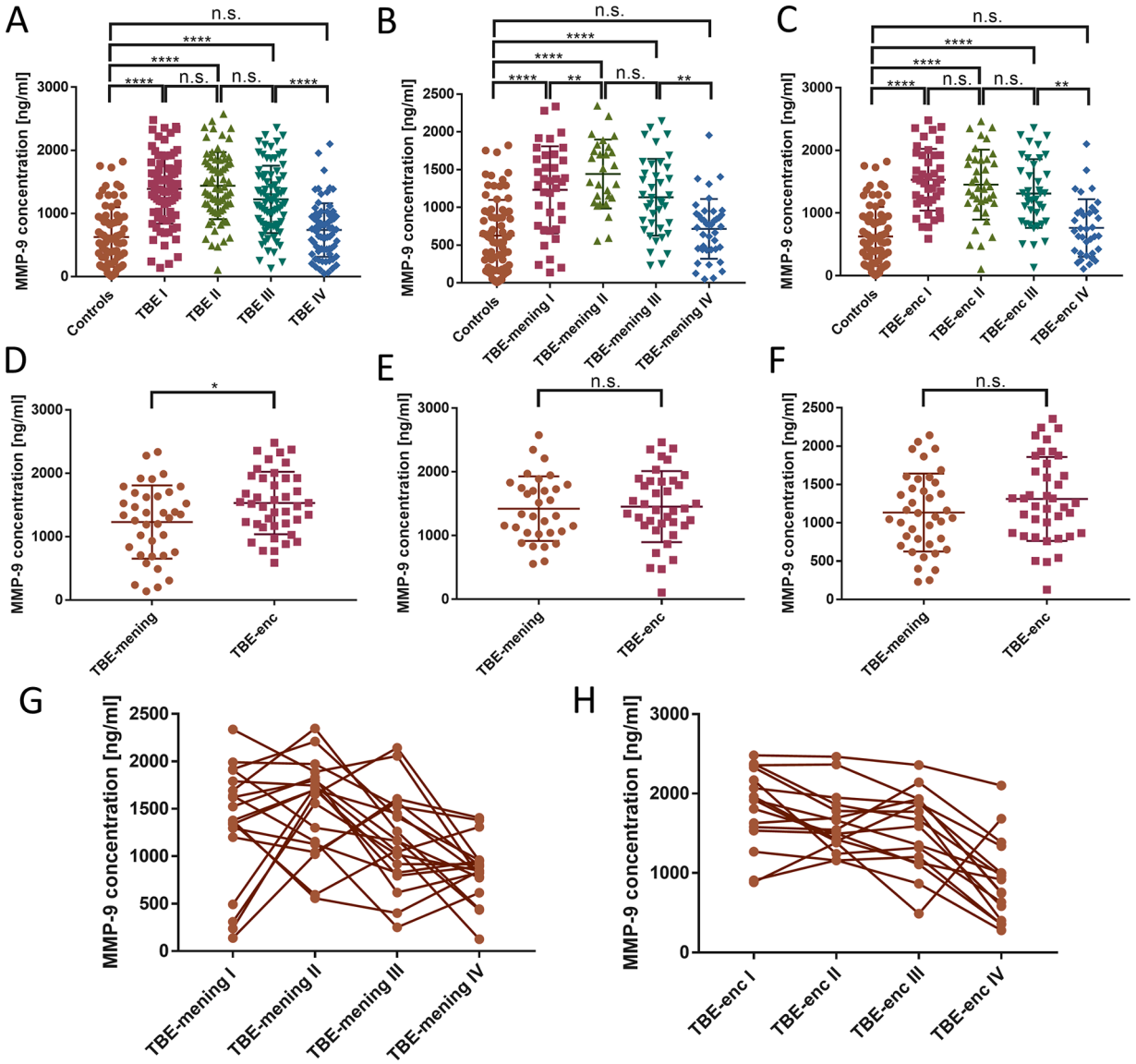

Fig. 2. Serum samples were collected from adult TBE patients and healthy blood donors (controls) and analysed by ELISA for MMP-9 levels. **(A)** Comparison of the MMP-9 levels in sera from controls and sera collected from TBE patients at different time points: hospital admission (TBE-I), 2 days after admission (TBE-II), at hospital discharge (4–22 days; TBE-III), and at first follow-up control (26–120 days; TBE-IV. **(B)** Comparison of serum MMP-9 levels in controls and TBE patients diagnosed with meningitis at different time points: at hospital admission (TBE-mening I), 2 days after admission (TBE-mening II), at hospital discharge (TBE-mening III) and at first follow-up control (TBE-mening IV). **(C)** Comparison of serum MMP-9 levels in controls and TBE patients with encephalitic CNS involvement at different time points: at hospital admission (TBE-enc I), 2 days after admission (TBE-enc II), at hospital discharge (TBE-enc III), and at first follow-up control (TBE-enc IV). **(D-F)** Comparison of serum MMP-9 levels in TBE patients with meningitis (TBE-mening) and with CNS involvement (TBE-enc) at hospital discharge **(D)**, 2 days after admission **(E)**, and at hospital discharge **(F). (G-H)** The kinetics of serum MMP-9 levels in serum samples from TBE-mening **(G)** and TBE-enc **(H)** patients. Data were analysed by Mann-Whitney *U* test or by Dunn's multiple comparison test. n.s., not significant; *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.0001$.

collected at the first follow-up (i.e., 26–120 days after hospitalisation) were similar to those in sera from healthy controls ($p > 0.05$) (Fig. 2A). Similar trends (i.e., significantly elevated MMP-9 levels at admission, during hospitalisation, and at discharge, but not at follow-up) were observed in the group of TBE patients with meningitis (TBE-mening) (Fig. 2B) or with CNS involvement (TBE-enc) (Fig. 2C). Most patients had the highest serum MMP-9 levels on admission or 2 days after admission, and then the serum MMP-9 concentration decreased to physiological levels at follow-up) (Fig. 2G-H). TBE-enc patients had significantly higher serum levels of MMP-9 than did TBE-mening patients on admission ($p < 0.05$) (Fig. 2D), but at later time points no statistical difference in serum MMP-9 concentration was seen between these two groups ($p > 0.05$) (Fig. 2E-F).

When comparing MMP-9 concentrations in serum and CSF, significantly higher concentrations were observed in serum samples, regardless of whether all TBE patients were analysed ($p < 0.0001$) or whether TBE patients were subdivided according to disease pattern (i.e., TBE-mening and TBE-enc; $p < 0.0001$) (Fig. 1C-D).

3.3. Serum levels of MMP-9 in paediatric TBE patients

Children had significantly lower serum MMP-9 levels than did adults, both in controls ($p < 0.0001$) and in TBE patients ($p < 0.0001$) (Fig. 3A). However, significantly higher serum MMP-9 levels were found in paediatric TBE patients than in negative controls ($p < 0.01$) or paediatric patients with acute aseptic meningitis of other etiologies ($p < 0.01$) (Fig. 3B).

3.4. MMP-9 gene single-nucleotide polymorphism analysis

To investigate the association between the SNP in the *MMP9* gene and predisposition to TBE and/or to different clinical forms of TBE, 101 TBE patients and 75 control subjects were analysed to determine genotype and allele frequencies for the SNP rs17576 (A/G; Gln279Arg) (Table 1). No significant differences in genotype or allele frequencies for rs17576 were found between the TBE patient and control groups ($p > 0.05$). Similarly, there was no difference in genotype or allele frequencies between the control group and TBE patients with CNS involvement ($p > 0.05$), but the difference between the control group (41.3%) and TBE patients with meningitis (60.0%) was statistically significant in the A/G genotype group ($p < 0.05$) (Table 1).

Next, we compared MMP-9 levels in CSF and serum in TBE-mening and TBE-enc as a function of genotype. While there was no genotype dependence between CSF MMP-9 levels in TBE-enc and TBE-mening ($p > 0.05$), differences in serum MMP-9 levels between TBE-mening and TBE-enc patients were dependent on *MMP9* genotype. Specifically, there were no differences in serum MMP-9 levels between TBE-mening and TBE-enc patients homozygous for the SNP rs17576; only heterozygotes for this SNP had significant differences in serum MMP-9 levels between TBE-enc and TBE-mening ($p < 0.05$) (Fig. 4).

Table 1
Genotypic and allelic frequencies for *MMP9* rs17576 single-nucleotide polymorphisms in adult TBE patients with different clinical forms and in the population control group.

Genotypes or alleles	Genotype (allele) frequency:% (number)[a]				*p* values [c]
	Control group	TBE patients			
		All	Meningitis	Disease with CNS damage	
rs17576					
GG	20.0 (15)	16.8 (17)	14.0 (7)	19.6 (10)	n.s.
AA	38.7 (29)	28.7 (29)	26.0 (13)	31.4 (16)	n.s.
GA	41.3 (31)	54.5 (55)	60.0 (30)	49.0 (25)	n.s.; <0.05 [d]
G	40.7	44.1	44.0	44.1	n.s.
A	59.3	55.9	56.0	55.9	n.s.
N [b]	75	101	50	51	

[a] Number of subjects with a given genotype.
[b] N, number of individuals.
[c] *p* values were calculated for comparisons between TBE patients (all) and the control group, and for comparisons between TBE patients with disease with CNS damage and TBE patients with meningitis, as well as between TBE patients with meningitis and the control group and TBE patients with disease with CNS damage and the control group.
[d] *p* value for comparison between TBE patients with meningitis and the control group.
n.s., not significant.

4. Discussion

Matrix metalloproteinases (MMPs) are enzymes that have several functions in physiological and pathological processes that occur during CNS infections. One of their functions is to increase BBB permeability by degrading components of the extracellular matrix and tight junctions in endothelial cells forming the BBB (Lakhan and Avramut, 2012; Malemud, 2006). Increased BBB permeability is a hallmark of TBE patients, and this might be linked to MMPs. Previously, we and others found that TBE patients have elevated levels of MMP-9 in their serum or CSF (Kang et al., 2013; Palus et al., 2014b), indicating the importance of MMP-9 in TBE pathophysiology. However, some important questions related to MMP-9 and other MMPs in TBE patients remained open. To our

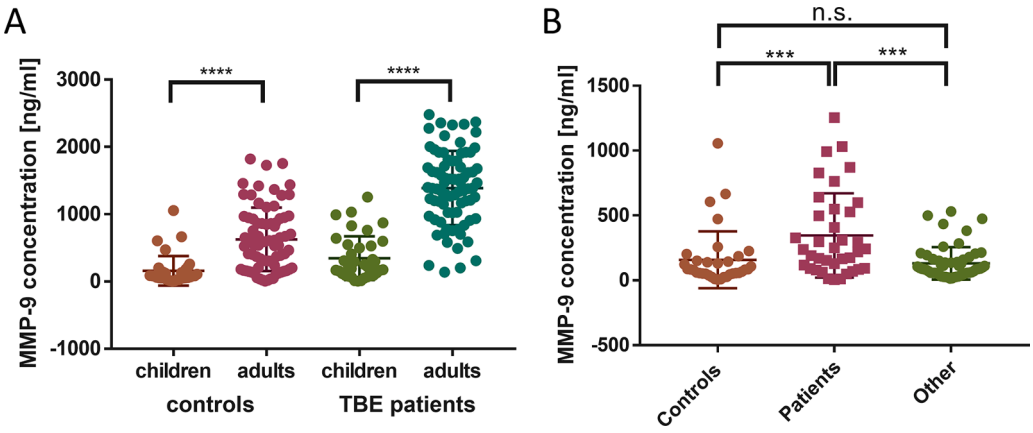

Fig. 3. **(A)** Serum MMP-9 levels in children and adults (controls and TBE patients) were compared. Data were analysed by Mann-Whitney *U* test. **(B)** Comparison of serum MMP-9 levels in paediatric TBE patients, healthy children (controls), and children with acute aseptic meningitis of other aetiology (other). Data were analysed by Mann-Whitney *U* test. n.s., not significant; ***, $p < 0.001$; ****, $p < 0.0001$.

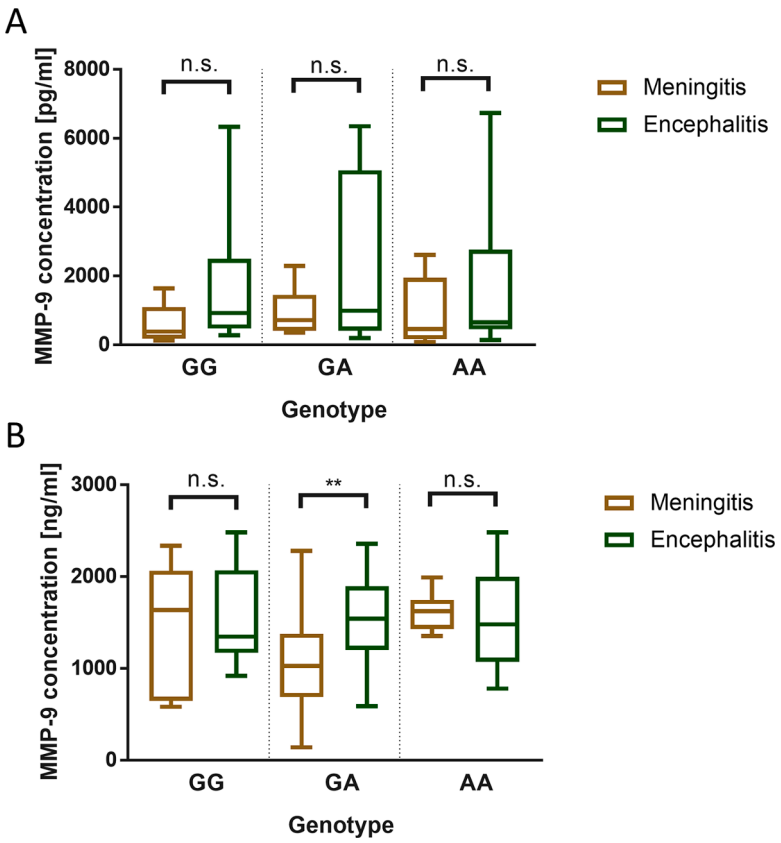

Fig. 4. CSF **(A)** and serum **(B)** MMP-9 levels in adult TBE patients according to their genotype at the rs17576 (A/G; Gln279Arg) single-nucleotide polymorphism in the *MMP-9* gene. CSF and serum samples were collected at admission. Data were analysed by Mann-Whitney *U* test. n.s., not significant; **, $p < 0.01$.

knowledge, this study is the first to investigate CSF levels of various MMPs in TBE patients. It also provides detailed information on serum MMP-9 levels in adult and paediatric patients, and the kinetics of serum MMP-9 levels during the acute and convalescent phases of TBE. In addition, it compares serum MMP-9 levels in TBE patients with meningitis or encephalitic CNS involvement. Finally, it investigates the association between a SNP in the *MMP9* gene and MMP-9 production in TBE patients.

Analysis of MMP-1, 2, 3, 7, 9, 10, 12, and 13 revealed that TBE patients had significantly increased levels of MMP-9 in CSF at hospital admission, whereas levels of MMP-12 were significantly decreased. No other MMPs examined were changed in the CSF of TBE patients. The finding of increased CSF MMP-9 levels is consistent with previous data in patients infected with Far Eastern TBEV (Kang et al., 2013). The authors concluded that elevation of CSF MMP-9 was closely associated with BBB dysfunction, brain inflammation, and disease severity. More specifically, of 72 patients in the Chinese study, 4 died as a result of TBE and those 4 patients had highly elevated levels of CSF MMP-9 (Kang et al., 2013). In our study, we divided the patients into subgroups of TBE patients with meningitis and with encephalitis on the basis of clinical presentation and compared the CSF MMP-9 values between these two groups. No statistically significant difference in CSF MMP-9 was found between the groups, suggesting that the levels of CSF MMP-9 do not allow discrimination between TBE meningitis and TBE encephalitis, and also that the use of CSF MMP-9 as a prognostic marker for the severity of TBE is rather limited.

The pathophysiological basis and consequences of the observed decrease in MMP-12 in the CSF of TBE patients on hospital admission are unknown. MMP-12 is expressed by both inflammatory and CNS resident cells during viral encephalitis (Zhou et al., 2005), but why it is decreased during TBE remains puzzling. In contrast with our study, increased MMP-12 levels were seen in CSF from COVID-19 patients with neurological complications (Mohammadhosayni et al., 2021). Based on this interesting observation, we will investigate the functional role of MMP-12 in TBE in our next studies. Similarly, we cannot exclude the possibility that other MMPs that were not altered in the CSF of TBE patients were also upregulated in the serum samples. Therefore, in our next study, we will analyse the full range of MMPs in the serum samples from TBE patients.

When we compared MMP-9 levels in paired CSF and serum of TBE patients at hospital admission, we found that patients had significantly higher serum MMP-9 levels, independent of clinical presentation (meningitis vs. encephalitis). Similarly, patients with various other neurological diseases such as bacterial meningitis, viral meningoencephalitis, neuroborreliosis, neurosyphilis, Guillain-Barré syndrome, and others have been previously found to have higher MMP-9 concentrations in serum than in CSF (Yushchenko et al., 2000). This suggests that MMP-9 detected in serum may be a better biomarker than MMP-9 detected in CSF. Similarly, in Japanese encephalitis virus–infected children, the concentration of MMP-9 in serum was significantly higher than in controls, while there was no observed MMP-9 increase in CSF from the patients compared with controls (Shukla et al., 2013). In general, biomarkers from serum are preferred over biomarkers from CSF because CSF collection is an invasive procedure that is usually performed only on admission, whereas blood samples can be collected routinely during hospitalisation (Shaw, 2015). Therefore, serum MMP-9 was investigated in more detail in TBE patients.

Comparison of serum MMP-9 levels in healthy children and adults revealed that children had significantly lower serum MMP-9 levels than adults. Children with TBE had higher serum MMP-9 levels than did healthy children, although the levels were still lower than those in adult TBE patients. This may be due to the overall lower production of MMP-9 in children and the generally more favourable clinical course of TBE in children than in adults (Bogovic and Strle, 2015; Kohlmaier et al., n.d.;

Krbková et al., 2015). Also, adult TBE patients had elevated levels of serum MMP-9, in agreement with our previous findings (Palus et al., 2014b). The elevated MMP-9 levels were observed throughout hospitalisation, and the levels remained at the same level or were slightly decreasing during hospitalisation. At the first follow-up, levels returned to normal and were similar to those of the controls. However, only serum samples collected at admission were consistent with the subsequent clinical course of the disease, because patients with TBE meningitis had lower serum MMP-9 levels than did patients with encephalitis. These results have important implications: increased serum MMP-9 concentration can be observed throughout hospitalisation, but only samples collected at admission reflect the overall disease severity.

Previously, in Russia, the frequency of the rs17576 G allele in the *MMP9* gene was found to be significantly higher in TBE patients with severe forms than it was in TBE patients with milder meningitis and in the control group of the population, suggesting that the *MMP9* gene may influence the genetic predisposition to severe TBE in the Russian population (Barkhash et al., 2018). Therefore, we screened our TBE patient cohort for the rs17576 SNP and compared allele frequencies between patients with different clinical manifestations and between TBE patients and the control population. Unlike Russian TBE patients, severity or predisposition to TBE did not appear to be associated with the rs17576 SNP in our patient cohort. The only significant difference was observed between the control group and the TBE patients with meningitis in the individuals with genotype A/G. These differences in genetic predisposition to severe TBE between the Russian and Czech populations could be explained either by their having different genetic backgrounds (Malyarchuk et al., 2002) or by the fact that Czech and Russian TBE patients are infected with different TBEV strains. Czech patients are infected with European TBEV strains, whereas most Russian TBE patients are infected with strains of Far Eastern or Siberian subtypes. Presumably, the patients included in the Russian study were infected predominantly by Siberian TBEV strains. Although strains with different virulence characteristics can be found within each TBEV subtype, it is generally accepted that infection with Far Eastern and to a lesser extent also Siberian TBEV strains is associated with more frequent occurrence of severe disease, whereas TBE caused by European strains is usually less severe (Ruzek et al., 2019). However, the SNP rs17576 was found to be associated with different serum MMP-9 levels in TBE-enc and TBE-mening patients. Only heterozygotes for this SNP had significant differences in serum MMP-9 levels between TBE-enc and TBE-mening patients, whereas there was no difference in serum MMP-9 levels between homozygous TBE-enc and TBE-mening patients. This suggests that the use of serum MMP-9 as a severity biomarker is appropriate for heterozygous rs17576 SNP patients, whereas the use of this biomarker may be limited in homozygous patients.

MMP-9 is known to play important roles in brain physiology and pathology. It is produced by various types of brain cells, including neurons, glia, astrocytes, and leukocytes. For example, we have previously observed a marked activation of MMP-9 production by TBEV-infected human primary astrocytes (Palus et al., 2014a). Under normal physiological conditions, the expression of MMP-9 in brain is low, but it can be activated under certain physiological or pathological stimuli (Vafadari et al., 2016). The increased MMP-9 production is not specific for TBE but similar trends might be observed also in brain infections of other aetiologies. The exact role of MMP-9 in TBE remains to be revealed, but it is likely involved in multiple processes, such as immune/inflammation responses, the activation of various cytokines and chemokines, and the disruption of the BBB (Palus et al., 2017, 2014b; Vafadari et al., 2016).

In conclusion, this study provides new evidence of elevated MMP-9 levels in the CSF and serum of TBE patients. To our knowledge, this is the first demonstration of elevated MMP-9 levels in not only adult but also paediatric TBE patients. Serum levels, but not CSF MMP-9 on admission, correlate with disease course and could serve as a good biomarker, especially in patients heterozygous for the rs17576 SNP of the *MMP9* gene. The role of MMP-9 in the pathophysiology of TBE should be further investigated as a potential target for new treatments.

Author contributions

The study was designed by Daniel Ruzek. Acquisition, analysis, or interpretation of data were done by Andrea Fortova, Vaclav Hönig, Jiri Salat and Martin Palus. Drafting of the manuscript was done by Daniel Ruzek. Statistical analysis was done by Vaclav Hönig, Martin Palus and Daniel Ruzek. Sample collection was done by Martina Pychova and Lenka Krbkova. Andrey V. Barkhash, Michal F. Kriha, Ales Chrdle and Marie Lipoldova contributed to study design and interpretation of the results. All authors contributed to the reviewing and approved the final version.

Declaration of Competing Interest

We declare no competing interests.

Data availability

Data will be made available on request.

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