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Serum and cerebrospinal fluid phosphorylated neurofilament heavy subunit as a marker of neuroaxonal damage in tick-borne encephalitis

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Abstract

Extensive axonal and neuronal loss is the main cause of severe manifestations and poor outcomes in tick-borne encephalitis (TBE). Phosphorylated neurofilament heavy subunit (pNF-H) is an essential component of axons, and its detection in cerebrospinal fluid (CSF) or serum can indicate the degree of neuroaxonal damage. We examined the use of pNF-H as a biomarker of neuroaxonal injury in TBE. In 89 patients with acute TBE, we measured CSF levels of pNF-H and 3 other markers of brain injury (glial fibrillary acidic protein, S100B and ubiquitin C-terminal hydrolase L1) and compared the results to those for patients with meningitis of other aetiology and controls. Serum pNF-H levels were measured in 80 patients and compared with findings for 90 healthy blood donors. TBE patients had significantly (P<0.001) higher CSF pNF-H levels than controls as early as hospital admission. Serum pNF-H concentrations were significantly higher in samples from TBE patients collected at hospital discharge (P<0.0001) than in controls. TBE patients with the highest peak values of serum pNF-H, exceeding 10000 pg ml^{-1}, had a very severe disease course, with coma or tetraplegia. Patients requiring intensive care had significantly higher serum pNF-H levels than other TBE patients (P<0.01). Elevated serum pNF-H values were also observed in patients with incomplete recovery (P<0.05). Peak serum pNF-H levels correlated positively with the duration of hospitalization (P=0.005). Measurement of pNF-H levels in TBE patients might be useful for assessing disease severity and determining prognosis.

INTRODUCTION

Tick-borne encephalitis (TBE) virus (TBEV; family *Flaviviridae*, genus *Flavivirus*) is a neurotropic virus that causes TBE, one of the most common human viral infections of the central nervous system (CNS) in Europe and northeast Asia [1]. More than 10000 TBE cases are reported annually, making the disease a significant cause of morbidity and mortality in endemic areas [2]. Most patients become infected following the feeding of an infected tick, but infection can also arise with consumption of unpasteurized goat, sheep, or cow dairy products [3]. The clinical picture of TBE can vary widely, from asymptomatic infection to a mild flu-like illness to neurological involvement. The neurological phase of TBE can manifest as meningitis, meningoencephalitis, or severe meningoencephalomyelitis [1]. In Europe, the mortality rate in TBE patients is <1% [4], and another 16–50% of patients suffer long-term sequelae such as headache, decreased concentration, tremor, ataxia and pareses of the extremities [4].

Diagnosis of TBE is usually based on clinical presentation suggestive of CNS inflammation and concurrent detection of TBEV-specific immunoglobulin (Ig)M and IgG antibodies in serum or cerebrospinal fluid (CSF) [5]. TBEV RNA also can be detected

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Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; CXCL, C-X-C motif ligand; ELISA, enzyme-linked immunosorbent assay; GFAP, glial fibrillary acidic protein; Ig, immunoglobulin; NF-H, neurofilament heavy chain; NSE, neuron-specific enolase; pNF-H, phosphorylated neurofilament heavy subunit; TBE, tick-borne encephalitis; TBE-enc, TBE cases with CNS involvement; TBE-mening, TBE cases with meningitis; TBEV, tick-borne encephalitis virus; UCHL1, ubiquitin C-terminal hydrolase isozyme L1.

in serum, blood, CSF, or urine [6–8], but only early in the course of the disease and/or in immunodeficient patients [9]. Thus, most patients admitted to the hospital at the onset of the neurological phase of TBE no longer have detectable levels of viral RNA.

In the initial phase of the disease, blood analysis reveals leukopenia, thrombocytopenia and moderately elevated C-reactive protein levels. Abnormal liver function tests may also be observed [10]. In the second phase of TBE, C-reactive protein levels and leukocyte counts are elevated [4, 10, 11]. Analysis of CSF samples from TBE patients usually shows pleocytosis and moderately elevated protein levels [12]. The protein/albumin ratio is also elevated, indicating increased blood–brain barrier permeability [13, 14].

In addition, several biomarkers for TBE have been identified in serum or CSF samples (reviewed in [15]), which may be useful for potential application in the diagnosis, prognosis, monitoring, or treatment of TBE [15]. For example, high-mobility group box 1 or chemokine (C-X-C motif) ligand (CXCL) 13 can be used to distinguish TBE meningitis from meningoencephalitis [15–17]. The levels of λ-free light chains, matrix metalloproteinase-9, or CXCL1 can be used as indicators of blood–brain barrier disruption [15, 18, 19]. Several other cytokines and chemokines, along with growth factors, monoamine neurotransmitters and other molecules, can be used as biomarkers for TBE [15, 20–27].

For disease prognosis and monitoring, biomarkers that indicate the extent of brain damage during TBE are needed. In particular, a biomarker that accurately reflects neuronal damage during TBE is critically needed to monitor disease progression and determine prognosis. Neurofilaments are abundant cytoskeleton proteins that are exclusively expressed in neurons. Pathological processes that involve axonal damage or neuronal cell death lead to the release of neurofilament proteins into the CSF and then into blood circulation. Thus, the level of neurofilaments in these body fluids reflects the extent of the neuronal damage [28, 29]. The neurofilament heavy chain (NF-H) is part of the neurofilament heteropolymer and its phosphorylated form (pNF-H) is specific to axons [30–32]. Thus, increased CSF and serum pNF-H values reflect the degree of neuroaxonal damage in various acute or chronic neurological diseases [28, 29, 32, 33].

Here, we aimed to examine pNF-H levels in CSF and serum samples to determine the extent of neuroaxonal damage in TBE patients and evaluate pNF-H as a biomarker of disease severity and prognosis. In addition, the levels of two proteins localized primarily in astrocytes [glial fibrillary acidic protein (GFAP) and S100B] and ubiquitin C-terminal hydrolase (UCHL1), which is found explicitly in neuronal perikarya and dendrites, were analysed in CSF from TBE patients.

PATIENTS AND METHODS

The patients and the controls were prospectively recruited during 2018–2020. 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, and an epidemiological link; (ii) CSF pleocytosis (>5 cells μl^{-1}); and (iii) the presence of TBEV-specific IgM and IgG antibodies in serum or TBE-specific IgM antibodies in CSF [5]. None of the patients had been vaccinated against TBE. All patients lived in the region of South Moravia, Czechia.

CSF sampling

CSF samples were collected from 89 adult patients with TBE (aged 19–86 years, median 49 years; 48 male) on admission to the hospital. Patients were classified into two subgroups based on TBE severity: those with meningitis (TBE-mening; *n*=44, 24 male, aged 20–77 years, mean 45.7 years) and those with CNS involvement (TBE-enc, i.e. meningoencephalitis, meningoencephalomyelitis; *n*=45, 26 male, aged 19–86 years, mean 53.5 years). CNS involvement was diagnosed based on consciousness disturbances and/or focal neurological signs detected during the neurological investigation. The data were collected prospectively. Control groups consisted of 28 age-matched patients with acute aseptic meningitis of other aetiology (matched positive controls; non-TBE meningitis; mostly cases of neuroborreliosis or varicella-zoster meningitis) and 71 age-matched controls with initially suspected CNS infection that was later excluded based on CSF analysis, serological investigation [serology for other neurotropic viruses (enteroviruses, herpes simplex virus 1 and 2, herpes hominis virus 6 and, in cases of cranial pareses, varicella-zoster virus and Epstein–Barr virus) and anti-borrelial antibodies], CSF analysis for intrathecal synthesis of specific anti-borrelial antibodies, polymerase chain reaction (PCR) examination of CSF (enteroviruses, parechovirus, herpes simplex virus 1 and 2, varicella-zoster virus, cytomegalovirus, herpes hominis virus 6 and 7) and analysis of C-reactive protein in serum (unaffected controls).

CSF samples were collected by lumbar puncture, centrifuged and kept frozen at −80 °C until analysis. CSF concentrations of GFAP, pNF-H, S100B and UCHL1 were measured using the Brain Injury 4-plex Human ProcartaPlex Panel (cat. no. EPX040-15827-901; Invitrogen). The assay was performed using a MAGPIX instrument (Luminex, Austin, TX, USA), according to the manufacturer's instructions.

Serum sampling

Serum samples were collected from 80 patients (aged 19–86 years, median 47 years) who were also divided into two subgroups: those with meningitis (TBE-mening; *n*=37, 16 male, aged 22–76 years, mean 47 years) and those with CNS involvement (TBE-enc; *n*=40, 26 male, aged 19–86 years, mean 48 years). The serum was taken from the patients from whom CSF had been collected,

except for 9 patients from whom only CSF was available. The first serum sample was collected on admission to the hospital (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 first follow-up control (26–120 days, mean 56.6 days). Not all samples were available for all patients; there were 65 samples at admission, 60 samples at +2 days, 61 samples at discharge and 55 samples at the follow-up control. For 30 patients, all 4 samples were available. Serum samples from 90 healthy blood donors were used as controls. pNF-H concentration was measured by Phosphorylated Neurofilament H Human ELISA (cat. no. RD191138300R; Biovendor, Czechia) according to the manufacturer's instructions.

Complete or incomplete recovery was evaluated at the time of hospital discharge after a thorough clinical and neurological investigation.

The data from the CSF samples were log-transformed and analysed via multiple *t*-tests with false discovery rate correction using the Holm–Šidák method (alpha=0.05). 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. The correlation between the length of hospitalization and serum pNF-H levels at discharge was tested using Spearman's rank correlation coefficient. Data were analysed with Statistica for Windows 13.1.

RESULTS AND DISCUSSION

Neurons represent a primary target for TBEV in the infected brain [34, 35]. Neuronal death after TBEV infection may be mediated directly by the infection or indirectly by induced secretion of neurotoxic proteins by resident glial cells or recruitment of peripheral immunocompetent cells (such as $CD8^+$ T lymphocytes) to the brain parenchyma [36, 37]. Axonal pathology and retrograde degeneration seem to lead to the death of TBEV-infected neurons [38]. Extensive axonal and neuronal loss is the primary cause of severe manifestations of TBE and poor outcome after the infection [1]. pNF-H is considered to be an excellent biomarker for acute or chronic axonal loss, and the concentration of this protein may therefore indicate the degree of neurological damage [32]. We evaluated pNF-H as a biomarker of neuroaxonal injury in CSF and serum samples collected from TBE patients.

We found that CSF concentrations of pNF-H were significantly higher in TBE patients than in controls (Fig. 1a, b); $P<0.001$), but the TBE-mening and TBE-enc groups did not differ significantly (Fig. 1b). CSF pNF-H concentrations were significantly higher in non-TBE meningitis versus unaffected controls (Fig. 1a; $P<0.05$), but did not differ between the TBE patients and the non-TBE meningitis group ($P>0.05$).

In patients with different clinical forms of TBE, Czupryna *et al*. examined CSF concentrations of neuron-specific enolase (NSE), another marker of brain damage not measured in our study [39]. They found that in the CSF of patients with meningoencephalitis, the NSE concentration was significantly higher than in the control group or in patients with meningitis, another indication of neuronal damage during the acute phase of TBE [39].

CSF concentrations also did not differ between controls and TBE patients overall for the other brain injury biomarkers examined, i.e. S100b, UCHL-1 and GFAP (Fig. 1a; $P>0.05$), suggesting that these analytes are not suitable biomarkers for assessing brain injury during TBE in the early neurological phase. Similarly, concentrations of these biomarkers did not differ between unaffected controls and non-TBE meningitis groups or between TBE patients and the non-TBE meningitis group ($P>0.05$).

S100b and GFAP are major proteins that arhe mainly localized in astrocytes. Indeed, we previously reported increased production of GFAP in human primary astrocytes infected with TBEV [40]. Thus, the release of S100b and GFAP primarily reflects damage to astrocytes that show low permissiveness to TBEV infection under normal conditions [40–43]. In agreement with our current study, another group found no increase in S100b concentrations in CSF in Polish TBE patients [39], and other authors have reported no difference in serum levels of S100b between TBE patients and controls [44]. Moderately elevated levels of GFAP in CSF were previously found in a small group of TBE patients [44]. UCHL1 is found explicitly in neuronal perikarya and dendrites, and its increased detection reflects neuronal loss, making this protein a good biomarker for CNS damage [32, 45]. However, it appears that neuronal loss or the extent of the damage in the early stages of neurological TBE is insufficient to increase UCHL1 levels in CSF.

Ideally, a good biomarker should be detectable in blood samples [32]. CSF collection is an invasive procedure, and in TBE patients is usually performed only at hospital admission. In contrast, blood samples are taken routinely during the hospitalization of TBE patients. For this reason, we also examined pNF-H levels in serum samples collected from TBE patients at different time points. The pNF-H concentrations in the sera were compared to those in sera from healthy blood donors.

We found no significant differences between the healthy blood donor and TBE patient samples collected at hospital admission and 2 days later (Fig. 2a, c and d; $P>0.05$). However, the pNF-H concentrations were significantly higher in samples collected from TBE patients at hospital discharge (Fig. 2a–d; $P<0.0001$). The pNF-H values also remained elevated during the first follow-up control compared to healthy individuals (Fig. 2a, c and d; $P<0.05$). pNF-H is highly resistant to proteases, so increased levels can be detectable for a relatively long time [32]. The TBE-mening and TBE-enc groups showed a similar

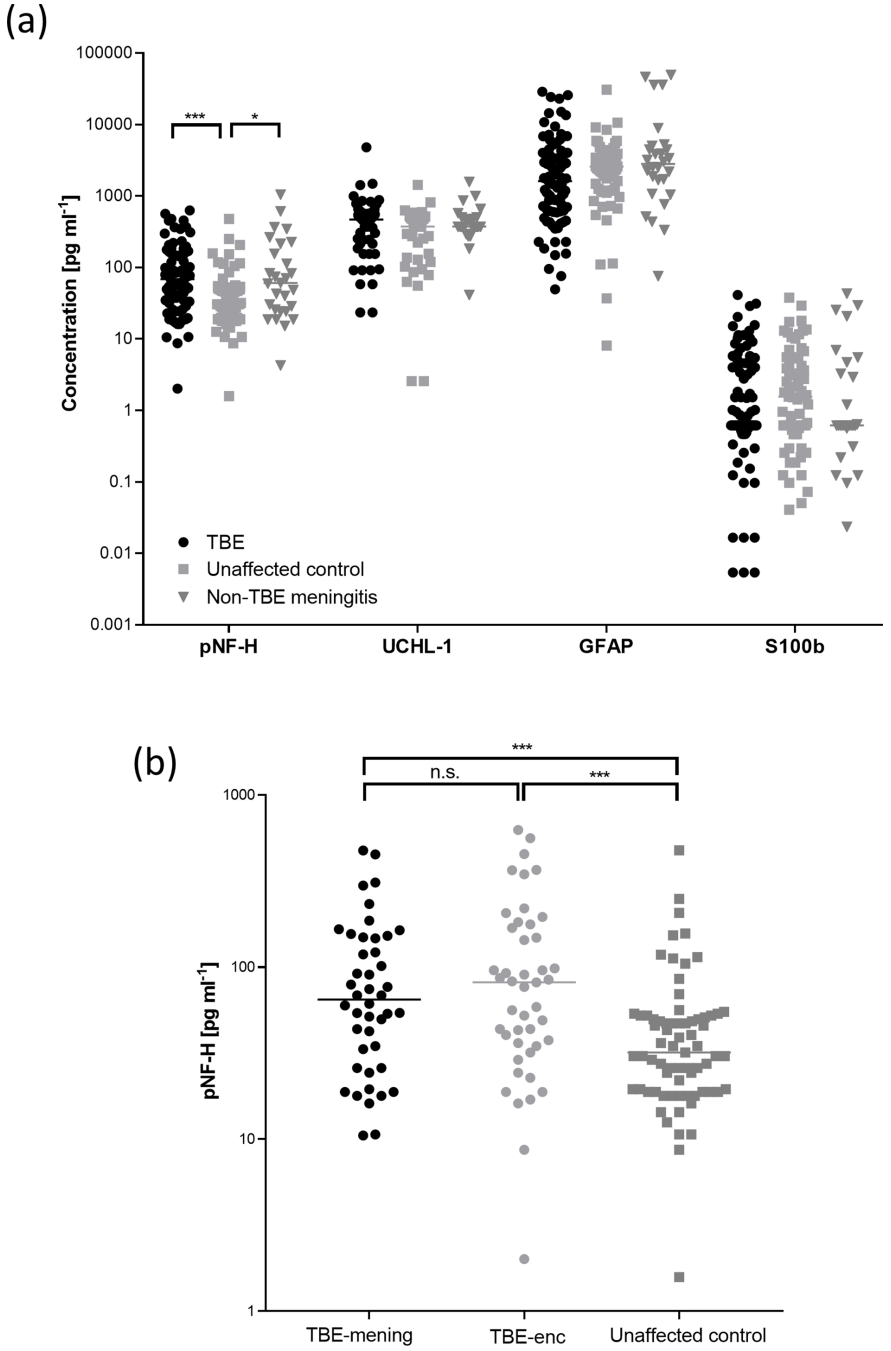

Fig. 1. Markers of brain injury in CSF from patients with TBE. CSF samples were collected from adult TBE patients by lumbar puncture at hospital admission. Control groups consisted of patients with acute aseptic meningitis of other aetiology (non-TBE meningitis) and those with initially suspected CNS infection that was excluded on CSF analysis (unaffected control). CSF concentrations of GFAP, pNF-H, S100B and UCHL1 were measured using the Brain Injury 4-plex Human ProcartaPlex Panel for the Luminex platform (a). Based on the severity of the TBE, the patients were classified into two subgroups: (i) patients with meningitis (TBE-mening) and (ii) patients with CNS involvement (TBE-enc). The CNS pNF-H levels were compared among TBE-mening, TBE-enc and non-TBE meningitis and unaffected controls (b). *, $P<0.05$; ***, $P<0.001$; NS, not significant.

trend in these concentrations, with no significant differences at any time point investigated (Fig. 2e; $P>0.05$). However, we did detect a tendency towards increasing serum pNF-H levels in encephalitis patients with a more severe course of the disease and only found peak values >5000 pg ml^{-1} in the TBE-enc group (Fig. 2e). Moreover, patients with the highest peak values (>10000 pg ml^{-1}) had a very severe course of the disease, with coma or tetraplegia.

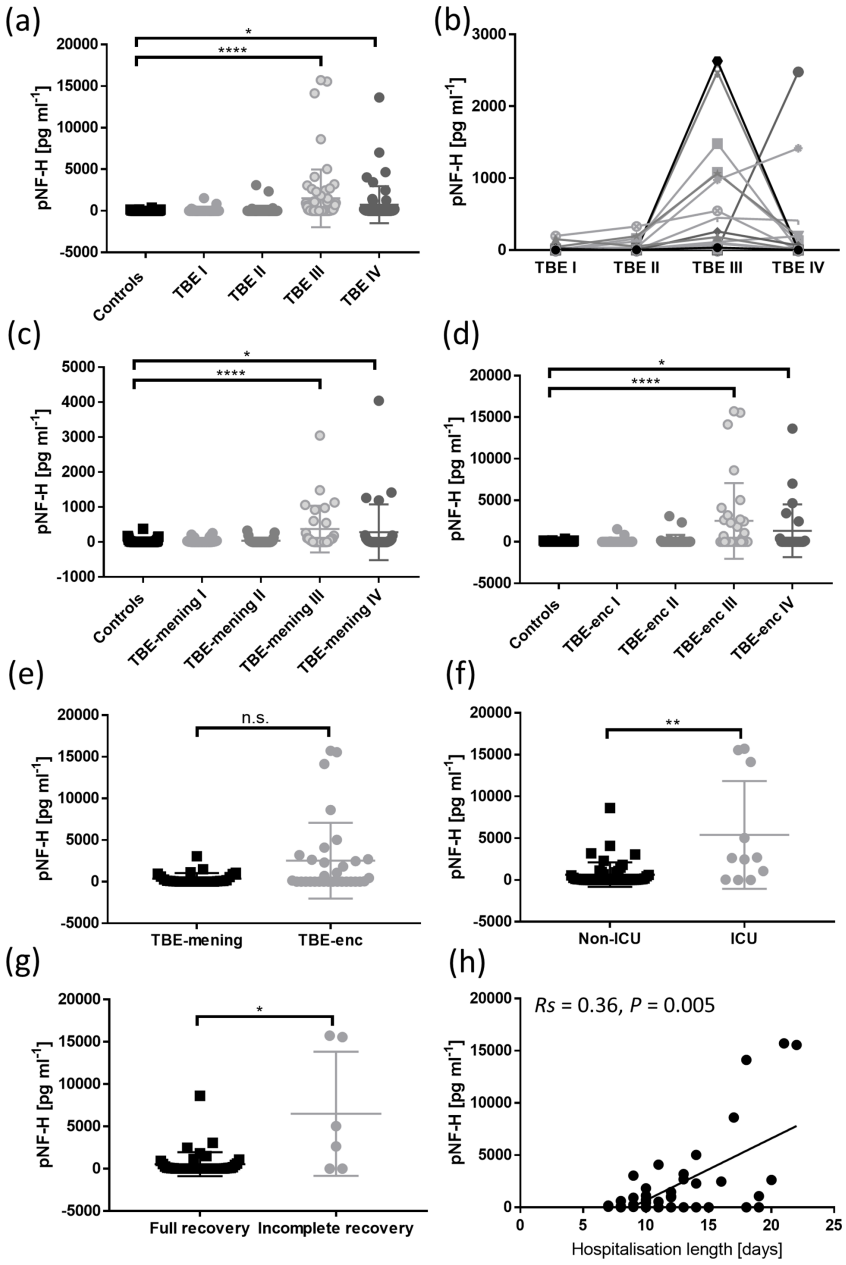

Fig. 2. Serum pNF-H levels in TBE patients. Serum samples were collected from TBE patients and healthy blood donors (controls) and analysed by ELISA for pNF-H levels. Comparison of the pNF-H levels in sera from controls and sera collected from TBE patients at different time points: hospital admission (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) (a). The kinetics of serum pNF-H levels in serum samples from TBE patients (n=30) (b). Comparison of serum pNF-H 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 pNF-H levels in controls and TBE patients with 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). Comparison of serum pNF-H levels in TBE patients with meningitis (TBE-mening) and with CNS involvement (TBE-enc) at hospital discharge (e). Comparison of serum pNF-H levels at hospital discharge in TBE patients requiring intensive care (ICU) and all other TBE patients (non-ICU) (f). Comparison of the serum pNF-H levels at hospital discharge in TBE patients with full versus incomplete recovery (g). Correlation between length of hospitalization and serum pNF-H levels at discharge (h). *, P<0.05; **, P<0.01; ****, P<0.0001; NS, not significant.

In encephalitis, the infection affects the brain parenchyma, whereas in meningitis, the infection is limited to the meninges with a much lower number of neurons involved. Therefore, a difference in pNF-H concentration between patients with meningitis and those with encephalitis would be expected, as seen in the case of serum and CSF neurofilament light chain in varicella-zoster virus CNS infection [46, 47]. Why there was no difference between the TBE-mening and TBE-enc groups

remains puzzling. However, it should also be noted that it is not always clinically possible to distinguish TBE cases with mild CNS involvement from those with pure meningitis.

TBE patients requiring intensive care also had significantly higher peak serum pNF-H levels than other TBE patients (Fig. 2f; $P<0.01$). In addition, we observed significantly elevated serum pNF-H values in patients with incomplete recovery compared with those who had good outcomes (Fig. 2g; $P<0.05$). Peak serum pNF-H levels correlated positively with the duration of hospitalization (Fig. 2h; Spearman's: $R_s=0.36$, $P=0.005$).

Taken together, our results indicate that serum pNF-H might be a useful marker for assessing neuroaxonal damage during TBE and can also be used as a predictive marker for outcomes after infection. We found that in TBE patients, pNF-H values increased in the CSF during the early neurological phase of the disease, but serum pNF-H values increased gradually during the hospitalization, peaking in patients with the most severe clinical course. Some patients had increased serum pNF-H levels even 1–4 months after acute TBE. Measurement of pNF-H levels in TBE patients might be useful for assessing disease severity, monitoring treatment responses and determining prognosis.

Data availability statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions
A.F.: investigation, formal analysis; V.H.: investigation, formal analysis; M.P.: data curation, visualization; J.S.: conceptualisation, data curation, writing – review and editing; M.P.: sample collection; L.K.: sample collection, project administration, writing – review and editing; T.V.: formal analysis; M.F.K.: data curation, writing – review and editing; A.C.: data curation, writing – review and editing; D.R.: conceptualization, project administration, methodology, writing – original draft.

Conflicts of interest
The authors declare that there are no conflicts of interest.

Ethical 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 agreed to their participation in the study and signed informed consent.

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