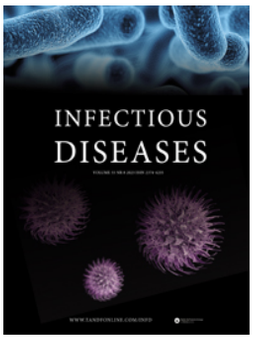

Infectious Diseases

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/infd20

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To cite this article: Anja Nääs, Peng Li, Clas Ahlm, Elisabeth Aurelius, Josef D. Järhult, Silvia Schliamser, Marie Studahl, Wenzhong Xiao, Jonas Bergquist & Gabriel Westman (2023): Temporal pathway analysis of cerebrospinal fluid proteome in herpes simplex encephalitis, Infectious Diseases, DOI: 10.1080/23744235.2023.2230281

To link to this article: https://doi.org/10.1080/23744235.2023.2230281

Taylor & Francis
Taylor & Francis Group

INFECTIOUS DISEASES,
2023; VOL. 0,
NO. 0, 1–12

https://doi.org/10.1080/23744235.2023.2230281

RESEARCH ARTICLE

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Temporal pathway analysis of cerebrospinal fluid proteome in herpes simplex encephalitis

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ABSTRACT

Objectives: We examined the temporal changes of the CSF proteome in patients with herpes simplex encephalitis (HSE) during the course of the disease, in relation to anti-N-methyl-D-aspartate receptor (NMDAR) serostatus, corticosteroid treatment, brain MRI and neurocognitive performance.

Methods: Patients were retrospectively included from a previous prospective trial with a pre-specified CSF sampling protocol. Mass spectrometry data of the CSF proteome were processed using pathway analysis.

Results: We included 48 patients (110 CSF samples). Samples were grouped based on time of collection relative to hospital admission – T1: $\leq$ 9 d, T2: 13–28 d, T3: $\geq$ 68 d. At T1, a strong multi-pathway response was seen including acute phase response, antimicrobial pattern recognition, glycolysis and gluconeogenesis. At T2, most pathways activated at T1 were no longer significantly different from T3. After correction for multiplicity and considering the effect size threshold, 6 proteins were significantly less abundant in anti-NMDAR seropositive patients compared to seronegative: procathepsin H, heparin cofactor 2, complement factor I, protein AMBP, apolipoprotein A1 and polymeric immunoglobulin receptor. No significant differences in individual protein levels were found in relation to corticosteroid treatment, size of brain MRI lesion or neurocognitive performance.

Conclusions: We show a temporal change in the CSF proteome in HSE patients during the course of the disease. This study provides insight into quantitative and qualitative aspects of the dynamic pathophysiology and pathway activation patterns in HSE and prompts for future studies on the role of apolipoprotein A1 in HSE, which has previously been associated with NMDAR encephalitis.

KEYWORDS
Herpes simplex encephalitis
proteomics
HSV-1
apolipoprotein A1

ARTICLE HISTORY
Received 16 January 2023
Revised 20 June 2023
Accepted 21 June 2023

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Supplemental data for this article can be accessed online at https://doi.org/10.1080/23744235.2023.2230281.

Introduction

Herpes simplex encephalitis (HSE) is a rare but severe manifestation of Herpes simplex virus, mainly caused by Herpes virus type 1 (HSV-1), with an incidence of 2–4 cases per million person-years [1,2]. HSE is a cytolytic infection causing an aggressive inflammation in brain tissue, mainly in the frontotemporal lobes. The major symptoms are fever, headache, altered mental status (confusion, reduced level of consciousness, changes in personality), seizures and focal neurological deficits. Many patients develop severe neurologic sequelae in spite of antiviral treatment, such as memory impairment, behavioral abnormalities, dysphasia and epilepsy [3,4]. It is not fully elucidated why some patients get struck by this severe manifestation of HSV-1 primary infection or reactivation, from a virus that in most people causes infection with mild skin symptoms, or no symptoms at all [5]. A minor part of HSE cases (ca 15%) can be linked to monogenic immune deficiencies, affecting TLR3 and interferon signalling [6–8].

Parameters known to be associated with poor outcome are the duration of symptoms before admission to the hospital, time to initiation of acyclovir treatment, older age, coma and brain MRI findings of extensive lesions [9,10]. We have previously shown that HSE triggers a strong intrathecal immune response, with several biomarkers of inflammation and brain injury being elevated for months after the acute phase of the disease, which also appear to correlate with the development of neuronal autoantibodies [11]. Further, increased levels of brain injury markers and development of anti-NMDAR antibodies correlates with inferior neurocognitive recovery [12] but it is otherwise unknown if there are additional factors that are predictive for the long-term prognosis.

A better understanding of the cellular pathways involved in the disease contributes to the understanding of the pathogenesis, such as individual susceptibility to the disease and the diversity in clinical outcome. The use of mass spectrometry allows for wide-band proteome profiling, which can lead to a deeper understanding of the cellular mechanisms involved in the pathogenesis of diseases in the central nervous system. As an example, previous studies of CSF proteome have revealed potential new biomarkers for distinguishing between different aetiologies in viral and bacterial meningitis [13–15].

The aim of this study was to examine the temporal changes in cerebrospinal fluid (CSF) proteome in HSE patients during the acute phase of the disease and throughout three months of follow-up, in relation to anti-N-methyl-D-aspartate receptor (NMDAR) serostatus, corticosteroid treatment, brain MRI findings and long-term neurocognitive performance.

Methods

Participants and sampling

A total of 48 adult patients with acute HSE were retrospectively included from a cohort originally generated for a prospective study (ClinicalTrials.gov number NCT00031486) investigating the effect of prolonged oral treatment with valacyclovir after initial standard of care with two to three weeks of intravenous acyclovir treatment [16]. Inclusion criteria included age above 12 years, clinical signs of encephalitis and HSV-1 DNA detected in CSF by PCR. Exclusion criteria were an anticipated life expectancy of less than 90 d, inability to swallow pills, creatinine clearance below 50 mL/min/1.73 m^2 and known immunosuppression. According to the study protocol, patients who presented with signs of elevated intracranial pressure were treated with corticosteroids at the investigator's discretion.

Patients were included in the cohort between 2001 and 2009 at five Swedish study sites. Study subjects underwent CSF and serum sampling and brain MRI during the first three months of follow-up, whereas neurological and cognitive evaluations were performed during a total of 24 months. CSF and blood samples were collected in the acute phase of the disease, after 14–21 d of intravenous acyclovir treatment (FU start), and after 3 months of follow-up (FU 3 M).

Informed consent was obtained from all study participants. The study was approved for all study sites by the Regional Ethical Review Board at Karolinska Institutet, Sweden (no. 99-409; 03-380).

Mass spectrometry

Mass spectrometry was performed to analyse the CSF proteome during the acute phase of HSE and at follow-up. The CSF samples were prepared as follows.

Sample preparation

Prior to nano LC-MS/MS analysis, the low abundant proteins were enriched through depletion of the seven most highly abundant proteins (albumin, IgG, alpha-1-antitrypsin, IgA, haptoglobin, transferrin, and fibrinogen) using a human Multiple Affinity Removal Spin Cartridge

- Hu-7 (Agilent Technologies, Palo Alto, CA, USA). Immunodepletion using the MARS spin cartridge was performed according to the manufacturer's instruction. Prior to depletion, an aliquot of 260 μL of each CSF sample was filtered through a 0.22 μm cellulose acetate spin filters (Agilent Technologies, Palo Alto, CA, USA) by centrifugation at 15,000 g for 2 min. To minimise any proteolytic degradation during the depletion, the samples were kept at 6 °C whenever possible. Next, an aliquot of 250 μL of each CSF sample was loaded onto the spin cartridge and the flow-through (FT) fraction was collected by centrifugation for 2 min at 100 g. Two successive wash steps with 400 μL of MARS-7 Buffer A were carried out to obtain the maximum yield. The flow-through and wash (W) fractions were combined. The spin cartridge was washed with 2 mL of MARS-7 Buffer B to remove bound proteins and was then re-equilibrated with Buffer A. The remaining fractions (FT + W) were dried using a SpeedVac (Thermo Scientific, Waltham, MA, USA).

The proteins in the depleted CSF sample were digested using a trypsin/Lys-C mixture. Briefly, the proteins were re-dissolved in 50 μL of digestion buffer (6 M urea, 50 mM NH4HCO3). 10 μL of 45 mM aqueous DTT was then added to all samples and the mixtures were incubated at 50 °C for 150 min to reduce the disulphide bridges. The samples were cooled to room temperature (RT) and 10 μL of 100 mM aqueous IAA was added before incubating the mixtures for an additional 15 min at RT in darkness in order to carbamidomethylate the cysteines. Finally, a volume of 50 μL of 100 mM NH4HCO3 was added to all samples followed by the trypsin/Lys-C mixture dissolved in 500 mM NH4HCO3, yielding a final trypsin/protein concentration of 5% (w/w). The tryptic digestion was performed overnight at 37 °C. Prior to MS analysis, the peptides were purified and desalted by Pierce C18 Spin Columns (Thermo Scientific). These columns were activated by 2 × 200 μL of 50% acetonitrile (CAN) and equilibrated with 2 × 200 μL of 0.5% trifluoroacetic acid (TFA). The tryptic peptides were adsorbed to the media using two repeated cycles of 40 μL sample loading and the column was washed using 3 × 200 μL of 0.5% TFA. Finally, the peptides were eluted in 3 × 50 μL of 70% ACN and dried.

LC-MS/MS

The samples were analysed using a QExactive Plus Orbitrap mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source. The peptides were separated by reversed-phase liquid chromatography using an EASY-nLC 1000 system (Thermo Fisher Scientific). A set-up of pre-column and analytical column was used. The pre-column was a 2 cm EASY-column (1D 100 μm, 5 μm C18) (Thermo Fisher Scientific) while the analytical column was a 10 cm EASY-column (ID 75 μm, 3 μm, C18; Thermo Fisher Scientific). Peptides were eluted with a 150 min linear gradient from 4% to 100% acetonitrile at 250 nL min^{-1}. The mass spectrometer was operated in positive ion mode acquiring a survey mass spectrum with resolving power 70,000 (full-width half maximum), $m/z = 400$–1750 using an automatic gain control (AGC) target of 3 × 106. The 10 most intense ions were selected for higher-energy collisional dissociation (HCD) fragmentation (25% normalised collision energy) and MS/MS spectra were generated with an AGC target of 5 × 105 at a resolution of 17,500. The mass spectrometer worked in a data-dependent mode.

Chemicals and reagents

Acetonitrile (ACN), methanol (MeOH), acetic acid (HAc), formic acid (FA) and ammonium bicarbonate (NH4HCO3) were obtained from Merck (Darmstadt, Germany). Acetone, protease inhibitor cocktail, phosphate-buffered saline (PBS), trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (St. Louis, MO, USA). For tryptic digestion iodoacetamide (IAA), urea and dithiothreitol (DTT) were obtained from Sigma Aldrich and trypsin/Lys-C mixture (Mass spectrometry grade; Promega, Mannheim, Germany). Ultrapure water was prepared by a Milli-Q water purification system (Millipore, Bedford, MA, USA).

Database search and protein quantification

The acquired data (RAW files) were initially processed in MaxQuant version 1.6.17.0 and database searches were performed using the implemented Andromeda search engine. MS/MS spectra were correlated to a FASTA database containing proteins from Homo sapiens extracted from the Uniprot database (release Feb 2021). A decoy search database, including common contaminants and a reverse database, was used to estimate the identification false discovery rate (FDR). An FDR of 1% was accepted. The search parameters included: maximum 10 ppm and 0.6 Da error tolerances for the survey scan and MS/MS analysis, respectively; enzyme specificity was trypsin; maximum one missed cleavage site allowed; N-terminal protein acetylation and methionine oxidation were selected as variable modifications. Carbamidomethylation of

cysteine residues was set as a fixed modification. Label-free quantification was performed using the standard LFQ settings (Stabilize large LFQ ratios: True, Require MS/MS for LFQ comparisons: True, iBAQ: False). The average area of the three most abundant peptides [17] for a matched protein was used to gauge protein abundances. The LFQ intensities were transformed using the log 2 function. Quantifiable proteins were further filtered by: (1) at least two peptides covered; (2) identified in at least 3 samples.

Statistical analysis and pathway analysis

Samples were assigned to one of three pre-specified time bins according to time from admission to sampling - T1: $\leq$ 9 d, T2: 13–28 d, T3: $\geq$ 68 d. The division into contrasting groups were also made by the following parameters: corticosteroid treatment vs no corticosteroid treatment, anti-NMDAR serostatus (analysed by the HEK293 assay as previously described [18]) and brain MRI findings of bilateral or extensive engagement vs minor MRI findings [9].

A moderated t-test was performed with the *limma* package version 3.14 [19] to calculate the significance of changes between multiple contrasts. Data were normalised using the quantile normalisation method across total proteins. In our analysis, we blocked the patient subject ID, which effectively incorporated a random effect for each individual subject in the linear model, accounting for the paired nature of the samples and controlling for within-subject variability. This approach is designed to handle unbalanced designs, such as those with missing data points, by efficiently estimating the relevant parameters for the linear model using the available data. Based on this methodology, the differentially expressed proteins were identified, facilitating the comparison between patient groups. The p values were corrected by the Benjamini–Hochberg algorithm and significant calls were made on the basis of an adjusted $p < .05$ and absolute fold change $>$ 1.5 in a given contrast.

Volcano plots of significantly differentially regulated proteins from *limma* were generated by the EnhancedVolcano R package [20].

Data were analysed through the use of QIAGEN's Ingenuity Pathway Analysis [21] using the default parameters. Input data correspond to significantly detected proteins with FDR $<$ 0.05 and absolute fold change $>$1.5. Significant pathways were filtered by Benjamini–Hochberg adjusted p-value less than .05 and Z-score larger than 1.

Heatmap analysis

Heatmaps were generated using the R package *pheatmap* [22]. Each row represents the protein abundance profile from one sample. The colour code presents protein abundances scaled at the column (individual protein) level. Both samples (rows) and proteins (columns) were clustered based on Pearson correlations.

Results

Participants

A total of 48 study subjects were included. A summary of their clinical characteristics is presented in Tables 1 and 2. Although 13 of 48 patients developed anti-NMDAR IgG antibodies in CSF during the course of HSE disease, signs of slower clinical recovery but few to no clear cases of clinical relapse was observed in this cohort [18]. Anti-NMDAR antibody titers are provided in Supplement 1.

The CSF samples were collected at three different time points, a complete dataset could not be obtained from all study subjects due to contraindications for lumbar puncture, loss to follow up or laboratory technical issues of the sample. The availability of CSF samples and clinical follow-up data is illustrated in Figure 1.

CSF proteomics

A total of 110 CSF samples were analysed, generating quantitative data on 801 proteins. In line with the original sampling protocol, all samples were assigned to one of three pre-specified time bins according to time from admission to sampling – T1: $\leq$ 9 d ($n = 25$), T2: 13–28 d ($n = 45$), T3: $\geq$ 68 d ($n = 40$), illustrated in Figure 2.

Table 1. Summary of clinical characteristics and investigations. Numerical values are given as proportions or median (range).

Clinical characteristics	$n = 48$
Age	56 (14–75)
Gender male:female	26:22
Clinical presentation	
Fever	46/48
Headache	40/48
Seizures in acute phase	15/48
Seizures in follow up period	7/48
RLS85 at admission to hospital	2 (1–3)
NIH stroke scale in acute phase	2 (0–19)
Investigations	
Development of CSF anti-NMDAR IgG	13/48
Major brain MRI abnormalities	10/48
Therapy	
Days of intravenous aciclovir therapy	18 (13–25)
Oral valaciclovir follow up therapy	23/48
Corticosteroid treatment	12/48

Table 2. Summary of CSF chemistry and neurocognitive follow-up.

	Acute phase (T1)	Follow up start 14–21 d (T2)	Follow up 3 months (T3)	Follow up 12 months	Follow up 24 months
CSF mononuclear cells	110 (0–726)	47 (0–226)	6 (0–32)	NA	NA
CSF polymorphnuclear cells	4 (0–388)	0 (0–17)	0 (0–1)	NA	NA
CSF albumin	393 (0.3–1680)	545 (0.19–1460)	348 (0.2–1640)	NA	NA
MMSE[a]	NA	27 (5–30)	29 (19–30)	29 (18–30)	29 (19–380)
MDRS[a]	NA	130 (82–144)	137 (73–144)	139 (113–144)	139 (10–144)

Numerical values are given as median (range).
[a]MMSE: Mini Mental State Examination; MDRS: Mattis Dementia Rating Score.

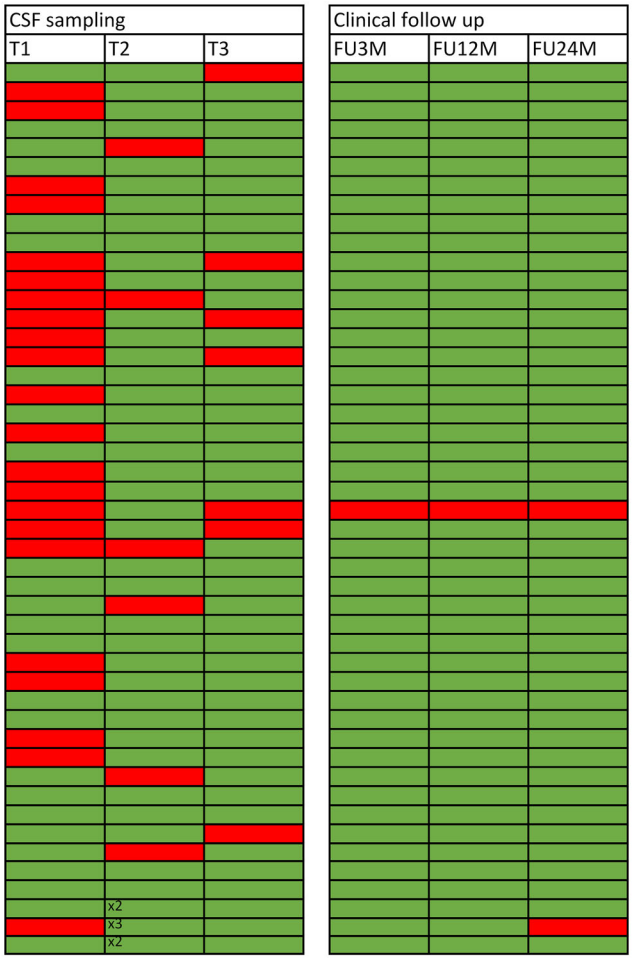

Figure 1. The availability of CSF samples at T1, T2, and T3 and clinical data at 3, 12 and 24 months of follow up. Each horizontal row represents an individual study subject, green cells illustrate available data and red cells missing data.

Given the semi-quantitative nature of mass spectrometry proteomics data, comparisons were made between protein scores at the three different time points to illustrate the dynamic changes during the acute phase of the disease and throughout the follow-up period. T3 was chosen as the baseline for contrasting findings at T1 and T2, as it was presumed that the biomarker profile and levels of pathway activation at the T3 time point, three months after the acute phase of the disease, is closest to the pre-morbid status, but without making any assumptions that T3 is fully comparable to a healthy state.

Temporal proteomics pathway analysis

Pathway activation in the acute phase of the disease (T1) was compared to the start of follow-up after 14–21 d (T2) and after three months of follow-up (T3) (Figure 3), showing a strong multi-pathway response at T1 including acute phase response, antimicrobial pattern recognition, coagulation, glycolysis and gluconeogenesis along with MSP-RON (Macrophage Stimulating Protein – Recepteur d'Origine Nantaise), 14-3-3, RHOA (Ras Homolog family member A), ERK5 (Extracellular signal-Related Kinase 5), PI3K/AKT (Phosphatidylinositol 3-kinases/protein kinase B) and GP6 (Glycoprotein VI) signalling. Furthermore, ARE-mediated mRNA degradation was downregulated along with decreased Hippo and CLEAR (Coordinated Lysosomal Expression and Regulation) signalling.

At the start of follow-up (T2) most pathways that were activated or inhibited at T1 were no longer significantly different from T3. However, a statistically significant decrease remained in the LXR/RXR (liver X receptors/retinoid X receptors) pathway, which regulates cholesterol, lipid and glucose metabolism.

All proteins contributing to the pathway signals observed are presented in Supplement 2.

Heatmap correlation analysis

The proteome from all samples, irrespective of sampling time, was arranged in a heatmap sorted by similarity by protein and sample (Figure 4). The heatmap was then matched to data on anti-NMDAR antibody development, corticosteroid treatment, size of brain MRI disease engagement, valaciclovir follow-up treatment and sampling time. Sampling time (i.e. phase of the disease) was clearly the variable with the strongest correlation to the total proteome fingerprint, particularly in the acute phase of the disease (T1).

Protein-level correlations

In a hypothesis-generating approach, individual protein levels were compared between contrasting patient groups in relation to anti-NMDAR serostatus, corticosteroid treatment, size of brain MRI lesion stratified

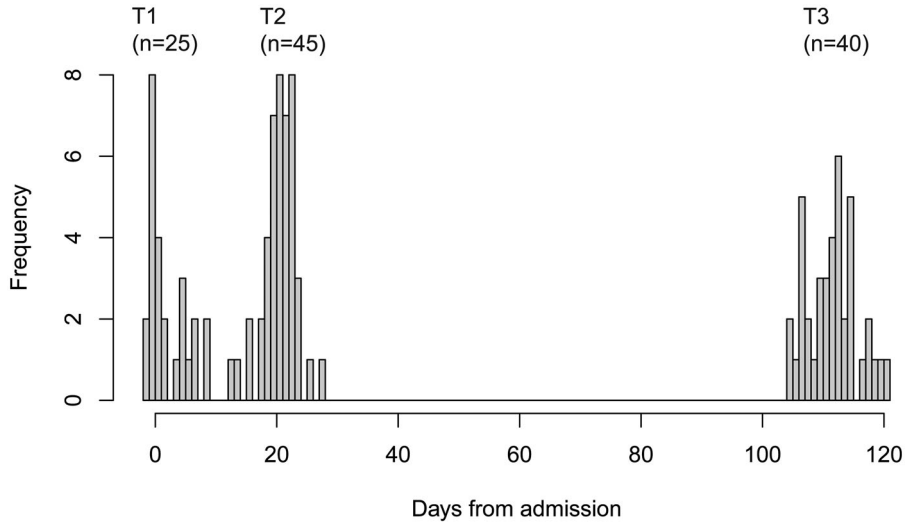

Figure 2. Sampling time in relation to admission date, showing three clusters corresponding to the T1, T2 and T3 time bins used in all statistical analyses.

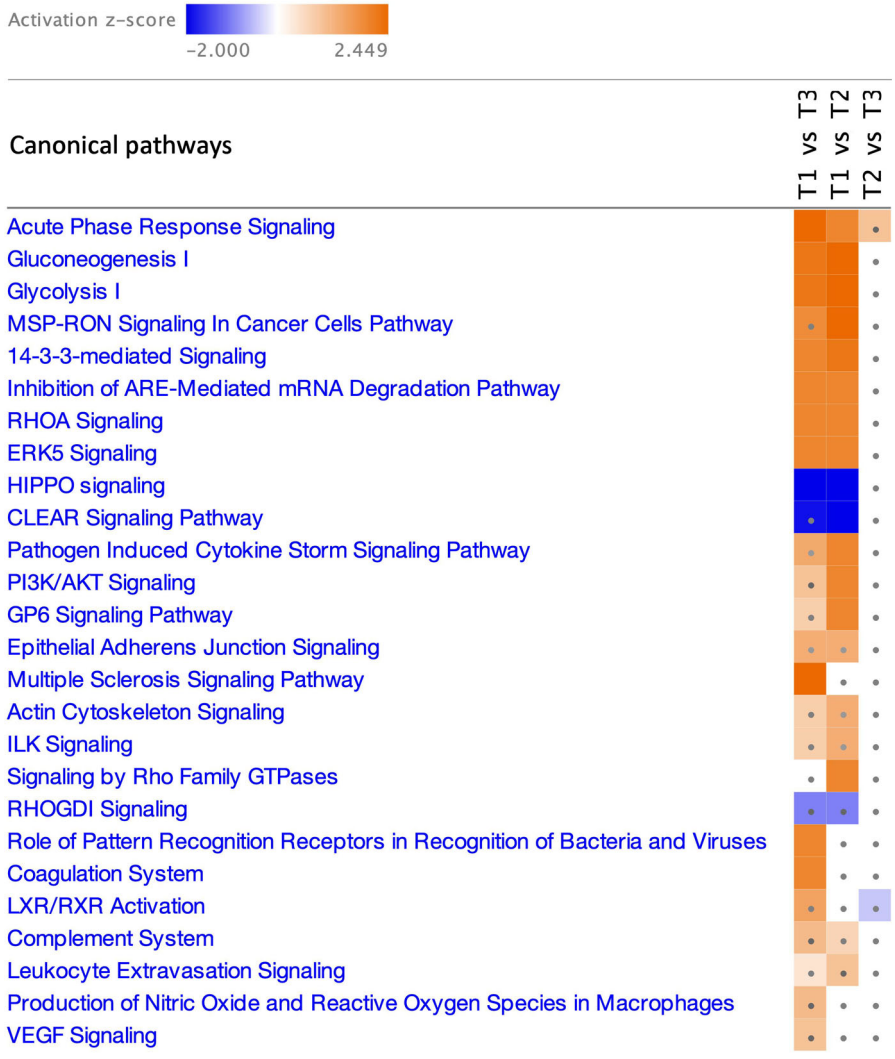

Figure 3. Pathway analysis of CSF samples at acute phase of herpes simplex encephalitis (T1), after 2–3 weeks of intravenous acyclovir Therapy (T2) and after 2–3 months of follow-up (T3). coloured cells indicate an adjusted *p*-value ≤ 0.05, orange indicates increased pathway activation and blue indicates decreased pathway activation. Dots indicate effect sizes below the pre-specified threshold (absolute *Z*-score < 2).

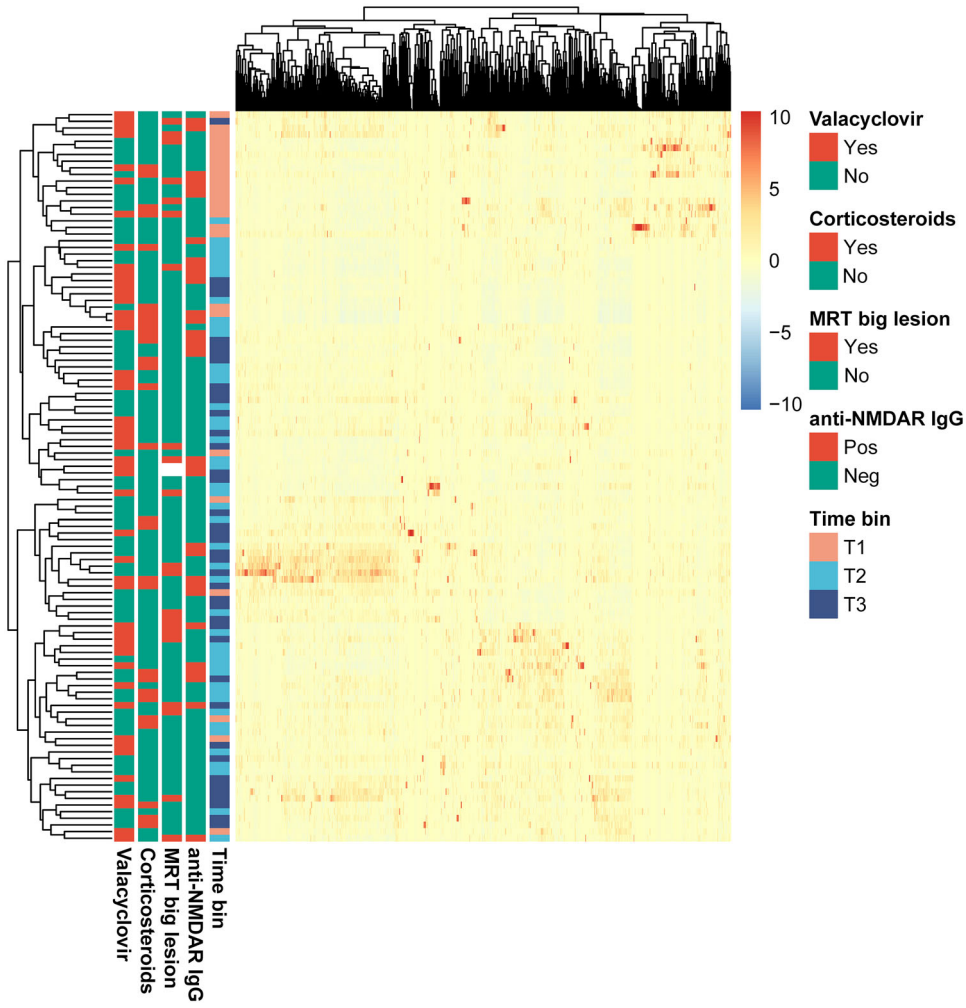

Figure 4. Proteome heatmap of all CSF samples in the study of herpes simplex encephalitis patients, sorted by protein similarity (x-axis) and sample similarity (y-axis). Subject-level data on corticosteroid treatment, anti-NMDAR antibody development, size of brain MRI disease engagement, prolonged antiviral treatment and sampling time is colour coded.

according to Sili et al. [9] and clinical outcome estimated by MDRS and MMSE scores after 3, 12 and 24 months of follow-up. To allow group-wise analyses of neurocognitive performance, data-driven clustering was used to assign patients to one of three groups for each time point.

After correction for multiplicity, six proteins showed significantly lower levels in the NMDAR seropositive group compared to the seronegative, differing among the three-time points as follows: At T1 – procathepsin H (adj. *p*-value .021), heparin cofactor 2 (adj. *p*-value .047), complement factor I (adj. *p*-value .038), protein AMBP (adj. *p*-value .021). At T2 – apolipoprotein A1 (adj. *p*-value .006) (Figure 6). At T3 – polymeric immunoglobulin receptor (adj. *p*-value .026). No substantial differences in these proteins could be observed in relation to anti-MNDAR IgG titres among the seropositive patients. No significant differences in individual proteins were found in relation to corticosteroid treatment, size of brain MRI lesion or neurocognitive performance.

Volcano plots of significantly differentially regulated proteins illustrate the significant changes in proteome over time compared to the smaller variations between anti NMDAR seronegative and anti-NMDAR seropositive groups (Figure 5). Given the previous findings of apoA1 in relation to NMDAR encephalitis, this protein was investigated in further detail (Figure 6).

Discussion

To the best of our knowledge, this is the first study to investigate the CSF proteome in infectious encephalitis using systematic and repeated CSF sampling. High-throughput proteomics using mass spectrometry allows characterisation of the pathophysiological processes in HSE in detail and in our case a view of the protein

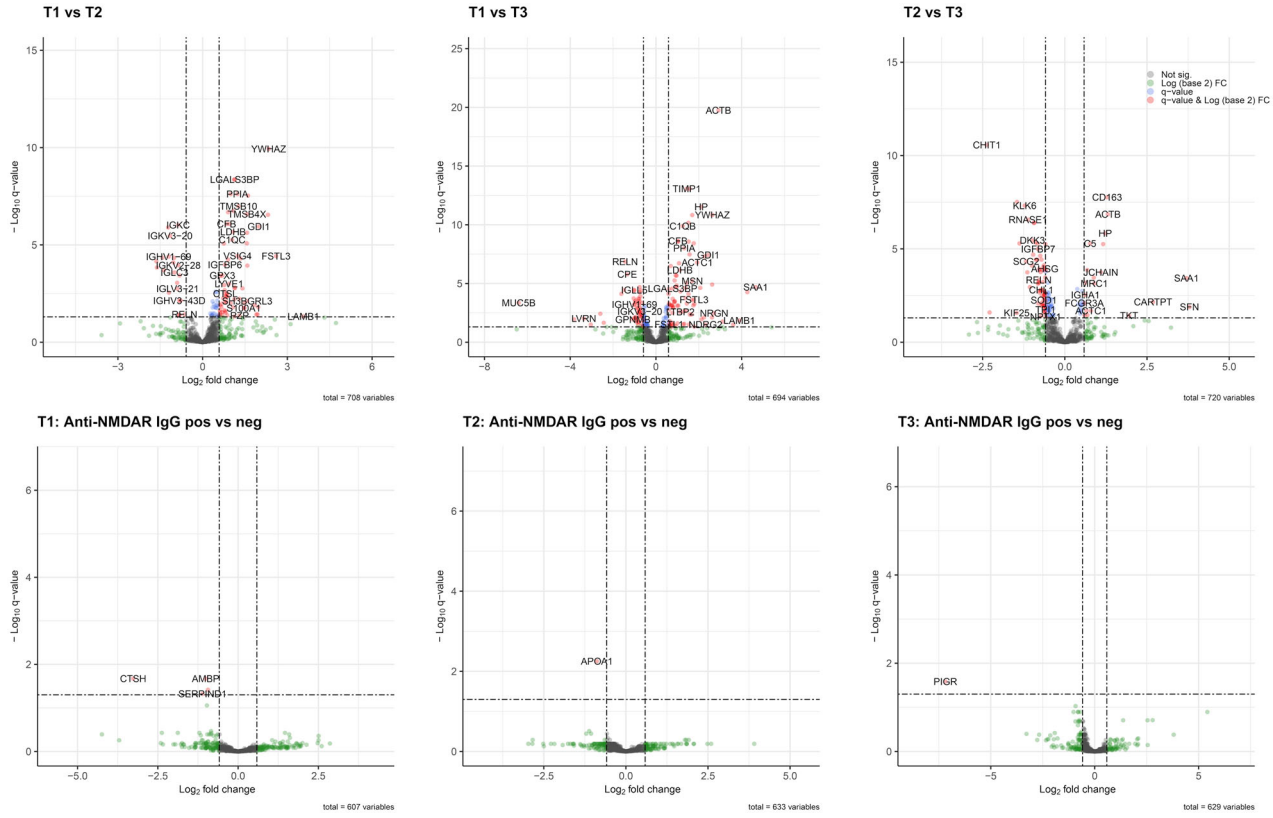

Figure 5. Volcano plots, showing proteins in relation to statistical significance and effect size for time- and group-wise comparisons. First row showing comparisons between different time points (T1–T3); second row showing comparisons between anti-NMDAR positive and negative subjects at each time point.

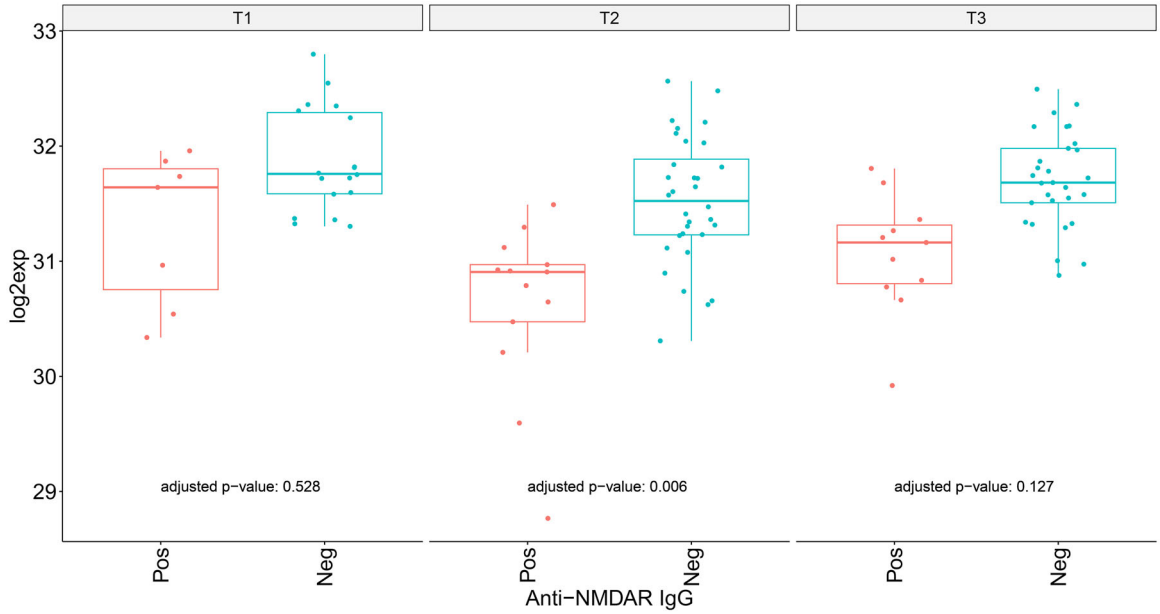

Figure 6. Apolipoprotein A1 levels in the anti-NMDAR seropositive (red) vs seronegative (blue) group in the acute phase (T1), after 14–21 d (T2) and after 3 months of follow-up (T3).

expression over time. We can show strong and statistically significant activation in a broad set of pathways, including acute phase response, coagulation, antimicrobial pattern recognition and several other pathways related to cell signalling, immune response and metabolism. The activated pathways overlap with those activated both in traumatic brain injury (for example complement system pathway and VEGF signalling) [23] as well as in Alzheimer's disease (leukocyte migration and cytokine-mediated signalling) [24].

A majority of the significantly activated pathways are involved in regulating the immune system and the response to infection. In some cases the role in the immune system is quite evident, like the Acute phase response pathway and the Leukocyte extravasation pathway [25,26]. Further, the MSP-RON, 14-3-3 and the Rho GTPase signalling are all involved in activating a variety of leukocytes including macrophages, T cells and neutrophils [27–29]. Further, ERK5, MSP-RON, ARE mediate mRNA degradation and Rho GTPase signalling are all involved in cytokine production [27,29–31]. From a CNS perspective, it is interesting to note that RhoA and the Multiple sclerorsis signalling pathway are linked to neurodegenerative disorders [32]. Appearing more general, some affected pathways are central for essential cell functions, such as the PI3K/Akt pathway, the ILK pathway and the Gluconeogenesis and Glycolysis pathways [33,34]. A common denominator of several of the pathways (e.g. MSP-RON, 14-3-3, RhoA, ERK5) is the harmful effects of dysregulated signalling where an overactivation can lead to extra inflammation and tissue damage [27,28,30,32].

In the study of CNS infections, proteomics has been used to find a pathogen-specific pattern in host response [14,35]. Few studies have focussed on longitudinal samples, but Bakochi et al. have analysed CSF from patients with acute bacterial meningitis and subarachnoid haemorrhage (SAH) for up to 13 days of follow-up, where the proteome in meningitis shows significant changes over time compared to the proteome in SAH, indicating that infections in the CNS result in a complex and dynamic host response [15].

Somewhat surprisingly, contradicting our previous findings of prolonged elevations of brain injury biomarkers and cytokines in HSE patients developing anti-NMDAR antibodies [12], these pathway activations appear to be to a large extent normalised already 2–3 weeks from HSE onset; at least to a level comparable to the activation state after 3 months of follow-up, in lack of a baseline fully representing the healthy state in this study. This illustrates that, although CSF is a relatively sparse biologic matrix, the finer details of anti-NMDAR-related inflammation previously described in this set of samples are difficult to capture using LC-MS/MS proteomics with the sample volumes that were available. Nevertheless, the pathway analysis showing a clear infection-induced dynamic response that validates the biologic relevance of the assay and our data provides new insight at protein-level detail.

The most prominent pattern in our data are the dynamic changes over time. When stratifying study subjects into contrasting groups based on the respective clinical parameters along with sampling time in a heatmap analysis, time appears to be the only parameter showing distinct clustering. In comparison, opposite patterns can be seen in patients with progressive neurologic diseases such as ALS and leptomeningeal metastases, where serial samples of CSF display a shift towards more pathologic pathway signalling over time [36,37].

When comparing the individual proteins between NMDAR seropositive and seronegative patients, six proteins show lower levels in the seropositive group at various time points. Out of these six (procathepsin H, heparin cofactor 2, complement factor I, protein AMBP apolipoprotein A1 and polymeric immunoglobulin receptor) we find apolipoprotein A1 to be of most interest in this biological context. Apolipoprotein A1 is the main component of the high-density lipoproteins (HDL), and aside from its well-known role in cholesterol metabolism it takes part in the immune response as well, having an anti-inflammatory effect [38]. There seems to be a link between apolipoprotein A1 levels and neurodegenerative disease, where a low level of serum apolipoprotein A1 correlates with a higher risk of dementia [39] and the severity of Alzheimer's disease [40,41]. A study by Liu et al. showed lower levels of serum apolipoprotein A1 in NMDAR encephalitis patients compared to healthy controls [42]. However, it should be noted that apolipoprotein A1 levels in serum compared to CSF does not always correlate, since it can be transported through transcytosis [43]. Our finding of lower levels in the anti-NMDAR seropositive group is in line with the hypothesis that apolipoprotein A1 has a neuroprotective effect; hence it should probably be interpreted as a potentially protective factor against the development of anti-NMDAR antibodies. No other single protein levels were significantly correlated to the level of brain MRI injury, corticosteroid treatment or neurocognitive status.

A study on CSF proteomics in Alzheimer's disease patients could define three different subtypes of proteomic profiles, and similar to our study the correlation between proteome and selected clinical parameters could not be statistically established [44]. On the other hand – a study performing CSF proteomics on patients with Parkinson's disease showed a correlation between 27 different proteins and the severity of illness, illustrating the potential in this analytic technique [45].

Previous *in vitro* studies on the proteome during HSV-1 infection have focussed on the early stages of the infection [46,47] while our study adds new knowledge about the molecular mechanisms in a clinical setting over a substantial length of follow-up.

A limitation to this study is the low number of study subjects, which is often the case working with this rare diagnosis. In combination with the high number of analytes captured using mass spectrometry, conclusions on individual proteins is limited by the need for strict application of multiplicity correction, since the variations can to some extent be part of normal inter/intra-individual differences. Another limitation is that the CSF protein levels were not compared to a control group of healthy individuals. A limitation of the proteomics method used is that the proteins that can be easily quantified with LC-MS/MS are those most abundant rather than those most significant in a biological context. For example, it cannot be ruled out that the lack of differences in protein expression related to corticosteroid treatment is a false negative finding, as we have previously shown that other types of assays can provide the resolution needed to detect significant differences in cytokine levels using the same stratification [12].

In conclusion, this study provides insight into both quantitative and qualitative aspects of the dynamic pathophysiology and pathway activation patterns in HSE and has generated a hypothesis for future studies on the role of apolipoprotein A1 in HSE. However, validation in an independent cohort is needed before firm conclusions can be drawn. Further investigations of the CSF proteome and anti-NMDAR serostatus in even later stages of recovery following HSE could provide additional insight into how the CNS is affected by this detrimental disease.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Funding

AN has received funding from the Olinder-Nielsen family foundation; CA has received funding from Umeå University and Region Västerbotten; WX has received funding from Open Medicine Foundation; JB has received funding from Open Medicine Foundation; GW and MS have received funding from the Swedish state under the ALF agreement.

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