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Cerebrospinal fluid biomarkers of brain injury, inflammation and synaptic autoimmunity predict long-term neurocognitive outcome in herpes simplex encephalitis

Gabriel Westman1*, Elisabeth Aurelius2#, Clas Ahlm3, Kaj Blennow4,5, Kristina Eriksson6, Liza Lind6, Silvia Schliamser7, Fredrik Sund1, Henrik Zetterberg4,5,8,9, Marie Studahl10,11#

1 Department of Medical Sciences, Section of Infectious Diseases, Uppsala University, Uppsala, Sweden
2 Unit of Infectious Diseases, Department of Medicine, Karolinska Institutet, and Department of Infectious Diseases, Karolinska University Hospital, Solna, Sweden
3 Department of Clinical Microbiology, Infection and Immunology, Umeå University, Umeå, Sweden
4 Department of Psychiatry and Neurochemistry, Institute of Neuroscience and Physiology, the Sahlgrenska Academy at the University of Gothenburg, Mölndal, Sweden
5 Clinical Neurochemistry Laboratory, Sahlgrenska University Hospital, Mölndal, Sweden
6 Department of Rheumatology and Inflammation Research, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden
7 Department of Clinical Sciences, Division of Infection Medicine, Lund University, Lund, Sweden
8 Department of Neurodegenerative Disease, UCL Institute of Neurology, London, United Kingdom
9 UK Dementia Research Institute at UCL, London, United Kingdom
10 Institute of Biomedicine, The Sahlgrenska Academy at the University of Gothenburg, Gothenburg, Sweden
11 Department of Infectious Diseases, Sahlgrenska University Hospital, Gothenburg, Sweden

*Corresponding author: Gabriel Westman, Department of Medical Sciences, Uppsala University, 751 85 Uppsala, Sweden. Tel +46 18 611 2403. Fax +46 18 611 56 50.
E-mail address: gabriel.westman@medsci.uu.se

#MS and EA contributed equally to this article, and both should be considered senior author.

Keywords: Herpes simplex encephalitis, HSV-1, NFL, cytokines, chemokines, NMDAR, antibodies
Abstract

Objectives

To investigate the correlation between biomarkers of brain injury and long-term neurocognitive outcome, and the interplay with intrathecal inflammation and neuronal autoimmunity, in patients with herpes simplex encephalitis (HSE).

Methods

A total of 53 adult/adolescent HSE patients were included from a prospective cohort in a randomized placebo-controlled trial investigating the effect of a 3-month follow-up treatment with valaciclovir. Study subjects underwent repeated serum/CSF sampling and brain MRI the first 3 months along with cognitive assessment by Mattis Dementia Rating Scale (MDRS) during 24 months. CSF samples were analyzed for biomarkers of brain injury, inflammation and synaptic autoimmunity. The pre-defined primary analysis was the correlation between peak CSF neurofilament protein (NFL), a biomarker of neuronal damage, and MDRS at 24 months.

Results

Impaired cognitive performance significantly correlated with NFL levels (rho = -0.36, p = 0.020). Development of IgG anti-N-methyl-D-aspartate receptor (NDMAR) antibodies was associated with a broad and prolonged proinflammatory CSF response. In a linear regression model, lower MDRS at 24 months was associated with previous development of IgG anti-NMDAR (beta = -0.6249, p = 0.024) and age (z-score beta = -0.2784, p = 0.024), but not CSF NFL, which however significantly correlated with subsequent NMDAR autoimmunization (p = 0.006).

Conclusions

Our findings show that NFL levels are predictive of long-term neurocognitive outcome in HSE, and suggest a causative chain of events where brain tissue damage increases the risk of NMDAR autoimmunisation and subsequent prolongation of CSF inflammation. The data provides guidance for a future intervention study of immunosuppressive therapy administered in the recovery phase of HSE.
Introduction

Herpes simplex encephalitis (HSE) affects approximately 2-4 individuals per million each year and often results in severe neurocognitive sequelae in spite of antiviral therapy [1-5]. As in most infectious diseases, the outcome is dependent not only on the pathogen and the antimicrobial drugs administered, but also on the character, intensity and timing of the immune response [6]. Aciclovir (ACV) treatment greatly improves clinical outcome but far from all patients reach full neurocognitive recovery [3, 7].

Based on indirect support of efficacy, adjunctive corticosteroid treatment has been used to modify the immune response but conclusive evidence from prospective clinical trials regarding the benefit/risk-balance of this intervention is still missing [8, 9].

Previous clinical studies of HSE have investigated various aspects of central nervous system (CNS) inflammation and brain injury during both the acute and recovery phases of the infection. In line with radiologic findings and clinical outcome, several biomarkers of brain injury are elevated and patients often present with long-term intrathecal inflammation [10-13]. Cerebrospinal fluid (CSF) neurofilament (NFL), a marker of axonal degeneration, is elevated in HSE with a maximum level approximately two weeks after onset of disease. Similarly, markers of astroglial cell damage, glial fibrillary acidic protein (GFAP) and S100B are also greatly elevated but reach their peak already in the first week of disease [14]. However, the response in neurodegeneration-related biomarkers such as the synaptic protein neurogranin (Ng) and the astroglial marker YKL-40 (chitinase 3-like protein 1) has not previously been characterized in HSE but correlates with negative outcome in other neuroinflammatory diseases [15, 16].

Herpes simplex virus type 1 (HSV-1) has been shown to trigger not only an antiviral immune response but can also elicit synaptic autoimmunity towards the N-methyl-D-aspartate receptor (NMDAR). This can cause a sterile relapse in clinical encephalitis, but also seems related to a more subtle impairment of neurocognitive recovery [17, 18]. However, in the absence of systematic CSF sampling together with long-term clinical follow-up, the biomarker kinetics and chain of causality in the pathophysiological process have been difficult to elucidate.
In this study, based on a pre-specified statistical analysis plan, we have investigated biomarkers of brain injury along with a broad panel of cytokines and chemokines, in relation to long-term cognitive performance, NMDAR autoimmunity and radiologic outcome in prospectively collected CSF and serum samples from HSE patients.

Materials and Methods

Study subjects, sampling and investigations

A total of 53 adult or adolescent patients with PCR-verified HSE were included from a cohort that was prospectively generated during a placebo-controlled randomized clinical trial investigating the effect of a 3-month follow-up treatment with oral valaciclovir vs. placebo after acute treatment with iv acyclovir for 14-21 days [19]. Adjunctive corticosteroid treatment was given if patients presented with clinical signs of elevated intracranial pressure.

Study subjects were recruited at five Swedish study sites during 2001-2009 and underwent serum/CSF sampling and brain MRI three times during the first 3 months along with systematic neurological and cognitive assessment during 24 months. Cognitive testing was performed using the Mattis Dementia Rating Scale (MDRS), a multi-domain cognitive test with a maximum total score of 144 points indicating good cognitive health [20].

CSF and serum samples were collected at onset of disease (Onset), at end of 14-21 days of iv ACV treatment (FU start) and after three months of follow-up (FU 3M) resulting in a total laboratory follow-up of 104-111 days. Relative to onset of disease, sampling windows were defined as ≤ Day 7 (Onset), Day >12-30 (FU start) and ≥ Day 90 (FU 3M). Only one within-window sample per study subject was included in the statistical analyses.

In addition to standard blood chemistry and haematological investigations, IgG anti-NMDAR was analyzed as previously described [17]. Four subjects did not participate in clinical follow-up. Also, CSF analyses were in some cases limited by sample availability. Written informed consent was
obtained from each patient or legal guardian. The study was approved by the Regional Ethical Review Board at Karolinska Institutet, Sweden.

Biomarkers of neuronal and synaptic injury, glial activation and inflammation

CSF GFAP concentration was measured using an in-house sandwich enzyme-linked immunosorbent assay (ELISA), as previously described [21]. Serum and CSF concentrations of S100B were measured on the Modular system using the S100 reagent kit (Roche Diagnostics, Basel, Switzerland). CSF NFL concentration was measured using an in-house sandwich ELISA, as previously described [22]. Serum NFL concentration was measured using an in-house digital ELISA on a Single molecule array (Simoa) platform (Quanterix, Lexington, MA), as previously described [23]. CSF tau concentration was measured using INNOTEST ELISA (Fujirebio, Ghent, Belgium), whilst serum tau concentration was measured by Simoa using the Human Total Tau 2.0 kit (Quanterix, Lexington, MA). CSF Ng concentration was measured using an in-house ELISA, as previously described [24]. CSF YKL-40 concentration was measured using a commercial ELISA (R&D Systems, Minneapolis, MN). All measurements were performed by board-certified laboratory technicians in one round of analyses using one batch of reagents. Intra-assay coefficients of variation were below 10%.

A total of 30 chemokines and 10 cytokines (CCL1-3, CCL7-8, CCL11, CCL13, CCL15, CCL17, CCL19-27, CX3CL1, CXCL1-2, CXCL5-6, CXCL8-13, CXCL16, GM-CSF, IFN-γ, IL1β, IL2, IL4, IL6, IL10, IL16, MIF and TNF-α) were quantified in duplicate samples of CSF as previously described [25].

Brain MRI

Poor radiologic outcome was pre-defined as bilateral or extensive involvement on any brain MRI examination, based on the classification previously used by Sili et al. [3].
Statistical analyses

Prior to opening the biomarker dataset, all primary and secondary statistical analyses were defined. The primary analysis was the correlation between the maximum level of CSF NFL and MDRS total score after 24 months of follow-up.

Secondary analyses were, without hierarchy, defined as:

- Correlation between brain tissue biomarkers and neurocognitive outcome
- Correlation between brain tissue biomarkers and CNS cytokine/chemokine patterns
- Correlation between brain tissue biomarkers and extension of brain MRI abnormalities
- Correlation between brain tissue biomarkers in CSF and serum
- Laboratory, radiologic and neurocognitive outcome in patients with and without adjunctive corticosteroid treatment
- Laboratory, radiologic and neurocognitive outcome in patients with and without anti-NMDAR IgG

Further details regarding data management and statistical analyses are presented in Supplement 1.

Results

Demographic and clinical characteristics

A summary of the study population demographics, clinical characteristics, pharmacotherapy and selected investigations is presented in Table 1. Of the 13 patients receiving adjunctive steroid treatment, 2 were given dexamethasone, 10 betamethasone, and 1 methyl prednisolone. The median time to start of steroid treatment was 1 day (range 0-4) and therapy was given for a median of 10 days (range 1-125). The patient receiving steroids for 125 days was an outlier case (second longest duration was 22 days) with a prolonged and multi-faceted clinical course.
Primary analysis

The primary pre-defined analysis in the study was the correlation between peak CSF NFL (FU start) and MDRS total score at 24 months from start of follow-up (FU 24M), tested by a two-sided Spearman's rank correlation test. Impaired cognitive performance measured by MDRS was significantly correlated with CSF NFL levels (rho = -0.36, p = 0.020, Figure 1).

To put this correlation into context, an exploratory normalized linear regression model predicting MDRS total score at FU 24M was created showing that, of the selected variables, CSF IgG anti-NMDAR status and age remain as statistically significant predictors of cognitive performance at FU 24M (Table 2).

Biomarkers of neuronal and synaptic injury, glial activation and inflammation

The levels of biomarkers related to brain injury and inflammation, number of analysed samples and individual profiles are presented in Figure 2 and Supplements 2-3. GFAP, Ng and S100B reached their maximum level already at onset of disease, while NFL, tau and YKL40 peak in the FU start sample window approximately two weeks later. Many proinflammatory and anti-inflammatory cytokines, including IFN-γ, TNF-α, IL6, GM-CSF, IL1b and IL10, also reached their maximum level in the first sampling window. In contrast, a subset of chemokines including CCL17, CCL21-CCL27 and CXCL12-13 peaked later in the course of disease.

Levels of brain injury biomarkers were stratified based on subsequent development of IgG anti-NMDAR in CSF. Subjects that later developed NMDAR autoantibodies presented with significantly higher levels of NFL (CSF and serum) and tau (CSF) at FU start, while GFAP, YKL40 and Ng were comparable between groups (Supplement 4). When stratifying for radiologic outcome, subjects with bilateral or extensive parenchymal changes on brain MRI presented with higher levels of S100B in CSF at FU3M and serum at FU start, while NFL was similar between groups. There were no
substantial differences between groups when stratifying for adjunctive corticosteroid treatment, length
of iv ACV therapy or VACV follow-up therapy.

The cytokine/chemokine response was analyzed in subgroups defined by IgG anti-NMDAR status,
extent of radiologic findings, adjunctive corticosteroid treatment and VACV follow-up treatment.
Subjects who developed IgG anti-NMDAR in CSF presented with a broad increase of overall
inflammatory response, with differences most prominent at FU 3M where levels of IFN-\(\gamma\), IL1b, IL2,
IL6, IL10, CCL1, CCL3, CCL11, CCL13, CCL17, CCL26, CXCL2, CXCL8 and CXCL9 were
significantly elevated compared to subjects without signs of synaptic autoimmunity (Supplement 5).

When stratifying for adjunctive corticosteroid treatment, subjects receiving steroids had an overall
lower inflammatory response at FU start, i.e. after steroids had been given (Supplement 6). Stratifying
for radiologic findings, subjects with bilateral or extensive parenchymal involvement on brain MRI
presented with significantly lower levels of IFN-\(\gamma\), IL1b, IL2, IL4, IL6, TNF-\(\alpha\), CCL17, CCL19-23,
CCL25, CXCL1, CXCL2, CXCL8, CXCL10 and CXCL10-13 at the end of the acute phase of disease
(FU start) (Supplement 7). This difference was not driven by a disproportionate fraction of these
subjects receiving adjunctive corticosteroid treatment, as only 2 of 13 subjects fulfilling the radiologic
criteria for bilateral/extensive lesions were among those receiving steroids. There were no clear
differences in cytokine/chemokine response with regards to VACV follow-up therapy.

The correlation between inflammation in the acute phase of disease and CSF NFL at FU start was
investigated in a linear regression model including selected pro- and anti-inflammatory cytokines, total
CSF leukocyte count and corticosteroid treatment (Table 2), showing that NFL is significantly
correlated with IL10 levels (positively) and total CSF leukocyte count (negatively) (Supplement 8). A
sensitivity analysis was performed to verify that age was not a significant confounder in the model
(data not shown).
Discussion

Despite seemingly effective antiviral treatment against HSE, many patients experience significant neurological sequelae. Here, we show that the long-term neurocognitive outcome after HSE correlates with CSF biomarkers of brain injury, inflammation and synaptic autoimmunity.

The pre-defined primary statistical analysis shows a significant correlation between NFL levels in CSF and long-term cognitive performance as measured by MDRS after 24 months of follow-up. This effect appears at least partially mediated through synaptic autoimmunization as high CSF levels of NFL strongly correlate with subsequent development of anti-NMDAR which, together with age, remain as an independent statistically significant predictor of long-term cognitive performance in the multivariable linear regression model. Furthermore, perhaps explaining the impaired recovery of neurocognitive performance related to NMDAR autoimmunisation previously observed [17], subjects developing IgG anti-NMDAR in CSF present with a significantly prolonged phase of intrathecal inflammation, illustrated by a broad-scale and highly significant elevation of both pro- and anti-inflammatory cytokines.

Our findings suggest a causative chain of events where the initial brain tissue damage, caused by the lytic HSV-1 infection, increase the risk of NMDAR autoimmunisation which in turn prolongs the CSF inflammation. This is in line with previous findings by Kamei et al. and Michael et al. [12, 13] and could also explain previous findings by Aurelius et al. of a pro-inflammatory state that extends even further in time [10, 11].

CSF levels of IL10 and total leukocyte count at onset of disease could serve as prognostic factors for peak CSF NFL level a few weeks later. Also, CSF NFL levels at the end of iv ACV therapy could potentially serve as a predictive biomarker for NMDAR autoimmunization, in addition to the primary analysis showing a correlation to long-term neurocognitive outcome. As we have previously shown, a reliable test for synaptic autoimmunity cannot be performed during the acute phase of HSE but it is
possible that a new lumbar puncture 2-4 weeks later would be suitable to screen for NMDAR
autoantibodies rather than waiting 3 months as was done in this study protocol [17, 18].

To address the remaining challenges in the treatment of HSE, a better understanding of how immune
modulation affects long-term clinical outcome is needed. In contrast to acute bacterial meningitis [26],
it is not clear whether adjunctive corticosteroid treatment in the acute phase of HSE contributes to
improved outcome or whether the effects of suppressing the early innate immune response could even
be detrimental. Today, corticosteroids are used on clinical indication when patients develop signs of
increased intracranial pressure (ICP) in the acute phase. A beneficial effect of corticosteroids has been
suggested in a retrospective study of humans [8] as well as in animal studies [9, 27]. The relation in
our dataset between lower CSF inflammation and bilateral/extensive brain MRI lesions raises the
question whether a strong pro-inflammatory state could be beneficial in early stages of disease,
serving to limit the spread of HSV-1 in the brain parenchyma. Steroid treatment was clearly associated
with lower CSF inflammation as illustrated by a significant reduction of a broad range of
inflammatory cytokines and chemokines. To fully clarify this issue and untangle possible
confounding, prospective randomized clinical trials are needed.

There are several limitations to this study. First, the availability of samples was a limiting factor
leading to a paucity of data for some biomarkers, and some measurements that were reported above
the upper limit of quantification had to be estimated rather than re-analyzed. However, non-parametric
statistical analyses have been used wherever possible to mitigate this issue, including the primary
study endpoint. Also, although samples have been stored in -70°C it cannot be excluded that the
absolute biomarker levels could be affected by time-dependent degradation. However, as our statistical
analyses are all within-study comparisons between subgroups the findings should be robust in this
aspect. Finally, the regression models were built after viewing the data and should be viewed as
exploratory. The selection of predictors was based on a scientific rationale, considering the size
limitations of the dataset, and although we believe the models provide a valuable understanding of the
interrelations in our data the findings could be driven by random effects and need to be independently
verified.
In conclusion, our findings illustrate the interplay between brain damage, synaptic autoimmunity, CNS inflammation and long-term clinical outcome. We believe that there now is sufficient data to support the initiation of a clinical trial investigating whether prolonged, low-dose corticosteroid therapy together with oral HSV-1 relapse antiviral prophylaxis, administered after the acute phase of disease, could reduce the risk of post-infectious neuronal autoimmunity and improve long-term clinical outcome. Also, we propose that CSF NFL measured at the end of iv ACV therapy could serve as a prognostic biomarker of clinical outcome in HSE and that such sampling could be coordinated with repeated analysis of HSV DNA.

Disclosures

This work was supported by grants from Fredrik och Ingrid Thurings stiftelse (GW), the Swedish state under the agreement between the Swedish government and the country councils, the ALF-agreement (GW, KE, KB, HZ), the Swedish Research Council (KE, KB, HZ), the Swedish Alzheimer Foundation (KB, #AF-742881), Hjärnfonden, Sweden (KB, #FO2017-0243) and the European Research Council (HZ, #681712). Furthermore, KB holds the Torsten Söderberg Professorship in Medicine at the Royal Swedish Academy of Sciences and HZ is a Wallenberg Scholar.

KB has served as a consultant or at advisory boards for Axon, Biogen, CogRx, Lilly, MagQu, Novartis and Roche Diagnostics, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg, all unrelated to the work presented in this paper.

HZ has served at scientific advisory boards for Roche Diagnostics, Wave, Samumed and CogRx, has given lectures in symposia sponsored by Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB, a GU Ventures-based platform company at the University of Gothenburg, all unrelated to the work presented in this paper.

The remaining authors have no disclosures.
Author contributions (CRediT)

Conceptualization - GW, MS, EA. Formal analysis - GW. Investigation - All authors. Resources -

GW, MS, HZ, KE. Writing, original draft - GW. Writing, review & editing - All authors. Visualization

- GW.
Table 1. Demographic and clinical characteristics of 53 patients with herpes simplex encephalitis. Data presented as medians (range) or proportions.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>59 (14-80)</td>
</tr>
<tr>
<td>Sex (male:female)</td>
<td>30:23</td>
</tr>
<tr>
<td>RLS at onset of disease</td>
<td>2 (1-4)</td>
</tr>
<tr>
<td>Duration of iv ACV therapy (days)</td>
<td>20 (13-25)</td>
</tr>
<tr>
<td>Adjunctive corticosteroid therapy</td>
<td>13/53</td>
</tr>
<tr>
<td>VACV follow-up therapy</td>
<td>26/53</td>
</tr>
<tr>
<td>Brain MRI with bilateral/extensive involvement</td>
<td>11/49</td>
</tr>
<tr>
<td>CSF IgG anti-NMDAR positivity</td>
<td>14/53</td>
</tr>
</tbody>
</table>

Table 2. Standardized (z-score) multivariable linear regression models of predictors for long-term neurocognitive outcome and CSF neurofilament levels.

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Estimate</th>
<th>2.5%</th>
<th>97.5%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mattis Dementia Rating Scale after 24 months of follow-up (FU 24M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CSF NFL (FU start)</td>
<td>-0.1401</td>
<td>-0.39765593</td>
<td>0.11755411</td>
<td>0.2773</td>
</tr>
<tr>
<td>Brain MRI bilateral/extensive</td>
<td>-0.2359</td>
<td>-0.78630262</td>
<td>0.31452980</td>
<td>0.3902</td>
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<tr>
<td>CSF IgG anti-NMDAR positivity</td>
<td>-0.6249</td>
<td>-1.16037057</td>
<td>-0.08936476</td>
<td>0.0235</td>
</tr>
<tr>
<td>Age</td>
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<td>-0.51844287</td>
<td>-0.03827104</td>
<td>0.0243</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Predictor</th>
<th>Estimate</th>
<th>2.5%</th>
<th>97.5%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF NFL at start of follow-up (FU start)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ (onset)</td>
<td>0.11902</td>
<td>-0.18424157</td>
<td>0.4222752</td>
<td>0.41408</td>
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<tr>
<td>TNF-α (onset)</td>
<td>0.15024</td>
<td>-0.23818103</td>
<td>0.5386518</td>
<td>0.42070</td>
</tr>
<tr>
<td>IL1b (onset)</td>
<td>0.19700</td>
<td>-0.03076934</td>
<td>0.4247605</td>
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<tr>
<td>IL10 (onset)</td>
<td>0.46428</td>
<td>0.14583882</td>
<td>0.7827224</td>
<td>0.00742</td>
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<tr>
<td>Adjunctive steroid therapy</td>
<td>-0.23169</td>
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<td>0.3127903</td>
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<tr>
<td>CSF total leukocyte count (onset)</td>
<td>-0.42709</td>
<td>-0.73509479</td>
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<td>0.01005</td>
</tr>
</tbody>
</table>

Onset, onset of disease
FU start, start of follow-up after 14-21 days if iv treatment
FU 24M, follow-up at 24 months
Figure 1. Correlation between CSF NFL at start of follow-up (FU start) and Mattis Dementia Rating Scale (MDRS) after 24 months of follow-up (FU 24M).

Figure 2. Brain injury biomarkers in patients with herpes simplex encephalitis, tracking individual subjects during the acute phase of disease and through 3 months of follow-up.

Supplement 1. Data management and statistical analyses.

Supplement 2. Summary of brain injury and inflammation biomarkers at onset of disease (Onset), at end of 14-21 days of iv ACV treatment (FU start) and after three months of follow-up (FU 3M).

Supplement 3. Inflammation biomarkers in patients with herpes simplex encephalitis, tracking individual subjects during the acute phase of disease and through 3 months of follow-up.

Supplement 4. Selected brain injury biomarkers, stratified for the presence of IgG anti-N-methyl-D-aspartate receptor (NMDAR) autoantibodies in herpes simplex encephalitis.

Supplement 5. Selected inflammation biomarkers, stratified for the presence of IgG anti-N-methyl-D-aspartate receptor (NMDAR) autoantibodies in herpes simplex encephalitis.

Supplement 6. Cytokine/chemokine response in relation to corticosteroid treatment at onset of disease (Onset), at end of 14-21 days of iv ACV treatment (FU start) and after three months of follow-up (FU 3M).

Supplement 7. Cytokine/chemokine response in relation to brain MRI bilateral/extensive involvement (MRI big lesion) at onset of disease (Onset), at end of 14-21 days of iv ACV treatment (FU start) and after three months of follow-up (FU 3M).

Supplement 8. Correlation between CSF IL-10 (left) and total leukocyte count (right) at onset of disease and CSF neurofilament protein (NFL) at start of follow-up (FU start).
References


