SARS-CoV-2 encephalitis is a cytokine release syndrome:
evidences from cerebrospinal fluid analyses

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Summary:

In this study, we applied an extensive panel of neuronal, glial and inflammatory cerebrospinal fluid biomarkers in a consecutive sample of SARS-CoV-2 related encephalitis. The neurochemical alterations strongly argued for a cytokine-induced neuroinflammation as underlying mechanism of SARS-CoV-2 related encephalitis.
ABSTRACT:

Background:

Recent findings indicated that SARS-CoV-2 related neurological manifestations involve cytokine release syndrome along with endothelial activation, blood brain barrier dysfunction, and immune-mediated mechanisms. Very few studies have fully investigated the CSF correlates of SARS-CoV-2 encephalitis.

Methods:

Patients with PCR-confirmed SARS-CoV-2 infection and encephalitis (COV-Enc), encephalitis without SARS-CoV-2 infection (ENC) and healthy controls (HC) underwent an extended panel of CSF neuronal (NfL, T-tau), glial (GFAP, TREM2, YKL-40) and inflammatory biomarkers (IL-1β, IL-6, IL-8, TNF-α, CXCL-13 and β2-microglobulin).

Results:

Thirteen COV-Enc, 21 ENC and 18 HC entered the study. In COV-Enc cases, CSF was negative for SARS-CoV-2 real-time PCR but exhibited increased IL-8 levels independently from presence of pleocytosis/hyperproteinorrachia. COV-Enc patients showed increased IL-6, TNF-α, and β2-microglobulin and glial markers (GFAP, sTREM-2, YKL-40) levels similar to ENC but normal CXCL13 levels. Neuronal markers NfL and T-Tau were abnormal only in severe cases.

Conclusions:

SARS-CoV-2-related encephalitis were associated with prominent glial activation and neuroinflammatory markers, whereas neuronal markers were increased in severe cases only. The pattern of CSF alterations suggested a cytokine-release syndrome as the main inflammatory mechanism of SARS-CoV-2 related encephalitis.

Keywords: Encephalitis, COVID-19, SARS-CoV-2, Cytokine storm syndrome; ICANS
INTRODUCTION

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is characterized predominantly by lower respiratory tract involvement. In addition to its pulmonary manifestations, growing evidence showed encephalitis as a possible manifestation of the disease [1-3]. While the number of reported cases of encephalitis in COVID-19 is rapidly increasing, the question whether SARS-CoV-2 may cause neurological manifestations through a direct neuropathic effect or by promoting a hyperinflammatory reaction in the host's immune system in the form cytokine release syndrome is still a theme of debate [1,4,5].

Few cases indeed exhibited SARS-CoV-2 virus [6-7] or autoantibodies in CSF [8], whereas the majority of cases appeared to be concomitant of infection and associated with abnormal neuroinflammatory parameters in CSF [2-5, 9,10].

Cytokine release syndrome (CRS) is a potentially fatal complication of various infectious (e.g. influenza, SARS, Epstein–Barr virus) and non-infectious diseases (e.g. multiple organ dysfunction syndrome, multiple sclerosis) and is triggered by an initial release of cytokines able to activate bystander immune cells and endothelial cells to produce proinflammatory molecules. CRS-based neurological disturbances have been also recently described following CAR-T cell therapy and are termed Immune effector cell-associated neurotoxicity syndrome (ICANS).

Disproportionately high concentrations of IL-6 and IL-8 and TNF alpha have been found in the CSF of patients with severe CRS and ICANS neurotoxicity, thought to be due to the combination of increased barrier permeability and intrathecal production by activated myeloid, astrocyte, and/or endothelial cells. According to this, glial cell activation has been
recently pointed out as one of most sensitive alterations leading to neuroinflammation in ICANS and CRS [11-13].

According to these findings, we aimed to investigate CSF abnormalities in SARS-CoV-2 related encephalitis in order to confirm the hypothesis that neurologic involvement during COVID-19 is due to cytokine release syndrome [4,11,14].

To this end, we examined CSF samples from patients with SARS-CoV-2-related encephalitis (Cov-Enc) using an extensive panel of cytokines/chemokines and neuronal/glial biomarkers, contrasting them with healthy controls (HC) and encephalitis not associated with COVID-19 infection (ENC).

METHODS:

Patients

The study was carried out at ASST Spedali Civili and ASST Cremona hospitals between February 20th and 30th June 2020 including COVID-19 patients consecutively admitted fulfilling criteria for encephalitis according to a full screening protocol [15]. The case definition for COV-Enc included any person with confirmed SARS-CoV-2 infection aged ≥18 admitted to hospital with altered mental status lasting ≥24 hours and the presence of two or more of the following criteria: i) generalized or partial seizures not fully attributable to a pre-existing epilepsy ii) new onset of focal neurologic findings iii) CSF white blood cell count ≥5/cubic mm³ iv) abnormality of brain parenchyma on neuroimaging suggestive of encephalitis that was either new from prior studies or appears acute in onset v) abnormality on electroencephalography consistent with encephalitis. Fever was not considered as supportive feature for encephalitis (as indicated by standard criteria[15], as it is highly prevalent in COVID-19 disease. Laboratory confirmation of SARS-CoV-2 infection was
carried out by RT-PCR procedure on throat swab and nasopharyngeal specimens in all patients.

For biomarker comparison, a cohort of 18 neurologically-healthy controls (HC) with normal MRI, neurological examination and biochemical CSF analyses and a group of 21 encephalitis (10 infectious and 11 autoimmune- including 4 associated with anti-NMDAR antibodies, 5 with anti-LG1 antibodies, 2 with anti-GAD antibodies) were retrospectively included. The Institutional Ethical Standards Committee on human experimentation at Brescia University Hospital provided approval for the study (NP 4067).

*Encephalitis assessment and diagnosis*

First-line testing included all commonly recognized causes of encephalitis according to current guidelines [15,16]. Each COV-Enc underwent brain magnetic resonance imaging, standard electroencephalography (EEG), thyroid function and antibodies (anti-thyroglobulin, anti-thyroid peroxidase), IgM and IgG for *Borrelia burgdorferi*. CSF viral screening included herpes simplex virus (HSV-1, HSV-2, HSV-6, HSV-8, CMV, Epstein-Barr virus, varicella zoster virus), adenovirus and enterovirus. SARS-CoV-2 virus in the CSF was tested by RT-PCR in all cases and additionally tested for four samples at the Department of Infectious Diseases, Italian Institute of Public Health. Briefly, an aliquot of CSF samples was used for the virus culture by standard methodology: the Vero cells which were cultured 1 × Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2% fetal bovine serum at 37 °C with 5% CO2; used for the inoculation of samples [17].

The illness severity of COVID-19 was defined according to the Brescia COVID Respiratory Severity Scale (BCRSS) for COVID-19 [18] and according to the quick Sequential Organ Failure Assessment (qSOFA)[19]. The severity of neurological symptoms was defined according to the ICANS grading proposed by Lee and coauthors [13].
CSF immunological and biochemical analyses

At enrollment, 3 milliliters of CSF from each participant were collected, centrifugated and firstly processed for standard biochemical analyses. Two milliliters of CSF were stored in cryotubes at −80°C before testing. All subjects underwent immunological screening including antibodies against NMDAR, LGI1, CASPR2, GABA\(_{\alpha}\)R, AMPAR, DPPX, Ri, Yo, Ma2, CV2, Hu, amphiphysin, titin (EUroleline and Mosaic kit, Euroimmun, Luebeck) and MOG (live cell-based assay) at the Neurology Unit, Department of Neurosciences, Biomedicine and Movement Sciences, University of Verona, Italy. In addition, to expand the analyses of unknown antigens or not commercially available autoantibodies, indirect immunofluorescence was performed in eight patients fulfilling criteria for possible autoimmune encephalitis [16] using two different fixation protocols on mouse tissue substrate (including CNS and non-CNS tissues in order to exclude patients with non-neural specific autoimmunity) in the Neuroimmunology laboratory at Mayo Clinic, in Rochester; serum was tested after absorption with liver powder at 1:240 and CSF was tested undiluted. Briefly, mouse cryosections were either fixed with 4% paraformaldehyde (1 minute), washed (phosphate-buffered saline [PBS]), permeabilized with 0.5% CHAPS (C32H58N2O7S, 1 minute), or directly fixed with 10% buffered formalin for 10 minutes. After washing with PBS, normal goat serum (10% diluted in PBS) was applied for 1 hour and sections were then incubated with patient’s samples (40 minutes), washed, incubated with anti-human IgG secondary antibodies for 30 minutes, washed and examined by two independent reviewers (AZ, AMcK). This extensive autoimmune screening did not reveal any abnormality in all COV-Enc cases included.

All CSF samples were analyzed for inflammatory and neuronal/glial markers at the Clinical Neurochemistry Laboratory at Sahlgrenska University Hospital (Möln达尔, Sweden). CSF cytokine concentrations (including IL-6, IL-8, TNF-\(\alpha\), IL-1\(\beta\)) were measured using a Mesoscale Discovery multiplexed immunoassay (Rockville, MD). CSF CXCL13 concentration was measured using a commercial ELISA (R&D Systems, Minneapolis, MN). CSF \(\beta\)2M concentration was measured using an
immunoassay on an Atellica instrument (Siemens Healthcare GmbH, Erlangen, Germany). CSF total tau (T-tau) concentration was measured by Lumipulse (Fujirebio, Ghent, Belgium). CSF NfL and GFAP concentrations were measured using in house enzyme-linked immunosorbert assays [20, 21]. CSF sTREM2 concentration was measured using an in-house immunoassay with electrochemiluminescent detection, as previously described in detail [22]. CSF YKL-40 concentration was measured using the Human Chitinase 3-like 1 Quantikine kit (R&D Systems, Minneapolis, MN). All analyses were performed by board-certified laboratory technicians who were blinded to clinical data.

Statistical analyses

Data are presented as median, interquartile ranges for continuous variables and number (%) for categorical variables. For subgroup comparisons, we used the Fisher exact test and nonparametric test (Kruskall Wallis test adjusted for the effect of age), when appropriate. SPSS 24 (IBM, Armonk, NY) was used for statistical analysis. Post-hoc analyses were performed using Bonferroni correction at p=0.05.

Data availability

All clinical and CSF analyses data are available from authors upon reasonable request.

RESULTS

Clinical characteristics of COVID-19 related encephalitis

The study recruited thirteen cases of SARS-CoV-2-related encephalitis. Clinical features, respiratory severity and final outcomes are highlighted in table 1. All cases presented almost concomitant the respiratory symptoms with onset altered mental status associated in six cases with aphasia and three with dysarthria (ICANS grading 2-4). EEG was abnormal in all cases,
showing generalized slow waves prominent on the frontal derivations in ten patients, while focal epileptic alteration was observed in three cases.

Brain imaging was normal in ten cases whereas three patients showed heterogeneous MRI alterations including multiple subcortical/cortical T2-hyperintensities, associated in one case to diffusion weighted imaging (DWI) hyperintensities (Table 1). Four cases (mean ICANS grade 3.75) deceased during hospitalisation. Spontaneous recovery was observed in five patients, while four patients clinically improved after high-dose methylprednisolone treatment (mean ICANS grade 2.75).

**CSF analyses**

CSF biochemical standard analyses showed mild pleocytosis (5 to 26 cells) in nine patients and increased protein levels in eight COV-Enc patients (Tables 1 and 2).

RT-PCR for SARS-CoV-2 was negative in the CSF of all COVID-19 patients. In four additional samples the virus culture showed no specific cytopathic effect after three days of inoculation (see methods for details).

Compared with HC, COV-Enc showed normal CXCL13 levels, significantly higher CSF levels of neuronal damage markers such as NfL and total tau, as well as glial-related markers such as GFAP, TREM2 and YKL-40, higher concentration of cytokines such as IL-1β, IL-6, IL-8, TNF-α (p<0.001 for all, Supplementary Table 1). COV-Enc exhibited comparable neuronal, glial and inflammatory markers but normal CXCL13 levels (p=0.026) compared to encephalitis not associated with SARS-CoV-2 infection (infectious and autoimmune, Figure 1 and supplementary Table 1).

All Cov-Enc cases showed increased IL-8 CSF levels, whereas eleven showed increased beta-2 microglobulin. Glial markers, namely GFAP and sTREM2, were abnormal in twelve and ten COV-Enc patients, respectively. Tau and NfL levels were abnormal in five and six
subjects with higher considering age-adjusted cut-offs [20]. Three out of four cases without pleocytosis exhibited increased glial markers, whereas all showed abnormal IL-8 CSF levels (table 2).

**DISCUSSION**

The present study demonstrated that SARS-CoV-2 encephalitis are associated with early IL-8 increases and glial alterations whereas neuronal damage markers were elevated in severe cases. Furthermore, the pattern of neuroinflammatory markers assessed is highly suggestive for a cytokine-release syndrome as driver of SARS-CoV-2 related encephalitis.

The sample included thirteen hospitalized patients affected by SARS-CoV-2 infection fulfilling diagnostic criteria for probable encephalitis [15]. Clinically, most patients presented with early language disturbances associated with altered mental status, in line with clinical features exhibited by most ICANS cases [11]. The CSF biochemical analyses showed mild increased protein levels indicating a blood-brain barrier damage and also mild pleocytosis. Patients had negative infectious and neuronal antibodies screening and tested negative for the presence of SARS-CoV-2 virus in CSF. Conversely, CSF neuronal damage markers appeared to be elevated in CSF compared to controls and similar to non COVID-19 encephalitis. ENC-Cov severe patients specifically exhibited very high levels of NfL, an axonal structural protein and a biomarker of neuronal injury, recently observed also in a subset of SARS-CoV-2 related encephalopathies [23]. In addition to this, COV-Enc showed increased CSF levels of markers of activation and damage of astrocytes and microglia, such as GFAP, TREM2 and YKL-40, arguing for a strong neuroinflammatory response similar to cases of non COVID-19 encephalitis. Specifically, we observed increased CSF levels in the membrane-bound receptor TREM-2, a known marker of microglia activation, and modulator of neuroinflammation in both chronic and acute CNS disorders [24]. COV-Enc showed also abnormal levels of YKL-40, a glycoprotein produced
by reactive astrocytes and microglia during neuroinflammation in different primary CNS diseases and encephalitis [25]. Of note, YKL-40 release could be activated by increased level of IL-6 and TNF-α [26], and thus plays an important role in CRS together with GFAP, an intermediate filament highly expressed in astrocytes [12]. Indeed, alterations within GFAP expression indicating involvement of astrocytes have been recently described widespread in subcortical and cortical regions in severe cases of SARS-CoV-2 infection [27]. GFAP increased levels have been also identified as early alterations of ICANS, when CRS was induced by T-cell therapy [11]. Of note, increased GFAP levels were presented by all but one COV-Enc patient, even in absence of pleocytosis or hyperproteinorrachia.

The analysis of CSF in COV-Enc patients additionally revealed increased level of several cytokines, consistent with an acute neuroinflammatory response and blood-brain barrier disruption [28]. Specifically, we observed a significant increase of CSF levels of IL-6, IL-8, β2M and TNF-α [28, 29] whereas levels of the chemokine CXCL13 were normal [31] at variance with other forms of encephalitis. In absence of CSF evidences of autoantibodies or SARS-CoV-2 virus, the normal levels of CXCL13 found in COV-ENC patients thus argue against a “classical” direct infectious as well as autoimmune based mechanisms in the pathogenesis of SARS-CoV-2-related encephalitis.

Additionally, COV-Enc exhibited very high levels of IL-8, a cytokine already associated with several inflammatory alterations but specifically high in both ICANS [11] and CRS complications in SARS-CoV-2 infection [32].

Thus, the biomarkers/cytokine alterations observed strongly support the claim of an inflammatory-induced mechanism causing SARS-CoV-2 encephalitis. This fits well with the risk of cytokine-induced syndrome in COVID-19 disease [10] and with clinical presentations observed after immune effector associated treatment[14]. This pathophysiology, supported by our extensive CSF data, could fit with the majority of encephalitis reported in the literature with a disease onset concomitant the respiratory infection [1-5,9,10]
The close known relationship between CRS and neurotoxicity suggests that systemic inflammatory mediators act directly across the blood-brain barrier with neuroinflammation induction via glial and astrocyte activation [12]. This can lead to secondary neuroinflammatory-mediated encephalitis highlighted by positive axonal injury biomarkers and stronger cytokine/interleukin changes in CSF than serum.

These findings have deep implications for the management of patients presenting with encephalitis related to SARS-CoV-2 infection, as high-dose corticosteroid treatment (i.e. methylprednisolone 1 g/day for five days) is the first-line therapy for severe neurotoxicity in CRS cases [13] after exclusion of co-infections- whereas additional immunomodulatory treatment could be proposed according to single case-specific cytokine profile.

We acknowledge that this study entails several limitations: First, due to the absence of autopsy, our cases can be classified as “probably associated” with SARS-CoV-2 infection [1] and further studies evaluating SARS-CoV-2 antibodies in CSF are urgently needed. Second, the study potentially exclude cases of SARS-CoV-2 encephalitis with severe respiratory COVID-19 who could not undergo a complete MRI, EEG and CSF assessment. Third, the study was cross-sectional in nature and the normalisation of the CSF markers after clinical improvement should be verified in on-going longitudinal studies.

Despite these limitations, the study demonstrated for the first time that encephalitis in COVID-19 are associated with alterations within neuronal/glial biomarkers with a specific cytokine pattern indicating inflammatory-mediated underpinning mechanisms in this complication to SARS-CoV-2 infection.
AUTHORS’ CONTRIBUTION


ACKNOWLEDGEMENT

The authors thank Stefano Fiore, Eleonora Benedetti, Concetta Fabiani, Dept of Infectious Diseases, Istituto Superiore di Sanità for technical assistance on SARS-CoV-2 virus cultivation experiments.

FUNDING

AP is supported by the Italian Ministry of Health and H2020-IMI initiative (#IMI2-2018-15-06).

KB is supported by the Swedish Research Council (#2017-00915), the Alzheimer Drug Discovery Foundation (ADDF), USA (#RDAPB-201809-2016615), the Swedish Alzheimer Foundation (#AF-742881), Hjärnfonden, Sweden (#FO2017-0243), the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986), and European Union Joint Program for Neurodegenerative Disorders (JPND2019-466-236).

HZ is a Wallenberg Scholar supported by grants from the Swedish Research Council (#2018-02532), the European Research Council (#681712), Swedish State Support for Clinical Research (#ALFGBG-...
the Alzheimer Drug Discovery Foundation (ADDF), USA (#201809-2016862), and the UK Dementia Research Institute at UCL.

COMPETING INTEREST

Andrea Pilotto is consultant and served on the scientific advisory board of Z-cube (Technology Division of Zambon Pharma), received speaker honoraria from Biomarin and Zambon Pharmaceuticals; and has received grants from H2020, Italian Ministry of Health, grants/research support from Zambon Italy, and personal fees from Biomarin, UCB, Zambon Italy, Z-cube, outside the submitted work.

Alessandro Padovani has received grants from H2020, Italian Ministry of Health, CARIPLO FUndation and Zambon Italy and has received other support from ROChe Pharma, ELI-Lilli, Actelion, Zambon Italy, and Z-cube, outside the submitted work.

AZ has a patent on PDE10A-IgG as a biomarker of neurological autoimmunity

KB has received personal fees while serving as a consultant, at advisory boards, or at data monitoring committees for Abcam, Axon, Biogen, JOMDD/Shimadzu. Julius Clinical, Lilly, MagQu, Novartis, Roche Diagnostics, and Siemens Healthineers, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program. KB reports grants from Swedish Research Council (#2017-00915), the Alzheimer Drug Discovery Foundation (ADDF), USA (#RDAPB-201809-2016615), the Swedish Alzheimer Foundation (#AF-742881), Hjärnfonden, Sweden (#FO2017-0243), the Swedish state under the agreement between the Swedish government and the County Councils, the ALF-agreement (#ALFGBG-715986), and European Union Joint Program for Neurodegenerative Disorders (JPND2019-466-236), outside the submitted work.
H.Z. has received personal fees while serving at scientific advisory boards for Denali, Roche Diagnostics, Wave, Samumed, Siemens Healthineers, Pinteon Therapeutics and CogRx, has given lectures in symposia sponsored by Fujirebio, Alzecure and Biogen, and is a co-founder of Brain Biomarker Solutions in Gothenburg AB (BBS), which is a part of the GU Ventures Incubator Program (outside submitted work). H.Z. has received grants from Swedish Research Council (#2018-02532), the European Research Council (#681712), Swedish State Support for Clinical Research (#ALFGBG-720931), the Alzheimer Drug Discovery Foundation (ADDF), USA (#201809-2016862), and the UK Dementia Research Institute at UCL.


Neuropathology of patients with COVID-19 in Germany: a post-mortem case series.
Lancet Neurol 2020; 0: 1–11.


Figure legend

**Figure 1.** Differences in neuronal, glial and inflammatory markers according to the clinical diagnosis. Boxplot indicate median and interquartile ranges.

**Abbreviations:** COV-ENC, encephalitis cases concomitant COVID-19 disease; CXCL13, chemokine (C-X-C motif) ligand 13; ENC, encephalitis without concomitant COVID-19; GFAP, Glial fibrillary acidic protein; HC, control group; IL-6, interleukin 6; IL-8, interleukin 8; NfL, neurofilament light chain; sTREM-2, triggering receptor expressed on myeloid cells 2; YKL-40, Chitinase-3-like protein 1; post-hoc comparison post-hoc comparison.
<table>
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<th>Age, sex</th>
<th>Onset, days Δ</th>
<th>Clinical features</th>
<th>ICANS grading</th>
<th>WBC, n/mm³</th>
<th>CRP, mg/L</th>
<th>Fibrinogen, mg/dL</th>
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<td>2</td>
<td>8.2</td>
<td>195</td>
<td>904</td>
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<td>1</td>
<td>CS,Y</td>
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<td>1</td>
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Table 1. Clinical features, imaging and CSF biochemical analyses of SARS-CoV-2 encephalitis cases. ICANS grading system is based on Lee and coauthors classification of 2019 (Lee et al. 2019), BCRSS grading is based on recent classification performed by Duca (Duca et al. 2020).

**Abbreviations:** AMS, altered mental state; BCRSS, Brescia COVID Respiratory Severity Scale; CRP, C-reactive protein, CS, corticosteroid treatment; DWI, diffusion-weighted imaging; F, female; Hyp, hyperintensities; ICANS, Immune effector cell-associated neurotoxicity syndrome; IVIg, immunoglobulin treatment; M, male; MRI, magnetic Resonance imaging; mRS, modified Ranking score; NEG, negative; qSOFA, quick Sequential Organ Failure Assessment; TREAT, treatment adopted including corticosteroid or immunoglobulin or no treatment (-) and positive (Y) or negative (N) response to treatment; WBC, white blood cell count; Δ number of days between neurological and respiratory symptoms (positive value indicated encephalitis presenting after the onset of respiratory symptomatology).* cell count per uL and protein levels in mg/dL.

| #13 | 74 F | 0 | Aphasia, confusion, seizures followed by AMS and non-convulsive SE | 4 | 12.3 | 3.3 | 293 | Bilateral temporal and Pulvinar T2-Hyp | 1 | 1 | CS, IVIg; N | 6 |

Table 1 shows the clinical features, imaging, and CSF biochemical analyses of SARS-CoV-2 encephalitis cases. The ICANS grading system is based on Lee and coauthors' classification of 2019, while the BCRSS grading is based on a recent classification performed by Duca. The table lists various clinical characteristics, imaging findings, and CSF analyses for each case.
Table 2. Clinical features, imaging and CSF biochemical analyses of COV-Enc cases. Values are expressed in pg/mL for all CSF neuronal, glial and cytokine levels if no otherwise indicated. **Abbreviations:** F, female; GFAP, Glial fibrillary acidic protein; IL-6, interleukin 6; ICANS, Immune effector cell-associated neurotoxicity syndrome; IL-8, interleukin 8; M, male; mRS, modified Ranking score; NfL, neurofilament light chain; prot, protein in the CSF; sTREM-2, triggering receptor expressed on myeloid cells 2, β-2-Mg, beta-2 microglobulin (levels in mg/dL) * cell count per uL # protein levels in mg/dL ¶ levels in mg/L

<table>
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<tr>
<th>ID</th>
<th>Age, sex</th>
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<th>Final mRS</th>
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Table 2. Clinical features, imaging and CSF biochemical analyses of COV-Enc cases. Values are expressed in pg/mL for all CSF neuronal, glial and cytokine levels if no otherwise indicated. **Abbreviations:** F, female; GFAP, Glial fibrillary acidic protein; IL-6, interleukin 6; ICANS, Immune effector cell-associated neurotoxicity syndrome; IL-8, interleukin 8; M, male; mRS, modified Ranking score; NfL, neurofilament light chain; prot, protein in the CSF; sTREM-2, triggering receptor expressed on myeloid cells 2, β-2-Mg, beta-2 microglobulin (levels in mg/dL) * cell count per uL # protein levels in mg/dL ¶ levels in mg/L
Figure 1

- NfL (pg/mL)
- tTAU (pg/mL)
- GFAP (pg/mL)
- S-TREM2 (pg/mL)
- YKL-40 (pg/mL)
- CXCL13 (pg/mL)
- IL-6 (pg/mL)
- B2 microglobulin (mg/L)
- IL-8 (pg/mL)