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MAJOR ARTICLE

Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Encephalitis Is a Cytokine Release Syndrome: Evidences From Cerebrospinal Fluid Analyses

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Background. Recent findings indicated that severe acute respiratory syndrome coronavirus 2 (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 cerebrospinal fluid (CSF) correlates of SARS-CoV-2 encephalitis.

Methods. Patients with polymerase chain reaction (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 (neurofilament light chain [NfL], T-tau), glial (glial fibrillary acidic protein [GFAP], soluble triggering receptor expressed on myeloid cells 2 [sTREM2], chitinase-3-like protein 1 [YKL-40]) and inflammatory biomarkers (interleukin [IL]-1β, IL-6, Il-8, tumor necrosis factor [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/hyperproteinorracchia. COV-Enc patients showed increased IL-6, TNF- α, and β2-microglobulin and glial markers (GFAP, sTREM2, 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.

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]. Although 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 cerebrospinal fluid (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].

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Cytokine release syndrome (CRS) is a potentially fatal complication of various infectious (eg, influenza, severe acute respiratory syndrome [SARS], Epstein-Barr virus) and noninfectious diseases (eg, 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 chimeric antigen receptor (CAR) T-cell therapy and are termed immune effector cell-associated neurotoxicity syndrome (ICANS).

Disproportionately high concentrations of interleukin (IL)-6 and IL-8 and tumor necrosis factor (TNF) α 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 the 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 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 20 February and 30 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 years admitted to hospital with altered mental status lasting ≥24 hours and the presence of 2 or more of the following criteria: (i) generalized or partial seizures not fully attributable to a preexisting epilepsy, (ii) new onset of focal neurologic findings, (iii) CSF white blood cell count ≥5/cubic mm^3, (iv) abnormality of brain parenchyma on neuroimaging suggestive of encephalitis that was either new from prior studies or appears acute in onset, and (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 reverse transcription polymerase chain reaction (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 magnetic resonance imaging (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 MRI, standard electroencephalography (EEG), thyroid function and antibodies (anti-thyroglobulin, anti-thyroid peroxidase), immunoglobulin M (IgM) and immunoglobulin G (IgG) for *Borrelia burgdorferi*. CSF viral screening included herpes simplex virus (HSV-1, HSV-2, HSV-6, HSV-8, cytomegalovirus [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 4 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 first 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, $GABA_bR$, AMPAR, DPPX, Ri, Yo, Ma2, CV2, Hu, amphiphysin, titin (Euroline 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 8 patients fulfilling criteria for possible autoimmune encephalitis [16] using 2 different fixation protocols on mouse tissue substrate (including CNS and non-CNS tissues in order to exclude patients with nonneural specific autoimmunity) in the Neuroimmunology laboratory

at Mayo Clinic, in Rochester, New York, USA; 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 2 independent reviewers (A.Z., A.McK.). 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 (Mölndal, Sweden). CSF cytokine concentrations (including IL-6, IL-8, TNF-α, IL-1β) were measured using a Mesoscale Discovery multiplexed immunoassay (Rockville, Maryland, USA). CSF CXCL13 concentration was measured using a commercial ELISA (R&D Systems, Minneapolis, Minnesota, USA). CSF β2M 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 neurofilament light chain (NfL) and glial fibrillary acidic protein (GFAP) concentrations were measured using in house enzyme-linked immunosorbent assays [20, 21]. CSF soluble triggering receptor expressed on myeloid cells 2 (sTREM2) concentration was measured using an in-house immunoassay with electrochemiluminescent detection, as previously described in detail [22]. CSF chitinase-3-like protein 1(YKL-40) concentration was measured using the Human Chitinase 3-like 1 Quantikine kit (R&D Systems, Minneapolis, Minnesota, USA). 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, New York, USA) was used for statistical analysis. Post hoc analyses were performed using Bonferroni correction at $P = .05$.

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 13 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 6 cases with aphasia and 3 with dysarthria (ICANS grading 2–4). EEG was abnormal in all cases, showing generalized slow waves prominent on the frontal derivations in ten patients, whereas focal epileptic alteration was observed in 3 cases.

Brain imaging was normal in 10 cases, whereas 3 patients showed heterogeneous MRI alterations including multiple subcortical/cortical T2-hyperintensities, associated in 1 case to diffusion weighted imaging (DWI) hyperintensities (Table 1). Four cases (mean ICANS grade 3.75) deceased during hospitalization. Spontaneous recovery was observed in 5 patients, whereas 4 patients clinically improved after high-dose methylprednisolone treatment (mean ICANS grade 2.75).

CSF Analyses

CSF biochemical standard analyses showed mild pleocytosis (5–26 cells) in 9 patients and increased protein levels in 8 COV-Enc patients (Tables 1 and 2).

RT-PCR for SARS-CoV-2 was negative in the CSF of all COVID-19 patients. In 4 additional samples the virus culture showed no specific cytopathic effect after 3 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 ($P < .001$ for all; Supplementary Table 1). COV-Enc exhibited comparable neuronal, glial and inflammatory markers but normal CXCL13 levels ($P = .026$) 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 11 showed increased beta-2 microglobulin. Glial markers, namely, GFAP and sTREM2, were abnormal in 12 and 10 COV-Enc patients, respectively. Tau and NfL levels were abnormal in 5 and 6 subjects respectively, considering age-adjusted cutoffs [20]. Three of 4 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

Table 1. Clinical Features, Imaging and Cerebrospinal Fluid (CSF) Biochemical Analyses of Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Encephalitis Cases

ID	Age, sex	Onset, days Δ	Clinical features	ICANS grading	WBC, n/mm3	CRP, mg/L	Fibrinogen, mg/dL	MRI	BCRSS	qSOFA	TREAT	Final mRS
1	70, F	10	AMS with aphasia, behavioral abnormalities, and agitation	2	8.2	195	904	NEG	1	1	CS,Y	2
2	60, M	0	AMS with severe akinetic mutism	3	6.3	1	340	NEG (2)	1	1	CS,Y	0
3	65, F	10	AMS with transitory left arm motor deficit, dysarthria	3	4.1	0.4	310	NEG	1	2	…	0
4	77, M	0	Seizure with AMS, dysarthria	3	5.9	2.5	317	NEG	1	1	…	0
5	60, F	0	AMS with psychosis with behavioral abnormalities and agitation	2	8.9	1.6	281	NEG (2)	0	0	CS, Y	0
6	78, F	0	AMS with aphasia and confusion	2	8.6	2.5	226	NEG	1	1	CS, Y	0
7	70, F	10	Confusion aphasia, followed by seizures and nonconvulsive SE	4	7.2	29.2	442	NEG (2)	1	2	CS, IVIg; N	6
8	52, M	−3	AMS with aphasia, transitory right facial-brachial motor deficit	4	5.7	242	315	NEG	1	1	…	0
9	61, M	4	AMS with transitory right leg deficit	4	9.2	67	586	NEG	1	2	…	0
10	50, M	0	Epileptic seizures, severe AMS with fluctuating agitation	3	23.6	48	487	NEG	1	2	…	2
11	60, M	0	AMS followed by nonconvulsive SE	4	7.1	7	415	Multiple frontotemporal T2-Hyp	3	2	CS, N	6
12	75, M	0	AMS with dysarthria	3	11.5	72	650	Focal temporo-parietal T2/DWI Hyp	1	1	IVIg; N	6
13	74, F	0	Aphasia, confusion, seizures followed by AMS and nonconvulsive SE	4	12.3	3.3	293	Bilateral temporal and pulvinar T2-Hyp	1	1	CS, IVIg; N	6

ICANS grading system is based on Lee and colleagues' [13] classification of 2019. BCRSS grading is based on recent classification performed by Duca [18]. Cell count per uL and protein levels in mg/dL.

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, intravenous immunoglobulin treatment; M, male; MRI, magnetic resonance imaging; mRS, modified ranking scale; 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).

Table 2. Clinical Features, Imaging, and Cerebrospinal Fluid (CSF) Biochemical Analyses of COV-Enc Cases.

		Cerebrospinal Fluid										
ID	Age, Sex	Cells[a]	Prot[b]	TAU	NfL	GFAP	sTREM-2	IL-6	IL-8	β-2-Mg[c]	ICANS Grade	Final mRS
1	70, F	5	34.2	189	707	300	2875	3.5	51	0.9	2	2
2	60, M	19	69.6	231	789	244	4609	2.3	1106	3.1	3	0
3	65, F	5	68.7	256	368	197	2499	1.5	57	1.3	3	0
4	77, M	1	49.5	322	837	253	917	0.6	74	1.8	3	0
5	60, F	9	46.7	344	150	184	469	1.7	72	0.8	2	0
6	78, F	1	36.2	344	1353	151	469	1.8	88	1.4	2	0
7	70, F	1	19.7	456	1394	492	1460	0.9	188	1.9	4	6
8	52, M	19	80.5	258	1825	607	4166	1.7	57	1.9	4	0
9	51, M	5	125.2	1044	3004	456	8955	2.1	92	5.9	4	0
10	50, M	10	77.0	1047	8382	280	4100	290	1106	1.3	3	2
11	60, M	26	74.0	1272	13 801	744	4867	531	1106	2.7	4	6
12	75, M	0	40.0	1245	19 082	789	2531	2.1	259	2.8	3	6
13	74, F	16	23.3	1741	19 126	358	1839	2.1	252	2.2	4	6

Values are expressed in pg/mL for all CSF neuronal, glial, and cytokine levels if not otherwise indicated. Abbreviations: F, female; GFAP, glial fibrillary acidic protein; ICANS, immune effector cell-associated neurotoxicity syndrome; IL, interleukin; M, male; mRS, modified ranking scale; NfL, neurofilament light chain; prot, protein in the CSF; sTREM2, soluble triggering receptor expressed on myeloid cells 2, β-2-Mg, beta-2 microglobulin (levels in mg/dL).

[a] Cell count per uL.

[b] Protein levels in mg/dL.

[c] Levels in mg/L.

assessed is highly suggestive for a cytokine-release syndrome as driver of SARS-CoV-2-related encephalitis.

The sample included 13 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]. Increased GFAP levels also have been 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

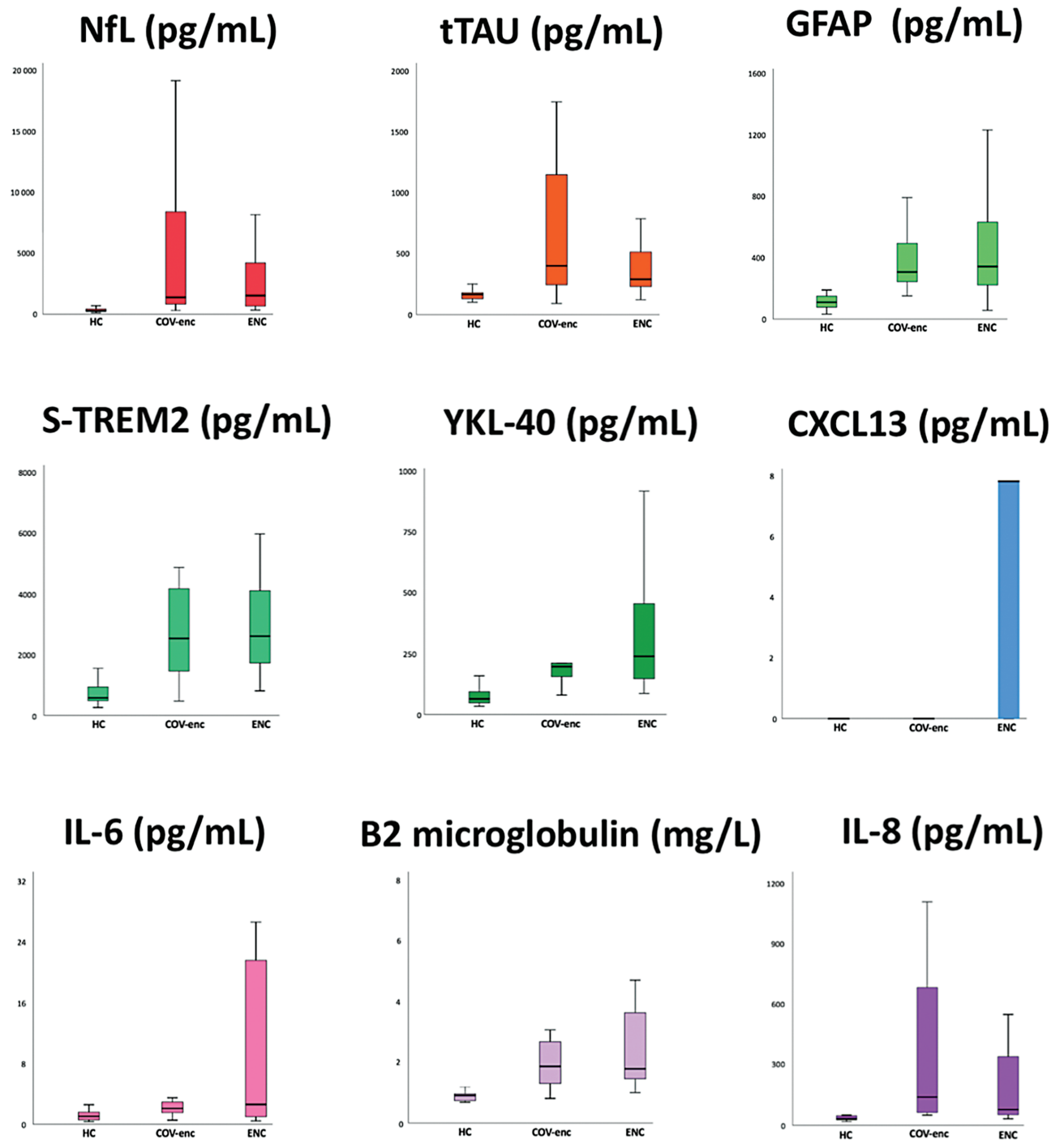

Figure 1. Differences in neuronal, glial, and inflammatory markers according to the clinical diagnosis. Boxplot indicate median and interquartile ranges. Abbreviations: COV-Enc, encephalitis cases concomitant coronavirus disease 2019 (COVID-19); CXCL13, chemokine (C-X-C motif) ligand 13; ENC, encephalitis without concomitant COVID-19; GFAP, glial fibrillary acidic protein; HC, healthy control group; IL, interleukin; NfL, neurofilament light chain; sTREM2, soluble triggering receptor expressed on myeloid cells 2; YKL-40, chitinase-3-like protein 1.

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 strong cytokine/interleukin changes in CSF.

These findings have deep implications for the management of patients presenting with encephalitis related to SARS-CoV-2 infection, as high-dose corticosteroid treatment (ie, methylprednisolone 1 g/day for 5 days) is the first-line therapy for severe neurotoxicity in CRS cases [13] after exclusion of coinfections, 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 excludes cases of SARS-CoV-2 encephalitis with severe respiratory COVID-19 that could not undergo a complete MRI, EEG, and CSF assessment. Third, the study was cross-sectional in nature, and the normalization of the CSF markers after clinical improvement should be verified in ongoing 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.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. A. Pilotto and A. Padovani contributed to the conception and design of the study. A. Pilotto, S. Masciocchi, I. V, V. D., F. C., S. Mariotta, S. F., S. B., A. I., B. R., E. P. A. B., E. F., F. C., G. Z., A. Z, A. M., N. J. A., K. B., H. Z., and A. Padovani contributed to the acquisition and analyses of data. A. Pilotto, S. M., and A. Padovani contributed to drafting the text.

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