




# Inflammatory Cytokine Patterns Associated with Neurological Diseases in Coronavirus Disease 2019

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Patients with coronavirus disease 2019 (COVID-19) can present with distinct neurological manifestations. This study shows that inflammatory neurological diseases were associated with increased levels of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-12, chemokine (C-X-C motif) ligand 8 (CXCL8), and CXCL10 in the cerebrospinal fluid. Conversely, encephalopathy was associated with high serum levels of IL-6, CXCL8, and active tumor growth factor  $\beta$ 1. Inflammatory syndromes of the central nervous system in COVID-19 can appear early, as a parainfectious process without significant systemic involvement, or without direct evidence of severe acute respiratory syndrome coronavirus 2 neuroinvasion. At the same time, encephalopathy is mainly influenced by peripheral events, including inflammatory cytokines.

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The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the etiologic agent of coronavirus disease 2019 (COVID-19), which is mainly characterized by fever, myalgia, diarrhea, and respiratory illness,<sup>1,2</sup> and has caused more than 30 million cases and 1 million deaths worldwide up to September 2020.<sup>3</sup> The SARS-CoV-2 infection has also been associated with several central nervous system (CNS) syndromes, including headache, encephalopathy, and inflammatory neurological diseases, such as encephalitis, meningoencephalitis, acute disseminated encephalomyelitis (ADEM), and acute myelitis.<sup>4,5</sup>

Possible mechanisms implicated in the pathogenesis of these neurological manifestations include neuronal injury due to immune-mediated processes, hyperinflammation as a result of cytokine release syndrome, para- or postinfectious inflammation, and even secondary events related to effects of systemic disorders, such as sepsis, hyperpyrexia, hypoxia, hypercoagulability, and critical illness.<sup>6-8</sup> However, although SARS-CoV-2 RNA can be detected in the brain tissue,<sup>9</sup> it is still unclear whether the mechanisms and events are secondary to neuroinvasion by SARS-CoV-2 or whether inflammatory cytokines from the periphery influence this process.

In this study, we sought to evaluate the levels of several cytokines in cerebrospinal fluid (CSF) and serum of patients with COVID-19. We show that patients with inflammatory and noninflammatory neurological conditions associated with COVID-19 have distinct cytokine profiles in the CNS and periphery.

## Patients and Methods

### Study Population and Samples

This study represents a case series of 48 patients with COVID-19 presenting with neurological manifestations admitted between April and June 2020 at reference hospitals in the cities of Rio de Janeiro (Rio de Janeiro State Servants Hospital and Evandro Chagas National Institute of Infectious Diseases) and Niterói (Niterói Hospital Complex), Brazil. All patients had COVID-19 confirmed by detecting SARS-CoV-2 RNA in nasopharyngeal swabs by quantitative reverse transcription polymerase chain reaction (RT-PCR), as previously described.<sup>10</sup>

CSF (n = 48) and serum (n = 29) samples were collected on the same day and immediately processed,

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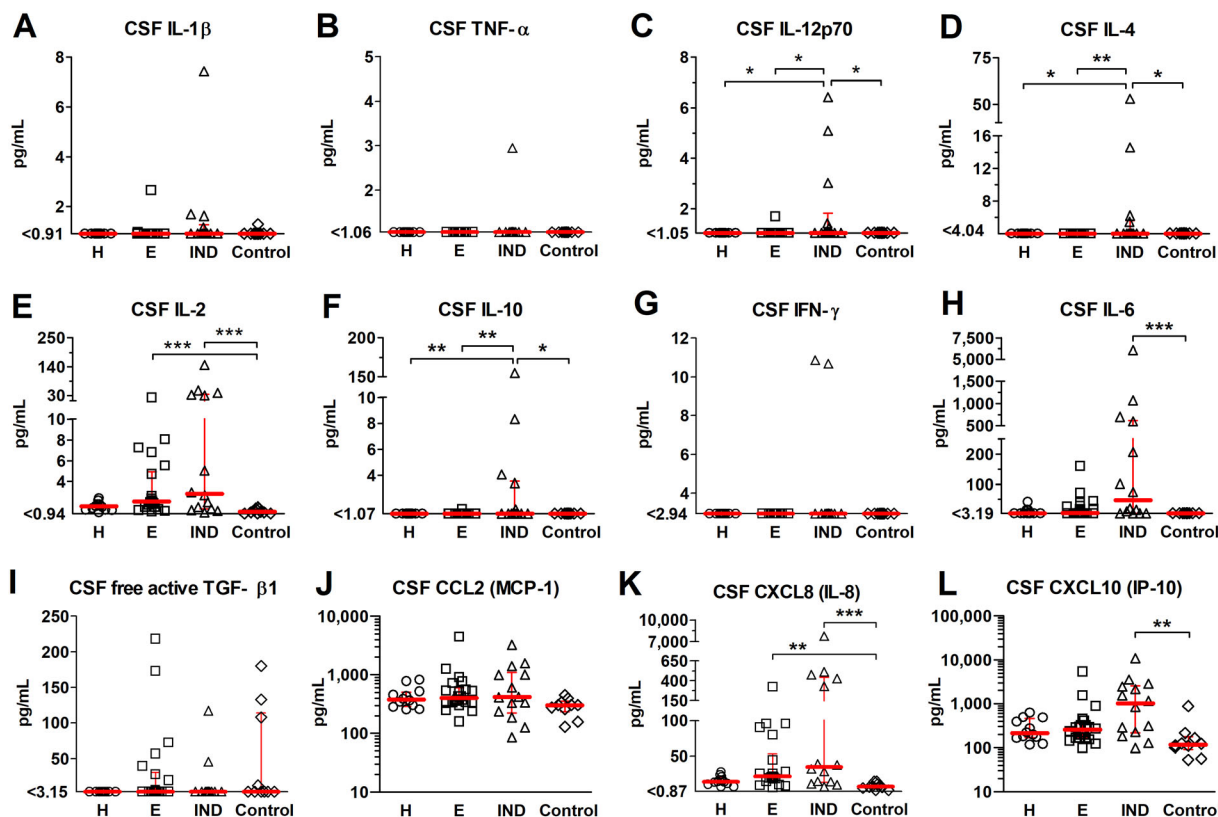
aliquoted, and kept at  $-80^{\circ}\text{C}$  until use. CSF samples were investigated for the presence of SARS-CoV-2 RNA and also tested by PCR for other neuropathogens.<sup>10</sup> Patients with CNS infection by pathogens other than SARS-CoV-2 were excluded. CSF ( $n = 10$ ) and serum ( $n = 5$ ) samples obtained from individuals with non-inflammatory, noninfectious neurological diseases before the emergence of COVID-19 in Brazil were used as controls. These controls had normal intracranial opening pressure ( $\leq 200\text{mmH}_2\text{O}$ ) and normal values for CSF cell count ( $<5$  cells/ $\text{mm}^3$ ) and total protein concentration ( $15\text{--}45\text{mg/dl}$ ). The study was approved by the Brazilian National Committee of Ethics in Research (Protocol number: 30611720.6.0000.5262), and informed consent was obtained from all participants.

Patients were grouped according to their primary neurological condition: (1) refractory headache ( $n = 12$ ) with or without visual symptoms as the predominant neurological complaints, excluding individuals with any clinical or laboratory evidence for meningitis or meningoencephalitis, such as

neck stiffness, altered consciousness, focal neurological signs, or inflammatory characteristics in CSF analysis; (2) encephalopathy ( $n = 22$ ), which was characterized by diffuse brain dysfunction, presenting with convulsions, alterations in cognition, consciousness, personality, or behavior, without signs of acute CNS inflammation, such as CSF pleocytosis and changes in brain magnetic resonance imaging (MRI); (3) inflammatory neurological diseases, including ADEM ( $n = 2$ ), encephalitis ( $n = 2$ ), meningitis ( $n = 2$ ), meningoencephalitis ( $n = 4$ ), acute myelitis ( $n = 3$ ), and neuromyelitis optica ( $n = 1$ ). Encephalitis was discriminated from encephalopathy by clinical evidence of acute brain inflammation, CSF pleocytosis, brain MRI changes, or focal abnormalities on electroencephalography.

### Quantification of Cytokines and Detection of SARS-CoV-2 Immunoglobulin M

Interleukin (IL)-1 $\beta$ , IL-2, IL-4, IL-6, IL-8 (CXCL8), IL-10, IL-12p70, IL-17A/F, interferon  $\gamma$  (IFN- $\gamma$ ), macrophage chemoattractant protein 1 (MCP-1/CCL2), IFN- $\gamma$ -induced



**FIGURE 1:** Cytokine concentration in the cerebrospinal fluid (CSF) of patients with coronavirus disease 2019 and neurological manifestations. Inflammatory cytokines were quantified in the CSF of patients with headache (H;  $n = 12$ ), encephalopathy (E;  $n = 22$ ), and inflammatory neurological diseases (IND;  $n = 14$ ) and controls ( $n = 10$ ) by cytometry bead-based multiplex assay. Comparative analysis was performed with Kruskal–Wallis and post hoc analysis with Dunn test with Bonferroni correction for multiple comparisons. Differences were considered significant at  $p < 0.05$ . \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . CCL = chemokine (C-C motif) ligand; CXCL = chemokine (C-X-C motif) ligand; IFN = interferon; IL = interleukin; IP = IFN- $\gamma$ -induced protein; MCP = macrophage chemoattractant protein; TGF = tumor growth factor; TNF = tumor necrosis factor. [Color figure can be viewed at [www.annalsofneurology.org](http://www.annalsofneurology.org)]

protein 10 (IP-10/CXCL10), free active tumor growth factor  $\beta$ 1 (TGF- $\beta$ 1), and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) were quantified using the cytometry bead-based multiplex assay LEGENDplex Human Essential Immune Response Panel 13-plex (BioLegend, San Diego, CA), following the manufacturer's instructions. CSF samples were used undiluted, and serum samples were diluted at 1:2. Data acquisition was carried out with a FACSCanto II cytometer (BD Biosciences, Franklin Lakes, NJ), and cytokine concentration was calculated in 5-parameter logarithmic curves using the software supplied with the assay.

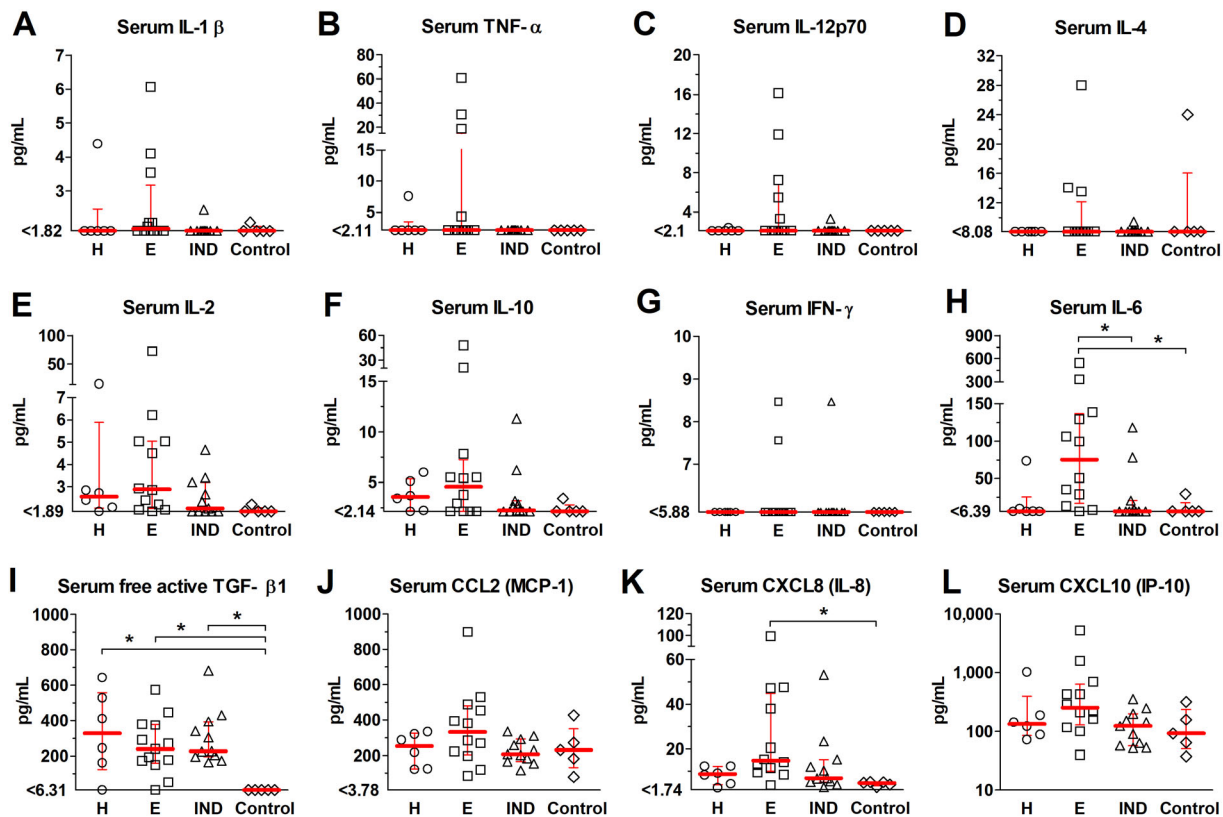
Detection of SARS-CoV-2 immunoglobulin M (IgM) in CSF samples was carried with the LIAISON SARS-CoV-2 IgM chemiluminescence immunoassay (DiaSorin, Saluggia, Italy) following the manufacturer's instructions for serum/plasma samples, because no commercial assay has been validated for CSF. This assay has specificity of >99% and sensitivity varying from 64.8% to 91.5% for serum/plasma collected  $\leq 7$  days and between 8 and 14 days from diagnosis of SARS-CoV-2 infection by RT-PCR, respectively, as shown by the manufacturer.

## Statistical Analysis

Data analysis was performed using Prism v5 (GraphPad Software, San Diego, CA) and R software v3.6.1. Data are shown as the median and interquartile range (IQR), and comparative analysis was carried out with Kruskal–Wallis and post hoc analysis with Dunn test with Bonferroni correction for multiple comparisons. Differences were considered significant at  $p < 0.05$ . Relative cytokine expression between groups was evaluated by heatmap analysis using the gplots package for R software.

## Results

Most patients enrolled in the study developed neurological manifestations as the first COVID-19 symptoms or within 14 days from COVID-19 onset, suggesting a parainfectious process. Isolated refractory headache was manifested after a median interval of 2 days (IQR = 0–10 days), encephalopathy after 3 days (IQR = 2–7 days), and inflammatory neurological diseases after 4 days (IQR = 0–14 days) from COVID-19 onset. Only 3 patients in the study manifested



**FIGURE 2:** Inflammatory cytokines in the serum of patients with coronavirus disease 2019 and neurological manifestations. Comparative analysis of cytokine concentration in the serum of patients with headache (H;  $n = 6$ ), encephalopathy (E;  $n = 12$ ), and inflammatory neurological diseases (IND;  $n = 11$ ) and controls ( $n = 5$ ) was performed with Kruskal–Wallis and post hoc analysis with Dunn test with Bonferroni correction for multiple comparisons. Differences were considered significant at  $*p < 0.05$ . CCL = chemokine (C-C motif) ligand; CXCL = chemokine (C-X-C motif) ligand; IFN = interferon; IL = interleukin; IP = IFN- $\gamma$ -induced protein; MCP = macrophage chemoattractant protein; TGF = tumor growth factor; TNF = tumor necrosis factor. [Color figure can be viewed at [www.annalsofneurology.org](http://www.annalsofneurology.org)]

neurological alterations over 14 days after COVID-19 initiation: one with encephalopathy, one with ADEM, and another with acute myelitis, starting at day 18, 19, and 29, respectively. Moreover, all patients had undetectable SARS-CoV-2 IgM in the CSF.

Inflammatory cytokines in the CSF of patients with isolated headache showed no significant difference from control individuals (Fig 1). One individual with COVID-19 and headache as the main neurological alteration had detectable SARS-CoV-2 RNA in the CSF, with no evidence of blood contamination. Despite intracranial hypertension (CSF opening pressure of 300mmH<sub>2</sub>O), brain MRI was unremarkable, and the CSF had no inflammatory changes, with normal cell counts, normal glucose and total protein levels, and absence of oligoclonal bands.

IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  levels were undetectable or negligible in the CSF of most patients (Fig 1), and IL-17A/F was undetectable (<1.17pg/ml) in the CSF of all

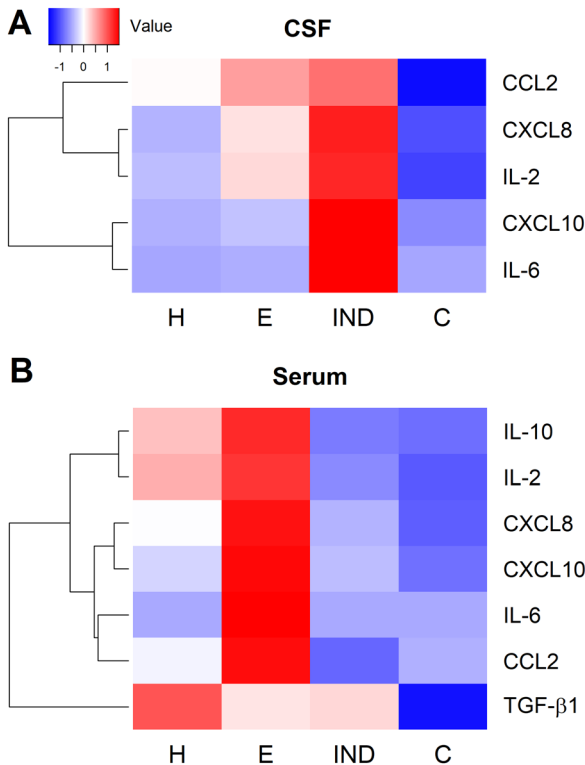
patients. Conversely, the CSF of patients with inflammatory neurological diseases had elevated levels of IL-2, IL-4, IL-6, IL-10, IL-12, CXCL8, and CXCL10 compared to controls, whereas patients with encephalopathy presented increased concentration of only IL-2 and CXCL8 (Fig 1). IL-1 $\beta$ , IL-4, IL-10, IL-12, IFN- $\gamma$ , and TNF- $\alpha$  were undetectable or at low levels in the serum of most patients (Fig 2), as was IL-17A/F, which was detectable (>2.35pg/ml) in the serum of only 2 patients with encephalopathy. Serum levels of IL-6, CXCL8, and active TGF- $\beta$ 1 were increased in patients with encephalopathy compared to controls, and only active TGF- $\beta$ 1 was elevated in the serum of patients with headache and inflammatory neurological diseases (Fig 2).

The relative expression of the main cytokines detected in the CSF and serum revealed 2 profiles: one associated with inflammatory neurological diseases, which presented a pronounced CNS inflammation (Fig 3A) with increased CSF levels of IL-6, CXCL8, CXCL10, and IL-2, and without significant systemic involvement (Fig 3B); and another associated with encephalopathy, showing an exacerbated systemic inflammatory response, with higher serum levels of IL-6, CCL2, CXCL8, CXCL10, IL-2, and IL-10 compared to the other groups (Fig 3B) but without correspondence in CSF findings (Fig 3A).

**Discussion**

Previous studies have reported increased inflammatory cytokines in the CSF of patients with COVID-19, although in a small number of cases.<sup>11,12</sup> In our study, patients with refractory headache did not display increased CSF levels of inflammatory cytokines. Otherwise, patients with encephalopathy and inflammatory neurological syndromes associated with SARS-CoV-2 infection showed distinct cytokine profiles in CSF and serum. Encephalopathy was associated with high serum levels of IL-6, a strong predictor of morbidity/mortality in COVID-19.<sup>13</sup> IL-2, a lymphocyte-derived cytokine known to stimulate T- and B-cell proliferation, was also increased in these patients' CSF. This corroborates our previous findings showing intrathecal IgG synthesis and type 4 oligoclonal bands (identical IgG bands in CSF and serum).<sup>10</sup> Thus, our data suggest that the development of encephalopathy in COVID-19 is multifactorial, and it can be driven by systemic inflammation and immune-mediated processes.

Inflammatory neurological diseases were associated with increased CSF levels of IL-6, CXCL8, and CXCL10, which are commonly secreted by activated astrocytes and microglial cells. Previously, we have shown the CSF of these patients was characterized by pleocytosis of mononuclear cells, mild increase in total protein concentration, and high levels of neurofilament light chain protein, revealing an active



**FIGURE 3:** Relative cytokine expression in cerebrospinal fluid (CSF) and serum of patients with coronavirus disease 2019 and neurological manifestations. The expression of the main cytokines altered in the (A) CSF and (B) serum of patients with headache (H), encephalopathy (E), and inflammatory neurological diseases (IND) and controls (C) was evaluated between groups by heatmap analysis. The expression was scaled for each cytokine (lines), varying from -1 (blue) to 1 (red), where white indicates the median (zero), shades of blue represent reduced expression, and shades of red indicate increased relative expression between groups. CCL = chemokine (C-C motif) ligand; CXCL = chemokine (C-X-C motif) ligand; IL = interleukin; TGF = tumor growth factor.

and exacerbated inflammatory process leading to injury of myelinated axons.<sup>10</sup> However, it is not clear whether this process was triggered by SARS-CoV-2 neuroinfection, as direct evidence was obtained in only one case of ADEM, and none of the patients had detectable levels of SARS-CoV-2 IgM. In addition, SARS-CoV-2 RNA was also detected in the CSF of a patient with isolated headache, presenting neither inflammatory changes in brain MRI and CSF analysis nor increased CSF cytokine levels. SARS-CoV-2 RNA detection in CSF is infrequent,<sup>14</sup> which is in line with the low SARS-CoV-2 RNA load reported in brain tissue biopsies.<sup>9</sup> Although the olfactory route has been suggested for neuroinvasion,<sup>15</sup> events associated with altered blood–brain barrier (BBB) function may not be excluded entirely. It was reported that patients with COVID-19 show increased albumin content in the CSF and increased serum levels of the astroglial protein S100B.<sup>16</sup> Here, all groups evaluated, including patients with persistent headache, encephalopathy, and inflammatory neurological diseases, had significantly elevated serum levels of active TGF- $\beta$ 1, a pleiotropic cytokine known to regulate vascular integrity, endothelial activation, and BBB permeability, which can be induced during hypoxia.<sup>17</sup> Thus, TGF- $\beta$ 1 may be involved in BBB dysfunction processes and consequent CNS involvement in COVID-19.

Neurological manifestations associated with COVID-19 have diverse CSF profiles, even within the same clinical condition,<sup>10</sup> and this was also observed in CSF and serum cytokine patterns. Overlapping patterns could be seen in a small group of patients with encephalopathy and inflammatory neurological diseases, suggesting that intrathecal immune activation also occurred in the encephalopathy group, although not as severe as shown in neuroinflammatory diseases. Nevertheless, clinical distinction between inflammatory neurological syndromes and encephalopathy can be challenging, and therefore laboratory information to support disease definition might be helpful.

In conclusion, our data indicate that parainfectious CNS inflammatory syndromes related to SARS-CoV-2 infection were associated with high CSF levels of IL-6, CXCL8, and CXCL10 rather than the high serum levels seen in encephalopathy, which was associated with acute systemic inflammation in most cases.

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## Author Contributions

M.T.T.S., O.M.E., A.C.C.B.L., M.A.S.D.L., and A.Q.C.A. contributed to the study concept and design.

O.M.E., R.C.T., Y.C.P.G., C.O.B., M.S., C.N.S., C.O.V., A.J.C.C., and G.T. performed data acquisition and analysis. O.M.E., Y.C.P.G., and M.T.T.S. drafted the manuscript and figures.

## Potential Conflicts of Interest

Nothing to report.

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