Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

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Intrathecal inflammatory responses in the absence of SARS-CoV-2 nucleic acid in the CSF of COVID-19 hospitalized patients

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ABSTRACT

Objective: Little is known about CSF profiles in patients with acute COVID-19 infection and neurological symptoms. Here, CSF was tested for SARS-CoV-2 RNA and inflammatory cytokines and chemokines and compared to controls and patients with known neurotropic pathogens.

Methods: CSF from twenty-seven consecutive patients with COVID-19 and neurological symptoms was assayed for SARS-CoV-2 RNA using quantitative reverse transcription PCR (RT-qPCR) and unbiased metagenomic sequencing. Assays for blood brain barrier (BBB) breakdown (CSF:serum albumin ratio (Q-Alb)), and proinflammatory cytokines and chemokines (IL-6, IL-8, IL-15, IL-16, monocyte chemoattractant protein −1 (MCP-1) and monocyte inhibitory protein – 1β (MIP-1β)) were performed in 23 patients and compared to CSF from patients with HIV-1 (16 virally suppressed, 5 unsuppressed), West Nile virus (WNV) ($n = 4$) and 16 healthy controls (HC).

Results: Median CSF cell count for COVID-19 patients was 1 white blood cell/μL; two patients were infected with a second pathogen (*Neisseria*, *Cryptococcus neoformans*). No CSF samples had detectable SARS-CoV-2 RNA by either detection method. In patients with COVID-19 only, CSF IL-6, IL-8, IL-15, and MIP-1β levels were higher than HC and suppressed HIV (corrected-$p < 0.05$). MCP-1 and MIP-1β levels were higher, while IL-6, IL-8, IL-15 were similar in COVID-19 compared to WNV patients. Q-Alb correlated with all proinflammatory markers, with IL-6, IL-8, and MIP-1β ($r \geq 0.6$, $p < 0.01$) demonstrating the strongest associations.

Conclusions: Lack of SARS-CoV-2 RNA in CSF is consistent with pre-existing literature. Evidence of intrathecal proinflammatory markers in a subset of COVID-19 patients with BBB breakdown despite minimal CSF pleocytosis is atypical for neurotropic pathogens.

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1. Introduction

Neurologic symptoms have been described in 30–60% of hospitalized patients with Coronavirus Disease 2019 (COVID-19) [1,2]. These include delirium and confusion, headache, and anosmia/hyposmia [1,3,4]. Prior clinical [5,6], animal and in vitro studies of other human coronaviruses indicate there is neurotropic potential of SARS-CoV-2 [7]. While autopsy studies show low levels of SARS-CoV-2 RNA in CNS tissue, definitive evidence of SARS-CoV-2 neuroinvasion into the CNS remains speculative [8].

The overwhelming majority of case series documenting CSF findings in patients with COVID-19 did not detect SARS-CoV-2 RNA in CSF by quantitative reverse transcriptase PCR (RT-qPCR) despite abnormal brain imaging or the presence of neurologic sequelae including altered mental status, seizure, stroke, weakness, or Guillain-Barré syndrome (see review [9]). While CSF protein, glucose, and cell counts are often normal in these patients, cases with CSF pleocytosis [10–12], elevated CSF protein [13–15], and positive oligoclonal bands [2] have been reported. Without definitive evidence of SARS-CoV-2 RNA in the CNS, other factors such as dysregulated intrathecal cytokine responses in the setting of a cytokine storm or silent hypoxia have been postulated to explain neurologic symptoms in COVID-19 patients.

Here we evaluated SARS-CoV-2 RNA in CSF from hospitalized COVID-19 patients using RT-qPCR and unbiased RNA metagenomic sequencing, enabling us to accurately determine viral burden even when SARS-CoV-2 RNA is highly fragmented or has mutations in the RT-qPCR target regions. Additionally, we evaluated intrathecal levels of proinflammatory cytokines and chemokines in COVID-19 patients compared to controls with no known neurological disease as well as patients infected with neurotropic RNA viruses including West Nile Virus (WNV) and HIV-1.

2. Methods

2.1. Human subjects ethical statements

The study was approved by the MGB Institutional Review Board and the requirement for obtaining written informed consent was waived for excess clinical samples (Protocol #: 2020P000468). RT-qPCR, sequencing, and analysis of samples at the Broad Institute was covered under Mass General Brigham (MGB) protocol 2015P001388.

2.2. Study design and patients

We prospectively identified 31 hospitalized COVID-19 patients who underwent a lumbar puncture for neurological symptoms between 03/10/2020–05/30/2020 at two academic hospitals, Massachusetts General Hospital (MGH) or Brigham and Women's Hospital in Boston, Massachusetts. Patients were identified by search queries in the electronic health record based on laboratory-confirmed COVID-19 and at least one CSF test ordered or completed. One patient had concurrent prion disease and was excluded from further study due to biosafety considerations [12], and three patients did not have excess CSF available for further testing (Fig. 1). Paired excess blood samples were not available for testing. Of the 27 patients included, 26 patients had a positive RT-qPCR test for SARS-CoV-2 from a nasopharyngeal (NP) swab specimen and one patient had serological evidence of COVID-19 infection with a positive anti-SARS-CoV-2 nucleocapsid total antibody test (Roche Diagnostics, Indianapolis, IN). A retrospective medical record review using the electronic medical health record was performed to characterize the clinical course including disease severity using the World Health Organization eight-category ordinal scale [16], radiologic results, and CSF and serum laboratory data of all patients.

Archived cryopreserved CSF samples were obtained through the Prospective Meningitis and Encephalitis study (PEMS), which aims to establish a collection of CSF samples from individuals with confirmed or suspected neurotropic infections. Samples from patients with HIV and detectable plasma viral load (VL) were obtained within 30 days of CSF sampling. We included samples from a group of participants with suppressed HIV on antiretroviral therapy (ART) defined as plasma VL <200 copies/ml, and a group of participants with a HIV VL >1000 copies/ml; one virally suppressed HIV participant had a varicella zoster virus infection at the time of CSF analyses. CSF was also obtained from a third infected group of participants who had WNV neuroinvasive disease. A total of 16 control CSF samples were obtained from the MGH LifeSPAN study, which includes healthy controls. All HIV, WNV, and control samples were obtained prior to 01/01/2020.

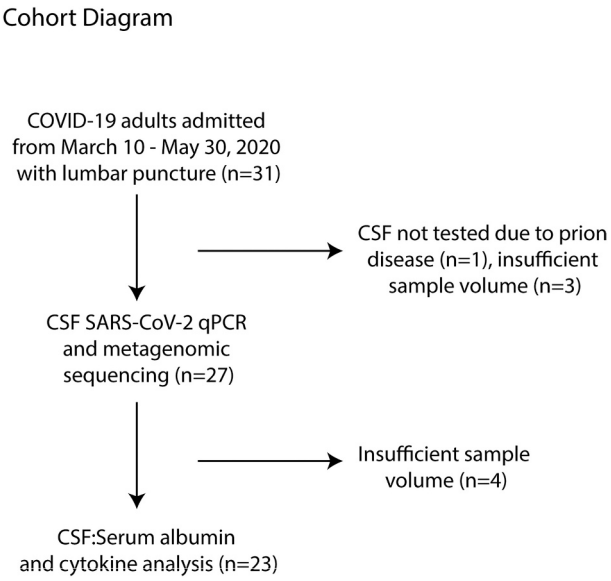

Fig. 1. A schematic of the study design. A brief summary of patients included and analyses performed in this study.

2.3. SARS-CoV-2 RT-qPCR and metagenomic sequencing

Nucleic acid was extracted from 280 μL of CSF using a QIAamp Viral RNA Mini kit (Qiagen) and RNA was purified by enzymatic digestion of DNA (ArcticZymes). Extracted samples were eluted in 40 μL of water, and each extraction batch included at least one water sample processed in parallel with CSF samples to serve as a negative control.

RNA samples were tested for the presence of SARS-CoV-2 RNA using a lab-developed probe-based RT-qPCR assay based on the CDC assays targeting two regions of the SARS-CoV-2 nucleocapsid gene [17]. The RT-qPCR assays were performed in triplicate with 1 μL of purified RNA per 10 μL reaction, using TaqPath™ 1-Step RT-qPCR Master Mix (Thermo Fisher Scientific). Forward and reverse primer sequences were, respectively: GACCCCAAAATCAGCGAAAT, TCTGGTTACTGCCAGTTGAATCTG (N1) and TTACAAACATTGGCCGCAAA, GCGCGACATTCCGAATAA (N2) (IDT). The RT-PCR assay was run with double-quenched fluorescent-labeled probes with the following sequences: 5′-FAM-ACCCCGCAT-ZEN-TACGTTTGGTGGACC-BHQ1–3′ (N1) and 5′-FAM-ACAATTTGC-ZEN-CCCCAGCGCTTCAG-BHQ1–3′ (N2) (IDT). RT-qPCR was performed on an Applied Biosystems QuantStudio 6, with reverse transcription (RT) run for 30 min at 48C, followed by 45 cycles of PCR at 95C for 10s, 60C for 45 s. A synthetic dsDNA fragment of the SARS-CoV-2 N gene was used as a positive control, and water was used as a negative control for the RT-qPCR assay. Quantification was performed using the Standard Curve module of the Applied Biosystems Analysis Software.

All samples underwent unbiased RNA metagenomic sequencing. 30 μL of the extracted total nucleic acid described above was depleted of DNA with Turbo DNAse (Thermo Fisher Scientific), cDNA was

synthesized using random primers (Thermo Fisher Scientific), and libraries were constructed using Nextera XT (Illumina), as described previously [18]. Samples were sequenced to a depth of approximately 1 million (0.5–3.9 million) paired-end reads each using MiSeq V3 (2 × 75 bp) and NovaSeq SP (2 × 100 bp) technologies (Illumina). For CSF from one subject (3844; Supplementary Table 1) and its associated water control, we also constructed a DNA metagenomic sequencing library from extracted DNA to confirm cryptococcal meningitis infection following a positive diagnostic cryptococcal rapid antigen test. The total nucleic acid sample was depleted of RNA with RNAse I (Lucigen), and libraries were constructed using Nextera XT (Illumina). These samples were sequenced to a depth of approximately 10 million paired-end reads on a NovaSeq SP (2 × 146 bp) (Illumina).

Sequencing data was analyzed using viral-ngs v2.0.21 (https://github.com/broadinstitute/viral-pipelines) as previously described [19,20]. Briefly, reads were demultiplexed, trimmed and quality filtered, then mapped and assembled to a SARS-CoV-2 reference genome (NC_045512.2) using the assemble_refbased workflow. Read pairs (not singletons, to reduce sequencing artifacts) that mapped to SARS-CoV-2 were confirmed by BLAST (NCBI) and quantified. Reads also underwent metagenomic sequence classification by kraken2 [21] using the classify_kraken2 pipeline in viral-ngs. Contigs assembled from reads mapping to pathogens of interest were confirmed by BLAST (NCBI) and pathogen reads were quantified. To confirm coinfections, we used the viral-ngs pipeline assemble_denovo_with_deplete with references corresponding to the pathogen of interest (for *Neisseria meningitidis*: NZ_CP021725.1;for *Cryptococcus neoformans*: CP025717.1-CP025730.1; for HIV: EU541617.1, MN090732.1, MN090390.1, MN090343.1, MN090336.1, MN090714.1, MN090383.1, MT191217.1, MT191109.1, MT191105.1, MT191069.1, MT191027.1, MT191009.1, MT191008.1, MT191007.1, MT190988.1) to identify and quantify reads mapping to common CNS pathogens that were clinically suspected or identified in the metagenomic classification analysis. We performed this analysis in parallel on the sample that was suspected to contain the pathogen and its associated negative control; negative controls did not contain reads mapping to pathogens described. A maximum likelihood phylogenetic tree of 772 previously reported SARS-CoV-2 genomes [22] was downloaded from NextStrain [23,24] and annotated in FigTree (v1.4.4).

2.4. Cytokine and chemokine profiling and CSF:serum albumin ratio quantification

A total of 23 COVID-19 patients had CSF available for profiling after SARS-CoV-2 RT-qPCR. CSF concentrations of 8 analytes (interleukin (IL)-6, IL-7, IL-8, IL-15, IL-16, MCP-1, MDC, MIP-1β) were examined using a custom U-PLEX electrochemiluminescent (ECL) immunoassay (Catalog number K15067L) and Meso-Scale Discovery (MSD) platform (Meso-Scale Diagnostics, LLC, Rockville, MD). Two analytes (IL-7 and MDC (CCL22)) had high coefficient of variation (>20%) in 70% of samples and were not analyzed further. The assay was performed according to manufacturer's specifications and samples diluted per assay requirements. Data from sample replicates analyzed in different batches was normalized to the average of each analyte across batches. Cytokine and chemokine concentrations are in pg/ml and values are $\log_{10}$-transformed. The Human Albumin ELISA kit from Abcam (Catalog number: ab108788) was used to measure albumin in CSF with an 8000× dilution factor. Paired serum samples were not available for albumin testing, thus, albumin values closest in time to CSF analyses performed for clinical reasons were obtained from the medical record (and the CSF:serum albumin ratio (Q-Alb) was calculated).

2.5. Statistical analysis

Descriptive statistics were used to characterize the cohort. Pearson correlations were used to evaluate relationships between $\log_{10}$-transformed plasma C-reactive protein (CRP) or $\log_{10}$-transformed erythrocyte sedimentation rate (ESR) and Q-Alb in COVID+ patients. CSF cytokine analyses used two-way analysis of variants (ANOVA) followed by Holm-Sidak post-hoc analyses to investigate differences in $\log_{10}$-transformed cytokines and chemokines between patients with COVID-19, WNV and HIV compared to healthy controls where appropriate (corrected $p < 0.05$ was considered significant). The heatmap showing the relative abundance of CSF cytokines and chemokines in patients with COVID-19 was generated using the 'ComplexHeatmap' package in R (version 4.0.3). Samples were clustered using unsupervised hierarchical clustering with Euclidean distances as the similarity measure and Ward's method as the clustering algorithm [25]. Statistical analyses for ANOVA and post-hoc analyses were performed with PRISM GraphPad. Pearson correlations were constructed in R.

2.6. Literature review

Systematic literature review of prior reports of SARS-CoV-2 testing in CSF was performed using NCBI PubMed search. NCBI PubMed search criteria included: ("SARS-CoV-2", "coronavirus" or "COVID-19") AND ("neurologic symptoms", "encephalopathy", "altered mental status", or "weakness"), ("SARS-CoV-2" "coronavirus" or "COVID-19") AND ("guillain-barre"), ("SARS-CoV-2" "coronavirus" or "COVID-19") AND ("stroke"), ("SARS-CoV-2" "coronavirus" or "COVID-19") AND ("myelopathy"), ("SARS-CoV-2" "coronavirus" or "COVID-19") AND ("encephalitis"). Results were included if they described reports of neurologic symptoms in patients with a confirmed positive NP swab for SARS-CoV-2 and CSF testing for SARS-CoV-2 RNA had been performed.

2.7. Data availability

Anonymized data will be made available to qualified external researchers with IRB approval, and requests will be reviewed and approved on the basis of scientific merit. Sequencing data will be publicly available on NCBI under BioProject accession PRJNA770695.

3. Results

3.1. Clinical characterization

For the 27 hospitalized COVID-19 patients with neurological

Table 1
Neurological findings in hospitalized patients with neurologic manifestations and COVID-19.

	Median or n	Range or %
Patient characteristics		
Age	56	24–82
Male:Female	21:6	n/a
WHO severity scale at lumbar puncture	3	3–7
Neurologic Syndrome prompting CSF (n = 27)		
Encephalopathy/Delirium	16/27	59%
Seizure	5/27	19%
Weakness/Sensory disturbance	6/27	22%
Anosmia	1/27	4%
Neurological imaging (MRI, n = 25)		
Acute ischemic infarct(s)	3/25	12%
White matter changes likely due to small vessel disease	2/25	8%
Leukoencephalopathy and microhemorrhages	3/25	12%
Enhancing white matter lesions (ADEM)	1/25	4%
Lumbar root enhancement	1/25	4%
SARS-CoV-2 or immunomodulatory treatments (n = 10)		
Tocilizumab	3/10	30%
Hydroxychloroquine	2/10	20%
Remdesivir	1/10	10%
Inhaled nitric oxide	1/10	10%
IVIG/Steroids	5/10	50%

symptoms and available CSF, the median age was 56 years (interquartile range (IQR) 41–67 years), and 21 (78%) were male (Table 1). The top reasons for LP were altered mental status including encephalopathy, headache, weakness and numbness. Other reasons are described in Supplementary Table 1. Disease severity for patients with CSF analyses ranged from having no or minimal respiratory symptoms (n = 16), requiring mechanical ventilation (n = 4), and requiring mechanical ventilation with other organ support (n = 7). The cohort included patients in the acute phase (within 7 days (n = 8)) and subacute-chronic (> 7 days (n = 19)) after SARS-CoV-2 infection (as measured by positive NP swab PCR).

The median CSF cell count for COVID-19 patients was 1 white blood cell/μL (WBC; range 0–7250), protein 42 mg/dL (range 16–750), and glucose 75 mg/dL (range 42–134). Five patients had CSF with WBC > 5 cells/ul, with three of these five patients diagnosed with or suspected to have bacterial or fungal co-infections (9644, 2044, 3844), one patient with suspected acute demyelinating encephalomyelitis (ADEM; 6429), and one patient with acute stroke following cardiac arrest in the setting of acute SARS-CoV-2 infection (2230).

3.2. Characterizing viral RNA in CSF by RT-qPCR and metagenomic sequencing

None of the patients had detectable SARS-CoV-2 RNA in CSF despite a wide range of clinical neurological presentations and evidence of neuroimaging abnormalities, including leukoencephalopathy. When we performed two distinct RT-qPCR assays, all replicates of all samples had undetermined cycle thresholds while in-assay positive and negative controls performed as expected. We also employed unbiased RNA metagenomic sequencing using a read depth of approximately 1 million paired end-reads per sample to determine if metagenomic sequencing could identify SARS-CoV-2 RNA missed in the targeted RT-qPCR approach. For 25/27 patients, we identified zero read pairs mapping to SARS-CoV-2 identified, and for the remaining two patient samples we identified 1 read pair/million. Owing to the potential for false positives, particularly in a research lab performing extensive SARS-CoV-2 sequencing, we set a threshold for evidence of SARS-CoV-2 presence at 3 reads mapping to distinct regions of the viral genome, as described previously [26]. No samples met this threshold; thus, we did not consider any patient as having detectable SARS-CoV-2 RNA by either RT-qPCR or metagenomic sequencing (Fig. 2A).

To detect potential alternative infections not identified in clinical testing that might account for CNS symptoms in COVID-19 patients, we performed metagenomic classification for 10 commonly-reported CNS pathogens (Enterovirus, Herpes Simplex virus 1, Herpes Simplex virus 2, Varicella-Zoster virus, *Streptococcus pneumoniae, N. meningitidis, Haemophilus influenzae, Listeria monocytogenes, Mycobacterium tuberculosis*, and *C. neoformans*) [27]. We found evidence for infections other than COVID-19 in CSF from 2/27 patients. *N. meningitidis* (1800 unique read pairs/million) was identified in RNA from the CSF of a patient with known *N. meningitidis* infection (9644). Similarly, *C. neoformans* (130 unique read pairs/million) was detected in the DNA metagenomic sequencing data from the CSF of a patient with a clinical diagnosis of cryptococcal meningitis (3844). In the same patient, a single read pair

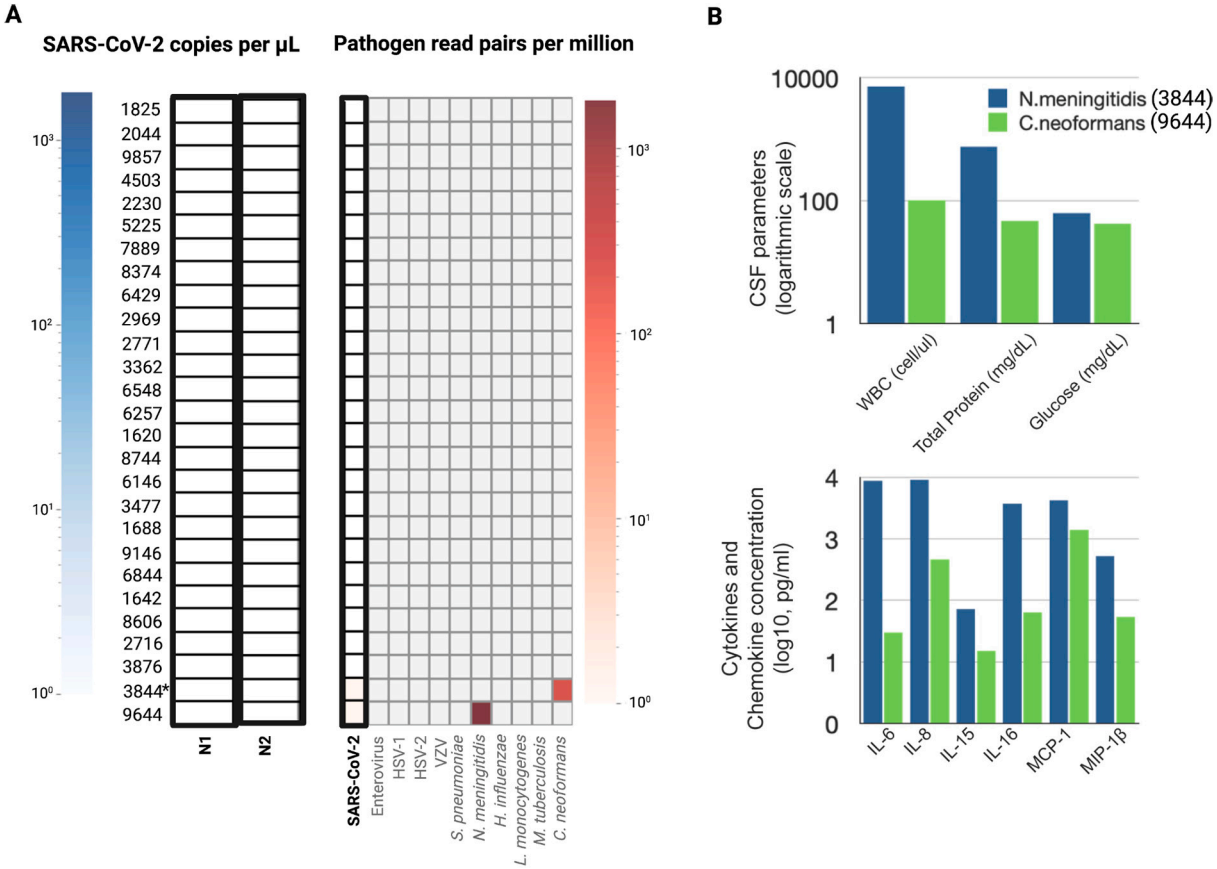

Fig. 2. Detection of pathogens in CSF from patients with COVID-19. A) Heatmap depicting the amount of SARS-CoV-2 RNA detected by RT-qPCR (blue) and RNA from SARS-CoV-2 and 10 common respiratory pathogens detected in metagenomic sequencing (red) (*DNA from 3884 was also profiled for pathogen reads). White boxes represents measured zero while grey boxes represent no measurement. B) CSF white blood cell count (WBC), total protein and glucose values in patients with *Neisseria meningitidis* (blue) or *Cryptococcus neoformans* (green) and COVID-19 infection (top panel). Values are shown on a logarithmic scale. Log10-transformed concentrations of IL-6, IL-8, IL-15, IL-16, MCP-1 and MIP-1β are shown for the two patients with CNS infections (bottom panel). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mapping to HIV was identified in the RNA metagenomic sequencing data (but not in that of negative controls); this patient was newly diagnosed with HIV-1 infection where ART had not yet been initiated. None of the other 25 patients' CSF samples, including from two patients with clinically suspected bacterial meningitis (2044, 7889), had evidence of reads mapping to any of the 10 common CNS pathogens targeted in our analyses (Fig. 2A, Supplementary Table 1).

3.3. Analysis of three SARS-CoV-2 genomes from NP swabs

In an independent study [22], NP swabs from three of the patients characterized here (2771, 3844, 7889) were sequenced and SARS-CoV-2 genomes were assembled (GenBank accessions: MT520526.1, MT873079.1, and MT873406.1 respectively). All three genomes carried the same Spike gene D614G mutation, which was commonly circulating at the time [28], and no other amino acid substitutions in that region. Altogether, these genomes were highly similar to strains circulating in this geographical region (greater Boston) at the time of sampling (March–May 2020) (Supplementary Fig. 1) [22].

3.4. Mixed intrathecal cytokine and chemokine profiles in COVID-19 patients compared to control and patients infected with other RNA viruses

To establish the potential role of cytokines in the neurological symptoms observed in COVID-19, we measured the CSF concentration of a select group of cytokines and chemokines (IL-6, IL-8, IL-15, IL-16, MCP-1 (CCL2), and MIP-1β (CCL4)) associated with severe or critical COVID-19 infection [11,29,30]. A total of 23/27 patients had enough remaining CSF for further testing after RT-qPCR and metagenomic sequencing. We compared CSF inflammatory profiles from COVID-19 patients to that of patients with virally suppressed (n = 16) and unsuppressed (n = 5) HIV infection, WNV (n = 4) and to patients without evidence of infection or other neurological disease (n = 16). Compared to control participants without neurological diseases, COVID-19 patients had significantly elevated CSF concentrations of $\log_{10}$ IL-6, IL-8, IL-15 and MIP-1β (Supplementary Fig. 2A).

Bacterial and fungal CNS infections are known to produce robust CSF cellular responses, which was underscored in this cohort by the observation that the patient with the highest concentration of IL-6, IL-8, IL-15, IL-6 and MIP-1β had CSF culture-positive *N. meningitidis* (9644). Thus, to minimize intrathecal responses due to factors other than COVID-19, we excluded the two patients with clinically identified CNS infections

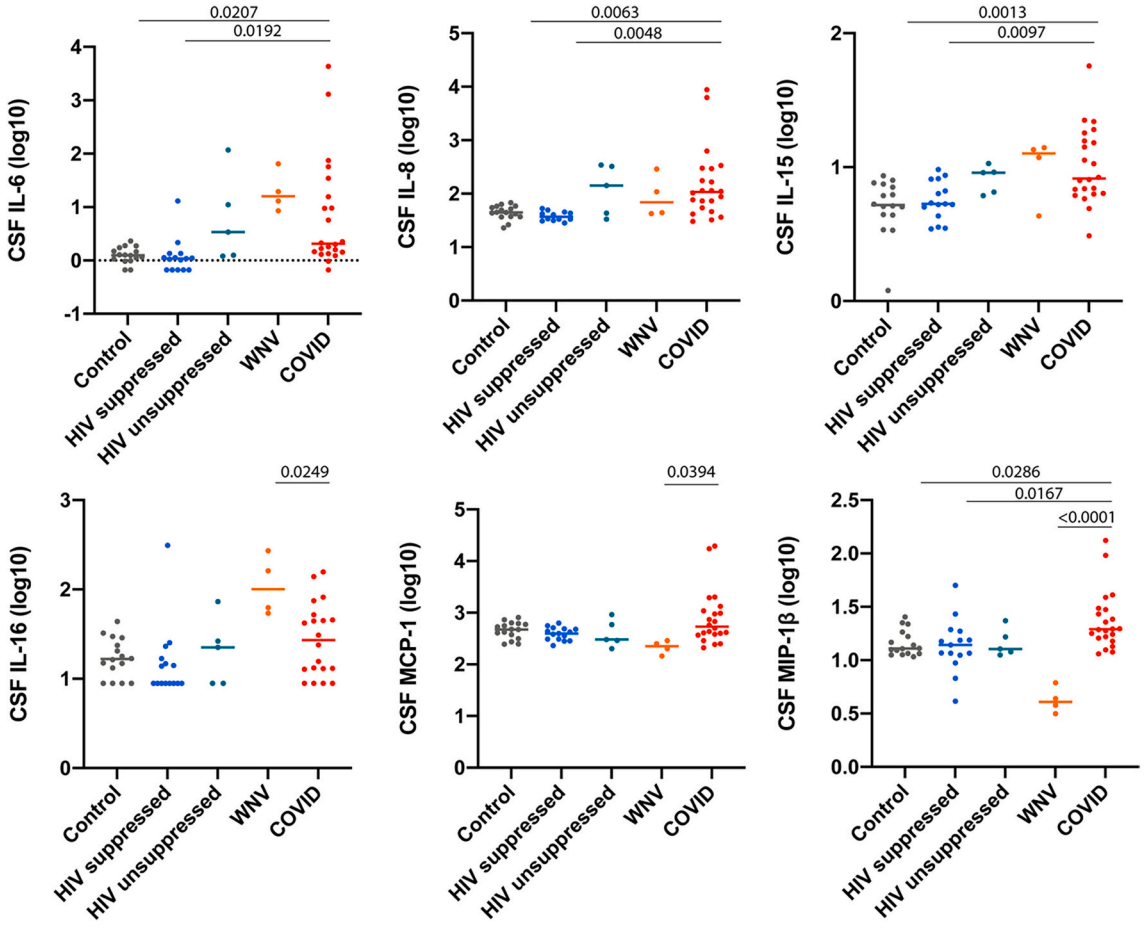

Fig. 3. Cytokine and chemokine expression in patients with COVID-19 and other infections and healthy controls. For five cohorts, including healthy controls (n = 16), patients diagnosed with suppressed HIV (n = 15), unsuppressed HIV (n = 5), WNV (n = 4) and COVID-19 (n = 21; two patients with confirmed CNS coinfections were omitted), CSF samples from each patient were profiled for cytokine and chemokine expression by multianalyte protein detection assay. Each figure panel characterizes a unique cytokine, with fluorescent signal (log base 10) representing cytokine expression (y-axis). Data points represent measurements for individual patients, and are categorized by cohort (x-axis); for each cohort, the mean fluorescent signal is represented by a horizontal line. For pairwise comparisons between the COVID-19 cohort and others with corrected p value less than 0.05 is displayed. There were significant differences between non-COVID-19 cohorts, including, for IL-6: between WNV and control (corrected p = 0.02), WNV and HIV suppressed (corrected p = 0.02); for IL-16: WNV and control (corrected $p < 0.01$), WNV and HIV suppressed (corrected $p < 0.001$), WNV and HIV unsuppressed (corrected p = 0.02); for MIP-1β: WNV and control (corrected $p < 0.001$), WNV and HIV suppressed (corrected $p < 0.001$), WNV and HIV unsuppressed (corrected $p < 0.01$) not shown on graphs.

(9644, 3844) confirmed by metagenomic sequencing when comparing intrathecal cytokine and chemokines (Fig. 2B). Despite their exclusion, COVID-19 patients still had increased CSF levels of specific cytokines and chemokines when compared to other cohorts (Fig. 3). The median concentrations of IL-6 (0.34 vs. 0.018, corrected $p = 0.02$), IL-8 (2.05 vs. 1.65, corrected $p = 0.01$), IL-15 (0.97 vs. 0.72, corrected $p = 0.001$), and MIP-1β (1.29 vs. 1.11, corrected $p = 0.03$) remained significantly increased in COVID-19 patients when compared to healthy controls (Fig. 3), an inflammatory response driven by a subset of patients. Patients with COVID-19 also had significantly increased levels of IL-6, IL-8, IL-15, and MIP-1β compared to HIV suppressed patients, though not when compared to patients with unsuppressed HIV infection (Fig. 3). Interestingly, MCP-1 and MIP-1β levels were higher in COVID-19 patients when compared to patients with WNV neuroinvasive disease, while IL-16 levels were lower in COVID-19 patients compared WNV, and there were no statistically significant differences in IL-6, IL-8 and IL-15. While IL-15 expression levels were significantly higher in COVID-19 patients with WHO ordinal severity scales of 4 or greater compared to WHO ordinal severity scale of 3, there were no statistically significant differences in the expression levels of other cytokines or chemokines, including IL-6 (Supplementary Fig. 2B).

3.5. Blood brain barrier disruption in SARS-CoV-2 infected patients

Increased CSF permeability and BBB breakdown has been postulated to be a mechanism for neurological impairment in COVID-19, potentially as a result of systemic proinflammatory cytokines rather than intrathecal cytokine production [31,32]. To further understand common factors that may increase intrathecal inflammatory responses in COVID-19 patients, we calculated the Q-Alb as a marker of BBB breakdown. This ratio is generally considered abnormal when greater than 9 in patients aged 40 or older [33].

One third of the examined hospitalized COVID-19 patients (8/23) had a Q-Alb >9, suggestive of BBB breakdown. Of these, two had severe COVID-19 leukoencephalopathy on MRI (1825, 2771, [59]; Supplementary Fig. 3), one was coinfected with *Neisseria meningitidis* (9644), and two had a history of neurosurgical intervention with clinically suspected co-infection with bacterial meningitis (2044, 7889). The final three patients with evidence of BBB breakdown were diagnosed with post-infectious complications including radiculoneuritis or myelitis (5225, 6257, 6975); one case diagnosed as acute disseminated encephalomyelitis, a diagnosis considered to be para-infectious or post-infectious, had a normal Q-Alb at the time of LP (i.e., 6429, Q-Alb = 6.4).

The majority of COVID-19 patients with neurological symptoms and CSF testing had normal Q-Alb ($n = 16$). Two out of the 16 had nonspecific MRI T2-FLAIR white matter hyperintensities (Q-Alb ≤1), and two had acute infarcts (Q-Alb ≤7). In all 7 COVID-19 patients with neurological symptoms suspected to be related to systemic disease (Supplementary Table 1), the Q-Alb was normal. In looking at our comparison samples, evidence of increased BBB breakdown as measured by Q-Alb was only observed in 1 patient with virally unsuppressed HIV infection (Q-Alb = 9.4) and two patients with virally suppressed HIV infection; one of these was co-infected with varicella zoster virus (Q-Alb = 47) and the other diagnosed with HIV dementia (Q-Alb = 14.3). No patients with WNV had Q-Alb values >9.

We hypothesized that clinical markers of systemic inflammation may correlate with BBB disruption; thus, where data was available, we tested the correlation between CRP and ESR obtained prior to LP and Q-Alb. While there was a trend towards positive correlation with Q-Alb and both systemic CRP levels ($r = 0.38$, $p = 0.08$) and ESR levels ($r = 0.43$, $p = 0.06$), the strength of the correlation decreased after removing the two patients with confirmed CNS infections (CRP ($r = 0.36$, $p = 0.11$) and ESR ($r = 0.41$, $p = 0.09$)), suggesting that there may not be a strong predictive relationship between systemic proinflammatory markers and BBB disruption in COVID-19. There were modest associations between CRP and intrathecal IL-15 ($r = 0.37$, $p = 0.04$) prior to correction. No other statistically significant relationships were identified between CRP and intrathecal cytokines. Serum IL-6 levels were available in only 8 patients, and there were no clear associations with Q-Alb or intrathecal cytokines or chemokines profiled in this dataset (data not shown).

In order to determine whether CSF proinflammatory cytokines were associated with increased BBB disruption irrespective of pathogen, we first compared levels of CSF IL-6, IL-8, IL-15, IL-16, MCP-1 and MIP-1β in all patients with Q-Alb>9 compared to those with Q-Alb ≤9. Levels of all CSF cytokines were significantly increased in patients with Q-Alb >9, indicating a correlation of BBB breakdown with increased cytokine levels in the CSF (Supplementary Fig. 4). Given the interest in identifying potential COVID-19-associated effects, we performed a Pearson correlation between Q-Alb and cytokine levels in the 22 COVID-19 patients with this data and without confirmed CNS infection. For all cytokines and chemokines assayed, the correlation between Q-Alb was moderate to strong, with correlation coefficients ranging from 0.57–0.79, and *p*-values all below 0.01(Fig. 4).

Using unsupervised hierarchical clustering, we found that intrathecal cytokine and chemokine levels were highly correlated within patients, with each exhibiting consistent levels across all cytokines (Fig. 5). Specifically, three COVID-19 patients had the top values of all cytokines analyzed, including a patient with catastrophic and fatal SARS-CoV-2 leukoencephalopathy without CSF pleocytosis (1825), a patient with refractory epilepsy and recent history of shunt placement (2044), and a patient with newly diagnosed epilepsy after COVID-19 infection (9857. CSF pleocytosis was minimal (CSF WBC <10 cells/ul) to absent in cases exhibiting relative elevations of inflammatory cytokines and chemokines (Fig. 5).

4. Discussion

In this study, we present CSF analysis of 27 hospitalized patients with COVID-19 infection using two RT-qPCR assays and metagenomic sequencing, and for 23 of these, further quantification of BBB disruption measured by CSF:serum albumin ratio and cytokine and chemokine levels. We did not identify SARS-CoV-2 RNA in the CSF by RT-qPCR assays, results that were reinforced by minimal detection by metagenomic sequencing, supporting the hypothesis that the neurologic symptoms seen in SARS-CoV-2 infection are unlikely to be due to active intrathecal viral replication. Our study alternatively suggests that intrathecal responses are heterogeneous and that in a small subset of patients with COVID-19, including those with COVID-19 leukoencephalopathy and post-infectious complications, there is an increase in proinflammatory cytokines or chemokines such as IL-6, IL-8, IL-15 and MIP-1β compared to control outpatient participants. This elevation occurred in the relative absence of CSF pleocytosis, and strongly correlated with a marker of BBB disruption. The majority of patients with HIV infection but no patients with WNV had evidence of elevated Q-Alb in this cohort. While WNV and HIV differ pathologically, including in their mechanism of neuroinvasion and CNS cell death, both viruses are capable of inducing profound CSF pleocytosis resulting in meningitis or encephalitis. These data along with other case series investigating CSF biomarkers in COVID-19 [9,34] indicate that CSF cellular responses in COVID-19 are minimal and that the intrathecal inflammatory cytokine and chemokine profiles in COVID-19 may differ from typical neurotropic RNA infections.

The lack of SARS-CoV-2 RNA detected in CSF here is consistent with numerous case series which find that detection in CSF is rare [35,36]. We did not detect any SARS-CoV-2 RNA by two RT-qPCR assays; while gold standards for the detection of CSF SARS-CoV-2 RNA are not definitively established, the assays used here demonstrated high sensitivity in spike-in studies, each with limits of detection reported at 10 copies/μL across several sample types [37]. We further demonstrated that SARS-CoV-2 RNA was not detectable in any of our samples by unbiased metagenomic sequencing, a pathogen detection method that is not dependent on pathogen sequence, making it an extremely robust

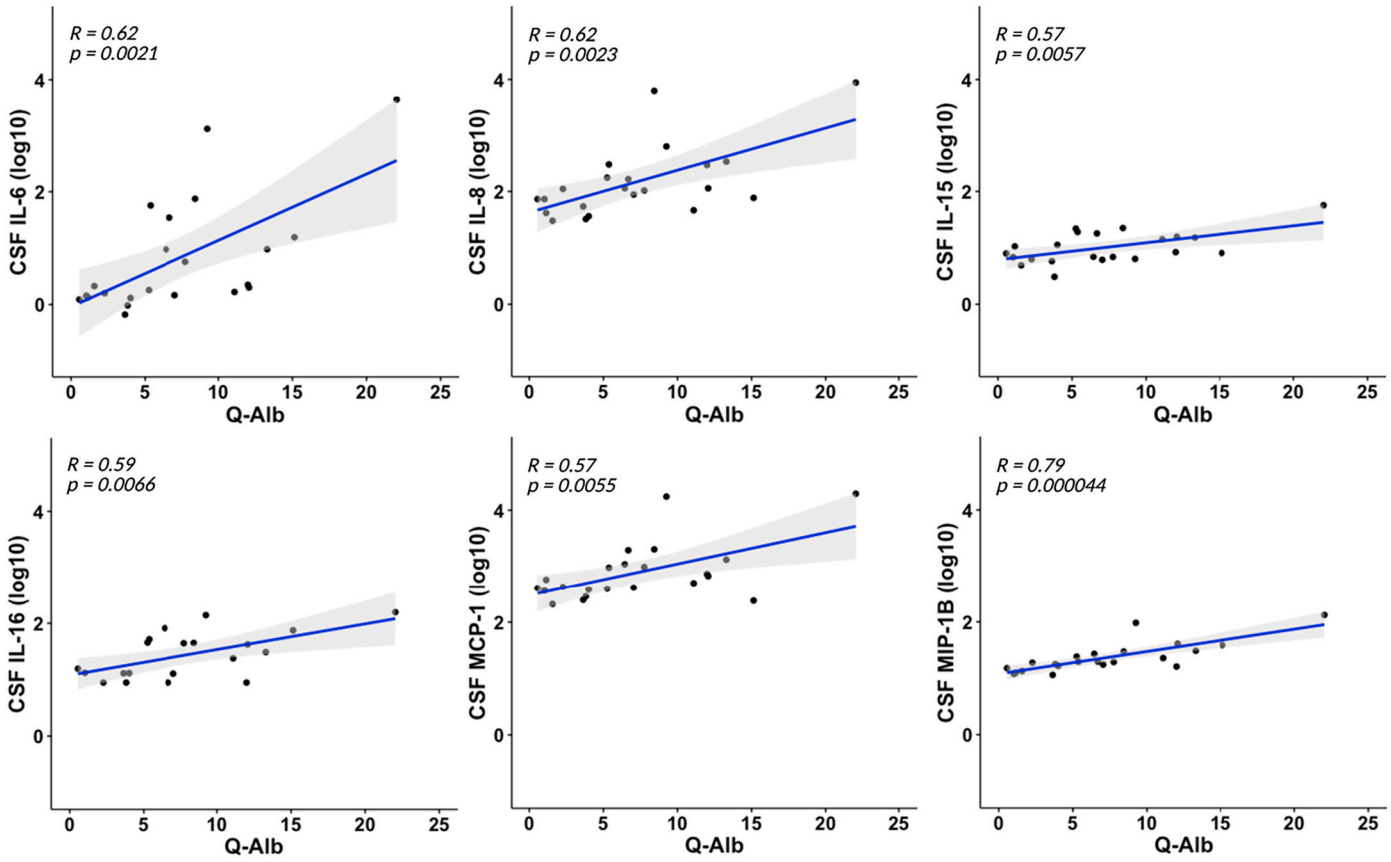

Fig. 4. Correlation between Q-Alb and cytokine or chemokine expression in COVID-19 patients. For COVID-19 patients included in this study ($n = 21$; two patients with confirmed CNS coinfections were omitted) cytokine or chemokine expression correlated with blood brain barrier integrity measured by CSF:serum albumin ratio (Q-Alb). Each figure panel characterizes a unique cytokine, with fluorescent signal (log base 10) representing cytokine expression. Data points represent cytokine expression (y-axis) and Q-alb (x-axis) measurements for individual patients. For each unique cytokine, the regression line is shown in blue and 95% confidence interval is shaded in grey; Pearson correlation coefficient (R) and significance (p) are also displayed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

technique to detect SARS-CoV-2 even in the context of viral mutations in RT-qPCR primer binding regions or significant RNA degradation. Thus, our data provide strong evidence against the presence of SARS-CoV-2 RNA in the CSF of our cohort of patients with COVID-19.

We compared our SARS-CoV-2 RNA results to all cases reported in the literature with COVID-19 and neurologic symptoms by the time of our cohort analysis. During the time interval of this study, there were 150 reported hospitalized cases with CSF testing due to neurological reasons, 6 of which had detectable SARS-CoV-2 RNA in CSF by RT-qPCR (Supplementary Table 2) [35,38–42], suggesting an estimated prevalence of CSF SARS-CoV-2 RNA in COVID-19 hospitalized patients of 3.6%. A recent detailed review of CSF analyses published between December 1, 2019 and November 18, 2020 reported CSF SARS-CoV-2 RNA was identified in 16 out of 303 patients with neurological symptoms (5%) [9]. However, given that RT-qPCR testing for SARS-CoV-2 is not covered under the FDA Emergency Use Authorization in the United States and remains inaccessible for most hospitals worldwide, there is limited CSF surveillance data, and current prevalence estimates are likely flawed due to reporting biases, RT-qPCR assay heterogeneity, and reliance on convenience sampling. The absence of nucleic acid does not exclude the possibility that SARS-CoV-2 is a neuroinvasive pathogen. For example, in the case of WNV neuroinvasive disease which is characterized by CSF pleocytosis and elevated total protein in 95% of patients, CSF RT-qPCR is relatively insensitive (57–70%) compared to WNV IgM [43]. Prior work with animal models expressing humanized ACE2 in brain and lung tissue and infected intranasally with SARS-CoV-2 demonstrated anti-SARS-CoV-2 antibodies in CSF and the brain [44], and a few reports in humans have shown high-titer anti-SARS-CoV-2 IgG in patients with COVID-19 and neurologic symptoms (reviewed in[9]). Specifically, an 8-person case series showed high-titers of CSF IgG in a subgroup of hospitalized patients with COVID-19 and encephalopathy despite absence of CSF SARS-CoV-2 RNA or intrathecal pleocytosis [31]. We did not quantify CSF anti-SARS-CoV-2 IgG and in the absence of paired serology, this would limit our ability to comment on CNS compartmentalization. Given recent literature also demonstrating that autoantibodies may be present in the CSF of patients with COVID-19 [44–49], further studies are required to determine whether compartmentalized anti-SARS-CoV-2 antibodies are present and if so, whether the presence of antibodies is associated with intrathecal inflammatory responses or blood brain barrier disruption despite absent pleocytosis or elevated protein.

Prior case series which examined CSF cytokine changes have shown elevated levels of IL-6, IL-1β, and IL-10 in some but not all patients with COVID-19 [11,36,50], though none of these included a control dataset for comparison. Rare studies [44,51,52] that included pre-pandemic CSF samples as controls have mixed results on COVID-specific compartmentalized immune responses. In this cohort, a relative increase in CSF cytokines (IL-6, IL-8, IL-15, MIP-1β) was observed in COVID-19 patients compared to controls and HIV suppressed patients, driven by a small subset of individuals. This relative increase of these specific innate-immunity secreted factors in SARS-CoV-2 infected patients is consistent with our understanding of the immunobiology of COVID-19, where macrophage-activation syndromes concurrent with systemic infection have been well-described [53–55]. However, in our study, whether the

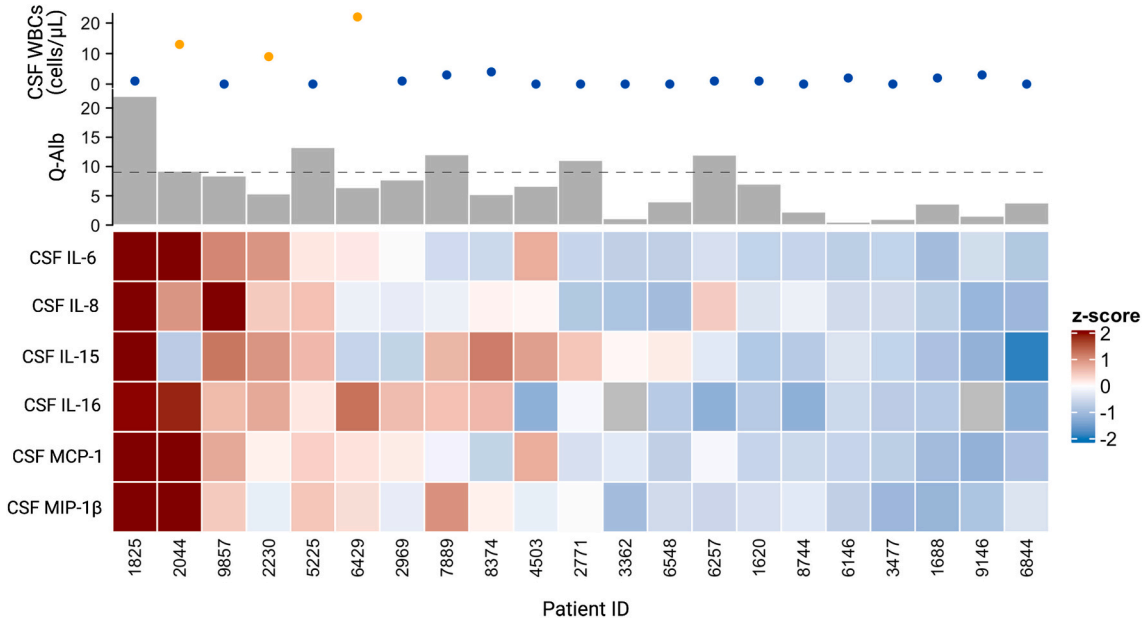

Fig. 5. Interrelationships between BBB permeability and CSF cytokines in COVID-19 patients. For each COVID-19 patient ($n = 21$; two patients with confirmed CNS coinfections were omitted), cytokine and chemokine expression levels were quantified (log base 10), and relative expression levels were z-score transformed across all samples and represented by colour. Patients and cytokines were ordered by unsupervised hierarchical clustering analyses of the cohort. Columns represent individual patients; rows, cytokine or chemokine. The CSF:serum albumin ratio (Q-Alb) is displayed by the height of the bars above each patient, where the dotted line (at Q-Alb = 9) represents the boundary between normal and abnormal. The data points displayed above the Q-Alb ratio show the CSF white blood cell count (WBC) for each patient where the orange indicates pleocytosis defined as CSF WBC > 5 cells/ul and blue indicates normal cell count. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

source of this elevated CSF IL-6, IL-8, IL-15, and MIP1β/CCL4, all of which are secreted by mononuclear-phagocytic system cells [56], is activated microglia, the tissue-resident macrophages of the CNS or circulating peripheral monocytes remains in question. Our data suggest that BBB breakdown occurs in a subset of patients and correlates with proinflammatory cytokine and chemokine responses; the directionality of this relationship remains to be defined. Systemic predictors of BBB breakdown in COVID-19 were not clearly identified in this small cohort, and further large-scale studies with longitudinal timed collection of CSF will be important to determine causality and ascertain relationship to clinical outcomes.

Our paper has several strengths. Our cohort was diverse, including patients with a wide spectrum of neurologic symptoms and radiological findings associated with COVID-19 adding substantial information to the literature. We included patients in the acute and subacute-chronic phases after SARS-CoV-2 infection. Additionally, by performing both RT-qPCR and unbiased metagenomic sequencing, we comprehensively profiled SARS-CoV-2 RNA in CSF. This study additionally adds to the case–control studies published in COVID-19, and provides unique comparisons with known neuroinvasive RNA pathogens.

Despite these strengths, our study has several notable limitations. First, our dataset was a convenience cohort of admitted patients with SARS-CoV-2 who presented with or developed neurologic symptoms during their hospitalization. The decision whether to proceed with lumbar puncture was based on staff preference with likely varying practices, and the majority of neurologic symptoms in our presentation were altered mental status or encephalopathy, which are nonspecific and may be subjective based on the examiner. The timing of lumbar puncture in relation to SARS-CoV-2 testing varied greatly based on clinical suspicion and timing of development of neurologic symptoms. While we captured a wide range of timing in this study, we may still miss the full breadth of the neurologic spectrum of COVID-19, most notably nonhospitalized patients. Additionally, we do not have complete clinical peripheral cytokine and laboratory data for all cases given this study retrospectively examined patients who had been hospitalized during the SARS-CoV-2 pandemic. Specifically, plasma samples from the time of hospitalization were not available from all patients and as a result, correlated levels of CSF and plasma cytokines are not available. Also, hypoxia has been associated with inflammation [57,58] and could be a confounding factor in our study. While CSF IL-15 was elevated in patients with WHO ordinal severity scale 4–7 (supplemental oxygen required) compared to WHO ordinal severity scale 3, other cytokines and chemokines, including IL-6, were not statistically different. Given the dynamic nature of oxygen saturation during hospitalization, our data lack the granularity to fully address this limitation. Additional data, including adequately measured arterial oxygen saturation during LP and comparison groups with respiratory infections or noninfectious causes of hypoxia are required to determine the modifying effects of hypoxia on intrathecal cytokine and chemokines responses. Finally, while our use of metagenomic sequencing allowed us to identify a wide range of potential co-infections that may have contributed to neurological symptoms (as were found in two cohort subjects), it is possible that other pathogens present at a very low-copy were missed by this analysis.

In the future, prospective, global surveillance studies that comprehensively evaluate the presence or absence of SARS-CoV-2 RNA, and protein profiling across time points in different clinical samples in patients presenting with neurologic symptoms are crucially needed. With the unfortunate accrual of additional COVID-19 patients in hospitals, and emergence of viral variants, identifying CNS compartment specific responses remains vital in our understanding of long-term neurological sequelae of disease.

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Declaration of Competing Interest

JEL has received consulting fees from Sherlock biosciences. PCS is a co-founder of, shareholder in, and scientific advisor to Sherlock biosciences, Inc., as well as a board member of and shareholder in Danaher corporation. MNA is a co-founder and equity holder of Day zero diagnostics, outside of the submitted work. All other others report no relevant declarations

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jns.2021.120023.

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