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Neuro-axonal injury in COVID-19: the role of systemic inflammation and SARS-CoV-2 specific immune response

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Abstract

Background: In coronavirus disease-2019 (COVID-19) patients, there is increasing evidence of neuronal injury by the means of elevated serum neurofilament light chain (sNfL) levels. However, the role of systemic inflammation and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)–specific immune response with regard to neuronal injury has not yet been investigated.

Methods: In a prospective cohort study, we recruited patients with mild–moderate ($n = 39$) and severe ($n = 14$) COVID-19 and measured sNfL levels, cytokine concentrations, SARS-CoV-2-specific antibodies including neutralizing antibody titers, and cell-mediated immune responses at enrollment and at 28($\pm$7) days. We explored the association of neuro-axonal injury as by the means of sNfL measurements with disease severity, cytokine levels, and virus-specific immune responses.

Results: sNfL levels, as an indicator for neuronal injury, were higher at enrollment and increased during follow-up in severely ill patients, whereas during mild–moderate COVID-19, sNfL levels remained unchanged. Severe COVID-19 was associated with increased concentrations of cytokines assessed [interleukin (IL)-6, IL-8, interleukin-1 beta (IL-1β), and tumor necrosis factor-alpha (TNF-α)], higher anti-spike IgG and anti-nucleocapsid IgG concentrations, and increased neutralizing antibody titers compared with mild–moderate disease. Patients with more severe disease had higher counts of defined SARS-CoV-2-specific T cells. Increases in sNfL concentrations from baseline to day 28($\pm$7) positively correlated with anti-spike protein IgG antibody levels and with titers of neutralizing antibodies.

Conclusion: Severe COVID-19 is associated with increased serum concentration of cytokines and subsequent neuronal injury as reflected by increased levels of sNfL. Patients with more severe disease developed higher neutralizing antibody titers and higher counts of SARS-CoV-2-specific T cells during the course of COVID-19 disease. Mounting a pronounced virus-specific humoral and cell-mediated immune response upon SARS-CoV-2 infection did not protect from neuro-axonal damage as by the means of sNfL levels.

Keywords: cytokines, immune response, neurofilament light chain protein, neurologic damage, SARS-CoV-2

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Background

Neurologic symptoms, including altered mental status, cerebrovascular disease, and anosmia, are reported in many severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)–infected individuals during the acute stadium of disease.[1]

Furthermore, there is increasing evidence for persistent neurologic symptoms such as chronic fatigue, memory impairment, and depression after acute coronavirus disease-2019 (COVID-19).[2] Neuropathological studies reveal evidence for central nervous system (CNS) damage in fatal COVID-19 cases.[3–5] A common finding in these studies was cerebral cell loss, either attributed to hypoxia[3,4] or inflammation[5] either with or without immunohistochemical evidence of viral CNS invasion.[3,4,6–8]

Members of the betacoronavirus genus are potent pathogens, usually associated with respiratory tract disease in humans [e.g. SARS-CoV-2, severe acute respiratory syndrome coronavirus (SARS-CoV), or Middle East respiratory syndrome (MERS)] and gastrointestinal, hepatic, and neurologic disease in animals.[9] Mouse hepatitis virus (MHV) belongs to the betacoronavirus genus and shows neurotropic properties. MHV-JHM and MHV-A59 have been studied for ages as a mouse model of virus-induced encephalitis, myelitis, and chronic progressive demyelination concurrent with axonal loss of the CNS.[9] The importance of the humoral immune response for clearance of betacoronaviruses (MHV-59 and MHV-JHM) from CNS in mice is well established.[10–12] In humans, humoral immune deficiencies have been associated with prolonged SARS-CoV-2 shedding and severe disease,[13–17] but the role of the SARS-CoV-2 specific immune response with regard to neuronal injury in COVID-19 patients has not yet been investigated.

Neurofilament light chain (NfL) is an intra-axonal cytoskeleton protein highly expressed in large caliber myelinated axons.[18] Serum neurofilament light chain (sNfL) measurements are used to detect and monitor CNS injury in various neurodegenerative conditions, including multiple sclerosis, Alzheimer's disease, and amyotrophic lateral sclerosis.[18] In moderate to severely ill COVID-19 patients, there is increasing evidence for neuronal injury by the means of elevated sNfL levels.[19–25]

The aim of this study was to examine in COVID-19 patients the association of neuro-axonal injury as by the means of sNfL measurements with disease severity and virus-specific immune response. We explored whether an insufficient immunological control of the virus might play a role in promoting neuro-axonal injury.

Methods

Study population

We prospectively included patients with confirmed SARS-CoV-2 infection from 5 March 2020 to 16 July 2020. Serum samples were collected at the time of enrollment in the study (baseline) and at follow-up 28 ± 7 days later. Patients with preexisting neuroinflammatory disorders were excluded from the analysis. COVID-19 disease severity was categorized according to the *COVID-19 WHO Ordinal Scale for Clinical Improvement*, ranging from 1 (*no symptoms*) to 8 (*fatal COVID-19*).[26] For analysis, patients with a score of 1–4 (patients without need for high-flow oxygen) were defined to have mild/moderate disease, and patients with a score of 5–8 (patients with need for high-flow oxygen, non-invasive ventilation, or mechanical ventilation) were defined to have severe disease. The present study is part of a larger COVID project (NCT04510012), and data of some patients were previously investigated regarding immune functionality in COVID-19.[27] The trial was approved by the Ethics Commission of the Canton of Bern, Bern, Switzerland, Nr. 2020-00877 and registered at ClinicalTrials.gov (NCT04510012). Patients were included after provision of informed consent. In case of lack of capacity and/or inability to provide consent, enrollment followed the procedures for research projects in emergency situations according to Swiss law.

Viral detection method

Patient nasopharyngeal sample was obtained using Copan FLOQSwabs and Copan UTM Viral Transport Medium (Copan, Brescia, Italy). For polymerase chain reaction (PCR) testing, three different methodologies were used: a laboratory developed workflow based on a published protocol,[28] and two commercial workflows, the Seegene Allplex 2019-nCoV Assay (Seegene, Seoul, Korea) and the Roche Cobas® SARS-CoV-2 Assay (Roche Diagnostics, Rotkreuz, Switzerland).

NfL and cytokine measurements

Cytokines [interleukin (IL)-6, IL-8, interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α)] and NfL were quantified in serum samples using an automated enzyme-linked immunosorbent assay (ELISA)–based microfluidic system (ELLA®, ProteinSimple, San Jose, CA, USA)

with dedicated cartridges according to the manufacturer's instructions. Samples were separated into triplicates that were automatically and independently processed. Raw data were analyzed using the manufacturer's software. A mean value derived from the three replicates was generated.

Humoral and cell-mediated immune response

Anti-spike IgG antibody concentrations in serum were measured using the DiaSorin LIAISON® SARS-CoV-2 S1/S2 IgG assay [DiaSorin, Saluggia, Italy; positive cutoff of >15 AU/ml (arbitrary units per milliliter)]. Anti-nucleocapsid antibody concentrations were determined using the Abbott SARS-CoV-2 IgG assay run on the Abbott ARCHITECT i2000 instrument [Abbott Diagnostics, Abbott Park, IL, USA; positive cutoff of >1.4 S/C Index (sample control index ratio)].

For the measurement of serum neutralizing SARS-CoV-2 antibody titers, 20,000 Vero E6 cells were seeded in a 96-well plate format. The following day, heat-inactivated sera were twofold serial diluted and mixed with 200 plaque forming units of SARS-CoV-2 virus, which was generated, rescued, and propagated as previously described.[29,30] After 1 h of pre-incubation at room temperature, the mixture was added to Vero E6 cells and incubated at 37°C. After 4 days, cells were fixed with 4% formalin and stained with crystal violet to analyze the reciprocal dilution at which SARS-CoV-2 was neutralized. The experiment was performed in the biosafety level-3 laboratories at the Institute for Infectious Diseases, University of Bern.

The cell-mediated immune (CMI) response was characterized using the human interferon-gamma (IFN-γ)/IL-2 SARS-CoV-2 FluoroSpot^{PLUS} kit (Mabtech AB, Nacka, Sweden) according to the manufacturer's instructions. In brief, after washing three times with phosphate-buffered saline (PBS), 250,000 cryopreserved peripheral blood mononuclear cells (PBMCs) per well were incubated (37°C, humified incubator with 5% CO_2) on pre-coated 96-well plates overnight and stimulated with S2N (spike-2 protein, nucleocapsid protein) and SNMO (spike protein, nucleocapsid protein, membrane protein, open reading frame proteins) peptide pools in the presence of costimulatory anti-CD28 antibodies. The SARS-CoV-2 S2N defined peptide pool contains 41 synthetic peptides binding to human HLA (human leukocyte antigen) derived from the S2 and N proteins of the SARS-CoV-2 virus (#3620-1, Mabtech AB). The SARS-CoV-2 SNMO defined peptide pool contains 47 synthetic peptides binding to human HLA, derived from the S, N, M ORF3a, and ORF7a proteins (#3622-1, Mabtech AB). Positive (anti-CD3 antibodies) and negative controls (neither anti-CD3 antibodies nor peptide pools) were included. Thereafter, cells were removed by emptying the plate and washing five times with PBS. Detection antibodies (anti-IFN-γ and anti-IL-2) were added and incubated for 2 h. After washing (5× with PBS), fluorophore conjugates (anti-BAM-490; SA-550) were added and incubated for 1 h. After washing (5× with PBS), fluorescence enhancer (IFN-γ: Fluorescein-5-isothiocyanate (FITC); IL-2: Cy3) was added for 10 min and then removed by flicking. Spot analysis was performed with an automated FluoroSpot reader (AID ispot EliSpot/FluoroSpot Reader, AID, Strassberg, Germany) equipped with filters for the fluorophores used. The results were expressed as the number of spot forming units (SFUs) per 250,000 seeded cells after subtracting the background spots of the negative control.

Statistics

Demographics were analyzed using descriptive statistics (Fisher's exact test). Mann–Whitney *U* tests were used to assess the associations between dichotomous COVID-19 severity (mild–moderate and severe), cytokine levels, antibody concentrations, CMI (SFU/250,000 cells), and sNfL concentrations. To account for the potential for confounding by age, corticosteroid use, and time from symptom onset to sample measurement, we repeated the analyses using multiple linear regression adjusted for those covariates. Variables of interest were log-transformed where appropriate to approximate a normal distribution. We compared outcomes between individuals with severe and mild–moderate COVID-19 using adjusted mean values, and calculated mean differences for untransformed and ratio of means for log-transformed variables.[31] Cytokine levels, antibody concentrations, CMI (SFU/250,000 cells), and sNfL concentrations in paired samples over time were compared using Wilcoxon signed-rank test.

Because sNfL levels increase with age,[18] which is also a major risk factor for severe COVID-19, we further refined our analyses by associating sNfL

changes (ΔsNfL: sNfL at follow-up minus sNfL at baseline; sNfL fold-change: sNfL at follow-up divided by sNfL at baseline) with the immune response. We used univariable and multivariable linear regression adjusted for age, corticosteroid use, and time from symptom onset to sample measurement to estimate the association between changes in sNfL levels and cytokine concentrations, antibody levels, and SFUs. sNfL fold-changes were modeled using log-transformation, and absolute sNfL differences were modeled using a cube-root transformation to account for the long tails in addition to positive and negative values.[32] Statistical significance was defined as a p value $<.05$. In this exploratory study, p values and widths of 95% confidence intervals were not adjusted for multiplicity.[33,34] Statistical analyses were performed using Stata software version 16.0 (Stata Corp., College Station, TX, USA) and R version 4.1.1 (R Core Team 2021, R: A language and environment for statistical computing, R Foundation for Statistical Computing, Vienna, Austria, URL https://www.R-project.org/). Figures were created using R and GraphPad, version 8.0 (LaJolla, CA, USA).

Results

Demographics

We enrolled 55 patients with symptomatic COVID-19 and excluded two patients from analysis due to the presence of an active neuroinflammatory disorder (one patient with Guillain–Barré syndrome; one patient with a facial palsy due to a suppurative otitis). Patients with mild to moderate disease ($n = 39$) were younger and had less comorbidities compared with severe COVID-19 cases ($n = 14$) (Table 1). The median duration of clinical symptoms before enrollment was 5 [interquartile range (IQR): 3–7] days for patients with mild to moderate disease and 7 (IQR: 6–8) days for patients with severe COVID-19 ($p = 0.071$). Most patients (64%; 25/39) with mild–moderate COVID-19 were treated as outpatients. All patients with severe disease were admitted to the intensive care unit (ICU) because of respiratory failure requiring mechanical ventilation. By chart review, we identified eight patients who developed neurologic complications after enrollment (six patients: confusion with the need for antipsychotic therapy, one patient: confusion with the need for antipsychotic therapy in combination with cerebral hemorrhage, one patient: critical illness polyneuropathy).

Cytokine response

All patients had baseline serum samples, and follow-up samples were available for 49/53 patients (2 patients died, 1 patient refused follow-up blood work). In unadjusted analysis, median serum cytokine concentrations at baseline were higher in patients with severe COVID-19 compared with those with mild–moderate disease (IL-6: 167.5 *versus* 3.3 pg/ml, $p < 0.001$; IL-8: 112.5 *versus* 14.7 pg/ml, $p < 0.001$; IL-1β: 0.657 *versus* 0.257 pg/ml, $p < 0.001$; TNF-α: 30.6 *versus* 15.1 pg/ml, $p < 0.001$; Figure 1(a)). After adjusting for age, time from symptom onset to sampling, and use of immunomodulatory drugs, IL-6, IL-8, and TNF-α levels at baseline remained significantly higher in patients with severe disease (Table 2). Cytokine levels decreased over time. In unadjusted analysis of follow-up samples, cytokines remained significantly elevated in severely ill patients compared with mild–moderate cases (IL-6: 8.6 *versus* 1.5 pg/ml, $p < 0.001$; IL-8: 41.4 *versus* 10.2 pg/ml, $p < 0.001$; IL-1β: 0.374 *versus* 0.176 pg/ml, $p = 0.034$; TNF-α: 18.2 *versus* 10.3 pg/ml, $p < 0.001$; Figure 1(a)). Concentrations of IL-8 and TNF-α in follow-up samples remained significantly higher in severely ill COVID-19 patients compared with patients with mild–moderate disease after adjusting for confounders (Table 2).

Antibody response

At baseline, most patients were seronegative for anti-spike IgG antibodies (severe COVID-19: 57.1% (6/14), mild–moderate COVID-19: 84.6% (33/39), $p = 0.060$) and anti-nucleocapsid IgG antibodies (severe COVID-19: 64.3% (9/14), mild–moderate COVID-19: 71.8% (28/39), $p = 0.736$). The majority of patients did not have detectable neutralizing antibody titers at baseline (severe COVID-19: 57.1% (8/14), mild–moderate COVID-19: 76.9% (30/39), $p = 0.182$). Anti-spike IgG, anti-nucleocapsid IgG, and neutralizing antibody titers increased significantly over time in both patient groups (Figure 1(b)). In unadjusted analysis, anti-spike and anti-nucleocapsid IgG levels along with neutralizing antibody titers were significantly higher in follow-up samples of patients with severe COVID-19 (Figure 1(b)), without any changes in the adjusted analyses (Table 2).

CMI response

PBMCs were available for 84.9% (45/53) of patients at baseline and for 86.8% (46/53) of

Table 1. Patient characteristics.

	Total ($N = 53$)	Mild/moderate COVID-19 ($n = 39$)	Severe COVID-19 ($n = 14$)	Between-group *p* value
Age, median (IQR), years	50.6 (32.7–65.3)	38.6 (28.5–62.0)	66.0 (57.1–69.4)	**0.002**
Gender (male, %)	33 (62.3%)	23 (59.0%)	10 (71.4%)	0.527
Symptom onset to first serum sample, median (IQR), days	6 (4–8)	5 (3–7)	7 (6–8)	0.071
Symptom onset to second serum sample, median (IQR), days	33 (30–37)	34 (32–36)	32 (30–37)	0.584
Comorbidities				
Diabetes (%)	8 (15.1%)	4 (10.3%)	4 (28.6%)	0.186
Hypertension (%)	15 (28.3%)	8 (20.5%)	7 (50.0%)	**0.046**
Cardiac disease (%)	9 (17.0%)	3 (7.7%)	6 (42.9%)	**0.007**
Cerebrovascular disease (%)	4 (7.6%)	3 (7.7%)	1 (7.1%)	1.000
Pulmonary disease (%)	6 (11.3%)	5 (12.8%)	1 (7.1%)	1.000
Renal disease (%)	4 (7.6%)	0 (0.0%)	4 (28.6%)	**0.003**
Immune disorder/immunosuppression (%)[a]	2 (3.8%)	0 (0.0%)	2 (14.3%)	0.066
Malignancy (%)	2 (3.8%)	2 (5.1%)	0 (0.0%)	1.000
COVID-19 treatment and outcome				
Hospital admission (%)	28 (52.8%)	14 (35.9%)	14 (100%)	**<0.001**
ICU admission (%)	16 (30.2%)	2 (5.1%)[b]	14 (100%)	**<0.001**
Mechanical ventilation (%)	14 (26.4%)	0 (0.0%)	14 (100%)	**<0.001**
Antiviral therapy (%)[c]	5 (9.4%)	1 (2.6%)	4 (28.6%)	**0.014**
Corticosteroid therapy (%)	6 (11.3%)	1 (2.6%)	5 (35.7%)	**0.004**
Confusion (%)[d]	7 (13.2%)	0 (0.0%)	7 (50.0%)	**<0.001**
Other neurologic complication (%)[e]	2 (3.8%)	0 (0.0%)	2 (14.3%)	0.066
Fatal COVID-19	3 (5.7%)	0 (0.0%)	3 (21.4%)	**0.016**

ICU, intensive care unit; IQR, interquartile range.
[a]One kidney transplant recipient, one HIV+ patient.
[b]Short-term ICU stay for evaluation.
[c]1 lopinavir/ritonavir (mild/moderate COVID-19 case), 2 hydroxychloroquine, 1 remdesivir/hydroxychloroquine, 1 atazanavir, 1 atazanavir/hydroxychloroquine.
[d]Defined as delirium in need of pharmacotherapy.
[e]1 cerebral hemorrhage, 1 critical illness polyneuropathy.
Significant *p* values (<0.05) are in bold.

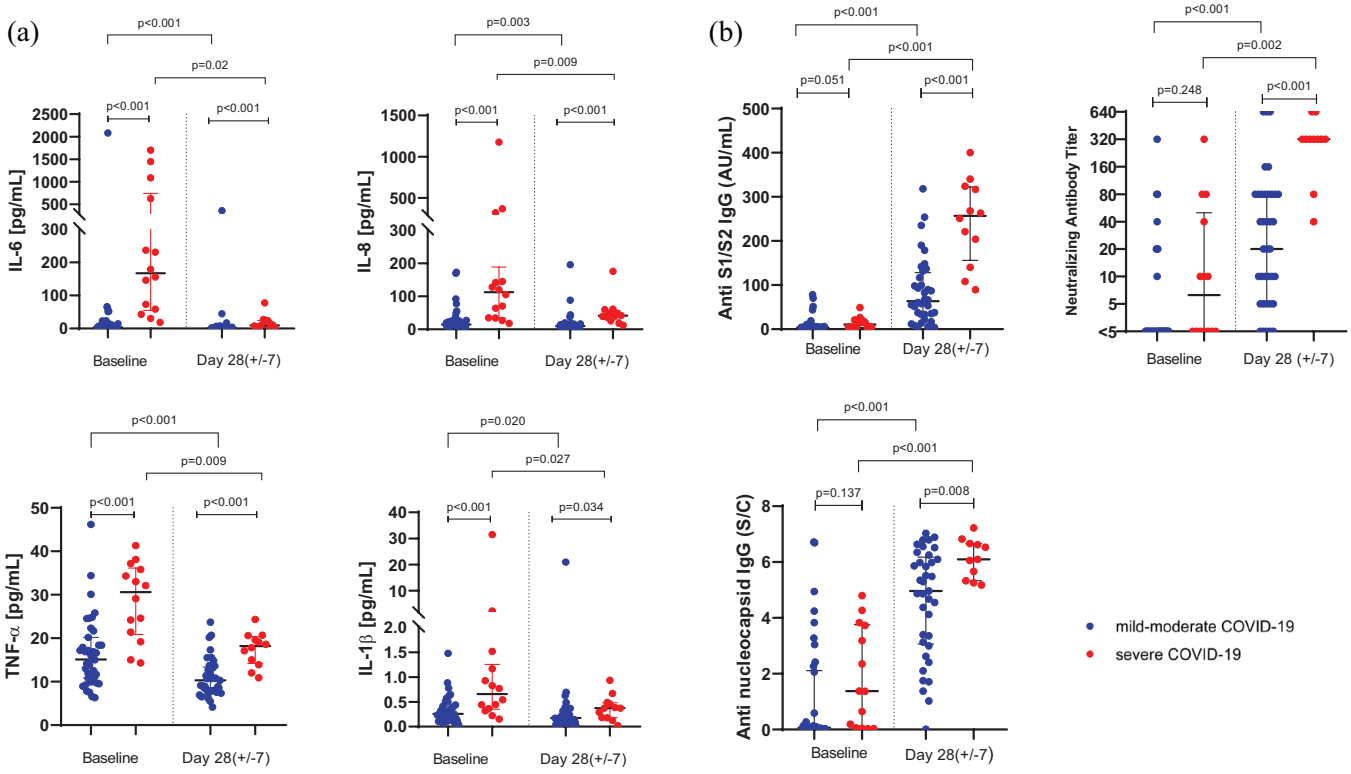

Figure 1. Serum cytokine and SARS-CoV-2-specific antibody concentrations in relation to COVID-19 severity. (a) Serum IL-6, IL-8, IL-1β, and TNF-α serum concentration at baseline and day 28(±7) in patients with severe (red) and mild–moderate (blue) COVID-19. (b) Anti-spike (S1/S2), neutralizing antibody, and anti-nucleocapsid antibody concentration at baseline and day 28(±7) in patients with severe (red) and mild–moderate (blue) COVID-19. Black horizontal bars indicate median values. Whiskers indicate interquartile ranges.
COVID-19, coronavirus disease-2019; IL-6, interleukin-6; IL-8, interleukin-8; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-alpha.

patients at follow-up. Dichotomized disease severity (mild–moderate *versus* severe) was not associated with the number of IFN-γ positive SFUs (unadjusted analyses: Figure 2(a) and (b); adjusted analyses: Table 2).

IL-2 SFUs significantly increased from baseline to follow-up upon stimulation with the S2N and SNMO peptide pool (Figure 2(a) and (b)). Upon cell stimulation with the SNMO peptide pool, patients with severe COVID-19 had significantly more IL-2-positive SFUs compared with individuals with mild–moderate disease at follow-up (unadjusted analyses: Figure 2(a); adjusted analyses: Table 2).

IFN-γ/IL-2 double-positive spots significantly increased from baseline to follow-up after cell stimulation with the SNMO peptide pool and in unadjusted analyses; patients with severe disease had more IFN-γ/IL-2 double-positive SFU at follow-up compared with those with mild–moderate disease (Figure 2(a)). Adjusted differences in IFN-γ/IL-2 double-positive SFUs at follow-up were no longer statistically significant (Table 2). Upon stimulation with the S2N peptide pool, IFN-γ/IL-2 double-positive spots increased from baseline to follow-up, but changes were only statistically significant in the larger mild–moderate COVID-19 group (Figure 2(b)). There was no statistically significant difference in IFN-γ/IL-2 double-positive spots at follow-up between mild/moderate and severe COVID-19 patients in unadjusted (Figure 2(b)) and adjusted (Table 2) analyses.

NfL concentrations

Serum NfL measurements were available for 98.1% (52/53) at baseline and for 92.5% (49/53) at day 28(±7).

In unadjusted analysis, median sNfL concentrations at baseline were higher in patients with

Table 2. Adjusted comparison of cytokine concentrations, antibody levels, virus-specific T cells, and sNfL concentrations between severe and mild–moderate COVID-19 cases.

	Severe *versus* mild–moderate COVID-19	***p* value**
Ratio of adjusted mean cytokine levels at baseline		
IL-6 (95% CI)	11.7 (4.3 to 32.2)	**<0.001**
IL-8 (95% CI)	3.3 (1.8 to 6.2)	**<0.001**
IL-1β (95% CI)	2.1 (1.1 to 4.1)	**0.024**
TNF-α (95% CI)	1.9 (1.3 to 2.8)	**0.003**
Ratio of adjusted mean cytokine levels at day 28(±7)		
IL-6 (95% CI)	1.9 (0.9 to 4.1)	0.117
IL-8 (95% CI)	2.3 (1.4 to 3.9)	**0.002**
IL-1β (95% CI)	1.4 (0.6 to 3.5)	0.404
TNF-α (95% CI)	1.4 (1.1 to 1.82)	**0.008**
Mean differences in adjusted antibody levels at day 28(±7)		
Anti-S1/S2 IgG (95% CI) [AU/ml]	113.2 (59.9 to 166.6)	**<0.001**
Anti-nucleocapsid IgG (95% CI) [S/C]	1.4 (0.1 to 2.7)	**0.037**
Ratio of adjusted mean neutralizing antibody titers at day 28(±7)		
Neutralizing antibodies (95% CI)	7.9 (2.3 to 26.4)	**0.001**
Mean differences in adjusted cell-mediated immune response at day 28(±7)		
IFN-γ-specific T-cells SNMO peptide pool (95% CI) [SFU/250,000 PBMCs]	10.2 (–18.7 to 39.0)	0.481
IFN-γ-specific T-cells S2N peptide pool (95% CI) [SFU/250,000 PBMCs]	–3.6 (–16.4 to 9.2)	0.571
IL-2-specific T-cells SNMO peptide pool (95% CI) [SFU/250,000 PBMCs]	46.7 (5.1 to 88.2)	**0.029**
IL-2-specific T-cells S2N peptide pool (95% CI) [SFU/250,000 PBMCs]	4.0 (–4.9 to 12.8)	0.371
IL-2/IFN-γ double-positive T-cells SNMO peptide pool (95% CI) [SFU/250,000 PBMCs]	2.3 (–1.9 to 6.4)	0.300
IL-2/IFN-γ double-positive T-cells S2N peptide pool (95% CI) [SFU/250,000 PBMCs]	–0.4 (–1.7 to 1.0)	0.589
Ratio of adjusted mean sNfL levels at baseline		
sNfL (95% CI)	1.65 (1.03 to 2.62)	**0.037**
Ratio of adjusted mean sNfL levels at day 28(±7)		
sNfL (95% CI)	3.68 (1.84 to 7.36)	**<0.001**

AU, arbitrary units; CI, confidence interval; COVID-19, coronavirus disease-2019; IFN-γ, interferon-gamma; IL-2, interleukin-2; IL-6, interleukin-6; IL-8, interleukin-8; IL-1β, interleukin-1β; PBMCs, peripheral blood mononuclear cells; S/C, sample control index ratio; SFU, spot forming unit; sNfL, serum neurofilament light chain; SNMO, spike protein, nucleocapsid protein, membrane protein, open reading frame proteins; S2N, spike-2 protein, nucleocapsid protein; TNF-α, tumor necrosis factor-alpha.
Mild-to-moderate and severe COVID-19 cases were compared using multivariable linear regression models, adjusted for age, corticosteroid use, and time from symptom onset to sample measurement. Comparisons are presented as adjusted mean differences when using models with untransformed data, and as ratio of adjusted mean values when using log-transformed data.
Significant *p* values (<0.05) are in bold.

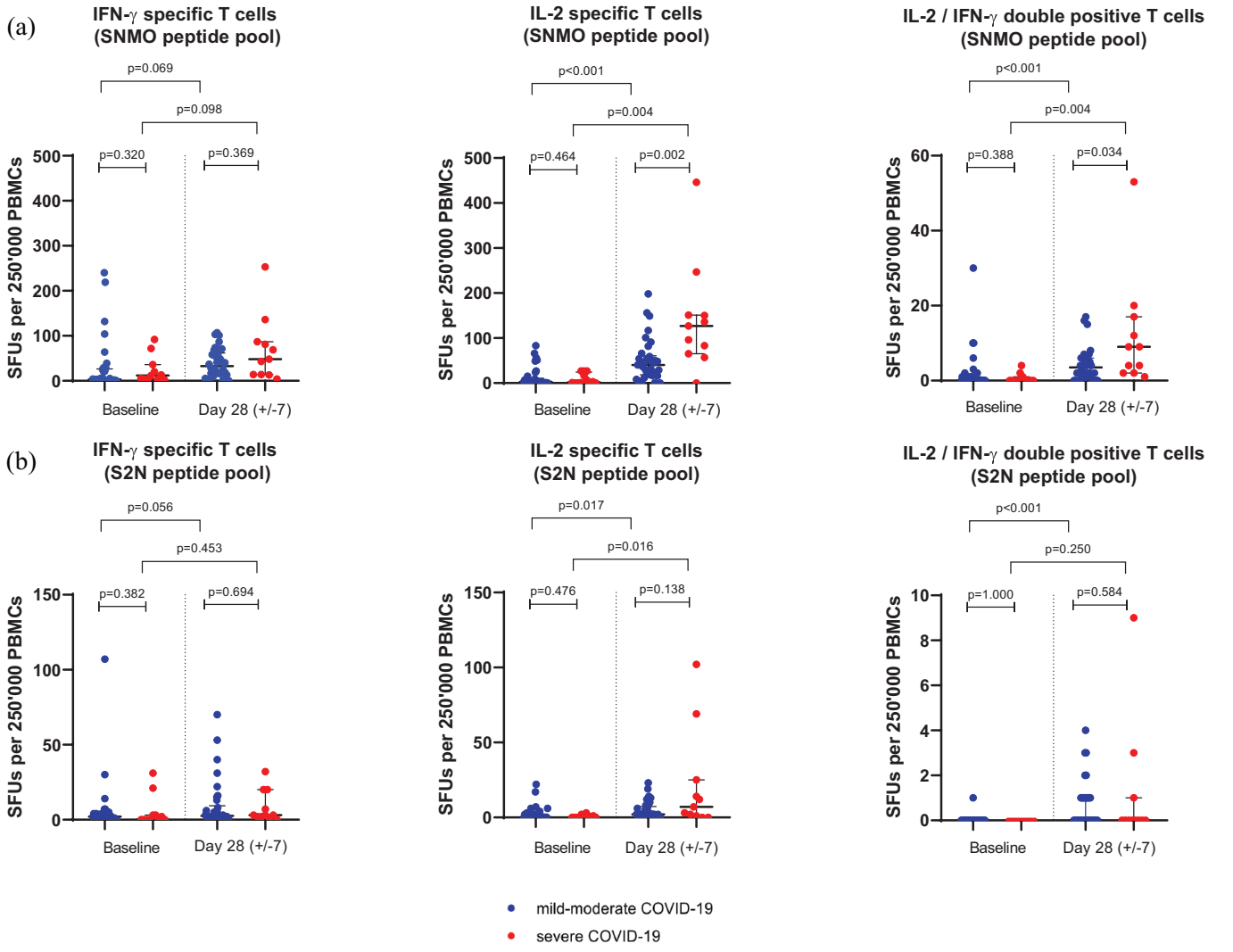

Figure 2. Cell-mediated immunity in relation to COVID-19 severity. (a) SFUs per 250,000 PBMCs upon stimulation with the SNMO peptide pool at baseline and day 28(±7) in patients with severe (red) and mild–moderate (blue) COVID-19. (b) SFUs per 250,000 PBMCs upon stimulation with the S2N peptide pool at baseline and day 28(±7) in patients with severe (red) and mild–moderate (blue) COVID-19. Black horizontal bars indicate median values. Whiskers indicate interquartile ranges.
COVID-19, coronavirus disease-2019; IFN-γ, interferon-gamma; IL-2, interleukin-2; PBMCs, peripheral blood mononuclear cells; SFUs, spot forming units; SNMO, spike protein, nucleocapsid protein, membrane protein, open reading frame proteins; S2N, spike-2 protein, nucleocapsid protein.

severe COVID-19 compared with those with mild–moderate disease (41.2 *versus* 13.4 pg/ml, $p < 0.001$; Figure 3). While in mild–moderate cases sNfL concentrations remained unchanged in follow-up samples (13.4 pg/ml, median difference of paired-sample sNfL concentration 0.8 pg/ml, $p = 0.317$), sNfL levels increased significantly in severe cases (165.5 pg/ml, median difference from baseline 130.0 pg/ml, $p = 0.002$; Figure 3). Both ΔsNfL and sNfL fold-changes were higher in severely ill COVID-19 patients (ΔsNfL – mild–moderate COVID-19: 0.8 pg/ml, severe COVID-19: 130 pg/ml, $p < 0.001$; sNfL fold-change – mild–moderate COVID-19: 1.0 fold; severe COVID-19: 4.3 fold, $p < 0.001$).

In adjusted analysis, sNfL levels at baseline were higher in severe COVID-19 patients compared with mild–moderate cases (adjusted mean 26.0 *versus* 15.8 pg/ml, $p = 0.037$). Similar to the unadjusted analysis, patients with severe COVID-19 had also higher sNfL levels compared with patients with mild–moderate disease at day 28(±7) (adjusted mean 26.3 *versus* 64.3 pg/ml, $p < 0.001$).

The results remained unchanged when excluding the patient who suffered a cerebral hemorrhage after the first and before the second serum sample was drawn (data not shown).

NfL dynamics and immune response

To further explore the association between neuro-axonal damage and the SARS-CoV-2-specific immune response, we correlated changes in sNfL (irrespective of COVID-19 disease severity scores) with cytokine levels, antibody concentrations, and the CMI response.

In unadjusted and adjusted analyses, IL-6, IL-8, and IL-1β concentrations at baseline significantly correlated with log10 sNfL fold-changes (Supplementary Figure 1A) and cube-root transformed ΔsNfL (Supplementary Figure 1B).

Increases in sNfL concentrations positively correlated with anti-spike IgG concentrations and neutralizing antibody titers in unadjusted and adjusted analyses (Figure 4(a) and (b)). There was a positive correlation for changes in sNfL concentrations and anti-nucleocapsid levels in the unadjusted analyses, but not in adjusted analyses (Figure 4(b)).

We did not observe significant correlations between CMI responses and sNfL changes (Supplementary Figure 2).

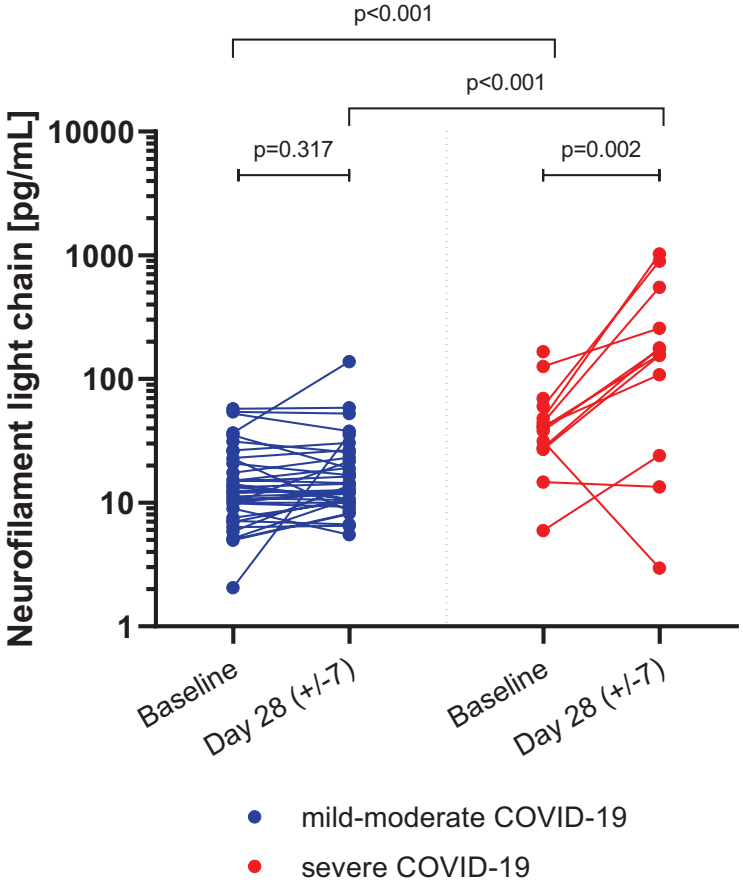

Figure 3. Serum neurofilament light chain concentrations (log10 transformed) in relation to COVID-19 severity (unadjusted analysis). COVID-19, coronavirus disease-2019.

Discussion

We examined the association of COVID-19 disease severity, cytokine levels, humoral and CMI response, and biochemical evidence for neuro-axonal injury. The major findings of our study were as follows: (a) We observed that elevated IL-6, IL-8, IL-1β, and TNF-α serum cytokine levels are a characteristic feature of severe COVID-19; (b) patients with severe COVID-19 elicit more pronounced anti-spike, anti-nucleocapsid, and neutralizing antibody responses; (c) some SARS-CoV-2-specific T-cell subsets are elevated in severely ill individuals; (d) severe COVID-19 is associated with subsequent neuronal injury as reflected by increased levels of sNfL; and (e) neuronal injury is not associated with inadequate SARS-CoV-2-specific humoral or CMI responses.

In accordance with previous findings, we observed elevated levels of pro-inflammatory cytokines in patients with severe COVID-19 compared with individuals with mild–moderate disease.[35,36] The degree of cytokinemia in our cohort was consistent with previous reports.[37] IL-6 levels of patients with severe COVID-19 lay within the reported range of individuals with severe bacterial pneumonia[38–40] but are markedly lower compared with patients with chimeric antigen receptor (CAR)-T-cell-induced cytokine release syndrome.[37]

Our findings corroborate the results of other groups, who reported elevated sNfL levels in patients with severe COVID-19 compared with patients with mild and moderate disease.[19,20,25] Our observations suggest effects of the systemic inflammatory response, as potential drivers of neuro-axonal injury in severe COVID-19. This hypothesis is also supported by the results of a recent study, which examined sNfL levels in patients with septic shock without primary CNS infection.[41] In this study, sNfL levels increased in

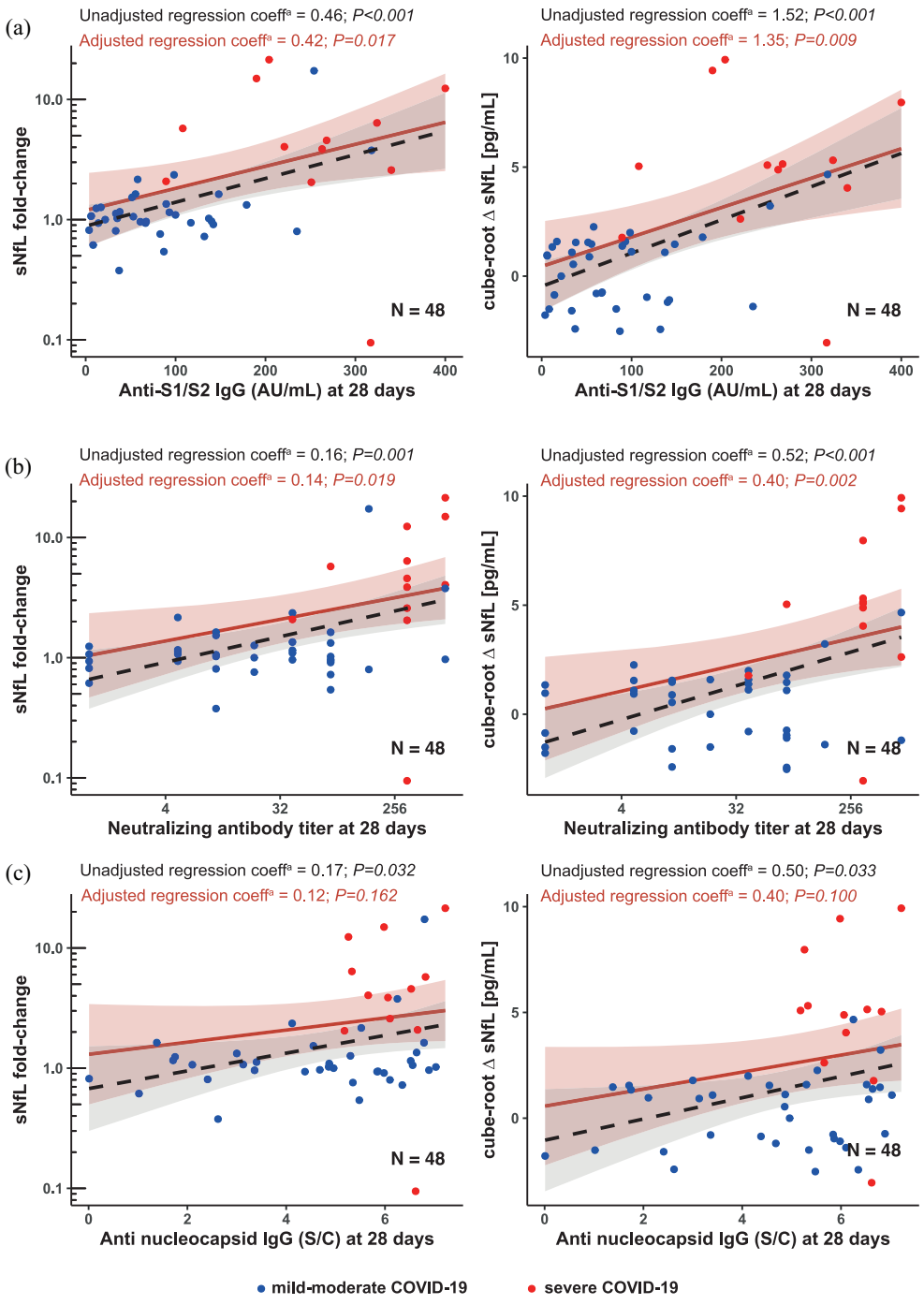

Figure 4. Unadjusted (black) and adjusted (red) associations between antibodies at day 28(±7) and changes in sNfL levels. (a) Changes in sNfL levels (log10 fold-change; cube-root transformed differences) in correlation to anti-spike IgG antibody concentration at day 28(±7). (b) Changes in sNfL levels (log10 fold-change; cube-root transformed differences) in correlation to neutralizing antibody titers at day 28(±7). (c) Changes in sNfL levels (log10 fold-change; cube-root transformed differences) in correlation to anti-nucleocapsid IgG antibody concentration at day 28(±7).

Dashed black line: regression line of the unadjusted linear regression model. Gray shade area: 95% confidence interval of the unadjusted linear regression model. Red solid line: regression line of the adjusted linear regression model. Red shade area: 95% confidence interval of the adjusted linear regression model. AU/ml, arbitrary units per milliliter; S/C, specimen/calibrator ratio; ΔsNfL, difference in serum neurofilament concentration at day 28(±7) to baseline; sNfL, serum neurofilament light chain.

[a]Regression coefficient per 100 AU/ml.

sepsis patients but remained stable in patients without sepsis.[41] It has been suggested that neurologic damage in sepsis develops along with an activation of the cerebral endothelium and an increase in the permeability of the blood–brain barrier (BBB).[42] Cytokines, which are known to increase the BBB permeability [e.g. IL-6,[43] IL-8,[44] interleukin-1b (IL-1b)[43]], are elevated in both severe SARS-CoV-2 infection and bacterial sepsis. Therefore, our findings might not be COVID-19 specific but rather reflect neuro-axonal injury seen in severely ill patients with systemic inflammation. However, without cerebrospinal fluid (CSF) analysis, it remains unclear to which extent sNfL levels originate from the CNS or from the peripheral nervous system as sNfL concentrations are also elevated in individuals with critical illness polyneuropathy.[45]

We used the ELLA platform (ProteinSimple) for quantification of sNfL concentrations in serum. The current reference method for sNfL quantification is the Single Molecular Array (Simoa, Quanterix Corp., Boston, MA, USA). However, both assays are using the same antibody for NfL detection, and a recent thorough validation study showed that the two platforms are equivalent.[46] We are therefore confident that our results can be reliably interpreted in the context of previous studies that used the Simoa technology for sNfL measurement in COVID-19 patients.[19–25]

Serum NfL shows distinct kinetics, with a delayed increase in serum levels and a peak 2 weeks after brain injury, followed by a slow decrease of serum concentrations for 3–9 months.[18] Based on the knowledge of sNfL dynamics, it is not surprising that the difference in sNfL levels among mild–moderate and severe COVID-19 patients was most evident in the follow-up serum sample after 28($\pm$7) days.

Severe COVID-19 was associated with higher antibody production and neutralizing titers in our cohort. This phenomenon has also been described in other cohorts.[35,47,48] One possible explanation for this finding could be that severe disease caused by hyper-inflammation or uncontrolled viral replication induces excess antibody production as surrogate marker of disease severity. This is supported by our finding that the group of severely ill COVID-19 patients had not only the highest neutralizing antibody titers but also the highest levels of pro-inflammatory cytokines. We observed a similar phenomenon for a subset of IL-2-specific and IL-2/IFN-γ double-positive T-cells (SNMO peptide pool stimulated only). There is conflicting evidence about the neuroinvasive capacity of SARS-CoV-2.[3,4,6–8,49] However, in mice, members of the betacoronavirus genus (MHV-JHM and MHV-A59) are clearly neurotropic and induce CNS infection.[10–12] In humans, humoral immune deficiencies have been associated with prolonged SARS-CoV-2 shedding and severe disease,[13–17] but the role of the SARS-CoV-2 antibody response with regard to neuronal injury in COVID-19 patients has not been investigated. In contrast to humans, the importance of the humoral and CMI response for clearance of betacoronaviruses (MHV-59 and MHV-JHM) from CNS in mice is well established.[10–12,50,51] A recent *in vitro* study showed that anti-viral antibodies from human CSF block SARS-CoV-2 infection of human brain organoids.[49] We therefore hypothesized that low neutralizing antibody titers or decreased CMI response might contribute to neuro-axonal injury in COVID-19. However, when correlating the humoral or cell-mediated SARS-CoV-2-specific immune response with sNfL increases over time, we did not find evidence to support this hypothesis.

A unique aspect of our study was the prospective design with collection of paired samples at uniform time points, which allows to analyze the dynamic change of sNfL concentration in the development of the disease. Here, we demonstrate for the first time an association between the systemic inflammatory response in severe COVID-19 and neuronal injury, as postulated previously.[19] Our cohort covered the whole spectrum of COVID-19 disease severity, from outpatients to mechanically ventilated patients on the ICU. Since sNfL levels highly depend on age,[18] which is also a risk factor for severe COVID-19, our study design allows to analyze the dynamic change of sNfL concentration, which is most likely age independent. In addition, we performed adjusted analyses corrected for age, time from symptom onset to sampling, and use of immunomodulatory drugs. One strength of our study includes the measurement of neutralizing antibody titers by using an authentic SARS-CoV-2 isolate for serum neutralization assays instead of using pseudovirus-based neutralization assays or solely ELISA-based methods. In contrast to surrogate methods, this approach ensures that neutralizing capacities of antibodies represent real

findings. Our study has important limitations that require discussion. Systematic clinical neurological and/or neurocognitive evaluation was not performed due to resource limitations and restrictions associated with the pandemic. Therefore, we cannot provide data on the correlation of sNfL levels and post-illness neurocognitive disorders. In addition, we did not systematically carry out neuroradiological imaging of our patients to correlate radiologic findings with sNfL measurements. Also, in this observational study, we did not perform lumbar punctures and cannot provide data on CNS inflammatory parameters or evidence for viral CNS invasion. Therefore, these aspects will have to be included in follow-up studies to assess their role in the association of neuro-axonal damage. Furthermore, we acknowledge that this is a small cohort. Including an independent validation cohort and a comparison group of healthy volunteers (without COVID-19) would have strengthened our findings.

In summary, we provide novel information indicating that systemic inflammation in severe COVID-19 disease is associated with ensuing neuro-axonal damage. Patients with more severe disease developed higher neutralizing antibody titers and higher counts of SARS-CoV-2-specific T cells during the course of COVID-19 disease. Mounting a pronounced virus-specific humoral and CMI response upon SARS-CoV-2 infection did not protect from neuro-axonal damage as by the means of sNfL levels.

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Ronald Dijkman: Investigation; Methodology; Writing – review & editing.

Stephen L. Leib: Conceptualization; Supervision; Writing – review & editing.

Conflict of interest statement

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Supplemental material

Supplemental material for this article is available online.

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