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Plasma biomarkers of brain injury in COVID-19 patients with neurological symptoms

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ABSTRACT

Objective: Neurological symptoms (NS) were often reported in COVID-19 infection. We examined the plasma levels of glial fibrillary acidic protein (GFAP) and S100B together, as brain injury biomarkers, in relation to persistent NS in a cohort of patients with COVID-19 during the acute phase of the disease.
Methods: A total of 20 healthy controls and 58 patients with confirmed COVID-19 were enrolled in this prospective study. Serum GFAP and S100B levels were measured by using enzymle linked immunoassay method from blood samples.
Results: Serum GFAP levels were found to be significantly higher in the severe group than in the controls ($p = 0.007$). However, serum S100B levels were similar between control and disease groups ($p > 0.05$). No significant results for GFAP and S100B were obtained between the disease groups depending on whether the sampling time was below or above 5 days ($p > 0.05$). We did not find a correlation between serum GFAP and S100B levels and the presence of NS ($p > 0.05$). However, serum S100B levels were slightly higher in patients with multiple NS than in those with a single symptom ($p = 0.044$).
Conclusions: Elevated GFAP was associated with disease severity but not with NS in COVID-19 patients. Whereas, high serum S100B was associated with the multipl NS in these patients. Our data suggest that GFAP and S100B may be of limited value currently in order to represent the neuronal damage, though serving a basis for the future work.

1. Introduction

The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is known to have a neuroinvasive potential [1,2]. Possible mechanisms for central nervous system (CNS) involvement have been proposed as direct effect of the virus or indirect effects of systemic infammation due to immune activation or hypoxia [3–5]. Accumulating data have described various neurological manifestations in coronavirus disease 2019 (COVID-19) [3,6–8]. Headache, ageusia, anosmia and dizziness were the most common neurological symptoms (NS) as a presentation of neuronal injury in SARS-CoV-2 infected patients [6,9–11]. It is still curious whether an increase in NS accompanied by an increase in neuronal injury markers as well. In contrast to cerebrospinal fluid (CSF) assessment [12,13], measurement of these markers in the plasma may provide a practical method of assessing brain injury during the acute phase of infection in COVID-19 [5,14–17].

Glial fibrillary acidic protein (GFAP) is a cytoskeleton glial protein mainly expressed in astrocytes which regulates the morphology and function of these cells in the CNS [18,19]. Serum GFAP levels are very low in healthy individuals, but increased GFAP levels due to astrocyte disintegration are known to indicate the neuronal damage [18,20] Therefore, GFAP is increasingly used as a serum biomarker of astroglial activation/injury [20,21]. Recent studies have shown increased levels of GFAP in COVID-19 patients and these levels were correlated with disease severity [14,15,22,23]. On the other hand, S100B protein is a cytosolic calcium binding biomarker, originally isolated in the CNS, where it is mainly expressed in astrocytes as well as in adipocytes [24,25]. S100B acts on dynamics of energy metabolism and calcium homeostasis, thus when released from damaged cells, extracellular signals could be activated by S100B [26,27]. Accordingly, S100B is

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considered to be involved in infammatory processes as a danger-associated molecular patterns molecule [27]. In certain conditions, S100B can be detected in biological fuids such as CSF, blood, urine, etc. [24]. Similar to GFAP, increased plasma levels of S100B were reported in the acute phase of COVID-19 patients [5,17,28]. However, data is limited regarding the serum levels of both GFAP [29] and S100B [30] together in the context of neurological signs of CNS dysfunction in SARS-CoV-2 infected patients.

In the present study, we have prospectively examined the relationship between the levels of plasma biomarkers of CNS injury (GFAP and S100B) and persistent NS in a cohort of patients with mild, moderate, or severe COVID-19 during the acute phase of the disease.

2. Methods

2.1. Patients and study design

This prospective single-center study included 78 patients, consecutively recruited from Kirsehir Ahi Evran University Hospital, Kirsehir, Turkey, from April 2021 up to October 2021. Study group comprised 20 healthy volunteers with no symptoms of COVID-19 and 58 patients with confirmed COVID-19 infection, who were then divided into 3 groups related to disease severity as 17 patients with mild (not requiring hospitalization), 18 with moderate (hospitalized and requiring oxygen supplementation), and 23 with severe (admitted to the intensive care unit) disease [31]. The demographic questionnaire included age, gender, body mass index and self-reported comorbid conditions for all pariticipants. Patients were neurologically evaluated by experienced neurologist as to have COVID-19-related NS (headache, ageusia, anosmia, vertigo, peripheral neuropathy, cranial nerve affection or cognitive deficits) experienced during the acute infection and onset date of these symptoms. The patients who had at least one of these new-onset NS were included in this study. Patients below age 18 and those who had chronic inflammatory disease; kidney, cardiac and liver failure; malignancy; pregnancy or documented neurologic and psychiatric disorders; as well as, those who were inability to complete the questionnaire were excluded.

The diagnosis of SARS-CoV-2 infection was confirmed with real-time reverse transcription-polymerase chain reaction analysis of nasal and throat swab specimens as previously reported [32]. Peripheral blood samples were collected during the acute phase of the COVID-19 infection; up to a median (min-max) of 5 days (0−13).

Informed consent was obtained from each participant. This study was approved by the Kirsehir Ahi Evran University Ethical Commitee (approval date 09/02/2021; approval number 2021–03/29).

2.2. Biomarker analyses

Blood samples were collected with gel flat serum tube of 5 mL (Becton Dickinson company) and centrifuged within 1 h at 2000*g* for 10 min at room temperature. Serum samples were immediately stored at −80 °C until analysis. Serum GFAP and S100B levels were measured using the Human GFAP ELISA kit (Elabscience, USA) and the Human S100B ELISA kit (Elabscience, USA) according to the manufacturer's protocols. Serum GFAP and S100B measurements were performed in the Clinical Neurochemistry Laboratory at the Kirsehir Ahi Evran University Hospital by using SPECTROstar Nano microplate reader (BMG LABTECH, Germany) analyzer. The levels of both biomarkers are reported as pg/mL.

2.3. Statistical analysis

Histogram and q–q plots were examined and Shapiro–Wilk's test was performed to assess the data normality. The Levene's test was used to assess the variance homogeneity. One Way Analysis of Variance (ANOVA)-Welch Test, Kruskal-Wallis Test or Mann-Whitney *U* Test were performed depending on the normality of the quantitive data. Tamhane's *t*-Test or Dunn Test were utilized as post-hoc tests for pairwise comparisons. Box-Plot graphs were given for parameters that has significant difference across groups. Cohen's d and Partial Eta Squared (η2) statistics were calculated to report effect sizes. One-way analysis of covariance (ANCOVA) was applied to compare the effect of headache symptom groups on biomarker values after adjusting age and BMI variables. Similarly, two-way ANCOVA was utilized to compare the headache and COVID severity groups on biomarker values after adjusting age and BMI values. Receiver operating characteristic (ROC) curve analysis was applied for GFAP. In this regard, Youden Index (YI) values were calculated to determine cut-off values for GFAP in discriminating severe COVID-19 patients and healthy individuals. The area under curve (AUC) measure was calculated with 95% confidence interval (CI) for GFAP. Values are expressed as frequencies (*n*) and percentages (%), means and standard deviations (SD), or medians (minimum and maximum). Two-sided *p*-values <0.05 were considered statistically significant. Analyses were performed using SPSS v.21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.)

Table 1
Demographic and laboratory data ($n = 78$).

Variables	Control (n = 20)	Mild (n = 17)	Moderate (n = 18)	Severe (n = 23)	p	η^{2***}
Age (years)	41.7 ± 12.72	34.59 ± 8.94	39.94 ± 9.88	45.39 ± 7.47	**0.003****	**0.139**
Gender (female)	10(50)	9 (52.9)	8(44.4)	13 (56.5)	0.891	
Body mass index (kg/m^2)	27.95 ± 4.52	26.07 ± 3.639	28.48 ± 5.63	30.64 ± 4.83	**0.029***	**0.114**
Comordities						
Diabetes mellitus	3(15)	1(5.9)	1(5.6)	4 (17.4)	0.538	–
Hypertension	2(10)	0(0)	1(5.6)	3(13)	0.456	–
Coronary artery disease	1(50)	0(0)	0(0)	1(50)	0.639	–
Others	2(10)	1(5.9)	1(5.6)	3(13)	0.814	–
Neurological symptoms						
Headache	–	10 (58.8)	17(94.4)	20(87)	**0.018**	–
Ageusia	–	11 (64.7)	13(72.2)	18 (78.3)	0.638	–
Anosmia	–	11 (64.7)	11(61.1)	14 (60.9)	0.965	–
Vertigo	–	1(5.9)	5(27.8)	6 (26.1)	0.199	–
Peripheral neuropathy	–	0(0)	0(0)	1(100)	0.384	–
Cranial nerve affection	–	0(0)	0(0)	1(100)	0.585	–
Cognitive deficits	–	0(0)	0(0)	1(100)	0.338	–
Biomarkers						
GFAP (pg/mL)	43.74 ± 23.40	63.72 ± 78.89	83.19 ± 57.70	86.43 ± 63.17	**0.007****	**0.086**
S100B (pg/mL)	21.26 ± 10.40	17.40 ± 13.82	16.97 ± 8.02	14.24 ± 7.18	0.158	0.067

Values are expressed as *n*(%) or mean ± SD. GFAP indicates glial fibrillary acidic protein.
** Bonferroni.
* Tamhane's T.
*** : Partial Eta Squared.

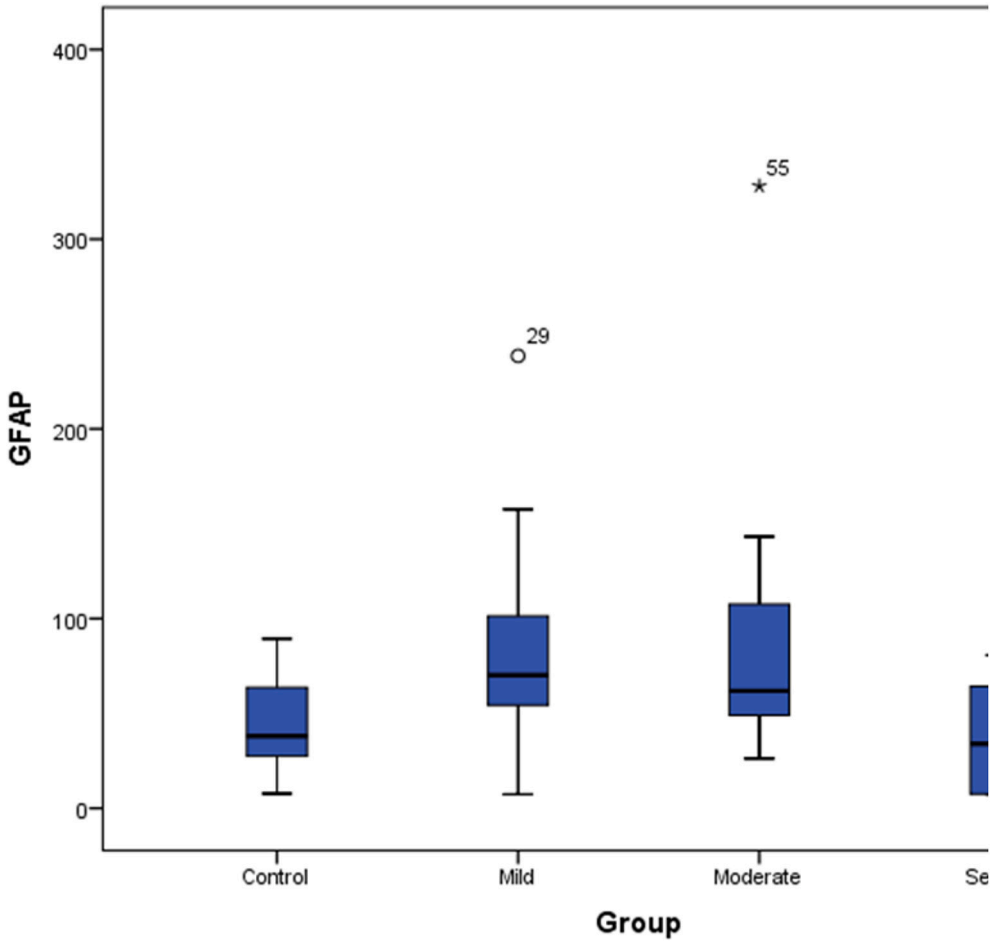

Fig. 1. Box-plot for glial fibrillary acidic protein (GFAP) between the groups.

Table 2
Serum levels of GFAP and S100B regarding sampling time and neurological symptoms.

Variables		GFAP				S100B			
		<5 days ($n = 27$)	≥5 days ($n = 31$)	p	Effect Size	<5 days ($n = 27$)	≥5 days ($n = 31$)	p	Effect Size
Sampling time (day)	**Mild** ($n = 17$)	38.39(7.23–249.09)	33.92(7.16–161.14)	0.813	0.316*	9.52(8.41–23.05)	16.88 (8.57–54.60)	0.193	0.837*
	Moderate ($n = 18$)	64.282(7.35–157.63)	82.99 (10.71–238.44)	0.315	0.538*	12.69 (8.09–30.88)	17.39 (9.36–36.66)	0.315	0.410*
	Severe ($n = 23$)	61.912 (26.23–328.01)	59.18 (39.58–143.25)	0.829	0.311*	12.85 (8.57–24.29)	12.29 (8.88–37.33)	0.999	0.244*
Neurological symptoms (number)	**One** ($n = 11$)	57.426(7.46–238.443)		0.853	0.112**	10.15(8.72–48.86)		**0.044**	0.036**
	Two ($n = 16$)	64.26(7.23–249.09)				12.61(8.57–31.08)			
	Three ($n = 12$)	72.93(7.16–157.63)				19.21(9.52–37.33)			
	Four ($n = 16$)	62.26(10.71–328.01)				10.94(8.41–54.60)			
	Five ($n = 3$)	54.29(7.35–59.07)				18.95(8.09–24.29)			

Values are expressed as median(min-max). GFAP indicates glial fibrillary acidic protein.
* Cohen's d.
** Partial Eta Squared (η^2).

3. Result

A total of 78 patients were included in the present study. The demographic and laboratory data were summarized in Table 1. There were no significant differences between the groups in sex or comorbidities ($p > 0.05$). However, advanced age ($p = 0.003$, $\eta 2 = 0.139$) and increased BMI ($p = 0.029$, $\eta 2 = 0.114$) were significantly more frequent in the severe group than in the controls. Among the NS, headache was significantly higher in the severe group than in non-severe groups ($p = 0.018$). Regarding biomarkers, serum GFAP levels were found to be significantly higher in the severe group than in the controls, yielding medium effect size across groups ($p = 0.007$; $\eta 2 = 0.086$; Fig. 1). However, serum

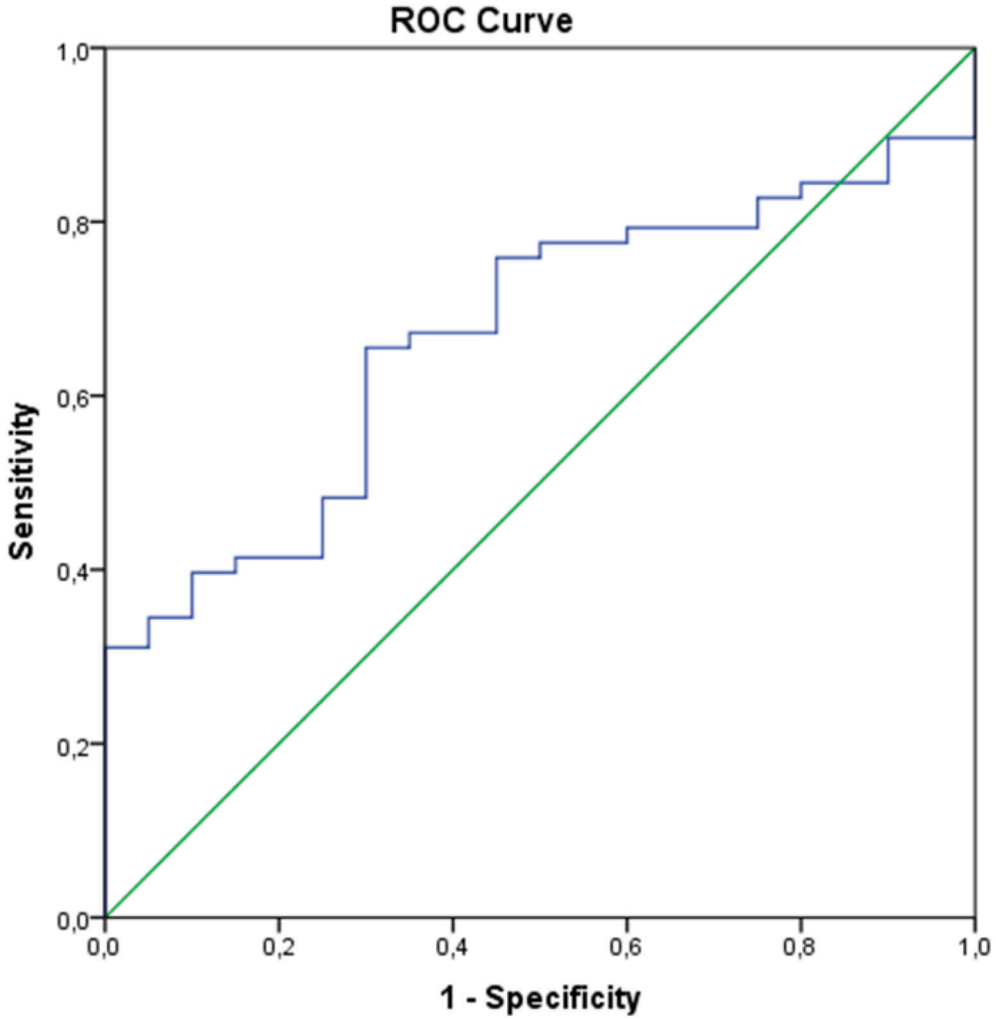

Fig. 2. Receiving operating characteristic (ROC) analysis for GFAP.

S100B levels were similar between control and disease groups ($p > 0.05$).

Serum levels of GFAP and S100B according to the sampling time and NS were shown in Table 2. Regarding sampling time point, no significant results for GFAP and S100B were obtained between the disease groups depending on whether the sampling time was below or above 5 days ($p > 0.05$). We did not find a correlation between serum GFAP and S100B levels and the presence of NS ($p > 0.05$). However, serum S100B levels were slightly higher in patients with multiple NS than in those with a single symptom ($p = 0.044$; $\eta 2 = 0.036$). Whereas serum GFAP levels were similar in this respect ($p > 0.05$).

ROC analysis was applied for GFAP. AUC value was found as 0.67 (0.54–0.79) with a cut-off value of 51.05 for GFAP was obtained to predict the clinical severity in COVID-19 patients with a sensitivity and specificity of 65.5, and 70.0%, respectively in the ROC analysis (Fig. 2).

One-way ANCOVA analysis results revealed that no statistically significant difference in GFAP was observed between headache status groups ($p = 0.573$, $\eta 2 = 0.006$) after controlling age and BMI. Similarly, S100B values did not statistically significantly differ across headache groups ($p = 0.711$, $\eta 2 = 0.003$) after adjusting age and BMI. On the other hand, two-way ANCOVA results have showed that neither GFAP ($p = 0.504$, $\eta 2 = 0.009$ for headache groups; $p = 0.841$, $\eta 2 = 0.007$ for COVID severity groups) nor S100B ($p = 0.417$, $\eta 2 = 0.013$ for headache groups; $p = 0.172$, $\eta 2 = 0.065$ for COVID severity groups) values statistically differ across groups after controlling age and BMI (Table 3).

4. Discussion

In this cross-sectional study, we investigated the levels of astroglial activation/injury markers of circulating GFAP and S100B in serum of COVID-19 patients with NS at various stages of the disease. Four main findings has emerged from the present study. First, advanced age and increased BMI were significantly more frequent in the severe group than in the controls. Second, serum GFAP levels were significantly higher in the severe group than in the controls. Third, serum S100B levels were slightly higher in patients with multiple NS. Fourth, a cut-off value of 51.05 for GFAP level with a sensitivity of 65.5% and a specificity of 70.0% was obtained in the ROC analysis.

Injury to the nervous system has been markedly reported in COVID-19. The SARS-CoV-2 virus attacks the brain with a wide range of NS, some of which were headache, ageusia, anosmia, dizziness [6,9–11]. Several mechanisms likely contribute to CNS involvement in COVID-19. These includes direct viral invasion as retrograde neuronal route and, indirect effects of systemic infammation due to immune activation or hypoxia as hematogenous route [3–5]. There is an increasing evidence that neuronal injury is mediated primarily via hyperinflammation [5]. The virus enters the endothelial cells of the blood-brain barrier (BBB) by binding to the angiotensin converting enzyme 2 receptor which damages the endothelial tight junctions. With this, many CSF studies showing absence of viral RNA suggest that the excessive systemic inflammatory response triggered by the viral infection rather than the virus itself, may disturb these junctions [5,30]. Finally, BBB breakdown facilitates the

Table 3
Adjusted and observed means and descriptive statistics across groups.

Biomarker*	Headache	Mean (Adjusted)	Std Error (SE)	Mean ± SD
GFAP	Yes	76.397	9.461	77.13 ± 64.282
	No	88.888	19.746	85.74 ± 76.676
S100B	Yes	16.254	1.453	16.28 ± 10.323
	No	14.994	3.033	14.9 ± 6.975

Biomarker**	Headache	COVID Group	Mean (Adjusted)	Std Error (SE)	**Mean ± SD**
GFAP	Yes	Mild	70.945	20.381	55.58 ± 70.726
		Moderate	83.46	15.558	84.71 ± 59.106
		Severe	73.103	15.065	81.48 ± 66.161
	No	Mild	87.196	22.1	75.35 ± 93.942
		Moderate	99.71	27.506	57.37***
		Severe	89.353	26.139	119.44 ± 20.895
S100B	Yes	Mild	20.357	3.037	21.46 ± 17.032
		Moderate	17.283	2.318	16.52 ± 8.032
		Severe	13.221	2.245	13.48 ± 6.621
	No	Mild	17.413	3.293	11.61 ± 2.816
		Moderate	14.339	4.098	24.57***
		Severe	10.277	3.895	19.35 ± 10.258

* One-way ANCOVA.
** Two-way ANCOVA.
*** Standard deviation can not be calculated since the sample size of the group is 1.

entry of SARS-CoV-2 into the brain tissue, possibly causing neuroinflammation in COVID-19 [33,34]. There is a growing evidence of the role for astrocytes in this inflammatory milieu prior to neuronal and astrocytic injury [12,29]. It has been suggested that these cells might be targets of SARS-CoV-2 [35] and play a key role in the control of the overexpression of inflammatory mediators [36,37]. Astrocytes release numerous neurotrophic and gliotrophic factors that play a role in CNS homeostasis [19]. Upon extensive cytokine activation, they release neurotoxic substances and increase the expression of GFAP which passes from the intracellular compartment into the blood stream [19]. GFAP is a cytoskeletal protein within glial cells and is expressed mainly by astrocytes in the CNS [18,19]. Several studies have shown that serum GFAP levels increase in different neurological conditions [38], as well in COVID-19 [14,15,22,23]. Kanberg et al. found high plasma levels of GFAP in moderate and severe stages of COVID-19 patients [14]. In another study by Cooper et al. plasma GFAP was two-fold higher in critically ill patients with COVID-19 with respect to controls [22]. They both suggested that GFAP concentrations were correlated with disease severity [14,22]. Similarly, recent studies by De Lorenzo et al. [23] and Aamodt et al. [15] reported that high blood GFAP levels are related to fatal outcomes. Despite these findings, data is scarce on the relation of biomarkers with the NS in COVID-19 patients. There is a single study noticeable in the literature, demonstrating increased plasma GFAP levels, parallel with CSF levels, in these patients but not comparing with the healthy population [29]. In the present sudy, we included COVID-19 patients whom all have at least one or more NS and compared with a control group in order to clarify the role of GFAP as a sign for astrocytic damage presenting with neurological deterioration. We found higher plasma GFAP levels in severe COVID-19 patients when compared to the controls, despite the lack of significance across the disease groups, which might be attributable to the small size. ROC analysis indicated greater AUC for serum GFAP levels (AUC = 0.663, 95% CI = 0.545–0.791). Serum GFAP concentration was measured as 51.05 pg/mL at 65.5% sensitivity and 70.0% specificity ($p = 0.026$) in our study. However, we did not find a correlation between serum GFAP levels and the presence of NS. We also detected similar GFAP levels in these patients with either a single or multipl NS. Taken altogether, we may speculate that increased GFAP can distinguish severe stages from healthy individuals, but high levels seem to be triggered by the viral infection rather than the neuronal injury.

S100B is an another protein known to participate in infammatory processes [26]. Previously, serum S100B levels have been shown to be elevated especially in traumatic brain injuries [39] and ischemic strokes [40], and these high levels have also been associated with poor outcomes. S100B is a calcium-binding protein localized in the astrocytic cytoplasm and, some in adipose tissue [24,25]. The raised plasma levels of S100B as an astrocytic injury marker have rarely been explored in relation to COVID-19 [5,17,28]. Mete et al. showed significantly higher S100B levels in patients with COVID-19 pneumonia with severe disease than in the controls [28]. Similarly, Aceti et al. found that serum S100B significantly correlated with clinical severity in COVID-19 patients, as in association with inflammatory markers of ferritin, C-reactive protein, procalcitonin, etc. [17]. Additionally, Savarraj et al. in his recent study, investigated brain and endothelial injury markers and found the significantly increased plasma levels of S100B in the acute phase of COVID-19 cohort compared to controls with no difference across the clinical severity [5]. They explained this lack of difference as an effect of the time-point sampling [5]. However, the relation between S100B and the neurological involvement in SARS-CoV-2 infected patients is limited. There is a single study being published on the overexpression of S100B protein in patients with neurological dysfunction [30]. Perrin et al. in his longitudinal study, included five patients with COVID-19, presenting with NS, some were confusion, tremor, cerebellar ataxia, aphasia, coma, and cranial nerve palsy [30]. While SARS-CoV-2 was undetectable in the CSF, serum S100B levels were increased at the time of cytokine release syndrome, reflecting an increased BBB permeability [24], and returned to their reference range when NS and signs of hyperinflammation regressed [30]. They linked this to the neuroinflammatory response accompanied by reactive gliosis and release of S100B protein [30]. On the contrary, we did not find any significant difference for serum S100B levels between the groups. Several factors can be considered. Patients were included at different time points because of the different admission times, which may have affected the results [5]. In fact, depending on the time-point at which the samples were drawn below or above 5 days, we did find similar results for GFAP and S100B levels; which means we excluded possible confounding effect of kinetics of these proteins [29]. Also, the samples were quickly put at -80 °C as soon as were taken since S100B has a short half-life of 30 min in serum [41]. Though not useful in discriminating different stages of COVID-19 infection, serum S100B levels were found to be slightly higher in patients with multiple NS -perhaps indicating the more injury- than in those with a single symptom. This may suggest a role for S100B to act together with severe neuronal injury rather than milder forms. Doubtless larger cohorts are needed to ascertain the contribution of S100B in underlying mechanisms of neurological manifestations in COVID-19 patients.

The present study has some limitations. First, it included a limited number of participants. Second, it is cross-sectional and thus can not determine causality. Third, we used a single S100B measurement at a single time point, whereas repeated measurements during the clinical follow-up may provide a more accurate assessment of the cerebral milieu to reflect the actual patient status. Fourth, neuroimaging and electrophysiological studies are lacking due to infection control measures, in which some neurological signs may be overlooked. Fifth, cognitive symptoms were self-reported and not confirmed by standardized cognitive tests.

5. Conclusions

Based on the present findings, elevated GFAP was associated with disease severity regardless of accompanied NS in COVID-19 patients. Whereas, serum S100B levels were similar between the groups, however it may have a minor role in COVID-19 patients with multipl NS. Taken together, GFAP and S100B may be of limited value currently in order to represent the neuronal damage, though serving a basis for the future work. More studies in larger samples are required to explore and understand the genesis of CNS injury and evaluate the usefulness of these biomarkers in patients with COVID-19.

Declaration of Competing Interest

None.

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