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The Association Between Serum Levels of Glial Biomarkers, Clinical Severity and Electro-encephalography Features in Idiopathic West and Lennox-Gastaut Syndromes

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ABSTRACT

Introduction: Although the contribution of enhanced glial activity in seizure induction is increasingly recognized, the role of glia-induced neuroinflammation in the physiopathology of epileptic encephalopathy (EE) has been scarcely investigated.

Methods: To delineate the contribution of glial activity in EE, we measured levels of glia-derived mediators with previously described biomarker value, including glial fibrillary acidic protein (GFAP), high mobility group box 1 (HMGB1), chitinase-3-like protein 1 (CHI3L1), soluble CD163 (sCD163) and triggering receptor expressed on myeloid cells 2 (TREM2) by ELISA in sera of patients with idiopathic West syndrome (WS, n=18), idiopathic Lennox-Gastaut syndrome (LGS, n=13) and healthy controls (n=31).

Results: Patients with EE showed significantly higher CHI3L1 levels compared to healthy controls. Levels of HMGB1, CHI3L1, sCD163 and TREM2 were higher in LGS patients than WS patients and/or healthy controls. One or more of the investigated mediators were associated with treatment responsiveness, disease severity and presence of pathological features on electroencephalography (EEG).

Conclusions: To our knowledge, our findings provide the initial patient-based evidence that astrocyte- and microglia-mediated neuroinflammation might be involved in the pathogenesis of LGS and WS. Moreover, glial mediators may serve as prognostic biomarkers in patients with idiopathic EE.

Keywords: Chitinase-3-like protein 1, epileptic encephalopathy, glia, Lennox-Gastaut syndrome, neuroinflammation, West syndrome

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INTRODUCTION

Epilepsy is a chronic central nervous system (CNS) disease characterized by repeated seizures. Ion channel dysfunction and increased neuronal excitability are believed to function as major factors in epilepsy. Nevertheless, the exact pathogenic mechanisms of epilepsy are unknown and anti-epileptic medications aimed to mitigate increased neuronal excitability fail to prevent seizures in a sizeable number of epilepsy patients (1). Glial cells, particularly astrocytes and microglia, have been increasingly recognized as major contributors to enhanced neuronal excitability in the pathophysiology of epilepsy (2). At the same time, repeated seizures have been shown to trigger glial activation and lead to the release of glia-derived pro-inflammatory mediators forming a vicious positive feedback loop and establishing a suitable microenvironment for continuation of epileptic seizures (3).

Highlights

- **Patients with EE showed significantly higher CHI3L1 levels compared to healthy controls.**
- **Levels of HMGB1, CHI3L1, sCD163 and TREM2 were higher in LGS patients.**
- **Findings are the first evidence of astrocytic/microglial neuroinflammation in EE.**
- **Glial mediators may serve as prognostic biomarkers in patients with idiopathic EE.**

Some of the major neuroinflammatory glial pathways involved in epilepsy are nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and mitogen-activated protein kinase (MAPK) signaling pathways activated through the interaction between toll-like receptors and high mobility group box 1 (HMGB1), inflammasome pathways, tumor necrosis factor-α (TNF-α)-TNF receptor 1 signaling pathway and various chemokine signaling pathways (3). Pro-inflammatory mediators released by glial cells interfere with glial functions thereby reducing the uptake of glutamate and the buffering of excess potassium released by firing of neurons, alter synaptic release of neurotransmitters and expression of

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neurotransmitter receptors and disrupt the blood-brain barrier allowing the access of additional inflammatory mediators from the peripheral blood into the brain parenchyma (4).

Epileptic encephalopathy (EE) comprises a group of relatively immunotherapy-responsive disorders, in which continuous epileptic seizures lead to progressive cerebral dysfunction, motor-mental retardation and behavioral abnormalities. West syndrome (WS) and Lennox-Gastaut syndrome (LGS) are among the most frequent EE syndromes in clinical practice (5). Based on the above-mentioned significant contribution of glia-derived mediators to seizure induction, neuroinflammation and neuronal dysfunction, it is reasonable to assume that glial cells participate in the pathophysiology of EE syndromes. Nevertheless, little is known about the involvement of glial cells in EE and most of our knowledge on this issue is limited to immunohistochemistry studies showing enhanced glial activation in the CNS of idiopathic WS and LGS patients (6,7).

In this study, to provide evidence regarding the involvement of glial cells and glia-induced neuroinflammation in EE pathophysiology and to assess the prognostic value of glial biomarkers in EE, we aimed to screen serum levels of a panel of glia-derived mediators with previously reported biomarker value in patients with idiopathic WS and LGS. For this purpose, we selected glial fibrillary acidic protein (GFAP), HMGB1, chitinase-3-like protein 1 (CHI3L1, also known as YKL-40), soluble CD163 (sCD163) and triggering receptor expressed on myeloid cells 2 (TREM2) due to their enhanced expression by astrocytes and/or microglia during neuroinflammation (8–12) and due to their previously demonstrated biomarker values in several CNS disorders characterized by neuroinflammation (13–18).

METHODS

Participants

Eighteen consecutive idiopathic WS patients, 13 consecutive idiopathic LGS patients who had been diagnosed with their typical clinical and EEG findings as per relevant criteria (19,20) and 31 age/gender-matched healthy controls were included (Table 1). Healthy controls consisted of children who came in for a health examination. During enrollment, all patients underwent peripheral blood sampling, EEG examination and cranial magnetic resonance imaging (MRI) (done with a 1.5 Tesla scanner). Recent EEG and MRI examinations were used for some patients, and for the rest, old records were used. Patients with symptomatic causes of encephalopathy (e.g. brain ischemia/hemorrhage, tumor, malformations, migration defects, cortical dysplasia and traumatic brain injury) and accompanying infectious, autoimmune or inflammatory disorders were excluded. To exclude additional metabolic causes of EE, a detailed routine investigation scheme was utilized for all patients and included investigations of biotidinase deficiency, pyridoxine deficiency, mitochondrial disorders, glycosylation defects, phenylketonuria and other aminoaciduria, peroxisomal and lysosomal disorders for differential diagnosis. Patients with neurocutaneous syndromes (e.g. tuberous sclerosis) were also excluded. A genetic screening failed to display commonly found genetic mutations in the included EE patients. Only patients with mild periventricular leukomalacia were not excluded. None of the patients had a remarkable perinatal, past or family medical history. All patients were under anti-epileptic treatment (ranging between 1–3 drugs) and 26 patients had received immunotherapy (adrenocorticotrophin hormone or steroids) during their disease course. None of the enrolled patients had received immunotherapy within the last three months of blood sampling. Favorable response to treatment was considered as >50% decline in seizure frequency when compared to baseline monthly seizure frequency. Seizure freedom was evaluated based on the International League Against Epilepsy classification, which is as follows: 1- Complete freedom from seizures (for at least one year), including auras / or 2- More than 50% reduction from baseline seizure days (at least for one year), which may or may not include auras (21). Prognosis during enrollment was determined as per Gross Motor Function, Manual Ability and Communication Function Classification Systems (22) and denoted on a scale between Level I-V. Background activity, spike-wave discharges and burst-suppression pattern were recorded during EEG examination.

The study was approved by Istanbul University, Istanbul Faculty of Medicine the institutional review board (date and number: 18/12/2020–257365) and written informed consent was obtained from all patients or their designated proxy. The study was conducted as per the regulations of Helsinki declaration.

ELISA

Peripheral blood samples of all participants were collected in the morning between 8–10 AM, sera were isolated and stored at -80°C until use. Serum levels of GFAP, HMGB1, sCD163 (Elabscience, Houston, TX, USA), CHI3L1 (R&D Systems, Minneapolis, MN, USA) and TREM2 (Cusabio, Houston, TX, USA) were measured by ELISA according to manufacturer's guidelines.

Statistical Analysis

Categorical variables were compared using the chi-square test. Following normallity evaluation of the continous variables for two-group comparisons Mann-Whitney U test was used. For multiple group comparisons, the non-parametric Kruskal-Wallis test was utilized together with Dunn's post-hoc analysis. Correlation statistics were conducted with Spearman's test. Statistical significance was defined as a p-value less than 0.05.

RESULTS

Clinical and Demographic Data

There were no significant differences among patients with EE (8.4±5.8 year-old) and healthy controls (9.1±4.7 year-old) in terms of age ($p=0.290$) and gender (14 female, 17 male in EE group versus 11 female, 20 male in healthy controls; $p=0.437$). Gender distribution was also comparable among LGS (8 female, 5 male) and WS patients (6 female, 12 male) ($p=0.119$). However, LGS patients (13.0±4.6 year-old) were significantly older than WS patients (5.1±4.0 year-old) ($p<0.001$) and had significantly higher disease duration ($p<0.001$). There were no significant differences among LGS and WS patients in terms of age of disease onset, seizure frequency and clinical severity and response to treatment during blood sampling (Table 1). All LGS patients displayed slow waves, whereas 3 WS patients had normal background activity on EEG done during blood sampling. While ratios of patients with burst-suppression EEG pattern were similar between WS and LGS patients, a significantly higher number of LGS patients showed spike-wave discharges compared to WS patients. Only 4 of 13 LGS and 5 of 18 WS patients showed mild periventricular leukomalacia on MRI (Table 1).

Levels of Glia-Derived Biomarkers in EE Subgroups

When patients with EE (combined cohort of WS and LGS patients) were compared with healthy controls in terms of serum levels of glial biomarkers, only CHI3L1 levels were found to be significantly increased in EE patients, whereas levels of GFAP, HMGB1, sCD163 and TREM2 were comparable among study groups (Figure 1). When EE subgroups were separately evaluated, WS patients showed significantly lower GFAP levels compared to both LGS and healthy control groups. On the other hand, LGS patients displayed significantly higher levels of CHI3L1 compared to healthy controls, higher sCD163 and TREM2 levels compared to WS patients and higher HMGB1 levels compared to WS patients and healthy controls (Figure 2).

Table 1. Demographic and clinical features of children with epileptic encephalopathy and healthy controls

	LGS (n=13)	WS (n=18)	HC (n=31)	p value
Gender Female Male	 8 5	 6 12	 11 20	0.212
Age (years)	13.0±4.6	5.1±3.9	9.1±4.7	0.580
Disease duration (years)	11.8±4.4	4.8±4.0		**<0.001**
Age of disease onset (months)	4.9±2.9	4.1±2.3		0.481
Seizure frequency of the last month None ≤1/week >1/week <1/day ≥1/day	 2 3 1 7	 8 1 2 7		0.237
Clinical severity of EE* Level I-III (mild-moderate) Level IV-V (severe)	 10 3	 11 7		0.353
Response to treatment** >50% decrease in seizure frequency ≤50% decrease in seizure frequency	 4 6	 10 6		0.263
Background activity on EEG Normal Slow waves	 0 13	 3 15		0.121
Burst-suppression EEG pattern Absent Present	 5 8	 5 13		0.723
Spike-wave discharges None 2–2.5 Hz 2.5–3 Hz >3 Hz	 2 10 1 0	 15 2 0 1		**0.001**
MRI Normal Periventricular leukomalacia	 9 4	 13 5		0.856

Continuous variables are denoted as mean ± standard deviation. Significant p values were denoted with bold letters.
*Clinical severity was determined as per Gross Motor Function, Manual Ability and Communication Function Classification Systems.
**Immunotherapy could not be administered to 5 patients due to lack of parental consent or side effects. Therefore, these patients were not included in the treatment response analysis.
EE: epileptic encephalopathy; EEG: electroencephalography; HC: healthy controls; LGS: Lennox-Gastaut syndrome; MRI: magnetic resonance imaging; WS: West syndrome.
Mann-Whitney U, chi-square and Kruskal-Wallis tests were used for statistical analysis, as required.

To assess the association between serum levels of glial biomarkers and clinical/laboratory features of EE, we compared levels of GFAP, HMGB1, CHI3L1, sCD163 and TREM2 among specific EE subgroups. Epileptic encephalopathy patients without seizures during blood sampling showed trends towards displaying lower serum levels of GFAP, HMGB1 and TREM2 without attaining statistical significance (Table 2). Similarly, patients with treatment response (>50% reduction in seizure frequency) showed significantly lower GFAP and TREM2 levels (Table 3). Epileptic encephalopathy patients with increased clinical severity (Level IV-V) had significantly higher serum levels of HMGB1, CHI3L1 and sCD163 compared to patients with relatively milder disease severity (Level I-III) (Table 4). Moreover, Spearman test showed a significant positive correlation (*p=0.006*, R=0.481) between serum CHI3L1 levels and Gross Motor Function, Manual Ability and Communication Function Classification Systems levels assessing the clinical severity of EE on a scale of Level I and V. No significant correlation was found between GFAP, HMGB1, sCD163 and TREM2 versus clinical severity. When glial biomarker levels were compared among EE subgroups determined as per EEG findings, EE patients with burst-suppression pattern were found to have significantly higher levels of HMGB1 and sCD163 compared to those without (Table 5). Similarly, presence of spike-wave discharges was associated with increased serum levels of HMGB1, CHI3L1, sCD163 and TREM2 (Table 6). There were no significant differences between EE patients with and without periventricular leukomalacia in terms of serum levels of glial biomarkers (data not shown). In addition, levels of glial biomarkers were not significantly correlated with age, disease duration and age of disease onset variables (*p=0.154–0.733*; R=0.064–0.262).

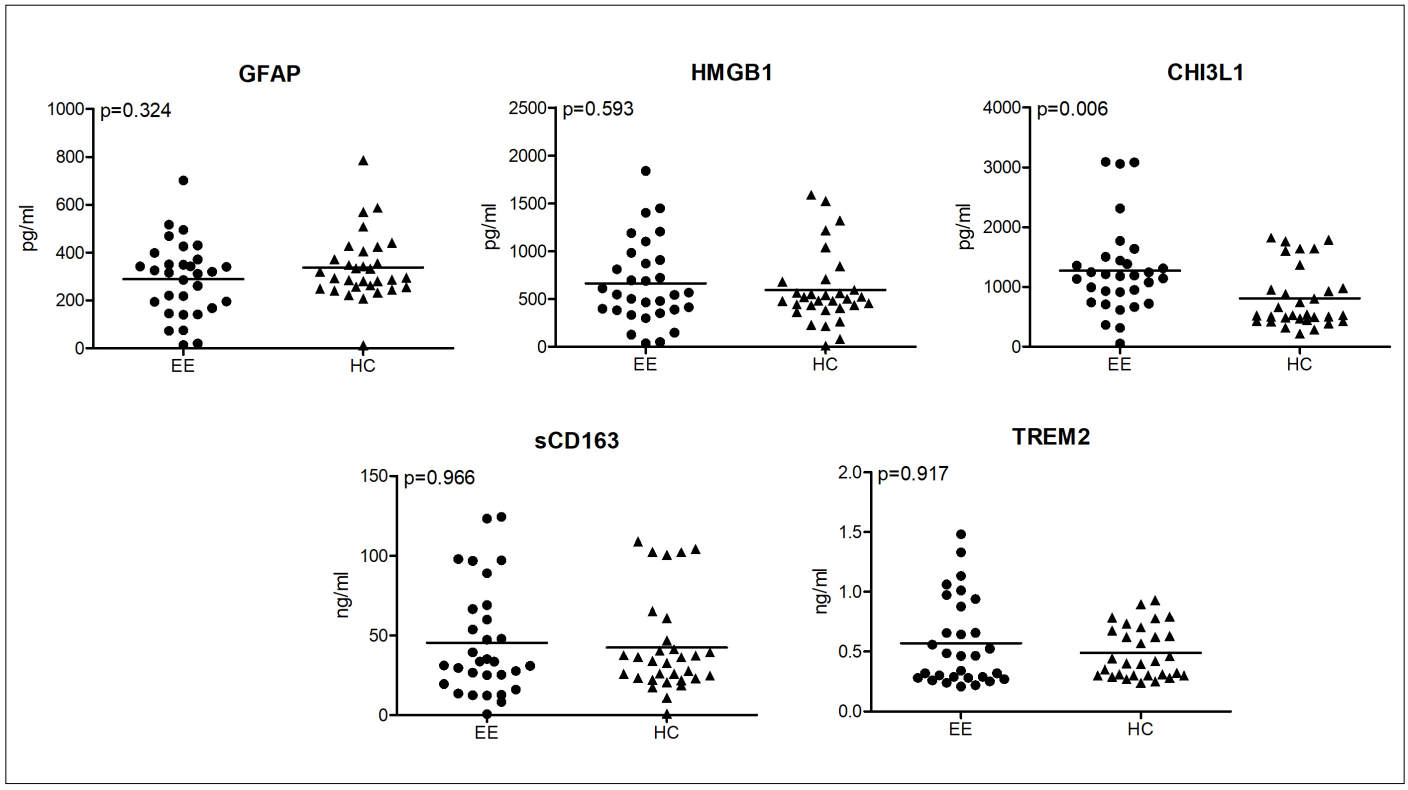

Figure 1. Comparison of serum levels of glia-derived biomarkers among patients with epileptic encephalopathy (EE) and healthy controls (HC). Glial fibrillary acidic protein, GFAP; high mobility group box 1, HMGB1; chitinase-3-like protein 1, CHI3L1; soluble CD163, sCD163; triggering receptor expressed on myeloid cells 2, TREM2. Horizontal lines indicate mean values. p values denoted on the upper left quadrant of each panel were obtained by Mann-Whitney U test.

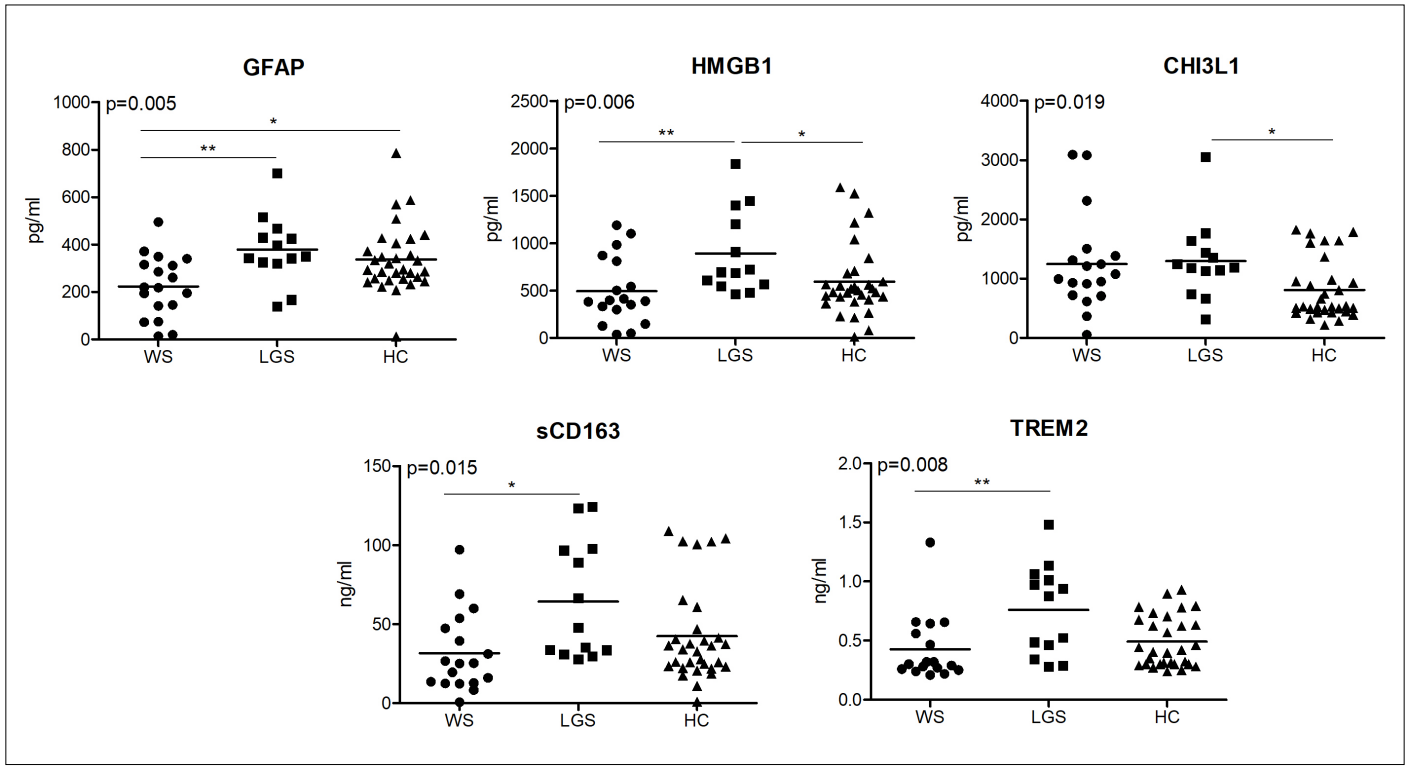

Figure 2. Comparison of serum levels of glia-derived biomarkers among patients with West Syndrome (WS), Lennox-Gastaut syndrome (LGS), and healthy controls (HC). Glial fibrillary acidic protein, GFAP; high mobility group box 1, HMGB1; chitinase-3-like protein 1, CHI3L1; soluble CD163, sCD163; triggering receptor expressed on myeloid cells 2, TREM2. Horizontal lines indicate mean values. p values denoted on the upper left quadrant of each panel were obtained by Kruskal-Wallis test. *, p<0.05 and **, p<0.01 by Dunn's post-hoc analysis.

Table 2. Comparison of glial biomarkers among EE patients with and without seizures during blood sampling

Seizure activity	Absent (n=10)	Present (n=21)	p value
GFAP (pg/ml)	225.0±143.1	319.3±154.8	0.056
HMGB1 (pg/ml)	530.1±393.7	723.1±446.2	0.118
CHI3L1 (pg/ml)	1151.6±750.9	1355.6± 836.3	0.252
sCD163 (ng/ml)	43.2±35.0	46.4±34.8	0.407
TREM2 (ng/ml)	0.5±0.3	0.7±0.5	0.095

Mann-Whitney U test was used for statistical analysis. Significant p values were denoted with bold letters.
CHI3L1: Chitinase-3-like protein 1; EE: epileptic encephalopathy; GFAP: glial fibrillary acidic protein; HMGB1: high mobility group box 1; sCD163: soluble CD163; TREM2: triggering receptor expressed on myeloid cells 2.

Table 3. Comparison of glial biomarkers among treatment-responsive and –resistant EE patients

	Resistant (n=12)	Responsive (n=14)	p value
GFAP (pg/ml)	344.2±125.7	234.2±124.2	**0.018**
HMGB1 (pg/ml)	825.5±496.6	608.8±395.6	0.119
CHI3L1 (pg/ml)	1300.8±892.4	1247.5±673.8	0.434
sCD163 (ng/ml)	55.3±38.2	41.3±35.4	0.173
TREM2 (ng/ml)	0.8±0.6	0.4±0.2	**0.010**

Mann-Whitney U test was used for statistical analysis. Significant p values were denoted with bold letters.
CHI3L1: Chitinase-3-like protein 1; EE: epileptic encephalopathy; GFAP: glial fibrillary acidic protein; HMGB1: high mobility group box 1; sCD163: soluble CD163; TREM2: triggering receptor expressed on myeloid cells 2.

Table 4. Comparison of glial biomarkers among EE patients with mild and severe clinical severity as determined by Gross Motor Function, Manual Ability and Communication Function Classification Systems

	Level I-III (n=21)	Level IV-V (n=10)	p value
GFAP (pg/ml)	306.3±127.9	252.4±204.7	0.229
HMGB1 (pg/ml)	496.8±309.8	738.9±467.3	**0.049**
CHI3L1 (pg/ml)	1069.0±449.5	1753.4±1160.9	**0.048**
sCD163 (ng/ml)	31.0±18.0	52.2±38.3	**0.023**
TREM2 (ng/ml)	0.7±0.5	0.5±0.4	0.106

Mann-Whitney U test was used for statistical analysis. Significant p values were denoted with bold letters.
CHI3L1: Chitinase-3-like protein 1; EE: epileptic encephalopathy; GFAP: glial fibrillary acidic protein; HMGB1: high mobility group box 1; sCD163: soluble CD163; TREM2: triggering receptor expressed on myeloid cells 2.

Table 5. Comparison of glial biomarkers among EE patients with and without burst-suppression EEG pattern during blood sampling

	Absent (n=10)	Present (n=21)	p value
GFAP (pg/ml)	291.0±227.6	287.9±113.5	0.485
HMGB1 (pg/ml)	448.6±258.0	761.9±467.1	**0.012**
CHI3L1 (pg/ml)	1211.9±812.3	1326.9±815.9	0.359
sCD163 (ng/ml)	32.9±23.9	51.3±37.9	**0.044**
TREM2 (ng/ml)	0.6±0.4	0.6±0.5	0.411

Mann-Whitney U test was used for statistical analysis. Significant p values were denoted with bold letters.
CHI3L1: Chitinase-3-like protein 1; EE: epileptic encephalopathy; GFAP: glial fibrillary acidic protein; HMGB1: high mobility group box 1; sCD163: soluble CD163; TREM2: triggering receptor expressed on myeloid cells 2.

Table 6. Comparison of glial biomarkers among EE patients with and without spike-wave discharges on EEG during blood sampling

	Absent (n=17)	Present (n=14)	p value
GFAP (pg/ml)	258.8±121.2	325.4±187.1	0.132
HMGB1 (pg/ml)	542.5±340.0	804.5±500.2	**0.045**
CHI3L1 (pg/ml)	988.5±500.0	1655.7±957.7	**0.015**
sCD163 (ng/ml)	34.2±24.9	59.0±39.9	**0.028**
TREM2 (ng/ml)	0.5±0.3	0.8±0.6	**0.030**

Mann-Whitney U test was used for statistical analysis. Significant p values were denoted with bold letters.
CHI3L1: Chitinase-3-like protein 1; EE: epileptic encephalopathy; GFAP: glial fibrillary acidic protein; HMGB1: high mobility group box 1; sCD163: soluble CD163; TREM2: triggering receptor expressed on myeloid cells 2.

DISCUSSION

In this study, we found elevated serum levels of CHI3L1 in the combined WS and LGS cohort and higher serum levels of HMGB1, sCD163 and TREM2 in LGS patients compared to WS patients and/or healthy controls. To our knowledge, these findings provide evidence for the enhanced glial activity and neuroinflammation particularly in LGS patients, for the first time.

The putative significance and contribution of glial cells have been confirmed mostly by using animal models of EE. Introduction of mutated variants of genes associated with EE (e.g. salt-inducible kinase 1, SCN8A and KCNMA1) into experimental animals induces enhancement of neural excitability, epileptic seizures and increased cortical expression of markers of astrocyte and/or microglia activity (23–25). Thus, our patient-derived findings further corroborate the significance of glial activity in EE.

The lack of increase in levels of GFAP, which is a characteristic astrocytic product, may lead to reservations about the role of astrocytes in EE. However, arguing against this notion, CHIC3L1, produced by both activated astrocytes and microglia (16), was significantly increased in LGS patients and correlated with several clinical features of EE. Glial fibrillary acidic protein levels are known to be increased by age (26) and this may explain reduced GFAP levels in WS patients, who were younger than the other two study groups. Although there was no correlation between GFAP levels and age of the patients, this may be explained with the low number of participants and reduced statistical power of our study. Finally, serum GFAP levels might not be properly reflecting the pathological alterations occurring in the CNS of EE patients and thus measurement of cerebrospinal fluid GFAP levels might be warranted for future research.

Nevertheless, GFAP levels were found to show increasing trends in patients having active seizures during blood sampling and in patients showing resistance to anti-epileptic medications. This is compatible with the genetic EE model studies showing enhanced numbers of GFAP positive astrocytes in parallel to the induction of seizures in animals (23–25). Also in favor of this notion, experimental animals treated with the astrocyte-inhibitor ONO-2506 exhibit reduced frequency of seizures and spike-wave discharges in parallel with decreased expression of GFAP in the brain (27).

HMGB1, CHI3L1 and sCD163 levels were associated with increased clinical severity scores rather than intensity of the seizures. A putative explanation is that the clinical scale used to assess the severity of EE takes into consideration the motor-mental development. Thus, these three glial mediators are more likely to be related with glia-induced neuronal destruction and associated neurological impairment. In support of this assertion, ventricular administration of recombinant HMGB1 into rats has caused cortical neuronal death, disturbance of neuronal migration and organization and increased cerebral glutamate content (28).

The association between HMGB1, CHI3L1 and sCD163 with the electrophysiological findings of suppression burst and spike-wave discharges provides evidence regarding the involvement of microglia and astrocytes in formation of pathological EEG findings in EE. Notably, intraventricular administration of the conditioned medium of stimulated microglia results in increased glial IL-1β and TNF-a production, enhanced cortical glutamate content, induction of seizures and spike-wave discharges (29). There are additional reports indicating the role of other cortical glial cells such as astrocytes in the formation of spike-wave discharges in epileptic patients (30–32).

CHI3L1 is a 40-kD glycoprotein abundantly expressed by several tissue types. Although its exact biological function is still not very well known, its potential as a biomarker of inflammation has been increasingly recognized. In neurology, CHI3L1 has been found to carry a great biomarker value particularly in amyotrophic lateral sclerosis, stroke and multiple sclerosis (16). Potential biomarker value of CHI3L1 has also been proposed in drug resistant epilepsy (33). However, its significance in most epileptic disorders including EE is largely unknown. In our study, CHI3L1 was increased in both LGS and WS patients and was correlated with the severity of motor/mental impairment and electrophysiological features. Therefore, the precise mechanisms by which CHI3L1 is involved in EE need to be further investigated.

Although our results have provided further support for involvement of glial cells in the physiopathology of EE, it should also be noted that HMGB1 is also produced by neurons and it is mostly a marker of neuron-glia crosstalk (34,35). Similarly, sCD163 and CH3L1 are produced by not only microglia but also monocytes and myeloid cell types (16,36). The contribution of peripheral blood monocytes to EE pathogenesis has been previously postulated (37). Thus, elevated levels of sCD163 and CH3L1 might be related with the activation and contribution of other myeloid cell types to the clinical features of EE, as well.

As a limitation of our study, we had a low case number especially in the LGS group, which might have reduced the statistical power and concealed some of the significant associations between biomarker levels and clinical/demographic features. Therefore, our study should be considered a preliminary exploration study, which should be followed by future studies conducted with a higher number of EE patients. Additionally, the high standard deviation of serum GFAP levels may make it difficult to use it as a marker in practice. Therefore, we recommend investigating alternative glial markers.

In brief, our results provide a "proof of concept" basis regarding the contribution of glial cells especially in LGS patients and thus confirm the findings of the previous animal model-based mechanistic studies and patient-based immunohistochemistry reports. Furthermore,

CHI3L1 came into prominence as a glial mediator and a potential biomarker associated with clinical severity, motor/mental retardation and electrophysiological findings of EE. Our findings also lend further support to choose glial cells as a potential target for future therapeutic interventions of EE.

Ethics Committee Approval: The study was approved by the ethics committee of Istanbul University, Istanbul Faculty of Medicine (date and number: 18/12/2020–257365).

Informed Consent: Written informed consent was obtained from all patients or their representatives.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept- PT; Design- ET, PT; Supervision- PT, ET, ZY; Resource- (-); Materials- (-); Data Collection and/or Processing- MC, SS, PT, CİK, MS; Analysis and/or Interpretation- PT, ET, MS, CİK, VY; Literature Search- MC, SS; Writing- MC, PT, ET; Critical Reviews- PT, ET, VY, ZY, CİK.

Conflict of Interest: The authors declared that there is no conflict of interest.

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