Original article

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HMGB-1, TLR4, IL-1R1, TNF-α, and IL-1β: novel epilepsy markers?

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ABSTRACT – *Aim*. The purpose of this study was to compare HMGB-1, TLR4, IL-1β, IL-1R1, and TNF-α levels in patients with mild and severe epilepsy with those in a healthy control group.
Methods. Children aged 4-17 years, diagnosed with epilepsy for at least three years and with no progressive neurological disease, metabolic disease or infection, were selected for the study. The severe epilepsy group consisted of 28 children with at least one episode a week despite receiving three or more antiepileptic drugs. The mild epilepsy group consisted of 29 children with no seizures in the previous year, receiving only one antiepileptic drug, while 27 healthy children were selected as the control group. HMGB-1, TLR4, IL-1R1, TNF-α and IL-1β levels were investigated in these three groups. The MRI findings and clinical characteristics of the patients in the epilepsy group were also compared with these markers.
Results. HMGB-1, TLR4, TNF-α, and IL-1β levels in the severe epilepsy group were higher than in the control group and the mild epilepsy group ($p<0.05$), and were higher in the mild epilepsy group than in the control group ($p<0.05$). IL-1R1 was also higher in the severe epilepsy group than in the control group ($p<0.05$).
Conclusion. In this first report to identity a possible correlation between HMGB-1, TLR4, IL-1β, IL-1R1, and TNF-α levels and severity of epilepsy, our data demonstrates that the serum level of these cytokines is higher in cases of drug-refractory epilepsy.

Key words: inflammation molecules, immune epilepsy, inflammation, cytokines, epilepsy, seizure

The term "neurogenic inflammation or neuroinflammation" has recently been recommended to describe neuroinflammatory mechanisms triggered by neuronal activity (Xanthos and Sandkuhler, 2014). Neuroinflammation may appear not only as a response to effects triggering inflammation, such as infection and autoimmunity, but also in response to factors that increase neuronal activity. Seizures have the greatest stimulatory effect on neuronal activity. Inflammatory mediators such as cytokines, chemokines, and prostaglandins secreted by

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brain cells during epileptic activity not only encourage inflammation, but also act as neuromodulators that affect neuronal function and stimulability (Vezzani and Viviani, 2015). Recent experimental studies have shown that specific inflammatory molecules make a significant contribution to seizure development, epileptogenesis, and even drug resistance (Vezzani *et al.*, 2011; Ravizza *et al.*, 2006; Terrone *et al.*, 2016).

The discovery of the association between inflammation, the immune system and epilepsy gave rise to significant new hope in the search for new treatments of epilepsy, and as a first step, it is important to establish the precise role of inflammatory or immune markers in epilepsy.

It has been suggested that some specific inflammatory molecules and their receptors may mediate neuronal cell loss and contribute to synaptic plasticity in epilepsy. Various factors, such as trauma, stroke, febrile seizure, status epilepticus, infection, and genetic mutations can lead to activation in microglia, astrocytes and neurons. The majority of studies on this subject have focused on markers to reveal this effect. The best known of these, in addition to interleukin (IL)-1β, is high mobility group box 1 protein (HMGB-1), which is involved in IL-1/toll-like receptor (TLR) signal activation. The release of these inflammation molecules occurs with the effects described, and together with an increase in calcium, this results in NMDA receptor activation, in turn, leading to seizures. IL-1 receptor, type 1 (IL-1R1) and TLR4 have been found to increase in microglia, astrocytes and neurons under conditions such as cell damage and seizure, in addition to physiological conditions, and this has been attributed to increased neuronal excitability (Ericsson *et al.*, 1995; Turrin and Rivest, 2004; Allan *et al.*, 2005; Ravizza and Vezzani, 2006; Maroso *et al.*, 2010; Peltier *et al.*, 2010; Zurolo *et al.*, 2011).

The purpose of this study was to investigate HMGB-1, TLR4, and IL-1R1 levels between patients with refractory epilepsy, a group whose seizures were under control, and a healthy control group.

In addition to these markers, we also investigated tumour necrosis factor-α (TNFα) and IL-1 (IL-1β) in epileptic patients. We selected IL-1β as a prominent cytokine in ischaemic injury based on both experimental stroke models and studies involving mice, since some studies have noted a relationship between this cytokine and epilepsy. For example, extended induction of IL-1β in prolonged febrile seizures in immature rats has been reported to lead to permanent epilepsy (Dube *et al.*, 2010). Immunohistochemical studies have also shown that IL-1β is induced during the acute phase of status epilepticus and during the chronic phase in spontaneous seizures in active microglia and astrocytes (Voutsinos-Porche *et al.*, 2004; Ravizza *et al.*, 2008). Rapid TNF-α induction has also been shown in the rat brain during limbic seizures (De Simoni *et al.*, 2000), and although immunohistochemical analysis of TNF-α in the hippocampus has revealed evidence of cytokine expression, there have been few studies on this subject.

Methods

Subjects

Fifty-seven children, aged 4-17 years, diagnosed with epilepsy and followed at the Karadeniz Technical University Pediatric Neurology Department, Turkey, between January 2018 and January 2019, were selected for the study. These children were divided into two groups. The first represented the severe epilepsy group. This consisted of 28 children diagnosed with epilepsy at least three years previously, and with at least one episode a week despite receiving three or more antiepileptic drugs. The second group constituted the mild epilepsy group. This consisted of 29 children diagnosed with epilepsy at least three years previously, with no seizures in the previous year and receiving only one antiepileptic. A control group was also established consisting of 27 children with a similar age group with no disease.

Study design

Epilepsy histories were taken from patients meeting the study criteria before blood collection, neurological examination, and examination of imaging and EEG records.

Written permission was obtained from the mothers or fathers of all children before inclusion in the study. Ethical approval was granted by the Karadeniz Technical University ethical committee.

Biochemical analyses

HMGB-1, TLR4, IL-1R1, TNF-α, IL-1β levels in human serum were determined using an enzyme-linked immunosorbent assay (ELISA) kit (Elabscience, Czech Republic); HMGB-1 (Cat No: E-EL-H1554); TLR4 (Cat No: E-EL-H1539); IL-1R1 (Cat No: E-EL-H1028); TNF-α (Cat No: E-EL-H0109); and IL-1β (Cat No: E-EL-H0149). HMGB-1, TNF-α and IL-1β were expressed as pg/mL, and TLR4 and IL-1R1 as ng/mL, in line with the manufacturers' protocols. The absorbance of samples was measured at 450 nm using a microplate reader (Molecular Devices, CA, USA).

ELISA measurements for each patient were performed in duplicate on the same day (intra-assay) according to the manufacturer's recommendations. The mean values of the results were calculated. The coefficient of variation (n: 10) (CV) was also measured and calculated

for each parameter, yielding an intra-assay CV of 5.3% for IL-1R1, 5.7% for TLR4, 4.3% for HMGB-1, 4.9% for TNF-α, and 5.6% for IL-1β.

Statistical analyses

Sample size calculation was performed using an effect size of 60% and 80% power at a 95% confidence interval. This showed that at least 72 patients would be required in total. Analysis of the data set included 84 samples. Statistical Program for the Social Sciences (SPSS, version 23) was used for statistical analysis.

The study data were expressed as mean ± SD. IL-1R1 and TLR4 were compared among the three groups (healthy control, mild epilepsy, and severe epilepsy) using analysis of variance (ANOVA) with post-hoc correction for multiple comparisons after the Kolmogorov-Smirnov test had been applied for normality. Statistically significant relationships between groups were indicated by the Tamhane post-hoc test corrected for unequal variances. In case of independent samples without normal distribution, HMGB1, TNFα and IL-1β were compared among the three groups using the Kruskal-Wallis test, while paired Mann-Whitney U tests were used for multiple comparisons.

In order to determine the diagnostic sensitivity of the patient and control groups, diagnoses of epilepsy based on HMGB1, TLR4, IL-1β, IL-1R1, and TNF-α receiver operating characteristic curve (ROC) analyses were calculated. Mild and severe epilepsy groups (n=57) and a control group (n=27) were used for ROC analysis.

Results

Patients and clinical findings

The patients included in the study were divided into three groups, severe epilepsy, mild epilepsy, and control, consisting of 28, 29 and 27 children, respectively. No significant differences were determined between the groups in terms of mean age or sex. Also, no significant difference was determined between the severe and mild epilepsy groups in terms of mean duration of disease. Patients' demographic data are shown in *table 1*.

Statistical results

ANOVA analysis revealed significant differences in IL-1R1 and TLR4 between the healthy control, mild epilepsy and severe epilepsy groups. Based on post-hoc analyses, this variation was observed only between the severe epilepsy and control groups for IL-1R1 (F=4,404, p=0.015), and among all three groups for TLR4 (F=36.78, p=0.0001) (p<0.05) (*table 2*).

The Kruskal Wallis-H test revealed significant differences among the three groups in terms of HMGB1 (χ^2 = 14.53, p = 0.001), TNFα (χ^2 = 30.98, p = 0.0001), and IL-1β (χ^2 = 23.14, p = 0.0001). Post-hoc analyses for each group showed that all paired differences were significant (p<0.05) (*table 2*).

Additionally, the area under ROC curves (AUC) for two classifiers (control and patient groups) is shown in *table 3* and *figure 1*. In order to quantify the performance characteristics of each classifier, AUC was computed for the validation data ROC curve. *Table 3* presents a summary of these AUC values for the two classifiers. As shown in *table 3*, the TLR4-based classifier is the better classifier with AUC=0.880 for the validation data ROC curves, while the ILR1-based classifier exhibited a lower performance (AUC=0.654).

Mean HMGB-1 values in the severe epilepsy group (1056.73±378.42) were statistically significantly higher than those in both the mild epilepsy group (859.40±267.36) and the heathy control group (721.40±239.72) (p=0.001) (*table 2*). Mean TLR4 values in the severe epilepsy group (8.35±1.48) were statistically significantly higher than those in both the mild epilepsy group (6.97±1.62) and the healthy control group (5.17±0.89) (p=0.0001) (*table 2*). Mean IL-1β values in the severe epilepsy group (70.04±14.17) were statistically significantly higher than those in both the mild epilepsy group (66.44±30.26) and the healthy control group (53.05±8.3) (p=0.0001) (*table 2*).

Table 1. Demographic data of groups.

	Severe epilepsy mean ± SD	Mild epilepsy mean ± SD	Healthy control mean ± SD
Age (years)	10.11 ± 4.16	11.48 ± 3.23	11.07 ± 3.47
Duration of disease (years)	6.04 ± 2.67	5.17 ± 2.36	-
Gender n (%)			
Females	15 (53.6)	14 (48.3)	14 (51.9)
Males	13 (46.4)	15 (51.7)	13 (48.1)

Table 2. Comparison of laboratory finding among patient and control groups.

	Healthy control	Mild epilepsy	Severe epilepsy	*p*	*p1*	*p2*	*p3*
HMGB1	721.40 ± 239.72	859.40 ± 267.36	1056.73 ± 378.42	0.001*	0.027*	0.0001*	0.029*
TLR4	5.17 ± 0.89	6.97 ± 1.62	8.35 ± 1.48	0.0001*	0.0001*	0.0001*	0.004*
IL-1β	53.05 ± 8.3	66.44 ± 30.26	70.04 ±14.17	0.0001*	0.002*	0.0001*	0.035*
IL-1R1	1.05 ± 0.41	1.23 ± 0.54	1.51 ± 0.71	0.015*	0.456	0.018*	0.278
TNF-alfa	8.96 ± 2.89	10.39 ± 2.67	15.07 ± 4.49	0.0001*	0.006*	0.0001*	0.0001*

Values are mean ± SD
p: within groups
p1: significance between healthy control and mild epilepsy groups
p2: significance between healthy control and severe epilepsy groups
p3: significance between mild epilepsy and severe epilepsy groups
* $p < 0.05$.

Table 3. Comparison of patient and control groups.

Classifier type	Area under ROC curve	*p*	Lower 95% CI	Upper 95% CI	Cut-off
HMGB1	0.723	0.001*	0.607	0.839	772.85
TLR4	0.880	0.0001*	0.810	0.950	6.785
IL-1β	0.799	0.0001*	0.699	0.899	54.9
IL-1R1	0.654	0.023*	0.533	0.775	1.635
TNF-α	0.793	0.0001*	0.692	0.894	9.835

$p < 0.05$ (cut-off based on values determined according to youden index).

Mean IL-1R1 values in the severe epilepsy group (1.51±0.71) were also higher than in both the other groups, although the difference was only statistically significant between the severe epilepsy group and the control group (1.05±0.41) (*p*=0.015) (*table 2*). Mean TNFα values in the severe epilepsy group (15.07±4.49) were also statistically significantly higher than those in both the mild epilepsy group (10.39±2.67) and the healthy control group (8.96±2.89) (*p*=0.0001) (*table 2*).

Clinical characteristics of patients in the severe epilepsy group

The mean age of the severe epilepsy group was 10.11±4.16 years (4-17), and the mean duration of epilepsy was 6.04±2.67 years (3-13). The patients in this group had been receiving three or more antiepileptic drugs. Seven patients were being treated due to hypoxic ischaemic encephalopathy, six due to an epileptic syndrome, four due to congenital malformations, two due to seizures commencing and persisting during encephalitis, one due to tuberous sclerosis, two due to seizures commencing after trauma, and one due to hydrocephaly. No underlying condition could be identified in five patients. No significant relationship was determined between underlying diseases and HMGB-1, TLR4, IL-1R1, TNF-α, or IL-1β values.

MRI was normal in 12 cases (43%). Sixteen patients had cranial MRI findings, and hypoxia-related changes were observed in 10 (62%) of these. Congenital malformations were present in five patients, and cortical tubers supporting neurocutaneous disease in one. No significant relationship was determined between MRI findings and HMGB-1, TLR4, IL-1R1, TNF-α, or IL-1β values. Clinical and imaging characteristics of the severe epilepsy group are summarized in *table 4*.

No underlying disease was present in any of the mild epilepsy group. Cranial MRI findings were normal apart from five patients in whom mild ventricular asymmetry, cavum vergae variant and non-specific gliotic changes were observed.

Discussion

Since previous studies have demonstrated that inflammatory factors may cause seizures or induce

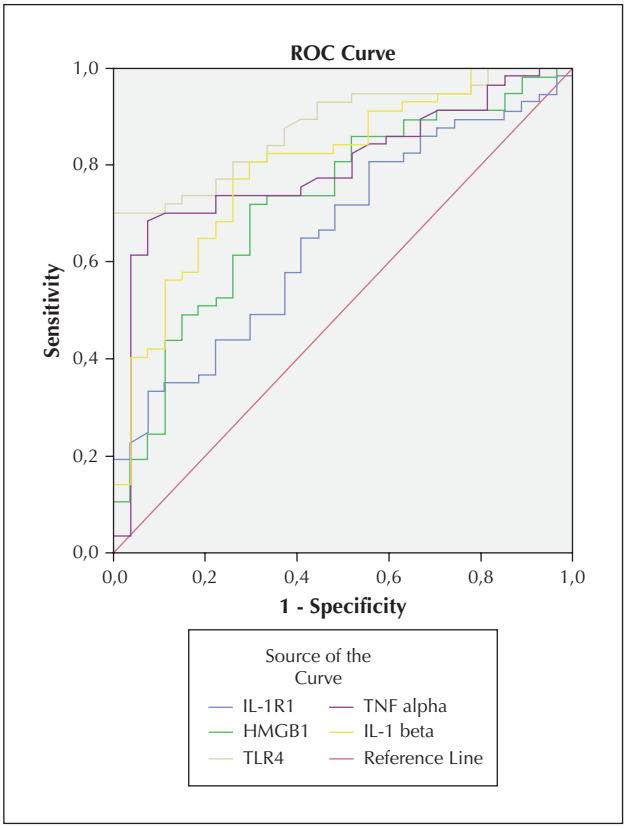

Figure 1. HMGB1, TLR4, IL-1beta, IL-1R1 and TNF-alpha receivers operating characteristic curve (ROC) analyses.

permanent seizures, based on cohorts of patients with severe epilepsy and mild epilepsy and healthy control groups, we chose to compare the levels of HMGB-1, TLR4, IL-1R1, TNF-α, and IL-1β cytokines among these three groups in the present study.

Our findings show significantly higher HMGB-1, TLR4, TNF-α, and IL-1β levels in the severe epilepsy group than in the other two groups. Additionally, the fact that the levels also differed significantly between the mild epilepsy and healthy control groups suggests that, in addition to their association with epilepsy, these four cytokines may be important markers of the condition. IL-1R1 was higher in the severe epilepsy group, but this was only statistically significant when matched against the healthy control group, suggesting that IL-1R1 may only be associated with severity of epilepsy.

Evidence and clinical observations from experimental studies in recent years supports the idea that inflammation in the brain, or neuroinflammation, is a common feature of refractory epilepsies (Vezzani *et al.*, 2011). According to these studies, inflammation not only lowers the seizure threshold, but is also an important factor determining progressive or recurrent seizure characteristics (Kulkarni and Dhir, 2009; Riazi *et al.*, 2010; Vezzani *et al.*, 2011). Events contributing to chronic inflammation, such as leukocyte infiltration in the blood, astroglial and microglial activation, and chemokine and cytokine release, are also processes underlying epileptogenesis (Vezzani *et al.*, 2013). The present study focused on the inflammatory mediators, HMGB-1, TLR4, IL-1β, IL-1R1 and TNF-α, which have been the focus of numerous previous experimental studies involving these processes.

HMGB-1 signaling activation has been shown in experimental models of acute seizures. It has also been shown in models of epilepsy acquired via trauma and in brain tissues in drug-resistant epilepsies (Balosso *et al.*, 2010; Zurolo *et al.*, 2011; Iori *et al.*, 2013; Chiavegato *et al.*, 2014; Pauletti *et al.*, 2017; Walker *et al.*, 2017; Zhao *et al.*, 2017). HMGB-1 is a ubiquitous nuclear protein released by glia and in response to neuron inflammation activation, and is one of the best characterized damage-associated molecular patterns (DAMPs). It activates the receptor for advanced glycation end-products (RAGE) and TLR4 in target cells. The resulting HMGB-1/TLR4 axis is an important trigger of neuroinflammation (Paudel *et al.*, 2018). TLR4 activation by HMGB-1 in neurons and astrocytes is a key mechanism in seizure development, and blocking the TLR4 signal with an antagonist can reduce the severity of epilepsy (Iori *et al.*, 2013). HMGB-1 leads to the phosphorylation of NMDA-NR2B receptors by activation of IL-IR/TLR signalling, and plays a key role, not only in seizure development through the proinflammatory cytokine pathway, but also in recurrence (Vezzani *et al.*, 2012; Zaben *et al.*, 2017).

Walker *et al.* showed an increase in HMGB-1 and its translocation from the nucleus to cytoplasm in neurons and glia in epileptic foci in patients with drug-resistant epilepsies and animal models. They also showed that HMGB-1 passes from the nucleus to the cytoplasm before the onset of spontaneous seizures in rats under continuous video-EEG monitoring (Walker *et al.*, 2017). We therefore believe that cerebral changes associated with HMGB-1 are not only the result of continuous seizure activity, but that increased levels in brain tissue, for an unknown reason, may also be responsible for an increase in seizures or resistance. The potential pathological role of HMGB-1 and its molecular isoforms in the development and recurrence of neuropathology has been confirmed through investigation in several experimental epilepsy models and in human drug-resistant epilepsies (Zurolo *et al.*, 2011; Iori *et al.*, 2013; Balosso *et al.*, 2014; Chiavegato *et al.*, 2014; Luan *et al.*, 2016; Fu *et al.*, 2017; Walker *et al.*, 2017; Zhao *et al.*, 2017). In the study of Walker *et al.* (2017), the authors examined the serum HMGB-1 levels of 65 patients with drug-resistant epilepsy, 20 patients with well-controlled epilepsy, and a

Table 4. Clinical and MRI features of the severe epilepsy group.

Age (year)	Duration of epilepsy	Number of treatments	Other disease/causal disease	MRI	Serum concentration				
					IL-1R1 (ng/mL)	HMGB-1 (pg/mL)	TLR4 (ng/mL)	TNF-α (pg/mL)	IL-1β (pg/mL)
9	5	4	congenital malformation, hydrocephalus	pontocerebellar hypoplasia	2.223	1154.3	10.11	16.65	54.37
16	7	4	neurocutaneous disease	cortical tubers	2.145	1050.3	9.88	16.15	54.72
12	7	5	normal	normal	2.168	1288.3	8.55	16.12	73.60
11	6	3	epileptic syndrome	hypoxic injury	0.878	989.9	9.83	14.77	65.14
6	5	3	seizures after encephalitis	hypoxic injury	1.890	806.6	7.15	7.67	49.88
13	8	4	epileptic syndrome	normal	1.409	750.6	9.41	16.54	62.93
11	7	5	normal	normal	1.121	1461.6	6.47	15.40	56.83
12	9	4	seizures after encephalitis	normal	2.071	873.1	10.13	29.53	97.44
4	3	3	hypoxic ischaemic encephalopathy	hypoxic injury	1.034	1087.1	8.60	12.63	96.86
4	3	3	epileptic syndrome	normal	2.259	1167.1	8.39	15.19	59.44
12	8	4	epileptic syndrome	normal	1.129	1259.3	8.23	16.44	64.01
14	4	3	post-traumatic	normal	1.588	965.3	7.68	14.49	60.79
7	4	4	normal	normal	1.377	1068.4	5.72	12.38	72.06
13	6	3	hypoxic ischaemic encephalopathy	hypoxic injury	3.616	927.0	8.80	23.04	69.32
6	5	5	epileptic syndrome	normal	2.278	1047.2	9.26	20.84	77.76
8	3	4	post-traumatic	normal	1.752	800.0	9.14	16.25	88.26
17	10	4	hypoxic ischaemic encephalopathy	hypoxic injury	1.092	1030.1	8.52	8.72	48.96
16	13	4	hypoxic ischaemic encephalopathy	hypoxic injury	0.226	1249.5	10.92	15.16	64.21

Table 4. Clinical and MRI features of the severe epilepsy group (*continued*).

Age (year)	Duration of epilepsy	Number of treatments	Other disease/causal disease	MRI	Serum concentration				
					IL-1R1 (ng/mL)	HMGB-1 (pg/mL)	TLR4 (ng/mL)	TNF-α (pg/mL)	IL-1β (pg/mL)
13	7	3	hypoxic ischaemic encephalopathy	hypoxic injury	0.414	610.2	4.29	15.81	73.34
13	9	4	congenital malformation	cerebral cerebellar atrophy	0.655	907.7	7.54	7.45	75.26
9	7	5	normal	normal	2.136	560.2	9.35	15.25	75.32
5	3	4	hypoxic ischaemic encephalopathy	hypoxic injury	1.434	513.3	8.63	16.81	50.30
8	4	3	hydrocephalus	microlizencephaly	1.707	525.3	6.12	8.83	75.62
12	5	3	normal	normal	1.720	647.3	8.55	12.67	83.41
17	11	4	epileptic syndrome	hypoxic injury	0.915	1462.0	6.90	14.89	87.71
4	3	4	congenital malformation	lizencephaly	1.107	2068.0	8.85	15.93	95.51
7	4	4	hypoxic ischaemic encephalopathy	hypoxic injury	0.988	1919.1	9.25	15.08	64.93
4	3	4	congenital malformation	simplified gyral pattern	1.008	1399.7	7.77	11.33	63.23

74-member healthy control group. The patients with drug-resistant epilepsy had higher total HMGB-1 levels than both the healthy controls and the well-controlled epilepsy group. In this study, patients with abnormal cerebral MRI had significantly elevated HMGB-1 levels. HMGB-1 values were significantly higher in the severe epilepsy group than in both the control group and mild epilepsy group in the present study. This suggests that HMGB-1 may be a good marker for epilepsy. An increase in IL-1R1, observed only in severe epilepsy patients, strengthens the correlation between this cytokine and severity. In addition, the presence of additional pathologies, associated with preparing the framework for refractory epilepsies, such as injury and congenital malformation, in patients in the severe epilepsy group, may indicate that increased HMGB-1 release following tissue damage can render seizures permanent.

The seizure triggering effects of HMGB-1 in neurons and astrocytes are known to be mediated by TLR4. Additionally, the HMGB-1/TLR4 axis is an important initiator of neuroinflammation. Blocking TLR4 signalling using an antagonist can reduce the severity of epilepsy (Ivory *et al.*, 2013). Moreover, TLR4 or RAGE knockout mice are less susceptible to seizures and epilepsy development (Maroso *et al.*, 2010). IL-1R-1/TLR4 signalling is associated with a generally poorer prognosis and the development of drug-resistant seizures, and is thought to be activated in epilepsy cases with cerebral malformations or lesions (Pitkanen and Sutula, 2002; Sarkis *et al.*, 2012; Schmidt and Sillanpaa, 2013; Iori *et al.*, 2017).

The finding that TLR4 levels were significantly higher in the severe epilepsy group compared to both the mild epilepsy and control groups in our study suggests that TLR4 may represent an important marker in drug-resistant epilepsies in particular. This therefore makes the IL-1R1 and TLR4 axes attractive, not only as markers for prognosis but also targets for treatment.

The role of IL-1β in epilepsy is, however, rather more complex. IL-1β was formerly regarded as solely a proinflammatory cytokine but subsequently linked to various diseases of the central nervous system (CNS) in addition to its physiological effects on the CNS (Claycomb *et al.*, 2012). Although there is some evidence to suggest that endogenous IL-1 has proconvulsive properties in acute seizures (Vezzani *et al.*, 1999; 2000; 2002; Plata-Salaman *et al.*, 2000; Ravizza *et al.*, 2008), some authors suggest that IL-1β has acute anticonvulsive properties (Miller *et al.*, 1991; Sayyah *et al.*, 2005). One study of transgenic mice with deletion in IL-1β or its signal receptors focused on the anticonvulsive properties of IL-1β, showing that epileptic activity was more severe in these mice with a similar effect to that of the COX-2 inhibitor, rofecoxib (Claycomb *et al.*, 2012).

In another study, IL-1β injection into rodent brains shortly before kainic acid- or bicuculline-triggered seizures resulted in pro-convulsive effects, while intracerebral injection of an IL-1β antagonist exhibited powerful anticonvulsive effects (Vezzani *et al.*, 1999; 2000; 2002; Dube *et al.*, 2005).

The level of IL-1β in this study was higher in the severe epilepsy group than in both the mild epilepsy and control groups. However, it is unclear whether this is a cause or effect, and further studies involving pre- and post-seizure measurements would be required to clarify this.

Similar to IL-1β, the situation is also not entirely clear for TNF-α. TNF-α mRNA in the rodent brain is induced rapidly during limbic seizures, and its expression then declines to basal values 72 hours after onset of status epilepticus. Immunohistochemical analysis of TNF-α has also shown expression in the rodent hippocampus (De Simoni *et al.*, 2000; Lehtimaki *et al.*, 2003; Turrin and Rivest, 2004; Dhote *et al.*, 2007; Kuteykin-Teplyakov *et al.*, 2009; Weinberg *et al.*, 2013). Studies have reported increases in neurons and astrocytes in tissue specimens from patients with temporal lobe epilepsy and tuberous sclerosis, although levels were below detectable limits in autoptic control tissues. A decrease in seizures has been observed with TNFR2 activation following injection of TNF-α into the mouse hippocampus, but an increase in seizures with TNFR1 activation. High expression of TNF-α is a marker of neurological dysfunction, including seizures, and decreased seizure susceptibility was observed in transgenic mice with low to moderate TNF-α in astrocytes. These findings emphasize the importance of the subreceptors of this cytokine and the cerebral concentrations in determining the effect on seizures (Maldonado *et al.*, 2003; Balosso *et al.*, 2005; Lu *et al.*, 2008; Weinberg *et al.*, 2013).

In a study by Steinborn *et al.*, the authors compared serum IL-1β, IL-2, IL-6, and TNFα levels before and after valproate treatment and recorded only a significant decrease in IL-6 levels, along with a decrease in seizures (Steinborn *et al.*, 2019). Although valproate treatment also led to a decrease in seizures in the mild epilepsy group in our study, we cannot directly compare the two studies due to differences in their design.

TNF-α levels in the present study were higher in the severe epilepsy group than in the mild epilepsy and control groups. A promising pilot study in which TNF-α antibodies were used for therapeutic purposes (Lagarde *et al.*, 2016) led us to select this cytokine as one of the markers in the present study, and the results are encouraging in terms of future treatment strategies.

The association between inflammation and epilepsy is a subject that has recently led to new hope in the treatment of patients with refractory epilepsy. Although numerous experimental animal studies have been performed, the number of studies involving clinical observations is limited. Our aim in the present study was to perform rigorous clinical observation by combining all these markers that have previously been evaluated separately in different studies. We hope that the finding of significantly high levels of cytokines in the severe epilepsy group will encourage further research which may lead to new hope for the treatment of patients with refractory epilepsy.

In this study we intended to evaluate the relationship between MRI findings and the above-mentioned cytokines, and all patients' cranial MRI results were accessed for that purpose. However, since significant anomalies in MRI findings were only observed in the severe epilepsy group, it was not possible to establish new groups on the basis of the imaging results and to subject these to statistical analysis. Detailed clinical data and MRI findings in the severe epilepsy group are summarized in *table 4*. Hypoxic injury, frequently determined as an MRI finding in these patients, was particularly notable.

One of the limitations of our study is the low patient number, together with the limited number of markers studied. However, although several animal studies on the subject have previously been reported, the number of previous clinical studies is limited and we hope that our research will serve as a source of inspiration for future studies. □

Supplementary data.
Summary didactic slides are available on the www.epilepticdisorders.com website.

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TEST YOURSELF

(1) What is neuroinflammation?

(2) Why is neuroinflammation important?

(3) Based on this study, which are the most important inflammatory markers in neuroinflammation and epilepsy?

Note: Reading the manuscript provides an answer to all questions. Correct answers may be accessed on the website, www.epilepticdisorders.com, under the section "The EpiCentre".