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Evaluation of serum HMGB-1 and serum P-glycoprotein levels in epileptic patients: Associations with clinical features, seizure patterns, and drug resistance

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ABSTRACT

This study aimed to evaluate the association of serum HMGB-1 and serum P-gp levels with clinical characteristics, seizure patterns, and drug resistance in patients with epilepsy. Patients were divided into monotherapy (n=40) and polytherapy (n=40) groups, and their serum HMGB-1 and serum P-gp levels were analysed using the ELISA technique. In the multinomial logistic regression model, serum P-gp levels were significantly higher in the polytherapy group compared to the healthy control group (p=0.021). Additionally, to the best of our knowledge, this is the first study to analyse the correlation between serum HMGB-1 and serum P-gp levels. In this study, a moderate positive correlation

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was found between serum HMGB-1 and serum P-gp levels in the monotherapy group of epilepsy patients.

Keywords: drug resistance, epilepsy, HMGB-1, P-glycoprotein, serum

INTRODUCTION

Epilepsy is a chronic neurological disease that affects a significant portion of the global population, approximately 70 million individuals[1]. The development of at least two unprovoked febrile seizures within 24 hours, a single unprovoked seizure with more than 60% risk of re-seizure or the presence of an epileptic syndrome is defined as epilepsy[2]. According to World Health Organization (WHO) reports, 5 million people are diagnosed with epilepsy every year[3]. Besides causing problems such as loss of consciousness, impairment in motor functions and sensory impairment, it may also lead to sudden unexpected death (SUDEP). It has been reported that the rate of SUDEP in patients with epilepsy is 23 times higher than in the general population of the same age[4]. This makes epilepsy a serious health problem and it is crucial to understand the mechanisms playing a role and to develop effective treatment regimens.

Although a large number of antiepileptic drugs are currently used to treat epilepsy, a significant proportion of people with epilepsy (about 30%) do not achieve seizure freedom with pharmacotherapy despite the use of two or more appropriately selected antiepileptic drugs, singly or in combination[5]. This patient group is referred to as drug-resistant epilepsy patients[6].

Inflammation is known to be one of the major mechanisms involved in epileptogenesis and the development of resistant epilepsy[7,8]. As a result of inflammatory response in pathological conditions, physiological and functional changes occur in neuronal and glial cells through cytokines and immune cells[9,10]. Clinical research and experimental animal studies provide evidence that inflammation triggers acute and chronic seizures and negatively impacts prognosis[11-15]. Moreover, the presence of active inflammation has been detected in surgically resected epileptic tissues from patients with refractory epilepsy[8,16-19]. Thus, inflammation may be considered as an important target mechanism for new drug development research in epilepsy.

Therefore, inflammatory markers have been one of the focus of epilepsy studies for many years[20-22]. High mobility group box 1 (HMGB-1) is one of the proteins with proinflammatory activity that mediates systemic inflammatory response[23]. While HMGB-1 is found in neuronal nuclei under physiological

conditions, it is expressed from microglia and astrocytes depending on seizure activity and transported to the cytoplasm[13]. HMGB-1-mediated signalling cascades play a role in neuronal dysfunction, cognitive impairment, oxidative stress and NMDA receptor activation, and have been associated with seizure severity, drug resistance and seizure triggering in epilepsy[10,24-26].

Another effective mechanism in the development of multidrug resistance in epilepsy patients is ATP-binding cassette (ABC) transporter proteins. These transmembrane proteins utilize the energy derived from ATP hydrolysis to actively efflux a wide range of substrates across the cellular membrane[27]. P-glycoprotein (P-gp) is an important ABC transporter protein associated with multidrug resistance and has been determined to be overexpressed in refractory epilepsy in both clinical and *in vivo* studies[28]. Increased expression of P-gp in the blood brain barrier and in specific brain regions leads to subtherapeutic drug doses and lack of response to treatment[29,30].

Recent studies have investigated the potential relationship between HMGB-1 levels and P-gp expression[31]. Chen et al. reported that in a kainic acid-induced epilepsy model in mice, HMGB-1 administration increased P-gp expression in the brain, whereas HMGB-1 antagonist administration decreased P-gp expression[31]. To the best of our knowledge, this is the only study evaluating the relationship between HMGB-1 and P-gp specifically in epilepsy and there is no clinical study examining this potential relationship. It was thought that there may be a relationship between the serum levels of these two important proteins, which are associated with epilepsy pathogenesis and drug resistance under pathological conditions. This study aimed to determine serum HMGB-1 and P-gp levels in epilepsy patients and evaluate their potential associations with clinical characteristics, seizure patterns, and drug response. Furthermore, it is the first study to investigate the correlation between serum P-gp and HMGB-1 levels. This study is expected to improve our understanding of serum P-gp and HMGB-1 levels in the clinical profile of epilepsy patients and provide insight into whether the levels of these proteins may be predictive biomarkers.

METHODOLOGY

Study design and patient selection

Our study included patients diagnosed with epilepsy and healthy volunteers who applied to the Neurology Department of Haydarpaşa Numune Training and Research Hospital and Ümraniye Training and Research Hospital. The study included a total of 80 epilepsy patients and 40 healthy control volunteers. These 80 epilepsy patients were divided into monotherapy (n=40)

and polytherapy (n=40) groups. The age range of the patients and volunteers included in the study was determined as 18-50 years old men and women. Exclusion criteria were as follows; patients with cancer or haematological disease, patients with recent severe infection, patients with renal or hepatic dysfunction, patients taking glucocorticoids or immunosuppressive drugs.

Blood samples were collected by trained personnel early in the morning after 8 hours of fasting. Serum was obtained from whole blood by centrifugation and stored at -80°C. HMGB-1 and P-gp levels were analysed from serum samples. Correlation evaluation was performed between serum HMGB-1 and P-gp levels of the patients.

Our study was conducted in accordance with the Declaration of Helsinki and with the approval of Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee with the number E-10840098-772.02-7713. Informed consent forms were obtained from all patients and healthy volunteers included in the study.

Clinical assessment and data collection

The trained personnel performing the analyses were blinded to the clinical information. Parameters such as age, sex, antiepileptic drugs used, comorbidities, seizure types, seizure frequency (presence of seizures in the last 6 year or more than 3 seizures per month), and presence of resistant epilepsy were analysed using outcome data from patient electronic medical records. The presence of resistant epilepsy was considered in accordance with the definition of the ILAE drug-resistant as "failure of adequate trials of two tolerated and appropriately chosen AED schedules (whether as monotherapies or in combination) to achieve seizure freedom".

Determination of P-gp and HMGB-1 blood levels

Samples were stored at -80°C until the day of analysis. Each sample was analysed in duplicate according to the manufacturer's instructions. Serum HMGB-1 and P-gp levels were analysed using commercially available ELISA kit. For the HMGB-1 analysis, BT Lab ELISA Kit (Cat. No. E1635Hu, China) was used and the sensitivity was 0.24 ng/mL. For P-gp analysis, SunRed Company ELISA Kit (Cat. No. 201-12-7302, China) was used and sensitivity was 0.132 ng/mL.

Statistical analysis

Qualitative variables reported as frequencies and percentages (%) while quantitative ones are represented as means ± standard deviations (sd). The

normality assumption is checked using graphical methods, Kolmogorov-Smirnov and Shapiro-Wilk tests. The quantitative variables are not normally distributed. Comparison of two groups differences are computed using Mann-Whitney U test. The Kruskal-Wallis H test used to compare between three groups with Mann-Whitney U test and Bonferroni's correction. The chi-squared test (or Fisher's exact test) is used to compare between qualitative variables. Spearman's rank correlation is used to find correlations between serum levels. Logistic regression and multivariate logistic regression are performed to find adjusted p-values. Analyses are performed with IBM SPSS 24.0. Results are evaluated at the 5% significance level.

RESULTS and DISCUSSION

Patients and reference group

The demographic and clinical characteristics of each patient group including age, sex, comorbidity, duration of epilepsy diagnosis, antiepileptic drugs used in treatment, presence of refractory epilepsy, seizure types, seizure frequency, and seizure-free periods are presented in Table 1. In addition, serum P-gp and serum HMGB-1 values were analysed for both patient groups and healthy control groups. There was no significant difference between the patient groups and the control group in terms of serum HMGB-1 ($p=0.126$), but there was a significant difference between the groups in terms of serum P-gp levels ($p<0.001$).

Table 1. Demographic and clinical characteristics of the control group and patients groups in this study

Variable		Control	Monotherapy	Polytherapy	p-value
Age, years (mean ± SD)		37.58 ± 8.578	31.44 ± 9.99	35.48 ± 9.394	0.009
Sex	female, n (%)	23 (35.90%)	25 (39.10%)	16 (25.00%)	0.13
	male, n (%)	17 (28.10%)	16 (28.10%)	24 (42.10%)	
Comorbidities, n (%)			16 (40.00%)	20 (50.00%)	0.369
Duration of disease (years)	<10 years		14 (35.00%)	4 (10.00%)	0.007
	>10 years		26 (65.00%)	36 (90.00%)	
Antiepileptic drug resistance, n (%)	present		4 (12.50%)	28 (87.50%)	p<0.001
	not present		36 (75%)	12 (25%)	
Seizure types, n (%)	generalised		26 (65.00%)	13 (32.50%)	0.004
	focal to tonic-clonic		14 (35.00%)	27 (67.50%)	

Seizure frequency, n (%)	none		28 (70%)	12 (30.00%)	0.001
	rare		9 (22.50%)	12 (30.00%)	
	often		3 (7.50%)	7 (17.50%)	
	very often		0 (0.0%)	9 (22.50%)	
Seizure-free period, n (%)	>1 years		27 (67.50%)	13 (32.50%)	0.002
	<1 years		13 (32.50%)	27 (67.50%)	
HMGB-1, ng/ml (mean ± SD)		31.05 ± 15.03	45.32 ± 40.41	31.79 ± 24.4	0.126
P-glycoprotein, ng/ml (mean ± SD)		10.36 ± 8.95	13.96 ±13.81	16.08 ± 13.86	p<0.001

*Chi-square test was performed for comparisons between qualitative variables. The nonparametric Kruskal-Wallis test, with Bonferroni correction, was performed for comparisons involving three groups. The <0.05 p-value was considered statistically significant.

Patients were classified according to the number of epileptic seizures in the last year as >53 times/year (very often), 13-52 times/year (often), 1-12 times/year (rare) and no seizure (none)[32]. In our study, it was founded that the number of seizures in the last year was significantly higher in the polytherapy group compared to the monotherapy group (p=0.001) Moreover, it was found that when patients with more than three seizures per month were analysed, this rate was statistically significantly higher in the polytherapy group compared to the monotherapy group (p=0.018). Also, antiepileptic drug resistance was analysed in the patient groups. Drug resistance was significantly higher in the polytherapy group compared to the monotherapy group (0.125 ,0.875; p<0.001). In addition, the proportion of patients with epilepsy duration of more than 10 years was statistically higher in the polytherapy group than in the monotherapy group (0.65, 0.90; p=0.007). Moreover, the polytherapy and monotherapy groups were significantly different in terms of seizure type (p=0.004). In the monotherapy group, generalised seizures were seen in 65.2% of the patients, whereas in the polytherapy group, focal to tonic clonic seizures were seen in 67.5% of the patients. However, no significant difference was found between monotherapy and polytherapy patient groups in terms of comorbidity status (p=0.369). Recorded comorbidities included cardiac disorders, diabetes, depression, mood disorders, hypothyroidism, and asthma.

The percentages of drugs administered in monotherapy and polytherapy patient groups are presented in Table 2. The most commonly used drugs in the monotherapy group were levetiracetam (LEV/32.5%) and valproic acid (VPA/30.0%), while the most common combination in the polytherapy group was levetiracetam-carbamazepine (LEV & CBZ /42.5%). In the polytherapy group, all except one patient (LTG, LCM, PHE) were treated with dual drug therapy.

Table 2. Antiepileptic drug treatments used in monotherapy and polytherapy groups

Drug used in the monotherapy, n (%)	Drug combinations used in the polytherapy, n (%)
VPA, 12 (30%)	VPA &CBZ , 4 (10 %)
LEV, 13 (32.5)	VPA & LTG , 1 (2.5%)
LTG, 5 (12.5%)	LEV & CBZ , 17 (42.5%)
CBZ, 8 (20%)	LEV & VPA , 10 (25%)
PHT , 1 (2.5%)	LEV & ZNS , 2 (5%)
TPM, 1 (2.5%)	LEV & LCM , 1 (2.5%)
	LEV & TPM , 1 (2.5%)
	LEV & LTG , 1 (2.5%)
	LTG & LCM & PHT , 1 (2.5%)
	CBZ & LCM , 1 (2.5%)
	CBZ & LTG , 1 (2.5%)

*VPA Valproic acid, LEV Levetiracetam, LTG Lamotrigine, CBZ Carbamazepine, PHT Phenytoin, TPM Topiramate, ZNS Zonisamide, LCM Lacosamide

Association between serum HMGB-1, serum P-gp, and clinical variables in epilepsy patients

In our study, no significant difference was found in serum P-gp (p=0.924) or serum HMGB-1 levels (p=0.107) when patients were analysed according to seizure frequency (Table 3 and Table 4). Additionally, the seizure types of the patients were examined and classified as generalized or focal to tonic-clonic seizures. Statistical analyses revealed no significant difference in serum HMGB-1 and serum P-gp levels based on seizure type. Similarly, no significant association was found between drug resistance, seizure-free periods and duration of disease parameters and serum HMGB-1 or serum P-gp levels (Table 3 and Table 4).

Table 3. Factors associated with serum HMGB-1 levels

Factors associated with serum HMGB-1 levels						
Variables		**N**	**Mean**	**Standard Deviations (sd)**	**Min-Max**	**p-value**
Antiepileptic Drug resistance	present	32	33.6	26.74	13.77-94.96	0.065
	not present	48	41.86	37.79	17.08-140.42	
Seizure frequency	none	40	39.32	5.66	17.08-140.42	0.107
	rare	21	46.01	8.40	16.87-132.97	
	often	10	28.80	7.75	13.77-94.96	
	very often	9	28.60	5.84	17.03-60.88	
Seizure type	generalised	39	39.74	34.42	13.77- 140.42	0.07
	focal to tonic-clonic	41	37.43	33.72	16.87-124.26	
Seizure-free period	more than 1 year	40	38.74	33.58	17.08-140.42	0.191
	less than 1 year	40	38.37	34.58	13.77-132.97	
Duration of disease (years)	more than 10 year	62	37.27	32.49	13.77-140.42	0.454
	less than 10 year	18	42.97	38.93	17.03-132.97	

*The analyses reported in the table were performed for all epilepsy patients, including monotherapy and polytherapy (n=80). Mann Witney-U and Kruskal Wallis tests were used for statistical analyses. The $p<0.05$ value was considered statistically significant.

Table 4. Factors associated with serum P-gp levels

Factors Associated with Serum P-gp Levels						
Variables		**N**	**Mean**	**Standard Deviations (sd)**	**Min-max**	**p-value**
Antiepileptic Drug resistance	present	32	15.88	11.07	6.93-50.42	0.298
	not present	48	17.8	15.3	6.73-61.13	
Seizure frequency	none	40	15.23	13.86	2.08-60.21	0.924
	rare	21	15.07	15.39	3.01-50.94	
	often	10	13.89	12.09	4.92-43.92	
	very often	9	15.25	13.46	6.73-47.49	
Seizure type	generalised	39	15.44	14.15	2.08-59.94	0.567
	focal to tonic-clonic	41	14.62	13.58	3.01-60.21	
Seizure-free period	more than 1 year	40	13.94	11.81	2.08-50.23	0.769
	less than 1 year	40	16.01	15.58	3.01-60.21	
Duration of disease (years)	more than 10 year	62	14.75	13.02	2.47-50.94	0.765
	less than 10 year	18	15.94	16.52	2.08-60.21	

*The analyses reported in the table were performed for all epilepsy patients, including monotherapy and polytherapy (n=80). Mann Witney-U and Kruskal Wallis tests were used for statistical analyses. The $p<0.05$ value was considered statistically significant.

A logistic regression was performed to ascertain the effects of age, gender, seizure types, duration of disease and comorbidities, HMGB-1 and P-gp on the likelihood that patients have polytherapy group (Table 5). The logistic regression model was statistically significant, $\chi2(8)=34.850$, $p<0.0001$ (Hosmer and Lemeshow $\chi2(8)=13.492$, $p=0.096$). The model explained 47.1% (Nagelkerke R2) of the variance in patient groups and correctly classified 78.8% of cases. Seizure-free period was statistically higher monotherapy patients than polytherapy patients (OR=5.30; p=0.03). Serum HMGB-1 levels were statistically higher on monotherapy patients than polytherapy patients (OR=5.3; p=0.002) whereas serum P-gp levels were higher on polytherapy patients (OR=1.06; p=0.051).

Table 5. Factors associated monotherapy and polytherapy patient groups

	Estimate	Standard Error	Sig.	Odds Ratio (OR)	95% CI.for OR	
					Lower	Upper
Age	-0.002	0.031	0.943	0.998	0.939	1.06
Gender	1.017	0.582	0.081	2.764	0.883	8.653
Seizure type	1.518	0.6	0.011	4.561	1.408	14.778
Duration of disease	1.772	0.787	0.024	5.881	1.257	27.508
Comorbidities	-0.429	0.649	0.508	0.651	0.183	2.32
Seizure-free period	-1.665	0.628	0.008	0.189	0.055	0.648
HMGB-1 levels	-0.03	0.012	0.012	0.97	0.948	0.993
P-glycoprotein levels	0.056	0.029	0.051	1.058	1	1.119

* The $p<0.05$ value was considered statistically significant.

A multinomial logistic regression was used to create a model of the relationship between serum levels in the three groups (monotherapy, polytherapy, control) after adjusting age and gender (Table 6). The model is statistically significant ($\chi2(8)=24.328$, Nagelkerke $R^2=0.206$, $p<.001$). P-gp values are statistically higher on polytherapy group compared to healthy control group ($p=0.021$; OR=1.063).

Table 6. Factors associated with three groups

		Estimate	SEM	p-value	OR	95% Confidence nterval for OR	
Monotherapy	Age	-0.064	0.026	0.013	0.938	0.891	0.987
	HMGB-1	0.013	0.01	0.193	1.013	0.993	1.034
	P-gp	0.009	0.027	0.723	1.009	0.958	1.064
	Sex	0.108	0.483	0.823	1.114	0.432	2.871
Polytherapy	Age	-0.026	0.025	0.288	0.974	0.927	1.023
	HMGB-1	-0.017	0.012	0.178	0.984	0.96	1.008
	P-gp	0.061	0.026	0.021	1.063	1.009	1.119
	Sex	-0.777	0.474	0.101	0.46	0.182	1.164

* The $p<0.05$ value was considered statistically significant.

Correlation between serum HMGB-1 and serum P-gp levels in epilepsy patients

Figure 1 presents the correlation analyses between serum P-gp and serum HMGB-1 levels in our study groups. In the monotherapy patient group, serum P-gp levels showed a positive correlation with serum HMGB-1 levels. In the polytherapy group, a low positive correlation was found. However, no significant correlation was observed in the healthy control group.

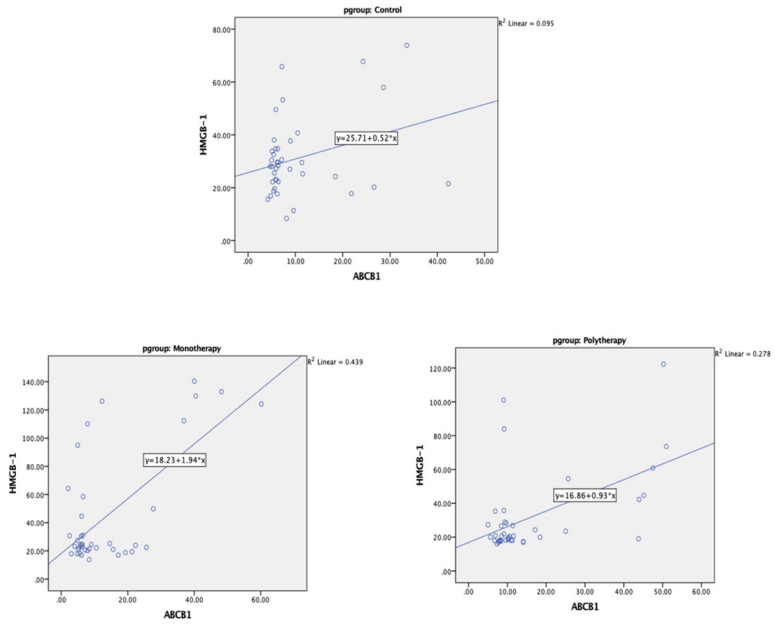

Figure 1. Correlations between serum P-gp levels and serum HMGB-1 levels

ABCB1 (serum P-glycoprotein level, ng/ml), HMGB-1 (serum HMGB-1 level, ng/ml). Spearman correlation coefficient was used because the variables were not normally distributed. For the correction, Pearson correlation analysis was used in the graphs.

HMGB-1 increases P-glycoprotein (P-gp) activity in the epileptic brain through activation of the TLR4/RAGE/NF-κB signalling pathway[33]. Activation of the TLR4 receptor complex leads to the stimulation of nuclear factor kappa B (NF-κB). HMGB-1 also binds to the receptor for advanced glycation end products (RAGE), thereby enhancing the biological effects mediated by NF-κB[34]. The RAGE/NF-κB plays a critical role in the regulation of inflammation[35]. NF-κB activation can induce the release of extracellular glutamate, which in turn stimulates N-methyl-D-aspartate (NMDA) receptors and may contribute to the overexpression of P-gp[31]. Consequently, HMGB-1 and P-gp expression are known to be linked through the TLR4/RAGE/NF-κB pathway[33]. By activating this pathway in brain endothelial cells, HMGB-1 acts a pro-epileptic effect and play a role in the development of drug resistance in epileptic brain regions characterized by P-gp overexpression[31,36,37]. In line with the findings obtained from our study, we report a moderate positive correlation between serum P-gp and serum HMGB-1 levels in epileptic patients for the monotherapy patient group and a low positive correlation for the polytherapy patient group. It is noteworthy

that, to the best of our knowledge, this is the first clinical study investigating the correlation between serum HMGB-1 and serum P-gp in epilepsy.

In our study, the rate of drug-resistant patients was significantly higher in the polytherapy group compared to the monotherapy group ($p<0.001$). This finding suggests a potential positive correlation between serum HMGB-1 and serum P-gp levels in patients with refractory epilepsy. Previous studies have suggested that HMGB-1 levels may influence P-gp expression in various *in vivo* and *in vitro* disease models[31,33,38]. It has been reported that increased HMGB-1 levels can promote drug resistance by upregulating P-gp expression in human gastric adenocarcinoma cells, while high levels of HMGB-1 in a xenograft lung cancer model exhibited similar co-localization with P-gp in the cytoplasm[38,39]. Furthermore, overexpression of P-gp in the epileptic rat brain has been shown to be regulated by HMGB-1 through the RAGE/NF-κB signalling pathway[35]. In another study, different results were observed between blood HMGB-1 and tissue P-gp levels in kidney and liver tissues in a lipopolysaccharide-induced inflammation model in mice[40]. In this model, the increase in blood HMGB-1 was associated with upregulation of P-gp in the kidney, whereas no such association was found in the liver.

When compared to the polytherapy group, long-term seizure freedom was significantly higher (0.67, $p=0.002$) and seizure frequency was significantly lower ($p=0.001$) in the monotherapy group. Additionally, drug resistance was observed in 12.5% of the monotherapy group and 87.5% of the polytherapy group. All these findings explain the need for more than one drug in the treatment of polytherapy group patients. A clinical study reported that serum P-gp levels were significantly higher in the pharmacoresistant group in systemic lupus erythematosus compared to both the healthy and drug-responsive groups[41]. Also, several clinical and *in vivo* studies have reported that P-gp is overexpressed at the blood–brain barrier and various brain regions in drug-resistant forms of epileps[42-45]. In a clinical study, P-gp expression was found to be significantly higher in the temporal cortex of drug-resistant MTLE patients[42]. Similarly, Liu et al. found a highly localised P-glycoprotein overexpression in the hippocampus of refractory epilepsy patients[46]. The results of our study are in a similar line with this study. However, no significant correlation was found between drug response and serum P-gp or serum HMGB-1 levels. It is hypothesized that the limited sample size in the patient groups and wide variation of drugs in the polytherapy group may have contributed to these results. Therefore, further studies with larger sample sizes are needed to investigate the potential biomarker properties of serum P-gp and serum HMGB-1 levels in epilepsy.

STATEMENT OF ETHICS

This study was conducted in accordance with the Declaration of Helsinki and with the approval of Istanbul Medipol University Non-Interventional Clinical Research Ethics Committee, with the number E-10840098-772.02-7713 on December 12,2023. Informed consent forms were obtained from all patients and healthy volunteers included in the study.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

Design, EA; acquisition of data, EN, TO, GG; analysis, EA; drafting of the manuscript, EA, statistical analysis, PY; critical revision of the manuscript, GG, PY, AAS, YC; technical or financial support, Istanbul Medipol University Scientific Research Projects (BAP) Committee; supervision, AAS, YC.

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