■ Original article

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Epileptic Disorders

Relationship between seizure type, metabolic profile, and inflammatory markers in blood samples of patients with epilepsy

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ABSTRACT

Objective. We investigated the metabolic profile, reactive species production, and inflammatory parameters in patients with epilepsy. Furthermore, we investigated whether there is any relationship between these parameters and seizure type.

Methods. Patients with epilepsy (n=43) and healthy subjects (control group; n=41) were recruited to participate in the study. Initially, the participants were submitted to a clinical questionnaire and patients with epilepsy were classified according to seizure type. Metabolic markers and inflammatory and oxidative factors were also measured in specific blood samples. We compared these results with data from the control subjects.

Results. Statistical analyses showed that patients with epilepsy presented with higher levels of glycolipid, oxidative stress, and inflammatory parameters compared to the control subjects. Interestingly, patients with generalized seizures presented with higher MnSOD activity and metabolic parameters (total cholesterol, low-density lipoprotein, glucose and triglyceride levels) compared to the partial seizure and control groups. Furthermore, patients with generalized epilepsy demonstrated a significant correlation between TNF-α and caspase 8 (p<0.05), caspase 3 (p<0.05), and Picogreen (p<0.001).

Significance. This study supports evidence that the levels of inflammatory, glycolipid, and oxidative factors are higher in epilepsy patients, especially those with generalized epilepsy.

Key words: apoptosis; DNA damage; epilepsy; inflammation; metabolism; oxidative stress

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Epilepsy is usually characterized by recurrent spontaneous seizures and results from a complex series of pathophysiological events, including increased reactive species generation [1], inflammation [2], and apoptotic cell death [3]. Furthermore, studies have demonstrated significant associations between epilepsy with cardiovascular and cerebrovascular comorbidities [4, 5]. In fact, considerable interest in seizure-related cardiac abnormalities has emerged, particularly because previous studies have shown a relationship between cholesterol levels and epilepsy [6, 7], which likely alters markers of vascular risk [8]. In this context, Hermann *et al*. [7] demonstrated that patients with chronic epilepsy exhibit multiple abnormalities in metabolic, vascular, and inflammatory systems. In fact, a relationship between pro-inflammatory cytokine levels and

seizure type has been demonstrated [9]. Furthermore, Lehtimäki *et al*. [9] observed that generalized seizures were associated with higher cytokine levels than partial seizures, suggesting that inflammation is related to seizure activity and intensity. While the interaction between inflammation and lipid accumulation is well described in neurovascular diseases, such as stroke [10], molecular mechanisms and the link between these events with epilepsy are not fully understood.

In line with this view, and since inflammation is related to lipid levels [10], the antiepileptic activity of drugs with cholesterol-lowering effects has also been studied. In fact, statin treatment reduces picrotoxin-, pilocarpin-, and kainic acid (KA)-induced seizures as well as inflammation and cell death in the hippocampus [11-13], implying that there is an important relationship between lipid metabolism and inflammation in epilepsy. In addition, recent studies also revealed that anti-inflammatory and antioxidant effects of statins decreased the risk of hospitalization in epilepsy patients [14-16].

Another point of interest in epilepsy pathophysiology is the relationship between intracellular reactive species (RS) and signal transduction associated with TNF-α following binding to its receptor. Moreover, some important discoveries have further highlighted the critical role of RS in TNF-α-mediated activation of cell death pathways [17]. Although some studies using experimental models have demonstrated a bidirectional link between epilepsy and apoptosis [8], the complex signalling pathways leading to neuronal apoptosis in epilepsy have not yet been completely elucidated.

Despite the increased vascular risks in patients with epilepsy being well known, there have been few attempts to reduce such risk [18] and elucidate the factors that may be associated with metabolic abnormalities in epilepsy patients. Thus, even though metabolic markers (glycolipid levels) also appear to be involved in the inflammatory pathways in epilepsy, the molecular mechanisms and link between metabolic alteration and seizures have yet to be determined. Thus, the aim of this study was to investigate whether the glycolipid profile and inflammatory, apoptotic, oxidative stress, and DNA damage biomarkers are related to epilepsy and whether these biochemical parameters are related to seizure type.

Methods

Study of the population

We prospectively recruited 43 patients with probable symptomatic epilepsy from June 2013 to July 2017 at our institution. No aetiology was found after detailed analysis of history and physical, laboratory, and imaging data. Major exclusion criteria were history of autoimmune, liver, kidney, and inflammatory diseases, allergic response, immune deficiency disorder, diabetes, psychiatric illness, malignancy, smoking or systemic or central nervous system (CNS) infection two weeks before sample collection. Epilepsy was diagnosed by two experienced neurologists according to the 2010 International League Against Epilepsy (ILAE) classification [19]. All patients were evaluated for seizure frequency using seizure diaries [20]. Afterwards, the epilepsy patients and healthy subjects received a protocol number, and blood samples were identified using the protocol number of each individual. The study protocol was approved by the local institutional review board at the authors' affiliated institutions. Informed consent was obtained from all the subjects or their legal guardians. The work described was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

- ***Epilepsy group***

The seizure type for the epilepsy group (*n*=43) was confirmed through interviews with the patients and their relatives, as well as EEG analysis and tomography or magnetic resonance imaging. In the epilepsy group with generalized seizures (*n*=23), 20 patients had no identified aetiology, one patient with genetic aetiology met the criteria for Marden-Walker syndrome, and one patient had an episode of encephalitis. In the epilepsy group with partial seizures (*n*=20), 15 patients had no identified aetiology, four had focal ischaemic lesion, and one had a tumour.

Data on seizure frequency and status of seizure control with medication were also obtained. Forty-one patients were in remission except for two patients who were diagnosed with refractory epilepsy. All patients with epilepsy had normal neurological examinations except for one who presented with tetra paresis secondary to a spinal cord lesion, another patient with dysarthria, and one patient with right upper limb paresis. Thirty-six patients had normal 1.5T MRI imaging, four had a focal ischaemic lesion, one had a tumour, one patient had encephalitis as a sequela, and one patient had right and left hippocampal sclerosis.

- ***Control group***

Forty-one sex-matched healthy volunteers were included in the control group.

Laboratory analyses

Samples were collected at least seven days from the last seizure attack [21]. After 12 hours of overnight fasting, blood samples were collected by venipuncture using purple, green, and red top Vacutainers® (BD Diagnostics, Plymouth, UK) tubes with ethylenediamine tetra acetic acid (EDTA), heparin or no anticoagulants, respectively. Specimens were routinely centrifuged within one hour of collection for 15 minutes at 2,500 g and aliquots of the serum samples and supernatant

were saved and stored at -80 °C for subsequent laboratory analyses according to specific methods. Plasma thiobarbituric acid reactive substances (TBARS) were measured according to the modified method of Jentzsch *et al.* [22] The carbonylation of plasma proteins was determined according to Reznick and Packer [23]. Protein was measured by the method of Bradford using bovine serum albumin as standard. The levels of alpha tumour necrosis factor (TNF-α) (eBIOSCIENCE, San Diego, USA) were measured in serum by enzyme-linked immunosorbent assay (ELISA) as instructed by the manufacturers. Caspase 8 and 3 activities were measured in serum by Assay Kits, Fluorimetric (BioVision, Mountain View, CA). The Picogreen test was determined in plasm according to Há *et al.* [24]. Catalase activity was measured in whole blood using the method of Aebi, by measuring the rate of H_2O_2 decomposition at 240 nm. Whole-blood superoxide dismutase activity was measured, as described by McCord and Fridovich. Glucose levels were measured in plasma while total cholesterol, low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), and triglycerides (TRI) were measured in serum. These techniques were performed using standard enzymatic methods with Ortho-Clinical Diagnostics® reagents on a fully automated analyser (Vitros 950® dry chemistry system; Johnson & Johnson, Rochester, NY, USA).

Statistical analyses

Data were analysed by unpaired *t* test when appropriate and expressed as mean and standard error of the mean (SEM). Statistical analyses were performed using the SPSS (Statistical Package for the Social Sciences) software in a PC-compatible computer. Correlation analyses were carried out using Spearman's correlation coefficient. Differences between groups according to seizure type and the investigated parameters of the epilepsy group were evaluated by analysis of variance (one-way ANOVA) followed by Tukey's Multiple Comparison Test. Statistical significance was assumed when $p<0.05$.

▼ **Table 1.** Clinical characteristics of epileptic and control groups.

Characteristic	Epilepsy group (*n*=43)	Control group (*n*=41)
Gender (%)		
Male	20 (46.51%)	21 (51.21%)
Female	23 (53.48%)	20 (48.78%)
Mean age (years)		
Male	38.5	40.6
Female	33.6	45.5
Mean age at onset (years)	8 (23.25%)	0
Mean duration of seizure (minutes)	7.5 (18.60%)	0
AED use (%)		
No treatment	0	41 (100%)
One drug	15 (34.88%)	0
≥ Two drugs	28 (65.11%)	0

AED: Antiepileptic drug. Data are expressed as percentage.

Results

Eighty-four subjects were enrolled in this study, which consisted of 43 patients with epilepsy (Epilepsy group) and 41 controls (Control group). Baseline characteristics and clinical aspects of the participants are described in *table 1*. Statistical analyses showed a significant association ($p<0.05$) between TNF-α, caspase 8, caspase 3, and Picogreen and cholesterol, glucose, and LDL in the epilepsy group (*table 2*). We did not observe any significant statistical differences regarding an association between TRI and TNF-α, caspase 8, caspase 3, or Picogreen ($p<0.05$).

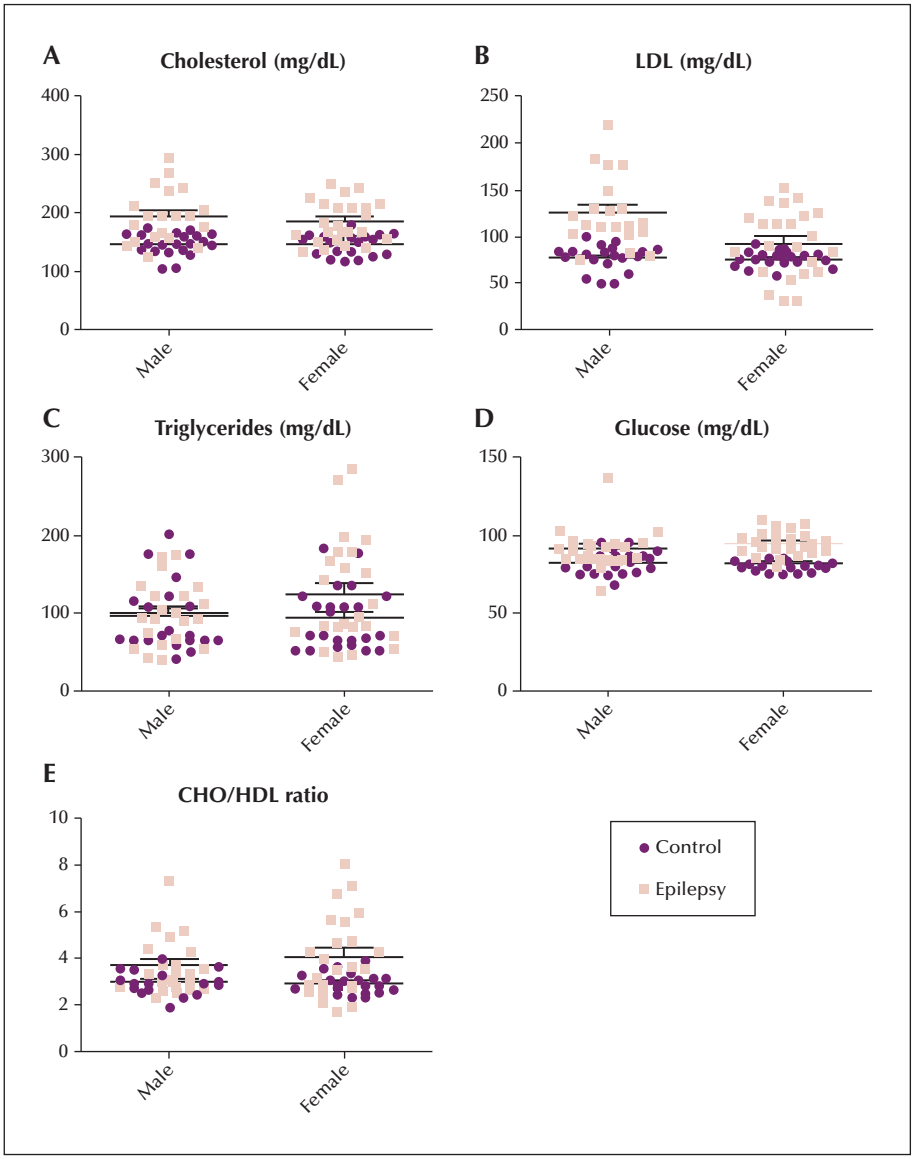
▼ **Table 2.** Association between Picogreen, caspases and TNF-α and cholesterol, LDL, glucose and triglycerides in epilepsy patients.

	TNF-α	CASP8	CASP 3	PG
CHO	[F (2.73) = 812.2 p<0.0001]*	[F (2.68) = 783 *p*<0.001]*	[F (2.68) = 783 *p*<0.001]*	[F (2.57) = 891.2 *p*<0.001]*
TG	[F (2.67) = 1970]	[F (2.68) = 2365]	[F (2.68) = 2365]	[F (2.57) = 2441]
GLU	[F (2.72) = 90.57 *p*<0.0001]*	[F (2.72) = 90.57 *p*<0.0001]*	[F (2.72) = 90.57 *p*<0.0001]*	[F (2.61) = 101.9 *p*<0.001]*
LDL	[F (2.68) = 606.2 *p*<0.0001]*	[F (2.68) = 606.2 *p*<0.0001]*	[F (2.68) = 606.2 *p*<0.0001]*	[F (2.57) = 711.6 *p*<0.0001]*

PG: Picogreen; CASP8: caspase 8; CASP3: caspase 3; TNF: tumour necrosis factor; CHO: Total cholesterol, TG: triglycerides, GLU: glucose. Data are expressed as mean ± standard error of the mean (SEM). *Significant values when p < 0.05.

Assessment of metabolism parameters (*figure 1*)

The epilepsy group presented with increased levels of total cholesterol (CHO) (189.7 ± 6.11 mg/dL) (t=6.497, df=59, $p<0.0001$) vs. controls (145.9 ± 2.83), LDL (109.8 ± 6.05 mg/dL) (t=5.26, df=47, $p<0.0001$) vs. controls (76.63 ± 1.77), TRI (113.7 ± 8.94 mg/dL) (t=1.62 df=76, $p<0.05$) vs. controls (95.61 ± 6.63), and glucose (93.47 ± 1.6 mg/dL) (t=5.65 df=70, $p<0.0001$) vs. controls (82.27 ± 1.06). No significant results were noted for HDL when the epilepsy group was compared with the controls (data not shown). Furthermore, a significant CHO/HDL ratio was observed in patients with epilepsy (3.863 ± 0.26) (t=3.51 df=72, $p<0.001$) when compared to the control group (2.958 ± 0.07).



■ **Figure 1.** Comparison of metabolic parameters (level of cholesterol, LDL, triglycerides and glucose) in epilepsy and control groups (female and male). The epileptic group had higher levels of CHO (A), LDL (B), triglycerides (C) and glucose (D) compared to the control group. A significant CHO/HDL ratio (E) was observed in the epilepsy group when compared to the control group. Significance was considered at $p<0.05$, according to the Student's t-test.

Assessment of inflammatory, apoptotic, and DNA damage markers (*figure 2*)

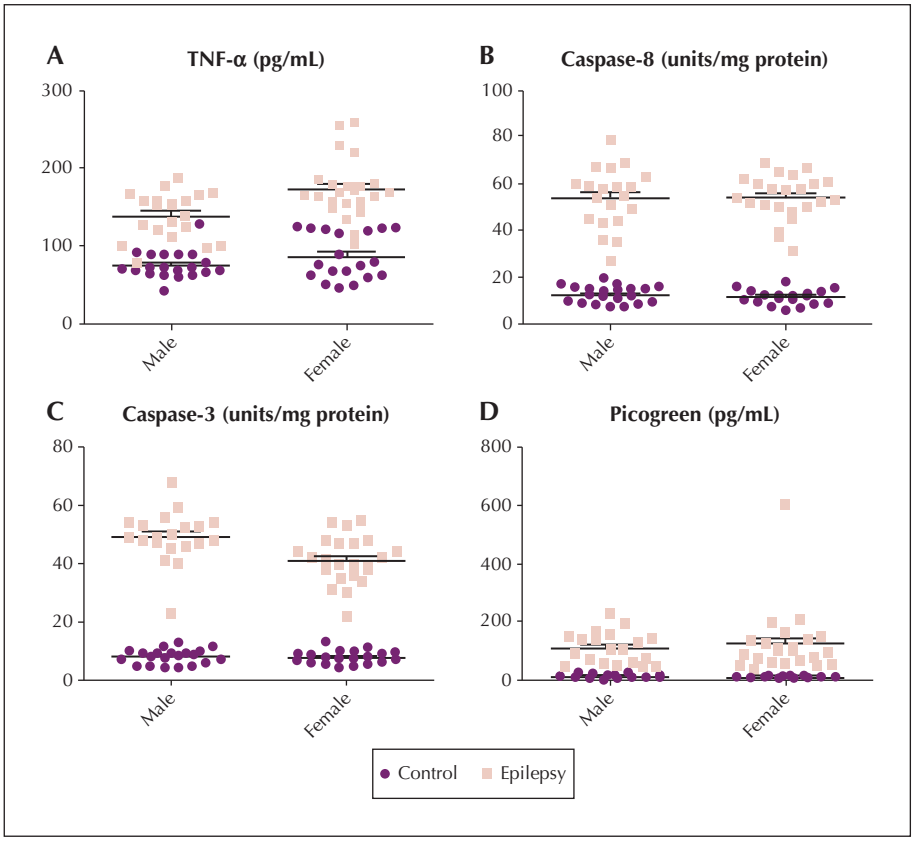
The epilepsy group presented with increased levels of TNF-α (154.7 ± 5.370 pg/mL) (t=11.34; df=82, $p<0.0001$) vs. controls (79.59 ± 3.778), caspase 8 (52.60 ± 1.517 units/mg protein) (t=24.76 df=82, $p<0.0001$) vs. controls (11.97 ± 0.5397), caspase 3 (44.33 ± 1.282 units/mg protein) (t=26.64 df=82, $p<0.0001$) vs. controls (8.107 ± 0.3595), and Picogreen (124.8 ± 13.83 pg/mL) (t=7.900 df=82, $p<0.0001$) vs. controls (12.79 ± 0.6157).

Assessment of oxidative stress markers (*figure 3*)

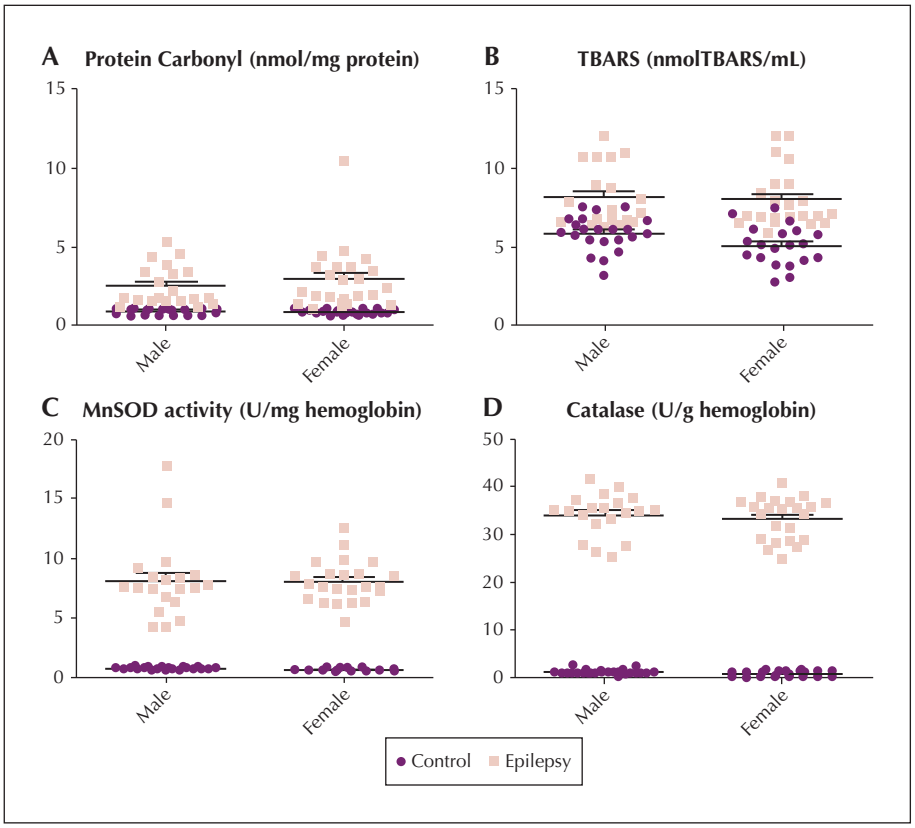
The epilepsy group presented with significantly increased protein carbonyl (2.856 ± 0.2592 nmol/mg protein) (t=7.173; df=82, $p<0.0001$) vs. controls (0.9444 ± 0.02421), TBARS (8.193 ± 0.4143 nmol TBARS/mL) (t=4.770 df=42, $p<0.0001$) vs. controls (5.846 ± 0.2427), manganese superoxide dismutase activity (8.595 ± 1.057 U/mg haemoglobin) (t=11.53; df=41, $p< 0.0001$) vs. controls (0.6848 ± 0.02674), and catalase activity (36.89 ± 4.718 U/g haemoglobin) (t=7.546; df=59, $p<0.0001$) vs. controls (0.6848 ± 0.02674).

Correlation between inflammatory, apoptotic, and DNA damage markers and epilepsy (*figure 4*)

Statistical analyses demonstrated a significant correlation between inflammatory, apoptotic, and DNA damage parameters in the epileptic group: TNF-α vs. caspase 8 (r=0.4173; $p<0.01$), TNF-α vs. caspase 3 (r=0.3780; $p<0.05$), and TNF-α vs. Picogreen (r=0.3281; $p<0.05$). In view of the association of these factors in epilepsy patients, we decided to investigate whether these factors correlate with seizure type.
The statistical analyses also demonstrated a significant correlation between TNF-α vs. caspase 8 (r=0.4618; $p<0.05$), TNF-α vs. caspase 3 (r=0.4765; $p<0.05$), and TNF-α vs. Picogreen (r=0.7276; $p<0.001$) (*figure 4F*) in



■ **Figure 2.** Comparison of inflammatory (TNF-α) and apoptotic parameters (caspase 8 [CASP8] and caspase 3 [CASP3]) and DNA damage (Picogreen) in epilepsy and control groups (female and male). The group with epilepsy presented with higher levels of TNF-α (A), CASP8 (B), CASP3 (C), and Picogreen (D) when compared to the control group. Significance was considered at $p<0.05$, according to the Student's t-test.



■ **Figure 3.** Comparison of oxidative stress (protein carbonyl [PC], TBARS, MnSOD and catalase) in the epilepsy and control groups (female and male). The group with epilepsy presented with higher levels of PC (A), TBARS (B), MnSOD activity (C), and catalase activity (D) when compared to the control group. Significance was considered at $p<0.05$, according to the Student's t-test.

the epilepsy group that presented with generalized seizures. No correlation was found between these markers and epilepsy patients with partial seizures (data not shown).

Correlation between metabolic parameters and generalized seizures (*figure 5*)

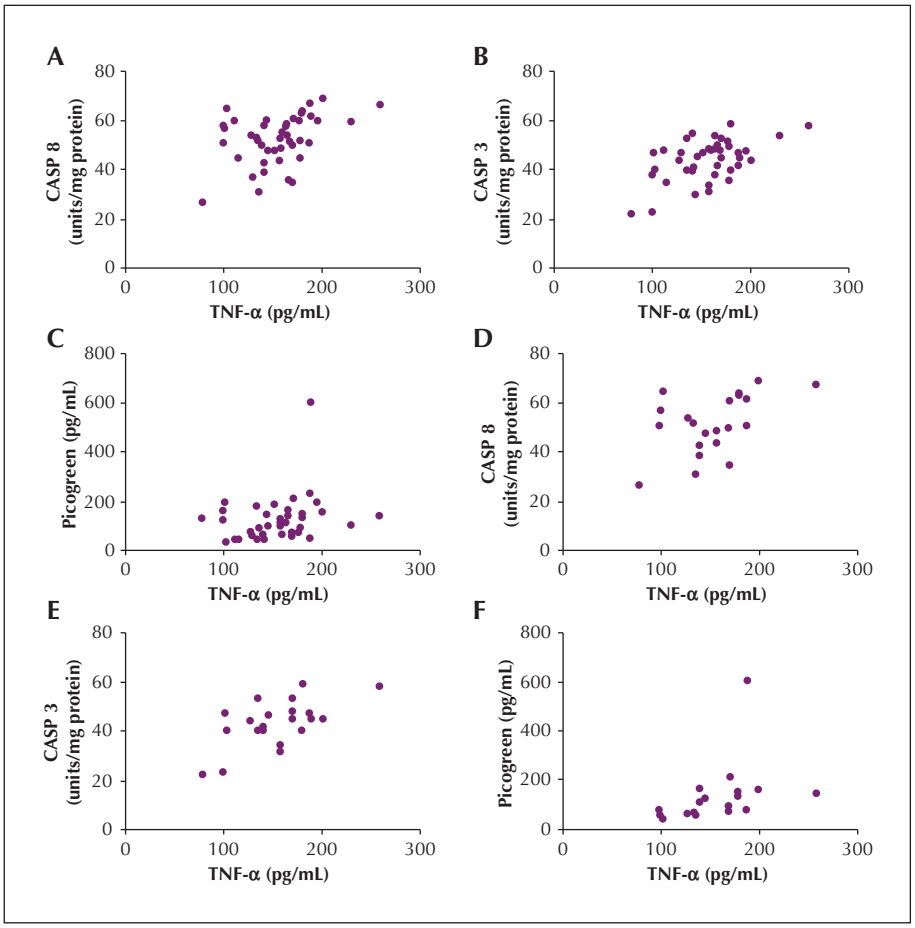
Since we identified a correlation between inflammatory and apoptotic factors in patients who presented generalized seizures, we decided to investigate whether generalized seizures also correlate with metabolic parameters.
Statistical analyses also demonstrated a significant correlation between lipid markers in patients with epilepsy and generalized seizures: CHO vs. TRI (r=0.5; $p<0.05$) and CHO vs. LDL (r=0.7; $p<0.01$). No significant association was observed for the other markers or for partial seizures (data not shown).

Correlation between generalized epileptic seizures and increased antioxidant enzyme activity (*figure 6*)

The epileptic group with generalized seizures exhibited increased MnSOD activity [F (2, 40) = 117.1; $p<0.05$] compared to those with partial seizures. There was no statistical difference in catalase activity, TBARS, or carbonyl protein content relative to seizure type (data not shown).

Discussion

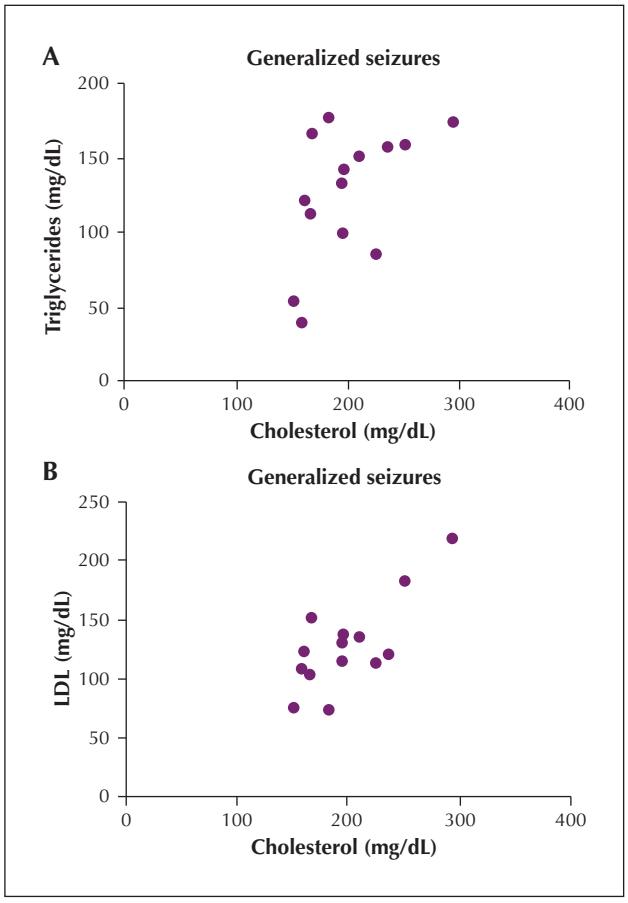
In the present study, we show that patients with epilepsy presented with higher levels of glycolipid, inflammatory, oxidative, and apoptotic factors, especially in patients with generalized seizures.
Epilepsy has been associated with a higher risk of cardiovascular disease (CVD) and related events [25-27].



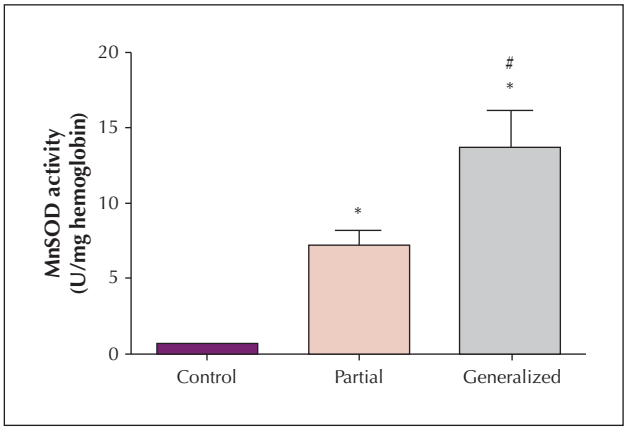
■ **Figure 4.** Correlation between TNF-α and apoptotic markers (caspases 8 and 3) and DNA damage (Picogreen) in the epilepsy group. In the epilepsy group, increased levels of TNF-α correlated with activation of caspase 8 (CASP8) (r=0.4173; $p<0.01$) (A), caspase 3 (CASP3) (r=0.3780; $p<0.05$) (B), and Picogreen (r=0.3281; $p<0.05$) (C). In the epileptic group with generalized seizures, increased TNF-α levels correlated with activation of CASP8 (r=0.4618; $p<0.05$) (D), CASP3 (r=0.4765; $p<0.05$) (E), and Picogreen (r=0.7276; $p<0.001$) (F).

In fact, epidemiological studies have demonstrated that patients with epilepsy have a higher mortality rate compared to the general population with CVD, indicating that epilepsy is a factor in premature death [28, 29]. In this context, neuronal injury during epileptic seizures may influence cholesterol homeostasis in the brain [30], in particular, the oxidation of LDL since reactive oxygen species cause endothelial dysfunction, leading to foam cell formation. On the other hand, HDL plays a protective role against lipoprotein oxidation due to increased paraxonase activity, preventing inflammation and enhancing cholesterol efflux to the liver [31]. Our results demonstrate that the epilepsy group presented higher levels of cholesterol, LDL, TRI, and glucose compared to the control group [32]. Regarding HDL, despite the epilepsy group presenting with low HDL levels compared to the control group, this was not statistically significant. These results corroborate those of Hermann *et al*. [7], who reported lower HDL levels in the epilepsy group. Although HDL has been known to protect the endothelium through different complex mechanisms [33], not all HDL are referred to as "good cholesterol" [34]. Alterations in HDL composition and metabolism, resulting from inflammation, may not only render HDL ineffective as an anti-inflammatory and antioxidant, but also actually have a pro-inflammatory and pro-oxidant role by promoting LDL oxidation [35].

Inflammatory and oxidative processes within the brain may constitute a common and crucial mechanism in the pathophysiology of seizures and epilepsy [2]. Clinical and experimental evidence has shown that severe inflammatory mediators, such as cytokines, are synthesized during epileptic activity in



■ **Figure 5.** Correlation between metabolic parameters (CHO, TRI, LDL) and generalized seizures. Increased levels of CHO correlated with TRI (r=0.5; $p<0.05$) (A) and LDL (r=0.7; $p<0.01$) (B). Significance was considered at $p<0.05$, according Pearson's correlation.



■ **Figure 6.** Level of enzymatic antioxidant (MnSOD) associated with different epileptic seizures (generalized vs. partial) and in the control group. The epileptic group with generalized seizures showed increased MnSOD activity when compared to patients with partial seizures and the control group. Statistical significance was considered at $p<0.0001$, according to one-way ANOVA.

the regions of the brain where seizures initiate and spread [36]. Furthermore, data from clinical research has shown that various cytokines, such as IL-1β and TNF-α, are also present in the cerebrospinal fluid (CSF), peripheral blood mononuclear cells, and plasma of patients with epilepsy [37]. Furthermore, patients with epilepsy have been shown to have more oxidative damage and higher pro-inflammatory cytokine levels in the peripheral blood [38-40], which was further confirmed by our results (increased TNF-α, PC, and TBARS).

Our findings corroborate current evidence indicating that cytokines, particularly TNF-α, increase neuronal excitability by activating TNF receptors (TNFR) [41] and induce excitotoxicity, increasing reactive species generation [42] and caspase activation, such as caspase 8 and 3 [43, 44]. Moreover, these events may increase lipid peroxidation, protein carbonylation, and DNA damage [45]. Adding to these data, we found a significant correlation between DNA damage (Picogreen), inflammation, and apoptotic parameters in the epilepsy group (*figure 4*).

Since there are different epileptic seizure types and some authors have demonstrated that inflammatory activity is worse in generalized seizures [38], we investigated possible correlations between inflammatory/oxidative pathways and glycolipid parameters and generalized and partial seizures in the epilepsy group. Interestingly, the results reveal that TNF-α positively correlated with Picogreen, caspase 3, and caspase 8 in patients with generalized seizures. No correlation was found between partial seizures or the control group and Picogreen, caspase 3, or caspase 8. Hence, the metabolic markers (CHO, TRI, and LDL) also demonstrated significant correlation in the epilepsy group with generalized seizures. Furthermore, SOD activity is reported to increase in patients with generalized seizures relative to partial seizures or the control group. In fact, Yis *et al.* [46] also found increased levels of this enzyme in patients with epilepsy, corroborating our results.

One hypothesis to account for these results is that seizures may cause changes in the neuronal tissue micro-environment, causing glial cell release of cytokines [9] and ROS [47], consequently activating caspase pathways [44] and causing DNA damage [48].

In the case of generalized seizures, these changes are more widespread (than partial seizures) throughout the nervous system, leading to a more robust increase in these parameters in peripheral blood. All these data suggest that these parameters may be useful to evaluate the degree of impairment caused by convulsive seizures, since the differences were greater in patients with generalized seizures than in those with partial seizures. Moreover, McCoy *et al*. [41] reported that elevated soluble TNF levels may be a hallmark of neuro-inflammation in a number of neurodegenerative conditions. In addition, Lagarde *et al*. [49] showed that anti-TNFα therapy improved seizure frequency in patients with Rasmussen's encephalitis.

We also show that levels of oxidative, inflammatory, and apoptotic markers did not correlate with use of antiepileptic drugs (AEDs), suggesting that AEDs do not influence the analysed markers. Furthermore, there was no difference in oxidative stress, inflammatory, or apoptotic parameters when comparing patients on monotherapy ($p>0.05$) or polytherapy ($p>0.05$) treatment. We therefore suggest that these parameters are independent of AED administration in patients with epilepsy. Similar results have been published in treated and untreated epilepsy patients, showing no differences in oxidative and inflammatory markers [39, 50, 51].

Conclusion

Despite our cohort being small, the results show a strong statistical difference between the epilepsy and control groups, suggesting a relationship between inflammation and metabolic markers in patients with epilepsy. In conclusion, we suggest that the events detected in this study may lead, at least in part, to neuronal dysfunction, contributing to seizures in patients with epilepsy. The data support the inclusion of routine evaluation of metabolic parameters (cholesterol, TRI, and glucose) and cardiovascular examination in most patients presenting with epilepsy. The findings also emphasize the need to be aware of the glycolipid profile when assessing presumed treatment failure of AEDs to control the convulsive events. In addition, we show relevant statistical data demonstrating an association between inflammatory and metabolic markers and generalized seizures, suggesting that higher levels of these parameters are related to seizure activity. Thus, we propose that the use of drugs with anti-inflammatory and anti-dyslipidaemic potential represent a new paradigm in the development of medical strategies, which may involve neuro-modulation of the nervous system. However, further studies are required to investigate the relationship between these aspects in a larger population. ■

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The authors have no conflit of interest to declare.
Informed consent was obtained from all individual participants included in the study. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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