Apoptotic Markers Are Increased in Epilepsy Patients: A Relation with Manganese Superoxide Dismutase Ala16Val Polymorphism and Seizure Type through IL-1β and IL-6 Pathways

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1. Introduction

Epilepsy is one of the most common neurological disorders [1] characterized by an enduring predisposition to generate seizures [2] affecting more than 65 million people worldwide [3]. Despite progress in pharmacological and surgical treatments of epilepsy, it is not clear about the processes leading to the generation of seizures and about the mechanisms; whereby, a healthy brain is rendered epileptic [4]. Apoptosis [5], neuroinflammation [4], and oxidative stress [6] are some relevant factors implicated in epilepsy pathophysiology. Many works have acknowledged the role of neuroinflammation in the pathogenesis of seizures, but little is known about the mechanisms that start the inflammatory
process in epilepsy [7]. The microglia constitute the primary CNS immune cells and are quickly activated in response to an insult. However, the excessive activation of microglia may be harmful, promoting the development of neuronal diseases by producing large amounts of inflammatory molecules, such as IL-6 [8], IL-1β, and reactive oxygen species (ROS) [9]. In epilepsy, there is a complex cascade of molecular and cell mechanisms involved in excitotoxicity [10], oxidative stress [11], and inflammation [4] beyond cytotoxicity mediated by cytokines [8] and cell death pathway activation [12]. In fact, when the brain is affected by brain diseases (i.e., epilepsy), the microglia cells are activated [13], and this activation may lead to production of inflammatory cytokines as IL-β [14] and IL-6 [15]. Interestingly, some antioxidant molecules were reported to decrease the levels of proinflammatory mediators by scavenging ROS [16]. Therefore, the redox balance is thought to regulate a series of neuroinflammatory processes mediated by microglia [9]. Manganese superoxide dismutase (MnSOD) antioxidant enzyme is the only known major defense against reactive oxygen species within mitochondria [17]. Furthermore, MnSOD is reportedly induced in the CNS under inflammatory conditions [9]. Regarding the relevance of MnSOD, numerous factors can impact on the effectiveness of antioxidant enzymes, including enzymatic polymorphism [18]. Two main MnSOD SNPs have been described in the literature, one of which is Ala16Val [17]. The change of alanine (Ala) to valine (Val) at the 16th amino acid (Ala16Val) of the signal sequence of MnSOD is suggested to change the structure of the protein. The alanine-to-valine substitution produces a β-sheet secondary structure instead of an α-helix structure, decreasing the enzyme transport efficiency into the mitochondria and compromising the antioxidant potential [19]. The Ala16Val MnSOD SNPs generate three possible genotypes: AA, AV, and VV. Sutton et al. [20] reported that the Val allele results in reduced expression and production of an unstable and VV. Sutton et al. [20] reported that the Val allele 

2. Materials and Methods

2.1. Study Design

2.2. Participants. We performed a case-control study and a total of 90 subjects were recruited and allocated into two groups: epilepsy group (n = 47) and control group (healthy subjects, n = 43). No etiology was found after detailed history, physical, laboratory, and imaging studies. Major exclusion criteria were history of autoimmune, liver, kidney, and inflammatory diseases; allergic response; immune deficiency disorder; diabetes, psychiatric illness; malignancy; smoking; or a systemic or central nervous system (CNS) infection 2 weeks before sample collection. The study protocol was approved by the local institutional review boards at the authors’ affiliated institutions. Informed written consent was obtained from all the subjects or their legal surrogates. The work described has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

2.3. Epilepsy Group. The epilepsy patients were recruited from the University Hospital of Santa Maria and invited to volunteer for the study. Epilepsy was diagnosed by two experienced neurologists according to the 2010 International League Against Epilepsy (ILAE) Classification, revised in 2017 [26]; Fisher et al., 2017; Scheffer et al., 2017]. All patients were evaluated for seizure frequency using seizure diaries [27]. The seizure type from epilepsy group (n = 47) was confirmed through interviews with the patients and their relatives as well as EEG analysis and tomography or magnetic resonance imaging (MRI). For a diagnosis of generalized epilepsy, the patient would typically show generalized spike-wave activity on EEG. Individuals with generalized epilepsies may have a range of seizure types including absence, myoclonic, atonic, tonic, and tonic–clonic seizures. The diagnosis of generalized epilepsy is made on clinical grounds, supported by the finding of typical interictal EEG discharges. Caution needs to be exercised for a patient with generalized tonic–clonic seizures and a normal EEG. In this case, supportive evidence would need to be presented to make a diagnosis of generalized epilepsy, such as myoclonic jerks or a relevant family history. Focal epilepsies include unifocal and multifocal disorders as well as seizures involving one hemisphere. A range of seizure types can be seen including focal aware seizures, focal impaired awareness seizures, focal motor seizures, focal nonmotor seizures, and focal to bilateral
tonic–clonic seizures. The interictal EEG typically shows focal epileptiform discharges, but the diagnosis is made on clinical grounds, supported by EEG findings. The term “unknown” is used to denote where it is understood that the patient has epilepsy, but the clinician is unable to determine if the epilepsy type is focal or generalized because there is insufficient information available.

In our study, forty-five patients were in remission except for two patients who were diagnosed with refractory epilepsy. All epilepsy patients had normal neurological examinations except for one who presented tetra paresis secondary to spinal cord lesion. All epilepsy had normal 1.5 T MRI; one patient had right and left hippocampal sclerosis.

2.4. Study Variables. Sex (dichotomous): male and female
Age (quantitative): years
Antiepileptic drugs (quantitative): number of drugs used for each patient
MnSOD Ala16Val genotype AA, AV, and VV (quantitative): frequencies (%)
Epilepsy type (dichotomous): generalized, focal, and unknown
Protein carbonyl (quantitative): nmol/mg protein
SOD2 activity (quantitative): U/mg hemoglobin
IL-1β (quantitative): pg/mL
IL-6 (quantitative): (pg/mL)
Caspase-3 (quantitative): mg/mL
Caspase-1 (quantitative): mg/mL

2.5. Laboratory Analyses. Samples were collected at least 7 days from the last seizure attack (Mao et al., 2013). After 12 h of overnight fasting, blood samples were collected by venipuncture using purple, green, and red top Vacutainer® (BD Diagnostics, Plymouth, UK) tubes with ethylenediamine tetra acetic acid (EDTA), heparin, or no anticoagulants, respectively. The specimens were routinely centrifuged at -80°C for subsequent laboratory analysis, according to specific methods.

2.6. Protein Carbonyl (PC). The analysis of protein carbonyl was in accordance with [28].

2.7. Manganese Superoxide Dismutase (MnSOD). The manganese superoxide dismutase activity was performed in accordance with [29].

2.8. Caspase Determination. Caspase-1 and caspase-3 activities were determined by Fluorimetric Assay Kits (BioVision, Mountain View, CA). The fluorescence intensity was recorded at wavelength of 400 nm for excitation and at wavelength of 505 nm for emission for both. The activity was then calculated as fluorescence intensity (FI)/min/mL = ΔFlt/(t × v), where ΔFlt is the difference in fluorescence intensity between time zero and time t minutes, t is the reaction time in min, and v is the volume of sample in mL.

2.9. Cytokine Determination. The cytokines were assessed by ELISA using commercial kits for human IL-1β and IL-6 (eBioscience, San Diego, USA).

2.10. DNA Damage. The alkaline DNA comet assay as described by Pereira. Genomic DNA was isolated from peripheral blood leukocytes using a DNA Mini Kit Purification (Mo Bio).

2.11. MnSOD Ala16Val Genotyping. Genomic DNA was isolated from peripheral blood leukocytes using a DNA Mini Kit Purification (Mo Bio). MnSOD Ala16Val SNP was detected by PCR-RFLP according to Taufet et al. PCR amplifications were performed in a total volume of 50 μl containing 5 μl of 10x buffer, 1 μl of 25 mM MgCl2, 1.25 μl of 10 mM dNTP, 0.5 μl of Taq polymerase (Gibco Inc., Co.), 1 μl of each primer (40 pmol), 3 μl of genomic DNA (0.25 μg), and 34.5 μl of ddH2O. The amplification primers (Gibco Inc., Co.) for a 110 bp fragment of the human MnSOD gene were 5′-ACACAGGGGCACCTTG CGCCGG-3′ (sense strand) and 5′-GCGGTGATGTG AGGTTCGAG-3′ (antisense strand) with the following thermocycler parameters: an initial cycle of 95°C for 5 min followed by 35 cycles at 95°C for 1 min and 61°C for 1 min. The final cycle was followed by an extension period of 2 min at 72°C. The PCR product (10 μl) was digested with Hae III (15 U; 37°C; 6 h; Gibco Inc., Co.). Digested products (23 and 85 bp) were visualized on a 4% agarose gel (Amersham Biosciences Inc., Co.) stained with ethidium bromide. A mutation was introduced by a primer mismatch to create a restriction cut site for Hae III in the -9 codon, and the following genotypes were observed: -9Ala/Ala (23 and 85 bp); -9Ala/Val (23, 85, and 110 bp); and -9Val/Val (110 bp).

2.12. Sample Size. As there is no comparison in the literature of apoptotic, inflammatory, and oxidative levels with MnSOD polymorphism in epilepsy patients, an adequate calculation if the sample size is not possible. Considering a significant difference of a standard deviation between the two groups and using the PEPI software, considering a study power of 90% and an alpha error of 0.05, 46 patients would be needed.

2.13. Statistical Methods. Data were analyzed by analysis of variance (two-way ANOVA) followed by Tukey’s multiple comparison test. Statistical analysis was performed using the SPSS (Statistical Package for the Social Sciences) software in a PC-compatible computer. Correlation analyses were carried out using the Pearson correlation coefficient. Statistical significance was assumed when p < 0.05. Chi-square test was used to calculate sex, age, and genotype frequencies.

3. Results

Baseline characteristics of the participants are described in Table 1. According to Chi-square analysis, no statistically difference was observed between the epilepsy group and control group relating with sex (p = 0.5) and age (p = 0.6).
Analysis of the Ala16Val MnSOD gene yielded three variants of the genotype: AA (wild type), AV (heterozygous), and VV (homozygous). The Ala16Val MnSOD genotype frequencies were calculated and are presented in Table 2. In the epilepsy group, the genotype frequencies were 31.9% for AA, 21.2% for AV, and 46.8% for VV. The frequencies for AA, AV, and VV genotypes were 39.5%, 32.5%, and 27.9%, respectively, in the control group. According to Chi-square analysis, no statistically difference in Ala16Val MnSOD genotype frequencies was observed ($p = 0.1$).

3.1. Protein Carbonyl (PC). A two-way ANOVA demonstrated increased protein carbonyl levels in the epilepsy group when compared to the control group ($F(1, 84) = 36.48$, $p < 0.0001$). Post hoc analysis with Tukey’s test for multiple comparisons revealed increased levels of PC in the epilepsy group (AA, AV, and VV genotypes) when compared to their genotypes from the control group, respectively (Figure 1).

3.2. Manganese Superoxide Dismutase (MnSOD). A two-way ANOVA demonstrated increased MnSOD enzyme activity in the epilepsy group when compared to the control group ($F(1, 81) = 617.5$, $p < 0.0001$). Post hoc analysis with Tukey’s test for multiple comparisons revealed increased MnSOD activity in the epilepsy group (AA, AV, and VV genotypes) when compared to their genotypes from the control group, respectively. Furthermore, in the epilepsy group, the homozygous VV genotype presented decreased enzyme activity when compared to AA genotype (Figure 2).

3.3. IL-1β. A two-way ANOVA demonstrated increased IL-1β levels in the epilepsy group when compared to the control group ($F(2, 85) = 6.2$, $p < 0.01$). Post hoc analysis with Tukey’s test for multiple comparisons revealed increased IL-1β levels in the epilepsy group (AA, AV, and VV genotypes) when compared to their genotypes from the control group, respectively. Furthermore, in the epilepsy group, the homozygous VV genotype presented increased levels when compared to AA genotype (Figure 3).

3.4. IL-6. A two-way ANOVA demonstrated increased IL-6 levels in the epilepsy group when compared to the control group ($F(2, 77) = 4.6$, $p < 0.05$). Post hoc analysis with Tukey’s test for multiple comparisons revealed increased IL-6 levels in the epilepsy group (AA, AV, and VV genotypes) when compared to their genotypes from the control group, respectively. Furthermore, in the epilepsy group, the homozygous VV genotype presented increased levels when compared to AA genotype (Figure 4).
3.5. Caspase-3. A two-way ANOVA demonstrated increased caspase-3 levels in the epilepsy group when compared to the control group ($F(2, 77) = 6.9, p < 0.01$). Post hoc analysis with Tukey’s test for multiple comparisons revealed increased caspase-3 levels in the epilepsy group (AA, AV, and VV genotypes) when compared to their genotypes from the control group, respectively. Furthermore, in the epilepsy group, the homozygous VV genotype presented increased levels when compared to AA genotype (Figure 5).

3.6. Caspase-1. A two-way ANOVA demonstrated increased caspase-1 levels in the epilepsy group when compared to the control group ($F(2, 77) = 3.8, p < 0.05$). Post hoc analysis with Tukey’s test for multiple comparisons revealed increased caspase-1 levels in the epilepsy group (AA, AV, and VV genotypes) when compared to their genotypes from the control group, respectively. Furthermore, in the epilepsy group, the homozygous VV genotype presented increased levels when compared to AA genotype (Figure 6).

3.7. Comet Assay. A two-way ANOVA demonstrated increased amount of DNA damage in the epilepsy group when compared to the control group ($F(1, 84) = 1282, p < 0.0001$). Post hoc analysis with Tukey’s test for multiple comparisons revealed increased amount of DNA damage in epilepsy group (AA, AV, and VV genotypes) when compared to their genotypes from the control group, respectively (data not shown).

3.8. Correlations

3.8.1. IL-1β vs. Caspase-1. Pearson’s analysis demonstrated an interesting correlation between IL-1β and caspase-1 ($r = 0.7, p < 0.001$) in the epilepsy group (VV genotype) (Table 3).

3.8.2. IL-6 vs. Caspase-3. Pearson’s analysis demonstrated an interesting correlation between IL-6 and caspase-3 ($r = 0.5, p < 0.05$) in the epilepsy group (VV genotype) (Table 3).

3.8.3. Seizure Type vs. Polymorphism. Pearson’s analysis demonstrated in the epilepsy group which presented generalized seizures (VV genotype), an interesting correlation between inflammatory and apoptotic parameters: IL-1β vs. caspase-1 ($r = 0.7, p < 0.05$) and IL-6 vs. caspase-3 ($r = 0.6, p < 0.05$). Furthermore, the results demonstrated an increased in caspase-1 levels in the epilepsy group which presented generalized seizures (VV genotype) ($t = 2.89, p < 0.05$). The other parameters did not demonstrate significant alteration in relation to generalized or partial seizures (data not shown) (Table 3).

3.8.4. Seizures’ Duration Time (Minutes). The statistical analysis revealed that the epilepsy group which presented generalized seizures (VV genotype) presented longer seizure time.

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**Figure 3:** Comparison of Ala16Val MnSOD polymorphism genotypes (AA, AV, and VV) from control and epilepsy groups in relation to IL-1β. The epilepsy group presented increased levels of IL-1β when compared to control group. *$p < 0.05$ when compared to respective control group; †$p < 0.05$ when compared to epilepsy group (VV vs. AV and AA).

**Figure 5:** Comparison of Ala16Val MnSOD polymorphism genotypes (AA, AV, and VV) from control and epilepsy groups in relation to caspase-3. The epilepsy group presented increased levels of caspase-3 when compared to control group. *$p < 0.05$ when compared to respective control group; †$p < 0.05$ when compared to the epilepsy group (VV vs. AA).
oxidant enzyme (MnSOD) in epilepsy. Of such interest, we
proposed a role in mitochondrial dysfunction [31]. Fur-
thermore, the oxidative stress can alter/influence factors
leading to neuronal death, and, consequently the DNA
damage [32]. The intense seizure activity can lead to cyto-
toxic effects mediated by oxidative stress. The superoxide
anion ($O_2^-$) is the central mediator of oxidative stress,
influencing both physiological and pathological processes
[33]. While there are some evidences confirming that oxida-
tive stress manifest as a consequence of the first insult,
which turns out later to become the cause of epileptogen-
esis [34], other studies support the influence of oxidative
stress in epilepsy. In accordance with Patel [34], oxidative
stress is the cause or consequence of epileptic seizures.

Protein oxidation is an irreversible oxidative damage,
considered to be a marker for severe oxidative stress [28].
Our results demonstrated increased levels of protein car-
bonyl when compared with the epilepsy vs. control group.
Accordingly, Sudha et al. [35] described protein carbonyl
increased levels in epilepsy patients than in controls. Of such
importance, when analyzing MnSOD, the results suggested
that the polymorphism plays an influence on its perfor-
mance, in view that the homozygous VV epilepsy group
demonstrated decreased activity when compared to AV and
AA genotypes.

The apoptotic pathway occurs primarily through the
extrinsic and intrinsic pathways [36]. In relation to intrinsic
(or mitochondrial) pathway, the apoptosis can be initiated
by cytokines such as IL-6. In this pathway, the mitochondria
release cytochrome c, activating the caspase-3, leading to cell
death [37]. Of note, our results demonstrated increased IL-6
and caspase-3 levels in the epilepsy group when compared to
the control group. Accordingly, Peltola et al. [38] reported
increased levels of IL-6 in plasma and cerebrospinal fluid
(CSF) of epilepsy patients when compared to nonepilepsy
patients. Increased caspase-3 in brain tissues has been found
in animal models of epilepsy [12] and epilepsy patients [39].
Studies also relate increased serum caspase-3 with traumatic
brain injury (TBI). However, caspase-3 has been scarcely
explored in blood of epilepsy patients [40]. The Ala16Val
MnSOD polymorphism also revealed a significant impor-
tance when associated with the genotype: the VV epilepsy
polymorphism also revealed a significant importance when
associated with the genotype: the VV epilepsy group
presented increased levels when compared to other genotypes
(AV and AA). In this context, an interesting correlation between IL-6 vs. caspase-3 was observed. When compared to seizure type, a positive correlation was obtained when related to generalized sei-

| Correlation | $r$ value | $p$ value |
|-------------|-----------|-----------|
| VV genotype–epilepsy group | | |
| IL-1$\beta$ vs. caspase-1 | 0.7 | $<0.001$ |
| IL-6 vs. caspase-3 | 0.5 | $<0.05$ |
| VV genotype–epilepsy group (generalized seizures) | | |
| IL-1$\beta$ vs. caspase-1 | 0.7 | $<0.05$ |
| IL-6 vs. caspase-3 | 0.6 | $<0.05$ |

(minutes) than the epilepsy group which presented partial
seizures (VV genotype) ($t = 2.46, p < 0.05$).

4. Discussion

The novel finding of the study is the influence of Ala16Val
MnSOD gene polymorphism–VV genotype on inflamma-
atory (IL-6, IL-1$\beta$), apoptotic (caspases -1 and -3) and anti-
oxidant enzyme (MnSOD) in epilepsy. Of such interest, we
observed an interesting correlation (caspase-1 vs. IL-1$\beta$)
and (caspase-3 vs. IL-6) in VV epilepsy patients. Further-
more, the generalized seizures were impacted by the VV
genotype in relation to the referred parameters and with
relation to seizures’ duration time. The burst firing neurons
associated with epileptic discharges could lead to changes
with events of cascades at the cellular level [30]. The com-
plex mechanism of epileptogenesis remains largely unclear.
However, oxidative stress by free radical generation does
indeed play a role in mitochondrial dysfunction [31]. Fur-
thermore, the oxidative stress can alter/influence factors
leading to neuronal death, and, consequently the DNA
damage [32]. The intense seizure activity can lead to cyto-
toxic effects mediated by oxidative stress. The superoxide
anion ($O_2^-$) is the central mediator of oxidative stress,
Ala16Val MnSOD polymorphism has an important role on apoptotic, and oxidative stress biomarkers, suggesting that the Ala16Val MnSOD polymorphism in epilepsy [25, 47]. We found some important results when analyzed AA, AV, and VV genotypes in the epilepsy group. It was observed a decreased MnSOD activity in the VV epilepsy group when compared to the AA epilepsy group. In this sense, our results are in accordance with that the ValVal could be less efficient than the AlaAla genotype to control the oxidative stress [17]. The V allele presents increased superoxide radical levels than the A allele due to its lower efficiency to dismutate this molecule into H₂O₂ [45]. Accordingly, the superoxide anion (O₂⁻) is the central mediator of oxidative stress, this anion could lead to mitochondrial destabilization resulting in cell apoptosis activation [46]. Although the small sample size of the study, there are few studies indicating the association/inactivation [46].

The date of approval is November 20, 2012.

The ethical approval code is CAAE 10554612.1.0000.5346.

5. Conclusion

Finally, the result demonstrated influence of Ala16Val MnSOD polymorphism, mainly of VV genotype in epilepsy patients. Accordingly, studies have shown that the polymorphism of some genes may be related to the efficacy, tolerability, and action of antiepileptic drugs [48, 49].

In this sense, prolonged seizures or status epilepticus in epilepsy patients can become a serious problem due to their consequences on the quality life from this population [50]. Seizures can have devastating consequences and, as result, suffer bodily injury requiring hospitalization. Others have shortened life span due to the increased risk of unexpected sudden death that is associated with uncontrolled seizures [50–52]. Studies have shown that patients with epilepsy can be significant neuropsychological, psychiatric, and social impairments that limit employment, reduce marriage rates, and decrease quality of life [51–53]. Thus, genetic polymorphism becomes an “ally” to help discover the cause of drug refractoriness and provides insight into the type and magnitude of clinic-laboratorial manifestations may have across individuals, helping to determine the best treatment and improve the quality of life of patients with neurological diseases, such as epilepsy.

Data Availability

The data availability data used to support the findings of this study are included within the article.

Ethical Approval

The ethical approval code is CAAE 10554612.1.0000.5346. The date of approval is November 20, 2012.

Conflicts of Interest

The authors declare there are any potential conflicts of interest. The authors declare they have no actual or potential competing financial interests.

Authors’ Contributions

All authors contributed equally in the study. All authors read and approved the final manuscript.

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