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Increased oxidative stress toxicity and lowered antioxidant defenses in temporal lobe epilepsy and mesial temporal sclerosis: associations with psychiatric comorbidities.

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Abstract

Oxidative stress toxicity (OSTOX), as well as lowered antioxidant defenses (ANTIOX), play a role in temporal lobe epilepsy (TLE). Nevertheless, the associations between OSTOX/ANTIOX and psychiatric comorbidities in TLE are largely unknown.

Thus, this study examines plasma malondialdehyde (MDA), lipid hydroperoxides (LOOH), advanced oxidation protein products (AOPP), nitric oxide metabolites (NOx), total radical trapping antioxidant parameter (TRAP) and sulfhydryl (-SH) groups in Depression due to TLE (n=25); Anxiety Disorders due to TLE (n=27); Psychotic Disorder due to TLE (n=25); "pure TLE" (n=27); and healthy controls (n=40).

TLE and mesial temporal sclerosis (MTS) were characterized by significant increases in OSTOX (MDA, AOPP, LOOH) and lowered ANTIOX (-SH groups, TRAP). The discrimination of pure TLE from controls yielded a significant area under the ROC curve for MDA (0.999), AOPP (0.851), -SH groups (0.899) and the OSTOX/ANTIOX ratio (0.996). Seizure frequency is significantly associated with increased MDA and lowered LOOH and NOx levels. Increased MDA was associated with the severity of depressive and physiosomatic symptoms, whilst increased AOPP levels predicted suicidal ideation. Depression and anxiety disorders co-occurring with TLE showed significantly lower MDA levels than TLE without any comorbidities. The psychotic and negative symptoms of TLE are associated with increased MDA levels and excitation with increased LOOH and lowered TRAP levels.

These results indicate that oxidative stress toxicity especially protein oxidation and aldehyde formation coupled with lowered -SH groups play a key role in the pathophysiology of

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TLE/MTS. Increased aldehyde formation also impacts psychopathology, psychosis, as well as negative and depressive symptoms.

Key words: oxidative stress, neuroimmunomodulation, major depression, inflammation, neurotoxicity, schizophrenia

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Introduction

Temporal lobe epilepsy (TLE) is the most common type of epilepsy with an incidence rate of 10.4 per 100,000 (1945 – 1964) [1] and is characterized by recurrent focal seizures that originate in the temporal lobes [2-4]. Mesial temporal sclerosis (MTS) or hippocampal sclerosis, which consists of neuronal cell loss, gliosis and sclerosis in the dentate gyrus, CA1, 3 and CA4 regions, is the most common etiology that accounts for 43%-73% of TLE cases [5-7]. Moreover, TLE is characterized by a high prevalence of comorbid neuropsychiatric syndromes (around 54.1%) with a high prevalence of depression (42.9%) and anxiety (18.4%), especially generalized anxiety disorder (GAD), while psychosis shows a lower prevalence [8,9]. In another study [10], the lifetime prevalence of psychiatric disorders in TLE was as high as 70.0% with mood disorders showing a prevalence of 49.3%, anxiety disorders 42.5% and psychosis 5.5%. Those comorbidities between TLE and psychiatric disorders have a negative impact on health-related quality of life (HR-QoL) [11], although no significant associations were observed between those psychiatric comorbidities and TLE features including age at onset and response to treatment with antiepileptic drugs (AEDs) [8].

There is now evidence that increased production of reactive oxygen (ROS) and nitrogen (RNS) species, lowered antioxidant defenses and increased oxidative stress toxicity play a role in epilepsy and TLE [12-14]. Increased nitro-oxidative stress may be the consequence of epileptic seizures, especially recurrent seizures, but may also contribute to epileptogenesis and treatment resistance [12-15]. In fact, epileptogenesis is accompanied by increased ROS production and lipid peroxidation, as well as hippocampal neurodegeneration and neuronal network reorganization with reactive gliosis, which together increase vulnerability to new

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seizures [15]. Following induction of experimental TL status epilepticus, increased production of superoxide, nitric oxide (NO) and, consequently, peroxynitrite may contribute to apoptotic cell death in hippocampal neuronal cells through activation of the caspase-3 signaling pathway [16,17]. Indicants of oxidative stress toxicity in epilepsy are increased levels of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) (indicating lipid peroxidation with consequent aldehyde formation), protein carbonyls (indicating protein oxidation), nitro-tyrosine (indicating increased nitration of proteins with production of immunogenic neoantigens) and 8-hydroxy-2-deoxyguanosine (indicating oxidative damage to DNA) [18-21]. Lowered levels of superoxide dismutase [22], catalase and glutathione peroxidase [19], vitamin E, and sulfhydryl (-SH) groups [23] are observed in patients with epilepsy. On the other hand, some papers did not detect alterations in advanced oxidation protein products (AOPP) and sulfhydryl or thiol (-SH) groups in epilepsy while NO levels were significantly lowered [21,24]. Interestingly, in a small-n study, some of the redox variables were associated with epilepsy characteristics including frequency of seizures, age at onset of seizures and number of drugs [21]. Nevertheless, despite the changes in ROS/RNS and indices of oxidative stress toxicity in epilepsy, it remains largely unknown whether comorbid psychiatric syndromes could contribute to changes in peripheral redox parameters in patients with MTS.

Recently, it was shown that psychiatric disorders including major depression and GAD are accompanied by increased ROS/RNS as well as oxidative stress toxicity as indicated by increased levels of MDA and AOPP, and lowered levels of -SH groups and total radical trapping antioxidant parameter (TRAP) [25-27]. The association between redox status and psychosis is more complex, with no or minimal changes being observed in first-episode

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psychosis [28,29] and in chronic schizophrenia [30], although deficit, but not non-deficit schizophrenia, is accompanied by increased levels of AOPP and lipid hydroperoxides (LOOH), but not MDA, and lowered levels of -SH groups and TRAP (Maes et al., in preparation). A recent study showed that MDA levels were significantly higher in TLE patients with depression than in those without [31], suggesting that this comorbidity may increase oxidative stress toxicity.

Thus, the aims of the present study were to examine a) whether TLE and MTS are characterized by increased levels of LOOH, MDA, AOPP, and NOx (NO metabolites), and lowered levels of TRAP and -SH groups; and b) whether these redox parameters are associated with comorbid depressive, anxiety and psychotic symptoms. The a priori hypotheses are that TLE and MTS and especially TLE with comorbid psychiatric disorders are accompanied by increased MDA, AOPP, LOOH and NOx and lowered TRAP and sulfhydryl levels.

Subjects and methods

Participants

In this case-control study, we recruited 104 patients with TLE and 40 healthy controls. The TLE outpatients were admitted to the Comprehensive Epilepsy Unit of the King Chulalongkorn Memorial Hospital, Bangkok, Thailand from December 2013 – December 2014. All patients were diagnosed as suffering from TLE by a senior neurologist specialized in epilepsy based on a history of seizure clinical characteristics, EEG record and magnetic resonance imaging (MRI). Moreover, the study group of patients with TLE was divided into 4 subgroups based on the presence of psychiatric comorbidities diagnosed using DSM-IV-TR criteria, namely a) Mood Disorders Due to TLE with depressed features (n=25); b) Anxiety Disorder Due to TLE with panic attacks, GAD or obsessive-compulsive symptoms (n=27); c)

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Psychotic Disorder Due to TLE with delusions or hallucinations (n=25); and d) "pure TLE" when those (or other) psychiatric comorbidities were absent (n=27).

Exclusion criteria for healthy controls were a diagnosis of epilepsy including febrile seizures in children or any axis-1 diagnosis of psychiatric disorders and a family history of epilepsy, mood or psychotic disorders. We excluded TLE patients when a) they presented axis I disorders other than Mood, Anxiety or Psychotic Disorders Due to TLE; b) suffered from inter-ictal dysphoric disorder (IDD) according to Blumer's criteria [32], and c) suffered from a recent seizure including aura (last week prior to the study). Additional exclusion criteria for Mood Disorders due to TLE were: the presence of anxiety and psychosis; for Anxiety Disorders due to TLE: the presence of mood disorders or psychosis; for Psychotic Disorder due to TLE: the presence of mood disorders or anxiety; and for "pure TLE" patients: the presence of any psychiatric comorbidity. Exclusion criteria for both patients and controls were: a) neurodegenerative/neuroinflammatory disorders including multiple sclerosis, stroke, and Parkinson's, Huntington's or Alzheimer's disease; b) (auto)immune disorders including diabetes, psoriasis, rheumatoid arthritis, inflammatory bowel disease, and chronic obstructive pulmonary disease; c) an immune, inflammatory or allergic response three months before the study; d) a lifetime history of treatment with immunomodulatory drugs including glucocorticoids; e) use of therapeutic doses of antioxidants or ω3-polyunsaturated fatty acid supplements three months before inclusion in the study; and f) pregnant or lactating women.

All participants provided written informed consent to take part in the study. Approval for the study was obtained from the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (IRB number 305/56), which is in compliance

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with the International Guideline for Human Research protection as required by the Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization on Good Clinical Practice (ICH-GCP).

Measurements

Semi-structured interviews were conducted by the senior neurologist and a senior psychiatrist specialized in epilepsy comorbidities. The neurologist collected socio-demographic data and rated epilepsy-related characteristics including age at onset of TLE, lesion location, type of epilepsy, presence of aura and type of aura, frequency and type of seizures, family history of epilepsy, post-ictal confusion, precipitating factors, and use of AEDs. Epilepsy semiology was performed and the diagnosis TLE was made based on a history of partial seizures and registration of epileptiform activity over one or both temporal regions. MRI results were used to make the diagnosis of TLE with MTS, other/undefined TLE, and TLE with tumoral origin and the radiologist and senior neurologist verified the diagnosis of MTS. The senior psychiatrist assessed patients and controls for depression, anxiety and psychotic symptoms using DSM-IV-TR criteria of a) Mood Disorders Due to TLE with depressed features, which included patients with ictus-related depression and major or minor depression who were in remission, partial remission or acute episode. b) Anxiety Disorder Due to TLE with panic attacks, generalized anxiety or obsessive-compulsive symptoms, which includes ictus-related anxiety (fear, horror, déjà-vu, and panic). c) Psychotic Disorder Due to TLE with delusions (being possessed, persecutory, ideas of reference, paranoid) or hallucinations (visual, auditory, tactile, gustatory, and olfactory) with or without severe disorganized behaviors. Patients allocated to this diagnosis also may show ictus-related psychoses as defined by Kanchanatawan et al. [33]

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including pre-ictal psychosis, post-ictal psychosis, peri-ictal psychoses, psychotic aura, ictal psychosis, and inter-ictal psychosis or schizophrenia-like psychosis. It should be underscored that the following symptoms were not regarded as psychosis: horror, fear, deja-vu, deja-vecu, going mad, forced thinking, autoscopic phenomena, and out-of-body experiences. In addition, the senior psychiatrist scored the Hamilton Depression (HAM-D) and Anxiety (HAM-A) rating scale and the Brief Psychiatric Rating Scale (BPRS) [34-36] in patients and controls. Smoking behavior was assessed using the Fagerstrom rating scale [37]. Body mass index was computed as body weight (in kg) divided by length (meter)^2.

Assays

Blood for the assay of the nitro-oxidative stress biomarkers was sampled at 8.00 a.m. after an overnight fast. Serum was aliquoted and stored at −80 °C until thawed for assay. The biomarkers measured include MDA, AOPP, LOOH, NOx, TRAP, -SH groups. The methods were described previously [26,27]. "MDA levels were measured through complexation with two molecules of thiobarbituric acid using MDA estimation through high-performance liquid chromatography (HPLC Alliance e2695, Waters', Barueri, SP, Brasil) [38]. Experimental conditions included the use of a column Eclipse XDB-C18 (Agilent, USA); mobile phase consisting of 65% potassium phosphate buffer (50 mM pH 7.0) and 35% HPLC grade methanol; flow rate of 1.0 mL/minute; temperature of 30 ºC; wavelength of 532 nm. MDA concentration in the samples was quantified based on a calibration curve and are expressed in mmol of MDA/mg proteins." AOPP was quantified in a microplate reader (EnSpire, Perkin Elmer, USA) at a wavelength of 340 nm [39,40] and is expressed in mM of equivalent chloramine T. LOOH

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was quantified by chemiluminescence in a Glomax Luminometer (TD 20/20), in the dark, at 30 °C for 60 min [41,42] and the results are expressed in relative light units (RLU). NOx was assessed in a microplate reader (EnSpire®, Perkin Elmer, USA) at a wavelength of 540 nm by measuring the concentration of nitrite and nitrate [43] and results are expressed as μM. TRAP was evaluated in a microplate reader (Victor X-3, Perkin Elmer, USA) and results are expressed in μM Trolox [44]. -SH groups were evaluated in a microplate reader (EnSpire®, Perkin Elmer, USA) at a wavelength of 412 nm and results are expressed in μM". [45,46]

Statistics

Analysis of variance was employed to check differences in continuous variables among scale variables while analysis of contingency tables (χ^2-tests) was employed to check relationships between nominal variables. Binary regression analysis (automatic, step-up) was used to assess the best biomarker prediction of TLE (or subgroups) as dependent variables and controls as a reference group. Multivariate general linear model (GLM) analysis was used to delineate the associations between biomarkers and diagnosis while adjusting for possible intervening variables including age, sex, BMI, smoking and the drug state. Tests for between-subject effects were used to delineate the relationships between diagnosis and each of the biomarkers and, subsequently, we computed model-generated estimated marginal mean (SE) values and carried out protected pair-wise comparisons among group means. We employed p-corrections for false discovery rate (FDR) to adjust for multiple statistical tests [47]. Multiple regression analysis (automatic, stepwise) was employed to delineate the significant biomarkers which are associated with the BPRS, HAM-D, HAM-A and MMSE scores. All regression

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analyses were checked for multicollinearity using VIF and tolerance values. All results are additionally bootstrapped using 5000 samples and the bootstrapped results are shown in case of discrepant results. All tests were two-tailed and a p-value of 0.05 was used for statistical significance. IBM SPSS25 for windows was used to analyze the data.

Using the scores of the BPRS, HAM-D, and HAM-A we have computed different symptom domain scores: psychosis was computed as the sum of 4 BPRS items, namely item 4 (conceptual disorganization), item 11 (suspiciousness), item 12 (hallucinations) and item 15 (unusual thought content); excitation was computed as sum of 2 BPRS items, namely item 8 (grandiosity) + item 17 (excitation), and negative symptoms as the sum of 2 BPRS items, namely item 3 (emotional withdrawal) + item 16 (blunted affect). The HAM-D physiosomatic subdomain score was computed as the sum of 5 HAM-D items, namely item 11 (somatic anxiety), item 12 (somatic symptoms, gastro-intestinal), item 13 (somatic symptoms general), item 14 (genital symptoms) and item 15 (hypochondriasis). We used item 3 of the HAM-D to assess suicidal ideation. An index of general psychopathology was assessed as the sum of the z values of the BPRS (z BPRS), + z HAM-D + z HAM-A.

Results.

Demographic and clinical data

Table 1 displays the demographic data of the patients and controls who participated in the present study. There were no significant differences in age, BMI, marital status, and smoking among the 5 diagnostic classes. There were somewhat more women in the TLE study groups with depression and anxiety as compared with the TLE group with psychotic features.

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Patients with TLE were somewhat less well educated than healthy controls. Therefore, we have statistically adjusted the results of multiple regression analyses for the putative effects of education (e.g. the MMSE and neurocognitive test results). This table also shows that there are no significant differences among the 4 TLE subgroups in the frequency of seizures, the age of onset of epilepsy, a history of aura and status epilepticus. Definite MTS was diagnosed in 55 of the TLE patients.

The HAM-D score was significantly higher in patients with TLE with depression than in the other 4 diagnostic categories. Table 1 also shows that the HAM-A and BPRS scores were significantly different between the 5 diagnostic categories with the lowest levels in healthy controls and the highest values in TLE + anxiety and TLE + psychosis diagnostic categories, respectively.

Associations between ONS biomarkers and TLE with and without comorbidities

Table 2 shows the results of multivariate GLM analysis, which examined the associations between biomarkers and diagnosis while adjusting for sex, age, BMI and smoking. We found a highly significant association between diagnosis and the biomarkers with an effect size of 0.321 and there were also significant effects of sex and BMI but not age or smoking. Tests for-between-subject effects showed significant associations between diagnosis and TRAP, -SH groups, MDA and AOPP with a very strong association between MDA and diagnosis (effect size of 0.689). The second GLM analysis in Table 2 shows that there were strong associations between diagnosis and the OSTOX, ANTIOX and OSTOX/ANTIOX indices. Table 3 shows that TRAP, -SH groups and the ANTIOX index were significantly lower in the 4 TLE groups than in controls, whereas MDA, AOPP, OSTOX, and OSTOX/ANTIOX

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index were significantly increased in all 4 TLE groups as compared with controls. Moreover, MDA was higher in "pure TLE" than in Mood Disorder due to TLE with depressive features or Anxiety Disorder due to TLE; and significantly higher in Psychotic Disorder due to TLE than in Anxiety Disorder due to TLE. LOOH was significantly higher in "pure TLE", Psychotic Disorder due to TLE and Mood Disorder due to TLE than in normal controls. The OSTOX and OSTOX/ANTIOX indices were significantly higher in all TLE subgroups than in controls and higher in "pure TLE" than in Anxiety Disorder due to TLE.

Figure 1 shows the 6 O&NS biomarkers as well as the three indices in healthy controls versus TLE patients. TRAP (F=31.97, p<0.001, effect size: 0.189), -SH groups (F=47.13, p<0.001, effect size: 0.256), and the ANTIOX index (F=78.43, p<0.001, effect size: 0.364), were significantly lowered in TLE as compared with controls, while MDA (F=233.31, p<0.001, effect size: 0.630), AOPP (F=51.06, p<0.001, effect size: 0.271), LOOH (F=8.70, p=0.004, effect size: 0.060), and the OSOX (F=160.50, p<0.001, effect size: 0.540), and OSTOX/ANTIOX (F=214.15, p<0.001, effect size: 0.610) indices were significantly higher in TLE than in controls (all results of GLM analyses with age, sex, BMI and TUD as covariates). Moreover, MTS and pure TLE with MTS showed the same pattern of disorders in the biomarkers. ROC analysis showed a highly significant separation of pure TLE versus controls for MDA (AUC ROC=0.999, SE=0.002, p<0.001), AOPP (AUC ROC=0.851, SE=0.046, p<0.001), -SH groups (AUC ROC=0.899, SE=0.039, p<0.001), TRAP (AUC ROC=0.743, SE=0.063, p=0.001), OSTOX (AUC ROC=0.981, SE=0.013, p<0.001), ANTIOX (AUC ROC=0.920, SE=0.031, p<0.001), OSTOX/ANTIOX (AUC ROC=0.996, SE=0.004, p<0.001) whereas LOOH was less significant (AUC ROC=0.665, SE=0.067, p=0.024).

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Effects of putative confounding variables.

As shown in Table 2 there were significant effects of sex and BMI on the biomarkers. Tests for between-subject effects showed significant effects of sex on TRAP ($F=12.92$, $df=1/134$, $p<0.001$), and LOOH ($F=6.85$, $df=1/134$, $p=0.010$) with higher TRAP and LOOH values in men than in women. Analysis of parameter estimates showed that BMI was associated with AOPP only ($t=+4.47$, $p<0.001$). The effects of antiepileptic drugs (AEDs) and other treatments were examined using the GLM analyses shown in Table 2 which considered the effects of phenytoin ($n=38$), valproate ($n=34$), phenobarbital ($n=26$), carbamazepine ($n=61$), lamotrigine ($n=27$), levetiracetam ($n=38$), topiramate ($n=12$), clonazepam ($n=10$), gabapentin ($n=8$), clobazam ($n=58$), antipsychotics ($n=9$), antidepressants ($n=16$), anxiolytics ($n=10$), CaCo3 ($n=13$) and folic acid ($n=27$). Multivariate and univariate GLM analyses (even without p correction for FDR) showed no significant effects of AEDs or other drugs. There were no significant associations (Spearman rank-order correlations) between the number of AEDs the patients were taking and any of the biomarker data even without p correction for FDR.

Best prediction of TLE and subtypes using biomarkers

Table 4 shows the results of automatic binary logistic regression analyses with TLE or TLE subtypes as dependent variables and biomarkers as explanatory variables. MDA was the single best biomarker predictor of TLE ($\chi2=148.34$, $df=1$, $p<0.001$) with a Nagelkerke value of 0.935, an Odd's ratio of 154.75 and a sensitivity of 99.1% and specificity of 94.7%. MDA combined with the ANTIOX index were the best predictors of MTS ($\chi2=102.09$, $df=2$, $p<0.001$;

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Nagelkerke = 0.852, sensitivity of 98.5% and specificity of 97.1%) and "pure TLE" (without comorbidities) + MTS ($\chi2$=48.33, df=2, p<0.001; Nagelkerke = 0.835, sensitivity of 93.8% and specificity of 97.4%). A history of post-ictal confusion was also associated with MDA and the ANTIOX index ($\chi2$=45.51, df=1, p<0.001; Nagelkerke = 0.366, sensitivity of 71.4% and specificity of 69.7%). There was a significant albeit weak association between TRAP levels and a history of status epilepticus with a Nagelkerke value of 0.068 ($\chi2$=6.00, df=1, p<0.014). MDA was also significantly associated with a history of aura ($\chi2$=56.57, df=1, p<0.001; Nagelkerke = 0.435, sensitivity of 76.3% and specificity of 70.3%).

Biomarker predictors of seizure frequency and psychiatric rating scale scores.

In order to examine the associations between biomarkers and seizure frequency, we have performed automatic multiple regression analyses with seizure frequency as dependent variable and biomarkers are explanatory variables while allowing for the effects of age and sex (**Table 5**). We found that 30.0% of the variance in seizure frequency was explained by MDA (positively) and TRAP, NOx and age (inversely). In the restricted study sample of TLE patients, we found that 18.2% of the variance in TLE seizure frequency was explained by age at onset, LOOH and NOx (all inversely). We found that 28.8% of the variance in the BPRS score and 10.6% in psychosis was explained by MDA (positively) and education (negatively). 9.1% of the variance in excitation was explained by LOOH (positively) and TRAP (negatively). A large part of the variance in negative symptoms (30.1%) was predicted by MDA (positively) and AOPP (inversely), male sex, and lower education and age. MDA was also the best predictor of the HAM-D score together with education (inversely associated) and female sex. Furthermore,

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MDA was the single best predictor of physiosomatic symptoms explaining 14.0% of the variance in this symptom domain. Suicidal ideation was positively associated with AOPP levels, while the HAM-A score was predicted by MDA (positively) and TRAP (negatively) which together explained 14.6% of the variance in anxiety levels. Elevated MDA levels and lowered education together explained 14.6% of the variance in the psychopathology index.

Prediction of MDA and AOPP levels

Table 6 shows the results of automatic regression analyses with MDA and AOPP as dependent variables. We found that 34.5% of the variance in MDA could be explained by LOOH (positively) and -SH groups, TRAP, and NOx (all negatively), while 9.1% of the variance in AOPP is explained by lowered -SH groups.

Discussion

The first major finding of this study is that TLE (with or without comorbidities) and MTS are characterized by increased oxidative stress toxicity as assessed with MDA, LOOH and AOPP and lowered antioxidant defenses as assessed with TRAP and -SH groups. The discrimination of TLE (without comorbidities) from controls is highly significant with an AUC ROC curve for MDA of 0.999 with a sensitivity of 99.1% and specificity of 94.7% for TLE. In fact, TLE is accompanied by a huge increase in MDA of 260% and a difference of 2.2 SDs in MDA levels between TLE and controls. Moreover, also the AUC ROC curves for AOPP (AUC ROC=0.851), -SH groups (AUC ROC=0.899) and the oxidative stress toxicity / antioxidant ratio (AUC ROC=0.996) were all highly significant. These results indicate that TLE and MTS are

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characterized by highly specific peripheral changes in aldehyde formation (increased MDA levels) as well as protein oxidation (increased AOPP) and lowered antioxidant defenses (especially reduced sulfhydryl groups). As such, MDA alone or the oxidative stress toxicity / antioxidant ratio may be used as external validating criterion for the diagnosis of TLE and MTS versus healthy controls. These findings extend those of a previous paper reporting increased levels of MDA in patients with TLE and MTS [21]. Animal models of TLE also show indicants of lipid peroxidation and aldehyde formation and lowered levels of antioxidants including vitamin E, although other antioxidants may be increased including GSH, SOD, and catalase [48-54]. In patients with epilepsy, increased levels of MDA and 4-HNE (both indicating increased lipid peroxidation and aldehyde formation), protein oxidation (as assessed with protein carbonyls) and lowered antioxidant defenses including sulfhydryl groups, superoxide dismutase, catalase, GSH and vitamin E and C were frequently observed [21,18-20,22,23,55]. Only a few papers examined AOPP levels in epilepsy and reported negative findings in drug-resistant partial complex seizures and idiopathic epilepsy syndrome [21,56]. There are also some negative findings on sulfhydryl groups in epilepsy [57]. We could not find any changes in NOx levels between TLE patients and controls, findings that are in agreement with the negative report by [58] while other authors reported lower NO levels in epilepsy [21].

It should be stressed that our results were controlled for possible effects of background variables including age, sex, smoking, and BMI, which all may affect oxidative and antioxidant biomarkers [26,27]. Some studies reported significant effects of AEDs on MDA, NO levels and -SH groups [59-65]. Therefore, we controlled our results for possible effects of AEDs. No significant effects of AEDs on the biomarkers were found while there were no associations between the number of AEDs taking by the patients and the biomarkers. These results extend

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the findings of [58] who reported no significant differences in MDA, protein carbonyls and NO levels between subjects on AED-monotherapy and polytherapy. Menon et al. [58] also reported higher levels of MDA and protein carbonyls (and no changes in NO) in untreated patients with epilepsy than in controls, suggesting that the increased oxidative stress toxicity is not induced by AEDs. Moreover, there were no significant differences in MDA, protein carbonyls and NO levels between both AED-treated and untreated patient groups [58], suggesting that AEDs do not affect these three biomarkers. Finally, as in our study, Menon et al. [58] were unable to find any effects of individual AEDs namely carbamazepine, valproate and phenytoin on the biomarkers. Other studies were also unable to find differences in oxidative biomarkers including carbonyls, lipid peroxidation, and antioxidant enzymes between treated and untreated patients [62,66].

The second major finding of this study is that not only TLE and MTS are characterized by highly increased oxidative biomarkers, but also that some features of TLE are associated with those biomarkers. Thus, seizure frequency was significantly associated with increased MDA but lowered LOOH, indicating that seizure frequency increases with aldehyde formation but not lipid peroxidation per se. Animal models of epilepsy show that reducing ROS production through the administration of corilagin is associated with lowered seizure frequency [67]. Moreover, activation of Nrf2 following administration of RTA 408 attenuates ROS production in association with a reduction in late spontaneous seizures [68] while sub-acute treatment with a cannabinoid agonist (WIN 55,212-2) attenuates recurrent seizures while normalizing the thiol redox state. On the other hand, the administration of vitamin E, which reduced oxidative damage (protein carbonyl levels), had no significant effect on seizure frequency [69]. Our results that NOx levels are inversely associated with seizure frequency

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suggest a causal association with increased NO use whereby NO may be consumed by increased nitration (e.g. the formation of 3-nitrotyrosine) and nitrosylation, which both may induce neurodegenerative processes [25,70-72]. As such, our findings extend the results of a previous report that in epilepsy patients, seizure frequency is significantly associated with the expression of 3-nitrotyrosine, a consequence of enhanced nitration processes [21]. All in all, it may appear that aldehyde formation (rather than protein oxidation or lipid peroxidation) and increased NO consumption, through nitration/nitrosylation processes, are associated with seizure frequency. Moreover, we also observed that increased aldehyde formation is associated with aura, while lowered TRAP is associated with a history of status epilepticus.

The third major finding of this study is that in the study sample of patients and controls combined, increased MDA predicts the severity of depressive and physiosomatic symptoms whilst increased AOPP levels predict suicidal ideation. These results are in agreement with the knowledge that depression and physiosomatic symptoms are accompanied by increased oxidative stress including lipid peroxidation and aldehyde formation and lowered antioxidant defenses as well [73-75]. Previously, a significant association between suicidal behaviors and oxidative toxicity including elevated AOPP levels was reported [76]. Moreover, de Araujo Filho et al. [31] observed that MDA levels were significantly increased in comorbid TLE patients and depression. Nevertheless, in our study, mood disorders due to TLE with depressive symptoms and anxiety disorders due to TLE are accompanied by somewhat lowered MDA levels, while anxiety disorders due to TLE also show a lower lowered oxidative stress toxicity index than patients with TLE without these comorbidities. All in all, while increased aldehyde formation is associated with depression and anxiety disorder due to TLE, it appears that, in patients with those comorbidities, MDA levels are somewhat lower than in pure TLE, suggesting that

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oxidative stress toxicity is somewhat lower when those two comorbidities are present. These findings contrast our a priori hypothesis that both comorbidities are accompanied by increased oxidative stress toxicity which would reflect cumulative effects of increased levels in both TLE and affective disorders. Moreover, comorbidities between depression and neuroinflammatory disorders including multiple sclerosis and stroke are associated not only with increased morbidity and mortality but also with increased inflammatory and oxidative stress biomarkers [25,77-79]. Therefore, our findings could indicate that depression and anxiety disorder due to TLE have a different pathophysiology than major depression and anxiety disorders such as GAD [26,27].

The current study also shows that psychotic disorder due to TLE is associated with significantly higher MDA levels as compared with healthy controls and anxiety disorders due to TLE. Moreover, psychotic and negative symptoms are associated with increased MDA levels, while excitation is associated with increased LOOH and lowered TRAP levels. Likewise, indices of general psychopathology were strongly associated with increased MDA levels. Previously, it was detected that first episode psychosis and chronic schizophrenia are not associated with increased nitro-oxidative stress [28-30]. Nevertheless, previous studies showed that hallucinations-delusions and excitation, assessed over a broader range of schizophrenia syndromes, are associated with increased LOOH and lower TRAP/-SH groups, while negative symptoms are associated with increased AOPP and lowered TRAP/-SH groups (Maes et al., in preparation). Thus, the oxidative pathophysiology of psychotic disorder due to TLE appears to be different from the oxidative stress profile observed in schizophrenia, suggesting that psychosis and negative symptoms in both conditions are not the same nosological entities.

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In our study, increased aldehyde formation in TLE was strongly predicted by increased LOOH, and lowered antioxidant levels (-SH groups and TRAP) and NOx. These findings reflect that MDA formation is a consequence of lipid peroxidation and peroxyl radical propagation and that formation of peroxynitrite may aggravate lipid peroxidation and MDA formation, explaining the inverse association between NOx levels and MDA [26]. Moreover, lowered antioxidant defenses increase vulnerability to oxidative stress toxicity and, therefore, the direct toxicity exerted by MDA including signaling pathway alterations, ATP depletion, mitochondrial dysfunctions, oxidative DNA damage and mutagenicity, disruption of cellular homeostasis, apoptosis and cell death, immune activation, and neurodegenerative processes [25]. The formation of AOPP may further aggravate this direct MDA-induced toxicity for example by causing new ROS/RNS formation, conformational changes in proteins, loss of functional activity of proteins, modulation of gene expression and intracellular signaling, induction of apoptosis and necrosis [25]. Moreover, lowered -SH groups may indicate formation of sulfide bonds with alterations in secondary and tertiary protein structure, which may lead to increased susceptibility to proteolysis [26]. There is now evidence that increased oxidative stress including in the hippocampus increases susceptibility to seizures and that peripheral activation of immune-inflammatory responses may induce oxidative stress in the hippocampus [20]. There is also some evidence that inflammatory responses and associated redox mechanisms may initiate or augment seizures and play a role in disease progression [80,81]. In this respect, it is interesting to note that the administration of pilocarpine and kainic acid, two models of TLE [82,83] may induce increases in hippocampal thiobarbituric acid reactive substances (TBARS), an assay used to assess MDA production [84]. Therefore, it may be posited that the highly specific increase in peripheral MDA and AOPP formation may contribute to epileptogenesis and MTS.

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The results of this study should be discussed with regard to its limitations. First, this is a case-control study and therefore no causal inferences can be made. Secondly, it would have been more interesting if we also had measured myeloperoxidase and xanthine oxidase as well as antioxidant enzymes including catalase and superoxide dismutase. Future research should focus on possible differences or similarities between psychosis/depression/anxiety due to TLE and the same symptom domains in schizophrenia, major depression or anxiety disorders. In addition, future research should examine disabilities and HR-QoL in depression and anxiety disorder due to TLE versus "pure TLE". Indeed, based on our findings that pure TLE is accompanied by higher MDA levels and the knowledge that MDA is associated with a lower HR-QoL [85] one could predict (albeit counterintuitively) a worse HR-QoL in pure TLE when no comorbidities with affective symptoms are present.

All in all, TLE and MTS are associated with increased LOOH, MDA, and AOPP levels and lowered TRAP and -SH groups. Increased MDA coupled with lowered LOOH and NOx levels are associated with seizure frequency, indicating a key role of aldehyde formation and NO-related mechanisms. Increased MDA formation is also associated with the severity of depressive and physiosomatic symptoms while increased AOPP is associated with suicidal ideation. Psychotic and negative symptoms in TLE are associated with increased MDA levels and excitation with increased LOOH coupled with lowered TRAP levels. Oxidative stress toxicity and lowered antioxidant defenses play a key role in the pathophysiology of TLE and MTS and comorbid psychopathologies.

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Conflict of interest

The authors have no conflict of interest with any commercial or other association in connection with the submitted article.

Author's contributions

All the contributing authors have participated in the manuscript. BK and MM designed the study. BK and CL recruited patients and completed diagnostic interviews and rating scale measurements. MM carried out the statistical analyses. All authors contributed to interpretation of the data and writing of the manuscript. All authors approved the final version of the manuscript.

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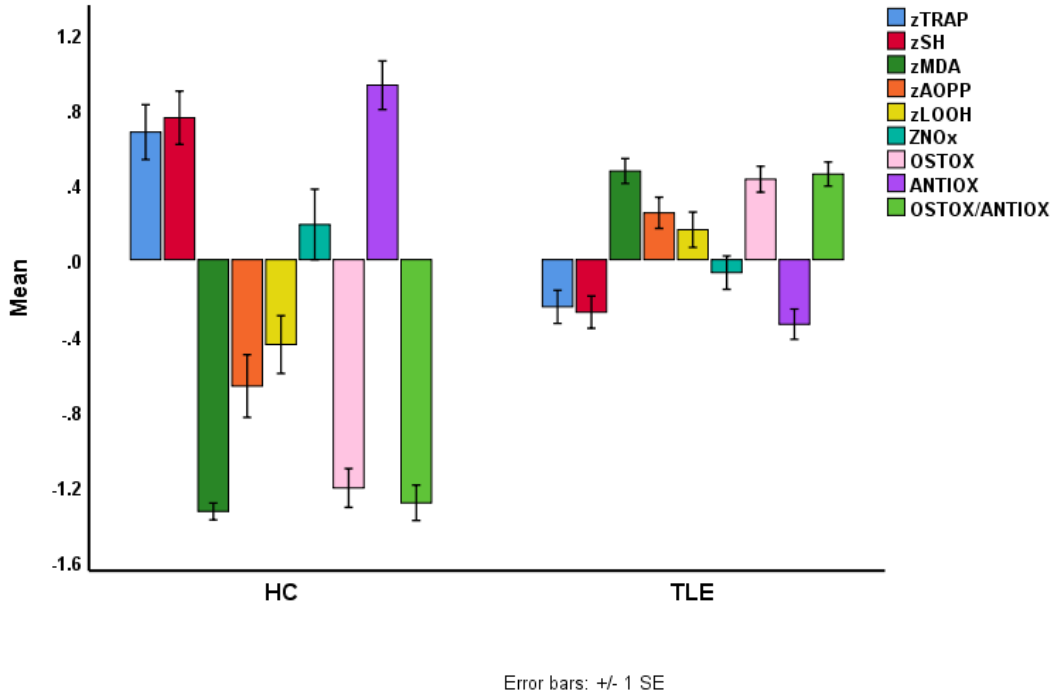

Figure 1. Nitro-oxidative stress biomarkers in patients with temporal lobe epilepsy (TLE) and healthy controls (HC).

TRAP: total radical-trapping antioxidant parameter; -SH: sulfhydryl groups; MDA: malondialdehyde; AOPP: advanced oxidation protein products; LOOH: lipid hydroperoxides; NOx: nitric oxide metabolites. OSTOX: index of oxidative stress toxicity; ANTIOX: index of antioxidant activity; OSTOX/ANTIOX: ratio of oxidative stress toxicity / antioxidant activity.

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Table 1. Sociodemographic and clinical data of healthy controls (HC) and patients with temporal lobe epilepsy (TLE) with psychotic disorder (TLE+PSY), depression (TLE+DEP) and anxiety disorder (TLE+ANX) due to TLE

Variables	**HC** [a]	**Pure TLE** [b]	**TLE+PSY** [c]	**TLE+DEP** [d]	**TLE+ANX** [e]	**F/Ψ/X²**	**df**	**P**
Age (years)	37.4 (12.8)	40.0 (12.8)	37.9 (10.5)	39.0 (10.7)	37.0 (8.2)	0.34	4/141	0.849
Sex (♂/♀)	10/30	11/16	13/14	4/21	5/22	10.31	4	0.036
BMI (kg/m²)	24.0 (4.3)	24.1 (4.0)	23.5 (3.7)	23.9 (4.3)	22.4 (4.3)	0.79	4/140	0.535
Married (No/Yes)	26/14	18/9	20/7	20/5	15/11	Ψ=3.58	-	0.466
Education (years)	14.2 (4.9) [b,c,d,e]	11.4 (4.7) [a]	9.4 (4.4) [a]	10.3(5.4) [a]	10.8 (4.5) [a]	5.14	4/141	0.001
Smoking (N/Y)	38/2	24/3	23/4	21/4	23/4	Ψ=0.136	-	0.607
Number of seizures	-	29.1 (84.7)	19.1 (40.7)	8.0 (17.0)	9.7 (11.0)	0.99	3/89	0.402
Age onset (years)	-	17.8 (12.6)	12.2 (10.1)	17.6 (8.9)	16.1 (8.8)	1.75	3/100	0.162
Definite MTS	-	16	20	11	18	-	-	-
Aura (No/Yes)	-	6/21	5/22	7/18	8/19	1.15	3	0.766
Status epilepticus (No/Yes)	-	24/3 [c]	14/11 [b]	21/4	13/9	10.75	3	0.013
BPRS	18.3 (1.1) [b,c,d,e]	23.6 (3.3) [a,c,d,e]	41.3 (5.9) [a,b,d,e]	32.9 (6.7) [a,b,c,e]	29.4 (5.0) [a,b,c,d]	115.64	4/141	<0.001

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HAM-D	0.6 (2.0) [b,c,d,e]	4.8 (2.5) [a,d,e]	5.8 (2.9) [a,e]	19.8 (4.9) [a,b,c,e]	10.3 (3.8) [a,b,c,d]	145.21	4/140	<0.001
HAM-A	2.6 (5.4) [b,c,d,e]	7.8 (3.9) [a,c,d,e]	11.6 (6.7) [a,b,c,e]	18.9 (8.8) [a,b,c,e]	23.8 (5.4) [a,b,c,d]	59.69	4/141	<0.001

All values are shown as mean (SD); BMI: body mass index; MTS: mesial temporal sclerosis.

BPRS: Brief Psychiatric Rating Scale; HAM-D: Hamilton Depression Rating Scale; HAM-A: Hamilton Anxiety Rating Scale; Pure TLE: TLE without any psychiatric comorbidities

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Table 2. Results of multivariate GLM analysis examining the differences between diagnostic groups (diagnosis), namely healthy controls, temporal lobe epilepsy with and without depression, psychosis, or anxiety.

Tests	**Dependent variables**	**Exploratory variables**	**F**	**df**	**p**	**Partial Eta Squared**
Multivariate	All 6 biomarkers: TRAP, -SH, LOOH, MDA, AOPP, Nox	Diagnosis	10.48	24/528	<0.001	0.321
		Sex	3.85	6/129	0.001	0.152
		Age	1.37	6/129	0.231	0.060
		BMI	4.30	6/129	0.001	0.167
		Smoking	1.37	6/129	0.233	0.060
Between-subject effects	TRAP	Diagnosis	8.56	1/134	<0.001	0.204
	-SH	Diagnosis	13.44	1/134	<0.001	0.286
	MDA	Diagnosis	74.05	1/134	<0.001	0.689
	AOPP	Diagnosis	13.07	1/134	<0.001	0.290
	LOOH	Diagnosis	2.27	1/134	0.065	0.063
	NOx	Diagnosis	2.05	1/134	0.091	0.058
Multivariate	All 3 composite scores:	Diagnosis	23.19	8/266	<0.001	0.411
		Sex	6.91	2/133	0.001	0.094
		Age				

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		BMI Smoking	2.00 1.51 0.29	2/133 2/133 2/133	0.139 0.224 0.7456	0.029 0.022 0.004
Between-subject effects	OSTOX ANTIOX OSTOX/ANTIOX	Diagnosis Diagnosis Diagnosis	43.60 19.95 57.06	1/134 1/134 1/134	<0.001 <0.001 <0.001	0.565 0.373 0.630

Diagnosis: five diagnostic groups, namely Psychotic Disorder due to temporal lobe epilepsy (TLE), Mood Disorder due to TLE with depressive features, Anxiety Disorder due to TLE, Pure TLE (that is no comorbidities) and healthy controls

TRAP: total radical-trapping antioxidant parameter; -SH: sulfhydryl groups; MDA: malondialdehyde; AOPP: advanced oxidation protein products; LOOH: lipid hydroperoxides; NOx: nitric oxide metabolites.

OSTOX: index of oxidative stress toxicity; ANTIOX: index of antioxidant activity; OSTOX/ANTIOX: ratio of oxidative stress toxicity / antioxidant activity.

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Table 3. Model-generated estimated marginal means (SE) of nitro-oxidative and antioxidant biomarkers in healthy controls (HC) and patients with temporal lobe epilepsy (TLE) with and without psychosis, depression or anxiety.

Variables	HC [a]	Pure TLE [b]	TLE+PSY [c]	TLE+DEP [d]	TLE+ANX [e]
TRAP (µmol Trolox)	995.2 (23.7) [b-e]	835.6 (27.1) [a]	852.5 (26.9) [a]	874.9 (29.6) [a]	816.5 (29.0) [a]
-SH (µmol/L)	321.8 (9.4) [b-e]	233.0 (10.8) [a,e]	250.7 (10.7) [a]	241.7 (11.8) [a]	268.4 (11.5) [a,b]
MDA (µM/mg protein)	2.32 (0.16) [b-e]	6.06 (0.19) [a,d,e]	5.54 (0.19) [a,e]	5.03 (0.21) [a,b]	4.79 0.20 [a,b,c]
AOPP (µmol/L/eq. cloramin T)	208.9 (30.0) [b-e]	409.3 (31.9) [a]	351.7 (31.7) [a]	427.7 (34.9) [a]	337.7 (34.2) [a]
LOOH (RLU)	1175 (53) [b,c,d]	1329 (60) a	1369 (60) [a]	1329 (66) [a]	1298 (65)
NOx (µmol/L)	7.67 (0.99)	4.49 (1.13)	6.03 (1.13)	7.69 (1.24)	6.91 (1.22)
OSTOX	-1.123 (0.111) [b-e]	0.717 (0.126) [a,e]	0.535 (0.126) [a]	0.466 (0.138) [a]	0.203 (0.135) [a,b]
ANTIOX	1.097 (0.136) [b-e]	-0.438 (0.155) [a]	-0.193 (0.154) [a]	-0.189 (0.169) [a]	-0.170 (0.166) [a]
OSTOX/ANTIOX	-1.304 (0.104) [b-e]	0.681 (0.119) [a,e]	0.430 (0.118) [a]	0.387 (0.130) [a]	0.221 (0.127) [a,b]

TLE+PSY: Psychotic Disorder due to TLE; TLE+DEP: Mood Disorder due to TLE with depressive features; TLE+ANX: Anxiety Disorder due to TLE; Pure TLE: TLE without ant psychiatric comorbidities

TRAP: total radical-trapping antioxidant parameter; -SH: sulfhydryl groups; MDA: malondialdehyde; AOPP: advanced oxidation protein products; LOOH: lipid hydroperoxides; NOx: nitric oxide metabolites.

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OSTOX: index of oxidative stress toxicity; ANTIOX: index of antioxidant activity; OSTOX/ANTIOX: ratio of oxidative stress toxicity / antioxidant activity.

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Table 4. Results of automatic binary logistic regression analyses with temporal lobe epilepsy (TLE) and phenotypes as dependent variables.

Dependent variables	Explanatory variables	B	SE	Wald	df	P	Odd's ratio	95% CI interval
TLE	MDA	5.04	1.441	12.83	1	<0.001	154.75	9.18-2609
MTS	MDA	3.32	0.704	22.19	1	<0.001	27.56	6.93-109.52
	ANTIOX index	-1.83	0.727	6.35	1	0.012	0.160	0.038-0.666
Pure TLE + MTS	MDA	1.85	0.974	7.52	1	0.006	6.34	1.69-23.74
	ANTIOX index	-2.84	0.392	4.16	1	0.041	0.06	0.00-0.89
Hx of post-ictal confusion	MDA	0.856	0.318	7.25	1	0.007	2.35	1.26-4.39
	ANTIOX index	0.917	0.332	7.62	1	0.006	2.50	1.31-4.80
Hx of status epilepticus	TRAP	-0.562	0.242	5.39	1	0.020	0.57	0.35-0.92
Hx of aura	MDA	1.62	0.273	35.15	1	<0.001	5.04	2.95-8.59

TLE: temporal lobe epilepsy; MTS: mesial temporal sclerosis; Pure TLE: TLE without any psychiatric comorbidities

Hx: a history of

MDA: malondialdehyde; TRAP: total radical trapping antioxidant parameter; ANTIOX index: index of antioxidant activities computed as z value of TRAP + z value of thiol (-SH) groups

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Table 5. Results of multiple regression analysis with seizure frequency and rating scale scores as dependent variables and nitro-oxidative and antioxidant biomarkers as explanatory variables.

Dependent variables	Explanatory variables	β	t	P	F model	df	p	partial Eta Squared
Seizure frequency	**Model**				14.50	4/135	<0.001	0.3000
	MDA	0.360	4.71	<0.001				
	TRAP	-0.229	-3.04	0.003				
	Age	-0.161	-2.23	0.027				
	NOx	-0.163	-2.22	0.028				
Seizure frequency in TLE	**Model**				7.10	3/96	<0.001	0.182
	Age at onset	-0.226	2.45	0.016				
	LOOH	-0.283	-3.06	0.003				
	NOx	-0.220	-2.38	0.019				
BPRS	**Model**				28.50	2/141	<0.001	0.288
	MDA	0.448	6.10	<0.001				
	Education	-0.202	-2.74	0.007				
Psychosis	**Model**				8.32	2/141	<0.001	0.106
	MDA	0.239	2.90	0.004				
	Education	-0.167	-2.03	0.044				

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Excitation-grandiosity	**Model** TRAP LOOH	 -0.225 0.187	 -2.80 2.32	 0.006 0,022	7.06	2/141	0.001	0.091
Negative symptoms	**Model** Female sex Education Age MDA AOPP	 -0.359 -0.331 -0.149 0.254 -0.219	 -4.95 -4.29 -1.98 3.18 -2.75	 <0.001 <0.001 0.049 0.002 0.007	11.86	5/138	<0.001	0.301
HAM-D	**Model** MDA Education Female sex	 0.317 -0.205 0.161	 4.04 -2.59 2.10	 <0.001 0.011 0.037	11.42	3/139	<0.001	0.198
Physiosomatic symptoms of HAM-D	**Model** MDA	 0.374	 4.81	 <0.001	23.09	1/142	<0.001	0.140
Suicidal ideation	Model AOPP	 0.261	 3.22	 0.002	10.36	1/142	0.002	0.068
HAM-A	**Model** MDA TRAP	 0.271 -0.201	 3.33 -.247	 0.001 0.015	12.05	2/141	<0.001	0.146

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Psychopathology index	**Model** MDA Education	 0.415 -0.219	 5.54 -2.92	 <0.001 0.004	25.42	2/140	<0.001	0.146

BPRS: Brief Psychiatric Rating Scale; Psychosis: sum of 4 BPRS items, namely item 4 (conceptual disorganization), item 11 (suspiciousness), item 12 (hallucinations) and item 15 (unusual thought content); Excitation-grandiosity: sum of 2 BPRS items, namely item 8 (grandiosity) and item 17 (excitement); Negative symptoms: sum of 2 BPRS items, namely item 3 (emotional withdrawal) and item 16 (blunted affect)

HAM-D: Hamilton Depression Rating Scale; Physiosomatic symptoms of the HAM-D: sum of 5 HAM-D items, namely item 11 (anxiety somatic), item 12 (somatic symptoms), item 13 (somatic symptoms general), item 14 (genital symptoms) and item 15 (hypochondriasis); Suicidal ideation: item 3 of the HAM-D; HAM-A: Hamilton Anxiety Rating Scale; Psychopathology index: index of overall severity of psychopathology computed as z value BPRS (z BPRS) + z HAM-D + z HAM-A.

MDA: malondialdehyde; TRAP: total radical-trapping antioxidant parameter; LOOH: lipid hydroperoxides; AOPP: advanced oxidation protein products

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Table 6. Results of multiple regression analyses with oxidative stress toxicity biomarkers as dependent variables.

Dependent variables	Explanatory variables	β	t	p	F model	df	P	partial Eta Squared
MDA	**Model**				18.34	4/139	<0.001	0.345
	-SH	-0.450	-6.32	<0.001				
	NOx	-0.203	-2.92	0.004				
	TRAP	-0.193	-2.77	0.006				
	LOOH	0.174	2.48	0.014				
AOPP	**Model**				14.24	1/141	<0.001	0.091
	-SH	-0.302	-3.77	<0.001				

MDA: malondialdehyde; AOPP: advanced oxidation protein products; -SH: sulfhydryl groups; NOx: nitric oxide metabolites; TRAP: total radical-trapping antioxidant parameter; LOOH: lipid hydroperoxides