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# Antiepileptic drugs affect lipid oxidative markers-neuroprostanes and F<sub>2</sub>-dihomo-isoprostanes- in patients with epilepsy: differences among first-, second-, and third-generation drugs by UHPLC-QqQ-MS/MS

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Metabolites of the non-enzymatic lipid peroxidation of polyunsaturated fatty acids (DHA, *n*-6DPA, and AdA), neuroprostanes (NeuroPs), and F<sub>2</sub>-dihomo-isoprostanes (F<sub>2</sub>-dihomo-IsoPs) have become important biomarkers for oxidative stress (OS) in neurodegenerative diseases, particularly in epilepsy. These biomarkers were measured by UHPLC-QqQ-MS/MS in the urine of 30 epileptic patients treated with different antiepileptic drugs (AEDs) (old treatment (*n* = 15) or new-generation treatments (*n* = 15)), in comparison with 15 healthy controls. After treatment with new-generation AEDs (levetiracetam, eslicarbazepine acetate, lacosamide, and zonisamide) a decrease was observed in the total urinary neuronal membrane degradation markers (NeuroPs) and neuromotor system degradation markers (F<sub>2</sub>-Dihomo-IsoPs), in comparison with the old treatment (carbamazepine, phenytoin, valproic acid) and control (no pharmacological treatment) groups. These results suggest that new-generation AEDs reduce the total NeuroPs/F<sub>2</sub>-Dihomo-IsoPs to levels similar to those of the control group and hence play an important role in antioxidant systems in epileptic patients. Unfortunately, little is known currently about the neuroprotective effect of second- and third-generation AEDs; so, more clinical studies need to be performed to clarify the relationships among chronic treatment with newer AEDs, oxidative stress, and epilepsy.

## 1. Introduction

Epilepsy is one of the most common and diverse neurological disorders and it is defined by a state of recurrent, spontaneous seizures, which can be convulsive or non-convulsive episodes.<sup>1</sup> It has been suggested that oxidative damage and neuronal cell death are common pathological processes that can contribute to epileptogenesis.<sup>2</sup> It has been reported that the increased generation of free radicals can cause some forms of epilepsy, and also increase the risk of seizures recurrence.<sup>3</sup> Antiepileptic drugs (AEDs) are widely used in the treatment of epilepsy, as well as in various other neurological and psychiatric disorders.<sup>4</sup>

The AEDs are considered the first-line drugs (carbamazepine (CBZ), phenobarbital (PB), and valproic acid (VPA)) which increase free radical formation; this causes oxidative damage within neuronal cells, increases the peroxidation of neuronal membrane lipids, and reduces the protective effects of antioxidants.<sup>5</sup> Due to this, during the last three decades, 14 new AEDs have been licensed for clinical use. They can be divided into new second-generation (felbamate (FBM), gabapentin (GBP), lamotrigine (LTG), levetiracetam (LEV), oxcarbazepine (OXC), pregabalin (PGB), rufinamide (RFN), stiripentol (STP), tiagabine (TGB), topiramate (TPM), vigabatrin (VGB), and zonisamide (ZNS)) and third-generation (eslicarbazepine acetate (ESL) and lacosamide (LCM)) AEDs. Compared with first-generation AEDs, second- and third-generation AEDs interact less (pharmacokinetics and pharmacodynamics), and so lead to less complicated therapeutic outcomes and fewer complications for patients.<sup>4</sup>

In recent years, important advances have been made in the diagnosis of epilepsy. However, the understanding of the molecular mechanisms underlying epileptogenesis is still incomplete. Among various factors supposed to play a role in this disorder, the role of oxidative stress (OS) in neurological

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diseases has recently emerged.<sup>6</sup> Oxidative stress is a biochemical state in which reactive oxygen species (ROS) are generated and it has been associated with pathological states including epilepsy; therefore, oxidative injury (OI) may play a crucial role in the initiation and progression of this disease.<sup>7</sup> Therein, neuroprostanes (NeuroPs) and F<sub>2</sub>-dihomo-isoprostanes (F<sub>2</sub>-dihomo-IsoPs) – a series of compounds formed non-enzymatically through free radical induced peroxidation of DHA, n-6DPA, and AdA, respectively – are implicated in the pathophysiological status of epilepsy.<sup>8</sup>

The aim of this study was to evaluate the levels of urinary NeuroPs/F<sub>2</sub>-dihomo-IsoPs in epileptic patients, and then to determine whether treatment with first-(classic treatment) or second/third-generation (new-generation treatment) AEDs influences the urinary NeuroPs/F<sub>2</sub>-dihomo-IsoPs levels.

## 2. Materials and methods

### 2.1 Chemicals and reagents

Nine NeuroPs – 4(*RS*)-F<sub>4t</sub>-NeuroP; d<sub>4</sub>-4(*RS*)-F<sub>4t</sub>-NeuroP; 10-*epi*-10-F<sub>4t</sub>-NeuroP; d<sub>4</sub>-10-*epi*-10-F<sub>4t</sub>-NeuroP; 10-F<sub>4t</sub>-NeuroP; d<sub>4</sub>-10-F<sub>4t</sub>-NeuroP; 4-F<sub>4t</sub>-NeuroP; 4-*epi*-4-F<sub>3t</sub>-NeuroP; and 4-F<sub>3t</sub>-NeuroP – as well as four F<sub>2</sub>-dihomo-IsoPs – *Ent*-7(*RS*)-7-F<sub>2t</sub>-dihomo-IsoP; *Ent*-7(*S*)-7-F<sub>2t</sub>-dihomo-IsoP; 17-F<sub>2t</sub>-dihomo-IsoP; and 17-*epi*-17-F<sub>2t</sub>-dihomo-IsoP – were synthesized according to our published procedures.<sup>9–12</sup> The β-glucuronidase, type H2, from *Helix pomatia*, and BIS-TRIS (bis-(2-hydroxyethyl)-amino-tris(hydroxymethyl)-methane) used in this study were from Sigma-Aldrich (St. Louis, MO, USA). All LC-MS grade solvents were from J.T. Baker (Phillipsburg, NJ, USA). Chlorhydric acid was purchased from Panreac (Castellar del Vallés, Barcelona, Spain), and the Strata X-AW (100 mg 3 mL<sup>-1</sup>) SPE cartridges from Phenomenex (Torrance, CA, USA).

### 2.2 Selection of study participants

Thirty ambulatory epileptic patients from the Epilepsy Unit of the Virgen de la Arrixaca Hospital (Murcia, Spain) were enrolled in this study. Based on the type of AED treatment, the patients were divided into two groups, 15 patients receiving first-generation AEDs (classic treatment (CE)) and 15 receiving second- or third-generation AEDs (new-generation treatment (NGE)). All volunteers with epilepsy were seizure-free and without any changes in the daily dose of monotherapy AEDs for the last six months prior to their inclusion. All these patients had been diagnosed with epilepsy, based on clinical criteria supported by electroencephalographic recordings and cerebral magnetic resonance imaging (MRI). Standard EEG was performed at seizure onset and, if it was not conclusive, video-EEG monitoring was used. A cerebral MRI scan (1.5T or 3T when available) was performed in every patient in order to detect any structural lesion, such as cortical dysplasia, hippocampal sclerosis, brain tumors, or vascular lesions. Epilepsy was then classified, according to the International League Against Epilepsy (ILAE) criteria, as genetic, structural, or of unknown origin. The physical characteristics and clinical profiles of the subjects are summarized in Table 1. The control subjects (C; *n* = 15) were

age-matched individuals without clinical evidence of epilepsy or other neurological diseases, being otherwise healthy subjects. Importantly, all individuals in this study were non-smokers and had no dietary restrictions; during the study, the women were not menstruating. The control group did not receive any medication or drug intake (we noted the specific absence of the acute administration of anti-inflammation drugs). All patients gave written informed consent for the experiment. This study was approved by the Bioethics Committee of the Virgen de la Arrixaca Hospital (EPA-SP (IRE-CBZ-2014-01)) and the research was carried out in compliance with the Declaration of Helsinki for all human experimental investigations.

### 2.3 Standard and sample preparation

**2.3.1 Calibration standards.** Stock solutions of NeuroPs/F<sub>2</sub>-dihomo-IsoPs were diluted with methanol–water (1 : 1, v/v) to obtain the appropriate working solutions containing 10 analytes at a concentration of 1000 nmol L<sup>-1</sup>. For the determination of the calibration curves, 12 successive dilutions were prepared. All solutions were stored at –80 °C. The NeuroPs/F<sub>2</sub>-dihomo-IsoPs concentrations were calculated from standard curves freshly prepared each day.

**2.3.2 Urine sampling.** Twenty-four-hour urine samples were collected from the control group and after the AEDs treatment periods (classic treatment and new-generation treatment). They were collected in sterile, dark polystyrene pots with screw caps. Urine samples were collected from all patients and the control group: they were centrifuged, aliquoted, and stored at –80 °C. The 24 h urine was used for the absolute calculation of the amounts of NeuroPs/F<sub>2</sub>-dihomo-IsoPs excreted. The urinary excretion of these markers was analyzed using the previously described method.<sup>8</sup> This method showed the importance of the enzymatic hydrolysis of the urine samples, due to the fact that NeuroPs and F<sub>2</sub>-dihomo-IsoPs are excreted in urine as glucuronide and sulfate conjugates. Removing these conjugates, 1 mL of urine was added to 250 μL 0.1 M acetate buffer (pH 4.9) and 55 μL enzyme β-glucuronidase from *Helix pomatia*. The mixture was incubated at 37 °C for 2 hours. After this step, a protein precipitation was carried out with 500 μL of MeOH/HCl 200 mM. Then, samples were centrifuged at 10 000 g during 5 minutes. Briefly, the urine samples were applied to Strata X-AW SPE cartridges from Phenomenex (Torrance, CA, USA) previously conditioned and equilibrated and washed with 4 mL of water. The analytes were eluted with 1 mL of MeOH and dried using a SpeedVac concentrator (Savant SPD121P, Thermo Scientific, MA, USA). The extracts were reconstituted with 200 μL of solvent A/B (90 : 10, v/v) and filtered through a 0.45 μm filter of PVDF (Millipore, MA, USA). Then, 20 μL were analyzed in a UHPLC-QqQ-MS/MS.

### 2.4 UHPLC-QqQ-MS/MS analyses

The separation and quantification of the NeuroPs and F<sub>2</sub>-dihomo-IsoPs in the urine were performed using a UHPLC coupled with a 6460 QqQ-MS/MS (Agilent Technologies, Waldbronn, Germany), and the analytical method previously described.<sup>8</sup> Briefly, chromatographic separation was carried out on an ACQUITY BEH C18 column (2.1 × 50 mm, 1.7 μm pore

**Table 1** Physic characteristics and clinical profile of the all study participants<sup>a</sup>

	Control group (n = 15)	Epileptic CE (n = 15)	Epileptic NGE (n = 15)
Age (years)	32 ± 8	38.5 ± 11.8	36.1 ± 17.7
Gender male (n)	10	12	11
BMI (kg m <sup>-2</sup> )	22.5 ± 3.2	27.4 ± 4.7	24.5 ± 4.8
Epilepsy type (n)			
• Genetic	—	3	2
• Structural	—	8	7
• Unknown origin	—	4	6
Seizure type (n)			
• Generalized	—	5	7
• Focal	—	5	4
• Evolving to a bilateral convulsive seizures	—	5	4
Duration of epilepsy (years)	—	12.9 ± 8.1	7.3 ± 6.9
Duration of therapy (months)	—	104.9 ± 85.8	34.3 ± 22.9
AED (n (mean dose mg per d))			
• CBZ	—	6 (629)	—
• PHT	—	1 (300)	—
• VPA	—	8 (1169)	—
• LEV	—	—	11 (1325)
• LCM	—	—	1 (200)
• ESL	—	—	2 (1000)
• ZNS	—	—	1 (300)
Calcium (mg dL <sup>-1</sup> )	9.9 ± 0.4	9.6 ± 0.5	9.7 ± 0.2
Sodium (mEq L <sup>-1</sup> )	134.8 ± 16.9	141.2 ± 2.2	141.1 ± 2.5
Glucose (mg dL <sup>-1</sup> )	87.5 ± 6.9	98.1 ± 29.5	91.1 ± 6.7
Urea (mg dL <sup>-1</sup> )	32.5 ± 3.7	34.1 ± 10	31.0 ± 9.6
Creatinine (mg dL <sup>-1</sup> )	0.8 ± 0.1	0.8 ± 0.2	0.8 ± 0.1
Bilirubin total (mg dL <sup>-1</sup> )	0.7 ± 0.2	0.4 ± 0.2	0.8 ± 0.3
GOT (U L <sup>-1</sup> )	26.0 ± 6.1	21.3 ± 7.8	22.7 ± 12.2
GPT (U L <sup>-1</sup> )	24.0 ± 8.4	22.6 ± 8.6	22.7 ± 12.5
GGT (U L <sup>-1</sup> )	14.8 ± 3.8	33.2 ± 20.2	20.7 ± 13.2
FT4 (ng dL <sup>-1</sup> )	1.2 ± 0.1	1.1 ± 0.1	2.2 ± 0.7
TSH (uUI mL <sup>-1</sup> )	2.1 ± 0.7	2.5 ± 1.6	1.1 ± 0.1

<sup>a</sup> Data are presented as mean ± SD. Abbreviations: BMI (Body Mass Index); AED (Antiepileptic Drug); CBZ (Carbamazepine); ESL (Eslicarbazepine acetate); GGT (Gamma Glutamyl Transferase); GOT (Glutamic Oxaloacetic Transaminase); GPT (Glutamic Pyruvate Transaminase); LCM (Lacosamide); LEV (Levetiracetam); PHT (Phenytoin); TSH (Thyroid Stimulating hormone); FT4 (Free Thyroxine); VPA (Valproic Acid); ZNS (Zonisamide). No significant differences either between the control and AEDs treated groups or between the two treated (CE and NGE) groups were found.

size) (Waters, MA, USA). The mass spectrometry analysis was performed in the multiple reaction monitoring (MRM) mode, in the negative ionization mode. The mobile phases were solvent A (Milli-Q water/acetic acid, 99.99 : 0.01, v/v) and solvent B (methanol/acetic acid, 99.99 : 0.01, v/v). The flow rate was 0.2 mL min<sup>-1</sup>. The ESI conditions and ion optics were those previously described.<sup>8</sup> Data acquisition and processing were performed using MassHunter software version B.04.00 (Agilent Technologies, Walbronn, Germany). The quantitative analysis of NeuroPs/F<sub>2</sub>-dihomo-IsoPs was performed using the authentic markers synthesized by Durand's team.

## 2.5 Statistical analyses

The quantitative data are presented as mean ± SD (standard deviation). Specific differences in the amounts of NeuroPs and F<sub>2</sub>-dihomo-IsoPs excreted were calculated as ng per 24 h urine. The samples of the control and AEDs treatment groups were examined by one-way ANOVA (three groups: control, classic AEDs treatment, and new-generation AEDs treatment) followed by Tukey's HSD (Honestly Significant Difference) test. Statistical

analyses were performed using the SPSS 21.0 software package (LEAD Technologies Inc., Chicago, USA) and the level of statistical significance was set at *P* < 0.05.

## 3. Results

### 3.1 Characteristics of the study population

The study was performed in 30 patients diagnosed with epilepsy and 15 healthy volunteers (control group). The epileptic patients were divided into two groups of 15, depending on the AED received: classic and new-generation AEDs (Table 1). The mean age of the patients receiving classic AEDs was 38.5 ± 11.8 years, vs. 36.1 ± 17.7 years for patients on new-generation AEDs. Of the patients treated with classic AEDs, 53% had structural epilepsy and 26% had epilepsy of unknown origin, while 47% of those on newer AEDs were diagnosed with structural epilepsy and 40% had epilepsy of unknown origin. This classification was carried out under guidelines previously published by Berg and colleagues.<sup>13</sup> The duration of the epilepsy and therapy was greater in the classic AEDs group than with new-generation

drugs ( $12.9 \pm 8.1$  years *vs.*  $7.3 \pm 6.9$  years and  $104.9 \pm 85.8$  months *vs.*  $34.3 \pm 22.9$  months, respectively). Biochemical parameters are shown in Table 1.

### 3.2 Qualitative profile of neuroprostanes and F<sub>2</sub>-dihomo-isoprostanes in the epileptic subject urines

The analysis of NeuroPs/F<sub>2</sub>-dihomo-IsoPs allowed the detection of nine lipid oxidation markers. The separation of the

compounds – using the method developed by Medina and colleagues<sup>8</sup>– was satisfactory (Fig. 1). The only NeuroP not detected was 4(*S*)-F<sub>4t</sub>-NeuroP. Moreover, for the unequivocal identification of the target compounds, the most intensive MRM transition was selected (see Fig. 1 and its footnote). Some MRM transitions were not exclusive to a single compound; so, some compounds were differentiated by their distinct retention times.

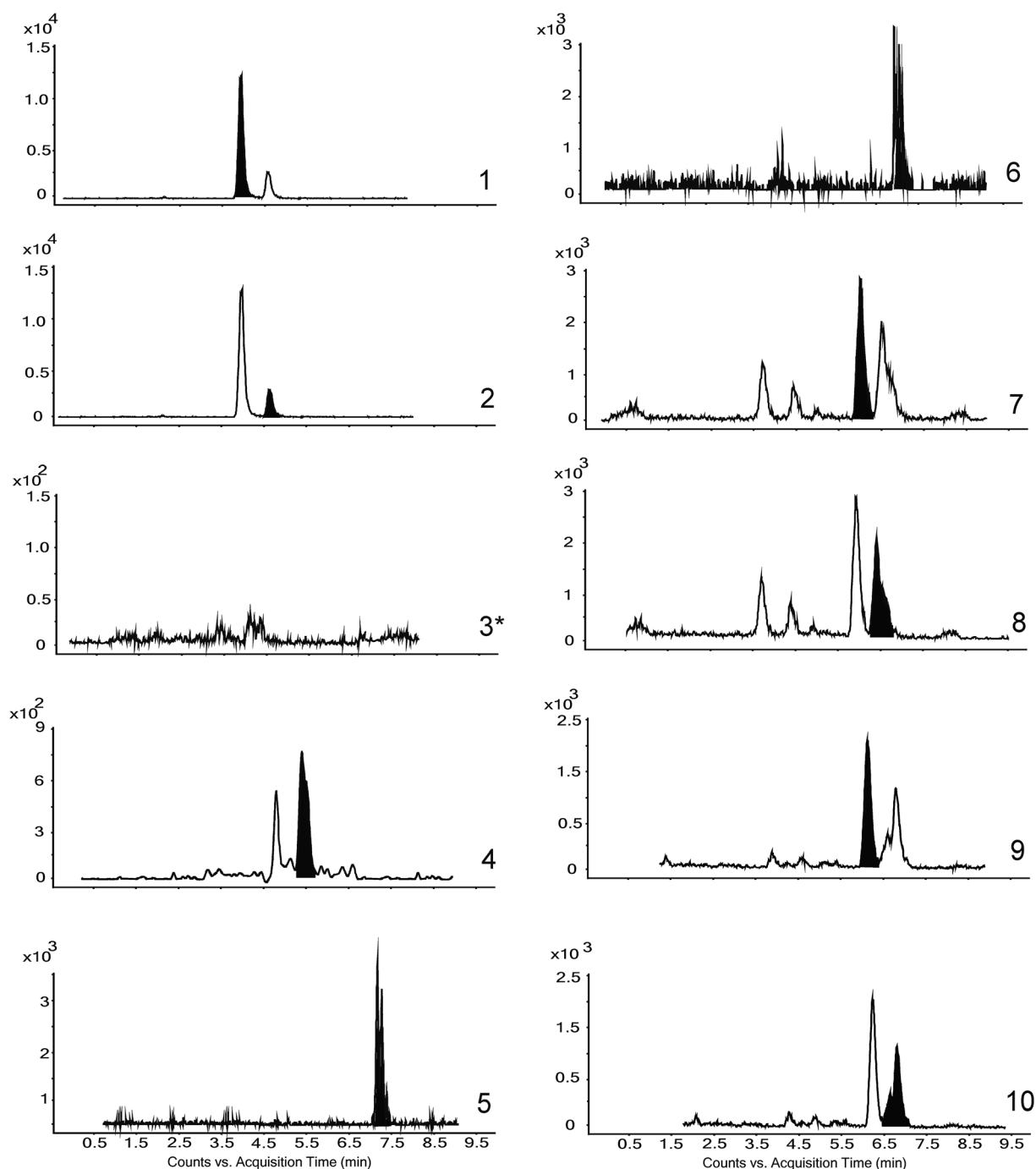


Fig. 1 UHPLC-QqQ-MS/MS chromatograms of neuroprostanes and F<sub>2</sub>-dihomo-isoprostanes in urine samples: (1) 10-F<sub>4t</sub>-NeuroP (377.1 → 152.9), (2) 10-*epi*-10-F<sub>4t</sub>-NeuroP (377.1 → 152.9), (3) 4(*S*)-4-F<sub>4t</sub>-NeuroP (377.1 → 333.1), (4) 4(*R*)-4-F<sub>4t</sub>-NeuroP (377.1 → 271.2), (5) 4-F<sub>3t</sub>-NeuroP (379.1 → 101.1), (6) 4-*epi*-4-F<sub>3t</sub>-NeuroP (379.0 → 219.0), (7) *Ent*-7(*R*)-7-F<sub>2t</sub>-Dihomo-IsoP (381.1 → 362.2), (8) *Ent*-7(*S*)-7-F<sub>2t</sub>-Dihomo-IsoP (381.1 → 362.2), (9) 17-*epi*-17-F<sub>2t</sub>-Dihomo-IsoP (381.0 → 337.1), (10) 17-F<sub>2t</sub>-Dihomo-IsoP (381.0 → 337.1). \* Not detected.

### 3.3 Effect of classic and new-generation antiepileptic drugs on urinary levels of lipid oxidation markers

The method of urine analysis developed by Medina and colleagues<sup>8</sup> allowed the quantification of nine lipid oxidation markers (five NeuroPs and four F<sub>2</sub>-dihomo-IsoPs) in subjects with epilepsy treated with different AEDs, classic (first-generation) and new generation (second- and third-generation), compared with the control group. There was a statistically significant increase ( $P < 0.05$ ) in the subtotal of neuronal membrane degradation markers (NeuroPs) in the epileptic patients treated with classic AEDs ( $27\,565.7 \pm 9306.9$  ng  $24\text{ h}^{-1}$ ), when compared with the new-generation AEDs treatment patients ( $15\,070.1 \pm 3398.1$  ng  $24\text{ h}^{-1}$ ) and the control group ( $13\,218.6 \pm 4683.2$  ng  $24\text{ h}^{-1}$ ). Similarly, the subtotal of F<sub>2</sub>-dihomo-isoprostanes (neuromotor

system degradation markers) was higher in patients receiving classic-generation drugs ( $31\,224.9 \pm 7497.1$  ng  $24\text{ h}^{-1}$ ) than in epileptic subjects treated with new-generation AEDs ( $20\,441 \pm 4968.7$  ng  $24\text{ h}^{-1}$ ) or the control group ( $24\,505.6 \pm 6025$  ng  $24\text{ h}^{-1}$ ).

Regarding the neuronal membrane degradation markers, two (*4-epi-4-F<sub>3t</sub>*-NeuroP and *10-epi-10-F<sub>4t</sub>*-NeuroP) did not show statistically significant differences among the groups. Moreover, one compound (*4(R)-F<sub>4t</sub>*-NeuroP) was only detected and quantified in the group of patients treated with first-generation AEDs.

The data show a decrease in four lipid oxidation markers (*4-F<sub>3t</sub>*-NeuroP, *17-F<sub>2t</sub>*-Dihomo-IsoP, *Ent-7(R)-7-F<sub>2t</sub>*-dihomo-IsoP, and *Ent-7(S)-7-F<sub>2t</sub>*-dihomo-IsoP) when the patients had

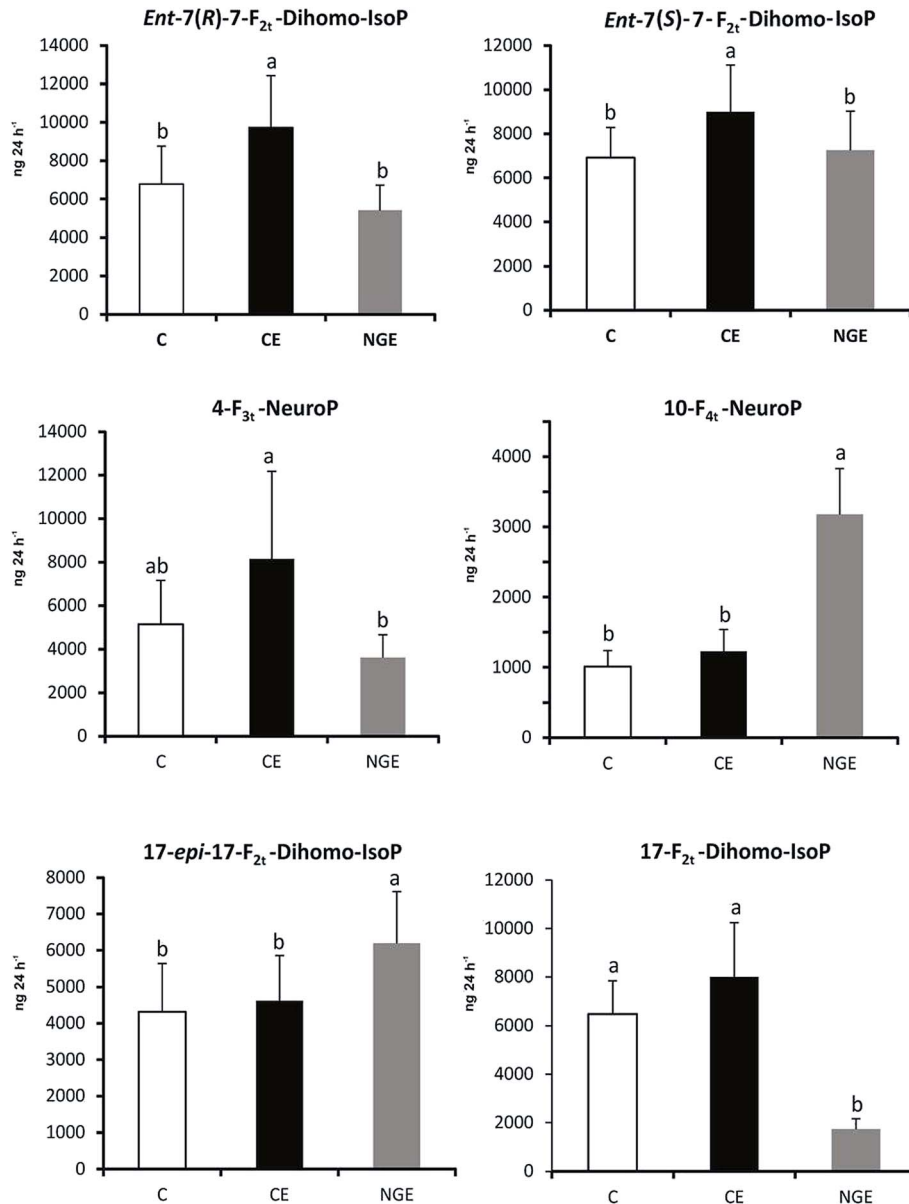


Fig. 2 Urinary excretion (ng  $24\text{ h}^{-1}$ ) of neuroprostanes and F<sub>2</sub>-dihomo-isoprostanes in the control group (C), epileptic patients with classical treatment (CE), and epileptic patients with new-generation antiepileptic drugs (NGE). Data are presented as mean  $\pm$  SD (standard deviation). Different lowercase letters mean significant differences at  $P < 0.05$ , according to the Tukey HSD Multiple Range Test.

received new-generation AEDs, compared with the classic treatment and control group (see Fig. 2). So, 4-F<sub>3t</sub>-NeuroP decreased by ~56% and ~37% (new-generation treatment and control, respectively) relative to the classic treatment. Also, the most marked reduction (~78%) was observed for 17-F<sub>2t</sub>-dihomo-IsoP, when compared with the classic treatment and new generation treatment groups. In the same sense, the amounts of *Ent-7(R)*-7-F<sub>2t</sub>-Dihomo-IsoP and *Ent-7(S)*-7-F<sub>2t</sub>-dihomo-IsoP decreased by ~43% and ~21%, respectively, comparing the new-generation treatment with the first-generation AEDs. Moreover, only two compounds (10-F<sub>4t</sub>-NeuroP and 17-*epi*-17-F<sub>2t</sub>-dihomo-IsoP) showed increases in the patients treated with new-generation drugs (rises of ~62% and ~26%, respectively), compared to the classical medication (Fig. 2). A very important fact about this study is that 11 of the 15 patients in the new AEDs group were on LEV (a drug with little interaction in the liver). However, the oxidative marker levels after treatment with other new AEDs (ESL, LCM, or ZNS) were statistically similar to those in the volunteers receiving LEV alone. In the same sense, in the volunteers treated with classic AEDs, the NeuroPs/F<sub>2</sub>-dihomo-IsoPs levels showed no statistical differences among the CBZ, PHT, or VPA treatments.

## 4. Discussion

Lipid peroxidation is one of the most biologically important free radical reactions and it has been pointed out as a key chemical event in the OS associated with several inborn and acquired pathologies.<sup>14</sup> The most widely used test for OS is measurement of malondialdehyde (MDA), a product of lipid peroxidation in a thiobarbituric acid-reacting substances (TBARS) assay. However, the use of this analysis to evaluate the OS status is problematic because MDA is not a specific product of lipid peroxidation and the TBARS assay is not specific for MDA.<sup>15,16</sup> The recently developed measurement of F<sub>2</sub>-IsoPs, NeuroPs, and F<sub>2</sub>-dihomo-IsoPs is the best available assay of lipid peroxidation in neurodegenerative diseases.<sup>8,17,18</sup>

In all analyses, one very important aspect is the technique used and its limitations. The GC/MS device was the one used first for the quantification of NeuroPs/F<sub>2</sub>-dihomo-IsoPs.<sup>19</sup> However, due to its handicaps (extensive sample preparation, weak specificity, and an inability to isolate isomers of compounds), other devices such as LC/MS-MS are gaining more attention.<sup>20</sup> As well as LC/MS, other techniques, such as enzyme-linked immunosorbent assay (ELISA) and radioimmunoassay (RIA), were developed for the determination of IsoPs (8-iso-PGF<sub>2α</sub>) in the urine of epileptic patients<sup>21,22</sup> and for the detection of NeuroPs/F<sub>2</sub>-dihomo-IsoPs.<sup>23</sup> So, immunological methods were chosen because these techniques are reproducible and easy to perform. However, the cross-reactivity among the different NeuroPs/F<sub>2</sub>-dihomo-IsoPs and their demonstrated overestimation constituted significant disadvantages compared to LC/MS-MS, making them inappropriate for clinical application.<sup>24</sup> Also, LC/MS (specifically UHPLC) is superior to other techniques (such as GC/NICI-MS and ELISA/RIA) in the identification of different regioisomers and diastereoisomers of NeuroPs/F<sub>2</sub>-dihomo-IsoPs;<sup>8</sup> moreover, this technique is much faster.

From a clinical point of view, previous studies reported that F<sub>4</sub>-NeuroPs were increased in patients with neurological diseases (such as aneurysmal subarachnoid hemorrhage, Rett syndrome, Alzheimer's disease, or atherosclerosis), compared to healthy control groups.<sup>25-28</sup> Indeed, in comparison with these reports, our study has detected differences among groups (C, CE, and NGE) and has quantified a greater amount of NeuroPs/F<sub>2</sub>-dihomo-IsoPs. This may be due to the greater suitability and selectivity of the UHPLC-MS technique applied in our study. As well, the enzymatic hydrolysis step was crucial for good and real quantification in the urine samples. A recently published study showed that these compounds are excreted as glucuronide conjugates; so, the hydrolysis of the urine samples could allow measurement of greater amounts of NeuroPs/F<sub>2</sub>-dihomo-IsoPs, compared with non-hydrolyzed samples.<sup>8</sup>

In recent years, important advances have been made in the diagnosis and treatment of neurological diseases in general and of epilepsy in particular. It has been shown that, apart from other known mechanisms in epileptogenesis, OS and generation of ROS are implicated in seizure disorders.<sup>21</sup> Similarly, increased levels of OS markers have been found in different experimental models of epilepsy.<sup>29</sup> So, lipid peroxidation markers (8-iso-PGF<sub>2α</sub>, also named 15-F<sub>2t</sub>-IsoP) were found to have increased in abundance in rats after VPA (classical AEDs) treatment.<sup>30</sup> In the same sense, in humans, Michoulas and colleagues showed increased urinary levels of 8-iso-PGF<sub>2α</sub> in epileptic children who were treated with VPA. This may indicate that VPA induces the OS in epileptic patients;<sup>31</sup> it indicates also that long-term use of AEDs may affect lipid oxidative damage and antioxidant defense in epileptic patients.<sup>32</sup> In our study, there was a difference in the treatment duration between the two groups (classic and new-generation AEDs); however, the effect of the AEDs treatment duration on the generation of OS markers is unclear. Chuang and colleagues reported that long-term monotherapy with diverse AEDs had no effect on lipid peroxidation markers (TBARS) or on inflammation markers such as hs-CRP.<sup>33</sup>

Regarding treatment with new-generation AEDs, only one study has reported LEV-induced formation of 8-iso-PGF<sub>2α</sub> in epileptic patients.<sup>22</sup> Several previous reports have studied the properties of different AEDs (VPA, CBZ, LEV, and ZNS) and their behavior in the diverse pathways of pro-oxidation/antioxidation. A problem that arises when trying to clarify the role of AEDs in this field is the heterogeneity in the samples studied: for example, blood or urine of epileptic patients,<sup>34</sup> rat astrocytes cultures,<sup>35</sup> plasma or tissues of rat,<sup>36</sup> or rodent hippocampus.<sup>37</sup> In a similar vein, there were also differences among studies performed *in vitro* and *in vivo* experiments, in most cases involving the same AEDs.<sup>38</sup>

The relationship between OS and AEDs is documented in the scientific literature in a poor and controversial way, especially regarding the differences between classic and new-generation drugs. The classic drugs such as VPA or CBZ could induce overproduction of ROS, whereas new-generation drugs could have a "scavenger" function in relation to ROS production in human and animal models.<sup>39</sup> So, VPA seems to have pro-oxidant activity: Martinez-Ballesteros and colleagues reported that

plasma samples of patients treated with this AED showed vulnerability to OS that was directly proportional to the plasma drug concentration.<sup>40</sup> On the other hand, previous studies showed that AEDs did not influence the oxidative markers, suggesting the presence of seizure-induced OS.<sup>41</sup> Besides, findings concerning the effects of AEDs and OS indicated that the pro-oxidant/antioxidant balance of epileptic patients was modified by AEDs therapy.<sup>42</sup> Therefore, knowledge of how these drugs could modulate this oxidative system will open a new therapeutic window for epilepsy treatments in which OS is one of the underlying mechanisms.<sup>43</sup> Indeed, in the report of French and colleagues,<sup>44</sup> it is cited that it is impossible to confirm that “all old AEDs are bad” or “all new AEDs are good”. However, the absence of hepatic enzyme induction/inhibition when most of the newer AEDs are used provides one more advantage.<sup>44</sup> So, this study provides mechanistic support for further research in this field. The first aspect that must be investigated is the true impacts of AEDs on the oxidative status of epileptic patients, being as several different factors, including pharmacogenetics, contribute to inter-individual variability in drug response.

## 5. Conclusion

The present findings show that treatment with new-generation AEDs globally reduces the excretion of NeuroPs/F<sub>2</sub>-dihomo-IsoPs to values similar to those in the control group, indicating a positive effect of these AEDs on the antioxidant status of epileptic patients. Unfortunately, little is known about the neuroprotective effect of second- and third-generation AEDs, so more investigation needs to be done in the clinical field to clarify the relationships among chronic treatment of AEDs, oxidative stress, and epilepsy. So, it would be of interest to conduct additional prospective studies specifically designed to clarify the effect of pharmacological treatment in patients with epilepsy.

## Abbreviations

AdA	Adrenic acid
AEDs	Antiepileptic drugs
CBZ	Carbamazepine
DHA	Docosahexaenoic acid
Dihomo-IsoPs	Dihomo-isoprostanes
DPA	Docosapentanoic acid
EEG	Electroencephalography
ELISA	Enzyme-linked immunosorbent assay
ESI	Electrospray ionization
ESL	Eslicarbazepine acetate
FBM	Felbamate
GBP	Gabapentin
GC-NICI-MS	Gas chromatography-negative ion chemical ionization-mass spectrometry
hs-CRP	High-sensitivity C-reactive protein
ILAE	International league against epilepsy
LC/MS-MS	Liquid chromatography tandem mass spectrometry
LCM	Lacosamide

LC-MS	Liquid chromatography mass spectrometry
LEV	Levetiracetam
LTG	Lamotrigine
MDA	Malondialdehyde
MRI	Magnetic resonance imaging
MRM	Multiple reaction monitoring
NeuroPs	Neuroprostanes
OI	Oxidative injury
OS	Oxidative stress
OXC	Oxcarbazepine
PB	Phenobarbital
PGB	Pregabalin
RFN	Rufinamide
RIA	Radioimmunoassay
SPE	Solid phase extraction
STP	Stiripentol
TBARS	Thiobarbituric acid-reacting substances
TGB	Tiagabine
TPM	Topiramate
UHPLC-QqQ-MS/MS	Ultrahigh pressure liquid chromatography-triple quadrupole-tandem mass spectrometry
VGB	Vigabatrin
VPA	Valproic acid
ZNS	Zonisamide

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