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# Relevance of 4-F<sub>4t</sub>-neuroprostane and 10-F<sub>4t</sub>-neuroprostane to neurological diseases

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## A B S T R A C T

F<sub>4</sub>-neuroprostanes (F<sub>4</sub>-NeuroPs) are non-enzymatic oxidized products derived from docosahexaenoic acid (DHA) and are suggested to be oxidative damage biomarkers of neurological diseases. However, 128 isomers can be formed from DHA oxidation and among them, 4(RS)–4-F<sub>4t</sub>-NeuroP (4-F<sub>4t</sub>-NeuroP) and 10(RS)–10-F<sub>4t</sub>-NeuroP (10-F<sub>4t</sub>-NeuroP) are the most studied.

Here, we report the identification and the clinical relevance of 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP in plasma of four different neurological diseases, including multiple sclerosis (MS), autism spectrum disorders (ASD), Rett syndrome (RTT), and Down syndrome (DS).

The identification and the optimization of the method were carried out by gas chromatography/negative-ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) using chemically synthesized 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP standards and in oxidized DHA liposome.

Both 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP were detectable in all plasma samples from MS (n = 16), DS (n = 16), ASD (n = 9) and RTT (n = 20) patients. While plasma 10-F<sub>4t</sub>-NeuroP content was significantly higher in patients of all diseases as compared to age and gender matched healthy control subjects (n = 61), 4-F<sub>4t</sub>-NeuroP levels were significantly higher in MS and RTT as compared to healthy controls. Significant positive relationships were observed between relative disease severity and 4-F<sub>4t</sub>-NeuroP levels (r = 0.469, P < 0.0001), and 10-F<sub>4t</sub>-NeuroP levels (r = 0.757, P < 0.0001). The study showed that the plasma amount ratio of 10-F<sub>4t</sub>-NeuroP to 4-F<sub>4t</sub>-NeuroP and the plasma amount as individual isomer can be used to discriminate between different brain diseases.

Overall, by comparing the different types of disease, our plasma data indicates that 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP: i) are biologically synthesized in vivo and circulated, ii) are related to clinical severity of neurological diseases, iii) are useful to identify shared pathogenetic pathways in distinct brain diseases, and iv) appears to be distinctive for different neurological conditions, thus representing potentially new biological disease markers. Our data strongly suggest that in vivo DHA oxidation follows preferential chemical rearrangements according to different brain diseases.

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**Abbreviations:** 4-F<sub>4t</sub>-NeuroP, 4(RS)–4-F<sub>4t</sub>-neuroprostane; 10-F<sub>4t</sub>-NeuroP, 10(RS)–10-F<sub>4t</sub>-neuroprostane; 15-F<sub>2t</sub>-IsoP, 15-F<sub>2t</sub>-isoprostane; ASD, autism spectrum disorders; DHA, docosahexaenoic acid; DS, Down syndrome; F<sub>4t</sub>-NeuroPs, F<sub>4</sub>-neuroprostanes; GC/NICI-MS/MS, gas chromatography/negative-ion chemical ionization tandem mass spectrometry; MS, multiple sclerosis; PGF<sub>2α</sub>-d<sub>4</sub>, tetraoxygenated prostaglandin F<sub>2α</sub>; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RTT, Rett syndrome

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## 1. Introduction

Isoprostanooids, the metabolites of non-enzymatic lipid peroxidation of polyunsaturated fatty acid (PUFA), are measured to assess in vivo oxidative stress status [1,2]. Among all the metabolites from PUFAs, 15-F<sub>2t</sub>-isoprostane (15-F<sub>2t</sub>-IsoP) derived from arachidonic acid (AA) has been largely studied in medical research [3–5]. Also, 15-F<sub>2t</sub>-IsoP (also referred to as 8-iso-PGF<sub>2α</sub>) has been identified to be the most abundant F<sub>2</sub>-IsoP isomer in the free radical-induced AA oxidation [6] and elevated levels are closely related for example, to inflammation and cardiovascular diseases. As a result, 15-F<sub>2t</sub>-IsoP is quantified by scientists to unveil pathological mechanisms associated to oxidative stress and recently, the measurement of 15-F<sub>2t</sub>-IsoP was classified in a meta-analysis for its role in several specific diseases associated to oxidative damage [7].

Despite this, 15-F<sub>2t</sub>-IsoP appears to be a non-specific metabolite to be determined in diseases related to neuronal damage [8]. One reason is the difference in PUFA content where docosahexaenoic acid (DHA) is more abundant compared to AA in the brain, in particular the gray matter. Non-enzymatic DHA oxidation generates 128 isomers from 8 regioisomer series (4, 7, 10, 11, 13, 14, 17 or 20) of which 4-series or F<sub>4</sub>-neuroprostanes (F<sub>4</sub>-NeuroPs) [9–11] is the most abundant. Moreover, because of the extra methylene bonds in the structure, DHA is more prone to oxidation than AA. F<sub>4</sub>-NeuroPs are very promising isoprostanooids and might have paramount applications in medical field, taking into account the abundance of DHA in the central nervous system [12–14]. In this regard, it was shown that NeuroPs and not IsoPs are the critical metabolites for neuronal damage. Among all the F<sub>4</sub>-NeuroP molecules, only a handful of the isomers are characterized in neuro-pathological conditions and so far, 4-F<sub>4t</sub>-NeuroPs and 10-F<sub>4t</sub>-NeuroPs are considered to be the most represented [8,15–25].

Previously, our group showed altered levels of plasma F<sub>4</sub>-NeuroPs in Rett syndrome (RTT) [26], and Down syndrome (DS) patients [27], as well as in RTT experimental mice models [28] and in a rodent model of neonatal hypoxic-ischemic encephalopathy [29]. Moreover, in RTT and DS diseases, elevated levels of F<sub>4</sub>-NeuroP have been shown to be intimately related to neurological severity [26] and cognitive performance [27], respectively. Other studies showed that elevated plasma F<sub>4</sub>-NeuroPs are in smokers [30], type 2 diabetes [31], ischemic-stroke [32], tick-borne encephalitis [33] and neuroborreliosis [34] but its chemical formation, the preferential formation of the type of F<sub>4</sub>-NeuroP isomers and the relevance to the type of diseases are not well characterized.

In this study, we aimed i) to optimize mass spectrometric analysis for 4(RS)–4-F<sub>4t</sub>-NeuroP (4-F<sub>4t</sub>-NeuroP) and 10(RS)–10-F<sub>4t</sub>-NeuroP (10-F<sub>4t</sub>-NeuroP) that were chemically synthesized as standards and in oxidized DHA liposome, and ii) to investigate the relevance of 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP in different types of neurological diseases, i.e. multiple sclerosis (MS), Down syndrome (DS), autism spectrum disorders (ASD), and Rett syndrome (RTT), where oxidative injury and fatty acid oxidation take part in the pathogenesis [27,35–48].

## 2. Materials and methods

### 2.1. Subjects

A total of 122 subjects were enrolled in this study. Of them, 16 patients (46.6 ± 12.1 years old, male to female ratio 7:9) were relapsing-remitting MS [49], 20 were RTT (10.3 ± 10.3 years old, all female) with proven *MECP2* mutations and typical clinical presentation [50] (Child Neuropsychiatry Unit, University Hospital, Siena Italy), 9 were autistic patients (13.5 ± 4.6 years old, male to female ratio 7:2) diagnosed according to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition [51], and 16 were DS subjects (24.9 ± 4.7 years old, male to female ratio 9:7) who had cariotypically confirmed trisomy 21. In addition, a total of 61 of age- and gender-matched healthy control

subjects were recruited. Written consent form was obtained by all the subjects enrolled, or by the patient guardian. This study was approved by the institutional review boards and was carried out in accordance to the rules expressed in the Declaration of Helsinki Ethical Principles for Medical Research involving Human Subjects (Brazil, 2013).

In addition, depending on the disease, different types of clinical tests were conducted on the patients, clinical severity was assessed by the Rett Clinical Severity Score (RCSS) [52] (disability range: 7–30) and Childhood Autism Rating Scale (CARS) [53] for ASD (disability range: 36–48). Expanded Disease Status Scale Score (EDSS) [54] for MS (disability range: 0.0–4.0), and Raven's Colored Progressive Matrices (CPM) [55] as measure of cognitive impairment for DS (disability range: 3–22) were also used.

### 2.2. Sample preparation

Platelet poor plasma samples were obtained by centrifugation (2400 × g for 15 min at 4 °C) of blood aliquots collected in heparinized tubes. As an antioxidant, butylated hydroxytoluene (BHT) (90 μM prepared in absolute ethanol) was added to each plasma samples, mixed and stored at –70 °C for “free” NeuroPs (i.e. not esterified to phospholipids) determination.

To identify the existence of the NeuroPs isomers in a diseased model, brain tissues of RTT mice (*Mecp2* stop/y model) and its wild-type were extracted [28].

All mice (n = 8; age 12–17 weeks) were obtained from Dr. J. Guy, Wellcome Centre for Cell Biology, University of Edinburgh, United Kingdom. The aggregate score of phenotypic severity of the mice was performed on weekly basis for symptoms arising from *Mecp2* deficiency. [56] After transcardial perfusion with saline, the mice were sacrificed and the brain tissues were removed and bisected on the sagittal plane. The brain hemispheres were immediately frozen in dry ice and stored at –80 °C until assay. In circulation, NeuroPs are found as “free” and “esterified” form but in tissues most of the NeuroPs are esterified to phospholipids. Therefore, the samples required a hydrolysis process. The tissue was homogenized (10% w/v) in phosphate-buffered saline, pH 7.4 with BHT. To an aliquot (1 ml) of the brain homogenate, aqueous KOH (1 mM, 500 μl) was added. After incubation at 45 °C for 45 min, the pH was adjusted to 3 by adding HCl (1 mM, 500 μl). Each sample was spiked with the internal standard, tetradeuterated prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>-d<sub>4</sub>, 500 pg in 50 μl ethanol) and then ethyl acetate (10 ml) was added to extract the lipid portion by vortex-mixing and centrifugation at 1000 × g for 5 min at room temperature.

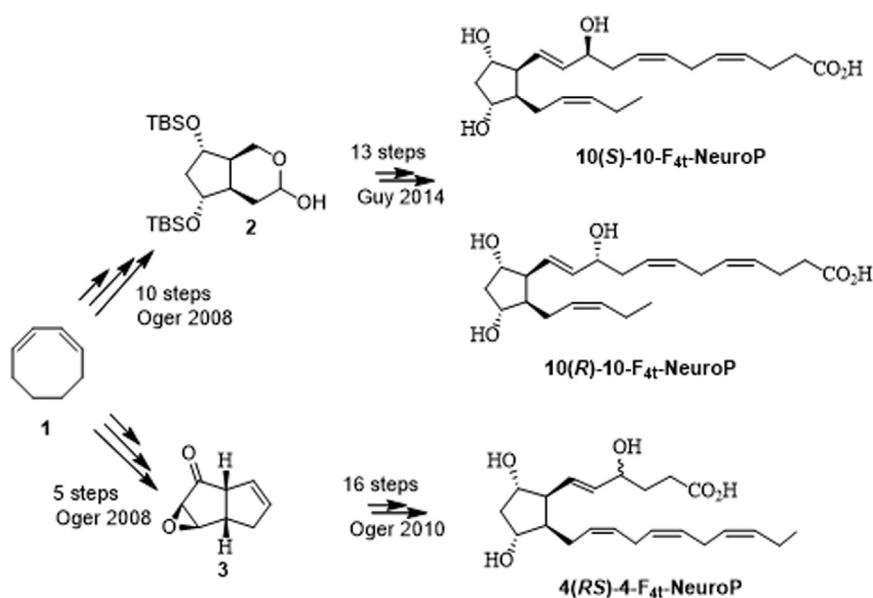
### 2.3. In vitro oxidation of DHA

Following a model of free radical-induced oxidation process reported [57], DHA was oxidized by 2,2'-azobis-(2-amidinopropane) hydrochloride (AAPH). DHA (20 mg) was vortexed with 20 ml of phosphate-buffered saline (10 mM potassium phosphate, 10 mM sodium chloride, pH 7.4, PBS). The dispersion was then ultrasonicated for 3 min and incubated in presence of 10 mM AAPH for 24 h at 37 °C. [29].

### 2.4. 4(RS)-F<sub>4t</sub>-NeuroP, and 10(R)–10-F<sub>4t</sub>-NeuroP and 10(S)–10-F<sub>4t</sub>-NeuroP synthesis

The synthesis of the two series of 4- and 10-F<sub>4t</sub>-NeuroPs [58–60] is summarized in Scheme 1. Starting from the commercially available 1,3-cyclooctadiene **1**, the two key bicyclic intermediates **2** and **3** were obtained in 10 and 5 steps respectively giving 8.8% (for intermediate **2**) and 18% (for intermediate **3**) yields. The introduction of α and ω chains was performed by using regioselective protections/deprotections, oxidations, Wittig elongation and cross metathesis coupling reactions as the main steps. The final step is the saponification of the methyl esters in the presence of LiOH to obtain free acids. The 4(RS)–4-F<sub>4t</sub>-NeuroP

Scheme 1. Chemical strategy for 4- $F_{4t}$ -NeuroP and 10- $F_{4t}$ -NeuroP.



was obtained starting from intermediate 3 that followed 16 steps of the main synthesis giving 9% yield while 10(*S*)-10- $F_{4t}$ -NeuroP and its 10-epimer were obtained in 13 steps from intermediate 2 giving 6.7% yield.

## 2.5. Extraction of $F_4$ -NeuroPs

$F_4$ -NeuroPs were extracted from the plasma using the following procedures. The internal standard (PGF<sub>2α</sub>-d<sub>4</sub>, 500 pg contained in 50 μl ethanol) was added to each plasma sample (1 ml) before extraction process. Thereafter, a volume of 2 ml acidified water (pH 3) were added to each sample and then applied on a C<sub>18</sub> solid phase extraction (SPE) cartridge followed by a NH<sub>2</sub> SPE cartridge for a series of clean-up procedures using known methods [56,60]. Briefly, the C<sub>18</sub> cartridge (500 mg Sorbent per Cartridge, 55–105 μm Particle Size, 6cc, Waters, USA) was preconditioned with methanol (5 ml) and water (5 ml), and sequentially washed after loading the sample with 10 ml water (pH 3), and 10 ml water: acetonitrile (85:15, v/v). Hexane: ethyl acetate: propan-2-ol (30:65:5 v/v/v, 5 ml) mix was used for the final eluate. Afterwards, the eluate was transferred to an NH<sub>2</sub> cartridge (500 mg Sorbent per Cartridge, 55–105 μm Particle Size, 6cc, Waters, USA), which was preconditioned with hexane (5 ml). After loading the eluate, it was sequentially washed with 10 ml of hexane: ethyl acetate (30:70, v/v), 10 ml acetonitrile: water (9:1, v/v) and 10 ml acetonitrile. The final elution was a mix of ethyl acetate: methanol: acetic acid (10:85:5, v/v/v, 5 ml). The collected eluate was first evaporated under nitrogen at 40 °C prior derivatization process.

The same procedures were used for the extraction of the oxidized DHA liposome. As for the brain tissue samples, the lipid portion obtained by extraction with ethyl acetate (as reported in *Sample preparation* paragraph) was directly transferred to an NH<sub>2</sub> cartridge and the subsequent procedures were the same as plasma samples.

## 2.6. Derivatization of $F_4$ -NeuroPs

In the derivatization process, the carboxylic group of the  $F_4$ -NeuroPs or PGF<sub>2α</sub>-d<sub>4</sub> was converted into pentafluorobenzyl ester while the hydroxyl group was converted to trimethylsilyl ethers [61]. To achieve this, the following procedures were made where 40 μl of pentafluorobenzyl bromide (10% in acetonitrile) and 20 μl of diisopropylethylamine (10% in acetonitrile) were added to each dried extract. It was incubated for 45 min at 40 °C and subsequently, the solvent was evaporated under a stream of nitrogen. Then, 50 μl of N,O-bis

(trimethylsilyl)trifluoroacetamide with 5 μl of diisopropylethylamine (10% in acetonitrile) were added and incubated at 45 °C for 1 h. The samples were dried and re-suspended in 50 μl of undecane containing bis(trimethylsilyl)trifluoroacetamide (10%) for gas chromatography/negative-ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis. The same procedure was carried out for the derivatization of chemically synthesized 4- $F_{4t}$ -NeuroP and 10- $F_{4t}$ -NeuroP, the oxidized DHA liposome, and plasma or brain tissue samples.

## 2.7. Gas chromatography-mass spectrometry determination of $F_4$ -NeuroPs

The derivatized  $F_4$ -NeuroPs and PGF<sub>2α</sub>-d<sub>4</sub> were determined using GC/NICI-MS/MS with ion trap. The prepared samples (2 μl) were injected into the gas chromatograph (Trace GC and PolarisQ, Thermo/Finnigan, USA) set at splitless mode (2 min). The injector temperature was 250 °C and the oven temperature was held at 175 °C for 3 min and then increased to 270 °C (30 °C/min). The transfer line was heated to 280 °C and the ion source temperature was maintained at 200 °C. Helium was used as the carrier gas, (1 ml/min) and the chromatography was performed using a SPB 1701 GC capillary column (Supelco, 30 m × 0.25 mm i.d., 0.25 μm film thickness). The reagent gas for the chemical ionization was methane set to 2.0 ml/min flow rate. The precursor ion isolation width was 1.5 Da, the multiplier was set to 1500 V, and the automatic gain control was performed. The collision energy was 1.3 eV and the scan event time was 0.87 s with maximum ion as 25 ms, and the number of microscans per ion was 9.

The mass ions determined were the product ions at  $m/z$  323 and  $m/z$  303 derived from the [M-181]<sup>-</sup> precursor ions of NeuroPs ( $m/z$  593) and PGF<sub>2α</sub>-d<sub>4</sub> ( $m/z$  573) respectively, that corresponds to the loss of CH<sub>2</sub>C<sub>6</sub>F<sub>5</sub> from the derivatization process. Quantification of the NeuroPs were determined by relating the analyte/internal standard peak area ratio ( $F_{4t}$ -NeuroP/PGF<sub>2α</sub>-d<sub>4</sub>) to the calibration curve constructed.

## 2.8. Calibration curve, accuracy and precision of the method

The calibration curves were constructed by measuring seven concentration points (in triplicates) ranging from 1 to 500 pg/ml of the synthesized 4- $F_{4t}$ -NeuroP and 10- $F_{4t}$ -NeuroP with 500 pg PGF<sub>2α</sub>-d<sub>4</sub> as the internal standard. Following the procedure described above, all samples were purified by SPE, derivatized, and analyzed by GC/NICI-MS/MS. The coefficients of correlation were above 0.99. Based on the standard deviation of the area ratio ( $F_{4t}$ -NeuroP/PGF<sub>2α</sub>-d<sub>4</sub>) for the lowest concentration point and the curve slope, the limits of detection

(LOD) and quantification (LOQ) were 0.36–1.21 and 0.33 – 1.08 pg/ml, for 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP, respectively. The intra-assay and inter-assay coefficients of variability, as well as the accuracy of the method, were determined by analyzing three samples containing 500 pg/ml of synthesized 10-F<sub>4t</sub>-NeuroP and 500 pg/ml of PGF<sub>2α</sub>-d<sub>4</sub>.

## 2.9. Data analysis

Differences between groups were evaluated by either one-way analysis of variance (ANOVA), Levene's test for equality of error variances, and Scheffé test for all pairwise comparisons. The association between variables were tested using the Spearman rank correlation at 95% confidence intervals (95% C.I.). The efficiency of 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP in discriminating ASD, DS, MS or RTT from either healthy control subjects, or in discriminating each neurological disease from each other, was evaluated using receiver operating characteristic (ROC) curve analysis. A two-tailed P < 0.05 was considered to indicate statistical significance. The MedCalc ver. 12.0 statistical software package (MedCalc. Software, Mariakerke, Belgium) was used for data analysis.

## 3. Results

### 3.1. Method optimization of F<sub>4t</sub>-NeuroPs

In this study, the pentafluorobenzyl bromide-esters and trimethylsilyl-ether derivatives of the chemically synthesized F<sub>4t</sub>-NeuroPs were characterized using the GC/NICI-MS/MS by obtaining the mass [M–181] (for the precursor ion) of F<sub>4t</sub>-NeuroP molecules (*m/z* 593) and internal standard PGF<sub>2α</sub>-d<sub>4</sub> (*m/z* 573). The detection of the product ions was carried out by microscans in the *m/z* range 322.5–323.5 (F<sub>4t</sub>-NeuroP isomers) and 302.5–303.5 (PGF<sub>2α</sub>-d<sub>4</sub>). The retention time for the peaks produced were 19.54–19.75 min for 4-F<sub>4t</sub>-NeuroP, and 21.27 and 21.44 min for the epimers of the 10-F<sub>4t</sub>-NeuroP. Afterwards, the chromatographic spectrum of the oxidized DHA liposome was scanned for 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP and the peaks produced matched with the peaks of the chemically synthesized F<sub>4t</sub>-NeuroPs (Fig. 1, panels B to E). The exact match between the chromatographic spectrum of the oxidized DHA for both 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP were further

confirmed by the retention time of the internal standard, PGF<sub>2α</sub>-d<sub>4</sub>, (Fig. 1, panel A). The relatively high signal-to-noise (S/N) ratio (103) of the 10-F<sub>4t</sub>-NeuroP ion chromatogram (Fig. 1, panel D), indicate an extremely good detection level by the method set up. However, the S/N ratio of the oxidized DHA ion chromatogram was lower (22) as it contains a mixture of non-purified isomers. The ions produced through the loss of trimethylsilyl hydroxide (HOTMS) were predominant in the MS/MS spectrum of F<sub>4t</sub>-NeuroPs. In particular, the ions at *m/z* 503, 413 and 323 were attributed by the elimination of one, two, or three trimethylsilyl hydroxide (HOTMS) groups, respectively, from [M–181] ions and an example of 10-F<sub>4t</sub>-NeuroP is depicted in Fig. 2. As the most abundant product ion was *m/z* 323, a narrower scan range was set at *m/z* 322.5–323.5 for F<sub>4t</sub>-NeuroPs and *m/z* 302.5–303.5 for PGF<sub>2α</sub>-d<sub>4</sub> which was adequate to ensure high selectivity and sensitivity of the MS/MS method.

### 3.2. Accuracy and precision of F<sub>4t</sub>-NeuroPs measurement in plasma

Fig. 3 displays a typical chromatogram of a plasma sample analysis where the peaks shown are related to 4-F<sub>4t</sub>-NeuroP (19.59 min), and 10-F<sub>4t</sub>-NeuroP (21.26 and 21.44 min) epimers with S/N ratio approximately 15 compared to the chromatographic peaks of chemically synthesized F<sub>4t</sub>-NeuroP with higher S/N (approximately 100) (Fig. 1 panels C to E).

The precision of the applied method was determined by the measurement of intra-assay coefficient of variability from three replicated measurements of 10-F<sub>4t</sub>-NeuroP in healthy control plasma and was found to be 4.80 ± 0.35%. The inter-assay coefficient of variability was measured by comparing three samples containing specific amounts of chemically synthesized 10-F<sub>4t</sub>-NeuroP and was found to be 5.51 ± 0.36%. The quantification accuracy that is defined as closeness of the measured value to the true quantity value was 96.89 ± 2.02% for 10-F<sub>4t</sub>-NeuroP. The reference values for F<sub>4t</sub>-NeuroP levels in plasma samples ranged from 0 to 0.9 pg/ml (n = 61, healthy control subjects) and in the investigated neurological diseases, the plasma values ranged from 1.1 to 29.8 pg/ml (n = 61).

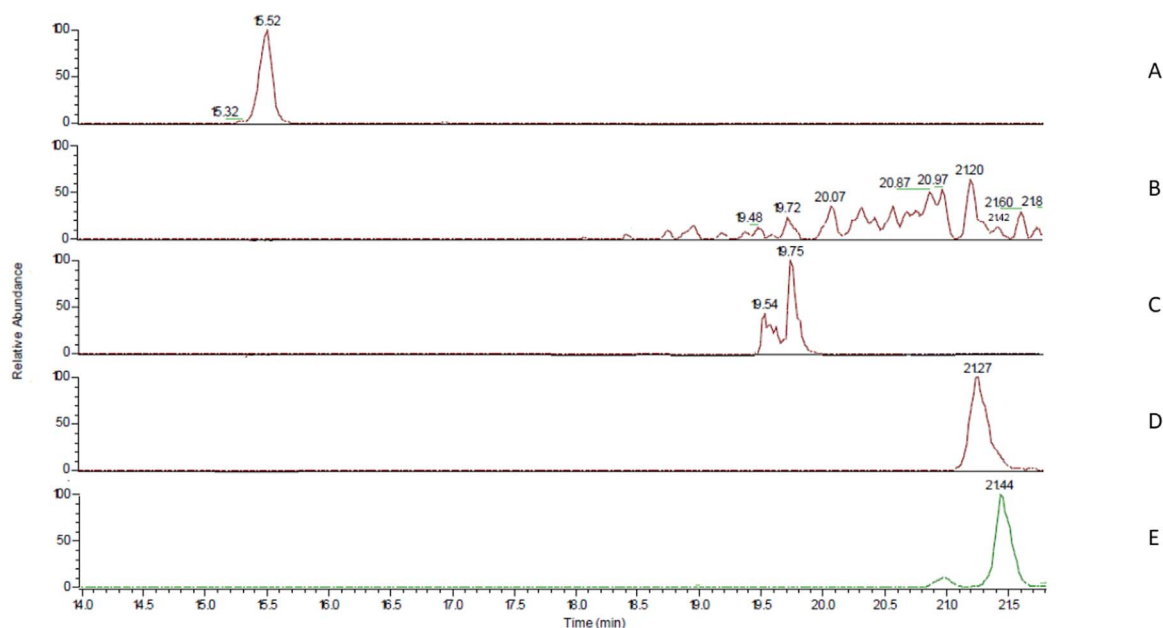
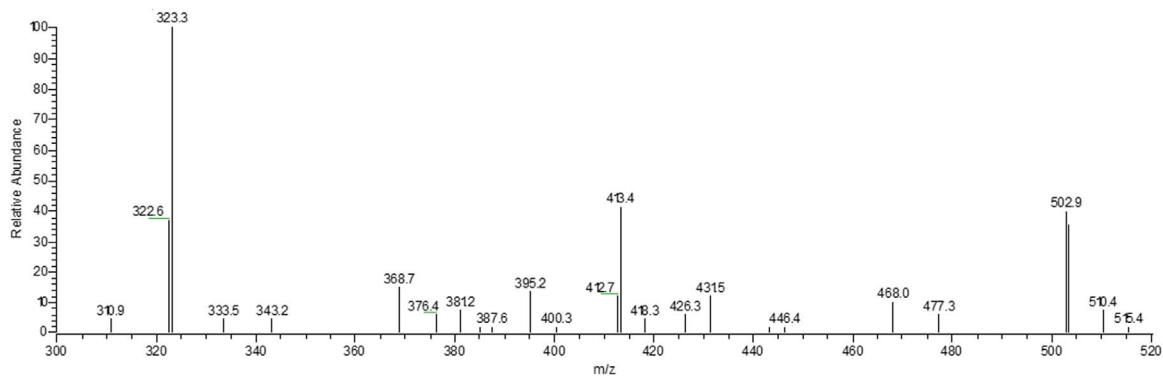
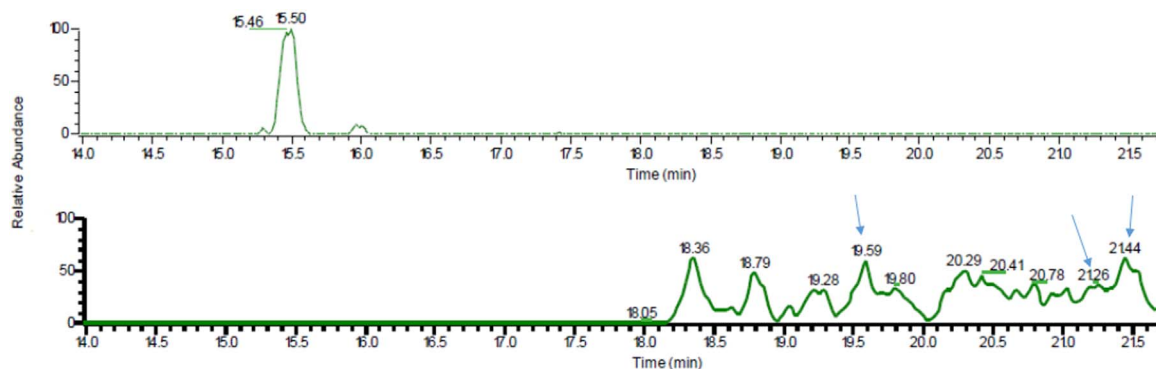


Fig. 1. Chromatogram obtained using GC/NICI-MS/MS conditions by selecting the species [M–181] as precursor ion. (A) internal standard PGF<sub>2α</sub>-d<sub>4</sub> (303 *m/z*, precursor ion *m/z* 573); (B) *in vitro* oxidized DHA liposome (323 *m/z*, precursor ion *m/z* 593); (C) 4(*RS*)-4-F<sub>4t</sub>-NeuroP (323 *m/z*, precursor ion *m/z* 593); (D) and (E); 10(*S*)-F<sub>4t</sub>-NeuroP and 10(*R*)-F<sub>4t</sub>-NeuroP, respectively; (323 *m/z*, precursor ion *m/z* 593). Retention times are indicated. The collision energy was 1.3 eV.



**Fig. 2.** MS/MS mass spectrum showing all the product ions of the derivatized 10-F<sub>4t</sub>-NeuroP. As precursor ion, the [M-PFB]<sup>-</sup> ion (*m/z* 593) was selected. The *m/z* range was 300.0–520.0. The collision energy was 1.3 eV. Ions at *m/z* 503, 413 and 323 are attributed by the elimination of one, two, or three trimethylsilyl hydroxide, respectively. As the most abundant product ion is at *m/z* 323, a narrow range scan over *m/z* 322.5–323.5 was sufficient to ensure high selectivity and sensitivity of the MS/MS method.



**Fig. 3.** Chromatogram of GC/NICI-MS/MS for F<sub>4t</sub>-NeuroPs obtained from plasma of a typical MS patient.

### 3.3. Levels in healthy controls and neurological disease patients

As compared to the corresponding healthy controls, plasma 10-F<sub>4t</sub>-NeuroP levels were significantly higher in the patients. On the other hand, plasma 4-F<sub>4t</sub>-NeuroP levels were found to be significantly higher only in MS and RTT patients (Fig. 4, panels A and B). Furthermore, the significant positive correlation ( $r = 0.676$ ; 95% C.I.: 0.566–0.762;  $n = 122$ ;  $P < 0.0001$ ) between the plasma levels of 10-F<sub>4t</sub>-NeuroP and 4-F<sub>4t</sub>-NeuroP demonstrate that the two isomers share the same pathway of DHA oxidation.

Even though 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP were detectable in all the plasma samples, 10-F<sub>4t</sub>-NeuroP level was more abundant in the ASD, DS, and RTT patients compared to the control group. Particularly, 10-F<sub>4t</sub>-NeuroP levels were greater compared to 4-F<sub>4t</sub>-NeuroP levels in all ASD and RTT patients, and in 13 out of 16 DS patients. However, in MS patients, 12 out of 16 plasma samples showed a major amount of 4-F<sub>4t</sub>-NeuroP. Moreover, 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP formation was shown to be influenced by MS disease progression. Specifically, 10-F<sub>4t</sub>-NeuroP levels were positively correlated with the onset age of the disease ( $r = 0.533$ ; 95% C.I.: 0.0509 to 0.814;  $n = 16$ ;  $P = 0.034$ ), while the ratio of 10-F<sub>4t</sub>-NeuroP to 4-F<sub>4t</sub>-NeuroP was negatively related to the disease duration ( $r = -0.517$ ; 95% C.I.: -0.806 to -0.0286;  $n = 16$ ;  $P = 0.04$ ).

Receiver operating characteristic (ROC) curves indicate that the ratio of F<sub>4t</sub>-NeuroP isomer, i.e. 10-F<sub>4t</sub>-NeuroP/4-F<sub>4t</sub>-NeuroP amounts, and the amount of individual 10-F<sub>4t</sub>-NeuroP and 4-F<sub>4t</sub>-NeuroP could be the discriminant between the brain disease group (ASD, DS, MS and RTT) from the healthy control subjects (Tables 1 and 2). Moreover, 10-F<sub>4t</sub>-NeuroP/4-F<sub>4t</sub>-NeuroP ratio, as well as 10-F<sub>4t</sub>-NeuroP and 4-F<sub>4t</sub>-NeuroP amounts were discriminants for different clinical conditions. Nevertheless, the comparisons between ASD and DS were not significant. As compared to DS, 10-F<sub>4t</sub>-NeuroP/4-F<sub>4t</sub>-NeuroP ratio and 4-

F<sub>4t</sub>-NeuroP amounts in RTT were found to be discriminants, but not for 10-F<sub>4t</sub>-NeuroP levels (Table 3).

Significant positive relationships were observed between relative disease clinical severity and 4-F<sub>4t</sub>-NeuroP or 10-F<sub>4t</sub>-NeuroP levels ( $r = 0.469$ ; 95% C.I.: 0.312–0.601;  $n = 114$ ;  $P < 0.0001$ ;  $r = 0.757$ ; 95% C.I.: 0.666–0.826;  $n = 114$ ;  $P < 0.0001$ , respectively). Furthermore, the relative clinical severity was significantly correlated with 10-F<sub>4t</sub>-NeuroP/4-F<sub>4t</sub>-NeuroP ratio ( $r = 0.820$ ; 95% C.I.: 0.726–0.883;  $n = 72$ ;  $P < 0.0001$ ) and 4-F<sub>4t</sub>-NeuroP/10-F<sub>4t</sub>-NeuroP ratio ( $r = -0.328$ ; 95% C.I. -0.513 to -0.114;  $n = 78$ ;  $P = 0.0033$ ).

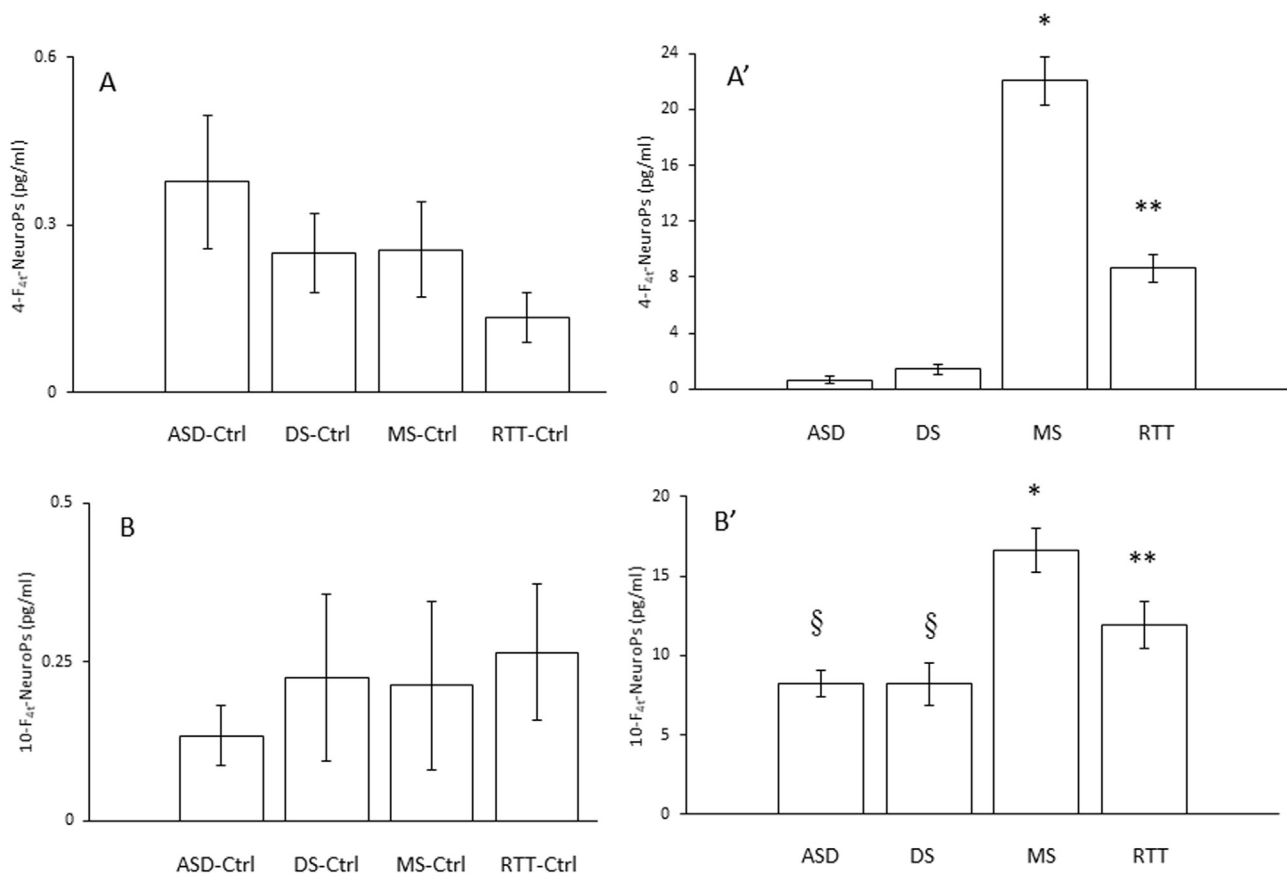
### 3.4. Levels in RTT mice brain tissue

The amount of 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP in brain tissue of RTT mice was significantly higher than the wild-type. Unlike plasma levels in RTT patients, the amount of 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP were similar in the brain tissues of RTT mice. Moreover, in our observation, 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP levels were significantly correlated to the phenotypic severity of the RTT mice (Table 4).

## 4. Discussion

In this study, it was apparent that NeuroPs, specifically 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP i) were biologically synthesized *in vivo* in different human neurological conditions, ii) were related to clinical severity, iii) were useful to identify shared pathogenetic pathways in distinct brain diseases, and iv) their relative plasma abundance appeared to be a distinctive feature for different neurological conditions.

Insofar, 15-F<sub>2t</sub>-IsoP derived from AA, is the most studied in medical research related to oxidative stress and disease progression. This is probably due to the availability of the standard and well characterized protocols using mass spectrometry. However, many of the findings were



**Fig. 4.** 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP plasma levels in neurological disease patients and healthy controls. MS: multiple sclerosis; DS: Down syndrome; ASD: autism spectrum disorders; RTT: Rett syndrome; ASD-Ctrl, DS-Ctrl, MS-Ctrl, and RTT-Ctrl: age- and gender-matched control population of the respective disease. Panels A and A': \*statistically different from ASD-Ctrl, DS-Ctrl, MS-Ctrl, RTT-Ctrl, ASD, DS and RTT; \*\*statistically different from ASD-Ctrl, DS-Ctrl, MS-Ctrl, RTT-Ctrl; ASD, DS and MS. Panels B and B': \* Statistically different from ASD-Ctrl, DS-Ctrl, MS-Ctrl, RTT-Ctrl, ASD, and DS; \*\* Statistically different from ASD-Ctrl, DS-Ctrl, MS-Ctrl, RTT-Ctrl; § Statistically different from ASD-Ctrl, DS-Ctrl, MS-Ctrl, RTT-Ctrl, and MS. Scheffé test for all pairwise comparisons: P < 0.05; Levene's test for equality of error variances: P < 0.001. One Way ANOVA test Significance level: P < 0.001.

not relevant to neurological diseases as described in recent reports [8,62] Therefore, investigations on F<sub>4</sub>-NeuroPs derived from DHA were worthy to be further explored, especially when taking into account their potential relevance in neurological disorders. As compared to F<sub>2</sub>-IsoPs, F<sub>4</sub>-NeuroPs could be related to the intrinsic mechanisms of brain diseases associated to more specific pathway [26] and it is well established that different oxidative stress biomarkers do not necessarily measure identical aspects of the disease progression [30].

The interest in the study of F<sub>4</sub>-NeuroP was first initiated from the fact that they are generated by free radical/ROS oxidation of DHA, which is highly concentrated in the neuronal membrane, that allows neurons to work at their best by avoiding afflictions of the nervous system [63–66]. The rate of oxidation is greater than AA due to extra methylene double bonds in the structure. Nevertheless, F<sub>4</sub>-NeuroPs have been quite recently indicated as a predictive variable of

atherosclerosis in a mouse model, and displayed protective mechanism in cardiac ischemia-reperfusion injury [67,68]. The selective role for the F<sub>4</sub>-NeuroPs in the brain tissue oxidative status was also evidenced by the major increase of F<sub>4</sub>-NeuroP levels, rather than F<sub>2</sub>-IsoPs, in brain injury [69,70]. Despite this, the number of scientific papers for F<sub>4</sub>-NeuroPs is relatively limited [8], and perhaps due to the lack understanding and international consensus in identifying the characteristic isomer among all the metabolites originating from DHA oxidation. Additionally, no commercial standards are available and no unanimous consensus has been reached for methodological protocol of F<sub>4</sub>-NeuroP analysis.

F<sub>4</sub>-NeuroPs are considered to be specific and accurate biomarkers when measurements are conducted in either brain tissue or cerebrospinal fluid [71]. For this reason, there is a limitation to further understand the NeuroP relevance in human neuro-pathology, and

**Table 1**

Discriminating power for 10-F<sub>4t</sub>-NeuroP/4-F<sub>4t</sub>-NeuroP ratio, and 10-F<sub>4t</sub>-NeuroP or 4-F<sub>4t</sub>-NeuroP content: summary of receiver operating characteristic (ROC) curve analysis.

	Variable	Cut-off value	Sensitivity (%)	Specificity (%)	+ PV (%)	- PV (%)	P value
<b>Disease group vs Healthy control</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	> 0.67	89.80	85.71	91.7	82.8	< 0.0001
	10-F <sub>4t</sub> -NeuroP	> 2.1 (pg/ml)	96.72	100	100	96.8	< 0.0001
	4-F <sub>4t</sub> -NeuroP	> 0.9 (pg/ml)	77.05	100	100	81.3	< 0.0001

The neurological disease group (i.e., all MS, ASD, RTT and DS patients) (n = 61) was compared to the age- and gender-matched healthy control population (n = 61). Plasma F<sub>4t</sub>-NeuroPs were measured as “free” forms.

+P.V.: positive predictive value; -P.V.: negative predictive value.

**Table 2**

Discriminating power for 10-F<sub>4t</sub>-NeuroP/4-F<sub>4t</sub>-NeuroP ratio, and 10-F<sub>4t</sub>-NeuroP or 4-F<sub>4t</sub>-NeuroP content for each neurological disease group - summary of receiver operating characteristic (ROC) curve analysis.

Comparisons	Variable	Cut-off value	Sensitivity (%)	Specificity (%)	+ P.V. (%)	- P.V. (%)	P value
<b>ASD vs Corresponding Healthy Control Group</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	> 1	100	100	100	100	< 0.0001
	10-F <sub>4t</sub> -NeuroP	> 2.1 (pg/ml)	100	100	100	100	< 0.0001
	4-F <sub>4t</sub> -NeuroP	> 0.5 (pg/ml)	44.44	91.67	66.7	81.5	N.S.
<b>DS vs Corresponding Healthy Control Group</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	> 1	100	100	100	100	< 0.0001
	10-F <sub>4t</sub> -NeuroP	> 2.1 (pg/ml)	87.50	100	100	92.3	< 0.0001
	4-F <sub>4t</sub> -NeuroP	> 0.9 (pg/ml)	50	100	81.8	75.9	0.0440
<b>MS vs Corresponding Healthy Control Group</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	> 0.4	100	60	80	100	0.0065
	10-F <sub>4t</sub> -NeuroP	> 2.1 (pg/ml)	100	100	100	100	< 0.0001
	4-F <sub>4t</sub> -NeuroP	> 0.9 (pg/ml)	100	100	100	100	< 0.0001
<b>RTT vs Corresponding Healthy Control Group</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	> 1	90	100	100	83.3	< 0.0001
	10-F <sub>4t</sub> -NeuroP	> 2.1 (pg/ml)	100	100	100	100	< 0.0001
	4-F <sub>4t</sub> -NeuroP	> 0.9 (pg/ml)	100	100	100	100	< 0.0001

Each neurological disease group was compared to the corresponding age- and gender-matched healthy control population. Plasma F<sub>4t</sub>-NeuroPs were measured as “free” forms. N. S.: not significant, referred to the area under the ROC curve; +P.V.: positive predictive value; -P.V.: negative predictive value.

**Table 3**

Receiver operating characteristic (ROC) curve analysis for 10-F<sub>4t</sub>-NeuroP/4-F<sub>4t</sub>-NeuroP ratio, or 10-F<sub>4t</sub>-NeuroP and 4-F<sub>4t</sub>-NeuroP content.

Pairwise Comparisons	Variable	Cut-off value	Sensitivity (%)	Specificity (%)	+ P.V. (%)	- P.V. (%)	P value
<b>ASD vs MS</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	> 1.19	100	100	100	100	< 0.0001
<b>ASD vs MS</b>	10-F <sub>4t</sub> -NeuroP	≤ 11.9 (pg/ml)	100	75	69.2	100	< 0.0001
<b>ASD vs MS</b>	4-F <sub>4t</sub> -NeuroP	≤ 2 (pg/ml)	100	100	100	100	< 0.0001
<b>ASD vs RTT</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	> 1.57	100	100	100	100	< 0.0001
<b>ASD vs RTT</b>	10-F <sub>4t</sub> -NeuroP	≤ 9.5 (pg/ml)	77.78	66.67	50	87.5	0.05
<b>ASD vs RTT</b>	4-F <sub>4t</sub> -NeuroP	≤ 2 (pg/ml)	100	100	100	100	< 0.0001
<b>ASD vs DS</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	> 4.2	75	88.89	50	72.7	N.S.
<b>ASD vs DS</b>	10-F <sub>4t</sub> -NeuroP	> 5.8 (pg/ml)	88.89	37.5	44.4	85.7	N.S.
<b>ASD vs DS</b>	4-F <sub>4t</sub> -NeuroP	≤ 2 (pg/ml)	100	31.25	45	100	N.S.
<b>MS vs RTT</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	≤ 1.19	100	90	88.9	100	< 0.0001
<b>MS vs RTT</b>	10-F <sub>4t</sub> -NeuroP	> 12.8 (pg/ml)	75	71.43	66.7	78.9	0.0033
<b>MS vs RTT</b>	4-F <sub>4t</sub> -NeuroP	> 16.7 (pg/ml)	75	100	100	84	< 0.0001
<b>MS vs DS</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	≤ 1.19	100	100	100	100	< 0.0001
<b>MS vs DS</b>	10-F <sub>4t</sub> -NeuroP	> 9.9 (pg/ml)	93.75	75	78.9	92.3	< 0.0001
<b>MS vs DS</b>	4-F <sub>4t</sub> -NeuroP	> 4.9 (pg/ml)	100	100	100	100	< 0.0001
<b>RTT vs DS</b>	10-F <sub>4t</sub> -NeuroP/4-F <sub>4t</sub> -NeuroP	≤ 1.57	100	88.89	95.2	100	< 0.0001
<b>RTT vs DS</b>	10-F <sub>4t</sub> -NeuroP	> 7.1 (pg/ml)	80.95	56.25	70.8	69.2	N.S.
<b>RTT vs DS</b>	4-F <sub>4t</sub> -NeuroP	> 4.9 (pg/ml)	80.95	100	100	80	< 0.0001

Plasma F<sub>4t</sub>-NeuroPs were measured as “free” forms.

N. S.: not significant, referred to the area under the ROC curve; +P.V.: positive predictive value; -P.V.: negative predictive value.

**Table 4**

4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP brain levels in symptomatic *Mecp2* stop/y mice and their correlation to phenotype severity.

Brain F <sub>4t</sub> -NeuroP isomer	Mouse group	Median (ng/g)	95% C.I. for median value	P value (two-tailed)
<b>4-F<sub>4t</sub>-NeuroP</b>	<i>Mecp2</i> stop/y (n = 4)	7.85	4.85 to 13.4	0.02
	wt (n = 4)	1.4	0.5 to 2.0	
<b>10-F<sub>4t</sub>-NeuroP</b>	<i>Mecp2</i> stop/y (n = 4)	10.45	9.05 to 17.75	0.02
	wt (n = 4)	1.9	0.7 to 3.05	
	<b>Correlation with phenotype severity<sup>b</sup></b>	<b>95% C.I. for rho</b>		<b>P value (two-tailed)</b>
<b>4-F<sub>4t</sub>-NeuroP</b>	0.944 <sup>a</sup>	0.72 to 0.99		0.0004
<b>10-F<sub>4t</sub>-NeuroP</b>	0.888 <sup>a</sup>	0.49 to 0.98		0.0032

Brain F<sub>4t</sub>-NeuroPs were measured as “free” plus “esterified” forms.

<sup>a</sup> Spearman's rho correlations coefficient. C.I.: confidence interval; wt: wild type.

<sup>b</sup> The phenotype severity was expressed as an aggregate score (Section 2).

subsequently, investigations could only reside to the availability of biological samples and in appropriate animal models. Our previous experiments on several RTT murine models corroborate the in vivo

plasma F<sub>4t</sub>-NeuroP results [28] and furthermore, in this report, we identified 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP in brain tissue whose levels were related to the phenotypic severity in RTT mice. Despite 4-F<sub>4t</sub>-NeuroP and 10-F<sub>4t</sub>-NeuroP brain levels in our observation was limited to one of the four pathological conditions examined in the patients, the data strongly emphasized one of the major key points which is the validity of plasma F<sub>4t</sub>-NeuroPs levels as a mirror for the ongoing DHA oxidation process in the brain/nervous system, and its close relationship with symptoms of neurological abnormalities.

Our group has repeatedly explored the relevance of plasma levels of NeuroPs in RTT [26] and other authors have confirmed the importance of monitoring the plasma levels of NeuroPs in Parkinson's disease [72] smokers [30], type 2 diabetes [31], ischemic-stroke [32], tick-borne encephalitis [33] and neuroborreliosis [34]. Also, NeuroPs have been determined in newborn serum plasma using ultra-high performance liquid chromatography coupled to tandem mass spectrometry [73], thus reinforcing the relevance of systemic blood products as appropriate samples for the NeuroPs detection. However, the transport of F<sub>4t</sub>-NeuroP levels in circulation to the brain, i.e. the ratio of plasma F<sub>4t</sub>-NeuroPs to brain F<sub>4t</sub>-NeuroPs, is still not known.

Although DHA is abundant in the brain, particularly in the gray matter, [74], the ability for the cerebral tissue to synthesize DHA from its omega-3 PUFA precursors are limited. Instead, most of the brain



DHA is from the plasma transported by fatty acid-binding proteins and the crossing of blood-brain barrier [75–77], and it is expected that the oxidized DHA like NeuroPs will be transported in similar manner.

In MS, the involvement of oxidative stress has been reported to affect both central and peripheral components [78], where the relationship between oxidative DNA damage (8-hydroxy-2'-deoxyguanosine values) and index of blood-brain barrier permeability, clinical severity of disease, and demyelinated brain lesion volume were identified [35,79]. Moreover, urinary 15-F<sub>2t</sub>-IsoP levels were found to be elevated [80], and the antioxidant capacity to be reduced [81].

Although, the participation of lipid peroxidation in MS cerebrospinal fluid is debatable [36], evidences for oxidative injury, including fatty acid peroxidation, were reported in the cerebellar gray matter [37]. Thus, elevated F<sub>4t</sub>-NeuroP plasma levels could denote a gray matter damage in the MS pathogenesis in the early phase of the disease that could become more prominent at severe stage [82,83].

Correlations between the F<sub>4t</sub>-NeuroP isomers and the relative severity scales were observed in this study. The data suggest that the relative abundance of the different F<sub>4t</sub>-NeuroP isomers along the cascade formation is influenced by the pathological conditions and/or contribute to disease severity given that oxidative stress impairment is a well-known fact involved in the investigated neurological diseases [27,44,84]. These data are strongly against the hypothesis in the formation of F<sub>4t</sub>-NeuroPs in vivo, which is random whenever DHA oxidation takes place. The major abundance of 4-F<sub>4t</sub>-NeuroP in MS samples is in line with other previous observations; compared to the other series of the F<sub>4t</sub>-NeuroP isomers, 4- and 20-series are different and could generate in greater amounts because the precursor cannot further cyclize in the chemical pathway [85]. This topic is of paramount relevance to the prospect of F<sub>4t</sub>-NeuroP quantification in DHA oxidant assessment in human clinical pathological conditions. Although our study did not investigate all the potential F<sub>4t</sub>-NeuroP isomers, which could be derived from DHA oxidation, 10-F<sub>4t</sub>-NeuroP and 4-F<sub>4t</sub>-NeuroP synthesis appears to take place in the investigated neurological diseases, and is associated to the relative clinical severity, and able to discriminate pathological condition, suggesting their involvement in disease mechanisms. This view is supported by the receiver operating curve, which is widely considered a test of accuracy for discriminating between different population. Hence, it appears that most of the patients' groups can be distinguished with confidence on the basis of the 10-F<sub>4t</sub>-NeuroP/4-F<sub>4t</sub>-NeuroP ratio, as well as by the absolute concentration of the individual isomer. Further, the biological relevance for the investigated F<sub>4t</sub>-NeuroP isomers is reinforced by their ability to discriminate the neurological disease. Intriguingly, ASD and DS (16–19% or higher) are easily distinguishable from the clinical phenotype, where a consistent proportion of the children with DS also shows ASD [86]. Likewise, in RTT autistic features are transient and lasting weeks to several months [87,88]. There is no doubt that the examined neurological conditions (i.e., RTT, ASD, MS, and DS) show a wide diversity in their neuropathological features. If we wished to identify a shared denominator among them, likely the loss of acquired neurological functions (i.e. “neuroregression”) would be the communal event occurring in three of the considered conditions (RTT, ASD, MS) and in about a fifth of the DS patients with autistic traits.

Our data strongly suggest that in vivo DHA oxidation follows preferential chemical rearrangements according to different neurological diseases, and that 10-F<sub>4t</sub>-NeuroP and 4-F<sub>4t</sub>-NeuroP are able to identify pathogenetic pathways in distinct brain diseases, thus representing potentially new approach in biological disease biomarkers.

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