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Relevance of 4-F_{4t}-neuroprostane and 10-F_{4t}-neuroprostane to neurological diseases

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ABSTRACT

F_4-neuroprostanes (F_4-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_{4t}-NeuroP (4-F_{4t}-NeuroP) and 10(*RS*) − 10-F_{4t}-NeuroP (10-F_{4t}-NeuroP) are the most studied.

Here, we report the identification and the clinical relevance of 4-F_{4t}-NeuroP and 10-F_{4t}-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_{4t}-NeuroP and 10-F_{4t}-NeuroP standards and in oxidized DHA liposome.

Both 4-F_{4t}-NeuroP and 10-F_{4t}-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_{4t}-NeuroP content was significantly higher in patients of all diseases as compared to age and gender matched healthy control subjects (n = 61), 4-F_{4t}-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_{4t}-NeuroP levels ($r = 0.469$, $P < 0.0001$), and 10-F_{4t}-NeuroP levels ($r = 0.757$, $P < 0.0001$). The study showed that the plasma amount ratio of 10-F_{4t}-NeuroP to 4-F_{4t}-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_{4t}-NeuroP and 10-F_{4t}-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.

Abbreviations: 4-F_{4t}-NeuroP, 4(*RS*) − 4-F_{4t}-neuroprostane; 10-F_{4t}-NeuroP, 10(*RS*) − 10-F_{4t}-neuroprostane; 15-F_{2t}-IsoP, 15-F_{2t}-isoprostane; ASD, autism spectrum disorders; DHA, docosahexaenoic acid; DS, Down syndrome; F_{4t}-NeuroPs, F_4-neuroprostanes; GC/NICI-MS/MS, gas chromatography/negative-ion chemical ionization tandem mass spectrometry; MS, multiple sclerosis; $PGF_{2\alpha}$-d_4, tetradeuterated prostaglandin $F_{2\alpha}$; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; RTT, Rett syndrome

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1. Introduction

Isoprostanoids, 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_{2t}-isoprostane (15-F_{2t}-IsoP) derived from arachidonic acid (AA) has been largely studied in medical research [3–5]. Also, 15-F_{2t}-IsoP (also referred to as 8-iso-$PGF_{2\alpha}$) has been identified to be the most abundant F_2-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_{2t}-IsoP is quantified by scientists to unveil pathological mechanisms associated to oxidative stress and recently, the measurement of 15-F_{2t}-IsoP was classified in a meta-analysis for its role in several specific diseases associated to oxidative damage [7].

Despite this, 15-F_{2t}-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_4-neuroprostanes (F_4-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_4-NeuroPs are very promising isoprostanoids 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_4-NeuroP molecules, only a handful of the isomers are characterized in neuro-pathological conditions and so far, 4-F_{4t}-NeuroPs and 10-F_{4t}NeuroPs are considered to be the most represented [8,15–25].

Previously, our group showed altered levels of plasma F_4-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_4-NeuroP have been shown to be intimately related to neurological severity [26] and cognitive performance [27], respectively. Other studies showed that elevated plasma F_4-NeuroPs are in smokers [30], type 2 diabetes [31], ischemic-stroke [323], tick-borne encephalitis [33] and neuroborreliosis [34] but its chemical formation, the preferential formation of the type of F_4-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_{4t}-NeuroP (4-F_{4t}-NeuroP) and 10(*RS*) − 10-F_{4t}-NeuroP (10-F_{4t}-NeuroP) that were chemically synthesized as standards and in oxidized DHA liposome, and ii) to investigate the relevance of 4-F_{4t}-NeuroP and 10-F_{4t}-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 cariotipically 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 $F_{2\alpha}$ ($PGF_{2\alpha}$-d_4, 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_{4t}-NeuroP, and 10(R) − 10-F_{4t}-NeuroP and 10(S) − 10-F_{4t}-NeuroP synthesis

The synthesis of the two series of 4- and 10-F_{4t}-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_{4t}-NeuroP