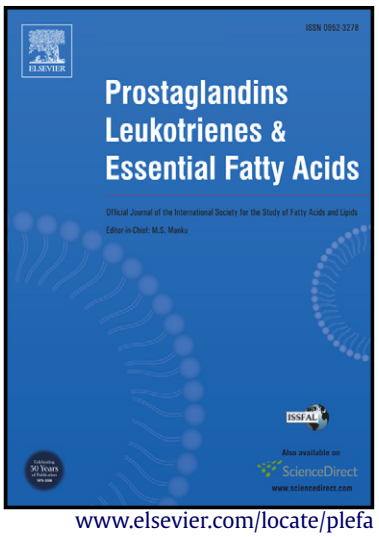

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Altered erythrocyte membrane fatty acid profile in typical Rett syndrome: effects of omega-3 polyunsaturated fatty acid supplementation☆

Cinzia Signorini[a*1], Claudio De Felice[b*1], Silvia Leoncini[a,c1], Thierry Durand[d], Jean-Marie Galano[d], Alessio Cortelazzo[c,e], Gloria Zollo[a,c], Roberto Guerranti[e], Stefano Gonnelli[f], Carla Caffarelli[f], Marcello Rossi[g], Alessandra Pecorelli[a,c], Giuseppe Valacchi[h], Lucia Ciccoli[a], Joussef Hayek[c]

[a]Department of Molecular and Developmental Medicine, University of Siena Via Aldo Moro, I-53100 Siena, Italy

[b]Neonatal Intensive Care Unit, University Hospital Azienda Ospedaliera Universitaria Senese (AOUS), Policlinico "S. M. alle Scotte, Viale M. Bracci 1, I-53100 Siena, Italy

[c]Child Neuropsychiatry Unit, University Hospital (AOUS), I-53100 Siena, Italy

[d]Institut des Biomolécules Max Mousseron (IBMM), UMR 5247-CNRS-UM I-UM II-ENSCM, BP 14491 34093, Montpellier, Cedex 5, France

[e]Department of Medical Biotechnologies, University of Siena, I-53100 Siena, Italy

[f]Department of Medicine, Surgery and Neuroscience, University of Siena, Italy

[g]Respiratory Pathophysiology and Rehabilitation Unit, University Hospital, AOUS, Viale M. Bracci 16, 53100 Siena, Italy

[h]Department of Life Science and Biotechnologies, University of Ferrara, I-44121 Ferrara, Italy

[*]Corresponding author.

[*]Corresponding author.

Email: cinzia.signorini@unisi.it
Email: geniente@gmail.com

Abstract

This study mainly aims at examining the erythrocyte membrane fatty acid (FAs) profile in Rett syndrome (RTT), a genetically determined neurodevelopmental disease. Early reports suggest a beneficial effects of omega-3 polyunsaturated fatty acids (ω-3 PUFAs) on disease severity in RTT. A total of 24 RTT patients were assigned to ω-3 PUFAs-containing fish oil for 12 months in a randomized controlled study (average DHA and EPA doses of 72.9, and 117.1 mg/kg b.w./day, respectively). A distinctly altered FAs profile was detectable in RTT, with deficient ω-6 PUFAs, increased saturated FAs and reduced *trans* 20:4 FAs. FAs changes were found to be related to redox

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[1] Co-first authors.

imbalance, subclinical inflammation, and decreased bone density. Supplementation with ω-3 PUFAs led to improved ω-6/ω-3 ratio and serum plasma lipid profile, decreased PUFAs peroxidation end-products, normalization of biochemical markers of inflammation, and reduction of bone hypodensity as compared to the untreated RTT group. Our data indicate that a significant FAs abnormality is detectable in the RTT erythrocyte membranes and is partially rescued by ω-3 PUFAs.

Keywords

Omega-3 polyunsaturated fatty acids; erythrocyte membrane; inflammation; isoprostanes; Rett syndrome

Introduction

In recent years, evaluation of lipid profile has assumed great relevance in neurodevelopmental and neuropsychiatric disorders [1-3]. In particular, the ratio between omega-3 polyunsaturated fatty acids (ω-3 PUFAs) and omega-6 polyunsaturated fatty acids (ω-6 PUFAs) has been widely investigated as a potential nutritional modulator for neuroprotection. General consensus exists on ω-6-/ω-3 ratios as close as possible to 1/1 or 2/1 being healthier than higher ones, although difficult to reach with the current western diet where ratios ranging from 10/1 to 20–25/1 are commonly reported [4]. Nutraceutical interventions with ω-3 PUFAs have therefore been widely tested as a candidate potential approach for the prevention of different human diseases, including cardiovascular and cognitive disorders [5-8].

In this context, Rett syndrome (RTT) seems to deserve a special place. RTT is a rare (1:10,000) neurodevelopmental disorder, firstly described in 1966 by the Austrian neuropediatrician Andreas Rett [9], affecting almost exclusively females and mainly caused by a functional deficiency of the Methyl-CpG-binding protein 2 (MeCP2), a nuclear protein that binds to methylated CpGs and regulates gene expression [10-12]. This human disease, known to be highly heterogeneous with the

typical 4-stage picture accounting for 80% of all the clinical presentations [13,14], represents a natural experimental model for a wide range neuroregressive disorders [15].

A complex alteration in the lipid metabolism [16-19] and a significantly increased lipid peroxidation [15,20-22] have been reported in both clinical and experimental RTT, along with the strong suggestion of a beneficial effects of ω-3 PUFAs supplementation on disease severity [23-28]. Therefore, lipidomics appear to be a promising field of research in RTT, where the fatty acids (FAs) composition of cell membranes has not been investigated.

The present work reports a longitudinal study performed in a RTT population before and after ω-3 PUFAs supplementation, in which the erythrocyte membrane FAs profile and its relationships with PUFA oxidized end-product metabolites, and systemic signs of inflammation were investigated.

Patients and Methods

Subjects

A total of 24 female patients with confirmed *MECP2* gene mutation and RTT typical presentation (mean age: 11.6 ± 3.5 years; range: 3-32), as well as 12 healthy female controls of comparable age (mean age: 11.8 ± 4.1 years; range: 3-32) were enrolled. All patients were consecutively admitted to the Child Neuropsychiatry Unit of the University Hospital of Siena (Head: J.H.). Z-scores of RTT patients were referred to validated Rett syndrome-specific growth charts [29]. Blood samplings in the control group were carried out during routine health checks. All the examined subjects were on a typical Mediterranean diet.

Study design

At admission, RTT patients were randomized to either oral supplementation with ω-3 PUFAs for 12 months or no treatment (n = 12 treated, mean age at supplementation time zero: 11.7 ± 3.9 years; n = 12 untreated, mean age at time zero: 11.8 ± 4.3 years). Omega-3 PUFAs were administered as fish oil in liquid form before food intake (Norwegian Fish Oil AS, Trondheim, Norway, Product

Number HO320-6; Italian importer: Transforma AS Italia. Forlimpopoli (FC). Italy; Italian Ministry Registration Code: 10 43863-Y) at a dose of 5 ml twice daily, corresponding to DHA, 72.9 ± 8.1 mg/kg b.w./day; EPA, 117.1 ± 13.1 mg/kg b.w./day; with a total ω-3 PUFAs, 246.0 ± 27.5 mg/kg b.w./day.

The study was conducted with the approval of the Institutional Review Board of the University Hospital Azienda Ospedaliera Universitaria Senese.

All informed consents were obtained from either the parents or the legal tutors of the enrolled patients or directly from healthy adults.

Erythrocyte membrane fatty acids (FAs) profile analysis

Two ml of blood were collected in presence of ethylene diaminetetraacetic acid (EDTA) as the anticoagulant. Erythrocyte membrane phospholipids were extracted [30] and subsequently converted into fatty acid methyl esters (FAME) by a transesterification procedure performed in presence of a methanol solution of 0.5M KOH at room temperature for 10 min [31].

Subsequently, FAME were analyzed by a gas chromatography instrumentation (GC) (Agilent 6850, Milan) equipped with a 60m × 0.25mm × 0.25μm (50%-cyanopropyl)-methylpolysiloxane column (DB23, Agilent, USA) and a flame ionization detector. Injector temperature: 230 °C. Temperature started from 195 °C, held for 26 min, followed by an increase of 10 °C/min up to 205 °C, held for 13 min, followed by a second increase of 30 °C/min up to 240 °C, and held for 10 min. A constant pressure mode (29 psi) was chosen with hydrogen as carrier gas. Methyl esters were identified by comparison with the retention times of authentic molecules.

Enzymes involved in the metabolism of essential PUFAs

Enzyme activities for delta desaturases 18:0/18:1, 20:4/20:3, 16:0/16:1 (Δ9D 18:0/9c - 18:1, Δ5D 20:4/20:3, and Δ9D 16:0/16:1, respectively), and for delta desaturase + elongase 18:2/20:3 (Δ6D + Elongase 18:2/20:3) were usually expressed as abundance ratios (enzyme substrates/metabolic

products for Δ9D 18:0/9c - 18:1, Δ9D 16:0/16:1, and Δ6D 18:2/20:3, or metabolic products/enzyme substrates for Δ5D 20:4/20:3).

Plasma isoprostanes (IsoPs) and neuroprostanes (NeuroPs) determinations

IsoPs are considered as the gold standard for the oxidative stress (OS) *in vivo* evaluation [32,33]. Specifically, F_2-IsoPs are the oxidation end-products of arachidonic acid (AA), F_3- IsoPs derive from oxidation of eicosapentaenoic acid (EPA), whereas F_4-NeuroPs are the end-products of docosahexaenoic acid (DHA), abundant in neuronal membranes [33,34].

Blood was collected in heparinized tubes and all manipulations were carried out within 2 h after sample collection. The blood samples were centrifuged at 2400g for 15 min at 4°C and the platelet poor plasma was collected. Butylated hydroxytoluene (BHT) (90 μM) was added as an antioxidant. The resulting plasma samples were stored at − 80 °C until assay for free, unesterified, F_2-IsoPs, F_3-IsoPs, and F_4-NeuroPs determinations.

Each plasma sample was spiked with tetradeuterated prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}-d_4$) (500 pg in 50 ml of ethanol), as an internal standard. After acidification (2 ml of acidified water, pH 3), the extraction and purification procedures were carried out. It consisted of two solid−phase separation steps: an octadecylsilane cartridge followed by an aminopropyl cartridge. In eluted samples, the carboxylic group was derivatized as the pentafluorobenzil ester, whereas the hydroxyl groups were converted to trimethylsilyl ethers [35]. Finally, isoprostane and neuroprostane determinations were carried out by gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis. In all determinations, the $[M-181]^-$ precursor ions were fragmented (collision energy 1.3eV) and the product ions identified. In particular, the measured ions were at *m/z* 299, *m/z* 297, and *m/z* 323 for F_2-IsoPs, F_3- IsoPs, and F_4−NeuroPs, respectively [23,25,35]. The ion specie identified for $PGF_{2\alpha}-d_4$ was at *m/z* 303.

Serum Cholesterol, HDL-Cholesterol, Triglycerides, Immunoglobulins, Complement components 3 and 4

Routine determinations for serum concentrations of total cholesterol, HDL-cholesterol, triglycerides, immunoglobulin class G, class A and class M (IgG, IgA, IgM), Complement component 3 (C3) and component 4 (C4), were performed on Cobas 6000 System (Roche Diagnostics).

Total cholesterol was assayed by an enzymatic method based on the determination of the Δ4-cholestenone following the enzymatic cleavage of cholesterol ester by cholesterol esterase, the transformation of cholesterol by cholesterol oxidase and the subsequent measurement, using the Trinder reaction of the hydrogen peroxide produced [36]. HDL Cholesterol was determined with homogeneous enzymatic colorimetric assay with cholesterol esterase, cholesterol oxidase, and 4-aminoantipyrine [37]. IgA, IgG and IgM were assayed with an immunoturbidimetric method based on the immunological agglutination with specific antibodies, respectively anti-IgA, anti IgG and anti IgM [38]. Triglycerides were measured by an enzymatic colorimetric method based on the oxidation of glycerol, obtained by their hydrolysis, to dihydroxyacetone phosphate and hydrogen peroxide, which, together with 4-aminophenazone and 4-chlorophenol form a colored product by peroxidase reaction [39]. C3, C4 were assayed with a immunoturbidimetric method with specific antibodies [40].

Serum fibrinogen

Routine determination of fibrinogen concentration was determined by analyzing the change in optical signal during prothrombin time determination using a derived fibrinogen calibration curve. The assay was performed by using Thromborel Reagent on BCS XP coagulation analyzer (Siemens Healthcare) [41].

Erythrocyte Sedimentation Rate (ESR)

ESR was assayed on a widely used automated system (i.e., "TEST 1") measuring the aggregation capacity of red blood cells (RBCs) by an infrared ray microphotometer with a light wavelength of 950 nm. Blood was directly drawn from the collection tube, distributed in a capillary, and centrifuged at about 20g. The sensing area temperature was maintained at 37°C. A mathematical algorithm converts the raw data obtained from evaluation of optical density signals into ESR results, which were transformed into conventional Westergren ESR values [42].

Blood Cells Counts (BCCs)

All routine clinical analytes assayed on Sysmex XT-2100 system [43].

RBC number, platelets (PLT) number, mean corpuscular volume (MCV), and hematocrit (HCT) were measured with electric resistance detecting method (impedance technology) with hydro dynamic focusing.

White blood cells (WBC) number was assayed by fluorescence flow cytometry with a 633 nm semi-conductor laser. For the measurement of neutrophils, lymphocytes, monocytes, and eosinophils, white cells were stained with fluorescent dyes that bind to both DNA and RNA. Side scatter was employed to determine size and granularity of the cells. Fluorescence and scatter measurements were combined to characterize white cell populations. Basophils were measured separately on the basis of cell size and side scatter properties. Hemoglobin (HGB) was measured photocolorimetrically using a cyanide-free method.

Bone densitometry

Amplitude-dependent speed of sound (AD-SoS) and bone transmission time (BTT) were evaluated by quantitative ultrasound (QUS) of the distal end of the first phalanx diaphysis of the last four fingers of the hand as previously described [44].

Statistical Data Analysis

All variables were tested for normal distribution (D'Agostino- Pearson test). Data were presented as medians ± semi-inter-quartile range or means ± standard deviation for non-gaussian continuous or normally distributed variables, respectively. Differences between different groups were evaluated either using Kruskal-Wallis analysis of variance and Mann-Whitney rank sum test or one-way analysis of variance and Student-Newman-Keuls post hoc test. Associations between variables were tested by either parametric (Pearson's coefficients) or nonparametric univariate (Spearman's rho) regression analysis. or, as appropriate). The MedCalc version 12.1.4 statistical software package (MedCalc Software, Mariakerke, Belgium) was used for data analysis and a two-tailed $P < 0.05$ was considered to indicate statistical significance after Bonferroni correction for multiple tests.

Results

Biometric data of RTT before *vs.* after ω-3 PUFA supplementation (height, RTT *z*-score for age, P= 0.081; Body weight, RTT *z*-score for age, P =0.9589; Body mass index, RTT *z*-score, P=0.7805) were comparable.

Baseline data

Fatty acids (FAs) profile in erythrocyte membranes and activities of enzymes involved in the metabolism of essential PUFAs

In RTT, a distinct FAs profile was detectable, with a relative deficiency of PUFAs ascribable to ω-6 PUFAs deficit, higher saturated FAs (SFAs) level, and reduced *trans* 20:4 FAs levels (Table 1). In particular, the erythrocyte membrane FAs profile of RTT patients showed a significant decrease of the percentage content for linoleic acid (LA), and AA, whereas the SFAs/PUFAs ratio was significantly increased (Table 1). Activities of enzymes involved in the metabolism of essential PUFAs (i.e., Δ9D 18:0/9c - 18:1, Δ5D 20:4/20:3, and Δ9D 16:0/16:1, and Δ6D + Elongase

18:2/20:3) in RTT patients were comparable to those of controls (Fig. 1). Data referring to the baseline status before supplementation (ω-3 PUFAs supplemented RTT patients 0 months) as well as untreated RTT subjects were compared to those of control population.

Lipid peroxidation

The susceptibility of the PUFAs oxidation was also evaluated by determining the plasma levels of IsoPs and NeuroPs specifically derived from AA (i.e., F_2-IsoPs), EPA (i.e., F_3-IsoPs), and DHA (i.e., F_4-NeuroPs). Levels of F_2-IsoPs, F_3-IsoPs, and F_4-NeuroPs were significantly increased in RTT patients before ω-3 PUFAs (ω-3 PUFAs supplemented RTT patients 0 months) and in untreated RTT patients (time 0 and time 12 months) as compared to healthy subjects (Fig. 2).

Bone density

Bone density, as assessed by quantitative ultrasound of the hand, was significantly reduced in RTT patients, RTT before supplementation (ω-3 PUFAs supplemented RTT patients 0 months) and untreated RTT population, as compared to healthy controls (Fig. 3).

Serum lipid profile

Serum total cholesterol and triglycerides were significantly higher in untreated RTT as compared to controls, whereas HDL-cholesterol and HDL-cholesterol/total cholesterol ratio were comparable (Fig. 4). The SFAs/PUFAs ratio was positively correlated to total cholesterol ($r = 0.658$; $P < 0.0001$) and total triglycerides ($r = 0.499$; $P = 0.001$). Similar relationships were observed between ω-6/ ω-3 PUFAs ratio and total cholesterol or triglycerides ($r = 0.435$; $P = 0.0001$, and $r = 0.569$; $P < 0.0001$, respectively).

Inflammatory status

In the untreated RTT population and in the RTT patients before ω-3 PUFAs supplementation (ω-3 PUFAs supplemented RTT patients 0 months), ESR values, as well as the levels of fibrinogen, C3, IgG, and platelet counts were significantly higher than those of healthy control population (Fig. 5). On the other hand, neutrophil, lymphocyte, monocytes, eosinophil and erythrocyte counts, hemoglobin concentration, MCV, MCH, and MCHC of untreated RTT patients were comparable to those of healthy subjects (Fig. 5 and Table 2).

Effects of supplementation with ω-3 PUFAs

Fatty acids profile and FAs metabolism-related enzyme activities

As the result of the ω-3 PUFAs supplementation, the ω-6 to ω-3 ratio was decreased with respect to the determination in the same patients before supplementation (ω-3 PUFAs supplemented RTT patients 0 months) and to untreated RTT subjects. Although a trend towards increased in EPA and DHA was evidenced, the SFAs/PUFAs ratio remained substantially unchanged. *Trans* 20:4 FAs were normalized following ω-3 PUFAs supplementation, and monounsatured fatty acids (MUFAs) were increased.

The Δ9D 16:0/16, Δ5D 20:4/20:3, and Δ6D + Elongase 18:2/20:3, were decreased in the RTT population after supplementation (ω-3 PUFAs supplemented RTT patients 12 months) as evidenced by the enzyme substrate abundance to metabolic products abundance ratio (16:0/16:1, 20:4/20:3 and 18:2/20:3, respectively) (Fig. 1). The other tested enzyme, i.e., Δ9D (18:0/9c - 18:1 ratio), remained unchanged.

Fatty acids peroxidation end-product metabolites

All the examined IsoPs (i.e., F_2-IsoPs, F_3-IsoPs, and F_4-NeuroPs) were decreased in RTT subjects following ω-3 PUFAs supplementation (Fig. 2). No significant correlation was observed between

EPA and plasma F_3-IsoPs levels (r = 0.069; P = 0.6516), whereas negative relationships were detectable for AA vs F_2-IsoPs (r = 0.294; P = 0.0380) and DHA vs F_4-NeuroPs (r = -0.372; P = 0.0072).

The SFAs/PUFAs ratio was positively related to F_4-NeuroPs plasma levels (r = 0.314; P = 0.0250), but not F_2-IsoPs or F_3-IsoPs (r = -0.062; P = 0.6677, and r = -0.054; P = 0.7249, respectively). Likewise ω-6 to ω-3 PUFAs ratio was positively related to plasma F_4-NeuroPs (r = 0.476; P = 0.0004).

Membrane lipidomics and bone density

Bone density *z*-score were significantly improved in supplemented RTT subjects, although no normalized (Fig. 3). SFAs/PUFAs ratio was inversely related to bone density in RTT patients as expressed either as AD-SOS z-score for age (r = -0.460; P = 0.0047) and BTT z-score for age (r = -0.475; P = 0.0045).

Serum lipids profile

Following ω-3 PUFA supplementation, serum total triglycerides were normalized, i.e. comparable to the control population values. In addition, serum HDL-cholesterol and HDL-cholesterol/ total cholesterol ratio were increased as compared to patients before supplementation (ω-3 PUFAs supplemented RTT patients 0 months), untreated RTT patients, and healthy control subjects, while the observed decrease in serum total cholesterol was not statistically significant (Fig. 4).

Membrane lipidomics and inflammatory status

After ω-3 PUFAs supplementation (ω-3 PUFAs supplemented RTT patients 12 months), ESR, fibrinogen, C3, IgG, and absolute lymphocyte, monocyte, eosinophil, and basophil counts, were normalized (i.e., comparable to values detectable in the healthy control population) (Fig. 5).

Total SFAs were positively related to ESR (r = 0.455; P = 0.0003). A significant negative relationship was observed between total PUFAS membrane content and ESR (r = -0.493; P = 0.001). Likewise, SFAs/PUFAs ratio was positively related to ESR (r = 0.311; P < 0.0175) whereas total ω-3 PUFAs content in the erythrocyte membrane was inversely related to ESR (r = -0.345; P = 0.0079). Of particular interest were the inverse relationship between EPA or DHA membrane contents and ESR (r = -0.365; P = 0.0048, and r = -0.309; P = 0.00183, respectively). *Trans* 20:4 FAs were also found to be inverse related to ESR (r = -0.062; P = 0.6677, and r = -0.054; P = 0.7249, respectively).

Following ω-3 PUFA supplementation, MCV, MCH, and MCHC values significantly increased, as compared to healthy control subjects and RTT patients before supplementation (ω-3 PUFAs supplemented RTT patients 0 months). Slightly reduced total RBC counts after ω-3 PUFA (ω-3 PUFAs supplemented RTT patients 12 months) were also observed (Table 2).

Discussion

Our data indicate, for the first time, the presence of an essential FAs deficiency in RTT erythrocyte membranes. Erythrocytes are considered as a model for cell membranes [45], and commonly used as standard cells for measuring the essential FAs profile in health and disease [31,46,47]. In particular, our findings demonstrate that the ω-6 PUFAs content is significantly reduced in the untreated patients, whereas SFAs concentrations appear to be increased, with a consequent increase of the SFAs/PUFAs ratio. The ω-6 /ω-3 ratio in the untreated RTT patients appears to be comparable to that of the healthy control counterparts. EPA and DHA amounts tend to slightly increase in the untreated patients, albeit below our selected statistical significance level. In addition, *trans* 20:4 FAs are significantly reduced as compared to the healthy population, a finding without an easy explanation. To this regard, *Trans* configuration of FAs has been previously related to use of hydrogenated fats of industrial origin [48]. Therefore, the latter finding may suggest that the abnormal lipidomics observed in RTT is likely not caused by dietary factors.

Three out of the four investigated enzyme activities related to FA metabolism were found to be decreased following ω-3 PUFAs supplementation in RTT patients. We speculate that the observed phenomenon could reflect an adaptation response to the increased abundance/availability of the final pathway metabolites.

Overall, this FAs pattern appears to be unique of RTT and, in particular, it significantly differs from the lipidomic profile previously reported in autism, where an increase in MUFAs, a decrease in EPA and DHA with a consequently increased in ω6/ω3 ratio are described [31].

To this regard, while RTT has long been considered to be a component of the autism spectrum disorders (ASDs), only recently the American Psychiatric Association considers RTT as a nosologically distinct neurodevelopmental disorder [49]. Actually, commonalities and differences between RTT and autism are known to exist and range from the clinical to the molecular level [50], being the detection of *MECP2* gene mutations the major critical difference with autism. Therefore, our data on the lipidomics of the RTT erythrocyte membranes further add a new distinctive feature at the molecular level to the clinical differences with autism that have lead to the reclassification of these disorders.

The mechanisms behind the observed FAs distribution abnormality in RTT remain unclear to date. Particularly, the underlying reasons for the ω-6 PUFAs deficiency observed in our RTT population could be theoretically related to either insufficient intake or reduced intestinal absorption. Although an essential fatty acid deficiency (EFAD) has been previously reported in special patients, either morbidly obese or on total parental nutrition, no evidence for EFAD exists in individuals on balanced spontaneous nutrition, with the single exception of experimental dermatitis [51-54]. Although nutritional difficulties are known in RTT, it seems unlikely that the low ω-6 PUFAs content observed in the erythrocyte membranes of our patients population could be related to a primary selective nutritional deficiency. On the other hand, gastrointestinal dysmotility commonly occurs in RTT (about 92%) and accounts for the majority of the gastrointestinal symptoms in the

disease [55-57] followed by chewing and swallowing difficulties (about 81%), and biliary tract disorders (about 3%).

Moreover, the effects of ω-3 PUFAs supplementation strikingly differ between RTT and ASD. While cumulating evidence indicates a beneficial effects of ω-3 on the disease severity of RTT [23-28], to date insufficient evidence exists in order to draw any conclusions about the usefulness of ω-3 PUFAs in ASD or specific learning disorders [58-60] even if maternal fat intake before or during pregnancy has been associated with a higher risk for ASDs in the offspring.

The effects of exogenous ω-3 PUFAs on the end-peroxidation product metabolites of endogenous ω-6 PUFAs, together with either exogenous or endogenous ω-3 PUFAs, is a current focus of intense research from several laboratories.

According to some authors, exogenous ω-3 PUFAs administration leads to increased peroxidized end-product metabolites [61-63]. However, administration of exogenous ω-3 PUFAs is followed by a decrease in isoprostanes [64-69], also in RTT patients [23,25,27].

In the present study, the levels of peroxidized end-product metabolites of AA, EPA, and DHA were found to be normalized (i.e. comparable to those observed in the healthy control population) following an oral supplementation with ω-3 PUFAs-containing fish oil at a high dose for one year. These data seem to add new evidence to the hypothesis of the "fatty acid paradox" in RTT [27].

Lipid rafts, specialized membrane structures involved in the signal transduction, are considered to play a role in the neurotransmission both in the presynaptic and postsynaptic sites, and to be related to the synaptic deficits in ASDs [70]. The relevance of the membrane-lipid arrangement in synaptic plasticity has been suggested for some human neuropathologies [71-73]. Recently, it has been showed that the loss of Mecp2 disrupts cholesterol homeostasis, thus suggesting the involvement of an abnormal cholesterol metabolism in the RTT pathogenic mechanisms [19]. Thus, the membrane-lipid arrangement, as well as the serum lipid profile, appears to be a promising target issue in RTT.

The triglyceride-lowering effect observed in RTT population following ω-3 PUFAs supplementation is in line with the described PUFAs actions [74-76]. However, the increase in the

serum HDL-cholesterol and HDL-cholesterol/total cholesterol ratio is rather unexpected and suggests, although indirectly, that no irreversible enzymatic pathway errors should be present in RTT patients. This point, of course, is in need of further investigation both at the clinical and molecular level.

Bone disease in RTT is a serious condition whose mechanism are still unknown while a specific cure does not exist [77]. Vitamin D deficiency is supposed to play a role, but it is detectable in only 20% of the cases [78]. Based on the observed modulation of AD-SoS and BTT by ω-3 PUFAs dietary supplementation, we speculate that PUFAS deficiency may play a role in the pathogenesis of the bone pathology in RTT.

Our recent reports indicate the presence of a previously unrecognized, subclinical inflammatory status in typical RTT [79,80]. In the last decade a number of effects of ω-3 PUFAs on inflammatory events have been reported including g leucocyte chemotaxis, leucocyte-endothelial adhesive interactions, production of eicosanoids from arachidonic acid, production of inflammatory cytokines and T cell reactivity [81]. The findings of the present study confirm the modulatory effects of ω-3 PUFAs on the persistent inflammation in RTT girls.

Our data in progress on the effects of ω-3 PUFAs on the plasma cytokines patterns in RTT (Leoncini *et al.*, manuscript in preparation) confirm that ω-3 PUFAs are actually able to modulate the abnormal Th1/Th2 balance observed in classical (i.e., *MECP2* mutation-related) and atypical (i.e., *CDKL5* mutation-related) RTT disorders. Therefore, our preliminary data appear to be consistent with the concept that ω-3 PUFAs in RTT are likely to act by exerting a modulatory effect on the subclinical inflammatory events intimately linked to the disease.

The gut-brain axis is the bi-directional communication between the gut and the brain which occurs through multiple pathways that include hormonal, neural and immune mediators [82]. The signals along this axis can originate in the gut, the brain, or both, with the objective of maintaining normal gut function and appropriate behavior. In recent years, the study of gut microbiota has become one of the most important areas in biomedical research, as there is evidence that the functional status of

gut is related to the condition of the brain [83] while the reverse also seems to be true [84]. Consequently, manipulation of gut microbiota [85] appears to be a novel frontier in major neurological and neuropsychiatric conditions, such as autoimmune encephalopathies (i.e., experimental autoimmune encephalomyelitis) [86], and hepatic encephalopathy [87,88], acute stress response /anxiety [89,90] major depression [90,91] and even autism [92].

Therefore, at this time, it cannot be ruled out that at least some of the effects of the ω-3 PUFAs supplementation could be mediated by related changes in the gut microbiota [93,94].

Overall, the mechanisms of action of ω-3 PUFAs remains a vast and stimulating field of research. Cumulating evidences indicates that oxygenated metabolites of PUFAs are the real actors on the scene [95,96]. A full host of chemical families have been detected in recent years including maresins, protectins, and lipoxins. Therefore, it is plausible that these, or other yet to be identified, secondary metabolites are the real effectors of the beneficial effects observed in our ω-3 PUFAs treated RTT population.

Conclusions

Our study reveals, for the first time, the presence of an altered FAs distribution in RTT erythrocyte membrane. Moreover, the observed beneficial effects of omega-3 PUFAs on the redox status and inflammatory status point out the key relevance of the cell membrane lipid arrangement as a new target issue in the pathogenesis of the disease.

Conflict of interest statement

The authors declare that there are no conflicts of interests regarding the publication of this article.

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Figure Legends

Figure 1. Fatty acids metabolism-related enzyme activities in patients with Rett syndrome as a function of 12 months high dose ω-3 PUFAs supplementation.

Baseline enzyme activities in the RTT group (ω-3 PUFAs supplemented 0 mo.s, and untreated RTT) are comparable to those of healthy subjects. Omega-3 PUFAs supplementation (12 mo.s) leads to significantly suppressed activities of Δ5Desaturase 20:4/20:3, Δ9Desaturase 16:0/16:1, and Δ6Desaturase + Elongase 18:2/20:3. See Methods section for further details on ω-3 PUFAs dose. Data are expressed as medians ± semi-interquartile ranges (See text for more details). Two-tailed P-values refer to Kruskal-Wallis analysis of variance. Asterisks denote significant post-hoc pairwise tests. Legend: mo.s, months.

Figure 2 Plasma isoprostanes concentrations in patients with Rett syndrome as a function of 12 months high dose ω-3 PUFAs supplementation.

Baseline levels of F_2-IsoPs, F_3-IsoPs, and F_4-NeuroPs are significantly increased in the RTT group (ω-3 PUFAs supplemented RTT patients 0 mo.s, and untreated RTT) as compared to healthy subjects. Omega-3 PUFAs supplementation (12 mo.s) leads to normalized levels of F_2-IsoPs, F_3-IsoPs and a significant decrease of F_4-NeuroPs. See Methods section for further details on ω-3 PUFAs dose. Data are expressed as medians ± semi-interquartile ranges (See text for more details). Two-tailed P-values refer to Kruskal-Wallis analysis of variance, and asterisks denote significant post-hoc pairwise tests. Legend: mo.s, months.

Figure 3. Bone density in patients with Rett syndrome as a function of 12 months high dose ω-3 PUFAs supplementation.

Omega-3 PUFAs supplementation leads to improvement, but not normalisation, of the bone density in Rett girls as measured as quantitative hands ultrasound (see Methods section for ω-3 PUFAs dose and bone density details). Data are expressed as medians ± semi-interquartile ranges (See text for

more details). Two-tailed P-values refer to Kruskal-Wallis analysis of variance, and asterisks denote significant post-hoc pairwise differences. Legend: mo.s, months.

Figure 4. Serum lipids profile in patients with Rett syndrome as a function of 12 months high dose ω-3 PUFAs supplementation.

Baseline total cholesterol and total TG concentrations are found to be higher in the RTT group (ω-3 PUFAs supplemented RTT patients 0 mo.s, and untreated RTT) as compared to healthy subjects, whereas HDL/total cholesterol ratio is decreased and HDL-cholesterol is comparable to controls. Omega-3 PUFAs supplementation (12 months) leads to a normalisation of total TG, a significant increase in HDL-cholesterol and HDL/total cholesterol ratio. Total cholesterol level appears to be slightly decreased albeit not significantly. See Methods section for further details on ω-3 PUFAs dose. Data are expressed as medians ± semi-interquartile ranges (See text for more details). Two-tailed P-values refer to Kruskal-Wallis analysis of variance, and asterisks denote significant post-hoc pairwise tests. Legend: mo.s, months.

Figure 5. Routine chemistry biomarkers for systemic inflammation in patients with Rett syndrome as a function of 12 months high dose ω-3 PUFAs supplementation.

Baseline ESR, fibrinogen, complement 3 fragment, IgG , and platelets count are significant increased in the RTT group (ω-3 PUFAs supplemented RTT patients 0 mo.s, and untreated RTT) as compared to healthy subjects, whereas absolute eosinophils count and absolute basophils count is decreased. Absolute lymphocyte count is comparable to that of controls. Omega-3 PUFAs supplementation leads to normalization of fibrinogen, complement component 3 (C3), IgG, and platelet count, whereas a significant decrease in absolute eosinophils count, absolute basophils count, absolute lymphocyte count, absolute monocytes count is observed. See Methods section for further details on ω-3 PUFAs dose. Data are expressed as medians ± semi-interquartile ranges (See text for more details). Two-tailed P-values refer to Kruskal-Wallis analysis of variance, and asterisks denote significant post-hoc pairwise tests. Legend: mo.s, months.

Table 1. **Fatty acids profiles in red blood cell membrane from patients with Rett syndrome as a function of 12 months high dose ω-3 PUFAs supplementation.** The data mainly underly a relative deficiency of PUFAs, in particular of the ω-6 series, faced with increased SFAs content and consequently unfavourable SFAs / PUFAs ratio. Supplementation with ω-3 PUFAs is unable to reverse total PUFAs and SFAs/PUFAs ratio, although significantly improves ω-6 PUFAs / ω-3 PUFAs ratio, and leads to increased MUFAs and *trans* 20:4 fatty acids contents.

Fatty acid profil	Healthy controls	ω–3 PUFAs supplemented RTT patients (0 mo.s)	ω–3 PUFAs supplemented RTT patients (12 mo.s)	Untreated RTT patients (0 mo.s)	Untreated RTT patients (12 mo.s)	P-value (Kruskal-Wallis test)
LA (18:2 ω–6) (%	**12.72±1.07^{a,b,c,d}**	**10.13±0.84^{a}**	**8.55±1.62^{b}**	**10.22±1.07^{c}**	**10.42±0.94^{d}**	**<0.001**
AA (20:4 ω–6) (%	**18.3±0.48^{a}**	**16.48±2.01**	**15.15±2.29^{a}**	**15.97±2.29^{a}**	**15.93±1.7^{a}**	**0.0149**
EPA (20:5 ω–3) (	0.47±0.049	1.73±0.77	2.9±2.51	2.17±0.91	1.56±0.79	0.0897
DHA (22:6 ω–3) (%)	4.26±0.75	5.72±2.61	6.25±1.41	5.93±2.69	5.13±2.47	0.199
Total ω–6 PUFAs (%)	**33.18±1.1^{a,b,c,d}**	**28.8±3.17^{a}**	**26.15±5.48^{b}**	**28.38±3.69^{c}**	**29.03±3.2^{d}**	**0.0001**
Total ω–3 PUFAs (%)	4.73±0.77	7.45±1.47	9.15±3.91	8±5.82	6.72±1.49	0.2128
ω–6 PUFAs/ ω–3 PUFAs ratio	**7.2±1.36^{a}**	**5.38±2.5**	**3.7±2.19^{a}**	**5.19±2.59**	**6.05±2.62**	**0.011**
SFAs (%)	**43.3±0.4^{a,b,c,d}**	**44.37±1.12^{a}**	**44.7±1.25^{b}**	**44.35±1.08^{c}**	**44.73±1.09^{d}**	**0.0291**
MUFAs (%)	**18.5±0.8^{a,b,c,d}**	**19.22±1.08^{a}**	**19.95±0.16^{b}**	**19.11±1.3^{c}**	**19.44±1.14^{d}**	**0.0267**
SFAs/MUFAs rati	2.36±0.098	2.32±0.11	2.25±0.05	2.34±0.12	2.31±0.108	0.0905
SFAs/PUFAs ratio	**1.14±0.033^{a,b,c,d}**	**1.23±0.094^{a}**	**1.27±0.092^{b}**	**1.22±0.099^{c}**	**1.26±0.092^{d}**	**0.0027**
Trans 20:4 (%)	**0.102±0.01^{a,b,c}**	**0.067±0.014^{a}**	**0.102±0.01**	**0.058±0.015^{b}**	**0.042±0.014^{c}**	**0.0027**
Total PUFAs (%)	**37.92±0.88^{a,b,c,d}**	**36.25±2.09**	**35.3±1.57**	**36.38±2.32**	**35.72 ±2.08**	**0.0036**

Data are medians ± semi-interquartile ranges. Bold characters indicate significant inter-group differences. Superscript letters denote significant post-hoc pairwise tests. ; mo.s, months. LA: Linoleic Acid, AA: Arachidonic Acid; EPA: Eicosapentaenoic Acid, DHA: Docosahexaenoic Acid, PUFAs: Polyunsaturated Fatty Acids; SFAs: Saturated Fatty Acids; MUFAs: Monounsaturated Fatty Acids; See Methods section for further details on ω-3 PUFAs dose.

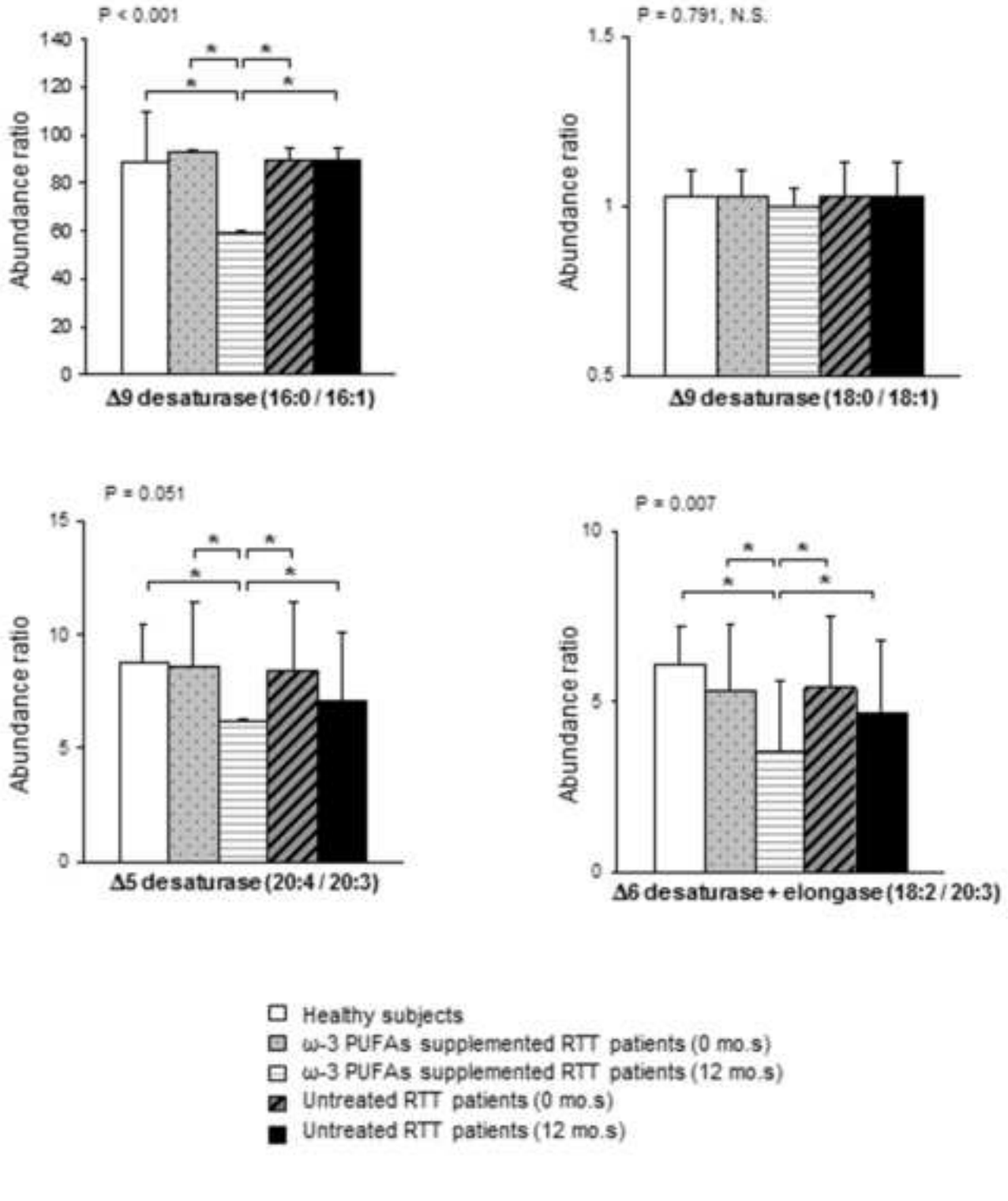
Table 2. **Red blood cells counts variables in patients with Rett syndrome as a function of 12 months high dose ω-3 PUFAs supplementation**.

RBC variables	Healthy controls	ω–3 PUFAs supplemented RTT patients (0 mo.s)	ω–3 PUFAs supplemented RTT patients (12 mo.s)	Untreated RTT patients (0 mo.s)	Untreated RTT patients (12 mo.s)	P-value (Kruskal-Wallis test)
RBC count (10^6/mm^3)	**4.89±0.36**[a]	**4.57±0.41**	**4.40±0.15**[a,b,c]	**4.76±0.44**[b]	**4.79±0.37**[c]	**0.0055**
Hb (g/dL)	13.19±1.00	12.25±1.79	13.55±0.15	13.01±1.86	13.45±1.34	0.0972
MCV (fL)	**81.34±12.95**[a]	**82.22±4.45**[b]	**90.7±0.10**[a,b,c,d]	**82.92±4.96**[c]	**85.7±4.53**[d]	**<0.0001**
MCH (pg/cell)	**27.43±1.53**[a]	**26.89±1.89**[b]	**30.80±0.73**[a,b,c,d]	**27.30±1.85**[c]	**28.30±1.46**[d]	**<0.0001**
MCHC (g/dL)	**33.79±1.09**	**29.34±6.86**[a]	**33.90±0.78**[a,b,c]	**28.08±8.03**[b]	**29.08±7.02**[c]	**<0.0001**

The data show an increase in Hb content and changes in mean corpuscular volume. See Methods section for further details on ω-3 PUFAs dose.

Data are medians ± semi-interquartile ranges. Bold characters indicate significant inter-group differences. Superscript letters denote significant post-hoc pairwise tests.

Figure 1



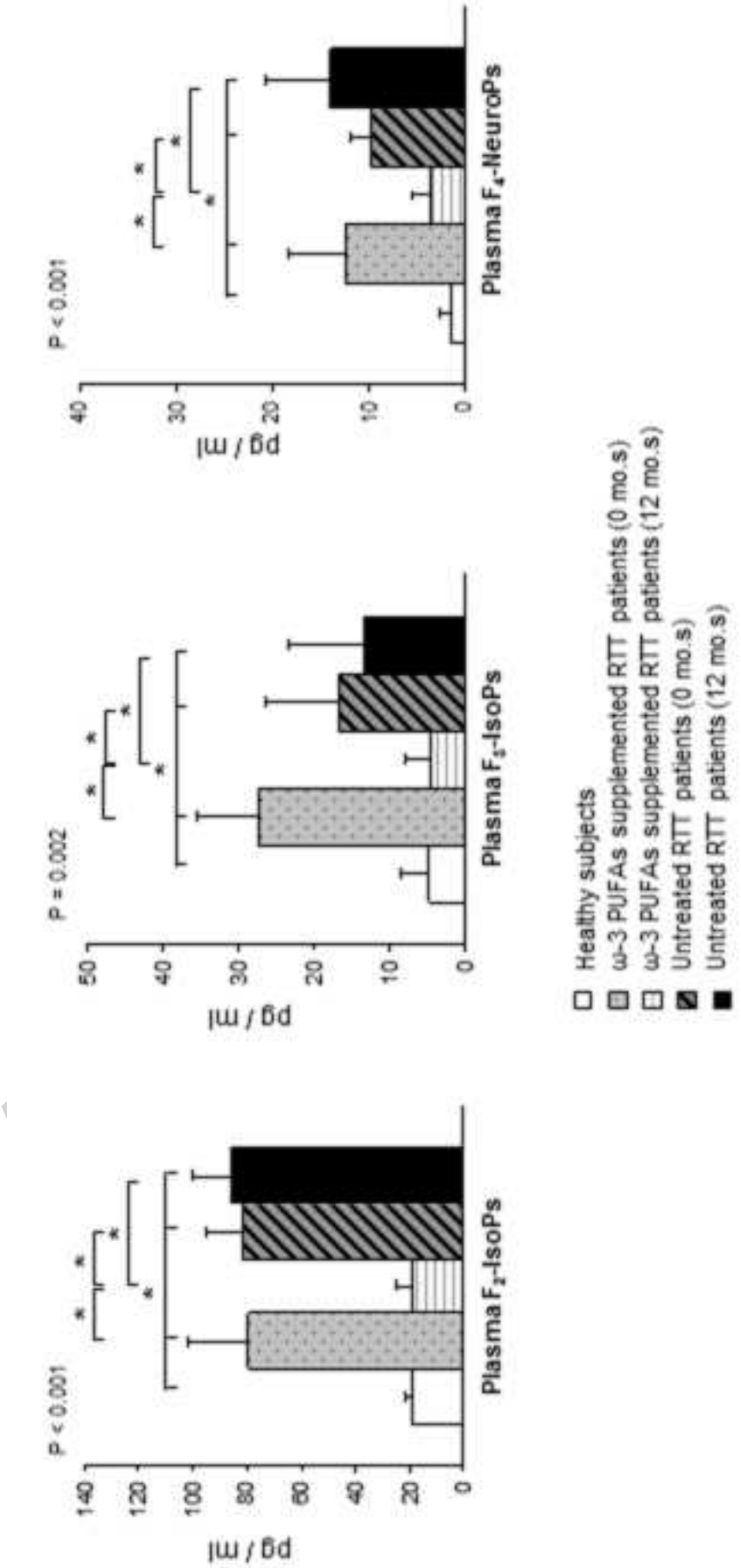

Plasma F4-NeuroPs
P < 0.001
pg / ml
0
10
20
30
40
Plasma F2-IsoPs
P = 0.002
pg / ml
0
10
20
30
40
50
Plasma F2-IsoPs
P < 0.001
pg / ml
0
20
40
60
80
100
120
140
Healthy subjects
ω-3 PUFAs supplemented RTT patients (0 mo.s)
ω-3 PUFAs supplemented RTT patients (12 mo.s)
Untreated RTT patients (0 mo.s)
Untreated RTT patients (12 mo.s)

Figure 2

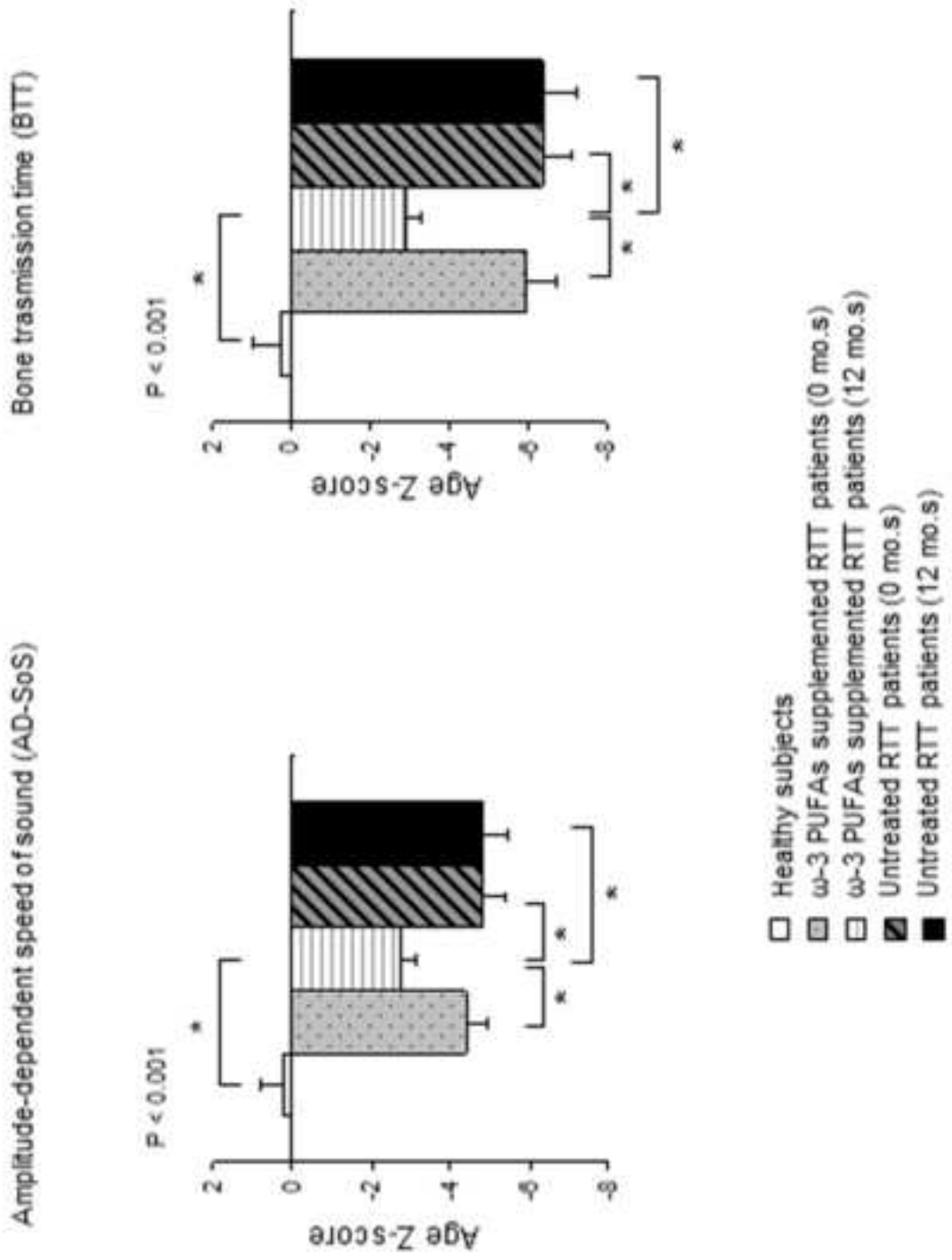

Amplitude-dependent speed of sound (AD-SoS)
Bone trasmission time (BTT)
P < 0.001
P < 0.001
Age Z-score
Age Z-score
2
0
-2
-4
-6
-8
*
Healthy subjects
ω-3 PUFAs supplemented RTT patients (0 mo.s)
ω-3 PUFAs supplemented RTT patients (12 mo.s)
Untreated RTT patients (0 mo.s)
Untreated RTT patients (12 mo.s)

Figure 3

Figure 4

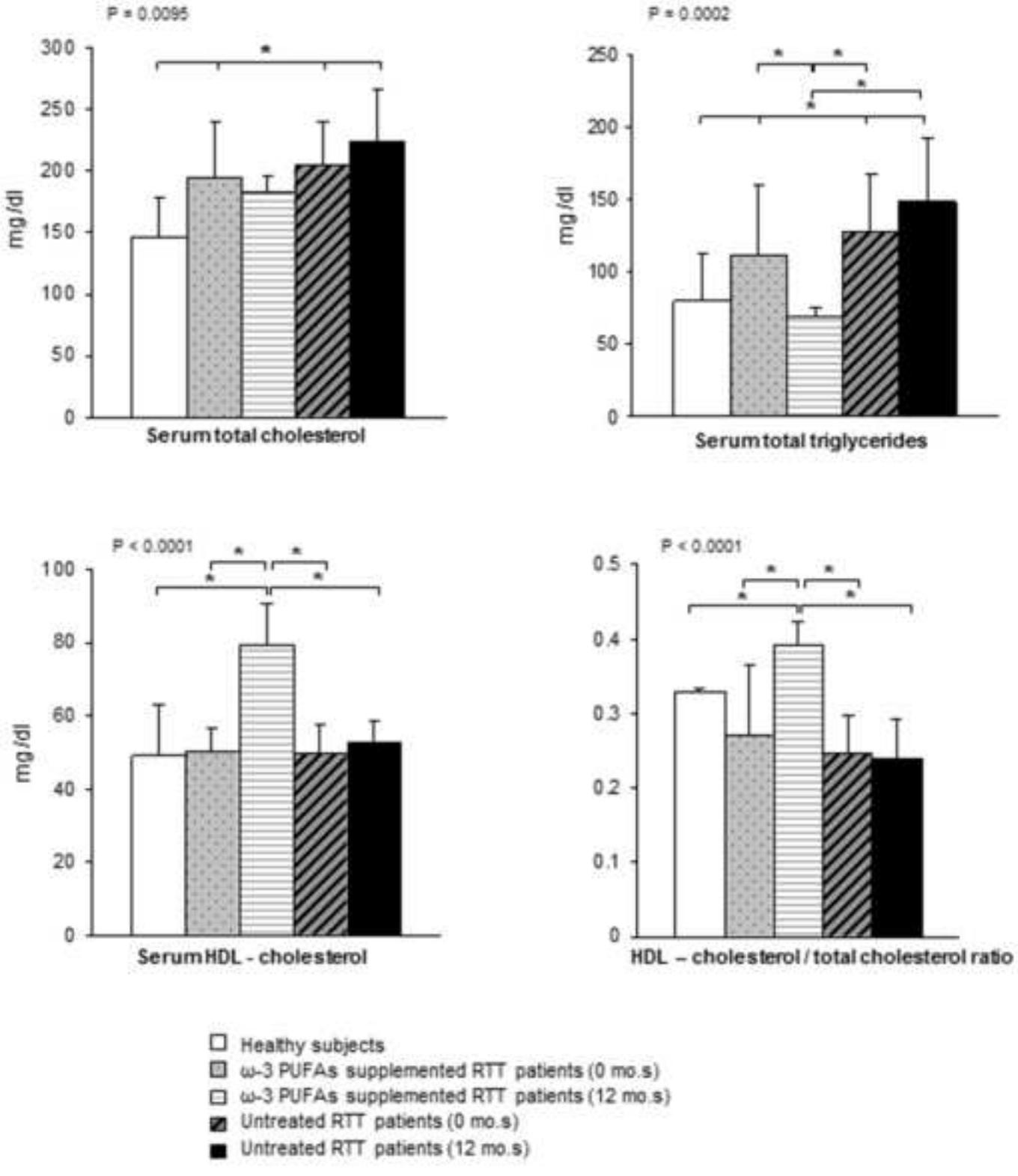


Figure 5