Elsevier Editorial System(tm) for Clinical Nutrition
Manuscript Draft

Manuscript Number:

Title: Protective effect of ω-3 PUFAs against unexplained sudden death in Rett syndrome

Article Type: Full Length Article

Keywords: Omega-3 fatty acids; Omega-3 index; Isoprostanes; Sudden death; Rett syndrome

Corresponding Author: Dr.Med. Claudio De Felice, M.D.

Corresponding Author's Institution: University Hospital AOUS, Policlinico “S. M. alle Scotte

First Author: Claudio De Felice, M.D.

Order of Authors: Claudio De Felice, M.D.; Cinzia Signorini; Silvia Leoncini; Alessio Cortelazzo; Gloria Zollo; Thierry Durand; Jean-Marie Galano; Camille Oger; Jetty Chung-Yung Lee; Jean-Yves Le Guennec; Silvia Maffei; Alessandra Pecorelli; Giuseppe Valacchi; Lucia Ciccoli; Joussef Hayek

Abstract: Background & aims: Rett syndrome , although considered a rare disease, is the second commonest cause of severe mental retardation in the female gender. The disease is mainly caused by mutations in the gene encoding the MeCP2 protein. Of note, in RTT a 300- folds increased risk of sudden death has been reported. This fatal event is to be considered as unexplained as its causes are unknown, although a cardiac event has been previously postulated. In the present study, a protective effect of ω-3 fatty acids against the phenomenon of the unexplained sudden death in Rett syndrome was tested. Methods: A cohort of patients on a regular clinical and biochemical follow-up. (n=214, all females, mean age: 15.4 ± 7.5 years) were examined. A supplementation with ω-3 fatty acids at high dosage (250 ± 45 mg/kg b.w./day) was proposed and the number of sudden death during 5-years were recorded. Fatty acids profile in erythrocytes and systemic levels of non-protein-bound iron (plasma and intraerythrocyte) and isoprostanoid oxidative stress biomarkers, (i.e., F2-isoprostanes F2-dihomo-isoprostanes, and F4-neuroprostanes) were measured.Results: A significantly lower risk of sudden death was observed for the ω-3 fatty acids-supplemented population (O.R.: 115.143; 95% CI 14.33 to 924.80, P <0.0001).The reduced risk of sudden death in the ω-3 fatty acids-supplemented group was found to be associated with increased blood docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and ω-3 index, as well as decreased systemic levels of oxidative stress biomarkers.Conclusions: Our data indicate that ω-3 fatty acids protect RTT patients from sudden death by alleviating oxidative stress.

Opposed Reviewers:

COI

Click here to download ICMJE Conflict of Interest: COI.pdf

Siena, 15 October, 2014

Prof. N.E.P. Deutz, MD, PhD
Clinical Nutrition
Dept. of Health and Kinesiology, Texas A&M University,
4243 TAMU, College Station, 77843, USA

Dear Editor,

Please find enclosed a manuscript titled *"**Omega 3 PUFAs protect against unexplained sudden death in Rett syndrome"*** by De Felice, et al. The submitted work is intended for possible publication in ***Clinical Nutrition*** as a Full Length Article.

Rett syndrome , although considered a rare disease, is the second commonest cause of severe mental retardation in the female. It is mainly caused by mutations in the gene encoding the MeCP2 protein. Of note, in RTT a 300- fold increased risk for sudden death has been reported. In the present study, a protective effect of ω-3 fatty acids against the phenomenon of the unexplained sudden death in Rett syndrome was explored in a cohort of patients on a regular clinical and biochemical 5-year-long follow-up. A significantly lower risk for sudden death was observed for the ω-3 fatty acids-supplemented population (O.R.: 115.143; 95% CI 14.33 to 924.80, *P* <0.0001).The reduced risk for sudden death in the ω-3 fatty acids-supplemented group was found to be associated with increased blood docosahexaenoic acid, eicosapentaenoic acid, and omega-3 index, as well as decreased systemic levels of non-protein-bound iron (plasma and intraerythrocyte) and isoprostanoid oxidative stress biomarkers, (F_2-isoprostanes F_2-dihomo-isoprostanes, and F_4-neuroprostanes). Our data indicate that ω-3 fatty acids protect RTT patients from sudden death by alleviating oxidative stress. Our findings confirm and extend the data by Albert et al. (N Engl J Med. 2002 Apr 11;346:1113-8) on the protective effects of long-chain n-3 fatty acids and the risk of sudden death.

Although RTT is a rare disease, it offers a unique setting to explore pathogenetic mechanisms that could be involved in other neurological human diseases with higher social impact.

Therefore, we believe that the *Clinical Nutrition* readership would be potentially interested to our study.

All the authors declare that:
there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.
the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed
the order of authors listed in the manuscript has been approved by all of us.

Author Contributions
Concept: **CDF, CS, SL, JH**
Experimental design: **CDF, CS**
Clinical follow up**: JH, CDF, SM**
Sample blood preparation: **CS, SL, GZ**
Isoprostanes and neuroprostanes assays: **CS**
NPBI assays**: SL, GZ**
Isoprostanes synthesis: **TD, CO, JMG, AG, VBP;**

Data analysis: **CDF, CS, SL, GZ**
Data interpretation: **All the Authors**
Manuscript drafting: **All the Authors**
Approval: **All the Authors**

We thank you in advance for kind consideration of this manuscript and, on behalf of all the co-authors, we look forward to hearing from you in due course.

Yours sincerely,

The Corresponding Authors

Claudio De Felice, MD, Neonatal Intensive Care Unit, University Hospital AOUS, Policlinico "S. M. alle Scotte, Viale M. Bracci 1, Siena 53100, Italy; E-mail: geniente@gmail.com

Cinzia Signorini, PhD, Department of Molecular and Developmental Medicine University of Siena Via Aldo Moro, I-53100 Siena Italy, *E-mail:* cinzia.signorini@unisi.it

List of Potential reviewers:

William S. Harris, expert in omega-3 PUFAs and human health and disease (inventor of the Omega-3 index)
Sanford School of Medicine, University of South Dakota and OmegaQuant Analytics, LLC, Sioux Falls, SD, USA 2329 N. Career Ave, Ste 113, Sioux Falls, SD 57107, USA Tel.: +1 605 275 8783.
E-mail address: bill@omegaquant.com (W.S. Harris)

Philip C. Calder, expert in omega-3 PUFAs and human health and disease
Human Development and Health Academic Unit, Faculty of Medicine, University of Southampton, Southampton, UK NIHR Southampton Biomedical Research Centre, University Hospital Southampton NHS Foundation Trust and University of Southampton, Southampton, UK. Department of Biological Sciences, Faculty of Science, King Abdulaziz University, Jeddah, Saudi Arabia.
E-mail: pcc@soton.ac.uk.

John Christodoulou, expert in Rett disorders
NSW Centre for Rett Syndrome Research, Western Sydney Genetics Program, Children's Hospital at Westmead, Sydney, Australia; Discipline of Paediatrics & Child Health, University of Sydney, Australia; Discipline of Genetic Medicine, Sydney Medical School, University of Sydney, Australia
E-mail : john.christodoulou@health.nsw.gov.au

Sarojini Budden, expert in therapeutic approaches in Rett syndrome
Division of Developmental Pediatrics, Oregon Health & Science University, Portland, OR 97239, USA
E-mail address: sbudden@comcast.net

Renee C. Wachtel, expert in therapeutic approaches in Rett syndrome
Children's Hospital Oakland Research Institute Developmental Behavioral Pediatrics
15250 Hesperian Blvd Suite 100 San Leandro CA 94578
E-mail address: drrwachtel@aol.com

Kazuo Miyashita, expert in the biochemistry of Omega-3 PUFAs and oxidative stress
Faculty of Fisheries Sciences, Hokkaido University, Hakodate, Hokkaido, Japan
E-mail address: kmiya@fish.hokudai.ac.jp

AUTHOR DECLARATION

On behalf of all authors,
I wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.
I confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. I further confirm that the order of authors listed in the manuscript has been approved by all of us.
I confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing I confirm that we have followed the regulations of our institutions concerning intellectual property.
I further confirm that any aspect of the work covered in this manuscript that has involved human patients has been conducted with the ethical approval of all relevant bodies.
All authors understand that the Corresponding Author is the sole contact for the Editorial process (including Editorial Manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs.
I confirm that I have provided a current, correct email (geniente@gmail.com)

Siena, October 15, 2014

Claudio De Felice

Protective effect of ω-3 PUFAs against unexplained sudden death in Rett syndrome

Claudio De Felice [a*], Cinzia Signorini [b*], Silvia Leoncini [b,c], Alessio Cortelazzo [c,d], Gloria Zollo [b,c], Thierry Durand [e], Jean-Marie Galano [e], Camille Oger [e], Jetty Chung-Yung Lee [f], Jean-Yves Le Guennec [g], Silvia Maffei [h], Alessandra Pecorelli [b,c], Giuseppe Valacchi [i], Lucia Ciccoli [b], Joussef Hayek [c]

[a]Neonatal Intensive Care Unit, University Hospital Azienda Ospedaliera Universitaria Senese (AOUS), Policlinico "S. M. alle Scotte", Viale M. Bracci 1, I-53100 Siena, Italy; [b]Department of Molecular and Developmental Medicine, University of Siena Via Aldo Moro, I-53100 Siena, Italy; [c]Child Neuropsychiatry Unit, University Hospital (AOUS), I-53100 Siena, Italy; [d]Department of Medical Biotechnologies, University of Siena, I-53100 Siena, Italy; [e]Institut des Biomolécules Max Mousseron (IBMM), UMR 5247-CNRS-UM I-UM II-ENSCM, BP 14491, 34093, Montpellier, Cedex 5, France; [f]School of Biological Sciences, The University of Hong Kong, Hong Kong, China; [g]Inserm U1046 Physiologie & Médicine Experimentale du Coeur et des Muscles, University Montpellier 1 and Montpellier 2, Montpellier, France; [h]Department of Cardiology, University Hospital (AOUS), Viale M. Bracci 16,53100 Siena, Italy; [i]Department of Life Science and Biotechnologies, University of Ferrara, I-44121 Ferrara, Italy

*Claudio De Felice and Cinzia Signorini contributed equally to this work.

All authors declare no conflict of interest. In particular, the authors are not aware of any affiliations, memberships, funding, or financial holdings that might affect the objectivity of this paper.

Corresponding Authors:

Claudio De Felice, MD, Neonatal Intensive Care Unit, University Hospital AOUS, I-53100 Siena, Italy, *E-mail:* geniente@gmail.com

Cinzia Signorini, PhD, Department of Molecular and Developmental Medicine University of Siena Via Aldo Moro, I-53100 Siena Italy, *E-mail:* cinzia.signorini@unisi.it

SUMMARY

Background & aims: Rett syndrome , although considered a rare disease, is the second commonest cause of severe mental retardation in the female gender. The disease is mainly caused by mutations in the gene encoding the MeCP2 protein. Of note, in RTT a 300- folds increased risk of sudden death has been reported. This fatal event is to be considered as unexplained as its causes are unknown, although a cardiac event has been previously postulated. In the present study, a protective effect of ω-3 fatty acids against the phenomenon of the unexplained sudden death in Rett syndrome was tested.

Methods: A cohort of patients on a regular clinical and biochemical follow-up. (n=214, all females, mean age: 15.4 ± 7.5 years) were examined. A supplementation with ω-3 fatty acids at high dosage (250 ± 45 mg/kg b.w./day) was proposed and the number of sudden death during 5-years were recorded. Fatty acids profile in erythrocytes and systemic levels of non-protein-bound iron (plasma and intraerythrocyte) and isoprostanoid oxidative stress biomarkers, (i.e., F_2-isoprostanes F_2-dihomo-isoprostanes, and F_4-neuroprostanes) were measured.

Results: A significantly lower risk of sudden death was observed for the ω-3 fatty acids-supplemented population (O.R.: 115.143; 95% CI 14.33 to 924.80, *P* <0.0001).The reduced risk of sudden death in the ω-3 fatty acids-supplemented group was found to be associated with increased blood docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and ω -3 index, as well as decreased systemic levels of oxidative stress biomarkers.

Conclusions: Our data indicate that ω-3 fatty acids protect RTT patients from sudden death by alleviating oxidative stress.

Keywords

Omega-3 fatty acids; Omega-3 index; Isoprostanes; Sudden death; Rett syndrome

Non-standard abbreviations: OS, oxidative stress; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFAs, polyunsaturated fatty acids. P-NPBI, plasma non-protein-bound iron; IE-NPBI, intraerythrocyte non-protein-bound iron; F_2-IsoPs, F_2-isoprostanes; F_2-dihomo-IsoPs, F_2-dihomo-isoprostanes; F_4-NeuroPs, F_4-neuroprostanes;

Introduction

Rett syndrome (RTT) is a rare (1:10000 female live births) genetically determined and devastating neurological disorder, affecting almost exclusively females that is mainly caused by mutations in the gene encoding the X-linked methyl-CpG-binding protein 2 (MECP2) [1]. Mental retardation, microcephaly, language impairment, epilepsy, breathing abnormalities, stereotypes and loss of purposeful use of hands are the major clinical features of typical RTT, whereas oxidative stress (OS) status has been well established by our group in both blood samples and primary skin fibroblasts cultures from patients with RTT [2-4]. Recently, we have indicated oxidative brain damage as a previously unrecognized hallmark feature of murine RTT [5]. Omega-3 polyunsaturated fatty acids (ω-3 PUFAs) - predominantly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) - which contribute to human health and well-being [6], have been reported to ameliorate phenotype severity and improve lipid composition of erythrocyte membranes [7,8], as well as reduce the sub-clinical acute phase response [9] and normalize redox balance in RTT [8,10]. These results paved the way for our rationale of using ω-3 PUFAs in RTT.

Interestingly, in RTT a 300- fold increased risk of sudden death has been reported. This fatal event is to be considered as unexplained given that its causes remain unknown. Nevertheless, a cardiac cause has been previously postulated [11]. In the present study, the influence of ω-3 PUFAs prolonged supplementation on the phenomenon of unexplained sudden death in RTT was investigated.

Methods

Population

A total of 214 female patients (mean age 15.4 ± 7.5 years) with proven *MECP2* gene mutation with RTT typical presentation were enrolled in a regular 5-year-long follow-up clinical study, along with medical checks performed every 6 months (Child Neuropsychiatry Unit). A regular feedback was established with the families (by telephone, e-mail and/or social networks in order to notify the clinicians about any significant variations possibly occurring in intervals between examinations. All patients were following a standard Mediterranean diet. None of the patients had known congenital heart disease, or arrhythmias or surrogate markers of possible arrhythmias (specifically, long QT syndrome). RTT clinical severity was assessed using the compressed clinical severity score (CCSS), a validated clinical rating scoring system specifically devised for RTT [12] that measures clinical features common in the disease. Supplementation with ω-3 PUFAs was proposed to the examined patients' cohort in the form of fish oil (Norwegian Fish Oil AS, Trondheim, Norway, Product Number HO320-6; Italian importer: Transforma AS Italia, Forlì Italy; Italian Ministry Registration Code: 10 43863-Y) Use of EPA plus DHA in RTT was approved by the AOUS Ethical Committee. A total of n=6 sudden deaths during the selected follow-up period were recorded.

The work was carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). The informed consent was obtained for experimentation. The privacy rights of human subjects was always observed.

Blood sampling and laboratory analyses

Blood sampling was carried out after the overnight fasting. Blood was collected in heparinized (heparin blood) tubes, and the platelet poor plasma (2,400 g for 15 min) was saved for plasma nonprotein-bound iron (P-NPBI) (pro-oxidant factor) [13] and isoprostanoid biomarkers including free F_2-isoprostanes (F_2-IsoPs) oxidation products of arachidonic acid (AA) and an oxidative damage biomarker in vivo of systemic lipid peroxidation [14], free F_2-dihomo-isoprostanes (F_2-

dihomo-IsoPs) peroxidation products of adrenic acid and a potential biomarker of glia membrane damage in brain white matter [15], and free F_4-neuroprostanes (F_4-NeuroPs) peroxidation products of DHA and a biomarker of neuronal membrane damage of brain gray matter [10] determination. For all isoprostanoid determinations, 90 μM of butylated hydroxytoluene (BHT) was added to plasma as an antioxidant to prevent development of artefacts and all the determinations were carried out by a gas chromatography/negative ion chemical ionization tandem mass spectrometry analysis. Erythrocytes were resuspended (50%, vol/vol) in Ringer solution (pH 7.4) for intra-erythrocyte nonprotein-bound iron (IE-NPBI) analysis [13]. Both IE-NPBI and P-NPBI were determined by high performance liquid chromatography method [13]. Erythrocyte membrane fatty acids profile analysis was performed by gas chromotography method as previously reported [4] and the ω-3 index was calculated [16]. All clinical and biochemical data were determined every 6 months and average results used for data analysis.

Statistical Analyses

Differences between the sudden death *vs.* living RTT population were tested by chi-square statistics of Fisher's exact test, one-way analysis of variance (ANOVA), Student–Newman–Keuls post-hoc test, or Kruskal–Wallis test. The efficiency in discriminating the sudden death group from living patients was evaluated using Receiver Operating Characteristic (ROC) analysis. Associations between variables were tested by univariate regression, univariate logistic regression, multivariate logistic regression and multiple regression analyses with "sudden death" as a dependent variable. Two-tailed P values of less than 0.05 were considered significant. The statistical software MedCalc ver. 12.0 (MedCalc. Software, Mariakerke, Belgium) was used.

Results

A total of 188/214 patients (87.8%) were supplemented with ω-3 PUFAs during the examined follow-up time period. In the supplemented patients, the dose of ω-3 PUFAs corresponded to DHA 75 ± 15 mg/kg b.w./day and EPA 120 ± 25 mg/kg b.w./day (ratio 1:1.6), with a total ω-3 PUFAs intake of 250 ± 45 mg/kg b.w./day. Average duration of ω-3 PUFAs supplementation was 44.2 ± 20.5 months. Univariate analysis showed that the sudden death group differed from the remaining RTT cohort for significantly higher ω-3 PUFA supplementation and OS biomarkers blood levels (*P* ≤0.006; **Table 1**). Erythrocyte ω-3 PUFA levels were significantly lower in the sudden death group as compared to living patients (DHA: -39.09%; EPA: -68.06%, and ω-3 PUFAs index: -52.14%; *P* ≤0.0001), while levels of P-NPBI (+59%), IE-NPBI (+58.7%), free F_2-IsoPs (+63.5%), and free F_2-dihomo-IsoPs (+121.4%), and free F_4-NeuroPs (+97.1%) were significantly increased (*P* ≤0.0063).

Conversely, no differences were observed concerning age and phenotype severity. After calculating the cut-off values of potential predictors by ROC curve analysis (**Figure 1** and **2**), univariate logistic regression results (**Table 2**) indicate that a supplementation with ω-3 PUFA was highly protective against sudden death (O.R.: 0.019; 95% CI 0.004 to 0.090, *P* <0.0001). In particular, an average of 115-folds lower risk of sudden death in ω-3 PUFAs supplemented RTT girls was observed (O.R.: 115.143; 95% CI 14.33 to 924.80, *P* <0.0001). The results of a multivariate logistic regression (**Table 3**) and multiple regression (**Table 4**) indicate that a low DHA content in the erythrocyte membrane and a low ω-3 PUFA index are independent predictors for sudden death in the observed RTT patient cohort. While all the OS biomarkers were not included in the final regression model, plasma free F_4-NeuroPs was included as significant predictor for sudden death in RTT in the final multiple regression model (**Table 4**).

Discussion

The ω-3 PUFAs found in fish are strongly associated with a reduced risk of sudden death among adult men (interval age 40-84 years) without evidence of prior cardiovascular disease. [17]. The findings of the present study indicate a protective effect of ω-3 PUFAs against sudden death in a relatively large cohort of RTT patient, followed –up for 5 years in a National Rett Reference Centre. In our study, ω-3 PUFAs were given at high dose and for a prolonged period of time. Supplementation with marine ω-3 PUFAs were associated with a significant decrease in the blood levels of several OS biomarkers, therefore supporting the cumulating evidence for a lack of increased lipid peroxidation in biological systems following increased EPA and DHA intake [6,18,19].

Pathophysiologically, marine ω-3 PUFAs have been reported to protect against many human diseases and pathological conditions, including hypertension, hypertriglyceridemia, cardiac arrhythmias, cardiovascular disease, immune dysfunction, inflammation, allergy, neurodegenerative diseases or ageing, bone loss, and even cancer [6]. Physiologically, marine ω-3 PUFAs (especially DHA) promote optimal brain growth, visual and neural function [6].

On the other hands, mean adult intakes of marine ω-3 PUFAs are low [20]. Increasing EPA and DHA - i.e., the main fatty acids in fish oil- intake increases the EPA and DHA content of blood lipids, blood cells, and many tissues including liver, heart, skeletal muscle, in which the effect is known to be dose, time and tissue dependent [6].

The mechanisms of action of the ω-3 PUFAs remain a vast and very stimulating field of research. Cumulating evidence indicates that oxygenated metabolites of PUFAs are the real activators in the Rett syndrome patients [21,22]. In addition, ω-3 PUFA supplementation has been reported to change gene expression, in particular of nuclear receptor peroxisome proliferator-activated receptor-alpha, nuclear transcription-factor kappa B, redox balance, and activation of the OS response mediated by nuclear factor (erythroid-derived 2)-like 2 [23].

The reasons for the observed associations appear to be linked to mitigation of the redox alteration coexisting with the disease. When accounting for possible confounding variables, a close association with both plasma F_4-NeuroP levels and chronological age was observed.

Isoprostanes are considered as the gold standard for *in vivo* OS [4, 24]. Specifically, F_4-NeuroPs are known to be the end-products of DHA, abundant in neuronal membranes. Subsequently, our data suggest the involvement of neuronal OS in the pathogenesis of sudden death in RTT. This is particularly evident in a very recent study [25] on biomarkers of OS, where a series of omega-3 and omega-6 oxidized lipid products have been measured in the brain prefrontal cortex of preterm pigs, F_4-NeuroPs and dihomo-isoprostanes were found to be strong indicative biomarkers for oxidative brain damage, with the interrelating role of adrenic acid and DHA appearing to be relevant in neurological diseases.

The high dihomo-isoprostanes levels in RTT shown by our group [26] and the mitigation by fish oil supplementation, and potentially by F_4-NeuroPs, demonstrate the importance of fish oil in the RTT diet. In support, it has been also shown that F_4-NeuroPs have other bioactive roles, as they have been recently shown to possess anti-arrhythmic effects [24,27], as well as to be linked to atherosclerosis [28].

Although further studies are certainly needed in order to better understand the protective mechanisms of ω-3 PUFA in RTT, our data strongly indicate that a continued supplementation with these fatty acids at high dosage in RTT patients is not only able to improve clinical severity and quality of life [8], but appears to be potentially live-saving. Furthermore, our findings confirm and add new evidence on the protective effects of long-chain ω-3 fatty acids on the risk of sudden death.

Acknowledgements

We wish to dedicate the paper in memoriam of Prof. Mario Comporti (Siena, September 8th 1935 – October 3rd 2014) great Maestro and internationally renowned pioneer for the studies on oxidative stress and human disease. We also would like to sincerely thank professional singer Matteo Setti

(Reggio Emilia, Italy, official web site: www.matteosetti.com) for his many charity concerts and continued interest in the scientific aspects of our research. ("*Il respiro della musica*" / "*The breath of music* "*project*).

This research is also dedicated to the Rett girls and their families.

Statement of Authorship

All the authors declare that: i) the manuscript has been read and approved by all named authors; ii) there are no other persons who satisfied the criteria for authorship but are not listed; iii) the order of authors listed in the manuscript has been approved by all of us.

Author Contributions

Concept: **CDF, CS, SL, JH;** Experimental design: **CDF, CS;** Clinical follow-up**: JH, CDF, SM;** Sample blood preparation: **CS, SL, GZ;** Isoprostanes and neuroprostanes assays: **CS;** NPBI assays**: SL, GZ;** Isoprostanes synthesis: **TD, CO, JMG, AG, VBP;** Data analysis: **CDF, CS, SL, GZ;** Data interpretation: **All the Authors;** Manuscript drafting: **All the Authors;** Approval: **All the Authors**

Conflict of Interest Statement

All authors declare no conflict of interest. In particular, the authors are not aware of any affiliations, memberships, funding, or financial holdings that might affect the objectivity of this paper.

Funding sources

We thank the Tuscany Region [Bando Salute 2009, "Antioxidants (ω-3 Polyunsaturated Fatty Acids, lipoic acid) supplementation in Rett syndrome: A novel approach to therapy"], Italy for partial funding of the study.

Figure legends

Figure 1. **Receiver operating characteristic (ROC) analysis for erythrocyte ω-3 PUFAs profile in discriminating RTT-related "sudden death" from living patients.**

Abbreviations list: AUC: Area under the curve; SE: standard error; C.I.: confidence interval; L.R.: likelihood ratio.

* in: Ref. [17]

Figure 2. Receiver operating characteristic (ROC) analysis for oxidative stress biomarkers in discriminating RTT-related "sudden death" from living patients.

Abbreviations list: AUC: Area under the curve; SE: standard error; C.I.: confidence interval; L.R.: likelihood ratio.

References

1 Chahrour M, Zoghbi HY. The story of Rett syndrome: from clinic to neurobiology. Neuron. 2007;56(3):422-37.

2. De Felice C, Signorini C, Leoncini S, Pecorelli A, Durand T, Valacchi G, Ciccoli L, Hayek J. The role of oxidative stress in Rett syndrome: an overview. Ann N Y Acad Sci. 2012;1259:121-35

3. Ciccoli L, De Felice C, Paccagnini E, Leoncini S, Pecorelli A, Signorini C, Belmonte G, Valacchi G, Rossi M, Hayek J. Morphological changes and oxidative damage in Rett Syndrome erythrocytes. Biochim Biophys Acta. 2012;1820(4):511-20.

4. Signorini C, Leoncini S, De Felice C, Pecorelli A, Meloni I, Ariani F, Mari F, Amabile S, Paccagnini E, Gentile M, Belmonte G, Zollo G, Valacchi G, Durand T, Galano JM, Ciccoli L, Renieri A, Hayek J. Redox imbalance and morphological changes in skin fibroblasts in typical Rett syndrome. Oxid Med Cell Longev. 2014;2014:195935.

5. De Felice C, Della Ragione F, Signorini C, Leoncini S, Pecorelli A, Ciccoli L, Scalabrì F, Marracino F, Madonna M, Belmonte G, Ricceri L, De Filippis B, Laviola G, Valacchi G, Durand T, Galano JM, Oger C, Guy A, Bultel-Poncé V, Guy J, Filosa S, Hayek J, D'Esposito M. Oxidative brain damage in Mecp2-mutant murine models of Rett syndrome. Neurobiol Dis. 2014;68:66-77.

6. Calder PC. Marine omega-3 fatty acids and inflammatory processes: Effects, mechanisms and clinical relevance. Biochim Biophys Acta. 2014 Doi: 10.1016/j.bbalip.2014.08.010.

7. Signorini C, De Felice C, Leoncini S, Durand T, Galano JM, Cortelazzo A, Zollo G, Guerranti R, Gonnelli S, Caffarelli C, Rossi M, Pecorelli A, Valacchi G, Ciccoli L, Hayek J. Altered erythrocyte membrane fatty acid profile in typical Rett syndrome: Effects of omega-3 polyunsaturated fatty acid supplementation. Prostaglandins Leukot Essent Fatty Acids. 2014. Doi: 10.1016/j.plefa.2014.08.002.

8. De Felice C, Signorini C, Durand T, Ciccoli L, Leoncini S, D'Esposito M, Filosa S, Oger C, Guy A, Bultel-Poncé V, Galano JM, Pecorelli A, De Felice L, Valacchi G, Hayek J. Partial rescue of Rett syndrome by ω-3 polyunsaturated fatty acids (PUFAs) oil. Genes Nutr. 2012 Jul;7(3):447-58.
9. Cortelazzo A, De Felice C, Guerranti R, Signorini C, Leoncini S, Pecorelli A, Zollo G, Landi C, Valacchi G, Ciccoli L, Bini L, Hayek J. Subclinical inflammatory status in Rett syndrome. Mediators Inflamm. 2014;2014:480980.
10. Signorini C, De Felice C, Leoncini S, Giardini A, D'Esposito M, Filosa S, Della Ragione F, Rossi M, Pecorelli A, Valacchi G, Ciccoli L, Hayek J. F4-neuroprostanes mediate neurological severity in Rett syndrome. Clin Chim Acta. 2011 Jul 15;412(15-16):1399-406.
11. Kerr AM, Armstrong DD, Prescott RJ, Doyle D, Kearney DL. Rett syndrome: analysis of deaths in the British survey. European Child & Adolescent Psychiatry. 1997;6(supplement 1):71–74
12. Neul JL, Fang P, Barrish J, Lane J, Caeg EB, Smith EO, Zoghbi H, Percy A, Glaze DG (2008) Specific mutations in methyl-CpGbinding protein 2 confer different severity in Rett syndrome. Neurology 70:1313–1321
13. De Felice C, Ciccoli L, Leoncini S, Signorini C, Rossi M, Vannuccini L, Guazzi G, Latini G, Comporti M, Valacchi G, Hayek J. Systemic oxidative stress in classic Rett syndrome. Free Radic Biol Med. 2009 Aug 15;47(4):440-8.
14. Signorini, C., De Felice, C., Durand, T., Oger, C., Galano, J.M., Leoncini, S., Pecorelli, A., Valacchi, G., Ciccoli, L., Hayek, J., 2013. Isoprostanes and 4-hydroxy-2-nonenal: markers or mediators of disease? Focus on Rett syndrome as a model of autism spectrum disorder. Oxid. Med. Cell. Longev. 2013, 343824.
15. VanRollins M, Woltjer RL, Yin H, Morrow JD, Montine TJ. F2-dihomo-isoprostanes arise from free radical attack on adrenic acid. J Lipid Res. 2008;49(5):995-1005.
16. Harris WS, Von Schacky C. The Omega-3 Index: a new risk factor for death from coronary heart disease? Prev Med. 2004 Jul;39(1):212-20.

17. Albert CM, Campos H, Stampfer MJ, Ridker PM, Manson JE, Willett WC, Ma J. Blood levels of long-chain n-3 fatty acids and the risk of sudden death. N Engl J Med. 2002 Apr 11;346(15):1113-8.

18. Miyashita K. Paradox of omega-3 PUFA oxidation. European Journal of Lipid Science and Technology 2014. In press. DOI: 10.1002/ejlt.201400114

19. Ottestad I, Vogt G, Retterstøl K, Myhrstad MC, Haugen JE, Nilsson A, Ravn-Haren G, Nordvi B, Brønner KW, Andersen LF, Holven KB, Ulven SM. Oxidised fish oil does not influence established markers of oxidative stress in healthy human subjects: a randomised controlled trial. Br J Nutr. 2012 Jul;108(2):315-26.

20 Meyer BJ, Mann NJ, Lewis JL, Milligan GC, Sinclair AJ, Howe PR. Dietary intakes and food sources of omega-6 and omega-3 polyunsaturated fatty acids. Lipids. 2003 Apr;38(4):391-8

21. A.Guy,C.Oger, J.Heppekausen, et al., Oxygenated metabolites of n-3 polyunsaturated fatty acids as potential oxidative stress biomarkers: total synthesis of 8-F(3t)-IsoP,10-F(4t)-NeuroPand[D(4)]-10-F(4t)-NeuroP, Chemistry 20(2014)6374–6380.

22. S. Judé, S.Bedut, S.Roger, et al., Peroxidation of docosahexaenoic acid is responsible for its effects on ITO and ISS intraventricular myocytes, Br.J. Pharmacol. 139(2003)816–822.

23. Rudkowska I, Paradis AM, Thifault E, Julien P, Tchernof A, Couture P, Lemieux S, Barbier O, Vohl MC. Transcriptomic and metabolomic signatures of an n-3 polyunsaturated fatty acids supplementation in a normolipidemic/normocholesterolemic Caucasian population. J Nutr Biochem. 2013;24(1):54-61.

24. Galano, J.M., Mas, E., Barden, A., Mori, T.A., Signorini, C., De Felice, C., Barrett, A., Opere, C., Pinot, E., Schwedhelm, E., Benndorf, R., Roy, J., Le Guennec, J.Y., Oger, C., Durand, T., 2013. Isoprostanes and neuroprostanes: total synthesis, biological activity and biomarkers of oxidative stress in humans. Prostaglandins Other Lipid Mediat. 107, 95–102.

25. La Torre A, Lee YY, Oger C, Sangild PT, Durand T, Lee JC, Galano JM Synthesis, discovery, and quantitation of dihomo-isofurans: biomarkers for in vivo adrenic acid peroxidation. Angew Chem Int Ed Engl. 2014 Jun 10;53(24):6249-52. doi: 10.1002/anie.201402440.

26. De Felice C, Signorini C, Durand T, Oger C, Guy A, Bultel-Poncé V, Galano JM, Ciccoli L, Leoncini S, D'Esposito M, Filosa S, Pecorelli A, Valacchi G, Hayek J. F2-dihomo-isoprostanes as potential early biomarkers of lipid oxidative damage in Rett syndrome. J Lipid Res. 2011;52(12):2287-97

27. Le Guennec JY, Galano JM, Oger C, Thireau J, Roy J, Bultel-Poncé V, GuyA, Durand T, Methods and pharmaceutical composition for the treatmentand prevention of cardiac arrhythmias. Patent n. WO/2014/086819. Publication date 06/12/2014

28 Gladine C, Newman JW, Durand T, Pedersen TL, Galano JM, Demougeot C, Berdeaux O, Pujos-Guillot E, Mazur A, Comte B. Lipid profiling following intake of the omega 3 fatty acid DHA identifies the peroxidized metabolites F4-neuroprostanes as the best predictors of atherosclerosis prevention. PLoS One. 2014 Feb18;9(2):e89393.

Tables

Table 1. Comparisons between sudden death *vs.* living RTT patients during a 5-years follow-up.

Variable	Rett Syndrome Patients		*P*-value
	Sudden death	Living	
N	6	208	
Age (years)	19.5 ± 9.0	13.2 ± 8.9	0.0889
Phenotype severity (CCSS)	8.2 ± 1.9	7.9 ± 2.0	0.7787
Omega-3 PUFAs supplementation	1/6 (16.6%)	187/208 (89.9 %)	< 0.0001
DHA (%)	3.88 ± 1.62	6.37 ± 0.99	< 0.0001
EPA (%)	1.22 ± 0.50	3.82 ± 1.63	0.0001
Omega-3 PUFA index *	4.59 ± 2.06	9.59 ± 2.78	< 0.0001
P-NPBI (nmol/ml)	0.97 ± 0.15	0.61 ± 0.27	0.0014
IE-NPBI (nmol/ml)	1.27 ± 0.39	0.80 ± 0.37	0.0025
Plasma free F_2-IsoPs (pg/ml)	73.30 ± 24.80	44.84 ± 24.90	0.0063
Plasma free F_2-dihomo-IsoPs (pg/ml)	24.71 ± 14.18	11.16 ± 3.14	< 0.0001
Plasma free F_4-NeuroPs (pg/ml)	18.21 ± 4.84	9.24 ± 1.27	< 0.0001

Abbreviations: CCSS: compressed clinical severity score; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; PUFAs: polyunsaturated fatty acids; P-NPBI: plasma non-protein-bound iron; IE-NPBI: intraerythrocyte non-protein-bound iron; F_2-IsoPs, F_2-isoprostanes; F_2-dihomo-IsoPs, F_2-dihomo-isoprostanes; F_4-NeuroPs, F_4-neuroprostanes.

* In: Ref. [17]

Table 2. Univariate logistic regression for ω-3 PUFA supplementation, clinical severity, erythrocyte fatty acids profile, and OS markers on the dependent variable "sudden death" in RTT patients

Variable	O.R.	95% C.I.	P-value	% Correct	Null model 2 Log Likelihood	Full model -2 Log Likelihood	χ^2 statistics	D.F.	Overall model fit *P*-value
Age > 13 years [§]	2.254	0.668-7.600	0.1900	89.23	88.832	86.975	1.858	1	0.1729
CCSS >7 [§]	1.693	0.468-6.122	0.4217	78.79	68.211	67.537	0.674	1	0.412
Omega-3 PUFAs supplementation	0.0188	0.0039-0.0899	93.67	<0.0001	104.349	65.414	38.935	1	<0.0001
Erythrocyte DHA ≤ 5.15%	119..00	21.59-655.90	<0.0001	94.24	90.812	43.292	47.520	1	<0.0001
Erythrocyte EPA ≤ 2.3 %	122.42	14.715-1019.2	<0.0001	90.65	90.812	46.081	44.731	1	<0.0001
Omega-3 Index ≤ 6.7% (*)	25.25	5.297-120.369	0.0001	94.24	90.812	65.556	25.255	1	<0.0001
P-NPBI > 0.7 nmol/ml [§]	25.381	3.105-207.449	0.0026	81.58	72.613	54.685	17.928	1	<0.0001
IE-NPBI > 0.8 nmol/ml [§]	17.483	2.162-141.346	0.0073	82.93	74.955	61.325	13.600	1	0.0002
Plasma free F_2-IsoPs > 43.2 pg/ml [§]	6.175	1.527-24.971	0.0106	78.46	67.731	59.905	7.825	1	0.0052
Plasma free F_2-dihomo-IsoPs > 4.3 pg/ ml [§]	6.30	1.639-24.212	0.0055	74.0	59.295	51.580	7.716	1	0.0074
Plasma free F_4-NeuroPs > 6.5 pg/ml [§]	13.619	1.635-113.469	0.0158	75.44	63.551	53.461	10.090	1	0.0015

Abbreviations: O.R.,odds ratio; C.I., confidence interval; D.F., degrees of freedom; CCSS, compressed clinical severity score; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; PUFAs: polyunsaturated fatty acids; P-NPBI, plasma non-protein-bound iron; IE-NPBI, intraerythrocyte non-protein-bound iron; F_2-IsoPs, F_2-isoprostanes; F_2-dihomo-IsoPs, F_2-dihomo-isoprostanes; F_4-NeuroPs, F_4-neuroprostanes; PUFAs, polyunsaturated fatty acids.

[§] Cut-off values as calculated by ROC curve analyses

(*****) Ref. [17]

Table 3. Stepwise multivariate logistic regression for the dependent variable "sudden death" in RTT patients

Predictor Variables	O.R.	95% C.I.	*P*-value	% Correct	Null model 2 Log Likelihood	Full model -2 Log Likelihood	χ^2 statistics	D.F.	Overall model fit *P*-value
Erythrocyte DHA ≤ 5.15% [§]	**61.102**	**5.207-717.04**	**0.0011**	**92.0**	**59.295**	**20.062**	**39.234**	**1**	**<0.0001**
Omega-3 Index ≤ 6.7% (*) [§]	**20.273**	**1.726-238.19**	**0.0167**						
Erythrocyte EPA ≤ 2.3 % [§]	N.I.	-	-						
P-NPBI > 0.7 nmol/ml [§]	N.I.	-	-						
IE-NPBI > 0.8 nmol/ml [§]	N.I.	-	-						
Plasma free F_4-NeuroPs > 6.5 pg/ml [§]	N.I.	-	-						
Plasma free F_2-IsoPs > 43.2 pg/ml [§]	N.I.	-	-						
Plasma free F_2-dihomo-IsoPs > 4.3 pg/ ml [§]	N.I.	-	-						

Abbreviations: O.R.,odds ratio; C.I., confidence interval; D.F., degrees of freedom; CCSS, compressed clinical severity score; DHA: docosahexaenoic acid; EPA: eicosapentaenoic acid; P-NPBI, plasma non-protein-bound iron; IE-NPBI, intraerythrocyte non-protein-bound iron; F_2-IsoPs, F_2-isoprostanes; F_2-dihomo-IsoPs, F_2-dihomo-isoprostanes; F_4-NeuroPs, F_4-neuroprostanes; PUFAs, polyunsaturated fatty acids.
N.I.: Not included in the model
[§] Cut-off values as calculated by Receiver Operating Characteristic analyses
(*) In: Ref. [17]

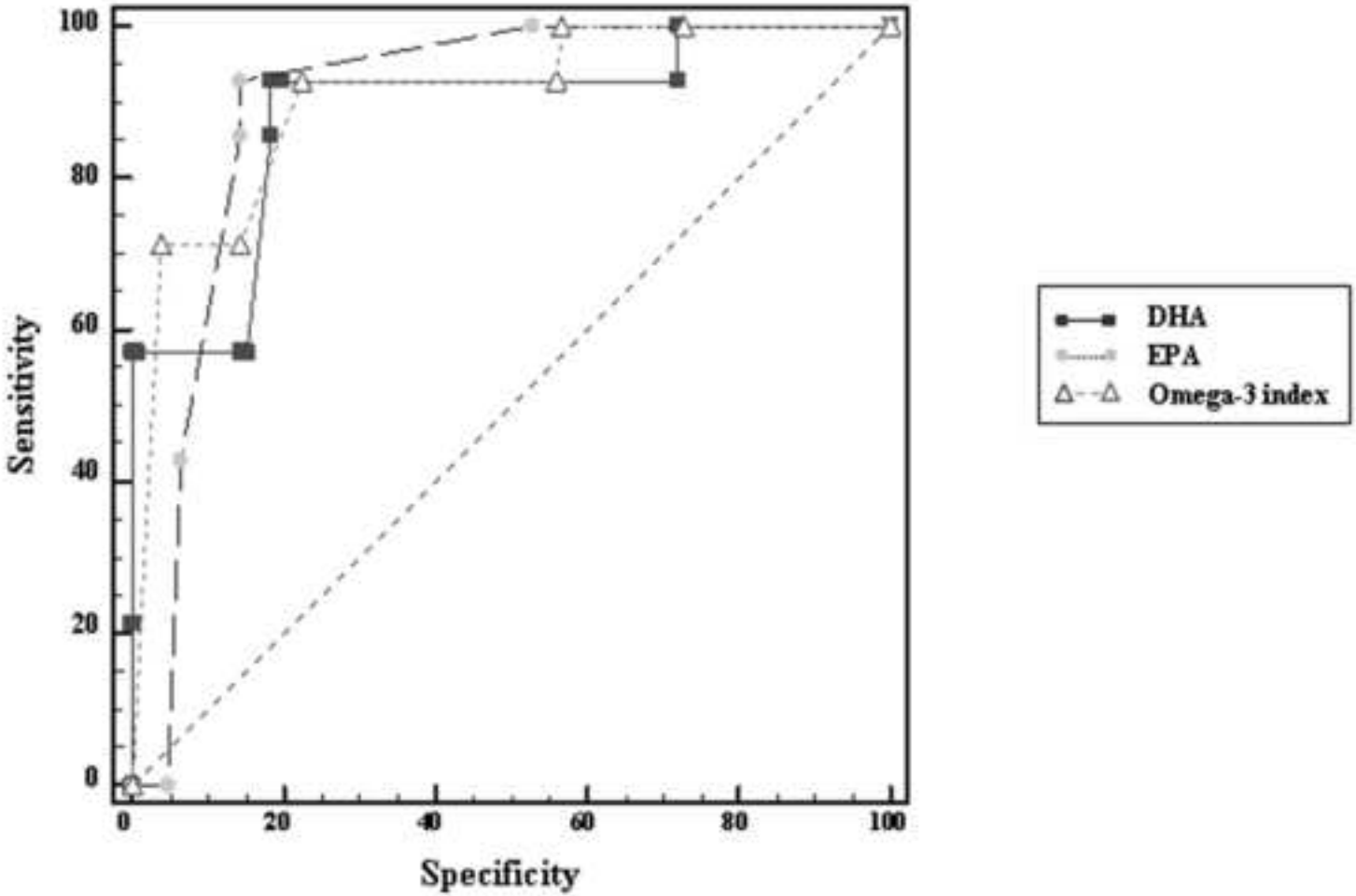
Table 4. Stepwise multiple regression for the dependent variable "sudden death" in RTT patients

Predictor Variables	Coeff.	S.E.	*r* partial	*t*	*P*-value	R^2	Adj. R^2	Multiple corr. coeff.	ANOVA *P*-value
(Constant)	**-0.1293**					**0.7774**	**0.7629**	**0.8817**	**<0.0001**
Erythrocyte DHA ≤ 5.15% [§]	**0.5331**	**0.08855**	**0.6639**	**6.021**	**<0.0001**				
Omega-3 Index ≤ 6.7% (*) [§]	**0.3312**	**0.08174**	**0.5129**	**4.052**	**0.0002**				
Plasma free F_4-NeuroPs > 6.5 pg/ml [§]	**0.2457**	**0.06587**	**0.4819**	**3.730**	**0.0005**				
Erythrocyte EPA ≤ 2.3 % [§]	N.I.	-	-	-	-				
P-NPBI > 0.7 nmol/ml [§]	N.I.	-	-	-	-				
IE-NPBI > 0.8 nmol/ml [§]	N.I.	-	-	-	-				
Plasma free F_2-IsoPs > 43.2 pg/ml [§]	N.I.	-	-	-	-				
Plasma free F_2-dihomo-IsoPs > 4.3 pg/ ml [§]	N.I.	-	-	-	-				

Abbreviations: O.R.,odds ratio; C.I., confidence interval; D.F., degrees of freedom; CCSS, compressed clinical severity score; DHA: ; EPA: ; P-NPBI, plasma non-protein-bound iron; IE-NPBI, intraerythrocyte non-protein-bound iron; F_2-IsoPs, F_2-isoprostanes; F_2-dihomo-IsoPs, F_2-dihomo-isoprostanes; F_4-NeuroPs, F_4-neuroprostanes; PUFAs, polyunsaturated fatty acids.
N.I.: Not included in the model
[§] Cut-off values as calculated by Receiver Operating Characteristic analyses
(*) In: Ref. [17]

Figure 1

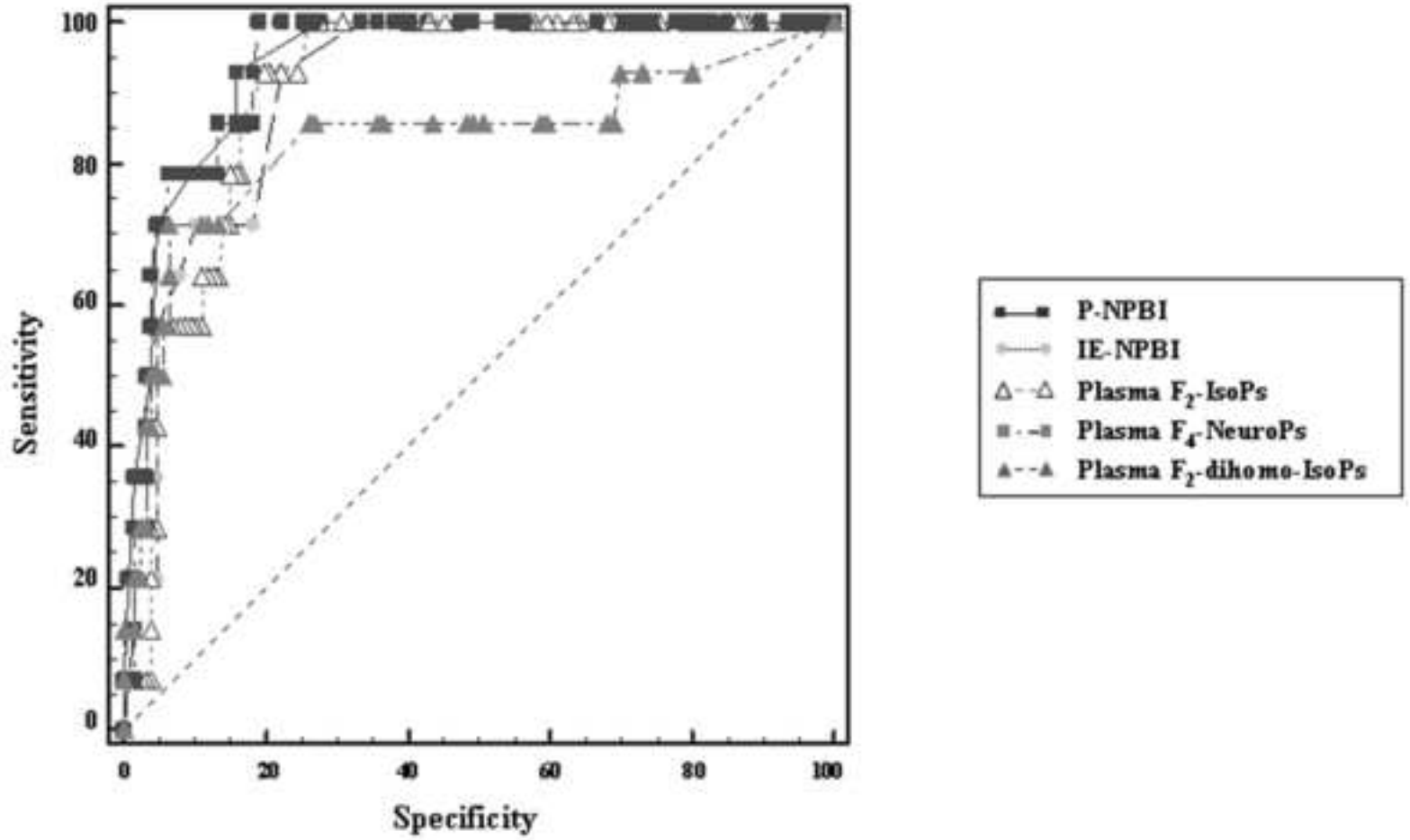
Click here to download high resolution image



Variable	Area under the curve				Criterion value and coordinates				
	AUC	SE	95%CI	*P* value	Cut-off	Sensitivity	Specificity	+LR	-LR
DHA	.887	.0533	.823-.935	<.0001	≤ 5.15%	92.86	81.60	5.05	0.088
EPA	.897	.0291	.834-.944	<.0001	≤ 2.3 %	92.86	85.60	6.45	0.083
Omega-3 Index *	.906	.0426	.854-.949	<.0001	≤ 6.7%	92.86	77.60	4.15	0.092

Figure 2

Click here to download high resolution image



Variable	Area under the curve				Criterion value and coordinates				
	AUC	SE	95%CI	P value	Cut-off	Sens.	Spec.	+LR	-LR
P-NPBI	.943	.0219	.891-.955	<.0001	>0.7 nmol/ml	92.86	84.13	5.85	0.085
IE-NPBI	.906	.0296	.846-.949	<.0001	>0.8 nmol/ml	92.86	77.78	4.18	0.092
Plasma free F_2-IsoPs	.906	.0276	.845-.949	<.0001	> 43.2 pg/ml	100	73.81	3.82	0.00
Plasma free F_2-dihomo-IsoPs	.834	.0755	.762-.892	<.0001	> 4.3 pg/ ml	71.43	93.65	11.25	0.31
Plasma free F_4-NeuroPs	.941	.0220	.888-.974	<.0001	> 6.5 pg/ml	100	80.95	5.25	0.00