Lipidomics to analyze the influence of diets with different EPA:DHA ratios in the progression of Metabolic Syndrome using SHROB rats as a model

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Abstract

The role of specific proportions of ω–3 EPA and DHA, in the modulation of inflammation and oxidative stress markers associated to the progression of Metabolic Syndrome was investigated. Potential inflammatory eicosanoids and docosanoids were discussed together to biomarkers of CVD, obesity, inflammation and oxidative stress in an animal model of metabolic disorders. Results evidenced a noteworthy health effect of 1:1 and 2:1 EPA:DHA proportions over 1:2 EPA:DHA based diets through a down–regulation in the production of strong pro–inflammatory ω–6 eicosanoids, a decrement of biomarkers of oxidative stress, and a modulation of fatty acid desaturase activities and plasma and membrane PUFAs towards greater anti–inflammatory profiles. Outcomes contribute to the general knowledge on the health benefits of marine lipids and their role on the progress of MetS, inflammation and oxidative stress. Results shed light on controversial protective mechanisms of EPA and DHA to better design dietary interventions aimed at reducing MetS.

Keywords

Lipid mediators, EPA, DHA, metabolic syndrome, SHROB, inflammation, oxidative stress.

1. Introduction

A sedentary lifestyle, malnutrition and low physical activity are risk factors associated to the progression of Metabolic Syndrome (MetS) (Morrow, Minton and Roberts 1992). MetS is defined as a cluster of risk factors for cardiovascular disease (CVD) and type–2–diabetes, which include decrease of HDL levels, hyperglycemia, hypertension, hypertriglyceridemia, insulin resistance and obesity. MetS is characterized by a chronic

47 inflammation grade below the threshold of pain, which raises the concentration of
48 inflammatory markers in the systemic circulation (Sears 2005). In addition, oxidative
49 stress generated by an overproduction of reactive oxygen species (ROS) has been also
50 linked to the promotion of inflammation processes and MetS (Greene *et al*. 2011; Sears
51 and Ricordi 2012). Therefore, biomarkers for the diagnosis of these metabolic
52 dysfunctions would be desirable to early prevention or appropriate therapeutic
53 programs. A new approach for biomarker identification is the metabolic profiling that
54 comprises the identification and quantification of small molecular weight lipids
55 involved in the development and progression of many inflammatory conditions directly
56 correlated with MetS (Massey and Nicolaou 2013). Specific examples include the
57 strong pro–inflammatory eicosanoids and isoprostanes derived from ω–6 arachidonic
58 (ARA) acid (i.e. PGE_2, $PGF_{1\alpha}$, $PGF_{2\alpha}$, TXA_2, $8isoPGF_{2\alpha}$, and so on) (Gao *et al*. 2006;
59 Massaro *et al*. 2008; Sears and Ricordi 2012), together to eicosanoids and docosanoids
60 derived from the ω–3 eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids that
61 are considered less inflammatory compounds than ARA derivates (Kelly *et al*. 2011;
62 Waddington *et al*. 2001). Moreover, novel studies have suggested a potent anti–
63 inflammatory and cellular protective activity of EPA and DHA resolvins (i.e., RvE_1 and
64 RvD_1) and protectins (i.e., PD_1) (Lee 2012; Serhan *et al*. 2006). In addition, other
65 indexes have been associated with an increasing prevalence of chronic inflammatory
66 diseases related to metabolic disorders (McDaniel, Massey and Nicolaou 2011). One of
67 the most selective markers of cellular inflammation is the ratio ω–6/ω–3 in blood, the
68 higher ratio the greater pro–inflammatory conditions (Sears 2005). Moreover, fatty acid
69 desaturases (FAD) are important regulators of the endogenous fatty acid (FA)
70 metabolism. A high desaturase activity related to oleic acid, palmitoleic acid, and ARA
71 production has been associated with obesity, hypertriacylglycerolemia, and insulin

resistance. In contrast, the enhancement of FAD activity associated with EPA and DHA biosynthetic pathways indicates insulin sensitivity, and the decrease of MetS and CVD (Vessby *et al*. 2013).

Regular consumption of marine ω–3 polyunsaturated fatty acids (PUFAs), mainly EPA and DHA, results in metabolic health benefits by modulating specific lipid biomarkers of cellular inflammation and oxidative stress and reducing CVD risk factors (Brahmbhatt *et al*. 2013; McDaniel, Massey and Nicolaou 2011; Neilson *et al*. 2012). Despite the growing evidence of the benefits of ω–3 supplements, there is no agreement on a dietary recommended proportion of DHA and EPA (Harris *et al*. 2009). Therefore, this investigation was aimed to delve deeper into the benefits exerted by the intake of specific EPA:DHA ratios, targeting the formation of anti–inflammatory and pro–inflammatory lipid mediators on an animal model affected by metabolic abnormalities associated to MetS. The Koletsky model, a genetically obese hypertensive rat (SHROB), was used since it is a well–accepted pattern for studying MetS (Xu *et al*. 2008). The formation of lipid mediators was considered together to biomarkers of oxidative stress and inflammation like FAD activity, plasma and membrane FA profile, and ω–6/ω–3 ratio. Animals were fed standard diets enriched with fish oil containing three different EPA:DHA ratios (1:1, 2:1, and 1:2), and were compared with animals fed control diets based on soybean and linseed oils. Soybean oil is a rich source of ω–6 PUFAs, dietary precursors of pro–inflammatory lipid mediators; whereas, linseed oil is a rich source of vegetable ω–3 PUFAs like linolenic acid (ALA), which slightly derives to EPA and DHA. Lipidomic results were linked to previous research that presented the effect of marine dietary interventions on biometric and clinical parameters of spontaneously hypertensive obese rats (Molinar–Toribio *et al*. 2015).

97

98 2. Materials and Methods

99 *2.1 Animals and diets*

100 Thirteen–week old female SHROB (*n* 35, Charles River, USA) rats were used as animal
101 models. Body weight was recorded on arrival (200–300g) and weekly thereafter. All the
102 specimens were kept in an isolated room with a constantly regulated temperature an
103 controlled humidity (22±2 ^{o}C, 50±10 % humidity) on a 12h light/dark cycle with *ad*
104 *libitum* access to water and standard pelleted A04 chow for rodents (16% protein, 60%
105 carbohydrate, 3% fat, 4% fiber and 5% ash; Harlan Iberica, Barcelona, Spain). Animals
106 were randomized in five dietary groups: soybean, linseed, EPA/DHA 1:1, EPA/DHA
107 2:1, and EPA/DHA 1:2 group. Each group was fed a single weekly dose of 0,8 ml/kg of
108 the assigned oil supplement as listed in Supplementary Material S1. Oil supplements
109 were prepared as previously described and they had a similar fat and energy content
110 (Méndez *et al.* 2013). Briefly, 1:1, 2:1 and 1:2 EPA/DHA supplements were prepared
111 by blending adequate proportions of the commercially available fish oils from
112 AFAMPES 121 EPA (AFAMSA, Vigo–Spain), EnerZona Omega 3 RX (ENERZONA,
113 Milano–Italy) and Oligen liquid DHA 80% (IFIGEN–EQUIP 98, Barcelona–Spain).
114 Soybean oil, obtained from cold pressing unrefined organic soybean oil, was from
115 Clearspring Ltd. (London, UK) and linseed oil, obtained from first cold pressing
116 unrefined organic flax oil, was from Biolasi S.L. (Ordizia, Guipuzcoa–Spain). The five
117 oils contained similar total amount of PUFA (55–66 mg per 100 mg of total fatty acid).
118 However, they significantly differed in the proportion of individual PUFAs. In fish oil
119 mixtures containing EPA/DHA ratios of 1:1, 2:1 and 1:2, EPA and DHA were the most
120 abundant PUFA; together they amounted to approximately 50 mg per 100 mg of total
121 fatty acids. The sum of EPA and DHA in the orally administered soy and linseed oils

122 only reached 1.75% of total fatty acids. In linseed oil, the short–chain omega–3 ALA and
123 omega–6 LA that made up 50% and 16.8% of total fatty acids respectively, were the
124 most abundant PUFA. In soybean oil, LA was the most abundant PUFA (47.5%)
125 whereas the content of ALA was 4%. After a 2–week adaptation period, the oils were
126 administrated for 13 weeks and the experiment ended when rats were 28 weeks old.
127 Then, rats were fasted overnight, anesthetized and sacrificed by exsanguination.
128 Handling and killing of the animals were in full accordance with the European Union
129 guidelines for the care and management of laboratory animals and the pertinent
130 permission was obtained from the CSIC Subcommittee of Bioethical Issues (ref.
131 AGL2009–12 374–C03–03).
132
133 *2.2 Fatty acid analysis of the oil supplements*
134 To determine the fatty acid composition of the oil supplements, 0.6 mg of lipid were
135 methylated following the method of Lepage and Roy (Lepage and Roy 1986). The fatty
136 acid nonadecanoic acid was used as an internal standard. The FA methyl esters were
137 analyzed by gas chromatography–flame ionization detector (GC–FID). Results are
138 shown in Supplementary Material S1.
139
140 *2.3 Plasma and erythrocyte sampling for FA analysis*
141 Blood samples were collected via cardiac puncture into polypropylene tubes containing
142 EDTA (1 mg/ml), and centrifuged for 15 min, at 4 °C and 850 g. After that, the buffy
143 coat was removed and the packed erythrocyte cells were washed according to the
144 protocol developed by Sonenberg (Tsukamoto and Sonenberg 1979). Erythrocytes were
145 collected into clean polypropylene tubes, frozen and kept at –80 °C until required. Then,

146 erythrocyte free plasma was supplemented with 5 mM PMSF (protease inhibitor) and
147 immediately stored at –80 °C until required.
148
149 *2.4 Extraction and analysis of plasma TFA and FFA*
150 Plasma samples for the analysis of total FA (TFA) (30 µL) and free FA (FFA) (100 µL)
151 were first spiked with an internal standard of nonadecanoic acid (Larodan Fine
152 Chemicals, Malmö–Sweden), and then, extracted with a dichloromethane: methanol:
153 water mixture (2:2:1, v/v) using a Bligh and Dyer procedure (Puttmann *et al.* 1993).
154 TFA were directly analyzed in the organic phase after dryness under a stream of
155 nitrogen gas. Then, samples were transesterified and analyzed by GC–FID (Clarus 500,
156 Perkin–Elmer) following the method of Lepage and Roy (Lepage and Roy 1986). To
157 isolate the FFA fraction, the resulting lipid mixture obtained from the Bligh and Dyer
158 extraction was subjected to solid phase extraction (SPE) on aminopropyl cartridges
159 (500mg, 6mL, Biotage, Uppsala–Sweden) as Kaluzny et al. previously described
160 (Kaluzny *et al.* 1985). Solvent was removed under a stream of nitrogen and then
161 subjected to transesterification and GC–FID analysis. Results are shown in Tab.1.
162
163 *2.5 Extraction and analysis of FA from erythrocyte membranes*
164 To study the profile of FA from the erythrocyte membranes, lyophilized erythrocyte
165 samples (50 mg) were extracted by Bligh and Dyer procedure (Bligh and Dyer 1959).
166 The organic layer was dried under nitrogen and the lipid content was gravimetrically
167 quantified. Finally, an aliquot of organic phase containing 0.15 mg of lipids was dried,
168 the internal standard was spiked and transesterification and GC analysis were done as
169 before indicated to plasma FA (Tab.1).
170

171 *2.6 Fatty acid desaturase indexes measurement*

172 Desaturase activities in dietary supplemented groups were measured using a validated
173 methodology from the TFA data (Warensjo *et al*. 2009). FAD indexes were calculated as
174 product/precursor ratio for: Stearoyl-CoA (SCD-16 or SCD-18) = [palmitoleic (16:1 ω-
175 7)/palmitic (16:0)] or [oleic (18:1 ω-9)/stearic (18:0)]; Δ5D = [ARA (20:4 ω-6)/DGLA
176 (20:3 ω-6)] and [DHA (22:6 ω-3)/DPA (22:5 ω-3)]; Δ6D = [DGLA (20:3 ω-6)/LA (18:2
177 ω-6)]; and Δ5/6D = [EPA (20:5 ω-3)/ALA (18:3 ω-3)]. Results are shown in Tab.2.

178

179 *2.7 SPE method for lipid metabolites isolation from plasma samples*

180 ARA, EPA and DHA oxidized lipid mediators were extracted from plasma samples by
181 following a previously developed SPE method (Dasilva *et al*. 2014). Briefly, samples
182 (90 µL) were diluted with 30% cold methanol (v/v) to a final volume of 1 mL, and then
183 spiked with the internal standard 11HETE-d_8. After centrifugation (1800g, 10 min, 4°
184 C), samples were loaded into conditioned Oasis-HLB cartridges (60mg, 3mL, Waters,
185 MA-USA). Then, cartridges were washed with 5mL 15% methanol (v/v), 5 mL Milli-Q
186 water and 2.5 mL hexane in succession. After sorbent dryness, analytes were eluted
187 with 2 mL methyl formate. Extracts were evaporated to dryness, re-dissolved in 30 µL
188 ethanol, and analyzed by LC-ESI-MS/MS. Standard solutions of lipid mediators were
189 purchased from Cayman Chemicals (Ann Arbor, MI, USA). Methanol and Water,
190 Optima LC-MS, were purchased from Fisher Scientific (New Jersey, USA).

191

192 *2.8 Analysis of lipid metabolites by LC-ESI-MS/MS*

193 Lipid mediators were quantified according to the previously developed methodology
194 (Dasilva *et al*. 2014) and results are shown in Tab.3. Briefly, analyses of SPE extracts
195 were carried out on an Agilent 1260 Series (Agilent, Palo Alto, CA) coupled to a linear

196 ion trap mass spectrometer LTQ Velos Pro with ESI (Thermo Fisher, Rockford, IL,
197 USA). A Waters C18–Symmetry column, 150×2.1 mm, 3.5 µm (Milford, MA, USA)
198 protected with a 4×2 mm C18 guard cartridge provided by Phenomenex (Torrance, CA,
199 USA) was used. A binary eluent system of water (A) and methanol (B), both with
200 0.02% (v/v) of formic acid, was used as mobile phase. The injection volume was set to
201 10 µL, ESI source operated in negative ion mode, and MS/MS conditions has been
202 reported in detail elsewhere (Dasilva *et al.* 2014). The quantification of target
203 compounds was made using the most intense, or selective, ions in their product ion scan
204 MS/MS spectra. Retention times for target compounds and individual MS/MS
205 parameters are summarized in Supplementary Material S2.
206
207 *2.9 Statistical analysis*
208 Data presented are expressed as mean ± SD. Statistical analyses were performed by one–
209 way analysis of variance (ANOVA) with R free software (version 386 3.1.0). Non–
210 parametric Kruskal Wallis analyses were required when data distribution did not fit a
211 Gaussian model or heterogeneity was found in variances. The means were further
212 compared by the post–hoc test Fisher least square difference (Fisher LSD) and
213 significant differences were set at $p<0.05$. Pearson test was used in order to determine
214 statistical correlations between metabolites and PUFA intake, CRP, SOD, GPX and
215 CAT levels.
216
217 3. Results
218 Weekly measurements demonstrated that there were not significant differences in food
219 intake, body weight gain (final body weight ranged between 570–610g for all animals)
220 or adiposity (ranged over 6.3–7.8 %) between experimental groups that could cause

differential effects between diets. Considering that the amount of total ω–3 in the marine enriched diets was similar, 50% of the total fat amount, the results here described strictly illustrate the effects produced by the different EPA/DHA dietary proportions compared with a control ω–6 diet and control ω–3 ALA diet. The measured parameters were correlated with the development/prevention of inflammatory processes, oxidative stress and metabolic disorders (McDaniel, Massey and Nicolaou 2011; Vessby *et al*. 2013).

3.1 Effect on plasma FFA profile, FA incorporation into tissues, and ***ω6/ω3*** *ratios.*

Noticeable differences were observed in FA profiles in plasma and FA incorporated into erythrocyte membranes between animals fed with 1:1 and 2:1 EPA/DHA diets when compared with 1:2 EPA/DHA, linseed and soybean groups. In detail, a significant enrichment of ω–3 EPA and DHA levels in plasma and incorporated into membranes was observed by 1:1 and 2:1 treatments, meanwhile the lowest levels were achieved by soybean one ($p<0.05$). The opposite trend was observed in ω–6 PUFAs levels. Animals fed 1:1 and 2:1 fish diets produced the lowest total amount of ω–6 PUFA (i.e., ARA), meanwhile soybean treatment significantly enhanced the production of total ω–6 PUFA ($p<0.05$) in both, plasma and erythrocyte membranes. Intermediate levels of ω–3 and ω–6 PUFAs were found in 1:2 and linseed diets. It is remarkable that the intervention with 1:2 EPA:DHA did not enhance DHA level neither in plasma nor in membranes as initially expected according to the high content of this PUFA on the diet. The main ω–6 PUFA from soybean oil (linoleic acid, LA) was found higher in plasma from soybean group, but poorly incorporated into membranes when compared with other treatments ($p<0.05$). ALA level (main ω–3 PUFA from linseed) in plasma significantly increased by linseed oil intervention as expected, but it was only incorporated into erythrocyte

membranes by animals fed linseed oil. Results also highlighted a significant reduction of plasma oleic acid by three EPA/DHA interventions compared with soybean and linseed ones ($p<0.05$). As a consequence of the FA modulation by the diet, the inflammatory index ω–6/ω–3 in plasma and membranes was found significantly reduced in animals fed 1:1 and 2:1 supplements ($p<0.05$) compared with the other groups. Soybean treatment produced the highest ratio; meanwhile intermediate values were obtained for 1:2 and linseed groups ($p<0.05$) (Tab.1).

3.2 Effect on FAD activity

Results outlined that the studied supplements did not exert a different influence on SCD–16 [palmitoleic/palmitic], and Δ6D [DGLA/LA] activities (Tab.2). Nevertheless, SCD–18 [oleic/stearic] was significantly reduced by 1:1 and 2:1 supplementations when compared with soybean diet ($p<0.05$). Moreover, ω–3 EPA/DHA diets were effective in down regulating Δ5D [ARA/DGLA] activity when compared with soybean and linseed diets ($p<0.05$). On the other hand, results highlighted the ability of EPA and DHA intake on harnessing Δ5/6D [EPA/ALA] and Δ5D [DHA/DPA] activities in comparison with linseed and soybean groups ($p<0.05$).

3.3 Formation of plasma lipid mediators depending on the ingested amount of ARA, EPA and DHA

Several hydroxyl and hydroperoxyl derivates, thromboxane and prostaglandin metabolites from ARA, EPA and DHA were quantified (Tab.3). In detail, four EPA eicosanoids from the family of hydroxides (12HEPE), hydroperoxides (15HpEPE and 12HpEPE), and a thromboxanes (TXB_3) were identified. From DHA, the 17HDoHE (hydroxide) and 17HpDoHE (hydroperoxide) were detected. One hydroxide and one

271 prostaglandin derived from ARA, 11HETE and PGE_2 respectively, were found in
272 plasma samples. Finally, isoprostanes, leukotrienes, resolvins, and protectins were not
273 detected. The quantitative and statistical analysis between groups revealed that dietary
274 interventions with linseed, 1:1 and 2:1 EPA/DHA oils generally decreased the overall
275 levels of lipid mediators when compared with 1:2 and soybean groups.
276
277 In detail, levels of EPA eicosanoids, 12HEPE and TXB_3, significantly increased in 1:2
278 diet compared with other diets ($p<0.05$). Levels of EPA hydroperoxides, 12 and
279 15HpEPE, were significantly lower in 1:1, 2:1 and linseed diets when compared with
280 1:2 and soybean ones ($p<0.05$). A similar pattern was found for docosanoids derived
281 from DHA: animals fed 1:2 diet enhanced the production of 17HDoHE compared with
282 other groups ($p<0.01$); and the level of 17HpDoHE was found to be significantly higher
283 in 1:2 and soybean groups than 1:1, 2:1 and linseed ones ($p<0.001$). The strong pro–
284 inflammatory prostaglandin derived from ARA, PGE_2, was significantly less–produced
285 by dietary 1:1, 2:1 and linseed interventions; meanwhile soybean and 1:2 diets produced
286 the highest concentration ($p<0.05$). In addition, 11HETE (ARA hydroxide) level was
287 strongly reduced by EPA/DHA and linseed diets when compared with soybean one
288 ($p<0.05$). Therefore, dietary 1:1 and 2:1 fish oil interventions reduced more the
289 production of inflammatory hydroxides, hydroperoxides, and prostaglandins than 1:2,
290 soybean and linseed ones.
291
292 The analysis of the PUFA precursors (EPA, DHA, and ARA) showed the highest levels
293 by 1:1 and 2:1 fish diets when compared with 1:2, linseed and soybean groups
294 ($p<0.001$). Finally, other searched compounds as 15HEPE, 5HEPE, 8iso–$PGF_{3\alpha}$, 8iso–
295 $PGF_{2\alpha}$, PGD_3, PGE_3, 11HDoHE, 4HDoHE, RvD_1, PD_x, and LTB_4 were not produced by

any of the diets or were under the detection limits of the method.

The correlation analysis between lipid mediator levels in plasma and the dietary intake of EPA and DHA, showed that the intake of EPA (20C FA) exerted a different effect on metabolite production when compared with 22C DHA (Tab.4). In detail, an increase in the ingested amount of 20C FA produced an enhancement in plasma levels of free EPA and DHA (positive Pearson indexes over 0.7–0.8), however an increase in the ingested amount of 22C was weakly correlated with a higher level of EPA and DHA in plasma (positive indexes over 0.3–0.4). Interestingly, the correlation between the uptake of EPA and DHA and the production of their primary (hydroperoxides) and secondary (hydroxides and thromboxane) oxidation metabolites follow the opposite tendency: an increase in the ingested 20C produced a decrease of these metabolites (negative correlation indexes); meanwhile, an increase in the ingested 22C produced positive correlations with the exception of 12HpEPE. In addition, high inverse correlations were found between levels of strong pro-inflammatory metabolites from ω-6 ARA (11HETE and PGE_2) and EPA uptake (negative Pearson indexes over 0.84–0.98). Nevertheless, the decrease of ARA metabolites was weakly correlated with the increase of 22C uptake (negative Pearson indexes over 0.3–0.5). Therefore, results suggested that an increase in the ingested amount of EPA produced a down-regulation of PUFA oxidation, being particularly significant a minor production of ARA metabolites. However, the increase of DHA reduced this effect and even enhanced the production of oxidized metabolites.

3.4 Dietary interventions effect on general biochemical parameters.

Biochemical parameters related with MetS were previously reported to describe the influence of dietary interventions on phenotypic characteristics of genetically obese and

hypertensive rats (Molinar–Toribio *et al*. 2015). Several CVD risk factors, inflammation markers and oxidative stress parameters are here compared: TFA, total triglycerides (TG), total cholesterol (TC), high/low density lipoproteins (HDL, LDL), glycated hemoglobin (HbA1c), reactive C–protein (CRP), and antioxidant enzymes activity of the studied animals (Supplementary Material S3).

Briefly, it was observed that dietary interventions with 1:1 and 2:1 EPA:DHA ratios exerted a significant reduction of plasma TFA, TG, TC, LDL, and CRP levels ($p<0.05$) when compared with the soybean group (intermediate levels were achieved with 1:2 and linseed diets). The correlation analysis showed that the increase of the inflammatory CRP index was closely correlated with higher hydroperoxides and ω–6 inflammatory eicosanoids production (positive Pearson indexes over 0.8–0.99) and lower EPA and DHA concentrations (negative Pearson indexes over 0.8–0.9). No differences between diets were observed in HDL content and 1:1 and 2:1 groups enhanced HDL/LDL ratio compared with soybean ($p<0.05$); meanwhile, 1:2 and linseed animals produced intermediate levels. The glycaemia results showed a significant decrease in the HbA1c in all animals supplemented with ω–3 fish and linseed oils ($p<0.05$). Finally, the concentration of antioxidant enzymes in erythrocyte membranes showed lower levels in animals fed soybean oils. In detail: superoxide dismutase (SOD) was enhanced by ω–3 fish and linseed supplements; glutathione peroxidase (GPX) values were higher for 1:1 and 2:1 meanwhile 1:2 and linseed animals produced similar levels as soybean one. Catalase (CAT) was highly produced by 2:1 diet and intermediate values were achieved by 1:1, 1:2 and linseed diets. Generally, positive correlation indexes were found between these enzymes concentrations and EPA and DHA levels; meanwhile, negative indexes correlated the enzymes concentration and PUFA oxidation products.

346

347 4. Discussion

348 This investigation noteworthy suggests a health effect of 1:1 and 2:1 EPA/DHA
349 interventions by reducing inflammatory eicosanoids and docosanoids synthesis from ω–
350 3 EPA and DHA and ω–6 ARA, and modulating the FFA profile and FA incorporation
351 into tissues to reduce the inflammatory index ω–6/ω–3. These results are in agreement
352 with a reduced level of several inflammation and CVD risk factors, and an increased
353 level of relevant endogenous antioxidant enzymes. These data are consistent with a
354 higher anti–oxidant and anti–inflammatory capacity of EPA *vs* DHA, and fish ω–3 oils *vs*
355 vegetal ω–3 and ω–6 oils previously observed in a healthy animal model of Wistar rats
356 (Dasilva *et al*. 2015).

357

358 Lipoxygenase (LOX) enzymes are involved in the pathways of formation of primary
359 and secondary oxidation products (hydroperoxides and hydroxides, respectively) from
360 EPA and DHA. Through cyclooxygenase (COX) activity, TXB_3 from EPA and the
361 strong pro–inflammatory compounds derived from ARA (11HETE and PGE_2), are
362 produced (Massey and Nicolaou 2013). Results from the fish oil groups revealed that
363 1:1 and 2:1 diets produced lower levels of these metabolites in addition to linseed diet.
364 Therefore, these three diets seems to be more effective down–regulating the activity of
365 LOX and COX enzymes responsible of the synthesis of pro–inflammatory derived
366 eicosanoids and docosanoids. Hydroperoxides and hydroxides from ω–3 EPA and DHA
367 have been described as weaker inflammatory substances than the corresponding ω–6
368 ARA derivates (Sears and Ricordi 2012; Shearer *et al*. 2010). In the same way, TXB_3 is
369 related to the inflammatory response and is a competitor product of COX enzymes that
370 also produce strong pro–inflammatory series–2 thromboxanes from ARA (McDaniel,

371 Massey and Nicolaou 2011). ARA eicosanoids, PGE_2 and 11HETE, have been widely
372 studied and identified as key pro–inflammatory signaling molecules with pro–
373 aggregating, vasoconstrictive and immunosuppressive properties closely correlated with
374 the development of MetS (Ferreiro–Vera *et al*. 2011).
375
376 The antioxidant endogenous system described by SOD, GPX and CAT enzymatic
377 activities was significantly enhanced by fish diets, and specially by 1:1 and 2:1
378 EPA:DHA ratios. Accordingly, Pearson indexes show a strong positive correlation
379 between SOD, GPX and CAT concentrations and EPA and DHA levels in plasma;
380 meanwhile, the production of eicosanoids and docosanoids was reduced. Both findings
381 suggested an antioxidant effect in 1:1 and 2:1 diets and a decrease of oxidative stress.
382 Other studies have also found an improvement of the antioxidant SOD (Garrel *et al*.
383 2012; Yessoufou *et al*. 2006) and CAT (Chapman, Morgan and Murphy 2000) activities
384 after ω–3 PUFA supplementation. Therefore, the ability of 1:1 and 2:1 supplemented
385 diets to ameliorate the production of inflammatory oxidized lipids could be closely
386 correlated with the enhancement of the antioxidant system. As a consequence, the free
387 PUFA levels were higher and lipid metabolites concentrations lower in these groups
388 than in the other ones. The fish–enriched diet with the 1:2 EPA:DHA ratio was less
389 effective decreasing levels of inflammatory metabolites compared to 1:1 and 2:1, and it
390 even produced the highest concentrations for some compounds compared to controls.
391 Interestingly, 1:2 diet showed lower levels of antioxidant enzymes than 1:1 and 2:1, and
392 a major production of lipid oxidized metabolites that can be associated to a higher up–
393 regulation of LOX and COX activity.
394
395 Richard et al. (2008) demonstrated that supplementations with EPA and DHA (and

specially EPA) resulted in lower formation of ROS and in higher inhibition of superoxide anion than ω-6 enriched diets in cell models. In addition, levels of free fatty acids (ARA, EPA and DHA) were found higher after fish oil treatments with a balanced proportion EPA/DHA 1:1 or higher EPA (2:1). It seems that these diets produce a higher preservation of PUFA from oxidation that leads to a lower production of the corresponding lipid mediators as it was pointed above. In concurrence with these findings, the statistical correlation analysis between the weekly intake of EPA and DHA and further production of lipid metabolites, suggested that an increase in the amount of DHA in the diet may enhance the oxidation of PUFAs, and thus, stimulates the production of lipid oxidized derivates. Nevertheless, a higher dose of EPA leads to a protective effect on PUFAs oxidation and produced less derived inflammatory metabolites. According to Di Nunzio et al., the propensity of fatty acids to oxidation is theoretically proportional to the degree of instauration (Di Nunzio, Valli and Bordoni 2011). Considering the higher unsaturation level of DHA molecule (n=6) than EPA one (n=5), fish oil diet with a higher proportion of DHA (1:2 EPA:DHA ratio) could generate stronger oxidative conditions that activate LOX enzymes; and thus, the production of inflammatory metabolites.

Fatty acid desaturases are one of the main targets for the treatment of metabolic related disorders, and the rate-limiting step in the biosynthesis of different FFA. FFA released from adipose tissue into plasma are important because they exert relevant functions as signaling molecules participating in inflammation processes and oxidative stress (Martins *et al*. 2012). According to FAD indexes, diets rich in ω-3 fish oils up-regulated Δ6 and Δ5 desaturases involved in the transformation of ALA into EPA and DHA, meanwhile the activity of these enzymes to produce ARA from LA was reduced.

421 Moreover, results reveal a down-regulation of SCD-18 in 1:1 and 2:1 diets that is
422 responsible of the biosynthesis of oleic acid, a key factor in the development of obesity
423 (Mutch *et al*. 2007). These results are in agreement with FFA composition of plasma
424 and FA incorporated into erythrocyte membranes. FA profiles were enriched in ω-3
425 EPA and DHA by 1:1 and 2:1 diets; meanwhile ω-6 ARA and ω-9 oleic acid levels
426 were reduced in these treatments. Surprisingly, 1:2 diet did not enhance DHA level as
427 expected due to the high DHA dose uptake. As a consequence of the lipid modulation
428 associated to diets, animals fed 1:1 and 2:1 diets exhibited significantly lower plasma
429 and erythrocyte ω-6/ω-3 ratios. The ratio between ω-6 and ω-3 FA in blood and tissues
430 is one of the most selective markers of cellular inflammation. Indeed, elevated ratios
431 have been associated with an increasing prevalence of chronic inflammatory diseases
432 and MetS (Sears 2005).
433
434 According to the previous discussed results (Molinar-Toribio *et al*. 2015), diets 1:1 and
435 2:1 were the most effective to reduce levels of the main CVD risk factors like: TFA, TG,
436 TC, LDL, HbA1c, and significantly enhanced the HDL:LDL ratio. Levels of the
437 inflammatory marker CRP were found decreased by these treatments too. The statistical
438 analysis showed a strong correlation between the increase of CRP values and the
439 production of pro-inflammatory eicosanoids from ARA and ω-3 hydroperoxides.
440 Benefits suggested for 1:1 and 2:1 diets reducing levels of CVD risk factors and the
441 inflammatory CRP index, were clearly correlated with a reduction of inflammatory
442 eicosanoids and docosanoids and a global improvement in the lipid profiles discussed
443 above.
444

As a conclusion, dietary interventions with marine ω-3 PUFAs seems to be more effective in ameliorating the progression of MetS when compared with vegetable ω-6 LA and ω-3 ALA provided by linseed and soybean oils. It is important to highlight that the proportion between marine ω-3 EPA and DHA is crucial to obtain the best outcomes. Animals developing MetS from a spontaneous mutation show better results with ratios 1:1 and 2:1 EPA/DHA, and that may be explained by the lower susceptibility to oxidation of EPA *vs* DHA that leads to lower levels of inflammatory metabolites and oxidative stress, and subsequently, an improvement of MetS indexes. Generally, diets 1:1 and 2:1 showed an improvement on CVD and inflammation clinical parameters, strong pro-inflammatory eicosanoids, an activation or preservation of the antioxidant endogenous defenses, and a shift towards greater anti-inflammatory profiles associated to FA composition in plasma and membranes. These results show the need of drawing a new insight into the correct design of fish oil supplements, with proper proportions of EPA and DHA.

Acknowledgements

This work was supported by the Spanish Ministerio de Economía y Competitividad (AGL2009-12374-C3-1, -2, and -3, and AGL2013-49079-C2-1,2-R). The Consejo Superior de Investigaciones Científicas (CSIC) and the University of Santiago de Compostela (USC) are gratefully acknowledged for the doctoral fellowship to Gabriel Dasilva. Xunta de Galicia and European Social Fund are also thankfully recognized for the financial support of the postdoctoral contracts to M. P and E.G-E., and ISCIII for the postdoctoral contract "Sara Borrell" to J.P.-J. (CD09/00068).

Conflict of interests

There are not previous reports, financial or other relationships that lead to a conflict of interest with the present work. The manuscript has not been considered for publication in another journal.

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594

595 Table legends:

596 Tab. 1: Plasma and erythrocyte membranes (EM) composition of FA from SHROB rats
597 supplemented with soybean, linseed and fish oil mixtures (EPA/DHA 1:1, 2:1 and 1:2).
598 FAs were extracted using a Bligh and Dyer procedure and then analysed by GC–FID.
599 Results are expressed as percentage of total fatty acids (mg/100mg of TFA). Values are
600 shown as means ± SD. Levels of FA in plasma and membranes were statistically
601 compared between dietary groups. Significant differences for every FA at $p<0.05$ are
602 shown by different superscript letters. **16:0 (palmitic acid), 16:1 ω7 (palmitoleic acid,**
603 **PA), 18:0 (stearic acid), 18:1 ω9 (oleic acid, OA), 18:1 ω7 (vaccenic acid), 18:2 ω6**
604 **(LA), 18:3 ω3 (ALA), 20:3 ω6 (DGLA), 20:4 ω6 (ARA), 20:5 ω3 (EPA), 22:5 ω3**
605 **(DPA), 22:6 ω3 (DHA)**

606

607 Tab. 2: Fatty acid desaturases activity indexes from SHROB rats supplemented with
608 fish oil mixtures (EPA/DHA 1:1, 2:1 and 1:2), linseed and soybean oils. FAD indexes
609 were calculated as product/precursor ratio from the TFA data. Values are shown as
610 means ± SD. FAD levels were statistically compared between dietary groups.

611 Significant differences for every index at $p<0.05$ are shown by different superscript
612 letters.
613
614 Tab. 3: Levels of lipid mediators in plasma derived from ARA, EPA and DHA
615 obtained from the dietary interventions with SHROB rats. Eicosanoids and docosanoids
616 were isolated and concentrated from biological samples by SPE and then analysed by
617 LC–MS/MS. Results are expressed as ng/mL for derived eicosanoids and docosanoids,
618 and µg/mL for PUFAs. Values are shown as means ± SD. Concentration of lipid
619 derivates were statistically compared between dietary groups. Significant differences for
620 every metabolite at $p<0.05$ are shown by different superscript letters.
621
622 Tab. 4: Correlation Pearson indexes between lipid mediator levels in plasma and
623 dietary dose of 20C FA (EPA) and 22C FA (DHA), and CRP, SOD, GPX, and CAT
624 levels in SHROB rats supplemented with fish oil mixtures (EPA/DHA 1:1, 2:1 and 1:2),
625 linseed and soybean oils. Positive correlations mean an increase of lipid mediator levels
626 when the dose of EPA and DHA is higher, or levels of CRP, SOD, GPX, and CAT
627 increase. Negative indexes show a decrease of lipid mediators when the rest of
628 parameters increase. P–values lower than 0.05^{*} and 0.01^{**} show that the Pearson index
629 is significantly different to zero at 95% and 99% of confidence, respectively.
630
631
632 Supplementary electronic material legends:
633 S1: Fatty acid composition of soybean, linseed and fish oil mixtures (EPA/DHA 1:1,
634 2:1 and 1:2) supplemented to SHROB rats. FA were methylated and analysed by GC–
635 FID. Results are expressed as percentage of total fatty acids (mg/100mg of Total FA).

636 Weekly dose of EPA, DHA, ALA, ARA and LA expressed as mg/kg rat. Values are
637 shown as means ± SD.
638
639 S2: Retention times, collision energies and MS/MS transitions for ESI–LC–LIT/MS/MS
640
641 S3: Biochemical parameters related with MetS from supplemented SHROB groups with
642 EPA/DHA 1:1, 2:1 and 1:2; linseed and soybean oil. Data from previous work
643 (Molinar–Toribio *et al.* 2015).
644

Table(s)

Tab. 1.

	RATIO 1:1 (EPA/DHA)				RATIO 2:1 (EPA/DHA)				RATIO 1:2 (EPA/DHA)				Soybean				Linseed			
	FFA (Plasma)		FA (EM)		FFA (Plasma)		FA (EM)		FFA (Plasma)		FA (EM)		FFA (Plasma)		FA (EM)		FFA (Plasma)		FA (EM)	
	MEAN	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*
16:0 (palmitic)	31.79^{a}	1.1	23.45^{a}	0.7	31.66^{a}	0.8	23.99^{a}	1.1	31.43^{a}	0.8	23.50^{a}	0.7	31.28^{a}	1.3	22.78^{a}	0.4	30.20^{a}	1.4	22.49^{a}	0.7
16:1 **ω**7 (PA)	10.94^{a}	1.6	1.83^{ab}	0.5	10.49^{a}	0.8	1.40^{a}	0.2	10.55^{a}	1.2	1.52^{a}	0.2	9.12^{a}	2.2	1.28^{a}	0.4	10.54^{a}	2.4	2.24^{b}	0.2
18:0 (stearic)	8.60^{a}	0.9	14.32^{a}	1.3	7.93^{a}	0.4	15.34^{a}	0.4	8.27^{a}	0.9	15.14^{a}	0.6	7.90^{a}	0.9	16.10^{a}	0.9	8.14^{a}	1.7	14.06^{a}	1.3
18:1 **ω**9 (OA)	21.92^{a}	1	10.82^{ab}	2.4	22.42^{a}	0.9	9.00^{a}	0.6	24.42^{ab}	1.3	9.78^{a}	0.9	26.04^{b}	2.3	9.56^{a}	1	25.09^{b}	1	13.05^{b}	2.2
18:1 **ω**7 (vaccenic)	2.31^{a}	1.3	1.98^{a}	0.1	2.28^{a}	0.1	1.97^{a}	0.1	2.65^{a}	0.1	2.07^{a}	0.1	2.64^{a}	0.2	1.99^{a}	0.1	2.58^{a}	0.1	2.15^{b}	0.1
18:2 **ω**6 (LA)	9.54^{a}	0.8	8.21^{a}	0.3	10.26^{ab}	0.9	7.67^{ab}	0.2	10.56^{ab}	0.9	7.52^{bc}	0.3	11.29^{b}	0.5	6.74^{c}	0.8	10.68^{ab}	1.4	8.24^{ab}	0.5
18:3 **ω**3 (ALA)	0.99^{a}	0.1	n.d.	n.d.	1.05^{a}	0.1	n.d.	n.d.	0.94^{a}	0.1	n.d.	n.d.	0.90^{a}	0.2	n.d.	n.d.	1.97^{b}	0.4	0.37	0.1
20:3 **ω**6 (DGLA)	n.d.	n.d.	1.20^{a}	0.1	n.d.	n.d.	1.27^{a}	0.2	n.d.	n.d.	1.35^{a}	0.2	n.d.	n.d.	1.20^{a}	0.2	n.d.	n.d.	1.27^{a}	0.3
20:4 **ω**6 (ARA)	3.01^{a}	0.5	14.22^{a}	1.1	3.03^{a}	0.6	15.05^{a}	0.5	3.25^{a}	0.5	16.78^{b}	0.4	4.65^{b}	1.1	19.65^{c}	1.1	3.86^{ab}	0.8	16.55^{b}	0.6
20:5 **ω**3 (EPA)	1.34^{a}	0.3	2.20^{a}	0.4	1.30^{a}	0.2	2.21^{a}	0.3	0.66^{b}	0.1	1.24^{b}	0.2	0.45^{b}	0.1	0.77^{c}	0.2	0.81^{b}	0.2	1.47^{b}	0.3
22:5 **ω**3 (DPA)	1.74^{a}	0.3	3.10^{a}	0.3	2.36^{a}	0.1	3.80^{b}	0.1	1.15^{c}	0.1	2.70^{ac}	0.3	0.84^{d}	0.2	2.41^{c}	0.4	1.36^{ac}	0.2	3.13^{a}	0.5
22:6 **ω**3 (DHA)	5.44^{a}	0.9	9.11^{a}	0.7	5.00^{a}	0.5	8.22^{a}	0.4	3.95^{b}	0.3	8.47^{a}	0.1	2.85^{c}	0.4	6.70^{b}	0.6	2.76^{c}	0.4	6.36^{b}	0.3
∑ ω3	9.51^{a}	0.4	14.41^{a}	0.4	9.71^{a}	0.2	14.22^{a}	0.3	6.70^{b}	0.2	12.40^{b}	0.2	5.04^{c}	0.2	9.88^{c}	0.4	6.90^{b}	0.3	11.34^{d}	0.3
∑ ω6	12.55^{a}	0.6	23.63^{a}	0.5	13.29^{ab}	0.8	23.99^{a}	0.3	13.81^{b}	0.7	25.65^{b}	0.3	15.94^{c}	0.8	27.59^{c}	0.7	14.54^{bc}	1.1	26.06^{b}	0.4
∑ SFA	40.39^{a}	1.1	37.77^{ab}	1	39.59^{a}	0.6	39.33^{a}	0.8	39.70^{a}	0.9	38.64^{a}	0.6	39.18^{a}	1.2	38.88^{a}	0.6	38.34^{a}	1.5	36.56^{b}	1
∑ MUFA	35.17^{a}	1.3	14.63^{a}	1	35.19^{a}	0.6	12.36^{b}	0.3	37.62^{a}	0.9	13.36^{ac}	0.4	37.80^{a}	1.6	12.83^{bc}	0.5	38.21^{a}	1.5	17.44^{d}	0.8
∑ PUFA	22.06^{a}	0.5	38.04^{a}	0.5	23.00^{a}	0.4	38.21^{a}	0.3	20.51^{b}	0.4	38.06^{a}	0.3	20.98^{b}	0.4	37.47^{a}	0.6	21.44^{ab}	0.6	37.4^{a}	0.4
ω6/ω3	1.32		1.64		1.37		1.69		2.06		2.07		3.16		2.79		2.11		2.30	

Tab. 2

	RATIO 1:1 (EPA/DHA)		RATIO 2:1 (EPA/DHA)		RATIO 1:2 (EPA/DHA)		Soybean		Linseed	
	MEAN	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*
SCD16[palmitoleic/palmitic]	0.3^{a}	0.04	0.2^{a}	0.02	0.3^{a}	0.03	0.2^{a}	0.05	0.2^{a}	0.04
SCD-18 [oleic/stearic]	2.3^{a}	0.5	2.2^{a}	0.3	2.9^{b}	0.7	3.2^{b}	0.8	2.7^{b}	0.7
Δ5D [ARA/DGLA]	8.1^{a}	0.7	9.3^{a}	1.3	8.7^{a}	1.5	12.9^{b}	2.6	10.1^{c}	1.6
Δ6D [DGLA/LA]	0.1^{a}	0.01	0.1^{a}	0	0.1^{a}	0.01	0.1^{a}	0.01	0.1^{a}	0
Δ5/6D [EPA/ALA]	4.3^{a}	0.6	4.6^{a}	0.5	2.4^{b}	0.2	1.4^{c}	0.3	1.4^{c}	0.4
Δ5D [DHA/DPA]	5.3^{a}	0.8	3.7^{b}	0.5	5.3^{a}	0.3	4.9^{ab}	0.6	3.1^{b}	0.3

Tab. 3

	RATIO 1:1 (EPA/DHA)		RATIO 2:1 (EPA/DHA)		RATIO 1:2 (EPA/DHA)		Soybean		Linseed	
	MEAN	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*
Eicosanoids from EPA (ng/mL)										
5HEPE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12HEPE	401.7^{ab}	252.3	237.4^{a}	73.7	625.1^{b}	304.1	284.7^{a}	210.1	254.8^{a}	224
15HEPE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12HpEPE	1507.8^{a}	293.1	1385.3^{a}	242.9	2512.8^{b}	106	4393.6^{c}	1121	1584.4^{a}	537.8
15HpEPE	n.d.	n.d.	239^{a}	130.6	494.6^{b}	171.7	n.d.	n.d.	240.2^{a}	108.8
8iso-PGF3a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TXB3	5.1^{a}	1.3	4.5^{a}	0.3	6.8^{b}	1.7	5^{ab}	1	4.8^{a}	2.1
PGD3/E3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Docosanoids from DHA (ng/mL)										
17HDoHE	150.7^{a}	105.5	85.3^{a}	32.4	306.9^{b}	102.9	159.5^{a}	81.3	106.1^{a}	77.6
11HDoHE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
4HDoHE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17HpDoHE	2580.7^{a}	431.6	2317^{a}	203.9	4208.3^{b}	839	3819.8^{b}	1216.6	2726^{a}	786
RvD1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PDx	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Eicosanoids from ARA (ng/ml)										
11HETE	15.5^{a}	11	5.1^{a}	5.3	16.6^{a}	9.1	30.3^{b}	18.3	9.6^{a}	10.4
PGE2	13.7^{a}	10.2	15.6^{a}	6.8	81.7^{b}	55.1	83.4^{b}	35.4	26^{a}	14.3
LTB4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8isoPGF2a	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PUFA precursors (ng/uL)										
EPA	9.7^{a}	1.5	6.8^{b}	0.8	2.9^{c}	0.7	1.7^{d}	0.8	2.5^{cd}	0.7
DHA	51.9^{a}	5.5	33.5^{b}	7.2	22.7^{c}	6.3	17.3^{cd}	4	15.1^{d}	3.8
ARA	68.3^{a}	12.4	48.1^{bc}	3.8	38.2^{c}	10.2	50.8^{b}	10.3	37.5^{c}	3.6

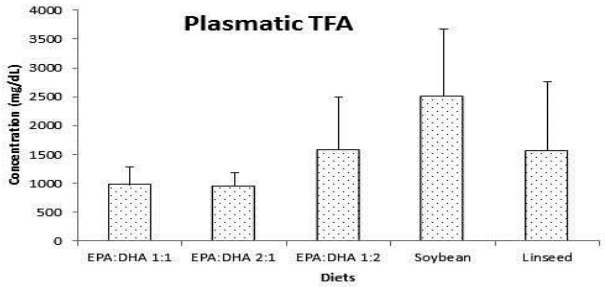
Tab. 4

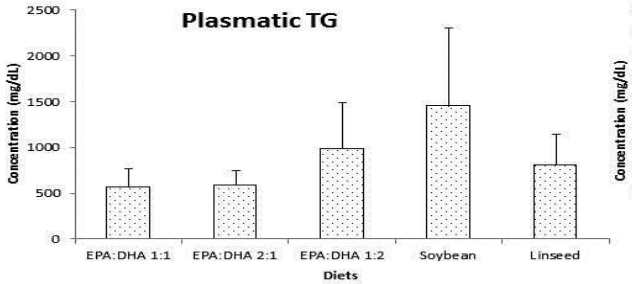
	Intake of EPA		Intake of DHA		CRP concentration		SOD concentration		GPX concentration		CAT concentration	
	Pearson index	*P-value*	*Pearson index*	*P-value*	*Pearson index*	*P-value*	*Pearson index*	*P-value*	*Pearson index*	*P-value*	*Pearson index*	*P-value*
12HEPE	-0.06	0.49	0.79	0.11	0.07	0.47	0.35	0.33	-0.62	0.19	-0.34	0.33
12HpEPE	-0.98	0.009**	-0.65	0.17	0.99	0.006**	-0.95	0.02*	-0.72	0.14	-0.76	0.12
15HpEPE	-1.00	0.001**	1.00	0.001**	1.00	0.001**	-1.00	0.001**	-1.00	0.001**	-1.00	0.001**
TXB3	-0.22	0.39	0.66	0.17	0.26	0.37	0.15	0.42	-0.71	0.15	-0.40	0.30
17HDoHE	-0.34	0.33	0.57	0.21	0.37	0.32	0.04	0.48	-0.79	0.11	-0.50	0.25
17HpDoHE	-0.76	0.12	0.04	0.48	0.81	0.09	-0.50	0.25	-0.92	0.04*	-0.71	0.15
11HETE	-0.98	0.01*	-0.54	0.22	0.90	0.05	-0.83	0.08	-0.82	0.09	-0.94	0.03*
PGE2	-0.84	0.08	-0.18	0.41	0.91	0.04*	-0.68	0.16	-0.86	0.07	-0.69	0.16
EPA	0.78	0.11	0.34	0.33	-0.90	0.04*	0.76	0.12	0.65	0.17	0.48	0.26
DHA	0.68	0.16	0.38	0.31	-0.84	0.08	0.74	0.13	0.50	0.25	0.32	0.34

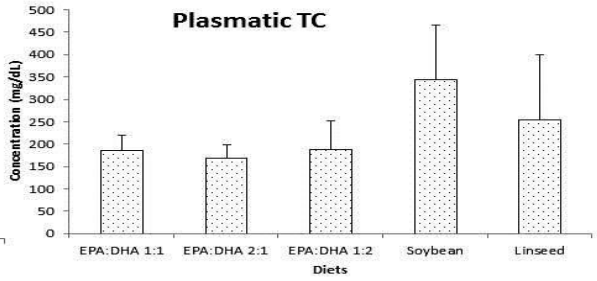
S1

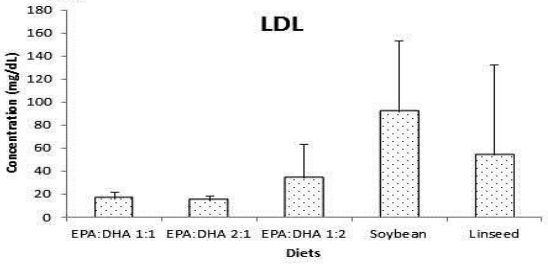
	RATIO 1:1 (EPA:DHA)		RATIO 2:1 (EPA:DHA)		RATIO 1:2 (EPA:DHA)		SOYBEAN		LINSEED	
FATTY ACID	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*	*MEAN*	*SD*
14:0	4.37	0.05	3.97	0.01	4.73	0.02	0.96	0.02	0.06	0.01
16:0	10.15	0.16	9.09	0.03	10.98	0.13	17.78	0.10	5.73	0.03
16:1 ω7 (PA)	4.99	0.04	4.57	0.02	5.39	0.03	0.90	0.03	0.11	0.01
18:0	2.94	0.03	2.95	0.00	2.97	0.01	2.07	0.01	4.75	0.02
18:1 ω9 (OA)	6.41	0.06	6.18	0.00	6.61	0.04	18.75	0.03	21.37	0.06
18:1 ω7	1.91	0.03	1.93	0.02	1.95	0.02	1.52	0.02	1.11	0.01
18:2 ω6 (LA)	0.65	0.01	0.61	0.00	0.65	0.02	47.55	0.01	16.76	0.03
20:0	0.32	0.01	0.39	0.01	0.20	0.00	0.00	0.00	0.00	0.00
18:3 ω3 (ALA)	0.36	0.01	0.32	0.00	0.33	0.02	4.00	0.04	50.02	0.10
20:1 ω9	0.98	0.03	1.39	0.01	0.63	0.02	1.43	0.09	n.d.	n.d.
18:4 ω3	1.51	0.02	1.56	0.02	1.64	0.00	0.15	0.00	n.d.	n.d.
20:2 ω6	0.21	0.00	0.28	0.01	0.17	0.01	0.20	0.05	n.d.	n.d.
20:3 ω6	0.22	0.01	0.27	0.01	0.15	0.00	0.00	0.00	n.d.	n.d.
20:4 ω6 (ARA)	1.68	0.04	1.98	0.03	1.16	0.02	0.40	0.02	n.d.	n.d.
22:1 ω11	1.14	0.01	1.58	0.02	0.45	0.01	1.08	0.00	n.d.	n.d.
22:1 ω9	0.28	0.03	0.37	0.03	0.19	0.02	0.25	0.02	n.d.	n.d.
20:4 ω3	1.02	0.02	1.31	0.02	0.75	0.01	0.20	0.03	n.d.	n.d.
20:5 ω3 (EPA)	25.09	0.10	32.43	0.06	17.33	0.03	0.70	0.02	n.d.	n.d.
24:1 ω9	0.38	0.00	0.55	0.02	0.25	0.01	0.28	0.05	n.d.	n.d.
22:5 ω3 (DPA)	4.30	0.05	5.24	0.02	2.60	0.12	0.26	0.01	n.d.	n.d.
22:6 ω3 (DHA)	25.70	0.21	17.98	0.03	34.85	0.10	1.15	0.03	n.d.	n.d.
Total ω3 PUFA	57.97	0.07	58.84	0.03	57.51	0.05	6.47	0.02	50.02	0.10
Total ω6 PUFA	2.76	0.01	3.14	0.01	2.14	0.01	48.15	0.02	16.76	0.03
Total SFA	18.52	0.22	17.05	0.02	19.68	0.12	21.17	0.10	10.63	0.06
Total MUFA	17.22	0.12	17.14	0.07	17.43	0.11	24.21	0.11	22.59	0.06
Total PUFA	64.26	0.33	65.81	0.08	62.90	0.24	54.62	0.03	66.78	0.11
weekly dose of EPA	159.30 mg/kg rat		205.84 mg/kg rat		110.03 mg/kg rat		4.45 mg/kg rat			
weekly dose of DHA	163.12 mg/kg rat		114.15 mg/kg rat		221.24 mg/kg rat		7.31 mg/kg rat			
weekly dose of ARA	10.65 mg/kg rat		12.55 mg/kg rat		7.37 mg/kg rat		2.54 mg/kg rat			
weekly dose of ALA	2.29 mg/kg rat		2.06 mg/kg rat		2.09 mg/kg rat		25.39 mg/kg rat		317.56 mg/kg rat	
weekly dose of LA	4.13 mg/kg rat		3.88 mg/kg rat		4.15 mg/kg rat		301.86 mg/kg rat		106.39 mg/kg rat	

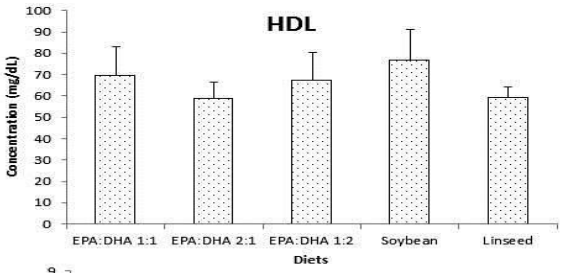
Compound	Retention Time (min)	LIT parameters	
		Collision energy (eV)	Quantification transition (*m/z*)
8iso-PGF$_{3\alpha}$	6.79	30	351>253
TXB_3	6.97	19	367>195
PGD_3/PGE_3	7.67	19	349>313
8iso-PGF$_{2\alpha}$	8.28	28	353>299
PGE_2	9.09	20	351>315
RvD_1	9.80	25	375>141
PD_x	12.89	30	359>153
LTB_4	13.79	27	335>195
15HpEPE	17.36	20	333>315
12HpEPE	17.80	25	333>315
15HEPE	17.92	27	317>219
12HEPE	18.72	27	317>179
5HEPE	20.47	25	317>255
17HpDoHE	21.90	26	359>341
17HDoHE	21.94	27	343>245
11HETE	22.09	30	319>167
12HETEd_8	22.66	30	325>307
11HDoHE	23.20	27	343>149
4HDoHE	23.64	27	343>281
EPA	24.43	27	301>257
DHA	24.93	30	327>283
ARA	25.10	30	303>259

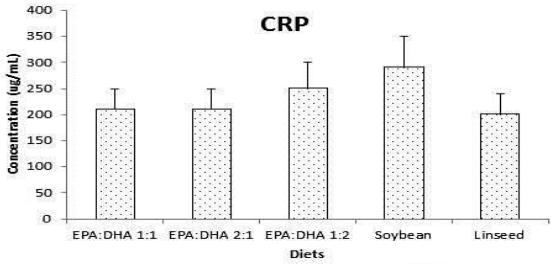

Plasmatic TFA
Concentration (mg/dL)
4000
3500
3000
2500
2000
1500
1000
500
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets

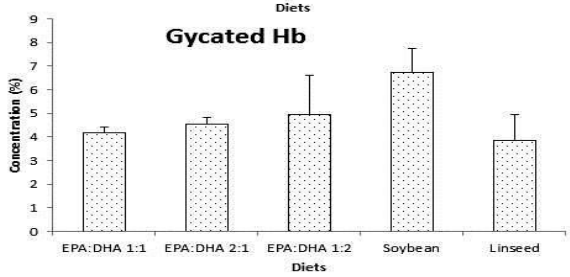

Plasmatic TG
Concentration (mg/dL)
2500
2000
1500
1000
500
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets

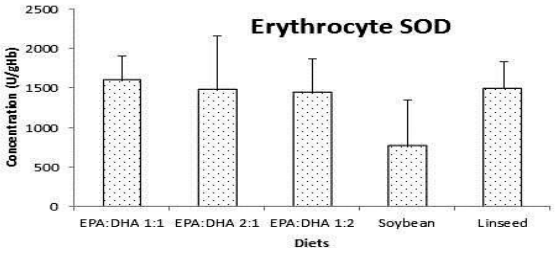

Plasmatic TC
Concentration (mg/dL)
500
450
400
350
300
250
200
150
100
50
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets

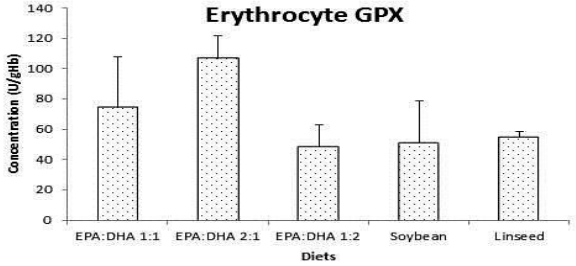

LDL
Concentration (mg/dL)
180
160
140
120
100
80
60
40
20
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets

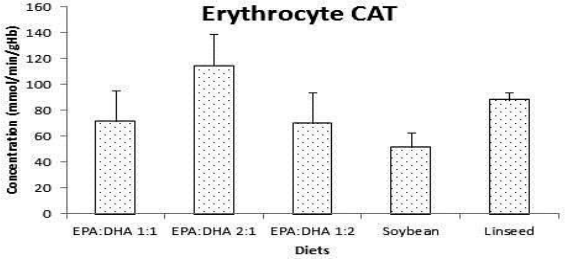

HDL
Concentration (mg/dL)
100
90
80
70
60
50
40
30
20
10
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets


CRP
Concentration (ug/mL)
400
350
300
250
200
150
100
50
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets


Gycated Hb
Concentration (%)
9
8
7
6
5
4
3
2
1
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets


Erythrocyte SOD
Concentration (U/gHb)
2500
2000
1500
1000
500
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets


Erythrocyte GPX
Concentration (U/gHb)
140
120
100
80
60
40
20
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets


Erythrocyte CAT
Concentration (mmol/min/gHb)
160
140
120
100
80
60
40
20
0
EPA:DHA 1:1
EPA:DHA 2:1
EPA:DHA 1:2
Soybean
Linseed
Diets