A lipidomic study on the regulation of inflammation and oxidative stress targeted by Marine ω-3 PUFA and Polyphenols in high- fat high-sucrose diets

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Abstract

The ability of polyphenols to ameliorate potential oxidative damage of ω-3 PUFAs when they are consumed together and then, to enhance their potentially individual effects on metabolic health are discussed through the modulation of fatty acids profiling and the production of lipid mediators. For that, the effects of the combined consumption of fish oils and grape seed procyanidins on the inflammatory response and redox unbalance triggered by high-fat high-sucrose (HFHS) diets were studied in an animal model of Wistar rats. A standard diet was used as control. Results suggested that fish oils produced a replacement of ω-6 by ω-3 PUFAs in membranes and tissues, and consequently they improved inflammatory and oxidative stress parameters: favored the activity of 12/15-lipoxygenases on ω-3 PUFAs, enhanced glutathione peroxidases activity, modulated pro-inflammatory lipid mediators synthesis through the cyclooxygenase (COX) pathways and down-regulated the synthesis *de novo* of ARA leaded by Δ5 desaturase. Although polyphenols exerted an anti-oxidative and anti-inflammatory effect in the standard diet, they were less effective to reduce inflammation in the HFHS dietary model. Contrary to the effect observed in the standard diet, polyphenols up-regulated COX pathways towards ω-6 pro-inflammatory eicosanoids as PGE_2 and 11-HETE, and decreased the detoxification of ω-3 hydroperoxides in the HFHS diet. As a result, additive effects between fish oils and polyphenols were found in the standard diet in terms of reducing inflammation and oxidative stress. However, in the HFHS diets, fish oils seem to be the main responsible of the positive effects found in the combined group.

Keywords

Eicosanoids; docosanoids; fish oils; polyphenols; inflammation; oxidative stress.

1. Introduction

Regular consumption of ω-3 PUFAs, mainly EPA and DHA, has been associated to the prevention and palliation of diabetes, cardio vascular diseases (CVD), chronic inflammation and oxidative stress processes closely linked with obesity, insulin resistance, dyslipidemia, and hypertension [1–4]. Although no agreement has been reached on the recommended proportion of these PUFAs in the diet [5], dietary designs with an optimal proportion of EPA and DHA seem to potentiate these health benefits. In previous works, we have suggested that fish oils having a balanced proportion between EPA:DHA (1:1) produce better anti-inflammatory response modulating eicosanoid and docosanoids pathways [6], down-regulate oxidative stress and protein damage [7], and improve biochemical parameters related to metabolic disorders [8,9], compared to oils with unbalanced 1:2 EPA:DHA. Recently, other authors have found that EPA:DHA at 1:1 ratio reduces in higher extent the atherosclerotic plaque area in an animal model of atherosclerosis [10].

Despite the beneficial effects of ω-3 supplements, higher amount of PUFAs in diet leads to greater levels of oxidizable substrate that may deregulate redox balance and increase oxidative stress [11]. Oxidative stress has also been associated with the development of inflammation [12,13]. In this regard, the use of antioxidants is a common strategy to relieve lipid oxidation [14,15]. Polyphenols like grape seed procyanidins (GSP) extracts are known to act as anti-oxidants as well as anti-inflammatory compounds that regulate gene transcription factors and cytokines secretion associated with inflammation [12], reduce C-reactive protein (CRP) [16], and down-regulate the expression of proteins involved in phospholipase A_2 (PLA_2) synthesis, a key enzyme in inflammation [17]. GSP have also prevented oxidative injury by modulating the expression of antioxidant glutathione enzymes [18], regulating glucose homeostasis [19], decreasing plasma levels of triglycerides, low-density lipoprotein (LDL), and circulating free fatty acids (FFA) in plasma [20], and down-regulating the expression of proteins related to *de novo* synthesis of long chain FA [17]. Others have attributed health benefits including vasodilatory, antioxidant, and anti-inflammatory effects by modulating enzyme activities that trigger eicosanoid synthesis [21]. There is a great deal of evidence that antioxidant properties of polyphenols can be exerted by acting at cellular and non-cellular level. Polyphenols act via inhibition of LDL oxidation [22]. At cellular levels, polyphenols can inhibit NADPH oxidase, a major catalyst for the formation of ROS [23], and consequently they down-regulate isoprostanes and thromboxane synthesis [24] in a dose-dependent manner [22]. From a mechanistic point of view, some studies have suggested that the capacity of polyphenols to inhibit dose-dependently protein kinase C (PKC) mediates their effect down-regulating NADPH oxidase activity [22,24]. PKC is an enzyme that activates NADPH oxidase via phosphorylation of $p47^{phox}$. Lipid mediators from ω-3 PUFAs like resolvins, protectins and lipoxins also reduce NADPH oxidase activity inhibiting the $p47^{phox}$ subunit membrane translocation [25]. The potential benefits of combining ω-3 and polyphenols have been barely described. Maestre *et al*. [26] showed that the inclusion of polyphenol-rich grape seed extracts

during the digestion of fish lipids decreased their oxidation. They also related the addition of polyphenols with greater uptake of DHA into a Caco-2 enterocyte model suggesting a protective role on ω-3 PUFAs which favors their intestinal bioavailability. Sekhon *et al*. [27] have found additive properties of both supplements to diminish CRP and cytokine factors in diet-induced inflammation studies.

High-fat high-sucrose (HFHS) diets are well-known model diets to intentionally promote inflammation processes and metabolic disorders [16,17]. Zhang *et al*. [28] provided novel evidences that high dietary fat intakes promoted neuronal oxidative stress and inflammation through NADPH oxidase activation and ROS generation, and PGE_2 production in cerebral cortex. The main goal of the anti-inflammatory nutrition has been focused on reducing dietary components that may induce inflammatory responses, such as ω-6 PUFAs, saturated fats, and refined carbohydrates, and increasing ω-3 PUFAs and antioxidants that activate endogenous mechanisms to reduce inflammation [12]. In this context, oxygenated lipid mediators derived from the metabolism of marine ω-3 PUFAs, EPA and DHA, have been suggested as one of the most efficient mechanisms to stop inflammation [29]. Additionally to the effects of ω-3 PUFAs on the regulation of gene expression through interacting with nuclear receptors and transcription factors [12], the modulation of the amount and type of mediators derived from PUFAs seems also to be an anti-inflammatory response key achieved by fish oils [30]. For instance, ω-6 ARA eicosanoids are considered strong pro-inflammatory molecules linked to hypercholesterolemia, type-2 diabetes, and obesity [12,25,31], ω-3 eicosanoids and docosanoids from EPA and DHA are weak inflammatory compounds [32,33], and resolvins and protectins from EPA and DHA are potent anti-inflammatory mediators [34,35]. Previous research on genetically obese and hypertensive rats, an animal model for Metabolic Syndrome, demonstrated the effectiveness of fish oils having balanced proportions of EPA and DHA to down-regulate the production of strong pro-inflammatory ω-6 eicosanoids [36].

The present study evaluated the role that fish oils, polyphenols, and the potential cooperative effect of both, in the prevention of inflammation and oxidative stress underlying metabolic disorders associated to the consumption of HFHS diets. For this scope, Wistar rats were fed a HFHS diet and compared with controls fed a standard diet. Marine PUFAs in a balanced 1:1 EPA:DHA proportion and GSP were employed as dietary supplements. A lipidomic platform based on SPE-LC-MS/MS was applied to determine the influence of each supplement on the synthesis of ω-3 and ω-6 eicosanoids and docosanoids related to inflammatory and metabolic processes. The regulation of lipid mediators was discussed together to the incorporation of fatty acids into membranes and tissues, fatty acid desaturase activities, and the rise in the plasma concentrations of circulating levels of FFAs. The PUFAs composition of cells has been described to influence inflammation by modifying intracellular signals and altering the pattern of lipid mediators produced [29]. FFAs are signaling molecules participating in oxidative stress and inflammatory processes and have been associated with an increase in fat mass and insulin resistance [37,38].

2. Materials and Methods

2.1 Animals and diets

Eight to ten week old female Wistar-Kyoto rats (*n* 56,Charles Rivers Lab., Wilmington, MA, USA) weighting 123-168 g were used. All animals were housed in cages (*n* 2-3/cage) with a 12 h light/dark cycle and constantly regulated humidity (50±10 %) and temperature (22±2 ^{o}C). The animals were given *ad libitum* access to feed and water. Animals were randomized in two dietary groups: a standard group (*n* 28) fed a standard diet (a modification of Teklad Global 2014, Harlan Teklad Inc., IN, USA) used as a control healthy model, and a HFHS group (*n* 28) fed a high-fat high-sucrose diet (a modification of TD 08811, Harlan Teklad Inc.) used as a risk model to develop chronic inflammation and metabolic disorders. Both groups were also subdivided into four supplemented subgroups (*n* 7): control, fish oils (ω3), grape seed proanthocyanidins (GSP), and fish oil with GSP (ω3&GSP). Each subgroup was fed the corresponding standard or HFHS diet enriched with the assigned supplement as listed in Supplementary Material S1. The experimental diets were pelletized in-house by lyophilization from frozen emulsions in order to incorporate each supplement. EPA:DHA 1:1 oil was obtained by blending adequate proportions of the commercially available fish oils from AFAMPES 121 EPA (AFAMSA, Vigo-Spain), EnerZona Omega 3 RX (ENERZONA, Milano-Italy) and Oligen liquid DHA 80% (IFIGEN-EQUIP 98, Barcelona-Spain). Soybean oil, obtained from cold pressing unrefined organic soybean oil, was from Clearspring Ltd. (London, UK). Grape seed extract Grajfnol (≥ 95% total oligomeric proanthocyanidins, ≥ 60% procyanidins dimer B_2) was from JF-Natural product (Tianjin, China). It is important to point out that fatty acids composition of ω3 and ω3&GSP supplemented feed had higher proportions of EPA, DPA, DHA, and ARA than control and GSP supplements. In order to compensate the caloric content between the four groups, control and GSP feeds were enriched with soybean oil as explained. Thereby, the fatty acids composition of control and GSP supplements had higher proportions of palmitic and oleic acids, LA, and ALA than ω3 and ω3&GSP supplements (Supplementary Material S2). After 25 weeks of nutritional intervention, rats were fasted overnight, anesthetized intraperitoneally with ketamine and xylazine, and killed by exsanguination. Handling and killing of the animals adhered strictly to the European Union guidelines for the management and care of laboratory animals and were approved by the CSIC Bioethical Issues Subcommittee (ref. AGL2009-12 374-C03-03).

2.2 Sample processing

The biological samples for fatty acids and metabolites analysis in plasma and erythrocytes were prepared according to previously described methods [6,36]. Briefly, on collection blood was added to EDTA containing tubes (1 mg/ml). After centrifugation erythrocytes were removed, packed and washed following Sonenberg protocol [39]. Then, PMSF (protease inhibitor) was added to plasma samples (erythrocyte free) and kept at -80 ºC until used. Liver and adipose tissue samples were collected, frozen in liquid N_2 and stored at - 80 ºC until used.

2.3 Total fatty acids analysis in liver, plasma, adipose tissue, erythrocytes, and circulating FFA analysis in plasma

Methods used for the analysis of total fatty acids (TFA) and FFA in plasma and erythrocytes were widely described in previous works [6,36]. Accordingly, a Bligh and Dyer [40] protocol was applied to plasma, liver, and adipose samples using dichloromethane:methanol:water (2:2:1, v/v) as extraction solvent. To analyze TFA, the organic phase was transesterified as Lepage and Roy [41] and TFA were determined by gas chromatography (GC/FID, Clarus 500, Perkin–Elmer). In the case of FFA in plasma, the organic phase was subjected to solid phase extraction (SPE) using aminopropyl cartridges (500 mg, 6 mL, Biotage, Uppsala-Sweden) as Kaluzny *et al*. [42] and then transesterified and analyzed by GC/FID. Similarly, a Bligh and Dyer modified procedure [43] was applied to erythrocyte samples and TFA were determined as indicated above. Results are summarized in Tables1-5.

2.4 Fatty acid desaturase (FAD) indexes

FAD indexes were calculated from plasma and liver TFA data [44,45]. Stearoyl-CoA Desaturases SCD-16 and SCD-18 regulate the desaturation from palmitic acid (16:0) into palmitoleic acid (16:1 ω-7) and stearic acid (18:0) into oleic acid (18:1 ω-9), respectively. Desaturases Δ4, Δ5, and Δ6 modulate the formation of ARA, EPA and DHA from LA and ALA. In detail, Δ4D regulates the desaturation from DPA (22:5 ω-3) into DHA (22:6 ω-3). Δ5D regulates the desaturation from DGLA (20:3 ω-6) into ARA (20:4 ω-6). Δ6D regulates the desaturation from LA (18:2 ω-6) into DGLA (20:3 ω-6). The pathway from ALA (18:3 ω-3) into EPA (20:5 ω-3) is mediated by the action of both Δ5D and Δ6D, firstly Δ6D desaturates ALA into SDA (18:4 ω-3) which is elongated and further desaturated by Δ5D into EPA. Therefore, several FAD indexes were determined as the ratio between product and precursor as follows: SCD-16 = [16:1 ω-7/16:0], SCD-18 = [18:1 ω-9/18:0], Δ4D = [22:6 ω-3/22:5 ω-3], Δ5D = [20:4 ω-6/20:3 ω-6], Δ6D = [20:3 ω-6/18:2 ω-6], and Δ5/6D = [20:5 ω-3/18:3 ω-3]. Results are shown in Tab. 6.

2.5 Lipid peroxidation, CRP and TNFα levels in liver, protein carbonylation, ORAC and antioxidant enzymes in plasma

Lipid peroxidation levels in liver were measured through conjugated dienes hydroperoxides (intermediate lipid oxidation product) and malondialdehyde (MDA, end lipid oxidation product). For that, liver lipids were extracted [43] and quantified [46]. Then, conjugated dienes were measured following the American Oil Chemists Society method [47]. Total MDA in liver was derivatized with thiobarbituric acid (TBA) after protein hydrolysis [48] and precipitation [49], and determined by HPLC-fluorescence according to Fukunaga *et al*. [50].

Protein oxidation in plasma was measured by determining the level of total protein carbonylation by a fluorescence-based assay as previously described [7]. Briefly,

carbonyls groups were labelled with fluorescein-5-thiosemicarbazide and detected on one-dimensional SDS-PAGE gels through a densitometric analysis. Plasma antioxidant capacity was measured as the oxygen radical absorbance capacity (ORAC) [51]. Total superoxide dismutase (SOD) activity was measured in plasma following the method of Misra and Fridovich [52]. Glutathione peroxidase (GPX) activities in plasma were determined according to Wheeler *et al*. [53]. ELISA kits from Cusabio Biotech (Hubei, China) were used to measure CRP and TNFα in liver. Results are shown in Tab. 6.

2.6 Analysis of lipid metabolites by SPE-LC-ESI-MS/MS

The methodology developed by Dasilva *et al*. [54] was applied to extract and determine ω-3 and ω-6 oxidized lipid mediators in plasma and results are shown in Tab. 7. Briefly, plasma samples were subjected to SPE using Oasis-HLB cartridges (60mg, 3mL, Waters, MA-USA) and extracts analyzed by LC-ESI-MS/MS on a Agilent 1260 Series (Agilent, Palo Alto, CA) coupled to a dual-pressure linear ion trap mass spectrometer LTQ Velos Pro with ESI (Thermo Fisher, Rockford, IL, USA). Instrument control and data acquisition were done with Xcalibur software. Retention times for target compounds and individual MS/MS parameters are summarized in Supplementary Material S3.

2.7 Statistics

Statistical analyses were performed by one-way and two-way analysis of variance (ANOVA) with R free software (version 3.2.4). Normal distribution and heterogeneity were evaluated and non-parametric Kruskal Wallis analyses were required when data distribution did not fit a Gaussian model or heterogeneity was found in variances. The means were further compared by the post-hoc test Fisher least square difference (Fisher LSD) and significant differences were set at $p<0.05$. Data presented are expressed as mean ± SD.

3. Results

Results are shown according to the two dietary groups. Firstly, the work shows results of animals fed standard diets which were used as a control healthy model. Then, results corresponding to the group fed HFHS diets associated to the development of chronic inflammation and metabolic disorders are presented [55]. Animals from each dietary experiment were divided in four supplemented subgroups: control, fish oils (ω3), grape seed proanthocyanidins (GSP), and fish oil with GSP (ω3&GSP). The regulation of plasma, membrane and tissues FAs profile, ω6/ω3 ratios, FADs activity, and several eicosanoid and docosanoids pathways were addressed. Results of eicosanoids/docosanoids and fatty acids profiling were related with lipid peroxidation levels in liver, carbonylation of plasma albumin, and with previous measurements in the same animals for the plasma antioxidant capacity, antioxidant enzymes activities in plasma (SOD and GPX) and inflammation markers in liver (CRP and TNFα).

3.1 Effect of fish oils and polyphenol supplements on the Standard diet

3.1.1 Fatty acids profile in tissues and plasma

3.1.1.1 TFA incorporation into liver

Supplementation with fish oils largely modulated the incorporation of PUFAs in liver in concordance with the FA composition of the supplements. In detail, the proportion of ω-3 EPA, DPA, and DHA and ω-6 DGLA increased in ω3 and ω3&GSP groups related to control, and it was observed a specific proportional decrease in the amount of ω-6 ARA, γ-ALA, and ω-9 oleic acid. Supplementation with polyphenols did not influence the incorporation of FA into liver; and thus, TFA profiles in GSP and control groups were similar. As a consequence, the inflammatory index ω-6/ω-3 in liver was significantly reduced after fish oils treatments compared with control and GSP groups. Animals fed fish oils (EPA:DHA 1:1) accumulated these PUFAs into liver in a proportion of EPA:DHA 1:4. However, animals from control and GSP groups showed EPA:DHA ratios in liver around 1:40 – 1:30, suggesting a higher accumulation and production of DHA synthesized from EPA. Finally, it was observed that all groups accumulated around 50% of PUFAs, 10% of MUFAs, and 40% of SFAs in liver without significant differences due to the supplementation. Results are shown in Tab. 1.

3.1.1.2 TFA profile in plasma

Regarding to the modulation of TFA in plasma due to the supplements (Tab. 2), fish oils increased amounts of ω-3 EPA, DPA, and DHA and ω-6 DGLA with the corresponding proportional decrease of ω-6 ARA and γ-ALA as observed in liver. GSP group showed a similar profile to control group except for a significant increase of DGLA. The inflammatory index ω-6/ω-3 was also significantly reduced after fish oils treatments compared with control and GSP groups as observed in liver. Percentages of PUFAs, MUFAs, and SFAs in plasma followed the same trend as described in liver. Therefore, the profile of TFA observed in plasma for all groups was similar to the profile of TFA in liver as expected due to plasmatic lipids mostly come from liver secretion in starvation [56]. However, differences were observed in the relative percentage of DHA and ARA between plasma and liver: the amount of DHA in plasma was found decreased for all groups compared with the amount accumulated in liver; similarly, the proportion of ARA was higher in plasma than in liver. In addition, the EPA:DHA ratio found in plasma in animals fed fish oils was 1:1,5, with lower proportion of DHA than ratio found in liver. The same trend was observed in control and GSP groups: EPA:DHA ratio was 1:10 showing a minor proportion of DHA than in liver.

3.1.1.3 TFA incorporation into erythrocyte membranes

The analysis of FA incorporated into erythrocyte membranes (Tab. 3) showed that the proportion of PUFAs was significantly affected by fish oils similarly to the effect observed in liver and TFA of plasma. In detail, the incorporation of EPA, DPA, and DHA was increased in the ω3 and ω3&GSP groups accompanied with the specific proportional decrease in the incorporation of ARA. ALA and γ-ALA were not incorporated into membranes in the four groups, and DGLA incorporation was similar

between all groups and not modulated by supplements. Finally, polyphenols did not influence the incorporation of FA into erythrocytes. Therefore, the ω-6/ω-3 ratio in membranes was significantly reduced in the ω3 and ω3&GSP groups compared with control and GSP. The proportion of PUFAs, MUFAs, and SFAs incorporated in membranes was similar than plasma profiles. However, the ratio EPA:DHA showed a higher proportion of DHA *vs.* EPA in membranes compared with ratio found in plasma. Animals fed fish oils incorporated these PUFAs in a proportion of EPA:DHA 1:3, and animals from control and GSP groups showed ratios around 1:16 – 1:20.

3.1.1.4 TFA accumulation in adipose tissue

The concentration of myristic and palmitic saturated acids, and EPA, DPA, and DHA PUFAs was increased in the ω3 and ω3&GSP groups related to control group simultaneously with a decrease of oleic, ARA and LA levels. The accumulation of DGLA was not modulated by supplements. PP group showed a similar fatty acid profile than control. Then, ω-6/ω-3 ratio found in adipose tissue was significantly reduced in ω3 and ω3&GSP groups compared with control and GSP groups. Adipose tissue was characterized by a higher content of oleic and LA acids and a lower content of stearic, ARA and DHA acids in all groups compared with the TFA profile observed in plasma, liver and erythrocytes. Accordingly, the proportion of PUFAs, MUFAs, and SFAs accumulated in adipocytes showed different values than plasma profiles towards a higher accumulation of MUFAs and lower amounts of PUFAs and SFAs. The ratio EPA:DHA 1:3 was similar to that found in erythrocytes in ω3 and ω3&GSP groups. Animals from control and GSP groups showed EPA:DHA 1:6. Results are shown in Tab. 4.

3.1.1.5 Circulating FFAs profile in plasma

Circulating FFAs in plasma (Tab. 5) are mostly derived from adipose tissue secretions. Accordingly, fish oils showed the same modulation of FFA profile that had been observed in adipose, and polyphenols did not influence the composition of FFA. In detail, it was found an increase in the proportion of myristic, palmitic, EPA, DPA, and DHA, with the subsequent proportional reduction of oleic, ALA, LA and ARA in the ω3 and ω3&GSP groups compared with control. GSP group showed a similar fatty acid profile than control. As a consequence, the ω-6/ω-3 ratio was significantly reduced in ω3 and ω3&GSP groups compared with control and GSP. Although the effect of supplements was similar in both adipose tissue and circulating FFA profiles, the proportion of circulating palmitic acid (SFA) was higher in plasma than adipose, and the contrary was observed for oleic acid (MUFA), suggesting a preference of adipose to accumulate MUFAs instead of SFAs in the standard diet. Regarding to the ratio EPA:DHA found in circulating fatty acids, it was observed a proportion 1:2 EPA:DHA in ω3 and ω3&GSP groups, different than that found in adipose tissue which indicated a greater accumulation of DHA *vs* EPA in adipose (ratio 1:3). Control and GSP groups showed EPA:DHA 1:6 and 1:9, respectively. The total concentration of FFA in plasma showed a trend to lower values for ω3 and GSP groups, and interestingly this parameter resulted to be significantly lower for the combined ω3&GSP group.

3.1.2 FADs indexes

FAD indexes were calculated regarding to the TFA data in plasma [44].The effects of supplementation of fish oils in ω3 and ω3&GSP groups related to control showed the following results: (a) significant lower values of Δ5D [ARA/DGLA], (b) and significant higher values of Δ4D [DHA/DPA] and Δ5/6D [EPA/ALA], and a tendency towards higher values of Δ6D [DGLA/LA]. It is remarkable that the increase of Δ5/6D [EPA/ALA] was significantly higher than the increase of Δ4D [DHA/DPA] after fish oils supplementation.

Interestingly, the supplementation with polyphenols resulted in a modulation of FAD indexes related to control: (a) lower values of Δ5D [ARA/DGLA] and Δ4D [DHA/DPA] in GSP (b) and higher values of Δ6D [DGLA/LA] in GSP and ω3&GSP groups. That means a significant accumulation of DGLA related to control and a trend to lower amounts of ARA, EPA and DHA. It is also remarkable the additive effect observed on the reduction of Δ5D [ARA/DGLA] for the ω3&GSP group which showed the lowest level compared with control, GSP and ω3 groups. The studied supplements did not exert any significant influence on SCD-16 and SCD-18. Results are shown in Tab. 6.

In high agreement with FAD indexes calculated in plasma, these indexes calculated in liver (Tab. 6) also showed decrease of Δ5D [ARA/DGLA], and increase of Δ4D [DHA/DPA], Δ5/6D [EPA/ALA], and Δ6D [DGLA/LA] in ω3 and ω3&GSP groups related to controls. GSP group in liver showed lower values of Δ4D [DHA/DPA] and a tendency to decrease Δ5D [ARA/DGLA] compared with control.

3.1.3 Eicosanoid and docosanoids levels

A total of nine compounds derived from PUFA oxidative metabolism were identified and quantified in plasma, five EPA eicosanoids, two DHA docosanoids, and two ARA eicosanoids (Tab. 7). The primary oxidation products found were the hydroperoxides 12-HpEPE, 15-HpEPE, and 17-HpDoHE. The secondary metabolites identified are hydroxides (12-HEPE, 15-HEPE, 17-HDoHE, and 11-HETE), a thromboxane (TXB_3), and a prostaglandin (PGE_2). Finally, other searched compounds as 5-HEPE, 8iso-$PGF_{3\alpha}$, 8iso-$PGF_{2\alpha}$, PGD_3, PGE_3, 11-HDoHE, 4-HDoHE, RvD_1, PD_x, and LTB_4 were under the detection limits of the method.

Data revealed that dietary interventions with fish oils in the ω3 group compared with control: (a) significantly decreased the concentration of 12-HpEPE and enhanced 15-HpEPE (EPA primary metabolites) coming from 12-LOX and 15-LOX activity respectively, (b) and significantly increased levels of the corresponding secondary metabolites from EPA: 12-HEPE produced from 12-HpEPE, and 15-HEPE from 15-HpEPE; (c) largely decreased the production of 17-HpDoHE (DHA primary metabolite) mediated by 15-LOX, (d) and increased 17HDoHE, secondary metabolite of DHA

produced from 17-HpDoHE; (e) and reduced the synthesis of secondary ARA metabolites (11-HETE and PGE_2) resulting from COX activity. The intervention with polyphenols in the GSP group compared with control: (a) reduced levels of primary metabolites from EPA and DHA produced by 12-LOX and 15-LOX (12-HpEPE, 15-HpEPE, and 17-HpDoHE), (b) did not significantly affect their secondary metabolites (12-HEPE, 15-HEPE, and 17-HDoHE) although it was observed a tendency to decrease their levels; (c) and significantly decreased COX secondary metabolites from ARA. Finally, TBX_3 resulting from COX activity on EPA was not detected in the GSP group, and no differences in its levels were observed between control, ω3, and ω3&GSP groups.

Interestingly, the consumption of fish oils together with polyphenols in the ω3&GSP group produced: (a) a significant decrease in the concentration of primary metabolites, 12-HpEPE and 17-HpDoHE, compared to control group showing an additive effect between both fish oils and polyphenols supplements over 12-LOX and 15-LOX activities, (b) a significant enhancement of 15-HpEPE compared to control as observed in the ω3 group; however, the concentration of 15-HpEPE in the ω3&GSP group was reduced related to the ω3 group in agreement with the reducing effect exerted by polyphenols in this pathway mediated by 15-LOX; (c) a trend to increase levels of 12-HEPE, 15-HEPE, and 17-HDoHE (secondary metabolites) was observed in ω3&GSP related to control although not statistically different, showing again a combined effect of both supplements (up-regulation of the formation of these secondary metabolites due to fish oils and down-regulation produced by polyphenols); (d) and a specially interesting decrease of pro-inflammatory secondary ARA metabolites (11-HETE and PGE_2) synthesis, according to the combined down-regulation effect of individual supplements.

3.1.4 Oxidative stress parameters

Levels of conjugated diene hydroperoxides and TBARS in plasma were under detection limits (data not shown). Lipid peroxidation in liver showed that the level of conjugated diene hydroperoxides was slightly lower in the ω3 and ω3&GSP groups related to control, whereas the concentration of secondary oxidation byproducts measured as TBARS was significantly higher. The GSP group did not modify conjugated dienes levels related to control and showed a trend to reduce the production of TBARS. Consequently, the combination of both supplements (ω3&GSP group) showed lower amount of conjugated diene hydroperoxides than control and slightly lower amount TBARS than ω3 group as a result of polyphenols effect. Data revealed an inhibition of protein carbonylation measured in plasma in the GSP group related to control and no effects on the groups supplemented with fish oils were observed. Finally, plasma antioxidant capacity by ORAC and the activity of some antioxidant enzymes was measured in previous studies executed in the same animals. In detail, no significant differences in ORAC index were recorded between groups, although a tendency towards higher values was observed in the ω3&GSP group compared to other groups. SOD (involved in the detoxification of O_2^-) and GPX (involved in the detoxification of hydroperoxides to give hydroxides) activities were associated with the synthesis of lipid

mediators. SOD showed a trend to be lower in the ω3 and GSP groups than in control, and the additive effect was observed when both supplements were combined leading to significant lower values. GPX was found increased in ω3 and GSP groups, and the additive effect was also observed in ω3&GSP compared with control. Results are shown in Tab. 6

3.2 Effect of fish oils and polyphenol supplements on the HFHS diet

3.2.1 Fatty acids profile in tissues and plasma

3.2.1.1 TFA incorporation into liver

As observed in the standard diet, supplementation with fish oils largely modulated the incorporation of PUFAs in liver in the HFHS diet. Similarly, the proportion of ω-3 EPA, DPA, and DHA increased in ω3 and ω3&GSP groups related to control, and a proportional decrease of ω-6 ARA and γ-ALA was observed. Supplementation with polyphenols did not influence the TFA profile compared with control group. Therefore, the ω-6/ω-3 index was found significantly reduced in ω3 and ω3&GSP compared with control and GSP groups as in the standard diet. Contrary to results observed in the standard diet, the accumulation of DGLA and oleic acid in liver was not significantly modulated by fish oils supplements in the HFHS dietary context. In addition, all groups with HFHS diet significantly accumulated higher proportions of oleic acid in liver and lower proportions of DHA than corresponding groups fed standard diet. EPA:DHA ratios found in liver were similar to that found in animals fed with standard diet and fish oils supplements. In detail, ω3 and ω3&GSP groups accumulated EPA:DHA 1:4 as in the standard diet, and control and GSP groups showed higher accumulation and production of DHA (EPA:DHA 1:20 – 1:16). Interestingly, control and GSP groups accumulated/produced half of the proportion of DHA under the HFHS diet under the standard diet (EPA:DHA 1:40 – 1:30). Finally, all groups accumulated similar percentages of PUFAs (45%), MUFAs (15%), and SFAs (40%) as in the standard diet experiment, without significant differences due to the supplementation. Results are shown in Tab.1.

3.2.1.2 TFA profile in plasma

TFA profile in plasma (Tab. 2) in the HFHS diet was similar to that found in the standard experiment and in agreement with the profile found in liver. Fish oils increased amounts of ω-3 EPA, DPA, and DHA and ω-6 DGLA with the corresponding proportional decrease of ω-6 ARA and γ-ALA as observed in liver. As in the standard diet, GSP group did not show significant differences in the proportion of TFA in plasma related to control group with the exception of DGLA which showed a significant enrichment compared to control. The inflammatory index ω-6/ω-3 was significantly reduced in ω3 and ω3&GSP groups compared with control and GSP groups. The profile of TFA observed in plasma and percentages of PUFAs, MUFAs, and SFAs for all groups in the HFHS diet were quite similar to the profiles found in liver, with the exception of the relative amount of ARA and DHA. The relative proportion of DHA in plasma was minor than accumulated in liver; and similarly, the proportion of ARA was

higher in plasma, as it had been observed in the standard diet. Finally, the EPA:DHA ratio was modulated by fish oils supplements similarly to the standard diet. In detail, the ω3 and ω3&GSP groups showed 1:1.5 EPA:DHA in plasma. And thus, the proportion of DHA was lower in plasma than the ratio found in liver. Regarding to control and GSP groups, they showed EPA:DHA 1:5, and again a minor proportion of DHA than in liver. Similarly to results observed in liver, control and GSP groups accumulated/produced half of the proportion of DHA in the HFHS diet than these groups in the standard diet (EPA:DHA 1:10).

3.2.1.3 TFA incorporation into erythrocyte membranes

The incorporation of FA into erythrocyte membranes (Tab. 3) in the HFHS diet followed the same trend as in the standard diet. Only the incorporation of PUFAs was significantly affected by fish oils. In the ω3 and ω3&GSP groups, the incorporation of EPA, DPA, and DHA increased, and ARA proportionally decreased related to control. ALA and γ-ALA were not incorporated and DGLA proportions were not modulated by the supplements. Polyphenols did not modify the profiles of FA in membranes. Consequently, erythrocyte membranes showed decreased ω-6/ω-3 ratios in the ω3 and ω3&GSP groups compared with control and GSP. The proportion of PUFAs, MUFAs, and SFAs between membranes and plasma were similar as observed in the standard diet. In the same trend as in plasma and liver, the ratio EPA:DHA in erythrocytes showed minor proportion of DHA in control and GSP groups in the HFHS diet (EPA:DHA 1:10) compared with the standard one (EPA.DHA 1:16-1:20). The same was observed in ω3 and ω3&GSP groups in the HFHS diet (EPA:DHA 1:2) and standard diet (EPA.DHA 1:3).

3.2.1.4 TFA accumulation in adipose tissue

Regarding to the influence of the supplements in the accumulation of fatty acids in the adipose tissue, results were similar to the standard diet. The proportion of myristic, EPA, DPA, and DHA was increased in the ω3 and ω3&GSP group related to control group with the subsequent decrease of oleic, ARA and LA. The accumulation of DGLA was not modulated by the supplements and GSP group showed a similar fatty acid profile than control. Therefore, the inflammatory index ω-6/ω-3 was significantly reduced in the ω3 and ω3&GSP groups compared with control and GSP ones. The profile of TFA in the adipose tissue was richer in oleic acid and less rich in stearic, ARA and DHA acids in all groups compared with plasma, liver and erythrocytes as in the standard diet. The ratio EPA:DHA found in ω3 and ω3&GSP groups was around 1:3; meanwhile control showed EPA:DHA 1:6 and GSP did not accumulate EPA. Results are shown in Tab. 4.

3.2.1.5 Circulating FFAs profile in plasma

The influence of fish oils in the proportion of circulating FFA in plasma (Tab. 5) was similar to that found in adipose, as it had been observed in the standard diet. The proportion of stearic, EPA, DPA, and DHA increased proportionally to the reduction of oleic, LA and ARA in ω3 and ω3&GSP groups compared with control. Polyphenols did

not influence the profile of circulating FFA. Therefore, fish oils treatments showed the lowest values for the inflammatory index ω-6/ω-3. Similarly to the standard diet, the proportion of circulating oleic acid (MUFA) was lower in plasma than adipose, and the contrary was observed for palmitic and stearic acids (SFAs). In the ω3 and ω3&GSP groups the proportion EPA:DHA was 1:2 as in the standard experiment, and control and GSP groups showed a proportion of EPA:DHA 1:3.5. In a different way to the standard diet, the total concentration of FFA in plasma showed larger values for the GSP group compared with control, and a trend to lower values for both ω3 and ω3&GSP groups (this last trend was also observed in the standard diet).

3.2.2 FADs indexes

Comparative results between HFHS and standard diets showed a similar influence of supplements on FAD indexes measured in plasma (Tab. 6). In ω3 and ω3&GSP groups related to control it was observed: (a) a significant decrease of Δ5D [ARA/DGLA], (b) a significant increase of Δ4D [DHA/DPA], Δ5/6D [EPA/ALA], and SCD-18 [oleic/stearic], (c) and a tendency to higher values of Δ6D [DGLA/LA]. As observed in the standard diet, the increase of Δ5/6D [EPA/ALA] in fish oils groups was significantly higher than the increase of Δ4D [DHA/DPA]. The effect of polyphenols related to control group showed: (a) lower values of Δ5D [ARA/DGLA] (b) and higher SCD-18 [oleic/stearic]. Interestingly, the ω3&GSP group showed an additive effect on the reduction of Δ5D [ARA/DGLA]. The studied supplements did not exert a different influence on SCD-16. As occurs in the standard diet, these FAD indexes calculated in liver (Tab. 6) mostly agree with FAD indexes in plasma for the ω3 and ω3&GSP groups. In detail, liver indexes also showed the decrease of Δ5D [ARA/DGLA], and increase of Δ4D [DHA/DPA] and Δ5/6D [EPA/ALA] in ω3 and ω3&GSP groups related to controls. However, FADs in liver did not show differences between GSP and control groups.

3.2.3 Eicosanoid and docosanoids levels

The lipidomic analysis revealed the production of the same compounds in almost all groups as in the standard diet experiment (Tab. 7). Data showed similar tendencies in the formation of eicosanoid and docosanoids in both standard and HFHS experiments when diets were supplemented with fish oils. Briefly, in the ω3 group compared with control, results showed: (a) a significant decrease of 12-LOX EPA product (12-HpEPE), and enhance of 15-LOX EPA product (15-HpEPE) and secondary metabolites of EPA and DHA (12-HEPE, 15-HEPE, and 17HDoHE), (b) a tendency to decrease the production of 15-LOX product of DHA (17-HpDoHE); (c) and a slightly reduction of the synthesis of secondary ARA metabolites (11-HETE and PGE_2). It is remarkable that the COX product of EPA (TBX_3) was only detected in fish oils groups.

Interestingly, the influence that polyphenols exerted on metabolites synthesis showed some differences between both standard and HFHS experiments. Specifically, the GSP group in the HFHS diet (a) did not significantly affect secondary metabolites of EPA and DHA in concordance with the standard diet. However, contrary to the standard diet

(b) polyphenols did not reduce levels of primary metabolites produced by 12-LOX and 15-LOX from EPA and DHA, and a tendency was even observed to produce higher values of 12-HpEPE and 17-HpDoHE in the HFHS diet; (c) and polyphenols significantly increased the synthesis of ARA metabolites derived from COX activity in the HFHS diet. The different influence of polyphenols between the HFHS and the standard experiment was also observed when polyphenols and fish oils were combined. (a) Levels of primary metabolites from EPA and DHA slightly increased in the ω3&GSP group related to the ω3 one (significant for 15-HpEPE) in the HFHS diet in agreement with the effect observed in the GSP group and suggesting a tendency to up-regulate 12-LOX and 15-LOX, contrary to results observed in the standard diet. (b) Secondary metabolites from EPA and DHA also tended to increase in the ω3&GSP group related to the ω3 one, contrary to the standard diet; (c) and secondary metabolites from ARA did not decrease in the ω3&GSP group related to ω3 as it had been found in the standard diet. Therefore, it seems that polyphenols down-regulated the activity of LOXs and COXs involved in the synthesis of these lipid mediators in the standard diet; however, they turned to up-regulate or slightly increase the activity of these enzymes in the HFHS diet. The two-ways ANOVA analysis showed significant interaction between diet (HFHS and Standard) and supplement group for several compounds due to the different influence exerted by polyphenols depending on the diet.

3.2.4 Oxidative stress parameters

As in the standard diet, levels of conjugated diene hydroperoxides and TBARS in plasma were under detection limits (data not shown). No differences were observed in conjugated diene hydroperoxides in liver between groups, and the concentration of TBARS in liver followed the same trend as in the standard diet: increased in ω3 and ω3&GSP groups related to control, and GSP group reduced the production of TBARS compared with control. Consequently, the combination of both supplements (ω3&GSP group) resulted in a lower production of TBARS than ω3 group. Data of protein carbonylation in plasma revealed an inhibition of protein oxidation in the ω3 and GSP groups that was clearly additive in the combined ω3&GSP group. Finally, regarding the antioxidant parameters: no significant differences were observed in ORAC although a tendency towards higher values in the ω3&GSP group was recorded as in the standard diet; SOD showed a trend to be lower in the ω3 group and significantly lower in GSP and ω3&GSP groups than in control; GPX was found significantly increased in ω3 and GSP groups, and the combined effect was also observed in ω3&GSP compared with control. Results are shown in Tab. 6.

4. Discussion

Both ω-3 PUFAs and polyphenols are well stablished for their individual health benefits. However, their combined effects are not well documented [27]. In previous works, we have suggested that diets supplemented with fish oils were able to modulate inflammation, oxidative stress and CVD risk markers, especially in a balanced proportion 1:1 EPA:DHA [6,36]. Considering that an increase of oxidizable substrates in the diet like PUFAs supplement may deregulate the redox balance and increase

oxidative stress [11], polyphenols have been used in this work in combination with fish oils having a proportion 1:1 EPA:DHA to potentially protect lipids from oxidation processes [14,15] and thus, enhance their health benefits. The potential mechanisms related to the influence on the regulation of lipid profiles in plasma, tissues and membranes, and eicosanoid and docosanoids synthesis have been suggested. Lipid profiles and further metabolites from fatty acids have been closely associated with inflammation, oxidative stress and the endogenous antioxidant system. Interestingly, some differences in the functionality of studied supplements were observed depending on the diet: standard *vs.* HFHS.

4.1 Fish oils influence

Outcomes suggested that supplementation with fish oils lead to a general improvement of fatty acids and lipid metabolites profiling in both, standard and HFHS diets. Interestingly, the influence of fish oils in the diet was similar in both caloric contexts, standard and HFHS. The replacement of ARA with EPA and DHA in membranes, plasma and tissues in fish oil diets, with the consequent enrichment on ω-3 long chain PUFAs brings a change on the fatty acid profiling accompanied by a modulation on the activity of the LOX and COX enzymes probably due to a competence mechanism. As a result, the production of lipid mediators is being affected and the uptake of ω-3 PUFAs modulates the synthesis *de novo* of ARA through elongases and desaturases.

After digestion, some fatty acids are transported in chylomicrons and directly accumulated in the adipose tissue or incorporated into liver. *Synthesis de novo* of fatty acids occurs in the liver and fatty acids are transported again as chylomicrons in blood to be used in different processes, incorporated into membranes or stored into the adipose tissue [56]. Accordingly, it was observed in this work that the modulation of the hepatic fatty acid profile by the fish oils supplement was also reflected in the TFA fraction of plasma and then in the incorporation into erythrocyte membranes, adipose tissue and circulating FFA in plasma. Fatty acids released from the adipose tissue become circulating FFA in plasma which are reflecting the influence of the diet and synthesis *de novo* in the accumulation of fat in adipocytes. These circulating FFAs in blood are considered signaling molecules participating in oxidative stress and inflammatory processes [38].

In this work, it was observed in both dietary backgrounds that the addition of fish oils (ω3 and ω3&GSP groups), with the subsequent increase of EPA, DPA, and DHA proportion in the diet, was reflected in a higher accumulation of these PUFAs in liver , adipose tissue and plasma compared with control and GSP groups. Interestingly, although ARA intake was higher in the diet supplemented with fish oils, the proportion of ARA detected in all samples was reduced related to control and GSP groups. Therefore, these results confirmed the preferential substrate competition of Δ5D, which modulates *de novo* synthesis of EPA and ARA for ω-3 PUFAs over ω-6 PUFAs [45]. In this regard, the inhibition of ARA synthesis in fish oils groups was generally accompanied by an increment of the concentration of its precursor DGLA.

In agreement with these findings, Sears *et al* [12] have also found a direct association between ω-3 dietary interventions and FADs modulation towards down-production of ARA and its inflammatory derivates. Others have also found that interventions in the diet with fish oils enriched FFA fraction with ω-3 PUFAs [57]. Here, it was noteworthy that the analysis of the FAD indexes in plasma [44] was highly consistent with these indexes calculated in liver. Both ω3 and ω3&GSP groups showed lower indexes of Δ5D modulating the synthesis of ARA, higher values of Δ4D related to DHA formation, and higher Δ5/6D to produce EPA, compared with control and GSP groups. As a final consequence, the ω6/ω3 ratio in plasma, circulating FFAs, membranes and tissues was lower in both standard and HFHS dietary contexts in the ω3 and ω3&GSP groups compared with control groups. The ω6/ω3 ratio is considered an excellent clinical marker for cellular inflammation [58], and higher levels have been associated with increasing prevalence of chronic inflammatory diseases [3].

Interestingly, adipose tissue showed a high enrichment in oleic acid compared with liver, TFA in plasma and erythrocytes in both dietary contexts regardless of the specific supplement. Accordingly, circulating fatty acids showed a high proportion of oleic acid as well. However, this accumulation of oleic acid in the adipose tissue was significantly reduced when fish oils were uptake. As a consequence, circulating oleic acid in plasma was minor in ω3 and ω3&GSP groups than in control and GSP groups in both dietary contexts. The increase of oleic acid in plasma and tissues has been associated with obesity [59], hypertriacylglycerolemia, and the risk of developing insulin resistance [37]. These conditions are usually accompanied by low-grade chronic inflammation [60] and increased oxidative stress [61]. It is remarkable that HFHS diet tended to accumulate higher proportions of oleic acid and lower PUFAs than the standard diet in all groups. In this regard, in the HFHS context more prone to increase oleic acid in adipose, fish oils were effective not only to reduce the accumulation in the adipose tissue as pointed above, but ω3 and ω3&GSP groups also showed a decrease of the desaturase index SCD-18 measured in plasma indicating a possible decrease of the synthesis *de novo* mediated by the desaturase. It has been published that increased SCD-18 activity was associated with insulin resistance, fatty liver and metabolic disorders [62].

The replacement of ARA by EPA, DPA and DHA in erythrocyte membranes in groups supplemented with fish oils was evident in both dietary experiments. Therefore according with literature [45], the synthesis of membrane phospholipids demonstrated to be highly modulated by a substrate competition between ω-3 EPA, DPA and DHA and ω-6 ARA. Consequently, the inflammatory ω6/ω3 ratio in erythrocytes composition was also lower in both standard and HFHS diets supplemented with fish oils, and this fact can lead the production of lipid inflammatory mediators as observed in this work.

Consequently, the analysis of lipid metabolites produced through enzymatic pathways from EPA, DHA and ARA showed a strong concordance with the fatty acid

composition of erythrocytes. These PUFAs are released from membrane phospholipids into cytosol by the PLA_2 and further oxidized by enzymatic processes [63]. Lipoxygenase (LOX) enzymes regulate the oxidation of EPA, DHA, and ARA to the primary and unstable metabolites (hydroperoxides) [64–66]; glutathione peroxidases (GPX) are responsible for the detoxification of deleterious of oxygen radicals [67] and reduce hydroperoxides into secondary metabolites like hydroxides [68]; cyclooxygenases (COX) produce secondary metabolites like TXB_3 from EPA and strong pro-inflammatory compounds from ARA, 11-HETE and PGE_2 [30]. The partial replacement of ARA by EPA and DHA in membranes in ω3 and ω3&GSP groups was consistent with a higher production of derived metabolites from EPA and DHA than derived from ARA. *In vitro* studies showed that the specificity of 12-LOX [69] and PLA_2 [70] was comparable between EPA and ARA; however interventions in the diet with fish oils have shown to favor ω-3 PUFAs, specially EPA, over ω-6 ARA for the same phospholipase enzymes by competence mechanisms [70]. Consequently, in groups supplemented with fish oils, and with lower ω6/ω3 ratios in membranes, the release of PUFAs by PLA_2 was favored towards EPA over ARA. Therefore, the production of eicosanoids was modulated towards the ω-3 hydroxides from EPA instead of the particularly pro-inflammatory ω-6 ARA metabolites, leading to a less inflammatory background compared with controls, probably by substrate competence mechanisms instead of a higher specificity of the enzymes for ω-3 PUFAs. In detail, the ω-3 metabolites have been considered weak-inflammatory compounds [32–35]; meanwhile, the corresponding ω-6 ones have been widely associated with strong pro-inflammatory activities [13,30,31,71], even 10 times more inflammatory than ω-3 derivates [72]. Then, although both fish oils enriched diets showed higher levels of secondary ω-3 metabolites which are considered weak inflammatory compounds, at the same time there was a significant decrease of corresponding strong pro-inflammatory ω-6.

It is remarkable that the proportion EPA:DHA found in control and GSP groups in erythrocytes membranes, as in other tissues and plasma, was highly moved towards greater amounts of DHA *vs.* EPA. Accordingly, metabolites from DHA produced by 15-LOX (17-HpDoHE) were significantly more concentrated than EPA metabolites mediated by 15-LOX (15-HpEPE) in control groups. However, supplementation with fish oils modulated EPA:DHA towards more balanced ratios in both standard and HFHS diets. In agreement, the higher increase of EPA than DHA in membranes produced a competence for 15-LOX that preferably increased the pathway of formation of EPA metabolites instead of DHA. Additionally to the substrate competence, *in vitro* studies have shown that the production of LOX and COX metabolites from EPA is favored over DHA which hardly fits into the active catalytic site of the enzyme due to its larger spatial size [70]. It was also proved that the production of 15-LOX metabolites derived from EPA (15-HpEPE) in fish oils supplemented groups was much favored than the production of 12-LOX metabolites (12-HpEPE). Finally, COX activity on EPA was less favored than LOX activity. In fact, the production of COX derived TXB_3 was not increased related to control in the standard diet as could be expected due to the higher concentration of EPA in the membranes. To the best of our knowledge, the catalysis of

ARA by LOXs and COXs has been studied [73,74], but little is known about kinetic rates in ω-3 substrates or comparative results *in vivo* related with the specificity of these enzymes between ω-6 *vs.* ω-3 PUFAs, and ω-3 EPA *vs.* ω-3 DHA.

Interestingly, higher levels of 17HDoHE were found after supplementation with fish oils compared with controls in both dietary contexts. 17HDoHE is the precursor of powerful anti-inflammatory eicosanoids as protectins (e.g. PD_x) and resolvins (e.g. RvD_1). These protectins and resolvins have been described as pro resolution targets in several inflammatory conditions as cardiovascular diseases, dry eyes or even Alzheimer [34]. Although searched RvD_1, and PD_x were not detected in plasma or were under the limit of detection, other tissues like brain or adipocytes ought to be analyzed as they have been described as potential staging areas of systemic cellular inflammation [75,76]. Additionally, the production of 17HDoHE from DHA and 18-HEPE from EPA has been associated with the suppression of TNFα, and then, with the down-regulation of pro-inflammatory interleukins synthesis [77].

This balance towards a lower inflammatory situation described by fatty acid profiles and lipid mediator synthesis in fish oils supplemented groups compared with controls was in highly agreement to the lower values of TNFα and CRP found in liver (Tab. 6). Lower values of CRP levels have been associated with general decrease of inflammation linked to CVD [78]. It has been also suggested a strong association between the increase of CRP and the synthesis of ARA derivates like PGE_2 and 11-HETE as biomarkers of inflammation [36] as it was found in this work. These results were consistent in the standard diet and in the HFHS diet targeting metabolic disorders.

A set of mechanisms might be implicated in this anti-inflammatory response that may explain data obtained in this work. Firstly, at high dietary intakes, the ω-3 PUFAs substrate can compete *vs* ω-6 PUFAs substrate for the same enzymes, as well as dilute out the concentration of ω-6 in the cell membrane [25,29], thereby decreasing its potential of being converted into pro-inflammatory eicosanoids as it was observed in this work. Secondly, dietary nutrients like ω-3 EPA and DHA can alter the innate immune system towards the enhancement of anti-inflammatory pathways, modulating toll-like receptors like peroxisome proliferator-activated receptor-γ (PPARγ) and gene transcription factors like nuclear factor kB (NF-kB) [12]. In detail, ω-3 EPA and DHA can inhibit the binding of saturated fats with toll-like receptors of membranes by direct competition for these sites. The binding with saturated fats would activate NF-kB gene transcription factor, which induces inflammatory responses through COX and cytokine synthesis [79]. In concurrence, it was observed in this work that fish oils supplementation down-regulated the synthesis of strong pro-inflammatory COX products [3] like PGE_2, 11-HETE, and TXB_3 [80–86] compared with controls.

Finally, the anti-inflammatory response described in ω3 and ω3&GSP groups was accompanied by an improvement of the antioxidant system in these groups. Although MDA is a secondary oxidation product from PUFAs, the enhancement of TBARS in

liver in fish oils supplements was not accompanied by a deleterious of oxidative stress markers. In detail, data of conjugated dienes produced in liver did not show an increase in the ω3 and ω3&GSP groups, on the contrary, they revealed a slightly lower production in the standard diet and no differences in the HFHS one. Additionally, data obtained for plasma antioxidant enzymes (Tab. 6) showed lower levels of SOD, and higher GPX in the ω3 and ω3&GSP groups compared with controls in both standard and HFHS diets. Although not statistically different, plasma antioxidant capacity measured by ORAC showed higher levels in ω3&GSP groups in both standard and HFHS diets. Carbonylation scores of plasma proteins showed a general decrease in the ω3 and ω3&GSP groups in the HFHS diet. Decreasing protein carbonylation levels [7] and SOD activity [87], and increasing ORAC index [88] and GPX activity [89,90] have been associated with the enhancement of the antioxidant system. Accordingly, GPX is the enzyme involved in the detoxification of hydroperoxides to give hydroxides. The up-production of secondary EPA and DHA metabolites mediated by GPX in fish oils groups is in complete agreement with the significant increase observed in the activity of this enzyme in ω3 and ω3&GSP groups related to controls. The increment of TBARS in ω3 and ω3&GSP groups is in agreement with the high dose of PUFAs incorporated with fish oils which are considered oxidizable substrates [11]. MDA is a side PUFA product by enzymatic processes during the biosynthesis of thromboxanes and hydroxyheptadecatrienoic acid catalyzed by COX and prostacyclin hydroperoxidase, respectively [91]. COX activity on ARA was down-regulated in ω3 and ω3&GSP groups compared with control; however TXB_3 pathway produced by COX on EPA was not reduced in the standard diet and even up-regulated in the HFHS diet. Therefore, the increasing EPA substrate of COX in fish oils may be in agreement with higher MDA levels detected. Therefore, the final outcome was a higher endogenous antioxidant activity, especially on GPX, in ω3 and ω3&GSP groups, which influenced the transformation of primary into secondary eicosanoid and docosanoids [67] and decreased post-translational oxidative damages of proteins indicating lower oxidative stress.

4.2 Polyphenols influence

Fish oils exerted the same anti-inflammatory and anti-oxidant effects in both dietary contexts; however, polyphenols showed differences in the efficacy to reduce inflammation depending on the caloric context of the diet. On one hand, it was observed an anti-inflammatory and anti-oxidant bioactivity of polyphenols compared with the control group in the standard diet as commonly described *in vivo* [92,93]. This effect was similar to that found after intervention with fish oils and the combination of both supplements produced an additive response in most of the parameters studied. On the other hand, in the HFHS diet, polyphenols were less effective to reduce inflammation compared with control and even lead to an up-regulation of some pro-inflammatory biomarkers.

The inclusion of polyphenols did not modify composition of feeds compared with controls in both standard and HFHS diets. Accordingly, profiles of liver, adipose tissue,

erythrocyte membranes, and circulating FFA in plasma were similar between control and GSP groups as expected. However, polyphenols exerted an effect modulating desaturase activities in plasma which was not observed in controls. Such effect was consistent in both, standard and HFHS diets. Δ5D and Δ4D indexes showed lower values in GSP group than in controls related to the synthesis of ARA and DHA, respectively. As a consequence, the concentration of DGLA (precursor of ARA) was increased related to controls and the index Δ6D (DGLA/LA) too. The synthesis of EPA regulated by Δ5D and Δ6D tended to decrease as well. Therefore, the FAD indexes suggested a decrease of Δ5D and Δ4D activities due to polyphenols. In this regard, Matsui *et al*. [94] have found that high-fat fed rats supplemented with polyphenols decreased the expression of genes for enzymes involved in fatty acid synthesis in liver and adipose tissue. Although the synthesis *de novo* modulated by these FAD could be affected by the presence of polyphenols, the effect was not reflected in profiles of fatty acids incorporated into phospholipid membranes, adipocytes, or circulating in plasma.

The composition of erythrocyte membranes was not affected by polyphenols, and thus, the substrate of LOX and COX in erythrocytes either. However, polyphenols seemed to modulate the pathways of formation of eicosanoid and docosanoids. Interestingly, their influence was dependent on the background of the diet. In the standard diet the GSP group generally down-regulated the production of ω-3 and ω-6 eicosanoid and docosanoids mediated by LOX, COX, and GPX activities compared with the control group. However, in the HFHS diet, polyphenols showed a tendency to increase products of LOX and GPX, and significantly enhanced the production of ARA eicosanoids mediated by COX.

Several mechanisms could be implicated in the general decrease of PUFAs metabolites production observed in the standard diet. Firstly, flavonoids and other polyphenols have been found to inhibit PLA_2 activity [17,95] which cleaves phospholipids to produce fatty acids, the substrate of LOX and COX. Other studies have suggested a direct influence of grape seed procyanidins inhibiting the activity of LOX [21] and COX [96] at transcriptional and enzyme level; and thus, polyphenols may indirectly block the synthesis of derived eicosanoids as it was observed in this work. Secondly, it was also described the influence of polyphenols up-regulating the nuclear factor-erythroid 2-related factor 2 (Nrf_2) which can activate the expression of antioxidant enzymes like GPX [97,98]. Accordingly, GPX levels were significantly increased after polyphenols interventions in this experiment (Tab. 6) which was directly associated with hydroperoxides detoxification. Polyphenols were also found to down-regulate Nf-kB [99] which activates the expression of COX-2 as explained before; and then, it would explain the decrease of COX products and the total inhibition of TXB_3 synthesis. Finally, polyphenols are well-known for their antioxidant activity [100] as scavengers of ROS [101,102], inhibitors of LDL oxidation and NADPH oxidase activation [22,88], although some outcomes are inconsistent [103,104]. These ROS are initiators of free radical oxidation mechanisms that also lead to the non-enzymatic formation of lipid eicosanoids [105]. Therefore, polyphenols could inhibit lipid peroxidation by

scavenging of ROS. In this regard, the possible mechanisms implicated in the down-regulation of oxidative processes due to polyphenols, agree with the significant enhancement of the antioxidant system (increase of GPX and decrease of SOD), the amelioration of protein carbonylation and reduction of TBARS in liver in GSP group compared with control.

As discussed above, the decrease of most eicosanoid and docosanoids studied is usually associated with the improvement of the inflammatory conditions. Polyphenols have been found to activate PPARγ enhancing anti-inflammatory pathways [101]. The combination of fish oils and polyphenols in the standard diet produced an additive effect giving rise to the minor levels of pro-inflammatory ω-6 metabolites. Accordingly, GSP and ω3&GSP groups also showed lower levels of pro-inflammatory markers (TNFα and CRP) (Tab. 6) compared with control group in the standard diet. In this regard, both polyphenols and ω-3 PUFAs through different mechanisms can inhibit NADPH oxidase activity and ROS generation, which activate NF-kB and the production of several inflammatory molecules. ORAC index (Tab. 6) showed a trend to higher values when fish oils and polyphenols were combined. This index measures the oxidative degradation of fluorescein due to free radical generators and ROS, and higher values may be correlated with lower amounts of ROS in plasma.

Curiously, in the HFHS diet, the presence of procyanidins significantly increased pathways of formation of ω-6 pro-inflammatory products of COX, and tended to increase the production of ω-3 eicosanoids and docosanoids from LOX and GPX, contrary to the effect exerted in the standard dietary context. Thus it suggests an induction to inflammatory conditions compared with controls. Taking into consideration that the Δ5D activity to rend ARA is diminished and COX activity seems to be increased related to control, both findings bring a situation where the production of ARA is down regulated by polyphenols together to an up-regulated specificity for ARA derivatives oxidation mediated by COX. As a consequence, the HFHS context might reduce the effectiveness of procyanidins to improve inflammation observed in the standard diet. In concordance, the pro-inflammatory markers TNFα and CRP in the HFHS experiment (Tab. 6) were not significantly improved in the GSP group as observed in the standard diet. Consequently, the cooperative effect observed in the ω3&GSP group in the standard diet decreasing COX activity on ARA and inflammatory parameters was not found in the HFHS diet due to the different modulation exerted by polyphenols.

Other important outcome was reflected in the amount of circulating FFA. Increasing values have been associated to potentiate and develop an inflammatory status [38] and metabolic disorders associated with lipid overload in non-adipose tissues [106]. Levels of FFA in the standard diet, decreased in GSP group compared with control, and the combined action with ω3 produced an additive decreasing effect. However, in the

HFHS context, values of FFA increased in the GSP group compared with control, and this effect was corrected when they were combined with ω-3.

Bioactivity and mechanism related to inflammation and polyphenols were reviewed by Yeyi Gu *et al* [21]. A number of *in vitro* studies have reported an inhibitory effect of polyphenols on COXs and LOXs mediated eicosanoid production as we described in the standard diet. The combined effect of ω-3 PUFAs and procyanidins inhibiting COX-1 and COX-2 activity *in vitro* was also observed [107]. In addition, animal models fed standard or high fat diets demonstrated a general preventive role of polyphenols in metabolic disorders. However, results on inflammatory markers in the context of Metabolic Syndrome are limited, and the effects on plasma total cholesterol levels are ambiguous. Moreover, recent works have described a strong activation *in vitro* [108] and *in vivo* [109] of COXs catalytic activity by flavonoids. They found a dose-dependent up-regulation *in vivo* of PGE_2 formation, increasing the production at lower doses of polyphenols. A molecular modeling study shed light about the mechanism of activation, and some flavonoids seem to act as cofactors binding tightly to the active site of the COX enzymes facilitating their re-activation [110]. Jenny *et al*. [111] have also found that polyphenols from white tea extracts up-regulated 15-LOX and PPAR-γ signaling pathways, and correlated these findings with the induction of apoptosis in cell lung culture studies. And finally, several works showed that the bioavailability of polyphenols depends on the degree of polymerization, food processing, the presence of other food components, and doses administrated; and then, the bioavailability of different species of polyphenols may influence their bioactivity [112]. In fact, Martinez-Micaelo *et al*. [107] showed specific bioactivities of procyanidins depending on the molecule.

Questions remain underlying mechanisms of action of polyphenols related to inflammation, and conclusions on the bioavailability and bioactivity of phenolic compounds may depend on the synergistic effects between polyphenols and food matrix, as fat or sugar. Therefore, we hypothesized a lower bioavailability of polyphenols in the HFHS diet compared with the standard context. And then, the lower uptake of some polyphenols may be associated with the up-regulation of COX-mediated eicosanoids from ARA and the lower anti-inflammatory effects observed compared with the standard diet.

As regards to the anti-oxidative properties of polyphenols, they seem to work in a similar way in both, standard and HFHS, diets. Carbonylation scores of plasma proteins highlighted the efficiency of dietary polyphenols, and especially when combined with ω-3, to reduce *in vivo* damage of proteins due to oxidative stress. These data also agree with the significant down-regulation of plasma SOD activity, enhancement of plasma GPX and ORAC, and reduction of liver TBARS in the GSP and ω3&GSP groups compared with control (Tab. 6).

5. Conclusion

Fish oils were effective to enhance the anti-inflammatory response and exert an anti-oxidant activity regardless the fat and sucrose composition and content of the diet. Mechanisms related with substrate competition for the same enzymes were suggested to explain the modulation of lipid profiles in tissues, membranes, and plasma, and regulate the synthesis of eicosanoids and docosanoids. As a result, supplementation with fish oils leaded to an anti-inflammatory situation clearly associated to a lower ω-6/ω-3 index in plasma and membranes, a lower production of ARA pro-inflammatory lipid mediators, an up-regulation of desaturases related to EPA and DHA synthesis, and a down-regulation of these desaturases to synthesize ARA. Such situation matches with a decrease of biomarkers of inflammation as TNFα and CRP. The anti-oxidant action exerted by fish oils seemed to be clearly associated to an activation of the antioxidant system, noteworthy detected on the major production of lipid mediators derived from GPX activity, and the decrease of SOD activity and protein carbonylation. However, polyphenols bioactivity resulted to be tentatively influenced by the fat and sugar content of the diet. In a standard diet they seemed to modulate enzymes towards an anti-inflammatory and anti-oxidant response, and the combination with fish oils exerted an interesting cooperative influence: down-regulating Δ5D related with ARA synthesis, decreasing COX activity on ARA, enhancing the antioxidant enzymes, and decreasing total FFA in plasma. Similarly, the combination of both supplements also produced a significant improvement in the antioxidant balance and oxidative stress in HFHS diets. However, the efficacy of polyphenols to reduce inflammation was lower in the HFHS diet and some pro-inflammatory pathways were found even up-regulated. Therefore, fish oils seem to be the main responsible of the anti-inflammatory effects observed in the combined ω3&GSP group in the HFHS diet. As a final outcome, the combination of both bioactive supplements can result in a significant improvement of metabolic health in both standard and HFHS diets by acting on inflammation and oxidative stress pathways.

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Table captions

Tab. 1: Liver TFA composition from animals supplemented with standard (ST) and high-fat high-sucrose (HFHS) diets divided in supplemented subgroups: control (C), fish oils (ω3), grape seed proanthocyanidins (GSP), and fish oil with GSP (ω3&GSP)..

Results are expressed as percentage of total fatty acids (mg/100mg of TFA). Values are shown as means ± SD. Values with different superscript letters in the same row indicate significant difference at $p<0.05$ between dietary groups.

Tab. 2: Plasma TFA composition from animals supplemented with ST and HFHS diets divided in supplemented subgroups: C, ω3, GSP, and ω3&GSP. Results are expressed as percentage of total fatty acids (mg/100mg of TFA). Values are shown as means ± SD. Values with different superscript letters in the same row indicate significant difference at $p<0.05$ between dietary groups.

Tab. 3: FA composition of erythrocyte membranes from animals supplemented with ST and HFHS diets divided in supplemented subgroups: C, ω3, GSP, and ω3&GSP. Results are expressed as percentage of total fatty acids (mg/100mg of TFA). Values are shown as means ± SD. Values with different superscript letters in the same row indicate significant difference at $p<0.05$ between dietary groups.

Tab. 4: TFA composition of the adipose tissue from animals supplemented with ST and HFHS diets divided in supplemented subgroups: C, ω3, GSP, and ω3&GSP. Results are expressed as percentage of total fatty acids (mg/100mg of TFA). Values are shown as means ± SD. Values with different superscript letters in the same row indicate significant difference at $p<0.05$ between dietary groups.

Tab. 5: Circulating FFA composition of plasma from animals supplemented with ST and HFHS diets divided in supplemented subgroups: C, ω3, GSP, and ω3&GSP. Results are expressed as percentage of total fatty acids (mg/100mg of TFA). Values are shown as means ± SD. Values with different superscript letters in the same row indicate significant difference at $p<0.05$ between dietary groups.

Tab. 6: FAD indexes from TFA data of plasma and liver calculated as product/precursor ratio. Conjugate dienes, TBARS, CRP*, and TNFα* calculated in liver. Percentage of total albumin carbonylation index, ORAC*, SOD*, and GPX* activities calculated in plasma. Values are shown as means ± SD. Values with different superscript letters in the same row indicate significant difference at $p<0.05$ between dietary groups. *These parameters have already been studied in the same animals.

Tab. 7: Levels of lipid mediators in plasma derived from ARA, EPA and DHA obtained from the dietary interventions. Results are expressed as ng/mL. Values are shown as means ± SD. One-way and two-way ANOVA analyses were done. Different superscript letters between the subgroups of the same diet indicate significant differences at $p<0.05$ among them. Superscript * indicates significant differences between ST and HFHS diets at $p<0.05$. Superscript # indicates significant interaction between diet (ST and HFHS) and supplement (C, ω3, GSP, ω3&GSP) at $p<0.05$.

Supplementary electronic material captions

S1: Diet composition and experimental design. ST (standard diet), HFHS (high-fat high-sucrose diet), C (control group); ω-3 (fish oils group); GSP (proanthocyanidins group); ω3&GSP (fish oils and proanthocyanidins group). [a]Standard flour (Tekland Global 2014). [b]High fat-high sucrose flour (TD 08811). [c]Grape seed proanthocyanidin extract. [d]27% from sucrose.

S2: Fatty acid composition of supplemented groups in the standard and HFHS diets: C (control group); ω-3 (fish oils group); GSP (polyphenols group); ω3&GSP (fish oils and polyphenols group). Results are expressed as a percentage of total fatty acids (mg/100mg of Total FA).

S3: Retention times, collision energies and MS/MS transitions for ESI-LC-LIT/MS/MS

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Table 1	ST DIET								HFHS DIET							
Liver TFA	C		ω3		GSP		ω3&GSP		C		ω3		GSP		ω3&GSP	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
14:0	0,2[a]	0,02	0,2[ab]	0,02	0,2[ab]	0,01	0,2[b]	0,01	0,5[a]	0,03	0,7[b]	0,004	0,5[a]	0,10	0,7[ab]	0,14
15:0	0,2[a]	0,03	0,1[a]	0,02	0,1[a]	0,03	0,1[a]	0,01	0,3[a]	0,02	0,3[b]	0,005	0,3[a]	0,02	0,4[b]	0,01
16:0	18,5[a]	0,30	19,4[a]	0,35	19,3[a]	0,43	18,3[a]	0,80	20,4[a]	0,75	22,6[b]	0,32	19,9[a]	0,68	22,8[b]	0,32
16:1ω7	0,6[a]	0,08	0,7[a]	0,07	0,8[a]	0,23	0,7[a]	0,04	1,0[a]	0,15	1,1[a]	0,12	0,8[a]	0,14	1,0[a]	0,18
17:0	0,6[a]	0,06	0,5[a]	0,07	0,5[a]	0,10	0,5[a]	0,10	0,6[a]	0,03	0,5[a]	0,02	0,6[a]	0,03	0,5[a]	0,01
17:1ω7	0,2[a]	0,02	0,1[b]	0,00	0,2[a]	0,01	0,1[b]	0,02	0,2[a]	0,02	0,2[a]	0,01	0,2[a]	0,01	0,1[b]	0,01
18:0	18,8[a]	0,34	19,6[a]	1,59	17,6[a]	0,51	19,3[a]	0,57	16,6[a]	1,13	13,6[b]	0,11	17,9[a]	0,93	12,6[b]	0,79
18:1ω9	6,7[a]	0,38	4,9[b]	0,29	7,2[a]	1,09	5,1[b]	0,70	14,3[a]	1,10	13,0[ab]	0,02	12,6[ab]	1,51	12,9[b]	0,11
18:1ω7	1,9[a]	0,02	1,7[a]	0,05	1,9[a]	0,09	1,6[a]	0,11	1,5[a]	0,03	1,4[a]	0,03	1,6[a]	0,09	1,3[a]	0,08
18:2ω6	16,5[a]	0,27	17,1[a]	0,06	17,1[a]	2,14	17,4[a]	0,67	15,4[a]	0,79	13,7[a]	0,08	14,7[a]	0,37	14,3[a]	0,46
18:3ω6	0,3[a]	0,06	0,1[b]	0,01	0,3[a]	0,01	0,1[b]	0,02	0,3[a]	0,04	0,1[b]	0,02	0,2[a]	0,01	0,1[b]	0,01
18:3ω3	0,4[a]	0,02	0,3[b]	0,01	0,5[a]	0,12	0,3[b]	0,02	0,6[ab]	0,06	0,7[a]	0,04	0,5[b]	0,05	0,7[a]	0,07
20:1ω9	0,1[a]	0,003	0,1[a]	0,01	0,1[a]	0,01	0,1[a]	0,01	0,3[a]	0,05	0,4[a]	0,01	0,3[a]	0,03	0,4[a]	0,01
20:2ω6	0,3[a]	0,01	0,2[b]	0,02	0,3[a]	0,03	0,2[b]	0,01	0,1[a]	0,01	0,1[b]	0,02	0,2[a]	0,02	0,1[b]	0,01
20:3ω6	0,2[a]	0,01	0,4[bc]	0,06	0,2[ab]	0,03	0,5[c]	0,15	0,3[a]	0,06	0,3[a]	0,02	0,3[a]	0,003	0,2[a]	0,03
20:4ω6	21,3[a]	0,29	11,8[b]	0,69	20,4[a]	0,68	12,1[b]	0,21	17,9[a]	1,08	7,9[b]	0,15	19,6[a]	1,33	7,6[b]	0,26
20:5ω3	0,3[a]	0,04	4,2[b]	0,49	0,3[a]	0,04	3,8[b]	0,23	0,5[a]	0,08	4,3[b]	0,51	0,4[a]	0,04	4,0[b]	0,36
24:0	0,1[a]	0,02	0,1[a]	0,01	0,2[b]	0,02	0,2[b]	0,01	0,1[a]	0,01	0,1[a]	0,01	0,2[b]	0,03	0,2[b]	0,005
24:1ω11	0,5[a]	0,05	0,1[b]	0,01	0,6[a]	0,11	0,1[b]	0,01	0,3[a]	0,03	0,2[b]	0,03	0,3[a]	0,04	0,2[b]	0,02
22:5ω3	1,5[a]	0,18	1,7[a]	0,04	1,8[a]	0,23	1,7[a]	0,13	1,8[a]	0,06	2,6[b]	0,19	1,8[a]	0,16	2,7[b]	0,21
22:6ω3	10,8[a]	0,05	18,1[b]	0,48	9,0[a]	0,21	17,2[b]	1,33	6,9[a]	0,30	16,1[b]	0,45	7,1[a]	0,36	17,0[b]	0,93
ω6/ω3	3,0[a]	0,1	1,2[b]	0,03	3,4[a]	0,03	1,3[b]	0,1	3,5[a]	0,1	0,9[b]	0,01	3,5[a]	0,1	0,9[b]	0,01
Σω3	12,9[a]	0,3	24,3[b]	0,1	11,6[a]	0,2	23,1[b]	1,6	9,7[a]	0,3	23,7[b]	0,3	9,9[a]	0,5	24,5[b]	0,3
Σω6	38,6[a]	0,5	29,6[b]	0,8	39,3[a]	0,3	30,3[b]	0,9	34,1[a]	0,7	22,0[b]	0,1	35,0[a]	1,1	22,3[b]	0,2
ΣSFA	38,4[a]	0,1	40,0[a]	1,4	38,3[a]	0,5	38,8[a]	0,2	38,4[a]	0,4	37,9[a]	0,5	39,5[a]	0,3	37,2[a]	0,4
ΣMUFA	10,0[a]	0,5	7,6[b]	0,4	10,9[a]	1,3	7,8[b]	0,9	17,8[a]	1,3	16,4[b]	0,1	15,7[ab]	1,7	16,0[b]	0,3
ΣPUFA	51,5[a]	0,6	53,9[b]	0,8	50,9[a]	0,5	53,5[b]	0,8	43,8[a]	1,0	45,8[b]	0,4	44,8[ab]	1,6	46,8[b]	0,6

Table 2	ST DIET								HFHS DIET							
Plasma TFA	C		ω3		GSP		ω3&GSP		C		ω3		GSP		ω3&GSP	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
14:0	0,4[a]	0,03	0,5[a]	0,04	0,4[a]	0,04	0,4[a]	0,03	0,9[a]	0,14	1,1[a]	0,10	1,0[a]	0,14	1,1[a]	0,12
15:0	0,3[a]	0,03	0,3[a]	0,07	0,3[a]	0,03	0,3[a]	0,05	0,4[a]	0,03	0,5[b]	0,05	0,5[ab]	0,04	0,5[a]	0,04
16:0	21,2[a]	0,5	23,7[b]	0,83	21,7[ab]	0,62	21,6[ab]	1,12	21,6[a]	0,93	25,3[b]	1,74	23,2[ab]	0,72	22,8[ab]	0,65
16:1ω7	1,0[a]	0,1	1,1[a]	0,18	1,0[a]	0,26	1,2[a]	0,23	1,0[a]	0,18	1,3[a]	0,11	1,2[a]	0,08	1,2[a]	0,20
17:0	0,5[a]	0,04	0,4[a]	0,05	0,4[a]	0,04	0,4[a]	0,03	0,5[a]	0,01	0,5[a]	0,03	0,4[a]	0,05	0,4[a]	0,04
18:0	15,8[a]	1,2	14,5[a]	1,14	13,3[a]	0,98	13,5[a]	1,37	17,3[a]	1,17	14,0[b]	0,93	13,3[b]	0,76	12,8[b]	0,71
18:1ω9	6,6[a]	0,8	6,4[a]	0,96	7,8[a]	1,26	6,4[a]	1,35	9,3[a]	1,07	10,3[ab]	1,20	14,0[b]	0,59	10,1[a]	0,67
18:1ω7	1,2[a]	0,1	1,1[a]	0,05	1,3[a]	0,12	1,1[a]	0,13	0,9[a]	0,05	0,8[a]	0,06	1,1[a]	0,02	0,9[a]	0,04
18:2ω6	18,3[a]	1,0	20,4[a]	1,07	19,9[a]	1,67	21,2[a]	1,77	14,0[a]	0,92	15,1[a]	0,44	16,5[b]	0,15	16,2[ab]	0,55
18:3ω6	0,4[a]	0,1	0,2[b]	0,07	0,4[a]	0,04	0,2[b]	0,03	0,4[a]	0,04	n.d.		0,4[a]	0,03	n.d.	
18:3ω3	0,4[a]	0,02	0,4[a]	0,08	0,5[a]	0,11	0,4[a]	0,08	0,4[a]	0,08	0,4[a]	0,06	0,6[b]	0,04	0,4[a]	0,05
20:3ω6	0,4[a]	0,03	0,6[b]	0,04	1,0[c]	0,11	1,4[d]	0,16	0,6[a]	0,08	0,9[abc]	0,44	1,0[b]	0,11	1,4[c]	0,14
20:4ω6	27,5[a]	0,8	13,1[b]	1,25	26,4[a]	2,36	14,4[b]	2,02	27,6[a]	1,72	12,4[b]	0,77	22,3[a]	0,82	13,4[b]	1,38
20:5ω3	0,5[a]	0,1	6,5[b]	0,70	0,4[a]	0,05	6,6[b]	0,76	0,7[a]	0,10	6,1[b]	1,13	0,6[a]	0,02	6,5[b]	0,78
22:5ω3	0,8[a]	0,05	1,3[b]	0,07	1,0[a]	0,05	1,5[b]	0,11	1,1[a]	0,04	1,6[b]	0,14	1,1[a]	0,09	1,7[b]	0,20
22:6ω3	4,7[a]	0,4	9,3[b]	0,35	3,9[a]	0,49	9,4[b]	0,48	3,2[a]	0,16	9,7[b]	0,74	3,0[a]	0,23	10,6[b]	0,83
ω6/ω3	7,3[a]	0,6	2,0[b]	0,1	8,3[a]	0,5	2,1[b]	0,2	8,1[a]	0,4	1,6[b]	0,1	7,7[a]	0,3	1,6[b]	0,1
Σω3	6,4[a]	0,4	17,5[b]	0,8	5,8[a]	0,4	17,9[b]	1,1	5,3[a]	0,2	17,8[b]	1,8	5,2[a]	0,3	19,2[b]	0,7
Σω6	46,6[a]	0,5	34,4[b]	0,6	47,7[a]	1,2	37,1[c]	1,4	42,7[a]	1,4	28,4[b]	1,2	40,1[a]	0,7	31,0[b]	1,2
ΣSFA	37,6[ab]	1,0	39,5[b]	0,7	36,1[a]	0,5	36,2[a]	1,3	40,6[ab]	1,4	41,4[a]	2,3	38,3[ab]	1,5	37,6[b]	0,5
ΣMUFA	9,4[a]	1,0	8,6[a]	1,2	10,4[a]	1,6	8,7[a]	1,7	11,4[a]	1,1	12,4[a]	1,3	16,4[b]	0,7	12,2[a]	0,7
ΣPUFA	53,0[ab]	0,3	51,9[a]	1,0	53,5[ab]	1,3	55,0[b]	1,5	48,0[ab]	1,5	46,2[b]	2,6	45,4[b]	1,0	50,2[a]	0,8
TFA ug/mL	3242,9[a]	380,9	2873,8[a]	343,7	3426,7[a]	338,5	2770,9[a]	438,6	2442,2[a]	245,0	2111,7[a]	305,7	3638,4[b]	183,4	2101,4[a]	231,1

Table 3 Erythrocite TFA	ST DIET								HFHS DIET							
	C		ω3		GSP		ω3&GSP		C		ω3		GSP		ω3&GSP	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
14:0	0,3[a]	0,1	0,6[b]	0,1	0,5[ab]	0,1	0,4[a]	0,02	0,7[bc]	0,1	0,8[c]	0,1	0,7[bc]	0,1	0,7[bc]	0,1
15:0	0,3[a]	0,02	0,3[a]	0,1	0,3[a]	0,04	0,3[a]	0,05	0,4[b]	0,03	0,5[b]	0,02	0,4[b]	0,1	0,5[b]	0,03
16:0	28,1[a]	0,9	29,2[a]	1,6	28,5[a]	0,4	28,2[a]	0,4	25,5[b]	0,7	27,1[ab]	0,6	25,4[b]	0,6	27,0[ab]	0,6
16:1 ω7	0,4[a]	0,1	0,5[a]	0,05	0,4[a]	0,04	0,4[a]	0,02	0,5[a]	0,1	0,5[a]	0,1	0,4[a]	0,04	0,5[a]	0,1
17:0	0,7[a]	0,1	0,7[a]	0,1	0,6[a]	0,1	0,6[a]	0,04	0,7[a]	0,1	0,7[a]	0,03	0,7[a]	0,1	0,7[a]	0,03
18:0	18,5[a]	1,3	17,8[a]	0,8	19,0[a]	0,5	18,0[a]	0,4	19,2[a]	0,3	16,8[a]	0,2	18,8[a]	0,5	17,1[a]	0,4
18:1 ω9	6,7[a]	0,4	6,2[ab]	0,3	6,6[a]	0,1	6,1[b]	0,1	8,6[c]	0,2	8,7[c]	0,1	8,4[c]	0,3	8,6[c]	0,2
18:1 ω7	2,2[ab]	0,1	2,0[a]	0,1	2,2[b]	0,0	1,9[a]	0,05	1,7[c]	0,1	1,6[c]	0,1	1,7[c]	0,04	1,6[c]	0,1
18:2 ω6	10,0[a]	1,1	10,5[a]	0,4	9,5[a]	0,4	10,5[a]	0,2	10,1[a]	0,5	9,8[a]	0,2	10,1[a]	0,4	10,1[a]	0,3
18:3 ω6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:3 ω3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:3 ω6	0,7[ab]	0,1	0,7[ab]	0,03	0,6[a]	0,05	0,8[b]	0,02	0,9[c]	0,1	0,9[c]	0,01	0,9[c]	0,05	0,9[c]	0,1
20:4 ω6	24,2[a]	0,9	15,4[bc]	0,7	24,7[a]	0,3	16,1[b]	0,4	24,0[a]	0,5	14,2[c]	0,5	24,8[a]	0,3	14,6[c]	0,2
20:5 ω3	0,3[ac]	0,1	3,0[b]	0,3	0,2[a]	0,03	3,1[b]	0,2	0,4[c]	0,02	4,0[d]	0,3	0,4[c]	0,02	3,9[d]	0,4
22:5 ω3	2,5[a]	0,2	4,2[b]	0,2	2,2[a]	0,2	4,5[b]	0,1	3,3[c]	0,2	5,1[d]	0,1	3,3[c]	0,1	5,1[d]	0,3
22:6 ω3	4,9[a]	0,7	8,7[b]	0,4	4,4[a]	0,4	8,9[b]	0,2	3,8[c]	0,2	9,2[b]	0,2	3,6[c]	0,3	8,7[b]	0,4
ω6/ω3	4,6[a]	0,7	1,7[b]	0,1	5,1[a]	0,2	1,7[b]	0,04	4,7[a]	0,1	1,4[b]	0,1	4,9[a]	0,2	1,4[b]	0,03
Σω3	7,7[a]	0,9	15,9[b]	0,8	6,9[a]	0,3	16,4[b]	0,3	7,4[a]	0,2	18,3[c]	0,3	7,4[a]	0,3	17,7[c]	0,3
Σω6	35,2[a]	1,1	26,8[bc]	0,9	35,1[a]	0,6	27,6[b]	0,4	35,3[a]	0,7	25,1[c]	0,7	36,1[a]	0,4	25,7[c]	0,4
ΣSFA	47,9[ab]	0,7	48,7[a]	1,6	48,9[a]	0,6	47,5[ab]	0,4	46,4[bc]	0,8	45,8[c]	0,7	46,0[b]	0,6	45,9[c]	0,4
ΣMUFA	9,3[a]	0,5	8,6[ab]	0,4	9,2[a]	0,1	8,4[b]	0,2	10,8[c]	0,2	10,8[c]	0,2	10,5[c]	0,4	10,6[c]	0,2
ΣPUFA	42,9[a]	0,5	42,7[a]	1,4	42,0[a]	0,6	44,1[a]	0,5	42,8[a]	0,8	43,4[a]	0,7	43,4[a]	0,5	43,4[a]	0,5

Table 4	ST DIET								HFHS DIET							
Adipose TFA	C		ω3		GSP		ω3&GSP		C		ω3		GSP		ω3&GSP	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
14:0	0,8[a]	0,01	1,2[b]	0,02	0,7[a]	0,02	1,3[b]	0,03	4,5[a]	0,06	5,3[b]	0,24	4,4[a]	0,28	5,4[b]	0,26
14:1ω5	0,1[a]	0,01	0,1[b]	0,01	0,1[a]	0,01	0,1[ab]	0,01	0,4[ab]	0,02	0,5[b]	0,05	0,4[a]	0,04	0,5[b]	0,04
15:0	0,3[a]	0,02	0,3[a]	0,07	0,2[a]	0,05	0,3[a]	0,05	0,7[a]	0,02	0,8[a]	0,03	0,7[a]	0,02	0,8[a]	0,04
16:0	19,4[a]	0,02	21,4[b]	0,65	19,6[a]	0,52	22,4[b]	0,81	23,9[a]	0,55	26,2[b]	0,77	24,7[ab]	0,63	25,3[ab]	1,57
16:1ω7	2,1[a]	0,22	3,2[b]	0,10	2,3[a]	0,21	3,4[b]	0,30	3,2[ab]	0,24	4,0[a]	0,65	2,7[b]	0,29	3,5[ab]	0,66
17:0	0,4[ab]	0,06	0,5[a]	0,05	0,3[b]	0,03	0,5[a]	0,04	0,6[a]	0,01	0,7[b]	0,04	0,6[ab]	0,05	0,7[b]	0,03
17:1ω7	0,2[a]	0,01	0,3[a]	0,02	0,2[a]	0,01	0,3[a]	0,03	0,4[a]	0,01	0,4[a]	0,04	0,4[a]	0,01	0,4[a]	0,04
18:0	3,2[a]	0,15	3,2[a]	0,08	2,9[a]	0,21	3,3[a]	0,21	5,4[a]	0,38	5,9[a]	0,63	5,7[a]	0,21	6,0[a]	0,39
18:1ω9	28,4[a]	0,57	25,0[b]	0,69	29,2[a]	0,81	24,9[b]	0,54	37,5[a]	0,25	35,2[b]	1,03	37,6[a]	0,38	35,2[b]	2,02
18:1ω7	3,1[a]	0,04	3,0[a]	0,10	3,2[a]	0,08	2,9[a]	0,13	2,3[a]	0,02	2,1[a]	0,06	2,3[a]	0,11	2,2[a]	0,18
18:2ω6	38,0[a]	0,47	33,3[b]	0,71	37,5[a]	1,07	32,7[b]	1,08	18,3[a]	0,27	14,5[b]	0,48	17,8[a]	0,22	14,9[b]	0,36
18:3ω6	0,1[a]	0,01	0,1[a]	0,01	0,1[a]	0,01	0,1[a]	0,01	n.d.		n.d.		n.d.		n.d.	
20:0	0,1[a]	0,01	0,1[a]	0,01	0,1[a]	0,01	0,1[a]	0,02	0,1[a]	0,01	0,1[a]	0,005	0,1[a]	0,003	0,1[a]	0,03
18:3ω3	1,6[a]	0,05	1,5[a]	0,01	1,6[a]	0,02	1,5[a]	0,03	1,2[a]	0,004	1,1[a]	0,03	1,1[a]	0,03	1,1[a]	0,19
20:1ω9	0,4[a]	0,03	0,5[a]	0,03	0,4[a]	0,07	0,5[a]	0,07	0,8[a]	0,01	0,9[a]	0,03	0,8[a]	0,02	0,9[a]	0,04
20:2ω6	0,3[a]	0,02	0,2[a]	0,02	0,2[a]	0,02	0,2[a]	0,03	0,1[a]	0,01	0,1[a]	0,01	0,1[a]	0,01	0,1[a]	0,01
20:3ω6	0,1[ab]	0,01	0,2[b]	0,01	0,1[a]	0,003	0,2[b]	0,002	0,1[a]	0,01	0,1[a]	0,004	0,1[a]	0,01	0,1[b]	0,01
20:4ω6	0,6[a]	0,09	0,4[b]	0,03	0,6[a]	0,07	0,4[b]	0,02	0,3[a]	0,02	0,2[b]	0,02	0,3[a]	0,03	0,2[ab]	0,01
20:5ω3	0,1[a]	0,01	0,7[a]	0,06	n.d.		0,8[b]	0,07	n.d.		0,3[a]	0,02	n.d.		0,3[a]	0,06
24:1ω11	0,05[a]	0,01	0,2[b]	0,01	0,2[b]	0,03	0,2[b]	0,02	0,1[a]	0,01	0,1[a]	0,01	0,1[a]	0,01	0,1[a]	0,00
22:5ω3	0,3[a]	0,03	1,5[b]	0,14	0,2[a]	0,02	1,3[b]	0,16	0,1[a]	0,01	0,6[b]	0,07	0,1[a]	0,02	0,8[b]	0,07
22:6ω3	0,4[a]	0,04	2,6[b]	0,20	0,2[a]	0,02	2,5[b]	0,24	0,1[a]	0,003	0,8[b]	0,12	0,1[a]	0,01	1,2[b]	0,13
ω6/ω3	17,1[a]	0,8	5,3[b]	0,2	20,2[a]	0,2	5,3[b]	0,5	14,3[a]	0,3	5,3[b]	0,3	14,6[a]	0,6	4,5[b]	0,6
Σω3	2,3[a]	0,1	6,5[b]	0,3	1,9[a]	0,1	6,3[b]	0,5	1,3[a]	0,04	2,8[b]	0,2	1,2[a]	0,1	3,5[b]	0,3
Σω6	39,1[a]	0,6	34,3[b]	0,8	38,6[a]	1,2	33,5[b]	1,1	18,7[a]	0,3	14,9[b]	0,5	18,2[a]	0,2	15,4[b]	0,4
ΣSFA	24,3[a]	0,2	26,9[b]	0,5	24,0[a]	0,3	27,9[b]	0,6	35,3[a]	0,7	39,1[b]	1,3	36,3[a]	0,4	38,4[b]	1,9
ΣMUFA	34,4[a]	0,7	32,4[b]	0,6	35,6[a]	1,0	32,3[b]	0,7	44,7[a]	0,5	43,2[a]	1,7	44,2[a]	0,6	42,8[a]	1,8
ΣPUFA	41,4[a]	0,6	40,7[a]	1,1	40,5[a]	1,2	39,8[a]	1,2	20,0[a]	0,3	17,7[b]	0,7	19,5[a]	0,3	18,8[b]	0,2

Table 5
Circulating plasma FFA

	ST DIET								HFHS DIET							
	C		**ω3**		**GSP**		**ω3&GSP**		**C**		**ω3**		**GSP**		**ω3&GSP**	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
14:0	1,6[a]	0,04	2,0[b]	0,2	1,9[b]	0,1	2,3[b]	0,3	4,6[c]	0,3	5,1[c]	0,2	3,8[d]	0,2	5,8[e]	0,3
15:0	0,6[a]	0,05	0,7[a]	0,1	0,4[a]	0,4	0,7[a]	0,2	1,0[b]	0,1	1,1[b]	0,1	0,9[a]	0,00	1,1[b]	0,1
16:0	29,5[a]	0,2	32,2[bc]	0,6	31,2[b]	0,6	32,1[bc]	1,7	33,0[c]	0,8	33,9[c]	0,5	33,1[c]	0,1	33,7[c]	1,0
16:1 ω7	3,6[a]	0,2	3,4[a]	0,6	3,5[a]	0,5	3,5[a]	0,9	3,7[a]	0,6	4,2[a]	0,4	3,3[a]	0,2	4,5[a]	0,7
17:0	1,6[a]	1,0	1,6[a]	1,2	1,2[a]	1,2	1,6[a]	1,4	1,5[a]	1,2	2,0[a]	1,4	1,3[a]	1,0	1,8[a]	1,5
18:0	7,3[a]	0,3	9,2[ab]	1,3	8,5[ab]	0,9	10,2[bc]	1,4	10,7[bc]	1,3	12,7[c]	0,9	9,6[b]	0,2	12,5[c]	1,6
18:1 ω9	18,7[a]	0,4	13,5[b]	1,0	17,2[a]	0,7	12,8[b]	1,7	21,6[cd]	1,9	18,4[ac]	2,5	22,8[d]	1,0	17,6[a]	1,4
18:1 ω7	2,3[a]	0,2	1,7[bc]	0,1	2,2[ab]	0,3	1,7[bcd]	0,4	1,4[cd]	0,2	1,2[d]	0,2	1,5[c]	0,04	1,2[d]	0,1
18:2 ω6	27,0[a]	0,6	22,1[b]	1,4	26,4[a]	1,0	21,9[b]	1,1	15,1[c]	0,6	10,8[d]	0,2	16,7[c]	0,1	11,2[d]	1,3
18:3 ω6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
18:3 ω3	1,4[a]	0,1	1,2[b]	0,1	1,4[ab]	0,1	1,2[bc]	0,1	1,2[b]	0,1	1,0[c]	0,1	1,3[ab]	0,1	1,0[c]	0,1
20:3 ω6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
20:4 ω6	4,1[a]	0,4	2,6[b]	0,5	4,3[a]	0,7	2,7[b]	0,4	4,2[a]	0,6	1,9[b]	0,2	4,1[a]	0,1	2,0[b]	0,2
20:5 ω3	0,2[a]	0,1	2,3[b]	0,3	0,1[a]	0,01	2,3[b]	0,2	0,2[a]	0,1	2,1[b]	0,6	0,2[a]	0,1	2,0[b]	0,2
22:5 ω3	0,5[a]	0,1	1,8[b]	0,3	0,5[a]	0,2	1,8[b]	0,3	0,6[a]	0,1	1,5[b]	0,5	0,7[a]	0,1	1,4[b]	0,3
22:6 ω3	1,2[a]	0,1	5,3[b]	0,3	0,9[c]	0,1	4,8[b]	1,0	0,7[c]	0,1	4,0[b]	0,9	0,7[c]	0,03	3,9[b]	0,7
ω6/ω3	9,5[ac]	1,2	2,3[b]	0,1	10,8[a]	1,2	2,5[b]	0,4	7,3[c]	0,7	1,6[d]	0,4	7,3[c]	0,7	1,6[d]	0,2
Σω3	3,3[a]	0,4	10,7[b]	0,4	2,9[a]	0,2	10,1[b]	1,4	2,7[a]	0,3	8,6[b]	2,1	2,9[a]	0,3	8,4[b]	1,0
Σω6	31,1[a]	0,9	24,7[b]	1,0	30,8[a]	1,2	24,5[b]	1,0	19,2[c]	1,2	12,8[d]	0,2	20,8[c]	0,2	13,2[d]	1,2
ΣSFA	40,7[a]	0,9	45,7[bc]	1,2	43,3[ac]	2,3	47,0[bc]	2,2	50,9[bd]	1,6	54,7[d]	1,0	48,7[b]	0,8	54,8[d]	2,7
ΣMUFA	24,9[a]	0,3	18,9[b]	1,1	23,1[a]	1,4	18,3[b]	3,2	27,2[c]	2,8	24,0[a]	2,5	27,6[c]	0,9	23,6[a]	2,1
ΣPUFA	34,4[a]	0,7	35,4[a]	1,1	33,6[a]	1,0	34,7[a]	1,5	21,9[b]	1,3	21,3[b]	2,1	23,7[b]	0,2	21,6[b]	1,8
Total FFA (μg/mL)	212,0[a]	22,1	164,2[ab]	32,0	166,6[ab]	24,2	147,5[b]	30,0	169,4[ab]	30,0	140,2[ac]	15,9	187,8[b]	4,5	136,6[c]	25,2

Table 6	ST DIET								HFHS DIET							
	C		ω3		GSP		ω3&GSP		C		ω3		GSP		ω3&GSP	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
FAD indexes in plasma																
SCD-16 [palmitoleic/palmitic]	0,05[a]	0,005	0,05[a]	0,006	0,05[a]	0,01	0,05[a]	0,009	0,05[a]	0,01	0,1[a]	0,01	0,1[a]	0,005	0,1[a]	0,01
SCD-18 [oleic/stearic]	0,4[a]	0,09	0,4[a]	0,1	0,6[a]	0,1	0,5[a]	0,1	0,5[a]	0,10	0,7[ab]	0,1	1,1[c]	0,1	0,8[b]	0,1
Δ5D [ARA/DGLA]	62,9[a]	4,1	20,8[b]	1,8	26,9[c]	2,9	10,7[d]	0,8	43,0[a]	4,9	15,6[b]	4,5	23,7[c]	3,2	9,7[d]	1,3
Δ6D [DGLA/LA]	0,02[a]	0,002	0,03[a]	0,004	0,1[b]	0,008	0,1[b]	0,01	0,05[a]	0,01	0,1[ab]	0,03	0,1[a]	0,01	0,1[b]	0,01
Δ6/5D [EPA/ALA]	1,2[a]	0,3	17,2[b]	4,3	0,8[a]	0,2	17,3[b]	6,4	2,0[a]	0,5	14,3[b]	3,8	1,0[a]	0,06	15,3[b]	1,9
Δ4D [DHA/DPA]	5,7[a]	0,7	7,0[b]	0,3	4,1[c]	0,6	6,5[ab]	0,8	3,1[a]	0,1	6,3[b]	0,5	2,7[a]	0,3	6,3[b]	0,9
FAD indexes in liver																
SCD-16 [palmitoleic/palmitic]	0,03[a]	0,004	0,04[a]	0,003	0,04[a]	0,01	0,04[a]	0,003	0,05[a]	0,01	0,05[a]	0,01	0,04[a]	0,01	0,04[a]	0,01
SCD-18 [oleic/stearic]	0,4[a]	0,01	0,3[b]	0,01	0,4[a]	0,1	0,3[b]	0,03	0,9[a]	0,1	1,0[a]	0,01	0,7[a]	0,1	1,0[a]	0,1
Δ5D [ARA/DGLA]	107,0[a]	4,8	31,9[b]	2,9	92,5[a]	12,1	26,5[b]	8,5	64,3[a]	8,7	31,4[b]	1,5	71,2[a]	4,3	31,4[b]	4,2
Δ6D [DGLA/LA]	0,01[a]	0,0004	0,02[b]	0,003	0,01[a]	0,002	0,03[b]	0,007	0,02[a]	0,004	0,02[a]	0,001	0,02[a]	0,001	0,02[a]	0,002
Δ6/5D [EPA/ALA]	0,6[a]	0,07	13,7[b]	1,5	0,6[a]	0,08	12,1[b]	1,3	0,8[a]	0,2	6,3[b]	0,4	1,0[a]	0,2	5,9[b]	0,3
Δ4D [DHA/DPA]	7,4[a]	0,9	10,6[b]	0,5	4,9[c]	0,6	9,9[b]	0,8	3,9[a]	0,1	6,3[b]	0,7	3,9[a]	0,2	6,3[b]	0,8
Lipid oxidation parameters in liver																
Conjugated dienes (mmol hydroperoxides/kg of lipid)	19,6[a]	0,9	17,2[b]	1,1	20,3[a]	1,8	16,2[b]	2,1	27,5[a]	1,5	29,8[a]	2,9	27,7[a]	2,2	27,5[a]	2,8
TBARS (mg MDA/kg of liver)	4,5[ac]	1,4	10,0[b]	2,4	3,2[c]	0,2	7,6[ab]	1,7	3,6[a]	0,7	10,7[b]	2,0	2,7[a]	0,3	6,6[c]	1,4
Oxidative stress parameters in plasma																
Total albumin carbonylation index (%)	36,8[a]	2,6	43,2[a]	4,1	28,1[b]	1,9	34,3[ab]	9,1	41,8[a]	3,3	37,3[a]	3,2	30,3[b]	2,8	24,3[b]	4,3
ORAC (µmol Trolox/mL plasma)	17,6[a]	8,3	18,8[a]	4,4	16,6[a]	5,1	22,1[a]	2,9	17,7[ab]	7,9	10,7[a]	5,7	19,3[ab]	5,0	24,0[b]	3,5
SOD (U/g Hb)*	3698[a]	512	3277[ab]	395	3033[ab]	274	2285[b]	1083	4688[a]	1344	3856[ab]	689	2619[b]	1338	2787[ab]	1462
GPX (U/g Hb)*	0,4[a]	0,1	1,2[a]	0,7	6,1[b]	0,5	19,2[c]	3,01	0,4[a]	0,04	3,8[a]	0,9	13,4[b]	1,9	36,7[c]	8,5
Inflammation parameters in liver																
CRP (mg/mL)*	91,4[a]	21,6	45,4[b]	15,7	49,7[b]	7,9	56,1[b]	26,5	93,9[a]	53,7	64,3[a]	18,9	76,7[a]	41,7	60,3[a]	46,9
TNFα (mg/mL)*	116,0[a]	72,5	63,8[b]	26,3	73,1[b]	66,7	62,6[b]	47,6	91,5[a]	47,8	55,2[b]	49,3	85,2[ab]	59,5	54,5[b]	65,4

Table 7	ST DIET								HFHS DIET							
	C		ω3		GSP		ω3&GSP		C		ω3		GSP		ω3&GSP	
	mean	SD	mean	SD	mean	SD	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.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
12HEPE	80[a]	44	468[b]	433	34[a]	15	195[ab]	191	35[a]	18	312[b]	176	19[a]	10	370[b]	96
15HEPE[*]	3,4[ac]	1,2	7,5[b]	3,5	1,6[a]	0,1	5,6[bc]	1,5	1,6[a]	0,2	4,6[b]	1,7	1,6[a]	0,2	5,0[b]	1,7
12HpEPE[#*]	593[a]	188	208[b]	66	200[b]	95	117[b]	81	270[a]	178	46[b]	23	387[a]	181	99[b]	32
15HpEPE[#*]	219[a]	131	2752[b]	594	86[a]	36	2269[b]	539	86[a]	43	376[b]	121	24[a]	6	511[c]	127
$8isoPGF_{3\alpha}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
TXB_3	3,4[a]	0,2	4,4[a]	1,5	n.d.	n.d.	3,6[a]	0,6	n.d.	n.d.	3,7[a]	0,6	n.d.	n.d.	4,0[a]	1,0
PGD_3/E_3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	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[#]	55[a]	20	148[b]	104	35[a]	12	77[a]	51	23[a]	11	101[b]	45	13[a]	5	170[c]	73
11HDoHE	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	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.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
17HpDoHE[#*]	2062[a]	434	1437[b]	367	1466[b]	333	1269[b]	195	1752[a]	476	1677[a]	313	1857[a]	298	1856[a]	598
RvD_1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
PD_x	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	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[#]	7,7[a]	4,5	2,5[b]	2,4	3,2[b]	1,6	0,5[b]	0,3	2,4[a]	0,6	1,0[a]	0,7	8,2[b]	4,8	1,4[a]	1,0
PGE_2[#]	16[a]	12	7[b]	4	7[b]	3	4[b]	1	6[a]	2	4[a]	2	10[b]	6	4[a]	1
LTB_4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
$8isoPGF_{2\alpha}$	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

S1

Dietary groups	ST-C	ST- ω3	ST-GSP	ST- ω3&GSP	HFHS-C	HFHS- ω3	HFHS-GSP	HFHS-ω3&GSP
Diet composition								
Flour (g)	1000[a]	1000[a]	1000[a]	1000[a]	1000[b]	1000[b]	1000[b]	1000[b]
Porcine gelatine (g)	25	25	25	25	25	25	25	25
Soybean lecithin (g)	6	6	6	6	22	22	22	22
Polyphenol extract[c] (mg)	-	-	880	880	-	-	1090	1090
Oil (mL)	19 mL soybean	19 mL EPA:DHA 1:1	19 mL soybean	19 mL EPA:DHA 1:1	24 mL soybean	24 mL EPA:DHA 1:1	24 mL soybean	24 mL EPA:DHA 1:1
Macronutrients (% weight)								
Protein	16.4	16.4	16.4	16.4	21.7	21.7	21.7	21.7
Fat	6.2	6.2	6.2	6.2	24.1	24.1	24.1	24.1
Carbohydrates	46.6	46.6	46.5	46.5	45.0	45.0	44.9	44.9
Macronutrients (% caloric value)								
Protein	21,3	21.3	21.3	21.3	17,9	17.9	17.9	17.9
Fat	18,2	18.2	18.2	18.2	44,9	44.9	44.9	44.9
Carbohydrates	60,5	60.5	60.5	60.5	37,2[d]	37.2[d]	37.2[d]	37.2[d]
Total energy density (kcal/g)	3.1	3.1	3.1	3.1	4.8	4.8	4.8	4.8

S2

	C and GSP groups		ω3 and ω3&GSP groups	
	Standard diet	**HFHS diet**	**Standard diet**	**HFHS diet**
14:0	0,017	11,5	0,075	11,6
16:0	14,8	32,2	14,6	32,0
16:1ω7	0,015	1,7	0,08	1,7
18:0	2,9	13,7	2,9	13,7
18:1ω9	20,5	26,4	20,3	26,2
18:1ω7	0,026	0,67	0,033	0,68
18:2ω6	58,6	9,7	57,8	8,7
20:0	n.d.	n.d.	0,0055	0,0069
18:3ω3	2,9	1,1	2,9	1,1
20:1ω9	0,025	0,031	0,017	0,021
18:4ω3	0,0026	0,0032	0,026	0,033
20:2ω6	0,0034	0,0043	0,0036	0,0045
20:3ω6	n.d.	n.d.	0,0038	0,0048
20:4ω6	0,0069	0,0086	0,029	0,036
22:1ω11	0,019	0,023	0,02	0,024
22:1ω9	0,0043	0,0054	0,0048	0,006
20:4ω3	0,0034	0,0043	0,018	0,022
20:5ω3	0,012	0,015	0,43	0,54
24:1ω9	0,0048	0,0060	0,0065	0,0082
22:5ω3	0,0045	0,0056	0,074	0,093
22:6ω3	0,02	0,024	0,44	0,56
Total SFAs	17,5	59,4	18,3	60,5
Total MUFAs	21,1	29,3	20,3	28,4
Total PUFAs	61,1	10,8	61,0	10,7
Total ω3	3,3	1,6	3,2	1,4
Total ω6	58,8	10,4	58,9	10,6

S3

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