Changes in liver proteins of rats fed standard and high-fat and sucrose diets induced by fish omega-3 PUFAs and their combination with grape polyphenols according to quantitative proteomics

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Running title: Joint action of fish oil & polyphenols on protein regulation

Abstract

This study considered the physiological modulation of liver proteins due to the supplementation with fish oils under two dietary backgrounds: standard or high in fat and sucrose (HFHS), and their combination with grape polyphenols. By using a quantitative proteomics approach, we showed that the capacity of the supplements for regulating proteins depended on the diet; namely, 10 different proteins changed into standard diets, while 45 changed into the HFHS diets and only scarcely proteins were found altered in common. However, in both contexts, fish oils were the main regulatory force, although the addition of polyphenols was able to modulate some fish oils' effects. Moreover, we demonstrated the ability of fish oils and their combination with grape polyphenols in improving biochemical parameters and reducing lipogenesis and glycolysis enzymes, enhancing fatty acid beta-oxidation and insulin signaling and ameliorating endoplasmic reticulum stress and protein oxidation when they are included in an unhealthy diet.

Keywords: fish oils; grape polyphenols; high-fat high-sucrose diet; bioactive compounds; liver protein regulation; Wistar Kyoto rat

1. Introduction

Diet composition exerts a critical role in the maintenance of human health and the progression of pathologic states, because nutrients can interact and modulate molecular mechanisms which govern physiological functions [1]. It has been largely accepted that consumption of marine foods and their long-chain ω-3 fatty acids as well as intake of plant-based foods rich in bioactive compounds as grape polyphenols are involved in health promotion and disease risk reduction. In fact, fish oils and polyphenolic compounds are commonly used as nutraceutical products for both preventive and palliative strategies [2, 3].

Regarding to ω-3 PUFAs of marine origin, habitual consumption of EPA and DHA have been implicated in both prevention and palliation of metabolic disorders, diabetes and cardiovascular disease (CVD), principally due to their ability to decrease blood pressure and levels of insulin, triglycerides, cholesterol, free fatty acids or total lipids [4]. They modulate these parameters via different mechanisms: EPA and DHA can alter membrane fluidity, are substrates for enzymes including cyclooxygenase, lipoxygenase and cytochrome P450 and can regulate the expression of genes involved in lipid, protein and carbohydrates metabolisms [5, 6]. Although it is not fully known, marine omega-3 PUFAs control gene expression by reducing the levels of sterol regulatory element binding proteins (SREBPs) and the carbohydrate response element binding protein (ChREBP) or by direct interaction with at least 4 metabolic nuclear receptors: PPAR (peroxisome proliferator activated receptor), LXR (liver X receptor), HNF-4α (hepatic nuclear factor 4) and farnesol X receptor (FXR) [7]. Also, these PUFAs have showed anti-inflammatory properties by inhibiting the nuclear factor kb (NF-κB) and rising to reduce cytokine production [8]. A potential role of marine omega-3 acting against oxidative stress, a key factor subjacent in mostly metabolic disorders, has been recently suggested as well [9].

Moreover, the composition of marine oil supplements in terms of the proportion of EPA and DHA seems to be clue for the action of these supplementations on metabolic disorders. Indeed, in the last years several studies have demonstrated that a balanced proportion between EPA and DHA (EPA:DHA 1:1) provides a higher reduction on cardiovascular disease (CVD) risk markers, oxidative stress parameters in healthy Wistar Kyoto rats [10, 11] and also in an animal model of metabolic syndrome (SHROB rats) [12, 13].

Despite the huge variety of the beneficial health effects above exposed for marine omega-3 supplementation, dietary EPA and DHA may result in a high susceptibility to lipid oxidation under high fat diet or obesity [14], physiological conditions known to prone oxidative stress [15]. On the other hand, lipid peroxidation products such as 4-hydroxynonenal (4-HNE) trigger fat accumulation [16], and proteasome dysfunction associated to oxidative stress compromises insulin sensitivity in human obesity [17]. The incorporation of antioxidants on diet is a common strategy to cope with lipid oxidation. Specially, natural polyphenolics are well-known for preventing the peroxidation of fish oils *in vitro* [18] and *in vivo* [19] conditions leading to an improvement on the intestinal absorption of omega-3 PUFA. The consumption of grape polyphenols *per se* has also been associated in some epidemiological studies with a minor risk of several diseases [3]. In particular, the “French paradox” [20] states that the consumption of red wine in certain French regions with diets high in saturated fats can explain the low prevalence of CVD in these regions. These findings have prompted the interest in grape polyphenols, and specially in flavonoids (flavan-3-ols and their oligomeric forms called proanthocyanidins) which exhibit different beneficial health effects by acting as antioxidant, anticarcinogen, cardioprotective, antimicrobial, antiviral and neuroprotective agents [3]. Recently, it has been

reported that grape polyphenols down-regulates SREBP1c and lipogenic enzymes in the liver of rats suffering metabolic syndrome [21].

Because different effects on health have been attributed to these bioactive compounds, a proteome-wide approach to map proteins from different pathways can provide a complementary and global vision. But to date, few studies have addressed protein regulation linked to fish oils [22] or grape polyphenols in liver rats [21]. The current study is aimed to better investigate the molecular mechanisms behind the beneficial action of a fish oil mixture (FOM) with high proportion of omega-3 PUFAs with EPA:DHA 1:1 together with proanthocyanidin-rich grape seed extract (GSE) for the prevention and mitigation of metabolic disorders. Then, the experimental design includes rats supplemented with FOM or GSE or the combination of both into the background of healthy and unhealthy diets. A hepatic proteome analysis to map proteins which are targets of these bioactive components trying to dig deeply into the effect on the metabolic pathways regulated by such proteins is presented. For such scope, liver proteins from female Wistar Kyoto rats fed for 24 weeks standard (STD) diets (STD-control, STD-FOM, STD-GSE and STD-FOM&GSE) and liver proteins from rats fed high-fat and high-sucrose diets (HFHS) (HFHS-control, HFHS-FOM, HFHS-GSE and HFHS-FOM&GSE) have been studied by iTRAQ-labeled coupled with nano LC-MS/MS and complemented with 2D-DIGE. To our knowledge, this is the first hepatic proteome analysis of the combined effect of fish oil and grape polyphenols in liver rats fed either healthy or unhealthy diets.

2. Materials and Methods

2.1. Animals and Diets

Fifty-six female Wistar Kyoto rats (Charles Rivers Laboratories, Wilmington, MA, USA) aged 8-10 weeks, were housed in animal cages (*n* 2–3/cage) with a constantly regulated temperature (22 ± 2 ºC) and humidity (50 ± 10%) with a 12 h light-12 h dark cycle.

The rats were randomly assigned to one of two dietary groups: a STD group (n = 28) fed a standard diet base on the reference diet Teklad Global 2014 (Harlan Teklad Inc., Indianapolis, IN, USA) and a HFHS group (n=28) fed a high-fat and high-sucrose diet base on the TD. 08811 diet (Harlan Teklad Inc).

Both groups were also divided in four dietary subgroups (n = 7): Control (C), which fed a STD or HFHS diet supplemented with soybean oil; Fish Oil Mixture(FOM), which fed a STD or HFHS diet supplemented with a fish oil mixture with a ratio of 1:1 EPA:DHA; Grape Seed Extract (GSE), which fed a STD or HFHS diet supplemented with proanthocyanidin-rich grape seed extract and finally; Fish Oil Mixture and Grape Seed extract (FOM&GSE), which fed a STD or HFHS diet supplemented with a combination of EPA:DHA 1:1 and grape seed extract.

EPA:DHA 1:1 oil was obtained by mixing appropriate quantities of the commercial fish oils AFAMPES 121 EPA (A.F.A.M.SA., Vigo, Spain), EnerZona Omega 3 RX (Milan, Italy) and Oligen liquid DHA 80% (IFIGEN-EQUIP 98, SL, Barcelona, Spain). Soybean oil, obtained from unrefined organic soy oil (first cold pressing) was from Clearspring Ltd. (London, UK). The fatty acid composition of the oil supplements was previously determined [10]. Grape seed extract Grajfnol® (≥95% proanthocyanidins, 85% oligomers) was from JF-Natural Product (Tianjin, China). The GSE dose used was 30 mg proanthocyanidin/kg body weight of rat which would be equivalent to a daily dose of 4.9 mg/kg body weight in humans, i.e. 340 mg/day for a 70-kg adult [23], and the median daily polyphenol intake in humans is from about 150 to nearly 500 mg/p/day [24]. The eight subgroups are shown in Table 1. The experimental diets were pelletized

in-house by lyophilization from frozen emulsions in order to incorporate each supplement. All groups had *ad libitum* access water and food.

After 24 weeks of experiment, the rats were fasted overnight, anesthetized intraperitoneally with ketamine and xylacine (80 mg/kg and 10 mg/kg body weight, respectively), and killed by exsanguination. Livers were excised, washed with 0.9% NaCl solution, weighed and immediately frozen in liquid nitrogen upon sacrifice. All samples were stored at −80 ºC until analysis.

All the procedures strictly followed the European Union guidelines for the care and management of laboratory animals, striving to minimize suffering, and were approved by the CSIC (Spanish Research Council) Subcommittee of Bioethical Issues (ref. AGL2009-12 374-C03-03).

2.2. Biochemical Measurements

Plasma total fatty acids (TFA) and free fatty acids (FFA) were analyzed as previously described [25, 26]. The level of protein oxidation in plasma and liver was measured following a method previously described [10]. Adiposity index: ((total abdominal fat × 100)/body weight) and hepatosomatic index: ((liver weight × 100)/body weight) were also calculated. Plasma triglycerides, total cholesterol and insulin concentrations joint to the percentage of glycated hemoglobin (HbA1c) in blood were measured in the same animals by following protocols previously described [27-29] and the results are currently published [30,31] . In order to facilitate the reading of the present work, a summary of those are showed in the Supplementary Tables S1 and S2, which also provide the rest of biochemical measurements done. Data were reported as mean and standard deviation (S.D.). Statistical analysis was performed by using the one-way analysis of variance followed by Tukey's Post Hoc Test with the IBM SPSS Statistics 22

software (Statsoft, Tulsa, OK). Significant difference was set at $p<0.05$. The one-way ANOVA test was applied to determine any significant difference between the treatments and, if any were detected, the Tukey's Post Hoc Test was used to compare all the different pairs of the treatments. According to proteomics design, comparisons among different supplements in the present research were made independently inside STD or HFHS backgrounds. The physiological effects of STD and HFHS, with or without supplements, were deeply discussed in the references [30] and [31].

2.3. Liver Protein Extraction

200 mg of liver were homogenized in a sodium phosphate buffer as previously described [10] and the concentration of the resulting protein extract was measuring by using the Micro BCA™ Protein Assay Kit (Thermo Scientific) [32].

2.4. Proteomics approaches to analyze the effect of FOM and GSE on liver protein regulation

2.4.1. iTRAQ approach: Experimental Design and Statistics

To evaluate the effect of FOM or GSE or both on liver protein regulation in the two different dietary backgrounds (STD and HFHS), two independent iTRAQ experiments were performed. The first one was made to analyze the effects of FOM, GSE and both into the context of STD healthy diets using the commercial reference chow-fed group supplemented with soybean oil (STD-C) as control group. The second one was made to analyze these effects into the context of HFHS unhealthy diets, using the commercial HFHS chow-fed group supplemented with soybean oil (HFHS-C) as control group. The experimental design is showed in the Table 1.

2.4.1.1. Protein digestion

50 µg of protein from each condition was precipitated by methanol/chloroform method, to concentrate and purify the protein sample. The pellet was resuspended and denatured in 20 µL 6 M guanidine hydrochloride/100mM HEPES, pH 7.5, (SERVA Electrophoresis GmbH), reduced with 1 µL of 50 mM Tris(2-carboxyethyl) phosphine (TCEP, AB SCIEX), pH 8.0, at 60°C for 30 min and followed by 2 µL of 200 mM cysteine-blocking reagent (methyl methanethiosulfonate) (MMTS, Pierce) for 10 min at room temperature. Samples were diluted up to 120 µL to reduce guanidine concentration with 50 mM TEAB. Digestions were initiated by adding 2.5 µL (1 µg/µL) sequence grade-modified trypsin (Sigma-Aldrich) to each sample in a ratio 1:20 (w/w), which were then incubated at 37°C overnight on a shaker.

2.4.1.2. Peptide labelling

In each iTRAQ experiment, the resulting peptides of tryptic digestion were labeled using iTRAQ 4-plex kit (AB SCIEX, Foster City, CA, USA) according to the manufacturer's instructions as follows: STD-C, tag-114; STD-FOM, tag-115; STD-GSE, tag-116 and STD-FOM&GSE, tag-117, for the first STD experiment; and HFHS-C, tag-114; HFHS-FOM, tag-115; HFHS-GSE, tag-116 and HFHS-FOM&GSE, tag-117, for the second HFHS experiment (Table 1).

After labeling, the samples were pooled, dried and desalted using a SEP-PAK C18 Cartridge (Waters). Finally cleaned tryptic peptides were evaporated to dryness and stored at –20°C for further analysis.

2.4.1.3. Nano-LC and MS Analysis

A 2 µg aliquot of the resulting mixture was subjected to nano LC ESI-MSMS analysis using a nano liquid chromatography system (Eksigent Technologies nanoLC Ultra 1D plus, AB SCIEX, Foster City, CA) coupled to high speed Triple TOF 5600 mass spectrometer (AB

SCIEX, Foster City, CA) with a Nanospray III source. The analytical column used was a silica-based reversed phase column C18 ChromXP 75 µm × 15 cm, 3 µm particle size and 120 Å pore size (Eksigent Technologies, AB SCIEX, Foster City, CA). The loading pump delivered a solution of 0.1% formic acid in water at 2 µL/min. The nano-pump provided a flow-rate of 300 nL/min and was operated under gradient elution conditions, using 0.1% formic acid in water as mobile phase A, and 0.1% formic acid in acetonitrile as mobile phase B. Peptides with iTRAQ labels were separated using a 150 minutes gradient ranging from 2% to 90% mobile phase B (mobile phase A: 2% acetonitrile, 0.1% formic acid; mobile phase B: 100% acetonitrile, 0.1% formic acid). Injection volume was 5 µl. Experiments were performed in triplicate.

Data acquisition was performed with a TripleTOF 5600 mass spectrometrer (AB SCIEX , Foster City, CA). Data was acquired using an ionspray voltage floating (ISVF) 2800 V, curtain gas (CUR) 20, interface heater temperature (IHT) 150, ion source gas 1 (GS1) 20, declustering potential (DP) 85 V. All data was acquired using information-dependent acquisition (IDA) mode with Analyst TF1.5.1 software (AB SCIEX, USA). For IDA parameters, 0.25 s MS survey scan in the mass range of 350–1250 Da were followed by 15 MS/MS scans of 250 ms in the mass range of 100–1800 (total cycle time: 4.04 s). Switching criteria were set to ions greater than mass to charge ratio (m/z) 350 and smaller than m/z 1250 with charge state of 2–5 and an abundance threshold of more than 70 counts (cps). Former target ions were excluded for 20 s. IDA rolling collision energy (CE) parameters script was used for automatically controlling the CE.

2.4.1.4. Database Searches and Statistics

MS and MS/MS data obtained for pooled samples were processed using Analyst® TF 1.5.1 Software (AB SCIEX). The raw files in each experiments of peptides were analyzed in triplicate by nano-LC-ESI-MS/MS. Raw data file conversion tools generated mgf files were independently

searched against the *Rattus norvegicus* UniProtKB/Swiss-Prot database (2012/10) containing 27765 protein coding genes and their corresponding reversed entries using the Mascot Server v. 2.3.1 (Matrix Science, London, UK). Search parameters were set as follows: enzyme, trypsin; allowed missed cleavages, 1; fixed modifications, iTRAQ 4-plex (N-term and K) and beta-methylthiolation of cysteine; variable modifications, oxidation of methionine. Peptide mass tolerance was set to ± 25 ppm for precursors and 0.05 Da for fragment masses. The confidence interval for protein identification was set to ≥ 95% ($p<0.05$) and only peptides with an individual ion score above the 1% False Discovery Rate (FDR) threshold were considered correctly identified. For quantitative analysis, only the correctly identified proteins having at least two quantitated peptides were considered in the quantitation. To obtain iTRAQ protein ratios the median was calculated over all distinct peptides assigned to a protein subgroup in each replicate. Then, each iTRAQ channel was normalized by dividing each protein ratio by the median of ratio in each channel. This normalized median in each replicate was used to obtain the final geometric media of the corresponding protein. After calculating log2 of geometric media, frequency distribution histograms were obtained from Excel 2010. Log2 protein ratios were fitted a normal distribution using least squares regression. Mean and standard deviation values derived from the Gaussian fit were used to calculate p-values and FDR (at quantitation level) [33]. The FDR for quantitation were then calculated as the FDR = (E value/protein rank), being E value = (p value*total number of quantified proteins) and the protein rank the individual position of the specific protein after ordered it by its p value. A 5% quantitation FDR threshold was estimated to consider the significant differentially expressed proteins.

2.4.2. 2D-DIGE approach: Experimental Design and Statistics.

2.4.2.1. 2D-DIGE experimental design

Differential protein regulation among four HFHS groups was evaluated by DIGE. Two or three technical replicates were performed in this study. 25 µg of each protein sample solubilized in standard cell lysis buffer were randomly labeled either with Cy3 or Cy5 Dyes. The experimental design (Table 2) determined that none of the differences observed was due to preferential labeling. The mixed internal standard methodology was used in this experiment. For this purpose, the internal standard was prepared by pooling equal amounts of proteins (6.25 µg) from each sample in the experiment and labeled with Cy2 Dye.

Additionally to the comparisons of HFHS-FOM, -GSE and -FOM&GSE with regards to control, the 2D-DIGE experimental design also allowed the realization of the rest of comparisons among the four different diets, i.e., HFHS-FOM vs. -GSE or FOM vs. -FOM&GSE or -GSE vs. -FOM&GSE and then, to add information to the iTRAQ results.

2.4.2.2. 2D-DIGE separation

Proteins were labeled according to the CyDyes manufacturer (GE Healthcare). Briefly, 25 µg of protein for each group (HFHS-C, -FOM, -GSE, -FOM&GSE) were minimally labeled with 200 pmol of the N-hydroxysuccinimide esters of Cy3 or Cy5 fluorescent cyanine dyes on ice in the dark for 30 min. All experiments comprised an internal standard containing equal amounts of each group of HFHS dietary intervention, which was labeled with Cy2 dye. The labeling reaction was quenched with 1 µL of 10 mM lysine on ice for 10 min in the dark. The individual groups and the internal standard protein samples were combined in pairs as shown in Table 2 and run in a single gel (75 µg total proteins). Protein extracts were diluted volume to volume in Rehydration Buffer 2X (7 M urea, 2 M thiourea, 4% CHAPS, 20 mM DTT 1,0% carrier ampholites pH 3-11NL,) up to final volume of 100 µl, and applied by Cup Loading to 24 cm IPG strips pH 3-11 NL (GE Healthcare) previously rehydrated with 440 µl of Rehydration Buffer (7

M urea, 2 M thiourea, 4% CHAPS, 0,5% carrier ampholites pH 3-11NL, 1,2% DeStreak). The first dimension was run at 0.05 mA/IPG strip in the IPGphor IEF System (GE Healthcare) following a voltage increase in 5 steps: 300 V/h for 3h, linear gradient to 1000V in 6h, linear gradient to 8000 V in 3h, 8000 V/h until 64000 V/h were reached. After the first dimension, strips were equilibrated in the dark with SDS Equilibration Buffer (50 mM Tris pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, traces of bromophenol blue) containing 1% (w/v) DTT for 15 min and thereafter in SDS Equilibration Buffer containing 4% (w/v) iodoacetamide for 15 additional min. Then the proteins were separated on home-casted 12.5% Tris–glycine gels using an Ettan Dalt Six device (GE Healthcare) at 20ºC until the tracking dye had migrated off the bottom of the gel.

2.4.2.3. Image acquisition and analysis

After electrophoresis, the gels were scanned with a Typhoon 9400 scanner (GE Healthcare) at 100 µm resolution using appropriate wavelengths and filters for Cy2, Cy3 and Cy5 dyes. Relative protein quantification across groups was performed using DeCyder software v 7.0. First, a Differential In-gel Analysis (DIA) module was used to co-detect the 3 images of a gel (internal standard and two samples) to measure accurate spot ratios of the Cy3 and Cy5 spot volumes referring to the standard Cy2 volume on each gel. Background subtraction, quantification and normalization were automatically applied with low experimental variation. Then those images individually processed with the DIA module were matched between gels with the Biological Variation Analysis (BVA) module, using the internal standard for gel-to-gel matching. BVA revealed the differences between experimental groups across all the gels. Each difference was calculated as average ratios for each spot (>= +1.5 or <= –1.5, were considered protein of

interest). The Student's t test was used to compare average ratios for each spot between groups. P values less than 0.05 were considered significant.

After imaging for CyDye components and DeCyder analysis, the gels were fixed in 10% methanol, 7% acetic acid for 30 min and then incubated overnight in the dark with Sypro Ruby (Molecular Probes). Sypro Ruby images were acquired on the same imager. For picking-up of the spots, the Sypro image was compared to the Master gel with DeCyder software, which translate individual coordinates for each spot of interest to the Sypro gel for automatically picking-up of the spots by the Spot Picking Robot (GE Healthcare). The gel was re-imaged after spot cutting out to ensure accurate protein excision.

2.4.2.4. In-gel protein automatic digestion and protein identification

After picking-up of the spots of interest, tryptic digestions were made by using an automatic protein digestor (Proteineer DP, Bruker Daltonics. Bremen, Germany). Protein identification of each spot of interest was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry using an AB Sciex 4800 MALDI-TOF/TOF mass spectrometer. A 0.8 µl aliquot of each peptide mixture was deposited onto a 384-well OptiTOF^{TM} Plate (AB SCIEX, Foster City, CA) and allowed to dry at room temperature. A 0.8 µl aliquot of matrix solution (3 mg/mL α-Cyano-4-hydroxycinnamic acid in MALDI solution) was then deposited onto dried digest and allowed to dry at room temperature.

For MALDI-TOF/TOF analysis, samples were automatically acquired in positive ion reflector mode (the ion acceleration voltage was 25 kV to MS acquisition and 2 kV to MSMS) using the 4000 Series Explorer Software v3.6. PMF and MSMS fragment ion spectra were smoothed, corrected to zero baseline and internally calibrated with the mass signals of trypsin autolysis ions. To submit the combined PMF and MS/MS data to MASCOT software v.2.3.1

(Matrix Science, London, UK), GPS Explorer v4.9 was used, searching in the previous protein database from Uniprot/Swiss-Prot. The following search parameters were used: enzyme, trypsin; allowed missed cleavages, 1; carbamidomethyl cystein as fixed modification; variable modifications, oxidation of methionine; mass tolerance for precursors was set to ± 50 ppm and for MS/MS fragment ions to ± 0.3 Da. The confidence interval for protein identification was set to ≥ 95% ($p < 0.05$) and only peptides with an individual ion score above the identity threshold were considered correctly identified.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [34] via the PRIDE partner repository with the dataset identifier PXD003061 and 10.6019/PXD003061" for iTRAQ analysis and with PXD003080 and 10.6019/PXD003080" for 2D-DIGE approach.

2.5. Gene ontology (GO) and network analysis

The final list of proteins differentially regulated by treatments was submitted to PANTHER program (http://www.pantherdb.org/), for the classification based on two main types of annotations: protein class and biological process. Network analysis was performed submitting the proteins of interest to the STRING (Search Tool for the Retrieval of Interacting Genes) software (v.9.0) (http://string-db.org/). A confidence score was fixed to 0.4 (medium level). Cluster networks were created using the Markov Cluster (MCL) Algorithm which is included in the STRING website and a value of 2 was selected for all the analyses.

3. Results

3.1. Outcomes under STD diets

3.1.1. Protein identification and quantification of liver proteins by iTRAQ analysis

The comparative proteomics analysis using iTRAQ 4-plex methodology evidenced different effects of the FOM or GSE or their combination (FOM&GSE) on liver protein regulation in rats fed STD diets. Given the identification filters described in the 2.4.1.4. section, 1076 proteins were identified considering the sum of the total different proteins identified in each of the three replicates 830 proteins were identified in the first replicate, 848 proteins were identified in the second one and 831 proteins were identified in the third one. Considering the 1076 whole different proteins, a total of 633 identified proteins were matched in all the three replicates, meanwhile 167 proteins were found in two replicates. Hence, the remained 276 identified proteins were found only in one of the individual replicates (Supplementary Figure S1A). Regarding quantification, a total of 692 proteins could be successfully quantified considering all the three replicates. To strengthen the conclusions of this work, only the proteins which were quantified in two or three replicates were employed for the analysis, being finally 569 proteins, which suppose the 82% of the total quantified proteins computed. Among these 569 proteins, the most of them (477 proteins, almost the 84%) were quantified in each of the three replicates, reinforcing the quantification confidence. All the identified and quantified proteins are listed in the Supplementary Table S3. Peptide proteomics data from the three replicates are presented in Supplementary Tables S4-6.

3.1.2. Up and Down Regulation of liver proteins by iTRAQ analysis

As Table 3 shows, the supplementation with FOM caused a significant alteration in 6 proteins versus the control group, with a minimal decrease of 21% and a minimal increase of 40% (FDR 3.1%). Five of them were significantly down-regulated: the glycolytic fructose-bisphoshate aldolase A (ALDOA), the protein Zadh2 (ZADH2), the mitochondrial glyoxalase domain-containing protein 4 (GLOD4), the T-complex protein 1 subunit beta (CCT2) and the

cysteine sulfinic acid decarboxylase (CSAD). Finally, only the protein G3bp1 (G3BP1), involved in Ras signaling, was highly up-regulated by the FOM diet.

The supplementation with GSE changed the amount of 2 proteins versus the control group with a minimal decrease of 35% and a minimal increase of 39% (FDR 0.2%). As well as FOM, GSE supplementation significant reduced the cellular amount of the ALDOA. The second protein was the mitochondrial glutaminase liver isoform (GLS2), which was also reduced by GSE (Table 3).

Finally, the combination of FOM&GSE altered 6 proteins versus the control group (with a minimal decrease of 24% and a minimal increase of 36%, FDR 4.4%), the same number but not exactly the same proteins altered by the supplementation with only FOM (Table 3). Considering the six altered proteins, ALDOA was found down-regulated by the combined FOM&GSE and by the FOM and GSE, as well. However, although FOM&GSE reduced the ALDOA amount in –32% did not display any additive effect, since the decrement was virtually the same than the exerted by FOM or GSE by their own (–35 and –36%, respectively). Two proteins were down-regulated by FOM and also by the combination FOM&GSE: GLOD4 and CSAD and therefore, the combination FOM&GSE showed three unique target proteins not affected in the other two diets: the long-chain-fatty-acid-CoA ligase 1 (ACSL1), the subunit beta of coatomer (COPB1) and the tubulin-specific chaperone A (TBCA) (Table 3). These findings may suggest a synergistic effect of the joint intake FOM and GSE on ACSL1, COPB1 and TBCA.

3.1.3. Functional information of differentially regulated regulated proteins: gene ontologies and network analysis

PANTHER analysis revealed the presence of 8 different protein classes. About a 20% were chaperones and the rest classes included oxidorreductases, ligases, lyases, hydrolases, signaling molecules, nucleic acid binding and membrane traffic proteins.

With regard to their biological function, PANTHER revealed 3 main biological functions. A 66.7% of proteins were involved in metabolic processes (mainly carbohydrate, protein, cellular amino acid and lipid metabolism), 25.0% in localization/transport processes and 8.3% in cellular component organization or biogenesis processes.

STRING database analysis showed a network composed by 33 nodes (or proteins) and enriched in interactions or edges (62 interactions, but only one direct interaction between two proteins of interest: Copb1 and Cct2) (Fig. 1). The topological analysis of the network revealed the existence of five sub-networks, in concordance with the main biological functions. Three of the sub-networks were highly interconnected through Cct2, Cct6a, Tcp1 and Sphk1 from protein metabolism, Pgk1, Gapdhs and ENSMUSG00000073212 from carbohydrate metabolism and Hras1, Nras, Caprin1 and Copb1 involved in transport and biological signaling. The lipid and cellular amino acid sub-networks were isolated to each other. Interestingly, Acsl1 from lipid metabolism was closely related to Ppara and also to proteins involved in both lipid biosynthesis (Acadm and Acadl) and degradation (Hadhb, Dci and Derc1).

3.2. Outcomes under HFHS diets

3.2.1. Protein identification and quantification of liver proteins by iTRAQ analysis

Considering the identification filters described in the 2.4.1.4. section, the sum of protein identified in the three replicates was 1036 proteins. Individually, 820 proteins were identified in the first replicate, 832 proteins were identified in the second one and 804 proteins were identified in the third one. Among the 1036 total proteins, 628 proteins were identified in common in all

the three replicates, other 164 proteins were found in two replicates and the rest, 244 proteins, were detected in only one replicate (Supplementary Figure S1B). With regard to quantification and according to the set filters the sum of proteins which could be quantified in the three replicates was 678 proteins. Among these proteins, 448 proteins were quantified in common in the three replicates and 115 proteins were quantified at least in two of them. Therefore, more than the 83% of proteins could be quantified in at least two replicates and used for our study. All the identified and quantified proteins are listed in the Supplementary Table S7. Peptide proteomics data from the three replicates are presented in the Supplementary Tables S8-10.

3.2.2. Up and Down Regulation of liver proteins by iTRAQ analysis

Quantitative iTRAQ analysis revealed a different effect of long time intake of FOM or GSE or a combination of both on liver proteins in the background of HFHS diet. Results are presented in the Table 3.

A total of 24 proteins were affected by FOM supplementation versus the control group with a minimal decrease of 23% and a minimal increase of 44% (FDR 4.9%). The 34% of altered proteins belonged to lipid metabolism. Among them, fish oil supplementation significantly down-regulated proteins belonging to lipid synthesis, such as the ATP-citrate synthase (ACLY), the enzyme fatty acid synthase (FASN) and the farnesyl pyrophosphate synthase (FDPS), and lipid transport, the epidermal fatty acid-binding protein (FABP5) and the acyl-CoA binding protein (ACBP). On the other hand, one protein related to lipid degradation, the peroxisomal acyl-CoA oxidase (ACOX2) was found to be strongly up-regulated by the effect of fish oil supplementation.

The decarboxylating enzyme 6-phosphogluconate dehydrogenase (PGD), associated to the pentose phosphate pathway and therefore a key enzyme in both lipid and carbohydrate

metabolisms, was significantly down-regulated by FOM. Likewise, FOM supplementation also decreased the levels of both the acyl-protein thioesterase 1 (ACP1), involved in acyl-CoA metabolism and protein lipidation, and the 60 kDa lysophospholipase (ASPG), involved in phospholipid and asparagine metabolisms.

Other group of proteins significantly altered by FOM belonging to protein and amino acid metabolism; indeed they constitute almost the 34% of differentially regulated proteins. These proteins were mainly involved in either protein translation (the protein (NARS) and the cytoplasmic tyrosine-tRNA ligase (YARS), both down-regulated, and the eukaryotic translation initiation factor 4B (EIF4B), which was strongly up-regulated) or protein degradation (proteasome subunit beta type-8 (PSMB8), dipeptidyl peptidase 1 light chain (CTSC) and L-serine dehydratase/L-threonine deaminase (SDS), all of them down-regulated) or protein transport (fish oil down-regulated the subunit beta of coatomer (COPB1) and up-regulated the actin-related protein 2 (ACTR2) and the protein G3BP1), or protein folding (such as the protein disulfide-isomerase A6 (PDIA6), which was down-regulated).

FOM also modulated the level of several proteins involved in diverse functions such as the liver response to oxidative stress by increasing the enzyme carbonic anhydrase 3 (CA3), the vitamin and steroid hormone metabolism, the biological adhesion, the ferric ion binding and the immune system process by decreasing, respectively, the levels of the catechol O-methyltransferase (COMT), the lumican (LUM), the light chain of ferritin (FTL1) and the protein IGHM.

The supplementation with GSE changed the amount of 4 proteins versus the control group (Table 3) with a minimal decrease of 21% and a minimal increase of 33% (FDR 4.8%). Two of them, related to lipid metabolism, were significantly down-regulated: the subunit beta of the

mitochondrial trifunctional enzyme (HADHB) and ACP1 also down-regulated by FOM as mentioned before; one from protein metabolism, the nuclear transport factor 2 (NUTF2) and finally, one from carbohydrate metabolism, the protein Tsta3 both down-regulated as well.

The supplementation with FOM&GSE altered 17 proteins versus the control group (Table 3), with a minimal decrease of 26% and a minimal increase of 46% (FDR 4.7%). The combination of fish oil and grape polyphenols resulted in an important dysregulation of proteins related to lipid metabolism (40% of the altered proteins). Six of them change in a similar way that found with only fish oils: ACLY, FASN, FABP5, ACBP and PGD decreased and ACOX2 increased. Additionally, only the combination FOM&GSE significantly up-regulated the enoyl-Co delta isomerase 1 (ECI1), an enzyme involved in fatty acid beta-oxidation.

In agreement with the high number of proteins related to protein and amino acid metabolisms altered by FOM, almost the 40% of the total proteins modulated by FOM&GSE belonged to this class as well. But the proteins affected were not the same, and in fact, only two proteins were altered in common by the two supplementations: the EIF4B and the PSMB8. A third protein belonging to protein metabolism, the transporter NUTF2, was found to be down-regulated by both FOM&GSE and GSE supplementations. The rest proteins of this subgroup appeared significantly down-regulated uniquely by FOM&GSE and were: the protein GTP cyclohydrolase 1 feedback regulatory (GCHFR), involved in the protein complex assembly, the protein Ppp2r1a (PPP2R1A) and the ubiquitin-conjugating enzyme E2N (UBE2N), both involved in protein modification and one protein (Uniprot code M0R7Y9) still uncharacterized. By using BLAST software, we found that this protein displayed 100% homology with the putative thymosin beta-4-like protein 6, involved in actin binding.

Regarding carbohydrate metabolism, one of the glycolysis regulatory enzymes, the pyruvate kinase PKLR (PKLR), suffered a significant reduction as consequence of the FOM&GSE supplementation. Finally, two proteins involved in immune system process were found altered: the prothymosin alpha (PTMA) and the protein LOC679748, resulting respectively up- and down-regulated. By using BLAST software, we found that this protein LOC679748 displayed 96% of homology with the rat macrophage migration inhibition factor (MIF).

3.2.3. Analysis of 2D-DIGE images of rat liver of HFHS diet

2D-DIGE analysis was also performed on rats fed HFHS diets aiming to complement and reinforce the iTRAQ results. The use of this alternative method of quantitative proteomics, which is complementary to iTRAQ, allowed us to confirm some of iTRAQ results but also it could be more adequate to quantify protein with high similarity of sequence. Moreover, 2D-DIGE design allowed us to perform direct comparisons among all groups in the same experiment. Our iTRAQ data revealed important effects of FOM, GSE and FOM&GSE supplementations against the unhealthy outcomes of HFHS diets. However, scarcely alteration of liver protein was found among the group fed the standard diets, so that we decide to perform the 2D-DIGE analysis with the rats fed HFHS diets.

The analysis of gel images of HFHS-fed rats (Figure 2) revealed significant changes in the protein quantity of 8 spots (corresponding to 5 different proteins) identified between FOM and control group. There were 15 spots (corresponding to 12 different proteins) changed when the FOM&GSE group was compared with control and only 1 change associated to the protein moesin (MSN) (+51% increased by GSE) when GSE was compared to the control HFHS-C. Between FOM and GSE gels, there were 4 different spots (corresponding to 3 proteins) and between FOM&GSE and GSE gels, 9 spots with different protein amount (corresponding to 8

different proteins). The comparison between proteins in FOM and FOM&GSE gels did not show any difference. Proteins differentially regulated are listed in the Table 4. Protein identification data are detailed in the Supplementary Table S11.

As compared to HFHS-C, FOM significantly down-regulated proteins involved in carbohydrate metabolism (the glucose-6-phosphate 1-dehydrogenase (G6PD) and the PKLR) and in cellular amino acid metabolic processes (the carbamoyl-phosphate synthase [ammonia], mitochondrial precursor (CPS1), the argininosuccinate synthase (ASS1) and the homogentisate 1,2-dioxygenase (HGD). Similar results were obtained from the comparison of the gels corresponding to FOM and GSE, in concordance with the slight differences found between GSE and control. In fact, FOM also significantly down-regulated G6PD, PKLR, ASS1 and HGD as compared to GSE. Moreover, the enzyme PGD was also down-regulated by FOM when it was confronted with GSE group.

When FOM&GSE gels were compared to HFHS-C, it has been revealed that the FOM&GSE significantly down-regulated proteins involved in lipid metabolism (ACLY, the bile acid-CoA:amino acid N-acyltransferase (BAAT) and the mitochondrial delta(3,5)-delta(2,4)-dienoyl-CoA isomerase (ECH1)), in carbohydrate metabolism (PGD and G6PD), in cellular amino acid metabolic processes (CPS1, ASS1 and HGD), altered proteins from protein metabolism (up-regulated the 78 kDa glucose-regulated protein (HSPA5) and MSN, and down-regulated the heat shock cognate 71 kDa protein (HSPA8)) and up-regulated the catalase (CAT) involved in cellular response to oxidative stress. Again, similar results were obtained when FOM&GSE were compared with GSE gels. Likewise, the combination of FOM&GSE reduced the protein quantity of ACLY, PGD, G6PD, ASS1, HGD and HSPA8. Additionally, PKLR was found down-regulated by FOM&GSE as compared with GSE group.

3.2.4. Altered proteins identified in both iTRAQ and DIGE methods

It should be noted that three proteins were overlapping between both methods: PGD, ACLY and PKLR. Both DIGE and iTRAQ data found that PGD and ACLY were significantly down-regulated by the combination of FOM&GSE as compared with the HFHS control. Additionally, DIGE demonstrated that these proteins were also reduced by the mix with respect to GSE-supplemented diet. Similarly, PKLR was also found reduced in both methods due to FOM&GSE supplementation, although with DIGE only when FOM&GSE were compared with GSE gels, a group which did not show differences with HFHS-C in the spots corresponding to PKLR.

ACLY and PGD also were found down-regulated by FOM in comparison with HFHS-C in the iTRAQ analysis. In contrast, DIGE did not find differences among group for ACLY and for PGD only when it was confronted to GSE group. However, because of neither differences between FOM and FOM&GSE nor between GSE and HFHS-C gels were found in ACLY and PGD protein spots, the tendency to down-regulation by FOM with regards to control could be assumed. DIGE also found down-regulated two spots identified as PKLR by FOM regarding to HFHS-C and GSE gels. However iTRAQ did not find significant altered PKLR by FOM. Although the total protein amount of PKLR was likely reduced by FOM supplementation, in view of DIGE results it is also possible that FOM provoked changes at posttranslational level which are easier to detect with DIGE methodology.

The results obtained from the two complementary quantitative proteomics approaches, iTRAQ and 2D-DIGE, were in concordance in terms of the metabolic pathways altered and the tendency of protein changes and will be discussed jointly in the 4.2. section.

3.2.5. Functional information of differentially regulated proteins in HFHS-fed rats: gene ontologies and network analysis

PANTHER analysis revealed the presence of 21 different protein classes (Supplementary Figure S2A). The most relevant classes were transferases (15.2%), oxidoreductases (13.9%), ligases (11.4%), hydrolases (10.1%), lyases (7.6%) and isomerases (7.6%). The elevated number of oxidoreductases affected by FOM or/and GSE supported the known role of these bioactive compounds in the homeostasis redox.

With regard to their biological function, PANTHER revealed 11 biological functions (Supplementary Figure S2B). Metabolic processes were the most relevant biological processes, representing for the global dataset the 49.4% of the data. Among them, the 89.5% were proteins involved in primary metabolic processes such as lipid metabolic process (38.2%), protein metabolic process (35.3%), carbohydrate metabolic process (26.5%) cellular amino acid metabolic process (23.5%), nucleobase-containing compound metabolic process (11.8%) and the tricarboxylic acid cycle (2.9%).

STRING database analysis showed a network composed by 74 nodes (or proteins) and enriched in interactions or edges (147 interactions, 22 direct interactions among the proteins of interest, $p = 2.18\ e^{-6}$) (Fig. 3). The topological analysis of the network revealed the existence of several sub-networks, in concordance with the main biological functions. Inside the sub-networks of lipid metabolism and cellular amino acid metabolism, the proteins of interest showed highly connected interactions. Moreover, both sub-networks were highly interconnected through Baat and Slc27a5 (from lipid metabolism) and Oct, Cps1 and Hgd (from cellular amino acid metabolism), and sparsely interconnected through Acly and Asl, respectively.

It is worth noting that the protein Srebf1 (a transcription factor that plays a central role in hepatic lipid and glucose metabolism, which stimulates lipogenic enzymes upon activation by glucose and insulin) is closely connected with three proteins of interest from lipid sub-network

(Fasn, Acly and Fdps) and one from carbohydrate metabolism sub-network (Pklr), stating a closely relation between the two sub-networks.

Meanwhile, the cellular amino acid metabolism sub-network was connected with Yars, a protein of interest belonging to protein metabolism.

The last important sub-network was the protein metabolism one. In this sub-network, the Psmb8 and the rest of proteasome subunits constituted a central interplay with highly interconnections and several mildly and sparsely direct interactions with other proteins of the same sub-network, such as Ppp2r1a, Eif4g1, Hspa5, Hsp8, Ube2n and with proteins from some of the others sub-networks, i.e. Acly (lipid metabolism) Copb1 and Cope, (localization/transport) and Sod1 (response to oxidative stress). Overlooking proteins corresponding to proteasome, the proteins from this sub-network presented slightly direct interactions with proteins from almost all the rest of sub-networks (except to the carbohydrate metabolism sub-network).

4. Discussion

In this study, we showed that the protein regulation exerted by the supplementation of diet with FOM as well as GSE and FOM&GSE was highly dependent on dietary background. Taken together, our results revealed a larger number of altered proteins by the three supplements in rats fed unhealthy HFHS diets, and only very few proteins overlapping between both backgrounds. Changes in the bioavailability/bioactivity of nutrients could increase their response to the different supplementations and explain our data.

On the other hand, in both dietary contexts FOM or GSE or FOM&GSE exhibited a different individual efficiency in inducing changes on liver proteome as compared to soybean controls, the two diets with high amount of fish oils being the main responsible of the changes. In fact, only 2 proteins were altered by GSE versus the 6 proteins altered by FOM and also by FOM&GSE in

the context of STD diet. Similarly, considering the 45 differentially regulated proteins found in HFHS-diet background by the two complementary quantitative methods, iTRAQ and DIGE, only 7 proteins were altered by GSE versus the 31 and the 28 proteins differentially regulated by FOM and FOM&GSE respectively.

It should be noted that the combined action of fish oil and polyphenols in both STD and HFHS dietary backgrounds was not the result of their simple addition, supporting a possible synergistic effect of these bioactive compounds.

4.1. Effect of fish oils and polyphenols on lipid and carbohydrate metabolisms

Our results revealed an important effect of fish oils, with or without polyphenols, in regulating proteins involved in lipid and carbohydrate metabolisms, especially under the unhealthy diet. In fact, FOM and FOM&GSE added to the HFHS diet were able to simultaneously down-regulate proteins involved in lipid synthesis, trafficking and store, and glycolysis and to up-regulate proteins involved in fatty acid oxidation. In comparison, the effect of supplements into the healthy diet was weaker considering the total number of proteins and pathways altered.

Effect into STD diets. iTRAQ analysis detected one protein, the ALDOA, down-regulated by the three supplements as compared to control. This finding could indicate a strong regulation of ALDOA by food components at least into STD context. Although ALDOA is a minority in liver and the aldolase B is the main one [33], on the basis of their kinetic properties it has been suggested that aldolase B have a role in gluconeogenesis while ALDOA is more effective participating in glycolysis [34]. Therefore, the down-regulation of ALDOA could reduce the glycolysis of carbohydrates from diet and decrease the production of acetyl-CoA for triglyceride synthesis in the liver. It is noteworthy that only FOM&GSE also down-regulated ACSL1, which

plays an important role in fatty acid metabolism and triacylglycerol (TAG) synthesis. Dietary supplementation with polyunsaturated fatty acids has proven to increase the mRNA of ACSL1 [35], because PUFAs are PPAR-α agonists and ACSL1 is a PPAR-α target gene in liver. The down-regulation of ACSL1 protein found in our study is not necessary in disagreement with the agonist effect of FOM and PPAR-α, because it has been reported that mRNA and protein amount ACSL1 are dissociated in liver [36]. Accordingly, FOM&GSE-STD showed a significant reduction of FFA in plasma as compared to STD-C, and also similar trends were found in FOM and GSE diets.

Effect into HFHS diets. Both FOM and FOM&GSE added to the HFHS diet down-regulated simultaneously proteins involved in lipid synthesis, trafficking and store, but showed more differences with respect to fatty acid oxidation. Important lipogenic proteins, such as ACLY, FASN and FDPS, targets of SREBPs, were clearly down-regulated in the two diets rich in fish oil. These results are in agreement with previous studies which reported repression of ACLY and FASN gene after fish oil supplementation or their enzyme activities [39] or their protein amounts [22]. Moreover, both FOM and FOM&GSE significantly reduced PGD and G6PD, enzymes from the pentose phosphate pathway involved in the generation of NADPH needed for both cholesterol and TG synthesis [7, 40, 41].

On the other hand, non-esterified polyunsaturated fatty acyl-CoA , especially of ALA, EPA and DHA, can directly bind to HNF-4α and affect carbohydrate metabolism by decreasing the transcription of genes such as the glycolytic PKLR [42]. Supporting this inhibitory effect of HNF-4α by omega-3 PUFA, our results revealed a significant reduction of PKLR in both FOM and FOM&GSE supplemented groups. Other authors have reported high glycolytic activity in rats fed high saturated fat diets or high glucose diets [21, 43].

Lipid trafficking and storage were also down-regulated by FOM and FOM&GSE, by controlling FABP5 and ACBP. Previous studies have demonstrated that the gene expression of FABP5 are positively related to Western-type diet feeding, obesity, fat liver accumulation and type 2 diabetes [44] and even the lacking of FABP5 could protect from diet-induced obesity and accumulation of fat in the liver [45]. For these reasons, FABP5 has been suggested as a novel hepatic SREBP target gene [44]. The second protein was ACBP, also target of SREBP and PPARs [46]. Although the specific biological function of ACBP in cells is presently unknown, it seems to stimulate the synthesis of long-chain fatty acyl-CoA and it has been reported that its levels increased in rat liver after consumption of a high-fat diet [47]. The levels of ACBP in tissues also showed a positive correlation with the concentration of triglycerides in rats' liver, suggesting a role for this protein in intermediary regulation of lipid metabolism [48]. These fatty acid and acyl-CoA binding proteins (FABPs and ACBPs) could be potential candidates to transcription factors that could transduce the fatty acid signals to DNA [42] and therefore contribute to explaining omega-3 mechanism of action in our case.

With regards to fatty acid oxidation, both FOM and FOM&GSE strongly up-regulated the enzyme ACOX2, involved in peroxisomal fatty acid β-oxidation and PPAR signaling. In contrast, a reduced gene expression of ACOX2 has been previously reported by high saturated fat diets [49]. However, only the combination FOM&GSE altered enzymes involved in the mitochondrial fatty acid β-oxidation. Indeed, FOM&GSE up-regulated the levels of mitochondrial protein ECI1 and down-regulated the levels of ECH1, both proteins targets of PPARs [22]. Meanwhile ECI1 are the key enzyme for the mitochondrial β-oxidation of all types of unsaturated fatty acids [50], ECHI is involved in alternative pathways for oxidation of specifically unsaturated fatty acid with cis-double bond at odd-numbered position [51], such as

oleic acid or araquidonic acid but also ALA, EPA and DHA. Our results showed that FOM&GSE might promote the main pathway for UFA β-oxidation in the mitochondria, but could reduce, at least in part, the oxidation of fatty acids such as EPA and DHA, which were in relatively high concentration in the FOM&GSE diet. Wrzesinski et al. have reported that only the combination of fish oil with an antioxidant (TTA, a synthetic fatty acid) increased the ECI1 levels when were included in a high fat diet. However, they found that protein amount of ECH1 increased with the combination of fish oil with TTA [22]. The different behavior of these two antioxidants could respond to a different way of modulation on the effect of FOM on liver protein such as ECH1 by the antioxidant. Interestingly, their antioxidant (TTA) also increased the level of the subunit alpha of mitochondrial trifunctional enzyme, also involved in the long chain FA β-oxidation [22]. On the contrary, our results showed that the GSE supplementation reduced the level of HADHB. Baiges et al. showed that grape polyphenols up-regulated the level of several proteins of β-oxidation but down-regulated others, such as the mitochondrial 2,4-dienoyl-CoA reductase 1, when there were included in a "cafeteria" diet [21].

Therefore, FOM and FOM&GSE showed similar trends for repressing lipid synthesis under HFHS diets, FOM&GSE being more effective in provoking changes in the proteins involved in the mitochondrial fatty acid β-oxidation. These protein changes induced by the FOM and above all by FOM&GSE were well correlated with their lower plasma total cholesterol and FFA levels as compare to HFHS control diet. Taking into account these findings, it seems clear that FOM and GSE exert a tight control on unsaturated fatty β-acid oxidation, and hence their combination could display a specific and stronger action on this pathway.

4.2. Effect of fish oils and polyphenols on protein and amino acid metabolisms

Considering an overview of the total proteins differentially regulated, FOM, GSE and FOM&GSE supplementations showed higher differences on the pathways associated with protein and amino acid metabolism, in both healthy and unhealthy diets.

Effect into STD diets. FOM and FOM&GSE groups showed lower level of CSAD, involved in taurine biosynthesis from cysteine , which could indicate that glutathione synthesis was favoured in rats fed FOM diet rather than taurine synthesis, since cysteine is the limiting amino acid for both biosynthetic processes [52]. Accordingly, FOM groups tended to increase glutathione reduced levels [30]. Meanwhile, GSE diet down-regulated GLS2. Previous studies found that hepatic GLS2 could be regulated by feeding conditions and diet composition and increased during starvation and diabetes [53].

Effect into HFHS diets. The effect of supplements on amino acid metabolism into HFHS diets seemed to be the result of the control of PPAR-α and hence SREBPs, which regulate amino acid metabolism by suppressing genes involved in transamination and deamination as well as genes involved in urea cycle [22]. Accordingly, FOM and FOM&GSE significantly down-regulated two key enzymes in the urea cycle (CPS1 and ASS1). Additionally, both diets down-regulated HGD, involved in the catabolism of tyrosine and phenylalanine, and FOM down-regulated ASPG and SDS also involved in amino acid catabolism, findings that could support the agonistic effect of fish oil and PPAR-α [54].

Both diets containing FOM also significantly reduced PSM8, FOM&GSE being the most effective one. Proteasome has a key role in insulin signaling, because it has been found that an enhanced proteasomal degradation of insulin signaling proteins cause insulin resistance in mouse fed high saturated diet [49]. Therefore, the down-regulation of proteasome by FOM and FOM&GSE could improve the insulin sensitivity of these HFHS fed-rats. Accordingly, the

insulin plasma levels and the HbAc in these groups tended to be reduced and these rats also showed a significantly reduction of FFA in plasma as compared to HFHS control diet. Moreover, since insulin regulated cytoskeleton activities [55], the changes found in our study in proteins related to cellular organization and especially to actin cytoskeleton (moesin, the putative thymosin beta-4-like protein 6 and probably NUTF2 which were found altered by FOM&GSE, and ACTR2 and COPB1, altered by FOM) support the influence of FOM on insulin signaling.

The role of GSE in controlling insulin signaling by regulating protein metabolism was scarce on they own. However, they appeared to be clue in modulating FOM activity. The down-regulation of PPP2R1A only by FOM&GSE could improve insulin signaling because it negatively regulates insulin' metabolic pathway by inhibiting Akt activity [56]. Additionally, this down-regulation of PPP2R1A by FOM&GSE could reduce ChREBP activation [42]. and therefore PKLR levels [57], as reflected in our results described before. Other authors found that PPP2R1A amounts were increased by a diet high in saturated fat [58].

4.3. Effect of fish oils and polyphenols on response to oxidative stress and inflammation

Only under HFHS, the fish oil supplements were able to modulate the amount of proteins involved in the cellular response to oxidative stress. However, the supplementation with only GSE did not alter these pathways at least through controlling the concentration of protein targets.

Both fish oil rich diets improved liver antioxidant status by up-regulating antioxidant proteins. FOM significantly up-regulated the enzyme CA3, a participant in the liver response to oxidative stress [59] and down-regulated ferritin, a protein which can increase oxidative stress that worsens mitochondrial dysfunction in liver [60]. Similar trends were found with the combination FOM&GSE, which also increased CAT and PTMA levels. Among the different roles of the hormone PTMA, it has been noted that it attenuates lipid peroxidation in rats [61].

As a result, FOM and FOM&GSE rats showed lower protein oxidation in liver compared with controls. Moreover, both diets clearly up-regulated the EIF4B and this up-regulation could indicate lower oxidative stress in the rats fed fish oil diets. Under stress conditions such as oxidative stress, cytoplasmic stress granules are formed to inhibit translation initiation and it is precisely the reduction of the levels of the eukaryotic initiation factors such as EIF4B which trigger the formation of these stress granules [62]. Therefore, although fish oils improved antioxidant status, the specific mechanisms to achieve that improvement depended on the presence of polyphenols.

A possible synergism between FOM and GSE can be observed from the finding that only FOM&GSE increased the protein levels of the chaperone HSPA5. This finding is very important because HSPA5 has a critical role in the unfolded protein response (UPR) [63]. The accumulation of unfolded or misfolded proteins in the endoplasmic reticulum (ER) caused by pathophysiological conditions such as elevated levels of fatty acids or cholesterol, oxidative stress, high or low glucose levels, inflammatory cytokines or hypoxia, induces ER stress and activates the UPR chronically [64]. Protein levels of HSPA5 are the key factors that regulate the UPR signaling. High levels of functional HSPA5 proteins in the liver mitigate the UPR, alleviate ER stress and improve chronic inflammation [65, 66]. It has been reported that HSPA5 overexpression inhibited activation of the SREBP-1c, reducing the expression of lipogenic genes and improving glucose homeostatic control in obese mice [67]. In contrast, HSPA5 protein levels were significantly reduced in a model of fatty liver [68]. In the present study, higher level of HSPA5 protein could be an index of lower ER stress in the liver of FOM&GSE rats. The lower carbonylation protein level found in this group could lead to lower accumulation of misfolding proteins, supporting these results. Moreover, the activation of UPR pathways leads to

inflammation by activation of NF-κB [69]. In this regard FOM&GSE decreased UBE2N, which normally participates in the induction and expression of NF-κB and MAPK-responsive inflammatory genes [70], and LOC679748, likely a pro-inflammatory cytokine belonging to MIF superfamily [71]. The down-regulation of the chaperone HSPA8 in FOM&GSE group, whose gene is stimulated after the activation of UPR signaling [72], also supports the alleviation of endoplasmic reticulum stress by the synergy of FOM and GSE.

4.4. Effect of fish oils and polyphenols on Ras signaling

Our results suggest an important role of FOM in Ras signaling pathway which can be modulated by the GSE addition. It is noteworthy that this effect seemed to be independent of dietary context.

In both STD and HFHS diets, the supplementation with FOM alone drastically up-regulated the protein G3BP1, directly associated with the SH3 domain of GTPase-activating protein (GAP or RASA1), a Ras inhibitor [73]. Moreover, FOM also down-regulated the chaperone CCT2, closely related to proteins involved in that pathway [74] under the STD diet, and APT1, an enzyme involved in the regulation of Ras function by depalmitoylation of several isoforms of Ras [75], in the HFHS background. Although APT1was found down-regulated by FOM and GSE supplementations, surprisingly the combination of both of them did not altered APT1 levels. Other authors demonstrated that omega-6 fatty acids such as linoleic acid, which are presented in high concentration in the soybean oil used in our study [10], and the omega-3 fatty acids, specially DHA, modulate Ras signaling differentially [76]. Although polyphenols also have showed an important role on Ras signaling [77], our data showed a scarcely individual effect of GSE on this pathway but an important role as modulators of FOM action.

To sum up, our findings reveal a role of the consumption of fish oils, especially visible under a background of high fat high sucrose diets, in regulating liver proteins resulting in an improvement on liver metabolic pathways. The figure 4 summarizes the main changes on liver metabolism induced by supplements into the context of HFHS diet. Proteomics analysis revealed that omega-3 PUFA from fish oils were the main force in the modulation of proteins from Ras signaling (G3BP1, CCT2 and APT1), the down-regulation the hepatic lipogenesis (ACLY, FASN, FDPS, PGD and G6PD) and glycolysis (ALDOA and PKLR) and the up-regulation fatty acid beta-oxidation (ACOX2) and antioxidant system (CA3). Moreover, proteomics data showed a potential synergistic effect when fish oils are consumed together to polyphenols on proteins such as ACLS1, TBCA, COPB1, GFRP, PPP2R1A, UBE2N, APT1, PTMA, LOC686548, BAAT, ECH1, ECI1, HSP5, HSPA8 and CAT, and therefore the addition of polyphenols to fish oils increased their capacity for regulation the fatty acid beta-oxidation, ameliorated ER stress, the liver protein oxidation levels, plasma lipid profile and improved insulin signaling into unhealthy diets. In conclusion, the fat quality in terms of fatty acid composition plays a critical role on liver protein regulation, its action being highly dependent on the dietary background.

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Conflicts of interest: none.

Abbreviations: 4-HNE, 4-hydroxynonenal; BCA, bicinchonic acid; BVA, biological variation analysis; CE, collision energy; ChREBP, carbohydrate response element binding protein; CUR, curtain gas; CVD, cardiovascular disease; DHA, docosahexaenoic acid; DIA, differential in-gel analysis; DP, declustering potential; EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; FDR, false discovery rate; FFA, free fatty acids; FOM, fish oil mixture; FXR, farnesol X receptor; GSE, proanthocyanidin-rich grape seed extract, GS1, ion source gas 1; HbA1c, glycated hemoglobin; HFHS, high-fat and high-sucrose diet; HNF-4α, hepatic nuclear factor 4; IDA, information-dependent acquisition; IHT, interface heater temperature; ISVF, ionspray voltage floating; iTRAQ, isobaric tag for relative and absolute quantitation; LXR, liver X receptor; MCL, Markov Cluster Algorithm; MMTS, methyl methanethiosulfonate; NF-κB, nuclear factor κB; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acids; SHROB, genetically obese spontaneously hypertensive rat; SREBPs, sterol regulatory element binding proteins; STD, standard diet; TCEP, tris(2-carboxyethyl) phosphine; TEAB, triethylammonium bicarbonate; TFA, total fatty acids; UPR, unfolded protein response

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FIGURE LEGENDS

Figure 1. Biological network analysis of the differentially regulated protein by FOM, GSE and/or FOM&GSE supplementations in the STD background by using the STRING software. The proteins of interest were the 10 differentially regulated proteins from Table 3. Lines indicate the known interrelationships and stronger associations are represented by thicker lines.

Figure 2. 2D-DIGE analysis of the regulation of liver proteins of HFHS-fed rats. A). Representative image of a 2D-DIGE gel showing spot identification numbers (refer to those in Table 4) for proteins found differentially regulated by supplements in the HFHS background. B). Representative images of 2D-DIGE gels according to experimental design showed in the Table 2. C) 3D view of ACLY, PGD, PKLR, CPS1, ASS1, HGD, HSPA5, HSPA8, G6PD, BAAT, ECH1 and CAT.

Figure 3. Biological network analysis of differentially regulated protein by FOM, GSE and/or FOM&GSE supplementations in the HFHS background by using the STRING software. The proteins of interest were the 45 differentially regulated proteins from Tables 3 and 4. Lines indicate the known interrelationships and stronger associations are represented by thicker lines.

Figure 4. Main metabolic liver pathways altered by the supplementation of HFHS diets with FOM, GSE or both as compared to HFHS control diet.

Table 1. Diet composition and iTRAQ experimental design.

	iTRAQ tag	Group	Flour (g)		Oil (mL)		Polyphenol extract (mg)[c]	Macronutrients (% caloric value)			Total energy density (kcal/g)[e]
			STD[a]	HFHS[b]	Soybean	EPA: DHA 1:1		Protein	Fat	Carbohydrate	
iTRAQ-STD	114	STD-C	100	-	1.9	-	-	19	19	62	3.0
	115	STD-FOM	100	-	-	1.9	-	19	19	62	3.0
	116	STD-GSE	100	-	1.9	-	88	19	19	62	3.0
	177	STD-FOM&GSE	100	-	-	1.9	88	19	19	62	3.0
iTRAQ-HFHS	114	HFHS-C	-	100	2.4	-	-	15	51	34[d]	5.0
	115	HFHS-FOM	-	100	-	2.4	-	15	51	34[d]	5.0
	116	HFHS-GSE	-	100	2.4	-	109	15	51	34[d]	5.0
	177	HFHS-FOM&GSE	-	100	-	2.4	109	15	51	34[d]	5.0

STD, standard; HFHS, high-fat high-sucrose; C, control; FOM, fish oil mixture with EPA:DHA 1:1; GSE, proanthocyanidin-rich grape seed extract; FOM&GSE, fish oil and grape polyphenols.

[a] Standard flour (Teklad Global 2014), containing wheat middlings, ground wheat, ground corn, corn gluten meal, calcium carbonate, soybean oil, dicalcium phosphate, iodized salt, L-lysine, vitamin E acetate, DL-methionine, magnesium oxide, choline chloride, manganous oxide, ferrous sulphate, menadione sodium bisulphite complex (source of vitamin K activity), zinc oxide, copper sulphate, niacin, calcium pantothenate, calcium iodate, pyridoxine hydrochloride, riboflavin, thiamin mononitrate, vitamin A acetate, vitamin B12 supplement, folic acid, cobalt carbonate, biotin and vitamin D3 supplement

[b] High-fat high-sucrose diet (Tekland TD 08811), containing sucrose, anhydrous milkfat, casein, maltodextrin, corn starch, cellulose, mineral mix AIN-93G-MX, soybean oil, vitamin mix AIN-93G-VX, L-cystine, choline, bitartrate, green food colour, tert-butylhydroquinone

[c] Proanthocyanidin-rich grape seed extract (Grajfnol®) dose was adjusted to provide a daily proanthocyanidin dose of 30 mg/kg body weight (body weight was higher in rats following a HFHS diet)

[d] 27% from sucrose

[e] Energy density is estimated as metabolizable energy based on the Atwater factors, assigning 4 kcal/g to protein, 9 kcal/g to fat and 4 kcal/g to carbohydrate, including dietary fibre

Table 2. Experimental design for 2D-DIGE study on the liver proteomics effects of different diet composition. 25 μg of protein liver extracts from rats fed one of the different kinds of HFHS diets was labeled with 200 pmoles of the indicated CyDye, mixed together as shown on 2D gels.

GEL NUMBER	Cy2 Standard	Cy3	Cy5
1	HFHS-C+FOM+GSE+FOM&GSE	HFHS-C	HFHS-FOM
2	HFHS-C+FOM+GSE+FOM&GSE	HFHS- FOM&GSE	HFHS-C
3	HFHS-C+FOM+GSE+FOM&GSE	HFHS-C	HFHS-GSE
4	HFHS-C+FOM+GSE+FOM&GSE	HFHS-FOM	HFHS- FOM&GSE
5	HFHS-C+FOM+GSE+FOM&GSE	HFHS-GSE	HFHS- FOM&GSE

Table 3. List of liver proteins differently regulated by FOM, GSE or FOM&GSE supplementation in the background of STD or HFHS diets found by using iTRAQ analysis**.** The protein relative abundance ratios are showed in the last six columns, corresponding to STD-FOM/STD-C, STD-GSE/STD-C, STD-FOM&GSE/STD-C, HFHS-FOM/HFHS-C, HFHS-GSE/HFHS-C and HFHS-FOM&GSE/HFHS-C and were calculated from the averages of three technical replicates from each sample group.

				EFFECT ON STD-C			EFFECT ON HFHS-C		
Identification	**UniProt code**	**Gene name**	**Function or biological process**	**FOM**	**GSE**	**FOM&GSE**	**FOM**	**GSE**	**FOM&GSE**
LIPID METABOLISM									
Enoyl-CoA delta isomerase 1, mitocondrial	P23965	Eci1	Fatty acid beta-oxidation	1,31	0,91	1,35	1,34	1,03	1,49*
Acyl-coenzyme A oxidase	F1LNW3	Acox2	Peroxisomal FA b-oxidation	1,06	0,84	1,15	1,51*	1,12	1,48*
Trifunctional enzyme subunit beta, mitocondrial	Q60587	Hadhb	Mitochondrial FA b-oxidation	1,00	0,91	1,03	0,85	0,79*	0,91
ATP-citrate synthase	P16638	Acly	Lipid biosynthetic process Tricarboxylic acid cycle	0,85	1,02	0,79	0,67*	0,89	0,66*
Fatty acid synthase	P12785	Fasn	Fatty acid biosynthetic process	0,93	0,89	0,82	0,74*	0,99	0,71*
Farnesyl pyrophosphate synthase	P05369	Fdps	Cholesterol biosynthetic process	1,05	1,09	1,03	0,76*	1,03	0,78
Fatty acid-binding protein, epidermal	P55053	Fabp5	Lipid transport	1,02	1,40	0,83	0,60*	0,99	0,65*
Acyl-CoA-binding protein (ACBP)	P11030	Dbi	Lipid transport	1,19	1,29	1,19	0,66*	0,97	0,76*
Acyl-protein thioesterase 1 (ACP1)	P70470	Lypla1	Acyl-CoA Metabolism Protein lipidation	1,05	1,15	1,12	0,67*	0,79*	0,94
60 kDa lysophospholipase	O88202	Aspg	Phospholipid and asparagine metabolic processes	n.q.	n.q.	n.q.	0,71*	0,91	0,92

Long-chain-fatty-acid--CoA ligase 1	P18163	Acsl1	Fatty acid transport	0,89	0,74	0,71*	n.q.	n.q.	n.q.
CARBOHYDRATE METABOLISM									
6-phosphogluconate dehydrogenase, decarboxylating	P85968	Pgd	Pentose phosphate shunt	1,06	1,13	0,99	0,77*	1,05	0,72*
Pyruvate kinase PKLR	P12928	Pklr	Glycolysis	0,97	1,05	0,90	0,80	1,01	0,74*
Protein Tsta3	B0BNN0	Tsta3	Glycogen metabolic process Cholesterol metabolic process	0,94	1,03	0,92	0,96	0,75*	0,89
Fructose-bisphosphate aldolase A	P05065	Aldoa	Glycolysis/Gluconeogenesis	0,65*	0,64*	0,68*	1,09	0,99	0,94
Protein Zadh2	D4A264	Zadh2	Carbohydrate metabolism	0,78*	0,92	1,00	n.q.	n.q.	n.q.
Glyoxalase domain-containing protein 4	Q5I0D1	Glod4	Carbohydrate metabolism	0,71*	1,05	0,73*	1,07	0,96	1,03
PROTEIN AND AMINO ACID METABOLISMS									
Protein disulfide-isomerase A6	Q63081	Pdia6	Protein folding	1.01	1.02	1.08	0.76*	1.07	1.16
Tubulin-specific chaperone A	Q6PEC1	Tbca	Protein folding	1,04	1,06	0,76*	1,03	1,08	0,97
T-complex protein 1 subunit beta	Q5XIM9	Cct2	Protein folding	0,79*	0,96	0,90	0,93	0,91	0,84
Protein Nars	F1LML0	Nars	Protein translation	0,99	1,11	1,12	0,79*	0,94	0,80
Tyrosine--tRNA ligase, cytoplasmic	Q4KM49	Yars	Protein translation	1,03	0,91	0,98	0,73*	0,89	0,81
Eukaryotic translation initiation factor 4B	Q5RKG9	Eif4b	Protein translation	n.q.	n.q.	n.q.	1,63*	1,04	1,47*
Dipeptidyl peptidase 1	P80067	Ctsc	Proteolysis	1,10	1,07	1,13	0,77*	0,81	0,89

Proteasome subunit beta type-8	P28064	Psmb8	Proteolysis	n.q.	n.q.	n.q.	0,79*	0,89	0,69*
GTP cyclohydrolase 1 feedback regulatory protein	P70552	Gchfr	Protein complex assembly Biological regulation	n.q.	n.q.	n.q.	0,81	0,85	0,74*
Protein Ppp2r1a	Q5XI34	Ppp2r1a	Protein dephosphorylation Biological regulation	0,92	0,89	0,88	0,83	0,88	0,76*
Glutaminase liver isoform, mitocondrial	P28492	Gls2	Glutamine metabolism and reactive oxygen species	0,81	0,65*	0,84	0,91	0,93	0,99
Coatomer subunit beta	P23514	Copb1	Protein transport	0,98	0,86	0,72*	0,78*	0,89	0,87
Actin-related protein 2	Q5M7U6	Actr2	Protein transport	0,90	0,88	0,89	1,36*	1,03	1,07
Nuclear transport factor 2	P61972	Nutf2	Protein transport	n.q.	n.q.	n.q.	0,80	0,77*	0,73*
Ubiquitin-conjugating enzyme E2 N	Q9EQX9	Ube2n	Ubiquitin-dependent protein catabolic process	0,95	1,06	0,94	0,82	0,87	0,76*
Cysteine sulfinic acid decarboxylase	Q64611	Csad	Sulfur amino acid metabolic process	0,75*	1,01	0,72*	1,00	1,00	1,08
L-serine dehydratase/L-threonine deaminase	F1LMK6	Sds	Amino acid metabolism	0,85	0,75	0,82	0,79*	0,91	0,88
MISCELLANEUS									
Protein G3bp1	D3ZYS7	G3bp1	Transport Ras protein signal transduction	1,40*	1,28	0,92	1,54*	1,10	1,17
Carbonic anhydrase 3	P14141	Ca3	Response to oxidative stress	1,14	1,05	1,12	1,45*	0,98	1,31
Catechol O-methyltransferase	P22734	Comt	Steroid hormone biosynthesis	1,03	1,14	0,99	0,76*	1,04	0,79

Lumican	P51886	Lum	Biological adhesion	n.q.	n.q.	n.q.	0,77*	0,82	0,77
Ferritin light chain 1	P02793	Ftl1	Ion binding/ferric ion binding	1,07	1,25	1,03	0,78*	0,81	0,79
Prothymosin alpha	P06302	Ptma	Nucleobase-containing compound metabolic process	0,95	1,11	1,17	1,13	1,27	1,40*
Protein Ighm	F1LN61	Ighm	Immune system process Antigen binding Immune system process	0,91	0,74	0,77	0,74*	0,84	0,94
UNCHARACTERIZED PROTEIN									
Protein LOC679748	D3ZE63	LOC686548	Predicted from sequence, "macrophage migration inhibition factor-like" Immune system process Pro-inflammatory cytokine	1,10	1,34	1,04	0,93	0,86	0,69*
Uncharacterized protein	M0R7Y9	-	Cellular component organization or biogenesis Actin binding	1,25	1,28	0,86	0,93	0,91	0,74*

* A value with a statistically significant difference (FDR<5% at quantification level) between STD-C or HFHS-C quantity and each diet supplemented with FOM, GSE or FOM&GSE quantity values.

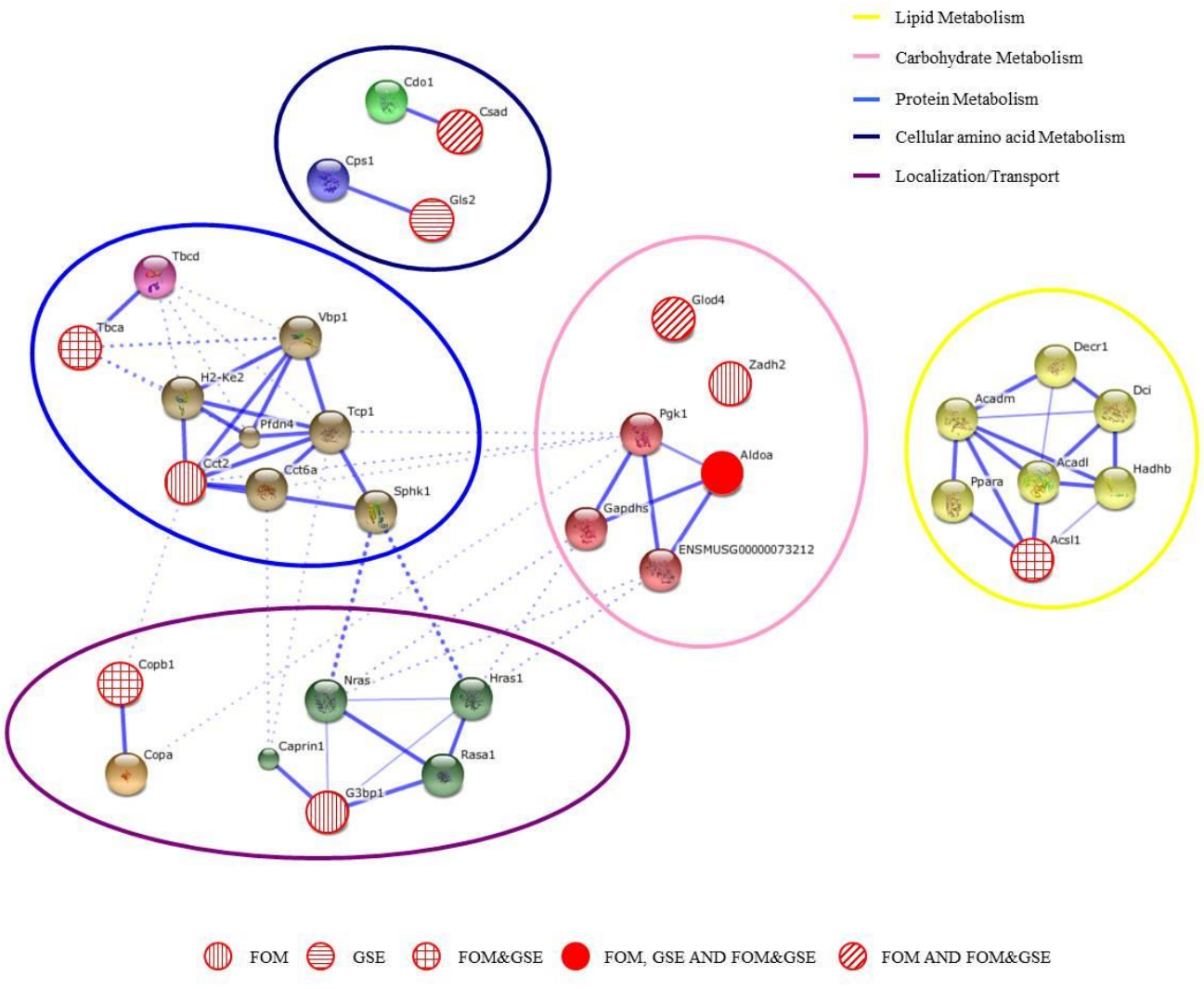
Table 4. List of liver proteins differently regulated by FOM, GSE or FOM&GSE supplementation in the background of HFHS diets found by using 2D-DIGE analysis. Protein spots with a significant ($p<0.05$) value < -1.5 or > 1.5 in relative abundance between diets are listed in the table.

						EFFECT OF FOM		EFFECT OF FOM&GSE	
SSP	**Identification**	**Gene name**	**Uniprot code**	**MASCOT Score**	**Matches**	**ON HFHS-C**	**ON HFHS-GSE**	**ON HFHS-C**	**ON HFHS-GSE**
	LIPID METABOLISM								
770	ATP-citrate synthase	Acly	P16638	62	7	n.s.	n.s.	-2,31	-1,63
1983	Bile acid-CoA:amino acid N-acyltransferase	Baat	Q63276	137	10	n.s.	n.s.	-1,51	n.s.
2627	Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitocondrial	Ech1	Q62651	179	10	n.s.	n.s.	-1,62	n.s.
	CARBOHYDRATE METABOLISM								
1825	6-phosphogluconate dehydrogenase, decarboxylating	Pgd	P85968	64	5	n.s.	-1,64	-1,55	-1,71
1580	Glucose-6-phosphate 1-dehydrogenase	G6pdx	P05370	221	14	-1,71	n.s.	-2,01	-1,64
1585	Pyruvate kinase PKLR	Pkrl	P12928	156	12	-1,74	-1,8	n.s.	-1,51
1606	Pyruvate kinase PKLR	Pkrl	P12928	60	4	n.s.	-1,55	n.s.	n.s.
	CELLULAR AMINO ACID METABOLISM								
741	Carbamoyl-phosphate synthase [ammonia], mitochondrial	Cps1	P07756	102	10	-2,47	n.s.	-1,95	n.s.
762	Carbamoyl-phosphate synthase [ammonia], mitochondrial	Cps1	P07756	95	11	-3,72	n.s.	n.s.	n.s.
2055	Argininosuccinate synthase	Ass1	P09034	239	20	-2,65	-2,68	-2,6	-2,62

1963	Argininosuccinate synthase	Ass1	P09034	507	24	n.s.	n.s.	-1,67	n.s.
1825	Homogentisate 1, 2-dioxygenase	Hgd	Q6AYR0	62	6	-1,54	n.s.	-1,7	-1,58
	PROTEIN METABOLISM								
1208	Moesin	Msn	O35763	66	3	n.s.	n.s.	1,58	n.s.
1199	78 kDa glucose-regulated protein	Hspa5	P06761	164	11	n.s.	n.s.	1,67	n.s.
1299	Heat shock cognate 71 kDa protein	Hspa8	P63018	283	21	n.s.	n.s.	-1,53	-1,58
	RESPONSE TO OXIDATIVE STRESS								
1548	Catalase	Cat	P04762	363	22	n.s.	n.s.	1,66	n.s.
	MISCELLANEUS								
871	Carbamoyl-phosphate synthase [ammonia], mitochondrial	Cps1	P07756	108	21	-4,49	-2.62	-6,24	-4,09
	10-formyltetrahydrofolate dehydrogenase	Aldh1l2	D3ZTP0	80	13				
878	Macrophage-capping protein	Capg	Q6AYC4	132	10	-2,39	n.s.	-3,21	-2,71
	Carbamoyl-phosphate synthase [ammonia], mitochondrial	Cps1	P07756	132	23				
	Cytosolic 10-formyltetrahydrofolate dehydrogenase	Aldh1l1	P28037	106	13		n.s.	-6,24	

n.s.=no significant value.

Figure 1.


Lipid Metabolism
Carbohydrate Metabolism
Protein Metabolism
Cellular amino acid Metabolism
Localization/Transport
Cdo1
Csad
Cps1
Gls2
Tbcd
Tbca
Vbp1
H2-Ke2
Pfdn4
Tcp1
Cct2
Cct6a
Sphk1
Glod4
Zadh2
Pgk1
Aldoa
Gapdhs
ENSMUSG00000073212
Decr1
Acadm
Dci
Acadl
Hadhb
Ppara
Acsl1
Copb1
Copa
Nras
Hras1
Caprin1
Rasa1
G3bp1
FOM
GSE
FOM&GSE
FOM, GSE AND FOM&GSE
FOM AND FOM&GSE

17 **Figure 2.**

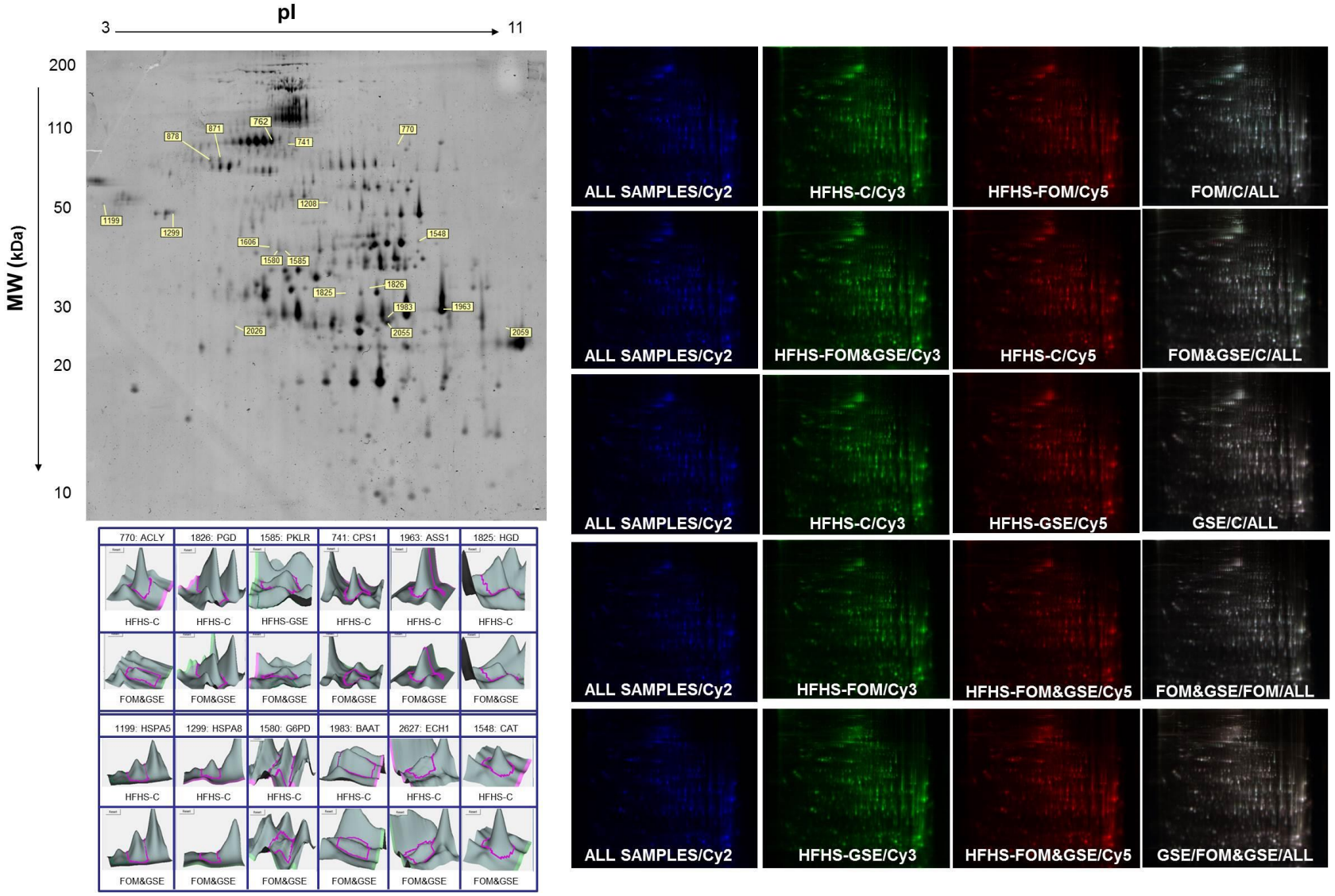

pI
3
11
MW (kDa)
200
110
50
30
20
10
762
871
878
741
770
1208
1199
1299
1606
1580
1585
1548
1825
1826
1983
1963
2026
2055
2059
770: ACLY
1826: PGD
1585: PKLR
741: CPS1
1963: ASS1
1825: HGD
HFHS-C
HFHS-GSE
FOM&GSE
1199: HSPA5
1299: HSPA8
1580: G6PD
1983: BAAT
2627: ECH1
1548: CAT
ALL SAMPLES/Cy2
HFHS-C/Cy3
HFHS-FOM/Cy5
FOM/C/ALL
HFHS-FOM&GSE/Cy3
HFHS-C/Cy5
FOM&GSE/C/ALL
HFHS-GSE/Cy5
GSE/C/ALL
HFHS-FOM/Cy3
HFHS-FOM&GSE/Cy5
FOM&GSE/FOM/ALL
HFHS-GSE/Cy3
HFHS-FOM&GSE/Cy5
GSE/FOM&GSE/ALL

Figure 3.

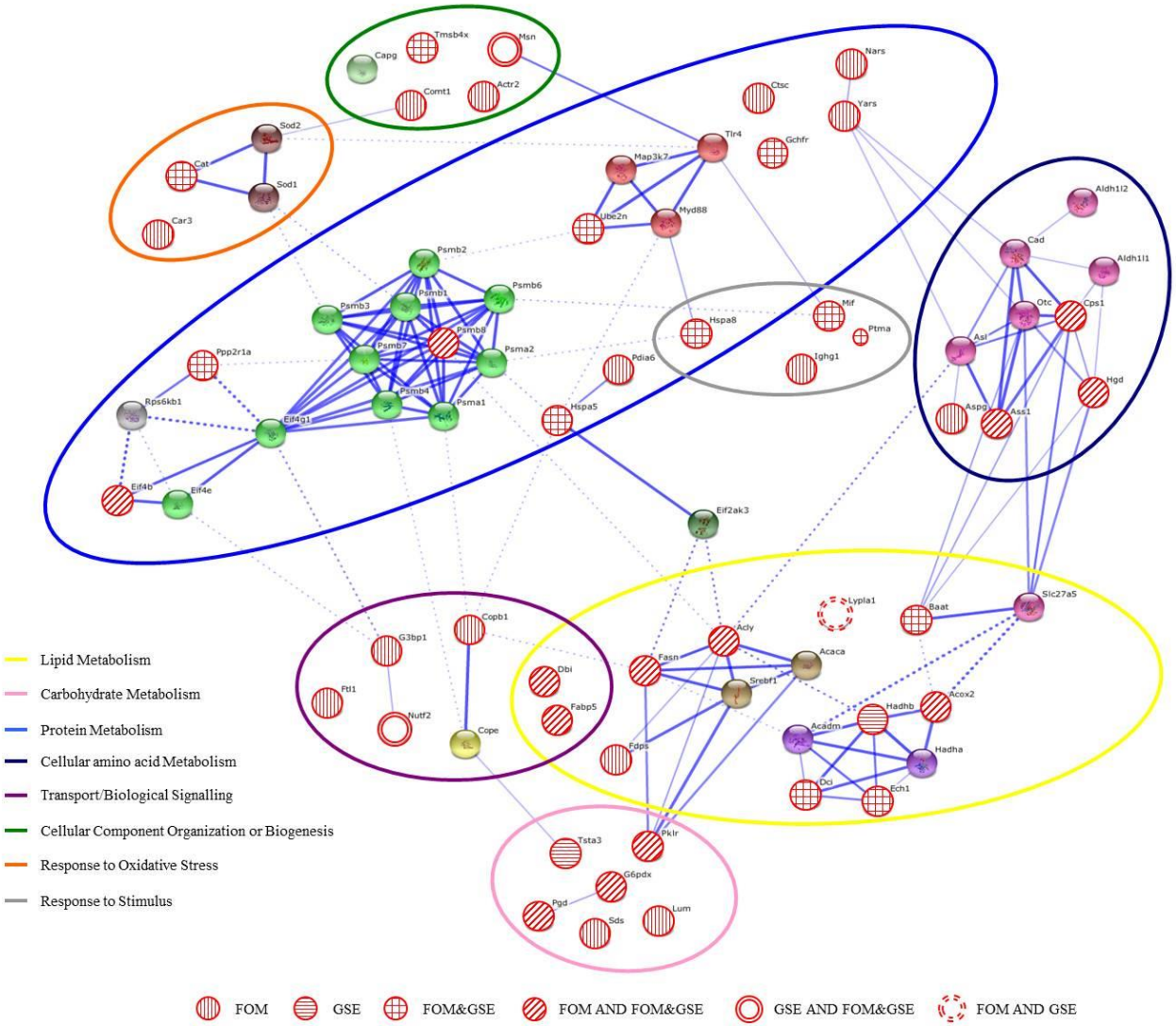
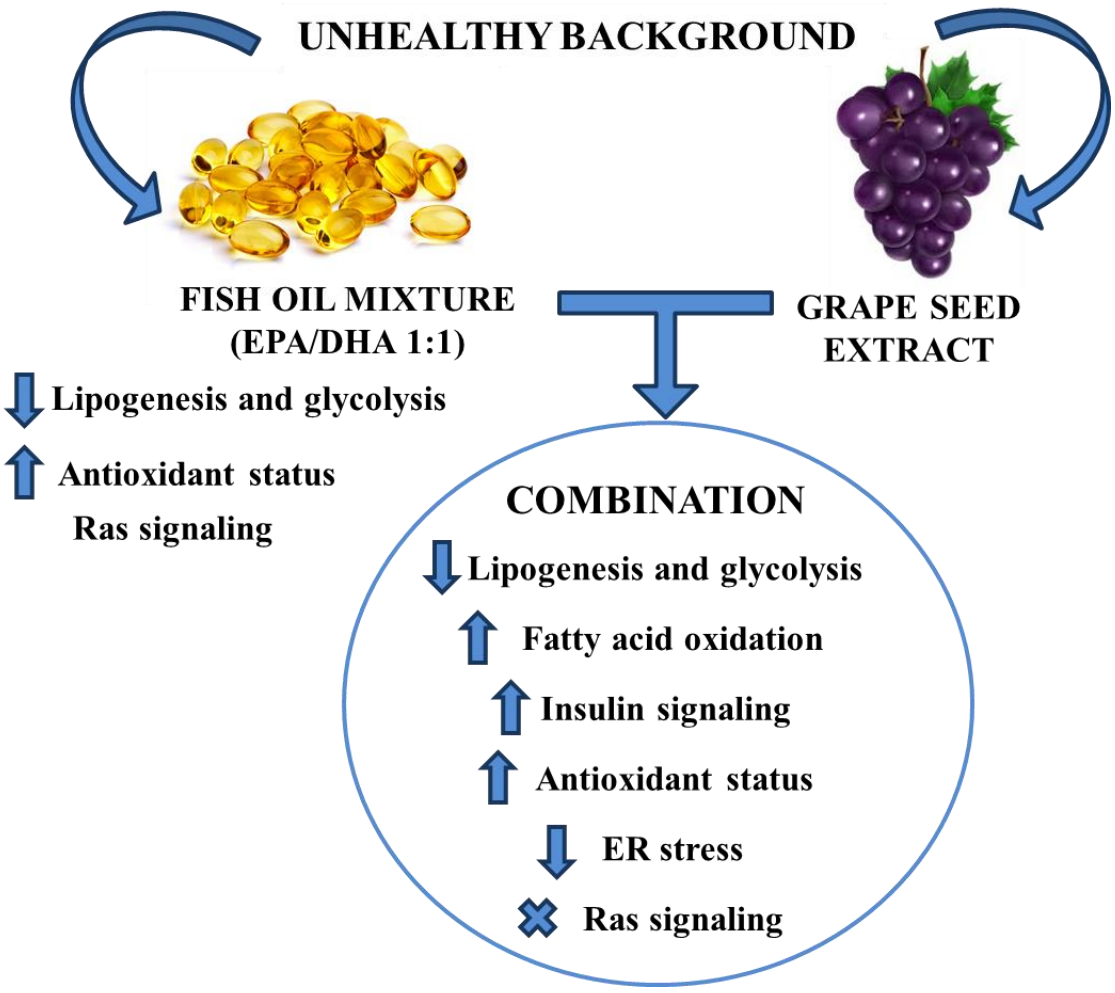

Tmsb4x
Msn
Capg
Comt1
Actr2
Sod2
Cat
Sod1
Car3
Nars
Ctsc
Yars
Tlr4
Gchfr
Map3k7
Ube2n
Myd88
Aldh1l2
Cad
Aldh1l1
Otc
Cps1
Asl
Hgd
Aspg
Ass1
Psmb2
Psmb6
Psmb1
Psmb3
Psmb8
Psmb7
Psma2
Psmb4
Psma1
Ppp2r1a
Rps6kb1
Eif4g1
Eif4b
Eif4e
Hspa8
Mif
Ptma
Ighg1
Pdia6
Hspa5
Eif2ak3
Copb1
G3bp1
Ftl1
Nutf2
Cope
Dbi
Fabp5
Lypla1
Baat
Slc27a5
Acly
Acaca
Fasn
Srebf1
Fdps
Hadhb
Acox2
Acadm
Hadha
Dci
Ech1
Tsta3
Pklr
G6pdx
Pgd
Sds
Lum
Lipid Metabolism
Carbohydrate Metabolism
Protein Metabolism
Cellular amino acid Metabolism
Transport/Biological Signalling
Cellular Component Organization or Biogenesis
Response to Oxidative Stress
Response to Stimulus
FOM
GSE
FOM&GSE
FOM AND FOM&GSE
GSE AND FOM&GSE
FOM AND GSE

Figure 4.


UNHEALTHY BACKGROUND
FISH OIL MIXTURE
(EPA/DHA 1:1)
GRAPE SEED
EXTRACT
Lipogenesis and glycolysis
Antioxidant status
Ras signaling
COMBINATION
Lipogenesis and glycolysis
Fatty acid oxidation
Insulin signaling
Antioxidant status
ER stress
Ras signaling