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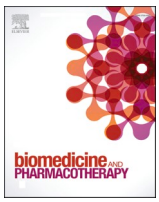

Fish oil supplementation counteracts the effect of high-fat and high-sucrose diets on the carbonylated proteome in the rat cerebral cortex

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ABSTRACT

High daily intake of saturated fats and refined carbohydrates, which often leads to obesity and overweight, has been associated with cognitive impairment, premature brain aging and the aggravation of neurodegenerative diseases. Although the molecular pathology of obesity-related brain damage is not fully understood, the increased levels of oxidative stress induced by the diet seem to be definitively involved. Being protein carbonylation determinant for protein activity and function and a main consequence of oxidative stress, this study aims to investigate the effect of the long-term high-fat and sucrose diet intake on carbonylated proteome of the cerebral cortex of Sprague-Dawley rats. To achieve this goal, the study identified and quantified the carbonylated proteins and lipid peroxidation products in the cortex, and correlated them with biometrical, biochemical and other redox status parameters. Results demonstrated that the obesogenic diet selectively increased oxidative damage of specific proteins that participate in fundamental pathways for brain function, i.e. energy production, glucose metabolism and neurotransmission. This study also evaluated the antioxidant properties of fish oil to counteract diet-induced brain oxidative damage. Fish oil supplementation demonstrated a stronger capacity to modulate carbonylated proteome in the brain cortex. Data indicated that fish oils did not just decrease carbonylation of proteins affected by the obesogenic diet, but also decreased the oxidative damage of other proteins participating in the same metabolic functions, reinforcing the beneficial effect of the supplement on those pathways. The results could help contribute to the development of successful nutritional-based interventions to prevent cognitive decline and promote brain health.

1. Introduction

High daily intake of saturated fats and refined carbohydrates, which characterizes the western diet pattern, often leads to obesity and overweight. Both health conditions have become pandemic in the 21st century, quickly spreading worldwide [1]. Obesity and overweight have been recognized by the World Health Organization as major risk factors for several chronic diseases, such as diabetes, cardiovascular diseases, and cancer [2–4]. They have also been associated with cognitive decline during aging [5] and are independent risk factors for diverse neurophatologies, including Alzheimer's disease and vascular dementia, Parkinson's and Huntington's diseases and multiple sclerosis [6].

Many efforts have been made to understand the molecular mechanisms behind the detrimental effects of the regular intake of obesogenic diets. Of special interest are those connecting an unhealthy diet with cognitive decline, because of the dramatic increase in the global burden of neurological disorders in the last decades [7]. Overall, it has been postulated that oxidative stress, neuroinflammation, insulin resistance and changes to vascularization/blood brain barrier (BBB) integrity are among the most important factors that underlie obesity-induced cognitive decline [8,9]. Several reasons, including enrichment in unsaturated lipid, mitochondria, calcium, glutamate, glucose, modest antioxidant

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defence, redox active transition metals and neurotransmitter auto-oxidation, explain that vulnerability [10].

Particularly, the enrichment in unsaturated fatty acids and high oxygen consumption make the brain prone to lipid peroxidation [11]. Lipid peroxidation drives the formation of reactive carbonyl species (RCS), such as 4-hydroxy-2-nonenal (HNE) or malondialdehyde (MDA). RCS, which have greater half-life than most reactive oxygen species (ROS) and longer migration distances, react with nucleophilic groups in different macromolecules like proteins, DNA and aminophospholids, to form protein carbonyls, adducts and intra and intermolecular cross-links [12]. But protein carbonyls can also be formed by reaction with RCS generated from the oxidation of reducing sugars [13], by direct attack of ROS, by the metal-catalyzed oxidation of the side chains of several amino acids in the presence of H_2O_2 and by direct oxidation of tryptophan [14].

Protein carbonylation is a non-enzymatic, non-reversible post-translational modification that often results in functional losses, protein fragmentation, aggregation, and enhanced susceptibility to proteolytic digestion. Moreover, abnormal protein aggregation further increases oxidative stress through ROS production and mitochondrial dysfunction, which provokes neuronal death, the leading cause of neurodegenerative diseases [15]. Thus, protein carbonylation is a well-documented consequence and measurable indicator of oxidative stress in multiple neuropathologies [16], such as Alzheimer's [17,18], and Parkinson's diseases [19], amyotrophic lateral sclerosis [20] and multiple sclerosis [21]. It should be noted that some proteins are more sensitive than others to carbonylation, depending on factors such as their amino acid sequence, tridimensional conformation, cellular location, and function and the oxidative environment of the cell. This fact gives carbonylation an important role in metabolic control systems and several signaling pathways involving the carbonylation of specific proteins have also been identified [22]. Previous studies have demonstrated that the intake of diets high in saturated fat and sugars induced selective oxidative damage in specific proteins in peripheral organs, such as the liver and the kidney, which led to the alteration of critical metabolic and signaling pathways for the correct function of these organs [23–25]. The alterations were associated with several phenotypic changes, mainly insulin resistance and a poor antioxidant and anti-inflammatory status. Several studies have reported that the intake of high-energetic diets increases lipid peroxidation and protein carbonylation levels in the brain [26,27]. However, the effects of these diets on modulating the carbonylation of specific brain proteins, especially in the cerebral cortex, which might shed light on some of the detrimental outcomes of obesogenic diets, remain largely unknown.

In any case, the alarming spread of diet-induced obesity that fuels the risk of premature age-related neuropathologies has prompted the development of preventive health interventions. Nutrition-based strategies have gained a lot of attention in the last years [28]. Among nutrients assessed for brain health, the omega-3 polyunsaturated fatty acids (ω-3 PUFAs), specifically the eicosapentaenoic (EPA) and docosahexaenoic (DHA) fatty acids, which are mainly found in fish and other seafood, must be highlighted.

There are several reasons behind that interest in marine ω-3 PUFAs for promoting brain health. The brain is the major sink for ω-3 PUFAs, notably DHA. The proper functioning and homeostasis of the central nervous system (CNS) is dependent on the balance between ω-6 PUFAs and ω-3 PUFAs [29]. However, the current westernized diets that contain 15–20 times higher ω-6 PUFAs than ω-3, increase the risk of DHA deficiencies and brain malfunctioning. This is because the brain concentration of DHA depends on dietary DHA content or its shorter chain nutritionally essential precursor, α-linolenic acid (ALA), which can be converted to DHA in the liver [30], although this process is poorly efficient (less than 1% of ALA converted) [31]. Since the local capacity of the brain to synthesize DHA is also appreciably low (less than 0.2% of ALA entering into the brain) [32], the peripheral blood circulation must supply DHA, and other PUFAs, to fulfill the brain requirement. Therefore, PUFAs must be imported across the BBB by passive diffusion of albumin-associated free fatty acids, the transcytosis pathway (involving LDL receptors) and transporting using transmembrane proteins [33], concretely MFSD2A in the case of DHA [34].

Once in the brain, ω-3 PUFAs and their bioactive derivatives regulate various processes within the CNS, such as neuroinflammation, neurotransmission, synaptic plasticity, neurogenesis and neurodegeneration [35]. It has been reported that DHA and EPA have strong anti-inflammatory and inflammation-resolving effects, and antagonizing the pro-inflammatory effects of ω-6 PUFAs derivatives [36]. ω-3 PUFAs also have antioxidant capacity because they activate antioxidant defences by: (1) regulating the nuclear factor erythroid 2 like 2 (NFE2L2) and heme-oxygenase-1 (HO-1), (2) enhancing the synthesis and activity of antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), G-glutamyl-cysteinyl ligase and glutathione reductase (GR), and (3) significantly increasing reduced glutathione production (GSH), with or without stimulation of glutathione metabolism enzymes [28]. Therefore, due to this antioxidant effect, ω-3 PUFAs can modify cellular levels and profiles of both RCS and ROS and control protein carbonylation. Some studies have reported the capacity of ω-3 PUFAs for ameliorating brain oxidative stress in aged rats [37], in a rat model of Schizophrenia [38] or in a mice model of Alzheimer's disease [39]. They also decreased lipid and protein oxidation in the brain of rats fed a high-fat diet [40]. Previous studies have demonstrated that fish oil supplementation selectively decreased the level of carbonylation of target proteins in the liver and the kidney, which led to an improvement in several metabolic and signaling pathways [24,41,42]. However, the effect of ω-3 supplemention on carbonylation level of specific cortex proteins is mostly unknown.

Therefore, the present study has two main objectives: 1) to determine the effect of oxidative stress produced by the long-term consumption of a high-fat high-sucrose (HFHS) diet on protein carbonylation in the brain cortex in a model of prediabetic male Sprague-Dawley rats; and 2) to determine if the supplementation with a fish oil rich in EPA and DHA can counteract the effect of the obesogenic diet on the carbonylated proteome. The study uses a redox proteomic approach to identify and quantify specific carbonylated proteins and hence, the metabolic and signaling pathways affected in the cortex by the diet and the fish oil supplement. This study provides novel insight into the molecular oxidative-related alterations in the brain cortex in response to a HFHS diet and the potential benefits of fish oil intake.

2. Material and methods

2.1. Animal model, diets and experimental design

Thirty-six male Sprague-Dawley rats of 8–9-weeks-old and with an initial weight of about 300 g (Harlan Laboratories Ltd., Derby, UK) were kept in an insulated room in a 12 h artificial light cycle. Humidity (50 ± 10%) and temperature (22 ± 2 °C) were constantly regulated. It was made a random assignment of animals to four groups (n = 9): a control standard diet group (STD-C), fed a standard diet (Teklad Global 14% Protein Rodent Maintenance Diet, Harlan Laboratories, Derby, UK); a group fed the STD diet and supplemented with a fish oil (STD-ω3), which was composed by EPA/DHA in 1:1 ratio representing the 50% of the total fatty acids in oil; a control high-fat high-sucrose diet group (HFHS-C), fed a high-energetic diet (TD.08811 45% kcal Fat Diet, Harlan Laboratories, Derby, UK); and a group fed the HFHS diet and supplemented with the fish oil (HFHS-ω3). Fish oil supplementation was administered by oral gavage (0.8 mL/Kg body weight) once per week. Control groups STD-C and HFHS-C were supplemented with the same dose of soybean oil to compensate for the caloric intake among groups. Details of diet composition and fatty acid diet profiles are respectively provided in Tables S1 and S2. Rats had ad libitum access to water and food, which were registered daily during the experiment. After 21 weeks, rats were intraperitoneally anesthetized with ketamine

chlorhydrate and xylacine (80 mg/Kg and 10 mg/Kg body weight, respectively), and then sacrificed by exsanguination. The cerebral cortex was extracted at 4 °C, washed with a solution of 0.9% NaCl, weighed and immediately submerged in liquid nitrogen previously to store it at −80 °C. All procedures and animal experiments strictly followed the European Union guidelines for the care and management of laboratory animals. The Research Council (CSIC) Subcommittee of Bioethical Issues (ref. AGL2013–49079-C2–1-R) approved the animal study protocol, and the regional Catalan authorities (reference no. DAAM7921) licensed it.

Rats were originally from an investigation of the effects of the HFHS diet and fish oil supplementation on peripheral tissues, i.e. adipose tissue [43], liver [42], kidney [41], modulation of microbiota and glucose metabolism [44,45] and systemic oxidative status [46]. Thanks to those studies, the health status of the animal model used in the current study has already been characterized. Details of the most relevant biometrical and biochemical parameters that define the model are summarized in Table S3. In short, rats fed the HFHS diet developed a prediabetic state, which was characterized by elevated insulin levels needed to maintain blood glucose concentration [44,45]. That prediabetic state was supported by the excessive gain of body weight, a significantly higher adiposity index and the characteristic increment in fat ectopic accumulation [41–43]. Additionally, the intake of HFHS diets increased general oxidative stress of the rats (lower oxygen radical absorbance capacity (ORAC) and higher GSSG/GSH ratio [46] and plasma, liver and kidney protein carbonylation [41,42]). Supplementation of the HFHS diet with the fish oil rich in EPA and DHA ameliorated the prediabetic state of the rats, decreasing plasma insulin levels [44,45], weight gain and ectopic fat accumulation [41–43]. Fish oil also inhibited the general oxidative damage provoked by HFHS intake, since rats supplemented with this oil showed more ORAC capacity, and lower lipid and protein carbonylation in plasma and adipose tissue, liver and kidney [41,42,46].

2.2. Lipid extraction and fatty acid profiling

The Bligh and Dyer protocol [47] was applied to extract lipids from the cerebral cortex. A mixture of dichlorometane, methanol and water in 2:2:1 ratio (v/v) was used as the extraction solvent. Lipid fraction was determined by gravimetric quantification using a Sartorius Precision Balance (2023, Sartorius AG). Data was normalized by total g tissue. Fatty acid profiles were analyzed according to Lepage and Roy [48]. A total of amount of 0.6 mg of the organic phase were transesterificated and then, analyzed by gas chromatography (GC/FID, Clarus 500, Perkin–Elmer, MA, USA), using nonadecanoic acid (19:0) as internal standard.

2.3. Analysis of lipid peroxidation products

Lipid fractions obtained in Bligh and Dyer extraction were used to measure lipid peroxidation levels. Conjugated dienes hydroperoxides were determined by following the method developed by the American Oil Chemists' Society (AOCS) [49].

Measures of oxidized phospholipids (oxPL), 4-HNE and MDA were obtained by dot-blot immunoassay. Briefly, 5 μg lipid extract was put onto a PVDF membrane. After sample drying, the membrane was blocked (1 h at room temperature) and then, incubated with either the antibody anti-oxPL (1:500), or the antibody anti-4-HNE (1:1400) or the antibody anti-MDA (1:20000). After overnight incubation at 4 °C, the oxPL-membrane was washed and directly exposed to UV light. The HNE- and MDA-membranes were further incubated 1 h at room temperature with the corresponding FITC-labeled secondary anti-bodies (1:1000 in both cases). After washing, HNE- and MDA-membranes were exposed to UV light as well.

To determine the levels of those lipid peroxidation products that are bound to proteins, the remaining protein pellets obtained from the Bligh and Dyer lipid extraction were resuspended in urea buffer (7 M urea, 2 M thiourea, 2% CHAPS, and 0.4% DTT) and quantified by the Bradford's method [50]. Then, 5 μg of protein sample were put onto PVDF membranes, which were kept wet in PBS during sample loading, and then, protein samples were left to dry. Measures of oxPL-, HNE- and MDA-protein adducts were made by the same dot-blot protocol described previously for the analysis of unbound oxPL, HNE and MDA.

2.4. Analysis of carbonylated proteome

2.4.1. Measurements of total and specific protein carbonylation

Measures of total and specific protein carbonylation were performed as previously described [41,51]. Briefly, 200 mg of cerebral cortex were homogenized in 25 volumes (w/v) of 20 mM sodium phosphate buffer pH 6.0, 1 mM EDTA, 0.5 mM $MgCl_2$, 10 uL/mL of Proteoblock™ Protease Inhibitor Cocktail and 5 mM of PMSF, using an Ultra Turrax© T10 high-performance disperser (IKA®-Werke GmbH & Co. KG). The homogenate was fractionated in cytosolic and myofibrillar proteins by centrifugation at 100,000g (60 min at 4 °C). Cytosolic proteins, mostly presented in the supernatant, were quantified by BCA method [52] and then stored at −80 °C until used. The corresponding pellets were homogenized in 10 volumes of 10 mM Tris-HCl buffer pH 7.2, 0.6 M NaCl, 10 uL/mL of Proteoblock™ Protease Inhibitor Cocktail and 5 mM of PMSF, using the Ultra Turrax©. Homogenates were then centrifuged at 16000g (15 min at 4 °C), and supernatants, mostly containing myofibrillar proteins, were recovered, quantified by the BCA method and stored until used at −80 °C.

After protein extraction, carbonyl moieties were labeled with the fluorescence probe FTSC by incubating protein samples with 1 mM FTSC at 37 °C for 2.5 h, protected from light, as previously described [51]. After incubation, proteins were precipitated with a solution of TCA at 20% (v/v), centrifuged at 16,000g (20 °C, 10 min) and pellets were washed with 1:1 ethanol/ethyl acetate to eliminate the excess of FTSC. Samples were finally resuspended in 2-D buffer (7 M urea, 2 M tiourea, 2% Chaps, 0.5% Pharmalyte 3–10, 0.5% IPG 3–10 buffer, and 0.4% DTT). Protein concentration was measured by the Bradford's method [50].

Total protein carbonylation in samples was evaluated by resolving the 10 μg of each FTSC-labeled sample in a monodimensional (1-D) 10–12% SDS-polyacrylamide gel electrophoresis (PAGE) and run in a Mini-PROTEAN 3 cell (Bio-Rad, Hercules, CA). To study the level of carbonylation of specific proteins, FTSC-labeled proteins were resolved in bidimensional (2-D) electrophoresis gels. Briefly, 7-cm IPG dry strips with a pH range of 3–10 were loaded with 100 μg of protein sample. Isoelectric focusing (IEF) was performed at 20 °C with a PROTEAN i12 IEF System (Bio-Rad). IPG strips were put onto handmade 12% SDS-PAGE resolving gels and run in a Mini-PROTEAN 3 cell (Bio-Rad, Hercules, CA). Before running the second dimension, cysteine residues were reduced and alkylated.

Both 1D and 2D gels were exposed to UV light to visualize the FTSC-tagged proteins and then, the gels were stained overnight with Coomassie dye PhastGel Blue R-350 (GE Healthcare Science) to visualize the total protein content. For the accurate comparison of protein carbonylation levels among the different dietary groups, the protein carbonylation index was calculated [53]. This parameter is the result of dividing the signal given by spot/band/lane in the FTSC-labeled protein gel by its respective signal in the Coomassie-stained protein gel.

2.4.2. Identification of carbonylated proteins

For protein identification, FTSC-labeled proteins were isolated by 2-D electrophoresis. 500 μg of proteins were loaded onto 18-cm IPG dry strips with a pH range of 3–10. Isoelectrofocusing was performed on the Ettan IPGphor II isolectric focusing system (GE Healthcare Science) at 20 °C according to manufacturer's instructions. After reducing and alkylating cysteine residues, the second dimension was run on the Ettan DALTsix electrophoresis system (GE Healthcare Science) at 15 °C. Carbonylated protein spots were manually excised from 2D gels and trypsin-digested with 0.5 μM trypsin in 50 mM NH_4HCO_3 buffer, pH 8

(overnight, at 37 °C).

Peptide analysis was made by nano-LC ESI-IT-MS/MS using a Dionex UltiMate 3000 Series (ThermoFisher, Rockford, IL, USA) equipment coupled to a dual-pressure linear ion trap mass spectrometer LTQ Velos Pro with electrospray ionization (ESI) (Thermo Fisher, Rockford, IL, USA). Prior to loading onto the analytical column, 3 μL of sample was concentrated and cleaned using a μ-precolumn cartridge (μ-Precolumn C18 PepMap; 300 μm i.d. × 5 mm) (Thermo Scientific, San Jose, CA, USA) at a flow rate of 10 μL/min using 0.1% formic acid in water as loading solvent. Chromatographic separation of tryptic peptides was achieved in an analytical C18 column (Acclaim PepMap RSLC C18, 2 μm, 100 Å, 75 μm i.d. × 15 cm) and a flow rate of 300 nL/min. Mobile phase A was 0.1% formic acid in water, and mobile phase B was 0.1% formic acid in acetonitrile. Peptides were separated in a 20-min linear gradient elution from 5% to 40% B.

Tandem mass spectrometry (MS/MS) analysis of peptides was performed in a LTQ Velos Pro mass spectrometer, operating in data-dependent acquisition (DDA) mode. Full MS scans were acquired in positive mode and mass range of 400–1600 Da, and the six most-intense precursor ions with ≥ 2 charge state were subjected to MS/MS analysis. Fragmentation was made in collision-induced dissociation (CID) mode, fixing an isolation width of 2 Da and 35% normalized collision energy. It was stablish a dynamic exclusion of 30 s for fragmented masses after the second fragmentation event. Xcalibur 2.0 and Tune 2.2 software (Thermo Fisher Scientific, Inc.) were used for data acquisition and instrument control.

Protein identification was achieved by searching the raw data resulting from the MS analysis against the Uniprot/Swiss-Prot Rattus norvegicus database using the PEAKS DB (Bioinformatics Solutions Inc., Waterloo, ON, Canada) software. Searching criteria were trypsin as a proteolytic enzyme, oxidation of methionine and carbamidomethylation of cysteine as variable modifications, a maximum of 2 missed cleavages per peptide and a mass tolerance of ± 1 Da for precursor and ± 0.6 Da for product ion scans. For all the identifications, a false discovery rate (FDR) below 0.1% was fixed.

2.5. Gene Ontology (GO) and KEGG pathway functional enrichment analysis of the carbonylated proteome

Functional enrichment analysis of the carbonylated proteome in the rat cerebral cortex was conducted by submitting the corresponding gene list of the carbonylated protein to the freely online STRING (Search Tool for the Retrieval of Interacting Genes) software version 11.5 (http://stringdb.org/ (accessed on 30 March 2023) and selecting Rattus norvegicus as organism. Significant enrichment in the GO terms and KEGG pathways were considered if FDR < 0.05, which is the p-value corrected for multiple testing using the Benjamini–Hochberg procedure [54].

2.6. Image analysis

Dot-blot membranes for the analysis of lipid peroxidation products (both free and bound to proteins) and 1D and 2D gels for the analysis of protein carbonylation were exposed in the UVP BioDoc-It2 Gel Imaging System UV transilluminator (Analytik Jena AG, Upland, CA, USA), with a 520-nm band-pass filter (520DF30 62 mm).

LabImage 1D (Kapelan Bio-Imaging Solutions, Halle, Germany) was used for the analysis of the 1D gel images to obtain total protein carbonylation data. Dot-blot membrane images, for lipid peroxidation product determination, and 2D gel images for specific protein carbonylation measures, were analyzed using PDQuest software version 7.4 (Bio-Rad, Hercules, CA, USA).

2.7. Statistics

Data in tables and figures were shown as mean and standard deviation (SD). Two-way ANalysis Of VAriance (ANOVA) was conducted to evaluate the effects of the diet (D) and the supplement (S) or their interaction (DxS) with the freely available Jamovi Version 2.3.18.0 (retrieved from https://www.jamovi.org). Normal distribution and homogeneity of variance were previously verified by Shapiro–Wilk's and Levene's tests, respectively. When the assumptions of Two-way ANOVA were violated, nonparametric Kruskal–Wallis test were conducted. The post hoc test Tukey HD was used to compare the means among them, and significant differences were considered when $p < 0.05$.

2.8. Materials and reagents

Fish oil was prepared by mixing two commercial fish oils: AFAMPES 121 EPA (AFAMSA, Vigo, Spain) and EnerZona Omega 3 RX (Milan, Italy). The final EPA and DHA content represented 50% of the total fatty acids in the fish oil mixture, with EPA and DHA at a 1:1 ratio. Soybean oil, which was obtained from unrefined organic soy oil, was bought from Clearspring Ltd. (London, UK).

Dithiothreitol (DTT), phenylmethylsulfonyl fluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), iodoacetamide (IA), Tris Hydrochloride (Tris–HCl), N,N,N′,N′-Tetramethyl ethylenediamine (TEMED), trichloroacetic acid (TCA), 3-((3-cholamidopropyl) dimethylammonio)− 1-propanesulfonate (CHAPS) detergent and bicinchoninic acid (BCA) assay were obtained from Sigma (St. Louis, MO, USA).

BlueBlock Blocking Solution, Phosphate-Buffered Saline (PBS) 10X solution and PBS with 0.05% TweenTM-20 (PBST) 10X solution were purchased from Serva (SERVA Electro-phoresis GmbH, Germany). Protease inhibitor ProteoBlock™ was acquired from Thermo Fisher Scientific Inc. (Rockford, IL, USA). Ketamine-HCl was purchased from Merial Laboratorios S.A. (Barcelona, Spain) and xylazine (Rompun 2%) from Química Farmacéutica S.A. (Barcelona, Spain). Fluorescein-5-thiosemicarbazide (FTSC) was bought from Invitrogen (Carlsbad, CA). Trypsin sequencing-grade used for protein digestion was acquired from Promega (Madison, WI, USA).

E06 mouse monoclonal antibody (IgM), TopFluor® labeled anti-oxidized phospholipid (oxPL) was supplied by Avanti® Polar Lipids, Inc. (Croda international Plc, Birmingham, AL, USA). Primary antibodies anti-MDA (goat polyclonal antibody to malondialdehyde), anti-4-HNE (mouse monoclonal (HNEJ-2) antibody to 4-HNE), and secondary antibodies donkey anti-goat IgG (H+L) (FITC) and goat anti-mouse IgG (H+L) (FITC) were purchased to Antibodies.com (Europe AB, Stockholm, Sweden). Bio-Rad Laboratories (Hercules, CA, USA) provided Bio-Rad protein assay, acrylamide and bis-N,N-methylene-bis-acrylamide and PVDF membranes. 2-D electrophoresis IPG strips Immobiline DryStrip gels for isoelectric focusing (IEF) of pH range 3–10 and lengths of 7 and 18 cm were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden), as well as IPG buffer 3–10 pH and pharmalyte 3–10 pH. Cytiva (Marlborough, USA) provided bromophenol blue PlusOne.

3. Results

3.1. Effects of long-term consumption of high-fat and high-sucrose diet and fish oil supplementation on lipid and protein oxidation in the brain cortex

Table 1 shows the levels of ARA, DHA, and total PUFAs ω-3 and ω-6 in brain cortex after dietary intervention. The supplementation with fish oil caused an enrichment in the ratio PUFAs ω-3/ω-6 in both STD and HFHS frameworks. The oxidative stress induced in rats fed the HFHS diet was measured in the cerebral cortex and shown in Table 1, as well. Data demonstrated an increase in lipid peroxidation products in the cerebral cortex of HFHS-fed rats as compared to STD-fed ones. This increase was evidenced by the general trend toward the major formation of conjugated dienes hydroperoxides and the significant increment in the levels of specific oxidative compounds such as HNE and MDA. Noteworthy, HFHS diet tended to increase the formation of oxidized

Table 1
Oxidative stress-related measures and percentage of arachidonic acid (ARA), docosahexaenoic acid (DHA) and total polyunsaturated fatty acids (PUFAs) in the brain cortex of Sprague-Dawley rats fed a standard diet, supplemented either with soybean oil (STD-C) or fish oil (STD-ω3), and Sprague-Dawley rats fed a high-fat and high-sucrose diet, supplemented either with soybean oil (HFHS-C) or fish oil (HFHS-ω3).[1].

	STD-C		STD- ω3		HFHS-C		HFHS- ω3	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Conjugated dienes (mmoles hydroperoxide/kg Lipid)	5.78[a]	0.87	5.33[a]	0.81	6.82[a]	1.92	6.08[a]	0.53
HNE (a.u./μg Lipid)[$]	74.3[a]	9.45	80.46[a]	6.20	99.34[b]	4.65	74.17[a]	11.29
MDA (a.u./ μg Lipid)[$]	230.88[a]	21.13	300.77[b]	17.96	309.14[b]	5.59	256.63[a]	32.95
OxPC (a.u./ μg Lipid)	178.61[a]	18.21	177.86[a]	23.47	213.83[a]	15.33	166.31[a]	22.24
HNE-Protein (a.u./ μg Protein)	218.91[a]	65.37	212.14[a]	3.51	225.95[a]	22.17	208.37[a]	9.30
MDA-Protein (a.u./ μg Protein)	258.11[a]	10.25	233.73[a]	32.06	244.87[a]	10.78	232.53[a]	22.42
OxPC-Protein (a.u./ μg Protein)	772.42[a]	104.87	973.07[a]	201.01	873.91[a]	274.19	786.56[a]	203.52
Cytosolic protein carbonylation index (a.u/μg Protein)[$,#]	65.39[a]	12.33	93.55[b]	13.38	87.60[b]	7.97	86.79[b]	9.05
Myofibrillar protein carbonylation index (a.u/μg Protein)[$]	91.23[a]	13.31	69.90[b]	9.11	92.79[a]	4.85	79.42[b]	9.88
Total protein carbonylation index (a.u/μg Protein)[*]	78.31[a]	7.05	81.72[ab]	7.97	90.20[b]	4.19	83.10[ab]	6.62
% Arachidonic acid (ARA) (g/100 g fatty acid)[#]	9.97[a]	0.58	10.25[a]	0.46	10.20[a]	0.43	9.28[a]	0.69
% Docosahexaenoic acid (DHA)(g/100 g fatty acid)[$,#]	11.66[a]	0.75	13.12[b]	0.89	13.29[b]	0.76	13.26[b]	0.93
% ω-3 (g/100 g fatty acid)[$,#]	11.86[a]	0.81	13.56[b]	0.91	13.50[b]	0.78	13.53[b]	1.00
ω-6 (g/100 g fatty acid)[*,$,#]	15.48[ab]	0.90	15.47[ab]	0.49	15.05[bc]	0.43	13.51[c]	1.08
ω-3/ω-6 (g/100 g fatty acid)[*,$]	0.77[a]	0.03	0.88[b]	0.04	0.90[b]	0.03	1.00[c]	0.05
% Total PUFAs (g/100 g fatty acid)	27.34[a]	1.65	28.75[a]	1.38[a]	28.55[a]	1.18	27.04[a]	1.95

[1] Two-way ANOVA analyses were conducted.
[*] $p < 0.05$ significant differences given by the factor "diet" (STD and HFHS).
[$] $p < 0.05$ significant differences given by the factor "supplement" (CONTROL, ω-3).
[#] indicates significant interaction ($p < 0.05$) between the factors diet and supplement. Means with different superscript indicate significant differences ($p < 0.05$) (analyzed by post-hoc Tukey HSD). HNE: 4-hydroxy-2-nonenal; MDA: malondialdehyde; oxPC: oxidized phosphatidylcoline; HNE-Protein, MDA-Protein, oxPC-Protein are adducts between the corresponding lipid peroxidation product and proteins. a.u.: arbitrary units.

phosphatidylcholines (oxPC). Regarding protein oxidation, the intake of HFHS diet significantly increased carbonylation of brain cortical proteins, being cytosolic proteins particularly vulnerable to that, as shown in Table 1. The HFHS-C diet did not increase the formation of lipid peroxidation products-protein adducts with respect to the STD-C diet.

The incorporation of the fish oil supplement to the HFHS diet significantly decreased the levels of lipid peroxidation products, HNE, MDA and oxPC, and myofibrillar carbonylated proteins. Finally, the higher proportion of DHA in animals fed STD diet supplemented with fish oils, led to a slight increase in MDA and cytosolic protein

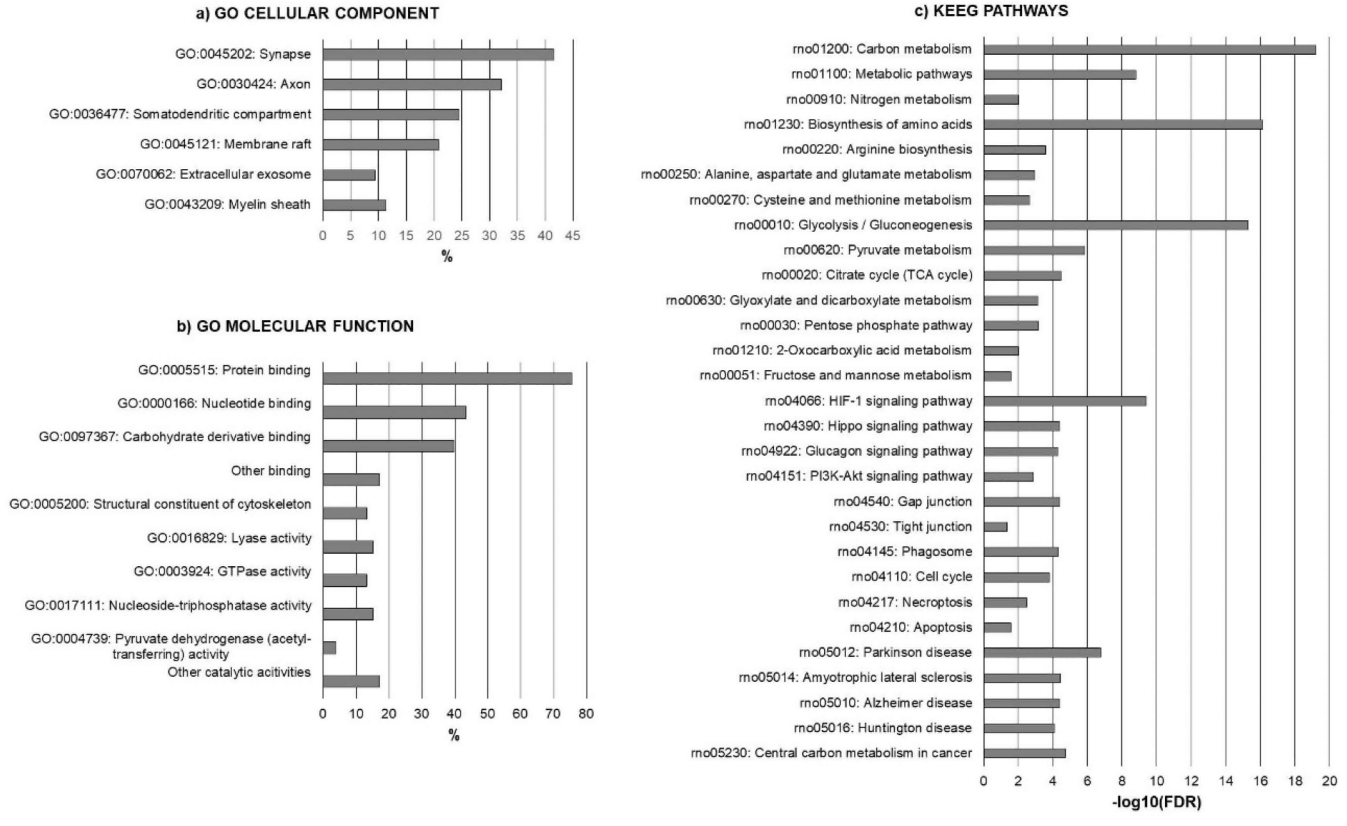

Fig. 1. GO and KEGG Functional enrichment analysis for carbonylated proteins identified in the cortex. (a) Significantly enriched GO cellular component (b) molecular function and (c) KEGG pathways. Analysis were conducted by using STRING. FDR: False Discovery Rate. Significant enrichment at FDR < 5%.

carbonylation, and a strong decrease in myofibrilar protein carbonylation.

3.2. Targets of protein carbonylation in cortex and functional enrichment analysis

The study of cortex carbonylome revealed 62 carbonylated spot targets (38 of them in the cytosolic fraction (spots Nº 1–38) Fig. S1) and 24 in the myofibrillar one (spots Nº 39–62, Fig. S2). They corresponded to 53 different carbonylated proteins, which created a protein carbonylation pattern qualitatively shared by all dietary groups. Protein identification are shown in the Table S4.

Functional enrichment analysis of all these carbonylated proteins were conducted for the Gene ontology (GO) terms of cellular component and molecular functions (Fig. 1).

According to the cellular component results shown in Fig. 1a, the 42% of total carbonylated proteins were proteins from the synapse, and more precisely, proteins from presynapse, postsynapse and postsynaptic actin cytoskeleton. Moreover, carbonylated proteins were essentially axonal proteins (32%), mainly from the axon terminus and the growth cone, and somatodendritic proteins (25%), especially from the neuronal body. There were also significant enrichments in proteins from the membrane raft (21%), the extracellular exosome (9%) and the myelin sheat (11%).

The 28% of those proteins belong to cytoskeleton, the 11% to the mitochondrial matrix and the 54% were cytosol proteins. Interestingly, the 51% of carbonylated proteins were part of protein-containing complex, with significant enrichments in protein complexes such as the mitochondrial pyruvate dehydrogenase complex, the mitochondrial proton-transporting ATP synthase complex, catalytic sector f(1), the phosphopyruvate hydratase complex, the hemoglobin complex and the haptoglobin-hemoglobin complex.

Fig. 1b shows the main molecular functions exhibited by carbonylated proteins identified in rat cortex. Data analysis indicated that 94% of carbonylated proteins identified possesed binding capacity. More than the 75% of carbonylated proteins can bind to proteins, with significant enrichments in functions such as identical protein binding, protein domain specific binding, cytoskeletal protein binding, angiostatin binding, ion channel binding, and enzyme binding. Among the latter, kinase, protease and ubiquitin protein ligase binding functions were significantly enriched. The 43% of the carbonylated proteins can bind to nucleotides, such as ATP, GTP, ADP and NAD, while the 40% of them had carbohydrate derivative binding function. Magnesium ion binding (9%) and carboxylic acid binding (5%) were other binding functions exhibited by the carbonylated proteins.

Besides binding functions, other molecular functions were significantly enriched in carbonylated proteins. Therefore, the 13% of proteins were structural constituent of cytoskeleton, especially of the postsynaptic actin cytoskeleton. The 64% of carbonylated proteins had catalytic activity, with significant enrichment in proteins with several lyase activities (15%), hydrolases with GTPase activity (13%) and nucleoside-triphosphatase activity (15%) and oxidorreductases with pyruvate dehydrogenase (acetyl-transferring) activity (4%).

Since carbonylation is an irreversible PTMs that can modulate protein function and activity, the application of KEGG functional enrichment analysis allowed the identification of pathways potentially modulated in rat cortex. The pathways found significantly enriched in carbonylated proteins are shown in Fig. 1c.

Results indicated that protein carbonylation is implicated in diverse metabolic pathways and signaling processes in brain cortex. Significant enrichments in proteins participating in the carbon metabolism were found. Among them, proteins involved in the amino acid metabolism (biosynthesis of amino acids, cysteine and methionine metabolism, alanine, aspartate and glutamate metabolism, arginine biosynthesis) and energy production (glycolysis/gluconeogenesis, pyruvate metabolism, TCA cycle, glyoxylate and dicarboxylate metabolism, pentose phosphate pathway, 2-oxocarboxylic acid metabolism, fructose and mannose metabolism). Additionally, several signaling pathways, which are critical for cortex function, resulted potentially under carbonylation control. Those pathways were: hypoxia-inducible factor (HIF)− 1 signaling pathway, hippo signaling pathway, glucagon signaling pathway and PI3K-Akt signaling pathway. Other critical pathways identified were the gap and tight junctions, the phagosome, the cell cycle and the necroptosis and apoptosis processes in brain. Interestingly, results indicated that cortex carbonylated proteins are specially involved in critical pathways related to several neurodegenerative diseases, including Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease and Huntington's disease, and in cancer, as well (central carbon metabolism in cancer).

3.2.1. Effects of long-term feeding of high-fat and high-sucrose diet on protein carbonylation patterns in brain cortex

Interestingly, long-term feeding of HFHS diet modified protein carbonylation level in 18 protein spots, which corresponded to 17 different proteins (Table 2). Those proteins were: aconitate hydratase mitochondrial (ACON), fructose-bisphosphate aldolase C (ALDOC), creatine kinase B-type (CKB), dihydropyrimidinase-related protein 2 (DPYSL2), alpha-enolase (ENOA), gamma-enolase (ENOG), glutamine synthetase (GLUL), hemoglobin subunit alpha-1/2 (HBA), heat shock cognate 71 kDa protein (HSPA8), pyruvate dehydrogenase E1 component subunit beta mitochondrial (PDHB), pyruvate kinase PKM (PKM), peptidyl-prolyl cis-trans isomerase A (PPIA), triosephosphate isomerase (TPIS), Rab GDP dissociation inhibitor alpha (GDIA), 60 kDa heat shock protein mitochondrial (HSPD1), malate dehydrogenase cytoplasmic (MDHC) and phosphoglycerate kinase 1 (PGK1). All proteins, with the exception of PDHB, PPIA and HBA, significantly increased carbonylation due to the HFHS diet.

Functional enrichment analysis of the proteins modulated by HFHS diet revealed that the altered carbonylation occurred preferentially in proteins from the axon, specially the distal axon, the cell body, the membrane raft and myelin sheat. A significant enrichment in proteins from the synapse and the postsynaptic cytosol were also found. Finally, HFHS diet significantly affected the phosphopyruvate hydratase complex in cortex brain. Regarding the molecular functions, the 88% of proteins had catalytic activity, especially carbon-oxygen or hydro-lyase activities, and had ADP and magnesium ion binding capacities.

According to the KEGG enrichment analysis (Fig. 2a), HFHS-feeding caused significant alterations in critical metabolic pathways by changing the carbonylation level of those specific proteins. HFHS diet preferentially affected the carbon metabolism, showing a strong influence on glycolysis/gluconeogenesis, pyruvate metabolism, citrate cycle (TCA cycle) and the metabolism of the glyoxylate and dicarboxylate. HFHS diet also altered the biosynthesis of amino acids. Remarkably, the cortex of rats fed the HFHS diet suffered significant alterations in carbonylated proteins participating in HIF-1 signaling pathway. The central carbon metabolism in cancer was also affect by the HFHS diet.

3.2.2. Effects of fish oil supplementation on protein carbonylation patterns in brain cortex

Supplementation with a fish oil rich in EPA and DHA quantitatively altered the pattern of cortex protein carbonylation in both STD and HFHS dietary frameworks (Table 2). Results indicated that the effect of the supplementation was slightly stronger when fish oil was added to the HFHS diet (20 proteins responded to fish oils in STD vs. 23 in HFHS). Noteworthy, the effect of fish oil to modulate cortex protein carbonylation was generally independent of background diet. Therefore, 20 proteins in both STD and HFHS diets significantly responded to fish oil supplementation by decreasing their carbonylation level, as compared to control groups. Those proteins were: ACON, ALDOC, CKB, DPYSL2, ENOA, ENOG, GLUL, HBA, HSPA8, PDHB, PKM, PPIA, TPIS, fascin (FSCN1), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), glutamate dehydrogenase 1 mitochondrial (GLUD1), aspartate

Table 2
Proteins whose carbonylation level was modulated by the high-fat and high-sucrose diet, the fish oil supplementation or both in the cerebral cortex of Sprague-Dawley rats.[1].

Spot Nº	Figure	Protein Abbreviation	Protein Description	Specific protein carbonylation (a.u./µg protein)			
				STD-C	STD-ω3	HFHS-C	HFHS-ω3
5	S1	**ACON**	Aconitate hydratase mitochondrial	$0.05^{a} \pm 0.02$	$0.03^{a} \pm 0.02$	$0.09^{b} \pm 0.01$	$0.06^{ab} \pm 0.01$
21	S1	**ALDOC**	Fructose-bisphosphate aldolase C[*,$]	$0.03^{a} \pm 0.01$	$0.02^{a} \pm 0.01$	$0.05^{b} \pm 0.02$	$0.02^{a} \pm 0.01$
22	S1	**ALDOC**	Fructose-bisphosphate aldolase C[*,$]	$0.07^{a} \pm 0.02$	$0.04^{a} \pm 0.01$	$0.11^{b} \pm 0.01$	$0.06^{a} \pm 0.01$
23	S1	**ALDOC**	Fructose-bisphosphate aldolase C[*,$]	$0.05^{ab} \pm 0.02$	$0.04^{a} \pm 0.01$	$0.09^{b} \pm 0.02$	$0.05^{a} \pm 0.00$
14	S1	**CKB**	Creatine kinase B-type[*,$]	$0.04^{a} \pm 0.01$	$0.03^{a} \pm 0.01$	$0.09^{b} \pm 0.04$	$0.07^{ab} \pm 0.01$
53	S2	**CKB**	Creatine kinase B-type[*,$]	$0.22^{ab} \pm 0.08$	$0.19^{ab} \pm 0.10$	$0.24^{b} \pm 0.03$	$0.18^{a} \pm 0.03$
3	S1	**DPYL2**	Dihydropyrimidinase-related protein 2[*,$]	$0.03^{ab} \pm 0.00$	$0.02^{a} \pm 0.00$	$0.04^{b} \pm 0.01$	$0.03^{ab} \pm 0.01$
16	S1	**ENOA**	Alpha-enolase[*,$]	$0.02^{a} \pm 0.01$	$0.01^{a} \pm 0.00$	$0.04^{b} \pm 0.00$	$0.02^{a} \pm 0.00$
12	S1	**ENOG**	Gamma-enolase[*,$]	$0.02^{a} \pm 0.00$	$0.03^{ab} \pm 0.01$	$0.05^{b} \pm 0.01$	$0.03^{ab} \pm 0.01$
51	S2	**FSCN1**	Fascin[$]	$0.14^{a} \pm 0.04$	$0.07^{b} \pm 0.02$	$0.12^{ab} \pm 0.02$	$0.09^{ab} \pm 0.02$
20	S1	**GAPDH**	Glyceraldehyde-3-phosphate dehydrogenase[#]	$0.08^{ab} \pm 0.03$	$0.13^{a} \pm 0.02$	$0.11^{ab} \pm 0.01$	$0.09^{b} \pm 0.07$
61	S2	**GAPDH**	Glyceraldehyde-3-phosphate dehydrogenase[$]	$0.30^{a} \pm 0.09$	$0.15^{b} \pm 0.04$	$0.21^{ab} \pm 0.04$	$0.12^{b} \pm 0.02$
6	S1	**GDIA**	Rab GDP dissociation inhibitor alpha[*]	$0.03^{a} \pm 0.01$	$0.03^{a} \pm 0.01$	$0.06^{a} \pm 0.02$	$0.07^{a} \pm 0.02$
46	S2	**GLUD1**	Glutamate dehydrogenase 1 mitochondrial[$]	$0.20^{a} \pm 0.03$	$0.09^{b} \pm 0.03$	$0.17^{ab} \pm 0.02$	$0.12^{ab} \pm 0.04$
18	S1	**GLUL**	Glutamine synthetase[*,$,#]	$0.03^{a} \pm 0.00$	$0.04^{a} \pm 0.00$	$0.09^{b} \pm 0.00$	$0.05^{c} \pm 0.02$
57	S2	**GLUL**	Glutamine synthetase[$]	$0.17^{a} \pm 0.05$	$0.06^{b} \pm 0.04$	$0.11^{ab} \pm 0.02$	$0.11^{ab} \pm 0.06$
17	S1	**GOT1**	Aspartate aminotransferase cytoplasmic[$,#]	$0.06^{ab} \pm 0.03$	$0.05^{ab} \pm 0.00$	$0.06^{a} \pm 0.01$	$0.03^{b} \pm 0.01$
35	S1	**HBA**	Hemoglobin subunit alpha-1/2[*,$]	$0.09^{a} \pm 0.03$	$0.05^{ab} \pm 0.01$	$0.05^{ab} \pm 0.01$	$0.03^{b} \pm 0.02$
38	S1	**HBB1**	Hemoglobin subunit beta-1[$]	$0.10^{ab} \pm 0.06$	$0.09^{ab} \pm 0.03$	$0.14^{a} \pm 0.03$	$0.03^{b} \pm 0.02$
2	S1	**HSPA8**	Heat shock cognate 71 kDa protein[*]	$0.01^{a} \pm 0.00$	$0.02^{ab} \pm 0.01$	$0.02^{b} \pm 0.00$	$0.02^{ab} \pm 0.00$
39	S2	**HSPA8**	Heat shock cognate 71 kDa protein[#]	$0.08^{a} \pm 0.01$	$0.05^{a} \pm 0.02$	$0.06^{a} \pm 0.01$	$0.08^{a} \pm 0.02$
42	S2	**HSPD1**	60 kDa heat shock protein mitochondrial[*]	$0.09^{a} \pm 0.02$	$0.08^{a} \pm 0.02$	$0.11^{a} \pm 0.04$	$0.15^{a} \pm 0.04$
27	S1	**MDHC**	Malate dehydrogenase cytoplasmic[*]	$0.01^{ab} \pm 0.00$	$0.01^{a} \pm 0.00$	$0.02^{b} \pm 0.01$	$0.02^{ab} \pm 0.00$
56	S2	**PDHA1**	Pyruvate dehydrogenase E1 component subunit alpha somatic form mitochondrial[$]	$0.24^{a} \pm 0.03$	$0.10^{b} \pm 0.03$	$0.23^{a} \pm 0.04$	$0.14^{b} \pm 0.02$
59	S2	**PDHB**	Pyruvate dehydrogenase E1 component subunit beta mitochondrial[#]	$0.19^{a} \pm 0.02$	$0.07^{b} \pm 0.02$	$0.11^{b} \pm 0.04$	$0.16^{a} \pm 0.03$
19	S1	**PGK1**	Phosphoglycerate kinase 1[$,#]	$0.06^{a} \pm 0.01$	$0.07^{ab} \pm 0.03$	$0.10^{b} \pm 0.00$	$0.04^{a} \pm 0.01$
8	S1	**PKM**	Pyruvate kinase PKM[*,$]	$0.01^{ab} \pm 0.00$	$0.01^{a} \pm 0.00$	$0.02^{b} \pm 0.00$	$0.01^{ab} \pm 0.01$
37	S1	**PPIA**	Peptidyl-prolyl cis-trans isomerase A[$]	$0.23^{a} \pm 0.12$	$0.10^{b} \pm 0.00$	$0.18^{a} \pm 0.02$	$0.08^{b} \pm 0.02$
36	S1	**PPIA**	Peptidyl-prolyl cis-trans isomerase A	$0.24^{a} \pm 0.10$	$0.09^{b} \pm 0.02$	$0.13^{b} \pm 0.02$	$0.08^{b} \pm 0.02$
25	S1	**SYN1**	Synapsin-1	$0.17^{ab} \pm 0.08$	$0.05^{a} \pm 0.01$	$0.20^{b} \pm 0.01$	$0.10^{ab} \pm 0.07$
33	S1	**TPIS**	Triosephosphate isomerase	$0.15^{a} \pm 0.04$	$0.21^{a} \pm 0.03$	$0.36^{b} \pm 0.02$	$0.30^{ab} \pm 0.11$
34	S1	**TPIS**	Triosephosphate isomerase	$0.83^{a} \pm 0.21$	$0.43^{ab} \pm 0.15$	$0.79^{ab} \pm 0.04$	$0.40^{b} \pm 0.17$
32	S1	**TPIS**	Triosephosphate isomerase	$0.14^{a} \pm 0.03$	$0.11^{a} \pm 0.04$	$0.14^{a} \pm 0.02$	$0.09^{a} \pm 0.03$

[1] Two-way ANOVA analyses were conducted. Data are means ± standard deviation.
[*] $p < 0.05$ significant differences given by the factor "diet" (STD and HFHS);
[$] $p < 0.05$ significant differences given by the factor "supplement" (CONTROL, ω-3).
[#] indicates significant interaction ($p < 0.05$) between the factors diet and supplement. Means with different superscript indicate significant differences ($p < 0.05$) (analyzed by post-hoc Tukey HSD). Spot Nº refers to the numbered spots in 2-DE gel images shown in Figs. S1 and S2. a.u.: arbitrary units.

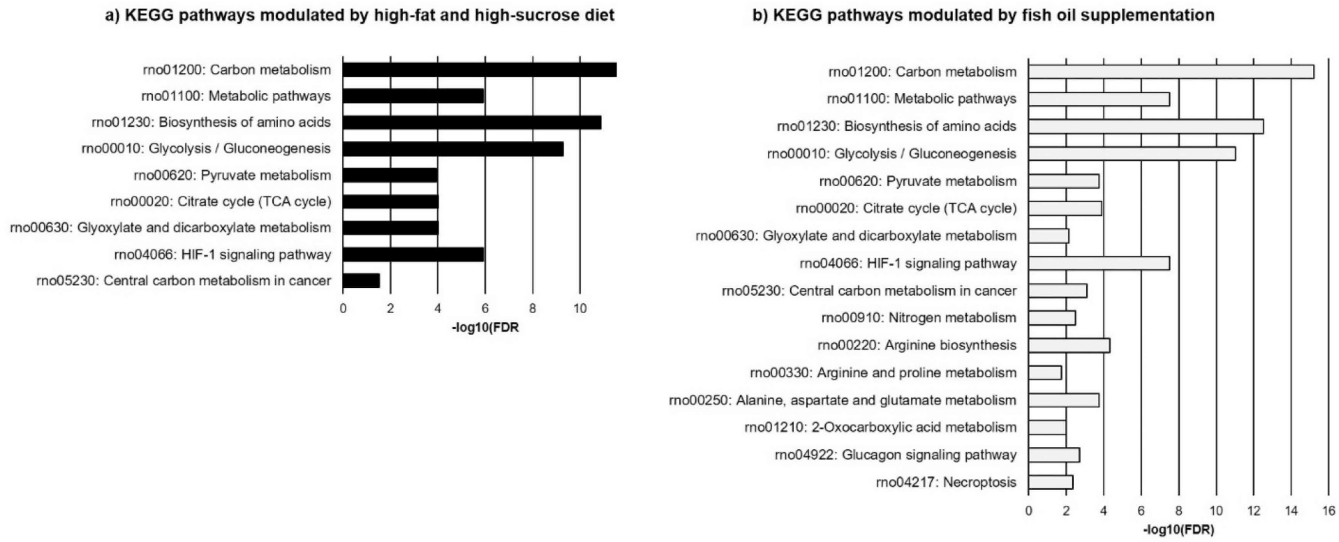

Fig. 2. KEGG pathways modulated either by (a) the effect of high fat and high sucrose-diet intake or (b) the fish oil supplementation through changes induced on carbonylated proteins in brain cortex. Significant enrichment at FDR < 5%.

aminotransferase cytoplasmic (GOT1), hemoglobin subunit beta-1 (HBB1), pyruvate dehydrogenase E1 component subunit alpha somatic form mitochondrial (PDHA1) and synapsin-1 (SYN1). Some proteins as HSPA8, PDHB, GAPDH, GLUL, GOT1, PGK1 and PPIA showed some interaction between the factor diet and supplement. The effect of fish oil over carbonylation for FSCN1 and PDHB were potenciated by the STD diet, while the HFHS diet potenciated the effect of fish oil over HSPA8, CKB, GAPDH, GLUL, GOT1, PGK1, PPIA.

Among the proteins already described above which result sensitive to HFHS intake, 65% of those proteins were also sensitive to fish oil supplementation. Then, similar GO functional enrichments can be described. Accordingly, carbonylated proteins responding to fish oil were proteins from axon (40%), especially the distal and termininus axon, the somatodendritic compartment (30%), preferentially the cell body, synapse (50%), both pre and postsynapse, and proteins from different complexes (30%), including the phosphopyruvate hydratase complex but also the mitochondrial pyruvate dehydrogenase complex. Regarding molecular functions, besides the ones found for carbonylated proteins sensitive to HFHS, specific significant enrichments in proteins with pyruvate dehydrogenase (acetyl-transferring) activity, phosphopyruvate hydratase activity, oxygen carrier activity were described, and additional binding capacities were significantly enriched, such as binding to carboxylic acid and identical proteins.

Finally, the supplementation of the diet with fish oil revealed the capacity of these marine PUFAs to regulate several KEGG pathways in brain cortex, in both STD and HFHS diets (Fig. 2b). Results indicated that fish oil counteracted the effect of HFHS diet intake, by lowering carbonylation of proteins involved in the same pathways found altered by the obesogenic diet. Those pathways were mainly metabolic pathways related to the carbon metabolism, biosynthesis of amino acids, glycolysis/gluconeogenesis, pyruvate metabolism, citrate cycle (TCA cycle), glyoxylate and dicarboxylate metabolism, HIF-1 signaling pathway and central carbon metabolism in cancer (Fig. 2b). Additionally, supplementation with fish oil provoked significant changes in carbonylation level of proteins corresponding to other pathways, such as the nitrogen metabolism, the alanine, aspartate and glutamate metabolism, the arginine biosynthesis, and interestingly, the glucagon signaling pathway and the necroptosis. All those pathways are brought under control of fish oil in both STD and HFHS diets. In an interesting manner, fish oil supplemented during the long-term HFHS intake distinctively controlled the 2-oxocarboxylic acid metabolism and the metabolism of both arginine and proline.

4. Discussion

This study examined the effects of long-term consumption of a diet high in fat and sugar, as a realistic model of the westernized diets [55], on the carbonylated proteome of the cerebral cortex of male Sprague-Dawley rats. Rats developed a prediabetic state that was accompanied by a general elevated level of oxidative stress and the accumulation of oxidatively damaged molecules, i.e. lipid peroxidation products and carbonylated proteins, in the cerebral cortex. This oxidative stress observed in the brain was found at systemic level as well (in plasma, blood and peripheral organs). Increased state levels of oxidative molecular damage in the brain cortex has been previously associated with the consumption of HFHS diets [56,57].

The present study further demonstrated that there was a selective modulation of carbonylated proteome by the obesogenic diet. The HFHS diet increased the oxidative damage of proteins participating in the energy metabolism in the brain cortex, concretely, glycolisis, pyruvate metabolism and TCA cycle, which are a series of coupled processes necessary to fulfill the ATP requirement of cells. More oxidative damage in glycolic proteins is especially relevant in brain, because glucose is the exclusive energy substrate for the brain in normal conditions [58]. Moreover, HFHS diet increased carbonylation of the CKB, an enzyme which participates in the rapid ATP production from phosphocreatine reservoirs, as an alternative source of ATP to glycolysis, TCA cycle and respiration [59]. Higher carbonylation of CKB was reported during aging and impaired brain creatine kinase activity was also found in neurodegenerative diseases as Huntington's disease [59]. Interestingly, the significant alteration in proteins from the TCA would compromise the homeostatic response of the brain to hypoxia, because TCA cycle intermediates modulate the HIF-1 signaling pathway. In normoxia, reduced levels of the TCA cycle intermediate 2-oxoglutarate induces HIF-1-dependent transcription [60]. A previous study found that the intake of a high-fat diet induced the overexpression of HIF-1a protein, impairing the autophagic flux and influencing brain damage [61]. Malfunction of the HIF-1 signaling pathway has been associated with neurodegenerative diseases [62]. Related to the O_2 homeostasis, results indicated that the HFHS diet decreased carbonylation of hemoglobin A. Its role in the nervous system is not well-known yet, but probably is involved in the oxygen transport or in regulation of cytosolic neuronal O_2 [63].

In line with energy production, HFHS diet also altered pathways devoted to the biosynthesis of amino acids. Besides worsening the ATP

production, this effect might be detrimental to neurotransmission, since amino acids are important precursors, neurotransmissers or neuromodulators [64]. Noteworthy, HFHS diets increased carbonylation of glutamine synthetase (GLUL), which catalizes the biosynthesis of glutamine from L-glutamate, the master excitatory neurotransmitter [65]. Additionally, GLUL located in astrocyte protects neurons against excitotoxicity by taking up excess ammonia and glutamate and converting it into glutamine [66]. An altered Glu-Gln cycling has been linked to high fat diet [67], and the expression of GLUL has been found downregulated in the hippocampus of high-fat-fed mice [68]. In closely related to TCA and glutamate metabolism, the obesogenic diet alterated the glyoxylate and dicarboxylate metabolism as well [69]. Alterations in the glyoxylate and dicarboxylated metabolism have been found in rats suffering from Alzheimer's disease [70] and in mice with learning and memory impairment [71].

Additionally, HFHS diet seemed affect neurotransmission by other mechanisms. HFHS diet preferentially damaged proteins involved in synapse and axonal proteins. Among those, HFHS diets increased carbonylation of DPYSL2, an enzyme critical in the fundamental processes of brain formation and organization. In humans, pathogenic genetic variants of the gene have been directly associated with intellectual disability and brain malformations [72]. Finally, there was an increased carbonylation in the chaperones HSPA8 and HSPD1, which are involved in proteostasis and control the targeting of proteins for subsequent degradation [73]. Considering that the nonenzymatic oxidative modifications are generally linked to functional losses, the potential inactivation of the carbonylated chaperones would promote the cellular oxidative stress found in these HFHS-fed rats.

Summing up, the HFHS diet mainly increased carbonylation of proteins involved in the energy metabolism, neurotransmission and proteostasis, the same functions particularly affected during brain aging [12]. These results could help understand some specific mechanisms behind the detrimental effects of the HFHS diet in accelerating and aggravating the effect of aging and maybe various other neuropathologies.

The second aim of the current study was to evaluate if the supplementation of the diet with a fish oil could counteract the effects of the long-term feeding of HFHS diets on the carbonylated proteome in brain cortex. Results demonstrated that fish oil significantly decreased the formation of lipid peroxidation products and protein carbonylation induced by the HFHS, while preventing the progression of the development of insulin resistance. It has been well documented that the antioxidant capacity of fish oils is mediated by their high content in ω-3 PUFAs, which can enhance activity of both enzymatic and non-enzymatic antioxidant systems in the brain [28]. Results showed that this antioxidant effect of the fish oil led to selective protection from carbonylation of specific cerebral proteins. This is because the precise composition of the fish oil supplement created a unique cell oxidative environment, which determined the pattern of protein carbonylation. Since redox homeostasis is a very complex phenomenon, there must be multiple factors that can explain the selective action of the fish oil on the carbonylated proteome. For instance, together with the promotion of the antioxidant system, it is well-known that the consumption of fish oil changes the cell lipidome [74]. Thus, it is expected that the profile of reactive carbonyl species (RCS) coming from lipid peroxidation will change too. Fish oil also modifies cellular profiles of ROS, because modulates different ROS-generating metabolic processes and systems [75]. ROS and RCS are highly herogenous and show very different reactivities, half-life and migration distance, facts that make some proteins more vulnerable to oxidation than other proteins, resulting in a characteristic protein carbonylation pattern. Previous studies have demonstrated this selective action of fish oil supplementation on carbonylated proteome in different tissues [24,41,42], but this topic has been neglected studied in the brain.

As an important outcome, the fish oil supplementation almost perfectly counteracted the effects of HFHS diets on carbonylated proteome by decreasing carbonylation of the proteins involved in the same pathways affected by the obesogenic diet. In fact, fish oil especially modulated carbonylation of proteins devoted to the energy production, amino acid metabolism and HIF-1 signaling pathway. Interestingly, fish oil showed a strong influence on glucose regulation in the brain cortex. Several studies have stated the hypothesis that ω-3 PUFAs are essential factors for optimal brain glucose metabolism, since they participate in regulating key steps in the delivery of blood glucose to neural cells [76]. The current study proporcionated an additional mechanism based on the redox control of glucose metabolism exerted by the fish oil, which decreased carbonylation of proteins in pathways devoted to ATP production from glucose.

Noteworthy, among the proteins selectively protected from oxidation by the fish oil supplement, there was the glycolitic enzyme GAPDH [77]. Some peculiarities of GAPDH make the effect of fish oil on it of special relevance. First, GAPDH is presented in cells at high concentration and has very functional diversity. Secondly, its activities may be regulated by redox reactions. Finally, GAPDH possesses pro-apoptotic functions that include the GAPDH-mediated cell death by protein aggregation as a result of oxidative stress [78,79]. These protein aggregates can accelerate pathologies such as Alzheimer's, Huntington's and Parkinson's diseases, diabetes and many others [77]. Since fish oil may modulate the formation of oxidative PTMs on GADPH, it could have an important role in regulating oxidative stress-induced apoptosis. Additionally, results also indicated that fish oil supplementation could modulate necroptosis in brain by altering the carbonylation level of specific proteins. Other authors have reported that ω-3 PUFAs could inhibit both necroptosis and neuroinflammation, at least in early brain damage after traumatic brain injury [80].

Fish oil supplementation seemed to affect the glucagon signaling pathway as well, which could be of special significance in the context of HFHS diet. During the last decades, the brain has increasingly been recognized as an important glucagon-sensitive organ [81]. Some studies have reported that activation of glucagon receptors in conjunction with other G protein-coupled receptors in the central glucagon system, especially the hypothalamus, is metabolically advantageous in diabetes and obesity, because it reduces hepatic glucose production and food intake [81,82]. The enrichment in DHA of membranes, at least in the STD context, which could potentiate the activity of G-protein-coupled signaling [83], and the lower protein carbonylation caused by fish oil in both STD and HFHS dietary backgrounds, could improve glucagon signaling.

Besides the strong effect on the regulation of energy metabolism, fish oil decreased carbonylation of several key proteins in neurotransmission. On one hand, fish oil diminished the level of carbonylation of glutamine synthetase involved in glutamate metabolism, counteracting the HFHS-diet effect. On the other hand, fish oil also diminished carbonylation of proteins that participate in aspartate, arginine and alanine metabolism. Aspartate can act as an excitatory neuromodulator and probably a neurotransmisser, and has been implicated in hormone release, neurogenesis, learning and memory [64]. Moreover, fish oil decreased carbonylation of the synaptic protein SYN1. Previous studies found that DHA supplementation increased the amount of SYN1 in the cortex of gerbils [84] and in rat hippocampal neurones [85]. Finally, fish oil supplementation led to lesser carbonylation of FSCN1, a protein that participates in neurotransmission because organizates actin filaments. Previous authors found that the intake of high-fat diet decreased the amount of FSCN1 in mice hippocampus [86]. Finally, fish oil supplementation also showed certain activity on chaperones, and the HSPA8 was less carbonylated in these groups. The capacity of fish oil to decrease carbonylation level of HSPA8 was also described in some tissues besides the brain [24]. Thus, this effect of fish oil could assist to restore proteostasis and counteract the detrimental effect of the obesogenic-diet consumption.

5. Conclusions

The long-term consumption of high-fat and high-sucrose diets increases body oxidative stress and causes oxidative damage in specific proteins in the brain cortex, while inducing a prediabetic state. The main protein targets affected by the obesogenic diet are involved in critical brain processes, such as glucose metabolism and neurotransmission. The supplementation of the diet with fish oil ameliorates the oxidative damage induced by the obesogenic diet in the brain. Additionally, fish oil supplement has a stronger influence on cortical carbonylated proteome, which could contribute to slowing down the natural progression of brain aging by protecting brain cells from oxidative insults. To the best of our knowledge, this is the first study that identified the carbonylated proteome in the brain cortex of rats and, remarkably, the pool of carbonylated proteins that responded to the intake of high fat and sucrose diet and to the supplementation of the diet with fish oil. The results could help the development of successful nutritional-based interventions to prevent brain oxidative damage and potentiate brain health.

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CRediT authorship contribution statement

Lucía Méndez, Isabel Medina: Conceptualization, Supervision. **Francisco Moreno, Lucía Méndez, Ana Raner, Bernat Miralles-Pérez, Sara Ramos-Romero**: Methodology, Validation, Formal analysis, Investigation, Data curation. **Marta Romeu, Josep Lluís Torres, Isabel Medina**: Resources. **Francisco Moreno, Lucía Méndez, Isabel Medina**: Writing – original draft preparation. **Francisco Moreno, Lucía Méndez, Ana Raner, Bernat Miralles-Pérez, Marta Romeu, Sara Ramos-Romero, Josep Lluís Torres, Isabel Medina**: Writing – review & editing. **Francisco Moreno, Lucía Méndez, Ana Raner, Isabel Medina**: Visualization. **Marta Romeu, Josep Lluís Torres, Isabel Medina**: Project administration. **Marta Romeu, Josep Lluís Torres, Isabel Medina**: Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.115708.

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