Iron-induced derangement in hepatic Δ-5 and Δ-6 desaturation capacity and fatty acid profile leading to steatosis: Impact on extrahepatic tissues and prevention by antioxidant-rich extra virgin olive oil

Cynthia Barrera[a], Rodrigo Valenzuela[a,*], Miguel A. Rincón[b], Alejandra Espinosa[c], Sandra López-Arana[a], Daniel González-Mañan[d], Nalda Romero[e], Romina Vargas[f], Luis A. Videla[f]

[a] Department of Nutrition, Faculty of Medicine, University of Chile, Independencia 1027, Casilla, Santiago 70000, Chile
[b] Institute of Nutrition and Food Technology, University of Chile, Santiago, Chile
[c] Department of Medical Technology, Faculty of Medicine, University of Chile, Santiago, Chile
[d] Núcleo de Química y Bioquímica, Facultad de Ciencias, Universidad Mayor, Chile
[e] Department of Food Science and Chemical Technology, Faculty of Chemical Sciences and Pharmacy, University of Chile, Santiago, Chile
[f] Molecular and Clinical Pharmacology Program, Institute of Biomedical Sciences, Faculty of Medicine, University of Chile, Santiago, Chile

ARTICLE INFO

ABSTRACT

The administration of iron induces liver oxidative stress and depletion of long-chain polyunsaturated fatty acids (LCPUFAs), n-6/n-3 LCPUFA ratio enhancement and fat accumulation, which may be prevented by antioxidant-rich extra virgin olive oil (AR-EVOO) supplementation. Male Wistar rats were subjected to a control diet (50 mg iron/kg diet) or iron-rich diet (IRD; 200 mg/kg diet) with alternate AR-EVOO for 21 days. Liver fatty acid (FA) analysis was performed by gas-liquid chromatography (GLC) after lipid extraction and fractionation, besides Δ-5 desaturase (Δ-5 D) and Δ6-D mRNA expression (qPCR) and activity (GLC) measurements. The IRD significantly ($p < 0.05$) increased hepatic total fat, triacylglycerols, free FA contents and serum transaminases levels, with diminution in those of n-6 and n-3 LCPUFAs, higher n-6/n-3 ratios, lower unsaturation index and Δ5-D and Δ6-D activities, whereas the mRNA expression of both desaturases was enhanced over control values, changes that were prevented by concomitant AR-EVOO supplementation. N-6 and n-3 LCPUFAs were also decreased by IRD in extrahepatic tissues and normalized by AR-EVOO. In conclusion, AR-EVOO supplementation prevents IRD-induced changes in parameters related to liver FA metabolism and steatosis, an effect that may have a significant impact in the treatment of iron-related pathologies or metabolic disorders such as non-alcoholic fatty liver disease.

1. Introduction

Iron, an essential micronutrient, induces pathological conditions at cellular concentrations outranging physiological levels, such as exacerbation of alcoholic liver disease [1] and development and progression of non-alcoholic fatty liver disease (NAFLD) [2] that involve iron overloading. NAFLD is a common metabolic disorder associated with type 2 diabetes, obesity and the side adverse effects of some drug therapies [2,3]. Iron-induced liver pathology is associated with its involvement in oxidation-reduction processes leading to the generation of reactive oxygen species (ROS), with the concomitant oxidative deterioration of biomolecules and loss of their functions [4]. In iron overloading conditions, the metal is primarily sequestered by cellular ferritin, with the progressive increase of the labile iron pool (LIP) [5]. This

entity consists of Fe^{2+} and Fe^{3+} ions loosely bound to cellular components, whose expansion triggers ROS generation [4–6].

Recent studies by our group revealed that administration to rats of an iron-rich diet (IRD; 200 mg iron/kg diet) for 21 days induces an hepatic pro-lipogenic response compared to animals receiving a control diet (50 mg iron/kg diet) [7]. Concomitantly, depletion of long-chain polyunsaturated fatty acids (LCPUFAs) and production of a mild micro- and macrovesicular steatosis were induced by IRD, the latter effect of IRD being histologically evidenced by a steatosis score of 1.8 [7], graded on a scale of 0 to 3 as previously established [8]. Diminution of liver n-3 LCPUFAs is related to several factors including utilization by lipid peroxidation processes due to their interaction with ROS, which is favoured by their high degree of unsaturation [9]. In the case of extrahepatic tissues, IRD-induced fall in n-3 LCPUFA levels [7] is

associated with a diminished transport from the liver through very low-density lipoprotein and albumin, considering that n-3 LCPUFAs are mainly synthesized in the liver through a pathway involving desaturation and elongation reactions [10]. Under conditions of n-3 LCPUFA depletion, the liver undergoes a major change in the pattern of lipid metabolism, favouring de novo lipogenesis over FA oxidation (FAO) [11]. This view is based in that diminished n-3 LCPUFA levels activate the lipogenic factor sterol responsive-element binding protein 1c (SREBP-1c), controlling FA and triacylglycerol (TG) synthesis [12], whereas peroxisome proliferator-activated receptor α (PPAR-α) favouring FA oxidation is down-regulated [13,14], thus triggering liver steatosis.

In view of these considerations, the aim of this study was to assess the influence of an antioxidant intervention on liver parameters related to hepatic steatosis and lipid metabolism altered by an IRD. For this purpose, rats subjected to IRD were supplemented with antioxidant-rich olive oil (AR-EVOO) [15]. This AR-EVOO type mitigated HFD-induced liver oxidative stress and diminution in the synthesis and accretion of n-3 LCPUFAs, when compared to other EVOO types with lower levels of antioxidants, which point toward a threshold in the antioxidant content of EVOOs to achieve useful outcomes [15]. Total levels of PUFAs and those of the n-6 and n-3 series were determined in liver and extrahepatic tissues, with respect to hepatic Δ-5 desaturase (Δ-5 D) and Δ6-D mRNA expression and activity.

2. Materials and methods

All experimental procedures were performed in accordance with the National Institute of Health (NIH) Guide for the Care and Use of Laboratory Animals, and approved by the Bioethics Committee for Research in Animals, Faculty of Medicine, University of Chile (CBA protocol 0580 FMUCH).

2.1. Animal treatments

Male Wistar rats (Animal Facility of the Nutrition Department, Faculty of Medicine, University of Chile) with an initial weight of about 90 g were used, growing animals that exhibit adequate iron homeostasis due to the efficient regulation of the iron transporters divalent metal-iron transporter-1 (DMT1) and ferroportin in the small intestine [16]. Under unrestricted water access, animals were subjected to a control diet (CD) that containing 10% fat (only canola oil), 20% protein and 70% carbohydrate and either 50 mg iron/kg diet (CD) or 200 mg iron/kg diet (IRD) for 21 days. The complete composition of the diet was previously described [17]. Table 1 shows the fatty acid composition of the experimental diets. During this period, groups of control rats and animals subjected to IRD received simultaneously a diet which replaced all canola oil by antioxidant-rich extra virgin olive oil (AR-EVOO) conforming four experimental groups: a) control, b) AR-EVOO, c) IRD and d) AR-EVOO + IRD. Daily controls of body weight, diet and iron intakes were performed during the entire study, at the end of which the animals were fasted overnight and anaesthetized by

Table. 1
Fatty acid composition of experimental diets.

Content (g/100 of diet)	Control	AR-EVOO	IRD	AR-EVOO + IRD
ΣSFA	0.6	0.9	0.6	0.9
ΣMUFA	6.4	6.7	6.4	6.7
Oleic	6.0	6.5	6.0	6.5
ΣPUFA	3.0	2.4	3.0	2.4
Σn-6 PUFA	2.6	2.2	2.6	2.2
LA	1.9	2.1	1.9	2.1
Σn-3 PUFA	1.0	0.2	1.0	0.2
ALA	0.9	0.1	0.9	0.1
n-6/n-3 PUFA ratio	2.0	11.0	2.0	11.0

intraperitoneally zolazepam (25 mg/mL) and tiletamine chlorhydrate (25 mg/mL) mixture (Zoletil 50). Blood serum and liver samples were collected and placed into chilled sample vials and frozen at −80 °C for subsequent assays. Serum aspartate transaminase (AST) and alanine transaminase (ALT) activities (units/L) were determined using specific kits (Biomerieux SA, Marcy l'Etoile, France). Contents of liver total fat (mg/g), TGs (mg/g) (Wiener Lab, Argentina) and free FAs (FFAs; μmol/g) (Cayman Chemical Co., Ann Arbor, MI, USA) were measured by specific kits according to manufacturer's instructions. Besides, liver samples were fixed in phosphate-buffered formalin, paraffin embedded, stained with haematoxylin-eosin, analysed by optical microscopy for evaluation of the steatosis score according to Brunt et al. (Grade cero for none: 1 for up 33% of affected hepatocytes; 2 for 33%–66%; and 3 for >66% steatosis) [8].

2.2. Analysis of polyphenols, α-tocopherol, antioxidant capacity of AR-EVOO

Determination of total polyphenols content in the EVOO (Kalamata variety) was assessed according to IOOC, 2009 and Mateos et al. [18,19]. Quantification of α-tocopherol was evaluated according to American Oil Chemistry Society official method [20]. Antioxidant capacity was assessed by oxygen radical antioxidant capacity—fluorescein according to a previously described method [21].

2.3. Liver lipid extraction and fractionation for fatty acid analysis

Quantitative extraction and separation of total lipids from erythrocytes, liver, adipose tissue, brain, heart, testicle, muscle, intestine and kidney was carried out according to Bligh and Dyer [22]. Briefly, erythrocytes and tissue samples were homogenized in ice-cold chloroform/methanol (2:1 v/v) containing 0.01% butylated hydroxytoluene (BHT) in an Ultraturrax homogenizer (Janke & Kunkel, Stufen, Germany). Total lipids from erythrocytes and tissues were extracted with chloroform/isopropanol (2:1 v/v), and phospholipids were separated from total lipid extracts by thin layer chromatography (TLC) on silica gel plates (aluminium sheets 20 × 20 cm, silica gel 60F-254; Merck), using hexane/diethyl ether/acetic acid (80:20:1 v/v/v) as mobile phase. After development and solvent evaporation, lipid spots were visualized by exposing the plates to a Camag UV (250 nm) lamp designed for TLC. The solvent system allows the separation of phospholipids, cholesterol, triacylglycerols and cholesterol esters according to their relative mobility. Phospholipid spots were extracted from the silica with both diethyl ether and chloroform/methanol (2:1 v/v), according to Ruiz-Gutierrez et al. [23].

For FA analysis of fatty acid methyl ester (FAME) derivatives from erythrocyte and tissue phospholipids, samples were prepared according to a previously described method [24], and FAMEs were separated and quantified by gas-liquid chromatography, by comparing the retention times and the peak area values (%) of the unknown samples with those of a commercial lipid standard (Nu-Chek Prep Inc, Elysian, MN, USA) [15]. C23:0 was used as internal standard and a Hewlett-Packard Chemstation Data System was employed for processing [15]. The amount of each FA is expressed as mole percentage of the total FA content.

2.4. Gene expression assays

Total RNA was isolated from liver samples using Trizol (Invitrogen, Carlsbad, CA, USA), according to the supplier's protocols. Purified RNA (2 μg) was then treated with DNAase I (DNA free kit; Ambion, Austin, TX, USA) and used to generate first-strand cDNA with M-MLV reverse transcriptase (Invitrogen), utilizing random hexamers (Invitrogen) and dNTP mix (Bioline, London, United Kingdom), according to the manufacturer`s protocol. The resultant cDNA was amplified with specific primers for mice in a total volume of 20 μL. Gene specific primer

Table. 2
Gene specific primer sequences used in the study.

mRNA	Forward primer	Reverse primer	Melting T° (°C)	Product length	Gen Bank code
Δ-5 desaturase	GATGAACCATATCCCCATGC	TTGGCGCACAGGCATTG	58°	182	NM_053445.2
Δ-6 desaturase	GGAACCATCGACATTTCCAG	TCTTTATGTCGGGGTCCTTG	58°	164	NC_005100.4
β-actin	ACTGCCGCATCCTCTTCCTC	CTCCTGCTTGCTGATCCACATC	62°	149	NC_005111.4

Sequences are listed in the 5` → 3` direction.

sequences used are shown in Table 2. Primers were optimized to yield 95%–100% of reaction efficiency with PCR products by development in agarose gel to verify the correct amplification length. The quality of the DNA extracted was checked by agarose gel (1%) electrophoresis according to Yu Leen et al. 2012 [25]. Briefly, samples were mixed with Gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol and 30% glycerol). Electrophoresis was run at 75 Volt for 20 min. DNA was stained using GelRed® Nucleic Acid Gel Stain (Biotum, Fremont, CA, USA). Real Time PCR was performed in a Stratagen Mx3000P System using Brilliant II SYBR® Green QPCR Master Mix (Agilent Technologies, La Jolla, CA, USA) following the manufacturer`s recommendations (Applied Biosystems, Foster City, CA, USA). All the expression levels of target genes under study were normalized by the expression of β-actin as internal control (Applied Biosystems, Foster City, CA, USA). Fold changes between groups was calculated by the $2^{(-\Delta\Delta Ct)}$ method, as established by Pfaffl [26] and Livak and Schmittgen [27].

2.5. Determination of liver Δ-5 and Δ-6 desaturase activities

Liver samples (500 mg) frozen in liquid nitrogen were homogenized in a buffer solution pH 7.9 containing 10 mmol/L HEPES, 1 mmol/L EDTA, 0.6% Nonidet P-40, 150 mmol/L NaCl, and protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL aprotinin, 1 μg/mL leupeptin, and 1 mmol/L orthovanadate). Hepatic homogenates were centrifuged at 4 °C, first at 2000 g for 30 s, followed by centrifugation of the supernatants at 5000 g for 5 min, and finally at 100,000 g for 60 min, to obtain the extracts for the assessment of desaturase activities. Protein content was measured by the method of Lowry et al. [28]. Δ-5 Desaturase activity was determined by the amount of dihomo-gamma-linolenic acid (DHGLA, C20:3 n-6) being converted into arachidonic acid (AA, C20:4 n-6) using albumin-bound FA precursors (LA and DHGLA) whereas Δ-6 desaturase activity was obtained by measuring the amount of γ-linolenic acid (GLA, C18:3 n-6) produced from linolenic acid (LA, C18:2 n-6) [29]. Desaturase activity was assayed using 1 mL of incubation medium containing 4 μmol ATP, 0.1 μmol coenzyme-A, 1.28 μmol NADH, 2.42 μmol N-acetylcysteine, 0.5 μmol nicotinamide, 5 μmol MgCl2, 62.5 μmol NaF, and 62.5 μmol phosphate buffer pH 7, supplemented with 100 nmol with the respective albumin-bound FA precursor and 1mg protein of cytosolic extract in a total volume of 100 μL, incubated at 37 °C for 30 min with shaking. Δ-5 and Δ-6 desaturase assays were conducted simultaneously. The reaction was stopped by adding 6 mL of a methanol:chloroform mixture (2:1 v/v). Eptadecanoic acid (C17:0; 99%+ pure) was added (20 μg) as internal standard. Δ-5 and Δ-6 Desaturase activities measured as net decrease in DHGLA production and net increase in GLA production were calculated from gas–liquid chromatography results as differences between baseline values and those obtained after 30 min incubation, and results were expressed as nmol/mg protein/min [15].

2.6. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 software (GraphPad Prism Software, Inc. San Diego, CA, USA). Data presented are means ± SEM for 10 rats per experimental group. Evaluation of normality of data distribution was performed using the Shapiro Wilk test. Statistical analysis was performed by two-way ANOVA and the Bonferroni's post-test ($p < 0.05$ was considered significant). The association between different variables was assessed by the Pearson correlation coefficient.

3. Results

3.1. Antioxidant characteristics of AR-EVOO

AR-EVOO (Kalamata variety) contained 860 mg/kg of polyphenols, 1.2 mg/kg of hydroxytyrosol, 5 mg/kg of tyrosol, 456 mg/kg of oleuropein and 227 mg α-tocopherol/kg oil, with an antioxidant capacity of 7.2 μmol of Trolox equivalents/L.

3.2. General, dietary and biochemical parameters in the serum and liver of control rats and animals after AR-EVOO and/or IRD supplementation

AR-EVOO supplementation did not modify general, dietary, serum and hepatic parameters compared to the control group, whereas IRD increased final iron intake by 302%, elicited higher contents of total liver fat, TGs and FFAs by 29%, 40% and 22%, respectively, with 7.4-fold enhancement in the steatosis score compared to control values ($p < 0.05$) (Table 3). These IRD-induced changes occurred concomitantly with 31% and 44% enhancements in the serum values of AST and ALT over those in controls ($p < 0.05$) (Table 3). With the exception of the final iron intake, all of the above changes were abolished in animals subjected to the combined IRD plus AR-EVOO protocol, with values being comparable to those in the control and AR-EVOO groups (Table 3). Besides, the hepatic steatosis score exhibited significant correlations with total liver fat (r = 0.98; $p < 0.01$), TGs (r = 0.99; $p < 0.002$) and FFAs (r = 0.98; $p < 0.02$) along with serum ALT (r = 0.97; $p < 0.01$) and AST (r = 0.92; $p < 0.002$).

3.3. Content of palmitic acid (PA), stearic acid, oleic acid (OA), and most relevant phospholipid n-6 LCPUFAs in erythrocytes, liver and extrahepatic tissues from control rats and animals after AR-EVOO and/or IRD supplementation

In all tissues studied, the content of PA (C16:0) was not altered by AR-EVOO alone or combined with IRD, however, PA levels were significantly increased ($p < 0.05$) in IRD-supplemented rats over those in the control group (Fig. 1A). Stearic acid (C18:0) and OA (C18:1 n-9) content were not significantly modified in all the tissues studied (Fig. 1B and C). The content of linoleic acid (LA; C18:2 n-6) remained unaltered in most tissues analysed from control rats and animals subjected to AR-EVOO and AR-EVOO plus IRD, with the exception of erythrocytes in which IRD induced a 37% diminution (Fig. 2A). Levels of both arachidonic acid (AA; C20:4 n-6) (Fig. 2B) and docosapentaenoic acid n-6 (DPA; C22:5 n-6) (Fig. 2C) after AR-EVOO and AR-EVOO combined with IRD were comparable to controls, whereas IRD supplementation elicited significant decreases of these n-6 LCPUFAs in all tissues (Fig. 2B and C), with the exception of brain DPAn-6 that increased by 175% (Fig. 2C).

Table. 3

General, dietary and biochemical related parameters in rats subjected to control diet (CD), antioxidant-rich extra virgin olive oil (AR-EVOO), short-term iron-rich diet (IRD) and AR-EVOO plus IRD.

	Experimental groups			
	CD (a)	AR-EVOO (b)	IRD (c)	AR-EVOO + IRD (d)
A. General parameters				
Initial body weight (g)	91.8 ± 5.9	90.7 ± 8.8	93.4 ± 6.9	90.2 ± 5.7
Final body weight (g)	227.6 ± 14.3	228.9 ± 13.0	225.2 ± 10.9	231.1 ± 14.7
Liver weight (g)	9.54 ± 0.8	9.75 ± 1.1	9.42 ± 0.9	9.63 ± 1.2
Visceral adipose tissue (g)	3.72 ± 0.5	3.91 ± 0.6	4.02 ± 0.8	3.88 ± 0.6
B. Dietary parameters				
Initial diet intake (g/day)	4.83 ± 1.1	4.92 ± 1.3	4.90 ± 1.2	4.85 ± 1.1
Final diet intake (g/day)	17.8 ± 2.8	18.3 ± 2.9	17.6 ± 2.5	18.0 ± 2.7
Initial iron intake (mg/day)	0.25 ± 0.05[c,d]	0.26 ± 0.04[c,d]	0.97 ± 0.1[a,b]	0.98 ± 0.1[a,b]
Final iron intake (mg/day)	0.88 ± 0.1[c,d]	0.89 ± 0.01[c,d]	3.54 ± 0.8[a,b]	3.50 ± 0.6[a,b]
C. Serum parameters				
AST (U/L)	111.5 ± 12.2[c]	109.6 ± 9.2[c]	145.7 ± 19.2[a,b,d]	113.2 ± 10.5[c]
ALT (U/L)	47.8 ± 6.4[c]	45.6 ± 6.1[c]	68.6 ± 7.8[a,b,d]	46.5 ± 7.0[c]
D. Liver parameters				
Total fat (mg/g liver)	35.9 ± 3.1[c]	33.7 ± 3.4[c]	46.2 ± 3.7[a,b,d]	36.3 ± 3.2[c]
Triacylglycerols (mg/g liver)	31.3 ± 3.2[c]	30.6 ± 3.1[c]	43.8 ± 3.8[a,b,d]	32.5 ± 3.5[c]
Free fatty acids (μmol/g liver)	260 ± 20[c]	251 ± 18.6[c]	316 ± 24[a,b,d]	265 ± 21[c]
Steatosis score	0.25 ± 0.05[c]	0.21 ± 0.02[c]	2.11 ± 0.07[a,b,d]	0.27 ± 0.04[c]

ALT, alanine aminotransferase; AST, aspartate aminotransferase.

Values represent means ± SEM for 10 rats per experimental group. The groups were compared by two-way ANOVA and the Bonferroni's post-test ($p < 0.05$), with significant differences being indicated by the letter identifying each experimental group.

3.4. Content of most relevant phospholipid n-3 LCPUFAs in erythrocytes, liver and extrahepatic tissues from control rats and animals after AR-EVOO and/or IRD supplementation

Rats subjected to treatment with AR-EVOO alone or joined with IRD showed comparable contents of α-linolenic acid (ALA; C18:3 n-3) (Fig. 3A), eicosapentaenoic acid (EPA; C20:5 n-3) (Fig. 3B), docosapentaenoic acid n-3 (DPA; C22:5 n-3) (Fig. 3C) and docosahexaenoic acid (DHA; C22:6 n-3) (Fig. 3D) compared to controls. IRD elicited significant decreases in ALA ($p < 0.05$) in erythrocytes, liver and adipose tissue, without changes in the rest of the tissues studied (Fig. 3A). Under conditions of IRD supplementation, EPA, DPA n-3 and DHA declined in all tissues ($p < 0.05$) over control values (Fig. 3B–D).

3.5. Liver content of total PUFAs, n-6 LCPUFAs, n-3 LCPUFAs, n-6/n-3 LCPUFA ratios and Δ5-desaturase (Δ5-D) and Δ6-D activity and mRNA expression in control rats and animals after AR-EVOO and/or IRD supplementation

In the experimental protocol studied, control and AR-EVOO groups exhibited comparable contents of hepatic total PUFAs, LCPUFAs of the n-6 and n-3 series and the respective n-6/n-3 ratios and the unsaturation index (Fig. 4A–E). Contrarily, IRD induced a significant 68% decrease in the total content of total PUFAs of the liver (Fig. 4A), comprising 49% and 74% diminutions in that of n-6 and n-3 LCPUFAs (Fig. 4B and C), with 127% increment in the n-6/n-3 LCPUFA ratio ($p < 0.05$) (Fig. 4D) and 50% reduction in the unsaturation index (Fig. 4E). IRD combined with AR-EVOO recovered to control values all changes induced by IRD alone (Fig. 4A–D).

Assessment of the FA desaturation capacity of the liver revealed lack of changes in Δ5-D and Δ6-D activities as well as in the mRNA expression of these enzymes in AR-EVOO compared to control values (Fig. 4F–I). However, IRD decreased the activity of Δ5-D and Δ6-D by 68% and 73% ($p < 0.05$), respectively (Fig. 4F and G), whereas Δ5-D and Δ6-D mRNA levels were enhanced by 50% compared to values in the control group (Fig. 4H and I). These changes in liver desaturation parameters were abolished by combined AR-EVOO plus IRD supplementation (Fig. 4E–I)

The analysis of correlations between variables showed significant direct associations between n-3 LCPUFAs and Δ5-D (r = 0.99;

$p < 0.001$) and Δ6-D activities (r = 0.98; $p < 0.01$), n-6 LCPUFAs versus Δ5-D (r = 0.95; $p < 0.03$) and Δ6-D activities (r = 0.97; $p < 0.02$) and between Δ5-D and Δ6-D activities (r = 0.96; $p < 0.05$).

4. Discussion

As a typical hormetic agent [30], low iron concentrations produce beneficial effects in association with signalling and metabolic regulation, whereas at high levels the formation of the LIP is favoured, with enhanced production of ROS and development of oxidative stress [31,32] (Fig. 4). Under conditions of redox unbalance, IRD led to a significant loss of hepatic total content of PUFAs, which is correlated with that of n-6 and n-3 LCPUFAs. This depleting effect of IRD is higher for the n-3 than the n-6 series of LCPUFAs, thus enhancing n-6/n-3 LCPUFA ratio with diminution in the unsaturation index of the liver. Under these conditions, IRD-induced liver n-3 LCPUFA depletion is likely to alter the signalling processes of key transcription factors involved in lipid metabolism, promoting (i) a lower activation of PPAR-α with downregulation of FA oxidation [33]; and (ii) activation of lipogenic SREBP-1c due to deficient inhibition of its processing [12], thus inducing hepatic steatosis (Table 3; Fig. 5). The latter conclusion is supported by the significant direct correlations established between liver steatosis scores with the levels of hepatic lipids and the activity of serum transaminases. Diminution of liver FA oxidation due to PPAR-α downregulation by IRD may be contributed by iron-induced mitochondrial dysfunction, which is related to LCPUFA peroxidation in phospholipids and protein oxidation, with decreased rates of mitochondrial respiration [34] and PA accumulation (Fig. 1A). In addition, depletion of n-3 LCPUFAs is likely to alter the membrane FA composition in the liver and extrahepatic tissues, which may modulate cellular-associated functions by lowering the degree of unsaturation of membrane phospholipids, with consequent untoward results [31,35]. In this respect, a recent study on the dysmetabolic iron overload syndrome (DIOS) and obesity showed that an iron/fat-enriched diet triggers testicular dysfunction [36]. This is characterized by decreased levels of testosterone (T) due to downregulation of T-converting enzymes, in association with diminished expression of mitochondrial biogenesis proteins (peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) and mitochondrial transcription factor A (mtTFA), whereas the expression of those related to endoplasmic reticulum (ER) stress is
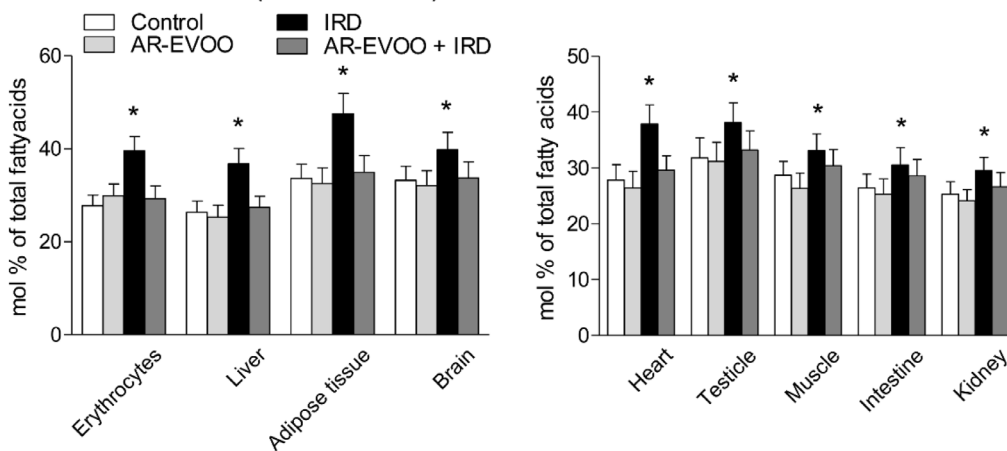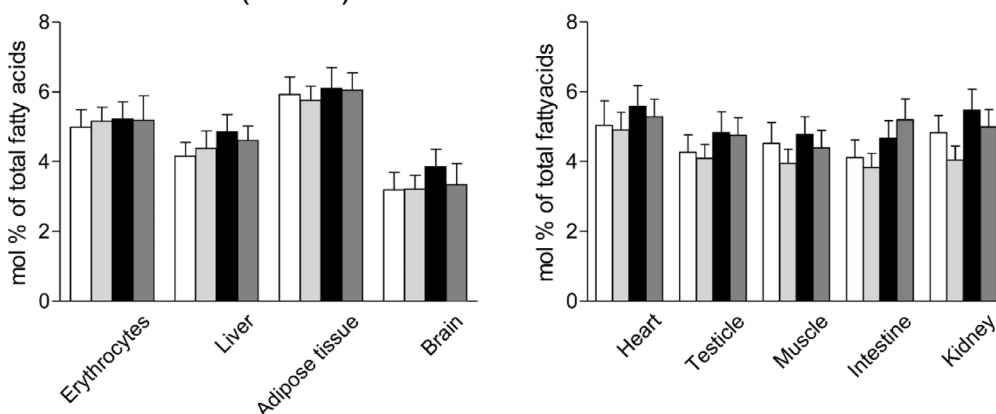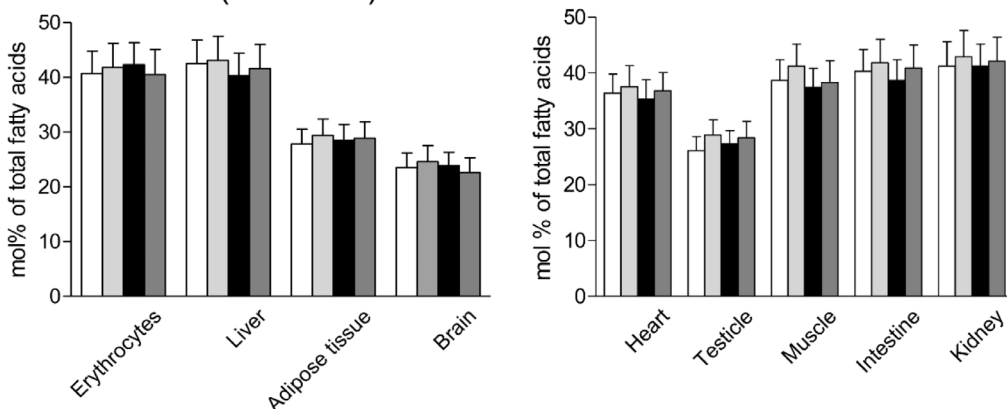
Fig. 1. Levels of palmitic acid (A), stearic acid (B) and oleic acid (C) in the liver and extrahepatic tissues of rats subjected to control diet, antioxidant-rich extra virgin olive oil (AR-EVOO), iron rich diet (IRD) and AR-EVOO plus IRD. Values are expressed as means ± SEM for 10 rats per group, and differences between groups were analysed by two-way ANOVA and the Bonferroni's post-test, with significant differences ($p < 0.05$) being indicated by asterisks (*).

upregulated [27].

Besides n-3 LCPUFA depletion, IRD-induced lipogenic effects in the liver may involve contributing factors that trigger ER stress [37], such as oxidative stress and PA accumulation [38,39] (Fig. 5). IRD-induced oxidative stress resulted in 75% enhancement in hepatic protein carbonylation [7], which leads to protein unfolding triggering ER stress, with the consequent lipogenic response through SREBP-1c, PPAR-γ and C/EBPαβ upregulation [40]. Secondly, IRD-induced PA accumulation in the liver is known to disrupt ER architecture by forming tripalmitin, an insoluble TG that is retained by the ER [39], and PA-saturated phospholipids, which enhanced ER stress-dependent lipogenic

signalling [41]. Moreover, oxidative stress induced by IRD can determine enzyme inactivation through direct free-radical-protein interactions or as consequence of protein unfolding marking enzymes for degradation [42], with diminution in Δ5-D and Δ6-D activities as an alternate factor promoting liver n-3 LCPUFA depletion and steatosis (Fig. 5).

AR-EVOO exhibiting a high antioxidant capacity contains oleic acid (OA) as the most abundant component, 4%–20% of PUFAs and a small proportion (1%–2%) of components such as tocopherols and hydroxytyrosol (HT) among others, which allow the modulation of signalling pathways in hepatocytes affording prevention or resolution of liver

A. Linoleic acid (LA - C18:2n-6)
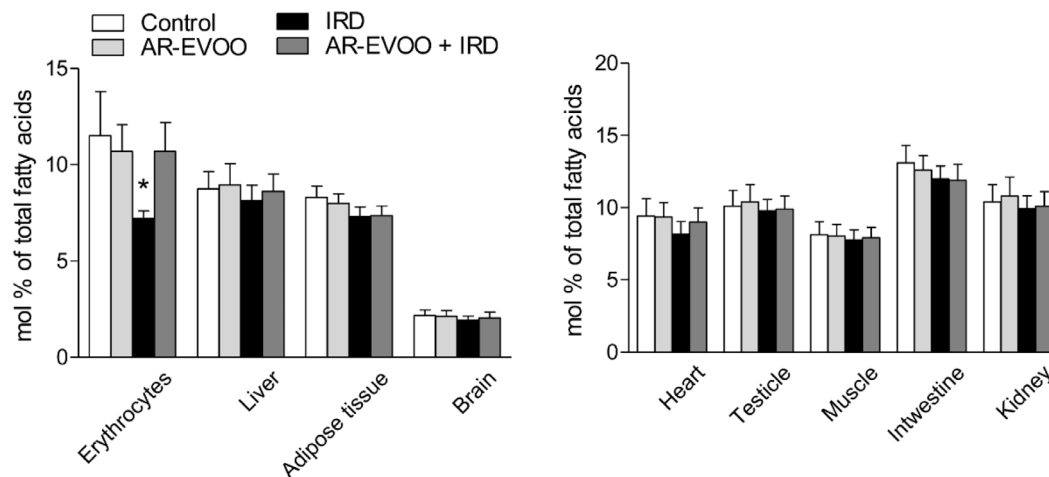
B. Arachidonic acid (AA - C20:4n-6)
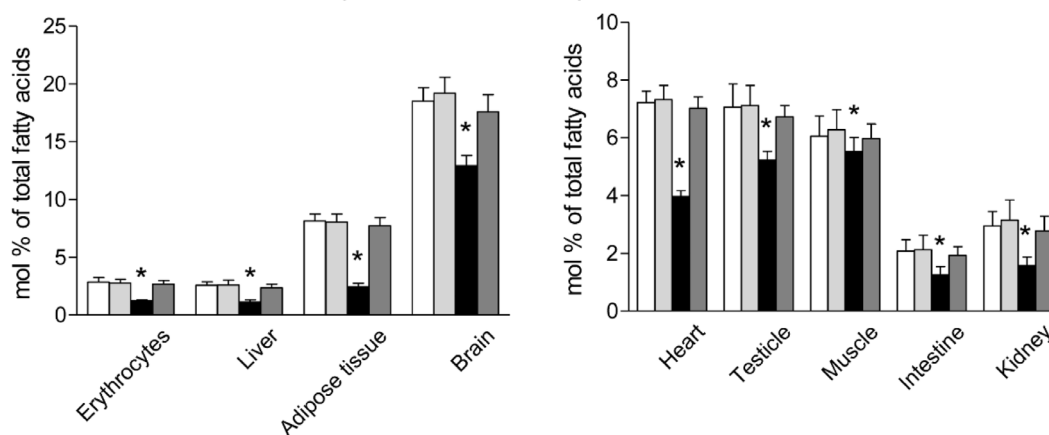
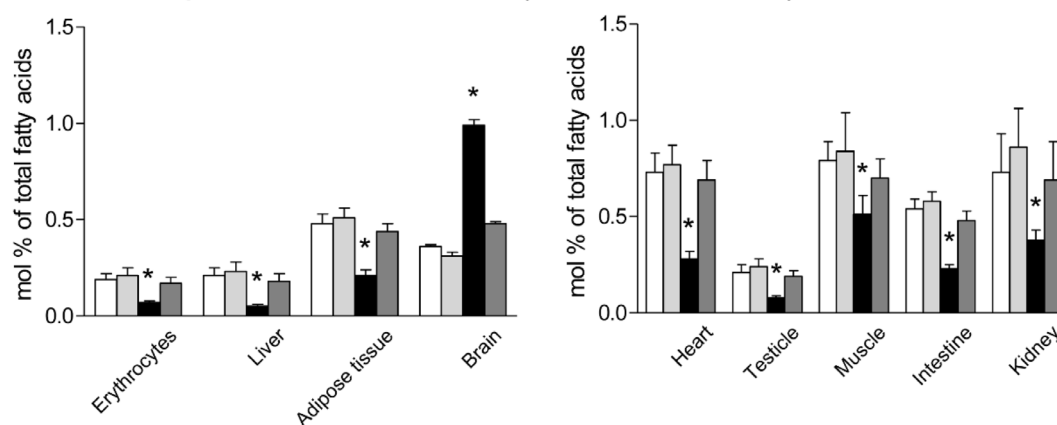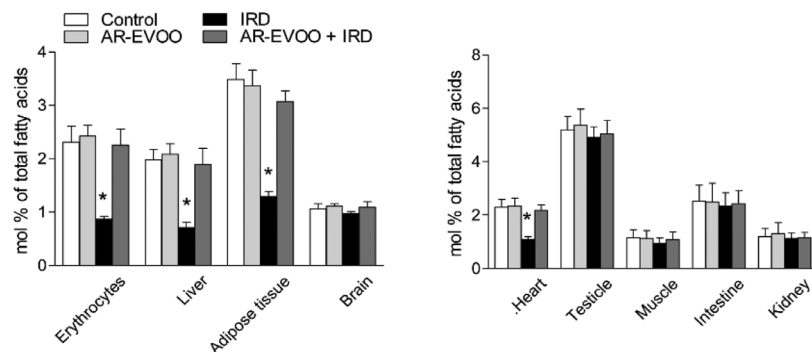## C. Docosapentaenoic acid n-6 (DPA - C22:5n-6)

Fig. 2. Levels of linoleic acid (A), arachidonic acid (B) and docosapentaenoic acid n-6 (C) in the liver and extrahepatic tissues of rats subjected to control diet, antioxidant-rich extra virgin olive oil (AR-EVOO), iron rich diet (IRD) and AR-EVOO plus IRD. Values are expressed as means ± SEM for 10 rats per group, and differences between groups were analysed by two-way ANOVA and the Bonferroni's post-test, with significant differences ($p < 0.05$) being indicated by asterisks (*).

injury [43]. Data presented indicate that AR-EVOO recovers PUFAs and LCPUFAs to baseline levels with normalization of the desaturation capacity of the liver and its membrane unsaturation degree, which are altered by IRD. This is shown by the normal activities of Δ5-D and Δ6-D and the diminution in the content of total fat and TGs increased by IRD (Table 3; Fig. 5). The protective mechanisms of AR-EVOO in the liver include (i) repletion of n-3 LCPUFA levels that are depleted by IRD; (ii) direct interception of ROS by tocopherols and HT abrogating oxidative

stress [44]; (iii) activation of nuclear factor-erythroid 2-related factor 2 (Nrf2) by n-3 LCPUFA-derived J3-isoprostanes [45] increasing the cellular antioxidant potential [46]; (iv) upregulation of the mRNA expression of Δ5-D and Δ6-D as an adaptive response to the decreased desaturase activity found [15]; (v) inhibition of the ER stress-related PERK pathway preventing the associated lipogenic response [47]; and (vi) PPAR-α upregulation favouring FA oxidation over lipogenesis, which comprises HT-induced PPAR-α mRNA expression [48] and OA-
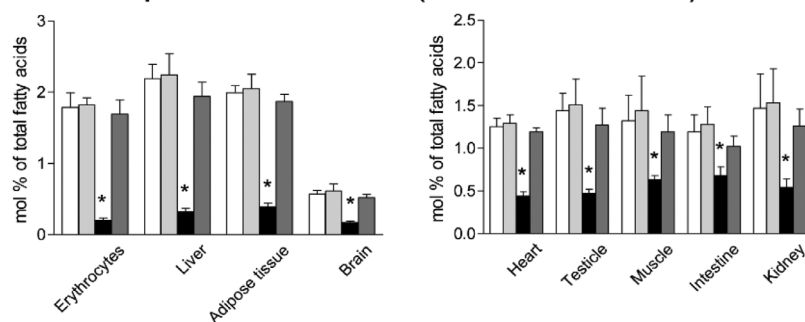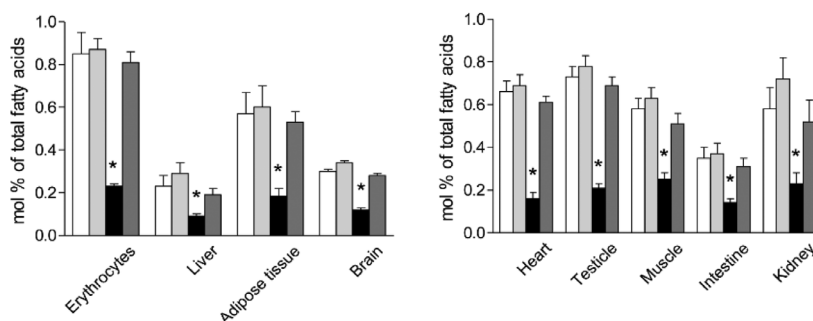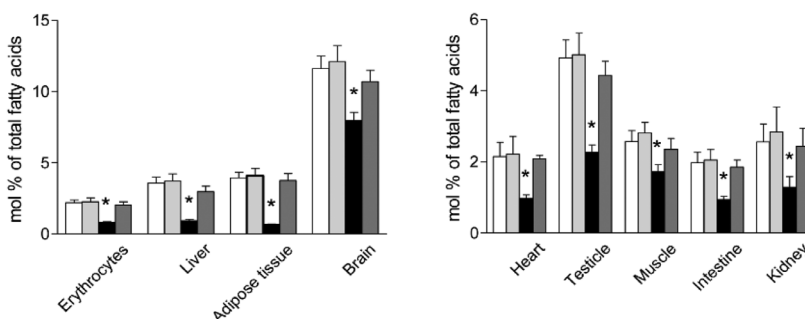
Fig. 3. Levels of α-linolenic acid (A), eicosapentaenoic acid (B), docosapentaenoic acid n-3C) and docosahexaenoic acid (D) in the liver and extrahepatic tissues of rats subjected to control diet, antioxidant-rich extra virgin olive oil (AR-EVOO), iron rich diet (IRD) and AR-EVOO plus IRD. Values are expressed as means ± SEM for 10 rats per group, and differences between groups were analysed by two-way ANOVA and the Bonferroni's post-test, with significant differences ($p < 0.05$) being indicated by asterisks (*).

dependent synthesis of oleoylethanolamide acting as high affinity PPAR-α agonist [49]. In this regard, AR-EVOO prevents IRD-induced liver steatosis, oxidative stress and mitochondrial dysfunction, effects that are directly related to the normalization of the activity of PPAR-α, SREBP-1c and Nrf2 [49], benefits that may be involved in the regularization in the activity of hepatic desaturases and n-3 LCPUFAs'

levels. In agreement with these views, n-3 LCPUFA levels exhibit a significant correlation with Δ5-D and Δ6-D activities, whereas inverse associations were reported between Δ5-D and Δ6-D activities and oxidative stress-related parameters [7]. An interesting finding in the experimental protocol used was the significant increase in the DPA n-6 content of the brain after IRD (Fig. 1D), in agreement with previous
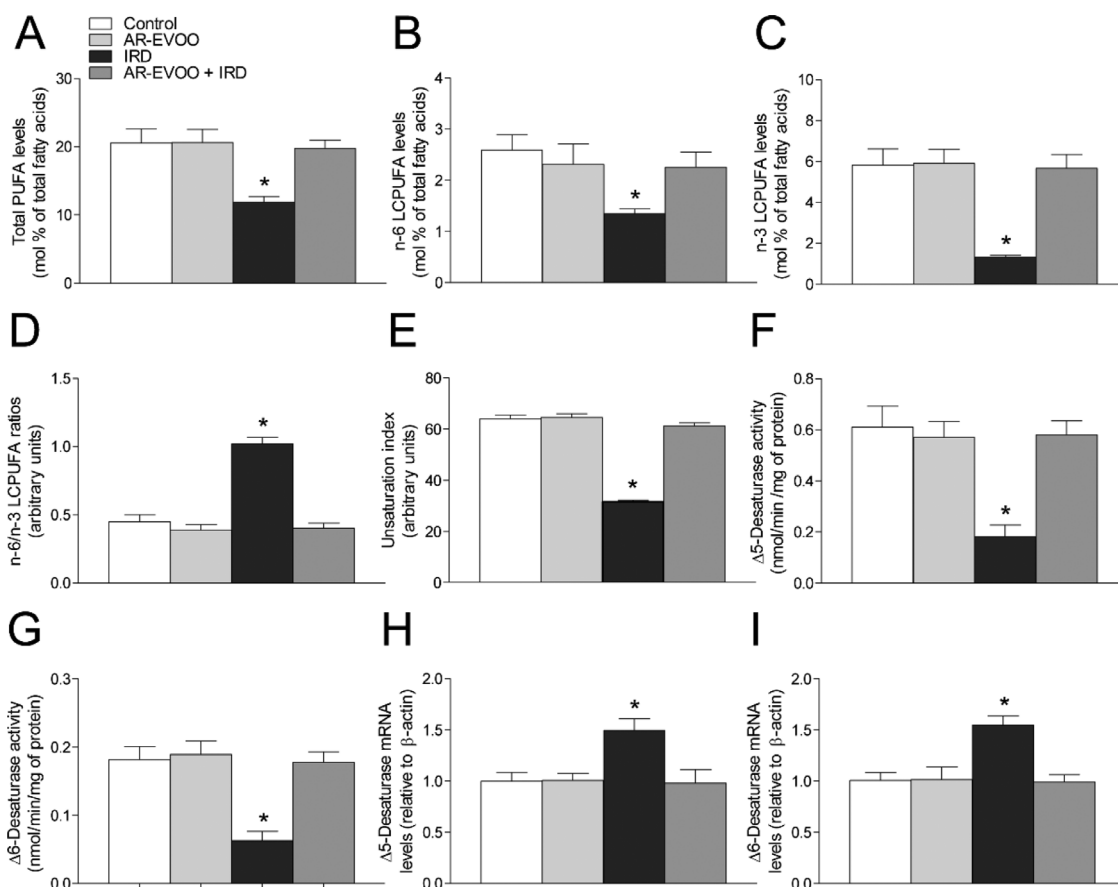
Fig. 4. Levels of liver total polyunsaturated fatty acids (PUFAs) (A), n-6 long-chain PUFAs (LCPUFAs) (B) and n-3 LCPUFAs (C), n-6/n-3 LCPUFA ratios (D) and unsaturation index (E), activity of Δ5-desaturase (F) and Δ6-desaturase (G), mRNA expression of Δ5-desaturase (H) and Δ6-desaturase (I) in rats subjected to control diet, antioxidant-rich extra virgin olive oil (AR-EVOO), iron rich diet (IRD) and AR-EVOO plus IRD. Values are expressed as means ± SEM for 10 rats per group, and differences between groups were analysed by two-way ANOVA and the Bonferroni's post-test, with significant differences ($p < 0.05$) being indicated by asterisks (*). Polyunsaturated fatty acids (PUFAs) correspond to C18:2 n-6, C18:3 n-3, C20:4 n-6, C20:5 n-3, C22:5 n-3 and C22:6 n-3; n-6 long-chain PUFAs are C20:4 n-6 and C22:5 n-6; n-3 LCPUFAs are C20:5 n-3, C22:5 n-3 and C22:6 n-3; n-6/n-3 LCPUFA ratios correspond to C20:4 n-6/(C20:5 n-3 + C22:5 n-3 + C22:6 n-3). The unsaturation index relates to the sum of [18:2 n-6]x2, [18:3 n-3]x3, [20:4 n-6]x4, [20:5 n-3]x5, [22:5 n-3]x5, [22:5 n-6]x5 and [22:6 n-3]x6.

studies with diets high in iron [7] or fat [50]. This finding may represent an adaptation to the diminution in the content of both n-6 and n-3 LCPUFAs in the brain, especially DHA [51,52], a result that requires further investigation.

Considering our results it is possible to establish important protective effects of AR-EVOO, specially the conservation of liver desaturation capacity and prevention of steatosis. In this regard, the specific polyphenols that are present in AR-EVOO (especially HT and tyrosol) could modulate cytoprotective responses in the liver [53,54]. Besides, the α-tocopherol content in AR-EVOO is also important, because this molecule is another specific component of EVOO with hepatoprotective

effects [43]. Therefore, these results can be used to propose new studies with isolated active components present in AR-EVOO. Currently, different pharmacological therapies, dietary and/or specific nutritional interventions have been proposed to prevent or attenuate liver steatosis. However, the results are controversial in human's clinical trials [55–57]. Therefore, studying the specific components present in a given EVOO with high antioxidant content could be a new individual or combined strategy for the treatment of NAFLD.
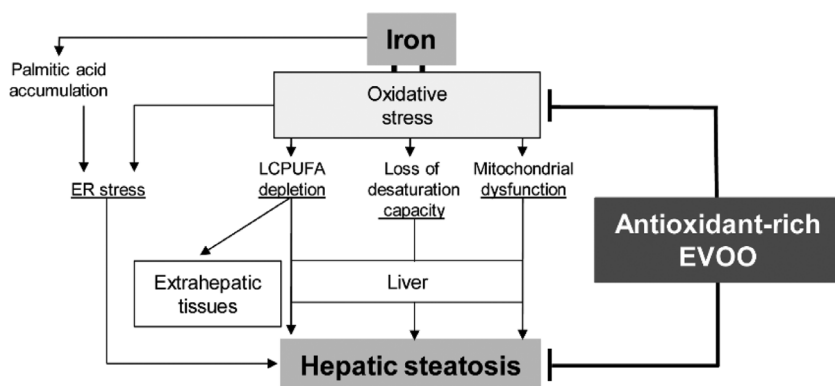

Fig. 5.. Iron-induced liver oxidative stress-dependent diminution in long-chain polyunsaturated fatty acids (LCPUFA) levels and desaturation capacity leading to steatosis and their prevention by antioxidant-rich olive oil (AR-EVOO) supplementation. Contribution by concomitant palmitic acid- and oxidative stress-dependent endoplasmic reticulum (ER) stress (inhibition).

5. Conclusions

The effects of AR-EVOO against IRD-induced hepatic steatosis, which are specifically associated with its active components OA, PUFAs, tocopherols and HT, induce the recovery of the desaturation capacity and n-3 LCPUFA status of the liver that triggers FA oxidation over lipogenesis, thus preventing fatty liver (Fig. 5). Normalization of the desaturation capacity and the LCPUFA status of the liver by AR-EVOO allows the recovery of not only the n-3 LCPUFAs EPA, DPA n-3 and DHA, but also that of n-6 LCPUFAs AA and DPA n-6 in extrahepatic tissues, which lack a significant competence for their biosynthesis, thus protecting tissues against iron toxicity. The presence of different active components in AR-EVOO may allow the use of low doses and/or supplementation periods that trigger different defensive processes or similar signalling mechanisms exhibiting synergism, representing a suitable preconditioning strategy with possible future clinical applications. The anti-steatotic effect of AR-EVOO is particularly important to avoid progression into more aggressive and irreversible states such as steatohepatitis, cirrhosis and hepatocellular cancer, which currently lack of effective pharmacological approaches to treat pathologies related to DIOS [58] or NAFLD [43,47]. Although the beneficial effects of EVOO are also related to cardioprotection, cancer prevention and neurodegeneration, high-quality human trials are required in order to claim health benefits in the clinical setting [59,60].

Limitations

Our study demonstrates that extra virgin olive oil, with a high antioxidant content, prevents (i) the diminution of the hepatic synthesis of n-6 and n-3 LCPUFAs; (ii) the depletion of n-6 and n-3 LCPUFAs in different tissues; and (iii) the increase in liver fat, generated by a diet with a high iron content. However, it is necessary to carry out studies in humans under iron overloading conditions, especially in liver, to confirm the benefits generated by the AR-EVOO on the metabolism of LCPUFAs and hepatic steatosis.

Funding

CRediT authorship contribution statement

Cynthia Barrera: Formal analysis, Investigation, Methodology. **Rodrigo Valenzuela:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Miguel A. Rincón:** Formal analysis, Investigation, Methodology. **Alejandra Espinosa:** Formal analysis, Investigation, Methodology. **Sandra López-Arana:** Formal analysis, Investigation, Methodology. **Daniel González-Mañan:** Formal analysis, Investigation, Methodology. **Nalda Romero:** Formal analysis, Investigation, Methodology. **Romina Vargas:** Formal analysis, Investigation, Methodology. **Luis A. Videla:** Conceptualization, Formal analysis, Investigation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.plefa.2020.102058.

References

[1] A. Pietrangelo, Iron-induced oxidant stress in alcoholic liver fibrogenesis, Alcohol 30 (2003) 121–129.

[2] U. Ahmed, P.S. Latham, P.S. Oates, Interactions between iron and lipid metabolism with possible relevance to steatohepatitis, World J. Gastroenterol. 18 (2012) 4651–4658.

[3] N. Anderson, J. Borlak, Molecular mechanisms and therapeutic targets in steatosis and steatohepatitis, Pharmacol. Rev. 60 (2008) 311–357.

[4] S Puntarulo, Iron, oxidative stress and human health, Mol. Aspects Med. 26 (2005) 299–312.

[5] O. Kakhlon, Z.I. Cabantchik, The labile iron pool: characterization, measurement, and participation in cellular processes, Free Radic. Biol. Med. 33 (2002) 1037–1046.

[6] M Kruszewski, Labile iron pool: the main determinant of cellular response to oxidative stress, Mutat. Res. 531 (2003) 81–92.

[7] R. Valenzuela, M.A. Rincón-Cervera, F. Echeverría, C. Barrera, A. Espinosa, M.C. Hernández-Rodas, M. Ortiz, A. Valenzuela, L.A. Videla, Iron-induced prooxidant and pro-lipogenic responses in relation to impaired synthesis and accretion of long-chain polyunsaturated fatty acids in rat hepatic and extrahepatic tissues, Nutrition 45 (2018) 49–58.

[8] E.M. Brunt, C.G. Janney, A.M. Di Bisceglie, B.A. Neuschwander-Tetri, B.R. Bacon, Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions, Am. J. Gastroenterol. 94 (1999) 2467–2474.

[9] L.A. Videla, Combined docosahexaenoic acid and thyroid hormone supplementation as a protocol supporting energy supply to precondition and afford protection against metabolic stress situations, IUBMB Life 71 (2019) 1211–1220.

[10] S.I. Rapoport, M. Igarashi, F. Gao, Quantitative contributions of diet and liver synthesis to docosahexaenoic acid homeostasis, Prostaglandins Leukot. Essent. Fatty Acids 82 (2010) 273–276.

[11] M.T. Nakamura, B.E. Yudell, J.J. Loor, Regulation of energy metabolism by long-chain fatty acids, Prog. Lipid Res. 53 (2014) 124–144.

[12] X. Deng, Q. Dong, D. Bridges, R. Raghow, E.A. Park, M.B. Elam, Docosahexaenoic acid inhibits proteolytic processing of sterol regulatory element-binding protein-1c (SREBP-1c) via activation of AMP-activated kinase, Biochim. Biophys. Acta (2015) 1521–1529 *1851*.

[13] M.C. Hernández-Rodas, R. Valenzuela, F. Echeverría, M.Á. Rincón-Cervera, A. Espinosa, P. Illesca, P. Muñoz, A. Corbari, N. Romero, D. Gonzalez-Mañan, L.A. Videla, Supplementation with docosahexaenoic acid and extra virgin olive oil prevents liver steatosis by a high-fat diet in mice through PPAR-α and Nrf2 upregulation with concomitant SREBP-1c and NF-κB downregulation, Mol. Nutr. Food Res. 61 (2017) 1700470.

[14] F. Echeverría, M. Ortiz, R. Valenzuela, LA. Videla, Long-chain polyunsaturated fatty acids regulation of PPARs, signaling: relationship to tissue development and aging, Prostaglandins Leukot. Essent. Fatty Acids 114 (2016) 28–34.

[15] M.A. Rincón-Cervera, R. Valenzuela, M.C. Hernández-Rodas, M. Marambio, A. Espinosa, S. Mayer, N. Romero, C. Barrera, A. Valenzuela, L.A. Videla, Supplementation with antioxidant-rich extra virgin olive oil prevents hepatic oxidative stress and reduction of desaturation capacity in mice fed a high-fat diet: effects on fatty acid composition in liver and extrahepatic tissues, Nutrition 32 (2016) 1254–1267.

[16] B. Lönnerdal, Development of iron homeostasis in infants and young children, Am. J. Clin. Nutr. 106 (2017) 1575S–1580S.

[17] D. González-Mañán, G. Tapia, J.G. Gormaz, A. D'Espessailles, A. Espinosa, L. Masson, P. Varela, A. Valenzuela, R. Valenzuela, Bioconversion of α-linolenic acid to n-3 LCPUFA and expression of PPAR-alpha, acyl coenzyme A oxidase 1 and carnitine acyl transferase I are incremented after feeding rats with α-linolenic acid-rich oils, Food Funct. 3 (2012) 765–772.

[18] International Olive Council (IOC), Determination of Biophenols in Olive Oils by HPLC, IOOC, 2009.

[19] R. Mateos, J.L. Espartero, M. Trujillo, J.J. Ríos, M. León-Camacho, F. Alcudia, A. Cert, Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-performance liquid chromatography with diode array ultraviolet detection, J. Agric. Food Chem. 49 (2001) 2185–2192.

[20] American Oil Chemists' Society, Official Methods and Recommended Practices of the American Oil Chemists' Society, American Oil Chemists' Society, Champaign, IL, 1993.

[21] R.L. Prior, H. Hoang, L. Gu, X. Wu, M. Bacchiocca, L. Howard, M. Hampsch-Woodill, D. Huang, B. Ou, R. Jacob, Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples, J. Agric. Food Chem. 51 (2003) 3273–3279.

[22] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.

[23] V. Ruiz-Gutierrez, A. Cert, J.J. Rios, Determination of phospholipid fatty acid and triacylglycerol composition of rat caecal mucosa, J. Chromatogr. 575 (1992) 1–6.

[24] W.R. Morrison, L.M. Smith, Preparation of fatty acid methyl esters and dimethylacetals from lipids with boron fluoride-methanol, J. Lipid Res. 5 (1964) 600–608.

[25] P.Y. Lee, J. Costumbrado, C.Y. Hsu, Y.H. Kim, Agarose gel electrophoresis for the separation of DNA fragments, J. Vis. Exp. 62 (2012) 3923.

[26] M.W. Pfaffl, A new mathematical model for relative quantification in real-time RT-PCR, Nucleic Acids Res. 29 (2001) e45.

[27] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method, Methods 25 (2001) 402–408.

[28] O.H Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[29] H.M. Su, J.T. Brenna, Simultaneous measurement of desaturase activities using stable isotope tracers or a nontracer method, Anal. Biochem. 261 (1998) 43–50.

[30] S.I.S. Rattan, Hormesis in aging, Ageing Res. Rev. 7 (2008) 63–78.

[31] TS Koskenkorva-Frank, G Weiss, WH Koppenol, S Burckhardt, The complex interplay of iron metabolism, reactive oxygen species, and reactive nitrogen species: insights into the potential of various iron therapies to induce oxidative and nitrosative stress, Free Radic. Biol. Med. 65 (2013) 1174–1194.

[32] NE Piloni, JC Perazzo, V Fernandez, LA Videla, S Puntarulo, Sub-chronic iron overload triggers oxidative stress development in rat brain: implications for cell protection, Biometals 29 (2016) 119–130.

[33] L.A. Videla, P. Pettinelli, Misregulation of PPAR functioning and its pathogenic consequences associated with nonalcoholic fatty liver disease in human obesity, PPAR Res. 2012 (2012) 107434.

[34] C. Saporito-Magriñá, R. Musacco-Sebio, J.M. Acosta, S. Bajicoff, P. Paredes-Fleitas, A. Boveris, M.G. Repetto, Rat liver mitochondrial dysfunction by addition of copper (II) or iron(III) ions, J. Inorg. Biochem. 166 (2017) 5–11.

[35] R.R. Crichton, S. Wilmet, R. Legssyer, R.J. Ward, Molecular and cellular mechanisms of iron homeostasis and toxicity in mammalian cells, J. Inorg. Biochem. 91 (2002) 9–18.

[36] A.L. Kurniawan, Y.C. Lee, C.K. Shih, R.H. Hsieh, S.H. Chen, J.S. Chang, Alteration in iron efflux affects male sex hormone testosterone biosynthesis in a diet-induced obese rat model, Food Funct 10 (2019) 4113–4123.

[37] L.X. Lou, B. Geng, Y. Chen, F. Yu, J. Zhao, C.S. Tang, Endoplasmic reticulum stress involved in heart and liver injury in iron-loaded rats, Clin. Exp. Pharmacol. Physiol. 36 (2009) 612–618.

[38] J. Fujii, T. Homma, S. Kobayashi, H.G. Seo, Mutual interaction between oxidative stress and endoplasmic reticulum stress in the pathogenesis of diseases specifically focusing on non-alcoholic fatty liver disease, World J. Biol. Chem. 9 (2018) 1–15.

[39] C.L. Gentile, M.A. Frye, M.J. Pagliassotti, Fatty acids and the endoplasmic reticulum in nonalcoholic fatty liver disease, Biofactors 37 (2011) 8–16.

[40] Z. Zheng, C. Zhang, K. Zhang, Role of unfolded protein response in lipogenesis, World J. Hepatol. 2 (2010) 203–207.

[41] A.K. Leamy, R.A. Egnatchik, J.D. Young, Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease, Prog. Lipid Res. 52 (2013) 165–174.

[42] E.R. Stadtman, Metal-ion catalyzed oxidation of proteins: biochemical mechanism and biological consequences, Free Radic. Biol. Med. 9 (1990) 315–325.

[43] S.A. Soto-Alarcón, R. Valenzuela, A. Valenzuela, L.A. Videla, Liver protective effects of extra virgin olive oil: interaction between its chemical composition and the cell-signaling pathways involved in protection, Endocr. Metab. Immune Disord. Drug Targets 18 (2018) 75–84.

[44] M.H. Gordon, F. Paiva-Martins, M. Almeida, Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols, J. Agric. Food Chem. 49 (2001) 2480–2485.

[45] L. Gao, J. Wang, K.R. Sekhar, H. Yin, N.F. Yared, S.N. Schneider, S. Sasi, T.P. Dalton, M.E. Anderson, J.Y. Chan, J.D. Morrow, M.L. Freeman, Novel n-3 fatty acid oxidation products activate Nrf2 by destabilizing the association between Keap1 and Cullin3, J. Biol. Chem. 282 (2007) 2529–2537.

[46] R. Valenzuela, L.A. Videla, Crosstalk mechanisms in hepatoprotection: Thyroid hormone-docosahexaenoic acid (DHA) and DHA-extra virgin olive oil combined protocols, Pharmacol. Res. 132 (2018) 168–175.

[47] C. Pirozzi, A. Lama, R. Simeoli, O. Paciello, T.B. Pagano, M.P. Mollica, F. Di Guida, R. Russo, S. Magliocca, R.B. Canani, G.M. Raso, A. Calignano, R. Meli, Hydroxytyrosol prevents metabolic impairment reducing hepatic inflammation and restoring duodenal integrity in a rat model of NAFLD, J. Nutr. Biochem. 30 (2016) 108–115.

[48] K.J. Bowen, P.M. Kris-Etherton, G.C. Shearer, S.G. West, L. Reddivari, P.J.H. Jones, Oleic acid-derived oleoylethanolamide: a nutritional science perspective, Prog. Lipid Res. 67 (2017) 1–15.

[49] C. Barrera, R. Valenzuela, M.A. Rincón, A. Espinosa, F. Echeverría, N. Romero, D. Gonzalez-Mañan, L.A. Videla, Molecular mechanisms related to the hepatoprotective effects of antioxidant-rich extra virgin olive oil supplementation in rats subjected to short-term iron administration, Free Radic. Biol. Med. 126 (2018) 313–321.

[50] R. Valenzuela, F. Echeverria, M. Ortiz, M.Á. Rincón-Cervera, A. Espinosa, M.C. Hernandez-Rodas, P. Illesca, A. Valenzuela, L.A. Videla, Hydroxytyrosol prevents reduction in liver activity of Δ-5 and Δ-6 desaturases, oxidative stress, and depletion in long chain polyunsaturated fatty acid content in different tissues of high-fat diet fed mice, Lipids Health Dis. 16 (2017) 64.

[51] S.M. Alashmali, K.E. Hopperton, R.P. Bazinet, Lowering dietary n-6 polyunsaturated fatty acids: interaction with brain arachidonic and docosahexaenoic acids, Curr. Opin. Lipidol. 27 (2016) 54–66.

[52] L.E. Lin, C.T. Chen, K.D. Hildebrand, Z. Liu, K.E. Hopperton, R.P. Bazinet, Chronic dietary n-6 PUFA deprivation leads to conservation of arachidonic acid and more rapid loss of DHA in rat brain phospholipids, J. Lipid. Res. 56 (2015) 390–402.

[53] F. Echeverría, M. Ortiz, R. Valenzuela, LA Videla, Hydroxytyrosol and cytoprotection: a projection for clinical interventions, Int. J. Mol. Sci. 18 (2017) E930 pii.

[54] A.M Karković, J. Torić, M. Barbarić, C.B.Hydroxytyrosol Jakobušić, Tyrosol and derivatives and their potential effects on human health, Molecules 24 (2019) E2001.

[55] A. Eshraghian, Current and emerging pharmacological therapy for non-alcoholic fatty liver disease, World J. Gastroenterol. 23 (2017) 7495–7504.

[56] M.C. Hernandez-Rodas, R. Valenzuela, L.A Videla, Relevant aspects of nutritional and dietary interventions in non-alcoholic fatty liver disease, Int. J. Mol. Sci. 16 (2015) 25168–25198.

[57] R. Valenzuela, M. Ortiz, M.C. Hernández-Rodas, F. Echeverría, L.A. Videla, Targeting n-3 polyunsaturated fatty acids in non-alcoholic fatty liver disease, Curr. Med. Chem. (2019), https://doi.org/10.2174/0929867326666190410121716.

[58] M. Ruivard, F. Lainé, T. Ganz, G. Olbina, M. Westerman, E. Nemeth, M. Rambeau, A. Mazur, L. Gerbaud, V. Tournilhac, A. Abergel, P. Philippe, Y. Deugnier, C. Coudray, Iron absorption in dysmetabolic iron overload syndrome is decreased and correlates with increased plasma hepcidin, J. Hepatol. 50 (2009) 1219–1225.

[59] M.C. Crespo, J. Tomé-Carneiro, A. Dávalos, F. Visioli, Pharma-nutritional properties of olive oil phenols. Transfer of new findings to human nutrition, Foods 7 (2018) 90.

[60] D Huang, Dietary antioxidants and health promotion, Antioxidants 7 (2018) 9.