Gene pathways associated with mitochondrial function, oxidative stress and telomere length are differentially expressed in the liver of rats fed lifelong on virgin olive, sunflower or fish oils☆

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Abstract

This study investigates the effect of lifelong intake of different fat sources rich in monounsaturated (virgin olive oil), n6 polyunsaturated (sunflower oil) or n3 polyunsaturated (fish oil) fatty acids in the aged liver. Male Wistar rats fed lifelong on diets differing in the fat source were killed at 6 and at 24 months of age. Liver histopathology, mitochondrial ultrastructure, biogenesis, oxidative stress, mitochondrial electron transport chain, relative telomere length and gene expression profiles were studied. Aging led to lipid accumulation in the liver. Virgin olive oil led to the lowest oxidation and ultrastructural alterations. Sunflower oil induced fibrosis, ultrastructural alterations and high oxidation. Fish oil intensified oxidation associated with age, lowered electron transport chain activity and enhanced the relative telomere length. Gene expression changes associated with age in animals fed virgin olive oil and fish oil were related mostly to mitochondrial function and oxidative stress pathways, followed by cell cycle and telomere length control. Sunflower oil avoided gene expression changes related to age. According to the results, virgin olive oil might be considered the dietary fat source that best preserves the liver during the aging process.
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1. Introduction

Senescence involves the progressive decline in the cell capacity to respond against oxidative damage, this decline ending in cell death [1,2]. Although liver has usually been described as a well-preserved organ during the aging process, rats reportedly develop few hepatocytes of greater volume. Likewise, at the cell level, a loss of smooth endoplasmic reticulum has also been noted, together with an increase in dense body-compartment volume (including secondary lysosomes, residual bodies and lipofuscin), and greater polyploidy [3]. In 1980, Ludwig et al. introduced the concept of nonalcoholic steatohepatitis (NASH) to describe liver histologic changes resembling alcoholic hepatitis in individuals without significant alcohol intake [4]. Nonalcoholic fatty liver disease (NAFLD) encompasses a continuum, from simple hepatic steatosis with moderate fatty infiltration to NASH with focal inflammation. A small portion of NAFLD may progress to advanced fibrosis, cirrhosis and eventually hepatocellular carcinoma [5]. The prevalence of NAFLD in the general population increases with

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age: from 1% to 3% in children, 5% in teenagers, 18% between 20 and 40 years, 39% in those aged 40 to 50 years and to over 40% in those older than 70 [6]. Moreover, aging in the liver reportedly accelerates the progression of NAFLD to NASH and fibrosis, thus raising the likelihood of mortality [7,8]. Aging may promote the development of NAFLD by several mechanisms such as promoting the onset of age-related obesity and diabetes, the cumulative effects of many years of lifestyle factors (e.g., overconsumption of an inadequate diet) or through physiological changes inherent to the process of aging such as altered autophagy or mitochondrial alterations and oxidative stress [9]. There is consistent evidence for a central role of mitochondrial dysfunction in the pathophysiology of NASH [10]. Recent findings suggest that continuous adaptation or "remodeling" of mitochondrial energetics, gene expression, morphology and content is key in the pathogenesis of simple steatosis/NASH [11]. Moreover, reactive oxygen species production by mitochondria has been consistently reported, and a number of studies in experimental models and humans indicate a strong association between the severity of NASH and degree of oxidative stress [12]. Moreover, oxidative stress, mainly at the mitochondrial level, has been associated with aging, and age-increased levels of protein carbonyl have been found in the liver of old mice [3] and rats [13].

Because the liver is the central organ of metabolism, changes in diet greatly affect this organ during aging [14]. Lifelong dietary changes, either in fat quality or in quantity, alter the fatty acid composition and make the liver more prone to damage and to developing NAFLD [8]. Regarding oxidative stress in the liver, the dietary fat source is known to strongly influence the lipid composition of the mitochondrial membrane, affecting the mitochondrial electron transport chain (mtETC) functions, oxidative damage and mtDNA alterations [15,16]. Thus, altered mitochondrial ultrastructure and function in the liver have been reported in aged rats fed sunflower oil vs. virgin olive oil, together with different levels of oxidative stress and mtDNA alterations [17–19].

In relation to the aging process in the liver, the present study investigates the effect of lifelong feeding on different dietary fat sources rich in unsaturated fatty acids: virgin olive oil, rich in monounsaturated fatty acids (MUFA); sunflower oil, rich in n6 polyunsaturated fatty acids (n6PUFA); and fish oil, rich in n3PUFA. Young (6 months) vs. old (24 months) rats were studied regarding histopathological and ultrastructural features, telomere length, mtETC function and oxidative stress status. Lastly, the gene expression profile was considered in an attempt to account for the changes observed in the aforementioned markers.

2. Materials and methods

2.1. Animals and diets

The rats were treated following the guidelines of the Spanish Society for Laboratory Animals, and the experiment was approved by the Ethics Committee of the University of Granada, Spain (permit number 20-CEA-2004). A total of 72 male Wistar rats (*Rattus norvegicus*) weighing 80–90 g were housed and maintained in a 12-h light/12-h darkness cycle, with free access to food and water. Individual rats were randomly assigned into three experimental groups and fed from weaning until 24 months of age on a semisynthetic and isoenergetic diet according to the AIN93 criteria [20] adjusted according to the fat source (virgin olive oil, sunflower oil or fish oil). The fatty acid profile of experimental diets is shown in Table 1. Twelve rats per group were killed by cervical dislocation followed by decapitation at 6 and 24 months from the beginning of the experiment. After exsanguination, livers were removed and preserved.

2.2. Mitochondrial isolation

Liver (1 g) was used for mitochondria extraction following Fleischer et al. [21]. The liver mitochondrial protein was determined following Lowry et al. [22].

2.3. Liver mitochondrial fatty acid profile

The liver mitochondrial fatty acid profile was determined using the method of Lepage and Roy [23]. A gas–liquid chromatograph Model HP-5890 Series II (Hewlett

Table 1
Fatty acid profile of experimental dietary fats (g/100 g)

Fatty acid or index	Virgin olive	Sunflower	Fish oil
C14:0	0.0	0.1	7.2
C16:0	8.3	6.4	17.1
C16:1n-9	1.1	0.1	9.6
C18:0	3.2	4.7	2.7
C18:1n-9	77.7	24.2	15.1
C18:2n-6	3.2	62.8	2.8
C20:3n-6	0.1	0.9	0.1
C20:4n-6	0.0	0.0	2.1
C20:5n-3	0.2	0.1	18.6
C24:0	0.0	0.1	0.3
C24:1n-9	0.0	0.0	0.9
C22:6n-3	0.0	0.0	10.5
Total saturated fatty acids	12.6	11.5	30.5
Total monounsaturated fatty acids	83.7	24.4	30.1
Total n-6 polyunsaturated fatty acids	3.3	63.7	8.2
Total n-3 polyunsaturated fatty acids	0.4	0.4	31.3
Total polyunsaturated fatty acids	3.7	64.1	39.4

Packard, Palo Alto, CA, USA) equipped with a flame ionization detector was used to analyze fatty acids. Chromatography was performed using a 60-m-long capillary column, 32 mm id and 20 mm thick, impregnated with SpTM 2330 FS (Supelco Inc., Bellefonte, Palo Alto, CA, USA). The injector and the detector were maintained at 250°C and 275°C, respectively, nitrogen was used as carrier gas, and the split ratio was 29:1. Temperature programming (for a total time of 40 min) was as follows: initial temperature, 160°C for 5 min, 6°C/min to195°C, 4°C/min to 220°C, 2°C/min to 230°C, hold 12 min, 1°C/min at 160°C.

2.4. Histopathological analysis of the liver

Immediately after the rats were killed, a fragment of a liver lobe (the same for all the animals) was placed in formol 3.9% at pH 7. After 24–48 h of fixation, each piece was cut and placed in a cassette for embedding in paraffin according to the conventional process, after dehydration in increasing alcohols to absolute ethyl alcohol. Subsequently, cuts at 4 μm were performed following by hematoxylin and eosin staining. Gomori trichromic, periodic acid–Schiff, Gomori's reticulin silver impregnation stains and Sirius red were used. Histological lesions were evaluated by the grading system proposed by Yeh and Brunt [24] for NASH analysis, and hepatic fibrosis was also studied. NASH grade was analyzed as follows: steatosis [grade 0: absence of or minimum (<5%) steatosis, grade 1: >5%–33% of observed area with steatosis, grade 2: >33%–66% of observed area with steatosis, grade 3: >66% of observed area with steatosis]; inflammation (grade 0=0/20 C20X, grade 1=1–2/20 C20X, grade 2=2–4/20 C20X, grade 3=>4/20 C20X); ballooning degeneration, which indicates the accumulation of fluid and other toxic substances in the cytoplasm of hepatocytes causing their swelling and rarefaction (grade 0: absence of ballooning, grade 1: minimum ballooning, grade 2: prominent ballooning). For liver fibrosis, slides stained with Sirius red were used. The area and the percentage of tissue positive for Sirius red (collagen) were calculated for each sample in comparison with hepatic parenchyma. Ten fields per sample were analyzed at a magnification of 10×. For the analysis, Olympus (Hamburg, Germany) AnaliSYS Image Processing software was used. To eliminate nuclear stain, color intensity caption at the picture was reduced both for collagen and for hepatic parenchyma by the capture of the picture with a 680×510 parameter.

2.5. Ultrastructural analysis of the liver

Briefly, a small piece of tissue was prefixed in 1.5% formaldehyde in 1% cacodylate buffer, pH 7.4, for 2 h at 4°C. After three washes in cacodylate buffer, extracts were fixed in 1% osmium tetroxide for 60 min at 0°C–4°C. The samples were dehydrated in graded ethanol and embedded in Epon resin. After overnight incubation at 65°C, ultrathin sections (70 nm) were cut with a diamond knife using an Ultracut S ultramicrotome and placed on 200-mesh copper grids. All sections were stained with uranyl acetate, counterstained with lead citrate and viewed using a Carl Zeiss (Oberkochen, Germany) EM10C electron microscope at 4000×, 7500× and 40,000× magnifications in the Biomedical Research Center at the University of Granada's Health Technology Park. The ImageJ 1.46r, a public domain program from the US National Institute of Health [25], was used to determine the mitochondrial area, perimeter and mitochondrial density of the liver.

2.6. Liver protein carbonyl assay

Liver protein carbonyl groups were assessed by a commercial kit (Cayman Chemical Company, Ann Arbor, MI, USA). Briefly, 100 μl of liver protein extract was transferred to two tubes, i.e., the sample tube and the control tube. After 400 μl of DNPH was added to the sample tube and 400 μl of 2.5 M HCl to the control tube, both were incubated in

darkness at room temperature for 1 h, with brief stirring every 15 min. Afterward, 0.5 ml of 20% trichloroacetic acid was added to each tube and incubated in ice for 5 min. This mixture was centrifuged at 10,000×g for 10 min and the resulting pellet was resuspended in 0.5 ml of 10% trichloroacetic acid and incubated in ice for 5 min and again centrifuged at 10,000×g for 10 min at +4°C. The pellet was resuspended in 0.5 ml of (1:1) ethanol/ethyl acetate mixture and centrifuged twice at 10,000×g for 10 min at +4°C. Finally, the pellet was resuspended in 250 μl of guanidine hydrochloride and centrifuged at 10,000×g for 10 min at +4°C, and 220 μl of the supernatant were transferred to a 96-well plate for a reading of the absorbance (SYNERGY HT, Multi-Detection Microplate Reader; BioTek Instruments, Inc., Winooski, VT, USA) at 370 nm. The total protein concentration in liver sample extracts was measured using Pierce BCA Protein Assay (Thermo Scientific, Rockford, IL, USA). The results were expressed as nmol of carbonyl proteins per mg of total proteins in the extract.

2.7. Western blot analysis of selected liver proteins

Liver protein (20 μg) was subjected to electrophoretic fractionation on a 4% to 12% Bis–Tris Criterion XT Precast gel (Bio-Rad Laboratories, Hercules, CA, USA). Separated fractions were transferred into a polyvinylidene difluoride 0.2-μm membrane (Bio-Rad Laboratories). Membranes were blocked with Tris-buffered saline and Tween 20 containing 5% nonfat milk and incubated with primary monoclonal antibodies against Pgc-1α and Tfam (Santa Cruz and Abcam). β-Actin (Abcam, Cambridge, UK) was used as the loading control. Specific horseradish-peroxidase-conjugated secondary antibodies from Bio-Rad Laboratories or Santa Cruz Biotechnology (CA, USA) were used. Chemiluminescence reagent Immun-Star HRP Chemiluminescence Kit (Bio-Rad Laboratories) was used to detect the protein signal. Quantification and recording were performed with a Luminescent Image Analyzer LAS-4000 mini (FUJIFILM Corp., Tokyo, Japan).

2.8. mtETC complex I activity

The Complex I Enzyme Activity Dipstick Assay Kit was acquired from Mitosciences (Eugene, OR, USA). Dipsticks contain a zone of anticomplex I mAb 18G12BC2 striped at 1 mg/ml. Dipstick assays were performed by inserting individual dipsticks into 50 μl of homogenized liver tissue. The entire sample was then allowed to pick up laterally through the membrane, passing through the zone where the target enzymes are immunocaptured and concentrated (ca 15 min). The dipsticks were then cleared by allowing 30 μl of wash buffer (50 mM Tris–Cl, 150 mM NaCl, pH 7.4) to pick up through the dipstick (ca 10 min). Complex I activity was revealed and measured by immersing each complex I activity dipstick in 500 μl of complex I reaction buffer [20 mM Tris–Cl, pH 7.4, containing 0.1 mg/ml NADH (Sigma) and 0.3 mg/ml Nitrotetrazolium blue (NBT, Bio-Rad Laboratories)] for 40 min, stopping the reaction by immersing the dipsticks in 300 μl of distilled water for 10 min and then measuring the amount of intensely colored, reduced NBT (NTBH), which precipitates at the site of immunocapture of functional complex I. A Hamamatsu MS-1000 immunochromatographic dipstick reader was used for densitometry.

2.9. Liver mitochondrial respiratory efficiency

The oxygen consumption rate (OCR) of liver mitochondria was measured with a XF24 Extracellular Flux Analyzer (Seahorse Bioscience, Billerica, MA, USA) as previously described [26]. To minimize variability between wells, the mitochondrial suspension was first diluted 10× in cold mitochondrial assay solution (MAS) buffer combined with 10 mM pyruvate, 2 mM malate and 4 μM carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) for the final concentration of 10 μg of mitochondria in 50 μl of MAS solution. Then, 50 μl of the diluted mitochondria solution were placed in each well (except for background correction wells) in an XF24 cell culture microplate (Seahorse Bioscience) while the microplate was on ice. The microplate was then transferred to a centrifuge equipped with a swinging bucket microplate adaptor and spun at 2000×g for 20 min at 4°C. After centrifugation, 450 μl of MAS + substrate was added to each well. The mitochondria were then placed at 37°C for 10 min in the XF Prep Station incubator (Seahorse Bioscience, Billerica, MA, USA). The plate was transferred to the XF24 instrument, and the experiment begun. Changes to the concentrations of dissolved oxygen in the media were measured in 2-s intervals by solid-state sensor probes. After determining the basal mitochondrial respiration, rotenone (2 μM), succinate (10 mM), antimycin A (4 μM) and ascorbate plus 1 mM TMPD (10 mM and 100 μM) were sequentially added to each well. XF software analyzed the data, presented as the average of three replicates per well±S.E. The results are expressed as OCR in picomoles of consumed oxygen per minute and were adjusted for protein concentration.

2.10. Relative telomere length analysis

DNA was isolated by a NucleoSpin Tissue kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany). Relative telomere length (RTL) was assessed by a quantitative reverse-transcription polymerase chain reaction (QRT-PCR) method developed by Cawthon et al. [27] with some modifications. In this relative quantification approach, the amount of telomere hexameric repeat (T) is measured and compared to the amount of a single-copy gene (S) that is assumed to be constant for the same sample to establish the ratio of telomere repeat copy number to the single gene copy number (T:S ratio).

Here, we chose the acidic ribosomal phosphoprotein PO (36B4) rat gene, as Cawthon et al. [27] did in human samples. This is a well-conserved gene located on chromosome 7 and has been used for gene dosage studies. Amplification of telomeric DNA (T), together with that of the single-copy genomic 36B4 gene (S) was performed on a MicroAmp Optical 384-well Reaction Plate (Applied Biosystems, Foster City, CA, USA) using Applied Biosystem's 7900HT Fast Real-Time PCR system. All samples were run in duplicate to account for possible technical variation. For this, 5 ng of liver-derived genomic DNA was dried overnight at room temperature in a 384-well plate placing two samples from each animal in adjacent wells and resuspending them in 10 μl of either the telomere or 36B4 PCR reaction mixture. The telomere reaction mixture consisted of 5 μl of Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 270 nM Tel-1b primer (5′-CGG TTT GTT TGG GTT TGG GTT TGG GTT TGG GTT TGG GTT-3′), 900 nM Tel-2b primer (5′-GGC TTG CCT TAC CCT TAC CCT TAC CCT TAC CCT TAC CCT-3′) and double-distilled H$_2$O. The specific reaction mixture for 36B4 was similar except that it included 300 nM forward primer (5′-CTG CAG ATT GGC TAC CCG AC-3′) and 500 nM reverse primer (5′-CAC AGA CAA AGC CAG GAC CC-3′). The reaction conditions were set at 95°C for 10 min followed by 40 cycles of data collection consisting of a denaturation step at 95°C for 15 s and an annealing/extension at the 54°C step for 4 min in the case of the telomere assay or at 58°C for 2 min in the case of the 36B4 assay. An additional melting curve analysis consisting of 95°C for 15 s, 60°C for 15 s and 95°C for 15 s was performed at the end of each reaction to verify specific amplification. To assess and compensate for interplate variations in PCR efficiency, each 384-well plate contained a 9-point standard curve from 0.23 to 30 ng in addition to the samples, using genomic rat DNA pool derived from our samples. For this, DNA was serially diluted using double-distilled H$_2$O, and each dilution was placed in 384-well plates per triplicate for both the 36B4 gene and telomere repeats.

Threshold values were set to 0.2 on Sequence Detector Systems version 2.4 software, and real-time PCR results were exported to an Excel (Microsoft, Redmond, WA, USA) spreadsheet for analysis. The standard curve was plotted from the mean Cq vs. the log of serial dilution concentrations, excluding points beyond the linear range, and only samples with quantification cycle (Cq) values inside curve were used in subsequent analyses. Cq values, mean and standard deviation (S.D.) were calculated for each sample, and those with an S.D. greater than 0.5 were disregarded in further analyses. Then the absolute concentration of the telomere hexameric repeat (T) was divided by the absolute concentration of the 36B4 gene (S) according to efficiency values calculated from their standard curves. The resulting value (T:S ratio) was divided by the T:S ratio determined from the calibrator DNA (one of our samples). The resulting ratio expresses the amount of telomere hexameric repeats, called RTL.

2.11. Liver DNA microarray

Gene expression analyses were performed in three samples from each group at both ages. Total RNA was extracted using the RNeasy Mini kit (Qiagen). RNA concentration was determined in a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). RNA integrity was assessed in an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The microarray analysis was performed using the Affymetrix Rat Genome 230 2.0 gene chip array. Chips and results were processed with the v7.3.1 Partek Genomics Suite (Partek Incorporated, Chesterfield, MO, USA). Expression values were calculated using the robust multiarray average algorithm (all microarray data were deposited in the Gene Expression Omnibus public repository: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE56166). A Bonferroni test was used to correct for multiple tests, considering $P<.05$ to be significant, and fold-change (FC) values were calculated for all comparisons. Gene expression statistical significance was identified by Student's t test for paired samples by the software GeneSpring GX 7.3 (Agilent Technologies). The FC expression value cutoff selected in the present study was 1.5, so expression changes lower than 1.5 were not considered in the analysis. The DNA microarray analysis was performed on liver from both young and old rats fed on the three different dietary fats. For each dietary group and gene, young and old groups were compared using the expression levels of the young animals as the baseline. After the comparative analysis of gene expression data (provided by DNA microarray analysis), a list of genes showing a differential expression in the three dietary treatments was created. On the other hand, a list of genes differently expressed but only in one or two of the dietary groups was also made. Genes in each list were called, respectively, "common genes" and "uncommon genes."

In the present study, only the uncommon genes were analyzed in-depth on the basis of a functional enrichment approach. For this, uncommon sequences were introduced into the Ingenuity Pathways Analysis (IPA) application (Ingenuity Systems, www.ingenuity.com) to perform a canonical pathways analysis. Such analysis identifies crucial pathways in the control of metabolism and cell function as well as processes that could be affected by changes in the expression of our genes of interest. The number of our selected genes located in a canonical pathway from IPA Knowledge Base was divided by the total number of genes belonging to the same canonical pathway. This ratio provides an idea about the percentage of genes in a pathway that were also found in the list of selected sequences (i.e., "uncommon genes"). In addition, Fisher's Exact Test was used to determine the probability of the association between the genes in our data set, and the canonical pathway was due exclusively to that chance. In other words, it refers to the probability that a canonical pathway might be affected by the experimental treatment. Thus, if a P value is very low, the pathway is more likely to be associated with the data set introduced. Both the calculated ratio and Fisher's Exact

Test *P* value were used to identify canonical pathways according to the significance of the association with our data set. A high ratio found for a pathway and a very low *P* value for the Fisher's Exact Test signify that this pathway is probably associated with the data and a large proportion of the pathway may be involved or affected. Therefore, such pathway would be a good choice for explaining the observed phenotype.

2.12. QRT-PCR validation of microarrays

Three samples, different from those used for the microarray, were used for RT-PCR validation [28]. RNA (150 ng) was reverse-transcribed into cDNA. The quantity and purity of the cDNA were determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific). Preamplification was performed with the QIAGEN Multiplex PCR Kit and the pool of all 20× TaqMan Gene Expression Assays as follows (with probe ID between brackets): Atp5d (Rn00756371_g1), Fis1 (Rn01480911_m1), Ndufa8 (Rn01438607_m1), Ndufa9 (Rn01462923_m1), Keap1 (Rn00589292_m1), Map2k6 (Rn00586764_m1), Pik3c2g (Rn00588317_m1), Rras (Rn01454699_g1), Prdx5 (Rn00586040_m1), Ccnd1 (Rn00432360_m1), Ppp2r1b (Rn01422402_g1), Xrcc6 (Rn00594589_m1) and Xrcc1 (Rn01457689_m1). Data analysis was performed by the $2^{-\Delta\Delta Ct}$ method [29]. Gapdh, 18S and B2m were used as the reference gene, and the most appropriate housekeeping gene was selected by GeNorm program (http://medgen.ugent.be/~jvdesomp/genorm/).

2.13. Statistical analysis

For all analyses, except for the DNA array experiment, the results were expressed as mean±standard error of the mean (S.E.M.) for six animals. Normal distribution and variance homogeneity were evaluated by Kolmogorov–Smirnov and Levene tests, respectively. Variables showing normal distribution were analyzed for differences between dietary treatments at 6 months and at 24 months by an analysis of variance with a Bonferroni *post hoc* test. Non-normal variables were analyzed by Kruskal–Wallis and Mann–Whitney *U* nonparametric tests. Tamhane's *T2* test was applied to variables with nonhomogeneous variances. To detect significant differences between age groups, for each dietary treatment, Student's *t* test was applied. In all analyses, significant differences were established at *P*<.05. Statistical analysis was performed with SPSS 24.0 for Windows (IBM, Chicago, IL, USA).

3. Results

3.1. Weight of rats and livers

No differences in body weight were found between treatments at 6 and 24 months (data not shown). Liver weight at 24 months of age was higher (*P*<.05) in the fish oil group (14.0±1.1 g) than in the virgin olive oil (11.2±0.3 g) or sunflower oil (11.6±0.8 g) groups.

3.2. Liver mitochondrial fatty acid profile

The results showed that for C18:1n-9 (oleic acid, the most representative fatty acid found in virgin olive oil), the highest percentage was found for the virgin olive oil group (14.4%±3.9%), values being significantly higher than those found for sunflower oil (8.8%±1.2%) and fish oil (7.2%±1.1%) groups. Concerning C18:2n-6 (linolenic acid, the most representative fatty acid found in sunflower oil), the sunflower oil group registered significantly higher percentages (9.1%±2.2%) than did the virgin olive oil (4.8%±0.5%) and fish oil (2.3%±0.3%) groups. Finally, for C22:6n-3 (docosahexaenoic acid, present mostly in marine species), the fish oil group gave significantly the highest percentage (16.2%±2.5%) compared with virgin olive oil (3.7%±0.9%) and sunflower oil (1.4%±0.9%) groups.

3.3. Histopathological study of the liver

At 24 months, all groups showed higher levels of steatosis (Fig. 1A) than at 6 months, with no differences between the three experimental groups. Similar results were found for the levels of centrilobular inflammation (Fig. 1B) and NASH grade (Fig. 1C). For liver fibrosis (Fig. 1D and E), no valuable levels were found in young animals. At 24 months, significantly higher levels were found in sunflower oil group.

3.4. Mitochondrial ultrastructural study

Aged animals fed on sunflower oil showed higher mitochondrial area and perimeter (Fig. 2). Sunflower oil led to lower mitochondrial density. Regarding fish oil, the liver response was closer to that reported for MUFA.

3.5. Pgc-1α and Tfam protein levels

PGC1α (Fig. 3A) did not exhibit differences at 6 or 24 months between any groups. For Tfam (Fig. 3B), at 24 months the fish oil group reported lower relative amounts than the virgin olive and sunflower oil groups. The aging effect on the sunflower oil group led to higher expression of Tfam than in the young animals.

3.6. Protein carbonyls levels

Protein carbonyls are shown in Fig. 3C. At 6 months, no differences were found between the experimental groups. At 24 months, virgin olive registered lower values than did the sunflower and fish oil groups.

3.7. mtETC complex I activity

No differences concerning complex I activity (Fig. 3D) in 6-month-old animals were found. At 24 months, the fish oil group showed lower activity than did the virgin olive oil and sunflower oil groups, which had higher activity than did their younger counterparts.

3.8. Liver mitochondrial respiratory efficiency

The OCR in isolated mitochondria is shown in Fig. 3E. Differences between groups were found at 6 months, with animals fed virgin olive oil exhibiting lower OCR than the sunflower and fish oil groups. At 24 months, rats fed fish oil showed lower values of OCR than those fed sunflower oil. Higher OCR values were found for aged animals fed virgin olive or sunflower oil compared with their younger counterparts.

3.9. Relative telomere length

The results for the relative telomere length are shown in Fig. 3F. No differences were found between groups at 6 months. Among old rats, the greatest length was found for the fish oil group. Differences between young and old animals were found only for the fish oil group, with lengths being greater in old animals than in young animals.

3.10. Gene expression analysis

Data from the microarray-based gene expression analysis were used to divide genes into two lists: "common genes" and "uncommon genes." Changes in the expression of the "common" genes were expected to be caused directly by aging, whereas the changes in the expression of "uncommon genes" were a consequence of the effects of a particular dietary fat, although modulated by aging. Our analysis of the data provided an "uncommon genes" list of 881 sequences that were introduced into IPA to perform a canonical pathways analysis. From the 82 canonical pathways identified by IPA, 4 were selected and arranged in descending order of significance to the study: "mitochondrial dysfunction," "oxidative stress," "cell cycle regulation" and "telomere length." Then, the "uncommon genes" included in these pathways were analyzed in detail. Several aspects were taken into account, including gene location, expression alteration extent and the relative position (in terms of IPA interest) for each canonical pathway and dietary fat. Next, 16 genes were selected for validation by QRT-PCR
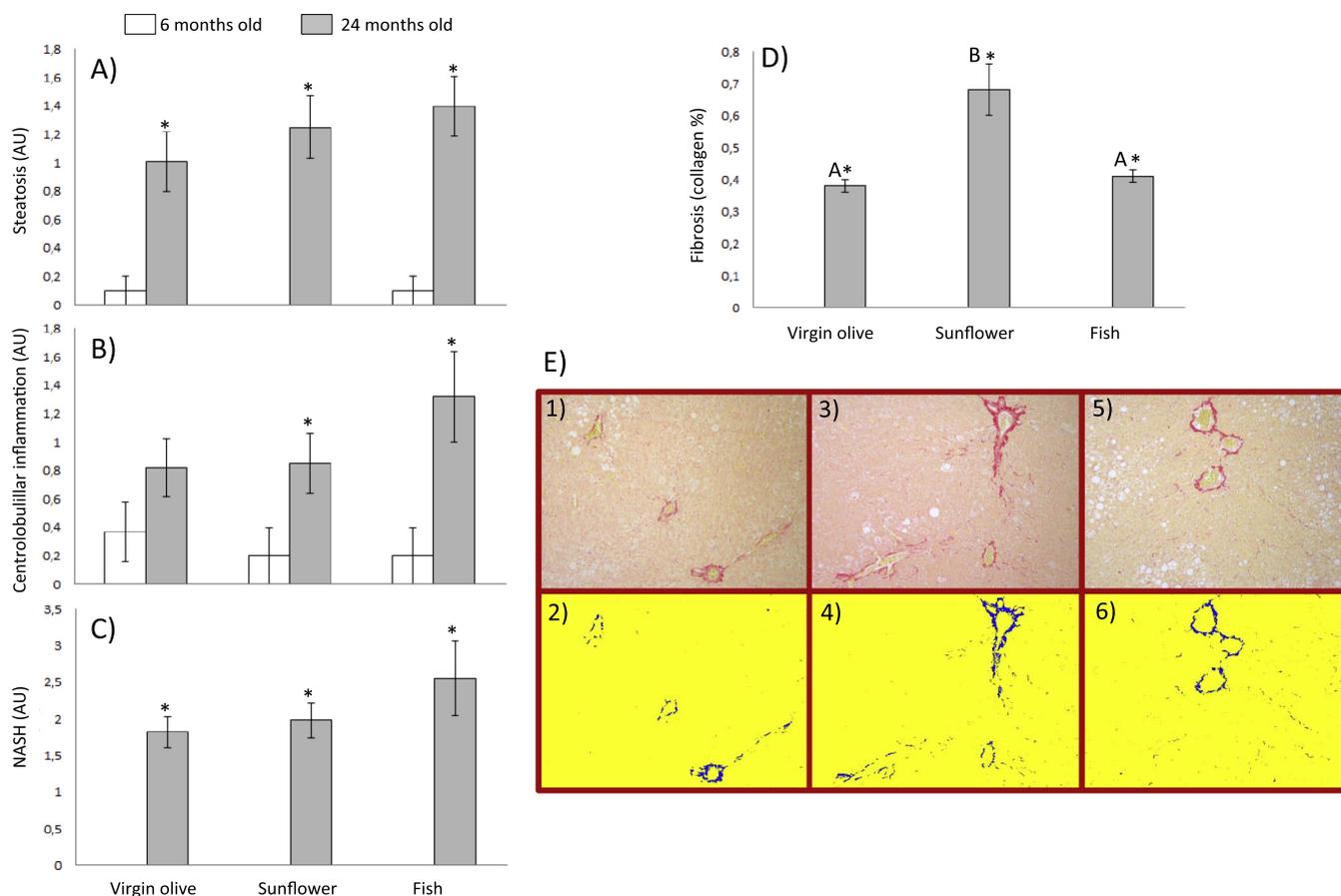
Fig. 1. Histology of rats fed on virgin olive oil, sunflower oil or fish oil for 6 or 24 months of age. (A) Steatosis. (B) Centrilobular inflammation. (C) NASH. (D) Fibrosis. (E) Liver fibrosis microscopy images and image quantification: (1, 3 and 5) Sirius red stained (10×). Note macro–microvesicular steatosis with the collagenous reinforcement in red. (2, 4, and 6) Quantification by image analysis showing collagenous deposit. For panels A to D, the results represent the mean±S.E.M. Some groups at 6 months had no bar since all animals from these groups showed the lowest category for the variable. Bar chart statistical symbols: Uppercase letters represent statistically significant differences between dietary treatments ($P<.05$) at 24 months of age. Asterisks (*) represent, for a dietary treatment, statistically significant differences ($P<.05$) between 6 and 24 months of age. Some data at 6 months.

(Table 2), and 13 genes were validated. In animals fed sunflower oil, no differences in gene expression were found. For animals fed virgin olive oil, eight genes displayed a higher expression in older rats: two associated with the mitochondrial dysfunction pathway (*Atp5d* and *Ndufa9*), four associated with the oxidative stress pathway (*Keap1, Map2k6, Pik3c2g* and *Rras*) and two associated with the telomere length pathway (*Xrcc6* and *Xrcc1*). Lastly, old rats from the fish oil group showed a higher expression for seven genes: three associated with the mitochondrial dysfunction pathway (*Atp5d, Fis1* and *Ndufa8*), two associated with the oxidative stress pathway (*Keap1* and *Prdx5*), two associated with the cell cycle pathway (*Ccnd1* and *Ppp2r1b*) and one associated with the telomere length pathway (*Xrcc1*).

4. Discussion

Although the liver is one of the organs least affected by aging, it has been found that lifelong changes in diet, either in quality or in quantity of fat, alter the fatty acid composition in liver and make the liver more or less susceptible to damage, leading it to develop NAFLD. The lipid profile was analyzed in the liver mitochondrial membranes since we have previously demonstrated that this is a good marker of dietary fat intervention at different tissue levels, including liver, brain, heart and skeletal muscle [19,30–33]. The lipid profile resembled that of the original composition of oils used in the diets, with animals fed on virgin olive oil showing the highest percentage on oleic acid, those fed on sunflower oil having the highest percentage on linoleic acid and animals fed on fish oil registering the highest percentage in

docosahexaenoic acid. These results indicate a proper adaptation of the rats to the different dietary fats, as expected based on previous studies [19]. In terms of histopathological features, the present results support the contention that aging augments physiological lipid accumulation in nonadipose tissues [34] that may compromise liver function by promoting lipotoxicity [35]. With liver fibrosis, the higher levels found in aged animals fed on sunflower oil are consistent with previously reported findings that thermally oxidized sunflower oil induces liver fibrosis [36,37]. Regarding ultrastructure alterations, aged animals fed sunflower oil showed greater mitochondrial area and perimeter, a typical aging marker related to a loss of function through swelling and Ψmt loss [38]. Sunflower oil also led to lower mitochondrial density, in agreement with previous studies based on dietary fat delivery at 8% w/w [19]. Lower mitochondrial density might be offset by the stimulation of biogenesis [39]. In the present study, aged animals fed sunflower oil had a higher expression of Tfam than did young animals, suggesting that sunflower oil stimulates biogenesis during aging.

With respect to oxidative stress status, protein carbonyls at 24 months indicated that rats fed on virgin olive had lower values than did those fed on sunflower and fish oils. These results agree with a previously reported effect of polyunsaturated fat on oxidative stress when compared with monounsaturated oils [16,19,31,32,40]. Studies in rats show that aging in the liver leads to chronic dysfunction of the mitochondrial respiratory chain at either complex I or III [41]. Aoun et al. [42] reported that fish-oil-rich diets at 30% w/w decreased complex I activity in the liver of rats in comparison to other diets. At 24 months,
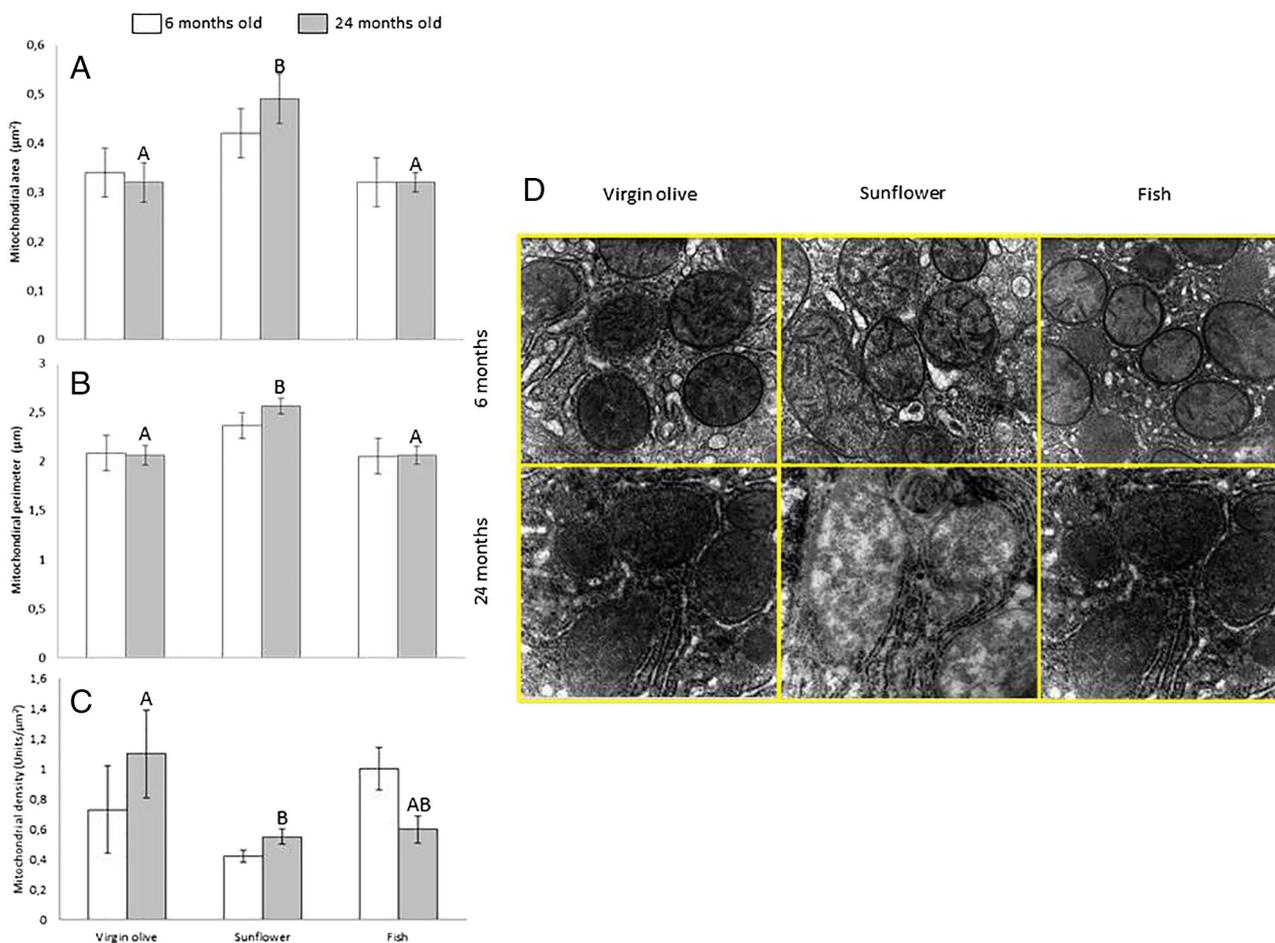
Fig. 2. Ultrastructural analysis of the liver of rats fed on virgin olive oil, sunflower oil or fish oil for 6 or 24 months of age. (A) Mitochondrial area. (B) Mitochondrial perimeter. (C) Mitochondrial density. (D) Representative TEM images (40,000×) of the different groups of rats. For panels A to C, results represent mean±S.E.M. Bar chart statistical symbols: Uppercase letters represent statistically significant differences between dietary treatments ($P<.05$) at 24 months of age.

the fish oil group showed lower activity than the virgin olive oil and sunflower oil groups. These results on fish oil might be related to differences found in Tfam expression or oxidative stress in these animals. Aged animals fed sunflower oil also reported high oxidative stress but not reduced complex I activity. With respect to the OCR, aged rats fed virgin olive oil or sunflower oil showed higher values than did their younger counterparts, while the fish oil group registered lower values than did the sunflower oil group. Decreased mitochondrial respiratory function and mtETC activity were reported in the liver of old rats, which might mean that the mitochondria are not well coupled and the mitochondrial membrane loses its integrity during aging [43]. Mitochondrial function in the liver of senescence-accelerated mice shows a higher respiration rate, probably as a compensatory mechanism or simply as part of the pathological aging process [44]. Mice fed for 8 weeks on diets enriched with fish oil reportedly registered lower rates of oxygen consumption than did those fed on lard [45]. Other studies have found that liver mitochondrial oxygen consumption was higher in animals supplemented with linoleic acid [46]. Other authors detected no differences when investigating dietary fat quality in the liver of young rats fed 5% and 30% fat for 12 weeks [42].

In short, the present research demonstrates that lifelong feeding of different dietary fats varying in fatty acid profile (MUFA, n6 PUFA or n3 PUFA-rich diets) had different effects on liver aging in relation to structural features, mitochondrial functionality and oxidative stress level. Animals fed virgin olive oil (MUFA rich) presented some

qualitative advantages over the other two dietary fat sources (sunflower oil, n6 PUFA rich; and fish oil, n3 PUFA rich) despite the fact that a similar lipid accumulation during aging was found for all three fats. MUFA fat induced no structural alterations associated with age, from the standpoint of either inflammation or fibrosis. The ultrastructure of the tissue was also normal and resulted in the lowest levels for oxidative stress in comparison with the other dietary fats. Conversely, the highest degree of fibrosis was found in the animals fed sunflower oil, whereas fish oil led to the highest proinflammatory phenotype. Both types of alterations were followed by changes in mitochondrial function and higher oxidative stress.

The study of the gene expression profile in the liver of the animals provided information to delve into the mechanisms associated with the findings discussed above. Regarding genes related to "mitochondrial dysfunction pathway," these encode for three mtETC subunits (Atp5d, Ndufa8 and Ndufa9), a mitochondrial fission complex promoter component (Fis1) and a member of the PRX family of antioxidant enzymes (Prdx5). It has been reported that *Atp5d* expression decreased [47], whereas *Fis1* expression increased [48], in relation to aging. Likewise, a reduction of PRX protein levels has been described in rat liver [49]. The results of the present study reveal greater expression for some of the genes involved in mitochondrial function in old animals compared with their younger counterparts, namely, Atp5d and Ndufa9 for the virgin olive oil group and Atp5d, Fis1, Ndufa8 and Ndufa9 for fish oil group. This suggests that these fats could, to some extent, prevent deleterious effects of aging through the
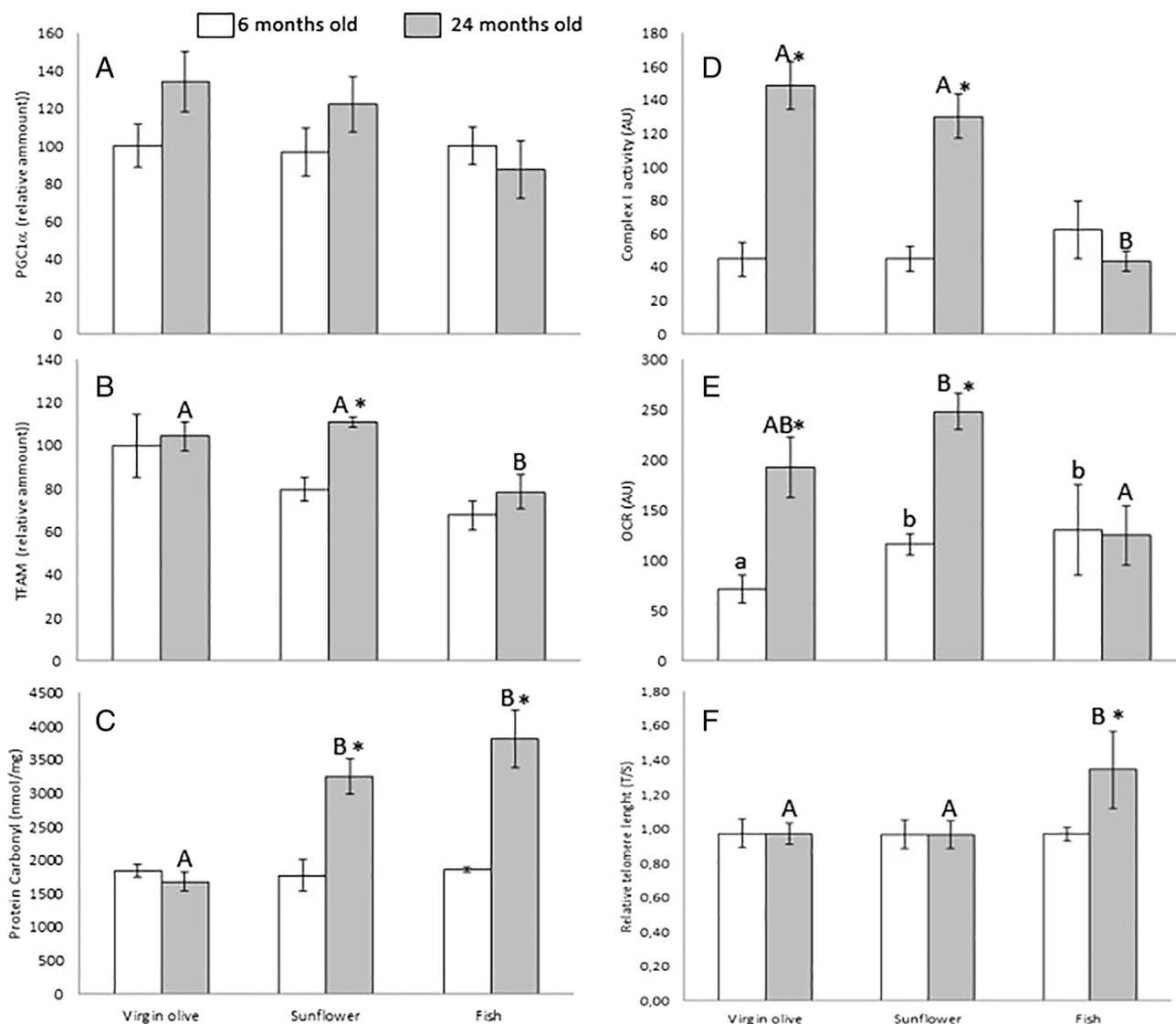
Fig. 3. PGC11α protein levels (A), TFAM protein levels (B), protein carbonyl concentration (C), mitochondrial complex I activity (D), mitochondrial oxygen consumption ratio (E) and relative telomere length (F) in the liver of rats fed on virgin olive oil, sunflower oil or fish oil for 6 or 24 months of age. Results represent mean±S.E.M. Bar chart statistical symbols: Lowercase letters represent statistically significant differences between dietary treatments ($P<.05$) at 6 months of age. Uppercase letters represent statistically significant differences between dietary treatments ($P<.05$) at 24 months of age. Asterisks (*) represent, for a dietary treatment, statistically significant differences ($P<.05$) between 6 and 24 months of age.

Table 2
RT-PCR validation results with the selected housekeeping (B2m)

Symbol	Pathway	Virgin olive oil (old vs. young)					Sunflower oil (old vs. young)					Fish (old vs. young)				
		Array		RT-PCR		Change with age	Array		RT-PCR		Change with age	Array		RT-PCR		Change with age
		FC	P	FC	P		FC	P	FC	P		FC	P	FC	P	
Atp5d	Mitochondrial dysfunction	0.42	.004	0.44	.004	↑					–	0.36	.000	0.53	.034	↑
Fis1	Mitochondrial dysfunction					–					–	0.40	.002	0.49	.002	↑
Ndufa8	Mitochondrial dysfunction					–					–	0.37	.002	0.52	.026	↑
Ndufa9	Mitochondrial dysfunction	0.39	.002	0.46	.007	↑					–	0.37	.001	0.65	.143	–
Keap1	Oxidative stress	0.40	.001	0.48	.046	↑					–	0.39	.002	0.36	.031	↑
Map2k6	Oxidative stress	0.14	.002	0.12	.020	↑					–					–
Pik3c2g	Oxidative stress	0.20	.000	0.30	.013	↑					–					–
Rras	Oxidative stress	0.38	.000	0.44	.004	↑					–					–
Prdx5	Oxidative stress					–					–	0.32	.000	0.44	.003	↑
Ccnd1	Cell cycle					–					–	101.34	.002	5.82	.032	↑
Ppp2r1b	Cell cycle					–	5.28	.000	1.03	.380	–	10.51	.000	2.73	.005	↑
Xrcc6	Telomere length	0.36	.002	0.37	.001	↑					–	0.34	.001	0.60	.43	–
Xrcc1	Telomere length	0.37	.003	0.53	.049	↑					–	0.36	.003	0.05	.045	↑

↑ = age-related overexpression. – = nongene expression change associated with age.

overexpression of these genes. One or more feedback mechanisms could explain variations in the amount or activity of mtETC complexes leading to the gene expression induction of mtETC components to try to compensate for changes in energy production and/or requirements in the mitochondria [50]. Overall, these feedback mechanisms might be directly or indirectly related to aging itself or to the interactions between the aging process and the age-related lipid accumulation in the liver. A striking result is the lack of response by sunflower-oil-fed animals to variations in the gene expression level with respect to mitochondrial function. It might be hypothesized that, under tolerable stress levels (oxidation, inflammation and maybe others), mitochondria could activate the necessary machinery to compensate for changes in bioenergetic requirements. However, under situations in which stress levels exceed the response capacity of mitochondria, as might be occurring for sunflower-oil-fed animals, another response or fate of the cell might be expected.

Six genes associated with the oxidative stress pathway were chosen to be verified. These included genes encoded for the negative regulator of *Nrf2* expression Keap1, two mitogen-activated protein kinases (Mapk6, Map2k9), a subunit of the phosphoinositide 3-kinase (PI3K) (Pik3c2g) and the transductor Rras, which activates PI3K. Age-associated changes in the expression of such genes were expected since mitochondrial dysfunction is closely related to reactive oxygen species production [1]. In this sense, boosted Map2k6 activity in the liver of aged mice has been previously reported [51]. Among the above-mentioned proteins, Keap1 would exert the most direct effect on genes involved in antioxidant defense [52]. Virgin olive and fish oils increased the expression of Keap1 in aged animals, suggesting an adaptation to oxidative stress associated with aging. Moreover, virgin olive oil led to an age-associated overexpression in Map2k6 and Pik3c2g. This might be the consequence of the greater protection against oxidation exerted by this dietary fat through aging, as demonstrated by protein carbonyl determination.

Two validated genes were related to cell cycle control pathway, namely, Ccnd1 and Ppp2r1b. Ccnd1 gene is a marker of proliferation, and aging is related to an increase in its expression [53]. Moreover, it has been shown that a high-fat diet decreased the gene expression of Ccdn1 [54]. Changing the n3:n6 fatty acid ratio in the diet through feeding n3 PUFA decreased Ccnd1 and reduced cell proliferation in a rat model of chemically induced mammary carcinoma [55]. In our experiment, Ccnd1expression was down-regulated in rats fed on fish oil. Ppp2r1b encodes a constant regulatory subunit of protein phosphatase 2, which is one of the four major Ser/Thr phosphatases, and it is involved in the negative control of cell growth and division. Mutations in Ppp2r1b lead to carcinogenesis and likely other human diseases [56]. Downstream regulation of its expression has been related to mitochondrial apoptosis and the development of liver injury [57,58]. Fish oils led to Ppp2r1b overexpression in the present study, probably leading to an induction of cell cycle. Lastly, related to the cell cycle, telomere length pathway genes included to be validated encoded for two proteins involved in DNA damage repair and subsequent prevention of telomere shortening (Xrcc1 and Xrcc6) [59]. Xrcc1 plays a key role in the DNA repair pathway. It encodes 633 amino acid proteins that act as a scaffold to stabilize the base excision repair (BER) proteins in both single-strand break repair and BER [60]. Both virgin olive oil and fish oil overexpressed this gene in the present study. Xrcc6 encodes a single-stranded DNA-dependent ATP-dependent helicase. Lower XRCC6 mRNA and protein expression has been found in HCC samples [59]. Both virgin olive oil and fish oil overexpressed this gene in the microarray study, but validation was positive only for animals fed virgin olive oil. This gene expression might indicate that both virgin olive oil and fish oil tend to protect the age-related telomere shortening, which was not detected in animals fed on sunflower oil. It bears noting that although changes concerning genes associated with telomere length were found for animals fed

virgin olive oil and fish oil, RTL proved higher during aging only for animals fed on fish oil. This might be related to other inductive signals for telomere extension in the fish oil group apart from those determined by the genes studied. Among these other signals, maybe the lower protein expression related to biogenesis machinery, the lower mtETC activity and the higher oxidative stress or proinflammatory state found in aged animals fed on fish oil might be included [61]. According to these data and given that fish-oil-fed animals registered a set of gene expression changes during aging, fish oil appeared to help the liver adapt to aging and to the lifelong feeding on this dietary fat. Notably, although a set of alterations was also found in sunflower-fed animals, including fibrosis, higher oxidative stress and lower mitochondrial density, no induction of telomere length was found, and no gene expression changes concerning their younger counterparts were reported. This lack of changes might indicate the ablation of the capacity of adapting to age exerted by this dietary fat during lifelong feeding.

In summary, unsaturated fats led to an age-related lipid accumulation in the liver of the rats. However, differences between the three dietary fat sources, namely, virgin olive, sunflower and fish oils, have been described concerning liver morphology and ultrastructure, as well as in relation to oxidative stress status and mitochondrial function. Only two of the fats studied, namely, virgin olive and fish oils, helped the liver to adapt to aging and diet through changes in the gene expression. Taking into consideration all the results, we conclude that virgin olive oil was the dietary fat source that best preserved the liver during the aging process since it led to the lowest number of tissue alterations and moreover allowed proper adaptation to aging in the liver through changes at the level of gene expression profile.

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