RESEARCH ARTICLE

Polyphenol-rich virgin olive oil reduces insulin resistance and liver inflammation and improves mitochondrial dysfunction in high-fat diet fed rats

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Scope: Virgin olive oil is an essential component of the Mediterranean diet. Its antioxidant and anti-inflammatory properties are mainly linked to phenolic contents. This study aims to evaluate the beneficial effects of a polyphenol-rich virgin olive oil (HPCOO) or olive oil without polyphenols (WPOO) in rats fed high-fat diet (HFD).

Methods and results: Male Sprague-Dawley rats were divided into four groups based on the different types of diet: (I) standard diet (STD); (II) HFD; (III) HFD containing WPOO, and (IV) HFD containing HPCOO. HPCOO and WPOO induced a significant improvement of HFD-induced impaired glucose homeostasis (by hyperglycemia, altered oral glucose tolerance, and HOMA-IR) and inflammatory status modulating pro- and anti-inflammatory cytokines (TNF-α, IL-1, and IL-10) and adipokines. Moreover, HPCOO and less extensively WPOO, limited HFD-induced liver oxidative and nitrosative stress and increased hepatic fatty acid oxidation. To study mitochondrial performance, oxidative capacity and energy efficiency were also evaluated in isolated liver mitochondria. HPCOO, but not WPOO, reduced H_2O_2 release and aconitase activity by decreasing degree of coupling, which plays a major role in the control of mitochondrial reactive oxygen species emission.

Conclusion: HPCOO limits HFD-induced insulin resistance, inflammation, and hepatic oxidative stress, preventing nonalcoholic fatty liver disease progression.

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1 Introduction

Nonalcoholic fatty liver disease (NAFLD) is characterized by insulin resistance (IR), hepatic steatosis and frequently type 2 diabetes and is a major public health problem in industrialized countries affecting up to 20–30% of individuals [1]. NAFLD represents a multi-hit process in which the accumulation of triglycerides increases the

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Abbreviations: **AMPK**, AMP-activated protein kinase; **HFD**, high-fat diet; **HPCOO**, polyphenol-rich virgin olive oil; **IR**, insulin resistance; **MDA**, malondialdehyde; **NAFLD**, nonalcoholic fatty liver disease; **NASH**, nonalcoholic steatohepatitis; **OGTT**, oral glucose tolerance test; **RNS**, reactive nitrogen species; **ROS**, reactive oxygen species; **STD**, standard diet; **TNF-α**, tumor necrosis factor α; **WPOO**, olive oil without polyphenols

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susceptibility to inflammatory damage, with subsequent onset of oxidative stress, mitochondrial dysfunction, endotoxemia, and endoplasmic reticulum stress [2–4]. The excessive fatty acid oxidation and mitochondrial dysfunction, with production of reactive oxygen species (ROS), represent important features for NAFLD progression toward nonalcoholic steatohepatitis (NASH) [5]. Inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), and IL-6, play a pivotal role in NAFLD onset. [6]. Also adipokines, leptin and adiponectin, control inflammation and fat metabolism are implicated in molecular mechanisms of NAFLD [7]. The involvement of several pathways highlights the strict connection between NAFLD and other metabolic disorders, such as obesity, IR, and dyslipidemia [8,9]. To date, although many strategies have shown their efficacy in clinical trials, the large-scale management of NAFLD is very complex, underlining the need to identify more effective approaches [10].

Diet is a modifiable target in both prevention and treatment of NAFLD and the Mediterranean diet has gained ever-increasing importance for its capability to improve hepatic steatosis and IR [11], limiting the progression of steatosis in steatohepatitis. Virgin olive oil represents a major component of this dietary regimen and its beneficial effects on human health are mainly related to the polyphenols content [12]. Phenolic compounds of olive oil with remarkable biological activities are oleocanthal, oleuropein, and its derivatives hydroxytyrosol and tyrosol [13]. *In vitro* study demonstrated the anti-inflammatory effect of oleuropein by inhibiting toll-like receptor and mitogen-activated protein kinases signaling in lipopolysaccharide stimulated RAW 264.7 and in a zebrafish model [14]. The antiproliferative and anti-inflammatory effects of oleocanthal were previously examined in human multiple myeloma cell line ARH-77 and in chondrocytes, in which a downregulation of ERK1/2 and protein kinase B (AKT) signal transduction pathways has been demonstrated [15, 16]. More recently, Lee et al. [17] showed that tyrosol protected against β-cell dysfunction and inhibited ER stress-induced apoptosis in mouse insulinoma cells NT-1 through JNK signaling. Moreover, our recent study demonstrated the beneficial effects of hydroxytyrosol in NAFLD, reducing IR, inflammation, and oxidative stress in rats [18]. Nevertheless, the benefits of polyphenol-rich virgin olive oil (HPCOO) in NAFLD have not been explored yet.

To this purpose, the aim of this study was to evaluate the effect of a virgin olive oil with high content of polyphenols (HPCOO) in a rat model of NAFLD induced by high-fat diet (HFD). We investigated the effect of HPCOO in reducing IR, inflammation and oxidative stress, and pathological features of steatosis. In particular, we evaluated HPCOO supplementation on glucose homeostasis and liver mitochondrial performance, oxidative capacity, and energy efficiency. To this aim, we also considered the effect of olive oil containing lower tocopherol amount without polyphenols (WPOO), in order to address the contribution of the high polyphenol content contained in olive oil in limiting early events in NAFLD.

2 Material and methods

2.1 Ethics statement

All procedures involving animals and their care were conducted in conformity with international and national law and policies (EU Directive 2010/63/EU for animal experiments, ARRIVE guidelines and the Basel declaration including the 3R concept). The procedures reported here were approved by the Institutional Committee on the Ethics of Animal Experiments (CSV) of the University of Naples "Federico II" and by the Ministero della Salute under protocol no. 2013/0040363. As suggested by the animal welfare protocol, all efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.2 Diets and animal model design

Control pelleted diet (standard diet (STD), Global diet 2018) was purchased from Harlan Laboratories (Udine, Italy) and it was composed of 17% fat, 23% proteins, 60% carbohydrates, and 16.1 kJ/g diet of energy density. HFD was prepared as previously described by Surwit et al. [19] from Laboratorio Dottori Piccioni (Gessate, Milan, Italy) and contained 58,1% of fat, 16% proteins, and 25,5% carbohydrates. To HFD was added 1.3% w/w of a natural virgin olive oil rich in polyphenols and tocopherols (HPCOO) or an olive oil containing tocopherols without polyphenols (WPOO). The percentage composition and energy density of enriched HFDs, as well as fatty acid percentage and tocopherol content in HPCOO and WPOO, are shown in Table 1. In addition, HPCOO contained different polyphenolic components, absent in WPOO, as shown in Table 2. The daily intake of polyphenols in rats was about 0.290 mg/kg, which is the amount included in the range of polyphenol daily intake in human. It is expressed as "human equivalent dose" [20].

Male Sprague-Dawley rats (113.5 ± 1.1 g; Harlan, Corezzano, Italy) were housed in cages in a room kept at 22 ± 1°C on a 12/12 h light/dark cycle. Animals were divided into four groups ($n = 6$ for each group) based on the different types of diet, as following: (I) STD; (II) HFD; (III) HFD enriched with WPOO (HFD + WPOO), and (IV) HFD enriched with HPCOO (HFD + HPCOO).

In our experimental conditions, early events of NAFLD and IR were induced by fat overnutrition, through the administration of this fat diet in young rats for 6 wks, excluding age and gender influences [21, 22]. After 5 wks of treatment oral glucose tolerance test (OGTT) was performed. At the end of experiment (6 wks), overnight fasted animals were anaesthetized by enflurane followed by cervical dislocation and serum and tissues were collected.

Table 1. Composition of HFDs enriched with olive oils containing (HFD + HPCOO) or not (HFD + WPOO) polyphenols

Diet composition	HFD + HPCOO	HFD + WPOO
Lipid	59.4	59.4
Carbohydrate (%)	24,2	24,2
Protein (%)	16	16
Energy density kJ/g diet	22.8	22.8
Fatty acid composition (%)	HPCOO	WPOO
C 16:0 Palmitic	13.7	v
C 16:1 (ω9) Palmitoleic	0.1	0.1
C 16:1 (ω7) Palmitoleic	1.1	0.8
C 17:0 Eptadecenoic	0.0	0.1
C 17:1 Eptadecenoic	0.1	0.1
C 18:0 Tearic	1.8	2.7
C 18:1 (ω9) Oleic	65.4	70.7
C 18:1 (ω7) Vaccenic	4.7	4.0
C 18:2 (ω6) Linoleic	10.5	8.9
C 20:0 Arachic	0.3	0.4
C 18:3 (ω3) Linolenic	0.8	0.5
C 20:1 Eicosanoic	0.3	0.3
C 22:0 Behenic	0.1	0.1
Squalen	0.9	0.3
C 24:0 Lignoceric	0.1	0.1
ratio linolenic (ω3)/linoleic (ω6)	0.08	0.06
Tocopherols (mg/100 g of oil)	HPCOO	WPOO
α-tocopherol	43.3	16.5
β+γ tocopherol	3.2	2.1
δ tocopherol	nq	nq
Total phenolic content (mg/kg of oil)	HPCOO	WPOO
	404.1[a)]	–

Fatty acid percentage and tocopherol content in HPCOO and in olive oil without polyphenol (WPOO) are also shown.
a) For the phenolic content and composition see Table 2.

2.3 Body weight gain and fat mass

Body weight was monitored two times per week and body weight gain was calculated as difference between the last measure and the body weight recorded at the beginning of the experiment. Before euthanasia, bioelectrical impedance analysis was performed to body composition assessment using a BIA 101analyzer, modified for the rat (Akern, Florence, Italy). Fat-free mass was calculated using the bioelectrical impedance analysis (50 kHz) prediction equation of Ilagan et al. [23], and fat mass content was determined as the difference between body weight and fat-free mass. Total liver lipids were extracted as previously described [24].

2.4 OGTT and serum parameters

At the fifth week of the experimental period, fasted rats received glucose (2 g/kg; per os) and glycemia was measured at time 0, 30, 60, 90, and 120 min. Glucose levels were measured by the glucometer One Touch UltraSmart (Lifescan, Milpitas, CA). Serum ALT and triglycerides were analyzed by standard automated procedures, according to manufacturer's protocols (AST Flex® reagent cartridge, ALT Flex® reagent cartridge, Dade Behring Inc., Newark, DE). Commercially available ELISA kits were used to determine insulin (Mercodia, Uppsala, Sweden), adiponectin and leptin (B-Bridge International, Mountain View, CA), TNF-α, IL-1β, and IL-10, (Thermo Scientific, Rockford, IL), following the manufacturer's instructions. Glucose levels were determined using glucose monitor (BRIO, Ascensia, NY) calibrated for rats. As index of IR, HOMA-IR (homeostasis model assessment) was obtained, using the formula (HOMA = fasting glucose (mmol/L) × fasting insulin (μU/mL)/22.5).

2.5 Western blot analysis

Liver (100 mg wet tissue) was homogenized on ice in lysis buffer (20 mM Tris–HCl, pH 7.5, 10 mM NaF, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulphonyl fluoride, 1 mM Na_3VO_4, leupeptin, and trypsin inhibitor 10 μg/mL). After 40 min, total protein lysates were obtained by centrifugation at 14 000 × *g* for 15 min at 4°C. Protein concentrations were estimated by the Bio-Rad protein assay using BSA as standard and equal amount of protein (50 μg) were subjected to SDS-PAGE and electrotransferred onto a nitrocellulose membrane (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) using a Bio-Rad Transblot (Bio-Rad, Milan,

Table 2. Phenolic and polyphenolic composition of high-polyphenol content olive oil (HPCOO)

Phenolic and polyphenolic components of HPCOO (mg/kg of oil)							
Phenolic acids		Secoiridoids		Lignans	Secoiridoids		
OHTy	Ty	OHTyEDA	TyEDA	Pr	OHTyEA	TyEA	Total phenols
Hydroxytyrosol	Tirosol	Dialdehydic form of oleuropein aglycone	Oleochantal	Pinoresinol	Aldehydic form of oleuropein aglycone	Aldehydic form of ligstroside aglycone	404.1
4.4	6.0	179.2	92.6	40.2	62.3	5.9	

Italy). Membranes were blocked at room temperature in milk buffer (1× PBS, 10% w/v nonfat dry milk, 0.1% v/v Tween-20) and then incubated at 4°C overnight with anti-nitrotyrosine (Nox-Tyr) and anti-GLUT2 (Merck Millipore, Billerica, MA, USA), anti-phospsho-Akt, for the residue Thr308, and anti-Akt (Cell Signaling, Danvers, MA, USA). Subsequently, the membranes were incubated for 90 min at room temperature with peroxidase-conjugated appropriate antibodies (Jackson ImmunoResearch Laboratories, Baltimore Pike, West Grove, PA). Western blot for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Sigma-Aldrich, Milan, Italy) was performed to ensure equal sample loading.

2.6 RNA extraction and Real-time semi-quantitative PCR

Total RNA was extracted from liver tissues using TRIzol Reagent (Bio-Rad Laboratories), according to the manufacturer's instructions. cDna was synthesized using a reverse transcription kit (NucleoSpin®, MACHEREY-NAGEL GmbH & Co, Düren, Germany) from 2 μg total RNA. PCRs were performed with a Bio-Rad CFX96 Connect Real-time PCR System instrument and software (Bio-Rad Laboratories). The PCR conditions were 15 min at 95°C followed by 40 cycles of two-step PCR denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. Each sample contained 1–100 ng cDNA in 2× QuantiTect SYBR-Green PCR Master Mix and primers, *Tnf, Cox2* and Peroxisome proliferator-activated receptor gamma (*Ppara*) (Qiagen, Hilden, Germany) in a final volume of 50 μl. The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and data were analyzed according to the 2-ΔΔCT method.

2.7 Measurements of mitochondrial oxidative capacities and degree of coupling

Mitochondria isolation and oxygen consumption (polarographically measured using a Clark-type electrode) were carried out as previously reported [25]. Briefly, the livers were finely minced and washed in a medium containing 220 Mm mannitol, 70 mM sucrose, 20 mM N′-(2-hydroxyethyl) piperazine-N-2-ethanesulfonic acid (HEPES) (pH 7.4), 1 mM-EDTA, and 0.1 % w/v fatty acid free BSA. Tissue fragments were homogenised with the above medium (1:4, w/v) in a Potter Elvehjem homogeniser (Heidolph, Kelheim, Germany) set at 500 rpm (4 strokes/min). The homogenate was centrifuged at 1000 gav for 10 min and the resulting supernatant fraction was again centrifuged at 3000 × *g* for 10 min. The mitochondrial pellet was washed twice and finally resuspended in a medium containing 80 mM KCl, 50 mM HEPES (pH 7.0), 5 mM KH_2PO_4, and 0.1% w/v fatty acid free BSA. The protein content of the mitochondrial suspension was determined by the method of Hartree [26] using BSA as the protein standard. Isolated mitochondria were then used for the determination of respiratory parameters.

Oxygen consumption was measured in the presence of substrates and ADP (state 3), in the presence of substrates alone (state 4) and their ratio (respiratory control ratio, RCR) were calculated. The substrates used for liver respiration were 10 mM succinate + 3.75 μM rotenone or 40 μM palmitoylcarnitine + 2.5 mM malate for the determination of fatty acid oxidation rate.

The degree of coupling was determined in the liver by applying equation by Cairns et al. [27]: degree of coupling = $\sqrt{1-(\mathrm{Jo})_{\mathrm{sh}}/(\mathrm{Jo})_{\mathrm{unc}}}$ where $(\mathrm{Jo})_{\mathrm{sh}}$ represents the oxygen consumption rate in the presence of oligomycin that inhibits ATP synthase, and $(\mathrm{Jo})_{\mathrm{unc}}$ is the uncoupled rate of oxygen consumption induced by carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP), which dissipates the transmitochondrial proton gradient. $(\mathrm{Jo})_{\mathrm{sh}}$ and $(\mathrm{Jo})_{\mathrm{unc}}$ were measured as above using succinate (10 mmol/L) rotenone (3.75 μmol/L) in the presence of oligomycin (2 μg/mL) or FCCP (1 μmol/L), respectively.

The rate of mitochondrial H_2O_2 release was assayed by following the linear increase in fluorescence (ex 312 nm and em 420 nm) due to the oxidation of homovanillic acid in the presence of horseradish peroxidase [28]. Aconitase activity was measured spectrophotometrically (at 412 nm). Determination of aconitase specific activity was carried out in a medium containing 30 mM sodium citrate, 0.6 mM $MnCl_2$, 0.2 mM NADP, 50 mM TRIS-HCl pH 7.4, and two units of isocitrate dehydrogenase. The formation of NADPH was followed spectrophotometrically (340 nm) at 25°C. The level of aconitase activity measured equals active aconitase (basal level). Aconitase inhibited by ROS in vivo was reactivated so that total activity could be measured by incubating mitochondrial extracts in a medium containing 50 mM dithiothreitol, 0.2 mM Na_2S, and 0.2 mM ferrous ammonium sulphate [29].

2.8 ROS assay

The levels of ROS were determined as previously reported [30]. An appropriate volume of freshly prepared tissue homogenate was diluted in 100 mM potassium phosphate buffer (pH 7.4) and incubated with a final concentration of 5 μM dichlorofluorescein diacetate (Sigma–Aldrich) in dimethyl sulfoxide for 15 min at 37°C. The dye-loaded samples were centrifuged at 12 500 × *g* for 10 min at 4°C. The pellet was mixed at ice-cold temperatures in 5 mL of 100 mM potassium phosphate buffer (pH 7.4) and again incubated for 60 min at 37°C. The fluorescence measurements were performed with a HTS-7000 Plus plate reader spectrofluorometer (Perkin Elmer, Wellesley, MA, USA) at 488 nm for excitation and 525 nm for emission wavelengths. ROS were quantified from the dichlorofluorescein standard curve in dimethyl sulfoxide (0–1 mM).

2.9 MDA measurement

Malondialdehyde (MDA) levels in the liver were determined as an indicator of lipid peroxidation [31]. Tissues were homogenized in 1.15% KCl solution. An aliquot (200 μL) of the homogenate was added to a reaction mixture containing 200 μL of 8.1% SDS, 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% thiobarbituric acid, and 600 μL of distilled water. Samples were then boiled for 1 h at 95°C and centrifuged at 3000 × *g* for 10 min. The supernatant absorbance was measured by spectrophotometry at 550 nm and the concentration of MDA was expressed as micromolar MDA per milligram protein of cell homogenate. A standard curve was prepared using MDA bisdimethylacetal as the source of MDA.

2.10 Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by analysis of variance test for multiple comparisons followed by Bonferroni's test to evaluate significance in differences among all groups, using Graph-Pad Prism (Graph-Pad software, San Diego, CA, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1 Polyphenol-rich olive oil improves glucose and lipid homeostasis

Serum ALT and triglycerides were increased by HFD feeding. The addition of HPCOO to diet reduced significantly these biochemical parameters, typical of NAFLD (Table 3). Weight gain and fat mass of animals did not change among groups, while the total lipid content resulted significantly higher in HFD rats, but it was significantly reduced by HPCOO (Table 3). No variation in food intake was shown between the HFD and WPOO and HPCOO fed animals (108.7 ± 6.9 versus 105.9 ± 6.0 and 110.3 ± 6.6 g/week, respectively).

After 5 wks of HFD feeding the OGTT was performed. HPCOO and WPOO showed a trend of reduction on glycemia in HFD animals at all time examined, raising the significance analyzing the area under the curve (Fig. 1A); HPCOO effect was more evident than that of WPOO. After 6 wks, fasting serum glucose levels (Fig. 1B) were significantly higher in HFD rats than STD, WPOO and HPCOO and insulinemia showed a trend of reduction only for HPCOO (Fig. 1C). Accordingly, IR assessed by HOMA-IR index resulted significantly improved by HPCOO, more than WPOO (Fig. 1D).

To confirm the effect of HPCOO and WPOO on IR, we evaluated the phosphorylation of AKT and GLUT2 expression in liver (Fig. 2A and B). HPCOO, and less extensively WPOO, increased the levels of these parameters improving glucose homeostasis. We also examined AMP-activated protein kinase (AMPK) phosporilation and the transcription of its downstream gene, peroxisome proliferator-activated receptor-α (PPAR-α) (Fig. 2C and D), which confirmed the efficacy of HPCOO, and partially of WPOO, on fat metabolism in liver.

3.2 Effect of polyphenols on serum levels of inflammatory cytokines and adipokines

The unbalance between pro-inflammatory (TNF-α and IL-1) and anti-inflammatory mediators (IL-10) plays a key role in the liver damage and the pathogenesis of hepatic IR. HPCOO significantly improved the serum levels of these parameters altered by HFD (Fig. 3A–C). Consistently, HPCOO also positively modulated the serum levels of leptin and adiponectin, two of the most important pro- and anti-inflammatory adipokines, respectively (Fig. 3D and E). No significant difference on these parameters was revealed after addition of WPOO in diet (Fig. 3A–E).

3.3 WPOO and HPCOO reduce liver inflammation and oxidative stress

Liver inflammation induced by HFD was also evaluated (Fig. 4). HPCOO and WPOO were able to significantly reduce TNF-α and cyclooxygenase-2 (COX-2) mRNA levels (Fig. 4A and B). Moreover, HFD caused a significant increase of oxidative damage from ROS and reactive nitrogen species (RNS), which mainly contribute to the hepatic damage. WPOO and to greater extent HPCOO reduced nitrosilation of proteins and decreased ROS production (Fig. 4C and D). In addition, we evaluated the levels of MDA, as a marker of lipid peroxida-

Table 3. Body, serum, and liver parameters

	STD ($n = 6$)	HFD ($n = 6$)	HFD + WPOO ($n = 6$)	HFD + HPCOO ($n = 6$)
Body weight gain (g)	246.8 ± 27.22[a]	253.9 ± 13.13[a]	266.1 ± 8.60[a]	254.6 ± 8.11[a]
Fat mass (g)	76.7 ± 8.04[a]	83.1 ± 4.34[a]	77.2 ± 4.05[a]	64.0 ± 4.78[a]
Serum ALT (U/L)	32.1 ± 3.40[a]	40.4 ± 3.69[b]	39.4 ± 3.50[b]	33.8 ±2.24[a]
Serum tryglicerides (mg/dL)	50.8 ± 1.70[a]	71.4 ± 2.09[b]	69.6 ± 1.63[b]	62.2 ±0.97[c]
Total liver lipids (mg/g tissue)	35.6 ± 0.78[a]	57.9 ± 0.61[b]	57.6 ± 3.42[b]	49.9 ± 0.15[c]

Data reported in table are mean ± SEM of six rats per group. All results were considered statistically significant at $p < 0.05$. Differences among groups are indicated by letters (a, b, c, d).

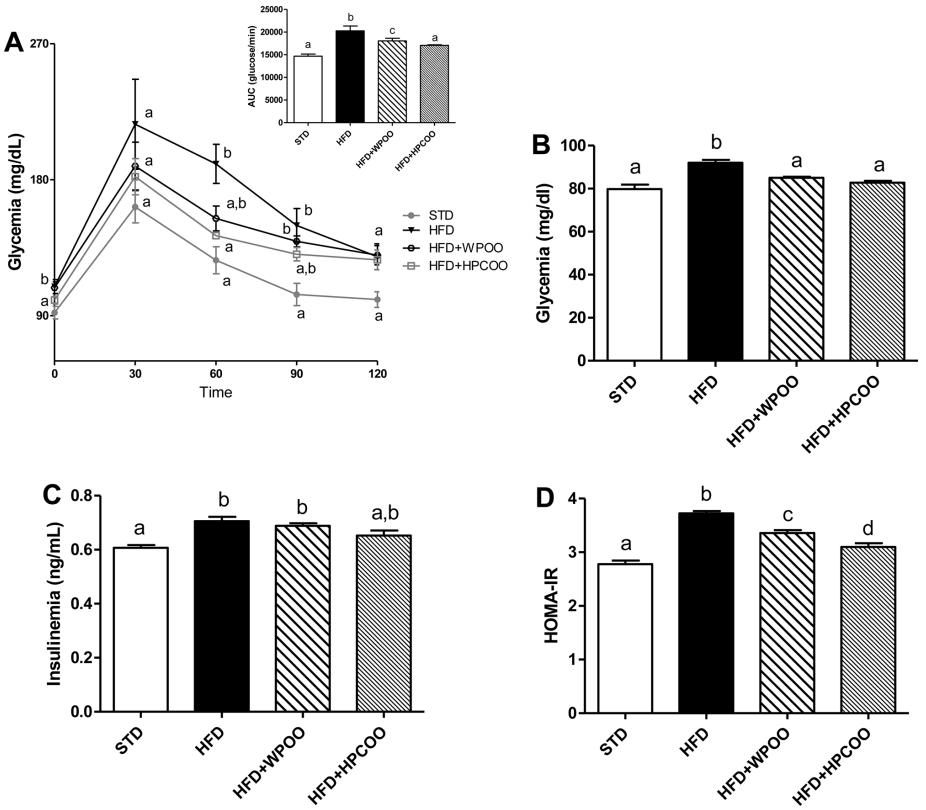

Figure 1. Effect of WPOO or HPCOO on glucose homeostasis and insulin resistance. OGTT in STD- and HFD-fed rats (n=6, each group) was performed and AUC evaluated (A). Fasting glucose (B), insulin levels (C), and HOMA-IR (D) were also reported (n=6, each group). All results were considered statistically significant at $p < 0.05$. Differences among groups are indicated by letters (a, b, c, d).

tion, and an increase of these levels in HFD-fed group than in STD group was revealed. WPOO and HPCOO significantly also decreased MDA levels (Fig. 4E). As demonstrated by a previous study [32], the presence of tocopherols in WPOO justifies its anti-oxidant effect, even if this capability is lower than that of HPCOO.

3.4 HPCOO increases fatty acid oxidation in hepatic mitochondria

Because mitochondria are the main site of the substrate oxidative process, we analyzed respiratory rates in isolated liver mitochondria. Oxidative mitochondrial respiratory activity was determined by using $FADH_2$-linked substrates (succinate plus rotenone) and palmitoyl-carnitine a substrate to assess the capacity of lipid substrate oxidation.

Mitochondria from HFD animals exhibited a significant lower state 3 respiration rate than STD and HFD + HPCOO in presence of succinate, as substrate (Fig. 5A). State 3, in presence of palmitoylcarnitine, was significantly higher in mitochondria of HFD, HFD + WPOO, and HFD + HPCOO than those measured in STD rats (Fig. 5B). Moreover HFD + HPCOO value significantly differed from the other ones. A not significant increasing trend in H_2O_2 levels in mitochondria was shown in HFD and HFD + WPOO fed groups compared to STD, HPCOO administered in the diet significantly reduced H_2O_2 levels compared to HFD group (Fig. 5C). Interestingly, even if the increase in palmitoylcarnitine amount indicates a more significant fatty acid oxidation in HFD + HPCOO, it is not related to an increase of H_2O_2 yield as result of oxidative stress. In fact, H_2O_2 was significantly reduced by the addition of HPCOO to diet. Similarly, a lower aconitase activity was found in HFD and HFD + WPOO compared to STD group but was significantly higher in the HFD + HPCOO than in the HFD (Fig. 5D). To test mitochondrial efficiency, we measured oxygen consumption in presence of oligomycin and FCCP. Oligomycin state 4 respiration showed a trend of reduction in HFD and WPOO animals than STD whereas was significantly increased in HFD + HPCOO (Fig. 5E), while no variation was found in maximal FCCP-stimulated respiration (Fig. 5F). As a consequence, hepatic mitochondrial energetic efficiency, assessed as degree of coupling, was significantly higher in HFD and WPOO animals than STD and significantly decreased in HFD + HPCOO (Fig. 5G).

4 Discussion

Evidences support the beneficial effects of Mediterranean diet on human health, specifically on NAFLD [33–36]. Among the components of this dietary regimen (cereals, vegetables, and legumes), olive oil and its phenolic content, which present important biological activities, are currently under investigation [37–39]. In our study, we comparatively evaluated the

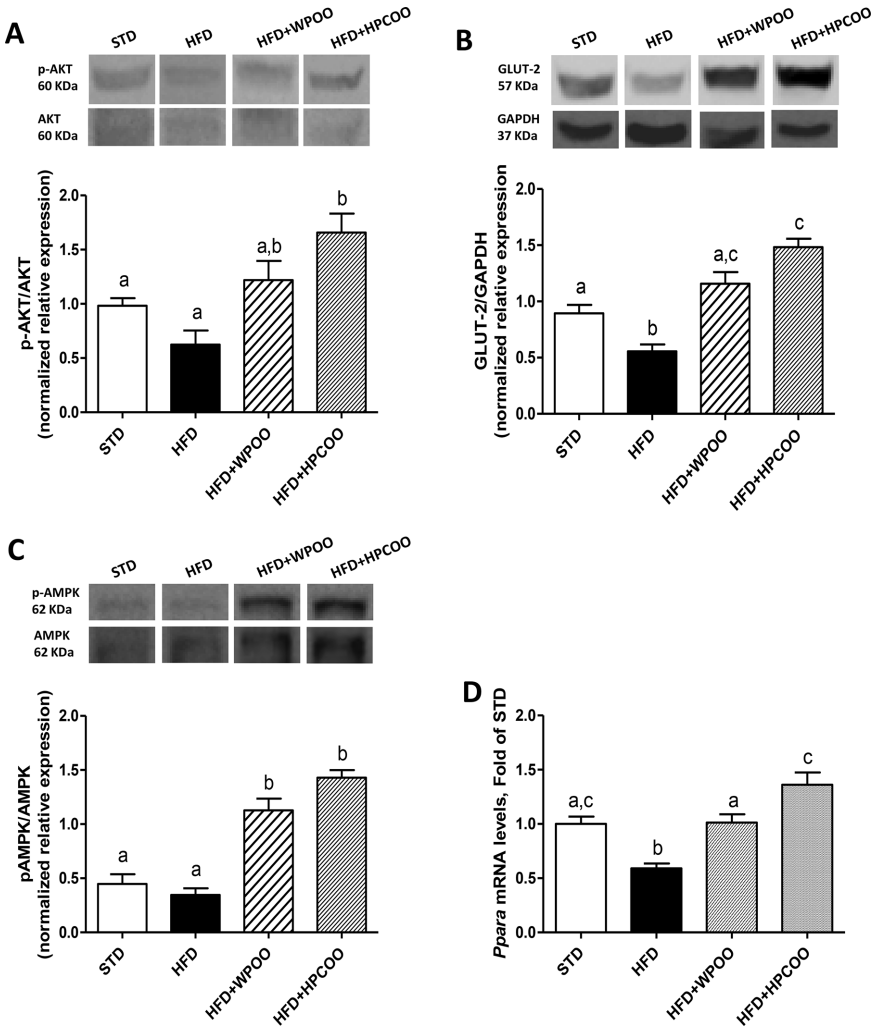

Figure 2. Effect of WPOO or HPCOO on metabolic impairment in liver from HFD fed rats. Levels of pAKT (A), GLUT2 (B) and pAMPK expression (C) in the liver lysates were measured by western blot analysis ($n = 6$ each group). A representative blot of pAKT and AKT, *p*-AMPK, and AMPK and the ratios from densitometric analysis of bands from all samples are also shown. mRNA transcriptional levels of *Ppara* were also shown (D). Data are mean ± SEM of six rats per group. All results were considered statistically significant at $p < 0.05$. Differences among groups are indicated by letters (a, b, c).

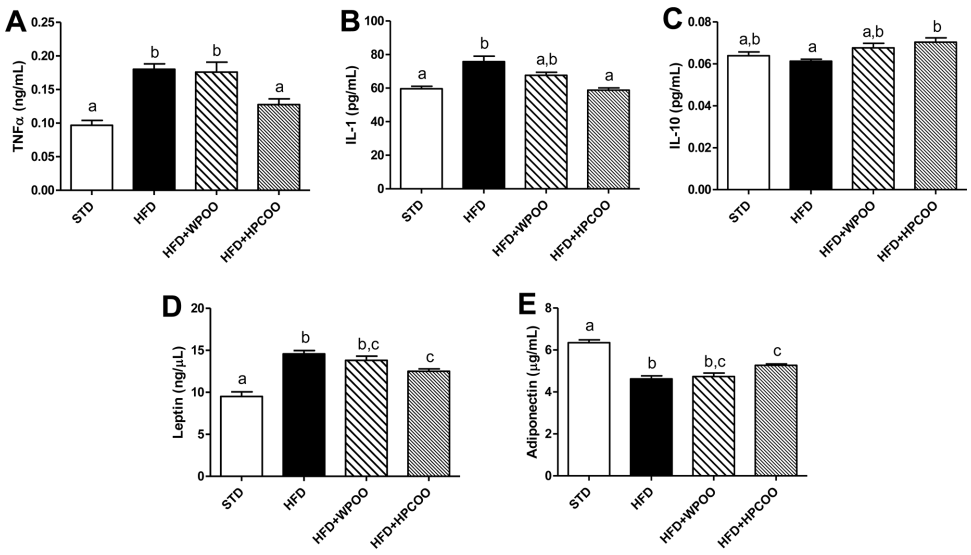

Figure 3. Effect of HPCOO on cytokine and adipokine serum levels. TNF-α (A) IL-1 (B), and IL-10 (C) are reported. Panels D and E show serum levels of leptin and adiponectin in all groups of rats. Data are mean ± SEM of six rats per group. All results were considered statistically significant at $p < 0.05$. Differences among groups are indicated by letters (a, b, c).

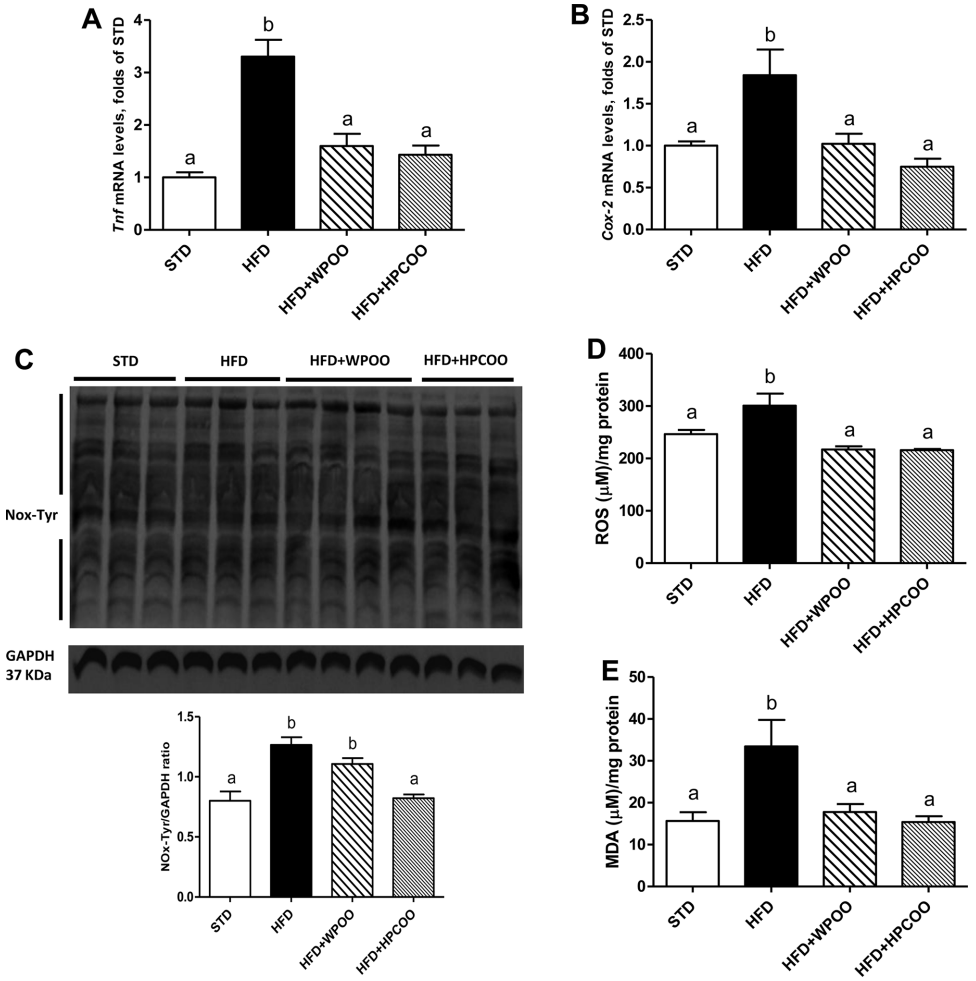

Figure 4. Effect of WPOO or HPCOO on *Tnf* and *Cox-2* mRNA levels and nitrosative and oxidative stress. *Tnf* (A) and *Cox-2* (B) mRNA was shown. Protein nitrosylation (C) in liver from STD and HFD rats fed or not with WPOO or HPCOO is shown. ROS production (D) and MDA content (E) are also reported. Values are reported as means ± SEM of at least six animals. All results were considered statistically significant at $p < 0.05$. Differences among groups are indicated by letters (a, b).

beneficial effects of HPCOO and WPOO in limiting the progression of early events of NAFLD. As we previously described [18], 6-wks HFD feeding is able to mimic pathogenetic mechanisms of hepatic fatty acid accumulation and glucose metabolism alteration. HPCOO and WPOO showed beneficial effects in restoring insulin sensitivity, reducing glycaemia, and HOMA-IR index. This effect on glucose homeostasis was more evident in HPCOO group than WPOO, underlining polyphenol contribution in limiting IR. Moreover, only HPCOO reduced also serum triglycerides and ALT, and all total liver lipids, parameters that characterize the HFD-induced NAFLD pattern.

The PKC/IRS/Akt signaling pathway plays a pivotal role in glucose response, whose impairment induces to accumulation of lipotoxic intermediates and development of IR [40]. In the liver, the activation of Akt leads to glucose uptake and gluconeogenesis inhibition, limiting NAFLD progression. In our experiment, we showed that WPOO and even more HPCOO are able to activate Akt and increase GLUT2 expression in liver, promoting glucose uptake and restoring insulin sensitivity, compromised in HFD rats. Consistently, it has been demonstrated that postnatal administration of resveratrol, a polyphenolic compound, modulated the metabolic profile of HFD-fed offspring, acting on PKC/IRS/Akt signaling [41].

The characteristic visceral adiposity of NAFLD is strictly correlated with a dysregulation of a variety of adipokines [42]. The unbalanced secretion of pro-steatosic and pro-inflammatory (leptin and TNF-α) and anti-steatosic and anti-inflammatory adipokines (adiponectin) is an important feature of the progression of NAFLD toward NASH [7]. In HFD-fed mice, a polyphenol-rich grape extract supplementation was able to reduce plasma leptin levels and to increase adiponectin ones [43]. Moreover, in a randomized controlled trial, Chen et al. [44] demonstrated that resveratrol reduced serum TNF-α and improved adiponectin levels in patients with NAFLD. In agreement with these data, we demonstrated that HPCOO, conversely to WPOO, was able to improve adipokine balance altered by HFD feeding.

The adipose tissue following overnutrition secretes inflammatory cytokines, such as TNF-α, IL-1β, and IL-6. These cytokines downregulate insulin sensitivity of hormone sensible organs, such as liver, whose metabolic activity is strongly influenced by insulin signaling. In fact, defects in the

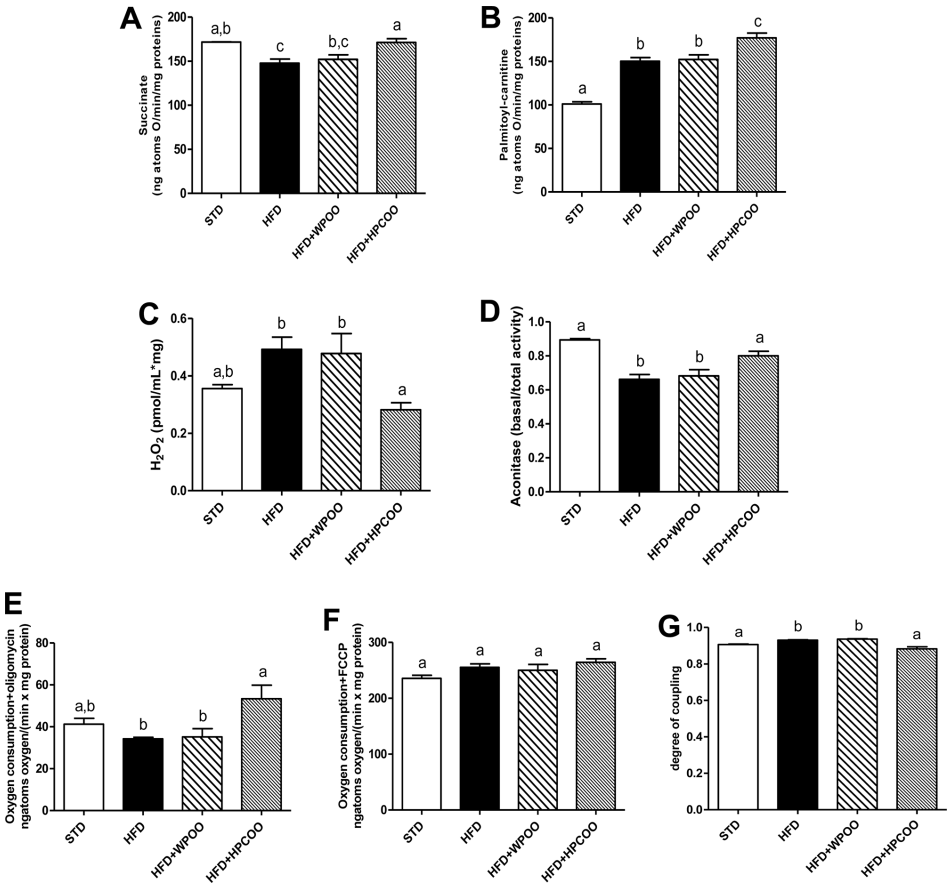

Figure 5. Effect of HPCOO on hepatic mitochondrial functions and energy efficiency. Mitochondrial respiration in the presence of succinate (A) or palmitoyl-carnitine (B) as substrates was determined in liver. H_2O_2 yield (C), aconitase activity (D), oxygen consumption in the presence of oligomycin (E), or uncoupled by FCCP (F), and degree of coupling values calculated from oxygen consumption in the presence of oligomycin and uncoupled by FCCP (G) are also shown. Values are reported as means ± SEM of at least six animals. All results were considered statistically significant at $p < 0.05$. Differences among groups are indicated by letters (a, b, c).

insulin signaling pathways contribute to accumulation of free fatty acids in organs and lead to the phosphorylation of several substrates [8]. In an *in vivo* study, resveratrol was able to decrease lipogenesis and inflammation induced by HFD, reducing TNF-α, IL-6, and NF-κB mRNA expression in the liver [45]. Moreover, the supplementation of this polyphenolic compound in healthy dietary regimen reduced serum inflammatory markers in patients with NAFLD [46]. In our study, the high content of polyphenols in virgin olive oil was able to significantly reduce the inflammation induced by HFD. Indeed, this beneficial effect was characterized by a decrease of TNF-α, IL-1, and an increase of IL-10 serum levels, while WPOO did not reach significant anti-inflammatory effect. As it happens for adipokines, the effects of HPCOO were higher than that induced by WPOO, underlining again the efficacy of polyphenols olive oil in limiting NAFLD. In addition, the two formulation of olive oil were able to reduce TNF-α and COX-2 transcription, confirming their anti-inflammatory activity in liver tissue.

The role of adiponectin, inhibited by TNF-α, IL-6 and resistin, in limiting IR is based on a stimulation of mitochondrial β-oxidation, by activating AMPK, and an inhibition of lipogenesis by downregulating SREBP-1c [47]. Experimental study demonstrated the reduction of phosphorylated AMPK protein expression induced by HFD [48], suggesting a strong involvement of AMPK signaling impairment in pathological onset of NAFLD. In LDL receptor deficient mice fed a HFD, it was demonstrated that polyphenols of rooibos extracts were able to prevent steatosis via AMPK activation [49]. Consistently with these findings, we showed that HPCOO positively acts on fatty acid metabolism and IR, inducing the activation of AMPK and the transcription of PPARα, its downstream gene. Interestingly, WPOO also plays a similar profile of activity, probably due to the presence of tocopherols. In fact, other studies demonstrated the beneficial effects of vitamin E on lipid metabolism and insulin sensitivity in murine models of steatosis and NASH [50, 51]. Here, WPOO and more significantly HPCOO are able to improve IR in HFD-fed animals via AMPK activation and restoration of Akt signaling in liver and increasing GLUT2 expression.

Another feature of pathogenetic mechanism of NAFLD is the increase of oxidative stress, caused by hepatic fatty acid accumulation and IR [5]. Oxidative damage is characterized by a redox unbalance with excessive release of ROS and RNS. The production of ROS triggers TNF-α secretion, which increases the damage of mitochondrial membrane, with the consequent release of cytochrome c, and following hepatocyte death [52]. The production of ROS occurred mainly in mitochondria leading to its dysfunction, key feature of NAFLD pathogenesis [53].

Here, oxidative capacity and energy efficiency were evaluated in hepatic mitochondria obtained from different rat groups, since both parameters could affect mitochondrial performance. We demonstrated that HPCOO improves fatty acid oxidation rate and reduces ROS (i.e. reduction of H_2O_2 release and increase of basal aconitase/total aconitase ratio) by decreasing degree of coupling that plays a major role in the control of mitochondrial ROS emission.

The presented results confirm the association between HFD-induced ectopic fat storage in the liver and mitochondrial alterations [54]. Accordingly, HFD hepatic mitochondria exhibited reduced respiratory capacity, as indicated by the decrease in succinate state 3 oxygen consumption, and increased oxidative stress, when the ability to utilize fat as a metabolic fuel was elevated, such as in HFD fed animals. Moreover, a further mechanism contributing to fat accumulation could be the enhanced mitochondrial efficiency, as shown by the increase in the degree of coupling in HFD rats. Increased mitochondrial energetic efficiency implies that less substrates need to be burned to obtain the same amount of ATP, while higher plasma triglycerides found in HFD rats fed dictate higher lipid substrate flux to the liver. As a consequence, it is possible that a condition of imbalance takes place in the hepatic cell, with substrate influx exceeding substrate burning and favoring ectopic lipid deposition.

The increase of fatty acids oxidation rate might play a crucial role in enhancing ROS production [55], as suggested by the increased H_2O_2 production observed in HFD rats. Moreover, higher degree of coupling should imply increased ROS production, in fact, one of the postulated roles of uncoupling is known to be the maintenance of mitochondrial membrane potential below the critical threshold for ROS production [56].

The improvement of respiratory capacity, fatty acid oxidation, oxidative stress, and the decreased mitochondrial efficiency, showed by HPCOO rats may be interpreted as the result of converging protective mechanisms against NAFLD, preventing its progression.

In our previous study, we demonstrated the anti-oxidant effect of hydroxytyrosol, a main metabolite of oleuropein, in liver steatosis induced in rat by HFD feeding [18]. Accordingly, in this study the polyphenol-rich olive oil was able to limit the oxidative and nitrosative stress, reducing the production of ROS and RNS and consequent lipid peroxidation. This effect was shown in both HPCOO and WPOO, indicating that the anti-oxidant activity can be linked not only to polyphenol content, but also to other substances, such as tocopherols, rather than omega-3 and omega-6, whose concentrations are similar in both oils examined. In fact, the beneficial effect of tocopherols has been already reported in experimental and clinical studies [32, 57].

In conclusion, we showed the protective effects of HPCOO against NAFLD induced by fat overnutrition. In particular, HPCOO, more than WPOO, is able to significantly reduce liver inflammation and mitochondrial oxidative stress and to restore insulin sensitivity. These data support the importance of polyphenolic content in olive oil, one of major components of Mediterranean diet, suggesting its pivotal role to limit liver injury and associated disorders in the onset of NAFLD and its progression in NASH.

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R.M. designed research, analyzed data, and wrote the paper. A.C. and R.B.C. designed research and reviewed manuscript. A.L and C.P. conducted research, analyzed data, and wrote the manuscript. G.T., G.C., and F.D.G. performed experiments. M.P.M. and G.M.R. analyzed data and reviewed the manuscript. All authors have read and approved the final manuscript.

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