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# Hydroxytyrosol prevents metabolic impairment reducing hepatic inflammation and restoring duodenal integrity in a rat model of NAFLD<sup>☆,☆☆,★</sup>

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## Abstract

The potential mechanisms of action of polyphenols in nonalcoholic fatty liver disease (NAFLD) are overlooked. Here, we evaluate the beneficial therapeutic effects of hydroxytyrosol (HT), the major metabolite of the oleuropein, in a nutritional model of insulin resistance (IR) and NAFLD by high-fat diet. Young male rats were divided into three groups receiving (1) standard diet (STD; 10.5% fat), (2) high-fat diet (HFD; 58.0% fat) and (3) HFD + HT (10 mg/kg/day by gavage). After 5 weeks, the oral glucose tolerance test was performed, and at 6th week, blood sample and tissues (liver and duodenum) were collected for following determinations. The HT-treated rats showed a marked reduction in serum AST, ALT and cholesterol and improved glucose tolerance and insulin sensitivity, reducing homeostasis model assessment index. HT significantly corrected the metabolic impairment induced by HFD, increasing hepatic peroxisome proliferator-activated receptor PPAR- $\alpha$  and its downstream-regulated gene fibroblast growth factor 21, the phosphorylation of acetyl-CoA carboxylase and the mRNA carnitine palmitoyltransferase 1a. HT also reduced liver inflammation and nitrosative/oxidative stress decreasing the nitrosylation of proteins, reactive oxygen species production and lipid peroxidation. Moreover, HT restored intestinal barrier integrity and functions (fluorescein isothiocyanate-dextran permeability and mRNA zona occludens ZO-1). Our data demonstrate the beneficial effect of HT in the prevention of early inflammatory events responsible for the onset of IR and steatosis, reducing hepatic inflammation and nitrosative/oxidative stress and restoring glucose homeostasis and intestinal barrier integrity.

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**Keywords:** Nonalcoholic fatty liver disease; Olive oil polyphenols; Glucose tolerance; Liver inflammation; Insulin resistance; Duodenal permeability

## 1. Introduction

The olive fruit and virgin olive oil are essential components of the Mediterranean diet, a nutritional regimen gaining ever-increasing recognition for its beneficial effects on human health [1–2]. The high

content of phenolic compounds in virgin olive oil has demonstrated antiinflammatory, antioxidant and antineoplastic activities [3–4]. Among these, hydroxytyrosol (HT), which is abundant in the aqueous fraction of olive pulp, is a simple phenolic compound with marked antioxidant activity [5–6]. A dose-dependent accumulation of HT and its metabolites has been demonstrated in target metabolic and vascular tissues [7], suggesting potential applications of HT to prevent hepatic and cardiovascular diseases.

The antioxidant activity of oleuropein, the precursor of HT, is able to reduce free-fatty acids (FFA)-induced hepatocellular steatosis in HepG2 cells reducing the number and size of lipid droplets and intracellular triglyceride accumulation [8]. Other *in vitro* studies have confirmed the hypolipidemic and antiinflammatory effect of HT [9,10], modulating the activity of key enzymes involved in fatty acid metabolism [i.e. acetyl-CoA carboxylase (ACC) and fatty acid synthase], triglyceride synthesis and cholesterologenesis in hepatocytes. This effect was higher than that of oleuropein or tyrosol [9]. HT showed also antiinflammatory mechanism, inhibiting inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2 expression, tumor

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necrosis factor (TNF)- $\alpha$  transcription and nitric oxide release in lipopolysaccharide-stimulated human monocytic THP-1 cell line [10].

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease. This condition is not only confined to liver, but there is now growing evidence that it is a multisystem disease [11]. The most important features of NAFLD are the aberrant lipid accumulation in hepatocytes and the inflammatory progression to steatohepatitis. Several mediators, as TNF- $\alpha$ , interleukin (IL)-6 and COX-2, play a key role in the onset of insulin resistance (IR) and liver inflammatory process, through inhibitory mechanisms on insulin signaling, hepatocytes apoptosis and increased production of reactive oxygen species (ROS) [12–13].

Some members of the nuclear receptor family are key regulators not only of hepatic lipogenesis but also of hepatic and systemic inflammation [14]. In particular, peroxisome proliferator-activated receptor (PPAR)- $\alpha$ , mainly expressed in the liver, is involved in the clearance of circulating and cellular lipids by controlling the expression of lipogenic genes. In virtue of that, it is critically involved in the onset and progression of steatosis and it currently represents a therapeutic target in this disease [15].

To study the efficacy and potential mechanisms of action of HT in NAFLD, we used an experimental model of steatosis, feeding young rats with a high-fat diet (HFD) [16]. Here, we demonstrate that HT is able to prevent the early hepatic damage underlying steatosis and IR onset, reducing inflammation and nitrosative/oxidative stress and modulating lipid metabolism. Finally, we evaluated the beneficial effect of HT on duodenal permeability impaired by fat over nutrition, limiting liver exposure to gut-derived detrimental factors.

## 2. Materials 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. 20138-0040363. Before killing and prior to serum and sample collection, animals, kept overnight fasted, were anesthetized by enflurane and euthanized by an intraperitoneal injection of a cocktail of ketamine/xylazine. 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

Standard (STD) and HFD were purchased from Laboratorio Dottori Piccioni (Gessate, Milan, Italy). STD had 15% fat, 22% proteins and 63% carbohydrates, while HFD had 58% of energy derived from fat, 18% from protein and 24% from carbohydrates. STD and HFD contained 4.06 kcal/g and 5.56 kcal/g, respectively. In our experiment, we administered HFD in young rats for 6 weeks to induce the early events of NAFLD due to fat overnutrition, excluding age and gender influences [16]. The polyphenol derivative HT was synthesized in the laboratory of Pharmaceutical Chemistry of the Department of Pharmacy of the University of Naples Federico II.

Male Sprague–Dawley rats (113.5  $\pm$  1.1 g; Harlan, Corazzano, Italy) were housed in cages in a room kept at 22  $\pm$  1°C on a 12/12 h light/dark cycle. After weaning, rats were divided into three groups (at least 6 rats for each group) as following: (1) STD, control group receiving STD and water as drug vehicle by gavage; (2) HFD-fed animals receiving vehicle; and (3) HFD-fed rats treated by gavage with HT (HFD + HT, 10 mg/kg/day). After 5 weeks, all rats were subjected to the oral glucose tolerance test (OGTT). After 6 weeks, blood sample was collected by cardiac puncture and serum was obtained. Liver and duodenum tissues were excised and immediately frozen for following determinations.

### 2.3. Histological analysis of liver tissue

Liver sections were stained with hematoxylin and eosin or Oil Red O for the morphological and intrahepatocyte lipid evaluations. Steatosis was graded on a scale of 0 (absence of steatosis), 1 (mild), 2 (moderate) and 3 (extensive). A double-blinded examination of the sections was made independently by two veterinary pathologists (OP and TBP) at a magnification  $\times$ 20.

### 2.4. OGTT and serum parameters

At the fifth week of treatment, fasted rats received glucose (2 g/kg; *per os*) and glycemia was measured at 0, 30, 60, 90 and 120 min after glucose administration. Glucose levels were measured by the glucometer One Touch UltraSmart (Lifescan, Milpitas, CA, USA).

All biochemical and hormonal parameters were measured in serum samples prepared from blood collected at sixth week. Serum AST, ALT, cholesterol and glucose were analyzed by standard automated procedures, according to manufacturer's protocols (AST Flex reagent cartridge and ALT Flex reagent cartridge; Dade Behring Inc., Newark, DE, USA). Insulin levels were also evaluated by ELISA assay (Millipore, Billerica, MA, USA). As index of IR, homeostasis model assessment (HOMA) was calculated, using the formula  $[HOMA = \text{fasting glucose (mmol/L)} \times \text{fasting insulin } (\mu\text{U/ml}) / 22.5]$ .

### 2.5. Real-time PCR

Total RNA, isolated from liver and small intestine, was extracted 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  $\mu\text{g}$  total RNA. PCRs were performed with a Bio-Rad CFX96 Connect Real-Time PCR System instrument and software (Bio-Rad Laboratories). The primer sequences are reported in Table 1. The PCR conditions were 10 min at 95°C followed by 40 cycles of two-step PCR denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. Each sample contained 1–100 ng cDNA in 2  $\times$  Power SYBRGreen PCR Master Mix (Bio-Rad Laboratories) and 200 nmol/L of each primer (Eurofins MWG Operon, Huntsville, AL, USA) in a final volume of 25  $\mu\text{l}$ . The relative amount of each studied mRNA was normalized to GAPDH as housekeeping gene, and the data were analyzed according to the  $2^{-\Delta\Delta\text{CT}}$  method. Before performing any reaction, the efficiency of primers was set through a standard curve in the tissue sample analyzed, and an amplification efficiency of 100% was obtained.

### 2.6. Western blot analysis

Livers were homogenized and total protein lysates were subjected to SDS-PAGE. Blots were probed with anti-nitrotyrosine (Nox-Tyr; Merck Millipore, Billerica, MA, USA), anti-phospho-acetyl-CoA-carboxylase (pACC), anti-ACC type 2 or  $\beta$  (Cell Signaling, Danvers, Massachusetts, USA) and anti-COX-2 (BD Transduction, Franklin Lakes, NJ, USA). Western blot for anti- $\alpha$ -tubulin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Sigma-Aldrich; Milan Italy) was performed to ensure equal sample loading.

### 2.7. ROS assay

The levels of ROS were determined as previously reported [17]. 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  $\mu\text{M}$  dichloro-fluorescein diacetate (Sigma-Aldrich) in dimethyl sulfoxide for 15 min at 37°C. The dye-loaded samples were centrifuged at 12,500g per 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, Massachusetts, USA) at 488 nm for excitation and 525 nm for emission wavelengths. ROS were quantified from the dichloro-fluorescein standard curve in dimethyl sulfoxide (0–1 mM).

### 2.8. MDA measurement

Malondialdehyde (MDA) levels in the liver were determined as an indicator of lipid peroxidation [18]. Tissues were homogenized in 1.15% KCl solution. An aliquot (200  $\mu\text{l}$ ) of the homogenate was added to a reaction mixture containing 200  $\mu\text{l}$  of 8.1% SDS, 1.5 ml of 20% acetic acid (pH 3.5), 1.5 ml of 0.8% thiobarbituric acid and 600  $\mu\text{l}$  of distilled water. Samples were then boiled for one hour at 95°C and centrifuged at 3000g for 10 min. The supernatant absorbance was measured by spectrophotometry at 550 nm and the concentration of MDA was expressed as micromoles of MDA per milligram of protein of cell homogenate. A standard curve was prepared using MDA bisdimethylacetal as the source of MDA. All solutions were freshly prepared on the day of the assay.

### 2.9. Measurement of gut permeability in vivo

In another set of experiments, after 6 weeks on HFD, rats were fasted for 6 h and then gavaged with 4000-kDa fluorescein isothiocyanate-labeled dextran (FITC-dextran; TdB Consultancy AB, Uppsala, Sweden) diluted in water (500 mg/kg, 125 mg/ml). After 2 or 24 h, blood (500  $\mu\text{l}$ ) was collected from intracardiac puncture and centrifuged (3000 rpm for 15 min at room temperature). FITC-dextran concentration in plasma was determined by spectrofluorimetry (excitation wavelength: 485 nm and emission wavelength: 535 nm; HTS-7000 Plus-plate-reader, Perkin Elmer), as previously described [19].

Table 1  
Real-time PCR primer sequences

Target gene	Forward primer (5'→3')	Reverse primer (3'→5')	Accession number
TNF- $\alpha$	5'-CATCTTCTCAAACTCGAGTGACAA-3'	3'-TGGGAGTAGATAAGGTACAGCCC-5'	NM_012675.3
IL-6	5'-ACAAGTGGGAGGCTTAATTACACAT-3'	3'-TTGCCATTGCACAACCTTTTC-5'	NM_012589.2
PPAR- $\alpha$	5'-GTGCCTGTCCGTCGGGATGT-3'	3'-GTGAGCTCGGTGACGGTCTC-5'	NM_013196.1
CPT1a	5'-CGTCATGGTCAACAGCAACTACT-3'	3'-CTCACGGTCTAATGTGCGACGA-5'	NM_031559.2
FGF21	5'-AGATCAGGGAGGATGGAACA-3'	3'-ATCAAAGTGAGGCGATCCATA-5'	NM_130752.1
ZO-1	5'-CCATCTTTGGACCGATTGCTG-3'	3'-TAATGCCCGAGCTCCGATG-5'	NM_001106266.1
GAPDH	5'-GGCACAGTCAAGGCTGAGAATG-3'	3'-ATGGTGTGAAGACGCCAGTA-5'	NM_017008.4

### 2.10. Statistical analysis

Data are presented as mean  $\pm$  SEM. Statistical analysis was performed by analysis of variance test for multiple comparisons followed by Bonferroni's test, using GraphPad Prism (GraphPad software, San Diego, CA, USA). Statistical significance was set at  $P < .05$ .

## 3. Results

### 3.1. Effects of HT on histological studies and serum parameters

Hepatic tissue analysis from rats fed with HFD stained with hematoxylin–eosin revealed a severe steatosis and inflammatory damage. The histological pattern was characterized by microvesicular steatosis and ballooning localized predominantly in zone 1, confirmed by Oil Red O staining (Fig. 1A and B).

Furthermore, the HFD-fed animals showed mild signs of liver inflammation characterized by the presence of mixed inflammatory cells, occasionally arranged in microgranulomas. No alterations were observed in the liver of the rats fed with the standard diet. Liver sections from animals fed with HFD and treated with HT showed almost a complete resolution of the microvesicular steatosis and ballooning and absence of liver inflammation.

Accordingly, the increase in serum AST, ALT and cholesterol in HFD group was reduced by the polyphenol (Fig. 1C–E).

Weight gain of animals did not significantly change among groups after 6 weeks (STD: 228.50  $\pm$  18.70 g, HFD: 234.60  $\pm$  32.7 g and HFD + HT: 207.30  $\pm$  16.15 g); accordingly, also fat mass did not vary among groups (STD: 80.30  $\pm$  7.18 g, HFD: 83.12  $\pm$  4.84 g and HFD + HT: 74.28  $\pm$  5.96 g). Moreover, food intake, expressed as grams of food

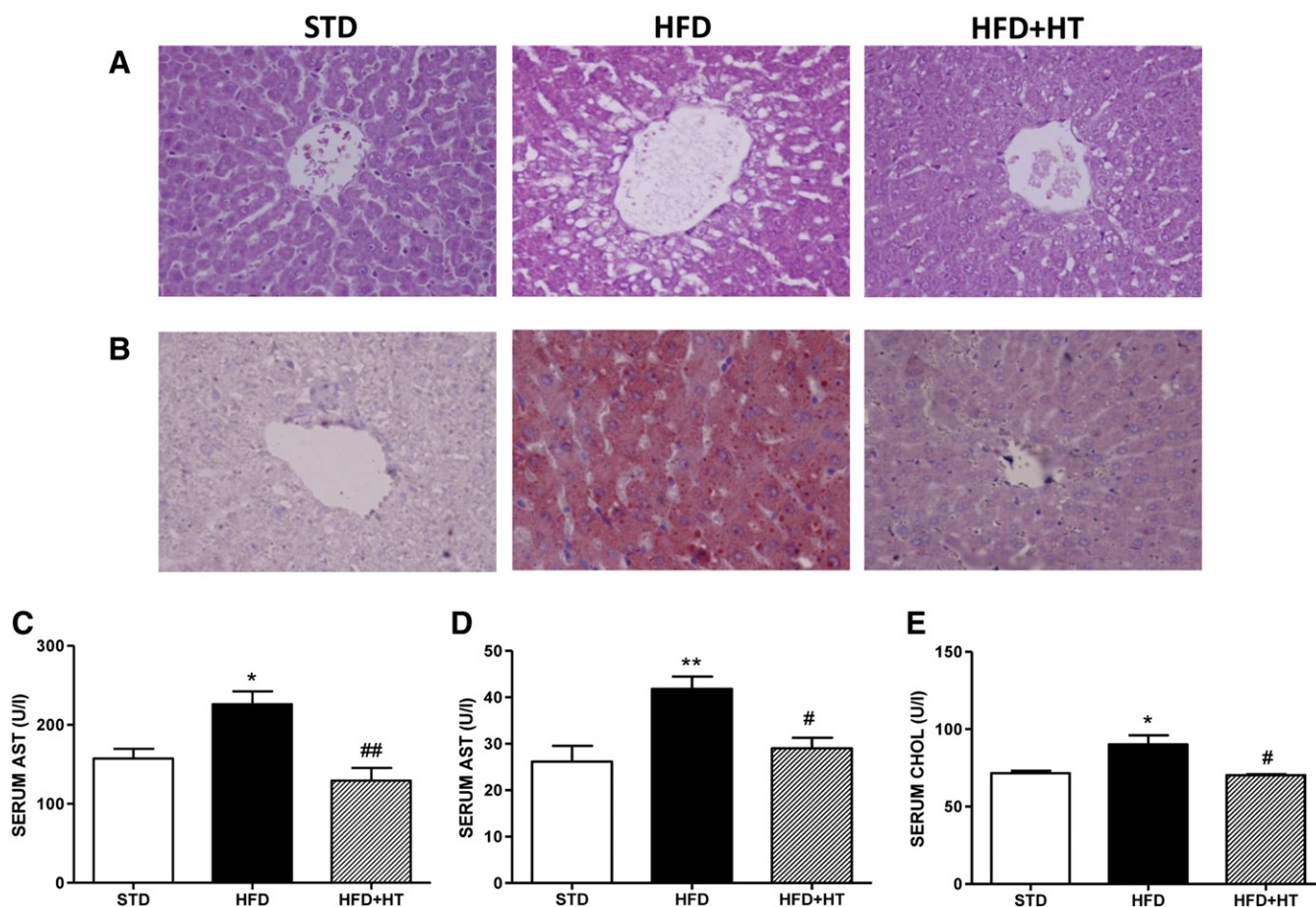


Fig. 1. HT effects on liver damage and lipid accumulation in HFD-fed rats. Paraffin-embedded sections of the liver ( $n = 4$  each group) were stained with hematoxylin–eosin (A) and Oil Red O (B). Micrographs are representative pictures with original magnification  $\times 20$ . Circulating AST (C), ALT (D) and CHOL (E) were measured ( $n = 6$ , each group). \* $P < .05$  and \*\* $P < .01$  vs. STD; # $P < .05$ , and ## $P < .01$  vs. HFD.



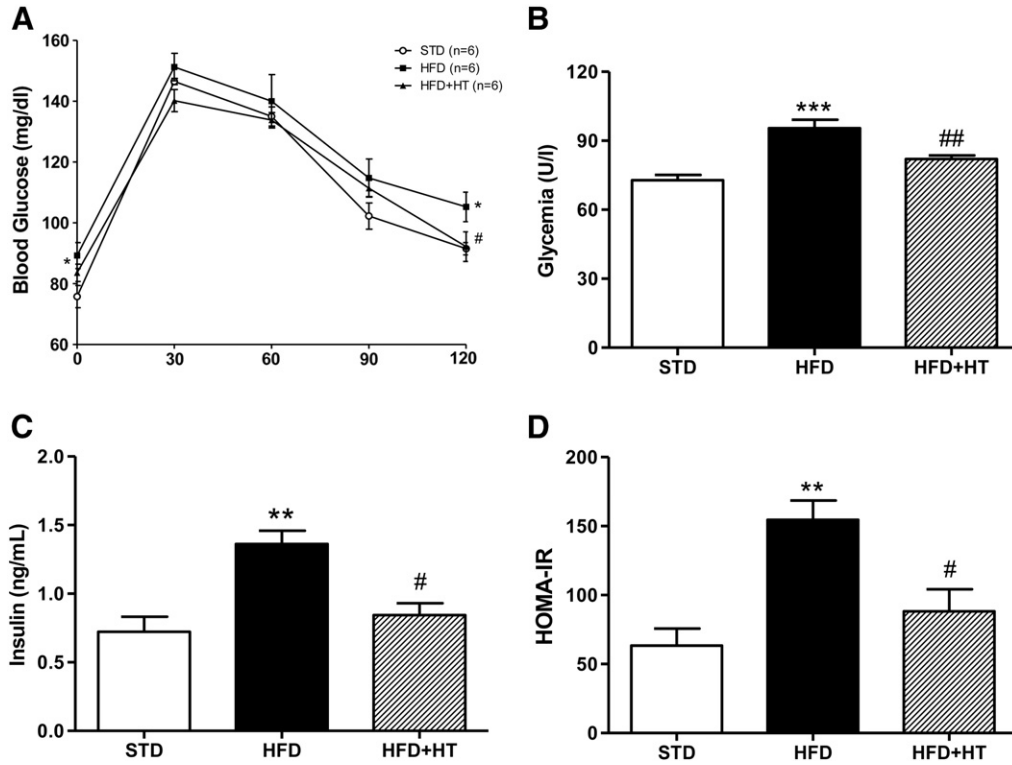


Fig. 2. Effect of HT on glucose homeostasis and IR. OGTT (A) in STD and HFD-fed rats ( $n = 6$ , each group) was performed. Fasting glucose (B), insulin levels (C) and HOMA IR (D) were also reported ( $n = 6$ , each group). \* $P < .05$ , \*\* $P < .01$  and \*\*\* $P < .001$  vs. STD; # $P < .05$ , and ## $P < .01$  vs. HFD.

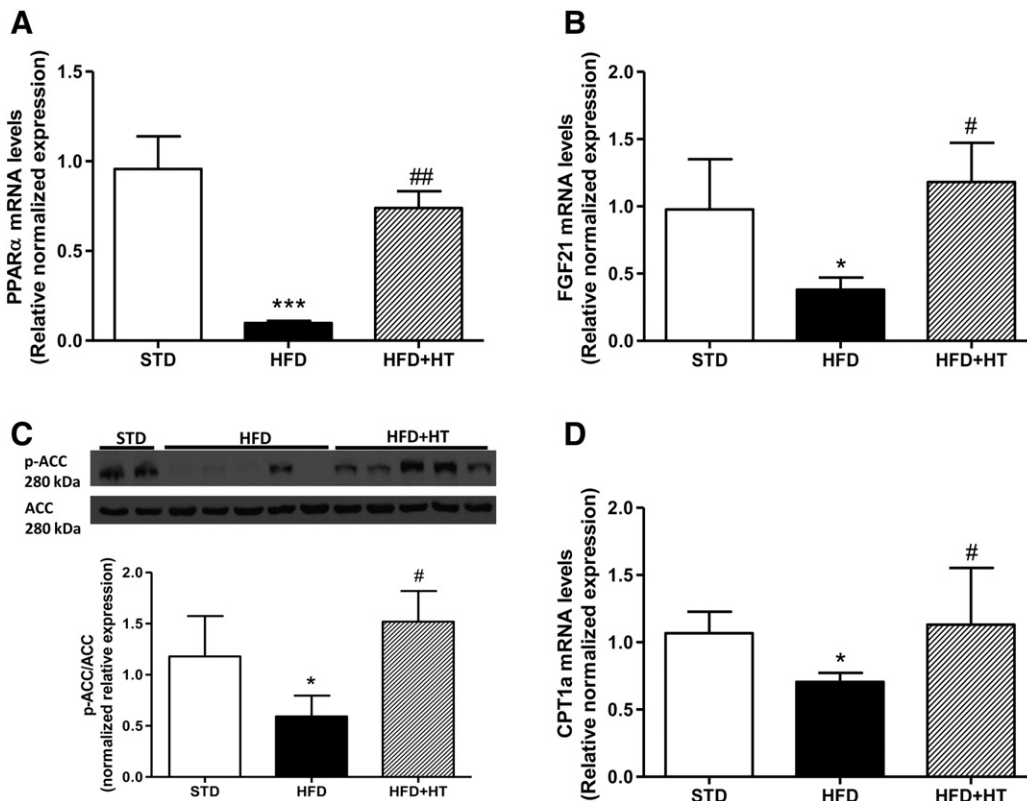


Fig. 3. Effect of HT treatment on metabolic impairment in liver from HFD-fed rats. PPAR- $\alpha$  (A) and FGF21 (B) mRNA abundance in the liver extracts were quantified by real-time PCR ( $n = 6$  each group). A representative blot of pACC and ACC and the ratio from densitometric analysis of bands from all samples are also shown (C). Liver CPT1a (D) mRNA (relative expression to STD) is also reported. Data are mean  $\pm$  SEM of 6 rats per group. \* $P < .05$  and \*\*\* $P < .001$  vs. STD; # $P < .05$  and ## $P < .01$  vs. HFD.

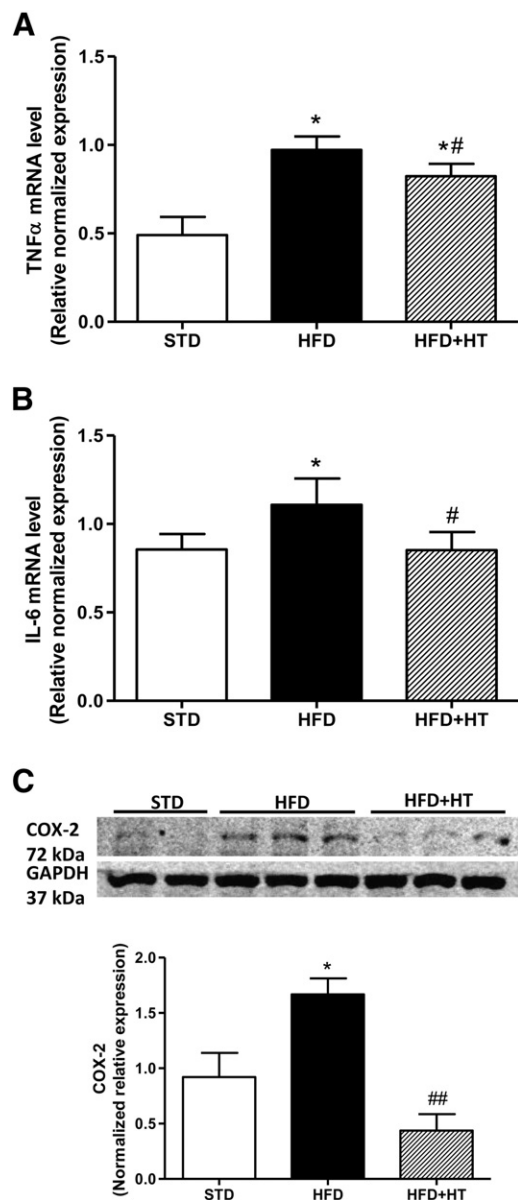


Fig. 4. HT treatment reduces TNF- $\alpha$  and IL-6 gene expression and COX-2 in liver. TNF- $\alpha$  (A) and IL-6 (B) mRNA expression (relative expression to STD) are reported ( $n=6$  each group). Panel C shows representative western blot analysis of COX-2 protein expression and densitometric analysis of bands from all samples. Data are mean  $\pm$  SEM of 6 rats per group. \* $P<.05$  vs. STD; # $P<.05$  and ## $P<.01$  vs. HFD.

taken daily, did not differ between untreated rats fed the HFD ( $14.54 \pm 0.58$  g/day/rat) or treated rats with HT ( $14.23 \pm 0.84$  g/day/rat).

### 3.2. Effect of HT on glucose homeostasis and IR

After the 5-week treatment, HT reduced basal glucose level measured before glucose load. In OGTT, HT treatment caused a significant decrease of glycemia at 120 min (Fig. 2A). At 6 weeks, fasting serum glucose (Fig. 2B) and insulin levels (Fig. 2C) were significantly higher in HFD rats than STD and HT normalized both parameters. Accordingly, IR assessed by HOMA index was significantly reduced by HT (Fig. 2D).

### 3.3. Modulation of hepatic lipid metabolism by HT

PPAR- $\alpha$  mRNA expression was deeply reduced by HFD and restored by HT (Fig. 3A). The evaluation of fibroblast growth factor FGF21 and liver carnitine palmitoyltransferase CPT1a, as a downstream target genes of PPAR- $\alpha$ , showed a similar profile of expression of their transcription factor (Fig. 3B and D). Accordingly, HT also increased the phosphorylation of ACC (Fig. 3C), indicating an increase in hepatic metabolism and oxidation of fatty acids in HT-treated rats.

### 3.4. HT treatment counteracts hepatic inflammation and oxidative stress

TNF- $\alpha$ , IL-6 and COX-2 are important proinflammatory mediators involved in the liver damage and the pathogenesis of hepatic IR. As shown in Fig. 4A and B, HFD induced an increase in TNF- $\alpha$  and IL-6 mRNA levels compared to STD group, and this effect was prevented by HT treatment. Similarly, the antiinflammatory effect of the polyphenol was confirmed by the reduction of COX-2 expression (Fig. 4C).

HFD caused a significant increase of oxidative damage from ROS and reactive nitrogen species (RNS), which mainly contribute to the hepatic damage. HT reduced nitrosylation of proteins and decreased ROS production (Fig. 5A and B, respectively). Moreover, we evaluated the levels of MDA, as a marker of lipid peroxidation, which were higher in HFD than in STD group. HT treatment significantly decreased MDA levels (Fig. 5C).

### 3.5. Effect of HT on gut permeability and intestinal barrier functions

As a consequence of HFD feeding, epithelial barrier integrity was compromised. Indeed, HFD group showed a significant increase in gut permeability measured *in vivo* and determined by plasmatic levels of FITC-labeled dextran at 2 and 24 h (Fig. 6A). This increased permeability was counteracted by HT and the effect was related with the restoration of zona occludens (ZO)-1 mRNA transcript in duodenum (Fig. 6B).

## 4. Discussion

The beneficial effects of the olive oil have been attributed to its several phenolic constituents (i.e. oleocanthal and oleuropein) [20–23]. Here, we focused on HT, the major metabolite of oleuropein [8,24], and showed its protective effect in limiting the metabolic impairment and early inflammatory events underpinning the onset of IR and hepatic steatosis in a rat model of NAFLD induced by HFD. To study the capability of HT in preventing liver steatosis progression on the onset of the disease, we chose 6-week feeding, as experimental time, that mimics the early phase of liver steatosis without a significant increase of body fat accumulation.

In our experiments, HT restores glucose homeostasis and insulin sensitivity, reducing fasting glycemia, insulinemia and HOMA index. Furthermore, this oleuropein derivative prevents the increase in hepatic damage markers, such as AST and ALT, and limits histological modifications, induced by fat overnutrition and early features of steatosis.

We have previously described this model characterized by an increase in serum lipid profile (nonesterified fatty acids, LDL and cholesterol) and lipid accumulation in the hepatocytes [16,25].

In NAFLD and related metabolic diseases, it has been demonstrated an alteration of sterol regulatory element-binding protein (SREBP)-1 and PPAR- $\alpha$  pathways, which regulate lipogenesis and lipid  $\beta$ -oxidation and catabolism, respectively [26–27]. In particular, data on PPAR- $\alpha^{-/-}$  mice fed with high-fat diet have shown abnormal lipid accumulation in hepatocytes, which is consistent with a crucial role for PPAR- $\alpha$  in hepatic lipid metabolism and fatty acid oxidation [28].

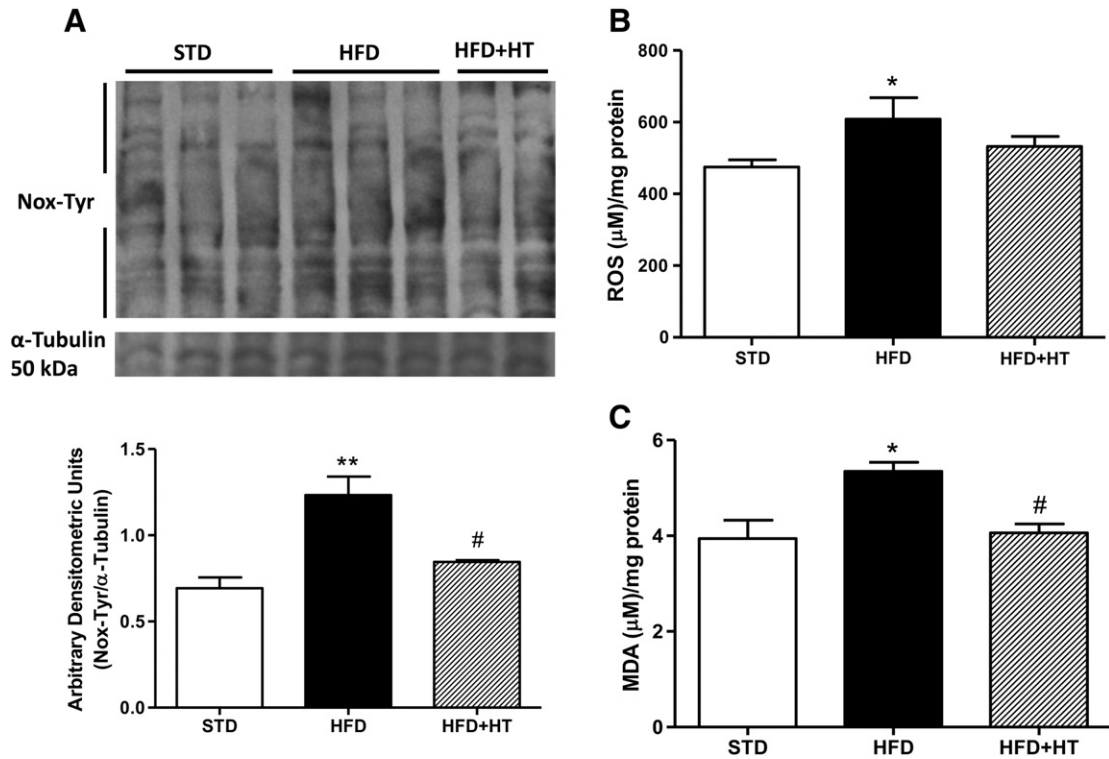


Fig. 5. Effect of HT on nitrosative and oxidative stress. Protein nitrosylation in liver from STD and HFD rats treated or not with HT is shown (A). ROS production (B) and MDA content (C) are also reported. Values are reported as mean  $\pm$  SEM of at least 6 animals. \* $P < .05$  and \*\* $P < .01$  vs. STD; # $P < .05$  vs. HFD.

We have also demonstrated that HFD feeding is associated with the reduction of PPAR- $\alpha$  expression in liver [16] and, according to our findings, the administration of a PPAR agonist or probiotics restores PPAR- $\alpha$  and improves hepatic steatosis [29–30]. Accordingly, here, we found that HFD feeding reduces PPAR- $\alpha$  mRNA liver expression and the phosphorylation of ACC, an enzyme involved in initial phase of fatty acid metabolism. This enzyme, indeed, belonging to the AMP-activated protein kinase (AMPK) pathway, is involved in the regulation of CPT1 activity [31].

Previously, a mechanistic study of the PGC1 $\alpha$  activation signaling pathway demonstrated that HT is an activator of AMPK and also up-regulates gene expression of PPAR- $\alpha$ , CPT1 and PPAR- $\gamma$  in adipocytes [32].

In particular, AMPK induces the phosphorylation of ACC, a modification that inactivates the enzyme, reducing the formation of malonyl-CoA. The decrease in malonyl-CoA synthesis, in turn, reduces liver CPT1 inhibition, thus prompting adequate shuttling of fatty acids into the mitochondria and hence their oxidation [26]. Here, we show that HT increases the phosphorylation of ACC and the transcription of liver CPT1a, both remarkably reduced by HFD. The restoration of CPT1 was consistent with the normalization of its transcriptional regulatory factor PPAR- $\alpha$ . Our results are in agreement with a previous study [33], in which resveratrol, another phenolic compound, triggering the phosphorylation of ACC and activating CPT1a, was able to increase liver fatty acid oxidation and to improve liver steatosis, induced by an obesogenic diet.

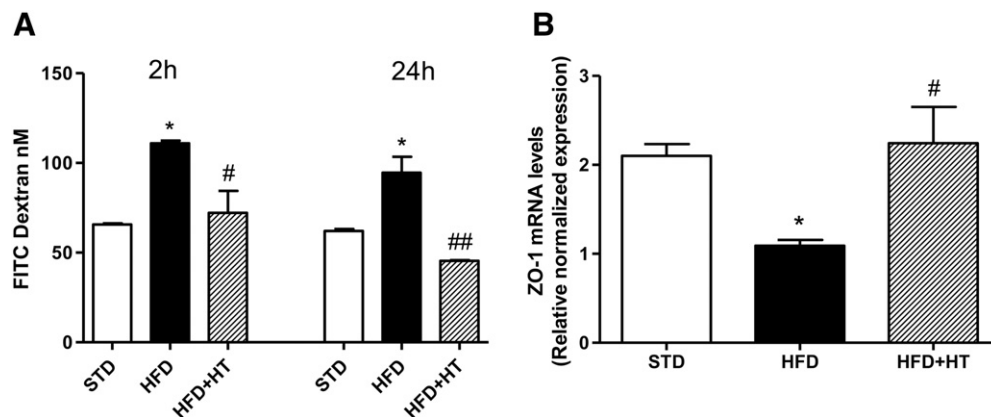


Fig. 6. HT treatment improves intestinal permeability and restores duodenal ZO-1 gene expression. Panel A shows the measurement of gut permeability by appearance of FITC-labeled dextran in plasma of STD, HFD and HFD + HT rats ( $n = 6$  each group) 2 and 24 h postadministration. mRNA expression for ZO-1 (B) in the duodenal tissue is also shown ( $n = 6$  each group). Values are reported as mean  $\pm$  SEM of at least 6 animals. \* $P < .05$  vs. STD; # $P < .05$  and ## $P < .01$  vs. HFD.

Here, we observed that HT treatment not only increases PPAR- $\alpha$  levels but also restores the expression of its downstream-regulated gene FGF21 in the liver. As known, liver production of FGF21, a cytokine/hormone whose transcription is regulated by PPAR- $\alpha$  [34], is required for the physiological activation of hepatic lipid oxidation and triglyceride clearance [35].

Our results confirm data previously reported by other authors in adipocytes [32], demonstrating that the strong effect of HT on phosphorylation of AMPK and ACC was consistent with the decrease in FFA and the increase in CTP1 and PPAR- $\alpha$  as indexes of increased fatty acid oxidation. Our data are consistent with these findings and indicate that HT might be potentially effective in increasing fatty acid oxidation and insulin sensitivity.

Liver and other tissues, such as skeletal muscle and adipose tissues, play a central role in metabolic homeostasis, predisposing whole body to inflammation when metabolism is compromised. In fact, inflammation is largely considered the driving force for progression or exacerbation of metabolic diseases, such as dyslipidemia and NAFLD.

We and others have demonstrated that PPAR- $\alpha$  is an important regulator of inflammatory process [14,36–37]. The antiinflammatory effects of all classes of PPARs rely primarily on genomic mechanisms of transrepression of transcriptional factors (i.e. NF- $\kappa$ B), which induce the reduction of proinflammatory cytokines and enzymes [38]. Therefore, similarly to PPAR- $\gamma$ , it is conceivable that PPAR- $\alpha$  activity could modulate metabolic disorders associated with inflammation either through its metabolic activity or its antiinflammatory effects.

Recently, the current knowledge concerning the main biological properties of HT was summarized, including its antiinflammatory and cardioprotective effects [24]. Here, we demonstrated that HT significantly reduces the expression of inflammatory cytokines (TNF- $\alpha$  and IL-6) and COX-2 in the liver, confirming its antiinflammatory activity, likely associated to its ability in controlling metabolic alterations through PPAR- $\alpha$  recover. Previous data examined the role of cytokine- and enzyme-induced inflammation and their link with IR and fatty liver [39–40]. Metabolic dysregulation, mitochondrial impairment and oxidative stress have a crucial role in determining hepatocyte damage, contributing to inflammatory process and NAFLD progression [41]. In particular, mitochondrial dysfunction is mainly related to the IR and lipotoxicity due to FFA excess [42]. Mitochondrial impairment causes enhanced ROS production, which in turn self-sustains organelle damage. In particular, products of cellular lipid peroxidation (i.e. MDA and 4-hydroxynonenal) associated to inflammatory cytokines (i.e. TNF- $\alpha$ ) contribute to mitochondrial dysfunction by interfering with mitochondrial respiratory chain and by forming superoxide anion [43]. Indirectly, TNF- $\alpha$  promotes mitochondrial dysfunction, increasing RNS as consequence of iNOS induction [44], and the administration of uric acid or anti-TNF antibodies was able to improve this dysfunction [45].

The antioxidant activity of HT has been well examined in physiological and pathological conditions [5–6,20,46–48], but its role in the oxidative damage in NAFLD is still largely unexplored. For this reason, we evaluated ROS- and RNS-induced stress in HFD-fed rats receiving HT. Here, HT significantly reduces ROS production, MDA levels and protein nitrosylation.

Our data are in agreement with recent data reporting neuroprotective effects of HT in type 2 diabetes animal model [49], where HT improves mitochondrial function and reduces oxidative stress potentially through activation of the AMPK pathway in the brain of db/db mice.

Another key role in the pathogenesis and ongoing of NAFLD is played by gut-derived endotoxin that reaches the liver when intestinal integrity and function are compromised, impairing liver homeostasis and sustaining tissue inflammation [50]. The polyphenol HT recovers the impairment of gut integrity, as evidenced by the reduction of plasmatic FITC-dextran levels and the increase in duodenal mRNA ZO-1, an important TJ protein that was significantly altered by HFD.

In summary, our data clearly demonstrate that HT protects the liver from damage caused by HFD. This effect was mediated by a reduction in hepatic fat accumulation (through increased fatty acid  $\beta$ -oxidation) and in liver oxidative stress (through the reduction of protein nitrosylation, ROS and MDA) and by inflammation (through modulation of enzyme and cytokines related to hepatic inflammation). Moreover, HT recovers the HFD-induced alteration of gut integrity, underpinned to the major exposure of the liver to gut-derived toxins and metabolites, which in turn contributes and increases the liver inflammation favoring the progression of steatosis in steatohepatitis.

In conclusion, our data show antioxidant, antiinflammatory and protective effect of the polyphenol HT in NAFLD, indicating the beneficial effects of virgin olive oil in Mediterranean diet likely related to this compound, among all its phenolic components. Indeed, consumption of virgin olive oil enriched in HT, combined with appropriate and balanced diet, could be considered an available strategy to prevent early events of liver steatosis and its related complications.

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