










Cite this: DOI: 10.1039/d5fo05134g

Oleacein prevents metabolic dysfunction-associated hepatic steatosis and dyslipidaemia in ApoE-KO mice

Beatriz Ormonde,  †^{a,b,c} Vânia Costa,  †^d Pedro Vieira,  ^{a,b,c} André Alves,  ^{a,b,c} Sofia Viana,  ^{a,b,c,e} Flávio Reis  *^{†,a,b,c} and Fátima Paiva-Martins  *^{†,d}

The modification of cardiometabolic risk factors is a major strategy to prevent metabolic dysfunction-associated fatty liver disease (MAFLD), given the current lack of approved pharmacological treatments. This study investigated whether oleacein (OLEA), a key polyphenol in olive oil, can prevent dyslipidemia and hepatic steatosis in apolipoprotein E knockout (ApoE-KO) mice, a model of atherosclerosis and metabolic disturbance. C57BL/6J wild-type (WT, $n = 16$) and ApoE-KO ($n = 16$) mice were assigned to four groups ($n = 8$) and fed for 10 weeks: WT mice received either a standard diet (WT + STD) or an atherogenic diet (WT + ATD), while ApoE-KO mice received the atherogenic diet without (ApoE-KO + ATD) or with OLEA supplementation ($50 \text{ mg kg}^{-1} \text{ day}^{-1}$, oral; ApoE-KO + ATD + OLEA). Metabolic parameters, serum lipid profile, and hepatic triglyceride (TG) content were determined, alongside markers of liver injury, oxidative status (SOD expression and activity), and lipid metabolism (including SCD1 expression). OLEA supplementation significantly improved the serum lipid profile and reduced hepatic TG accumulation ($p < 0.05$), effects associated with marked downregulation of hepatic SCD1, indicating inhibition of *de novo* lipogenesis, FABP1 and CPT1. In addition, OLEA restored hepatic SOD1 and SOD2 expression and enhanced serum SOD activity, suggesting reinforcement of antioxidant defenses. Overall, OLEA exerted strong anti-dyslipidemic and hepatoprotective effects in ApoE-KO mice, likely through the combined modulation of lipid synthesis and redox homeostasis. These findings highlight the potential of OLEA as a promising natural compound for preventing diet-induced metabolic and hepatic disorders associated with MAFLD.

Received 25th November 2025,
Accepted 28th April 2026

DOI: 10.1039/d5fo05134g

rsc.li/food-function

1. Introduction

Globally, over 35% of the population is affected by steatotic liver diseases (SLD) with approximately 25% diagnosed with metabolic dysfunction-associated steatotic liver disease (MAFLD), which is now recognized as the most prevalent form of chronic liver disease.^{1,2} MAFLD is a multifactorial condition associated with metabolic disturbances such as type 2 diabetes mellitus (T2DM), metabolic syndrome (MetS) and obesity. Its

development is strongly influenced by dietary factors, including excessive caloric intake and high-fat and sugar diets, while alcohol consumption represents a distinct but overlapping etiological pathway in liver steatosis.^{3,4} Over the years, steatosis has been associated to atherosclerosis and cardiovascular diseases (CVDs). Thus, the modification of CVDs risk factors has been recommended as a major approach to prevent MAFLD.⁵

Hepatic lipid accumulation can trigger oxidative stress and impair autophagic processes involved in the degradation of lipid droplets.⁶ The early stages of SLD are typically characterized by hepatic fat accumulation resulting from insulin resistance, excessive dietary fat intake and genetic susceptibility. Hyperinsulinemia further enhances lipolysis in peripheral tissues, increasing the influx of free fatty acids to the liver, where they are esterified and stored as triglycerides.⁷ This lipid overload promotes mitochondrial dysfunction and excessive production of reactive oxygen species (ROS), contributing to oxidative stress and inflammatory signaling.^{7,8} In experimental models of MAFLD, high-fat diets markedly increase hepatic oxidative stress and inflammatory cytokine expression, highlighting the central role of redox imbalance in disease patho-

^aInstitute of Pharmacology & Experimental Therapeutics, & Coimbra Institute for Clinical and Biomedical Research (ICBR), Faculty of Medicine, University of Coimbra, Coimbra, Portugal. E-mail: freis@fmed.uc.pt

^bCenter for Innovative Biomedicine and Biotechnology (CIBB), University of Coimbra, Coimbra, Portugal

^cClinical Academic Center of Coimbra (CACC), Coimbra, Portugal

^dREQUIMTE/LAQV, Department of Chemistry and Biochemistry, Faculty of Science, University of Porto, Rua do Campo Alegre 687, Porto, Portugal.

E-mail: mpmartin@fc.up.pt

^eFaculty of Pharmacy, University of Coimbra, Coimbra, Portugal

†These authors contributed equally and share first authorship.

‡These authors share senior authorship.



genesis.⁹ Oxidative stress can also induce endoplasmic reticulum stress and stimulate the release of pro-inflammatory mediators that activate hepatic stellate cells, promoting collagen deposition and progression toward liver fibrosis.⁶ Despite the high prevalence of this condition, no approved pharmacological therapies are currently available to effectively halt the progression of MAFLD to advanced fibrosis and its associated complications.^{10,11} Consequently, increasing attention has been directed toward dietary strategies and nutritional supplements with antioxidant properties as complementary approaches for the prevention or management of this condition.^{10–12}

Studies have shown that antioxidants may prevent CVDs and MAFLD.^{12–15} Compounds most frequently tested so far for MAFLD are α -tocopherol and silymarin polyphenolic compounds. By limiting oxidative injury to the cell membrane of hepatocytes, these antioxidants should theoretically decrease the recruitment of activated inflammatory cells, the oxidative injury to organelles, the activation of HSCs, and the impairment of membrane function, including insulin signalling.¹⁵ These antioxidants also act by increasing the activity of antioxidant enzymes like superoxide dismutase (SOD) and glutathione peroxidase (GPx),^{15–17} by inhibiting the inflammatory enzymes cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX),¹⁸ and by regulating the immune response.¹⁹ However, in clinical trials, diet and physical exercise seems to have more impact in the treatment of steatosis in children than this antioxidant supplementation²⁰ and, in adults, vitamin E supplementation used in a short period has shown limited or no benefits.^{12–15,21} Due to the lack of pharmacological approaches that clearly demonstrate a benefit in the prevention and/or treatment of MAFLD, both the American⁵ and European²² guidelines recommend lifestyle modification, namely the implementation of physical exercise and changes in eating habits, as the main means of treatment and prevention of MAFLD.

Among the dietary approaches evaluated in these patients, the adoption of the Mediterranean diet appears to be the one that produces the best results, with significant reductions in cardiovascular risk and hepatic fat accumulation.²³ Studies such as PREDIMED, shows that Mediterranean diet has a preventive/protective role against many diseases, such as CVDs, T2DM, MAFLD, among others. In addition, evidence from the PREDIMED study indicates that a Mediterranean diet supplemented with extra virgin olive oil (EVOO) is associated with a lower prevalence of hepatic steatosis, supporting a potential protective role of olive oil consumption in fatty liver disease.^{22–27} EVOO is one of the most important sources of lipids in this diet and, in addition to its particular lipid composition, rich in monounsaturated fatty acids, several studies have shown that its composition in minor compounds, such as phytosterols, squalene and phenolic compounds, also plays an important role to its biological effects and influence redox homeostasis.²⁸ Moreover, the European Food Safety Authority (EFSA) suggests that a daily dose of olive oil rich in hydroxytyrosol (HT) and its esters (5 mg of HT and esters/20 g of olive oil) provides protection against oxidative stress derived from

lipid oxidation, delaying the accumulation of oxidized low-density lipoprotein (LDL).²⁹ Among these HT derivatives, oleacein, a polyphenolic secoiridoid derived from the hydrolysis of the oleuropein glycoside and present as one of the most concentrated polyphenols in olive oil, has shown important *in vitro* antioxidant and anti-inflammatory properties.^{30,31} Recently, *in vivo* studies using WT mice treated with oleacein showed a protective effect against weight gain and fat deposition in the liver, accompanied by reduced macrophage infiltration.^{32,33} Moreover, it has been reported that oleacein presents neuroprotective effects in central nervous system in experimental autoimmune encephalomyelitis (EAE) mice.³⁴ However, the impact on animal models of cardiovascular and metabolic disease remains to be further elucidated, deserving further studies.

Wild-type mice are generally resistant to the development of atherosclerosis³⁵ due to their naturally high levels of high-density lipoprotein (HDL), low levels of low-density lipoprotein (LDL), and the absence of cholesteryl ester transfer protein (CETP), in contrast to humans who present higher LDL and lower HDL levels. The ApoE knockout (ApoE-KO) mouse is a genetically modified model in which the gene encoding apolipoprotein E (ApoE) is inactivated. ApoE plays a key role in lipid metabolism by acting as a ligand for LDL receptor family members, mediating the clearance of ApoB-containing atherogenic lipoproteins.^{36,37} It is also involved in cholesterol efflux, reverse cholesterol transport, and modulation of immune responses associated with atherosclerosis. In the absence of ApoE, impaired lipoprotein clearance leads to severe dyslipidaemia and rapid development of atherosclerotic plaques, particularly under an atherogenic diet.^{37–39}

There are several classes of anti-dyslipidemic drugs, including statins, bile acid scavengers, nicotinic acid and fibrates, among others. Statins are the most used and the most effective medicine to revert hyperlipidemia, especially by reducing c-LDL levels and improve vascular function, preventing plaque progression and reducing cardio-vascular risk and mortality caused by CV events.^{10,37} Despite this impact of statins on CVD, it has been reported for several years that statin intolerant patients develop side effects related to muscle and liver toxicity.³⁷

Given this context, the present study aimed to investigate whether oleacein, a bioactive polyphenol present in olive oil, exerts anti-dyslipidemic and hepatoprotective effects *in vivo* using the ApoE-KO mouse model fed an atherogenic refined diet.

2. Materials and methods

2.1. Oleacein isolation from olive oil leaves

Oleacein was isolated from olive leaves following the method described by Paiva-Martins and Gordon,⁴⁰ with minor modifications. Briefly, freshly harvested olive leaves (700 g), collected in October 2020, were stored under vacuum in sealed bags (50 × 25 cm) at 37 °C for 22 hours to promote the enzymatic con-



version of oleuropein into oleacein within the plant tissue. After this incubation, the leaves were macerated in 4 L of ethanol for five days at room temperature and in the dark. The resulting extract was filtered, and the solvent was removed under reduced pressure. The residue was redissolved in 500 mL of an acetone/water mixture (1 : 1, v/v), washed with *n*-hexane, and subsequently extracted with chloroform. The organic phase was dried over anhydrous sodium sulphate and evaporated to dryness. The crude extract, which contained 11.7 g of oleacein as determined by HPLC-DAD, was subjected to column chromatography on silica gel 60 (Merck, 230–400 mesh ASTM) using diethyl ether/methanol (35 : 1) as the eluent. The purified fractions yielded 4.9 g of oleacein (98% purity, analysed by HPLC-DAD – Fig. S1, SI), which was identified and confirmed by NMR spectroscopy (Fig. S2 and S3, SI). The purified compound was stored at –20 °C and protected from light until further use.

2.2. Animals, diets and experimental study design

Male *Mus musculus* mice aged 11 weeks were used in this study. A total of 32 animals were obtained from Charles River Laboratories (Paris, France), including 16 C57BL/6J wild-type (WT, 11 weeks old) mice, used as control groups, and 16 apolipoprotein E knockout (ApoE-KO) mice with a C57BL/6J background. Until the beginning of the experimental protocol, all animals were maintained on a standard chow diet (4RF21, Mucedola) with *ad libitum* access to food and water. They were housed in individually ventilated cages (20 × 36 × 18 cm, four animals per cage) under controlled environmental conditions: temperature (~22 °C), humidity (~60%), and a 12-hour light/dark cycle. All procedures involving animals were conducted in accordance with National (DL 113/2013) and European (2010/63/EU) Directives for the care and use of laboratory animals and were approved by the institutional Animal Welfare of the Institute for Clinical and Biomedical Research (iCBr) from the Faculty of Medicine of University of Coimbra (Ref: #22/2020), as well as by the National Authority (General Directorate of Food and Veterinary Affairs, DGAV), Ref: #421/2022.

Given that the consumption of unhealthy diets is a major risk factor for the development of hepatic steatosis and atherosclerosis, this experimental design aimed to evaluate the metabolic impact of chronic dietary intake over 10 weeks. Two diets were used: the standard unrefined chow diet (STD) and a commercially available atherogenic diet (ATD, TD.88137, Teklad). The nutritional compositions of both diets are detailed in Table 1.

To assess the presumed hepatoprotective effect of oleacein, the following four groups ($n = 8$ per group) were formed: group 1 (WT + STD) – WT mice fed with the standard diet; group 2 (WT + ATD) – WT mice fed with the atherogenic diet; group 3 (ApoE-KO + ATD) – ApoE-KO mice fed with the atherogenic diet; group 4 (ApoE-KO + ATD + OLEA) – ApoE-KO mice fed with the atherogenic diet and treated with oleacein (50 mg kg⁻¹) for 5 days a week for 10 weeks. The animals had free access to water and food, and the oral administration of oleacein was carried out using a new oral dosing technology

Table 1 Composition and energetic profile of ATD and STD dietary regiments. The table reports the macronutrient composition of the diets (% by weight) and caloric density (kcal g⁻¹), from which the relative energy contribution of fat, protein and carbohydrates can be derived

	ATD (TD.88137)	STD (Mucedola 4RF21)
Energy sources (% by weight)		
Protein	17.3	18.5
Carbohydrate	48.5	53.5
Fat	21.2	3.00
Energy (Kcal g⁻¹)		
Protein	0.69	0.74
Carbohydrate	1.94	2.14
Fat	1.91	0.27
Total	4.50	3.15
Proteins (% by weight)		
Casein	19.5	—
DL-Methionine	0.30	—
Carbohydrates (% by weight)		
Starch	—	53.5
Corn starch	15.0	—
Cellulose	5.0	—
Sucrose	34.1	—
Lipids (% by weight)		
Soybean oil	—	3.00
Anhydrous milkfat	21.0	—
Cholesterol	0.15	—
Vitamin/mineral mixes (% by weight)		
Mineral mix, AIN-76 (170915)	3.50	—
Vitamin mix, Teklad (40060)	1.00	—
Fatty acids (% by weight)		
Saturated fatty acids	61.8	22.0
Mono-unsaturated fatty acids	27.3	22.0
Poly-unsaturated fatty acids	4.70	56.0

(HaPILLness) that allows voluntary, stress-free, and accurate oral dosing (European patent EP4142698).⁴¹

2.3. *In vitro* monitoring

2.3.1. Body weight (BW), food and water consumption. Body weight (BW), food, and water consumption were measured once a week throughout the duration of the study. BW was recorded using an analytical balance (CQT251 Core® Portable Compact Balance, Adam Equipment, USA), while food intake was measured using a portable compact balance (CQT 2000 Core® Portable Compact Balance, Adam Equipment, USA). Food consumption was calculated by subtracting the remaining food weight from the initial weight placed in the cage at the end of each 7-day period. Beverage consumption was monitored using laboratory volumetric glassware.

2.3.2. Glucose tolerance test (GTT) and insulin tolerance test (ITT). In the first week of the study (T0), four animals from each group underwent an overnight fast (12 hours), followed by a glucose tolerance test (GTT). The remaining four animals from each group were fasted for 6 hours (morning fasting) and then underwent an insulin tolerance test (ITT). This experimental design was chosen to minimize biased metabolic outcomes and to adhere to the 3Rs Refinement principle (a gold-standard pillar of Laboratory Animal Sciences).

A glucose solution (Sigma-Aldrich, Merck, 1.5 g per kg BW) and an insulin solution (Novo Rapid, Novo Nordisk®, 0.5 U



per kg BW) were administered *via* intraperitoneal injection (i.p.), respectively. To measure glucose levels, blood samples were collected from the tail vein. The first drop of blood was discarded, and the second drop was used to measure baseline glucose levels using a portable commercial glucometer kit (GlucoMen® aero 2K, A. MENARINI diagnostics) at 0, 15, 30, 60, 90, and 120 minutes. On week 10 (T10) of the study, all animals underwent the same tests. The area under the curve (AUC) for both the GTT and ITT was calculated using the trapezoidal method as previously described.⁴²

2.3.3. Sample collection. At week 10, animals were anaesthetized in a saturated chamber with isoflurane (IsoFlo®, Abbott) followed by intraperitoneal injection of 150 mg kg⁻¹ of ketamine chloride (100 mg kg⁻¹; Imalgene®) in xylazine (10 mg kg⁻¹, Rompun®). Blood was immediately collected through heart puncture to serum tubes (BD Vacutainer SST II 47 Advance) and then centrifuged at 3500 rpm for 15 minutes (4 °C) and stored at -20 °C. Upon sacrifice, mice were submitted to transcardiac perfusion with ice-cold PBS 1X and the aorta and the liver were isolated, collected, washed and weighted. The tissue samples were divided into 3 sections for distinct purposes: the first one, for mRNA analyses, was immersed in RNA lather (R-0901, Sigma Aldrich); the second one was placed in OCT CryoMatrix (6769006, Thermo Scientific) for fluorescence microscopy and the third section was snap frozen in liquid nitrogen for protein analysis. Samples were stored at -80 °C until analysis.

2.4. *Ex vivo* analysis

2.4.1. Tissue Oil Red O staining of liver and aorta. Sections of fresh frozen liver and aorta tissue samples were cut to a thickness of 5 µm, mounted on slides, and allowed to dry for 30 minutes. The cryosections were then immersed in absolute propylene glycol for 2 minutes before being transferred to a 0.5% red oil in absolute propylene glycol solution for 10 minutes. The sections were differentiated in an 85% propylene glycol solution for 2 minutes, washed with distilled water, and counterstained with Hematoxylin Stain Solution Gill 1 (Sigma Aldrich, Missouri, USA) for 30 seconds. After rinsing in running water for 3 minutes, the sections were mounted using CC/Mount aqueous mounting medium (Sigma Aldrich, Missouri, USA). Lipids were stained bright red, while the nuclei appeared blue.

2.4.2. Tissue H&E staining of liver and aorta. Aorta and liver samples were fixed in formalin and embedded in paraffin wax. Cryosections (5 µm) were cut from each block and reviewed. Briefly, the tissue sections were deparaffinized in xylene and then hydrated through a decreasing series of ethanol solutions until reaching distilled water. The sections were subsequently immersed in Gill 1 Hematoxylin Stain Solution (Sigma Aldrich, Saint Louis, MO, USA) for 2 minutes, followed by rinsing in tap water. Next, the sections were counterstained with 0.5% aqueous eosin (Sigma Aldrich, MO, USA) for 30 seconds. Afterward, the sections were dehydrated, cleared, and mounted. All samples ($n = 8$ per group) were examined under light microscopy using a Zeiss Axioplan 2 microscope (Göttingen, Germany).

2.4.3. TAGs quantification in the liver tissue. After Oil Red-O staining, TAGs content in liver samples was quantified using an enzymatic colorimetric assay with a commercial kit (Ref. 1155010, Triglycerides MR, Cromatest®, Linear Chemicals, Barcelona, Spain). Briefly, 50 mg of frozen liver tissue was homogenized in 1 mL of isopropanol. The homogenate was sonicated and then centrifuged at 3000 rpm for 5 minutes at 4 °C. The supernatant was analysed following the manufacturer's instructions.

2.4.4. Gene expression analysis

2.4.4.1. RNA extraction from liver tissue. For liver samples, 35–50 mg of frozen liver tissue (stored in RNA Later Stabilization Solution, R-0901, Sigma Aldrich) was homogenized by mechanical dissociation using a Potter-Elvehjem homogenizer (Thomas Scientific, USA) in 1 mL of Trizol reagent (93289, Sigma). The sample was stored overnight at -80 °C. RNA extraction was performed using the RNeasy® Lipid Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol.

2.4.4.2. Complementary DNA (cDNA) synthesis. Complementary DNA (cDNA) synthesis was carried out using the Xpert cDNA Synthesis Mastermix (GK81.0100, Lot. 7E2709A, GRISP). For each reaction, 2 µg of RNA, 10 µL of Mastermix, and water were added to achieve a final volume of 19 µL. The cDNA synthesis was performed in a thermocycler (Bio-Rad T100TM Thermal Cycler, 1861096) according to the protocol provided by the Mastermix manufacturer. The synthesized cDNA samples were stored at -20 °C.

2.4.4.3. RT-PCR. A reaction mixture was prepared containing 10 µL of Sybr Green (iQaq Universal SYBR Green Supermix, 1725124, Bio-Rad), 0.4 µL of primer mix (Table 2), and 7.6 µL of autoclaved water. To each well, 18 µL of this mixture and 2 µL of cDNA sample were added. The RT-PCR protocol consisted of an initial denaturation step at 95 °C for 10 minutes, followed by 40 cycles of: 15 seconds at 95 °C, 45 seconds at 58–60 °C, and 30 seconds at 72 °C. Gene expression was normalized using the GeNorm algorithm, with Hypoxanthine Phosphoribosyltransferase (HPRT) and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serving as reference genes. Relative gene expression was calculated using the $\Delta\Delta Ct$ method ($2^{-\Delta\Delta Ct}$). Results are presented as a percentage of control.

2.5. Superoxide dismutase (SOD) activity

Serum and liver superoxide dismutase (SOD) activity was measured using colorimetric methods with the SOD colorimetric activity kit (EIASODC, ThermoFisher Scientific), which is designed to assess all types of SOD activity (Cu/Zn, Mn, and Fe superoxide dismutases). Briefly, blood samples were diluted 1 : 5 in 1X PBS. The homogenate was then centrifuged at 1500g for 10 minutes at 4 °C, and the supernatant was analysed according to the manufacturer's instructions.

2.6. Statistical analysis

Data are presented as means \pm standard errors of the mean (S.E.M.) and were analysed using GraphPad Prism® software,



Table 2 Primer sequences and real-time PCR conditions

Gene	Forward	Reverse	T/°C
GADPH	CGA CTT CAA CAG CAA CTC	TGT AGC CGT ATT CAT TGT	58
HPRT	TCC ATT CCT ATG ACT GTA	CAT CTC CAC CAA TAA CTT	58
CD36	GAT GAC GTG GCA AAG AAC AG	TCC TCG GGG TCC TGA GTT AT	58
FABP1	TAC CAA TTG CAG AGC CAG GA	ACT CAT TGC GGA CCA	58
SLC25a5	GCC ACA CCT CAT TTC ATC CG	GGT TCC TTC ACA CAC AGC CTG	58
CPT1	AAG AAC ATC GTG AGT GGC GTC	AGC ACC TTC AGC GAG TAG CG	58
CPT2	ATG CTC CGA GGC ATT TGT CA	GGT CAG CTG GCC ATG GTA TT	58
SCD1	CGT CTG GAG GAA CAT CAT TCT	CAG AGC GCT GGT CAT GTA GT	58
PPAR α	GGA GTG CAG CCT CAG CCA AGT T	AGG CCA CAG AGC GCT AAG CTG T	58
ACADL	AAA CGT CTG GAC TCC GGT TC	TCC GTG GAG TTG CAC ACA TT	60
MCP-1	GCC TGC TGT TCA CAG TTG C	GGT GAT CCT CTT GTA GCT CTC C	60
MMP-9	GCC CTG GAA CTC ACA CGA CA	TTG GAA ACT CAC ACG CCA GAA G	60
SOD-1	AAC CAG TTG TGT TGT CAG GAC	CCA CCA TGT TTC TTA GAG TGA	60
SOD-2	CAG ACC TGC CTT ACG ACT ATG	CTC GGT GGC GTT GAG ATT GTT	60

version 9.0.0 (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA), followed by Tukey's test for multiple comparisons, was applied when appropriate. For gene expression analysis in liver samples, an unpaired *t*-test was performed since only two groups were compared. A *p*-value of <0.05 was considered statistically significant.

3. Results and discussion

OLEA was administered orally at a dose of 50 mg kg⁻¹ using a method specifically developed and optimized for rodents, involving a semi-solid matrix form (HaPILLness, European patent EP4142698). This treatment was given five days per week over a period of 10 weeks. Previous studies have shown that administration of OLEA to mice, at a dose of 20 mg kg⁻¹ by oral gavage, can reduce lipid accumulation and BW.^{32,33} Oral administration is generally preferred for delivering substances, drugs, and other compounds due to its simplicity and ease of use. With this administration, digestive, metabolic, and absorptive processes in the stomach and intestines may necessitate higher dosages when compared with intravenous administration to achieve similar effects. OLEA has been shown to be stable under gastric acidic conditions and may be absorbed in the small intestine *via* passive diffusion, owing to its favourable partition coefficient.⁴⁰ Indeed, aglycone derivatives of oleuropein, such as oleacein, have been shown to be rapidly absorbed and at least partially hydrolysed into HT by carboxylesterases in the small intestine of both rats and humans.^{43–48} This enzymatic conversion leads to elevated concentrations of HT in plasma, the intestinal lumen, and various tissues, including the stomach, small intestine, and liver.⁴⁴ As a result, following the ingestion of virgin olive oil (VOO), the concentrations of HT and its metabolites in the body are significantly higher than would be predicted based solely on the native HT content of the oil.⁴³ Rather, these levels appear to correlate more closely with the overall phenolic content of the oil,^{46–50} thereby reinforcing the bioavailability of oleuropein aglycones. Moreover, several studies have reported the presence of low concentrations of unmetabolized oleacein in

plasma, alongside notable quantities of glucuronide and methyl conjugates.⁴⁹ Additionally, oleacein and its hydrated glucuronides have been identified in various rat tissues following oral administration of OLEA.^{44,47} The presence of two carbonyl groups in the oleacein structure—one of which is conjugated with a double bond—confers particular chemical reactivity toward amino groups. Consequently, oleacein is also expected to form covalent adducts with plasma and cellular proteins. These complex metabolic transformations and binding interactions complicate the accurate *in vitro* assessment of the specific mechanisms by which oleacein exerts its effects *in vivo* when consumed, as well as the precise identification of the metabolites responsible for mediating these biological activities. Moreover, recent evidence suggests that some of the metabolic effects of dietary polyphenols may also be mediated locally at the intestinal level. In particular, interactions between polyphenols and the intestinal mucosa may stimulate the release of gut-derived hormones and signalling molecules involved in the regulation of lipid and glucose metabolism. This emerging mechanism highlights the potential contribution of intestinal signalling pathways to the systemic metabolic effects attributed to olive oil phenolic compounds.⁵¹ Therefore, it is essential to conduct *in vivo* studies using routes of administration that are relevant to dietary intake in order to determine whether at least part of the health benefits attributed to olive oil can be specifically ascribed to oleacein.

3.1. *In vivo* oleacein effects

3.1.1. BW, food and beverage monitoring. In general, animals fed the ATD diet exhibited significantly lower food consumption compared to those on the standard diet (*p* < 0.001 *vs.* WT + STD). However, they displayed the highest caloric intake among the groups (*p* < 0.001 *vs.* WT + STD), which can be attributed to the higher caloric density of the ATD diet. Notably, the ApoE-KO + ATD + OLEA group demonstrated increased water intake, as shown in Table 3. Although the exact reason for this effect cannot be clarified, some hypothesis could be raised. The impact of oleacein on lipid metabolism might be associated with higher metabolic rate,



Table 3 Food, beverage and energy intake per week. Results are expressed as mean \pm S.E.M. of 8 animals per group. *** p < 0.001 vs. WT + STD; ## p < 0.01 vs. WT + ATD; ■■■ p < 0.001 vs. ApoE-KO + ATD + OLEA

Per week	WT + STD	WT + ATD	ApoE-KO + ATD	ApoE-KO + ATD + OLEA
Food intake (g)	24.0 \pm 0.5	19.9 \pm 0.3***	19.9 \pm 0.8***	20.39 \pm 0.30***
Beverage intake (ml)	39.3 \pm 1.3	36.6 \pm 1.9	35.8 \pm 0.8	43.88 \pm 0.77###, ■■■
Σ Total energy (kcal)	75.7 \pm 1.5	89.7 \pm 1.1***	89.6 \pm 1.1***	91.74 \pm 1.36***

which increases respiratory water loss and water demand; furthermore, the suggested effects on lipid oxidation can cause mild osmotic diuresis, leading to compensatory polydipsia (increased drinking).⁵² In addition, the oral sensory effects caused by oleacein, which is structurally related to compounds responsible for the throat irritation of extra virgin olive oil (similar sensory pathways as oleocanthal), can occasionally cause transient dry-mouth sensation and increased drinking after dosing.^{53,54}

BW was monitored weekly over the 10-week duration of the study (Fig. 1). Animals fed the ATD diet exhibited a statistically significant increase in BW compared to the WT + STD group as early as two weeks into the study (p < 0.05), with this difference becoming more pronounced over time and remaining significant through the end of the study (p < 0.001) (Fig. 1). Oleacein supplementation did not result in a statistically significant reduction in BW in the ApoE-KO + ATD group, in contrast to the findings of Lombardo *et al.* and Lepore *et al.*, likely due to the used of genetic modified animals in the present study.^{32,33}

3.1.2. Serum lipid profile and liver lipid deposition.

Dysregulation on lipid profile is a hallmark on MAFLD. So, we analyse in serum cholesterol associated with HDL and LDL lipoproteins (c-HDL and c-LDL, respectively), as well as total cholesterol (c-Total) and triglycerides (TAGs). ApoE-KO mice have a mutation that inactivates the gene coding for ApoE and, consequently, the inactivation of this gene leads to a state of severe hypercholesterolemia, much higher than that observed in the WT mice group also fed the ATD diet.^{36,45}

The WT + ATD group exhibited increased levels of c-HDL, c-LDL, and c-Total compared to the WT + STD group (p < 0.001

vs. STD) (Fig. 2), while showing a trend toward decreased TAGs levels, though this difference was not statistically significant. As expected, the ApoE-KO + ATD group showed a significant increase in c-Total, c-LDL, and TAGs, along with a decrease in c-HDL, when compared to the WT + ATD group. Supplementation with OLEA in the ATD diet resulted in a significant reduction in both c-Total and c-LDL (p < 0.01 vs. ApoE-KO + ATD) without affecting c-HDL levels. The most striking effect of oleacein supplementation was the reduction in serum TAGs levels, bringing them closer to the values observed in the WT + STD. Furthermore, the TAGs/HDL ratio—recognized as a reliable marker for identifying

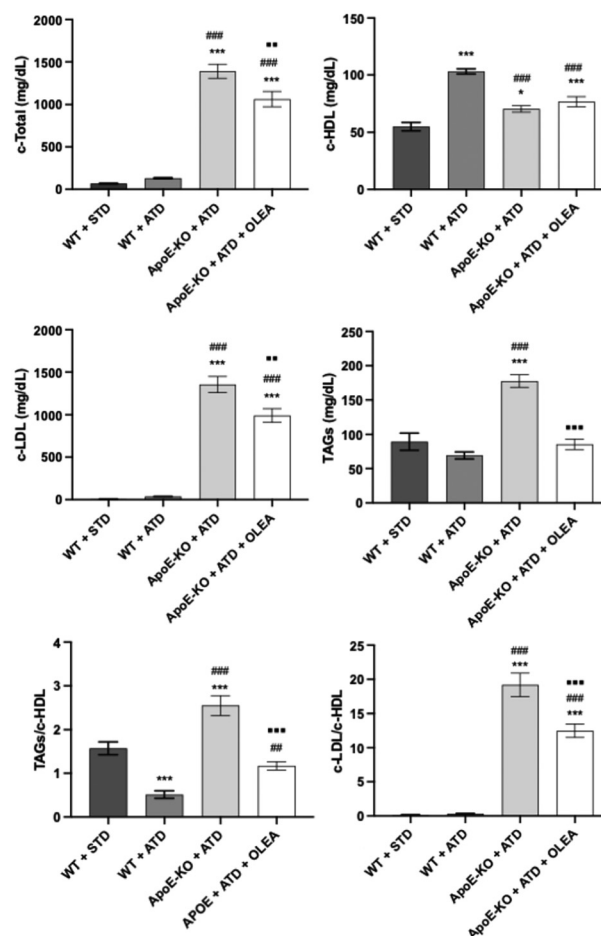


Fig. 2 Serum lipid profile. (A) c-LDL levels, (B) c-HDL levels; (C) c-Total levels, (D) TAGs levels, (E) TAGs/c-HDL ratio and (F) c-LDL/c-HDL ratio. Results are expressed as mean \pm S.E.M. (n = 6–8 per group). * p < 0.05 and *** p < 0.001 vs. WT + STD; # p < 0.01 and ## p < 0.001 vs. WT + ATD; ■ p < 0.01, ■■■ p < 0.001 vs. ApoE-KO + ATD.

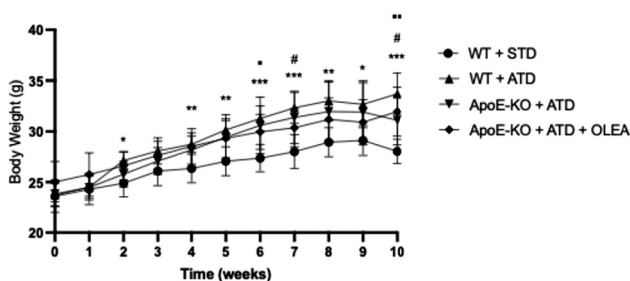


Fig. 1 Body weight evolution, in grams, of the 4 groups: WT + STD, WT + ATD, ApoE-KO + ATD and ApoE-KO + ATD + OLEA. Results are expressed as mean \pm S.E.M. (n = 8 animals per group). * p < 0.05, ** p < 0.01 and *** p < 0.001 WT + STD vs. WT + ATD; # p < 0.05 WT + STD vs. ApoE-KO + ATD; ■ p < 0.05, ■■ p < 0.01 WT + STD vs. ApoE-KO + ATD + OLEA.



individuals at high risk of coronary events and as a strong predictor of acute myocardial infarction in middle-aged women—was significantly reduced to levels comparable to those observed in the WT + STD group. This result is particularly relevant, as in humans the TAGs/HDL ratio constitutes a robust predictor of coronary artery calcification and the occurrence of first coronary events across all body mass index categories, being the sole predictor among normal-weight individuals.^{55–57}

OLEA supplementation also significantly reduced liver TAGs content ($p < 0.05$) when compared to the ApoE-KO + ATD group (Fig. 3). Although liver TAGs levels remained approximately double those observed in the WT + STD group, they decreased by more than 50% when compared to both the WT + ATD and ApoE-KO + ATD groups. These results are consistent with findings reported by Lombardo *et al.*,³² thus suggesting that oleacein primarily exerts its effects by influencing lipid metabolism.

3.1.3. Liver weight, enzymes and histomorphology.

Although statistically not significant, there was a trend to increased liver weights in the WT and ApoE-KO mice, this trend was absent in the OLEA-treated animals (Table 4). Elevated serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are commonly used as indicators of liver dysfunction and potential damage. While ALT is primarily found in the liver, AST is also present in other tissues, including cardiac and skeletal muscles, kidneys, lungs, brain, and blood cells.⁵⁸ No significant differences between the groups were observed regarding serum ALT and AST levels, as presented in Table 4. Previous studies have demonstrated that oleuropein significantly reduces the elevations in serum levels of AST, and ALT ($P < 0.05$) induced by doxorubicin (DXR), a well-known hepatotoxic agent, thereby supporting its protective effects against DXR-induced tissue

injury.⁵⁹ In agreement with these findings, in the present study, treatment with oleacein—a bioactive derivative of oleuropein—revealed that, although no statistically significant differences were observed among the three ATD groups, the ApoE-KO + ATD + OLEA group exhibited a trend toward lower ALT and AST levels compared with the ApoE-KO + ATD group. Furthermore, the oleacein dose employed in this study did not produce any signs of hepatic toxicity, reinforcing the safety of this compound under the experimental conditions used.

Representative images of H&E and Oil Red-O stained liver sections from all groups are shown in Fig. 4. Significant hepatic disturbances, characterized by fat vacuoles (indicated by black arrows), were observed in both the WT and ApoE-KO animals fed the ATD, in contrast to the normal liver profile seen in the WT + STD group (H&E). This was accompanied by increased Oil Red-O staining. Notably, Oil Red-O staining was most intense in the ApoE-KO + ATD group, followed by the WT + ATD group. In contrast, the OLEA-treated group exhibited less intense Oil Red-O staining and fewer, smaller fat vacuoles in H&E staining. These findings are consistent with the results observed for liver TAGs content, further supporting the effects of OLEA supplementation.

3.1.4. Aorta histomorphology. H&E staining images of the aorta (Fig. 5) revealed that the WT + ATD group did not develop atherosclerotic lesions (Fig. 5A and D), showing no significant changes in the vessel wall. In contrast, the ApoE-KO mice groups developed the atherosclerotic lesions described in the literature (Fig. 5B, C, E and F). Specifically, we observed endothelium denudation and foam cell formation. Both ApoE-KO + ATD groups, with and without OLEA supplementation, exhibited structural changes in the aorta, including necrotic cores and cholesterol clefts (Fig. 5E and F). While not definitive, the ApoE-KO + ATD + OLEA group showed less endothelium denudation, as well as reduced disorganization and thickening of the intima and media. However, further studies are necessary to fully evaluate the effects of oleacein on atherosclerotic plaque formation, particularly at higher doses, and additional observations are required to robustly assess its impact. Nevertheless, the significant effect on dyslipidaemia suggests a potential beneficial influence on the progression of atherosclerotic plaques.

3.2. Mechanisms underlying the *in vivo* effects of oleacein

To elucidate the mechanisms underlying the pronounced effects of oleacein on the plasma lipid profile—particularly triacylglycerol levels—and hepatic triacylglycerol accumulation, a series of studies were conducted, including glucose and

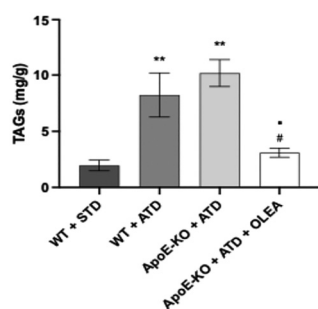


Fig. 3 Liver lipid deposition. Results are expressed as mean \pm S.E.M. ($n = 6–8$ per group). ** $p < 0.01$ vs. WT + STD; # $p < 0.05$ vs. WT + ATD.

Table 4 Liver weight (LW) and serum activities of liver enzymes ALT and AST. Results are expressed as mean \pm S.E.M ($n = 8$ animals per group). * $p < 0.05$ vs. WT + STD; # $p < 0.05$ vs. WT + ATD; ■ $p < 0.05$ vs. ApoE-KO + ATD

	WT + STD	WT + ATD	ApoE-KO + ATD	ApoE-KO + ATD + OLEA
LW (mg)	1375.8 \pm 43.8	1597.6 \pm 71.2	1559.4 \pm 114.4	1379.8 \pm 50.9
ALT (U L ⁻¹)	12.33 \pm 2.55	18.57 \pm 3.90	47.80 \pm 9.86*#	36.13 \pm 8.47
AST (U L ⁻¹)	62.60 \pm 8.81	78.57 \pm 7.67	98.20 \pm 10.35*	62.17 \pm 5.10■



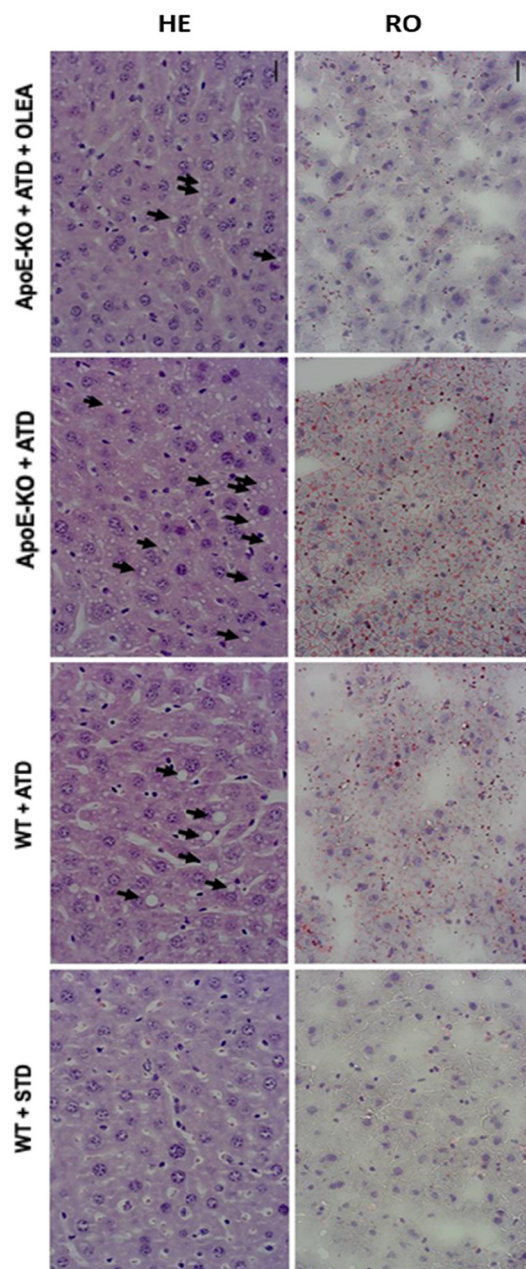


Fig. 4 Representative images of H&E and Oil Red-O staining in the liver. Black arrows depict hepatic liver deposition in fat vacuoles. Scale bar = 20 μ m.

insulin tolerance tests and the expression of mRNA of genes involved in lipid metabolism and antioxidant defence.

3.2.1. Glucose tolerance test (GTT) and insulin tolerance test (ITT). A GTT was conducted during the first and final weeks of the study (Fig. 6). In the first week, all groups—including the WT + STD group—demonstrated a comparable ability to regulate blood glucose levels following the glucose bolus administered during the test. A similar pattern was observed across all groups after 10 weeks on their respective

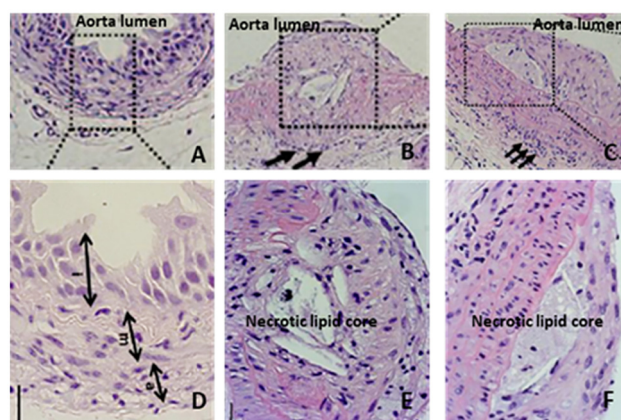


Fig. 5 Images of H&E staining in aorta rings. (A) and (D), aorta ring of the WT + ATD group, with the identification of the adventitia (a), media (m) and intima (i) layers; (B) and (E), aorta ring of the ApoE-KO + ATD group with atherosclerotic lesions; (C) and (F), aorta ring of the ApoE-KO + ATD + OLEA group. Squares in (B) and (C) indicate a necrotic core and cholesterol cleft; black arrows indicate inflammatory cell infiltration (scale bar = 20 μ m).

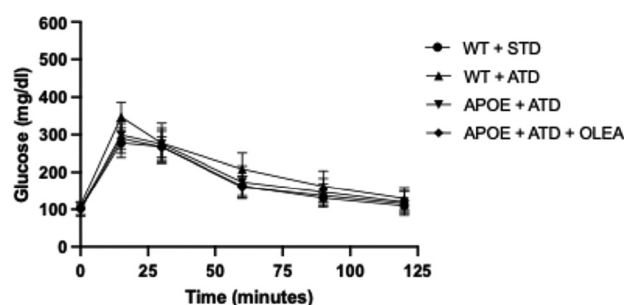


Fig. 6 Evolution of blood glucose values between 0 and 120 minutes after intraperitoneal injection of glucose solution (1.5 g per kg BW, GTT assay).

diets, indicating no significant differences in glucose handling over the course of the study.

Accordingly, the differences between the AUC values for weeks 1 and 10 do not show statistical significance (Fig. 7). Nevertheless, the absence of OLEA supplementation in the APOE-KO + ATD group was associated with a trend toward elevated fasting blood glucose levels after 6 hours of fasting at week 10 ($p = 0.07$), suggesting improved glycaemic control in the OLEA-supplemented group (Fig. 7).

A similar pattern was obtained for the ITT. All groups manage to recover the glucose levels after 2 hours (weeks 1 and 10), confirmed by the ITT AUC (Fig. 8 and 9).

The hypothesis proposed by previous studies—that oleacein can prevent metabolic dysfunction by improving insulin sensitivity and reducing circulating insulin levels—does not appear to align with the results observed in this study. Therefore, based on the data obtained, it is evident that the mechanism by which oleacein prevents dyslipidemia in treated mice is not likely related to the amelioration of insulin resistance. Thus,



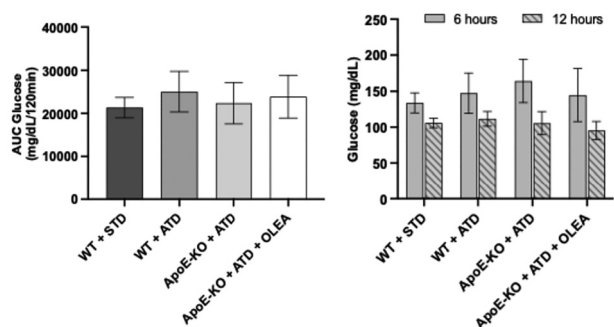


Fig. 7 AUC of GTT assay and glucose values after 6 h and 12 h fasting at week 10. Results are expressed as mean \pm S.E.M. of 4–8 animals per group.

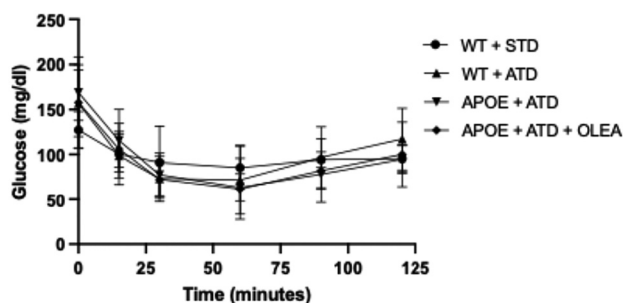


Fig. 8 Insulinemic profile. Evolution of blood glucose values between 0 and 120 minutes after intraperitoneal injection of insulin solution (0.5 U per kg BW, ITT assay). Results are expressed as mean \pm S.E.M. of 4–8 animals per group.

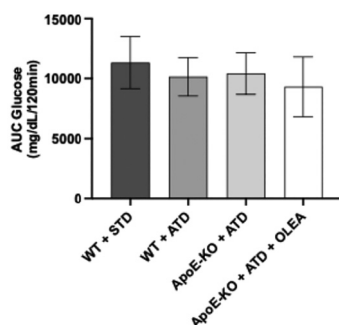


Fig. 9 AUC values of ITT assay (W1 and W10, respectively). Results are expressed as mean \pm S.E.M. of 4–8 animals per group.

oleacein does not appear to exert its beneficial effects through modulation of glucose levels, at least to a significant extent. The effects on glucose levels observed in a previous study in WT mouse may have been a consequence of oleacein's antidi-lipidemic activity rather than its cause.³²

3.2.2. SOD mRNA expression and activity in the liver and serum. To assess antioxidant capacity, the mRNA expression of SOD1 and SOD2 genes in the liver, as well as SOD enzymatic activity in both liver and serum, were evaluated. The WT + ATD group exhibited a non-significant decrease in hepatic SOD1

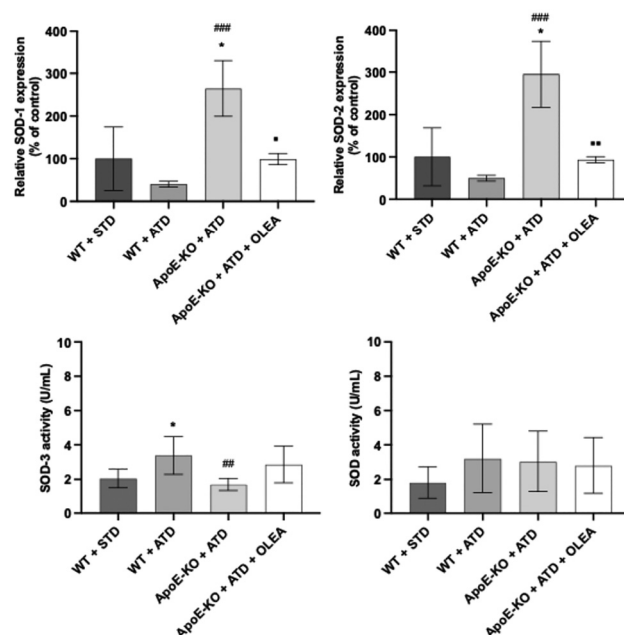


Fig. 10 SOD-1 (A) and SOD-2 (B) expression in the liver and SOD activity in the liver (C) and in the serum (D). Results are expressed as mean \pm S.E.M. of 6–8 animals per group. * p < 0.05 vs. WT + STD; ### p < 0.01 and #### p < 0.001 vs. WT + ATD; ■ p < 0.05 vs. ApoE-KO + ATD.

and SOD2 expression compared to the WT + STD group (p < 0.5), as shown in Fig. 10, but demonstrated significantly increased SOD activity in both liver and serum (p < 0.05 vs. STD). In contrast, the ApoE-KO + ATD group showed significantly higher SOD-1 and SOD2 gene expression levels compared to all other groups (p < 0.05 vs. WT + STD), while SOD activity in serum was significantly decreased (p < 0.01 vs. WT + ATD; Fig. 10). These findings are consistent with previous reports suggesting that increased SOD gene expression in ApoE-deficient mice may represent a compensatory response to elevated oxidative stress resulting from the absence of ApoE.¹⁰ As noted, ApoE deficiency induces hyperlipidemia, which is associated with increased oxidative stress. The ApoE-KO mice fed the ATD diet showed markedly reduced serum SOD activity (p < 0.001 vs. control group), supporting earlier observations.^{60,61}

Interestingly, oleacein supplementation normalized SOD-1 and SOD-2 mRNA expression to levels comparable with those of the WT + STD group and significantly increased serum SOD activity. This enhancement may contribute to the overall improvement in antioxidant defence capacity. Although SOD expression and activity are widely used indicators of antioxidant response, it should be noted that they represent indirect markers of oxidative status. The primary objective of the present work was to investigate the effects of oleacein on hepatic lipid metabolism and dyslipidaemia in the ApoE-KO model, with particular emphasis on molecular mechanisms related to lipid synthesis and accumulation. Consequently, the experimental design prioritized the evaluation of lipid metabolic pathways and their regulatory genes rather than a com-



prehensive characterization of oxidative stress biomarkers. Previous studies have also demonstrated the antioxidant benefits of some olive oil polyphenols in ApoE-KO mice, including reduced levels of oxidative stress markers such as F2-isoprostanes in serum.^{61,62} Additionally, other olive oil phenolics—such as oleocanthal and hydroxytyrosol—have been shown to enhance the activity of key antioxidant enzymes, including glutathione reductase (GSR), glutathione S-transferase (GST), and catalase (CAT), while simultaneously reducing various oxidative stress markers.^{12,63} Future studies including direct oxidative stress markers may further clarify the contribution of redox modulation to the biological effects of oleacein.

3.2.3. Expression of genes involved in lipid metabolism.

According to the literature, molecular components involved in the action of oleacein include key regulators of hepatic lipid metabolism, such as the transcriptional activator SREBP-1 and its target FAS, two lipogenic factors, as well as the inhibition of p-ERK in liver tissue.^{33,34} Some studies have also linked these hepatic markers to reduced weight gain associated with phenolic compounds.³³ To investigate the potential mechanisms underlying the lipid-lowering effects of OLEA, we analysed the expression of other genes involved in hepatic lipid metabolism.

While no statistically significant differences were observed between the untreated and OLEA-treated groups for most genes, a significant downregulation of SCD1 ($p = 0.0345$), FABP1 ($p = 0.0365$) and CPT1 ($p = 0.0365$) was detected. In addition, there was a trend toward reduced expression of MMP9 ($p = 0.551$) in the OLEA-treated group (Fig. 11).

The marked inhibition of SCD1 observed in our study—a key enzyme involved in *de novo* lipogenesis and triglyceride synthesis⁶⁴—strongly supports a reduction in hepatic lipid synthesis. This finding is in line with previous reports showing that SCD1 deficiency in mice leads to beneficial metabolic adaptations, including suppressed lipogenesis and enhanced β -oxidation.⁶⁴ Although we did not study the expression of upstream regulatory genes, previous studies of Lombardo *et al.*³² have demonstrated that oleacein supplementation downregulates SREBP-1, a central lipogenic transcription factor that regulates genes including SCD1.⁶⁴ Thus, the suppression of SCD1 expression in our model may reflect downstream effects of similar regulatory mechanisms, reinforcing the proposed role of OLEA in attenuating hepatic lipid accumulation through inhibition of lipogenesis.

The decrease in FABP1 expression is particularly relevant, as liver-specific knockdown of FABP1 has been associated with reduced liver weight and hepatic triglyceride accumulation. This effect is thought to result from both decreased fatty acid uptake and reduced intracellular fatty acid transport. Moreover, FABP1 deficiency has been shown to downregulate the expression of ACC and DGAT—key enzymes involved in fatty acid synthesis and triglyceride formation, respectively—ultimately suppressing *de novo* lipogenesis.⁶⁵ Thus, the observed reduction in FABP1 expression may contribute to the attenuation of hepatic steatosis in our model.

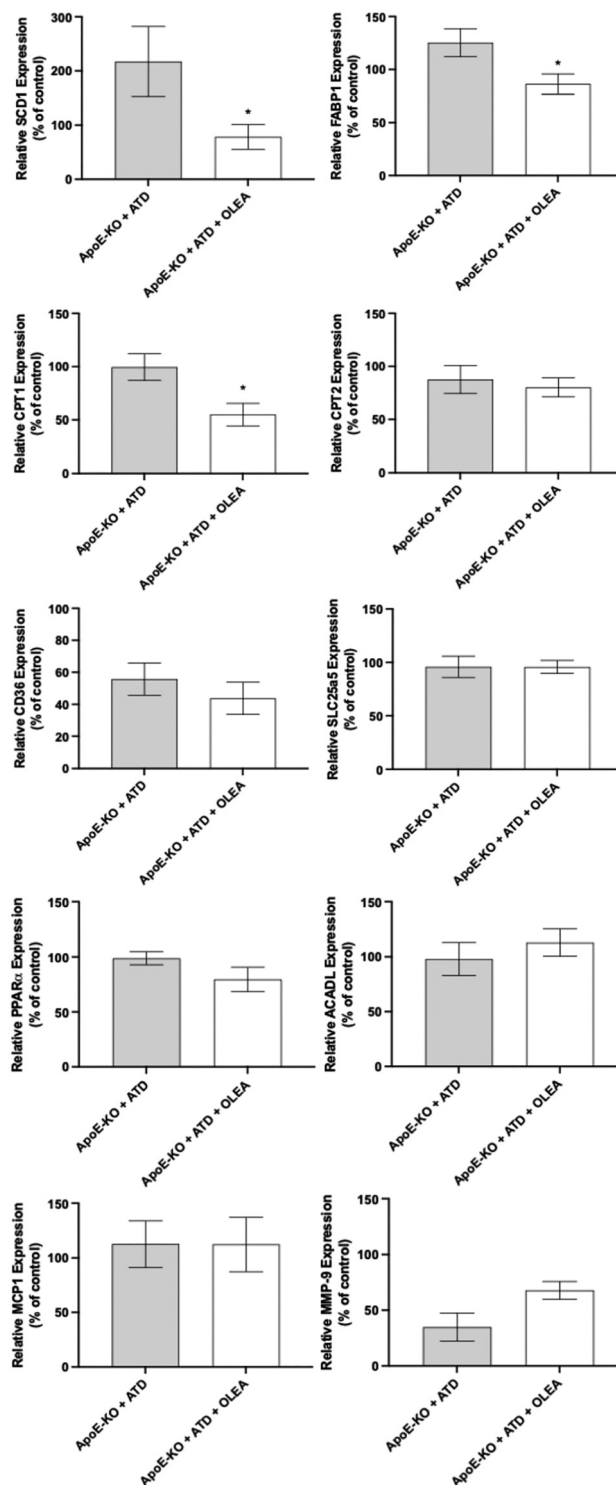


Fig. 11 SCD1, FABP1, CPT1, CPT2, CD36, SCL2a5, PPAR α , ACADL, MCP1 and MMP-9 expression from liver samples. Results are expressed as mean \pm S.E.M. of 8 animals per group.

Interestingly, although CPT1 downregulation is typically linked to impaired mitochondrial β -oxidation and increased lipid accumulation, the OLEA-treated mice showed reduced hepatic fat content. This apparent paradox might be explained



by enhanced CPT1 enzyme activity due to lower levels of malonyl-CoA—a known inhibitor of CPT1—or by compensatory upregulation of downstream β -oxidation enzymes.⁶⁶ Supporting this hypothesis, a modest increase in ACADL expression, which functions downstream of CPT1, was observed, suggesting a partial preservation or compensation of mitochondrial oxidative capacity.⁶⁶

Additionally, the mild upregulation of MMP9 ($p = 0.0551$), a matrix metalloproteinase involved in extracellular matrix remodelling and cytokine activation, may reflect adaptive tissue remodelling rather than deleterious inflammation (Fig. 11). Indeed, cell-specific overexpression of MMP9 has shown protective effects in certain contexts, although the precise mechanisms remain incompletely understood.⁶⁷

From a nutritional perspective, these findings further support the concept that phenolic compounds naturally present in extra virgin olive oil may contribute to the metabolic benefits traditionally associated with olive oil-rich dietary patterns such as the Mediterranean diet. It should be acknowledged that the experimental dose of oleacein used in this study is unlikely to be achieved through normal dietary consumption of olive oil and would more closely resemble a nutraceutical supplementation scenario. Nevertheless, the long-term and regular intake of olive oil across the lifespan may provide sustained exposure to a complex mixture of bioactive phenolics, whose cumulative biological effects on lipid metabolism and redox balance could contribute to the prevention of cardiometabolic disorders. From a clinical perspective, oleacein therefore emerges as a promising olive oil-derived bioactive compound that may merit further investigation as a nutraceutical strategy for the prevention or early management of dyslipidaemia-associated hepatic steatosis, although controlled studies in humans will be required to confirm efficacy and define appropriate intake levels.

Overall, our findings suggest that OLEA exerts a coordinated modulation of hepatic lipid metabolism, characterized by reduced fatty acid uptake, inhibition of *de novo* lipogenesis, and preservation or enhancement of fatty acid β -oxidation. These combined effects likely contribute to the improvement of hepatic metabolic homeostasis through multiple, complementary mechanisms.

4. Conclusions

Our study confirmed the hyperlipidemic effects of a refined atherogenic diet (ATD) in both WT and ApoE-KO mice, as demonstrated by significant increases in serum total cholesterol (c-Total), HDL cholesterol (c-HDL), LDL cholesterol (c-LDL), triglycerides (TAGs), and hepatic TAG content. Notably, OLEA supplementation exerted a marked anti-dyslipidemic effect in ApoE-KO mice fed the ATD. This was evidenced by a significant reduction in serum c-Total and c-LDL levels, as well as a pronounced decrease in serum TAGs and hepatic lipid accumulation. These biochemical findings were corroborated by histological analyses (H&E and Oil Red O staining),

which revealed that OLEA effectively prevented the development of hepatic steatosis. At the molecular level, a strong down-regulation of SCD1 and FABP1 was observed, suggesting an inhibition of *de novo* triglyceride synthesis as a key mechanism underlying the reduced hepatic lipid accumulation. Additionally, an increase in serum SOD levels was detected in OLEA-supplemented mice, consistent with a reduction in oxidative stress. These findings are consistent with previous evidence showing that hydroxytyrosol—one of the metabolites derived from oleacein—can ameliorate metabolic disturbances induced by high-fat diets through the modulation of key transcription factors involved in oxidative stress, inflammation and lipid metabolism. In particular, hydroxytyrosol supplementation has been shown to restore the activity of Nrf2 and PPAR- γ while attenuating the activation of NF- κ B and SREBP-1c, leading to reduced oxidative damage, decreased lipogenesis and improved metabolic function in adipose tissue.^{68,69}

Although this study did not fully elucidate the potential anti-atherosclerotic effects of OLEA, the robust lipid-lowering and antioxidant properties observed suggest a promising role in the prevention of atherosclerosis. Importantly, the beneficial effects do not appear to be primarily mediated by improvements in glucose tolerance and insulin resistance. Rather, it is plausible that the anti-dyslipidemic action of OLEA indirectly contributes to improved insulin sensitivity, as reported in a previous study.³² Furthermore, the observed effects seem to involve modulation of gene expression related to both β -oxidation and triglyceride synthesis.

Overall, these findings support the therapeutic potential of OLEA in the management of diet-induced dyslipidaemia and hepatic steatosis, while underscoring the need for further studies to clarify the molecular mechanisms involved and to assess dose-dependent responses.

Author contributions

Conceptualization: FPM and FR; methodology: FPM, SV and FR; formal analysis: FPM, FR, SV, VC and BO; investigation BO, VC, PV, AA; data curation: PV, AA, VC and BO; validation: FPM and FR; writing FPM, FR, VC and BO; project administration: FPM; supervision: FPM, FR, SV.

Conflicts of interest

There are no conflicts to declare.

Data availability

The data supporting this article have been included as part of the supplementary information (SI). Supplementary information is available. See DOI: <https://doi.org/10.1039/d5fo05134g>.



Acknowledgements

This work received financial support from the PT national funds (FCT/MECI, Fundação para a Ciência e Tecnologia and Ministério da Educação, Ciência e Inovação) through the strategic projects UID/50006/2025 from the Laboratório Associado para a Química Verde – Tecnologias e Processos Limpos, UIDP/04539/2025 and UIDB/04539/2025 from the Center for Innovative Biomedicine and Biotechnology (CIBB), and project PTDC/OCE-ETA/32492/2017-POCI-01-0145-FEDER-032492-PHENOLIVA, as well as funding from the European Union's Horizon Europe program (Excellence Hubs – HORIZON-WIDERA-2022-ACCESS-04-01), under the grant agreement #101087071. VC, PV and AA thanks FCT for the doctoral grants (2022.12302.BD, 2022.13182.BD and 2020.09481.BD, respectively).

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