# Effects of phenol-enriched olive oils on HDL and endothelial functions in cardiovascular risk individuals


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**DOCTORAL THESIS**

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# ANNEX

# 9. ANNEX

# Annex I: Publication nº 4

## Effect of phenol-enriched olive oils on HDL functionality in hypercholesterolemic subjects. A randomised, double-blind, crossover, controlled trial. The VOHF study.

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***SUBMITTED TO EDITOR***

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## Abbreviations:

ApoA-I: apolipoprotein A-I

CHD: coronary hearth disease

CoQ: coenzyme Q

EC: esterified cholesterol

FC: free cholesterol

FVOO: functional virgin olive oil

FVOOT: functional virgin olive oil with thyme

OO: olive oil

PA: physical activity

PC: phenolic compounds

PL: phospholipid

RCT: reverse cholesterol transport

TC: total-cholesterol

TG: triglyceride

UHPLC-ESI-MS/MS: high performance liquid chromatography-electrospray tandem mass UPLC/MS/MS: ultra-performance liquid chromatography coupled to tandem mass spectrometry
VOO: virgin olive oil

**Keywords:** Functional virgin olive oil, HDL functionality, cholesterol efflux, HDL antioxidants, HDL fluidity.

**ABSTRACT**

**Background:** High-density lipoprotein (HDL) functionality is currently considered to be a more important issue than the HDL-cholesterol circulating quantity. Consumption of olive oil phenolic compounds has beneficial effects on lipid profile and HDL function. Functional foods with complementary-antioxidants, according to their structure/activity relationship, could be a suitable option to obtain additional protective effects.

**Objective:** Our aim was to study whether enriched virgin olive oils, one enriched with its own phenolic-compounds (FVOO) and another with them plus additional complementary phenolic-compounds from thyme (FVOOT), could improve HDL functions.

**Design:** 33 hypercholesterolemic volunteers participated in a randomized, double blind, crossover, and controlled trial. Subjects ingested 25 mL/day of: 1) virgin olive oil (80 ppm), 2) FVOO (500 ppm), and 3) FVOOT (500 ppm, 250 from olive oil and 250 from thyme), for 3-weeks preceded by 2-week wash-out periods. Lipid-protein composition, antioxidant content, antioxidant capacity and oxidation resistance, monolayer fluidity, and cholesterol efflux capacity of HDL particle were analysed.

**Results:** An increase in HDL compounds with antioxidant activity was observed after both functional olive oil interventions ($p<0.05$). Ubiquinol, β-criptoxanthin, and lutein increased in HDL after both functional olive oil interventions ($p<0.05$). Retinol and hydroxytyrosol acetate sulphate increased in HDL only after FVOO ($p<0.05$). α-tocopherol, hydroxyphenylpropionic

acid sulphate, caffeic acid sulphate, and thymol sulphate only increased in HDL after FVOOT ($p < 0.05$). After FVOOT, HDL oxidation resistance and cholesterol efflux tended to increase versus its baseline ($p < 0.09$). In addition, cholesterol efflux increased after the FVOOT versus the FVOO one ($p < 0.05$). No changes were observed in the other analysed parameters.

**Conclusions:** Long-term consumption of phenol-enriched olive oils induced a better HDL antioxidant status. In addition, the enrichment of VOO with complementary phenols from olive and thyme (FVOOT) enhanced HDL cholesterol efflux capacity and HDL oxidation resistance, changes related with a more functional HDL with greater cardioprotective properties.

**Word count:** 300

## INTRODUCTION

Olive oil (OO) phenolic compounds (PC) have shown to promote beneficial effects on lipid profile, endothelial function, thrombosis, and haemostasis in humans [1-3]. Some studies demonstrated that OOPC could prevent coronary heart diseases (CHD), especially in humans with oxidative stress [4]. HDL-cholesterol (HDL-C) levels are inversely and independently related with cardiovascular disease (CVD) [5], so future pharmacological and natural product developments have been oriented to increase HDL-C concentrations. Nevertheless, the ineffectiveness or increased mortality risk of cholesteryl ester transfer protein (CETP) antagonists were observed in clinical trials [6-8], together with recent evidences that some genetic variables predisposing to high HDL-C levels are not associated with lower risk of suffering a coronary event [9], have resulted in the consideration that future therapeutic approaches should improve HDL functionality instead of its quantity [10]. Reverse cholesterol transport (RCT) is the main HDL biological function, which consist in extracting the excess of cholesterol from the peripheral cells (cholesterol efflux) and taking it to the liver for its further metabolism and excretion. This HDL

functional property has been tested in macrophage cell lines and has been inversely related to early atherosclerosis development and to high risk of experiencing a coronary event [11,12]. Moreover, HDL has other protective functions against the development of atherosclerosis: its antioxidant properties towards LDL [13], and its ability to protect the integrity of vascular endothelium [14,15]. HDL functions can be altered due to HDL physicochemical changes and inflammatory protein binding. These modifications can transform it into a dysfunctional particle [16-18]. Pharmacotherapy or functional foods oriented to improve HDL oxidative-inflammatory status would protect against the transformation into dysfunctional HDL.

Virgin olive oil (VOO) intervention trials have demonstrated some beneficial effects in HDL and lipid profile. The EUROLIVE study reported an increase of HDL-C concentration, and a decrease in *in vivo* lipid oxidative damage, in a dose-dependent manner with the PC-content of the OO administered [1]. In this context, in a subsample, we provided for the first-time, first-level evidence that VOO enhances (i) the cholesterol efflux, (ii) the HDL monolayer fluidity, and (iii) the HDL PC-content HDL, in healthy humans [19]. In addition, our group reported that OOPC enhance cholesterol efflux-related genes at post-prandial state [20].

PC-enriched foods could increase the healthy effects of some beneficial compounds without raising the fat content. However, enrichment with only a single antioxidant may have a dual action because antioxidants could also revert to pro-oxidants depending on the dose [21-23]. Functional foods with complementary-antioxidants, according to their structure/activity relationship, could be a suitable option to obtain greater beneficial health effects. Our aim was to test, in a randomized, double-blind, cross-over, and controlled trial, whether enriched VOOs, one enriched with its own PC (FVOO;500ppm from OO) and another with them plus complementary-PC from thyme (FVOOT;

250ppm from OO and 250 ppm from thyme), could improve HDL functionality, particularly antioxidant and cholesterol efflux functions.

## SUBJECTS AND METHODS

### *OO preparation and characteristics*

VOO with a low-phenolic content (80 ppm or mg total phenols/kg oil) was used as a control condition and as a matrix of enrichment to prepare two phenol-enriched OOs (FVOO and FVOOT;500 ppm). FVOO was enriched with its own PCs by addition of a phenol extract obtained from freeze-dried olive cake; and FVOOT was enriched with its own PC and complemented with thyme phenolics using a phenol extract obtained from a mixture of freeze-dried olive cake and dried thyme. The procedure to obtain the phenolic extracts and the enriched oils has been previously described [24]. Hence, FVOOT contained 50% of olive PC and 50% of thyme phenolics **(Supplemental material. Figure 1)**. The procedure to obtain the phenolic extracts and the enriched oils has been previously described [24]. For the wash-out period a common OO was used. The total phenolic-content of OOs was determined by Folin-Ciocalteau method [25]. The phenolic-composition of the OOs was analyzed by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC/MS/MS) [26].

### *Study design*

The VOHF-study was a randomized, double-blind, crossover, controlled trial with 33 hypercholesterolemic volunteers (total-cholesterol>200 mg/dL) (19 men), aged 35 to 80. Exclusion criteria included the following: BMI>35 Kg/m$^2$, smokers, athletes with high-physical activity (PA) (>3000 Kcal/day), diabetes, multiple allergies, intestinal diseases, or any other disease or condition that would worsen adherence to the measurements or treatment. The study was conducted at IMIM-Hospital del Mar Medical Research Institute (Spain) from April to September 2012.

Subjects were randomized to one of 3 orders of administration of raw OOs (1-VOO, 2-FVOO,3-FVOOT): Sequence1-FVOO,FVOOT,VOO; Sequence2-VOOT,VOO,FVOO; Sequence3-VOO,FVOO,FVOOT. The random allocation sequence was generated by a statistician, participant enrolment was carried out by a researcher, and participants' assignment to interventions according to the random sequence was done by a physician. Due to the fact that all participants received each one of the three VOOs, restrictions such as blocking were unnecessary. Intervention periods were of 3-weeks with an ingestion of 25 mL/day raw OO distributed along meals and preceded by 2-week wash-out periods with a common OO.

To avoid an excessive intake of antioxidants and PCs during the clinical trial period, participants were advised to limit the consumption of polyphenol-rich food. PA was evaluated by a Minnesota questionnaire at baseline and at the end of the study. A set of portable containers with the corresponding 25 mL of OO for each day of consumption were delivered to the participants at the beginning of each administration period. The participants were instructed to return the containers to the Centre after the corresponding OO consumption period in order to register the amount of OO consumed. Subjects with less than 80% treatment adherence (≥5 full OO containers returned) were considered non-compliant for this treatment.

The present clinical trial was conducted in accordance with the Helsinki Declaration and the Good Clinical Practice for Trials on Medical Products in the European Community. All participants provided written informed consent, and the local institutional ethics committees approved the protocol (CEIC-IMAS 2009/3347/I). The protocol is registered with the International Standard Randomized Controlled Trial register (www.controlled-trials.com;ISRCTN77500181) and followed CONSORT-guidelines.

***Dietary adherence***

24h-urine was collected at the start of the study and before and after each treatment. Urine samples were stored at -80ºC prior to use. We measured

urinary hydroxytyrosol-sulfate and thymol-sulfate, as biomarkers of adherence to the type of OO ingested in urine, by high performance liquid chromatography-electrospray tandem mass spectrometry (UHPLC-ESI-MS/MS) [27]. A 3-day dietary record was administered to the participants at baseline and before and after each intervention-period. A nutritionist personally advised participants to replace all types of habitually consumed raw fats with the OOs, and to limit their polyphenol-rich food consumption.

***Systemic biomarker analyses***

Blood samples were collected in a fasting state of at least 10 hours, at the start of the study and before and after each treatment. Plasma samples were obtained by centrifugation of whole blood directly after being drawn and were preserved at -80ºC until use. EDTA-plasma glucose, total-cholesterol (TC), and triglyceride (TG) levels were measured using standard enzymatic automated methods; and a polipoprotein A-I (ApoA-I) and ApoB100 by immunoturbidimetry in a PENTRA-400 autoanalyzer (ABX-Horiba Diagnostics,Montpellier,France). HDL-C was determined by an accelerator selective detergent method (ABX-Horiba Diagnostics). LDL-C was calculated by the Friedewald equation whenever TGs were <300 mg/dL. Plasma oxidized LDL (oxLDL) was analyzed by using ELISA (Mercodia AB, Uppsala, Sweden).

***HDL isolation and lipid-protein analyses***

HDL from the study volunteers of the study were isolated by a density gradient ultracentrifugation method [28] using prepared solutions of 1.006 and 1.21 density. TC, free-cholesterol (FC), and phospholipids (PL) in HDL were quantified by using automatic enzymatic methods (Spinreact,Barcelona,Spain). Esterified-cholesterol (EC) was calculated by subtracting FC from TC. TGs were determined in these samples by automatic enzymatic methods (ABX-Horiba Diagnostics, Montpellier, France). Apo-AI and Apo-AII were determined by automatic immunoturbidimetric methods (ABX-Horiba Diagnostics, and Spinreact, repectively). To assure the purity of HDL

fractions, ApoB100 and albumin levels were also determined in these samples by an automatic immunoturbidimetric methods (ABX-Horiba Diagnostics).

***HDL fatty acid analyses***

Lipids from HDL were transesterified by incubation of 5 mg of lyophilized HDL sample in 2 mL of methanol/acetyl chloride (93:7 v/v) at 75ºC for 90 min. After methanolysis 1 mL of saturated NaCl solution was added to stop the reaction and 0.75 mL of hexane were added to extract the fatty acid methyl esters. After 5 min of vortex, samples were centrifuged at 2212 g for 10 min and the supernatant was injected into the chromatographic system. The analysis of fatty acids was performed by gas chromatography (GC) (Agilent 7890A Series) using a capillary SP-2330 column (30 m x 0.25 mm x 0.2 μm) (Supelco, Bellefonte, USA), coupled to a flame ionization detector (FID). The column temperature was programmed at 100ºC rising by 8 ºC/min until it reached 200ºC then 3 ºC/min to 225ºC (total run time 23.8 minutes). Helium was the carrier gas (2 mL/min). Injection was carried out with a split injector (1:30) at 250ºC, detector temperature was 260ºC and 1 μL of the solution was injected into the GC/FID system. The identification and the relative percentage (area %) of the fatty acids were determined, in duplicate, using a reference mixture of methyl esters of fatty acids (Sigma-Aldrich, St. Louis, MO, USA).

***Analyses of HDL compounds with antioxidant properties***

*Fat-soluble antioxidants:*

All sampling procedures were performed under low ambient light conditions. For sample pre-treatment, 400 μL of HDL was added to 400 μL of ethanol containing internal standard (α-tocopherol acetate 100 mg/L) and butylated hydroxytoluene (BHT) (0.063%). Hexane phases were completely evaporated to dryness at room temperature under a nitrogen stream. The residue was re-dissolved in 75 μL of methanol and the fat-soluble antioxidants (carotenoids, retinol, ubiquinol, and tocopherols) were analyzed by liquid chromatography (HPLC) the same day of extraction. The HPLC system was made up of a Waters 717 plus Autosampler, a Waters 600 pump, a Waters 996 Photodiode

Array D etector a nd a Waters 2 475 F luorescence D etector m anaged by Empower software (Waters Inc., Milford, MA). A 150x4.6 mm i.d. YMC C30 analytical c olumn ( 3 μm) (Waters I nc., Mi lford, M A) w as u sed for t he separation of all components and HPLC analysis was performed following the procedure of Gleize et al. 2007 [29]. All compounds were identified by their retention time compared with pure standards, or, when unavailable (lutein and β-cripthoxanthin), w ith c ompounds obtained and pur ified i n t he l aboratory based on t he method of M inguez-Mosquera e t a l. (1992) [30], t he concentrations o f w hich were de termined by s pectrophotometry us ing t he molecular e xtinction c oefficient ( ε) of t he molecule. Ubiquinol, t he r educed form of Coenzyme Q10 detected in HDL, was quantified with the calibration curve of ubiquinone standard (oxidized form) using a correction factor (200:1) that was previously defined [31]. For the plasma quantification of each analyte, five-point standard curves were constructed with stock solutions individually prepared with appropriate solvents (correlation coefficients <0.99). They were run in duplicate.

*Phenolic and monoterpene metabolites:*

The phenolic and m onoterpene b iological m etabolites w ere e xtracted f rom HDL b y s olid-phase e xtraction (SPE) system us ing O ASIS H LB 60 m g cartridges (Waters C orp., Mi lford, M A). E xtractions w ere p erformed by loading 500 μL of HDL sample which had previously been mixed with 500 μL of d istilled w ater a nd 60 μL of pho sphoric a cid 85% to b reak t he bon ds between the pr oteins and p henolic c ompounds, a nd 10 0 μL o f c atechol as internal standard (IS). The retained phenolic compounds were eluted using 3 mL of methanol, which was evaporated to dryness under nitrogen flow. Prior to chromatographic a nalysis, t he s ample was r econstituted with 50 μL of methanol, prior t o c hromatographic a nalysis. T he a nalysis of the phenolic metabolites was carried out by UPLC/MS/MS based on the method described by R ubió, S erra, et a l. (2012) [32]. The s elected i on m onitoring ( SRM)

transitions, cone v oltage, a nd collision energy v alues w ere p reviously optimized in plasma for each phenol metabolite [27]. Only 6 were detected in HDL a mong a ll analysed p henolic m etabolites **(Supplemental material. Table 1)**. Most of the PC (mainly the native structures present in oils) were not found in H DL samples, t hus, quantification w as no t u ndertaken. The metabolites hydroxytyrosol sulfate (sulfHT) and thymol sulfate ( sulfTHY) were quantified, the rest o f t he metabolites, because t o t he l ack of r eference standards, w ere t entatively qua ntified w ith the c alibration c urves corresponding to their phenolic precursors or to similar metabolite compounds. In this r egard, the s ulfate c onjugates d erived from hydroxytyrosol, hydroxytyrosol acetate s ulfate (sulfHTAc) a nd homovanillic a lcohol s ulfate (sulfHVAlc) were quantified with the calibration curve of sulfHT. Caffeic acid sulfate ( sulfCA) a nd hydroxyphenylpropionic a cid s ulfate ( sulfHPPA) were tentatively q uantified b y us ing t he calibration curve of caffeic acid and 3 -(4-hydroxyphenyl)propionic a cid, respectively. A ll c alibration c urves w ere performed in HDL sample matrix. All analyses were run in duplicate.

***Direct measurement of HDL antioxidant capacity***

Prior t o e ach m easurement, H DL sam ples an d L DL f rom a p ool-control w ere defrosted a nd d ialyzed a gainst phos phate-buffered s aline, pH 7.4 a t 4° C, by passing t hrough a c olumn ( PD-10 D esalting C olumns, G E H ealthcare). A fter that, cholesterol content for each HDL and LDL sample was measured, in order to a djust f inal c oncentration. F inal c oncentrations o f 3 m g/dL f or H DL a nd 9 mg/dL for L DL were c hosen t o m aintain a phy siological r atio of 1 :3 f or H DL and LDL, respectively, comparable to conditions in the human body [33]. HDL and L DL co ncentrations were p laced in a 9 6-well p late in dup licate and $CuSo_4$ solution w as a dded a s ox idizing a gent ( at a f inal concentration of 5 µ M). Absorbance was registered, in duplicate, at 234 nm at 37°C each 5 minutes for 8 hours us ing a n I NFINITE M 200 r eader (Tecan G roup L td., Männedorf, Switzerland).

***Direct measurement of HDL resistance against oxidation***

Prior to each measurement, HDL samples were defrosted and dialyzed against phosphate-buffered saline, pH 7.4 at 4°C, by passing through a column (PD-10 Desalting Columns, GE Healthcare). After that, cholesterol content for each HDL sample was measured, in order to adjust final concentration. The final concentration of cholesterol in each well of the 96-well plate was 3 mg/dL and $CuSo_4$ solution was added as oxidizing agent (at a final concentration of 5 µM). Absorbance was measured, in duplicate, at 234 nm, 37°C each 5 minutes for 8 hours using an INFINITE M200 reader (Tecan Group Ltd., Männedorf, Switzerland).

***HDL monolayer fluidity determination***

The fluidity of the HDL particle was measured based on the determination of the steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), as previously described [34]. In brief, HDL fractions were incubated with DPH 1µM for 30 minutes at room temperature in constant agitation. After that, samples with the DPH probe were stimulated with a vertically polarized light at 360 nm. Fluorescent emission intensities were detected at 460 nm, in duplicate, in a Perkin-Elmer LS5OB spectrofluorometer (Perkin Elmer, Waltham, MA, USA), through a polarizer orientated in parallel and perpendicular to the direction of polarization of the emitted beam. Subsequently, we were able to measure the intensities of the perpendicular polarized fluorescence produced by the probe (*Ip*), which could vary depending on the sample fluidity. The steady-state fluorescence anisotropy (*r*) was calculated with these *Ip* values, and with the grating correction factor of the monochromator (*G*), using the following formula: $r = (Ivv-GIvh)/(Ivv+2GIvh)$. The steady-state anisotropy refers to the rigidity of the sample, therefore the inverse value of this parameter ($1/r$) is the fluidity index.

***HDL cholesterol efflux capacity determination***

Murine J-774A.1 monocytes were seeded at a density of 75000 cells/cm$^2$ and routinely grown for 24 hours in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Spain) supplemented with 10% heat-inactivated Fetal Bovine Serum

(FBS; BioWest, France), 100 U/mL penicillin (BioWest, France), and 100 mg/mL streptomycin (BioWest, France), at 5% $CO_2$, 95% $O_2$, 37ºC. To assess cholesterol efflux capacity, the fluorescent TopFluor-Cholesterol probe (Avanti Polar Lipids, USA), which consists of a BODIPY molecule anchored to the lipid moiety of the cholesterol molecule, was used. Confluent monolayers were labelled in DMEM containing 0.125mM total cholesterol, where the fluorescent cholesterol accounted for 20% of total cholesterol, complexed by sonication with methyl-β-Cyclodextrin (CD; Sigma-Aldrich, USA) at a molar ratio of 1:80 (CD: total cholesterol), for 1 hour. Labelled cells were subsequently washed twice with a warm medium and equilibrated with DMEM supplemented with 0.2% of fatty acid free BSA (Sigma-Aldrich, USA) in the presence of the non-steroidal LXR agonist TO-901317 (3µM; Sigma-Aldrich, USA) so that ABCA1 and ABCG1 reverse cholesterol transporter expression was up-regulated. Following 18 hours of equilibration, cells were incubated with DMEM containing volunteers' HDL (100 µgr/mL) previously dialyzed and 0.22 µm -filtered for 24 hours. All these incubations were performed in the presence of the Acyl-CoA cholesterol acyltransferase (ACAT) enzyme inhibitor Sandoz 58-035 (5µM; Sigma-Aldrich, USA). Afterwards, the media were collected and centrifuged to discard cellular debris, and cells were solubilized by shacking the plates at room temperature in the presence of 1% sheep cholic acid (Sigma-Aldrich, USA) for 4 hours. Media and cell fractions were pipetted into a black plate, and fluorescence intensity was monitored in the multi-detection Microplate Reader Synergy HT (BioTek Instruments; USA) at $\lambda_{Ex/Em}$=495/507nm. Cholesterol efflux capacity of HDL was calculated according to the following formula: [media fluorescence/(media fluorescence+cells fluorescence)] * 100. Background efflux (that observed in cholesterol-loaded cells incubated without HDL) was then subtracted from cholesterol efflux values obtained in the presence of HDL. All conditions were run in triplicate and data were pooled for each experiment.

***Sample size and power analyses***

The sample size of 30 individuals allows at least 80% power to detect a statistically significant difference among groups of 3 mg/dL of HDL-C, and a standard deviation of 1.9, assuming a drop out rate of 15% and a Type I error of 0.05 (2-sided).

***Statistical analyses***

Normality of continuous variables was assessed by normal probability plots. Non-normally distributed variables were log transformed if necessary. Non-compliance volunteers, as defined previously, were excluded from the analysis. To compare means (for normal distributed variables) or medians (for non-normal distributed variables) among groups, ANOVA and Kruskal-Wallis test were performed, respectively; whereas $\chi^2$ and exact F-test, as appropriate, were computed to compare proportions. Pearson and Spearman correlation analyses were used to evaluate relationships among variables. A general linear model for repeated measurements was used to assess the effect of intra- and inter-interventions. For binary variables recoded as being above or below a threshold level, a Mc Nemar test was performed to assess the statistical significance both within and between treatment effects. Presence of carry-over effect was assessed testing the period by treatment interaction significance under a mixed effects model introducing patient as a random intercept. Carry-over effect was discarded in all variables. A value of $p<0.05$ was considered significant. Statistical analyses were performed by SPSS18.0 software (IBN Corp) and R2.12.0 software (R Development Core Team).

**RESULTS**

***Participant characteristics and dietary adherence***

From 62 subjects who were assessed for eligibility, 29 were excluded. Finally, 33 eligible participants (19 men, 14 women) entered the study. **Figure 1** shows the flow of participants through the study. We could not identify any adverse effects related to OO intake. Participants' baseline characteristics are shown in

**Table 1**, no significant differences exist among orders. No changes in daily energy expenditure in leisure-time PA were observed from the beginning to the end of the study. No changes were observed in the main nutrients (data not shown) [35] and medication intake throughout the study, and participants' compliance was good as reflected in the phenolic metabolites analyzed in urine samples after OO interventions. Hydroxytyrosol sulfate (9.4±6.0) and hydroxytyrosol acetate sulfate (7.8±7.1) increased after FVOO intervention versus VOO one ($p<0.05$). Thymol sulfate (596.3±388.4), hydroxyphenylpropionic acid sulfate (342.5±200.5), and *p*-cymene-diol glucuronide (68.9±38.9) increased after FVOOT treatment versus FVOO and versus VOO ones ($p<0.05$) [27].

***Systemic biomarkers***

No changes were observed in blood pressure and BMI throughout the study. No changes were observed in lipid profile, glucose, and oxLDL throughout the study (data not shown).

***HDL lipid-protein and HDL fatty acids***

No changes were observed in TC, EC and FC, PL, TG, ApoA-I, and ApoA-II, in HDL particle, throughout the study **(Table 2)**. No changes were observed in HDL fatty acids throughout the study.

***HDL compounds with antioxidant properties***

Regarding fat-soluble antioxidants, an increase of HDL ubiquinol, β-criptoxanthin, and lutein was observed after both FVOOT and FVOO interventions from baseline ($p<0.05$). Ubiquinol and lutein were also significant after FVOO versus VOO ($p<0.05$). ß-criptoxanthin was also significant after FVOO versus VOO, and after FVOOT versus VOO ($p<0.05$). Additionally, α-tocopherol increased only after FVOOT from baseline, and retinol increased only after FVOO versus baseline and versus VOO and FVOOT interventions

($p<0.05$). Thymol sulfate, caffeic acid sulfate, and hydroxyphenylpropionic acid sulfate were the main phenolic compounds observed after FVOOT versus its baseline, and after FVOOT compared with VOO and FVOO ($p<0.05$). An increase of hydroxytyrosol acetate sulfate was observed after FVOO versus its baseline ($p<0.05$) **(Figure 2)**.

HDL antioxidant distribution showed cross-linked correlations with systemic biomarkers and with HDL composition. The HDL α-tocopherol post-value directly correlated with the HDL-C/LDL-C ratio and HDL TC/protein ratio after VOO, FVOO and FVOOT intake ($r>0.4$; $p<0.05$). In addition, the HDL α-tocopherol directly correlated with the HDL PL/protein ratio after FVOO and FVOOT intake ($r>0.4$; $p<0.05$). In contrast, the HDL retinol inversely correlated with the HDL-C/LDL-C ratio after VOO and FVOO interventions ($r<-0.5$; $p<0.05$). Furthermore, the increase in HDL retinol inversely correlated with the HDL TC/protein and the HDL PL/protein ratio after VOO, FVOO, and FVOOT interventions ($r<-0.6$; $p<0.05$).

***HDL antioxidant capacity and HDL oxidation resistance***

HDL antioxidant capacity did not change throughout the study. Regarding HDL oxidation resistance, a borderline decrease of oxidation rate (pre-FVOOT: 1.84±0.34; post-FVOOT: 1.72±0.45) ($p<0.09$), but no change in lag time, was only observed after FVOOT versus its baseline. No changes were observed in other interventions.

***HDL monolayer fluidity and HDL cholesterol efflux capacity***

FVOOT improved cholesterol efflux versus FVOO ($p<0.05$), and tended to increase versus its baseline ($p<0.09$) **(Figure 3)**. No changes were observed in HDL monolayer fluidity throughout the study.

**DISCUSSION**

We performed a randomized, double-blind, cross-over, controlled trial with a control VOO, and two phenol-enriched VOO, one with its own PCs, and another with them plus complementary ones from thyme. From our results, a functional OO, supplemented with complementary phenols from OO and thyme, improves HDL functionality, particularly, HDL cholesterol efflux capacity and antioxidant status. To the best of our knowledge this is the first time that this evidence is provided.

The antioxidant system is a complex network of interacting molecules. When an antioxidant is oxidized it is converted into a harmful radical, which needs to be turned back to its reduced form by complementary-antioxidants. It has been reported that supplementing high-risk individuals with a single type of antioxidant promoted rather than reduced lipid-peroxidation, while the combination of different antioxidants was effective in reducing atherosclerosis in human trials [36]. All of the above suggests that the enrichment of VOO with hydroxytyrosol derivatives combined with complementary-phenols from aromatic herbs, such as thyme, might be a good strategy to provide the optimum balance among the different kinds of flavonoids, simple phenols, monoterpenes, and phenolic acids [24].

Different antioxidants associated to HDL could improve its antioxidant function and preserve its structure. EUROLIVE study revealed that PC acquired through a high PC-VOO intervention can bind to HDL in a dose-dependent manner and this could contribute to the enhancement of HDL functionality [19]. In our work, after both phenol-enriched VOO interventions we found an increase in HDL of antioxidants with different activities. Furthermore, co-existence of lipo- and hydro-philic antioxidants linked to HDL may confer additional protection. Lipophilic antioxidants can act by scavenging aqueous peroxyl radicals at the surface of the membrane, and by scavenging lipid peroxyl radicals within it.

Lipophilic chain-breaking antioxidants in lipoproteins, such as α-tocopherol, retinol, and carotenoids, may play an important role in protecting lipids and proteins from oxidative damage [37,38]. It has been reported that a physiological concentration of β-carotene and coenzyme Q inhibits LDL and HDL oxidation *in vitro* [39,40]. Hydrophilic antioxidants, such as phenols, would be more effective if free radical injury occurs at the lipid/aqueous interphase. Some *in vitro* studies have shown that PC did not penetrate the phospholipid bilayer of the liposomes, probably as a consequence of their hydrophilic properties and their non-planar structures which give the phenols conformational mobility [41]. In the present study, both phenol-enriched VOOs increased lipophilic and hydrophilic antioxidants in HDL, and consequently both OOs improved the antioxidant state of the HDL particle.

A major issue in lipoprotein antioxidants is the rescue of vitamin E. Vitamin E (α, β, γ tocopherols) is the major antioxidant in human plasma, and is carried by HDL and LDL. α-tocopherol is the most potent antioxidant of the tocopherol family; it is the main initial chain-breaking antioxidant during lipid peroxidation and it is fully localized in the hydrophobic zone of the lipid bilayer [42]. CoQ recycles the resultant α-tocopherol phenoxyl back to its biologically active reduced form [43]. In this sense, we found an increase of α-tocopherol and CoQ after the FVOOT intervention, while after FVOO only CoQ was increased. In addition, some authors have reported that a fraction of highly active phenolic acids (as rosmarinic and caffeic ones) could regenerate α-tocopherol. Concretely, caffeic acid has been reported to protect α-tocopherol in LDL [44]. In our study, the FVOOT intervention increased rosmarinic acid biological metabolites (caffeic acid sulfate and hydroxyphenylpropionic acid sulfate), as well as α-tocopherol, which can suggest a better α-tocopherol regeneration and protection through this mechanism. In this regard, the FVOOT intervention could be better at improving HDL antioxidant activity and consequently preserving the HDL protein structures. Peruguini et al reported that HDL α-

tocopherol is related to the TC- and PL-/protein ratios [45]. In this sense, these correlations were also observed in the present work.

It has been described that an increment of antioxidants in biological membranes could increase fluidity [46], in contrast, others have reported that antioxidants could rigidify membranes thus avoiding oxidation transmission [47,48]. Regarding monolayer lipoprotein fluidity, Girona J et al. observed that HDL oxidation results in decreased HDL monolayer fluidity and less cholesterol efflux [17]. In addition, our team observed that VOO increases HDL antioxidant content, HDL monolayer fluidity, and cholesterol efflux in healthy volunteers [19]. In the present work, we did not observe an increase of HDL monolayer fluidity in any intervention; however, a non-significant linear increase through VOO, FVOO, and FVOOT interventions was determined. Moreover, we have recently published that FVOOT increased HDL PL/FC and HDL EC/FC ratios, suggesting a more fluid monolayer after this intervention [35].

In our study, an improvement of HDL resistance against oxidation after FVOOT was observed; nevertheless, we did not find a better HDL antioxidant capacity for LDL protection in none of the interventions. In agreement with this, we observed in a previous paper from the VOHF study an increase of PON1 activity after FVOOT intervention [35]. This better HDL antioxidant status after FVOOT may maintain the ApoA1 and other HDL protein structures and could improve the RCT. RCT is the main HDL biological function, it consists of extracting excess cholesterol from the peripheral cells (cholesterol efflux) and taking it to the liver to be metabolized and excreted. In the present study, an increase in cholesterol efflux after the FVOOT intervention has been established. This cholesterol efflux improvement can be related to the better HDL antioxidant status, reflected by HDL α-tocopherol and other lipid- and hydro-philic antioxidant content. Accordingly, a pooling sample proteomic approach, established an increase in afamin, which is related to tocopherol transport, after the FVOOT [49].

The antioxidant properties of OOPC *in vivo* are well-known. The EUROLIVE study showed a decrease *in vivo* in lipid oxidative damage and an increase of HDL-C in a dose-dependent manner with the PC of the OO administered [1]. In agreement with this, in a recent paper from the VOHF-study, an increment in the HDL-C was observed in the subsample of volunteers without hypolipidemic medication [35]. In 2011 the European Food Safety Authority recognized the PC-rich OO effects on protecting LDL from oxidation [50]. Nevertheless, in this study, no effect on the oxLDL was detected. The reduced sample, the hypercholesterolemic state, and pharmacological treatment could explain this result.

The strength of our study is its crossover and randomized design, which permitted the participants to consume all OO types and thus reduced the inter-individual variability. In addition, the three OOs have similar matrix (fat-soluble, vitamins, and fatty acids), so the differential character of the OOs is the PCs. Moreover, the laboratory analyses were centralized and all the time-series samples from the same volunteer were measured in the same run to minimize imprecision. A limitation of the study was its sample size, which could be responsible for reduced statistical power in some biomarkers with higher intra-individual variability. A synergistic effect on HDL-parameters provided by PC and other OO components is as yet unknown. The inability to assess potential interactions among the OOs and other dietary components and medication is also a limitation. In this sense, the medication and diet was controlled during all study and no change was registered.

In conclusion, long-term consumption of complementary phenol-enriched OO induced an improvement in HDL antioxidant status and HDL cholesterol efflux capacity. These results show that an enrichment of OO with complementary antioxidants promotes more benefits than an enrichment of OO with only its own phenolics. The enrichment of OO with PCs is a way of increasing its healthy properties whilst the same amount of fat is consumed. Our data suggest

that a complementary phenol-enriched OO could be a good nutraceutical for improving HDL functionality in cardiovascular high-risk individuals.

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Authors' contributions to manuscript were as following: M-IC, M-JM, RS, RdlT, and MFi design the research, MFa, S-FC, LR, SA, UC, M-PR, OC, AP, GB, DM-A, HS, and MFi were responsible for the execution of the study including hands-on conduct of the experiments and data collection; MFa, IS, and MFi analyzed data; MFa and MFi wrote the paper; M-JM, RS, and MFi had primary responsibility for final content. All authors read and approved the final manuscript.

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**Table 1.** Baseline characteristics of the participants.

| | Total[a] (n=33) |
|---|---|
| **GENERAL** | |
| **Sex: man** | 19 (57.6%) |
| **Age** | 55.21 ± 10.62 |
| **BMI (Kg/m$^2$)** | 26.64 ± 4.54 |
| **Hypolipidemic medication: no** | 19 (57.6%) |
| **Physical activity (Kcal/week)** | 2423.25 (897.38;4543.75) |
| **Diastolic blood pressure (mmHg)** | 70.76 ± 12.01 |
| **Systolic blood pressure (mmHg)** | 127.94 ± 17.37 |
| **SYSTEMIC LIPID PROFILE AND GLYCAEMIA** | |
| **Total-cholesterol (mg/dL)** | 226 ± 35 |
| **Triglycerides (mg/dL)** | 114 (85;145) |
| **Glucose (mg/dL)** | 91 ± 12 |
| **HDL-cholesterol (mg/dL)** | 53 ± 11 |
| **LDL-cholesterol (mg/dL)** | 148 ± 28 |
| **ApoA-I (g/L)** | 1.4 ± 0.2 |
| **Apolipoprotein-B100 (g/L)** | 1.1 ± 0.2 |

[a)] Values expressed as mean ± S.D. or median (25th to 75th percentile).

**Table 2.** HDL lipid-protein composition changes after the interventions.

| | Pre-VOO intervention[a] | Post-VOO intervention[a] | Pre-FVOO intervention[a] | Post-FVOO intervention[a] | Pre-FVOOT intervention[a] | Post-FVOOT intervention[a] | Inter-Intervention p-value |
|---|---|---|---|---|---|---|---|
| **HDL Total-cholesterol (mg/dL)** | 26 ± 4 | 26 ± 4 | 27 ± 4 | 27 ± 4 | 27 ± 4 | 26 ± 4 | 0.949 (VOO-FVOOT)<br>0.692 (FVOO-FVOOT)<br>0.829 (VOO-FVOO) |
| **HDL Triglycerides (mg/dL)** | 6.44 ± 1.96 | 6.54 ± 2.47 | 6.19 ± 1.70 | 6.30 ± 2.13 | 6.45 ± 1.95 | 6.71 ± 2.61 | 0.927 (VOO-FVOOT)<br>0.676 (FVOO-FVOOT)<br>0.748 (VOO-FVOO) |
| **HDL Apolipoprotein A-I (mg/dL)** | 56.02 ± 6.08 | 56.71 ± 6.53 | 56.28 ± 4.78 | 56.69 ± 5.84 | 57.00 ± 6.18 | 55.76 ± 6.00 | 0.319 (VOO-FVOOT)<br>0.189 (FVOO-FVOOT)<br>0.982 (VOO-FVOO) |
| **HDL Apolipoprotein A-II (mg/dL)** | 14.85 ± 3.00 | 14.94 ± 3.56 | 14.82 ± 3.58 | 15.08 ± 3.60 | 15.48 ± 3.12 | 15.19 ± 3.10 | 0.593 (VOO-FVOOT)<br>0.169 (FVOO-FVOOT)<br>0.637 (VOO-FVOO) |
| **HDL Free-cholesterol (mg/dL)** | 10.99 ± 3.79 | 11.25 ± 2.74 | 11.64 ± 3.64 | 11.43 ± 3.30 | 10.85 ± 3.93 | 10.60 ± 3.75 | 0.470 (VOO-FVOOT)<br>0.946 (FVOO-FVOOT)<br>0.338 (VOO-FVOO) |
| **HDL Esterified-cholesterol (mg/dL)** | 15.30 ± 4.15 | 15.17 ± 4.18 | 15.07 ± 4.35 | 15.07 ± 3.71 | 15.62 ± 3.80 | 15.77 ± 3.70 | 0.483 (VOO-FVOOT)<br>0.837 (FVOO-FVOOT)<br>0.408 (VOO-FVOO) |
| **HDL Phospholipids (mg/dL)** | 51.86 ± 2.73 | 51.53 ± 2.41 | 51.72 ± 2.54 | 51.55 ± 2.42 | 51.04 ± 2.45 | 51.17 ± 1.97 | 0.471 (VOO-FVOOT)<br>0.649 (FVOO-FVOOT)<br>0.859 (VOO-FVOO) |

[a)]Values expressed as pre- or post-intervention mean ± S.D.

No significant p-values.

Abbreviations: VOO, virgin olive oil; FVOO, functional VOO enriched with its own phenolic compounds; FVOOT, functional VOO enriched with its own phenolic compounds plus additional complementary ones from thyme.

**FIGURE LEGENDS**

Figure 1. Flowchart of VOHF-study.

[a]Non-intervention.

Figure 2. HDL compounds with antioxidant properties after the interventions.

Values represent pre- and post-interventions.

Values expressed as mean + SE or as median and 75th percentile.

* Intra-treatment p-value<0.05

| Inter-treatment FVOO-VOO p-value<0.05

· Inter-treatment FVOO-FVOOT p-value<0.05

# Inter-treatment FVOOT-VOO p-value<0.05

Figure 3. Cholesterol efflux changes after the interventions.

Values expressed as mean differences (post-treatment – pre-treatment) ± SE.

* p-value<0.05 between treatments.

^ p-value<0.09 within treatments.

**Figure 1. Flowchart of VOHF-study.**

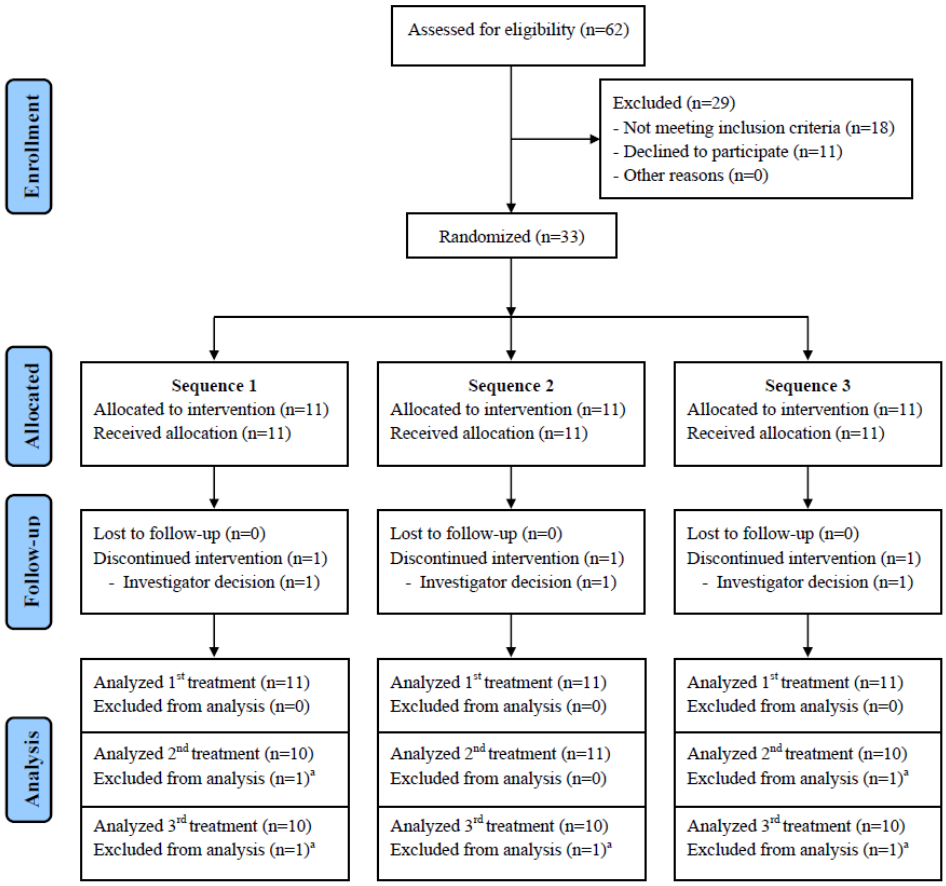


**Figure 2. HDL compounds with antioxidant properties after the interventions.**

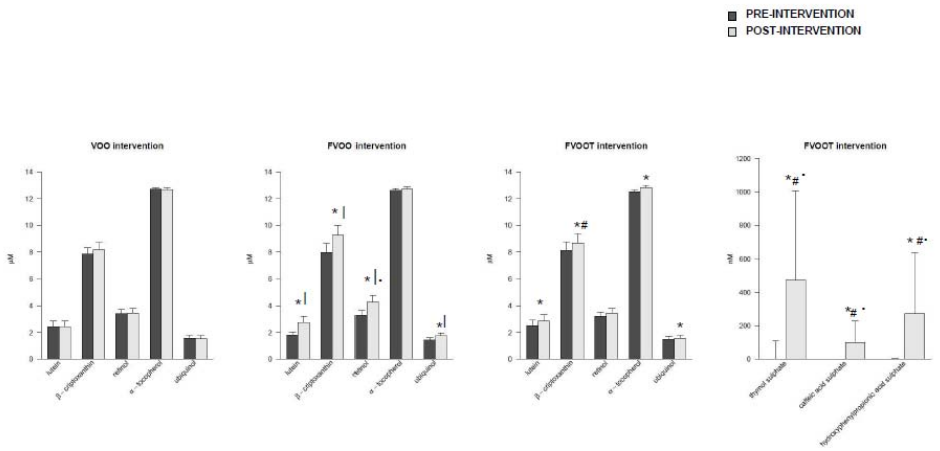
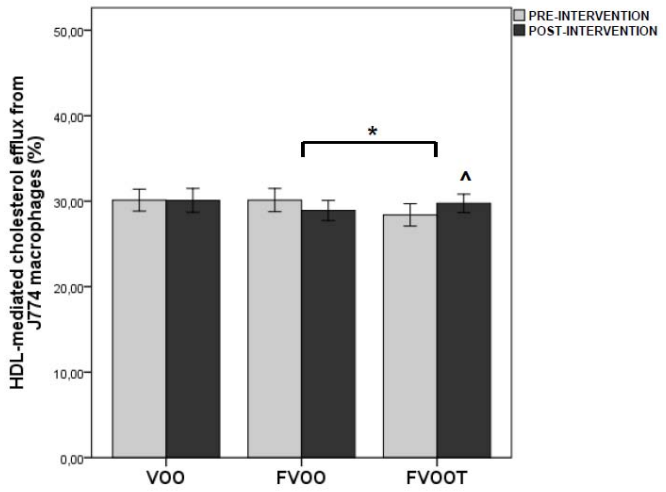


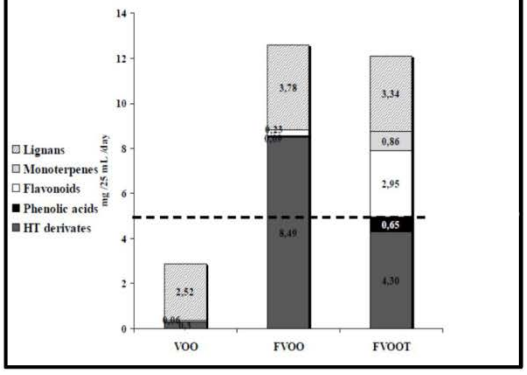

**Figure 3. Cholesterol efflux changes after the interventions.**



## SUPPLEMENTAL MATERIAL

**Figure 1 Supplemental material.** Chemical characterization of VOHF-study olive oils.

| | VOO | FVOO | FVOOT |
|---|---|---|---|
| **PHENOLIC COMPOUNDS (mg/25 mL/day)** | | | |
| hydroxytyrosol | 0.01 ± 0.00 | 0.21 ± 0.02 | 0.12 ± 0.00 |
| 3,4-DHPEA-AC | n.d. | 0.84 ± 0.06 | 0.39 ± 0.04 |
| 3,4-DHPEA-EDA | 0.04 ± 0.00 | 6.73 ± 0.37 | 3.43 ± 0.29 |
| 3,4-DHPEA-EA | 0.26 ± 0.04 | 0.71 ± 0.06 | 0.36 ± 0.03 |
| ***Total HT derivates*** | ***0.30*** | ***8.49*** | ***4.30*** |
| p-hydroxybenzoic acid | n.d. | 0.02 ± 0.00 | 0.06 ± 0.00 |
| vanillic acid | n.d. | 0.07 ± 0.00 | 0.13 ± 0.01 |
| caffeic acid | n.d. | 0.00 ± 0.00 | 0.06 ± 0.00 |
| rosmarinic acid | n.d. | n.d. | 0.41± 0.03 |
| ***Total phenolic acids*** | - | ***0.09*** | ***0.65*** |
| thymol | n.d. | n.d. | 0.64 ± 0.05 |
| carvacrol | n.d. | n.d. | 0.23 ± 0.02 |
| ***Total monoterpenes*** | - | - | ***0.86*** |
| luteolin | 0.04 ± 0.00 | 0.18 ± 0.02 | 0.21 ± 0.02 |
| apigenin | 0.02 ± 0.00 | 0.06 ± 0.00 | 0.10 ± 0.00 |
| naringenin | n.d. | n.d. | 0.20 ± 0.02 |
| eriodictyol | n.d. | n.d. | 0.17 ± 0.01 |
| thymusin | n.d. | n.d. | 1.22 ± 0.09 |
| xanthomicrol | n.d. | n.d. | 0.53 ± 0.06 |
| 7-methylsudachitin | n.d. | n.d. | 0.53 ± 0.09 |
| ***Total flavonoids*** | ***0.06*** | ***0.23*** | ***2.95*** |
| pinoresinol | 0.05 ± 0.00 | 0.12 ± 0.00 | 0.10 ± 0.05 |
| acetoxipinoresinol | 2.47 ± 0.19 | 3.66 ± 0.31 | 3.24 ± 0.28 |
| ***Total lignans*** | ***2.52*** | ***3.78*** | ***3.34*** |
| **FAT SOLUBLE MICRONUTRIENTS (mg/25 mL/day)** | | | |
| α-tocopherol | 3.27 ± 0.01 | 3.40 ± 0.02 | 3.44 ± 0.01 |
| lutein | 0.05 ± 0.00 | 0.06 ± 0.00 | 0.06 ± 0.00 |
| β-cryptoxanthin | 0.02 ± 0.00 | 0.03 ± 0.00 | 0.02 ± 0.00 |
| β-carotene | 0.01 ± 0.00 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| **FATTY ACIDS (relative area %)** | | | |
| Palmitic acid | 11.21 | 11.20 | 11.21 |
| Stearic acid | 1.92 | 1.92 | 1.92 |
| Araquidic acid | 0.36 | 0.36 | 0.36 |
| Behenic acid | 0.11 | 0.11 | 0.11 |
| ***Total saturated*** | ***13.75*** | ***13.74*** | ***13.75*** |
| Palmitoleic acid | 0.70 | 0.70 | 0.69 |
| Oleic acid | 76.74 | 76.83 | 76.75 |
| Gadoleic acid | 0.27 | 0.27 | 0.27 |
| ***Total monounsaturated*** | ***77.71*** | ***77.80*** | ***77.72*** |
| Linoleic acid | 7.43 | 7.36 | 7.43 |
| Timnodonic acid | 0.36 | 0.36 | 0.35 |
| Linolenic acid | 0.43 | 0.43 | 0.43 |
| ***Total polyunsaturated*** | ***8.22*** | ***8.15*** | ***8.22*** |




Values are expressed as means ± SD of mg/25 mL oil/day. The acidic composition is expressed as relative area percentage.

Abreviations: VOO, virgin olive oil; FVOO, functional VOO enriched with its own phenolic compounds; FVOOT, functional VOO enriched with its own phenolic-compounds plus additional complementary ones from thyme; 3,4-DHPEA-AC,4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA,dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA,oleuropein-aglycone.

**Table 1. Supplemental material.** Optimized SRM conditions used for the identification of phenolic compounds in HDL analysis.

| Phenolic compound | MW (g/mol) | SRM quantification | |
|---|---|---|---|
| | | Transition | Cone voltage (V) / Collision energy (eV) |
| **Olive Oil** | | | |
| 3,4-DHPEA-EDA | 320 | 319 > 195 | 40 / 5 |
| 3,4-DHPEA-EA | 378 | 377 > 275 | 35 / 10 |
| Acetoxypinoresinol | 416 | 415 > 151 | 45 / 15 |
| Alcohol homovanillic sulphate | 248 | 247 > 167 | 40 / 15 |
| Apigenin | 270 | 269 > 117 | 60 / 25 |
| Apigenin glucoside | 432 | 431 > 269 | 45 / 25 |
| Caffeic acid | 180 | 179 > 135 | 35 / 15 |
| Cinamic acid | 148 | 147 > 103 | 20 / 10 |
| Chlorogenic acid | 354 | 353 > 191 | 30 / 10 |
| Coumaric acid | 164 | 163 > 119 | 35 / 10 |
| Dihydroxyphenylpropionic acid | 182 | 182 > 137 | 20 / 10 |
| Elenolic acid | 242 | 241 > 139 | 30 / 15 |
| Ferulic acid | 194 | 193 > 134 | 30 / 15 |
| Homovanillic acid | 182 | 181 > 137 | 25 / 10 |
| Homovanillic acid glucuronide | 358 | 357 > 181 | 40 / 20 |
| Homovanillic acid sulphate | 262 | 261 > 181 | 40 / 15 |
| Homovanillic alcohol glucuronide | 344 | 343 > 167 | 40 / 20 |
| Homovanillic alcohol sulphate | 248 | 247 > 167 | 40 / 15 |
| Hydroxyphenylacetic acid | 152 | 151 > 107 | 20 / 10 |
| Hydroxyphenylpropionic acid sulphate | 346 | 245 > 165 | 35 / 15 |
| Hydroxyphenylpropionic acid glucuronide | 342 | 341 > 165 | 35 / 15 |
| Hydroxytyrosol | 154 | 153 > 123 | 35 / 10 |
| Hydroxytyrosol acetate | 196 | 195 > 135 | 30 / 10 |
| Hydroxytyrosol acetate sulphate | 276 | 275 > 195 | 35 / 15 |
| Hydroxytyrosol acetate glucuronide | 372 | 371 > 195 | 35 / 15 |
| Hydroxytyrosol glucuronide | 330 | 329 > 153 | 40 / 20 |
| Hydroxytyrosol sulphate | 234 | 233 > 153 | 40 / 15 |
| Ligstroside | 524 | 523 > 361 | 35 / 15 |
| Ligstroside derivate (1) | 336 | 335 > 199 | 40 / 10 |
| Ligstroside derivate (2) | 394 | 393 > 317 | 40 / 15 |
| Luteolin | 286 | 285 > 133 | 55 / 25 |
| Luteolin glucoside | 448 | 447 > 285 | 50 / 25 |
| Methyl 3,4-HPEA-EA | 410 | 409 > 377 | 30 / 5 |
| Methyl oleuropein aglycone | 392 | 391 > 255 | 35 / 15 |
| Oleuropein | 540 | 539 > 377 | 35 / 15 |
| Oleuropein derivate | 366 | 365 > 299 | 35 / 10 |
| *p*-HPEA-EA | 362 | 361 > 291 | 30 / 10 |
| *p*-HPEA-EDA | 304 | 303 > 285 | 30 / 5 |
| Pinoresinol | 358 | 357 > 151 | 40 / 10 |
| Rutin | 610 | 609 > 300 | 55 / 25 |
| Tyrosol | 138 | 137 > 106 | 40 / 15 |
| Tyrosol glucuronide | 314 | 313 > 137 | 25 / 30 |
| Tyrosol sulphate | 218 | 217 > 137 | 40 / 20 |
| Vanillic acid | 168 | 167 > 123 | 30 / 10 |
| Vanillin | 152 | 151 > 136 | 20 / 10 |
| **Thyme** | | | |

| | | | |
|---|---|---|---|
| Apigenin glucuronide | 446 | 445 > 269 | 40 / 25 |
| Apigenin rutinoside | 578 | 577 > 269 | 35 / 15 |
| Caffeic acid glucuronide | 356 | 355 > 179 | 40 / 15 |
| Caffeic acid sulphate | 260 | 259 > 179 | 35 / 15 |
| Carvacrol | 150 | 149 > 134 | 40 / 15 |
| Coumaric acid glucuronide | 340 | 339 > 163 | 35 / 15 |
| Coumaric acid sulphate | 244 | 243 > 163 | 35 / 15 |
| Dihidrokaempferol | 288 | 287 > 259 | 45 / 10 |
| Dihidroquercetin | 304 | 303 > 285 | 40 / 10 |
| Dihidroxanthomicol | 346 | 345 > 301 | 40 / 20 |
| Eriodictyol | 288 | 287 > 151 | 40 / 15 |
| Eriodictyol glucoside | 450 | 449 > 287 | 45 / 10 |
| Eriodictyol glucuronide | 464 | 463 > 287 | 40 / 20 |
| Eriodictiol rutinoside | 596 | 595 > 287 | 40 / 20 |
| Eriodictyol sulphate | 368 | 367 > 287 | 40 / 15 |
| Ferulic acid glucuronide | 370 | 369 > 193 | 35 / 15 |
| Ferulic acid sulphate | 274 | 273 > 193 | 35 / 15 |
| Hydroxyphenylpropionic acid | 166 | 165 > 121 | 20 / 10 |
| Hydroxyphenylpropionic acid glucuronide | 342 | 341 > 165 | 40 / 25 |
| Hydroxyphenylpropionic acid sulphate | 246 | 245 > 165 | 35 / 15 |
| Isorhamnetin glucoside | 478 | 477 > 315 | 45 / 20 |
| Isorhamnetin rutinoside | 624 | 623 > 315 | 55 / 25 |
| Kaempferol glucuronide | 462 | 461 > 285 | 40 / 25 |
| Kaempferol rhamnoside | 432 | 431 > 285 | 45 / 20 |
| Luteolin glucuronide | 462 | 461 > 285 | 40 / 25 |
| Methoxyluteolin | 300 | 299 > 119 | 35 / 15 |
| Methylsudachitin | 374 | 373 > 358 | 40 / 20 |
| Myricetin glucoside | 480 | 479 > 317 | 45 / 20 |
| Naringenin | 272 | 271 > 151 | 40 / 15 |
| Naringenin glucoside | 434 | 433 > 271 | 45 / 10 |
| Naringenin glucuronide | 448 | 447 > 271 | 40 / 25 |
| Naringenin rutinoside | 580 | 579 > 271 | 40 / 20 |
| Naringenin sulphate | 352 | 351 > 271 | 40 / 20 |
| *p*-cymene diol glucuronide | 342 | 341 > 165 | 40 / 25 |
| Quercetin | 302 | 301 > 151 | 40 / 15 |
| Quercetin arabinoside | 434 | 433 > 301 | 45 / 20 |
| Quercetin glucoside | 464 | 463 > 301 | 45 / 25 |
| Quercetin glucuronide | 478 | 477 > 301 | 40 / 20 |
| Quercetin rhamnoside | 448 | 447 > 301 | 40 / 15 |
| Quercetin sulphate | 382 | 381 > 301 | 40 / 20 |
| Rosmarinic acid | 360 | 359 > 161 | 40 / 20 |
| Rosmarinic acid glucuronide | 536 | 535 > 359 | 40 / 20 |
| Rosmarinic acid sulphate | 440 | 439 > 359 | 40 / 20 |
| Thymol | 150 | 149 > 134 | 40 / 15 |
| Thymol glucuronide | 326 | 325 > 149 | 20 / 25 |
| Thymol sulphate | 230 | 229 > 149 | 40 / 20 |
| Thymusin (1) | 330 | 329 > 286 | 40 / 25 |
| Thymusin (2) | 330 | 329 > 314 | 40 / 25 |
| Thymusin glucuronide | 506 | 505 > 329 | 40 / 20 |
| Thymusin sulphate | 410 | 409 > 329 | 40 / 20 |
| Xanthomicol | 344 | 343 > 328 | 40 / 20 |

MW: Molecular weight

3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycon; *p*-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol; *p*-HPEA-EA, ligstroside-aglycone.

## CONSORT 2010 checklist of information to include when reporting a randomised trial*

| Section/Topic | Item No | Checklist item | Reported on page No |
|---|---|---|---|
| **Title and abstract** | | | |
| | 1a | Identification as a randomised trial in the title | 1 |
| | 1b | Structured summary of trial design, methods, results, and conclusions (for specific guidance see CONSORT for abstracts) | 4,5 |
| **Introduction** | | | |
| Background and objectives | 2a | Scientific background and explanation of rationale | 6,7 |
| | 2b | Specific objectives or hypotheses | 7 |
| **Methods** | | | |
| Trial design | 3a | Description of trial design (such as parallel, factorial) including allocation ratio | 8, 9 |
| | 3b | Important changes to methods after trial commencement (such as eligibility criteria), with reasons | No applicable |
| Participants | 4a | Eligibility criteria for participants | 8 |
| | 4b | Settings and locations where the data were collected | 8 |
| Interventions | 5 | The interventions for each group with sufficient details to allow replication, including how and when they were actually administered | 7,8 |
| Outcomes | 6a | Completely defined pre-specified primary and secondary outcome measures, including how and when they were assessed | 9-15 |
| | 6b | Any changes to trial outcomes after the trial commenced, with reasons | No applicable |
| Sample size | 7a | How sample size was determined | 15 |
| | 7b | When applicable, explanation of any interim analyses and stopping guidelines | No applicable |
| Randomisation: | | | |
| Sequence generation | 8a | Method used to generate the random allocation sequence | 8 |
| | 8b | Type of randomisation; details of any restriction (such as blocking and block size) | 8 |
| Allocation concealment mechanism | 9 | Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned | 8 |
| Implementation | 10 | Who generated the random allocation sequence, who enrolled participants, and who assigned participants to interventions | 8 |
| Blinding | 11a | If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how | 8 |
| | 11b | If relevant, description of the similarity of interventions | 7 |
| Statistical methods | 12a | Statistical methods used to compare groups for primary and secondary outcomes | 15,16 |
| | 12b | Methods for additional analyses, such as subgroup analyses and adjusted analyses | No applicable |
| **Results** | | | |
| Participant flow (a diagram is strongly recommended) | 13a | For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome | Figure 1 |
| | 13b | For each group, losses and exclusions after randomisation, together with reasons | Figure 1 |
| Recruitment | 14a | Dates defining the periods of recruitment and follow-up | 8 |
| | 14b | Why the trial ended or was stopped | 8 |
| Baseline data | 15 | A table showing baseline demographic and clinical characteristics for each group | 31 (Table 1) |
| Numbers analysed | 16 | For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups | 16-18 |
| Outcomes and estimation | 17a | For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval) | 16-18 |
| | 17b | For binary outcomes, presentation of both absolute and relative effect sizes is recommended | - |
| Ancillary analyses | 18 | Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory | No applicable |
| Harms | 19 | All important harms or unintended effects in each group (for specific guidance see CONSORT for harms) | No applicable |
| **Discussion** | | | |
| Limitations | 20 | Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses | 22 |
| Generalisability | 21 | Generalisability (external validity, applicability) of the trial findings | 22 |
| Interpretation | 22 | Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence | 18-22 |
| **Other information** | | | |
| Registration | 23 | Registration number and name of trial registry | 2, 9 |
| Protocol | 24 | Where the full trial protocol can be accessed, if available | 9 |
| Funding | 25 | Sources of funding and other support (such as supply of drugs), role of funders | 2 |

*We strongly recommend reading this statement in conjunction with the CONSORT 2010 Explanation and Elaboration for important clarifications on all the items. If relevant, we also recommend reading CONSORT extensions for cluster randomised trials, non-inferiority and equivalence trials, non-pharmacological treatments, herbal interventions, and pragmatic trials. Additional extensions are forthcoming: for those and for up to date references relevant to this checklist, see www.consort-statement.org.

# Annex II: Publication nº 5

## Virgin olive oil enriched with its own phenolic compounds or complemented with thyme improves endothelial function in humans. A double blind, randomized, controlled, cross-over clinical trial. The VOHF study

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***SUBMITTED TO EDITOR***

# Virgin olive oil enriched with its own phenolic compounds or complemented with thyme improves endothelial function in humans. A double blind, randomized, controlled, cross-over clinical trial. The VOHF study

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*R-MV, MFa and AP contributed equally to the study.

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**Key words:** endothelial function, virgin olive oil, thyme, phenolic compounds, fat-soluble vitamins.

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**Abbreviations:** EF, endothelial function; EFSA, European Food Safety Authority; FVOO, functional virgin olive oil; GPx, glutathione peroxidase; HPLC, High performance Liquid Chromatography; hsCRP, high sensitivity C-reactive protein; ICAM-1, intercellular cell adhesion molecule; ICH GPC, International Conference of Harmonization; IRH, ischemic reactive hyperemia; MCP-1, monocyte chemotactic Protein; NOx, nitric oxide; OO, olive oil; PAI-1, plasminogen activator inhibitor type I; PC, phenolic compounds; TG, triglycerides; UPLC-MS/MS, Ultra Performance Liquid Chromatography-mass spectometry; VCAM-1, vascular cell adhesion molecule; VOO, virgin olive oil.

## ABSTRACT

**Scope:** To assess the effects in the endothelial function (EF) of different functional virgin olive oils (FVOOs) with varying phenolic compound (PC) classes and concentration. Furthermore, we aimed to determine whether FVOOs could increase the plasmatic concentrations of fat-soluble vitamins thus contributing to a synergistic effect on EF.

**Methods and results:** From the dose-response study (n= 12 healthy subjects), an enrichment of 500mg PC/kg oil was the optimal concentration for improving ischemic reactive hyperemia (IRH) and cardiovascular risk biomarkers. In the sustained study (n=33 hypercholesterolemic subjects), both FVOOs (500mg olive oil (OO) PC/kg oil and 500mg OO combined thyme PC/kg oil) increased IRH ($p<0.05$) and plasma concentrations of retinol, β-cryptoxanthin, and α-tocopherol ($p<0.01$), compared to control virgin OO.

**Conclusion:** FVOOs enriched with their own PC or combined with thyme PC, are a good choice to provide benefits on EF. The interaction between PC and systemic fat-soluble vitamins could influence the EF improvement.

**Clinical Trial Registration**: NCT01347515 and ISRCTN77500181.

## 1. Introduction

Endothelial dysfunction, characterized by an impairment of the endothelial-dependent vasodilatation, is a key mechanism involved in the onset and progression of atherosclerosis. It has emerged as a new risk factor for cardiovascular events before symptoms appear [1].

Dietary factors could play a key role on modulating endothelial function (EF). Some studies have suggested that dietary bioactive compounds, such as fat-soluble vitamins and phenolic compounds (PC), could have beneficial effects on EF through multiple complex mechanisms including: inhibition of monocyte adhesion, platelet activation, increased nitric-oxide production and improvement of vasodilatation [2, 3].

Virgin olive oil (VOO), which is a food item typical of the Mediterranean diet, has been related with a unique phenolic profile with specific biological properties on EF [4].

The consumption of a VOO with a high phenolic content has been related with an improvement of endothelial-dependent vasomotor function measured as ischemic reactive hyperemia (IRH) in humans in acute [5] and sustained studies [6]. However, the phenolic concentration in most of the VOOs available on the market is too low to provide the daily amount of PC needed to achieve the protection against LDL oxidation described in the health claim about olive oil, endorsed by the European Food Safety Authority (EFSA) (5mg of hydroxytyrosol and its derivatives per 20g of olive oil per day) [7]. Due to this, the enrichment of VOO with its own PC has been proposed as a possible approach to raise the PC consumption without increasing caloric intake [8, 9]. Recently, our group developed a functional VOO (FVOO) highly enriched with OOPC (961 mg/kg oil) capable to provide benefits on EF in hypertensive patients [10]. Nevertheless, the enrichment of VOO with its own PC (hydroxytyrosol and oleuropein and hydroxytyrosol derivatives, commonly named secoiridoids) could lead to its organoleptic taste being rejected by consumers related with high intensity in purgent and bitter attributes. Moreover, beneficial synergistic effects have been described when different classes of PC are combined [11, 12]. For these reason, the enrichment of a VOO by complementing its own phenols with thyme PC (flavonoids, rosmarinic and monoterpens) could improve the FVOO nutritional profile and organoleptic characteristics [9, 12]. To date, the effects of FVOOs enriched with their own PC and complemented with thyme PC on EF have not yet been evaluated.

Dietary PC are consumed together with other dietary antioxidants such as fat-soluble vitamins (carotenoids or tocopherols) which have been related to a protecting role against vascular dysfunction both in human and *in vitro* studies [13, 14]. It has been suggested that the intake of fat-soluble vitamins with other dietary antioxidants could protect fat-soluble vitamins against oxidative degradation thus enhancing their bioavailability [15]. Moreover, it has been reported that the presence of a dietary fatty matrix, such as VOO, could act as an absorption modifier resulting in an enhanced bioavailability of the fat-soluble

compounds [16, 17]. It has also been observed that the consumption of a Mediterranean diet rich in VOO increases the plasma concentrations of fat-soluble vitamins and decreases endothelial damage by mechanisms possibly associated with the protective synergistic effects of the antioxidant components of this dietary pattern [18].

In this context, we hypothesized that PC from VOO alone or mixed with PC from thyme protect the fat-soluble vitamins in plasma, which in turn would produce a synergistic effect on EF leading to its improvement. Our goal was first to assess the effects of different FVOOs that differ in PC concentration and composition in order to select which FVOO has the optimal PC profile for improving EF. Furthermore, we aimed to determine whether diets supplemented with these FVOOs could increase the plasmatic concentrations of fat-soluble vitamins, and whether this occurs it contributes to a synergistic effect on EF.

## 2. Materials and methods

The experiment design comprised 2 separate interventions: a dose-response intervention (dose-response study) and a 3-week intervention (sustained study). In the first place, a dose-response study was performed to assure olive oil (OO) PC bioavailability and to determine which concentration of OO PC was the optimal regarding EF. Subsequently, a sustained study was carried out to determine the effects of two different FVOOs, prepared with the optimal concentration of PC observed in the dose-response study, differing in PC composition (OO PC or OO PC plus thyme PC, and control VOO), on EF and plasma fat-soluble vitamins.

### 2.1 Dose-response study

Between April and September 2011 in Hospital Sant Joan de Reus, 6 men and 6 women (aged 20-70 years old) were recruited through a volunteer center database. Participants were considered healthy according to a physical examination and routine laboratory tests. Exclusion criteria included were LDL-

cholesterol (LDL-c) >4.9 mmol/L, triglycerides (TG) >3.97 mmol/L, or current hypolipemic treatment, diabetes mellitus, any chronic disease, and body mass index (BMI) >30 kg/m$^2$. Participants provided written informed consent prior to their enrollment in the trial. After a screening visit, eligibility was assessed by the attending physician on the basis of clinical records. The study was approved by the Clinical Research Ethical Committee of the Hospital Universitari Sant Joan de Reus (Ref 09-02-26/2proj2), Spain. The protocols were conducted according to the Helsinki Declaration and the Good Clinical Practice for Trials on Medical Products in the European Community and the International Conference of Harmonization (ICH GCP). The study is registered at ClinicalTrials.gov (Identifier: NCT01347515).

The procedure to obtain the phenolic extracts and the enriched oils has been previously

described [9]. Briefly, 3 functional phenol-enriched virgin olive oils (FVOO) with low PC-content (L-FVOO; 250 mg totals phenols/kg of oil), medium PC-content (M-FVOO; 500 mg totals phenols/kg of oil) and high PC-content (H-FVOO; 750 mg totals phenols/kg of oil) were prepared by the addition of an extract rich in the main OO PC (secoiridoid derivatives: 89,4%; phenyl alcohols: 3,5%; and flavonoids: 6,0%) to a VOO with low phenolic content (<80 mg/kg) used as enrichment matrix. This extract was obtained from olive cakes following the method described by Suárez M, et al. [19].

The dose-response study was randomized, controlled, double-blind and cross-over. Subjects participated in three one-day experimental sessions consuming the three treatment conditions separated by a 1-week washout period among interventions. Randomization was generated by using a web site (http://www.randomization.com) September 9$^{th}$ 2010 at 08:50:13 am. Participants received 30 mL single ingestion of each PC-enriched FVOO (L-FVOO, M-FVOO, H-FVOO) (Figure S1). These FVOOs were ingested with 80 g of bread after an overnight fast [9]. Participants rested for 8 h in a comfortable warm room.

Venous blood was collected at baseline (0 h) and at several time points after FVOOs intake (15 min, 30 min, 45 min, 1 h, 2 h, 4 h, and 6 h). Collection tubes were protected from the light with aluminum foil and centrifuged 15 min at 1500 g at 4ºC for the biological samples collection which were stored at -80ºC in the central laboratory' Biobanc.
(http://www.iispv.cat/plataformes_de_suport/biobanc_iispv_husjr.html bancmb@grupsagessa.com).

The endothelial-dependent vasomotor function was measured as IRH by a Laser-Doppler linear P eriflux 5 000 f lowmeter ( Perimed AB, J ärfälla, S tockholm, Sweden) as previously described [10]. The IRH was measured at baseline and at 2 h, 4 h, and 6 h after FVOOs intake.
Cardiovascular risk biomarkers were evaluated at baseline and at 1 h, 2 h, 4 h, and 6 h postprandial. Serum total cholesterol, TG, and glucose were measured by standardized enzymatic automated methods in a PENTRA-400 autoanalyzer (ABX-Horiba Diagnostics, Montpellier, France). HDL-cholesterol (HDL-c) was measured as a soluble HDL-c determined by an accelerator selective detergent method (ABX-Horiba Diagnostics, Montpellier, France), and high sensitivity C-reactive protein ( hsCRP) b y st andardized m ethods in a C obas M ira Plus autoanalyzer (Roche D iagnostics S ystems, Madrid, S pain). L DL-c w as calculated by t he F riedewald formula. EDTA plasma oxi dized L DL ( ox-LDL) was measured by ELISA kit (Mercodia AB, Uppsala, Sweden). The endothelial dysfunction b iomarkers were de termined a t b aseline, 2 h, 4 h a nd 6 h postprandial. S erum E ndothelin-1 a nd ni tric ox ide (NOx) w ere m easured by ELISA kits (R&D Systems, Minneapolis, USA). Plasminogen activator inhibitor type 1 (PAI-1) was measured in citrate plasma using an ELISA kit (Technoclone GmbH, Vienna, Austria).
Oxidative biomarkers were determined at baseline, 1 h, 2 h, 4 h a nd 6 h a fter FVOOs intake. E DTA plasma g lutathione pe roxidase ( GSH-Px) a ctivity w as assessed through glutathione oxidation-reduction measured by a Paglia and

Valentine method modification using cumene hydroperoxide as oxidant of glutathione (Ransel RS 505, Randox Laboratiories, Crumlin, UK) [20]. Reduced and oxidized glutathione (GSH and GSSG, respectively) were analyzed by high performance liquid chromatography (HPLC) [21, 22].

To verify dietary adherence plasma FVOO PC and their metabolites were measured as bioavailability biomarkers by Ultra Performance Liquid Chromatography-mass spectometry (UPLC-MS/MS) at baseline, 15 min, 30 min, 45 min, 1 h, 2 h, 4 h and 6 h after FVOOs intake as previously described [9].

**2.2 Sustained study**

From April to September 2012 in Hospital del Mar Medical Research Institute, 19 men and 14 women (aged 35-80 years old) were recruited. Participants were hypercholesterolemics (total cholesterol >200 mg/dL) and the exclusion criteria included were BMI > 35 kg/m$^2$, smokers, athletes with high physical activity (>3000 kcal/day), diabetes, multiple allergies, intestinal diseases, or any other condition that could worsen compliance. All participants provided written informed consent prior to their enrollment in the trial. The study was approved by the Clinical Research Ethical Committee of Institut Municipal d'Assistència Sanitària (IMAS) (CEIC-IMAS 2009/3347/I), Barcelona, Spain. The study was conducted in accordance to the

Helsinki Declaration and the ICHGCP. The study is registered at the International Standard Randomized Controlled Trial register (Identifier: ISRCTN77500181).

The procedure to obtain the phenolic extracts and the enriched oils has been previously described [23]. Briefly, VOO with a low phenolic content (80 mg totals phenols/kg of oil) was used as a control condition in the intervention and as an enrichment matrix for the preparation of the 2 functional phenol-enriched VOO. The first FVOO was enriched with its own PC (500 mg total phenols/kg of oil) by adding a phenol extract obtained from freeze-dried olive cake collected from a commercial olive mill in the olive-growing area of Les

Garrigues (Lleida, Catalonia, Spain). The FVOOT was enriched with its own PC (50%) and complemented with thyme PC (50%) (500 mg total phenols/kg of oil) using a phenol extract made up of a mixture of olive cake and commercially available dried thyme (*Thymus zyguis)*. The phenolic extracts used for enrichment were obtained in the laboratory using an accelerated solvent extractor (ASE 100 Dionex, Sunnyvale, CA).

The sustained study was randomized, controlled, double-blind, and cross-over. Subjects were randomly allocated to one of 3 sequences of administration of 25 mL/day of raw: VOO (80 mg/kg, FVOO (functional VOO enriched with its own PC) and FVOOT (functional VOO enriched both its own phenols and phenols from thyme). Intervention periods were of 3 weeks and VOOs were consumed daily distributed among meals. There was a 2-week washout period prior to VOO interventions during which a common OO kindly provided by Borges Mediterranean Group was consumed (Figure S2) [24].

The random allocation sequence was generated by a statistician, participant enrolment was carried out by a researcher, and participants' assignment to interventions according to the

random sequence was done by a physician. Due to the fact that all participants received

each one of the three VOOs, restrictions such as blocking were unnecessary.

The 3 VOOs tested in the sustained study had the same composition and concentration of fat-soluble vitamins as well as fatty acids (Table S1).

Blood samples were collected in fasting state at least of 10 h, at the start of the study and before and after each treatment. Plasma samples were obtained by centrifugation of whole blood directly after being drawn and were preserved at -80ºC until use (http://marbiobanc.imim.es/es/).

To analyze the clinical impact of the consumption of theses FVOOs, the following variables have been studied before and after each intervention: IRH, cardiovascular biomarkers (endothelin-1, hs CRP, PAI-1 and N Ox), and fat-

soluble vitamins ( retinol, β-crypthoxanthin, β-carotene, l utein, α- and γ-tocopherol).

IRH, lipid profile and cardiovascular biomarkers were performed following the same protocol and using the same equipment in the dose-response study.

Fat-soluble v itamins w ere d etermined in p lasma sam ples. R etinol a nd carotenoids (β-crypthoxanthin, β-carotene and lutein), were measured by HPLC and phot odiode a nd pho todiode a rray de tector a ccording t o G leize e t a l. [25]. Plasma α- and γ-tocopherol were determined by HPLC and fluorescence detector as described by Minguez-Mosquera et al. [26].

To v erify d ietary ad herence, t he compliance b iomarkers w ere d etermined i n plasma a nd 24 -h-urine be fore and after each V OO i ntervention p eriod. F or FVOO hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate were analysed; and for F VOOT t hymol s ulfate and hy droxyphenylpropionic a cid s ulfate w ere evaluated according to Rubió L, et al. [24].

### 2.3 Sample size

In t he dos e-response study, a sample size of 12 participants allowed at least ≥ 80% p ower to d etect a s tatistically si gnificant d ifference b etween g roups of 10 units o f I RH, a ssuming a dr opout rate o f 15 % a nd a t ype I e rror o f 0.05 ( 2-sided). The common standard deviation (SD) of the method is 11 units [5].

In t he s ustained study, a s ample s ize of 30 individuals a llowed a pow er o f a t least 8 0% p ower t o d etect a st atistically si gnificant d ifference am ong t hree groups of 3 m g/dL of HDL-c a nd a S D of 1. 9, u sing a n A NOVA t est a nd assuming a dropout rate of 15% and a Type I error of 0.05.

### 2.4 Statistics analysis

Data were expressed as the mean and SD for variables with normal distribution or percentages, according the type of variable. The geometric mean and antilog SD w ere us ed to d escribe l og-transformed v ariables w ith n ormal d istribution.

The Kolmogorov-Smirnov and Shapiro–Wilk's W test were used to verify the distributions of the variables.

In the dose-response study, kinetic parameters of the IRH and compliance biomarkers were calculated by means of pharmacokinetic functions (using Microsoft Excel).

The carry-over effect was discarded in all variables in both studies. Multiple Linear Regression analysis was used to predict post-intervention values adjusted for age, sex and pre-intervention values. Comparisons between groups were analyzed by General Linear Model. Paired T-test was used to test the changes post-pre intervention period on all studied variables in both studies.

Spearman correlation coefficients were calculated for relationships among IRH and cardiovascular biomarkers, fat-soluble vitamins, and PC compliance biomarkers. The level of statistical significance was set at $p<0.05$. All data were analyzed using the Statistical Package for the Social Sciences for windows (20.0 version; IBM Corp, Armonk, NY, USA).

## 3. Results

### 3.1 Dose-response study

*3.1.a Subjects*

13 participants were recruited, of these 12 (6 women) were eligible and completed the study. The 3 VOOs were well tolerated by all participants and no adverse events were reported. Participants' baseline characteristics are shown in Table S2.

*3.1.b.Endothelial function*

The postprandial time-course changes in IRH after ingestion of the 3 different VOOs are shown in Figure 1. The 3 VOOs increased IRH values during the

postprandial time with respect to their basal value. The L-FVOO increased IRH significantly at 6 h with respect to basal, 2 h, and 4 h after intake ($p<0.05$). The M-FVOO IRH time-course increased in a linear trend, at 4 h it was significantly higher with respect to baseline and, at 6 h is higher significantly with respect to basal, 2 h, and 4 h postprandial ($p<0.05$). The H-FVOO produced no significant increases in IRH values during the postprandial with respect to baseline.

*3.1.c.Cardiovascular risk biomarkers*

The time-course of the cardiovascular risk biomarkers is shown in Table S3. The L-FVOO significantly decreases the hsCRP, PAI-1, and NOx concentrations at each time-point with respect to baseline, and glucose concentrations at 1 h after intake ($p<0.001$). In addition, L-FVOO increased in a linear trend endothelin-1, and GSH concentrations, during the postprandial time-course with respect to baseline. Moreover, the total cholesterol, TG, and ox-LDL increased during the postprandial state from 1 h after intake ($p<0.002$).

The M-FVOO significantly decreases glucose concentrations during the postprandial state from 1 h and the PAI-1 values after 4 h from baseline ($p<0.001$). Also, M-FVOO increased TG concentrations during the postprandial time-course from 1 h, GSH values since 4 h from baseline and GSH/GSSG ratio at 4 h versus 2 h postprandial ($p<0.001$).

The H-FVOO significantly decreases the LDL-c, hsCRP, and PAI-1 concentrations ($p<0.001$) at each time-point with respect to baseline, glucose levels at 1 h after intake ($p<0.001$), and, NOx values 4 h versus 2 h postprandial ($p=0.048$). H-FVOO also significantly increases HDL-c, ox-LDL and ox-LDL/LDL-c ratio at 2 h with respect to 1 h postprandial ($p<0.002$), and endothelin-1 at 6 h postprandial with respect to the other time-points ($p=0.035$).

*3.1.d.Functional olive oil selection*

To select the best FVOO for the sustained study, we took into account the following aspects: a) M-FVOO increased IRH values linearly during the postprandial time and was the first to show at 4 h significant differences with respect to basal time; b) M-FVOO, compared to L-FVOO and H-FVOO, did not increase the endothelin-1 concentrations during the postprandial time and did not decrease the NOx values after intake. Moreover, M-FVOO increased the GSH and GSH/GSSG ratio at 4 h compared to L-FVOO and H-FVOO; and, c) only M-FVOO at 4 h after intake showed a negative borderline correlation for IRH AUC and ox-LDL (p=0.050). In addition, it showed, a positive borderline correlation for IRH AUC and GSH/GSSG ratio and with $C_{max}$ hydroxytyrosol sulfate (p=0.057) and $C_{max}$hydroxytyrosol acetate sulfate (p= 0.047).

Due to all these aspects, the FVOO with a PC concentration of 500 mg/kg oil (M-FVOO) was chosen as optimal, and was used as a reference to develop the FVOOs for the sustained study.

### 3.2. Sustained study

*3.2.a.Subjects*

The characteristics of the study participants at baseline, and segregated according to the sequence of VOO administration, are shown in Table S4. Significant differences were not observed among sequences of VOO administration. No changes were reported in the main nutrients and medication intake throughout the study. The 3 VOOs were well tolerated by all participants and no adverse events were reported. The participants' flow-chart is described in Figure S3.

*3.2.b.Endothelial function*

The changes in IRH values after each 3-week VOO intervention are shown in Figure 2. Both FVOOs, either enriched with their own PC or complemented with thyme PC, had higher post-intervention IRH values when compared to the

standard VOO ($p<0.05$). No significant differences were observed between both FVOOs. Post-intervention differences were not reported in any of the cardiovascular risk biomarkers analyzed.

A positive post-intervention relationship was observed for IRH values and HDL-c plasma concentrations ($r=0.369$, $p=0.001$). In contrast, an inverse correlation was described between IRH values and plasma concentrations of hsCRP ($p=0.001$).

*3.2.c.Fat-soluble vitamins*

The changes in plasma fat-soluble vitamin concentrations after the 3 VOO interventions are summarized in Table 1. The intervention with FVOOT allowed us to observe the greatest change. After FVOOT administration an increment of retinol, β-carotene, β-cryptoxanthin, lutein, and α-tocopherol was reported in plasma concentrations ($p<0.05$). The FVOO administration also increased the plasma concentrations of α- and γ- tocopherol ($p<0.05$). In contrast, after the control VOO intervention the plasma concentrations of retinol, lutein, and α-tocopherol decreased ($p<0.05$). Furthermore, we observed that after both FVOO interventions, the plasma concentrations of retinol, β-cryptoxanthin, lutein, and α-tocopherol were higher compared to the control VOO ($p<0.01$).

A positive post-intervention relationship was observed for IRH values and plasma concentrations of β-cryptoxanthin, lutein, and α-tocopherol ($p=0.043$; $p=0.008$; $p=0.024$, respectively).

*3.2.d.Phenol metabolites*

Homovanillic alcohol sulfate, which was identified in plasma and urine as a metabolite derived from OO PC and specifically from hydroxytyrosol, presented a positive relationship with IRH values at post-intervention state ($r=0.26$, $p=0.030$). Moreover, we observed positive correlations between phenol metabolites derived from OO (hydroxytyrosol sulfate, hydroxytyrosol acetate

sulfate, homovanillic alcohol sulfate, and homovanillic acetate sulfate) and retinol, β-carotene, β-cryptoxanthin and α- and γ- tocopherol ($p<0.05$; data not shown). On the other hand, thyme phenol metabolites (tymol sulfate, cafeic acid sulfate and hydroxyphenylpropionic acid sulfate) were positively correlated with retinol, β-carotene and α-tocopherol ($p<0.05$; data not shown).

## 4. Discussion

The present work combined the evaluation of the dose-response and sustained effect on EF of different FVOOs. From our results an acute intake of 30 mL of VOO enriched with its own PC, at 500 mg/kg, can provide additional benefits in the EF of healthy volunteers versus other VOO enriched with different PC concentrations (250 or 750 mg/kg). This finding confirms the results previously described by Valls RM, et al. in pre-/hypertensive subjects [10]. Furthermore, the 3-week sustained consumption of 25 mL of FVOOs containing 500 mg/kg PC, either enriched with their own PC or complemented with thyme PC, also improves EF by increasing IRH in hypercolesterolemic patients.

Previous studies have demonstrated the effect of VOO with high PC content on the EF improvement when compared to low PC ones [5, 10] or free-PC OO [6]. The challenge in the present studies is the use of a VOO (80 mg/kg) as matrix enrichment to prepare L-FVOO, M-FVOO and H-FVOO for the dose-response study, and to prepare FVOO and FVOOT for the sustained study. Thus, the composition of VOOs used in both studies was similar with the only difference in the PC content added through a phenolic extract obtained from olive paste. The EF improvement has been described to be mediated via reduction in oxidative stress and the increase of NOx metabolites [5]. In our study we did not observe differences in cardiovascular risk biomarkers after interventions. No differences in post-intervention endothelial dysfunction biomarkers analyzed (NOx, endothelin-1) were detected, so other mechanisms could involve in the vasodilation process such as gene expression. Several genes encoding for drug-

metabolizing or –transporting enzymes have been associated with blood pressure in humans. Environmental factors, such as diet or lifestyle may be modulators of the expression of these pharmacogenes [27].

However, after both FVOOs consumption a significant increment in plasma concentrations of fat-soluble vitamins was observed. The 3 VOOs tested in the sustained study had the same composition and concentration of fat-soluble vitamins and fatty acids. However, after the intervention periods with both FVOO and FVOOT we could observe a significant increment in plasma concentrations of fat-soluble vitamins. Post-intervention differences in fat-soluble vitamins could be related to variety and the different concentration of PC present in the 3 VOO tested. A high concentration of PC in OOs can lead to an increment in the absorption of fat-soluble vitamins and/or a preservation of their systemic levels, thus raising their plasma concentrations. Our results are in concordance with those of Marin C, et al. [18] who observed an increment in plasma concentrations of β-carotene after an intervention with a Mediterranean diet rich in VOO compared to another diet with the same percentage of dietary fat and β-carotene concentration. In addition, *in vitro* studies have suggested that flavonoids from citrus could enhance carotenoid uptake by intestinal Caco-2 cells through their iron-chelating activity [28]. In this regard, in our study we observed that FVOOT with flavonoids from thyme produced the greatest increment of carotenoids in plasma concentrations. Also, PC could modulate gut microbiota, enhancing the growth of specific beneficial bacteria strains and inhibiting the growth of some pathogenic bacteria, which in turn exerts a modulation of host metabolism and inflammation [29, 30]. It has also been described that when the phytoene dehydrogenase metabolic pathway, which is involved in the metabolism of lipid-soluble antioxidants, is enriched in the gut metagenome, the plasma concentrations of fat-soluble vitamins are also increased [31]. Thus, another hypothesis that could explain the observed increase in plasma fat-soluble vitamins after both FVOOs intake could be a possible increase of their synthesis by the microbiota. In this sense, positive

correlations in VOOs analysis amongst certain phenol metabolites, such as homovainillic alcohol sulfate or thyme metabolites (caffeic acid sulfate) and fat-soluble vitamins (β-carotene) have been observed.

Despite phenolic compounds being the major phytochemicals present in plant-derived products, studies on interactions between fat-soluble vitamins and PC remain scarce [28]. The mechanism by which PC could increase the absorption of fat-soluble vitamins and the subsequent increase in their plasma concentration needs to be studied.

We have also observed a positive correlation between IRH and plasma concentrations of certain fat-soluble vitamins. As a result, so that, a mechanism by which the IRH could be improved after both functional VOO interventions might be through the increase of these fat-soluble vitamins in plasma. Marin C, et al. [18] described positive correlations between β-carotene and circulating endothelial progenitor cells, which favor the regenerative capacity of the endothelium. Similarly, Karppi J, et al. [32] suggested that high plasma concentrations of β-cryptoxanthin, lycopene, and α-carotene may be associated with decreased intima-media thickness of the carotid artery wall.

It is known that a combination of bioactive compounds, rather than a single one, is most likely to have larger health benefits. For example, the combination of carotenoids and PC was revealed to have synergistic effects by preventing human LDL oxidation more effectively than carotenoids alone [33]. In this way, a synergistic effect of two different bioactive compounds, PC and fat-soluble vitamins, may have been the cause of the improvement of the IRH observed in the present sustained study.

In our sustained consumption study, a positive relationship was also observed between IRH and plasma concentrations of HDL-c, a result also described in hypercholesterolemic patients by Ruano J, et al [5]. The HDL particle has been described as exerting a protective effect on the vascular endothelium [34]. HDL has the ability to inhibit monocyte adhesion by inhibiting vascular cell adhesion molecule ( VCAM-1), intercellular cell adhesion molecule (ICAM-1), and E -

selectin expression, and also to suppress monocyte chemotactic protein-1 (MCP1) by inhibiting the chemokine secretion [34]. Accordingly, the increment of HDL particles could be another mechanism by which EF improvement occurs after the intake of both FVOOs in the sustained study. In the context of the same trial, it has been reported that the FVOOT intervention improved HDL subclass distribution and composition [35] and both FVOOs increased ApoA-1 concentrations [36] which can be reflected in a better HDL functionality.

One of the strengths of the present study is its design as a randomized, controlled, clinical trials which is able to provide the first level of scientific evidence. In addition, the cross-over design, in which each subject acts as the corresponding control, minimizes the interference of possible confounding variables. Also, this study evaluated both the acute and sustained effect through different phenol-enriched OO interventions, on EF, obtaining the same results. In addition, the OOPC effects on EF measured by IRH have been confirmed in different populations such as hyperlipemics [5], pre- and hypertensive [6, 10], and healthy subjects.

One potential limitation of the study was that, although the trial was blinded, some participants might have identified the type of olive oil ingested by its organoleptic characteristics. Another limitation is the inability to assess potential synergies and interactions among the VOOs and other diet components, although the controlled diet followed throughout the trial should have limited the scope of these interactions.

In summary, a FVOO enriched with its own PC or complemented with thyme PC, and with a PC concentration of 500 mg/kg, promoted greater additional improvements in human EF than that provided by a natural standard VOO. The interaction between OO and thyme phenol metabolites and fat-soluble vitamins appears to be possible mechanisms for explaining the improvement in EF after the sustained consumption of FVOOs. Thus, FVOOs tested in our study could be a new tool for cardiovascular disease prevention in hypercholesterolemic subjects.

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Authors' contributions to manuscript were as following: R-MV, MFi, RdlT, M-IC, M-JM, RS design research; R-MV, MFa, AP,SF-C, UC, LR, MR, MG, GT-S, MFi, RdlT, M-IC, M-JM, RS were responsible for the execution of the study including hands-on conduct of the experiments, data collection and interpretation of data; R-MV, AP, RS drafted the manuscript and MFi, M-IC, M-JM, RS revised the manuscript critically providing important intellectual content. RS has primary responsibility for final content. All the authors read and approved the final manuscript.

The authors have no conflicts of interest to declare.

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**FIGURE LEGENDS**

**Figure 1. Changes in IRH values at postprandial time, after FVOOs intake.**

L-FVOO, low phenolic compounds content functional virgin olive oil (250 mg totals phenols/kg of oil); M-FVOO, medium phenolic compounds content functional virgin olive oil (500 mg totals phenols/kg of oil); H-FVOO, high phenolic compounds content functional virgin olive oil (750 mg totals phenols/kg of oil).

* p < 0,05 with respect to basal time within the treatment; † p< 0,05 with respect to 2 h postprandial within the treatment; ¥ p< 0,05 with respect to 4 h postprandial within the treatment; a p< 0,05 between L-FVOO to M-FVOO at same postprandial time; b p< 0,05 between L-FVOO to H-FVOO at same postprandial time; c p < 0,05 between M-FVOO to H-FVOO at same postprandial time.

**Figure 2. Changes in IRH values after VOOs intervention.**

VOO, virgin olive oil with a low phenolic content (80 mg total phenols/kg of oil); FVOO, functional virgin olive oil enriched with its own phenolic compounds (500 mg total phenols/kg of oil); FVOOT, functional virgin olive oil (500 mg total phenols/kg of oil) enriched with its own phenolic compounds (50%) plus complementary phenols from thyme (50%).

* p< 0,05 with respect to VOO.

**Figure 1. Changes in IRH values at postprandial time, after FVOOs intake.**

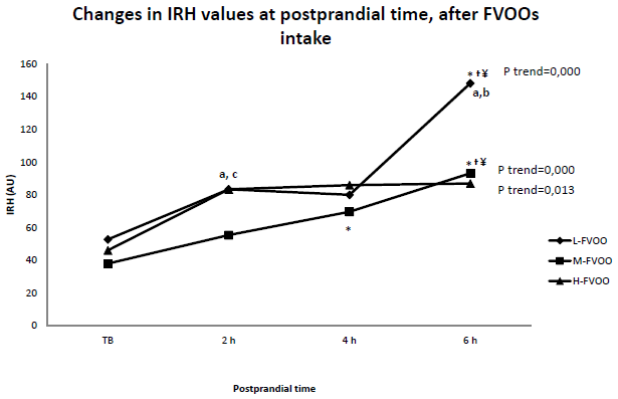



**Figure 2. Changes in IRH values after VOOs intervention.**

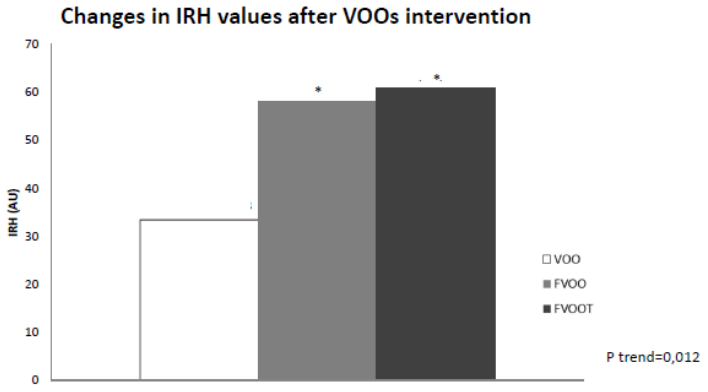


**Table 1.** Changes in plasma concentrations of fat-soluble vitamins after virgin olive oils interventions (n=33).

| | VOO | | | FVOO | | | FVOOT | | | Post-intervention differences between treatments, P value | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Variable** | **Baseline** | **Post-intervention** | **P value** | **Baseline** | **Post-intervention** | **P value** | **Baseline** | **Post-intervention** | **P value** | **VOO vs FVOO** | **VOO vs FVOOT** | **FVOO vs FVOOT** |
| Retinol, µmol/L | 20.59±4.08 | 18.12±3.48 | 0.001 | 19.95±4.63 | 19.74±5.14 | 0.770 | 20.03±3.92 | 22.39±4.49 | 0.002 | 0.000 | 0.000 | 0.001 |
| β-Carotene, µmol/L* | 4.40±2.28 | 4.14±2.33 | 0.508 | 4.38±2.37 | 4.74±2.49 | 0.413 | 3.83±2.24 | 5.14±2.10 | 0.029 | 0.795 | 0.036 | 0.165 |
| β-Cryptoxanthin, µmol/L* | 9.79±1.40 | 9.74±1.42 | 0.835 | 10.49±1.52 | 10.64±1.56 | 0.074 | 9.86±1.51 | 11.78±1.51 | 0.000 | 0.009 | 0.000 | 0.105 |
| Lutein, µmol/L* | 4.47±1.80 | 3.81±1.66 | 0.029 | 4.61±1.67 | 4.77±1.60 | 0.389 | 3.97±1.80 | 5.10±1.65 | 0.007 | 0.008 | 0.000 | 0.007 |
| α-Tocopherol, µmol/L | 16.93±1.48 | 16.57±1.41 | 0.010 | 17.04±1.69 | 17.59±1.99 | 0.016 | 16.50±1.89 | 18.50±2.28 | 0.000 | 0.000 | 0.000 | 0.002 |
| γ-Tocopherol, µmol/L* | 0.14±1.79 | 0.14±1.74 | 0.863 | 0.13±1.53 | 0.16±1.81 | 0.047 | 0.14±1.83 | 0.14±1.83 | 0.694 | 0.043 | 0.327 | 0.093 |

Values are expressed as mean±SD or *non normal variables are expressed by geometric mean±logSD.
Differences between baseline and post-intervention values for each treatment were analysed by T-Student test and the differences between treatments after olive oil intervention were analysed by repeated measures general lineal model test using the value adjusted by baseline value and gender.
Abbreviations: VOO, virgin olive oil with a low phenolic content; FVOO, functional virgin olive oil enriched with its own phenolic compounds; FVOOT, functional virgin olive oil enriched with its own phenolic compounds (50%) plus complementary phenols from thyme (50%).

# SUPPLEMENTAL MATERIAL

## TABLES

**Supplemental Table 1.** Virgin olive oils composition. Phenolic compounds, fat soluble micronutrients and fatty acids daily intake through 25 mL of VOO, FVOO and FVOOT.

| | VOO | FVOO | FVOOT |
|---|---|---|---|
| **PHENOLIC COMPOUNDS (mg/25 mL/day)** | | | |
| hydroxytyrosol | 0,01 ± 0,00 | 0,21 ± 0,02 | 0,12 ± 0,00 |
| 3,4-DHPEA-AC | n.d. | 0,84 ± 0,06 | 0,39 ± 0,04 |
| 3,4-DHPEA-EDA | 0,04 ± 0,00 | 6,73 ± 0,37 | 3,43 ± 0,29 |
| 3,4-DHPEA-EA | 0,26 ± 0,04 | 0,71 ± 0,06 | 0,36 ± 0,03 |
| ***Total hydroxytyrosol derivates*** | ***0,30*** | ***8,49*** | ***4,30*** |
| p-hydroxybenzoic acid | n.d. | 0,02 ± 0,00 | 0,06 ± 0,00 |
| vanillic acid | n.d. | 0,07 ± 0,00 | 0,13 ± 0,01 |
| caffeic acid | n.d. | 0,00 ± 0,00 | 0,06 ± 0,00 |
| rosmarinic acid | n.d. | n.d. | 0,41 ± 0,03 |
| ***Total phenolic acids*** | - | ***0,09*** | ***0,65*** |
| thymol | n.d. | n.d. | 0,64 ± 0,05 |
| carvacrol | n.d. | n.d. | 0,23 ± 0,02 |
| ***Total monoterpenes*** | - | - | ***0,86*** |
| luteolin | 0,04 ± 0,00 | 0,18 ± 0,02 | 0,21 ± 0,02 |
| apigenin | 0,02 ± 0,00 | 0,06 ± 0,00 | 0,10 ± 0,00 |
| naringenin | n.d. | n.d. | 0,20 ± 0,02 |
| eriodictyol | n.d. | n.d. | 0,17 ± 0,01 |
| thymusin | n.d. | n.d. | 1,22 ± 0,09 |
| xanthomicrol | n.d. | n.d. | 0,53 ± 0,06 |
| 7-methylsudachitin | n.d. | n.d. | 0,53 ± 0,09 |
| ***Total flavonoids*** | ***0,06*** | ***0,23*** | ***2,95*** |
| pinoresinol | 0,05 ± 0,00 | 0,12 ± 0,00 | 0,10 ± 0,05 |
| acetoxipinoresinol | 2,47 ± 0,19 | 3,66 ± 0,31 | 3,24 ± 0,28 |
| ***Total lignans*** | ***2,52*** | ***3,78*** | ***3,34*** |
| **FAT SOLUBLE MICRONUTRIENTS (mg/25 mL/day)** | | | |
| α-tocopherol | 3,27 ± 0,01 | 3,40 ± 0,02 | 3,44 ± 0,01 |
| lutein | 0,05 ± 0,00 | 0,06 ± 0,00 | 0,07 ± 0,00 |
| β-cryptoxanthin | 0,02 ± 0,00 | 0,03 ± 0,00 | 0,02 ± 0,00 |
| β-carotene | 0,01 ± 0,00 | 0,02 ± 0,00 | 0,02 ± 0,00 |
| **FATTY ACIDS (relative area %)** | | | |
| Palmitic acid | 11,21 | 11,20 | 11,21 |
| Stearic acid | 1,92 | 1,92 | 1,92 |
| Araquidic acid | 0,36 | 0,36 | 0,36 |
| Behenic acid | 0,11 | 0,11 | 0,11 |
| ***Total saturated*** | ***13,75*** | ***13,74*** | ***13,75*** |
| Palmitoleic acid | 0,70 | 0,70 | 0,69 |
| Oleic acid | 76,74 | 76,83 | 76,75 |
| Gadoleic acid | 0,27 | 0,27 | 0,27 |
| ***Total monounsaturated*** | ***77,71*** | ***77,80*** | ***77,72*** |
| Linoleic acid | 7,43 | 7,36 | 7,43 |
| Timnodonic acid | 0,36 | 0,36 | 0,35 |
| Linolenic acid | 0,43 | 0,43 | 0,43 |
| ***Total polyunsaturated*** | ***8,22*** | ***8,15*** | ***8,22*** |

Values expressed as mean ± standard deviation (SD). 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone.

**Supplemental Table 2.** Subjects' characteristics at inclusion visit of dose-response study (n=12).

| Variable | Total participants (n=12) |
|---|---|
| Gender (male/female) | 6/6 |
| Age, years | 50 ± 13 |
| Body weight, kg | 74.35 ± 13.88 |
| BMI, kg/m$^2$ | 27.20 ± 2.77 |
| SBP, mm Hg | 128.75 ± 21.34 |
| DBP, mm Hg | 79.08 ± 15.91 |
| Glucose, mg/dL | 92.44 ± 11.33 |
| LDL-c, mg/dL | 137.87 ± 32.39 |
| HDL-c, mg/dL | 52.78 ±11.75 |
| TG, mg/dL | 101.00 ± 47.95 |

Values are expressed as mean ± standard deviation (SD).
Abbreviations: BMI, body mass index, SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, HDL cholesterol; LDL-c, LDL cholesterol; TG, tryglicerides.

**Supplemental Table 3.** Postprandial time-course of cardiovascular risk biomarkers after different the 3 FVOOs interventions (n=12).

| Variable | Intervention | Baseline | 1 hour | 2 hours | 4 hours | 6 hours | P linear trend |
|---|---|---|---|---|---|---|---|
| Glucose, mg/dL | L-FVOO | 94.92 ± 9.91 | 109.67 ± 23.50$^{a}$ | 86.08 ± 17.55$^{ab}$ | 83.08 ± 0.95$^{ab}$ | 83.67 ± 2.88$^{ab}$ | 0.001 |
| | M-FVOO | 93.00 ± 8.26 | 111.00 ± 15.62$^{a}$ | 91.92 ± 11.80$^{b}$ | 86.33 ± 4.76$^{ab}$ | 82.42 ± 1.98$^{abcd}$ | 0.000 |
| | H-FPOO | 92.83 ± 9.23 | 111.17 ± 19.48$^{a}$ | 83.75 ± 11.48$^{b3}$ | 84.92 ± 3.52$^{ab}$ | 84.83 ± 0.97$^{ab3}$ | 0.001 |
| Total cholesterol, mg/dL | L-FVOO | 204.17 ± 31.47 | 195.92 ± 26.01$^{a}$ | 205.42 ± 27.08$^{b}$ | 205.08 ± 26.65$^{b}$ | 207.67 ± 27.19$^{ab}$ | 0.001 |
| | M-FVOO | 206.50 ± 34.95 | 198.08 ± 24.03$^{a}$ | 207.17 ± 26.82$^{b}$ | 207.92 ± 23.35$^{b}$ | 204.92 ± 22.68$^{bd}$ | 0.420 |
| | H-FPOO | 210.00 ± 29.65 | 197.33 ± 25.44$^{a}$ | 204.42 ± 23.97$^{b}$ | 199.17 ± 25.33$^{abc3}$ | 200.67 ± 27.99$^{ab}$ | 0.000 |
| HDL-c, mg/dL | L-FVOO | 52.83 ± 11.63 | 51.00 ± 10.70$^{a}$ | 53.78 ± 12.94$^{ab}$ | 53.40 ± 10.55$^{b}$ | 52.78 ± 10.14$^{bd}$ | 0.056 |
| | M-FVOO | 53.28 ± 11.55 | 50.52 ± 9.47$^{a}$ | 53.43 ± 10.38$^{b}$ | 51.81 ± 9.35$^{bc}$ | 52.46 ± 9.79$^{bcd}$ | 0.860 |
| | H-FPOO | 53.03 ± 10.19 | 48.67 ± 6.58$^{a}$ | 52.96 ± 10.34$^{b}$ | 50.83 ± 9.30$^{abc}$ | 50.61 ± 9.21$^{abc}$ | 0.002 |
| LDL-c, mg/dL | L-FVOO | 129.17 ± 28.42 | 121.67 ± 24.63$^{a}$ | 125.82 ± 24.33$^{ab}$ | 126.15 ± 25.86$^{b}$ | 129.75 ± 25.90$^{bcd}$ | 0.222 |
| | M-FVOO | 132.63 ± 30.76 | 125.82 ± 23.82$^{a}$ | 128.77 ± 26.28$^{a}$ | 129.74 ± 22.66$^{b}$ | 127.73 ± 19.14 | 0.449 |
| | H-FPOO | 134.91 ± 28.46 | 125.83 ± 23.25$^{a}$ | 126.48 ± 24.86$^{a}$ | 123.34 ± 23.77$^{ab}$ | 126.71 ± 25.51$^{ad}$ | 0.000 |
| TG, mg/dL | L-FVOO | 110.83 ± 48.47 | 116.25 ± 45.86$^{a}$ | 129.08 ± 46.57$^{ab}$ | 127.67 ± 36.41$^{ab}$ | 125.67 ± 48.51$^{ab}$ | 0.000 |
| | M-FVOO | 103.00 ± 34.00 | 108.75 ± 31.96$^{a}$ | 124.83 ± 32.72$^{ab}$ | 131.83 ± 17.27$^{ab}$ | 123.67 ± 45.05$^{ab}$ | 0.000 |
| | H-FPOO | 110.33 ± 43.32 | 114.17 ± 44.13 | 124.92 ± 46.93$^{ab}$ | 125.00 ± 52.96$^{ab}$ | 116.75 ± 53.88$^{ad}$ | 0.035 |
| ox-LDL, U/L | L-FVOO | 41.67 ± 17.60 | 37.51 ± 16.45$^{a}$ | 43.02 ± 9.60$^{b}$ | 45.69 ± 9.96$^{bc}$ | 45.05 ± 16.78$^{ab}$ | 0.000 |
| | M-FVOO | 47.05 ± 18.99 | 39.87 ± 8.18 | 43.13 ± 10.10$^{b}$ | 44.03 ± 6.84$^{b}$ | 49.71 ± 11.26$^{bcd}$ | 0.117 |
| | H-FPOO | 39.58 ± 12.05 | 33.36 ± 9.88 | 44.90 ± 9.83$^{ab}$ | 41.53 ± 11.24$^{bc}$ | 43.57 ± 9.38$^{ab}$ | 0.000 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Ratio oxLDL/LDL-c | L-FVOO | 0.33 ± 0.14 | 0.31 ± 0.12 [a] | 0.35 ± 0.90 | 0.36 ± 0.77 [bc] | 0.35 ± 0.12 [b] | 0.068 |
| | M-FVOO | 0.36 ± 0.15 | 0.32 ± 0.74 | 0.33 ± 0.82 [b] | 0.35 ± 0.85 [bc] | 0.39 ± 0.65 [bcd] | 0.156 |
| | H-FPOO | 0.31 ± 0.13 | 0.29 ± 0.14 | 0.36 ± 0.84 [ab] | 0.34 ± 0.86 [ab] | 0.36 ± 0.16 [ab] | 0.000 |
| GSH-Px, U/L | L-FVOO | 541.17 ± 92.57 | 537.58 ± 84.97 | 551.17 ± 78.58 [b] | 549.58 ± 64.90 | 568.25 ± 64.66 [bcd] | 0.113 |
| | M-FVOO | 563.83 ± 85.94 | 561.25 ± 48.81 | 565.17 ± 48.81 | 567.42 ± 58.97 [1] | 563.25 ± 68.97 | 0.938 |
| | H-FPOO | 559.75 ± 96.66 | 524.50 ± 98.02 [a] | 563.17 ± 60.35 [b] | 550.75 ± 58.64 [bc] | 559.75 ± 78.06 [b] | 0.360 |
| GSSG, µmol/L | L-FVOO | 0.61 ± 0.29 | 0.56 ± 0.18 | 0.59 ± 0.16 | 0.61 ± 0.15 [b] | 0.67 ± 0.15 [bcd] | 0.126 |
| | M-FVOO | 0.71 ± 0.29 | 0.65 ± 0.07 [1] | 0.67 ± 0.25 [a] | 0.86 ± 0.25 [b1] | 0.61 ± 0.18 [bcd] | 0.889 |
| | H-FPOO | 0.55 ± 0.15 | 0.61 ± 0.16 | 0.46 ± 0.11 [ab23] | 0.57 ± 0.09 [23] | 0.85 ± 0.66 [a] | 0.162 |
| GSH, µmol/L | L-FVOO | 1.71 ± 0.43 | 1.58 ± 0.26 | 1.64 ± 0.32 | 1.74 ± 0.47 | 2.33 ± 0.80 [abcd] | 0.001 |
| | M-FVOO | 1.83 ± 0.50 | 1.65 ± 0.18 | 1.62 ± 0.12 | 2.11 ± 0.12 [bc1] | 2.37 ± 0.77 [abc] | 0.000 |
| | H-FPOO | 1.60 ± 0.41 | 1.89 ± 0.89 | 1.59 ± 0.32 | 1.71 ± 0.19 [b] | 1.57 ± 0.16 [23] | 0.474 |
| Ratio glutathione reduced/oxidized | L-FVOO | 350.87 ± 205.45 | 390.84 ± 94.40 | 305.26 ± 130.00 [b] | 323.77 ± 138.16 [bc] | 385.77 ± 52.18 [a] | 0.976 |
| | M-FVOO | 280.17 ± 98.46 | 299.23 ± 31.94 [1] | 269.81 ± 34.60 [b] | 322.96 ± 20.23 [c] | 444.50 ± 145.97 [abcd] | 0.000 |
| | H-FPOO | 304.89 ± 91.92 | 382.66 ± 103.42 [2] | 356.49 ± 82.97 [a2] | 347.15 ± 71.82 [a] | 272.87 ± 122.06 [ab23] | 0.201 |
| TBARS, nmol/mL | L-FVOO | 1.16 ± 0.21 | 1.10 ± 0.16 | 1.17 ± 0.24 [b] | 1.18 ± 0.17 [b] | 1.11 ± 0.08 | 0.868 |
| | M-FVOO | 1.21 ± 0.19 | 0.47 ± 0.47 [a1] | 1.20 ± 0.19 [ab] | 1.16 ± 0.17 [ab] | 1.14 ± 0.21 [abc] | 0.000 |
| | H-FPOO | 1.22 ± 0.46 | 1.18 ± 0.22 [2] | 1.24 ± 0.33 | 1.21 ± 0.18 [b] | 1.10 ± 0.29 [bcd] | 0.212 |
| Endothelin-1, pg/mL | L-FVOO | 0.99 ± 0.47 | --- | 1.11 ± 0.42 [a] | 1.12 ± 0.38 [ac] | 1.30 ± 0.25 [ac] | 0.002 |
| | M-FVOO | 1.29 ± 0.26 [1] | --- | 1.05 ± 0.34 [a] | 1.07 ± 0.80 [a] | 1.36 ± 0.80 [cd] | 0.321 |
| | H-FPOO | 1.22 ± 0.54 | --- | 1.10 ± 0.30 | 1.13 ± 0.20 | 1.56 ± 0.26 [acd23] | 0.035 |
| hsCRP, mg/L | L-FVOO | 1.76 ± 1.90 | --- | 1.55 ± 1.81 [a] | 1.59 ± 1.91 [a] | 1.47 ± 1.82 [acd] | 0.000 |
| | M-FVOO | 1.08 ± 1.02 | --- | 1.00 ± 1.04 [a] | 1.06 ± 0.86 | 1.00 ± 0.85 [a] | 0.376 |
| | H-FPOO | 1.64 ± 1.39 | --- | 1.46 ± 1.43 [a] | 1.37 ± 1.43 [ac] | 1.44 ± 1.43 [ad] | 0.000 |
| PAI-1, ng/mL* | L-FVOO | 16.62 ± 1.95 | --- | 13.88 ± 1.23 [a] | 9.06 ± 1.24 [ac] | 8.46 ± 1.17 [acd] | 0.001 |
| | M-FVOO | 12.41 ± 2.16 | --- | 14.06 ± 1.91 [a] | 6.56 ± 3.16 [ac] | 7.80 ± 1.72 [ac] | 0.000 |
| | H-FPOO | 11.34 ± 1.89 | --- | 10.43 ± 1.91 [a2] | 5.02 ± 1.87 [ac2] | 7.40 ± 1.60 [acd] | 0.000 |
| NOx, µmol/L | L-FVOO | 23.00 ± 6.59 | --- | 19.23 ± 3.76 [a] | 18.00 ± 6.13 [a] | 18.43 ± 6.07 [a] | 0.000 |
| | M-FVOO | 19.50 ± 7.63 [1] | --- | 16.04 ± 4.46 [a1] | 15.51 ± 3.39 [a] | 18.41 ± 1.78 [c] | 0.504 |
| | H-FPOO | 21.83 ± 5.97 | --- | 22.97 ± 2.24 [23] | 20.00 ± 1.29 [c23] | 17.89 ± 1.28 [cd] | 0.048 |

Values are expressed as mean ± SD in normal variables and as geometric mean ± antilogSD in log-transformed variables with normal distribution*. The effects inter- and intra-treatments are analyzed adjusting the variables by gender and basal value. A general lineal model for repeated measurements is used to assess the effect intra- and inter-interventions. The level of statistical significance was set at $p<0.05$.

[a] $p<0.05$ versus baseline; [b] $p<0.05$ versus 1 hour; [c] $p<0.05$ versus 2 hours; [d] $p<0.05$ versus 4 hours; [1] $p<0.05$ between L-FVOO-M-FVOO treatments at the same time point; [2] $p<0.05$ between L-FVOO-H-FVOO treatments at the same time point; [3] $p<0.05$ between M-FVOO-H-FVOO treatments at the same time point.

Abbreviations: HDL-c, HDL cholesterol; LDL-c, LDL cholesterol; TG, triglycerides; ox-LDL, oxidized LDL; GSH-Px, glutathione peroxidase; GSSG, oxidized glutathione; GSH, reduced glutathione; TBARS, Thiobarbituric acid reactive substances; hsCRP, high sensitive C reactive protein; PAI-1, plasminogen activator inhibitor-1; NOx, Nitric oxide.

**Supplemental Table 4.** Characteristics of the study participants at baseline.

| Variable | Sequence 1 (n=11) | Sequence 2 (n=11) | Sequence 3 (n=11) |
|---|---|---|---|
| Gender (male/female) | 5/6 | 7/4 | 7/4 |
| Age, years | 54.91 ± 12.57 | 55.27 ± 11.88 | 55.45 ± 7.84 |
| Body weight, kg | 74.75 ± 16.80 | 74.60 ± 18.49 | 84.45 ± 17.74 |
| BMI, kg/m$^2$ | 25.63 ± 3.68 | 26.31 ±5.25 | 27.85 ± 4.71 |
| SBP, mm Hg | 125.09 ± 18.70 | 128.27 ± 16.69 | 130.45 ± 17.93 |
| DBP, mm Hg | 68.09 ± 13.53 | 72.27 ± 9.31 | 71.91 ± 13.43 |
| Glucose, mg/dL | 88.55 ± 11.63 | 93.00 ± 13.33 | 90.91 ± 10.53 |
| Total cholesterol, mg/dL | 228.36 ± 42.70 | 231.91 ± 32.70 | 218.82 ± 31.21 |
| LDL-c, mg/dL | 150.38 ± 32.33 | 152.08 ± 28.46 | 142.26 ± 25.72 |
| HDL-c, mg/dL | 52.78 ±11.75 | 52.96 ±12.82 | 53.39 ± 9.55 |
| TG, mg/dL | 94.00 (75.00; 149.00) | 119.00 (95.00; 168.00) | 117.00 (81.00; 126.00) |

Values expressed as mean ± standard deviation (SD) or median (25$^{th}$ to 75$^{th}$ percentile). Sequence 1= FVOO, FVOOT and VOO; Sequence 2= FVOOT, VOO and FVOO; Sequence 3= VOO, FVOO and FVOOT.

No significant differences between groups were observed. To compare means or medians among groups, ANOVA or Kruskal-Wallis test were performed, respectively; whereas $\chi 2$ and exact F-test, as appropriate, were computed to compare proportions.

Abbreviations: BMI, body mass index, SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-c, HDL cholesterol; LDL-c, LDL cholesterol; TG, tryglicerides.

**SUPPLEMENTAL FIGURE LEGENDS**

**Supplemental Figure 1.** Experimental protocol for the dose-response study.

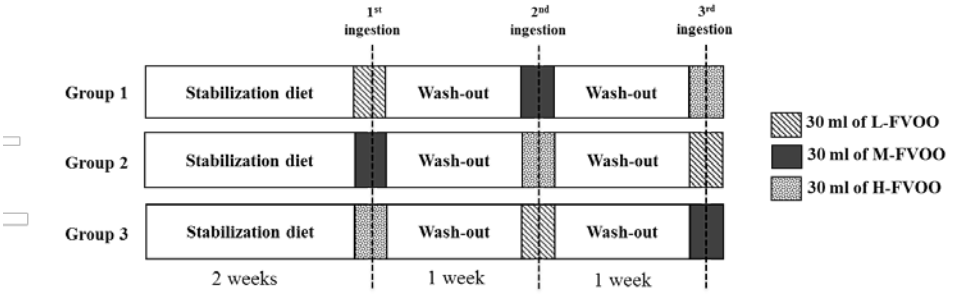
L-FVOO, low phenolic compounds content functional virgin olive oil (250 mg totals phenols/kg of oil); M-FVOO, medium phenolic compounds content functional virgin olive oil (500 mg totals phenols/kg of oil); H-FVOO, high phenolic compounds content functional virgin olive oil (750 mg totals phenols/kg of oil).

**Supplemental Figure 2.** Experimental protocol for the sustained study.

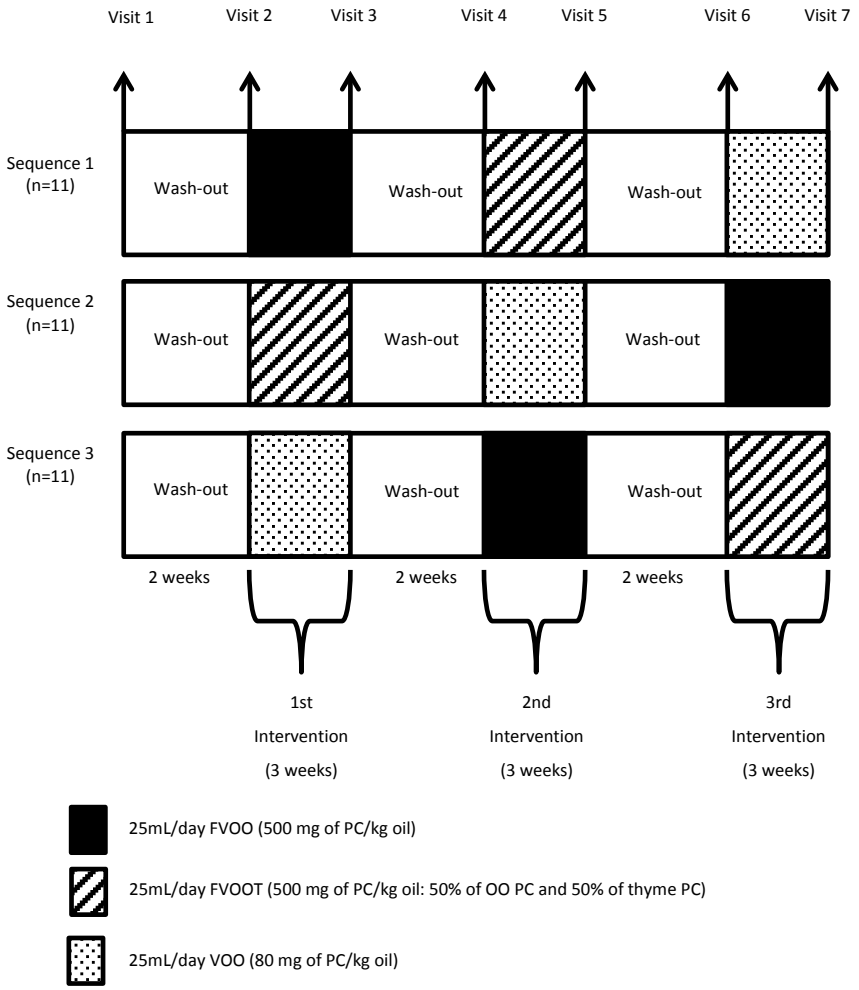
VOO: Virgin Olive Oil; FVOO: Functional Virgin Olive Oil enriched with its own PC; FVOOT: Functional Virgin Olive Oil enriched with its own PC plus complementary phenols from thyme. Sequence 1: FVOO, FVOOT and VOO; Sequence 2: FVOOT, VOO and FVOO; Sequence 3: VOO, FVOO and FVOOT.

**Supplemental Figure 3.** Participants Flow-chart based on post-interventions of IRH variable**.** Sequence 1: FVOO, FVOOT and VOO; Sequence 2: FVOOT, VOO and FVOO; Sequence 3: VOO, FVOO and FVOOT.

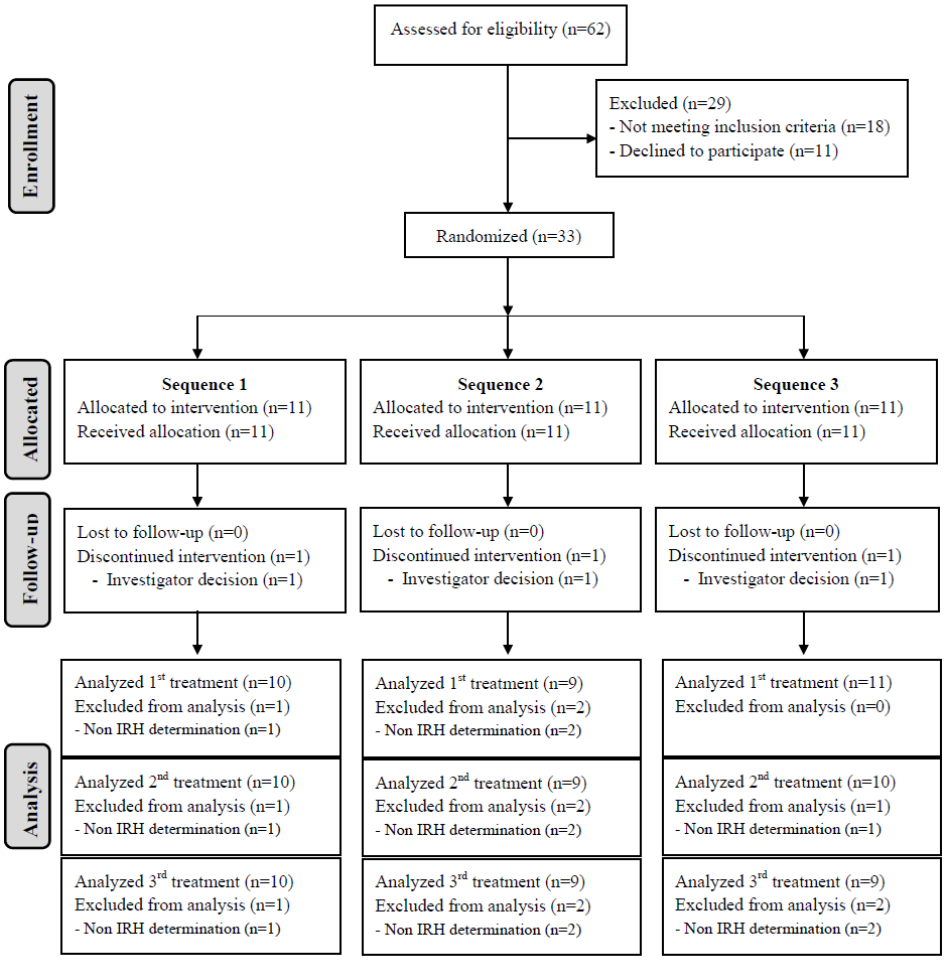
**Supplemental Figure 1.**




**Supplemental Figure 2.**



**Supplemental Figure 3.**



# Annex III: Publication nº 6

## HDL-Related Mechanisms of Olive Oil Protection in Cardiovascular Disease

José M. Lou-Bonafonte, Montse Fitó, María-Isabel Covas, Marta Farràs and Jesús Osada

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# HDL-Related Mechanisms of Olive Oil Protection in Cardiovascular Disease

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**Abstract:** The low incidence of cardiovascular disease in countries bordering the Mediterranean basin, where olive oil is the main source of dietary fat, and the negative association between this disease with high density lipoproteins has stimulated interest. This review summarizes the current knowledge gathered from human and animal studies regarding olive oil and high density lipoproteins. Cumulative evidence suggests that high density lipoprotein (HDL) cholesterol, and its main apolipoprotein A1, may be increased by consuming olive oil when compared with carbohydrate and low fat diets in humans. Conflicting results have been found in many studies when olive oil diets were compared with other sources of fat. The role of virgin olive oil minor components on its protective effect has been demonstrated by a growing number of studies although its exact mechanism remains to be elucidated. Dietary amount of olive oil, use of virgin olive oil, cholesterol intake, and physiopathological states such as genetic background, sex, age, obesity or fatty liver are variables that may offset those effects. Further studies in this field in humans and in animal models are warranted due to the complexity of HDL particles.

**Keywords:** Apolipoprotein, high density lipoprotein, olive oil, cardiovascular risk.

## INTRODUCTION

The Mediterranean diet is a nutritional pattern traditionally followed by the people of the Mediterranean basin. There are at least 16 countries bordering the Mediterranean Sea and food habits may vary among these countries according to culture, ethnic background, and religion. The traditional Mediterranean diet has been associated with a lower incidence of coronary heart disease (CHD) [1], it used to be the alimentary pattern of countries such as Spain, Italy, and Greece, and the region of southern France, during the decade of the sixties. The Mediterranean diet is largely vegetarian in nature and includes the consumption of large quantities of olive oil (OO) as the main source of calories [2].

## VIRGIN OLIVE OIL

Virgin OO is the juice from the fruit of *Olea europea* obtained by physical procedures and does not require any chemical extraction process compared to other seed oils. When the juice has certain organoleptic characteristics reaching a score > 6.5, verified by professional tasters, its acidity is <0.8 and peroxide content lower than 20 meq $O_2$/kg, it is denominated extra virgin OO. The constituents of virgin OO can be divided into 2 fractions, saponifiable and unsaponifiable [3-6]. The saponifiable fraction generally ranges from 98.5% to 99.5% of oil and is formed by triglycerides esterifying namely oleic acid, moderate quantities of palmitic and linoleic acids, and a low percentage of stearic and linolenic acids [3-6]. As shown in (Table **1**), the unsaponifiable fraction, also known as minor components of OO, contains a great variety of compounds that display a wide range of functions and maintain the stability of the oil and its organoleptic characteristics [7]. These minor components are lost to a great extent during the refining processes [8, 9] and thus are not present in olive oils unless enriched with virgin OO [6, 10]. As shown in (Table **1**), the terpenes represent one of the most abundant fractions and among these there are 2 alcohols, uvaol, and erythrodiol, and their corresponding acids, oleanolic and maslinic [10]. Phytosterols are also present as β-sytosterol, $\Delta^5$-avenasterol, and campesterol [11]. The main constituent of the hydrocarbons is squalene followed by the carotenes (lutein and β-carotene) in smaller amounts [9]. The phenolic compounds, representing the polar fraction, influence the stability and flavor of virgin OO [12, 13]. Among them, four main groups can be distinguished: a) simple phenols, either alcohols (tyrosol and hydroxytyrosol) or acids (p-cumaric, vanillic, caffeic, synaptic, protocatechuic, gallic, syringic); b) polyphenols (flavonoids: luteolin and apigenin); c) secoiridoids (ester derivatives of elenolic acid—glycosylated oleuropein or non-glycosylated—with hydroxytyrosol and tyrosol); and d) lignans: (+)-pinoresinol and (+)-1-acetoxypinoresinol [14]. Hydroxytyrosol is the most abundant phenolic compound. Part of the unsaponifiable fraction, are α-, β- and γ-tocopherols present in different quantities depending on the variety of olive grove [3, 15],

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Further details on minor components can be found in the literature [16, 17].

**Table 1. Composition of Virgin Olive Oils**

| Component | Content (g%) |
|---|---|
| **Fatty acids of triglycerides** | |
| Myristic (14:0) | 0.0-0.05 |
| Palmitic (16:0) | 7.5-20 |
| Palmitoleic (16:1n7) | 0.3-3.5 |
| Margaric (17:0) | 0-0.3 |
| Heptadecenoic (17:1) | 0.0-0.3 |
| Stearic (18:0) | 0.5-5.0 |
| Oleic (18:1n9) | 55-83 |
| Linoleic (18:2n6) | 3.5-21 |
| α-linolenic (18:3n3) | 0.0-0.9 |
| Arachidic (20:0) | 0.0-0.6 |
| Eicosenoic (20:1n9) | 0.0-0.4 |
| Behenic (22:0) | 0.0-0.2 |
| Lignoceric (24:0) | 0.0-0.2 |
| **Minor components** | |
| Terpene compounds | 0.1-0.3 |
| Phystosterols | 0.1-0.2 |
| Hydrocarbons | |
| Squalene | 0.1-0.8 |
| Carotenes | 0.05-0.1 |
| Phenolic compounds | 0.05-0.1 |

Adapted from [3-6, 9].

## HDL AND CARDIOVASCULAR RISK IN HUMANS

Several epidemiological studies have shown an inverse relationship between high density lipoprotein (HDL) cholesterol (C) concentrations and the risk of CHD [18, 19]. In the Framingham Heart Study, 43% to 44% of coronary events occurred in persons with HDL cholesterol (HDL-C) levels lower than 40 mg/dL (22% of the total study population). Those with 35 mg/dL had an 8-fold higher incidence of coronary disease compared with those having HDL-C levels of higher than 65 mg/dL [20, 21]. It was therefore proposed that an increase of 1 mg/dL of HDL-C levels was associated with a 2 and 3 % decrease of the risk for coronary artery disease in men and women, respectively [22]. In other studies, an even more pronounced decrease in the risk of coronary death (6%) was noted for a similar increase in HDL [23]. Remarkably, low HDL-C has been found as a predictor of cardiovascular disease in countries of low CHD incidence [24] and together with visceral obesity, insulin resistance, hypertension, hypertriglyceridemia are the cluster of disorders defining the metabolic syndrome [25]. Likewise, clinical trials or large-scale prospective studies have demonstrated that high HDL-C levels may prevent cardiovascular events [26, 27]. Furthermore, the use of image techniques in epidemiological studies has shown that the atherosclerotic plaque progression in individuals with carotid estenosis was smaller in those that presented high levels of HDL-C [28]. Current knowledge is still, however, insufficient to recommend desirable levels [29, 30].

## HDL CHARACTERISTICS

HDL, the smallest and most dense of all plasma lipoproteins, are a heterogeneous class of lipoproteins with subtypes identified on the basis of their density, electrophoretic mobility, size, lipid and apolipoprotein (APO) composition [31-33]. APOA1 and APOA2 are the major structural apolipoproteins of HDL. The former represents about 70% of the protein content, and is present on the majority of HDL particles, its plasma concentrations closely correlate with that of HDL-C. The latter, APOA2, is the second most abundant APO of HDL with 20% of the protein composition [34]. Recent proteomic analyses have shown that HDL also transport proteins involved in regulating the complement system and protecting tissue from proteolysis. In addition, other HDL binding proteins have anti-inflammatory properties. As a consequence, the HDL contribution to its antiatherogenic function has been amplified [35]. Furthermore, recent findings from genome-wide association studies, which have identified new genes influencing HDL-C levels, have expanded the complexity of HDL regulation [36].

HDL heterogeneity may affect its relationship with atherosclerosis [32, 33] since not all HDL particles share the same biological properties depending on the apolipoproteins and enzymes associated with them. For example, HDL enriched with APOA1, APOA4, or APOM are considered antiatherogenic [37-39], whereas HDL- APOA2 and HDL-APOJ are associated with pro-atherogenic effects [39, 40]. Thus, the HDL-C concentration *per se* may not adequately reflect the consequences of alterations in protein composition Fig. (**1**). In addition, the functionality of HDL particles may be altered by physicochemical modifications such as 1) non-enzymatic action due to the presence of free ion metals in the atherosclerotic plaques; 2) enzymatic action, such as that caused by myeloperoxidase and fosfolipase A2 activities; 3) incorporation into the HDL structure of acute phase reactants, such as the serum A amyloid or ceruloplasmin; and 4) metabolic modifications, such as those induced by hiperglycaemia, homocysteinylation, tyrosylation, and desialization. These modifications can affect the lipid and/or protein components of the lipoproteins, and alter their cardio-protective properties [41-44]. The importance of HDL as a protective factor for coronary disease is well-known, nevertheless the functionality of the HDL particle deserves to be investigated since it can become pro-inflammatory [33].

## FUNCTIONALITY OF THE HDL PARTICLE

The functionality of the HDL particle is not predicted by plasma levels of HDL-C. Whilst the best known biological function of HDL particles is reverse transport other beneficial

properties have also been described (antioxidant, antiinflamatory, antithrombotic/profibrinolytic, Table **2**).

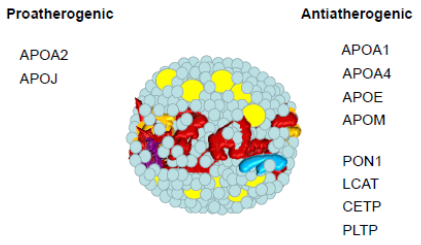


**Fig. (1).** Complexity of HDL with major apolipoproteins and enzymes reflected.

The anti-atherogenic properties of HDL have been mainly ascribed to the process of reverse transport, or flow of C taken from peripheral tissues by the circulating HDL to the liver, which also plays a key role in the regulation of the corporal C homeostasis. In the arterial wall, the removal C from macrophages by HDL may prevent its accumulation and transformation into foam cell, and thereby atherosclerosis [30, 45]. The first stage of the reverse transport is the C efflux from the cells to the fraction pre-β1 of the HDL, initiated by ABCA1-mediated C transport to lipid-poor APOA1. ABCG1 and ABCG4 may also participate. The free C caught by the pre-β-HDL particle is esterified with fatty acids by the lecithin cholesterol acyltransferase (LCAT), and the C ester is then internalized into the core of the particle, forming the mature HDL (α-migrating-HDL). These mature particles have at least three metabolic fates: firstly, the action of enzyme cholesterol ester transfer protein (CETP), which transfers the esterified C from HDL to very low density lipoprotein (VLDL) and low density lipoprotein (LDL), and facilitates the exchange of triglyceride and lipoperoxides from VLDL to HDL; secondly, the action of phospholipid transfer protein (PLTP) which remodels HDL into large and small particles by particle fusion and the dissociation of lipid-poor APOA1. HDL of greater size may incorporate APOE, thereby facilitating the reverse transport of C through binding to the HDL receptor [46]; and finally, direct transfer of cholesteryl esters mainly to the liver or to the steroidogenic tissues through the uptake by scavenger receptor B1 (CLA-1/SRB1), a step that is facilitated by the prior action of hepatic lipase. These two latter possibilities lead to the release of small HDL particles and APOA1, which then can newly acquire C in the peripheral tissues [34, 47]. Thus, cholesteryl esters carried by HDL may reach hepatocytes through a direct pathway involving SRB1 or an indirect way by the transference to VLDL and LDL (low density lipoprotein) exerted through CETP. The subsequent uptake of apolipoprotein B rich in cholesteryl esters, by hepatic LDL receptors may be responsible for up to 50% of reverse C transport [48]. The relevance of either pathway changes in different animal species depending on the CETP presence and activity [34, 47, 49].

The HDL particle has beneficial effects by prevention of LDL oxidation. This action involves a synergic inactivation of oxidized lipids present in LDL through one or more of their components: 1) proteins, such as APOA1, may reduce phospholipid hydro-peroxides by redox-active methionine residues with the formation of phospholipid hydroxides and methionine sulphoxides; other proteins partially associated with HDL (such as transferrin and ceruloplamin) are metal chelants; 2) enzymes-paraoxonase (PON1), platelet activating factor acetylhydrolase (PAF-AH), LCAT, and glutathion peroxidase (GSH-Px)- which hydrolyze short-chain oxidized phospholipids; 3) lipids, such as phospholipids, which could act as acceptors of the lipid oxidation products, stabilize the PON activity, and be essential for PON1 binding at lipoprotein surface; and 4) the high content of antioxidants (such as fat-soluble vitamins and phenolic compounds) present in the HDL also act as a depot system [47, 50, 51].

Other aspects of HDL include: 1) anti-inflammatory properties (diminishing the cellular and humoral mediators of the arterial inflammation) which are attributed to their sphingosine-1-phosphate content [52, 53]; 2) antithrombotic/profibrinolytic effects (reducing platelet aggregation and coagulation); 3) vasorelaxation effects (increasing the production of prostacyclin and nitric oxide); 4) proliferation and migration of the endothelial cells; and 5) apoptosis inhibition of the endothelial cells [30, 45, 48, 54].

**Table 2. HDL Properties**

| Properties | Responsible Components or Action Mechanisms | References |
|---|---|---|
| Antiatherogenic | APOA1, LCAT, CETP, PLTP, APOE | [34, 46, 47, 49] |
| Antioxidant | APOA1, PON1, PAF-AH, LCAT, GSH-Px, Phospholipids, Fat Soluble Vitamins, Phenolic Compounds | [47, 50, 51] |
| Anti-inflammatory | Sphingosine-1-phosphate | [52, 53] |
| Antithrombotic/profibrinolitic | Reducing Platelet Aggregation and Coagulation | [30, 45, 48, 54] |
| Vasorelaxation | Increasing the Production of Prostacyclin and Nitric Oxide | [30, 45, 48, 54] |
| Proliferation and migration of the endothelial cells | | [30, 45, 48, 54] |
| Inhibit the apoptosis of the endothelial cells | | [30, 45, 48, 54] |

The present review addresses the current available information regarding the effect of OO on HDL in humans and in different animal models.

## EFFECT OF OLIVE OIL CONSUMPTION ON HDL PARTICLES IN HUMANS

Many studies have been carried out in order to analyze the cardiovascular protective effect of OO in humans. In the present work, all those addressing HDL have been compiled and analyzed to gain more insight regarding their different outcomes. Some important aspects with reference to experiment design have been considered such as ethnicity, sex, genetic make-out, variety of OO, pathological state, etc.

### Epidemiological Studies

Cross-sectional studies have shown that greater adherence to the Mediterranean diet was found to be associated with elevated levels of HDL-C in plasma [55-58]. Even in high cardiovascular risk participants an inverse association between quartiles of adherence to the Mediterranean diet and the prevalence of metabolic syndrome (MS) was observed and persisted after adjusting for age, sex, total energy intake, smoking status and, physical activity. Participants with the highest adherence had fewer possibilities of having low HDL-C than those in the lowest quartile [59]. In children, consuming diets with less SFA was also associated with better LDL-C/HDL-C and APOB/APOA1 ratios [60]. These epidemiological studies of Mediterranean diets do not provide direct evidence of olive oil influence and should be evaluated with caution considering that adherence indices included consumption of fish, fruit, grains, legumes, nuts, vegetables, and wine in addition to olive oil.

### Outcome Depends on the Diet Used for Comparison. Clinical Trials

The information obtained through randomized clinical trials regarding the effects of olive oil intervention on lipid profile and HDL functionality, is summarized in (Tables **3** and **4**).

Both a high-fat diet (40% of energy), rich in OO and low in SFA, and a low-fat carbohydrate-rich diet, had similar C-lowering effects. However, in contrast to the carbohydrate-rich diet the OO diet did not lower HDL-C [61]. These results have been confirmed by other authors, where HDL-C, $HDL_2$, and APOA1 were found to be significantly increased in the olive-oil-enriched group compared to the high-carbohydrate one [62]. In addition, higher serum HDL-C and lower ratio of total to HDL C occurred after a OO diet compared with a high-carbohydrate or with low-fat diet in healthy free-living individuals following crossover interventions [63]. When Mesink *et al.* compared the influence of a high-fat, OO-rich diet or high carbohydrate-content diets after consuming a high-SFA diet, $HDL_2$ decreased in both groups but $HDL_3$ C was not modified, and APOA1 levels rose by 26 mg/L in the OO diet [64]. Likewise, a significant increase in APOA1, in this case accompanied by HDL-C, was observed in volunteers consuming 25 g/day of extra virgin OO for 3 weeks when compared with the same subjects consuming butter [65]. No differences in HDL-C levels were observed when comparing OO and different sources of SFA (butter, beef tallow and cocoa butter, fats with a high stearic acid content) [66, 67] or margarine [68]. Similar results were obtained for APOA1 levels in these types of diets [69]. Lichtenstein *et al.* corroborated these results for APOA1 and HDL-C by changing subjects from the saturated baseline to the OO-enriched diet [70, 71]. When subjects consuming palmolein were switched to OO, a reduction in HDL-C was observed [72]. The same effect was described after replacing the usual fats of Australian diets by OO [73].

Compared to low fat content diets (as those proposed by the National Cholesterol Education Program (NCEP)) a high fat diet containing OO did not lower neither HDL-C nor APOA1 levels [74, 75] whereas an increase was observed in both when virgin OO was used [76, 77]. This increase was observed after 3 months of intervention [78, 79] but it appeared to be diluted after one year of intervention when similar results were observed between virgin OO and low-fat diet groups [80]. In agreement with this observation, the decrease in HDL-C levels after low fat diets was recovered by OO supplements (10% energy as fat) [81] or virgin OO (30 ml/day) [82].

Controversial results have been obtained from the comparison with PUFA-contained oils. When 40 g of oil were given as sunflower or OO, the latter induced an increase in HDL-C and APOA1 in both sexes [83]. With 36% of calories as fat, significantly higher HDL-C and APOA1 levels were reported in women when they received an OO-rich diet compared with a sunflower-rich one [84]. In contrast, Casasnovas *et al.*, comparing these two oils, observed a 9.9% decrease in HDL-C although the total-C/HDL-C ratio increased 3.1% in young males consuming OO [85]. A similar finding was observed by Santi *et al.* in elderly people [86]. When comparing olive, rapeseed, and sunflower oils added as 50 g to a constant diet, no significant changes were found in total HDL-C, but $HDL_{2a}$ C was higher after OO and rapeseed oil compared to sunflower oil. The authors attributed these differences to the squalene and phytosterol contents of the oils [87]. A lesser supply of extra-virgin OO (20 g/day) in addition to the usual diet induced a decrease in the LDL-C/HDL-C ratio [88]. No differences were observed in neither HDL-C, nor APOA1 when extra-virgin OO, fish oil, fish oil esters of plant sterols, or sunflower oil esters of plant sterols were administered [89]. Likewise, APOA1 and HDL-C did not experience any change when comparing a mixture of soybean and olive oils *vs.* soybean-oil based emulsions in intravenous administration [90]. This evidence supports the fact that OO consumption maintains HDL-C levels when compared to SFA, and increases them in comparison to low-fat and high-carbohydrate diets [91, 92].

### Gender and Ethnic Differences

#### *Gender Differences*

In healthy middle-aged men and women, the replacement of animal fats for OO had no effect on HDL-C in men, but induced an increase in women [93]. A different response between genders was also observed by Mensink *et al.*, the changes in HDL-C were larger in men than in women when comparing carbohydrate- and OO- rich diets [61]. In a similar manner, when women and men consuming a diet rich in SFA were placed on diets containing olive and sunflower

**Table 3. Randomized Trials Studying the Effects of the Olive Oil on Lipid Profile in Healthy Humans**

| Duration of Intervention (days) | Intervention | Washout or Stabilization Period | Design of Study | Type of Olive Oil | Participants (n) | Dose | Effect | Reference |
|---|---|---|---|---|---|---|---|---|
| 21 | OO *vs.* rapeseed or sunflower oils | Washout periods of 5-12 weeks | Randomized, double-blinded and crossover study. | OO | 18 male (age range 20 to 28 y) | 50 g | $HDL_2$ was higher after OO and rapeseed oil compared with rapeseed oil | [87] |
| 21 | Refined OO *vs.* common OO *vs.* VOO | 2 weeks washout periods in which refined OO was used for raw and cooking purposes | Placebo-controlled, cross-over, double-blind, Randomized trial | Refined OO *vs.* common OO *vs.* VOO | 30 men from a religious center. 57 y | Raw daily dose of 25 ml of OO distributed over three meals | An increase was observed in HDL cholesterol levels after virgin olive oil administration | [130] |
| 21 | OO with low PC *vs.* OO with medium PC *vs.* OO with high PC | 2 weeks washout periods | Placebo-controlled, Randomized, crossover, double-blind trial | OO with low, medium or high PC compounds | 200 men. 20 to 60 y | 25 ml/d (22 g) | A linear increase of HDL was observed for three OO. TC/HDL-C ratio decreased linearly with the phenolic content of the OO | [132] |
| 21 | EVOO-enriched diet *vs.* VAO-enriched diet | Butter (25g/d) during 2 weeks (stabilization period) | Randomized and parallel. Two groups (30 subjects in each group) | EVOO | 60 men aged (20–43 y) | 25 g/d | OO diet and VAO diet increased APOA1 and HDL-C. LDL-C and APOB decreased in EVOO group as compared with the stabilization period. | [65] |
| 21 | OO-rich *vs.* butter-rich diet *vs.* beef tallow-rich diet *vs.* cocoa butter-rich diet (liquid formula diets) | Each dietary period was separated by 1 week of ad libitum feeding | Randomized and crossover. | OO | 10 men 51-72 y | | No differences in HDL-C | [66] |
| 24 | AAD *vs.* NCEP step II *vs.* high MUFA *vs.* high-MUFA (OO) diet *vs.* high-MUFA (peanut oil) *vs.* high-MUFA (peanuts and peanut butter) | No washout (only a short break lasting 4-11 days) | Randomized, double-blind, 5-period crossover study design | OO | 22 men and women (range 21 to 54 y) | 17-21% of energy of the OO and PO diets | Although not significant, there was a trend for HDL cholesterol to be higher in subjects consuming the high-MUFA diets than the Step II diet. | [74] |
| 26 | OO *vs.* soybean oil *vs.* dairy and cocoa butters | 4 weeks of washout between each experimental period | Randomized, crossover, double-blind trial | EVOO | 33 men with a mean age of 26 y | 81% of fat | HDL-C and APOA1 levels were unaffected by the experimental diets. The OO and SO diets were hypocholesterolemic compared with the CB diet | [69] |
| 28 | OO-rich diet *vs.* NCEP I diet | 25 days of stabilization period with NCEP I diet | Randomized and crossover design | VOO | 21 men. 21 ± 0.4 y | Oil was used for cooking, salad dressing and spread | OO-rich diet and sunflower-oil rich diet increased HDL-C and APOA1 compared with NCEP I diet. | [76] |

**(Table 3) contd….**

| Duration of Intervention (days) | Intervention | Washout or Stabilization Period | Design of Study | Type of Olive Oil | Participants (n) | Dose | Effect | Reference |
|---|---|---|---|---|---|---|---|---|
| 30 | OO-rich diet *vs.* palmolein oil-rich diet | Not reported | Randomized, two-period, crossover | EVOO | 21 men and women (range 19 to 44 y) | The amount given was individualized as ≥ 50% of their usual dietary fat intake | Both test oils decreased TC and HDL-C compared with the baseline. | [73] |
| 32 | Canola oil-rich NCEP diet *vs.* corn oil-rich NCEP diet *vs.* OO-rich NCEP diet | 1 to 2 weeks between phases | Randomized, double-blinded | OO | 15 subjects (8 women and 7 men) with a mean age of 61 y (range, 44 to 78 y) | 2/3 of the fat was provided by the test oil | No change in HDL-C in OO group. LDL APOB/APOA1 was reduced by vegetable oil enriched diets. | [70] |
| 36 | High fat diet (rich in OO) *vs.* low fat diet (rich in CH and fiber) | All subjects first received for 17 days a control diet (high in saturated fat) | Randomized and parallel. Two groups (in each group 12 women and 12 men) | OO | 48 men and women. 27 y | The proportion of energy diet from MUFA was 24% in OO-rich diet. | $HDL_2$ and LDL-C fell the same on both diets. $HDL_2$ increased on the OO diet. APOA1 and APOA1/APOB ratio rose in the OO diet. | [94] |
| 42 | Rapeseed oil on bread *vs.* OO on bread *vs.* butter and margarine on bread | No washout | Randomized, control and parallel. | OO | 57 middle-aged men and women | 17 g/d (mean) in rapeseed oil and 19 g/d (mean) in OO group | HDL-C remained unchanged in OO group. OO substitution decreased the LDL-C level. | [68] |
| 56 | OO-rich hypocaloric diet *vs.* high-hypocaloric CH diet | 3 months before starting the study body weight variation was assessed. Weight changes were required to be <1%. | Randomized and parallel | EVOO | 20 Caucasian women mildly obese. 30 y | 3 tablespoons/d | HDL-C increased significantly in the Hi-MUFA group, whereas a decreased level was observed in the Hi-Carbo group. | [62] |
| 84 | TMD+VOO *vs.* TMD+WOO *vs.* habitual diet | No washout | Randomized, parallel double-blind, and controlled | VOO, WOO | 90 men and women. 20 to 50 years old | 15 L (for all family) during the intervention (for cooking and dressing purposes) | HDL-C decreased after TMD *vs.* control. In the analysis of 3 groups, HDL-C, TC, LDL-C decreased after TMD+VOO | [145] |

Only those employing healthy subjects and lasting 3 weeks have been considered. AAD: American average diet; APO: apolipoprotein; C: cholesterol; CH: carbohydrate; CB: cocoa butter; d: day; EVOO: extra virgin olive oil; LDL-C: low density lipoprotein cholesterol; MUFA: monounsaturated fatty acid; NCEP: national cholesterol education; OO: olive oil; PC: phenolic compounds; SO: soybean oil; TC: total cholesterol; TMD: traditional Mediterranean diet; VAO: virgin argan oil; VLDL-C: very low density lipoprotein cholesterol; VOO: virgin olive oil; WOO: washed olive oil; y: year.

**Table 4. Randomized Trials in Healthy Humans Analyzing the Effects of the Olive Oil on HDL Characteristics**

| Intervention | Duration of Intervention (Days) | Washout or Stabilization Period | Design of Study | Type of Olive Oil | Participants (n) | Dose | Effect | Reference |
|---|---|---|---|---|---|---|---|---|
| All diets were isocaloric (54%: carbohydrate, 16%: protein, 30%: fat). 10% of the total energy was supplied by food structural fats, 4.4% by sunflower margarine, and 15.6% by one of the following fats: OO, soybean oil, corn oil or milk. | 35 | Same diet for several years before the start of the study (stabilization period) | Randomized, crossover trial with four interventions | OO | 12 nuns living in a convent, without any metabolic disturbances. 26-49 y | 35 g/day | Milk fat diet was the intervention with the highest increment on TC and HDL-C ($HDL_3$). The lowest triacylglycerol and phospholipid concentrations were obtained after corn oil. HDL from the OO intervention were the most fluid particles | [147] |

**(Table 4) contd….**

| Intervention | Duration of Intervention (Days) | Washout or Stabilization Period | Design of Study | Type of Olive Oil | Participants (n) | Dose | Effect | Reference |
|---|---|---|---|---|---|---|---|---|
| All diets were isocaloric (54%: carbohydrate, 16%: protein, 30%: fat). 10% of the total energy was supplied by food structural fats, 4.4% by sunflower margarine, and 15.6% by one of the following fats: OO, sunflower oil (PUFA n6), rapeseed oil (PUFA n3) or milk. | 21 | Same diet for several years before the start of the study (stabilization period) | Randomized, crossover trial with four interventions | OO | 12 nuns living in a convent, without any metabolic disturbances. 39 ± 1 y | | $HDL_3$ after the MUFA intervention reduced the content of intracellular cholesterol and enhanced $I^{125}$-LDL degradation. Also induced the greater [$H^3$] free cholesterol efflux due to its higher fluidity, cholesteryl ester content, linoleic/linolenic acid ratio in phospholipids, and smaller size. | [148] |
| Both diets contained 17% protein, 45% carbohydrate, and 38% fat (< 10% SFA, 6% PUFAs, and 22% MUFAs). a. MUFA diet: high-fat diet (38% fat); MUFAs (olive oil): 22% and 0.027 mg cholesterol/kJ b. MUFA-Cholesterol diet: same fat composition but containing 0.068 mg cholesterol/kJ. Cholesterol enrichment was provided by 2 eggs/d. | 24 | Initial 24-days period consuming an NCEP Step 1 diet calculated to provide <30% of total of energy as fat (SFA: <10%, MUFA: 14%, PUFA: 6%). After the intervention, all subjects consumed a 24-days cholesterol-enriched NCEP Step I diet (NCEP-C diet; 0.068 mg cholesterol/kJ) days. | Randomized, crossover trial with two interventions | OO | 15 healthy men 23 ± 5 y | | Total cholesterol, LDL-C, APOB and APOA1 decreased after the NCEP Step I and MUFA diets compared with the usual diet. The MUFA diet induced a better lipid profile than the NCEP Step I diet; however, the increase in the cholesterol content of both diets produced similar plasma lipid changes. The cholesterol in the NCEP Step I diet increased the cholesterol efflux induced by total serum. | [149] |
| Virgin argan oil group compared with extra virgin OO group | 21 | Controlled diet for two weeks as baseline (25g/day of butter) | Randomized, parallel trial with two interventions | EVOO | 60 Men 23 ± 4 y | 25 mL/day | PON1 activities increased in both groups. Lipoperoxides and LDL susceptibility to oxidation improved in both groups compared to baseline values. | [150] |

APO: apolipoprotein; C: cholesterol; EVOO: extra virgin olive oil; HDL: high density lipoprotein; LDL-C: low density lipoprotein cholesterol; MUFA: monounsaturated fatty acid; NCEP: national cholesterol education; OO: olive oil; PON: paraoxonase; PUFA: polyunsaturated fatty acid; TC: total cholesterol; TBARS: thiobarbituric acid reactive substances; VLDL-C: very low density lipoprotein cholesterol; y: year.

oils or sunflower oil alone, these two diets lowered the level of HDL-C slightly in men but not in women [94]. A more pronounced increase in HDL-C in women than in men was equally observed when they received OO- instead of sunflower-containing diets [95]. Remodeling of HDL lipoparticles was also influenced by diet and gender. In this way, concentrations of HDL containing only APOA1 (Lp A-I) were lower only in men consuming OO- and sunflower-containing diets than the palm oil-containing diet. Concentrations of lipoproteins containing both APOA1 and APOA2 (Lp A-I: A-II) were lower with both diets in women, but significantly higher in men [96].

### *Ethnic Differences*

Turkish people were found to have low levels of HDL-C (men, 34-38 mg/dL; women, 37-45 mg/dL) despite the consumption of OO-rich diets, what could be caused by a genetic factor not related to APOE isoforms [97]. Mensink *et al.* also reported that boys from rural Crete had serum-lipid levels similar to their counterparts from Western European countries in spite of their high OO intake [98]. In this sense, the beneficial effect of increasing OO intake on HDL and PON1 activity at population level was only observed in subjects carrying the R allele of the PON1-192 polymorphism [99]. Equally, genetic variability in the promoter of the APOA1 gene was found to change the response to dietary OO [100]. These examples illustrate the existence of diet-gene interactions that may explain the variability among individuals.

## Olive Oil in Pathological States

Although most of the studies have been carried out in healthy people, some of them have used patients suffering from different ailments, as shown below:

### *Hypercholesterolemia*

When hypercholesterolemic subjects consuming a baseline diet (36% kcal as fat: 15% SFA, 15% MUFA (monoun-

saturated fatty acids), and 6% PUFA; 180 mg C/1000 kcal) were switched to NCEP step 2 diets (30% kcal as fat containing 7% SFA, 80 mg C/Mcal), where two thirds of the fat was either olive, corn, or canola oil; HDL-C levels on the OO diet were maintained compared those of baseline diet [101]. Similar results were observed in moderate hypercholesterolemic [102], in type II hypercholesterolemic [103] and in high atherosclerosis risk patients [104]. Accordingly, in a randomized, cross-over study, the virgin OO intervention compared with a refined OO intervention showed no effect on HDL-C in stable CHD patients [105]. Contrarily, a low-fat diet supplemented with OO (26% of energy from fat) resulted in significantly less HDL-C lowering effect than the very-low-fat diet (10% of energy from fat) in this type of patients [106]. In mildly hypercholesterolemics, a daily supplementation of 4 g of extra virgin OO as capsules for 3 months showed a trend towards increase APOA1 [107]. In mild hypercholesterolemic children, the intake of OO-enriched skim milk caused significant increases in HDL-C and APOA1 in B1B1 carriers of CETP alleles [108]. Interestingly, a nutrient-drug interaction has also been observed since simvastatin was more effective hypolipemic drug in patients with high cardiovascular risk receiving OO-diets than in those on sunflower oil-diets [109].

In the line with the previous studies, several diet intervention studies in patients with peripheral vascular disease showed no effect on HDL-C concentration [110, 111]. In hyperlipidemic patients consuming one of three almond-, OO-, or dairy-based diets, the OO-based diet resulted in no significant changes in HDL [112]. Also in this type of patients, rapeseed or olive oils have similar effects on serum lipoprotein concentration [113]. Likewise, in mild dyslipidemic patients, 40 mL/day of either phenol-rich extra-virgin or phenol poor olive oils had no effect on plasma lipoprotein profile [114]. Nor was any difference observed in HDL oxidation of subjects consuming 18 mg per day of phenolic compounds from extra virgin OO [115].

*Hypertension*

In hypertensive women, a significant increase in plasma HDL-C concentration with regard to baseline was observed following diets enriched with high-oleic sunflower or olive oils. In contrast, the C to phospholipid ratio was raised significantly only in the erythrocyte membrane after the dietary OO. These data suggest that the differential effects of dietary OO are beyond the concentration of oleic acid [116] and may be influenced by triacylglycerol molecular species and the composition and amount of OO minor components [117].

*Diabesity*

In diabetic patients, dietary OO supplementation induced no change in the high-density lipoprotein levels compared to cod-liver oil that increased them [118]. Similar results were obtained by Mori *et al*. comparing supplements of olive and fish oils [119]. In overweight men no differences in HDL-C were observed between flaxseed oil and OO [120].

In first-degree relatives with high risk of developing type-2 diabetes, slightly higher levels of HDL-C and APOA1 were found in the OO diet compared to a carbohydrate-rich diet [121]. Likewise, ingestion of a virgin OO-based breakfast increased HDL-C concentration as compared with a carbohydrate-rich diet in offspring of obese and type 2 diabetes patients 7 [122]. These results indicate that in type of patients, HDL-C levels tended to be higher after an OO-rich diet with respect to carbohydrate-rich diet.

*Cancer*

In women previously diagnosed with invasive breast cancer, an OO supplement of 3 tablespoons of OO/day for 8 weeks resulted in higher HDL-C in comparison with the National Cancer Institute diet [123].

In conclusion, these results reveal that the underlying disease is also a source of variability regarding the influence of OO on HDL and a putative influence of some genetic make-up may participate.

**Role of Minor Olive Oil Components**

Explaining the discrepancy that existed between the classic experiments using OO, which found MUFA oils 'neutral', and some of the more recent experiments which found them more C-lowering than carbohydrates, has drawn a careful consideration of the non-saponifiable fractions of the different MUFA oils [124]. In this regard, several studies have also been carried out. Addition of a phytosterol mixture to an OO diet resulted in suppression of the significant differences in LDL-C concentrations between corn and OO although no significant differences in HDL-C were observed. However, when phytosterols were esterified to OO fatty acids and administered to hypercholesterolemic subjects, their consumption resulted in a significant decrease in LDL-C concentrations, total/ HDL-C and APOB100/APOA1 ratios in comparison with the administration of OO alone [125]. These results suggest that phytosterols are partly responsible for the differences observed in plasma C levels between different oils [126].

Squalene is a monoterpene abundant in OO that is at least partly absorbed and then quantitatively converted to C. A better understanding of the activity of this component is needed to reduce variability in diet studies and accurately assess the effects of oils [127]. Not many studies have analyzed the influence of monoterpenes. The relevance of these compounds on HDL was tested by Cook *et al*. who did not observe any significant effect on plasma HDL-C or APOA1 concentrations when OO was enriched with them [128].

On the contrary, the phenolic compounds of OO have got more attention. In this sense, a number of randomised, controlled, double-blind trials using similar olive oils with increasing phenolic compound concentration, have shown an increment of HDL-C with the phenolic content of the OO administered, in healthy humans both after a mid-term [129] and short-term period of OO consumption [130]. Also an increment of HDL-C was observed after a diet rich in OO in patients with vascular disease [131]. More recently, Covas *et al*. demonstrated an increase of HDL-C related to a high phenolic content in the OO, in healthy humans from six european centers. A randomised, controlled, double-blind trail with three OO differing in their polyphenol content (2.7, 164 and 366 mg/kg of OO), showed that all olive oils increased HDL-C and decreased triglycerides and total/HDL C

ratio. After three-weeks of intervention, the greatest effects on increasing HDL-C and decreasing lipid oxidative damage were observed after the high phenolic OO consumption [132]. The increasing linear trend of HDL-C occurred in a dose-dependent manner with the phenolic content of the olive oils. These results suggest that phenolic content of OO may play an important role in the plasma lipid [132] in addition to their anti-oxidative effects [133].

## MOLECULAR MECHANISMS OF THE OLIVE OIL EFFECT ON THE HDL PARTICLE IN HUMANS

The beneficial effects of the OO on the HDL particle could be produced by: 1) an increment of the HDL-C concentration as well as by 2): an improvement on the HDL functionality.

### Mechanisms of the HDL-C Increment

Several research works have focused on this aspect considering that the enhancement of HDL-C levels may prevent atherosclerosis development [134]. One of them proposes that the liver decrease in triglyceride accumulation by OO administration may improve insulin resistance and reduce nuclear factor-kappaB activation, what could be translated into higher levels of APOA1 and functional HDL [135]. Another proposed mechanism comes from postprandial experiments which have suggested that OO chylomicron remnants compete for hepatic lipase and prevent the decrease in HDL-C observed after consuming OO in comparison with soybean oil [136]. The latter mechanism has also found involved in intravenous administration of mixtures of 17% (wt:v) OO plus 3% soybean oil emulsion compared to that containing only 20% soybean oil, since the former one showed a slower elimination which was inversely related to the hepatic lipase activity [137] that would selectively decrease HDL-C while increase APOA1 [138]. A decrease in CETP that could increase HDL levels was observed by isoenergetic substitution of a saturated fatty acid diet with NCEP Step I or virgin olive oil diets [139].

Few studies have investigated minor HDL apolipoproteins in response to diet. In this regard, plasma concentrations of APOA4 increased when subjects consuming a SFA-containing diet were switched to diet enriched in refined OO, rapeseed oil or sunflower oil without differences among oils [140]. However, variation in APOA4 has been observed to modify the variability in HDL-C and APOA1 response to diets [141]. APOA2 and HDL-phospholipids decreased without HDL-C changes when extra virgin OO replaced a blend of OO plus sunflower oil [142].

### HDL Functionality and Olive Oil Consumption

OO can modulate this aspect by different mechanisms and minor compounds be involved. Some of them can directly counteract free radicals generated by different processes in the organism or in the case of MUFA are less prone to be oxidized [143]. In addition, these compounds can modulate the expression of atherosclerosis-related genes [144, 145].

A number of studies have investigated the C efflux to HDL from different cells, which is the first critical step of the reverse C transport. An early work of Esteva *et al*. described that [$^3$H]-C efflux from fibroblasts was correlated with the phospholipid fatty acid chain length of plasma HDL obtained from subjects fed different diets: peanut-, corn-, olive-, soybean-, low erucic acid rapeseed-oils or milk fats [146]. In this line, Solà *et al*. proved that the HDL isolated from women consuming 30% of the calories as OO were the most fluid particles compared to those coming from soybean, corn oils or milk fats. Percentages of triacylglycerol and oleic acid in HDL phospholipids were found responsible for the observed effects [147]. In fact, $HDL_3$ obtained after the OO diet induced the greatest cellular C efflux from cultured fibroblasts. This result could be due to its greater fluidity, higher cholesteryl ester content, elevated linoleic/linolenic acid ratio in phospholipids and its smaller size [148]. Later it was demonstrated that dietary C might modify the efflux capacity of HDL [149]. Furthermore, Cherki *et al*. have observed an increase of paraoxonase activities after consuming 25 g/day of extra virgin OO [150]. Recently, all these observations have been put together, since OO consumption increased HDL-phosphatidylcholine containing oleic acid, HDL-paraoxonase activities and PON1 stimulatory effect on HDL-mediated C efflux as compared to HDL before treatment. The phospholipid stimulatory effect was lost in a mutant form of PON1 lacking the first 20 amino acids. Thereby, these results suggest that this protein domain is crucial for the interaction with phosphatidylcholine and the increase induced by OO is mediated by this phospholipid [151]. *In vitro* oleuropein immerses into artificial membranes and could be responsible for a membrane fluidifying effect [152]. Whether these oleuropein actions may be present *in vivo* particle is an open question. Further support to a suggestive role for minor OO components in macrophage and fibroblast C efflux comes from the lack of changes using other MUFA-containing diets [153]. Overall, these data indicate that dietary manipulations in the chemical composition of HDL have an impact on their physicochemical properties and may influence cellular C efflux. Some minor OO components may also be involved in this action. The information obtained through randomized clinical trials on the effects of olive oil intervention on HDL functionality, is summarized in (Table **4**).

Despite the significant advances, the final proof about the specific mechanisms and contributing role of the different components of virgin OO to its beneficial effects still requires further investigations [143]. In the Covas' study, an improvement in the lipid oxidation status directly related to the phenolic content of the OO administered was observed [132]. To which extent HDL inactivation of peroxides was involved in such finding is an attractive hypothesis to be tested.

## EFFECT ON OLIVE OIL ON HDL IN DIFFERENT ANIMAL MODELS

The animal models, either available in nature or generated by genetic engineering, show interesting peculiarities of HDL as reflected in (Table **5**). The use of animals in olive oil studies provides a suggestive scenario to test different hypotheses and to look for mechanisms requiring confirmation in humans.

**Table 5. HDL Characteristics of Different Animal Models**

| Animal | Properties |
|---|---|
| **With CETP** | |
| Guinea pigs | Sex-differences in HDL |
| Hamster | Low CETP activity |
| Old world monkeys (Cynomolgus) | |
| Rabbit | Lack APOA2 and HL APOA1 is only expressed in intestine. |
| Dog | Low CETP activity |
| **Without CETP** | |
| Mouse | Lack APOA, APOD |
| APOE-deficient mice | Lack APOE |
| New world monkeys (Baboon) | |
| Pigs | Lack APOA2, low APOC3 |
| Rat | Lack APOA, APOD |

Adapted from [222]

### Primates

Baboons fed diets containing 1% C and 25% fat such as coconut, peanut or olive oils, for 20 weeks, showed a differential response of HDL-C to diets. The OO-fed group displayed an intermediate increase compared with the coconut oil one [154]. In male Cynomolgus monkeys, replacement of pork fat by OO induced a significant decrease in HDL-C associated with an increased activity of C ester transfer protein [155]. However, an exchange of 16:0 for 18:0 into diets, representing approximately 11 g per 2500 kcal, containing adequate polyunsaturated fatty acids (PUFA) amounts and 30% of total energy as fat, was not translated into plasma HDL-C variations [156]. These results indicate that only certain dietary maneuvers allow HDL-C to be modified in such animal models.

### Guinea Pigs

HDL-C levels did not differ among guinea pigs fed semi-synthetic diets containing 7.5 or 15% (wt/wt) of fats such as beef tallow, corn oil, OO, lard, palm kernel, or palm oil with or without 0.25% C [157-159]. With similar amounts of fat, and varying amounts of dietary C (0.0 to 0.3%), an interaction between dietary fat and C was seen for HDL-C concentrations. Saturated fatty acids (SFA) and the pharmacologic level of dietary C increased plasma HDL-C concentrations, whereas PUFA minimized the dietary C-mediated increase [160]. Despite the absence of changes in HDL levels, increased HDL binding to membranes was observed in animals fed PUFA and C [157]. A fact that indicates this process is regulated by both dietary C and fat type, and might indicate complex dynamics of HDL particles in this animal model.

### Dogs and Pigs

HDL-C increased following an OO diet in dogs fed a basal diet supplemented with either 14% OO or sunflower oil for six months [161].

In miniature swine fed two diets of 9% fat such as sunflower or olive oils, HDL-C were significantly higher in the sunflower group when compared with the olive oil one [162-164]. Such a difference was not observed in pigs fed high-fat diets containing 4% extra virgin olive or sunflower oils. Both diets increased plasma APOA4 concentrations, mainly in the lipoprotein-free fraction [165]. Despite the scarcity of studies using OO in these two models, they seem quite sensitive to dietary manipulations.

### Hamsters

#### *A Cholesterol Sensitive Animal Model*

Golden Syrian hamsters fed OO as 20% of fat with 0.1% C had significantly higher HDL-C concentrations than those fed corn oil. The effect was linked to lowered hepatic HDL binding and degradation [166]. Terpstra *et al*. also found that saturation of dietary fat resulted in increased concentrations of HDL-C and hepatic lipase activity which were significantly higher in palm- and olive-oil-fed hamsters compared with the corn group [167]. In the absence of dietary C, however, little influence of the nature of the dietary fat on HDL-C was observed [168-171]. Also, C depletion in F1B hamsters resulted in lower HDL-C levels paralleled with changes in expression of SRB1, APOA1, APOE, ABCA1, ABCG5, and ABCG8 [172]. These findings support the C requirement in HDL changes.

### Mice

#### *Influence of Cholesterol and Sex*

In absence of C, female APOE-knockout mice consuming 10% (wt/wt) OO showed increased plasma APOA1 levels, paralleled by hepatic expression [173]. In another study, the addition of 0.1% C to chow or OO-containing diets induced significant decreases of APOA1 in females and serum paraoxonase in males. These results demonstrate that the nutritional regulation of paraoxonase and APOA1 is dependent on sex and C [174].

#### *Role of Minor Components*

APOE-deficient mice received two diets containing 10% (w/w) as olive oils devoid of phenolic compounds. Both olive oils, differing in the content of linoleic, phytosterols, tocopherols, triterpenes, and waxes, did not elicit any change in HDL-C [175]. Similar results were observed with squalene administration [176]. On the other hand, enrichment of OO with green tea polyphenols resulted in increased serum HDL-C levels, paraoxonase, and stimulated C efflux from mouse peritoneal macrophages [177]. When the effects of Picual and Arbequina extra virgin olive oils (rich and poor in phenolic compounds, respectively) were compared to palm oil, a significant increase in HDL was observed in animals receiving the high phenolic olive oil [178]. However, when 10 mg/kg/day hydroxytyrosol, the main olive phenolic compound, was administered, no significant changes in

HDL-C or paraoxonase were observed [179]. These data may indicate that phenolic compounds, not necessarily hydroxytyrosol, might be involved in HDL effect.

***Funtionality of HDL Particles and Components***

BalbC mice consuming OO showed increased HDL phospholipids/protein ratio and paraoxonase activities. Furthermore, in the OO-fed mice PON1 contribution to HDL-mediated macrophage C efflux was higher than in controls [151]. In APOE-deficient mice, virgin OO induced a C-poor, APOA4-enriched lipoparticle that showed enhanced paraoxonase activity [180]. These results suggest an effect on HDL-associated paraoxonase 1 activity modulated by OO intake which would require chronic administration, since no change was observed in a postprandial regimen [181].

**Rabbits**

***Effect of Dietary Cholesterol and Strains***

Rabbits fed an atherogenic diet with 15% virgin OO showed an increase in HDL-C compared to the atherogenic diet alone [182]. Similarly, serum HDL-C was the highest in the rabbits fed olive and avocado oils compared to coconut or corn oils provided as 14% of fat in a semi-purified diet containing 0.2% C [183]. In absence of dietary C, Masi *et al.* also found an increase in HDL-C of rabbits receiving corn or olive oils as 10% (w/w) fat compared to the butter group [184]. However, Bayindir *et al.* did not observe those changes with similar study design [185]. Likewise, rabbits kept on a mean plasma C level of about 20 mM over a period of 12 weeks and then transferred to corn, margarine or OO did not experience any significant change in their levels [186, 187]. Likewise, no differences in HDL-C were observed in Watanabe heritable hyperlipidemic rabbits fed fish or olive oils [188]. The experimental design and strain of rabbits may influence the results of HDL.

***Effect of Minor Components***

HDL-C rose in rabbits consuming an atherogenic diet supplemented with 4 mg /kg body weight of hydroxytyrosol for two months [189]. This effect was not observed when 100 mg/kg of olive leaf extract containing hydroxytyrosol (22%), polyphenol (4%), and saccharide (67%) was administered [190]. A similar result was found using diets containing 1% C and 15% olive oils enriched in polar lipid, neutral lipid olive or pomace polar extracts, despite the increase in the platelet-activating factor-acetylhydrolase activity in the OO groups [191, 192].

**Rats**

***Dietary Fat and Cholesterol and Intervention Time***

Plasma HDL-C did not differ among groups receiving hazelnut, safflower, corn, olive or palm oils as 10% or 15% fat [193, 194]. However, compared to fish oil, coconut oil or chow diets, 10% or 15% OO increased HDL-C [195-197]. When 10% of fat with identical linolenic to linoleic acid ratios was administered, rats receiving OO showed higher HDL-C levels than those fed butter [198].

Dietary C also plays an important role in the regulation of HDL and the effect of different dietary oils. In a group of male Wistar rats maintained on a high fat diet containing 0.1% C and fat (40% w/w) either as coconut, corn or olive oils, the OO fed animals showed the highest levels of hepatic *Apoa1* mRNA [199, 200]. A threshold of dietary OO could be required since only the animals consuming higher percentages showed significant mRNA increases associated with plasma APOA1. Despite these increases, HDL-C levels of rats consuming 40% OO diet were lower than those corresponding to coconut oil and higher than those found in corn oil. These data indicate that different oils in varying quantities regulate APOA1 through differing mechanisms [201]. In contrast, HDL-C concentrations were significantly lower in male Wistar rats receiving C than in those fed 14 % olive or safflower oils [202]. In the presence of 1 or 2% C, the administration of different amounts of dietary OO (2% to 20%) resulted in decreased HDL-C levels compared to chow-fed rats [203, 204]. An effect that was more pronounced in males than females [205]. Plasma APOE and APOA1 concentrations were also decreased [206]. Surprisingly, in the exogenously hypercholesterolemic rat, a sub-strain from Sprague-Dawley rats with a high response to dietary C, a diet containing 10% OO, 2% C, and 0.4% sodium cholate elicited increased serum HDL and APOA1 levels after 32 weeks [207]. This was not observed in shorter interventions [208]. Collectively, these data indicate that the amount of fat and C and the length of intervention are influencing variables.

In addition, fatty liver seemed to modify the response, and serum HDL-C levels were highest in the sunflower compared with OO fed rats [209].

***HDL Phospholipids***

Male Wistar rats fed semi-synthetic diets enriched by 10% virgin or lampante oils showed decreased HDL-phospholipids [210]. In this regard, a high administration of OO (18%, w/w) induced a decrease in HDL sphingomyelin content attributed to changes in enzyme activities of sphingomyelin metabolism [211]. These results indicate that OO administration remodels HDL phospholipids and may contribute to a change in the secondary structure of plasma HDL [212].

***Role of Minor Olive Oil Components***

Several works have tackled this issue. Male Sprague-Dawley rats fed 12% fat as OO had significantly lower HDL protein and C concentrations than those receiving a similar amount of high oleic safflower or high linoleic safflower oils [213]. Furthermore, in Wistar rats fed a standard laboratory diet or a C-rich diet for 16 weeks, administration of 3 mg/kg of body weight of hydroxytyrosol, triacetylated hydroxytyrosol or 10 mg/kg of olive mill wastewater extract significantly increased serum HDL-C [214, 215]. A similar effect was observed with the administration of aqueous extracts of green and black olives [216], polyphenol-rich olive leaf extracts, oleuropein, oleuropein aglycone, and hydroxytyrosol-rich extracts [217]. When these OO minor constituents were added to virgin OO, increased HDL, and particularly $HDL_2$, C concentrations were observed [218, 219]. Faraq *et al.*, using a 1600 mg/kg dose that clearly exceeded that suggested by other authors -(10 mg per day) [220]-, showed some side-

effects such as increased plasma aminotransferase activities [219], and elevated lipid hydro-peroxide concentrations in cardiac muscle [221]. Data that suggest HDL is modified by these minor OO compounds.

## CONCLUSIONS

In Mediterranean diets, the major source of dietary fat has traditionally been OO. OO-enriched diets, compared with carbohydrate-rich and low fat diets, increase HDL-C. However, when those diets were compared with saturated and partially hydrogenated fat-rich diets, they did not reduce HDL-C but reduced LDL-C. Gender, gene-diet interactions, and the pathological characteristics of subjects add new aspects to be considered. Cumulative evidence suggests that HDL-C, and its main APOA1, may be increased by consuming virgin OO (Table **6**) though controversial results have been found and need to be clarified.

VOO is a complex mixture of components whose nutritional properties and bioavailability have not been completely elucidated. Furthermore, HDL particles are extraordinary diverse, and the complexity of their composition and properties following VOO administration needs to be explored.

**Table 6. Summary of Olive Oil Effect on HDL Levels and Functionality**

| Effect | Species | References |
|---|---|---|
| Increase CETP | Cynomolgus | [155] |
| Decrease CETP | Humans | [139] |
| Decrease Hepatic Lipase | Humans | [136] |
| Increase APOA1 | APOE-KO Mice<br>Rats<br>Humans | [173, 201]<br>[62, 64, 65, 76-79, 83, 84, 107, 108, 121] |
| Increase APOA4 | Human<br>Pig | [140]<br>[165] |
| Decrease APOA2 | Human | [142] |
| Increase PON1 | Balbc Mice<br>APOE-KO Mice<br>Human | [150, 151, 180] |
| Increase Cellular Cholesterol Efflux from Macrophages and Fibroblasts | Mice<br>Human | [148, 151, 177] |

## CONFLICT OF INTEREST

Declared none.

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## ABBREVIATIONS

APO = Apolipoprotein
C = Cholesterol
CETP = Cholesteryl Ester Transfer Protein
CHD = Coronary Heart Disease
FCHL = Familial Combined Hyperlipidemia
FPLC = Fast Protein Liquid Chromatography
HDL = High Density Lipoprotein
LCAT = Lecithin Cholesterol Acyltransferase
LDL = Low Density Lipoprotein
MS = Metabolic Syndrome
MUFA = Monounsaturated Fatty Acid
OO = Olive Oil
NCEP = National Cholesterol Education Program
PAF-AH = Platelet-Activating Factor-Acetylhydrolase
PON-1 = Paraoxonase 1
PLTP = Phospholipid Transfer Protein
PUFA = Polyunsaturated Fatty Acid
SFA = Saturated Fatty Acid
VLDL = Very Low Density Lipoprotein

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# Annex IV: Other scientific publications

· Hernáez Á, Farràs M, Fitó M. *Olive oil phenolic compounds and HDL function.* (Under revision)

· Buil-Cosiales P, Toledo E, Salas-Salvado J, Zazpe I, Farràs M, et al. *Association between dietary fiber intake and fruits or vegetables consumption and the risk of cardiovascular disease. Results from the PREDIMED trial.* (Under revision)

· Martín-Peláez S, Mosele J, Pizarro N, Farràs M, et al. *Effect of virgin olive oil and thyme phenolic compounds on blood lipid profile: implications of human gut microbiota.* (Under revision)

· Pedret A, Catalán Ú, Fernández-Castillejo S, Farràs M, et al. *Impact of virgin olive oil and phenol-enriched virgin olive oils on the high-density lipoprotein proteome in hypercholesterolemic subjects. A double blind, randomized, controlled, cross-over clinical trial (VOHF study).* PlosOne. In press.

· Hernáez Á, Remaley A, Farràs M, Fernández-Castillejo S, et al. *Olive oil polyphenols reduce LDL particle number and apolipoprotein B levels in humans: a randomized controlled trial.* Journal Nutrition. In press.

· Mosele JI, Martín-Peláez S, Macià A, Farràs M, et al. *Study of the catabolism of thyme phenols combining in-vitro fermentation and human intervention.* J Agric Food Chem. 2014. 62(45):10954-61.

· Mosele J I, Martin-Pelaez S, Macia A, Farràs M, et al. *Faecal microbial metabolism of olive oil phenolic compounds: in-vitro and in-vivo approaches.* Molecular Nutrition and Food Research. 2014. 58(9):1809-19.

· Hernáez A, Fernández-Castillejo S, Farràs M, Catalán Ú, et al. *Olive oil polyphenols enhance high-density lipoprotein function in humans. A randomized*

*controlled trial.* Arteriosclerosis, Thrombosis and Vascular Biology. 2014. 34(9):2115-9.

· Rubió L, Farràs M, de La Torre R, Macià A, et al. *Metabolite profiling of olive oil and thyme phenols after a sustained intake of two phenol-enriched olive oils by humans: Identification of compliance markers*. Food Research International. 2014.

# DISCUSSION

# 10. DISCUSSION

OO is a r ecognized h ealthy f ood. N evertheless, d ue to i ts h igh f at co ntent i t cannot be c onsumed i n l arge qua ntities. The en richment o f O O with P C is, therefore, a w ay o f increasing i ts b eneficial p roperties w hilst m aintaining t he same amount of fat. The aim of the present work was to assess whether FVOOs, enriched with their own PC o r w ith them p lus o thers, h ave p ositive e ffects o n both H DL and E F, in cardiovascular r isk hum ans. O utcomes on H DL characteristics, c holesterol e fflux pr omoted by H DL, t he e xpression of cholesterol efflux-related genes, and EF were, therefore, analyzed.

To achieve our goal, we designed two randomized, controlled trials. In the first study (OLIPA), we tested the post-prandial effect of an FVOO, enriched with its own PC (961 ppm), in pre-/hypertensive humans. In the second study (VOHF), we explored the l ong-term e ffect of di fferent F VOOs ( 500 ppm), on e enriched with its own PC, and another with them plus complementary ones from thyme, in hypercholesterolemic volunteers.

## 10.1. POSTPRANDIAL EFFECTS OF FUNCTIONAL VIRGIN OLIVE OIL, ENRICHED WITH ITS OWN PC, IN PRE-/HYPERTENSIVE HUMANS

In a f irst st udy, a p reliminary ap proach w as p erformed t o ass ess the ef fects o f PC-enriched VOOs on transcriptomics and endothelial dysfunction. Specifically, we e xplored the e ffects of a n F VOO e nriched w ith its ow n P C ( FVOO; 961 ppm) on cholesterol efflux gene expression and EF in pre/-hypertensive humans, in a post-prandial, randomised, double-blind, controlled trial.

### 10.1.1. EFFECTS ON CHOLESTEROL EFFLUX-RELATED GENES

In the initial manuscript, we observed for the first time, with the highest level of evidence, an enhancement of cholesterol efflux related gene expression after an FVOO (961 ppm) post-prandial intervention in pre- and stage 1 hypertensive subjects (315). We found an increase of ABCA-1, SR-B1, PPARα, PPARβ, PPARγ, PPARBP, CD-36, and COX-1 gene expression after the FVOO consumption versus the VOO one. Our data suggest that a PC-enriched VOO could promote atheroprotective molecular mechanisms.

The enhancement of ABC-A1 in a PC-enriched VOO versus VOO could be related to both the unsaturated fatty acids and polyphenols. A repression of ABC-A1 by unsaturated fatty acids, including oleic acid, in macrophages has been reported in experimental studies (316). This repression is mediated by histone deacetylase and some polyphenols have been shown to exert an histone deacetylase inhibition (317;318). It could be hypothesized that polyphenols present in the VOO were able to counteract the effects of oleic acid. In this regard, a direct relationship between plasma levels of HT acetate sulphate and ABCA1 expression was observed after the PC-enriched VOO intervention. In a previous study of our group, we did not observe significant differences in ABCA-1 expression after a Mediterranean diet plus VOO consumption (229), a fact that reinforces the results of the present work (315). Berrougui et al, in a lineal, non-randomized, non-controlled, long-term trial, detected an enhancement of ABCA-1 expression, and also ABCG-1, following extra-VOO consumption. Nevertheless, after the same consumption they observed a down-regulation of SR-B1 (99).

Nuclear receptor factors are involved in the expression of RCT-related genes. We studied the gene expression of the PPAR family due to the fact that polyphenols are ligands of PPARs. We observed an enhancement in PPARBP,

PPARα, PPARγ, and PPARδ after the PC-enriched VOO consumption (315). PPARBP, also referred to as MED-1, is a co-activator for PPARα and PPARγ. The increase of PPARBP expression had been previously described by our group after a polyphenol-rich VOO intervention (228). PPARα, PPARγ, and PPARδ ligands have been reported to stimulate cholesterol efflux in cultured macrophages by inducing ABCA-1 expression (319;320). PPARα has also been shown to up-regulate the expression of SR-B1. The ABCA-1 and SR-B1 enhancement observed in our study could have been mediated through PPAR up-regulation. In agreement with this, some authors also reported that polyphenols (e.g.anthocyanines and resveratrol) up-regulated PPAR expression. Whilst olive leaf extract has recently been found to augment hepatic PPARα gene expression in rats (321).Although HT treatment in some experimental studies has been reported to increase PPARα and PPARγ expression (227;322), contrasting results have been published (322).

CD-36 is a scavenger receptor that enhances uptake of oxLDL. PPARγ promotes lipid up-take by up-regulating CD-36 expression (319). An increment of CD-36 expression was observed after a PC-enriched VOO ingestion versus a VOO one (315). OxLDL has been reported to increase monocyte CD-36 expression (323). At the same time, this CD36 activation promotes PPARγ via the MAPK-dependent COX-2 pathway (324). In our study, oxLDL decreased after both treatments. This fact, together with the lack of COX-2 activation, supports the idea of an oxLDL-independent PPAR activation pathway. Furthermore, this hypothesis is supported by the direct relationship between the increase of PPARγ expression with that of antioxidant capacity (ORAC) and the decrease in oxLDL.

ABCA-1 expression can be enhanced via 27-hydroxylase-LXR. COX-1 inhibition down-regulates the expression of 27-hydroxylase (325). In our study, we observed an increase of COX-1 expression, which could also be involved in cholesterol efflux activation. The increase of COX-1 expression observed after a

PC-enriched VOO ingestion could be also via PPAR increment (326). It has recently been reported that HT, at nutritionally relevant concentrations and *in vitro*, reduces COX-2 protein expression and has no effect on COX-1 protein expression in peripheral blood mononuclear cells and activated human monocytes (U937). HT exerted a pre-translational effect reducing also COX-2 gene expression in U937 (327).

A number of studies have published a similar up-regulation of cholesterol efflux genes with different polyphenols. For example, it has been reported that chlorogenic acid enhances *in vitro* the expression of PPARg, LXRa, ABCA-1, and ABCG-1. Another aspect to take into account is the role of microRNAs at the post-transcriptional level. MiR-33 a/b has been described as playing a major role in the regulation of cholesterol homeostasis, modulating the ABCA-1 and ABCG-1. The de regulation of this non-coding RNA is related to metabolic diseases. An increase of miR-33 could promote atherosclerosis by decreasing the ABC transporter gene expression. Escudero et al. have reported that some polyphenols (e.g. grape seed, proanthocyanidins, and flavonoids) can repress miR-33 and thus enhance cholesterol efflux gene expression (328;329). Furthermore, the same authors have also observed that resveratrol increases miR-33 expression *in vitro*. It has been hypothesized that polyphenol binding on microRNA depends on the polyphenol's structure (329), further research is, however, needed in this field.

Lastly, it should be confirmed whether the enhancement of cholesterol efflux-related genes is produced with an improvement in cholesterol efflux at a 5-hour postprandial state. The nutrigenomic, protemomic, and functional effects do not always take place in a chronological sequence.

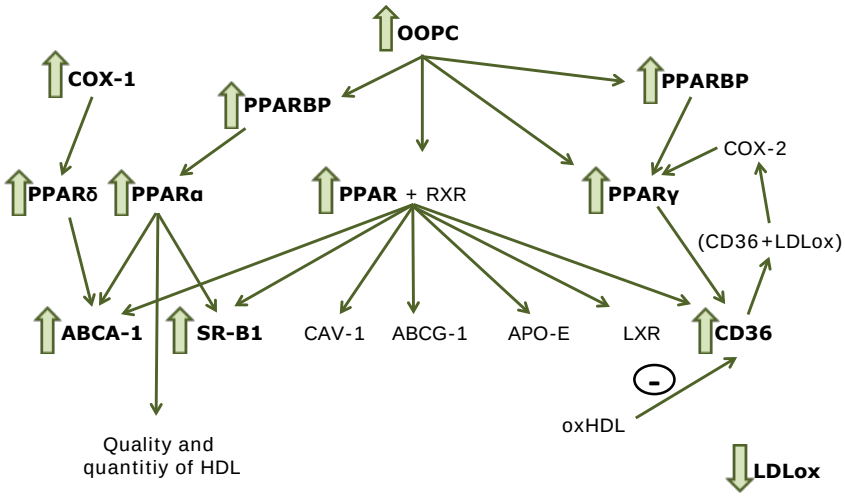



**FIGURE 8. Possible interactions between genes up-regulated after FVOO intervention in OLIPA study.**

***** In summary, an acute intervention with an FVOO enriched with its own PC enhanced EF and cholesterol efflux-related gene expression – ABC-A1, SR-B1, PPARBP, PPARα, PPARγ, PPARδ, CD-36, and COX-1 – compared with VOO. The observed improvement in EF after FVOO ingestion could be mediated by the increased OOPC biological metabolites in plasma, together with decreased levels of oxLDL. Changes in gene expression were also related to a decrease in oxLDL and an increment in ORAC and PC. OO, a recognized healthy food, cannot be consumed in large quantities, thus enrichment with its own PC is a way of increasing its beneficial properties whilst the same amount of fat is consumed. All of the above suggests that an FVOO enriched with

its own PC could be a useful dietary tool for improving EF and cholesterol efflux in pre-/hypertensive humans.

### 10.1.2. EFFECTS ON ENDOTHELIAL FUNCTION

In publication number 2 we reported an improvement in endothelium-dependent microvascular dilatation after post-prandial FVOO (961 ppm) consumption in participants with pre- and stage-1 hypertensive status (330).

Endothelial dysfunction, characterized by reduced bioavailability of NO, is considered the first pathological symptom of atherosclerosis (331), and recent evidence has suggested that oxidative stress may play a role (Heitzer T et al 2001). It has been reported that flavonoid consumption improves EF, after both acute and sustained ingestion in diabetic (332) and coronary heart disease patients (333). In addition, OO polyphenols have been demonstrated to improve EF in hyperlipemic volunteers at a post-prandial state (96) and hypertensive patients after a long-term intervention (107). These EF improvements could be mediated via reduction in oxidative stress and the increase of NO metabolites (96). Recently, Storniolo et al. has reported *in vitro* that high glucose, and linoleic and oleic acid treatments decrease endothelial NOS phosphorilation and, consequently, intracellular NO levels, with an increment of endothelin-1 (ET-1) synthesis by ECV304 cells. Such effects may be related to the stimulation of ROS production within the context of these experimental conditions. HT and a polyphenol extract from extra VOO partially reversed the above events (307). Nevertheless, in our study we did not observe differences in NO and endothelin 1 after VOO interventions, as a result, other mechanisms could be involved in the vasodilation process.

For most of the day western populations are in a non-fasting state. After a high-fat meal, oxidative stress occurs and this fact has been linked with a concomitant endothelial dysfunction (334). However, the consumption of fatty meals with

sources of antioxidants, such as red wine (335) and vitamin C (336), has been shown to minimize postprandial oxidative stress. In the study referred to in this thesis (330), consumption of FVOO reduced the postprandial hyperglycemia and hypertriglyceridemia peak compared with VOO in pre- and hypertensive patients. According to this, a reduction of oxLDL and PAI-I, LDL oxidation and inflammatory biomarkers, respectively, was observed after FVOO compared with VOO. Our team have previously described a decrease of oxLDL, related to the OOPC, with acute and sustained VOO consumption in healthy volunteers (337) (97). Moreover, we reported a decrease of oxLDL (110), IL-6, and hsCRP (106) after a VOO intervention compared to a refined OO one, in stable coronary heart disease patients. In this sense, the protection of FVOO consumption against LDL oxidation in the OLIPA study could be produced by the plasma OOPC metabolite (HT sulphate) increment after this intervention.
In the present study, an improvement in EF after FVOO treatment was inversely related to LDL oxidative damage. Thus, a decrease in both oxidative stress and inflammation, due to OOPC may contribute to the EF enhancement observed after FVOO ingestion.

## 10.2. LONG-TERM EFFECTS OF FUNCTIONAL VIRGIN OLIVE OILS, ENRICHED WITH ITS OWN PC OR OTHERS FROM THYME, IN HYPERCHOLESTEROLEMIC HUMANS

The antioxidant system is a complex network of interacting molecules. When an antioxidant is oxidized it is converted into a harmful radical, which needs to be turned back to its reduced form by complementary-antioxidants. It has been reported that supplementing high-risk individuals with a single type of antioxidant promoted rather than reduced lipid-peroxidation, while the combination of different antioxidants was effective in reducing atherosclerosis in

human trials (338). All of the above suggests that the enrichment of VOO with HT derivatives combined with complementary-phenols from aromatic herbs, such as thyme, might be a good strategy to provide the optimum balance among the different types of flavonoids, monoterpenes, and phenolic acids (141).

Due to this, in the second study we assessed the effect of different FVOOs (500 ppm), one enriched with its own PC (FVOO), and another with its PC plus thyme phenols (FVOOT), on HDL characteristics, HDL functionality, and EF, in hypercholesterolemic volunteers in a sustained, randomized, double-blind, controlled trial. Prior to this clinical trial we had developed a postprandial one to establish the PC concentration of the FVOOs.

### 10.2.1. EFFECTS ON HDL CHARACTERISTICS

In the third manuscript, after the FVOOT intervention, we observed an improvement of HDL characteristics: HDL-subclass distribution, HDL-composition, and enzymes related to HDL-metabolism and HDL-antioxidant capacity (339).

After the FVOOT intervention, an increment of LCAT activity was reported, and we also detected an increase of $HDL_2$-particle subclass percentage and a decrease of the $HDL_3$ one. Nevertheless, after the VOO intervention, we reported a decrease of LCAT activity and of $HDL_2$-particle subclass percentage. In addition, the increase of $HDL_2/HDL_3$ directly correlated with the increase of ApoA-I after the three interventions. Furthermore, an increment of CETP activity inversely correlated with $HDL_2/HDL_3$ after VOO and FVOO consumptions.

High cardiovascular risk patients usually present a profile characterized by low levels of large-HDLs, high levels of small-HDLs, and variable values of lipid-poor/lipid-free HDLs (pre-β fraction, the most effective one for cholesterol

efflux capacity) (340). The consumption of VOO has been shown to increase large-HDLs and to decrease the small ones in a randomized trial (36). The change towards greater HDL size has been confirmed after the consumption of FVOOT (339), a VOO-rich Mediterranean diet (176;177), and in a rat model after supplementation with OOPC (178). There is some controversy with respect to this issue. On the one hand, it has been reported that the small nascent pre-beta HDL and lipid-free APOA1 are the more functional particles (341). On the other hand, there are *in vitro* studies that reveal that small-HDLs have similar effects to large ones (153). Furthermore, increased levels of small-HDLs in plasma may indicate an aberration in HDL maturation and reduced reverse cholesterol transport (154). Large-HDLs also bind better to the SR-B1 cholesterol transporter (155), promoting cholesterol efflux via these receptors (156). Moreover, some HDL physicochemical modifications (e.g., inflammatory proteins bound to HDL) can transform the lipoprotein into a small, dysfunctional particle [16-18]. Due to all of the above, the interpretation of HDL size, without taking into account the overall biochemical context, is controversial. A similar antioxidant status between $HDL_2$ and $HDL_3$ has been described (157-159), however, in our study, an increase of $HDL_2$-subclass and an enhancement of antioxidant enzyme activities were observed after FVOOT intervention.

An increase in EC/FC and PL/EC in HDL, together with an increment of the LCAT activity, was observed after FVOOT consumption in hypercholesterolemic subjects (339). Our group had previously reported that VOO improved the fluidity of the HDL monolayer and the cholesterol efflux in a randomized, crossover, double-blind, controlled trial with healthy individuals (36). The aqueous diffusion cholesterol efflux mediates the bidirectional flux between the cell plasma membrane and HDL in the extracellular medium. The direction of net cholesterol mass transport is determined by the cholesterol concentration gradient as reflected by the proportions of FC and PL in the monolayer of donor and acceptor particles (161). In this regard, particles with

more PL/FC, such as those obtained after FVOOT consumption, may be more efficient in enhancing aqueous diffusion cholesterol efflux.

The antioxidant properties of OOPC *in vivo* are well-known. The EUROLIVE study showed a decrease *in vivo* in lipid oxidative damage and an increment in HDL-C in a dose-dependent manner with the OOPC administered (97). In this regard, an increment in the HDL-C was detected in the subsample of volunteers without hypolipidemic medication (339). In 2011, the European Food Safety Authority (EFSA) recognized the PC-rich OO effects on protecting LDL from oxidation (112). Moreover, dietary PC can also protect HDL antioxidant status. In agreement with this, an increase of PON activity and LCAT after the FVOOT intervention was observed in our study, reflecting an improved antioxidant status. In contrast, PON activity decreased after the FVOO intervention. In this context, Louded S et al. reported that 12-week extra-VOO consumption increased the anti-inflammatory activities of HDL and PON1 in humans (286). Furthermore, Bayram B et al described that a high OOPC diet increased serum PON1 activity in mice compared to a low one. They suggested that HT could be responsible for the PON1 increment via Nrf2 enhancement (342). In addition, it has been reported that resveratrol also increased LCAT and PON1 activities in elderly rats (343). It has recently been reported that a combination of date and pomegranate polyphenols was more efficient increasing serum PON1 activity and LCAT gene expression than these single-antioxidant interventions in mice models (344). This study, together with our research, suggests that a combination of different PC, which vary in structure and biological activity, is more efficient at enhancing antioxidant enzymes than a single-PC intervention. A less pro-inflammatory and oxidized-HDL could have a more efficient pleotropic function.

### 10.2.2. EFFECTS ON HDL FUNCTIONALITY

Following the improvement in HDL characteristics after FVOOT ingestion, our objective was to test the effect of these OOs on HDL functionality in the same clinical trial. In this regard, in publication number 4 (Annex I), we observed an improvement of HDL functionality after the FVOOT intervention. Specifically, we reported an enhanced HDL cholesterol efflux capacity and HDL oxidant resistance. Furthermore, both functional VOOs increased the HDL antioxidant content.

One of the functions of HDL is as an antioxidant, it protects LDL against oxidative modifications. This property is relevant since LDL oxidation is a key trigger for atherosclerotic plaque onset and development (291). A decrease in LDL oxidative status was found to be dose-dependently associated with the consumption of OOPC (97), and part of this protection could occur through an induction of the HDL antioxidant capacities. It has been reported that a consumption of a VOO-rich diet increases HDL antioxidant activity in apoE-deficient mice (175) and, therefore, it could also be expected in humans. The main proteins involved in HDL antioxidant capacity are ApoA-I and PON1, but others, such as LCAT and PAF-AH, also play a role. In this respect, in the third manuscript included in this thesis, an improvement of PON activity and LCAT mass after the FVOOT intervention was observed. In addition, HDL antioxidant content could also indirectly play a part in its antioxidant capacity (143;345) and thus preserve HDL protein structures. In the EUROLIVE study we observed that PC acquired through a high PC-VOO intervention could bind to HDL in a dose-dependent manner which could contribute to the enhancement of HDL functionality (36). In our study, we determined an increment of antioxidant compounds in the HDL particle, with different activities, after both phenol-enriched VOO interventions *(Farràs M et al submitted. Annex I.)*. Furthermore, co-existence of lipo- and hydro-philic antioxidants linked to HDL may confer additional protection. Lipophilic antioxidants can act by scavenging aqueous peroxyl radicals at the surface of the particle, and by scavenging lipid peroxyl

radicals within it. Lipophilic chain-breaking antioxidants in lipoproteins, such as α-tocopherol, retinol, and carotenoids, may play a major role in protecting lipids and proteins from oxidative damage (346;347). Hydrophilic antioxidants, such as phenols, would be more effective if free radical injury occurred at the lipid/aqueous interphase. In the present study, both phenol-enriched VOOs increased lipophilic and hydrophilic antioxidants in HDL and, consequently, both VOOs improved the antioxidative state of the HDL particle.

Vitamin E (α, β, γ tocopherols) is the major antioxidant in human plasma, and is carried by HDL and LDL, among others. Concretely, α-tocopherol is the most potent antioxidant of the vitamin E family; it is the main initial chain-breaking antioxidant during lipid peroxidation, and is localized in the hydrophobic zone of the lipid bilayer and monolayer (348). Coenzyme Q recycles the resultant α-tocopherol phenoxyl back to its biologically active reduced form. In this respect, an increase in HDL of α-tocopherol and coenzyme Q was observed after the FVOOT intervention, while after FVOO only coenzyme Q increased *(Farràs M et al submitted. Annex I)*. Furthermore, other authors have reported that a fraction of highly active phenolic acids (such as the rosmarinic and caffeic ones) could regenerate α-tocopherol. Specifically, caffeic acid has been reported to protect α-tocopherol in LDL (349). In our study, the FVOOT intake increased rosmarinic acid biological metabolites (caffeic acid sulfate and hydroxyphenylpropionic acid sulfate) and α-tocopherol in HDL, which could suggest a better α-tocopherol regeneration and protection through this mechanism. Thus the FVOOT intervention may be better at improving HDL antioxidative status and consequently preserving HDL protein structures. In concordance with this, a pooling sample proteomic approach, within the context of the same VOHF study, established an increase in afamin in HDL after the FVOOT intervention. Afamin is a protein related to tocopherol transport through cell membranes and this could also suggest a regulatory process to maintain α-tocopherol in the hydrophilic part of the HDL monolayer (166).

It has been reported that an augmentation of antioxidants in biological membranes could increase fluidity (170), in contrast, other authors have determined that antioxidants rigidify membranes thus preventing lipid oxidation propagation (171). Regarding monolayer lipoprotein fluidity, Girona J et al. observed that HDL oxidation promotes a decrease in HDL monolayer fluidity and cholesterol efflux in an *in vitro ex vivo* study (172). In this respect, our team has published that high PC-VOO increases HDL antioxidant content, HDL monolayer fluidity, and cholesterol efflux in healthy volunteers (36). In the VOHF study, we did not detect an increase of HDL monolayer fluidity in any intervention; however, a non-significant linear increase throughout the VOO, FVOO, and FVOOT interventions was observed. Moreover, in the third paper of this thesis we also determined that FVOOT increased HDL PL/FC and HDL EC/FC ratios, suggesting a more fluid HDL monolayer after this intervention (339).

In addition, an improvement of HDL resistance against oxidation after FVOOT was detected; nevertheless, we did not find an improved HDL antioxidant capacity for LDL protection in any of the interventions. We also observed a rise in PON1 activity following FVOOT consumption (339). The better HDL antioxidant status after FVOOT may maintain the ApoA1 and other HDL protein structures and could, therefore, improve cholesterol efflux, the main HDL biological function. In agreement with this, an enhancement in cholesterol efflux after the FVOOT intervention has been established although the results have not yet been published. In addition, cholesterol efflux can also be influenced by nutrigenomic effects, consequently further studies are needed to understand the overall process.

Besides the cholesterol efflux capacity from macrophages promoted by HDL, its capacity to deliver cholesterol via SR-BI must be also taken in account since this is the final step in randomized, controlled trials. Perhaps the HDL particle can enhance cholesterol efflux from macrophages, but is unable to deliver it properly via SR-BI in hepatocytes. In this respect, Nicod N et al., reported that the

treatment with some physiological concentrations of three polyphenols – from green tea, cocoa, and red wine – were not capable of affecting cholesterol uptake via SR-B1 in an *in vitro* study (350). More investigation is needed with regard to this issue.

### 10.2.3. EFFECTS ON ENDOTHELIAL FUNCTION

Apart from the cholesterol efflux capacity and antioxidant properties, HDL also has anti-inflammatory and vasoprotective functions. Endothelial dysfunction and the inflammatory responses of macrophages and endothelial cells are key factors for the perpetuation of the atherosclerotic plaque (291), and both seem to be counteracted by HDL (248). HDL has the ability to inhibit monocyte adhesion by inhibiting VCAM-1, intercellular cell adhesion molecule (ICAM-1), and E-selectin expression, and also to suppress monocyte chemotactic protein-1 by inhibiting chemokine secretion (351). Regarding the HDL anti-inflammatory property, the consumption of OOPC increases its capacity to block the secretion of intracellular adhesion molecule-1 and the adhesion of monocytes to endothelial cells (286). Furthermore, the HDL antioxidant and anti-inflammatory functions, which can be enhanced by OOPC, contribute to the integrity of the endothelial cells. It is noteworthy that HDL could act as a transporter of several OOPC derivatives, among other compounds, to the endothelial cells. There, these substances may prevent possible oxidative damage in cell mitochondria and preserve the production of NO, since OOPC have been shown to protect endothelial cells in several *in vitro* experiments (288). All of the above suggest that OOPC could contribute to an improvement of EF through HDL.

In manuscript number 5 (Annex II) of this thesis, we assessed the effect of these functional VOOs on EF within the context of the sustained clinical VOHF trial. We observed an improvement in EF after both FVOO sustained interventions *(Valls RM et al Submitted. Annex II)*. In this work, the dose-response effect on EF in healthy humans of an acute 30 mL intake of FVOO enriched with its own

PC (250, 500, 750 ppm) was determined. A 500 ppm-FVOO acute consumption provides additional benefits, such as a faster enhancement of EF, and an increase of endothelin-1 and the reduced/oxidized glutathione ratio, versus other FVOOs enriched with different PC concentrations (250 or 750 ppm). This finding showed an improvement of EF, after a functional VOO in pre-/hypertensive subjects, concurring with similar results previously described in the first manuscript of this thesis (330). Furthermore, the sustained consumption of 500 ppm-FVOOs, either enriched with their own PC or complemented with thyme PC, also improved EF by increasing IRH in hypercholesterolemic patients.

The effect of complementary vitamin supplementation on EF has been previously reported in randomized, controlled trials. A short WA et al. has recently published a systematic review concerning randomized, controlled trials with adult participants who were supplemented with vitamins C and E alone or in combination, for more than two weeks. Significant improvements in EF were observed in trials supplementing with vitamin C alone and vitamin E alone, whereas co-administration of both vitamins was ineffective (352). Nevertheless, in our study, EF improvement, measured as IRH, was observed after an OO enriched only with a single antioxidant and also after one enriched with two complementary antioxidants. The different ways to measure EF, the varying patient pathologies, and different types of complementary effects among the antioxidants could have had an influence on the contrasting results.

It has been reported that EF improvement is mediated via reduction in oxidative stress and the increase of NO metabolites (96). In the sustained trial of the VOHF study we did not observe differences in NO and endothelin-1 markers, as a consequence, other mechanisms could be involved in the vasodilation process. A mechanism by which the IRH could have been improved after both FVOO interventions might be through the increase of the PC and fat-soluble vitamins in plasma. In this regard, a positive correlation between IRH and systemic fat-soluble vitamins was observed *(Valls RM et al Submitted. Annex II)*. A positive correlation between β-carotene and circulating endothelial progenitor cells,

which favors the regenerative capacity of the endothelium has been reported (313). Karppi J, et al. suggested that high plasma concentrations of β-cryptoxanthin, lycopene, and α-carotene may be associated with a decreased intima-media thickness of the carotid artery wall (353). Crocetin, a carotenoid, inhibited Ang-II and VCAM-1 expression in HUVECs, and also reduced monocyte-endothelial cell adhesion in an *in vitro* study (354). Moreover, it has been described that tocopherol supplementation favorably influenced vascular function, circulating cytokine profile, and was effective in reducing atherosclerosis in apoE$^{-/-}$ mice with chronic kidney disease (355). In addition, it has been demonstrated that lutein (Fernández-Robredo P, Oxid Med Cel Longev, 2013) has shown some benefits in biomarkers related to EF in apoE$^{-/-}$ mice and humans, and beta-carotene, cryptoxanthin, and retinol exert the same effects in humans (356;357).

In our sustained consumption study, a positive relationship was observed between IRH and plasma concentrations of HDL-C, a result also described in hypercholesterolemic patients by Ruano J, et al (96). As previously mentioned, the HDL particle has been described as exerting a protective effect on the vascular endothelium. According to this, the improvement of HDL levels and/or function could be another mechanism by which EF improvement occurs after the intake of both FVOOs in the sustained study.

***** In summary, sustained and moderate consumption of complementary phenol-enriched VOO induced changes in the HDL profile related to low-cardiovascular risk and better HDL functionality, such as higher levels of large HDLs, lower levels of small ones, increased HDL EC/FC content, increased HDL-monolayer PL/FC, and increased HDL antioxidant and metabolism enzymes. In this regard, this intervention also improved HDL cholesterol efflux capacity. In addition, the sustained complementary phenol-enriched VOO intervention, and also the VOO enriched with its own PC, improved HDL antioxidant status

and EF. OO, a recognized healthy food, cannot be consumed in large quantities, thus, enrichment with its own PC is a way of increasing its healthy properties whilst maintaining the same amount of fat. These results show that a n enrichment of VOO with complementary antioxidants promotes greater benefits than with only its own PC. Our data suggest that a complementary phenol-enriched VOO could be a good nutraceutical product for improving HDL functionality, and thus a complementary tool for the management of hypercholesterolemic individuals.

## 10.3. STRENGTHS AND LIMITATIONS

One strength of our studies is its crossover, randomized, and controlled design, which permitted the participants to consume all types of VOO and thus eliminated inter-individual variability. Furthermore, the laboratory analyses were centralized and all the time-series samples from the same volunteer were measured in the same run to minimize imprecision. In addition, in each study, the VOOs had a similar matrix ( fat-soluble, vitamins, and fatty acids), as a result, their differential character was their PC content.

A limitation of these studies was their sample size, which could be responsible for reduced statistical power in some biomarkers with high inter-individual variability. Moreover, a synergistic effect between PC and other VOO components on analyzed parameters is as yet unknown. Another drawback was the inability to assess potential interactions among the VOOs and other diet components and medication. Nevertheless, medication and diet were controlled during the studies and no changes were registered. In addition, another potential limitation of the study was that, although the trials were blinded, some participants may have identified the type of VOO ingested by its organoleptic characteristics.

# CONCLUSIONS

# 11. CONCLUSIONS

1. A single 30 mL dose of functional virgin olive oil enriched with its own PC at 5-hour post-prandial in pre- and hypertensive humans, produces:
    - An improvement in endothelial function and a decrease in a number of biomarkers such as oxLDL, VCAM-1, hsCRP, and PAI-1. These results suggest a link between an enhancement in the oxidative-inflamatory state and that of endothelial function.
    - An improvement in cholesterol efflux gene expression. Specifically, the enhancement was produced in the nuclear receptor factors (PPARBP, PPARa, PPARd, PPARg) and cholesterol transmembrane transporter (ABCA-1, SR-B1, CD-36) genes in white blood cells.

2. A sustained, moderate 3-week consumption of 25 mL/day of a functional virgin olive oil enriched with its own phenolic compounds plus additional ones from thyme improves HDL-subclass distribution and composition, and antioxidant metabolism enzyme levels.

# CONCLUSIONS

1. Una dosi única de 30 mL d'oli d'oliva funcional enriquit amb els seus compostos fenòlics, a 5 hores post-prandial en humans pre- i hipertensos, produeix:
    - Una millora de la funció endotelial i una disminució en biomarcadors com oxLDL, VCAM-1, hsCRP, i PAI-1. Aquests resultats suggereixen un lligam entre una millora de l'estat oxidatiu/inflamatori i una millora de la funció endotelial.
    - Una millora en l'expressió gènica de l'eflux de colesterol. Concretament, l'activació va ser produïda en els gens dels factors receptors nuclears (PPARBP, PPARa, PPARd, PPARg) i dels transportadors transmembrana de colesterol (ABCA-1, SR-B1, CD-36) en cèl·lules sanguínies blanques.

2. Un consum sostingut, moderat durant 3 setmanes de 25 mL/dia d'oli d'oliva verge funcional enriquit amb els seus compostos fenòlics més adicionals de la farigola milloren la distribució de les subclasses d'HDL, la composició de l'HDL, i els nivells d'enzims antioxidants i del metabolisme.

# CONCLUSIONES

1. Una dosis única de 30 mL de aceite de oliva funcional enriquecido con sus compuestos fenólicos, a 5 horas post-prandial en humanos pre- e hipertensos, produce:
    - Una mejora de la función endotelial i una disminución en biomarcadors com oxLDL, VCAM-1, hsCRP, i PAI-1. Estos resultados sugieren un enlace entre una mejora del estado oxidativo/inflamatorio y una mejora de la función endotelial.
    - Una mejora en la expresión génica del flujo de colesterol. Concretamente, la activación fue producida va en los genes de los factores receptores nucleares (PPARBP, PPA Ra, PPA Rd, PPARg) y de los transportadores transmembrana de colesterol (ABCA-1, SR-B1, CD-36) en células sanguíneas blancas.

2. Un consumo sostenido, moderado durante 3 semanas de 25 mL/día de aceite de oliva virgen funcional enriquecido con sus compuestos fenólicos mádicionales del tomillo mejoran la distribución de las subclasses de HDL, la composición de la HDL, y los niveles de enzimas anitoxidantes y del metabolismo.


FUTURE PLANS

# 12. FUTURE PLANS

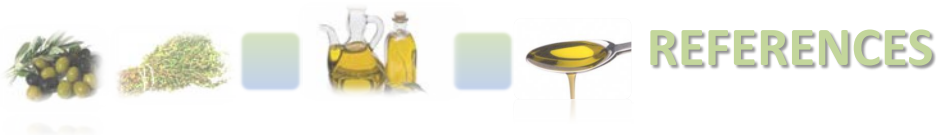
The present dissertation provides, for the first time, evidence of post-prandial and mid-term cardiovascular health-protective benefits in risk volunteers after the ingestion of a FVOO enriched with PCs. Furthermore, it has been demonstrated that a FVOO enriched with its own PCs, plus additional ones from thyme, is more beneficial than one enriched only with its own PCs for the markers analyzed in the present thesis. We are currently working on other manuscripts derived from the VOHF study to establish additional effects of both FVOOs, such as cholesterol efflux promoted by HDL (Annex 1), endothelial dysfunction (Annex 2), the oxidative/inflammatory state of the HDL particle, and the oxidative/inflammatory systemic state. We are also focused on the study of the subjacent mechanisms of these benefits through OMIC techniques, such as transcriptomics, proteomics, and metabolomics. In this regard, we are working in the results of the cholesterol efflux-related and CD-40 pathway-related gene expression in the volunteer's peripheral white blood cells.

Due the fact thact changes in gene expression do not necessarily imply that the codified proteins are also modified towards a health-protective mode. Proteomic, metabolomic and epigenomic approaches in the VOHF study will be of interest. Specifically, the analysis of miR-33, a miR involved in the cholesterol homeostasis could be of particular relevance. Such research would permit the establishment of a direct genotypic-phenotypic link which could explain, in more detail, the protective effects of FVOO with respect to CVD prevention.

The results of the present dissertation support the hypothesis that VOHF FVOOs could improve a number of HDL functions. In this regard, it is necessary to analyze HDL overall cholesterol efflux capacity after VOHF interventions, and also test cholesterol uptake via SR-B1 *in vitro*. In addition, other HDL properties should be investigated in the corresponding cellular models. We are, therefore, interested in the study of functional properties such as the anti-inflammatory

ones in endothelial and macrophage cells, insulin secretion in pancreatic cells, glucose uptake in muscular cells, and cholesterol uptake in hepatocytes.

Our results provide, for the first time and with the highest degree of evidence, that an enrichment of VOO with complementary antioxidants promotes greater benefits than an enrichment with own PC. The PCs enrichment is a way of increasing the healthy properties of VOO whilst maintaining the same amount of fat. Our findings suggest that FVOOT could be a useful dietary tool in the management of cardiovascular risk patients. Since the relevance of overall high-quality food patterns must be considered, further studies are needed to confirm the present ones and to study the intake of FVOOs in the context of a healthy diet. It is of note that synergies and cumulative effects among different foods and nutrients are behind the benefits of a healthy dietary pattern.



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