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Abstract

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Keywords	Functional virgin olive oil; phenol; HDL antioxidants; cholesterol efflux; HDL fluidity; HDL functionality

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Figure 1_HDL compounds with antioxidant properties after the interventions.pdf [Figure]

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Phenol-enriched olive oils improve HDL antioxidant content in hypercholesterolemic subjects. A randomised, double-blind, cross-over, controlled trial.

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ABBREVIATIONS

ApoA-I: apolipoprotein A-I

CoQ: coenzyme Q

EC: esterified cholesterol

FC: free cholesterol

FVOO: functional virgin olive oil

FVOOT: functional virgin olive oil with thyme

OO: olive oil

PC: phenolic compounds

PL: phospholipid

RCT: reverse cholesterol transport

TC: total-cholesterol

TG: triglyceride

VOO: virgin olive oil

Keywords: Functional virgin olive oil; phenol; HDL antioxidants; cholesterol efflux; HDL fluidity; HDL functionality.

ABSTRACT

At present, HDL-function is thought to be more relevant than HDL-cholesterol quantity. Consumption of olive oil phenolic-compounds (PC) has beneficial effects on HDL related markers. Enriched food with complementary antioxidants could be a suitable option to obtain additional protective effects. Our aim was to ascertain whether virgin olive oils (VOOs), enriched with (i) their own PC (FVOO) and (ii) with their own PC plus complementary ones from thyme (FVOOT) could improve HDL status and function. 33-hypercholesterolemic individuals ingested (25mL/day, 3 weeks) (i) VOO (80 ppm), (ii) FVOO (500 ppm), and (iii) FVOOT (500 ppm) in a randomised, double-blind, controlled, cross-over trial. A rise in HDL-antioxidant compounds was observed after both functional olive oil interventions. Nevertheless, α-tocopherol, the main HDL antioxidant, only augmented after FVOOT versus its baseline.

In conclusion, long-term consumption of phenol-enriched olive oils induced a better HDL-antioxidant content, the complementary phenol-enriched olive oil being the one which increased the main HDL antioxidant, alpha-tocopherol. Complementary phenol-enriched olive oil could be a useful dietary tool for improving HDL richness in antioxidants.

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1. INTRODUCTION

Olive oil (OO) phenolic compounds (PC) have been shown to prevent coronary heart disease, especially in humans with oxidative stress [1]. Due to the fact that HDL-cholesterol (HDL-C) levels are inversely and independently related with cardiovascular disease [2], pharmacological and natural product development has been oriented to the augmentation of their concentrations. Nevertheless, ineffectiveness, and even increased mortality risk of cholesteryl ester transfer protein antagonists have been reported in clinical trials [3, 4]. Such finding, combined with recent evidence that a number of genetic variables predisposing to high HDL-C levels are not associated with a lower risk of suffering a coronary event [5], have led to the consideration that future therapeutic approaches should improve HDL functionality rather than quantity [6]. Reverse cholesterol transport (RCT) is the main HDL biological function. It consists of extracting the cholesterol excess from the peripheral cells (cholesterol efflux) and taking it to the liver for further metabolism and excretion. This functional property has been tested in macrophage cell lines and shown to be inversely related to early atherosclerosis development and a high risk of experiencing a coronary event [7]. Increased HDL-C concentrations, and decreased *in vivo* lipid oxidative damage, in a dose-dependent manner with the PC content of the OO administered were reported in the EUROLIVE study [8]. In this regard, from a subsample of healthy humans we have, for the first time, first-level evidence that virgin olive oil (VOO) improves (i) cholesterol efflux, (ii) HDL monolayer fluidity, and (iii) HDL PC-content [9]. These characteristics can be altered by physicochemical changes and inflammatory protein binding resulting in a dysfunctional particle [10-12]. Protection against such a transformation could be provided by pharmacotherapy or functional foods oriented to improving HDL oxidative-inflammatory status. 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

produce a dual action because, depending on the dose, antioxidants could also revert to pro-oxidants [13,14]. One option to achieve greater beneficial health effects might be the development of functional foods with complementary-antioxidants, according to their structure/activity relationship. In a randomized, double-blind, cross-over, and controlled trial our objective was to ascertain whether VOOs enriched (i) with their own PC (FVOO; 500ppm from OO) and (ii) with their own PC plus additional ones from thyme (FVOOT; 250 ppm from OO and 250 ppm from thyme) could enhance HDL antioxidant content.

2. MATERIALS AND METHODS

2.1. OO preparation and characteristics

The two phenol-enriched OOs (FVOO and FVOOT; 500 ppm) were prepared using a low-phenolic content VOO (80 ppm) which also served as control. For the wash-out period a common OO was used. The procedure to obtain the phenolic extracts and the enriched oils has been previously described [15]. In short, VOO with a low phenolic content was used as a control treatment and as an enrichment matrix for the preparation of both phenol-enriched olive oils. FVOO was enriched with its own PCs by adding a phenol extract obtained from freeze-dried olive cake collected from a commercial olive mill in an olive-growing area (Les Garrigues, Lleida, Catalonia, Spain). FVOOT was enriched with its own PC (50%) plus those from thyme (50%) 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 **Supplemental material Fig. 1** shows the PCs, the fatty acid, and the fat soluble micronutrient daily intake with 25mL of VOO, FVOO, and FVOOT. The VOO phenolic profile was assessed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) as described Rubió et al. [15]. Tocopherol, fatty

acid, and carotenoid-contents in VOO, were analyzed using previously described methods [16,17].

2.2. Study design

Thirty-three hypercholesterolemic volunteers (total-cholesterol>200 mg/dL) participated in the VOHF study, a randomized, double-blind, crossover, controlled trial. Exclusion criteria were the following: BMI>35 Kg/m^2, smokers, athletes with high-physical activity (>3000 Kcal/day), diabetes, multiple allergies, intestinal diseases, or any other disease or condition that would worsen adherence to the measurements or treatment.

Participants were randomized to one of 3 orders of administration of raw OOs (VOO, FVOO, and FVOOT). Administration sequences were: 1) FVOO, FVOOT, VOO; 2) FVOOT, VOO, FVOO; and 3) VOO, FVOO, FVOOT. Intervention periods were of 3-weeks with an ingestion of 25 mL/day raw OO distributed along meals preceded by 2-week wash-out periods with a common OO.

Physical activity was evaluated by a Minnesota questionnaire at baseline and at the end of the study. Participants were asked to return the 21 containers at the end of each intervention period so that the daily amount of unconsumed olive oil could be registered. Those with less than 80% treatment adherence (≥5 full OO containers returned) were considered non-compliant. 24h-urine and blood samples were collected at fasting state at the start of the study and before and after each treatment. Plasma samples were obtained by whole blood centrifugation. Urine and plasma were preserved at -80ºC prior to use.

The trial was performed conforming to the Helsinki Declaration and the Good Clinical Practice for Trials on Medical Products in the European Community. Written informed consent was obtained from the participants. The protocol (CEIC-IMAS 2009/3347/I) was approved by the local ethics committees and filed with the International Standard Randomized Controlled Trial register (www.controlled-trials.com; ISRCTN77500181).

2.3. Dietary adherence

Urinary hydroxytyrosol-sulfate and thymol-sulfate were measured as biomarkers of adherence to the type of OO ingested. Measurements were performed by high performance liquid chromatography-electrospray MS/MS (UHPLC-ESI-MS/MS) [18]. Participants completed a 3-day dietary record at baseline and before/after each intervention. In addition, they received guidance from a nutritionist about replacing habitually consumed raw fats with the provided OOs and avoiding polyphenol-rich food (e.g. vegetables, fruit, coffee etc.).

2.4. Systemic biomarker analyses

EDTA-plasma glucose, total-cholesterol (TC), and triglyceride (TG) levels were determined using standard enzymatic automated methods; and apolipoprotein A-I (ApoA-I) and ApoB100 by immunoturbidimetry in a PENTRA-400 autoanalyzer (ABX-Horiba Diagnostics, Montpellier, France). HDL-C was measured by an accelerator selective detergent method (ABX-Horiba Diagnostics). LDL-C was computed by the Friedewald equation. Plasma oxidized LDL (oxLDL) was analyzed using ELISA (Mercodia AB, Uppsala, Sweden).

2.5. HDL isolation and lipid-protein analyses

HDL from the study volunteers were isolated by an ultracentrifugation with a density gradient preparation method [19], using at once two solutions of different densities, 1.006 g/mL and 1.21 g/mL. LDL and HDL fractions were isolated in a long ultracentrifugation tube which permits that the fractions are clearly separated after the ultracentrifugation. LDL is located in the upper half of the tube as a yellow-orange band, and HDL in the lower half as a wide-yellowish band; both ones are separated for a wide-colorless band. Each fraction was pippeted and aliquoted independently.

To assure the purity of HDL fractions, ApoB100 and albumin levels were also determined in these samples by automatic immunoturbidimetric methods (ABX-Horiba Diagnostics)

(Supplemental material Table 1). The lipid and protein composition of HDL has been previously described [20].

2.6. 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 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 8ºC/min until it reached 200ºC then 3ºC/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).

2.7. Analyses of HDL compounds with antioxidant properties

2.7.1. 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 Detector, and a Waters 2475 Fluorescence Detector managed by Empower software (Waters Inc., Milford, MA). A 150x4.6 mm i.d. YMC C30 analytical column (3 µm) (Waters Inc., Milford, MA) was used for the separation of all components and HPLC analysis was performed following the procedure of Gleize et al. (2007) [21]. All compounds were identified by their retention time compared with pure standards or, when unavailable (lutein and β-cripthoxanthin), with compounds obtained and purified in the laboratory, the concentrations of which were determined by spectrophotometry using the molecular extinction coefficient (ε) of the molecule. Ubiquinol, the reduced form of Coenzyme Q (CoQ) 10 detected in HDL, was quantified with the calibration curve of ubiquinone standard (oxidized form) using a correction factor (200:1) as previously defined [22]. 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.

2.7.2. Phenolic and monoterpene metabolites:

The phenolic and monoterpene biological metabolites were extracted from HDL by solid-phase extraction system using OASIS HLB 60 mg cartridges (Waters Corp., Milford, MA). Extractions were performed by loading 500 µL of HDL sample which had previously been mixed with 500 µL of distilled water and 60 µL of phosphoric acid 85% to break the bonds between the proteins and phenolic compounds, and 100 µL of catechol as internal standard. The retained phenolic compounds were eluted using 3 mL of methanol, which was evaporated to dryness under nitrogen flow. Prior to chromatographic analysis, the sample was reconstituted with 50 µL of methanol, before chromatographic analysis. The analysis of the phenolic metabolites was carried out by UPLC/MS/MS based on the method described

by Rubió et al. (2012) [23]. The selected ion monitoring (SRM) transitions, cone voltage, and collision energy values were previously optimized in plasma for each phenol metabolite [18]. Only 6 were detected in HDL among all the analysed phenolic metabolites **(Supplemental material. Table 2)**. Most of the PC (mainly the native structures present in the oils) were not found in HDL samples, thus, quantification was not undertaken. The metabolites hydroxytyrosol sulfate (sulfHT) and thymol sulfate (sulfTHY) were quantified, the rest of the metabolites, due to the lack of reference standards, were tentatively quantified with the calibration curves corresponding to their phenolic precursors or to similar metabolite compounds. In this regard, the sulfate conjugates derived from hydroxytyrosol, hydroxytyrosol acetate sulfate (sulfHTAc) and homovanillic alcohol sulfate (sulfHVAlc) were quantified with the calibration curve of sulfHT. Caffeic acid sulfate (sulfCA) and hydroxyphenylpropionic acid sulfate (sulfHPPA) were tentatively quantified by the calibration curve of caffeic acid and 3-(4-hydroxyphenyl)-propionic acid, respectively. All calibration curves were performed in HDL sample matrix. All analyses were run in duplicate.

2.8. HDL monolayer fluidity determination

The measurement of the HDL particle fluidity was based on the determination of the steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), as previously described [24]. 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.

2.9. HDL cholesterol efflux capacity determination

HDL cholesterol efflux was determined in a subsample of the study (n= 27). Murine J-774A.1 monocytes were seeded at a density of 75000 cells/cm^2 and routinely grown for 24 hours. 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. Labelled cells were subsequently washed 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 µg/mL). All these incubations were performed in the presence of the Acyl-CoA cholesterol acyltransferase (ACAT) enzyme inhibitor Sandoz 58-035 (5µM; Sigma-Aldrich, USA). Media and cell fractions were pipetted onto a black plate, and fluorescence intensity was monitored in the multi-detection Microplate Reader Synergy HT (BioTek Instruments; USA) at λEx/Em=485/528nm. 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.

2.10. 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).

2.11. Statistical analyses

Normality of continuous variables was evaluated by probability plots. Non-normally distributed variables were log transformed if necessary. Non-compliant participants, as defined previously, were excluded from analysis in these interventions. To compare means (for normal distributed variables) or medians (for non-normal distributed variables) among groups, ANOVA and Kruskal-Wallis tests were used, respectively; whereas χ^2 and exact F-test, as appropriate, were employed to compare proportions. To assess relationships among variables Pearson and Spearman correlation analyses were performed. A general linear model for repeated measurements was employed to evaluate the intra- and inter-intervention effects. 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 participant as a random intercept. Carry-over effect was discarded in all variables. A value of $p<0.05$ was considered significant. R2.12.0 software (R Development Core Team) and SPSS18.0 software (IBN Corp) were employed to perform the statistical analyses.

3. RESULTS

3.1. Participant characteristics, dietary adherence, and systemic biomarkers

From the sixty-two subjects evaluated, thirty-three eligible volunteers (19 men) were finally included. **Supplemental Figure 2** shows the flow of participants throughout the study. No adverse effects caused by OO ingestion were observed. Participants' baseline characteristics

are shown in **Table 1**, with no significant differences among orders. No changes in daily energy expenditure in leisure-time physical activity, main nutrients, and medication intake throughout the study were found [20]. Neither any alterations in blood pressure, BMI, glucose, oxLDL nor lipid profile (**Supplemental material. Table 3**) were reported. From the analysis of urinary phenolic metabolites it could be observed that the compliance of the participants was good. Hydroxytyrosol sulfate and hydroxytyrosol acetate sulfate increased after the FVOO intervention versus the VOO one ($p<0.05$). Thymol sulfate, hydroxyphenylpropionic acid sulfate, and *p*-cymene-diol glucuronide increased after the FVOOT treatment versus the FVOO and VOO ones ($p<0.05$) [18].

3.2. HDL fatty acids

No changes were observed in HDL fatty acids throughout the study.

3.3. 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 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 found after FVOO versus its baseline ($p<0.05$) **(Figure 1)**.

HDL antioxidant distribution showed cross-linked correlations with systemic biomarkers and with HDL composition. The HDL α-tocopherol post-value directly correlated with HDL cholesterol/protein ratio after VOO, FVOO, and FVOOT intakes ($r>0.6$; $p<0.001$). In

addition, HDL α-tocopherol directly correlated with the HDL PL/protein ratio after FVOOT intake (r=0.587; p=0.002).

3.4. HDL monolayer fluidity and HDL cholesterol efflux capacity

FVOOT improved cholesterol efflux versus FVOO (+1.353% ± 3.934 and -1.225% ± 3.854, respectively; p=0.019) but not versus VOO control group (-0.034% ± 5.421). Moreover, FVOOT tended to increase cholesterol efflux versus its baseline (pre-FVOOT: 28.394% ± 6.775 and post-FVOOT: 29.747% ± 5.638; p=0.086) **(Figure 2)**. No significant changes were found in HDL monolayer fluidity throughout the study (VOO= -0.036 AU ± 0.255; FVOO= +0.015 AU ± 0.217; FVOOT= +0.024 AU ± 0.198).

4. DISCUSSION

The VOHF study is a randomized, double-blind, cross-over, controlled trial with a VOO as control and two phenol-enriched ones: FVOO (enriched with its own PCs) and FVOOT (enriched with its own plus those from thyme). Our findings indicate that a functional OO, supplemented with complementary phenols from OO and thyme, improves HDL antioxidant content.

The antioxidant system is a complex network of interacting molecules. When an antioxidant is oxidized it is converted into a harmful radical that 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. In contrast, the combination of different antioxidants has been shown to be effective in reducing atherosclerosis in human trials [25]. 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 [15].

A number of antioxidants associated with HDL could improve its antioxidant function and preserve its structure. The EUROLIVE study revealed that PC acquired through a high PC-VOO intervention could bind to HDL in a dose-dependent manner and thus contribute to the enhancement of its functionality [9]. In our work, after both phenol-enriched VOO interventions we found an increase in HDL from antioxidants with various activities. Furthermore, the co-existence of lipo- and hydro-philic antioxidants linked to HDL may confer additional protection. On one hand, 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 a key role in protecting lipids and proteins from oxidative damage [26,27]. It has been reported that a physiological concentration of β-carotene and CoQ inhibits LDL and HDL oxidation *in vitro* [28,29]. On the other hand, hydrophilic antioxidants, such as phenols, would be more effective if free radical injury occurred at the lipid/aqueous interphase. Some *in vitro* studies have shown that PC do penetrate the phospholipid bilayer of the liposomes, probably as a consequence of their hydrophilic properties and their non-planar structures which confer conformational mobility [30]. 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 (α, β, γ tocopherols), the major antioxidant in human plasma, which is carried by HDL and LDL. The most potent antioxidant of the tocopherol family is α-tocopherol which is the main initial chain-breaking antioxidant during lipid peroxidation. It is fully localized in the hydrophobic zone of the

lipid bilayer [31]. In turn, CoQ recycles the resultant α-tocopherol phenoxyl back to its biologically active reduced form [32]. In this regard, we observed an augmentation 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 (such as rosmarinic and caffeic ones) could regenerate α-tocopherol. Specifically, caffeic acid has been reported to protect α-tocopherol in LDL [33]. In our study, the FVOOT intervention increased rosmarinic acid biological metabolites (caffeic acid sulfate and hydroxyphenylpropionic acid sulfate), as well as α-tocopherol, which might suggest a better α-tocopherol regeneration and protection through this mechanism. Thus, the FVOOT intervention could be better at improving HDL antioxidant activity and consequently preserving the HDL protein structures. Furthermore, Peruguini et al (2000) [34] reported that HDL α-tocopherol is related to the cholesterol- and PL-/protein ratios, correlations that were also reproduced in the present work.

It has been described that an increment of antioxidants in biological membranes could increase fluidity [35]. In contrast, other authors have reported that antioxidants could rigidify membrane cells thus hindering oxidation transmission [36]. Regarding monolayer lipoprotein fluidity, Girona et al. (2003) [11] observed that HDL oxidation results in decreased HDL monolayer fluidity and less cholesterol efflux in an *in vitro-ex vivo* experiments. In addition, our team observed that VOO increases HDL antioxidant content, HDL monolayer fluidity, and cholesterol efflux in healthy volunteers, in a crossover trial with two arms [9] (Hernáez et al., 2014). Nevertheless, in the present work with hypercholesterolemic subjects, we did not observe an increase of HDL monolayer fluidity or a significant increase of the cholesterol efflux in any intervention. It is of note that the reduced sample size and the three arms of intervention have conditioned less statistical power.

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 [8]. In concurrence, in a recent paper from the VOHF-study, an increment in HDL-C was observed in the subsample of volunteers without hypolipidemic medication [20]. The effects of PC-rich OO on protecting LDL from oxidation have been acknowledged by the European Food Safety Authority [37]. Nevertheless, in this study, although a decrease in the oxidized LDL was observed after three interventions, no significant change effect was detected. Hypercholesterolemic status and pharmacological treatment could explain such a result.

The crossover, randomized design of the study is a strength because it meant that inter-individual variability was reduced as the participants consumed all the kinds of OOs. In addition, the three OOs had a similar matrix (fat-soluble, vitamins, and fatty acids), with only their PC content varying. A further strength is the centralization of laboratory analyses and the time-series samples from the same participant being measured in the same run to minimize imprecision. The reduced sample size represents a possible limitation as it could have led to diminished statistical power in a number of biomarkers with increased intra-individual variability. A synergistic effect on HDL-parameters from PC and other OO constituents remains to be elucidated. The inability to assess potential interactions among the OOs and other dietary components and medication is also a limitation. In this regard, medication and diet was controlled throughout the study and no changes were registered.

In conclusion, long-term consumption of complementary phenol-enriched OO induced an improvement in HDL antioxidant content. These results show that an enrichment of OO with complementary antioxidants promotes greater benefits than an enrichment of OO with only its own phenolics in cardiovascular high-risk individuals.

CONCLUDING REMARKS

The greater benefits of complementary-phenol enriched olive oil consumption on HDL antioxidant content in hypercholesterolemic humans have been demonstrated for the first time, with the highest degree of evidence. Furthermore, such improvements can be achieved without increasing the individual's fat intake. These results indicate that a complementary phenol-enriched olive oil could be a useful dietary tool for improving the richness of HDL in antioxidants in cardiovascular high-risk individuals.

HIGHLIGHTS

- Phenol-enriched olive oils improve HDL antioxidant content.
- Complementary phenol-enriched olive oil increases α–tocopherol, the main HDL antioxidant.
- Our findings have been demonstrated in hypercholesterolemic individuals with the highest degree of evidence.
- Complementary phenol-enriched olive oil could be a useful tool for improving HDL profile in cardiovascular high-risk humans.

AUTHOR CONTRIBUTIONS

M-IC, M-JM, RS, RdlT, and MFi designed 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 participated in the writing, and read and approved the final manuscript.

ACKNOWLEDGEMENTS

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CONFLICT OF INTEREST STATEMENT

The authors have declared that no competing interests exist.

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FIGURE LEGENDS

Figure 1. 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 2. Mean change of cholesterol efflux after the interventions.

Values represent the mean differences of cholesterol efflux after the interventions.

Values expressed as mean ± SE

* Inter-treatment p-value<0.05

^ Intra-treatment p-value<0.09

TABLES

Table 1. Baseline characteristics of the participants.

	Total[a] (n=33)
GENERAL	
Sex: male	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

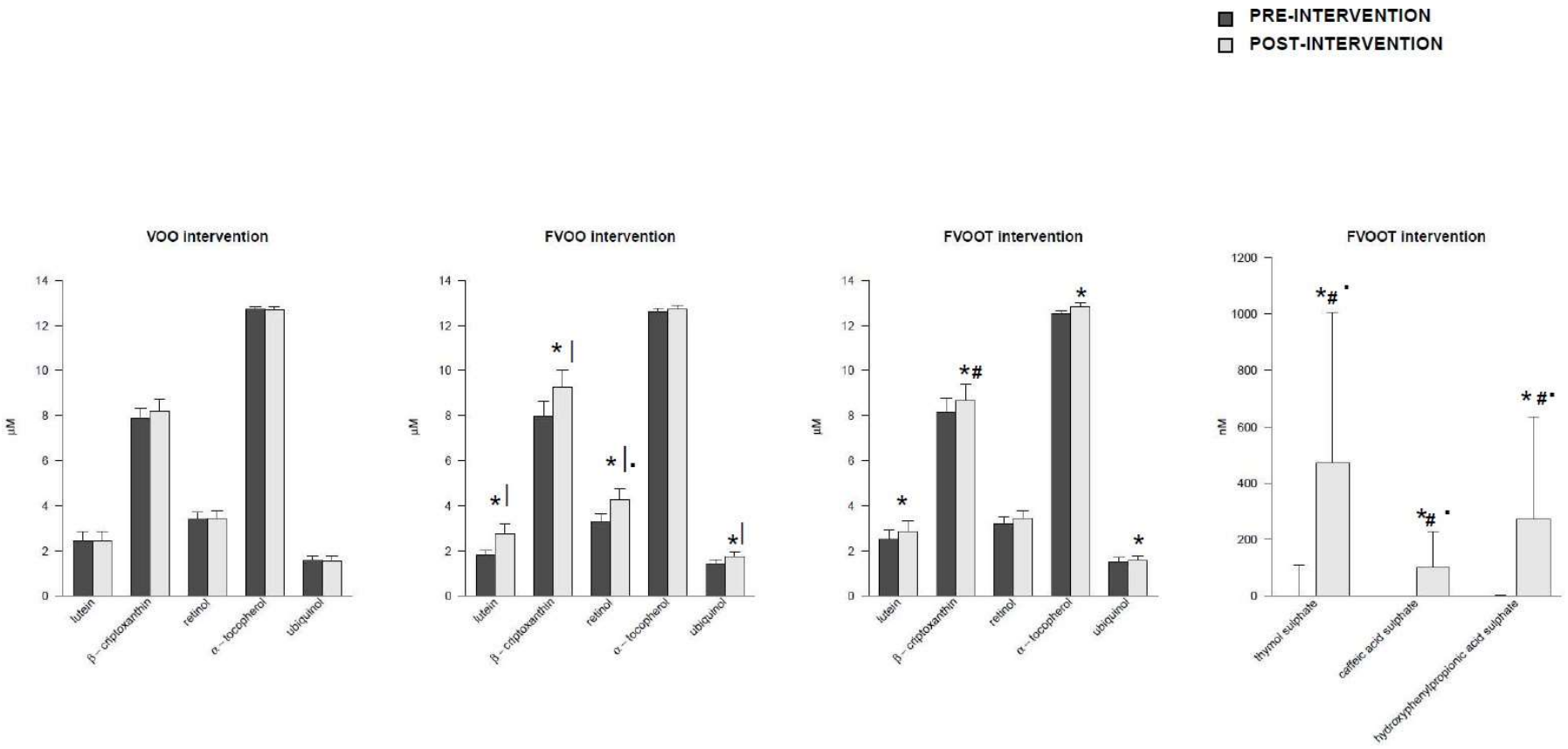
[a)] Values expressed as mean ± S.D. or median (25th to 75th percentile).

660 **FIGURES**

661

662 **Figure 1.**

663



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665 **Figure 2.**

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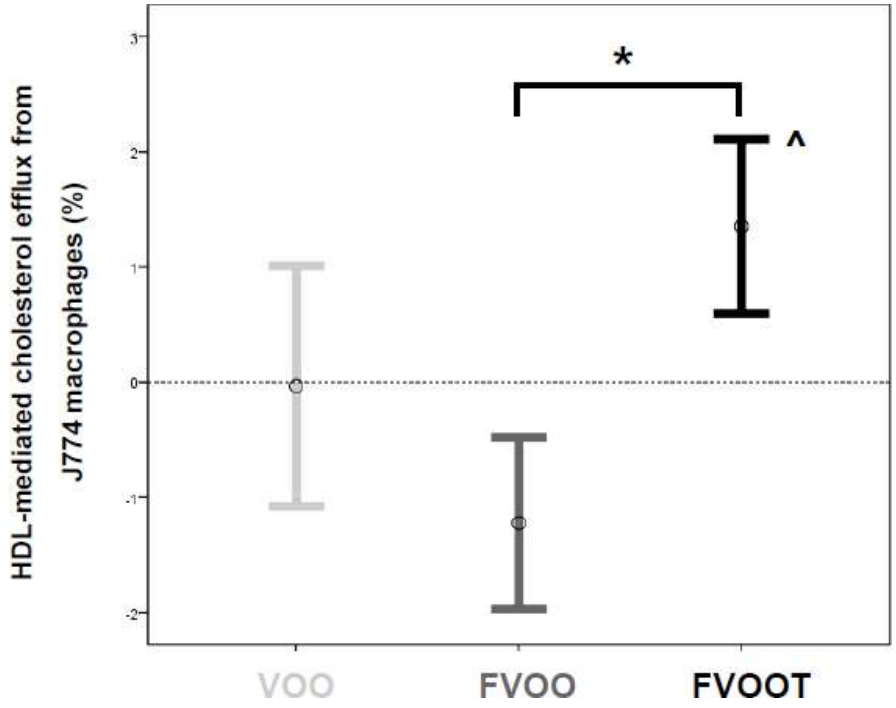

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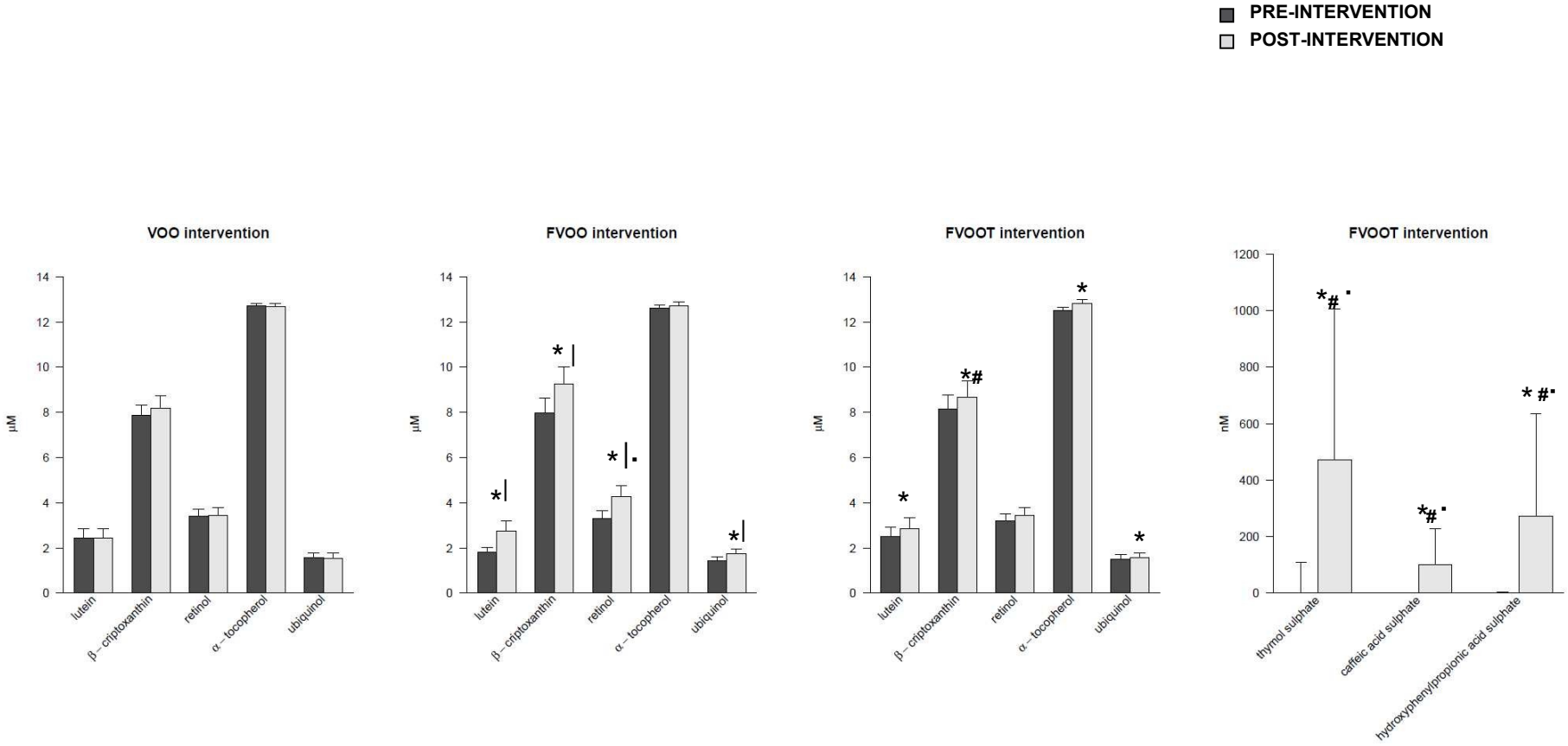
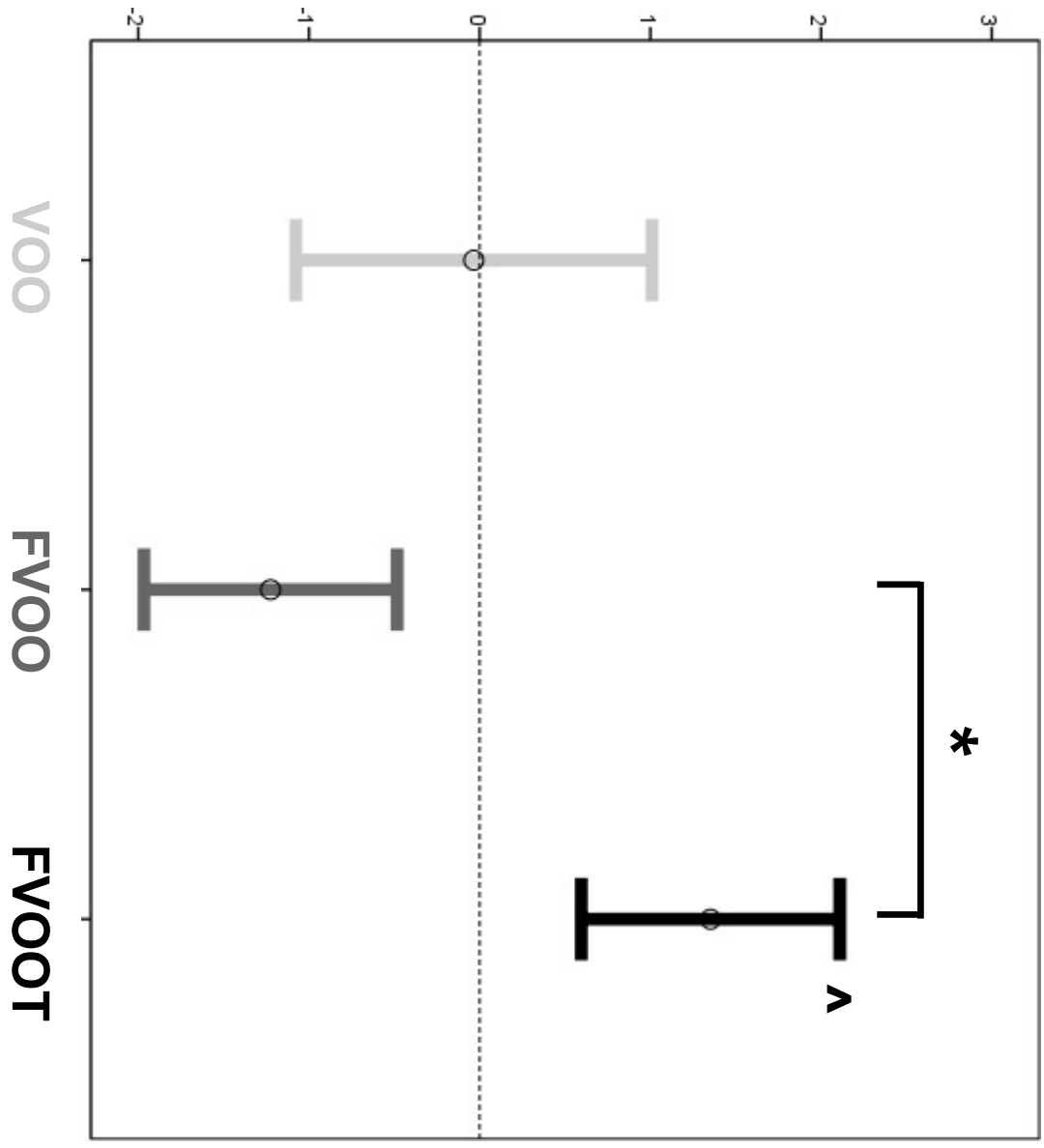

PRE-INTERVENTION
POST-INTERVENTION
VOO intervention
FVOO intervention
FVOOT intervention
FVOOT intervention
µM
nM
lutein
β−criptoxanthin
retinol
α−tocopherol
ubiquinol
thymol sulphate
caffeic acid sulphate
hydroxyphenylpropionic acid sulphate

HDL-mediated cholesterol efflux from J774 macrophages (%)



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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).

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***

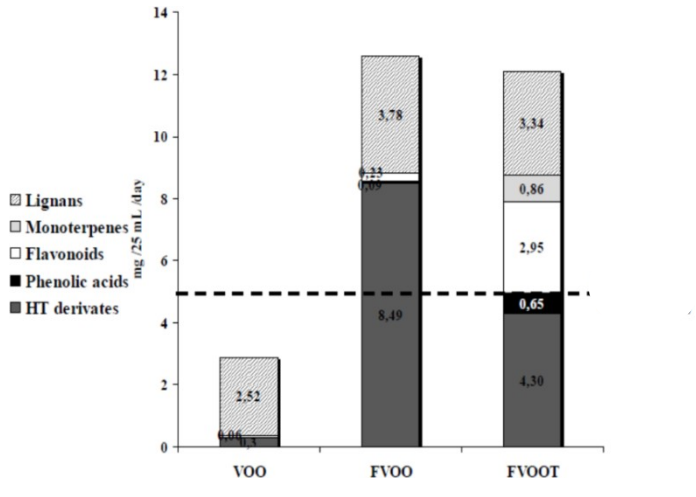
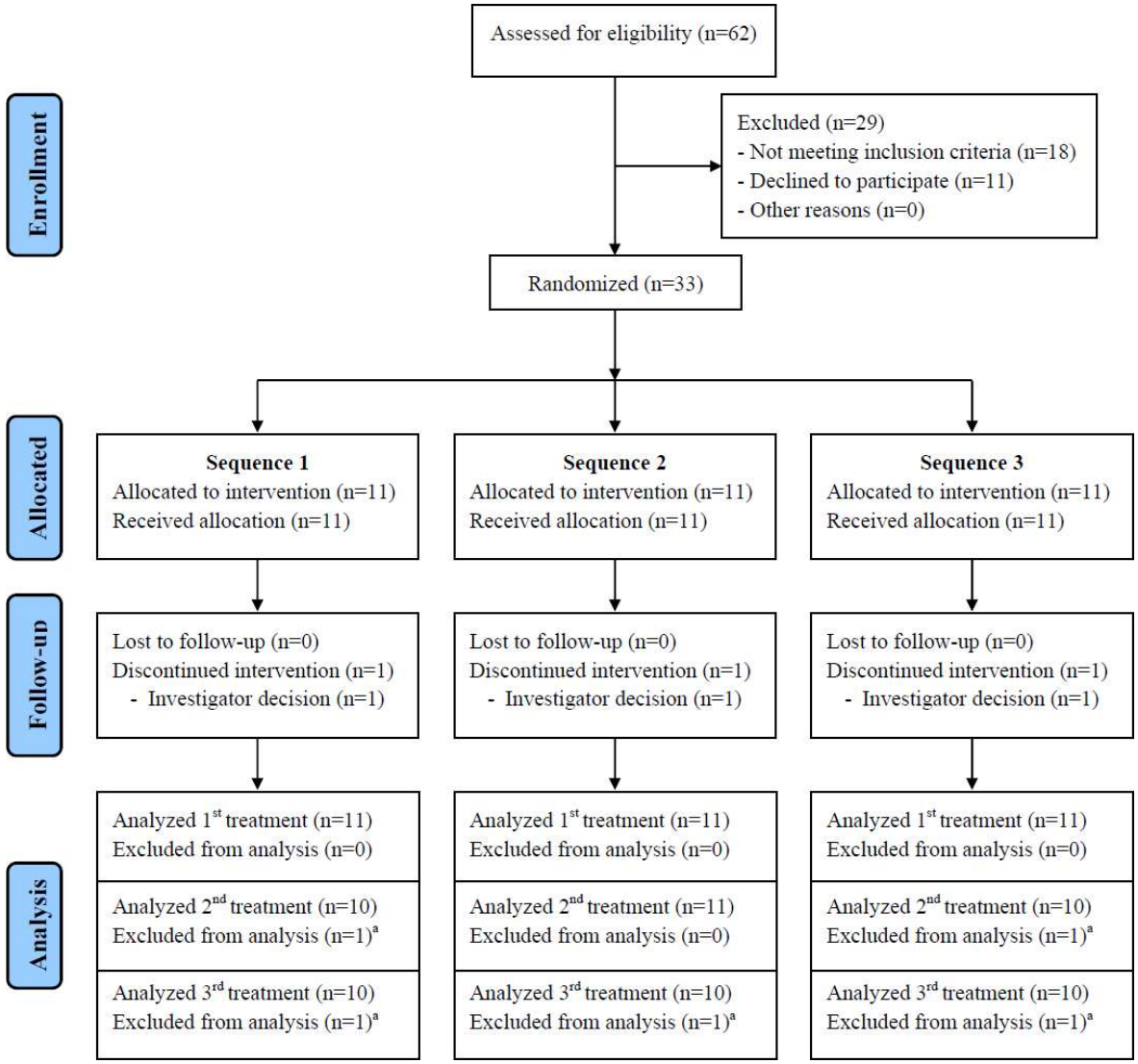


Figure 1 Supplemental material. Chemical characterization of VOHF-study olive oils.

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

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; 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.

Figure 2 Supplemental material. Flowchart of VOHF-study.



[a]Non-intervention

Table 1 Supplemental material. Apolipoprotein B and Albumin levels measured in HDL fraction.

	VOO		FVOO		FVOOT		
	Pre-intervention[a]	Post-intervention[a]	Pre-intervention[a]	Post-intervention[a]	Pre-intervention[a]	Post-intervention[a]	Inter-intervention p-value
Apolipoprotein B (g/L)	< 0.11	< 0.11	< 0.11	< 0.11	< 0.11	< 0.11	- - -
Albumin (g/L)	1.32 ± 0.85	1.22 ± 0.74	1.11 ± 0.50	1.10 ± 0.67	1.20 ± 0.62	1.29 ± 0.78	0.711 (VOO-FVOOT) 0.195 (FVOO-FVOOT) 0.404 (VOO-FVOO)

[a)] Values expressed as mean ± S.D.

Intra- and inter- intervention p-values were not significant.

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; HDL, high density lipoprotein; LDL, low density lipoprotein.

Table 2 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.

Table 3 Supplemental material. Lipid profile changes after the interventions.

	VOO		FVOO		FVOOT		
	Pre-intervention[a]	Post-intervention[a]	Pre-intervention[a]	Post-intervention[a]	Pre-intervention[a]	Post-intervention[a]	Inter-intervention p-value
Tryglicerides (mg/dL)	113 (93.75;137.5)	116.5 (94.25;145)	110 (78.75;150.5)	122 (71.25;155.75)	113 (83;149)	111 (91;148)	0.986 (VOO-FVOOT) 0.469 (FVOO-FVOOT) 0.660 (VOO-FVOO)
Total cholesterol (mg/dL)	217.33 ± 40.48	215.70 ± 33.01	221.21 ± 33.38	218.00 ± 33.23	214.33 ± 32.46	217.96 ± 35.07	0.828 (VOO-FVOOT) 0.405 (FVOO-FVOOT) 0.488 (VOO-FVOO)
HDL-cholesterol (mg/dL)	52.75 ± 11.22	52.07 ± 11.07	53.58 ± 12.51	53.37 ± 13.54	51.43 ± 11.84	52.81 ± 12.47	0.668 (VOO-FVOOT) 0.433 (FVOO-FVOOT) 0.650 (VOO-FVOO)
LDL-cholesterol (mg/dL)	139.43 ± 33.80	139.90 ± 30.04	144.13 ± 27.00	140.54 ± 24.44	138.86 ± 29.14	140.33 ± 27.95	0.836 (VOO-FVOOT) 0.406 (FVOO-FVOOT) 0.275 (VOO-FVOO)
Apolipoprotein A1 (g/L)	1.42 ± 0.24	1.45 ± 0.23	1.44 ± 0.24	1.42 ± 0.24	1.44 ± 0.25	1.44 ± 0.23	0.173 (VOO-FVOOT) 0.933 (FVOO-FVOOT) 0.211 (VOO-FVOO)
Apolipoprotein B (g/L)	1.09 ± 0.21	1.08 ± 0.19	1.12 ± 0.19	1.09 ± 0.18	1.07 ± 0.19	1.10 ± 0.20	0.497 (VOO-FVOOT) 0.186 (FVOO-FVOOT) 0.358 (VOO-FVOO)

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

Intra- and inter- intervention p-values were not significant.

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; HDL, high density lipoprotein; LDL, low density lipoprotein.