Complementary phenol-enriched olive oil improves HDL characteristics in hypercholesterolemic subjects. A randomised, double-blind, crossover, controlled trial. The VOHF study.

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Abbreviations:

ApoA-I: apolipoprotein A-I

ApoA-II: apolipoprotein A-II

ApoB100: apolipoprotein-B100

C: cholesterol

CETP: cholesteryl-esther transfer protein

CHD: coronary hearth disease

EC: esterified-cholesterol

FC: free-cholesterol

FVOO: functional virgin olive oil

FVOOT: functional virgin olive oil with thyme

GSH-Px: glutathione peroxidase

LCAT: lecithin-cholesterol acyltransferase

OO: olive oil

PA: physical activity

PAF-AH: platelet activating factor acetylhydrolase

PC: phenolic-compounds

PL: phospholipids

PON: paraoxonase/arylesterase

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 subclass, HDL composition, lecithin-cholesterol acyltransferase (LCAT), paraoxonase/arylesterase (PON).

Abstract

Scope: Consumption of olive oil (OO) phenolic-compounds (PC) has beneficial effects on lipid-profile. HDL functionality is currently considered to be a more important issue than its circulating quantity. Our aim was to assess whether functional virgin OOs (FVOOs), one enriched with its own PC (500 ppm;FVOO) and another with OOPC (250 ppm) plus additional complementary-PC from thyme (250 ppm) (total:500ppm;FVOOT), could improve HDL functionality-related properties versus a virgin OO-control (80 ppm;VOO).

Methods and results: In a randomized, double-blind, crossover, controlled trial, 33 hypercholesterolemic-volunteers received during 3-weeks 25 ml/day of: VOO, FVOO, and FVOOT. HDL-C increased 5.74% ($p<0.05$) versus its baseline after the FVOOT consumption in the participants without hypolipidemic-medication. We detected, after FVOOT, an increase of HDL_2-subclass (34.45,SD6.38) versus VOO (32.73,SD6.71). An increment of esterified-cholesterol/free-cholesterol and phospholipids/free-cholesterol in HDL were observed after FVOOT (1.73,SD 0.56;5.44,SD1.39) compared with VOO (1.53,SD 0.35;4.97,SD0.81) and FVOO (1.50,SD0.33;4.97,SD0.81). Accordingly, LCAT-mass increased after FVOOT (1228 ug/mL,SD130), compared with VOO (1160 ug/mL,SD144). An improvement in HDL oxidative-status was reflected after FVOOT versus its baseline, given an increment in the paraoxonase activity ($118 \cdot 10^3$ U/L,SD24).

Conclusions: FVOOT improves: HDL-subclass distribution and composition, and metabolism/antioxidant enzyme activities. FVOOT could be a useful dietary-tool in the management of high-cardiovascular risk patients.

1. Introduction

Olive oil (OO) phenolic-compounds (PC) exert anti-oxidant, anti-inflammatory properties, and chemoprotective activity in experimental studies [1]. Moreover, OOPC induce favourable changes of lipid-profile, improve endothelial function, modify the haemostasis, and have anti-thrombotic properties in humans [2-4]. Data from human studies show that OOPC are protective against risk factors for coronary heart disease (CHD), particularly in individuals with oxidative stress [5-7]. HDL cholesterol (HDL-C) levels are inversely and independently related with cardiovascular disease [8]. Low-levels of HDL-C are the most characteristic lipid feature in individuals with premature CHD [9]. Currently, pharmacological or natural agents which can increase HDL-C levels are being considered as key factors for future therapies [10]. Despite significant increases in HDL-C concentration through nicotinic acid, and cholesteryl-ester transport protein (CETP) inhibitor, these studies have been discontinued due to ineffectiveness or increased risk of mortality [11-13]. The unexpected association of torcetrapib, an agent that increases plasma HDL-C but also increased cardiovascular mortality, has led not only to the discontinuation of further trials involving this drug [13], but also to considering the functional quality of HDL as being a more important issue than its circulating quantity. In addition, Voight et al. recently reported in a mendelian-randomisation study that an increase in HDL-C concentration does not imply a reduction in the risk of suffering a myocardial infarction [14].

Results from the EUROLIVE study showed an increase of HDL-C levels, and a decrease in *in vivo* lipid oxidative damage, in a dose-dependent manner with the phenolic-content of the OO administered [15]. Furthermore, we provided for the first-time a first-level evidence of an HDL function enhancement by a virgin olive oil (VOO) in healthy humans [16]. In addition, our group recently reported that OOPC enhance cholesterol efflux-related genes in pre-/hypertensive subjects [17]. In this sense, Helal et al. reported in a linear, non-randomised, and non-controlled trial, that VOO consumption improved the capacity of HDL to mediate cholesterol efflux [18]. Moreover, some animal and humans studies have reported that OO has effects on some parameters related to HDL-functionality [19].

PC-rich foods, without increasing the amount of fat consumed, could have a dual action because antioxidants could also revert to pro-oxidants [20,21,22]. Functional food with complementary-antioxidants, according to their structure/activity relationship, could be a suitable option to obtain beneficial effects avoiding these harmful ones. Our aim was to test whether enriched VOOs (FVOOs;500 ppm), one enriched with its own PC (FVOO) and another with them plus additional complementary-PC from thyme (FVOOT), could improve properties related with a better low-cardiovascular risk HDL-profile, such as HDL size, metabolism, antioxidant status, and composition.

2. Materials and methods

2.1. OO preparation and characteristics.

VOO with a low-phenolic content (80 ppm) was used as a control condition and as a matrix of enrichment to prepare two functional OOs (FVOOs;500 ppm). Functional VOO (FVOO;500 ppm) was enriched with its own PCs by addition of a phenol extract obtained from freeze-dried olive cake. Functional virgin olive oil with thyme (FVOOT;500 ppm) 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. Hence, FVOOT contained 50 % of olive PC and 50 % of thyme phenolics (**Figure 1**). The phenolic compounds content is the main difference among the three olive oils, being the fatty acids and the fat soluble micronutrients very similar among them. The FVOO presents the highest amount of hydroxytyrosol derivatives whereas the FVOOT presents the highest one of flavonoids, lignans, and it is the only olive oil with detectable monoterpenes. The procedure to obtain the phenolic extracts and the enriched oils has been published [23]. For the wash-out period a common OO kindly provided by Borges Mediterranean Group was used. The total phenolic-content of OOs was determined by Folin-Ciocalteu method [24]. The phenolic-profile of the OOs was analyzed by ultra-performance liquid chromatography coupled to tandem mass spectrometry (UPLC/MS/MS) [25].

163

164 *2.2. Study design.*

165 The VOHF-study was randomized, double-blind, crossover, controlled trial with 33
166 hypercholesterolemic volunteers (total-cholesterol>200 mg/dL) (19 men), aged 35 to 80.
167 Exclusion criteria included the following: BMI>35 kg/m^2, smokers, athletes with high-physical
168 activity (PA) (>3000 kcal/day), diabetes, multiple allergies, intestinal diseases, or other disease or
169 condition that would worsen the adherence to the measurements or treatment. The study was
170 conducted at IMIM-Hospital del Mar Medical Research Institute (Spain) from April to September
171 2012.
172 Subjects were randomized to one of 3 orders of administration of raw OOs (1-VOO, 2-FVOO,3-
173 FVOOT): Sequence1-FVOO,FVOOT,VOO; Sequence2-FVOOT,VOO,FVOO; Sequence3-
174 VOO,FVOO,FVOOT. In the crossover design, intervention periods were of 3-weeks with an
175 ingestion of 25 mL/day raw OO distributed along meals and preceded by 2-week wash-out periods
176 with a common OO.
177 To avoid an excessive intake of antioxidants and PCs during the clinical trial period, participants
178 were advised to limit the consumption of rich-polyphenol food. PA was evaluated by a PA-
179 Questionnaire at the baseline and at the end of the study. A set of portable containers with the
180 corresponding 25 ml of OO for each day of consumption were delivered to the participants at the
181 beginning of each OO administration period. The participants were instructed to return the
182 containers to the centre after the corresponding OO consumption period in order to register the
183 amount of OO consumed in the period. Subjects with less than 80 % of treatment adherence (≥5
184 full OO containers returned) were considered non-compliant volunteers for this treatment.
185 The present clinical trial was conducted in accordance with the Helsinki Declaration and the Good
186 Clinical Practice for Trials on Medical Products in the European Community. All participants
187 provided written informed consent, and the local institutional ethics committees approved the
188 protocol (CEIC-IMAS 2009/3347/I). The protocol is registered with the International Standard
189 Randomized Controlled Trial register (www.controlled-trials.com;ISRCTN77500181) and followed
190 CONSORT-guidelines.

2.3. Dietary adherence.

24 h-urine was collected at the start of the study and before and after each treatment. Urine samples were stored at -80 ºC prior to use. We measured urinary hydroxytyrosol-sulphate and thymol-sulphate, 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) [26]. 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 rich-polyphenol food consumption.

2.4. Systemic biomarkers analyses.

Blood samples were collected in fasting state at least of 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 -80 ºC until use. EDTA-plasma glucose, total-cholesterol (TC), and triglyceride (TG) levels were measured 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 as a soluble HDL-C determined by an accelerator selective detergent method (ABX-Horiba Diagnostics,Montpellier,France). LDL-C was calculated by the Friedewald equation whenever TGs were less than 300 mg/dL. EDTA-plasma glutathione peroxidase activity (GSH-Px) was assessed through glutathione oxidation-reduction measured by a Paglia and Valentine method modification using cumene hydroperoxide as oxidant of glutathione (Ransel RS 505,Randox Laboratories,Crumlin,UK) [27]. Enzymatic activity of cholesteryl-ester transfer protein CETP (MBL,Woburn,USA) and mass concentration of lecithin-cholesterol acyl-transferase (LCAT) (American diagnostica GmbH,Pfungstadt,German) were analyzed in serum by fluorimetric kits. Platelet activating factor acetylhydrolase activity (PAF-AH) was measured in serum (Cayman chemical,Ann Arbor,Michigan). Finally, paraoxonase/arylesterase（PON) activity was determined in serum through the measurement of the capacity to cleavage of phenyl acetate resulting in

phenol formation (Zeptometrix Corporation,Buffalo,NY). A fluorimetric methodology based on the oxidation of HDL particle in the presence of dihydrorhodamine 123 (DHR) by measuring increasing fluorescence due to the production of reactive oxygen species over time was performed according to previous work [28]. The products of redox cycling are detected as time-dependent oxidation of the fluorogenic probe dihydrorhodamine 123 (DHR) to fluorescent rhodamine 123. The rate of DHR oxidation in the presence of HDL reflects the antioxidant activity of the particle.

2.5. HDL subclass distribution and composition analyses.

HDL-subclass distribution was measured in plasma by the Lipoprint-HDL System (Quantimetrix,Manhattan beach,California), in which following electrophoresis, lower-density lipoproteins (VLDL,LDL) remain at the beginning of the band (Rf=0.0) and the albumin moves to the front (Rf=1.0). Between these two points, we were able to find up to nine HDL bands with intermediate Rf values. The first three bands corresponded to a large-HDL subclass (HDL_2), and 4^{th} to 9^{th} bands corresponded to a small-HDL subclass (HDL_3). Taking all this into account, we calculated both proportions of HDL_2 and HDL_3 subtypes, as previously described [29]. HDL were isolated by a density gradient ultracentrifugation method [30] using preparation 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 substracting free-cholesterol from total-cholesterol. 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,Montpellier,France, and Spinreact,Barcelona,Spain, repectively). For assuring the purity of HDL fractions, ApoB100 and albumin levels were also determined in these samples by automatic immunoturbidimetric methods (ABX-Horiba Diagnostics,Montpellier,France).

2.6. APOE genotyping.

DNA was isolated from the buffy coat of blood collected into EDTA tubes using a standardized method (FlexiGene DNA Kit; Qiagen). Allelic discrimination of the APOE gene variants was performed with TaqMan PCR technology (QuantStudio™ 12K Instrument; Applied Biosystems) and Assay-on-Demand single nucleotide polymorphism genotyping assays (Applied Biosystems). The APOE haplotypes (E2/E2, E2/E3, E2/E4, E3/E3, E3/E4, and E4/E4) were determined from the alleles for the APOE single nucleotide polymorphisms rs7412 and rs429358.

2.7. 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).

2.8. Statistical analyses.

Normality of continuous variables was assessed by normal probability plots. Non-normally distributed variables were log transformed if it was necessary. Non-compliance volunteers were excluded from the analysis. To compare means (for normal distributed variables) or medians (for non-normal distributed variables) among groups, ANOVA or Kruskal-Wallis test were performed, respectively; whereas χ^2 and exact F-test, as appropiate, were computed to compare proportions. Pearson and Spearman correlation analyses were used to evaluate relationships among variables. Linear regression models were used to adjust post-intervention values for pre-intervention values. A general linear model for repeated measurements was used to assess the effect of intra- and inter-interventions. To check whether APOE4 carrier genotype modified the results, we tested the interaction of this variable (as between individual factor) with the treatment effect defined as the post-treatment value adjusted with its pre-treatment value (as within individual factor) in a GLM for repeated measurements. When interaction p-value was borderline or significant with a study variable, this variable was analysed without APOE4 carrier participants in GLM for repeated measurements. A value of $p<0.05$ was considered significant. Carry-over

273 effect was discarded in all variables. Statistical analyses were performed by SPSS13.0 software
274 (IBN Corp) and R2.12.0 software (R Development Core Team).
275
276 ## 3. Results
277
278 *3.1. Participant characteristics and dietary adherence.*
279 From 62 subjects who were assessed for eligibility, 29 were excluded. Finally, 33 eligible
280 participants (19 men, 14 women) entered the study. A discontinued single intervention was
281 occurred in three volunteers for the investigator decision. We could not identify any adverse effects
282 related to OO intake. Participants' baseline characteristics are shown in **Table 1**, no significant
283 differences exist between orders. No changes in daily energy expenditure in leisure-time PA were
284 observed from the beginning to the end of the study. No changes were observed in the main
285 nutrients (**Table 2**) and medication intake throughout the study, and participants' compliance was
286 good as reflected in the urinary phenols after OO interventions (**Table 3**).
287
288 *3.2. Systemic biomarkers.*
289 No changes were observed in blood pressure and BMI throughout the study. Although no changes
290 were observed in lipid profile in the entire sample, when the analyses were performed in the
291 participants without hypolipidemic medication, HDL-C increased 5.74 % ($p<0.05$) after FVOOT
292 consumption versus its baseline.
293 Changes in enzymes related with HDL metabolism and antioxidant status are shown in **Table 4**. An
294 improvement of LCAT activity was observed after FVOOT intervention compared with VOO
295 ($p=0.020$), while a borderline decrement was observed after VOO intake versus its baseline
296 ($p<0.09$). A raise in PON was observed after FVOOT consumption versus its baseline ($p<0.05$),
297 while a decrement reaching a borderline significance ($p<0.09$) was observed after VOO intake
298 versus its baseline. A borderline improvement of CETP activity was observed after FVOO
299 intervention compared with VOO ($p=0.089$). No changes were observed in PAF-AH activity. With

regard the DHR test, there were no significant changes in the intra- and inter-treatment analysis. In addition, an inverse correlation between the induced oxidation rate of DHR and the activity of the GSH-PX was observed after VOO and FVOOT treatments (rVOO=-0.49, rFVOOT=-0.57; $p<0.05$).

3.3. HDL subclass distribution and composition.

After the FVOOT intervention a rise in the HDL_2-subclass was observed ($p<0.05$). This increase was significant versus changes in VOO ($p=0.047$). HDL_3-subclass decreased after FVOOT ($p<0.05$) (**Figure 2**). Moreover, after FVOOT intervention an increment in HDL_2/HDL_3 was observed ($p<0.05$).

An increment in HDL EC/FC was observed after FVOOT consumption compared with VOO ($p=0.029$) and with FVOO ($p=0.007$). Also, an increment in HDL PL/FC was observed after the FVOOT compared with VOO ($p=0.028$) and compared with FVOO ($p=0.027$). No changes were observed in TC, TG, Apo-AI, Apo-AII, FC, EC, and PL in HDL after interventions. Nevertheless, a borderline decrease in HDL FC/TC after FVOOT versus VOO ($p=0.056$) and versus FVOO ($p=0.067$) was observed. Moreover, a borderline increase in HDL EC/TC after FVOOT versus VOO ($p=0.056$) and versus FVOO ($p=0.067$) was observed (**Table 5**).

Analyses after OOs intake of HDL-subclass distribution showed cross-linked correlations with HDL-composition and with HDL-metabolism enzymes. The increase in HDL_2/HDL_3 directly correlated with ApoA-I after OOs intake (rVOO=0.41, rFVOO=0.48, rFVOOT=0.44; $p<0.05$). The increase in HDL_2/HDL_3 directly correlated with the decrease of CETP after VOO ($r=-0.39$;$p<0.05$) and borderline correlated with the decrease of CETP after FVOO ($r=-0.30$;$p<0.09$).

3.4. APOE genotype.

66.67 % of the participants have APOE3/E3 genotype, 15.15 % have APOE2/E3 genotype, 15.15 % of volunteers have APOE3/E4 genotype, and 3.03 % have APOE4/E4 genotype. In the analyses of possible interaction among the studied variables and APOE4 carrier variable, only a

327 borderline interaction with HDL EC/FC, HDL PL/FC, and PAF-AH activity was observed ($p<0.09$).
328 When these variables were analysed without APOE4 carrier volunteers, similar results as entire
329 sample were obtained, except for HDL PL/FC increment after FVOOT consumption versus FVOO
330 one, which didn't reach significance ($p=0.053$).
331
332 **4. Discussion**
333
334 We performed a randomised, double-blind, cross-over, controlled trial with a VOO, an OO
335 enriched with its own PCs, and another with them plus complementary ones from thyme. From
336 our results, VOO enriched with its PC plus those of thyme improved parameters related with HDL
337 functional profile, such as: HDL-subclass distribution, HDL-composition, and enzymes related with
338 HDL-metabolism and antioxidant status. The additional benefits achieved with complementary
339 phenol-enriched OO consumption on the HDL functional profile in hypercholesterolemic
340 volunteers have been demonstrated with the highest degree of evidence, for the first time.
341 Moreover, such benefits can be obtained without increasing the individual's fat intake.
342
343 LCAT is an enzyme that mediates the esterification of the FC of nascent HDL monolayers to EC.
344 As a result, this hydrophobic EC move into the centre of the particles [31]. The cholesterol
345 esterification by LCAT mediates the gradual conversion of nascent discoidal HDL into small
346 spherical HDL (HDL_3) with two ApoA-I molecules/particle and, finally, into large spherical HDL
347 particles (HDL_2) that contain three ApoaA-I molecules [32,33]. Another enzyme intimately related
348 with the HDL-metabolism is CETP which extracts EC from the HDL core to TG-rich lipoproteins,
349 returning triglycerides from TG-rich lipoproteins to HDL [34]. These EC-poor-TG-rich particles are
350 substrates for hepatic lipase which hydrolyses the TGs. Hepatic lipase depletes the particles of core
351 lipids, generating a redundancy of surface constituents. A TG-poor HDL core may imply better
352 functional properties of the particle. When the HDL core is TG-rich, ApoA-I is more loosely bound to
353 the HDL surface [35]. Accordingly, after the intervention with FVOOT, which increases LCAT

activity, we observed an increase of HDL_2-particle subclass percentage and a decrease of the HDL_3 one, while after VOO intervention we detected a decrease of LCAT activity and of HDL_2-particle subclass percentage. Moreover, the increase of HDL_2/HDL_3 directly correlated with the increase of ApoA-I after the three interventions. In addition, an increase of CETP activity inversely correlated HDL_2/HDL_3 after VOO and FVOO intake. Numerous population studies have suggested that HDL_2-particles may be more cardioprotective than HDL_3 [36,37]. Low-levels of HDL_2 and/or high-levels of HDL_3 are present in CHD [38], ischemic stroke [39], type-II diabetes mellitus [40], and peripheral arterial disease patients [41]. Although there are also a number of *in vitro* studies that reveal that HDL_3 has some similar effects to HDL_2 [42], increased small HDL in serum may indicate an aberration in the maturation of HDL and further impaired reverse cholesterol transport (RCT) [43,44]. HDL_2-particles moreover bind better to SR-B1 [45], thus they are more effective in promoting cholesterol efflux via this receptor [46]. A similar antioxidant status between HDL_2 and HDL_3 has been described [47-49], nevertheless in our study, an increase of HDL_2-subclass and an increase of antioxidant enzyme activities were observed after FVOOT intake.

An HDL with an increase of EC/FC, and an HDL monolayer with more PL/FC, were observed after FVOOT consumption which also augmented LCAT activity. Hernáez et al. reported that VOO improves the fluidity of the HDL monolayer and the cholesterol efflux in a randomized, crossover, double-blind, controlled trial with healthy individuals [16]. Cholesterol efflux from lipid-loaded cells is a key athero-protective event that counteracts cholesterol uptake. The imbalance between cholesterol efflux and uptake determines the prevention or development of atherosclerosis. Four pathways for cholesterol efflux from cells to plasma are described: 1) aqueous diffusion-mediated, 2) the SR-BI-mediated, 3) the ABCA1-mediated, and 4) the ABCG1-mediated [50-53]. The aqueous diffusion one, 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 proportion of FC and PL content in the monolayer of donor and acceptor particles [50]. According to this, particles with more PL/FC, such as that which we obtained after FVOOT consumption, could be more efficient in enhancing

aqueous diffusion cholesterol efflux. Furthermore, other mechanisms can increase the cholesterol efflux mediated by other transporters. Viksdet R et al. described that the active form of PLTP can increase the cholesterol efflux from macrophage foam cells and that underlying mechanism involves PLTP-mediated HDL conversion into preß-HDL and large fused HDL particles, both of which are efficient acceptors for cellular cholesterol. These results suggested that an active form of PLTP could enhance ABCA-1 and ABCG-1 mediated efflux [54].

The antioxidant properties of OOPC *in vivo* are well-known. The EUROLIVE study showed a decrease *in vivo* in lipid oxidative damage in a dose-dependent manner with the phenolic-content of the OO administered [2]. Since an increment in HDL-C after a high polyphenol-olive oil intake was reported in healthy volunteers, a rise in HDL-C was expected. Accordingly an increment in the HDL-C was observed in the subsample of volunteers without hypolipidemic medication, although no changes in lipid profile were appreciated when all volunteers were included in the analysis. In 2011 the European Food Safety Authority recognized the PC-rich OO effects on protecting LDL from oxidation [55]. The PC acquired through diet can also directly or indirectly protect the HDL antioxidative status. An improvement of antioxidant status, reflected in an increase of PON and LCAT activities after the FVOOT intervention, was observed in the present study. In contrast, PON decreased after the FVOO intervention. PON1 which is associated to HDL exerts a protective effect against the oxidative damage of cells and lipoproteins. It also modulates the susceptibility of HDL and LDL to atherogenic modifications such as homocysteinylation [56]. A less proinflamatory and oxidized-HDL could be more efficient in its pleotropic function. In addition, LCAT is an enzyme related to HDL antioxidant activity and prevents the oxidation of LDL [57]. Hydroxytyrosol and the main phenols of the VOO, the secoiridoid group, have been described as acting in a similar manner to phenolic acids, inhibiting the lipid oxidation by trapping free and peroxy radicals. Moreover flavonoids, the main antioxidants of thyme, also help to control the extent of lipid peroxidation by chelating metal ions [58]. Furthermore, the enrichment of VOOs with hydroxytyrosol derivatives combined with complementary-phenols from aromatic herbs, such as thyme used for oil flavoring, might be a good strategy to provide the optimum balance amongst

410 the different kinds of OOPC such as flavonoids, monoterpenes, and phenolic acids [23] (**Figure**
411 **1**). In this sense, some non-human studies with flavonoid enriched olive oil have been performed.
412 Rosenblat et al. published that an extra VOO enriched with green tea polyphenols (mostly
413 epicatechin gallate) attenuates atherosclerosis development enhancing macrophage cholesterol
414 efflux in Apo-E-deficient mice more than an extra VOO. These olive oils also showed a decrease
415 in the serum oxidative stress in Apo-E-deficient mice compared with placebo treatment [59].
416 Recently, a study has reported that an OO-pomegranate sauce prolongs lipid oxidation and can
417 retard undesirable quality changes in anchovy [60].
418
419 One strength of our study is its crossover and randomised design, which permitted the
420 participants to consume all OO types and thus eliminated the inter-individual variability. Moreover,
421 the laboratory analyses were centralized and all the time-series samples from the same volunteer
422 were measured in the same run. It lacks a related-HDL oxidative status parameter to stand up the
423 association between the phenolic compound-enriched olive oil and the functionality HDL-related
424 characteristics. A limitation of the study was its sample size, which could be responsible for
425 reduced statistical power in some biomarkers with high inter-individual variability. A synergistic
426 effect on HDL-parameters between PC and other OO components is as yet unknown. Another
427 limitation is the inability to assess potential interactions among the OOs and other diet components
428 and medication, although the controlled diet and medication followed during all clinical trial should
429 have limited the scope of these interactions.
430
431 In conclusion, long-term consumption of complementary phenol-enriched OO induced changes in
432 the HDL-profile associated with low-cardiovascular risk, such as higher levels of large HDLs,
433 lower levels of small ones, increased HDL EC/FC content, increased HDL-monolayer PL/FC, and
434 increased HDL-antioxidant enzymes. OO, a recognized healthy food, cannot be consumed in large
435 quantities. Thus, the enrichment of OO with its PCs is a way of increasing its healthy properties
436 whilst the same amount of fat is consumed. Moreover, these results show that an enrichment of OO
437 with complementary antioxidants, according to their structure/activity relationship, promotes more

benefits than an enrichment of OO with only its own phenolics. Our data suggest that a complementary phenol-enriched OO could be a good nutraceutical to enhance the functionality of HDL-particles, and thus a complementary tool for the management of cardiovascular high-risk individuals.

Concluding remarks

The additional benefits achieved with complementary phenol-enriched OO consumption on the HDL functional profile in hypercholesterolemic volunteers have been demonstrated with the highest degree of evidence, for the first time. Moreover, such benefits can be obtained without increasing the individual's fat intake. These findings suggest that FVOOT could be a useful dietary tool in the management of high-cardiovascular risk patients.

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Conflict of interest statement

The authors have no financial conflicting interests to disclose.

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Figure legends

Figure 1. 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. HDL subclass distribution after interventions.

Values represent post-intervention mean ± SD.

Post-intervention values were adjusted with its pre-intervention ones.

$^{\#}$: Intra-intervention p-value<0.05; *: Inter-intervention p-value<0.05 compared with VOO intervention.

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.

Table 1. Baseline characteristics of the participants.

	Order 1[a] (n=11)	Order 2[a] (n=11)	Order 3[a] (n=11)	Total[a] (n=33)	p-value
GENERAL					
Sex: man	5 (45.6 %)	7 (63.6 %)	7 (63.6 %)	19 (57.6 %)	0.742
Age	54,91 ± 12,57	55,27 ± 11,88	55,45 ± 7,84	55.21 ± 10.62	0.856
BMI (kg/m^2)	25.63 ± 3.68	26.31 ± 5.25	27.85 ± 4.71	26.64 ± 4.54	0.529
Hypolipidemic medication: no	7 (63.6 %)	9 (81.8 %)	3 (27.3 %)	19 (57.6 %)	0.047
Physical activity (kcal/week)	3498.75 (1755.00;8092.50)	1188.75 (742.50;1687.50)	3322.50 (861.25;3663.75)	2423.25 (897.38;4543.75)	0.094
Diastolic blood pressure (mmHg)	68.09 ± 13.53	72.27 ± 9.31	71.91 ± 13.43	70.76 ± 12.01	0.678
Sistolic blood pressure (mmHg)	125.09 ± 18.70	128.27 ± 16.69	130.45 ± 17.93	127.94 ± 17.37	0.778
SYSTEMIC LIPID PROFILE AND GLYCAEMIA					
Total-cholesterol (mg/dL)	228 ± 43	232 ± 33	219 ± 31	226 ± 35	0.680
Triglycerides (mg/dL)	94 (75;149)	119 (95;168)	117 (81;126)	114 (85;145)	0.517
Glucose (mg/dL)	89 ± 12	93 ± 13	91 ± 11	91 ± 12	0.683
HDL-cholesterol (mg/dL)	53 ± 13	53 ± 13	53 ± 20	53 ± 11	0.992
LDL-cholesterol (mg/dL)	150 ± 32	152 ± 28	142 ± 26	148 ± 28	0.700
ApoA-I (g/L)	1.4 ± 0.2	1.4 ± 0.2	1.5 ± 0.2	1.4 ± 0.2	0.458
Apolipoprotein-B100 (g/L)	1.2 ± 0.2	1.2 ± 0.2	1.1 ± 0.2	1.1 ± 0.2	0.529

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

Table 2. Main nutrient diet changes.

	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
Energy, kcal	1975.07 ± 424.42	1882.66 ± 482.78	1916.48 ± 555.76	1913.81 ± 394.38	2019.96 ± 463.32	1899.11 ± 547.85	0.865 (VOO-FVOOT) 0.688 (FVOO-FVOOT) 0.559 (VOO-FVOO)
Carbohydrate, %	41.13 ± 5.01	42.38 ± 7.61	43.93 ± 9.20	42.61 ± 6.51	40.89 ± 6,19	42.55 ± 7.41	0.935 (VOO-FVOOT) 0.252 (FVOO-FVOOT) 0.293 (VOO-FVOO)
Protein, %	18.45 ± 3.68	19.80 ± 4.53	19.81 ± 4.49	19.76 ± 3.93	19.58 ± 4.60	19.72 ± 4.24	0.417 (VOO-FVOOT) 0.846 (FVOO-FVOOT) 0.380 (VOO-FVOO)
Total fat, %	41.12 ± 7,33	38.68 ± 8.49	38.29 ± 12.47	37.55 ± 7.70	41.55 ± 7.35	38.35 ± 7.52	0.823 (VOO-FVOOT) 0.542 (FVOO-FVOOT) 0.435 (VOO-FVOO)
Saturated fat, %	12.41 ± 3.88	11.33 ± 4.00	10.78 ± 2,98	11.60 ± 4.98	12.21 ± 3.06	10.71 ± 3.40	0.750 (VOO-FVOOT) 0.124 (FVOO-FVOOT) 0.087 (VOO-FVOO)
Monounsaturated fat, %	17.74 ± 3.19	16.23 ± 3.91	16.13 ± 5.31	16.22 ± 4.47	16.56 ± 3.73	16.09 ± 3.42	0.303 (VOO-FVOOT) 0.765 (FVOO-FVOOT) 0.135 (VOO-FVOO)
Polyunsaturated fat, %	4.84 ± 1.14	4.54 ± 0.88	4.88 ± 1.45	4.78 ± 1.08	4.69 ± 1.61	4.90 ± 1.32	0.231 (VOO-FVOOT) 0.388 (FVOO-FVOOT) 0.654 (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.

Table 3. Main compliance phenol markers changes.

	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**
Hydroxytyrosol sulphate (µmoles/24 hours-urine)	6.13 (3.66; 14.67)	5.50 (2.57;11.71)	6.11 (2.97;9.95)	11.09 (7.63;20.86)[b]	6.35 (3.57; 13.43)	9.08 (4.43;12.61)	0.402 (VOO-FVOOT) 0.328 (FVOO-FVOOT) 0.013 (VOO-FVOO)[d]
Thymol sulphate (µmoles/24 hours-urine)	38.89 (11.94;81.21)	30.93 (7.28;90.33)	59.16 (11.64;92.00)	36.81 (9.37;72.06)	83.53 (10.43; 108)	455 (120;880)[c]	0.000 (VOO-FVOOT)[d] 0.000 (FVOO-FVOOT)[d] 0.339 (VOO-FVOO)

[a)]Values expressed as median (25th to 75th percentile).

[b)]p-value for intra-intervention: p<0.05; [c)]p-value for intra-intervention: p<0.001; [d)]p-value for inter-intervention: p<0.001;

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.

716 **Table 4. Catalytic activities and concentration of enzymes related with HDL metabolism and antioxidant status after**
717 **interventions.**
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	Baseline[a]	Post-VOO intervention[a,b]	Post-FVOO intervention[a,b]	Post-FVOOT intervention[a,b]	Inter-intervention p-value
Paroxonase-arylesterase (10^3 U/L)	119 ± 30	116 ± 26	115 ± 25	118 ± 24[c]	0.287 (VOO-FVOOT) 0.174 (FVOO-FVOOT) 0.587 (VOO-FVOO)
Platelet activating factor acetylhydrolase (10^{-3} U/L)	15264 ± 3498	15321 ± 2874	15345 ± 3030	15239 ± 2374	0.861 (VOO-FVOOT) 0.821 (FVOO-FVOOT) 0.932 (VOO-FVOO)
Lecithin-cholesterol acyltransferase (ug/ml)	1161 ± 210	1160 ± 144	1202 ± 195	1228 ± 130	0.020 (VOO-FVOOT)[d] 0.341 (FVOO-FVOOT) 0.098 (VOO-FVOO)
Cholesteryl-ester transfer protein (10^3 U/L)	26117 ± 5983	25617 ± 3333	26717 ± 5350	26667 ± 4767	0.159 (VOO-FVOOT) 0.955 (FVOO-FVOOT) 0.089 (VOO-FVOO)
Glutathione peroxidase (U/L)	723 ± 105	703 ± 77	709 ± 79	717 ± 94	0.151 (VOO-FVOOT) 0.513 (FVOO-FVOOT) 0.584 (VOO-FVOO)
DHR Oxidation rate (Fluorescence units/s)	4.07 ± 1.47	4.07 ± 1.34	3.95 ± 1.37	4.21 ± 1.26	0.228 (VOO-FVOOT) 0.104 (FVOO-FVOOT) 0.494 (VOO-FVOO)

719
720 [a)]Values expressed as post-intervention mean ± S.D.
721 [b)]Post-intervention was adjusted by its pre-intervention.
722 [c)]p-value for intra-intervention: $p<0.05$; [d)]p-value for inter-intervention: $p<0.05$;
723 Abbreviations: VOO, virgin olive oil; FVOO, functional VOO enriched with its own phenolic-compounds; FVOOT, functional VOO enriched
724 with its own phenolic-compounds plus additional complementary ones from thyme; DHR, dihydrorhodamine 123.
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726

Table 5. HDL composition after interventions.

	Baseline[a]	Post-VOO intervention[a,b]	Post-FVOO intervention[a,b]	Post-FVOOT intervention[a,b]	Inter-Intervention p-value
HDL Total-cholesterol (mg/dL)	31 ± 11	31 ± 11	32 ± 11	31 ± 11	0.893 (VOO-FVOOT) 0.478 (FVOO-FVOOT) 0.445 (VOO-FVOO)
HDL Triglycerides (mg/dL)	7.48 ± 2.44	7.29 ± 1.19	7.25 ± 1.36	7.17 ± 1.46	0.628 (VOO-FVOOT) 0.749 (FVOO-FVOOT) 0.882 (VOO-FVOO)
HDL Apolipoprotein A-I (mg/dL)	66 ± 15	65 ± 11	66 ± 12	64 ± 14	0.271 (VOO-FVOOT) 0.150 (FVOO-FVOOT) 0.510 (VOO-FVOO)
HDL Apolipoprotein A-II (mg/dL)	16.85 ± 3.64	16.91 ± 2.20	17.61 ± 3.10	16.80 ± 3.38	0.818 (VOO-FVOOT) 0.152 (FVOO-FVOOT) 0.158 (VOO-FVOO)
HDL Free-cholesterol (mg/dL)	11.81 ± 5.38	12.98 ± 4.47	13.61 ± 5.90	12.53 ± 5.42	0.381 (VOO-FVOOT) 0.107 (FVOO-FVOOT) 0.303 (VOO-FVOO)
HDL Esterified-cholesterol (mg/dL)	19.49 ± 8.05	18.18 ± 6.30	18.25 ± 5.78	18.56 ± 7.36	0.624 (VOO-FVOOT) 0.625 (FVOO-FVOOT) 0.909 (VOO-FVOO)
HDL Phospholipids (mg/dL)	60.28 ± 17.04	59.73 ± 16.26	61.45 ± 14.89	59.49 ± 15.88	0.883 (VOO-FVOOT) 0.357 (FVOO-FVOOT) 0.395 (VOO-FVOO)
HDL Free-cholesterol/Total-cholesterol	0.38 ± 0.12	0.43 ± 0.06	0.42 ± 0.08	0.40 ± 0.08	0.056 (VOO-FVOOT) 0.063 (FVOO-FVOOT) 0.898 (VOO-FVOO)
HDL Esterified-cholesterol/Total-cholesterol	0.62 ± 0.12	0.57 ± 0.06	0.58 ± 0.08	0.60 ± 0.08	0.056 (VOO-FVOOT) 0.063 (FVOO-FVOOT) 0.898 (VOO-FVOO)
HDL Phospholipids/Free-cholesterol	5.39 ± 1.68	4.97 ± 0.81	4.99 ± 0.89	5.44 ± 1.39	0.028 (VOO-FVOOT)[c] 0.027 (FVOO-FVOOT)[c] 0.900 (VOO-FVOO)
HDL Esterified-cholesterol/Free-cholesterol	1.74 ± 0.77	1.53 ± 0.35	1.50 ± 0.33	1.73 ± 0.56	0.029 (VOO-FVOOT)[c] 0.007 (FVOO-FVOOT)[c] 0.604 (VOO-FVOO)

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

[b)]Post-intervention was adjusted by its pre-intervention.

Intra-intervention p-values were not significant. [c)]Inter-intervention p-value: $p<0.05$;

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.