Virgin olive oil enriched with its own phenolic compounds or complemented with thyme improves endothelial function: the potential role of plasmatic fat-soluble vitamins. A double blind, randomized, controlled, cross-over clinical trial.

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Abbreviations: 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; 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

The aim of the present study was to assess whether different functional virgin olive oils (FVOOs) with varying phenolic compounds (PC) could protect the plasmatic fat-soluble vitamins, which in turn could improve the endothelial function. In order to select the optimal phenolic dose in the improvement of ischemic reactive hyperemia (IRH), a dose-response study (n=12, healthy subjects) was performed and the enrichment of 500mg PC/kg oil was selected. In a 3-week cross-over sustained study (n=33 hypercholesterolemic subjects), the consumption of 25mL/day of two phenol-enriched olive oils (one enriched with its own PC and another combined with thyme PC) increased IRH and plasma concentrations of retinol, β-cryptoxanthin and α-tocopherol, compared to a control virgin olive oil. A positive post-intervention correlation was observed for IRH values and HDL-c, β-cryptoxanthin, lutein and α-tocopherol. Results suggest that preservation of plasmatic fat-soluble vitamins by PC from FVOOS could partially explain the endothelial function benefits.

Clinical Trial Registration: NCT01347515 and ISRCTN77500181.

Key words: endothelial function, fat-soluble vitamins, olive oil, phenol enrichment

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 (Libby, Ridker, & Hansson, 2011). Some studies have suggested that dietary bioactive compounds, such as fat-soluble vitamins and phenolic compounds (PC), could have beneficial effects on endothelial function through multiple complex mechanisms including inhibition of monocyte adhesion, platelet activation, increased nitric-oxide (NOx) production and improvement of vasodilatation (Brown & Hu, 2001; Sijtsma et al., 2014). 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 endothelial function (Storniolo, Roselló-Catafau, Pintó, Mitjavila, & Moreno, 2014). The consumption of a VOO with a high PC content has been related with an improvement of endothelial-dependent vasomotor function measured as ischemic reactive hyperemia (IRH) in humans in acute (Ruano et al., 2005; Valls et al., 2015) and sustained studies (Moreno-Luna et al., 2012). Thus, the enrichment of VOO with its own PC has been proposed as a possible approach to raise the PC consumption without increasing caloric intake (Rubió et al., 2012a; Suárez et al., 2011). Nevertheless, the enrichment of VOO with its own PC (hydroxytyrosol and its derivatives, commonly named secoiridoids) could lead to its organoleptic taste being rejected by consumers due to their high pungent and bitter attributes. Therefore, the enrichment of a VOO by complementing its own PC with thyme PC (flavonoids, phenolic acids and monoterpenes) was proposed as a novel approach to improve its organoleptic characteristics and the possible additional benefits on endothelial

function. Although our previous study demonstrated that the phenol-enriched VOO combining its own PC and thyme PC was better rated in the acceptance sensory test compared to the one enriched only with its own PC (Rubió, Farràs, et al., 2014a), their possible beneficial effects on endothelial function remain unknown.

In terms of bioavailability, a previous study in rats has shown that when olive PC were combined with thyme PC, an enhanced bioavailability of olive PC occurred (Rubió, Serra, et al., 2014). In agreement with these findings, when the volunteers from the VOHF (Virgin Olive and HDL Functionality) Project ingested VOO enriched with its own PC plus complementary PC from thyme, an slight enhance on bioavailability of olive PC was also observed (Rubió, Farràs, et al., 2014a). The combination of different PC sources might, therefore be a promising approach to improve not only the bioavailability but also a consequent enhancement of their biological effects.

Dietary PC are consumed together with other dietary antioxidants such as fat-soluble vitamins (carotenoids and tocopherols) which have been related to a protecting role against vascular dysfunction both in human and *in vitro* studies (Catalán et al., 2012; Kim et al., 2011). Moreover, 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 (Marin et al., 2011) suggesting that the interaction between PC and systemic fat-soluble vitamins could influence the endothelial function improvement.

In this context, the main aim of this study was to determine whether diets supplemented with PC from VOO alone or mixed with PC from thyme could protect the fat-soluble

vitamins in plasma, which in turn could improve the endothelial function in hypercholesterolemic subjects.

2. Materials and methods

2.1 Olive oils preparation and characterization

For the dose-response study, 3 functional phenol-enriched virgin olive oils (FVOO) were prepared: L-FVOO with a low phenolic content (250 mg total phenols/kg of oil), M-FVOO with a medium phenolic content (500 mg total phenols/kg of oil) and H-FVOO with a high phenolic content (750 mg total phenols/kg of oil). They were prepared by the addition of an extract obtained from freeze-dried olive cake rich in the main olive oil PC to a VOO with low phenolic content (80 mg total phenols/kg of oil) used as enrichment matrix. The process to prepare the FVOOs was previously described (Rubió et al., 2012b).

In the sustained study, the same VOO with a low phenolic content (80 mg total phenols/kg of oil) was used as a control condition and as an enrichment matrix for the preparation of two FVOO different in phenolic composition but with the same total amount of PC (500 mg total phenols/kg of oil). The first FVOO was enriched only with its own PC and the FVOOT was enriched with its own PC (50%) combined with thyme PC (50%) using a phenol extract made up of a mixture of olive cake and commercially available dried thyme (*Thymus zyguis)*. During washout periods a common olive oil kindly provided by Borges Mediterranean Group was consumed. The phenolic composition of the VOOs used in the dose-response and sustained study is shown in **Supplementary Table S1**.

2.2 Participants and experimental design

The experiment design comprised 2 separate interventions: a dose-response intervention (acute study) and a 3-week intervention (sustained study). Both studies were randomized, controlled, double-blind and cross-over designed (**Figure 1**). The dose-response study was performed to firstly determine which concentration of olive oil PC was the optimal regarding endothelial function and other related biomarkers. Subsequently, the sustained study (VOHF Project) was carried out to determine the effects of two different FVOOs containing the same phenolic content (500 mg/Kg oil) differing in PC composition (olive oil PC or combined with thyme PC) compared to a control VOO on lipid profile, endothelial function, cardiovascular risk biomarkers and plasma fat-soluble vitamins.

2.2.1 Dose-response study

Between April and September 2011 in Hospital Universitari 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.

Subjects participated in three one-day experimental sessions and received after an overnight fast 30 mL single ingestion of each phenol-enriched FVOO (L-FVOO, M-FVOO and H-FVOO) and the three treatment conditions were separated by a 1-week washout period. Venous blood was collected at baseline (0 h) and at several time points after FVOOs intake. 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.

The study was approved by the Clinical Research Ethical Committee of the Hospital Universitari Sant Joan de Reus (Ref 09-02-26/2proj2), Spain and was registered at ClinicalTrials.gov (Identifier: NCT01347515).

2.2.2 Sustained study

Volunteers from the VOHF Project (19 men and 14 women aged 35-80 years old) were recruited from April to September 2012 in Hospital del Mar Medical Research Institute as previously reported (Farràs et al., 2015). 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.

Subjects were randomly allocated to one of 3 sequences of administration of 25 mL/day of raw VOO, FVOO and FVOOT. Intervention periods were of 3 weeks and olive oils were consumed daily distributed among meals. There was a 2-week washout period prior to VOO interventions. A statistician generated the random allocation sequence, participant enrolment was carried out by a researcher and participants' assignment to interventions according to the random sequence was done by a physician. To avoid an excessive intake of antioxidants, such as PC, during the clinical trial period, participants were advised to limit the consumption of polyphenol-rich food. A 3-day dietary record was administered to the participants before and after each intervention period to control their habitual diet throughout the study. 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. The study was approved by the Clinical Research Ethical Committee of Institut Municipal d'Assistència Sanitària (IMAS) (CEIC-IMAS 2009/3347/I) (Barcelona, Spain) and registered at the International Standard Randomized Controlled Trial register (Identifier: ISRCTN77500181).

2.3 Lipid profile and glucose

Serum total cholesterol, triglycerides (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). LDL-c was calculated by the Friedewald formula.

2.4 Endothelial function

The endothelial-dependent vasomotor function was measured as IRH by a Laser-Doppler linear Periflux 5000 flowmeter (Perimed AB, Järfälla, Stockholm, Sweden) based on the backscatter of a beam of laser light which undergoes a change in wavelength when it encountered moving red blood cells. The laser was in direct contact with the skin leading to provide an index of perfusion referred to as flux (Anderson et al., 2015). IRH was determined as previously described (Valls et al., 2015). In the dose-response study at baseline, 2 h, 4 h and 6 h postprandial and before and after each intervention in the sustained study. The IRH was expressed in Arbitrary Units (AU).

2.5 Cardiovascular ~~risk~~ biomarkers

Serum Endothelin-1 and NOx were measured 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). These biomarkers were determined in the dose-response study at baseline, 2 h, 4 h and 6 h postprandial and before and after each intervention in the sustained study.

204 **2.6 Fat-soluble vitamins**

205 Fat-soluble vitamins were only determined in plasma samples of volunteers from the
206 sustained study before and after each intervention. Retinol and carotenoids (β-
207 crypthoxanthin, β-carotene and lutein), were measured by HPLC and photodiode and
208 photodiode array detector according to Gleize et al. (Gleize, Steib, André, & Reboul, 2012).
209 Plasma α- and γ-tocopherol were determined by HPLC and fluorescence detector as
210 described by Minguez-Mosquera et al. (Minguez-Mosquera, Gandul-Rojas, & Gallardo-
211 Guerrero, 1992).

212 **2.7 Phenolic compliance biomarkers**

213 To verify dietary adherence, the phenolic compliance biomarkers were determined by
214 UPLC-ESI-MS/MS in plasma and 24-h-urine before and after each intervention period in
215 the sustained study as previously described (Rubió, Farràs, et al., 2014a). According to this
216 previous study, hydroxytyrosol sulfate (HTS) and hydroxytyrosol acetate sulfate (HTAS)
217 were identified as compliance biomarkers for olive oil PC, whereas thymol sulfate (TS) and
218 hydroxyphenylpropionic acid sulfate (HPPAS) appeared to be the best compliance
219 biomarkers for thyme PC provided by FVOOT. Phenolic compliance biomarkers in plasma
220 were used in the present study to establish correlations with other measured outcomes.

221 **2.8 Sample size**

222 In the dose-response study, a sample size of 12 participants allowed at least ≥ 80% power
223 to detect a statistically significant difference between groups of 10 units of IRH, assuming a
224 dropout rate of 15% and a type I error of 0.05 (2-sided). The common standard deviation
225 (SD) of the method is 11 units (Ruano et al., 2005).

In the sustained study, a sample size of 30 individuals allowed a power of at least 80% power to detect a statistically significant difference among three groups of 3 mg/dL of HDL-c (according to the main aim of VOHF study) and a SD of 1.9, using an ANOVA test and assuming a dropout rate of 15% and a Type I error of 0.05.

2.9 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 were used to describe log-transformed variables with normal distribution. The Kolmogorov-Smirnov and Shapiro–Wilk's W test were used to verify the distributions of the variables.

In the dose-response study, Area Under the Curve (AUC) of the IRH was calculated by means of pharmacokinetic functions (using Microsoft Excel).

The carry-over effect was discarded in all variables in both studies analyzed by mix model and adjusting for multiple testing by Benjamini-Hochberg procedure. 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 repeated measures General Linear Model adjusted by baseline value and gender. 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 among IRH and the other studied parameters in both the dose-response study and sustained study. In the dose-response study correlations were stablished between IRH AUC and cardiovascular biomarkers in all time points and the maximum plasmatic concentration (Cmax) of phenolic compliance biomarkers. In the sustained study, correlations were calculated among post-treatment values of IRH and cardiovascular biomarkers, fat-soluble vitamins, and phenolic

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 Subjects and dietary adherence

13 participants were recruited for the dose-response study, of these 12 (6 women) were eligible and completed the study. The 3 FVOOs were well tolerated by all participants and no adverse events were reported. Participants' baseline characteristics are shown in **Supplementary Table S2.** The pharmacokinetic parameters in plasma of the phenolic biomarkers are reported in our previous work (Rubió et al. 2012a).

The participants' flow-chart of the sustained study is shown in **Supplementary Figure S1**. Participant's baseline characteristics, segregated according to the sequence of VOO administration, are shown in **Supplementary Table S3** and no significant differences exist among sequences. The subjects included and analyzed in sustained study were 33 (19 men and 14 women). Specifically, for IRH the subject number analyzed was n=27 in FVOO intervention; n=26 in FVOOT intervention and n=29 in VOO (control olive oil). As previously reported, no changes were observed in the main nutrients and medication intake throughout the study (Farràs et al., 2015). The 3 VOOs provided in the sustained study were well tolerated by all participants and no adverse events were reported. Participants' compliance was good as reflected in the 24-h urine and plasma phenolic compliance biomarkers after olive oil interventions (Rubió, Farràs, et al., 2014).

3.2 Lipid profile and glucose

In the dose-response study, the changes observed in lipid profile and glucose were due postprandial time-course but had no clinical impact (**Supplementary Table S4**). In the sustained study, no changes were observed in lipid profile and glucose as reported previously (Farràs et al., 2015).

3.3 Endothelial function

The postprandial time-course changes in IRH are shown in **Figure 2**. Only L-FVOO and M-FVOO produced significant increases in IRH values respect to their baseline. M-FVOO was the first to show a significant increase in IRH values already at 4 h and was linearly increased until 6 h. L-FVOO only presented a significant increase at 6 h. In this sense, M-FVOO (500 mg PC/kg oil) provided additional benefits in the endothelial function versus the low and high doses (250 or 750 mg PC/kg oil). So, in terms of endothelial function, 500 mg PC/kg oil appeared to be the optimal phenolic dose.

Changes in IRH values after each 3-week VOO intervention are shown in **Figure 3**. Both FVOOs, either enriched with their own PC or complemented with thyme PC, had a significant higher post-intervention IRH values when compared to the standard VOO ($p<0.05$). No significant differences were observed between both FVOOs, although an increasing trend was observed (p trend = 0.012).

3.4 Cardiovascular biomarkers

The time-course of the endothelial dysfunction biomarkers is shown in **Supplementary Figure S2**. In addition, oxidative biomarkers were also determined in dose-response study and detailed methods and results are shown in **Supplementary Table S5**. The L-FVOO significantly decreased the PAI-1 and NOx concentrations at each time-point with respect to baseline. Moreover, L-FVOO increased in a linear trend endothelin-1, and GSH

concentrations, during the postprandial time-course with respect to baseline. ox-LDL increased during the postprandial state from 1 h after intake ($p<0.002$).

The M-FVOO significantly decreased the PAI-1 values after 4 h from baseline ($p<0.001$). M-FVOO also increased 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 decreased PAI-1 concentrations ($p<0.001$) at each time-point with respect to baseline and NOx values 4 h versus 2 h postprandial ($p=0.048$). H-FVOO also significantly increased 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 ($p=0.035$).

In terms of cardiovascular risk biomarkers, M-FVOO showed a better postprandial response compared to L-FVOO and H-FVOO, as it did not increase the endothelin-1 concentrations during the postprandial time and did not decrease the NOx values after intake. Moreover, M-FVOO showed a negative borderline correlation for IRH AUC and ox-LDL 4 h after intake ($p=0.050$) and was also the only one that showed a positive borderline correlation for IRH AUC and C_{max} of the main compliance biomarkers (HTS and HTAS). So, the selection of M-FVOO (500 mg PC/kg oil) was not only supported by the additional benefits regarding endothelial function, but also by the added effects in the cardiovascular risk biomarkers.

Post-intervention differences were not reported in any of the cardiovascular risk biomarkers analyzed in the sustained study (Data not shown). However, a positive post-intervention relationship was observed for IRH values and HDL-c plasma concentrations ($r=0.369$, $p=0.001$) (**Table 1**).

3.5 Fat-soluble vitamins

Changes in plasma fat-soluble vitamins concentrations after the 3-week VOO interventions are shown in **Figure 4**. After FVOO intervention a significant increase was observed in α- and γ- tocopherol ($p<0.05$). After FVOOT intervention changes were observed with an increment of retinol, β-carotene, β-cryptoxanthin, lutein, and α-tocopherol ($p<0.05$). Furthermore, we observed that after FVOO or FVOOT interventions, the plasma concentrations of retinol, β-cryptoxanthin, lutein, and α-tocopherol were significantly 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 (**Table 1**).

3.6. Correlations among endothelial function and other parameters

In the dose-response study, correlations were established (data not shown) with IRH AUC and all data points of cardiovascular ~~risk~~ biomarkers and the maximum plasmatic concentration (C_{max}) of phenolic compliance biomarkers. We only observed a negative borderline correlation between IRH AUC and ox-LDL at 4 h after intake of M-FVOO ($p=0.050$). In addition, M-FVOO 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 ($p = 0.047$).

Correlations performed in the sustained study (**Table 1**) showed that homovanillic alcohol sulfate (HVAlcS), which was identified in plasma and urine as a metabolite derived from olive oil PC, presented a positive relationship with IRH values at post-intervention state. IRH post-intervention values also showed a positive correlation with retinol, β-cryptoxanthin and lutein.

In the sustained study we also observed positive correlations between several phenol metabolites derived from olive oil (HTS, HTAS and HVAlcS) and retinol, β-carotene, β-cryptoxanthin, α- and γ- tocopherol ($p<0.05$; data not shown). On the other hand, thyme phenolic metabolites (TS, HPPAS and cafeic acid sulfate) were also positively correlated with retinol, β-carotene and α-tocopherol ($p<0.059$; data not shown).

4. Discussion

The present work provides evidence that a sustained consumption of phenol-enriched VOOs with varying PC classes (olive oil and thyme PC) produce an increase in the plasmatic concentrations of fat-soluble vitamins and also an improvement in the endothelial function. In a preliminary stage, a dose-response study was performed to determine which concentration of PC was the optimal regarding the endothelial function improvement and other related cardiovascular biomarkers to further use this VOO in the sustained study. Results from the acute intake study revealed that VOO enriched at 500 mg/kg (M-FVOO) could provide additional benefits in the endothelial function versus the low and high doses (250 or 750 mg/kg) and showed a better postprandial response on the cardiovascular risk biomarkers. The selection of the dose of 500 mg/kg was also supported by our previous pharmacokinetic study (Rubió et al., 2012a), in which plasmatic phenolic metabolites did not show a complete linear response after 500 mg/kg and 750 mg/kg, indicating that a threshold could exist in olive oil phenolic absorption.

After the 3-week sustained consumption of 25 mL of VOOs containing 500 mg PC/kg, either enriched with its own PC (FVOO) or complemented with thyme PC (FVOOT), an improvement in endothelial function by increasing IRH was observed in

hypercholesterolemic patients. The olive oil PC beneficial effects on endothelial function measured by IRH have been confirmed in hyperlipemics in postprandial state (Ruano et al., 2005) and in pre- and hypertensive subjects at postprandial state (Valls et al., 2015) and after a sustained consumption (Moreno-Luna et al., 2012). In this context, the present trial is the first that have examined the sustained effects on the endothelial function of phenol-enriched olive oils with different and standardized phenolic content in a hypercholesterolemic population.

Previous studies have begun to uncover the potential mechanisms by which olive oil PC may induce endothelial improvements. The endothelial function enhancement has been described to be mediated via reduction in oxidative stress and the increase of NOx metabolites (Ruano et al., 2005). In both the present acute and sustained studies, no differences in post-intervention endothelial dysfunction biomarkers analyzed (NOx and endothelin-1) were detected, so other mechanisms could have been involved in the vasodilation process. One of the most relevant and novel findings of this trial was the significant increase of fat-soluble vitamins in plasma after both FVOO and FVOOT compared to control VOO, which could explain in part the improved endothelial function. The 3 VOOs tested in the sustained study had the same composition and concentration of fat-soluble vitamins and fatty acids so the significant increases observed in plasmatic levels was associated to the phenolic supplementation. So it was hypothesized that a supplementation of PC through VOO could lead to a preservation of the systemic levels of fat-soluble vitamins and in turn could improve the endothelial function.

A relation between the improvements in endothelial function and the increased levels of plasmatic fat-soluble vitamins is supported by the positive correlation observed

between IRH and plasma concentrations of certain fat-soluble vitamins. Our results are in concordance with those of Marin C, et al. (Marin et al., 2011) 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 this study they also described positive correlations between β-carotene and circulating endothelial progenitor cells, which favor the regenerative capacity of the endothelium. Similarly, Karppi J, et al. (Karppi, Kurl, Laukkanen, Rissanen, & Kauhanen, 2011) 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. In another study, plasmatic levels of carotenoids were inversely associated with endothelial dysfunction (Hozawa et al., 2007). In this context, the parallel significant increase of plasmatic fat-soluble vitamins with the plasmatic phenolic metabolites after FVOO and FVOOT consumption also supports the interaction between both micronutrients. Another alternative potential mechanism could be related to the microbial generation of bioactive PC metabolites in the gut and the consequent modulation of gut microbiota. PC have been shown to be capable of 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 (Dueñas et al., 2015; Etxeberria et al., 2013). In our previous study, reduction in blood ox-LDL after the phenol-enriched VOO consumption in the same subjects was proposed to be mediated by the increases in populations of bifidobacteria together with increases in phenolic microbial metabolites detected in faeces (Martín-Peláez et al., 2015). Another study has demonstrated that when the metabolic pathway involved in the metabolism of lipid-soluble antioxidants (phytoene dehydrogenase) is enriched in the gut metagenome, the plasma concentrations of

409 fat-soluble vitamins are increased (Karlsson et al., 2012). Thus, another hypothesis that
410 could explain the observed increase in plasma fat-soluble vitamins after both FVOOs intake
411 could be a possible modulation by increasing of their synthesis by the microbiota.

412 In the sustained consumption study, a positive relationship was also observed
413 between IRH and plasma concentrations of HDL-c, which was also described in
414 hypercholesterolemic patients after consumption of a VOO with a high PC content (Ruano
415 et al., 2005). The HDL particle can exert a protective effect on the vascular endothelium
416 (Birner-Gruenberger, Schittmayer, Holzer, & Marsche, 2014). This has been related to its
417 ability to inhibit monocyte adhesion by inhibiting vascular cell adhesion molecule (VCAM-
418 1), intercellular cell adhesion molecule (ICAM-1), and E-selectin expression, and also to
419 suppress monocyte chemotactic protein-1 (MCP1) by inhibiting the chemokine secretion
420 (Birner-Gruenberger et al., 2014). In the context of the same trial, it has been reported that
421 the FVOOT intervention improved HDL subclass distribution and composition (Farràs et
422 al., 2015) and both FVOOs increased ApoA-1 concentrations (Pedret et al., 2015), which
423 can lead to a better HDL functionality. Accordingly, the increment of HDL particles could
424 be another mechanism by which endothelial function improvement occurs after the
425 sustained intake of both FVOOs.

426 One of the strengths of the present study is its cross-over, randomized and
427 controlled design which enables to provide the first level of scientific evidence. The cross-
428 over design, in which each subject acts as the corresponding control, minimizes the
429 interference of possible confounding variables. Another challenge of the present studies is
430 the use of the same VOO (80 mg/kg) as the matrix enrichment to prepare all phenol-
431 enriched olive oils in the dose-response and sustained studies. This enabled to isolate the

effects of olive oil PC without complications of additional nutrient differences. 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, we report for the first time that a sustained consumption of phenol-enriched olive oils at a concentration of 500 mg PC/kg oil, either enriched with its own PC or complemented with thyme PC, is able to promote an increase in the plasmatic concentrations of fat-soluble vitamins and improvements in endothelial function compared to a natural standard VOO in hypercholesterolemic patients. Thus, FVOOs tested in our study could be a new tool for cardiovascular disease prevention in hypercholesterolemic subjects. Moreover, our results support a novel mechanism for explaining the improvement in endothelial function related to the preservation of the systemic fat-soluble vitamins by the supplemented PC provided with the phenol-enriched olive oils. However, further studies are needed to investigate the mechanisms explaining the role of plasmatic fat-soluble vitamins in the endothelial function modulation.

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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, LR drafted the manuscript and MFi, M-IC, M-JM, RS, LR 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. Study Design. **(a)** *Dose-response study*: randomized, controlled, double-blind and cross-over study performed to assess dosage regimens for the sustained study. Ischemic reactive hyperemia (IRH) and cardiovascular risk biomarkers were measured before and up to 6 h after consumption of three different olive oils enriched at different phenolic doses: L-FVOO with a low phenolic content (250 mg PC/kg of oil), M-FVOO with a medium phenolic content (500 mg totals phenols/kg of oil) and H-FVOO with a high phenolic content (750 mg totals phenols/kg of oil). (**b)** *Sustained study*: randomized, controlled, double-blind and cross-over study performed to determine the sustained effects of two different phenol-enriched olive oils containing the same phenolic content (500 mg/Kg oil) but differing in its composition: FVOO, enriched with olive oil PC and FVOOT, combining olive oil PC (50%) and thyme PC (50%), compared to a control olive oil (VOO, 80 mg /kg oil) on endothelial function and plasma fat-soluble vitamins.

Figure 2. Time course of acute changes in IRH upon ingestion of L-FVOO, M-FVOO and H-FVOO. For the detailed protocol of the dose-response study, refer to Figure 1. All values are expressed as mean ± standard deviation. The P linear trend to each FVOO intervention were L-FVOO, p=0,000; M-FVOO, p= 0,000 and H-FVOO, p=0,013. * p< 0,05 with respect to basal time within the treatment; ɨ 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 3. Changes in IRH values in the sustained study after the consumption of 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%). All values are expressed as mean ± standard deviation. * $p< 0,05$ with respect to VOO.

Figure 4. Changes in plasma concentrations of fat-soluble vitamins after after the consumption of 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%). The values of retinol and α-tocopherol are expressed as mean ± standard deviation. The values of β-Carotene, β-Cryptoxanthin, Lutein and γ-Tocopherol, non normal variables, are expressed by geometric mean ± logSD. * $p< 0,05$ with respect to VOO, ǂ $p< 0,05$ with respect to FVOO.

SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1 (S1). Flow chart diagram of sustained study participants.

Supplemental Figure 2 (S2). Changes in time course of acute changes in cardiovascular biomarkers upon ingestion of L-FVOO, M-FVOO and H-FVOO. For the detailed protocol of the dose-response study, refer to Figure 1.