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Analytical Methods

Effects of functional olive oil enriched with its own phenolic compounds on endothelial function in hypertensive patients. A randomised controlled trial

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ABSTRACT

The additional health-promoting properties of functional virgin olive oil (FVOO) enriched with its own phenolic compounds (OOPC) versus the parental virgin olive oil (VOO) must be tested in appropriate human clinical trials. Our aim was to assess the effects of FVOO on endothelial function in hypertensive patients. Thirteen pre- and stage-1 hypertensive patients received a single dose of 30 mL of FVOO (OOPC = 961 mg/kg) or VOO (OOPC = 289 mg/kg) in a postprandial randomised, double blind, crossover trial. Endothelial function, measured as ischemic reactive hyperemia (IRH) and related biomarkers, were followed for 5 h after consumption. Compared with VOO, FVOO increased IRH ($P < 0.05$) and plasma Cmax of hydroxytyrosol sulphate, a metabolite of OOPC 2 h postprandial ($P = 0.05$). After FVOO ingestion, oxidised LDL decreased ($P = 0.010$) in an inverse relationship with IRH AUC values ($P = 0.01$). FVOO provided more benefits on endothelial function than a standard natural virgin olive oil in pre- and hypertensive patients. Trial registration: isrctn.org. Identifier ISRCTN03450153.

1. Introduction

The Mediterranean diet, in which olive oil (OO) is the main source of fat, has been shown to be protective against cardiovascular diseases (López-Miranda et al., 2010). Apart from oleic acid, olive oil contains many bioactive components including polyphenols. In human studies, olive oil rich in polyphenols has been shown to improve antioxidant and anti-inflammatory effects, and to reduce the proliferation of cell adhesion molecules, compared with low-polyphenol olive oils (Covas, 2007; Fitó, De la Torre & Covas, 2007; López-Miranda et al., 2010). In 2011, the European Food Safety Authority (EFSA) endorsed a claim regarding the effectiveness olive oil polyphenols (5 mg/day) in protecting low-density lipoprotein (LDL) from oxidation (EFSA Panel, 2011).

Oxidative stress-mediated endothelial dysfunction is one of the characteristic features of essential hypertension (Ghiadoni, Taddei, & Virdis, 2012) and is one of the first pathological signs of atherosclerosis (Celermajer, Sorensen, Bull, Robinson, & Deanfield, 1994). In human studies, an improvement in endothelial function has been observed after the consumption of dietary flavanols (Balzer et al., 2008; Heiss et al., 2010) or natural virgin OO (VOO) versus a very low-polyphenol content oil (Ruano et al., 2005). The concentration of PC (PC) in an OO depends on factors such as the cultivar, climate, and ripeness of the olives at harvesting as well as agronomic and technologic aspects of oil preparation. A good strategy to ensure an optimal intake of polyphenols through the habitual

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diet is to enrich VOO with their own polyphenols (Suárez, Romero, Ramo, Macià, & Motilva, 2009). Also, although a recognised healthy food, OO cannot and should not be consumed in large quantities. Thus, enrichment of OO with its PC is a way of increasing its health-promoting properties whilst consuming the same or less fat. Currently, functional foods are developed to improve the properties of natural food components. However, functional foods must be tested in human clinical intervention trials with an appropriate design.

Endothelial-dependent vasodilatation is impaired during the postprandial state (Ghiadoni et al., 2012). Our aim was to test whether a high-polyphenol content functional virgin olive oil (FVOO) enriched with its own polyphenols, improved endothelial function in pre- and hypertensive subjects beyond the effects observed after the intake of a standard virgin olive oil (VOO) with moderate polyphenol content, in a postprandial randomised, crossover, controlled trial.

2. Materials and methods

2.1. OO characteristics

FVOO was prepared by the addition of a phenolic-rich extract (oleuropein complex or secoiridoids: 89.4%; hydroxytyrosol, tyrosol and phenyl alcohols: 3.5%; and flavonoids, 6.0%), obtained from the olive cake, to a natural VOO as previously described (Suárez et al., 2011). Briefly, olive cake phenolic extract (7 mg/mL oil) and 0.3% (p/v) of lecithin (Emulpur; Cargill, Barcelona, Spain) were dissolved in ethanol-water (50/50, v/v), and added to VOO, until fully homogenised, using a Polytron (Kinematica, Littau, Switzerland). To ensure the oils were as close as possible in composition, lecithin was added to VOO at the same concentration. Total polyphenol content of the OOs was 289 and 961 mg/kg oil for VOO and FVOO, respectively, measured by ultraperformance liquid chromatography coupled to a tandem mass detector (UPLC–ESI-MS/MS) as previously described by Suárez et al. (2011). Fatty acids were measured by gas chromatography. Table 1 shows the composition of the OOs used in the study.

2.2. Participants

Between January and July 2009, 22 participants (aged 20–75 years old) were recruited through a volunteer center database. Participants were community dwelling with pre-hypertension (systolic blood pressure (SBP) $\geqslant$120–139 mmHg, and/or diastolic blood pressure (DBP) $\geqslant$80–89 mmHg) or stage 1 hypertension (SBP $\geqslant$140–159 mmHg and/or DBP $\geqslant$90–99 mmHg (Chobanian et al., 2003), but not receiving anti-hypertensive treatment. This population was chosen because hypertensive patients have been shown to have a high degree of oxidative stress, which plays a key role in endothelial functionality (Fatehi-Hassanabad, Chan, & Furman, 2010). Exclusion criteria included: LDL cholesterol >4.9 mmol/L, triglycerides (TG) >3.97 mmol/L, or current hypolipemic treatment; diabetes mellitus; any chronic disease; and body mass index (BMI) >30 kg/m^2. Participants provided written informed consent prior to their enrollment in the trial. After a screening visit, eligibility or exclusion was assessed by the attending physician on the basis of the clinical records. The study was approved by the Clinical Research Ethical Committee of the Hospital Universitari Sant Joan de Reus (Ref 08-04-24/4proj5), Spain. Protocols were according to the Helsinki Declaration.

Table 1
Characteristics of the olive oils administered.

	VOO	FVOO
Quality parameters		
Free acidity, % of oleic acid	0.19	0.26
Peroxide value, mEqO_2/kg	16.76	6.10
Fatty acids, % of total		
Monounsaturated	72	72
Polyunsaturated	11	11
Saturated	17	17
Total polyphenols, mg/kg of olive oil	289	961
Free hydroxytyrosol	0.37	6.64
Free tyrosol	1.03	8.7
Secoroid derivatives	123	680
Vanillic acid	0.37	3.94
p-Coumaric acid	0.08	0.84
Vanillin	0.16	1.44
Pinoresinol	116	173
Luteolin	1.44	6.28
Apigenin	0.27	0.80

Abbreviations: VOO, natural virgin olive oil; FVOO, polyphenol enriched virgin olive oil.

2.3. Study design

The trial was randomised, controlled, double-blind and crossover. The washout period between interventions was of two weeks. The randomization plan was generated by using a web site (http://www.randomization.com) February 11th 2009 at 12:26:02 pm. Participants consumed 30 mL of each olive oil, VOO or FVOO, with bread (80 g; Fig. 1). The day before the intervention participants followed a polyphenol-free diet avoiding OO, olives, fresh fruit or juices, vegetables, legumes, soya, chocolate, coffee, tea, wine, and beer. One day polyphenol-free diet washout is enough, as our group has already demonstrated that after 5 h of ingestion of VOO, phenolic compounds in plasma reached basal concentration (Suárez et al., 2011). The peak of hydroxytyrosol in plasma after 25 mL VOO ingestion is 53 min, the half-life being around 2.4 h (Miró-Casas et al., 2003). During the week before the first test and the washout period (two weeks), the percentage of saturated fatty acids (SFA) in the diet was 10% within an isocaloric diet calculated using the Harris–Benedict equation and according the guidelines on cardiovascular disease prevention (Graham et al., 2007; Grundy et al., 2004). Compliance with the stabilization diet was assessed using a 3-day dietary record (2 working days and a holiday or weekend one) before the intervention day. Dieticians explained how these questionnaires should be completed. Participants were instructed to avoid intense physical activity three days prior to the intervention day. Physical activity was evaluated by the Minnesota Leisure Time Physical Activity Questionnaire validated for use in Spanish men and women (Elosua, Marrugat, Molina, Pons, & Pujol, 1994; Elosua et al., 2000). Anthropometric data were obtained by standardised methods. After 15 min of rest, three times at one-minute intervals using an automatic sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain). Venous blood was collected at the baseline (0 h) and at several time points after olive oil administration (Fig. 1). Serum and plasma were obtained by centrifugation of blood at 1500*g* at 4 °C for 20 min and stored at −80 °C.

2.4. Endothelial function

Endothelial-dependent vasomotor function was measured as ischemic reactive hyperemia (IRH) using a Laser-Doppler linear Periflux 5000 flowmeter (Perimed AB, Järfälla, Stockholm, Sweden). Measurements were performed with the patient lying in the supine position in a room with stable temperature (20–22 °C). Patients were at rest for 15–20 min before the test. The blood pressure cuff (Big Ben floor desing, Riester GmbH, Jungingen, Germany) was placed above the elbow of the dominant arm, while the laser

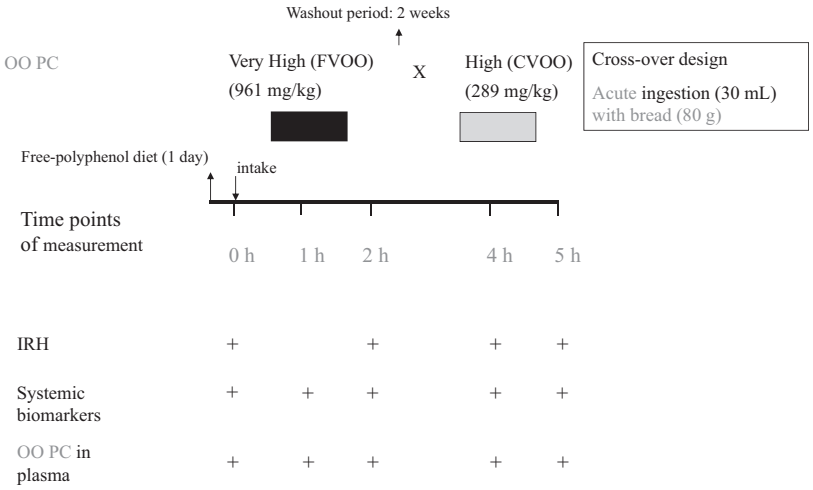

Fig. 1. Study design. OO PC, olive oil phenolic compounds; FVOO, functional virgin olive oil; VOO, virgin olive oil; IRH, ischemic reactive hyperemia.

probe was attached to the palmar surface of the second finger. After a 5 min resting period, basal capillary flow was measured for 1 min (t_0). Thereafter, 4 min distal ischemia was induced by inflating the cuff to suprasystolic pressure (220 mmHg). Subsequently, the cuff was deflated and, after 30 s, the flow was recorded during 1 min (t_d). Data were recorded and stored using the PeriSoft 2.5 software for Windows. The system monitor showed how the perfusion units (PU) fell regularly to reach comportment equal or similar to the basal situation. Results were expressed as arbitrary units (AU). Measurements were performed at baseline and at 2 h, 4 h, and 5 h after OO intake (Fig. 1). Calculations were performed using the formula: $\text{IRH} = ((\text{PU}t_d - \text{PU}t_0)/\text{PU}t_0) \times 100)$. The IRH value of the area under the curve (AUC) was calculated using Microsoft Excel for pharmacokinetic functions.

A reproducibility assay in a preliminary study, performed in ten healthy subjects with measurements two weeks apart, showed an inter-study variability of 9.05%. A total of 10 measurements within the same day in a healthy volunteer rendered an intra-study variability of 8.5%.

2.5. Systemic biomarkers

Cardiovascular risk biomarkers were measured at baseline (0 h) and at 2 h, 4 h, and 5 h after OO intake (Fig. 1). Serum total cholesterol, TG, HDL cholesterol, high sensitivity C-reactive protein (hsCRP), insulin, and glucose were measured by standardised methods using a Beckman autoanalyzer (Beckman Coulter-Synchron, Galway, Ireland). LDL cholesterol was calculated by the Friedewald formula (Friedewald, Levy, & Fredrickson 1972). Plasma EDTA circulating oxidised LDL (oxLDL; Mercodia AB, Uppsala, Sweden), vascular cell adhesion molecule type 1 (VCAM-1) and intercellular adhesion molecule type 1 (ICAM-1) (R&D Systems, Minneapolis, USA) were measured by ELISA. Plasminogen activator inhibitor type 1 (PAI-1) (Technoclone GmbH, Vienna, Austria) was measured in citrate plasma using ELISA kits.

Plasma OO polyphenols and their biological metabolites were measured by UPLC–MS/MS (Suárez et al., 2011) at baseline (0 h) and after 1 h, 2 h, 4 h, and 5 h.

2.6. Sample size and power analysis

A sample size of 13 participants allows at least greater than or equal to 80% power to detect a statistically significant difference between groups of 10 Units of IRH, assuming a dropout rate of 15% and a type I error of 0.05 (two-sided). The common standard deviation of the method is 11 Units (Ruano et al., 2005).

2.7. Statistical analyses

The test for normality of continuous variables, Pearson's correlation analyses, general linear models and paired Student *t*-test were performed using SPSS 17.0 software (SPSS Inc, Chicago, IL, USA). Mixed models were performed using SAS software (version 9.1.3; SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Study population

From the 22 participants recruited, 16 were eligible. Three participants dropped out before starting the study due to an incompatible work timetable, and 13 participants (7 men and 6 women) completed the study. Participants' baseline characteristics are shown in Table 2. No differences in baseline characteristics were observed between the two sequences of OO administration. No changes in blood pressure, weight, dietary habits, and physical activity were registered throughout the study.

3.2. Endothelial function

The postprandial time-course changes in IRH after ingestion of the OOs is shown in Fig. 2. IRH time-course increased in a linear

Table 2
Baseline characteristics of the participants.

Variable	Mean (SD)
Gender, male/female	7/6
Age (years)	50.6 (16.9)
Body mass index (kg/m^2)	25.7 (1.7)
Waist circumference (cm)	79.0 (6.0)
SBP (mmHg)	139 (13.6)
DBP (mmHg)	84 (10.2)
Total cholesterol (mmol/L)	5.06 (0.75)
LDL cholesterol (mmol/L)	2.84 (0.69)
HDL cholesterol (mmol/L)	1.70 (0.40)

Abbreviations: SBP, systolic blood pressure; DBP, diastolic blood pressure; LDL, low density lipoprotein; HDL, high density lipoprotein.

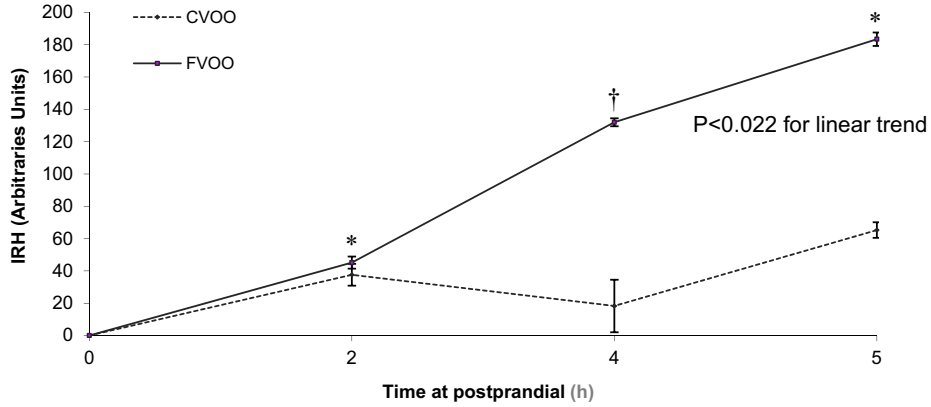

Fig. 2. Ischemic reactive hyperemia (IRH) at postprandial, after the intake of virgin olive oil (VOO) and functional olive oil enriched with its own phenolic compounds (FVOO). $^{*}P < 0.05$ versus baseline; $^{\dagger}P < 0.05$) versus VOO at the same time-point.

trend after FVOO ingestion from baseline to 5 h postprandial ($P = 0.022$). In this case, IRH increased more than three- and four-fold at 4 h and 5 h respectively compared to 2 h postprandial. When comparing both interventions, IRH values at 4 h postprandial after FVOO were significantly higher than those after VOO. An inverse relationship was observed only for IRH AUC and oxLDL plasma concentrations at 5 h after FVOO intake ($P = 0.014$).

3.3. Systemic biomarkers

The time-course of the systemic cardiovascular risk biomarkers is shown in Table 3. No carryover effect between the two intervention periods was detected. The postprandial increase in glucose and TG at 2 h was less following consumption of FVOO than VOO ingestion. Plasma oxidised LDL decreased in a linear trend after FVOO intervention ($P = 0.010$). Moreover, oxLDL concentrations after FVOO intake were significantly lower than baseline values in each time-point. Regarding VOO, only at 5 h after the intake, oxLDL values resulted statistically lower than its baseline. A decreasing linear trend was observed in PAI-1 and hsCPR concentrations after VOO ($P < 0.05$) and FVOO ($P < 0.01$). At 4 h postprandial, PAI concentration was lower after FVOO versus VOO treatment ($P < 0.05$). As shown in Table 3, no changes were observed in VCAM and ICAM. Also, no changes were observed in LDLc, HDLc or total cholesterol (data not shown). Hydroxytyrosol sulphate levels, the main biological metabolite of hydroxytyrosol, increased in a dose-dependent manner with the polyphenol content of the OO administered (FVOO or VOO). Similar kinetic trend was observed after both oils intake, as Hydroxytyrosol sulphate detected in plasma reached maximal levels at 2 h ($P < 0.05$) and these levels decreased to basal values at 5 h after oils intake. Nevertheless, FVOO produced higher Hydroxytyrosol sulphate levels than VOO at 1 and 2 h after the intake ($P < 0.05$) (Fig. 3).

4. Discussion

Our study shows that virgin OO enriched with its own PC (FVOO) can offer additional health benefits, as determined by human endothelial function, during the postprandial phase compared with a standard VOO with moderate polyphenol content. Postprandial values for glucose, TG and PAI-I were lower after FVOO in comparison with VOO ingestion. FVOO consumption improved the postprandial endothelium-dependent microvascular dilatation in patients with pre- and stage-1 hypertensive status in comparison with VOO.

Several studies have addressed the relationship between high versus low polyphenol intake and endothelial function assessment. Flavonoid consumption has been shown to improve the endothelial function, after both acute and sustained consumption in diabetic (Balzer et al., 2008) and coronary heart disease patients (Heiss et al., 2010). OO polyphenols have been shown to improve endothelial function in hyperlipemic (Ruano et al., 2005) and hypertensive (Moreno-Luna et al., 2012) patients. We performed a study comparing the benefits of a functional versus a natural VOO on endothelial function. We tested the effect of a FVOO versus a natural VOO. A meal containing high-phenolic VOO improves IRH during the postprandial state. This phenomenon might be mediated via reduction in oxidative stress and the increase of nitric oxide metabolites (Ruano et al. 2005).

In Western populations, we spend most of the time in a non-fasting state, with continuous fluctuations in plasma lipids throughout the day. Postprandial state is an active field of research in cardiovascular disease due to evidence indicating it influences on cardiovascular risk. Postprandial lipemia has been recognised as a risk factor for atherosclerosis development as it is associated with oxidative changes (López-Miranda et al., 2006; Roche & Gibney, 2000). After a high-fat meal, oxidative stress occurs that has been linked with concomitant impairment in the endothelial function (Ceriello et al., 2002). However, the consumption of fatty meals with sources of antioxidants, such as red wine (Natella, Ghiselli, Guidi, Ursini, & Scaccini, 2001) or vitamin C (Ling et al., 2002), has been shown to minimise postprandial oxidative stress.

In our pre-and hypertensive patients, consumption of FVOO reduced the postprandial hyperglycemia and hypertriglyceridemia peak in comparison with VOO. In agreement with this, a reduction of the LDL oxidation and inflammatory biomarkers was observed after FVOO versus VOO. Circulating oxLDL, one of the recognised methods for measuring oxidative damage mediated by reactive oxygen species, has been reported to be a predictor for development of cardiovascular disease (Meisinger, Baumert, Khuseyinova, Loewel, & Koenig, 2005). OxLDL produces pro-atherogenic effects in endothelial cells by inducing the expression of adhesion molecules, stimulating apoptosis, inducing superoxide anion formation, and impairing protective endothelial nitric oxide formation (Yu, Wong, Lau, Huang, & Yu, 2011). As we have previously reported, acute and sustained OO consumption, decrease oxLDL and hsCRP, linked to the polyphenol content of the OO, in healthy volunteers (Covas et al., 2006a, 2006b) and in stable coronary heart disease patients (Fitó et al., 2005, 2008). The protection against LDL oxidation linked to FVOO consumption in this study could be mediated by the increase in OO PC metabolites (i.e. hydroxytyrosol sulphate) observed in plasma. In previous studies,

Table 3
Postprandial time-course of systemic biomarkers after virgin olive oil (VOO) and polyphenol-enriched virgin olive oil (FVOO).

Variable	Intervention	Baseline	2 h	4 h	5 h	*P* linear trend
Glucose (mmol/L)	VOO	5.44 (0.51)	6.31 (1.57)†	5.19 (0.58)	5.07 (0.35)†	0.006
	FVOO	5.37 (0.34)	6.08 (1.07)†$	5.04 (0.41)†	5.07 (0.31)†	0.001
Insulin (pmoL/L)	VOO	36.7 (1.41)	99.4 (1.83)†	35.4 (2.09)	24.0 (1.80)†	<0.001
	FVOO	36.2 (1.41)	102.9 (1.89)	30.2 (1.41)†	23.1 (1.55)	<0.001
Triglycerides (mmol/L)*	VOO	1.03 (1.61)	1.39 (1.62)†	1.29 (1.76)†	1.17 (1.63)	0.008
	FVOO	0.98 (1.69)	1.29 (1.82)†$	1.32 (1.77)	1.40 (1.71)†	0.002
Oxidised LDL (U/L)	VOO	69.29 (18.37)	67.27 (11.61)	69.01 (22.59)	63.30 (14.09)†	0.057
	FVOO	70.59 (15.51)	67.71 (17.31)†	66.75 (15.49)†	65.41 (16.39)†	0.010
ICAM-1 (ng/mL)*	VOO	170.51 (1.25)	167.83 (1.25)	165.87 (1.35)	164.47 (1.29)	0.192
	FVOO	165.40 (1.25)	161.39 (1.32)	170.30 (1.37)	170.61 (1.31)	0.135
VCAM-1 (ng/mL)	VOO	612.29 (97.68)	609.56 (107.77)	626.27 (123.69)	635.71 (129.95)	0.180
	FVOO	633.28 (112.85)	589.70 (118.49)†	609.65 (107.58)	601.32 (97.29)	0.270
PA1-1 (ng/mL)*	VOO	13.18 (2.31)	14.40 (2.55)	6.56 (1.79)†	7.40 (1.59)	0.022
	FVOO	12.94 (2.34)	9.19 (1.55)	6.05 (2.09)†$	6.95 (1.81)†	0.002
hsCRP (ng/mL)*	VOO	0.71 (2.31)	0.66 (2.27)†	0.65 (2.30)†	0.66 (2.25)†	0.029
	FVOO	0.72 (1.85)	0.66 (1.84)†	0.64 (1.87)	0.64 (1.84)†	0.006

Abbreviations: ICAM, inter-cellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; PAI-I, plasminogen activator inhibitor-1; hsCRP, high sensitive C-reactive protein.
Values expressed as mean (SD).
* Non normal variables are expressed by Geometric mean (log SD). General linear mixed model.
† $P < 0.05$ versus baseline.
$ $P < 0.05$ between treatments at the same time point.

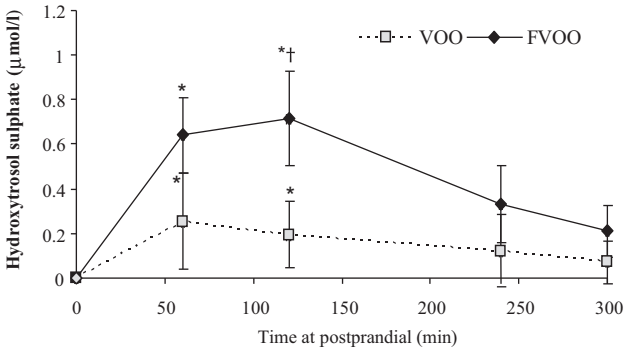

Fig. 3. Time-course of plasma concentrations of hydroxytyrosol sulphate (μmol/L) in human plasma after the intake of virgin olive oil (VOO) and functional olive oil enriched with its own phenolic compounds (FVOO). *$P < 0.05$ versus baseline; † $P < 0.05$ versus VOO at the same time-point.

we have observed a direct relationship between an increase in tyrosol and hydroxytyrosol concentrations in human plasma, after VOO ingestion, and LDL polyphenol content (Covas et al., 2006b), which has been shown to be inversely related to the degree of LDL oxidation (de la Torre-Carbot et al., 2010).

The term endothelial dysfunction implies the loss of homeostasis resulting from the complex interaction of vasodilatory and vasoconstrictive factors, on which diet exerts a crucial influence (Nettleton et al., 2006; Turner, Belch, & Khan, 2008). Generally, the literature is consistent with oxidative stress contributing to the five characteristic microvascular responses to inflammation, namely vasomotor dysfunction (impaired vessel dilation and constriction), leukocyte recruitment, increased vascular permeability, angiogenesis, and thrombosis (Nettleton et al., 2006). In the present study, improvement in endothelial function, reflected in an increase in IRH after FVOO, was inversely related to LDL oxidative damage. Thus, a reduction in both oxidative stress (decreased oxidative damage to LDL) and resulting inflammation could account for the improvement in the endothelial function observed after FVOO ingestion. Our results point to a key role for polyphenols in the improvement of the endothelial function in the pre- and hypertensive patients.

One strength of the study is its design. Randomised, controlled, clinical trials are able to provide first hand scientific evidence. The crossover design, in which each subject acts as their own control, minimises interferences from possible confounding factors unique to the individual. Our design, however, did not allow modelling of any first- and second-order carryover effects. One potential limitation of the study was that, despite the blinding, some participants might have identified the type of OO ingested because of their organoleptic characteristics. Another limitation is the inability to assess potential interactions between the oils and other diet components, although the controlled diet followed during the washout period should have limited the scope of these interactions.

In summary, the FVOO enriched with its own PC improved human endothelial function compared with VOO. The observed increase in biological metabolites of OO PC in plasma, hydroxytyrosol sulphate, together with decreased oxLDL, suggest possible mechanisms explaining the improved endothelial function after ingestion of the polyphenol-enriched FVOO. Based on these results, FVOO could be a useful tool for improving endothelial function in hypertensive individuals.

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