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

Marta Farràs Mañé

DOCTORAL THESIS

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Vista la instància presentada per **Marta Farràs Mañe** de sol·licitud de presentació de tesi doctoral com a compendi de publicacions,

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Acceptar la presentació de la tesi doctoral de **Marta Farràs Mañé** com a compendi de publicacions amb els articles següents com a part fonamental de la tesi:

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  "Olive oil polyphenols enhance the expression of cholesterol efflux related genes *in vivo* in humans. A randomized controlled trial".
  Journal of Nutritional Biochemistry (2013) 24, 1334-1339.

- Valls RM, Farràs M, Suárez M, Fernández-Castillejo S, Fitó M, Konstantinidou V, Fuentes F, López-Miranda J, Giralt M, Covas MI, Motilva MJ and Solà R.
  "Effects of functional olive oil enriched with its own phenolic compounds on endothelial function in hypertensive patients. A randomized controlled trial".
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- Farràs M, Castañer O, Martín-Pelaez S, Hernáez, A, Schröder H, Subirana I, Muñóz-Aguado D, Gaixas S, de la Torre R, Farré M, Rubió L, Díaz O, Fernández-Castillejo S, Solà R, Motilva MJ and Fitó M.
  "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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D'altra banda, les publicacions següents poden formar **part de la tesi com a annex** o part no fonamental, tot i que els treballs fets en aquestes publicacions es poden comentar en la discussió de resultats:

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  "HDL-related mechanisms of olive oil protection in cardiovascular disease".
  Current Vascular Pharmacology (2012) 10, 392-409 (Review).
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Contra aquesta resolució, que no esgota la via administrativa, les persones interessades poden interposar recurs d'alçada davant del Rector Magnífic de la UAB, en el termini d'un mes, a comptar des del dia següent a la recepció d'aquesta notificació o, si s'escau, des del dia següent de la seva publicació, de conformitat amb el que preveu l'article 115 de la Llei 30/1992, de 26 de novembre, de Règim Jurídic de les Administracions Públiques i del Procediment Administratiu Comú, modificada per la Llei 4/1999, de 13 de gener, i l'article 76 de la Llei 26/2010, de 3 d'agost, de Règim Jurídic i de Procediment de les Administracions Públiques de Catalunya de la Generalitat de Catalunya.

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The American Journal of Clinical Nutrition AJCN/2015/115949 (submitted).

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"Effect of phenol-enriched olive oils on HDL functionality in hypercholesterolemic subjects. A randomised, double blind, cross-over, controlled trial. The VOHF study".
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als meus pares

al meu germà

al Roger

“Let food be thy medicine, and medicine be thy food”

Hippocrates, ca. 400 BC




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1. ACKNOWLEDGEMENTS

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ABSTRACT

2. ABSTRACT

Dyslipidemia and hypertension are the classical risk factors of cardiovascular diseases related to atherosclerosis development. Due to the fact that high-density lipoprotein (HDL) cholesterol has been inversely related to atherogenesis, therapies have focused on increasing HDL-cholesterol. Clinical trials which augment HDL have shown an increased mortality risk, however, recent studies have attributed to side effects. Moreover, it has lately been reported that genetic variants predisposing to high HDL-C are not associated with a lower risk of suffering a coronary event. All of the above has led to HDL functionality being considered as a more important aspect than its quantity. Olive oil (OO) phenolic compounds (PC) are protective against risk factors for coronary heart disease by increasing HDL cholesterol concentration, decreasing *in vivo* lipid oxidative damage, enhancing HDL function, and improving endothelial function. Within this context, the enrichment of virgin olive oil (VOO) with PC is a promising strategy as it does not increase the amount of fat consumed, nevertheless such enrichment might have a dual action because an excess of antioxidants could also revert them to pro-oxidants. Functional virgin OOs with complementary antioxidants, according to their structure/activity relationship, could be a suitable option to obtain greater beneficial effects.

The scope of the present work was to investigate, in cardiovascular risk subjects, whether: (i) a functional virgin OO enriched with its own OOPC (FVOO; 961 ppm) modulates the cholesterol efflux-related gene expression and endothelial function, compared to a virgin OO (VOO; 289 ppm); and whether (ii) functional virgin OOs, one enriched with its own OOPC (FVOO; 500 ppm) and another with its own OOPC (250 ppm) plus additional complementary PC from thyme (250 ppm) (FVOOT; total: 500 ppm), could improve HDL functionality-related properties versus a virgin OO control (VOO; 80 ppm).

Two cross-over, randomized, double blind, control trial were performed: (i) 13 pre-/hypertensive subjects received a single 30 mL dose of VOO (289 ppm) and FVOO (961 p pm); and (ii) 33 hy percholesterolemic i ndividuals i ngested 25 mL/day dur ing 3 w eeks of V OO (80 ppm), F VOO (500 ppm), F VOOT (500 ppm).

The results i ndicate t hat a single d ose o f F VOO increased ch olesterol e fflux gene e xpression c ompared w ith VOO c onsumption, specifically A TP b inding cassette t ransporter-A1, scavenger r eceptor c lass B t ype 1 , p eroxisome proliferator-activated r eceptors (PPARBP, P PARα, PPA Rγ, PPA Rδ), a nd CD36. Mo reover, t his co nsumption augmented i schemic r eactive h yperemia versus t he V OO o ne. I n this r egard, a d ecrease o f ci rculating p lasminogen activator inhibitor-1 was observed after FVOO compared to VOO. A sustained intervention of FVOOT increased large HDL subclass percentage versus VOO, and also augmented esterified cholesterol/free cholesterol and phospholipids/free cholesterol ratios in HDL compared with the VOO and FVOO interventions. In addition, F VOOT c onsumption i ncreased lecithin-cholesterol a cyltransferase mass compared with VOO one.

In c onclusion, a pos tprandial c onsumption of a F VOO i mproves c holesterol efflux t ranscriptomics, e ndothelial function, a nd i nflammatory bi omarkers. Furthermore, the benefits achieved with FVOOT sustained consumption on the HDL functional profile have been demonstrated. Data from our studies suggest that F VOO a nd F VOOT c ould be a u seful di etary tool i n t he m anagement of cardiovascular risk patients.

RESUM

La dislipèmia i la hipertensió són factors clàssics de risc cardiovascular, relacionats amb el desenvolupament de l'arteriosclerosi. Donat que el colesterol de la lipoproteïna d'alta densitat (HDL) ha estat inversament relacionat a l'aterogènesi, les teràpies han estat focalitzades en incrementar el colesterol HDL. Assaigs clínics que han fet augmentar el colesterol HDL han estat associats a un increment de mortalitat, tot i que en estudis recents aquesta ha estat atribuïda a efectes secundaris de la medicació. A més, últimament s'ha publicat que variants genètiques que predisposen a tenir un colesterol-HDL elevat no estan associades a tenir menys risc de patir un event coronari. Tot això ha fet que la funcionalitat de l'HDL sigui considerada com un aspecte més important que la seva quantitat. Els compostos fenòlics (CF) de l'oli d'oliva (OO) són protectors front malalties coronàries del cor incrementant la concentració de colesterol HDL, disminuïnt *in vivo* el dany oxidatiu a lípids, activant la funció de l'HDL, i millorant la funció endotelial. En aquest context, l'enriquiment de l'oli d'oliva verge (OOV) amb CF és una estrategia prometadora que permet no incrementar el greix consumit, no obstant aquest enriquiment podria tenir una doble acció perquè un excés d'antioxidants podria també revertir-los a pro-oxidants. Olis d'oliva verge funcionals amb antioxidants complementaris, acord amb la seva relació estructura/activitat, podria ser una opció adequada per obtenir més efectes beneficiosos.

L'objectiu d'aquest present treball va ser investigar, en humans amb risc cardiovascular, si: (i) un OO verge enriquit amb els seus compostos fenòlics (OOVF; 961 ppm) modula l'expressió gènica de gens relacionats amb l'eflux de colesterol i la funció endotelial, comparat amb un OO verge (OOV; 289 ppm); i si (ii) OOs verge funcionals, un enriquit amb els seus CFOO (OOFV; 500 ppm) i un altre amb els seus CFOO (250 ppm) més CF de la farigola (250 ppm)

(OOFVT; total: 500 ppm), podria millorar propietats relacionades amb la funcionalitat de l'HDL en comparació amb un OO vergec ontrol (OOV; 80 ppm).

Dos assaigs clínics, creuats, aleatoritzats, doble cecs i controlats han estat realitzats: (i) 13 humans pre-/hipertensos van rebre una dosi única de 30 mL de OOV (289 ppm) i OOFV (961 ppm); i (ii) 33 individus hipercolesterolèmics van ingerir 25 mL/dia durant 3 setmanes de OOV (80 ppm), OOVF (500 ppm), i OOVFT (500 ppm).

Els resultats indiquen que una dosi única d'OOVF va incrementar l'expressió gènica de gens relacionats amb l'eflux de colesterol comparat amb el consum de OOV, concretament d'*ATP binding cassette transporter-A1, scavenger receptor class B type 1, peroxisome proliferator-activated receptors* (PPARBP, PPARα, PPARγ, PPARδ), i CD36. A més, aquest consum va augmentar la hiperèmia isquèmica reactiva comparat amb el d'OOV. En aquest sentit, una disminució de l'inhibidor de l'activador del plasminogen-1 va ser observada després de l'OOVF comparat amb l'OOV. Una intervenció sostinguda d'OOVFT va incrementar el percentatge de la subclasse gran d'HDL en comparació a la d'OOV, i també va augmentar els ratios colesterol esterificat/colesterol lliure i fosfolípids/colesterol lliure en HDL comparat amb les intervencions d'OOV i OOVF. A més a més, el consum d'OOVFT va incrementar la massa de lecitin colesterol acil transferasa comparat amb el d'OOV.

En conclusió, un consum postprandial d'OOVF millora la transcriptòmica de l'eflux de colesterol, la funció endotelial, i els marcadors de inflamació. També, els beneficis aconseguits amb el consum sostingut de OOVFT sobre el perfil funcional d'HDL han estat demostrats. Els resultats d'aquests estudis suggereixen que el OOVF i el OOVFT podrien ser eines dietètiques útils en el maneig de pacients amb risc cardiovascular.

RESUMEN

La dislipemia y la hipertensión son factores clásicos de riesgo cardiovascular, relacionados con el desarrollo de la arterosclerosis. Dado que el colesterol de la lipoproteína de alta densidad (HDL) ha estado inversamente relacionado con la aterogénesis, las terapias han estado focalizadas en incrementar el colesterol HDL. Ensayos clínicos que han hecho aumentar el colesterol HDL han sido asociados a un incremento de la mortalidad, aunque en estudios recientes esta ha sido atribuida a efectos secundarios de la medicación. Además, últimamente se ha publicado que variantes genéticas que predisponen a tener un colesterol HDL elevado no están asociadas a tener menos riesgo de sufrir un evento coronario. Todo esto ha hecho que la funcionalidad de la HDL sea considerada como un aspecto más importante que su cantidad. Los compuestos fenólicos (CF) del aceite de oliva (AO) son protectores frente enfermedades coronarias del corazón incrementando la concentración de colesterol HDL, disminuyendo in vivo el daño oxidativo de los lípidos, activando la función de la HDL, y mejorando la función endotelial. En este contexto, el enriquecimiento de aceite de oliva virgen (AOV) con compuestos fenólicos (CF) es una estrategia prometedora que permite no incrementar la grasa consumida, no obstante este enriquecimiento podría tener una doble acción porque un exceso de antioxidantes podría también revertirlos a pro-oxidantes. Aceites de oliva virgen funcionales con antioxidantes complementarios, acorde con su relación estructura/actividad, podría ser una opción adecuada para obtener más efectos beneficiosos.

El objetivo de este presente trabajo fue investigar, en sujetos con riesgo cardiovascular, si: (i) un AO virgen enriquecido con sus compuestos fenólicos (AOVF; 961 ppm) modula la expresión génica de genes relacionados con el eflujo de colesterol y la función endotelial, comparado con un AO virgen (AOV; 289 ppm); y si (ii) AOs vírgenes funcionales, uno enriquecido con sus CFAO (AOVF; 500 ppm) y otro con sus CFAO (250 ppm) más CF del tomillo (250

ppm) (AOVFT; total: 500 ppm), podría mejorar propiedades relacionadas con la funcionalidad de la HDL en comparación con un AO virgen control (AOV; 80 ppm).

Dos ensayos clínicos, cruzados, aleatorizados, doble ciegos y controlados han sido realizados: (i) 13 sujetos pre-/hipertensos recibieron una dosis única de 30 mL de AOV (289 ppm) i AOVF (961 ppm); y (ii) 33 individuos hipercolesterolémicos ingerieron 25 mL/día durante 3 semanas de AOV (80 ppm), AOVF (500 ppm), y AOVFT (500 ppm).

Los resultados indican que una dosis única de AOVF incrementó la expresión génica de genes relacionados con el eflujo de colesterol comparado con el consumo de AOV, concretamente de *ATP binding cassette transporter-A1, scavenger receptor class B type 1, peroxisome proliferator-activated receptors* (PPARBP, PPARα, PPARγ, PPARδ), y CD36. Además, este consumo aumentó la hiperemia isquémica reactiva comparando con el AOV. En este sentido, una disminución del inhibidor-1 del activador de plasminógeno fue observada después del AOVF comparado con el AOV. Una intervención sostenida de AOVFT incrementó el porcentaje de la subclase grande de HDL en comparación al AOV, también aumentó los ratios colesterol esterificado/colesterol libre y fosfolípidos/colesterol libre en HDL comparado con las intervenciones de AOV i AOVF. Además, el consumo de AOVFT incrementó la masa de lecitina colesterol acil transferasa comparado con el de AOV.

En conclusión, un consumo postprandial de AOVF mejora la transcriptómica del eflujo de colesterol, la función endotelial, y los marcadores de inflamación. También, los beneficios conseguidos con el consumo sostenido de AOVFT sobre el perfil funcional de HDL han sido demostrados. Los resultados de estos estudios sugieren que el AOVF y el AOVFT podrían ser herramientas dietéticas útiles en el manejo de pacientes con riesgo cardiovascular.

ABBREVIATIONS

4. ABBREVIATIONS

ABC: ATP-binding cassette transporter

Ang: angiotensin

Apo: apolipoprotein

CD-36: thrombospondin receptor

CETP: cholesteryl ester transport protein

COX: cyclooxygenase

CVD: cardiovascular disease

EC: esterified cholesterol

EF: endothelial function

ELISA: enzyme-linked immunosorbent assay

FC: free cholesterol

FVOO: functional virgin olive oil

FVOOT: functional virgin olive oil with thyme

HDL: high density lipoprotein

HDL-C: high density lipoprotein cholesterol

HPC: high phenolic content

hsCRP: high-sensibility C-reactive protein

HT: hydroxytyrosol

ICAM: intercelular adhesion molecule

IDL: intermediate density lipoproteins

IHD: ischemic heart disease

IL: interleukin

IRH: ischemic reactive hyperemia

LCAT: lecithin cholesterol acyltransferase

LDL: low density lipoprotein

LDL-C: low density lipoprotein cholesterol

LPL: lipoprotein lipase

LXR: liver X receptor

miR: microRNAs

MPC: moderate phenolic content

MUFA: monounsaturated fatty acids

NO: nitric oxide

NOS: nitric oxide syntase

oxLDL: oxidised LDL

OO: olive oil

PAF-AH: platelet-activating factor acetylhydrolase

PAI: plasminogen activator inhibitor

PC: phenolic compounds

PCR: polymerase chain reaction

PL: phospholipids

PLTP: phospholipid transfer protein

PON: paraoxonase

PPAR: peroxisome proliferator activated receptor

PUFA: polyunsaturated fatty acids

ROS: reactive oxygen species

RT-PCR: reverse transcription polymerase chain reaction

RXR: retinoid X receptor

SFA: saturated fatty acids

SRB1: scavenger receptor class B type 1

SREBP: sterol regulatory element binding protein

TG: triglycerides

VCAM-1: vascular cell adhesion molecule type 1

VLDL: very low density lipoprotein

VOO: virgin olive oil

INTRODUCTION

4. INTRODUCTION

4.1. CARDIOVASCULAR DISEASES

4.1.1. DEFINITION AND EPIDEMIOLOGY

Cardiovascular diseases (CVDs) are a group of disorders of the heart and blood vessels, including coronary heart disease, cerebrovascular disease and peripheral arterial disease. The World Health Organization (WHO, 2013) has identified CVDs as the leading cause of death worldwide and estimates that 17.3 million people died from them in 2008, representing 30% of all global deaths. The ratio of coronary heart disease mortality between low income countries, such as those from eastern Europe, and high income ones, for instance from western Europe, is now over four-fold in both sexes. In addition, within western Europe itself there is still an approximately two-fold difference between the highest rates for coronary heart disease in northern and central Europe, and the lowest ones in the Mediterranean countries (1).

4.1.2. CARDIOVASCULAR DISEASE RISK FACTORS

Atherosclerosis and coronary heart diseases are produced by a combination of different risk factors (**TABLE 1**). The major classical ones are hypertension, dyslipemia, obesity, diabetes, and smoking.

1) Dyslipemia:

Epidemiologic studies have demonstrated a dose response relationship between serum total cholesterol concentration and ischemic heart disease (IHD) risk (2;3). Nevertheless, the greatest atherogenic factor is low density lipoprotein (LDL). A high plasma low density lipoprotein cholesterol (LDL-C) concentration implies more substrate to be oxidised, to produce oxidised-LDL (oxLDL), and consequently to initiate atherosclerosis plaque. It has been

reported th at t he e xtent o f f atty s treaks, th e f irst v isible le sion in the development of atherosclerosis, occurring even in childhood, is associated with LDL-C prior to death (4). Moreover, LDL-C level in young adulthood predict coronary and cardiovascular event occurrence later in life (5-7).

In c ontrast, h igh density l ipoprotein cholesterol (HDL-C), ha s be en i nversely related t o a therogenesis (8-10). A n a nalysis of four prospective st udies demonstrated a c onsistent protective ef fect o f H DL-C, s uggesting t hat e ach 1mg/dl increment was associated with 2-3% decrease in coronary risk (11). In a primary pr evention t rial, b aseline l evels o f total ch olesterol, LDL-C, triglycerides (TG), and non-HDL-C were not predictive of IHD events, whereas HDL-C level was significantly p redictive a t b aseline and a lso w ith di fferent ratios (12;13).

2) Hypertension:

Both systolic and diastolic blood pressure have a continuous, independent, positive association with cardiovascular outcomes (14-16). Each increment of 20 mmHg in s ystolic blood pr essure or 10 mmHg i n di astolic bl ood p ressure doubles v ascular m ortality r isk a cross the e ntire range of b lood p ressure f rom 115/75 t o 185 /115 m mHg, i n subjects aged f rom 40 t o 70 y ears (17). T his relationship i s illustrated by t he fact t hat high b lood pressure v alues have b een associated w ith an i ncreased r isk o f C VD (18;19). Nonetheless, t he ass essment of h ypertension h as p laced g reater em phasis o n t he sy stolic component (20). Hypertension has b een s trongly asso ciated w ith the a therosclerosis o f b rain vessels as a major risk factor (21), and its association with IHD seems weaker (22).

3) Smoking:

Some studies have sh own a d ose-dependent r elationship be tween s moking a nd the risk of IHD (23;24). In fact, smoking cessation resulted in a rapid decline in risk for incident and recurrent IHD events (25) and for sudden cardiac death (26) in t hose of a ny a ge w ith a nd w ithout I HD (25;27). I n a ddition, it ha s be come

clear that not only is active smoking associated with increased risk of IHD, but passive smoking also raises the risk by 30% (28).

4) Obesity:

Obesity is an independent risk factor for all-cause mortality (29) and also for chronic metabolic disorder associated with numerous comorbidities such as CVD (30), type 2 diabetes (31), and hypertension (30).

5) Diabetes:

Diabetes is associated with a 2- to 4-fold increase in the likelihood of developing IHD (32), this increment being higher in women than in men (33;34). However, determination of cardiovascular risk in type 2 diabetes continues to be a topic for debate and a moving target.

MODIFIABLE RISK FACTORS	NON MODIFIABLE RISK FACTORS
Dyslipemia	Age
Hypertension	Sex
Diabetes	Family history / Genetics
Smoking	
Lifestyle risk factors (obesity, sedentarism, atherogenic diet)	

TABLE 1. Cardiovascular diseases classical risk factors. Adapted from Harrison's online 2009.

Some classical cardiovascular risk biomarkers for the general population tend to lose predictive power in specific populations, for instance the elderly (35). In addition, the systemic total cholesterol concentration and its fractions transported by lipoproteins, the role of LDL atherogenicity, high density lipoprotein (HDL) functionality and the lipoprotein particle-count subclasses play an increasingly major part (36). In addition, a percentage of individuals

who develop CVD have only one or none of the traditional CVD risk factors (37;38) and might even prove to have a worse prognosis (39). Thus, there is interest in emerging biomarkers (40), such as several cytokines and other markers of inflammation and oxidation, heart failure, renal and heart function.

4.1.3. PATHOPHYSIOLOGY OF ATHEROSCLEROSIS

Atherosclerosis is a chronic inflammatory disease arising from an imbalance in lipid metabolism and a maladaptive immune response driven by the accumulation of cholesterol-laden macrophages in the artery wall. Furthermore endothelial dysfunction takes place parallel to the inflammatory reactions. The different phases of atherosclerosis plaque formation are the following:

1) Infiltration and transformation of lipoproteins in the endothelial space

Among the processes involved in atherosclerosis, oxidative stress is of great relevance and is associated with many of the risk factors involved in its pathophysiology. Oxidative stress produces an excess generation of reactive oxygen species (ROS) which can trigger the onset of atherosclerosis plaque development. The two greatest deleterious effects of ROS include:

(i) Scavenging of endothelium-derived nitric oxide (NO): ROS reacts chemically with NO, and consequently NO levels are reduced. Endothelial dysfunction facilitates the infiltration of lipoproteins in the endothelial space. There, these particles are modified (for example: oxidation, cleavage, and aggregation) leading to their activating pro-inflammatory reactions of the endothelium.

(ii) Oxidation of LDL: LDL particles can be more or less resistant to oxidation. This property is related to the composition and content of the antioxidant compounds which will determine the particles' density and size. Smaller and denser LDL particles can be more easily oxidized. The first step in LDL oxidation is the generation of slightly modified LDL which activates the endothelium and has a strong capacity to induce

monocyte adhesion (41-43). After that, LDL with a higher oxidation capacity is generated. The properties of high grade oxidation LDLs are: a) induction of monocyte quimioatractant protein 1 expression and that of other adhesion molecules, such as interleukin (IL) 1, intercelular adhesion molecule (ICAM) 1, and P-selectin in endothelial cells (44), b) monocyte-to-macrophage differentiation, c) promotion of apoptosis in endothelial cells (45), d) dysfunction of NO production (45), e) proliferation of smooth muscle cells (46), f) modulation of intracellular signalling of inflammation in macrophages through the activation of nuclear kappa B factor (47), and g) platelet aggregation and thrombosis (48).

2) Monocyte immune response

OxLDL induces monocyte quimioatractant protein 1 expression and other adhesion molecules, such as interleuquin 1 (IL-1), ICAM-1, vascular cell adhesion molecule type 1 (VCAM-1), and P-selectin in endothelial cells (49). The monocytes use different chemokine-chemokine receptor pairs to infiltrate the intima, which is facilitated by endothelial adhesion molecules, including ICAM-1, V-CAM1, and selectin. This action leads to an immune response mediated by the monocyte recruitment into the subendothelial space.

3) Foam cell formation

The monocytes are transformed to macrophages in the subendothelial space. These cells have numerous scavenger receptors - scavenger receptor A, thrombospondin receptor (CD-36), MARCO, scavenger receptor member class B type 1 (SRB1), lectin-type oxLDL 1, scavenger receptor class E member 1 (SREC1), and scavenger for phosphatidylserine and oxLDL. When the monocytes are differentiated into macrophages, they take up oxidised and native LDL via scavenger receptors, such as CD-36, SR-A, and LDL-R. CD-36 and SR-A are the principal receptors responsible for the uptake of modified low density lipoprotein leading to lipid loading in macrophages (50). First the cholesteryl esters of the lipoproteins are hydrolyzed to free cholesterol (FC) and

fatty acids in the late endolysomal compartment, and then the FC is re-esterified to cholesteryl fatty acid esters in the endoplasmic reticulum. Finally, these macrophages are converted into cholesterol-laden macrophages or foam cells (51). The posterior accumulation in the intima of foam cells produces the formation of fatty streaks (52). In addition, foam cells secrete pro-inflammatory cytokines – IL-1, IL-6, tumor necrosis factor, chemokine ligand 2, chemokine ligand 5, and CXC-chemokine ligand 1 –, as well as macrophage retention factors (such as netrin 1 and semaphoring 3E), that amplify the inflammatory response.

4) Proliferation of smooth muscle cells

Smooth muscle cells in the media, activated by cytokines and growth factors, migrate to the intima and proliferate, contributing to lesion development (53). A feared complication of atherosclerosis is the destruction of the extracellular matrix by macrophage enzymes which this fact contributes to destabilising and future plaque rupture (53;54). Plaque rupture permits the contact of blood coagulation components with plaque material, including tissue factor, which triggers thrombosis, and can lead to myocardial infarction.

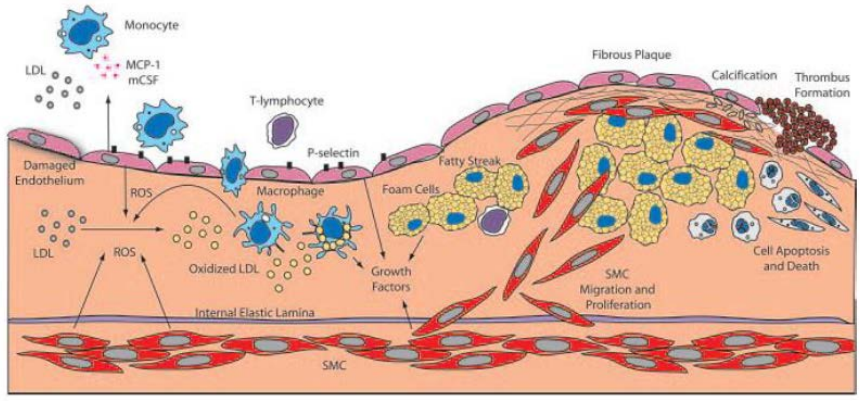


FIGURE 1. Pathophysiology of atherosclerosis (55).

4.1.4. PATHOPHYSIOLOGY OF HYPERTENSION

Blood pressure is regulated by a dynamic equilibrium of different complex mechanisms. The main factor determining systemic blood pressure is the blood arterial volume that, in turn, depends on cardiac output and vascular resistances. In addition to nervous and chemical factors, cardiac output is affected by mechanical features ensuring its adjustment to the venous return. Vascular resistance depends in part on the characteristics of the blood (viscosity) and on the diameter of the vascular lumen. The smooth muscle cell layer of the arteries may produce contraction or relaxation resulting in a parallel increase or decrease of blood pressure, respectively. Several mechanisms regulate vascular tone. Adrenergic sympathetic stimuli exert a vasoconstrictory effect through the activation of alfa1-adrenergic receptors of the vascular smooth muscle cells. In addition, paracrine hormonal and mechanical mechanisms contribute to the fine regulation of vascular tone to modulate blood pressure (56).

Hypertension is a complex, multifactorial disorder involving many organ systems (57;58). Key factors for the development of hypertension include activation of the sympathetic nervous system, up-regulation of the renin-angiotensin-aldosterone system, altered G protein-coupled receptor signaling, inflammation (59;60), and the immune system (61;62). A common feature of these processes is oxidative stress mainly due to an increased production of ROS, decreased NO levels, and reduced antioxidant capacity in the cardiovascular, renal, and central nervous systems (63;64).

Although ROS affect physiological functions through their modulation of the redox state of signalling molecules (65), a imbalanced increment of their levels can lead to deleterious effects in the human organism. ROS are involved in controlling endothelial function (EF) and vascular tone, in addition, they can exert a pathophysiological role in processes underlying endothelial dysfunction, hyperreactivity, inflammation, and vascular remodelling in CVDs, including hypertension (66-68). Nevertheless, a direct causative role of ROS in blood pressure elevation has yet to be demonstrated in humans. NADPH oxidase

(NOX) is the most relevant enzyme implied in hypertension development. NOX has a central role in the cardiac mechanosensing, endothelium dependent relaxation, and the redox signaling regulating tone of angiotensin-II (Ang-II) (56).

Oxidative stress induces acute and chronic inflammatory responses (69). Inflammatory cytokines upregulate inducible nitric oxide synthase (NOS) expression in macrophages and smooth cells. Inducible NOS is involved in compensating the downregulation of endothelial NOS produced by oxidative stress, while cytokines also activate polymorphonuclear lymphocyte NADPH oxidase which produces $O2^{-}$ (70-72). This excessive amount of $O2^{-}$ reacts with inducible NOS activity forming peroxynitrite. Furthermore, inducible NOS activity enhances arginase which reduces NO formation. OxLDL can also upregulates arginase activity and in turn reduce NO. Therefore, the combination of oxidative stress and inflammatory response leads to a decrease in NO biovailability causing endothelial dysfunction and a vasoconstrictive effect. A number of *in vivo* hypertensive models have shown the association of vascular inflammation, endothelial dysfunction, and hypertension (73-75).

4.2. OLIVE OIL

Olive oil (OO) is the primary source of fat in the Mediterranean diet. Its consumption assures an increase in monounsaturated fatty acids (MUFA) intake, without a significant raise of saturated fatty acids (SFA), and guarantees an appropriate ingestion of polyunsaturated fatty acids (PUFA).

4.2.1. OLIVE OIL COMPOSITION

The O O c omposition consists of m ajor a nd m inor c ompounds. The m ajor compounds or sap onificable fraction are the f atty acids which r epresents 98%. The m inor c ompounds o r t he u nsaponificable f raction represents 1 -2%, a nd includes a liphatic a nd t riterpenic a lcohols, s terols, hy drocarbons, v olatile compounds, and antioxidants (76) (**TABLE 2**).

TABLE 2. Chemical composition of OO.

SAPONIFIABLE FRACTION (about 98%)	UNSAPONIFIABLE FRACTION (about 2%)
MUFA Oleic acid (18:1n-9) (55-83%) **PUFA** Linolenic acid (18:3n-3) (0.0-1.5%) Palmitoleic acid (18:3n-3) (7.5-20%) Oleic acid (18:2n-6) (3.5-21%) **SFA** Palmitic acid (16:0) (7.5-20%) Myristic acid (16:0) (0-0.1%) Stearic acid (16:0) (0.5-5%)	Lipophilic phenolics *(tocopherols and tocotrienols)* Hydrophilic phenolics *(phenolic acids, phenolic alcohols, seicoroids, lignans and flavones)* Volatile compounds Pigments *(chlorophylls)* Hydrocarbons *(squalene, β-carotene, lycopene)* Sterols *(β-sitosterol, campesterol, estigmasterol)* Triterpene alcohols Aliphatic alcohols Non-glyceride esters *(alcoholic and sterol compounds, waxes)*

Adapted from Escrich et al 2007 Molecular Nutrition and Food Research (77).

The minor components of OO are classified into two types:

1) The nonpolar fraction: contains sq ualene, o ther t riterpenes, sterols, tocopherol, a nd p igments. T his pa rt c an be e xtracted w ith s olvents a fter saponification of the oil.

2) The polar/soluble fraction: includes the phenolic compounds (PC). The four major classes of OOPC that can be found in these oils are: flavonoids, lignans, simple phenols and secoiridoids. The highest concentrations of simple phenols and secoiridoids are found in OOs and indeed some of them are only present in these substances. The simple phenols are hydroxytyrosol (HT) (3,4-dihydroxy-phenyl-ethanol) and tyrosol (p-hydroxy-phenyl-ethanol), and their secoiridoid derivatives, such as oleuropein, make up around 90% of the total phenolic content of a VOO. The chemical composition of the phenolic fraction of OO has been studied extensively.

The content of the minor components in OO varies depending on cultivar, climate, olive ripeness at harvesting, and the processing system to produce the OO (78).

4.2.2. OLIVE OIL PHENOLIC COMPOUNDS

- **Phenolic acids:** these compounds encompass caffeic, vanillic, p-coumaric, protocatechuic, sinapic, p-hydroxybenzoic and gallic acids. Ferulic and cinnamic acids have also been quantified although in lesser quantities.

- **Flavonoids:** these compounds contain two benzene rings joined by a linear three carbon chain. Luteolin, apigenin and its glycoside forms are found in VOO.

- **Lignans:** they include (+)-pinoresinol and (+)-1-acetoxypinoresinol which are present in the olive fruit and transferred to VOO during the mechanical extraction process (79).

- **Phenolic alcohols:** they have a hydroxyl group attached to an aromatic hydrocarbon one. The main phenolic alcohols in VOO are HT, and p-hydroxyphenyl ethanol or tyrosol. They are at low concentrations in fresh oils,

but increase during storage (79). As they are released in large amounts in the stomach and small intestine they can have remarkable effects.

- **Seicoroids:** the main PC in VOO and present elenolic acid or elenolic acid derivatives in their structure. The main secoiridoids in VOO are the dialdehydic form of decarboxymethyl elenolic acid linked to HT or tyrosol termed 3,4-DHPEA-EDA and *p*-HPEA-EDA, oleuropein aglycon, and ligstroside aglycone. These compounds are intermediate structures of secoiridoid glucosides of the olive fruit oleuropein and ligstroside, originated during the crushing process. They are responsible for the particular bitter organoleptic attribute and the oxidative stability of VOO (80-82).

4.2.3. OLIVE OIL TYPES

Different processing methods produce virgin, ordinary, or pomace OO. Virgin OO (VOO) is obtained by direct pressing or centrifugation of the olives and it is rich in PC (around 150–400 ppm in those generally present on the market). VOO with a free acidity greater than 3.3 grams for every 100 grams (2.0 in the EU) is submitted to a refining process in which some components, mainly PC and to a lesser degree squalene, are lost (83). By mixing virgin and refined OO, an ordinary OO, with a lower phenolic content (around 50–150 ppm) (EU regulation 2568/1991) is produced and marketed. After VOO production, the rest of the olive drupe and seed is submitted to a refining process, after which a certain quantity of VOO is added, and the result product is pomace OO, with a low phenolic content (around 10–70 ppm) (78).

4.2.4. BENEFICIAL EFFECTS OF OLIVE OIL ON CARDIOVASCULAR DISEASES

The benefits on health of OO and the Mediterranean diet, rich in OO, have been extensively studied. The Mediterranean diet is associated with low CVD mortality and a decreased incidence of certain cancer types and neurodegenerative diseases (84;85). The EPIC study demonstrated an inverse association between OO consumption and CHD mortality (86-88). In addition, the Three City Study reported an inverse relationship between OO consumption and stroke risk in women (89). Recently, a primary prevention trial, the PREDIMED study, described that VOO consumption, within the framework of the Mediterranean diet, reduces atrial fibrillation risk (90). Moreover, this study showed with people at high cardiovascular risk that an unrestricted-energy Mediterranean diet, supplemented with extra-VOO or nuts, reduced the incidence of major cardiovascular events, with a relative risk reduction of approximately 30% and CVD mortality (84).

Many authors have provided evidence of the benefits of OO on secondary end points for CVD and on secondary risk factors for chronic degenerative diseases, such as lipid oxidation, lipid disorders, DNA oxidation, inflammation, EF, thrombotic factors, high blood pressure, and insulin sensitivity (85). Within this context, in 2004, the Federal Drug Administration of USA permitted a claim on OO bottle labels stating that two tablespoons (23 g) a day has benefits on CHD risk due to the OO MUFA content.

Currently, scientific studies have demonstrated that these effects should also be attributed to the phenolic fraction of OO. The OO with the highest PC content is the VOO. The main cardiovascular beneficial effects of OOPC demonstrated in randomized clinical trials included:

1) In post-prandial OO interventions:

(i) Antioxidant capacity improvement: an increase in the serum antioxidant capacity after a VOO ingestion in comparison with ordinary OO and corn oil (91).

(ii) Lipid oxidative damage reduction: the phenolic content of an OO modulates the degree of lipid and LDL oxidation; the lipid oxidative damage being lower after consumption of high rather than low phenolic content OO (92).

(iii) Anti-inflammatory and vasculoprotective effects: a decrease in plasma leukotriene B4 (LTB4) and thromboxane B2 (TBX2) (91), and a decrease in ICAM-1 and VCAM-1 (93) were observed after a VOO intervention versus a refined OO one.

(iv) Prothrombotic profile improvement: an improvement in haemostatic factors was observed after a VOO intervention in comparison with a refined OO one, in healthy subjects (94) and hypercholesterolemic patients (95).

(v) EF improvement: an enhancement of ischemic reactive hyperaemia (IRH) in hypercholesterolemic patients was reported following phenol-rich VOO consumption in comparison with a low phenolic one (96).

2) In sustained OO interventions:

(i) Lipid oxidative damage reduction: a decrease in lipid oxidative damage biomarkers such as plasma uninduced conjugated dienes and hydroxyl fatty acids was observed after a consumption of medium and high phenolic content OO, within the context of the EUROLIVE study (97). In addition, in a subsample of the PREDIMED study, after a Mediterranean diet enriched with VOO, a decrease in LDL oxidation was observed in a significant manner when compared with the control group (low-fat diet) (98).

(ii) HDL quantity increment: within the framework of EUROLIVE study, an increase in HDL-C was also observed in a linear relationship with the phenolic content of the OO consumed (97). Moreover, an

improvement of cholesterol efflux promoted by the HDL particle after the VOO consumption was reported (36;99).

(iii) LDL and LDL atherogenicity reduction: the consumption of olive oil polyphenols decreased plasma LDL concentrations and LDL atherogenicity in healthy young men (100).

(iv) DNA oxidation damage reduction: the protective effects of OOPC on in vivo DNA oxidation damage were observed in healthy male volunteers in a short-term study in which participants were submitted to a very low antioxidant diet (101). A protective effect of DNA was also found in postmenopausal women after a high-phenol VOO intervention (102). In the EUROLIVE study, however, all the OO interventions reduced DNA oxidation irrespective of their phenolic content (103).

(v) Anti-inflammatory and vasculoprotective effects: OO with high phenolic content have been shown to reduce the eiconsanoid inflammatory mediators derived from arachidonic acid, such as thromboxane B 2 and 6 -ketoprostaglandin F1α (104;105), as well as other inflammatory markers, such as high-sensibility C-reactive protein (hsCRP) and IL-6 (106;107).

(vi) EF improvement: an enhancement of EF has been observed after 4-month diet of PC-rich OO in subjects with early atherosclerosis (108).

(vii) Blood pressure reduction: after a VOO intervention, a decrease in systolic and diastolic blood pressure was reported in young woman with mild hypertension (107). Nevertheless, in other VOO interventions there was only observed a reduction in blood pressure effect in systolic blood pressure (SBP) in hypertensive women (109), hypertensive stable patients with CHD (110), and in healthy subjects, and diabetic individuals (111).

In 2011, the European Food and Safety Authority (EFSA) recognized a health claim for OO polyphenols contributing to the protection of LDL from oxidation,

for OO that contains at least 5 mg of HT and its derivatives (e.g. oleuropein complex and tyrosol) per 20 g of OO (112). These quantities, if provided through moderate amounts of OO, can be easily consumed in the context of a balanced diet.

4.3. FUNCTIONAL FOOD

4.3.1. FUNCTIONAL FOOD DEFINITION

The concept of functional food was first scientifically promoted in Japan in 1984. In the USA, evidence-based health and disease prevention claims have been allowed since 1990 by the Food and Drug Administration (FDA) (113). In the latter half of the 1990s, the European Commission funded "Functional Food Science in Europe" (FUFOSE) that produced a consensus report which has become widely used as a basis for discussion and further evolution of thought on the topic (114). The following definition was established: " A food can be regarded as functional if it is satisfactorily demonstrated to beneficially affect one or more target functions in the body beyond adequate nutritional effects in a way that is relevant to either an improved state of health and well-being or a reduction of disease risk". It was also laid down that functional food must remain food and demonstrate its effects when consumed in normally expected daily quantities. In 2006, in the European Union, Regulation (EC) No. 1924/2006 *On nutrition and health claims made on food*, appeared. It limited the use of nutrition and health claims by establishing the need for an extensive review of the strong scientific evidence submitted to the food authority. Four types of claims can be used by food manufacturers to communicate health effects on food labels: 1) nutrient content, 2) structure/function, 3) health, and 4) qualified health (115).

In practice, there is a wide range of possibilities related with the concept of functional foods. A functional food can be:

(i) an unmodified natural food;
(ii) a food in which a component has been enhanced through special growing conditions, breeding or biotechnological means;
(iii) a food to which a component has been added to provide benefits;
(iv) a food from which a component has been removed by technological or biotechnological means so that the food produces benefits not otherwise available;
(v) a food in which a component has been replaced by an alternative component with favourable properties;
(vi) a food in which a component has been modified by enzymatic, chemical or technological means to provide a benefit;
(vii) a food in which the bioavailability of a component has been modified;
(viii) or a combination of any of the above.
According to the various definitions, the main purpose of a functional food should be clear – to improve human health and well-being.
Within the context of CVD and diabetes mellitus type 2 there currently exists a long list of conventional foods with bioactive components that have benefits in the prevention of these diseases, including: whole grains, fruit, vegetables, legumes, dairy products, fish, green tea, OO, dark chocolate, garlic, cinnamon, turmeric, fenugreek, and red wine (116).
Moreover, the definition of functional food establishes a clear separation from nutraceuticals, which can be considered as diet supplements that deliver a concentrated form of a presumed bioactive component from a food, presented in a nonfood matrix, and used with the purpose of enhancing health in dosages that exceed those that could be obtained from natural source foods (117). The nutraceutical presentations are similar to drugs: pills, extracts, tablets, and the like (118).

4.3.2. FLAVOURED OLIVE OILS

In recent years, some OOs flavoured with herbs or spices have emerged on the market. Dry herbs or their extracts are used in oils and lipid-containing foods to retard oxidative deterioration. The most commonly employed spices and aromatic herbs as flavour enhancers in OOs within the Mediterranean area encompass garlic, hot pepper, rosemary, bay leaf, oregano, and thyme. These oils contain a wide variety of active phytochemicals, including flavonoids, phenolic terpenes and phenolic acids. Apart from enhancing flavour, these different bioactive compounds can provide many beneficial health effects. Improvements in lipid metabolism (119), efficacy as antidiabetics (120), antimicrobial action (121), efficacy as digestive stimulants (122), potential anticarcinogenic (123), antioxidant and anti-inflammatory properties (Srinivasan et al 2005) have all been described.

4.3.2.1. HERB AND SPICE PHENOLIC COMPOUNDS

- Phenolic terpenes: the largest class of natural products with > 55,000 known structurally diversified compounds. They form part of the secondary metabolism of plants and animals and are widely used in the industrial sector, perfumery, cosmetic products, and food additives as well as in the pharmaceutical industry where they are employed as active compounds of drugs. The wide range of their biological properties makes them potentially suitable interesting for clinical application. The biological activities described for terpenoids are cancer chemopreventive effects and antimicrobial, antifungal, antiviral, antihyperglycemic, analgesic, anti-inflammatory and antiparasitic activities (124). The most representative herb monoterpenes are thymol and carvacrol which are mainly present in thyme and oregano, respectively. They contribute to the herbs' characteristic aromas and have been described as potent antioxidants. A wider range of pharmacological properties for these compounds, such as analgesic capacity, has been reported (125). The main diterpenes in aromatic

herbs are carnosic acid and carnosol, together with rosmarinic acid, a hydroxycinnamic acid ester, are the principal antioxidant compounds in rosemary (126).

- **Hydroxycinnamates and phenylpropanoids:** the secondary plant metabolites of phenylalanine and tyrosine. Hydroxycinamic acids are precursors of many other complex phenols. They are found in almost all food groups, and at high levels in herbs and spices (127). A body of knowledge has demonstrated the *in vitro* and *in vivo* antioxidant capacity of hydroxycinnamates, they could, therefore, exert various health benefits in chronic diseases with associated oxidative damage. *p*-coumaric acid has a major presence in oregano, and eugenol (4-allyl-2 methoxyphenol), the principal component of cloves (128), is known for its aroma and medicinal values. Rosmarinic acid is typically found in Lamiaceae plants (basil, rosemary, thyme, mint and oregano) (129). It acid has two catechol structures, which is the structure with more antioxidant activity in PC.

- **Flavonoids:** the majority of flavonoid classes are found in herbs and spices. It has been reported that long-term administration of flavonoids can decrease, or at least, tend to decrease, CVD incidence and its consequences (130;131).

4.3.3. ANTIOXIDANT MECHANISMS OF PHENOLIC COMPOUNDS

ROS are essential for homeostasis in human organism and they appear to play a major role in the key intracellular signal transduction pathways (inflammation, proliferation, migration, differentiation, angiogenesis, ageing, and apoptosis) (132;133). Nevertheless, ROS are also products of oxidation reactions, and when they are in excess, for instance as a consequence of stress, exposure to environmental pollution, and ageing, they can trigger chain reactions leading to cell damage and death. ROS are free radicals and highly reactive oxidizers of DNA, lipids and proteins (134).

The biological oxidative effects of ROS in the organism are controlled by the antioxidant defence system (135). There are two kinds of antioxidant defenses:

- Endogenous antioxidant: in the cells and other structures such as lipoproteins. They can be classified as (i) enzymatic and (ii) non-enzymatic antioxidants. The major antioxidant enzymes directly involved in the neutralization of reactive species are: superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase, and glutathione reductase. The non-enzymatic antioxidants are produced by body metabolism (glutathione, L-arginine, coenzyme Q 10, melatonin, uric acid, bilirubin, metal-chelating proteins, transferrin, and the like).

- Exogenous antioxidants (or nutrient antioxidants): they cannot be produced in the body and must be provided through foods or supplements. The antioxidants of exogen source include tocopherols, retinol, carotenoids and PC among others. Tocopherols, retinol, and carotenoids play a key role in a number of functions in the organism and they need to be maintained at normal levels in the body. Diet PC are treated as xenobiotics in spite of exerting important antioxidant activity. It has been reported that despite their benefits, the intake of large amounts of nutrient antioxidants can also act as pro-oxidants by increasing oxidative stress (136;137). Therefore, overconsumption of antioxidant supplements could be harmful (138;139).

4.3.4. DEVELOPMENT OF THESIS FUNCTIONAL OLIVE OILS

1) OLIPA functional olive oils:

- Functional virgin olive oil (FVOO) / High phenolic content olive oil (HPC-OO) (961 ppm): 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 (140). 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 / Moderate phenolic content olive oil (MPC-

OO), until fully homogenised, using a Polytron (Kinematica, Littau, Switzerland).

2) VOHF functional olive oils:

- Functional virgin olive oil (VOO) (500 ppm): was enriched with its own PC by the addition of a phenol extract obtained from freeze-dried olive cake.

- Functional virgin olive oil with thyme (FVOOT) (500 ppm): enriched with its own PC and complemented with thyme phenolics using a phenol extract obtained from a mixture of freeze-dried olive cake (hydroxytyrosol derivates) and dried thyme (flavonoids, phenolic acids and monoterpenes). The resulting FVOOT contained 50% olive PC (250 ppm) and 50% thyme phenolics (250 ppm).

The main difference among the oils is their PC content, their fatty acids and fat-soluble micronutrients are very similar. The FVOO presents the highest amount of HT derivatives, whereas the FVOOT has the greatest amount of flavonoids, and lignans, and is the only OO with detectable monoterpenes. The procedure to obtain the phenolic extracts and enriched oils has been previously described (141).

4.4. HDL CHARACTERISTICS

4.4.1. LIPOPROTEIN STRUCTURE

Lipoproteins are particles with a central hydrophobic core of non-polar lipids (cholesterol esters and TG) and a hydrophilic monolayer which contains phospholipids (PL), FC, and apolipoproteins (Apos). The seven different classes of plasma lipoproteins are shown in the following table.

LIPOPROTEINS	DENSITY (g/ml)	SIZE (nm)	MAJOR LIPIDS	MAJOR APOPROTEINS
Chylomicrons	<0.930	75-1200	Triglycerides	Apo B-48, Apo C, Apo E, Apo A-I, A-II, A-IV
Chylomicron Remnants	0.930-1.006	30-80	Triglycerides Cholesterol	Apo B-48, Apo E
VLDL	0.930-1.006	30-80	Triglycerides	Apo B-100, Apo E, Apo C
IDL	1.006-1.019	25-35	Triglycerides Cholesterol	Apo B-100, Apo E, Apo C
LDL	1.019-1.063	18- 25	Cholesterol	Apo B-100
HDL	1.063-1.210	5- 12	Cholesterol Phospholipids	Apo A-I, Apo A-II, Apo C, Apo E
Lp (a)	1.055-1.085	~30	Cholesterol	Apo B-100, Apo (a)

TABLE 3. Lipoprotein classes. Adapted from Feingold KR 2015 (142).

4.4.2. HDL STRUCTURE

In comparison to other classes, HDL is a small, dense, protein-rich lipoprotein. Its mean size is 8–10 nm and density 1.063–1.21 g/ml (143). HDL particles are plurimolecular, quasi-spherical or discoid, pseudomicellar complexes composed mainly of polar lipids solubilized by apos. HDL also contains numerous other proteins, such as enzymes and acute-phase proteins, and could have small amounts of nonpolar lipids. Furthermore, HDL particles are highly heterogeneous in their structural, chemical and biological properties.

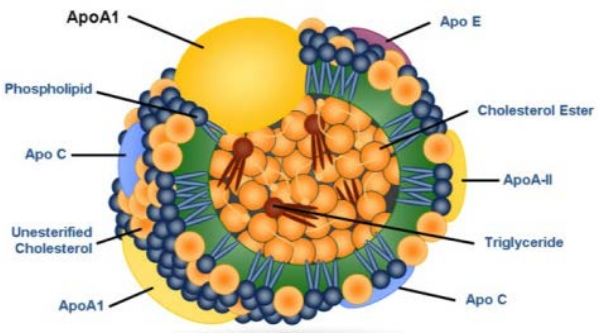


FIGURE 2. HDL structure. Reference: ***http://www.namrata.co/reverse-cholesterol-transport-and-the-role-of-hdlc/***

4.4.3. HDL SUBCLASSES

Differences in HDL subclass distribution were first described by Gofman et al by using analytic ultracentrifugation (144), the gold standard technique for HDL isolation. Two HDL subclasses were identified: the less and more dense were classified as HDL2 (1.063–1.125 g/mL), and HDL3 (1.125–1.21 g/mL), respectively. HDL2 and HDL 3 are relatively rich in lipids and protein, respectively.

Other methods based on size, charge, shape, and protein composition of the lipoprotein have been proposed to isolate them (see **TABLE 4**).

SEPARATION TECHNIQUES	HDL SUBCLASSES
Density (ultracentrifugation)	HDL2 HDL3
Size (GGE)	HDL2a HDL2b HDL3a HDL3b HDL3c
Size (NMR)	Large HDL Medium HDL Small HDL
Shape and charge (agarose gel)	α-HDL (spherical) Preβ-HDL (discoidal)
Charge and size	Preβ-HDL ($Pre\beta_1$ and $Pre\beta_2$)

(2D electrophoresis)	α-HDL (α_1, α_2, α_3 and α_4) Preα-HDL (preα_1, preα_2, preα_3)
Protein composition (electroimmunodiffusion)	LpA-I LpA-I:A-II

TABLE 4. Major HDL subclasses according to different isolation/separation techniques. Adapted from Kontush et al 2015 Structure of HDL: Particle Subclasses and Molecular Components (145).

The nomenclature and units of HDL subclasses vary among the different isolation methodologies. Experts have proposed a classification according to the physical properties of HDL particles, which integrates terminology from several methods and defines five HDL subclasses (very large, large, medium, small, and very small HDL) (146).

A number of studies have been focused on assessing the relationship between HDL subclasses and CVD, and the beneficial properties of each HDL subclass. Several population studies have suggested that HDL2 particles may be more cardioprotective than HDL3 (147;148). Low levels of HDL2 and/or high levels of HDL3 are present in CHD (149), ischemic stroke (150), type II diabetes mellitus (151), and peripheral arterial disease patients (152). Although there are also *in vitro* experiments that show similar effects of HDL3 and HDL2 (153), increased circulating small HDL could suggest an aberration in the maturation of HDL and further impaired reverse cholesterol transport (154). Moreover, HDL2 particles bind better to SR-B1 (155), thus they are more effective in promoting cholesterol efflux via this receptor (156). In addition, HDL2 have three ApoA1 in comparison to HDL3, which only contain two, suggesting better cholesterol efflux capacity for HDL2 particle also via ATP-binding cassette transporter A1 (ABCA-1). With reference to the antioxidant status, similarities between HDL2 and HDL3 have been described (157-159).

4.4.4. MOLECULAR COMPONENTS OF HDL

The composition of HDL could be related to its functionality. It has been described that HDL with a triglyceride-rich core has ApoA-1 more loosely bound to the HDL surface, and consequently, this particle has less capacity to enhance cholesterol efflux (160). An HDL surface with a lower cholesterol/PL ratio is also a characteristic related to a greater cholesterol efflux capacity via an aqueous diffusion pathway, because the direction of net cholesterol mass transport is determined by the cholesterol concentration gradient (161).

4.4.4.1. MAJOR PROTEIN COMPONENTS

Proteins form the major structural and functional component of HDL particles. HDL proteins can be classified into major subgroups which include Apos, enzymes, lipid transfer proteins, acute-phase response proteins, complement components, proteinase inhibitors and other protein components (145). Proteomic studies have permitted the reproducible identification of more than 100 proteins in human HDL (162-166). The major proteins of HDL are described in **TABLE 5**.

PROTEINS	CHARACTERISTICS AND MAJOR FUNCTION
APOs	
Apo A-I	Major structural and functional HDL protein (70% total protein). Main functions are: interaction with cellular receptors, activation of lecithin cholesterol acyltransferase (LCAT), and multiple anti-atherogenic activities.
Apo A-II	The second most abundant HDL protein (20% total protein). It is structural and functional Apo
Apo A-IV	Structural and functional Apo.
Apo C-I	Modulator of cholesteryl ester transport protein (CETP) activity, LCAT activator.
Apo C-II	Activator of lipoprotein lipase (LPL).
Apo C-III	Inhibitor of LPL. Furthermore, Apo C-III inhibits the interaction of triglyceride-rich lipoproteins with their receptors.
Apo C-IV	Regulates TG metabolism.
ApoD	Binding of small hydrophobic molecules.
ApoE	Structural and functional Apo, ligand for LDL receptor and LDL

	receptor related protein (LRP).
ApoF	An inhibitor of CETP.
ApoH	Binding of negatively charged molecules.
ApoJ	Binding of hydrophobic molecules, interaction with cell receptors
ApoL-I	A trypanolytic factor of human serum.
ApoM	Binding of small hydrophobic molecules
ENZYMES	
LCAT	Esterification of cholesterol to cholesteryl esters. In addition, LCAT is an enzyme related to HDL antioxidant activity and prevents the oxidation of LDL.
Paraoxonase (PON) 1	Calcium-dependent lactonase. PON1 is associated with HDL and exerts a protective effect against the oxidative damage of cells and lipoproteins.
Platelet-activating factor acetylhydrolase (PAF-AH)	Hydrolysis of short-chain oxidized PL.
Glutathione peroxidase 3	Reduction of hydroperoxides by glutathione
LIPID TRANSFER PROTEINS	
Phospholipid transfer protein (PLTP)	Conversion of HDL into larger and smaller particles, transport of lipopolysaccharide.
CETP	Heteroexchange of CE and TG and homoexchange of PL between HDL and apoB-containing lipoproteins

TABLE 5. Major protein components of HDL. Adapted from Kontush et al 2015 Structure of HDL: Particle Subclasses and Molecular Components. (145)

Moreover, HDL on its surface can also present acute phase proteins (serum amiloide A 1, serum amiloide A 4, Alpha-2-HSglycoprotein, and Fibrinogen alpha chain), complements components (C3), proteinase inhibitors (Alpha-1-antitrypsin, Hrp), and other proteins (transthyretin, serotransferrin, vitamin D-binding protein, alpha-1Bglycoprotein, hemopexin) (145).

4.4.4.2. LIPID COMPONENTS

The HDL lipidome (167), sphingolipidome, and phospholipidome were recently reported (168). The different HDL lipid classes are described in **TABLE 6**.

LIPIDS	CHARACTERISTICS AND MAJOR FUNCTION
PLs	
Phosphatidylcholine	The principal circulating PL that accounts for 32–35 mol % of total lipids in HDL. Phosphatidylcholine is a structural lipid, consistent with its even distribution across HDL subpopulations.
Lysophosphatidylcholine	A major PL subclass in HDL (1.4–8.1 mol % total lipids). It is derived from the regulated degradation of phosphatidylcholine by phospholipases, including LCAT, due to the preferential association of the latter with HDL particles.
Phosphatidylethanolamine	Moderately abundant in HDL (0.7–0.9 mol % total lipids), and its content tends to rise with increasing HDL hydrated density.
Plasmalogens	Contain a vinyl ether-linked fatty acid essential for their specific antioxidative properties. Phosphatidylcholine-plasmalogens are the most abundant species in HDL (2.2–3.5 mol %) although they represent less than 10 % of total phosphatidylcholine.
Isoprostanes	Well established as biomarkers of oxidative stress/inflammation and predominantly associated with HDL.
Phosphatidylinositol, phosphatidylserine, phosphatidylglycerol, phosphatidic acid, and cardiolipin	Negatively charged PL present in HDL which may significantly impact the net surface charge of HDL. The content of these lipids can thereby modulate lipoprotein interactions with lipases, membrane proteins, extracellular
SPHINGOLIPIDS	
Sphingomyelin	A structural lipid which enhances surface lipid rigidity, it is the major sphingolipid in circulating HDL (5.6–6.6 mol % total lipids), and largely originates from triacylglyceride-rich lipoproteins and to a minor extent from nascent HDL.
Lysosphingolipids	Among them sphingosine 1 phosphate is a bioactive lipid with key roles in vascular biology. More than 90 % of circulating sphingoid is found in HDL and albumin-containing fractions.
Ceramide	An intermediate sphingolipid involved in cell signaling, apoptosis, inflammatory responses, mitochondrial function and insulin sensitivity. This lipid is poorly transported by HDL.
NEUTRAL LIPIDS	
Unesterified (free) sterols	Present in the surface lipid monolayer of HDL particles and regulate fluidity. HDL sterols are dominated by cholesterol. Other sterols are located in lipoproteins at

	much lower levels.
Cholesteryl esters	Largely (up to 80 %) formed in circulating HDL as a result of transesterification of PL and cholesterol catalysed by LCAT. These highly hydrophobic lipids form the lipid core of HDL.
Triacylglicerides	Dominated by species containing oleic, palmitic and linoleic acid moieties and represent around 3 mol % of total HDL lipids.
Diacylglycerides, monoacylglycerides, and free fatty acids	Minor bioactive lipids present in HDL. The free fatty acids that predominant in HDL include palmitic, stearic and oleic acid-containing species.

TABLE 6. HDL lipid components. Adapted from Kontush et al 2015 Structure of HDL: Particle Subclasses and Molecular Components. (145)

4.4.5. HDL MONOLAYER FLUIDITY

The lipids in the surface monolayer determine its monolayer fluidity and other physical properties, which in turn influence apo composition, conformation, and binding. HDL monolayer fluidity could be an indicator of HDL functionality. In this regard, some studies have reported that the more fluid the monolayer is the greater the cholesterol efflux production (36;99). Furthermore, in accordance with that previously described, an HDL monolayer with less FC is more fluid (169), and consequently more functional in carrying out cholesterol efflux. Moreover, the antioxidant content can also modify HDL monolayer fluidity. It has been reported that an increment of antioxidants in biological membranes could increase fluidity (170), in contrast, others authors have reported that antioxidants could rigidify membranes thus avoiding oxidation transmission (171). Regarding monolayer lipoprotein fluidity, Girona J et al. observed that HDL oxidation produces a decrease in HDL monolayer fluidity and cholesterol efflux (172).

4.4.6. BENEFICIAL EFFECTS OF OLIVE OIL ON HDL CHARACTERISTICS

VOO-based interventions can modify HDL composition. It has been described that a VOO intervention in humans produces a trygliceride-poor core (36) and an increase of Apo-A1 (173;174) and ApoA-IV (166). This ApoA-IV increment has also been reported in apoE-deficient mice after a VOO-rich diet (175). In addition, it has been shown that VOO can increase HDL monolayer fluidity in healthy humans (36;99).

VOO consumption leads to changes in HDL subclass distribution and particle number. A short-term consumption of VOO in healthy individuals was reported to induce a non-significant increasing trend in HDL particle number (36). In addition, a VOO (36) and a VOO-rich Mediterranean diet (176;177) were observed to increase large HDL particles. This result was also found in rats after a supplementation with OOPCs (178).

4.5. HDL METABOLISM

4.5.1. LIPOPROTEIN METABOLISM

a) Exogenous lipoprotein pathway:

The exogenous pathway transport diet lipids to peripheral tissues and liver. Firstly, dietary fatty acids and monoacylglicerols are absorbed in the intestine and used to synthetize TG through monoacylglicerol acyltransferase and diacylglycerol transferase. In addition, most dietary cholesterol absorbed by the intestine is esterified by acyl-CoA cholesterol acyl transferase. The cholesterol esters and tryglicerides are packaged into chylomicrons in the endoplasmatic reticulum of enterocytes. Apo B-48 is required to create chylomicrons, and microsomal triglyceride transfer protein is needed to move lipids from the endoplasmic reticulum to the Apo B-48 (179).

Chylomicrons are secreted into the lymph and delivered to the circulation. The largest content of the chylomicrons is delivered to muscle and adipose tissue, which express high levels of LPL. This enzyme is activated by the Apo C-II of the chylomicrons and it hydrolyzes the chylomicron TG into free fatty acids, which are taken up by the muscle cells and adipocytes. Some free fatty acids from chylomicrons bind to albumin and can be transported to other tissues (180).

Chylomicrons remnants are enriched in cholesteryl esters and acquire ApoE. These particles decrease in size and PL and apos (ApoaA and C) on the chylomicron surface are transferred to other lipoproteins, mainly HDL. The transfer of Apo C-II from chylomicrons to HDL decreases the ability of LPL to continue hydrolyzing TG. The chylomicrons are taken up by the hepatocytes through the binding of the chylomicron ApoE to the LDL receptor and other hepatic receptors. Chylomicron cholesterol, in the liver, can be used for very low density lipoprotein (VLDL) formation, bile acids, or secreted back to the intestine (180).

b) Endogenous lipoprotein pathway:

Endogenous lipoprotein pathway transport liver lipids to peripheral tissues. In the liver, TG and cholesterol esters are transferred in the endoplasmic reticulum to newly synthesized ApoB-100. This process, mediated by microsomal triglyceride transfer protein, creates the VLDLs. These particles are transported to peripheral tissues where TG are hydrolyzed by LPL and fatty acids are released. The consequent VLDL remnants are called Intermediate Density Lipoproteins (IDL). These IDL particles are enriched in cholesterol esters and acquire ApoE from HDL. A part of these IDL (approximately 50%) is cleared in the liver via binding ApoE to LDL and LRP receptors. The remaining IDL TG are metabolized by hepatic lipase and IDL apos are transferred to other lipoproteins leading to the formation of LDL (181).

The production rate of LDL from VLDL is partially determined by hepatic LDL receptor activity. A high LDL receptor level increases LDL production due to an increase in IDL uptake. The LDL receptor levels in liver are regulated by the cell cholesterol content. A lower cholesterol content signifies that more LDL receptor levels are synthetized (181).

4.5.2. HDL METABOLISM

HDL particles are necessarily to transport the cholesterol from peripheral tissues to the liver in a process called reverse cholesterol transport. To generate mature HDL the synthesis of the main HDL protein, Apo A-I, is required. Apo A-I is synthetized and secreted in the liver and intestine where it acquires cholesterol and PL released from hepatocytes and enterocytes, among other cells. This transfer is mediated by ABCA-1. Later, HDL also acquires lipid from other tissues (muscle cells and adipocytes), lipoproteins (VLDL and trygliceride-rich lipoproteins), and chylomicrons (182;183).

The cholesterol effluxed from cells to HDL is FC and is situated on the HDL particle surface. In order to transforms small discoidal HDL to mature large spherical HDL particles with a core of cholesterol esters, cholesterol from the surface is esterified by LCAT and is transferred to the HDL core. ApoA1 is an activator of LCAT (182;183).

CETP extracts EC from the HDL core to TG-rich lipoproteins, returning TGs from TG-rich lipoproteins to HDL (143). These EC-poor-TG-rich HDL particles are substrates for hepatic lipase (HL) that metabolizes the TGs. HL depletes the particles of core lipids, generating a redundancy of surface. A TG-poor HDL core may imply better functional properties of the particle since when the HDL core is TG-rich, ApoA-I is more loosely bound to the HDL surface (160).
HDL cholesterol is mainly delivered to the liver, mediated by SR-B1. A smaller cholesterol-depleted HDL is returned to the circulation. Apo A-I is metabolized

independently of HDL-C by the kidneys, and the remainder by the liver (182;183).

Another enzyme involved in HDL metabolism is the PLTP. PLTP transfers PL between HDL and VLDL. Moreover, this enzyme remodels HDL into large and small particles (**FIG. 3**).

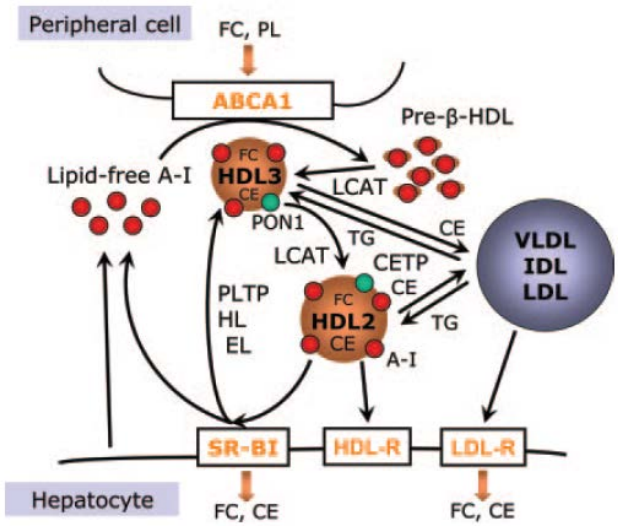


FIGURE 3. HDL metabolism. Reference: Kontush A, Pharmacological Reviews 2006 (143).

4.5.3. BENEFICIAL EFFECTS OF OLIVE OIL ON HDL METABOLISM

A number of studies have reported the effects of VOO on HDL metabolism in humans. After a sustained intervention of VOO during three weeks in healthy humans, CETP and LCAT did not show significant differences (36). Neither did the latter present changes after an OO sustained intervention of 60ml/day during two weeks in males with mild hypertension (184).

In animal models, similar effects have been reported. Dietary oleic acid versus corn oil had no effects on LCAT in mice (185). In this regard, after a cholesterol-enriched (1 g/kg) semipurified diet containing 200 g/kg of OO for 9 weeks in hamsters, non-differences were observed in LCAT, CETP, and PLTP (186). After a post-prandial 7 mL intervention of VOO in rats, hepatic mRNA expression of PLTP increased whilst LCAT gene expression remained unchanged (187). Moreover, squalene, the main hydrocarbon in the unsaponifiable fraction of VOO, increased LCAT hepatic expression after an administration of 1 g/kg in Apoa1- and Apoe- deficient mice (188).

4.6. REVERSE CHOLESTEROL TRANSPORT

4.6.1. REVERSE CHOLESTEROL TRANSPORT DEFINITION

Reverse cholesterol transport is the global process including the removal of excess cholesterol from peripheral cells and tissues, the transport of cholesterol to the liver for catabolism, its transformation into bile acids or other components, and elimination from the organism.

The first step in reverse cholesterol transport is cholesterol efflux, which consists of free-cholesterol efflux from the cell membrane to HDL. Cholesterol efflux from lipid-loaded cells forms part of the atheroprotective mechanism. An imbalance between the cholesterol efflux and its uptake determines atherosclerosis development and progress. The main cells that perform cholesterol efflux are the macrophages in which there are various pathways to carry out this process (189):

1) Aqueous diffusion: the main passive pathway (30% efflux) of cholesterol from the membrane to HDL. HDL particles with high fluid monolayer (shorter PL length and increased chain unsaturation) accept FC molecules from the cell

membrane at a faster rate than those containing highly organized lipid surfaces with restricted PL acyl chain mobility (190).

2) SR-B1: mediates an unspecific, slow, passive, and bidirectional cholesterol efflux (191;192). SR-B1 plays a role in the efflux of cholesterol from cells to mature HDL. At low HDL concentrations, binding of HDL to SR-B1 is critical, allowing bidirectional FC transit. This is because the FC concentration gradient between the bound HDL and the cell plasma membrane is contrary to that of CE. Furthermore, a high FC/PL ratio in cell membrane causes a transport of FC out of the cell. In this regard, increasing PL in HDL particles enhances FC efflux from the cell (193). It has been described that large HDL particles improve FC efflux more than smaller ones because they bind better to SR-B1 (156).

3) ABCG-1: intervenes in a specific, fast, active, and unidirectional cholesterol efflux (191;192). ABCG-1 mediates the FC and PL from cells to mature large spherical HDL2 and HDL 3 particles (194;195), but not to lipid-free apoA-1 (153;196). This transporter is located in endosomes, thus it can transport FC from the endoplasmic reticulum to cell membrane. Moreover, ABCG-1 promotes the efflux of 7-ketocholesterols and related oxysterols, preventing in this way the apoptosis of macrophages (197;198).

4) ABCA-1: brings about mediates an specific, fast, active, and unidirectional cholesterol eflux (191;192). ABCA-1 is responsible for transporting intracellular FC and PL to extracellular lipid poor pre-beta Apo A1, providing a key step in the formation of mature and spherical HDL. ABCA-1 actively transports phosphatidylcholine, phosphatidylserine, and sphingomyelin (199).

In addition, another mechanism that facilitates cholesterol efflux from macrophages is apoE secretion (200). It has been reported that apoE production by human and mouse macrophages activates cholesterol efflux to HDL. Moreover, another action that enhances cholesterol efflux is the inhibition of

acylCoA:cholesterol acyltransferase. This enzyme is responsible for the esterification for the cholesterol accumulated in macrophages (201).

Furthermore, Luo et al (202) describes that there is a caveolae transport centre. Caveolae are small invaginations of the plasma membrane and are rich in cholesterol and phosphosphingolipids, their formation and maintenance is primarily due to caveolin. Caveolae mediates transmembrane cholesterol transportation and the endocytosis and transitocytosis of lipoprotein (203;204). Specifically, the caveolin-1 complex system transports chlesterols from intracellular compartments into caveolae. After that, the ABCA1 and the SRB1 complex systems transfer the cholesterol from caveolae to HDL/ApoA1. Furthermore, it has been reported that caveolaes and caveolin-1 play a part in the endocytosis and transcytosis of oxLDL in endothelial cells (205).

The final efficiency of serum in accepting cellular cholesterol depends on HDL particle distribution and cell cholesterol transporter levels. Some pathways need different HDL subpopulations for an optimal function (189).

Once cholesterol is transferred from cells to HDL there are three pathways for the uptaken of cholesterol by the liver (142): 1) HDL can interact with hepatic SR-B1 resulting in the selective uptake of cholesterol from HDL particles, 2) CETP can transfer cholesterol from HDL particles to particles with ApoB which will then be uptaken by the liver, and 3) HDL can interact with HDL-R in the liver.

After that, cholesterol is eliminated by the liver through different pathways (142): 1) cholesterol is converted to bile acids which are then secreted into the bile; and 2) cholesterol is directly secreted into the bile.

4.6.2. REVERSE CHOLESTEROL TRANSPORT REGULATION

There are many nuclear factors that regulate the RCT-related gene expression:

- Peroxisome proliferator activated receptors (PPARs): there are three subtypes that constitute the PPAR family: PPARα, PPARβ/δ, and PPARγ. PPARα and PPARγ activators enhance SR-B1 transporter expression, and consequently activate the cholesterol efflux (206). In a mouse model PPARγ have also been reported to up-regulate ABCG-1 expression (207). Furthermore, PPARγ enhance CYP27A1 expression in human macrophages. CYP27A1 produces 27-hydroxycholesterol for the alternative bile acid synthesis pathway. At the same time, 27-hydroxycholesterol also up-regulates ABCA-1 and ABCG-1 in macrophages, and thus enhances cholesterol efflux (208). An activation of PPARβδ by its agonist GW501615 was shown to both up-regulate (induce ABCA-1 expression (209)) and down-regulate (decrease CY27A1 and ApoE, and increase CD36 and SR-A (210)) cholesterol efflux in Thp-1 macrophages. In mice, a PPARβδ enhancement caused increased circulating HDL levels promoting RCT (211). It has been observed that PPARα inhibits ACAT1 in primary human macrophages (212) and that PPARγ hinders ACAT1 in Thp-1 macrophages (213). Such an inhibition has been reported being able to stimulate cholesterol efflux (201).

- Liver X receptors (LXRs) and Retinoid X receptors (RXRs): LXRα and LXRβ are ligands of activated nuclear receptors and their role is the regulation of lipid metabolism and inflammation (214). These receptors form heterodimers with RXRs to bind LXR response elements (215). Oxidized or hydroxylized cholesterol metabolites are endogenous LXR-ligands, such as 22 (R)-hydroxycholesterol, 24(S)-hydroxycholesterol, 25-hydroxycholesterol, 27 -hydroxycholesterol and 24(S),25-epoxycholesterol (216). LXRα and LXRβ enhancement induces ABCA1 and ABCG1, and thus an increased cholesterol efflux. Activation of LXRs by agonists and oxysterols enhances ABCA1 expression and cholesterol efflux in healthy human macrophages (217). Furthermore, LXRs enhance ApoE expression in macrophages and adipose tissue but not the liver. ApoE plays a key role in the transport of cholesterol

from the periphery to the liver; it interacts with the LDLR and allows the uptake of lipoprotein with cholesterol by hepatocytes. In addition, LXRs induce PLTP and LPL in macrophages (218;219).

- Sterol regulatory element binding proteins (SREBPs): SREBPs, a basic-helix-loop-helix leucine zipper class of transcription factors, bind to the sterol regulatory element TCACNCCAC (220). These nuclear factors upregulate the expression levels of SR-B1, LDL-R, cholesterol synthesis enzyme, and HMG-CoA reductase; concomitantly, they downregulate the expression levels of ABCA-1, ABCG-1, ABCG-4, ABCG-5, ABCG-8, and SR-B1 (està a up-regulate també, check). Furthermore, SREBPs are regulated by LXR and PPARγ (221).

- Others: Recently, other studies have reported other nuclear receptors involved in the regulation of macrophage cholesterol homeostasis and the development of atherosclerosis, including pregnane X receptor, farnesoid X receptor, glucocorticoid receptor, retinoic acid receptors, and the NR4A nuclear receptor family members.

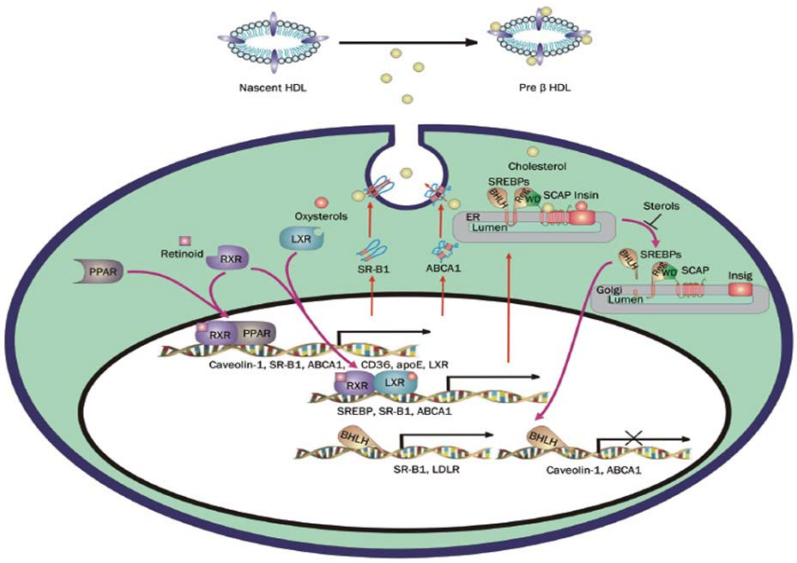


FIGURE 4. Cholesterol efflux nuclear receptor factors. Ref. (202) Luo Dx et al. Acta Pharmacol Sin. 2010 (10): 1243-57.

4.6.3. NUTRIGENOMIC TOOLS

Nutritional genomics has emerged as a relatively new field of research assessing the mechanisms by which nutrients and dietary patterns interact with the genome at different stages. It employs new technical and conceptual developments to study the interactions among nutrition, and its bioactive dietary components, the genome and health outcomes. Nutritional genomics embraces a systems biology approach to assess individual risk factors in the light of genetic diversity at the transcriptome, genetic, metabolome, and epigenome level.

Human dietary intervention studies have successfully used transcriptomics to show how diet induces alterations in gene expression. Transcriptomics is employed for three different purposes in nutrition research: 1) to provide information about the mechanism underlying the effects of a certain nutrient or diet; 2) to categorize genes, proteins, and metabolites that are altered in the pre-disease state and might act as molecular biomarkers; and 3) to identify and characterize pathways regulated by nutrients.

The molecular structure of each nutrient determines the specific signaling pathways that are affected. Small structural changes (e.g. SFA vs MUFA or cholesterol vs plant sterols) have a remarkable influence on the activation of a signaling pathway (222). Dietary components can regulate the expression of genes in the transcription (acting as transcriptor factors or interacting with them), mRNA processing, mRNA stability and trans- and post-translational modification stage. The effect of different nutrient metabolites acting as ligands of nuclear receptor transcription factors is well established. Nuclear receptors include: a) PPARα which binds to fatty acids; b) LXRα and RXR which bind oxidative sterols; and c) RXR which is mainly enhanced by retinoids (202). As previously mentioned, these nuclear factors play a key role in the regulation of atherosclerosis thus through nutrigenomics we can analyse the potential effect of nutrients in CVD.

The main genomic tools to study nutrigenomics are:

1) Microarray: Microarrays are high density arrays designed for quantitative and highly parallel measurements of gene expression (223). They consist of different nucleic acid probes that are chemically attached (hybridized) to a substrate, which can be a microchip, a glass slide or a microsphere-sized bead.

2) Quantitative Real-Time Reverse Transcription (RT) Polymerase Chain Reaction (PCR): PCR is a broadly used tool applied in parallel with microarray analysis in nutrigenomic studies. Real-time PCR approach follows the general principle of PCR but its key feature is that the amplified product is detected as the reaction progresses in real time, whereas in the traditional PCR, the product of the reaction is detected at the end. RT followed by quantitative PCR (qRT-PCR) is an extremely sensitive, cost-effective method for quantifying gene transcripts from cells. It combines the nucleic acid amplification and detection steps into one homogeneous assay and obviates the need for gel electrophoresis to detect amplification products. Its simplicity, specificity and sensitivity, together with its potential for high throughput analysis have made real-time RT-PCR the benchmark technology for the detection and/or comparison of RNA levels (224).

Epigenetics studies the heritable DNA modifications able to regulate chromosome architecture and modulate gene expression without changing the underlying sequence. Nutritional epigenetics is a novel mechanism underlying gene-diet interactions (225). Epigenetic phenomena are critical for the ageing process, from embryonic development to later adult life, and the complexity of integrating all these data is a huge multidisciplinary challenge for scientists.

Nutrimiromics has emerged as a subsidiary field of nutritional genomics assessing how nutrients affect microRNAs (miRs) and their function. MiRs are small non-coding RNA sequences of single sequences of 19-24 nucleotides located in intra- or inter- regions of protein coding genes (226). They are the

principal regulators of a great number of physiological processes including epigenetic regulators in CVD. To study miRNA target site polymorphisms as functional variants could contribute to a better understanding of the physio-pathology mechanisms of CVD, amongst others.

4.6.4. NUTRIGENOMIC BENEFICIAL EFFECTS OF OLIVE OIL ON REVERSE CHOLESTEROL TRANSPORT

There are few studies concerning the direct effect of OO compounds on reverse cholesterol transport gene expression. Nevertheless, HT, the main PC from OO, has been shown to be able to upregulate the gene expression of PPARα and PPARγ (227). In humans, an acute polyphenol-rich OO intervention increases the PPARBP, and also CD36 expression (228), and a sustained VOO intervention enhanced ABCA-1 and ABCG-1 expression, and reduced the SR-B1 one. Nevertheless, in a postprandial study performed by our group with VOO consumption and a Mediterranean diet no ABCA-1 differences were observed, and neither after a Mediterranean diet (229).

A large body of knowledge exists related with PC and reverse cholesterol transport. PC, and also fatty acids, are potent ligands of the PPARs family and other nuclear factors. Hydroxycinnamic acid derivatives, compounds present in OO, have been shown to be potent agonists of PPAR α/γ (230). In this regard, incubation of macrophages with anthocyanines induced, in a dose-dependent manner, cholesterol efflux, and also PPARγ and mRNA expression (231). Furthermore, resveratrol has been reported to up-regulate PPARα, PPARγ and PPARδ expression in macrophages (232). In agreement with this, both quercetin and resveratrol also attenuated the suppression of PPARγ mediated by tumor necrosis factor-α in human adipocytes (233). In addition, it has been described that chlorogenic acid, one of the polyphenols most present in the diet, increased PPARg and LXR expression in RAW264.7 cells (234).

It has been observed that PC can also modify directly or indirectly, through PPARs or other mechanisms, the expression of other genes involved in RCT. Phenolic-rich beverages, such as coffee, have been reported to enhance the cholesterol efflux from human macrophages to HDL, increasing the mRNA and protein levels of ABCG1 and SR-B1 (235). These effects were also found to be produced by caffeic and ferulic acid in experimental models (235). In addition, chlorogenic acid, one of the most common polyphenols in the diet, has been shown to increase ABCA-1 and ABCG-1 gene expression in RAW264.7 cells (234). The expression of ABCA-1 was also enhanced by anthocyanines in an *in vitro* model with macrophages (231). It has been reported that polyphenol-rich black chokeberry extract reduced the expression of NPC1L1 and SR-BI, and increased the expression of ABCA-1 and ABCG-8 (236). Furthermore, *Tamarindus indica* fruit pulp extract, with a high phenolic content, increased the expression of ApoA-1, ABCG-5, and LDL receptor gene expression (237).

4.7. HDL FUNCTIONALITY

HDL-C concentration is inversely and independently associated with CVD (238). Consequently, pharmacological or natural agents, which can increase HDL-C levels, have been considered as key factors for future therapies (239). In the past, clinical trials which showed an increment of HDL-C with CETP antagonists reported an increased mortality risk (240-242), recent studies, however, have attributed such a risk to side effects (243). The fact that current evidences has shown that genetic variants predisposing to high HDL-C are not always associated with lower risk of suffering a coronary event (244), has again highlighted HDL function instead of quantity (143).

HDL plays a central role in the first and key step of RCT, cholesterol efflux from peripheral cells. HDL cholesterol efflux capacity has been inversely related

to early atherosclerosis development and to the prediction of experiencing a coronary event, in human studies (245;246). In addition, HDL has other atheroprotective functions such as antioxidant properties towards LDL (247), anti-inflammatory properties, and vasoprotective capacity (248).

4.7.1. HDL CHOLESTEROL EFFLUX CAPACITY

The main function of HDL is its cholesterol efflux capacity. As the most clinically atheroprotective property of HDL, a disrupted cholesterol efflux rate from macrophages may reflect the presence of subclinical CVD better than HDL-C levels (245;249). Firstly, HDL crosses the endothelium to gain access to arterial intimal cells. Endothelial cells bind, internalise, and translocate HDL from the apical to the baso-lateral compartment by a mechanism involving SR-BI and ABCG1 (250;251). Transcytosis of lipid-free apoA-I also occurs involving ABCA1 and resulting in apoA-I lipidation (252;253). After that, HDL interacts with specific receptors on the cell surface, and with cellular surface lipids to activate the cholesterol efflux process (191). The active cholesterol efflux is mediated by ABCA-1 and ABCG-1, while the passive one is mediated by SR-B1 (191;192).

4.7.2. HDL ANTIOXIDANT ACTIVITY

Cholesterol-rich lipoproteins, mainly LDL, are retained in the arterial wall and oxidatively modified under the action of resident cells (254). Oxidation in the arterial intima results from local oxidative status, which represents an imbalance between prooxidants and antioxidants. Cellular oxidative systems involved in vivo include myeloperoxidase (MPO), NADPH oxidase, NOS and lipoxygenase. They produce a variety of reactive chlorine, nitrogen and oxygen species (255).

HDL can protect LDL and other lipoproteins from this oxidative stress. This fact has been reported *in vitro* on their co-incubation (256) and *in vivo* upon HDL

supplementation (257). The first step of HDL antioxidant capacity is to remove oxidised lipids from cells and LDL. PL hydroperoxides are rapidly transferred from ox LDL to HDL (258). The second step, is the inactivation of oxidized lipids in HDL. Depending on their structure, oxidized lipids can be reduced by apoA-I and other redox-active HDL components (258) or hydrolyzed by HDL hydrolytic enzymes (259). When this HDL capacity is overwhelmed, cholesteryl ester hydroperoxides can be removed from HDL via SR-B1 (260).

4.7.3. HDL ANTINFLAMMATORY CAPACITY

HDL particles have anti-inflammatory actions which may contribute to the suppression of chronic inflammatory response in the arterial wall which will later facilitate LDL-C deposition (261). Moreover, HDL particles reduce monocyte activation and adhesion in the endothelium decreasing adhesion molecule expression, chemokines, and chemokine receptors (262).

HDL particles also inhibit T-cell stimulation and the subsequent monocyte activation (263;264). Furthermore, HDL decreases neutrophil enhancement *in vitro* and *in vivo* (265). The HDL capacity to remove cholesterol from cells down-regulates the inflammatory macrophage phenotype expression, and thus decreases signaling via Toll-like receptors (266).

4.7.4. HDL VASOPROTECTIVE CAPACITY

HDLs is also considered a relevant vasoprotective agent. The endothelium has physiological activites involved in atheroprotection: it controls monocyte adhesion, regulates VSMC proliferation, maintains nonthrombogenic surfaces, and vasomotor tone (267). NO production is necessary for many of these functions. Thus, endothelial dysfunction, due to limited NO production, is a key

factor for the perpetuation of atherosclerosis (268). Furthermore, this endothelium dysfunction can be counteracted by HDL (248).

HDL particles have vasodilatory activity by stimulating the release of NO and prostacyclin in endothelial cells (269). Activation of NO production is initiated by the binding of HDL to SR-B1. The vasodilatory effects promoted by HDL are also due to cholesterol efflux via ABCG-1 of cholesterol and 7-oxysterols, which improves the formation of eNOS dimers and consequently, ROS production decreases (198). ROS inhibits NO, thus ROS decrement increase NO bioavailability, and improves vasodilation. HDL also reduces NADPH oxidase activity in endothelial cells (270).

4.7.5. OTHER CAPACITIES OF HDL

Other HDL functions have been described and included:

1) Cytoprotective actions: HDL protects macrophages and endothelial cells from apoptosis induced by loading with FC or oxLDL (198;271). In addition, HDL defends endothelial cells against cell death induced by chylomicrons remnants (272), TNF-alpha (273), complement system proteins (274), and growth factor withdrawal (275).

2) Modulation of glucose metabolism: HDL particles improve glucose metabolism through activating insulin secretion by pancreatic beta-cells, improving insulin sensitivity, and controlling cholesterol homeostasis (276).

3) Protection front infections: HDL binds circulating lipopolysaccharides and contributes to its hepatic clearance to the bile, and thus inhibits lipopolysaccharides-induced cellular activation (277) and protects against endotoxic shock in animal models (278).

4) Anti-thrombotic functions: HDLs exerts a reduction of platelet activity ex vivo in intervention trials (279;280). Moreover, in vivo anti-thrombotic HDL functions are the inhibitory actions in agonist-stimulated platelet aggregation, fibrinogen binding, granule secretion, and thromboxane A2 and 12-hydroxy-eicosatetraenoic acid production (280;281).

5) Regulation of gene expression by miRs: HDL transports small non-coding miRs which are key intracellular regulators of gene expression (282).

4.7.6. BENEFICIAL EFFECTS OF OLIVE OIL ON HDL FUNCTIONALITY

The consumption of different PCs activates cholesterol efflux in humans (235;237;283). Regarding, OOPC, some studies have demonstrated that they can enhance HDL cholesterol efflux capacity. In a 47-male subsample of the EUROLIVE study, we provided for the first-time, first-level evidence that 25 mL VOO during 3-weeks enhances the HDL cholesterol efflux capacity in healthy humans. This VOO intervention also improved HDL characteristics: incorporation of OOPC biological metabolites to HDL, higher large HDL (HDL_2) levels, decreased small HDL (HDL_3) levels, triglyceride poorer HDL core, and increased HDL monolayer fluidity (36). According to our findings, a better antioxidant protection, conferred by a higher content of OOPC metabolites in HDL, could have enhanced the HDL cholesterol efflux capacity. This antioxidant protection can avoid two important issues: 1) oxidative modifications of the ApoA-1, the main HDL protein involved in cholesterol efflux capacity, and other HDL protein structures; and 2) oxidative modifications of HDL lipids, making the lipoprotein more fluid and, thus, more functional (284). In this regard, Helal *et al.* showed similar results in a linear, non-randomised, and non-controlled trial. Specifically, VOO ingestion increased

the capacity of HDL to mediate cholesterol efflux and HDL monolayer fluidity (99).

Regarding antioxidant HDL function, it has been demonstrated that the consumption of OOPC was dose-dependently associated with a decrease in LDL oxidation status (97), and part of this protection could take place through an induction of HDL antioxidant capacities. An enhancement in this antioxidant activity has been reported in apoE-deficient mice with spontaneous atherosclerosis development, after the consumption of a VOO-rich diet (175). The main proteins involved in antioxidant function of HDL are ApoA-I and PON1. Some VOO-interventions in humans have increased PON1 activity (285) and ApoA-I concentrations (173;174). Two indirect mechanisms might explain this ApoA-I increment: 1) OOPC produces tryglyceride-poor core HDL, this composition has been associated with a more stable conformation of ApoA1 (160), and 2) OOPC metabolites in HDL could improve HDL antioxidant status (285;286) and reduce ApoA-I oxidative modifications. LCAT and PAF-AH enzymes are also related to HDL antioxidant capacity; nevertheless, no significant changes have been reported after OO consumptions.

With respect to antiinflammatory-vasoprotective HDL properties, VOO consumption has been shown to be highly protective for vascular response and endothelial integrity, as observed in several OOPC-rich interventions in humans (107;108;287). HDLs could act as a transporter of several OOPC derivatives to the endothelial cells, where they may provide protection from oxidative damage in mitochondria or conserve NO production, as described *in vitro* (107;288). Furthermore, in healthy humans, an OOPC intervention enhanced HDL capacity to reduce ICAM-1 secretion and monocyte adhesion to endothelial cells (286). It has been also reported that different OOPCs and a VOO-rich Mediterranean diet can reduce acute-phase proteins in HDL, promoting a less pro-inflamatory lipoprotein status.

4.8. ENDOTHELIAL FUNCTION

4.8.1. ENDOTHELIAL FUNCTION DEFINITION

The endothelium is a monolayer of cells lining the lumen of blood cells, its dysfunction is one of the initial factors of atherosclerosis. The endothelium has a protective effect on the vasculature by the release of NO, which derives from the transformation of L-arginine into citrulline through NOS. NO is produced under the stimulus of agonists acting on specific endothelial receptors, and of mechanical forces, such as stress (289;290). In pathological conditions, endothelium-derived contracting factors (such as thromboxane A2 and prostaglandin H2) and ROS, which decreases NO availability (290), produces a proatherosclerotic vascular phenotype, causing vasoconstriction and promoting platelet aggregation, vascular smooth muscle cell proliferation and migration, and monocyte adhesion (291).

4.8.2. METHODS TO ASSESS ENDOTHELIAL FUNCTION

Currently, there are different methods to evaluate EF:

1) Laser doppler flowmetry: a technique that monitors skin microvascular blood flow (292). The assumption is that the response observed in the cutaneous circulation is a window for the responses that may be observed in other vascular beds (293). Other techniques such as post-occlusive hyperaemia or local skin heating can also be employed. The major advantages are that laser doopler flowmetry is a non-invasive technique and measures the direct delivery of acetylcholine to the tissues.

2) Ischemic reactive hyperemia (IRH): is the method used in this thesis, and is measured by Laser doppler flowmetry technique. Measurements are performed with the patient lying in the supine position in a room at a stable temperature

(20–22 ºC). Patients are required to rest 15–20 min before the test. The blood pressure cuff is placed above the dominant arm elbow and the laser probe in the palmar surface of the second finger. Capillary flow is measured for 1 min. Then, the cuff is inflated to suprasystolic pressure (200 to 220 mmHg) and 4 min of distal ischemia is recorded. After that, the cuff is deflated and 30 secs later, the flow is recorded for 1 min. The system monitor show how the perfusion units (PU) fall regularly to reach a behaviour equal or similar to baseline. Results are expressed as arbitrary units (AU). Calculations are performed using the formula: IRH = (PUdistal-PU basal) / PUbasal x 100 (294).

3) Flow-mediated dilatation: measures changes in conduit artery diameter by ultrasound. This response reflects local bio-activity of endothelial-derived NO. The brachial artery is usually imaged. FMD is carried out in a quiet room. Subjects are asked to maintain a lying supine position for >10 min prior to image acquisition. A straight, non-branching segment of the brachial artery above the antecubital fossa is imaged in the longitudinal plane with the ultrasound probe securely fixed using a stereotactic clamp. A blood pressure cuff is placed 1-2 cm below the antecubital fossa and inflated to supra-systolic pressure. After cuff release, reactive hyperaemia results are quantified using Doppler. The arterial diameter is recorded at the final diastole using electrocardiographic gating during image acquisition, to determine the response of the conduit artery to rise in flow (295). Flow-mediated dilatation is expressed as a percentage change of the arterial diameter from the baseline vessel size.

4) Coronary EF: an invasive technique; nevertheless it remains a very used technique for evaluating EF of epicardial coronary arteries in routine clinical situations. It is applied a pharmacological stimuli to assess epicardial coronary vasodilation. For epicardial coronary vessels, quantitative coronary angiography measures changes in their diameter. Changes are usually compared with both baseline and vasodilation induced by endothelium independent drugs. Recently,

non-invasive quantitative coronary angiography has been developed by using computed tomography (296) and magnetic resonance imaging (297).

5) Venous occlusion plethysmography: it is based on the measurement of tissue blood flow by the assessment of the tissue volume change, induced by the inflation of a cuff proximally to the under evaluation tissue. The cuff is inflated up to that pressure which occludes venous outflow but allows arterial inflow. Consequently, the rate of the volume change is proportional to the rate of arterial inflow. A low-invasive, modified strain-gauge venous occlusion plethysmography method has been applied to research *in vivo* EF in human microcirculation (298).

6) Peripheral arterial tonometry: measurement of peripheral vasodilator response with fingertip peripheral arterial tonometry technology is appearing as a useful method for assessing vascular function (299). Despite the peripheral arterial tonometry signal being modulated by several factors, this parameter is also affected by the bioavailability of NO and, therefore, also depends on EF. In response to hyperaemic flow, digital pulse amplitude (and hence peripheral arterial tonometry signal amplitude) increases, a response that has been shown to depend in part on NO synthesis (300).

7) Pulse wave analysis: the arterial waveform contains key information about the stiffness of the large arteries and amount of wave reflection within the arterial system. Wave reflection occurs at sites of impedance mismatch, often branch points, and is usually quantified by measuring the augmentation index, which represents the difference between the first and second systolic peaks. Although the impedance of the large, elastic arteries is relatively static, impedance of the small arteries and arterioles is much more dynamic and depends to a large extent on smooth muscle tone and vessel size. Thus, changes in small artery tone affect wave reflection: vasodilatation reduces the augmentation index, whereas vasoconstriction increases it (301).

8) Biochemical markers and bioassays:

· Asymmetric dimethyl arginine: a product of arginine methylation, it acts as an endogenous inhibitor of endothelial NOS. Asymmetric dimethyl arginine is, however, not a specific endothelial NOS inhibitor, but also inhibits the other NOSs (302).

· OxLDL: contributes to endothelial dysfunction and atherosclerosis. Endothelial activation in response to oxLDL is possibly mediated by the lectin-like oxLDL receptor type 1 present in endothelial cells. OxLDL levels are not easy to assess *in vivo*, and in plasma there are probably only minimal oxLDLs present. Monoclonal antibodies have been used to characterize circulating oxLDL levels (303).

· Endothelial microparticles: are vesicles shed from plasma membranes following cell activation or apoptosis. In healthy plasma, 76 endothelial microparticles of different cellular origins have been described and include, platelets, leukocytes, red blood cells, and endothelial cells. Elevated circulating levels have been reported in patients with atherothrombotic diseases and nowadays these microparticles constitute an emerging surrogate marker of endothelial dysfunction (302).

· Endothelial progenitor cells: the reconstitution of the damaged endothelial monolayer may be a prerequisite for the prevention of endothelial dysfunction and atherosclerotic lesion formation. A growing body of knowledge suggests that circulating, bone marrow derived from endothelial progenitor cells plays a key role in endothelial cell regeneration. Endothelial progenitor cells can be measured from peripheral blood either by quantification using flow cytometry (304) or after cultivation of mononuclear cells (305).

· Endothelial glycocalyx: the glycocalyx forms a layer covering the endothelial lining, protecting endothelial cells from contact with circulating blood cells.

Endothelial glycocalyx has a major role in several crucial balances at the level of the vessel wall, including the vasoconstrictor–vasodilator, the pro- and anticoagulant, the pro- and anti-inflammatory, as well as the pro- and antioxidative balance (302).

4.8.3. BENEFICIAL EFFECTS OF OLIVE OIL ON ENDOTHELIAL FUNCTION

Nutrients could play a key role in modulating EF. Dietary bioactive compounds have been found to have beneficial effects on EF through multiple complex mechanisms including: inhibition of monocyte adhesion, platelet activation, increased NO production and improvement of vasodilatation (306).

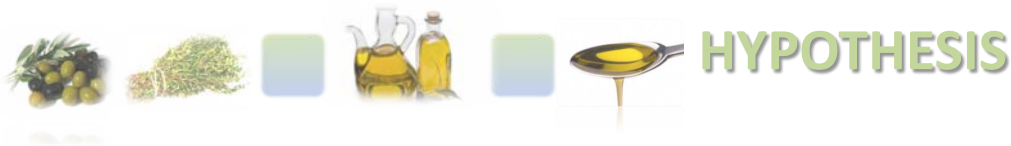
Several *in vitro* studies and *in vivo* animal models have shown the beneficial effects of OOPC on EF and related biomarkers. It has been reported that HT and polyphenol extract from VOO has a protective effect on endothelial dysfunction induced by hyperglycemia and free fatty acids in an *in vitro* model. Specifically, OOPC reversed such deleterious effects as the reduction of NO and the increment of endothelin-1 levels (307). Furthermore, in a study with rats, a long-term ingestion of a diet rich in extra-VOO produced obesity and insulin resistance but protected EF (308). The effects of pomace oil have also been tested in hypertensive rats and have demonstrated a reduction in blood pressure, an improvement in endothelium-dependent relaxation, an activation of vascular expression of endothelial NOS, and a reduction of tumor necrosis factor alpha, transforming growth factor beta, and collagen I (309).

In vivo and in humans, the consumption of a VOO with a high phenolic content has been related with an improvement of endothelial-dependent vasomotor function measured as IRH in both acute (96) and sustained clinical trials (107). Furthermore, a long-term OO treatment also enhanced EF and inflammatory biomarkers (soluble ICAM, white blood cells, monocytes, and lymphocytes) in

patients with early atherosclerosis (108). In addition, an OO intervention attenuated flow-mediated dilatation reduction caused by an environment with particulate matter, the most dangerous inhaled pollutant which causes endothelial dysfunction (310). Nevertheless, in a recent clinical trial with overweight and obese men, within the context of a high protein meal, neither the OO intervention nor the palmolein one modified post-prandrial EF (311).

Within the context of the Mediterranean diet, in the PREDIMED study, both Mediterranean diets, one enriched with VOO and other with nuts, increased NO and total polyphenol excretion after a one-year intervention, the increment in NO being associated with a reduction in systolic and diastolic blood pressure levels (312). It has also been observed that the consumption of a Mediterranean diet rich in VOO increases the plasma concentrations of fat-soluble vitamins and decreases endothelial damage by mechanisms possibly associated with the protective synergistic effects of the antioxidant components of this dietary pattern (313).

The beneficial effects of other components of OO, such as tocopherols, which have been related to a protective role against vascular dysfunction both in *in vitro* studies, have been reported (314).


HYPOTHESIS

5. HYPOTHESIS

1) A single dose of functional virgin olive oil enriched with its own phenolic compounds beyond a natural virgin olive oil, at a post-prandrial state, in pre- and hypertensive subjects, can produce:
    - A modulation of the cholesterol efflux transcriptome.
    - An improvement of the endothelial function.

2) A sustained consumption of a functional virgin olive oil enriched with its own phenolic compounds plus additional complementary ones from thyme could have more benefit effects on HDL characteristics-related with its functionality than a natural virgin olive oil in hypercholesterolemic subjects.

OBJECTIVES

6. OBJECTIVES

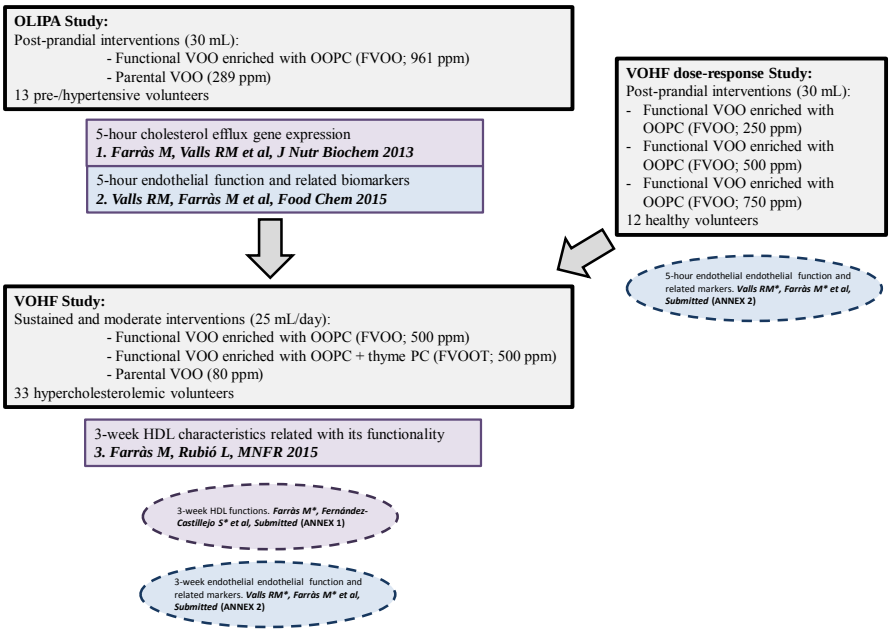
1) To assess whether a single dose of 30 mL functional virgin olive oil, enriched with its own phenolic compounds (961 ppm) beyond a parental virgin olive oil with moderate phenolic compounds content (289 ppm), in pre- and hypertensive volunteers, can produce a 5-hour post-prandrial state in a randomized, cross-over, double-blind, and controlled trial:
    - Amodulation of the expression of genes related with cholesterol efflux on momonuclear cells.
    - An improvement of the endothelial-dependent microvascular dilatation.

2) To test whether enriched functional virgin olive oils (FVOOs; 500 ppm), one enriched with its own PC (FVOO) and another enriched with them plus complementary ones from thyme (FVOOT), could improve HDL characteristics related with HDL functionality, versus a natural virgin olive oil. The HDL size, HDL metabolism-antioxidant enzymes, and HDL composition will be established in a randomised, cross-over, double-blind, and controlled trial.


METHODS

7. METHODS

This thesis encompasses two different projects. In both of them FVOOs were tested in cardiovascular risk subjects and a number of atherosclerosis-related biomarkers analyzed.



PC, phenolic compounds; VOO, virgin olive oil

FIGURE 5. Flowchart of thesis studies.

1) OO and hypertension study (The OLIPA study):

A cross-over, randomized, double blind, controlled trial was performed in 13 pre-hypertensive [systolic blood pressure (SBP) ≥120 mmHg to 139 mmHg and/or diastolic blood pressure (DBP) ≥80 mmHg to 89 mmHg] or stage 1 hypertensive (SBP ≥140 mmHg to 159 mmHg and/or DBP ≥90 mmHg to 99 mmHg) subjects, without hypertensive treatment. Exclusion criteria included the following: LDL-C >4.9 mmol/L, TG >3.97 mmol/L or current hypolipidemic treatment, diabetes mellitus, any chronic disease and body mass index (BMI)

>30 kg/m^2. Volunteers received a single 30 mL dose of VOO (289 ppm) and a functional VOO enriched with its own OOPC (FVOO; 961 ppm). Washout periods were of 2 weeks in which participants were instructed to follow a stabilization diet with 10% SFA during the week prior to the postprandial test. The day before the intervention, participants followed a polyphenol-free diet.

The analyzed biomarkers included:

- EF, measured as IRH, was performed using a laser-doppler linear Periflux flowmeter 5000.
- Glycaemia and lipid profile systemic biomarkers: total cholesterol, HDL-C, TG, and glucose were measured by standardised methods in a Beckman autoanalyzer. LDL-C was calculated by means of the Friedewald formula whenever TG were <300 mg/dl.
- Inflammation systemic biomarkers: hsCRP was analysed by a standardised method in a Beckman autoanalyzer; and plasminogen activator inhibitor type 1 (PAI-1), VCAM-1, and intercellular adhesion molecule type 1 (ICAM-1) were evaluated with enzyme-linked immunosorbent assay (ELISA) kits.
- Oxidation systemic biomarkers: oxidized LDL (oxLDL) and plasma oxygen radical absorbance capacity (ORAC) were measured by ELISA, and ferric reducing ability of plasma (FRAP) was determined by spectrophotometry.
- Cholesterol efflux-related genes: ABCA-1, ABCG-1, SRB1, PPARBP, PPARα, PPARγ, PPARδ, cyclooxygenase (COX) 1, COX-2, and CD36 gene expression were determined by real time RT-PCR.
- Bioavailability of PC: kinetic curves of the PC and their metabolites in urine were registered by ultra-performance liquid chromatography–tandem mass spectrometry.
- Questionnaires of physical activity and diet: the Minnesota leisure time physical activity questionnaire and a 3-day dietary record were administered to volunteers.
- Anthropometric measures: body mass index (BMI), blood pressure, and waist circumference were measured.

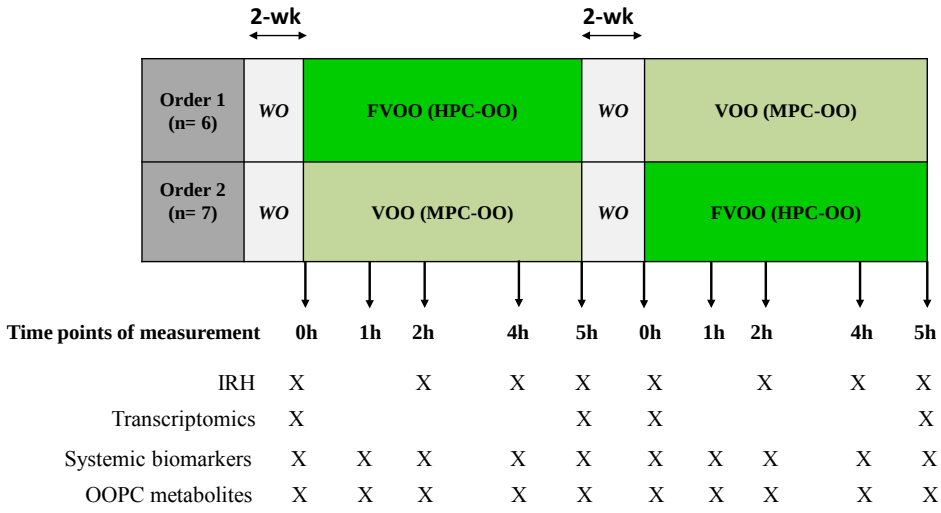


FIGURE 6. Experimental protocol of OLIPA study.

2) VOO and HDL Functionality study (The VOHF study):

A cross-over, randomized, double blind, controlled trial was performed in 33 hypercholesterolemic individuals (total cholesterol > 200 mg/dL), aged 35-80. Exclusion criteria included the following: BMI > 35 kg/m2, smokers, athletes with high physical activity (PA >3000 kcal/day), diabetes, multiple allergies, intestinal diseases, or other disease or condition that would worsen adherence to the measurements or treatment.

Volunteers ingested 25 mL/day during meals for 3 weeks of VOO (VOO; 80 ppm), functional VOO enriched with its own OOPC (FVOO; 500 ppm), and functional virgin olive oil enriched with its own OOPC (250 ppm) plus additional PC from thyme (250 ppm) (FVOOT; total: 500 ppm). The interventions were preceded by 2 week wash-out periods with a common OO (OO).

*The concentration of 500 ppm for the FVOOs was chosen according its bioavailability, sensory, and biomarkers in a

previous dose-response clinical trial (*Rubió L, Farràs M 2014*) (*Valls RM*, Farràs M*, Submitted*).

The analyzed biomarkers included:

- HDL characteristics related to its functionality:

  · HDL subclass distribution was analysed by Lipoprint HDL system 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, the first three bands corresponded to a large HDL subclass (HDL2), and fourth to ninth bands correspond to a small HDL one (HDL3).

  · HDL composition: composition was determined in HDLs isolated by a density gradient ultracentrifugation method [30] using preparation solutions of 1.006 and 1.21 density. Total cholesterol, PL, FC, and TG were quantified by enzymatic methods in a PENTRA-400 autoanalyser. Esterified cholesterol (EC) was calculated subtracting FC from TC. ApoA1, apoA2, apo-B100, and albumin were quantified by automatic immunoturbidimetric technique in a PENTRA-400 autoanalyser.

  · HDL metabolism-related enzymes: lecithin-cholesterol acyl transferase mass was measured by ELISA kit, PAF-AH by spectrophotometric assay, and CETP activity was analysed by fluorimetric kit.

  · HDL antioxidant status: paraoxonase/arylesterase activity was determined by measurement of the capacity for cleavage of phenyl acetate resulting in phenol formation, and dihydrorhodamine 123 oxidation rate by a fluorimetric method.

- Oxidative status systemic biomarkers: activity of glutathione peroxidase was measured by a Paglia and Valentine method modification using cumene hydroperoxide as oxidant of glutathione.

- Glycaemia and lipid profile systemic biomarkers: total cholesterol, glucose, and TG were measured using standard enzymatic automated methods, and

apoA1 and apoB-100 were analysed by immunoturbidimetric technique, in a PENTRA-400 autoanalyser. HDL-C was measured as a soluble HDL-C determined by an accelerator selective detergent method in a PENTRA-400 autoanalyser. LDL-C was calculated by means of the Friedewald formula whenever TG were <300 mg/dl.

- Bioavailability of PC: urinary HT sulfate and thymol sulfate were measured in urine by ultra-high performance liquid chromatography-electrospray ionization-tandem mass spectrometry.

- Questionnaires of physical activity (PA) and diet: the Minnesota physical activity short-questionnaire and a 3-day dietary record were administered to volunteers.

- ApoE haplotypes: allelic discrimination ApoE gene variants were performed with TaqMan PCR technology (QuantStudioTM 12K Instrument).

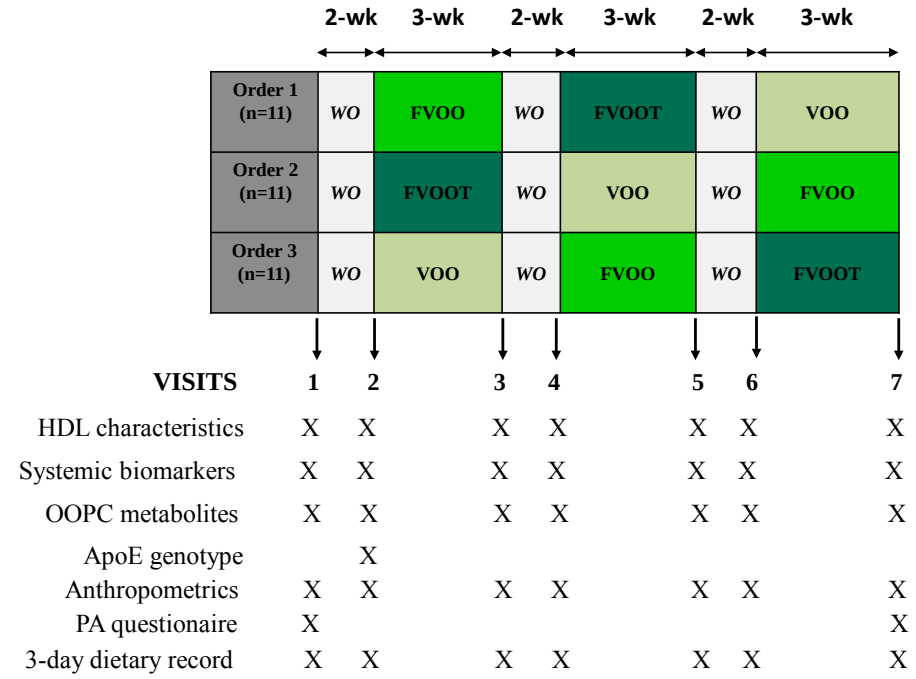


FIGURE 7. Experimental protocol of VOHF sustained study.


RESULTS

8. RESULTS

Publication nº 1

Olive oil polyphenols enhance cholesterol efflux related genes in hypertensive humans. A randomized controlled clinical trial. The VOHF study

Marta Farràs, Rosa M. Valls, Sara Fernández-Castillejo, Montserrat Giralt, Rosa Solà, Isaac Subirana, María-José Motilva, Valentini Konstantinidou, María-Isabel Covas*, Montserrat Fitó*.

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In brief, after an acute load of FVOO (961 ppm), we observed an enhancement of cholesterol efflux-related gene expressions *in vivo* at a 5-hour postprandial state, in pre-/hypertensive humans, in a randomized, double-blind, crossover, and controlled trial. We observed an increase in ABCA-1, SR-B1, PPARBP, PPARα, PPARγ, PPARδ, and CD-36 gene expression in white blood cells after an ingestion of FVOO compared with VOO (289 ppm) one. In addition a borderline increase of COX-1 was also observed after FVOO intervention versus VOO one. Cross-linked correlations among gene expression changes were observed after FVOOT consumption. Circulating oxLDL decreased after both interventions, and HT sulphate and HT sulphate acetate plasma concentrations increased in a dose-dependent manner with the OOPC content. Linear regression analyses showed that changes in gene expression were related to an improvement on oxidative stress-related markers such as oxLDL, ORAC, and OOPC metabolites.

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Olive oil polyphenols enhance the expression of cholesterol efflux related genes *in vivo* in humans. A randomized controlled trial

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Abstract

Both oleic acid and polyphenols have been shown to increase high-density lipoprotein (HDL) cholesterol and to protect HDL from oxidation, a phenomenon associated with a low cholesterol efflux from cells. Our goal was to determine whether polyphenols from olive oil could exert an *in vivo* nutrigenomic effect on genes related to cholesterol efflux in humans. In a randomized, controlled, cross-over trial, 13 pre/hypertensive patients were assigned 30 ml of two similar olive oils with high (961 mg/kg) and moderate (289 mg/kg) polyphenol content. We found an increase in ATP binding cassette transporter-A1, scavenger receptor class B type 1, peroxisome proliferator-activated receptor (PPAR)BP, PPARα, PPARγ, PPARδ and CD36 gene expression in white blood cells at postprandial after high polyphenol olive oil when compared with moderate polyphenol olive oil intervention ($P<.017$), with COX-1 reaching borderline significance ($P=.024$). Linear regression analyses showed that changes in gene expression were related to a decrease in oxidized low-density lipoproteins and with an increase in oxygen radical absorbance capacity and olive oil polyphenols ($P<.05$). Our results indicate a significant role of olive oil polyphenols in the up-regulation of genes involved in the cholesterol efflux from cells to HDL *in vivo* in humans. These results are in agreement with previous ones concerning the fact that benefits associated with polyphenol-rich olive oil consumption on cardiovascular risk could be mediated through an *in vivo* nutrigenomic effect in humans.
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Keywords: ATP binding cassette transporter-A1 (ABCA1); Olive oil polyphenols, gene expression; Peroxisome proliferator-activated receptor (PPAR); Scavenger receptor class B member-1(SR-B1); CD36 molecule (thrombospondin receptor) (CD36)

1. Introduction

Data from human studies show that olive oil polyphenols are protective against risk factors for coronary heart disease (CHD) [1], particularly in individuals submitted to an oxidative stress situation (i.e., hypertensive, CHD patients) [2,3]. A crucial event for the development of the atherosclerosis plaque is the accumulation of cholesterol in macrophages that leads to the formation of foam cells. In response to a lipid loading, macrophages activate a compensatory pathway for cholesterol efflux from cells to the high-density lipoprotein (HDL): the reverse cholesterol transport (RCT), in which accumulated cholesterol is removed from macrophages in the subintima of the vessel wall and collected by HDL and ApoA-I [4,5]. Several mechanisms are involved including passive diffusion and protein transmembrane transporters such as sterol 27-hydroxylase, the ATP-binding membrane cassette system or the scavenger receptor class B type 1 (SR-B1) [4,5]. Results of the European EUROLIVE study, performed in 200 healthy individuals, showed that olive oil consumption promotes an increase in plasma HDL cholesterol and a decrease in low-density lipoprotein (LDL) oxidative damage in a direct relationship with the polyphenol content of the olive oil administered [6].

We have previously reported that oxidation of HDL reduces the HDL functionality by impairing cholesterol efflux from macrophages [7] and that oleic acid consumption reduces HDL oxidation *in vivo* in humans [8]. In experimental studies, polyphenols from red wine have been shown to protect HDL and LDL from oxidation [9]. Polyphenols, however, can exert protective effects not only through the scavenging of free radicals but also by modulating signal transduction, cell signaling, gene expression and cellular communications in various pathways [10]. From our data and others, olive oil polyphenols modulate, towards a protective mode, the expression of inflammation-related genes [11–13], a common target of dietary intervention

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Table 1
Characteristics of the olive oils administered

	Type of olive oil	
	MPC	HPC
Quality parameters		
Free acidity (% of oleic acid)	0.19	0.26
Peroxide value (mEq O_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)	288.9	961.2
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	115.8	173.1
Luteolin	1.44	6.28
Apigenin	0.27	0.80

studies [14]. Concerning cholesterol efflux related genes, hydrocinnamic acid derivatives have proven to be potent dual peroxisome proliferator-activated receptor (PPAR) α/γ agonists [15]. Ingestion of a phenolic-rich beverage, such as coffee, has been shown to enhance the cholesterol efflux from human macrophages to HDL, while increasing the messenger ribonucleic acid (mRNA) and protein levels of ATP binding cassette transporter G1 (ABCG1) and SR-B1 [16]. These effects were also observed to be mediated by caffeic and ferulic acid in experimental models [16]. Therefore, we assessed the *in vivo* human transcriptome response related with cholesterol efflux after sustained consumption of similar olive oils, but with differences in their phenolic content, in a pre/hypertensive population.

2. Methods and materials

2.1. Olive oil preparation and characteristics

A virgin olive oil with a high phenolic content (HPC) was prepared, as previously described [17], by the addition of a phenolic-rich extract [oleuropein complex or secoiridoids (89.4%); hydroxytyrosol, tyrosol or phenil alcohols (3.5%); and flavonoids (6.0%)], obtained from the olive cake, to a natural virgin olive oil with a moderate content of phenolic compound (MPC). 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 the MPC using a Polytron (Kinematica, Littau, Switzerland) until full homogenization. The MPC was also submitted to the process of lecithin addition to ensure a similarity with HPC. Total polyphenol content of the olive oils was 289 and 961 mg/kg oil for MPC and HPC, respectively, measured by ultraperformance liquid chromatography coupled to a tandem mass detector as previously described [18]. Fatty acid type content of olive oils was measured by gas chromatography. Table 1 shows the composition of the olive oils used in the study.

2.2. Study design

2.2.1. Participants

Between January and July 2009, 22 participants, aged 20 to 75, were recruited through a volunteer center database. Participants were community dwelling with prehypertension [systolic blood pressure (SBP) ≥120 mmHg to 139 mmHg and/or diastolic blood pressure (DBP) ≥80 mmHg to 89 mmHg] or stage 1 hypertension (SBP ≥140 mmHg to 159 mmHg and/or DBP ≥90 mmHg to 99 mmHg) without antihypertensive treatment. We selected this type of patients because they are submitted to a higher oxidative stress than healthy individuals [19]. Due to this, pre- and hypertensive individuals could be more susceptible to receiving benefits from polyphenol-rich olive oil. Exclusion criteria included the following: 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 enrollment in the trial. After a screening visit, eligibility or exclusion was assessed by the attending physician based on a review of the clinical records. The study was approved by the Clinical Research Ethical Committee of the Hospital Universitari Sant Joan de Reus, Spain. Protocols were according to the Helsinki Declaration. This trial is registered with the International Standard Randomized Controlled Trial Number (identifier: ISRCTN03450153).

2.2.2. Randomization and intervention

The trial was randomized, controlled, double blind and crossover (Fig. 1). The randomization scheme was generated by using a Web site (http://www.randomization.com). Participants received 30 ml of one of the two types of olive oil, MPC or HPC, with bread (80 g). Washout periods were 2 weeks in which participants were instructed to follow a stabilization diet with 10% of saturated fatty acids during the week before the postprandial test. The day before the intervention, participants followed a polyphenol-free diet, avoiding olive oil, olives, fresh fruit or juices, vegetables, legumes, soya, chocolate, coffee, tea, wine and beer. Compliance was assessed using a 3-day dietary record (2 working days and a holiday or weekend one) before the intervention days. Trained dieticians explained to participants how to complete these questionnaires. Participants were instructed to avoid intense physical activity 3 days prior to the intervention day. Physical activity was evaluated by the Minnesota Leisure Time Physical Activity Questionnaire validated for its use in Spanish men and women [20,21]. Venous blood was collected at baseline of each intervention period (0 h) and at several periods after olive oil administration. Serum and plasma were obtained by centrifugation of blood at 1500*g* at 4°C for 20 min and stored at −80°C in the central laboratory's biobank. Whole blood was collected at baseline and at 5 h after olive oil intake in PAXgene tubes for gene expression analyses and stored at −80°C after 2 h at room temperature.

2.3. Systemic biomarkers

Anthropometric data were obtained by standardized methods. After 15 min of resting, blood pressure was measured in triplicate (1-min intervals) using an automatic sphygmomanometer (OMRON HEM-907; Peroxfarma, Barcelona, Spain).

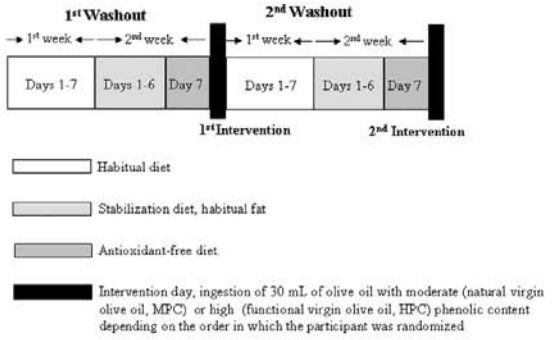

Fig. 1. Design of the crossover study.

Table 2
Baseline characteristics of the participants

Gender (male/female)	7/6
Age, years	51.15±16.67
BMI, kg/m^2	25.74±1.89
Waist circumference, cm	87.81±6.11
Systolic blood pressure, mmHg	136.46±19.76
Diastolic blood pressure, mmHg	81.77±10.19
Glucose, mmol/L	5.54±0.48
Total cholesterol, mmol/L	5.09±0.79
TG, mmol/L	0.86 (0.72 to 1.23)
HDL-C, mmol/L	1.70±0.40
LDL-C, mmol/L	2.88±0.68
Oxidized LDL, U/L	74.5±17.9
ORAC, μmol TE/ml	0.59±0.26
FRAP, μmol TE/ml	51.6±6.4
F_2-isoprostanes, pg/ml	82.3±60.4

Results are expressed as mean±S.D. or median (25th to 75th percentile) (n=13).

Serum total and HDL cholesterol, TG and glucose measurements were performed using standardized enzymatic methods in an autoanalyzer (Beckman Coulter-Synchron, Galway, Ireland). Low-density lipoprotein cholesterol (LDL-C) was calculated by means of the Friedewald formula whenever TG were <300 mg/dl. Plasma circulating oxidized LDL (Mercodia AB, Uppsala, Sweden) was measured by immunoassay. Olive oil polyphenols and their biological metabolites were measured in plasma, as markers of compliance, as previously described [18]. Ferric reducing ability of plasma (FRAP) was determined by 2,4,6-tripyridyl-s-triazine chelation at 593 nm in a UV–VIS spectrophotometer (Lambda 25, Perkin Elmer, Beaconsfield, UK). Plasma oxygen radical absorbance capacity (ORAC) was measured by peroxyl radical generation by 2,2′-azobis(2-amidinopropane) dihydrochloride using fluorescein as a fluorescence probe in a Fluoroskan Ascent fluorescence plate reader (Labsystems, Helsinki, Finland). F_2-isoprostanes were measured by enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI, USA).

2.4. Gene expression analyses

Total RNA was obtained from white blood cells (WBC) by using the PAXgene extraction kit (PreAnalytiX GmbH, Hombrechtikon, Switzerland). For mRNA expression analyses, isolation of total RNA (tRNA) was performed by a liquid–liquid method. RNA purity and integrity were assessed by Agilent (Agilent Technologies, Santa Clara, CA, USA). After cDNA conversion, gene expression was measured by real-time polymerase chain reaction with TaqMan® Low Density Microfluidic cards in triplicate and analyzed by the Sequence Detection System 2.1 software according to the manufacturer's instructions (Applied Biosystems-Life Technologies Corporation, Carlsbad, CA, USA). Changes in gene expression were calculated using the relative quantification method (RQ) and applying the $2^{-\Delta\Delta CT}$ formula. The selection of candidate genes was performed on the basis of their relationship with cholesterol efflux.

2.5. Sample size and power analyses

A total sample size of 16 participants allows for a more than 80% power to detect a significant difference between olive oil groups of 1 U of RQ in the gene expression of *ABCA1* measurement with consideration of a two-sided type I error of 0.05. This sample size takes into account a 20% dropout rate. Calculations were made from our previous data concerning the standard deviation of ABCA1 gene expression in healthy volunteers [13].

2.6. Statistical methods

Normality of continuous variables was assessed by normal probability plots. Nonnormally distributed variables were log transformed, and values were expressed as antilogarithm. Pearson correlation analyses were used to evaluate relationships among variables. A general lineal model was performed to assess the effect of each intervention compared to its baseline and to assess the effect between interventions for systemic and gene expression variables. No interactions with age and gender were observed. Due to this, models were performed without covariates. Multiple regression analyses were performed, adjusted by gender and age, in order to explore the relationship among systemic and gene expression variables. A value of P<.05 was considered significant. For gene comparison adjustment, a P<.017 was considered significant due to the fact that we explored three gene families (cholesterol transporters, PPAR nuclear receptor factors and prostaglandins). Statistical analyses were performed by SPSS 13.0 software.

3. Results

3.1. Participants and baseline characteristics

From the 22 participants recruited, 16 were eligible. Three participants dropped out before starting the study, two due to an incompatible work timetable and one for problems with blood collection. Finally, 13 participants (7 men and 6 women) entered the study. We could not identify any adverse effects related to olive oil intake. Participants' baseline characteristics are shown in Table 2. No changes were observed in dietary patterns throughout the study. No changes in daily energy expenditure in leisure-time physical activity were observed from the beginning to the end of the study.

3.2. Systemic biomarkers

Changes in cardiovascular risk biomarkers from baseline to 5 h postprandial are shown in Table 3. As expected, a postprandial hypertriglyceridemia occurred after olive oil ingestions. A significant decrease in oxidized LDL was observed after both interventions. Plasma concentration of hydroxytyrosol sulphate and that of hydroxytyrosol acetate sulphate, the main biological metabolite of hydroxytyrosol, increased in a dose-dependent manner with the polyphenol content of the olive oil (P<.05), reaching a peak at 120 min after ingestion of the oils. Areas under the curve from 0 to 5 h (AUC_{0-5h}) for hydroxytyrosol sulphate were (mean±standard deviation, μmol × min/L) 46±37 and 139± 33, and those for hydroxytyrosol acetate sulphate were 335±165 and 727± 352, for MPC and HPC, respectively (P<.005). No changes were observed in blood pressure, weight or waist circumference throughout the study.

3.3. Gene expression

The expressions of ABCA1, SR-B1, PPARBP, PPARa, PPARγ, PPARδ and CD36 genes increased significantly after the HPC intervention

Table 3
Changes in cardiovascular risk systemic biomarkers at 5 h postprandial after olive oil ingestion

	Olive oil interventions			
	MPC		HPC	
	Postintervention	Change	Postintervention	Change
Glucose, mmol/L	5.08±0.35	−0.362±0.344 *	5.07±0.31	−0.305±0.464 *
TG, mmol/L	1.23(0.75 to 1.53)	0.22(0.03 to 0.30) *	1.24 (0.99 to 1.77)	0.40(0.16 to 0.69) *
Cholesterol, mmol/L				
Total	5.09±0.84	0.029±0.218	4.99±0.85	0.031±0.192
HDL	1.71±0.39	0.004±0.094	1.65±0.36	−0.014±0.071
LDL	2.82±0.70	−0.023±0.078	2.70±0.70	0.026±0.175
Oxidized LDL, U/L	63.3±14.1	−5.98±8.52 *	65.4±16.4	−5.18±5.37 *
ORAC, μmol TE/ml	50.1±6.1	−1.34±7.38	50.1±7.02	1.91±8.95
FRAP, μmol TE/ml	0.57±0.25	−0.010±0.107	0.59±0.24	−0.013±0.099
F_2-isoprostanes, pg/ml	60.6±38.3	−15.6±42.0	64.9±38.0	−20.1±40.2

Values expressed as mean (S.D.) or median (25th to 75th percentile) (n=13).
* P<.05 versus its corresponding baseline.

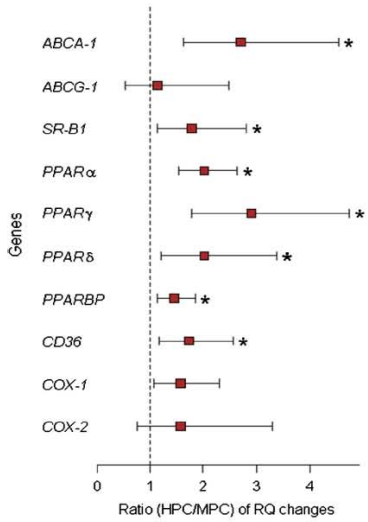

Fig. 2. Geometric mean (95% confidence interval) of the ratio (HPC olive oil/MPC olive oil) of RQ changes in gene expression after interventions. Dot axis displays the significance between olive oils at $P<.05$ level. *$P<.017$ significance after HPC versus MPC intervention.

when compared to the MCP one ($P<.017$), with changes in COX-1 reaching a borderline significance ($P=.024$). Fig. 2 shows the ratio of changes between the two interventions considering the dot axis at $P<.05$ to be of significance.

Analyses of gene expression changes after HPC showed cross-linked correlations among genes related with the cholesterol efflux cascade. The increase in the expression of ABCA1 directly correlated with that of PPARα. Changes in the expression of the PPARs gene family directly correlated among them, and those of PPARα with the increase in its coactivator, the PPARBP. The increase in SR-B1 expression directly correlated with that of CD36, COX-1 and COX-2, whose changes were also directly correlated ($P<.01$).

Multiple regression analyses showed that, after HPC intervention, values of ABCA1, PPARα and PPARγ gene expression increased with the decrease in oxidized LDL ($P<.05$). For each decrease in 1 U/L of oxidized LDL, there was a decrease of 18.2%, 5.2% and 13.8% in the gene expression of ABCA1, PPARα and PPARγ, respectively. Values of ORAC postprandial were directly related with the increase in PPARBP and PPARs gene expression ($P<.025$). For each increase of 1 μmol/L of ORAC, there was an increase of 9.6%, 7.2%, 18.8% and 41.3% in the gene expression of PPARBP, PPARα, PPARδ and PPARδ, respectively. A direct relationship was observed between the concentrations of hydroxytyrosol acetate sulphate at postprandial and changes in ABCA1 expression ($P=.036$). For each increase in 1 μmol/L of hydroxytyrosol acetate sulphate at 5 h postprandial, there was an increase of 43.2% in ABCA1 expression. These associations were not found after MPC ingestion.

4. Discussion

These outcomes showed that a randomized, crossover, controlled intervention with HPC olive oil increased the gene expression of ABCA1 and SR-B1, the main transmembrane transporters of cholesterol efflux, in WBC of pre- and stage 1 hypertensive individuals in comparison with MPC olive oil. Other related genes involved in the cholesterol efflux such as PPARα, PPARγ, their activator PPARBP, PPARδ/β, CD36 and COX-1 showed an increase in their expression when comparing HPC versus MPC intervention. To our knowledge, this is the first time changes in cholesterol efflux related genes linked to the polyphenol content of the olive oil have been reported *in vivo* in humans after a randomized trial. Our data also provide further first-level evidence that atheroprotective molecular mechanisms can be promoted by a polyphenol-rich olive oil intervention.

HDL-mediated cholesterol efflux is the natural rate-limiting step of reverse cholesterol transport [4,5]. Two main transmembrane transport systems are responsible for the removal of cholesterol from cells to the HDL lipoprotein: the ABC complex and the SR-B1 complex. ABCA1 and ABCG1 are responsible for cholesterol efflux. ABCA1 transports intracellular free unesterified cholesterol and phospholipids to the extracellular Apo-A1 in nascent HDL. ABCA1 mediates the efflux of cellular cholesterol to lipid poor apolipoproteins, but not to full HDL particles, a task performed by ABCG1. HDL presents or accepts cholesterol while anchored to plasma membranes via its receptor SR-B1 [4,5]. The density gradient of cholesterol between HDL and the cell surface determines whether HDL gives or accepts cholesterol. In perithelial cells, the main direction is the cholesterol efflux from cells [4]. In experimental models, high-fat–high-cholesterol diets decreased SR-BI expression [22], the posttranslational down-regulation of SR-BI occurring via the PDZ domain PDZK1 [23]. Genetic variants at the PDZK1-interacting domain of SR-BI interact with diet to influence the risk of metabolic syndrome in obese individuals with high polyunsaturated fatty acid and carbohydrate intakes [24]. The up-regulation of ABCA1 observed after HPC versus MPC intervention could be related to the unsaturated fatty acids/polyphenols ratio present in the olive oils tested. In fact, a repression of ABCA1 expression in macrophages by unsaturated fatty acids, including oleic acid [25], has been reported in experimental studies. The transcriptional repression of ABCA1 induced by unsaturated fatty acids has been shown to be abrogated by histone deacetylase (HDAC) inhibitors, which promote an increase in ABCA1 expression [25]. Polyphenols, such as those of green tea or quercetin, are HDAC inhibitors [26,27], a mechanism proposed as anticarcinogenic. Thus, we can hypothesize that, in HPC treatment, polyphenols present in the olive oil were able to counteract the repression induced by the oleic acid and other unsaturated fatty acids present in olive oil. This fact promoted an enhancement of ABCA1 gene expression linked to the polyphenol content of the olive oil. The direct relationship observed between plasma levels of hydroxytyrosol acetate sulphate and ABCA1 expression after HPC intervention would support this hypothesis. In a previous work with healthy volunteers [13], we did not detect significant differences in ABCA1 expression associated with a Mediterranean diet or virgin olive oil consumption. The fact that the virgin olive oil provided to participants had similar characteristics to those of the MPC reinforces the results obtained in the present study.

Many nuclear receptor factors are involved in regulating the expression of RCT-related genes. In this paper, we have focused on the PPAR family, comprising three homeotypic isomerides, PPARα, PPARγ and PPARδ, because fatty acids and also polyphenols are natural PPARs ligands [4,28]. PPARBP, also referred to as MED1, is a coactivator for PPARγ and PPARα [29]. The results obtained in this study are in agreement with our previous ones in which we observed an increase in both the expression of PPARBP and that of the lipoic acid synthase at 6 h after polyphenol-rich olive oil ingestion [30]. Lipoic acid is a powerful antioxidant that can activate PPARα and PPARγ [31]. Ligand-activated PPARs decrease inflammatory responses [32]. PPARγ and PPAR α ligands have been shown to induce a decrease in CD40, monocyte chemotactic protein MCP1 and vascular

endothelial growth factor (VEGF) secretion and to inhibit interferon γ (IFNγ) and intracellular cell adhesion molecule-1 (ICAM-1) expression in cultured cells [33]. In animal models, PPARδ agonists inhibit inflammatory gene expression including those of IFNγ, MCP1 and ICAM-1[33]. In agreement with this, and with the increase in the PPARs expression observed in our study, we have recently reported a down-regulation of CD40-ligand expression and its downstream and related products, such as VEGF, ICAM-1, IFNγ and MCP1, *in vivo* in humans linked to the polyphenol content of the olive oil [12]. A PPARδ agonist has been shown to increase HDL cholesterol *in vivo* in humans [34]. Thus, the fact that polyphenols from olive oil can increase PPARδ expression could explain the higher increase in HDL cholesterol (HDL-C) levels observed after polyphenol-rich olive oil sustained consumption in human studies [6]. PPARα, PPARγ and PPARδ ligands have been shown to stimulate cholesterol efflux in cultured macrophages by inducing the expression of ABCA1 [32,33]. PPARα has also been reported to up-regulate the expression of SR-B1 [4]. The olive oil polyphenol effect on ABCA1 and SR-B1 up-regulation observed in our study could be mediated through the enhancement of PPARs expression. In this sense, incubation of mouse peritoneal macrophages and macrophage-derived foam cells with anthocyanines led to dose-dependent induction in cholesterol efflux and in PPARγ and ABCA1 mRNA expression [35]. Resveratrol has been shown to up-regulate PPARα, PPARγ and PPARδ expression in macrophages [36]. Both quercetin and resveratrol attenuated the suppression of PPARγ mediated by tumor necrosis factor α in human adipocytes [37]. In some experimental studies, but not all [38], hydroxytyrosol, the main phenolic compound from olive oil, has been shown to be able to up-regulate the gene expression of PPARα and PPARγ [39]. In agreement with that referred to above, we observed an increase in PPARs, PPARBP, ABCA1 and SR-B1 expression after HPC olive oil ingestion.

CD36 is a scavenger receptor that promotes uptake of oxidized LDL. PPARγ promotes lipid uptake by up-regulating CD36 expression [32]. We observed an increase in CD36 expression after HPC versus MPC olive oil. This fact is in agreement with our previous results in which a single oral load of polyphenol-rich olive oil, besides an increase in the PPARγ co-activator, the PPARBP, promoted an increase in CD36 expression [30]. Oxidized LDL has been shown to increase monocyte CD36 expression [40]. This activation in turn promotes that of PPARγ via a MAPK-dependent COX-2 pathway [41]. In our setting, oxidized LDL was decreased after both treatments. This fact, together with a lack of COX-2 activation, supports the idea of an oxidized-LDL-independent PPARγ activation. Oxidized HDL decreases CD36 expression via PPARγ [40]. Whether a higher decrease in HDL oxidation after HPC versus MPC could enhance the PPARγ expression leading to an increase in that of CD36 is unknown. The direct relationship between the increase in PPARγ expression with an increase in the antioxidant capacity (ORAC) and the decrease in oxidized LDL would support this hypothesis. An increase in CD36 has been associated with atherosclerosis in experimental models [32]. However, recent data show that CD36 deficiency is associated with enhanced atherosclerotic cardiovascular diseases in humans [42].

Although ABC-dependent RCT is the primary mechanism of cholesterol removal, the 27-hydroxylase pathway provides an alternative to the apoA-I-dependent process [5]. At gene expression level, 27-hydroxycholesterol up-regulates ABCA1 expression via LXR receptor [43]. Selective COX-2 inhibition reduced the mRNA expression of cholesterol 27-hydroxylase and ABCA1, whereas selective COX-1 inhibition down-regulated the expression of 27-hydroxylase mRNA [44]. This interference with RCT has been proposed as an explanation for the fact that COX-2 inhibition elevates cardiovascular risk [45]. In experimental studies, olive oil polyphenols differed from an inhibition of COX-1 and COX-2 activity [46] to a noneffect [47] and also to a repression of the anoxia-induced COX-2 expression [48]. A PPARα and PPARγ control of COX1 and COX-2 expression in macrophages and platelet cultures has been reported, whereas PPARδ appears to be a target of COX-1[49]. Thus, one explanation for the increase in COX-1 expression observed in our study could be via the increase in PPAR expression.

In summary, an up-regulation of the expression of cholesterol efflux related genes — ABCA1, SR-B1, PPARBP, PPARα, PPARγ, PPARδ, CD-36 and COX-1 — in human white blood cells occurs after a polyphenol-rich olive oil ingestion versus a moderate one. Our results point out a significant role of olive oil polyphenols in the up-regulation of genes involved in an enhancement of the cholesterol efflux from cells. Changes in the gene expression were related to a decrease in oxidized LDL and an increase in ORAC and antioxidant polyphenols. Olive oil, a recognized healthy food, cannot be consumed in large quantities. Thus, the enrichment of olive oil with its phenolic compounds is a way of increasing its healthy properties whilst consuming the same amount of fat. Our results are in agreement with previous ones concerning the fact that benefits associated with polyphenol-rich olive oil consumption on cardiovascular risk could be mediated through a nutrigenomic effect. Long-term randomized intervention trials examining the effects of high-polyphenol olive oil diets on HDL functionality are warranted.

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Publication nº 2

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

Rosa-M. Valls, Marta Farràs, Manuel Suárez, Sara Fernández-Castillejo, Montserrat Fitó, Valentini Konstantinidou, Francisco Fuentes, José López-Miranda , Montserrat Giralt, María-Isabel Covas, María-José Motilva, Rosa Solà

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Briefly, in a randomized, crossover, double-blind, controlled trial, after a postpandrial intervention of a FVOO enriched with its own PC (FVOO; 961 ppm), an improvement in EF, measured as ischemia reactive hyperaemia, was observed at 5 hours compared to its baseline and the parental VOO (289 ppm) treatment, in pre-/hypertensive subjects. An inverse relationship between EF and oxidised LDL, was observed after the FVOO intervention. Furthermore, a decreasing linear trend was detected in PAI-1 and hsCRP after VOO consumption and FVOO one. PAI-1 decreased at 4 hours after both interventions versus its baselines, and the decrement was significative after the FVOO consumption versus after the VOO one. In addition, a decrease of VCAM-1 was detected at 2 hours after FVOO intervention compared with its baseline. A reduction in both oxidative stress (decreased oxidative damage to LDL) and resulting inflammation could account for the improvement in the EF observed after FVOO.

Moreover, plasma HT sulphate concentration, the main biological metabolite of hydroxytyrosol, increased in a dose-dependent manner with the OOPC content, with a C_{max} at 2 hours reached. The protection against LDL oxidation linked to FVOO consumption in this study could be mediated by the increase in OOPC metabolites (i.e. HT sulphate) observed in plasma.

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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.

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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) ⩾120–139 mmHg, and/or diastolic blood pressure (DBP) ⩾80–89 mmHg) or stage 1 hypertension (SBP ⩾140–159 mmHg and/or DBP ⩾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 1500g 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

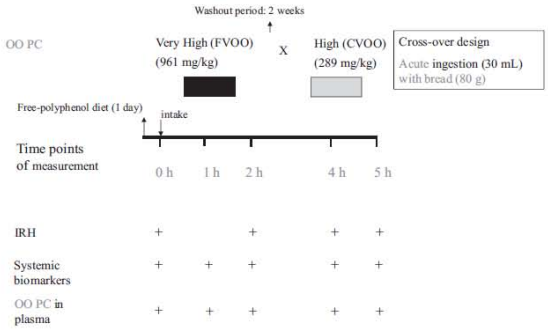

Fig. 1. Study design. OO PC, olive oil phenolic compounds; FVOO, functional virgin olive oil; VOO, virgin olive oil; IRH, ischemic reactive hyperemia.

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: $IRH = ((PUt_d - PUt_0)/PUt_0) \times 100$. 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.

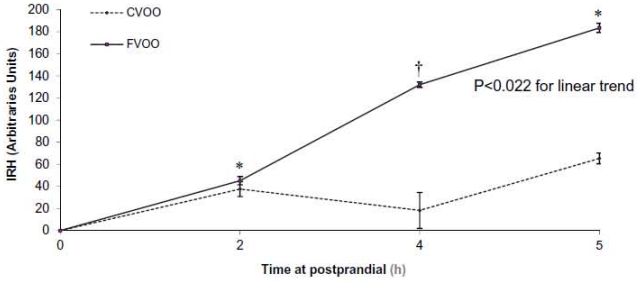

Fig. 2. Ischemic reactive hyperemia (IRH) at postprandial, after the intake of virgin olive oil (VOO) and functional olive oil enriched with its own phenolic compounds (FVOO). *$P < 0.05$ versus baseline; †$P < 0.05$) versus VOO at the same time-point.

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 (logSD). General linear mixed model.
† $P < 0.05$ versus baseline.
§ $P < 0.05$ between treatments at the same time point.

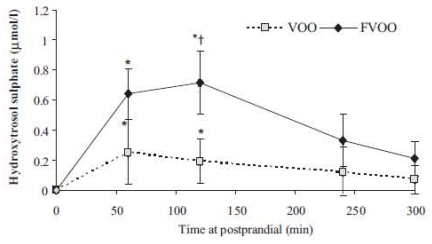

Fig. 3. Time-course of plasma concentrations of hydroxytyrosol sulphate (µmol/L) in human plasma after the intake of virgin olive oil (VOO) and functional olive oil enriched with its own phenolic compounds (FVOO). *$P < 0.05$ versus baseline; †$P < 0.05$ versus VOO at the same time-point.

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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Statement of Authorship: Authors' contributions to manuscript: R.S. and M.-J.M. designed research; R.-M.V., M.S., S.F.-C., F.F. and J.L.-M. conducted research; R.S., R.-M.V., M.F., M.S., S.F.-C., M.F., M.G., M.-I.C., and M.-J.M. provided essential reagents or provided essential materials; R.-M.V., M.-I.C., V.K. and M.F. analysed data or performed statistical analysis; R.S., R.-M.V., M.-I.C., M.F., M.F., M.-J.M. wrote paper; R.S. and M.-J.M. had primary responsibility for final content. All authors critically revised the manuscript for important intellectual content and approved the manuscript being submitted for acknowledgement to contributors who do not meet authorship criteria publication.

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Publication nº 3

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

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Molecular Nutrition and Food Research. 2015, 59, 1758–1770

In summary, after a sustained consumption of a FVOO enriched with its own PC (FVOO; 500 ppm) and plus additional complementary PC from thyme (FVOOT; 500 ppm) versus a natural VOO (80 ppm), improved HDL characteristics related to HDL functionality, in hipercholesterolemic subjects. A randomized, crossover, double-blind, controlled trial was designed. A rise in EC/FC and PL/FC in HDL were observed after FVOOT intervention versus VOO ingestion and FVOO one. Whereas HDL_2-subclass increased after FVOOT consumption versus its baseline and versus VOO one, HDL_3-subclass decreased after FVOOT intervention compared with its baseline. In accordance, LCAT mass increased also after FVOOT ingestion. Furthermore, an improvement in HDL oxidative status was reflected in an increment of PON activity after FVOOT intervention compared with its baseline. HDL subclass distribution showed cross-linked correlations with HDL composition and with HDL metabolism enzymes after OO interventions. In addition, HDL-C concentration increased after FVOOT intervention in the volunteers without hipolipidemic medication.

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RESEARCH ARTICLE

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

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Scope: Consumption of olive oil (OO) phenolic compounds (PCs) 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 olive oils (FVOOs), one enriched with its own PC (500 ppm; FVOO) and another with OOPC (250 ppm) plus additional complementary PCs from thyme (250 ppm) (total: 500 ppm; FVOOT (functional virgin olive oil with thyme)), 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 25 mL/day of VOO, FVOO, and FVOOT during 3 wk. HDL cholesterol increased 5.74% ($p < 0.05$) versus its baseline after the FVOOT consumption in the participants without hypolipidemic medication. We detected, after FVOOT consumption, an increase in HDL_2-subclass (34.45, SD = 6.38) versus VOO intake (32.73, SD = 6.71). An increment in esterified cholesterol/free cholesterol and phospholipids/free cholesterol in HDL was observed after FVOOT consumption (1.73, SD = 0.56; 5.44, SD = 1.39) compared with VOO intervention (1.53, SD = 0.35; 4.97, SD = 0.81) and FVOO intervention (1.50, SD = 0.33; 4.97, SD = 0.81). Accordingly, lecithin-cholesterol acyltransferase mass increased after FVOOT consumption (1228 μg/mL, SD = 130), compared with VOO consumption (1160 μg/mL, SD =

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Abbreviations: **ApoA-I**, apolipoprotein A-I; **CETP**, cholesteryl-ester transfer protein; **CHD**, coronary hearh disease; **DHR**, dihydrorhodamine 123; **EC**, esterified cholesterol; **FC**, free cholesterol; **FVOO**, functional virgin olive oil; **FVOOT**, functional virgin olive oil with thyme; **HDL-C**, HDL-cholesterol; **LCAT**, lecithin-cholesterol acyltransferase; **OO**, olive oil; **PA**, physical activity; **PAF-AH**, platelet-activating factor acetylhydrolase; **PC**, phenolic compound; **PL**, phospholipids; **PON**, paraoxonase/arylesterase; **TC**, total cholesterol; **TG**, triglyceride; **VOO**, virgin olive oil

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144). An improvement in HDL oxidative-status was reflected after FVOOT consumption versus its baseline, given an increment in the paraoxonase activity (118×10^3 U/L, SD = 24).
Conclusion: 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.

Keywords:
Functional virgin olive oil / HDL composition / HDL subclass / Lecithin-cholesterol acyltransferase (LCAT) / Paraoxonase/arylesterase (PON)

1 Introduction

Olive oil (OO) phenolic compounds (PCs) exert antioxidant, anti-inflammatory properties, and chemoprotective activity in experimental studies [1]. Moreover, OOPCs induce favorable changes in lipid profile, improve endothelial function, modify the hemostasis, and have antithrombotic properties in humans [2–4]. Data from human studies show that OOPCs 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 and also 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 randomization 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 in 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 OOPCs enhance cholesterol efflux related genes in pre/hypertensive subjects [17]. In this sense, Helal et al. reported in a linear, nonrandomized, and noncontrolled 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 [20–22]. 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 (functional virgin olive oils); 500 ppm), one enriched with its own PC (FVOO) and another with them plus additional complementary PCs from thyme (FVOOT (functional virgin olive oil with thyme)), 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 FVOOs (500 ppm). FVOO (500 ppm) was enriched with its own PCs by addition of a phenol extract obtained from freeze-dried olive cake. 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 (Fig. 1). The PCs content is the main difference among the three OOs, being the fatty acids and fat-soluble micronutrients very similar among them. The FVOO presents the highest amount of hydroxytyrosol derivatives, whereas the FVOOT presents the highest amount of flavonoids, lignans, and it is the only OO with detectable monoterpenes. The procedure to obtain the phenolic extracts and 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 ultraperformance LC-MS/MS [25].

2.2 Study design

The VOHF (Virgin olive Oil and HDL Functionality) study was randomized, double-blind, crossover, controlled trial with 33 hypercholesterolemic volunteers (total cholesterol (TC) > 200 mg/dL); (19 men), aged 35–80.

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

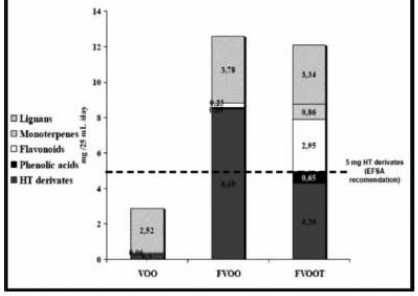

Figure 1. Chemical characterization of VOHF study olive oils. Values are expressed as means ± SD of mg/25 mL oil/day. The acidic composition is expressed as relative area percentage.
Abbreviations: VOO, virgin olive oil; FVOO, functional VOO enriched with its own phenolic compounds; FVOOT, functional 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.

Exclusion criteria included the following: BMI > 35 kg/m^2, smokers, athletes with high physical activity (PA; >3000 kcal/day), diabetes, multiple allergies, intestinal diseases, or other disease or condition that would worsen the adherence to the measurements or treatment. The study was conducted at IMIM-Hospital del Mar Medical Research Institute (Spain) from April to September 2012.

Subjects were randomized to one of three orders of administration of raw OOs (1-VOO, 2-FVOO, 3-FVOOT): Sequence 1-FVOO, FVOOT, VOO; Sequence 2-FVOOT, VOO, FVOO; Sequence 3-VOO, FVOO, FVOOT. In the crossover design, intervention periods were of 3 wk with an ingestion of 25 mL/day raw OO distributed along meals and preceded by 2-wk wash-out periods with a common OO.

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

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

2.3 Dietary adherence

Twenty-four-hour urine was collected at the start of the study and before and after each treatment. Urine samples were stored at –80°C prior to use. We measured urinary hydroxytyrosol sulfate and thymol sulfate as biomarkers of adherence to the type of OO ingested in urine by ultra-HPLC-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 polyphenol-rich food consumption.

2.4 Systemic biomarkers analyses

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

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of whole blood directly after being drawn and were preserved at −80°C until use. EDTA-plasma glucose, TC, and triglyceride (TG) levels were measured using standard enzymatic automated methods and, apolipoprotein A-I (ApoA-I) and apolipoprotein-B100 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). LDL-C was calculated by the Friedewald equation whenever TGs were less than 300 mg/dL. EDTA-plasma glutathione peroxidase activity 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 CETP (MBL, Woburn, MA, USA) and mass concentration of lecithin-cholesterol acyltransferase (LCAT) (American Diagnostica GmbH, Pfungstadt, Germany) were analyzed in serum by fluorimetric kits. Platelet-activating factor acetylhydrolase (PAF-AH) activity was measured in serum (Cayman Chemical, Ann Arbor, MI, USA). Finally, paraoxonase/arylesterase (PON) activity was determined in serum through the measurement of the capacity for cleavage of phenyl acetate resulting in phenol formation (Zeptometrix Corporation, Buffalo, NY, USA). 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 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, CA, USA), 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 fourth to ninth 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]. HDLs 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 subtracting FC from TC. TGs were determined in these samples by automatic enzymatic methods (ABX-Horiba Diagnostics). Apo-AI and Apo-AII were determined by automatic immunoturbidimetric methods (ABX-Horiba Diagnostics, and Spinreact, respectively). For assuring the purity of HDL fractions, apolipoprotein-B100 and albumin levels were also determined in these samples by automatic immunoturbidimetric methods (ABX-Horiba Diagnostics).

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 an SD of 1.9, assuming a dropout rate of 15% and a Type I error of 0.05 (two-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. Noncompliance 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 appropriate, were computed to compare proportions. Pearson and Spearman correlation analyses were used to evaluate relationships among variables. Linear regression models were used to adjust postintervention values for preintervention 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 posttreatment value adjusted with its pretreatment value (as within individual factor) in a general lineal model (GLM) for repeated measurements. When interaction *p*-value was borderline or significant with a study variable, this variable was analyzed without APOE4 carrier participants in GLM for repeated measurements. A value of $p < 0.05$ was considered significant. Carry-over effect was discarded in all variables.

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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 (years)	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/wk)	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
Systolic 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 ± SD or median (25th to 75th percentile).

Statistical analyses were performed by SPSS13.0 software (IBN Corp.) and R2.12.0 software (R Development Core Team).

3 Results

3.1 Participant characteristics and dietary adherence

From 62 subjects who were assessed for eligibility, 29 were excluded. Finally, 33 eligible participants (19 men, 14 women) entered the study. A discontinued single intervention was occurred in three volunteers for the investigator decision. We could not identify any adverse effects related to OO intake. Participants' baseline characteristics are shown in Table 1, no significant differences exist between orders. No changes in daily energy expenditure in leisure-time PA were observed from the beginning to the end of the study. No changes were observed in the main nutrients (Table 2) and medication intake throughout the study, and participants' compliance was good as reflected in the urinary phenols after OO interventions (Table 3).

3.2 Systemic biomarkers

No changes were observed in blood pressure and BMI throughout the study. Although no changes were observed in lipid profile in the entire sample, when the analyses were performed in the participants without hypolipidemic medication, HDL-C increased 5.74% ($p < 0.05$) after FVOOT consumption versus its baseline.

Changes in enzymes related with HDL metabolism and antioxidant status are shown in Table 4. An improvement of LCAT activity was observed after FVOOT intervention compared with VOO ($p = 0.020$), while a borderline decrement was observed after VOO intake versus its baseline ($p < 0.09$). A rise in PON was observed after FVOOT consumption versus its baseline ($p < 0.05$), while a decrement reaching a borderline significance ($p < 0.09$) was observed after VOO intake versus its baseline. A borderline improvement of CETP activity was observed after FVOO intervention compared with VOO ($p = 0.089$). No changes were observed in PAF-AH activity. With regard to the DHR test, there were no significant changes in the intra- and intertreatment analysis. In addition, an inverse correlation between the induced oxidation rate of DHR and the activity of the glutathione peroxidase 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 intervention ($p < 0.05$; Fig. 2). Moreover,

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Table 2. Main nutrient diet changes

	VOO		FVOO		FVOOT		Inter-intervention *p*-Value
	Preintervention[a)]	Postintervention[a)]	Preintervention[a)]	Postintervention[a)]	Preintervention[a)]	Postintervention[a)]	
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 ± SD.
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.

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Table 3. Main compliance phenol markers changes

	VOO		FVOO		FVOOT		Inter-intervention *p*-value
	Preintervention[a)]	Postintervention[a)]	Preintervention[a)]	Postintervention[a)]	Preintervention[a)]	Postintervention[a)]	
Hydroxytyrosol sulfate (μmoles/24 h-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 sulfate (μmoles/24 h-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.

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 FVOO ($p = 0.007$). Also, an increment in HDL PL/FC was observed after the FVOOT consumption compared with VOO ($p = 0.028$) and 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 consumption versus VOO ($p = 0.056$) and FVOO ($p = 0.067$) was observed. Moreover, a borderline increase in HDL EC/TC after FVOOT consumption versus VOO ($p = 0.056$) and 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 in CETP after VOO intake ($r = –0.39$; $p < 0.05$) and borderline correlated with the decrease in CETP after FVOO intake ($r = –0.30$; $p < 0.09$).

3.4 APOE genotype

A total of 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 borderline interaction with HDL EC/FC, HDL PL/FC, and PAF-AH activity was observed ($p < 0.09$). When these variables were analyzed without APOE4 carrier volunteers, similar results as entire sample were obtained, except for HDL PL/FC increment after FVOOT consumption versus FVOO, which did not reach significance ($p = 0.053$).

4 Discussion

We performed a randomized, double-blind, crossover, controlled trial with a VOO, an OO enriched with its own PCs, and another with them plus complementary PCs from thyme. From our results, VOO enriched with its PC plus those of thyme improved parameters related with HDL functional profile, such as HDL-subclass distribution, HDL composition, and enzymes related with HDL metabolism and antioxidant status. For the first time, 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. Moreover, such benefits can be obtained without increasing the individual's fat intake.

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Table 4. Catalytic activities and concentration of enzymes related with HDL metabolism and antioxidant status after interventions

	Baseline[a)]	Post-VOO intervention[a),b)]	Post-FVOO intervention[a),b)]	Post-FVOOT intervention[a),b)]	Inter-intervention *p*-value
Paraoxonase-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)	15 264 ± 3498	15 321 ± 2874	15 345 ± 3030	15 239 ± 2374	0.861 (VOO–FVOOT) 0.821 (FVOO–FVOOT) 0.932 (VOO–FVOO)
Lecithin-cholesterol acyltransferase (μg/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)	26 117 ± 5983	25 617 ± 3333	26 717 ± 5350	26 667 ± 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)

a) Values expressed as postintervention mean ± SD.
b) Postintervention was adjusted by its preintervention.
c) *p*-Value for intra-intervention: $p < 0.05$.
d) *p*-Value for inter-intervention: $p < 0.05$.
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; DHR, dihydrorhodamine 123.

LCAT is an enzyme that mediates the esterification of the FC of nascent HDL monolayers to EC. As a result, this hydrophobic EC move into the center of the particles [31]. The cholesterol esterification by LCAT mediates the gradual conversion of nascent discoidal HDL into small spherical HDL (HDL_3) with two ApoA-I molecules/particle and, finally, into large spherical HDL particles (HDL_2) that contain three ApoA-I molecules [32, 33]. Another enzyme intimately related with the HDL metabolism is CETP that extracts EC from the HDL core to TG-rich lipoproteins, returning TCs from TG-rich lipoproteins to HDL [34]. These EC-poor-TG-rich particles are substrates for hepatic lipase that hydrolyses the TGs. Hepatic lipase depletes the particles of core lipids, generating a redundancy of surface constituents. A TG-poor HDL core may imply better functional properties of the particle. When the HDL core is TG-rich, ApoA-I is more loosely bound to the HDL surface [35]. Accordingly, after the intervention with FVOOT, which increases LCAT activity, we observed an increase in HDL_2-particle subclass percentage and a decrease in the HDL_3 one, while after VOO intervention we detected a decrease in LCAT activity and in HDL_2-particle subclass percentage. Moreover, the increase in HDL_2/HDL_3 directly correlated with the increase in ApoA-I after the three interventions. In addition, an increase in 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 [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 in HDL_2-subclass and in antioxidant enzyme activities was observed after FVOOT intake.

An HDL with an increase in 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 atheroprotective 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: (i) aqueous diffusion mediated, (ii) SR-BI mediated, (iii) ABCA1 mediated, and (iv) ABCG1 mediated [50–53]. The aqueous diffusion-mediated pathway mediates the bidirectional flux between the cell plasma membrane and HDL in the

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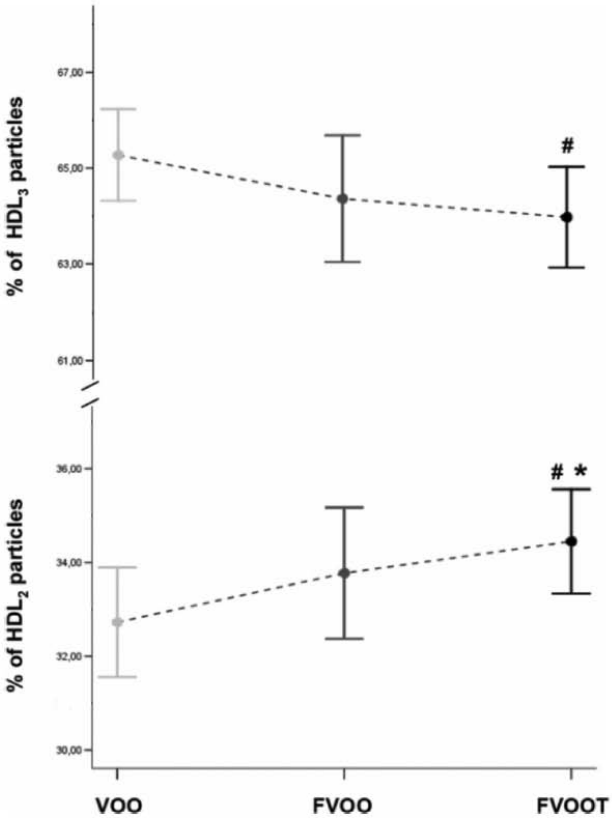

Figure 2. HDL subclass distribution after interventions. Values represent postintervention mean ± SD. Postintervention values were adjusted with its preintervention ones. #Intra-intervention *p*-value < 0.05; *Inter-intervention *p*-value < 0.05 compared with VOO intervention.
Abbreviations: VOO, virgin 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.

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 preβ-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 ABCA1- and ABCG1-mediated efflux [54].

The antioxidant properties of OOPC in vivo are well known. The EUROLIVE study showed a decrease in in vivo 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-OO 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 in PON and LCAT activities after the FVOOT intervention, was observed in the

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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 postintervention mean ± SD.
b) Postintervention was adjusted by its preintervention.
c) Inter-intervention *p*-value; $p < 0.05$.
Intra-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.

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present study. In contrast, PON decreased after the FVOO intervention. PON1, which is associated with 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 among the different kinds of OOPC such as flavonoids, monoterpenes, and phenolic acids [23] (Fig. 1). In this sense, some non-human studies with flavonoid-enriched OO have been performed. Rosenblat et al. published that an extra VOO enriched with green tea polyphenols (mostly epicatechin gallate) attenuates atherosclerosis development enhancing macrophage cholesterol efflux in Apo-E-deficient mice more than an extra VOO. These OOs also showed a decrease in the serum oxidative stress in Apo-E-deficient mice compared with placebo treatment [59]. Recently, a study has reported that an OO-pomegranate sauce prolongs lipid oxidation and can retard undesirable quality changes in anchovy [60].

One strength of our study is its crossover and randomized design, which permitted the participants to consume all OO types and thus eliminated the interindividual variability. Moreover, the laboratory analyses were centralized and all the time-series samples from the same volunteer were measured in the same run. It lacks a related-HDL oxidative status parameter to stand up the association between the PC-enriched OO and the functionality HDL-related characteristics. A limitation of the study was its sample size, which could be responsible for reduced statistical power in some biomarkers with high interindividual variability. A synergistic effect on HDL parameters between PC and other OO components is as yet unknown. Another limitation is the inability to assess potential interactions among the OOs and other diet components and medication, although the controlled diet and medication followed during all clinical trial should have limited the scope of these interactions.

In conclusion, long-term consumption of complementary phenol-enriched OO induced changes in the HDL profile associated with low cardiovascular risk, such as higher levels of large HDLs, lower levels of small ones, increased HDL EC/FC content, increased HDL monolayer PL/FC, and increased HDL antioxidant enzymes. OO, a recognized healthy food, cannot be consumed in large quantities. Thus, the enrichment of OO with its PCs is a way of increasing its healthy properties while the same amount of fat is consumed. Moreover, these results show that an enrichment of OO 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 high cardiovascular risk individuals.

In conclusion, for the first time, 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. 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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