EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS

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INTERNATIONAL DOCTORAL THESIS

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UNIVERSITAT ROVIRA I VIRGILI

Reus, Spain 2017

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FAIG CONSTAR que aquest treball, titulat "*Efectes d'olis d'oliva verge enriquits en compostos fenòlics sobre la funcionalitat de l'HDL en individus hipercolesterolèmics*", que presenta Sara Fernández Castillejo per a l'obtenció del títol de Doctor de menció internacional, ha estat realitzat sota la meva direcció al Departament de Medicina i Cirurgia d'aquesta universitat.

HAGO CONSTAR que el presente trabajo, titulado "*Efectos de aceites de oliva virgen enriquecidos con compuestos fenólicos sobre la funcionalidad de la HDL en individuos hipercolesterolémicos*", que presenta Sara Fernández Castillejo para la obtención del título de Doctor de mención internacional, ha sido realizado bajo mi dirección en el Departamento de Medicina y Cirugía de esta universidad.

I STATE that the present study, entitled "*Effects of phenol-enriched virgin olive oils on HDL functionality in hypercholesterolemic subjects",* presented by Sara Fernández Castillejo for the award of the degree of Doctor with international mention, has been carried out under my supervision at the Department of medicine and Surgery of this university.

Reus, 05 de Maig del 2017

El/s director/s de la tesi doctoral
El/los director/es de la tesis doctoral
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International thesis for the award of the degree of Biomedicine Doctor with International Mention at the *Universitat Rovira i Virgili*, defensed by Sara Fernández Castillejo:

- Degree in Biology at the *Universitat Pompeu Fabra* (1999-2004, Barcelona, Spain).
- Master of Clinical Nutrition and Metabolism at the *Universitat Rovira i Virgili* (2009-2010, Tarragona, Spain) awarded with special mention.

Funding sources: This work was supported by the Spanish Ministry of Economy and Competitiveness within the activities of the following projects: AGL2009-13517-C03, AGL2012-40144-C03 and AGL2016-76943-C2-2-R.

This work was also supported by the Universitat Rovira i Virgili, with the International Mobility Grant for PhD students of the Biomedicine Program 2013.

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A mi familia por estar siempre ahí de forma incondicional,

y en especial a Ricard y Valentina, las dos chispitas de mi vida.

A Jordi, por enseñarme todo lo que no sabía que existía.

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EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS
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Abbreviations

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ABC	ATP-binding cassette transporters
AHA/ACC	American College of Cardiology/American Heart Association
AHR	aryl hydrocarbon receptor
ANOVA	analysis of variance
Apo	apolipoprotein
Bd-Ch	BODIPY-cholesterol
BODIPY	boron dipyrromethene difluoride
CAD	coronary artery disease
CCL-2	chemokine (C-C motif) ligand 2
CE	cholesteryl esters
CEC	HDL cholesterol efflux capacity
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
ChE	cholesterol efflux
CON diet	control diet, commercial feed pellets
CoQ	coenzyme Q
CVD	cardiovascular disease
CVR	cardiovascular risk
EFSA	European Food Safety Authority
ELISA	Enzyme-Linked ImmunoSorbent Assay
EPIC	European Prospective Investigation into Cancer and Nutrition
ESI-MS/MS	electrospray tandem mass spectrometry
EUROLIVE	Effect of Olive Oils on Oxidative Damage in European Populations study
FA	fatty acids
FC	free cholesterol
FUFOSE	Functional Food Science in Europe
FVOO	functional VOO enriched with its own PC
FVOOT	functional VOO enriched with its own PC (50%) plus complementary ones from thyme (50%)
GC	gas chromatography
GSPx	glutathione selenoperoxidase
^{3}H-Ch	tritiated cholesterol
HDL	high-density lipoprotein

HDL-C	HDL cholesterol
HDL-P	HDL particle number
H-FVOO	high-functional virgin olive oil with 750 ppm of total phenolic content
HOMA	homeostatic model assessment
HPLC	high-performance liquid chromatography
HT	hydroxytyrosol
ICAM-1	intercellular adhesion molecule-1
IDL	intermediate-density lipoprotein
IRH	ischemic reactive hyperemia
LCAT	lecithin-cholesterol acyltransferase
LDL	low-density lipoprotein
LDL-C	LDL cholesterol
LDL-P	LDL particle number
L-FVOO	low-functional VOO with 250 ppm of total phenolic content
l-HDL	large-HDL
LP-IR	lipoprotein insulin resistance index
LXR	liver X receptor
MAPK	mitogen-activated protein kinases
MESA	Multi-Ethnic Study of Atherosclerosis
M-FVOO	medium-functional VOO with 500 ppm of total phenolic content
m-HDL	medium HDL
MPO	myeloperoxidase
MUFA	monounsaturated fatty acids
NMR	nuclear magnetic resonance
OO	olive oil
OO-PC	olive oil phenolic compounds
ORAC	oxygen radical absorbance capacity assay
oxLDL	oxidized LDL
PAF-AH	platelet-activating factor acetylhydrolase
PAGGE	polyacrylamide gradient gel electrophoresis
PC	phenolic compounds
PL	phospholipids
PLTP	phospholipid transfer protein

PON	paraoxonases
PPAR	peroxisome proliferator-activated receptor
ppm	parts per million, mg/Kg
PREDIMED	Prevención con Dieta Mediterránea study
PROCAM	Prospective Cardiovascular Münster study
PUFA	polyunsaturated fatty acids
RCT	reverse cholesterol transport
ROS	reactive oxygen species
SEC diet	control diet supplemented with phenolic compounds from olive oil, mainly secoiridoids
SEC+THY diet	diet supplemented with secoiridoids and thyme phenols extracts
SFA	saturated fatty acids
s-HDL	small-HDL
SMC	smooth muscle cells
SNP	single-nucleotide polymorphism
SR-BI	scavenger receptor type BI
T1D	type 1 diabetes
T2DM	type 2 diabetes *mellitus*
TBBL	5-thiobutyl butyrolactone
TG	triglycerides
Th-PC	phenolic compounds from thyme
THY diet	diet supplemented with thyme phenols extract
TNF-α	tumor necrosis factor-α
TPC	total phenolic content
UPLC/MS/MS	ultra-performance liquid chromatography-tandem mass spectrometer
VA-HIT	Veterans Affairs HDL Intervention Trial
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein
VOHF	Virgin Olive Oil and HDL Functionality study
VOO	virgin olive oil
WHO	World Health Organization

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Summary

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INTRODUCTION

Mounting epidemiologic evidence has confirmed that high-density lipoprotein (HDL) cholesterol levels are a strong negative risk marker for cardiovascular disease (CVD). However, conflicting data have cast doubt on such an association, as therapeutic strategies that increase HDL cholesterol (HDL-C) levels in the circulation have not yielded clinical benefits in CVD.

This paradoxical association between low HDL-C levels and high CVD risk (CVR) can be explained by the fact that the HDL pool encompasses a heterogeneous population of HDL particles that differ not only in physicochemical properties but also in functional quality, irrespective of their cholesterol content. Therefore, alternative therapeutic strategies should be developed to properly modify HDL functionality rather than HDL quantity to tackle CVD.

In November 2011, the European Food Safety Authority (EFSA) released a health claim concerning the beneficial properties of phenolic compounds (PC) from virgin olive oil (VOO) in the maintenance of normal HDL-C levels. However, the phenolic concentration in most VOOs available on the market is too low for sufficient consumption of VOO phenols to support the health claim. A good approach to ensure the optimal intake of PC in the context of a balanced diet is to tailor a phenol-enriched functional VOO. Combining PC from different classes and sources might allow increased phenol consumption, which will optimize the likely effects observed from the consumption of a single type of PC.

HYPOTHESIS

The sustained intake of functional VOOs enriched with their own PC, predominantly secoiridoids, such as hydroxytyrosol and its derivatives, or with their own PC plus additional complementary PC from thyme, such as flavonoids, may modify the physicochemical properties of HDL particles towards a cardioprotective mode and may promote changes in HDL subclass distribution, leading to the consequent enhancement of HDL functionality in hypercholesterolemic subjects. The secondary hypothesis is that several HDL physicochemical properties, such as composition,

fluidity, oxidative status, and size, might be the main determinants of the capacity of HDL to promote cholesterol efflux (ChE) from cells.

OBJECTIVES

Primary objective

To assess whether the sustained intake of phenol-enriched VOOs could enhance HDL functionality in hypercholesterolemic subjects by modifying the following:

1. HDL metabolism and maturation and therefore subpopulations distribution (Study 1: Fernández-Castillejo *et al*., 2016),
2. antioxidant content (*i.e.*, fat-soluble antioxidants and phenolic metabolites) (Study 2: Farràs & Fernández-Castillejo *et al*., under review),
3. ChE and HDL characteristics influencing ChE (*i.e.*, fluidity), (Study 2: Farràs & Fernández-Castillejo *et al*., under review) and
4. Paraoxonase (PON) enzyme family (Study 3: Fernández-Castillejo *et al*., 2017).

Secondary objectives

1. To assess the effect of the sustained intake of phenol-enriched VOOs on several CVR parameters, such as very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) subpopulation distribution, and LDL particles/HDL particles (LDL-P/HDL-P), HDL-C/HDL-P, and small-HDL/large-HDL (s-HDL/l-HDL) atherogenic ratios (Study 1: Fernández-Castillejo *et al*., 2016).

2. To devise a high-throughput *in vitro* ChE assay to efficiently screen large numbers of specimens, such in the case of clinical trials, avoiding the use of radiolabeled cholesterol (Study 2: Farràs & Fernández-Castillejo *et al*., under review).

3. To assess whether the acute intake of phenol-enriched VOOs could instigate short-term modifications in PON-related variables (Study 3: Fernández-Castillejo *et al*., 2017).

4. To investigate the mechanisms of action involved in the PON system in an *in vivo* animal model (Study 3: Fernández-Castillejo *et al*., 2017).

5. To define which parameters are the best predictors for HDL cholesterol efflux capacity (CEC) and its interrelations with the fluidity of HDL monolayer after VOO intake (Study 4: Fernández-Castillejo *et al*., under review).

METHODS

Two randomized, controlled, double-blind, crossover interventions were conducted within the framework of the "*Virgin Olive Oil and HDL Functionality*" (VOHF) study:

Acute-intake study

Twelve healthy participants ingested a single dose of 30 mL of raw VOOs enriched with their own PC, but differing in phenolic content:

a. Low-functional VOO (L-FVOO) enriched with 250 ppm of PC
b. Medium-functional VOO (M-FVOO) enriched with 500 ppm of PC
c. High-functional VOO (H-FVOO) enriched with 750 ppm of PC

A natural VOO with 80 ppm of PC was used as the matrix to prepare these phenol-enriched VOOs by adding a freeze-dried olive cake extract that was rich in PC from olive oil. Interventions were separated by a 1-week washout period.

Sustained-intake study

Thirty-three hypercholesterolemic participants ingested 25 mL/day of three VOOs, differing in PC source and content:

a. A natural control VOO with 80 ppm of PC
b. A functional VOO enriched with 500 ppm of PC from olive oil (FVOO)
c. A functional VOO enriched with 500 ppm of total PC from olive oil and complementary ones from thyme in a 1:1 ratio (FVOOT).

For the sustained-intake study, the same parental VOO used for the acute-intake study was used as a control condition and as a matrix to prepare both functional VOOs. The intervention periods were 3 weeks long and were separated by 2-week washout periods.

Study 1: *"Polyphenol rich olive oils improve lipoprotein particle atherogenic ratios and subclasses profile: A randomized, crossover, controlled trial"*

Lipoprotein particle counts and subclass distribution measurements were performed by nuclear magnetic resonance in a Vantera clinical spectrometer produced by LipoScience. The LipoProfile-3 algorithm was use to quantify the average particle size and particle concentrations of VLDL, LDL, and HDL. Subparticle concentrations were determined for three VLDL subclasses (large or chylomicrons: > 60 nm; medium: 35–60 nm; and small: 27–35 nm); three LDL subclasses (intermediate-density lipoprotein (IDL): 23–27 nm; large: 21.2–23 nm; and small: 18–21.2 nm); and three HDL subclasses (large: 8.8–13 nm; medium: 8.2– 8.8 nm; and small: 7.3–8.2 nm). The Lipoprotein Resistance Index (LP-IR), a lipoprotein particle-derived measure of insulin resistance, was also assessed. These analyses were performed in the samples from the sustained-intake study.

Study 2: "*Phenol-enriched olive oils improve HDL antioxidant content in hypercholesterolemic subjects. A randomised, double-blind, cross-over, controlled trial*"

HDL fatty acids, fat-soluble antioxidants and phenolic metabolites were analyzed by gas chromatography, liquid chromatography, and ultra-performance liquid chromatography-tandem mass spectrometer, respectively. HDL particle fluidity was measured by determination of the steady-state anisotropy of a 1,6-diphenyl-1,3,5-hexatriene fluorescent probe stimulated with a polarized light. ChE was assessed in an *in vitro* cellular model that implicates macrophage labelling with fluorescent cholesterol and incubation with HDL from the volunteers. All these analyses were performed in the samples from the sustained-intake study.

Study 3: "*Phenol-enriched olive oils modify paraoxonase–related variables: a Randomized, Crossover, Controlled Trial*"

PON3 and PON1 protein concentrations were measured in human sera by an in-house Enzyme-Linked ImmunoSorbent Assay with rabbit polyclonal antibodies. PON1-associated paraoxonase and lactonase activities were assessed in human sera by measuring the rate of hydrolysis of paraoxon and 5-thiobityl butyrolactone, respectively. These analyses were performed in the samples from both the acute- and the sustained-intake studies.

Mechanistic studies were carried out in rats that followed a diet enriched with the appropriate phenolic extract for 21 days: phenolic extracts from olive oil, from thyme, or from both olive oil and thyme. A control group was also included in the study. The activation of mitogen-activated protein kinases and additional related kinases was assessed in rat liver homogenates. Furthermore, gene expression of *Pon1*, *Pon3,* and *chemokine (C-C motif) ligand 2* (CCL-2), along with transcription factors *aryl hydrocarbon receptor*, *peroxisome proliferator-activated* (*PPAR*)*α*, *δ,* and *γ*, were also analyzed in rat liver homogenates.

Study 4: "*Determinants of HDL cholesterol efflux capacity after virgin olive oil ingestion: Interrelationships with fluidity of HDL monolayer*"

Post hoc analyses were performed with samples from participants in the sustained-intake VOHF study to assess the relationships in the 3-week changes in HDL-related variables after the three intervention periods. Mixed linear models, adjusted by age, sex, and the individual level of the test subjects as a random effect, were fitted with variables related to CEC and fluidity in univariate analyses.

RESULTS

Study 1: *"Polyphenol rich olive oils improve lipoprotein particle atherogenic ratios and subclasses profile: A randomized, crossover, controlled trial"*

FVOO decreased LDL-C and the LDL-P/HDL-P ratio and improved the lipoprotein subclasses by decreasing the total apolipoprotein (Apo)B100-containing lipoproteins, total LDL, small LDL, IDL and s-HDL particle concentrations, and LDL and VLDL particle sizes. Both FVOO and FVOOT decreased medium VLDL particles, increased l-HDL particles and HDL size, and decreased the s-HDL/l-HDL ratio, the HDL-C/HDL-P ratio and LP-IR.

Study 2: "*Phenol-enriched olive oils improve HDL antioxidant content in hypercholesterolemic subjects. A randomised, double-blind, cross-over, controlled trial*"

The sustained intake of FVOO and FVOOT increased both lipophilic and hydrophilic compounds with antioxidant properties in HDL. Regarding fat-soluble antioxidants, FVOO and FVOOT intake increased ubiquinol and the carotenoids lutein and β-cryptoxanthin. FVOO increased retinol, while FVOOT increased α-tocopherol. Concerning hydrophilic compounds, FVOOT intake increased the phenolic metabolites caffeic acid sulfate, hydroxyphenylpropionic acid sulfate, and thymol sulfate, while hydroxytyrosol acetate sulfate increased after FVOO intake. ChE tended to increase after FVOOT versus its baseline and increased versus FVOO. No significant changes were observed in HDL monolayer fluidity nor in fatty acids after any intervention.

Study 3: "*Phenol-enriched olive oils modify paraoxonase–related variables: a Randomized, Crossover, Controlled Trial*"

The acute and sustained intake of VOO and FVOO decreased PON1 protein and increased PON1-associated activities, while sustained FVOOT intake yielded the opposite results, not producing synergic effects. PON3 protein levels increased only after the sustained consumption of VOO.

Mechanistic studies performed in rat livers showed that the intake of isolated PC from olive oil or thyme modulates mitogen-activated protein kinases and PPAR, regulating PON synthesis, while a combination of these PC cancelled such regulation.

Study 4: "*Determinants of HDL cholesterol efflux capacity after virgin olive oil ingestion: Interrelationships with fluidity of HDL monolayer*"

An increase in HDL fluidity and the concentration of apolipoprotein (Apo) A-I in HDL and a decrease in HDL oxidative status were found to be the main determinants for CEC. Changes in HDL lipid composition (in particular, a reduction in free cholesterol (FC) and an increase in triglycerides (TG) in HDL) and a decrease in s-HDL particle number or an increase in HDL mean size were the main determinants of the fluidity of the HDL monolayer.

CONCLUSIONS

1. Sustained consumption of phenol-enriched VOOs, namely FVOO and FVOOT, shifted subclass distribution and associated ratios of lipoproteins to a less atherogenic pattern:

   1.1. Both phenol-enriched VOOs enhanced HDL maturation, since they modified HDL size and subclass distribution towards larger and more mature HDL particles.

   1.2. FVOO decreased the LDL and VLDL parameters commonly associated with coronary heart disease risk and dyslipemia.

   1.3. Both phenol-enriched VOOs decreased several atherogenic ratios.

   1.4. FVOO could be better at improving lipoprotein subclass distribution, since FVOOT had a milder impact on HDL and VLDL subclass distribution and atherogenic ratios than FVOO, and had no impact on LDL particle distribution.

2. Sustained consumption of both phenol-enriched VOOs improved the HDL antioxidant content, since they increased the lipophilic and hydrophilic antioxidants in HDL. The co-existence of these antioxidants linked to HDL may confer additional benefits by protecting lipids and proteins from oxidative damage via different antioxidant mechanisms. However, FVOOT intervention could be better at improving HDL antioxidant activity, as it increased α-tocopherol, the major antioxidant in

human plasma. Moreover, FVOOT also increased phenolic acids in HDL, which are known to regenerate α-tocopherol.

3. Acute and sustained intake of phenol-enriched VOOs modify the PON enzyme family towards a cardioprotective mode according to the phenolic content and source. In particular, FVOO intake may be better at modulating the PON enzyme family, as it promoted changes that are indicative of a proper oxidative balance, supporting HDL function enhancement. On the other hand, FVOOT induced opposite results due to the combination of PC from OO and thyme, in comparison to the sole intake of PC from thyme. Several mechanisms are involved in PON system modulation, such as PON synthesis, higher content of antioxidants in HDL, and enhanced HDL maturation.

4. Decreases in HDL oxidative status and increases in HDL monolayer fluidity and ApoA-I content in HDL are the major determinants of CEC. In turn, monolayer fluidity is determined by HDL lipid composition (in particular, by an increase in TG and a reduction in FC), together with a decrease in the concentration of s-HDL particles or an increase in HDL mean size.

5. Sustained intake of FVOOT increased ChE versus FVOO intake, despite the fact that no changes were observed in HDL monolayer fluidity.

6. Factors other than monolayer fluidity may be responsible for the ChE enhancement observed after FVOOT. These include a) a higher presence of antioxidants in HDL, which may confer better antioxidant protection and may therefore enhance ChE, b) a decrease in FC in the HDL monolayer, and c) the modulation of HDL subclass distribution.

GLOBAL CONCLUSION

The present thesis confirms the hypothesis that the sustained intake of functional VOOs enriched with their own PC or with their own PC plus additional complementary ones from thyme modifies the physicochemical properties of HDL particles towards a cardioprotective mode and promotes changes in HDL subclass distribution, leading to the consequent enhancement of HDL functionality in hypercholesterolemic subjects.

These changes occurred based on the phenol content and source in the tested VOOs.

The enrichment of VOOs with PC is a way of increasing the healthy properties of VOO without increasing the individual's caloric intake. Therefore, the tailoring of functional VOOs is an interesting and useful strategy for enhancing the functional quality of HDL, and thus, it is a complementary tool for the management of hypercholesterolemic individuals.

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Justification

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The role of HDL in cardiovascular disease

The understanding of the role of high-density lipoprotein (HDL) in cardiovascular disease (CVD) is currently under debate. In 1975, it was observed that plasma HDL cholesterol levels (HDL-C) levels were reduced in several conditions, which was associated with an increased risk of future CVD outcomes (Miller & Miller, 1975).

The importance of low HDL-C levels as an independent CVD risk (CVR) factor triggered decades of intense research to figure out the underlying mechanisms of this prognostic relationship, which would allow the development of new therapeutic strategies to raise HDL-C levels and therefore tackle CVD (Choi, Hafiane, Schwertani, & Genest, 2016; Karathanasis, Freeman, Gordon, & Remaley, 2017). On one hand, the Framingham Heart and Prospective Cardiovascular Münster (PROCAM) studies showed an increase in cardiovascular morbidity and mortality in individuals with low HDL-C levels compared to those with higher HDL-C levels (Assmann, Schulte, Von Eckardstein, & Huang, 1996; T. Gordon, WP, Hjortland, Kannel, & TR DAwber, 1977; Wilson, Abbott, & Castelli, 1988). On the other hand, a meta-analysis of prospective studies showed that there was a 2-3% increase in CVR for every 1 mg/dL decrease in plasma levels of HDL-C, irrespective of other CVR factors, including plasma low-density lipoprotein (LDL) cholesterol (LDL-C) levels (Gordon et al., 1989).

The strength of this evidence led to formulation of the “HDL hypothesis”: HDL plays a causal role in the pathogenesis of atherosclerotic CVD. Thus, it was assumed that interventions that increase plasma HDL-C concentrations would reduce the risk of coronary heart disease (CHD) (Wilson, Abbott, & Castelli, 1988).

However, conflicting evidence has cast doubt on the HDL hypothesis since therapeutic strategies that increase HDL-C levels in the circulation have not yielded clinical benefits in CVD (J.C. Escolà-Gil, Cedó, & Blanco-Vaca, 2013; Kingwell, Chapman, Kontush, & Miller, 2014; Lüscher, Landmesser, Von Eckardstein, & Fogelman, 2014; Salazar et al., 2015). Pharmacological treatment with HDL-C-rising agents by inhibiting or delaying HDL catabolism, such as cholesteryl ester transfer protein (CETP) inhibitors (Barter et al., 2007) or niacin (Boden et al., 2011), managed to increase

such HDL-C levels, but did not show clinical benefits. What is more, a clinical trial with the CETP inhibitor torcetrapib was called off in 2006 when excessive all-cause mortality was detected in the group receiving a combination of atorvastatin and torcetrapib versus the group taking atorvastatin alone (Barter et al., 2007; Pearson, 2006; Tall, Yvan-Charvet, & Wang, 2007). In the same way, hereditary syndromes characterized by extremely low HDL-C levels, such as Tangier disease and Apolipoprotein (Apo)A-I_{Milano}, surprisingly display a cardioprotective phenotype (Elshourbagy, Meyers, & Abdel-Meguid, 2014; Hafiane & Genest, 2015; Karathanasis et al., 2017; Salazar et al., 2015). Similarly, genetic deficiency of lecithin-cholesterol acyltransferase (LCAT) presents very low HDL-C levels, but only a modest increase in the risk for premature atherosclerosis (Ossoli, Simonelli, Vitali, Franceschini, & Calabresi, 2015). Finally, Mendelian genetic studies have failed in establishing causal relationships between gene variants associated with low HDL-C and increased CVR, raising concerns about the use of HDL-C as a target to tackle CVD (Frikke-Schmidt, 2008; Gugliucci & Menini, 2015; Hafiane & Genest, 2015).

Therefore, although the HDL hypothesis suggests that HDL has a protective role against CVD, its understanding has recently been challenged due to the evidence mentioned above.

Pursuing the paradoxical HDL hypothesis

The paradoxical association between low HDL-C levels and high CVR can be explained by the fact that HDL is a highly dynamic particle that undergoes constant remodeling and recycling. Rather than being homogeneous, the HDL pool encompasses a heterogeneous population of HDL particles that differ in physicochemical properties, such as shape, size, density, surface charge, lipid composition, and antigenicity (Ansell, Fonarow, & Fogelman, 2007).

This HDL physicochemical heterogeneity parallels the heterogeneity of HDL functional quality. That is, the heterogeneous nature of HDL composition is translated into several HDL subpopulations, which exhibit differences in functionality, and therefore atherogenicity, irrespective of their cholesterol content (Karathanasis et al., 2017). That is, the cholesterol content of HDL is not the factor that determines its biologic activity; rather, its functional quality determines this activity to such an

extent that HDL particle number (HDL-P) has been suggested as being a better surrogate of HDL function than HDL-C (Akinkuolie, Paynter, Padmanabhan, & Mora, 2014; Choi et al., 2016; K. Rye & Barter, 2014; Santos-Gallego, 2015).

It should be mentioned that the HDL hypothesis has always been related to HDL function rather than its quantity, *i.e.,* HDL-C, and simply raising levels of HDL-C cannot be directly inferred as improvements in HDL function. Thus, emerging therapies aimed to decrease CVD outcomes should focus their efforts on properly increasing not only HDL quantity but also quality, enhancing HDL functionality (Hafiane & Genest, 2015).

HDL holds atheroprotective properties

An essential function of HDL is to transport and deliver excess cholesterol from peripheral tissues to the liver, where it is excreted into the bile in a process known as reverse cholesterol transport (RCT). In the first step of RCT, HDL acts as an acceptor of cholesterol found in lipid-laden macrophages, a process known as ChE (Rader, Alexander, Weibel, Billheimer, & Rothblat, 2009).

HDL can also attenuate oxidative stress that is present in the arterial intima of the atherosclerotic lesion since this lipoprotein holds antioxidant properties down to the presence of several antioxidant proteins, such as paraoxonases (PON) and Apos, within its proteome. The PON enzyme family is widely considered the major contributing factor to the antioxidant potential of HDLs (Karlsson, Kontush, & James, 2015).

While ChE and antioxidant function are two processes that play a key role in the atheroprotective function of HDL, additional properties have been attributed to the HDL particle (Figure 1), such as the following:

1. anti-inflammatory,
2. antiapoptotic,
3. antithrombotic,
4. anti-infective,
5. vasoprotective,
6. effects on glucose metabolism, and
7. intracellular communication (Choi et al., 2016; Karathanasis et al., 2017; Kingwell et al., 2014).

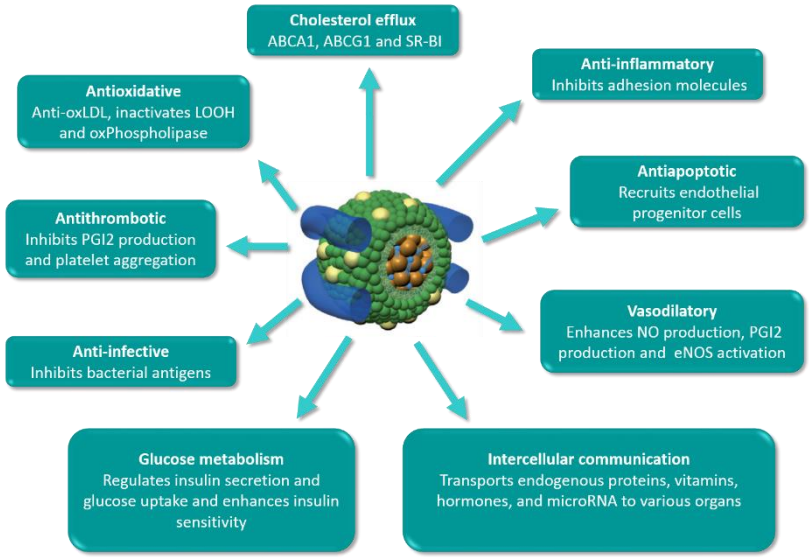

Figure 1 | Cardioprotective benefits of HDL. HDL holds pleiotropic activities in protection against arteriosclerosis. ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; eNOS, endothelial nitric oxide synthase; LOOH, lipid hydroperoxides; NO, nitric oxide; oxLDL, oxidized low-density lipoprotein; PGI2, prostaglandin I2; SR-BI, scavenger receptor class B type I. Source: adapted from Choi *et al.,* 2016.

Atheroprotective HDL: quantity or quality?

Recent research has questioned the HDL hypothesis, as the absolute HDL-C levels, which are typically used as a marker of atheroprotection, may be of less clinical significance compared to HDL quality or functional capacity (Karathanasis et al., 2017; Santos-Gallego, 2015). Both experimental and observational studies note that rather than HDL-C, HDL lipoprotein profile (defined as HDL-P number, size, and subclass distribution) might be an appropriate parameter for assessing the quality of HDL and therefore its atheroprotective function. As a matter of fact, HDL lipoprotein profile has been found to be more strongly associated with CVR than HDL-C (Qi et al., 2015; Rhee, Byrne, & Sung, 2017).

HDL subclasses do not all protect against atherosclerosis equally well, as subgroups of particles boast distinct antiatherogenic benefits. Broadly speaking, small HDL_2 are considered more functional than HDL_3 (Movva & Rader, 2008; K.-A. Rye, Bursill, Lambert, Tabet, & Barter, 2009; Salazar et al., 2015). For instance, patients with the same HDL-C levels may present different CVR depending on the number and size of HDL-P (Figure 2). In this sense, the presence of massive cholesterol-overloaded HDL-P is currently considered a potentially new measure of HDL cardioprotective function, as it has been shown to be directly related to atherosclerosis progression in a CVD-free population (Qi et al., 2015).

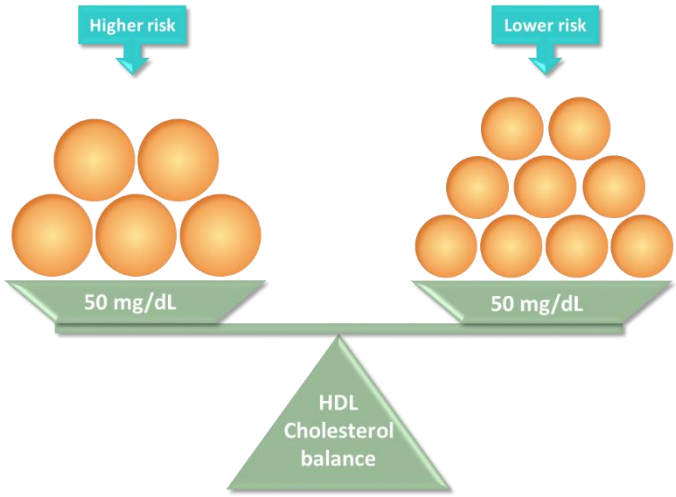

Figure 2 | Patients with the same HDL-C levels may present different CVR depending on the number and size of HDL-P. In this sense, the presence of cholesterol-overloaded HDL-P has been associated with the progression of carotid atherosclerosis in a CVD-free population. Source: created by the author.

Born out of this heterogeneity in HDL quality, a growing interest in identifying HDL subclasses and their related functions has emerged. Emerging techniques, such as proton nuclear magnetic resonance (NMR) spectroscopy, allow us to directly measure lipoproteins particle number, size, and subpopulation distribution (Anastasius et al., 2016; Eren, Yilmaz, & Aydin, 2012).

Strategies to tackle CVD by modifying HDL quality: olive oil and phenolic compounds

The Mediterranean diet has been broadly claimed to be one of the reasons for the low incidence of CVD registered in the Mediterranean area, despite the high prevalence of CVR factors (Lou-Bonafonte, Gabás-Rivera, Navarro, & Osada, 2015; Pauwels, 2011). The Mediterranean dietary pattern is characterized by the high intake of fruits and vegetables, along with the use of virgin olive oil (VOO) as the main source of fat consumption. Most of the beneficial effects of the Mediterranean diet are attributed to the presence of minor components in VOO, *i.e.*, phenolic compounds (PC) (Lou-Bonafonte et al., 2015).

These PC are naturally present not only in VOO but also in fruits and vegetables, and they hold atheroprotective benefits (M.-I. Covas, Konstantinidou, & Fito, 2009; María Isabel Covas, 2007; María Isabel Covas, de la Torre, & Fitó, 2015; Álvaro Hernáez et al., 2014; J. López-Miranda et al., 2010). In fact, the European Food Safety Authority (EFSA) released a health claim concerning the effects of a daily intake of 5 mg of the PC hydroxytyrosol (HT) and its derivatives on LDL protection from oxidation and on the maintenance of normal blood HDL-C levels (EFSA Panel on Dietetic Products. Nutrition and allergies & (NDA)., 2011). Nevertheless, the phenolic content in some VOO available on the market is too low to allow the recommended consumption of HT and its derivatives within the context of a balanced diet.

The enrichment of VOO with its own PC has been postulated as an interesting strategy to increase daily phenolic intake without increasing caloric intake. However, the enrichment of olive oil (OO) with its own PC has a bitter and pungent taste, which could lead to rejection by consumers (Rubió, Motilva, Maclà, Ramo, & Romero, 2012). Moreover, extensive PC enrichment could have a dual action since antioxidants can also act as oxidants. A suitable approach is to enrich VOO with its own PC, together with complementary ones from other sources (Rubió, Valls, et al., 2012; Suárez et al., 2011). Thyme (*Thymus zyguis*) is the herb selected in the present study for VOO flavoring, as it is one of the richest sources of PC flavonoids (Rubió, Motilva, et al., 2012). . Our previous studies have

reported that PC from thyme (Th-PC) enhance the bioavailability of the PC from OO (Rubió, Farràs, et al., 2014; Rubió, Serra, et al., 2014) and that a thyme-enriched VOO intervention improves HDL oxidative status (Farràs et al., 2015; Martín-Peláez et al., 2017) and DNA protection against oxidation (Romeu et al., 2016) and exerts a cardioprotective impact on the HDL proteome (Pedret et al., 2015).

Taking all these data into consideration, effective methods to increase not only HDL quantity but also quality will be developed in future studies, resulting in possible protection against CVD events.

UNIVERSITAT ROVIRA I VIRGILI
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Introduction

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1. CARDIOVASCULAR DISEASE

1.1 Definition and epidemiology

CVD includes disorders of the heart and blood vessels and comprises CHD, cerebrovascular disease, stroke, peripheral arterial disease, rheumatic heart disease and other related conditions (WHO, 2016).

According to the World Health Organization (WHO), CVD are the world's leading cause of death, as more people die annually from CVD than from any other cause. In fact, it is estimated that 17,5 million people die each year from CVD, which represents a 31% of all deaths worldwide, and this figure is expected to keep increasing globally, reaching 23,3 million of deaths by 2030. Within these deaths, an estimated 7,4 million are due to CHD, and 6,7 million are due to stroke. In other words, four out of five CVD deaths are due to heart attacks and strokes (WHO, 2016).

It is worth highlighting that CVD were once considered diseases that occur more frequently in industrialized countries. However, there has been a substantial increase in CVR factors in recent years, including obesity, hypertension, hyperlipidemia and diabetes. In fact, approximately three quarters of CVD deaths are registered in low- and middle-income countries, surely because inhabitants of these areas often do not have the benefit of integrated primary health care programmes for early detection, management, and treatment compared to people in high-income countries (WHO, 2016).

1.2 Pathophysiology of atherosclerosis

Atherosclerosis is the main biological process causing both ischemic stroke and myocardial infarction and is, therefore, the major cause of cardiovascular death. Atherosclerosis is defined as a progressive, chronic, inflammatory disease of the arterial wall arising from an imbalance in lipid metabolism. The understanding of the pathophysiology of atherosclerosis has been the main objective for CVD prevention for decades, since the implementation and development of new strategies is essential to tackling CVD. The atherosclerotic process is initiated by lipid retention, oxidation,

and modification, which provoke chronic inflammation, which in turn leads to plaque formation inside the arteries. Plaque is made of cholesterol, fatty substances, cellular waste products, calcium, and fibrin. When a piece of this plaque breaks off, or a thrombus is formed on the plaque's surface, the affected artery is blocked and a heart attack or stroke may result (American Heart Association, 2017).

The normal artery contains three layers. The inner layer, or tunica intima, is built up by a monolayer of endothelial cells that is in contact with blood. The middle layer, or tunica media, contains smooth muscle cells (SMCs) embedded in a complex extracellular matrix. The outer layer, or tunica adventitia, contains mast cells, nerve endings, and microvessels (Figure 3A).

The first process of atherosclerosis involves activation of the monolayer of endothelial cells in contact with the bloodstream. Once activated, endothelial cells express adhesion proteins on their membrane to adhere leukocytes (in particular, monocytes and T-lymphocytes) to the endothelial monolayer. Although vascular cell adhesion molecule-1 (VCAM-1) is one of the most important adhesion molecules involved in the interaction between the endothelium and leukocytes, other adhesion molecules, such as E-selectin, P-selectin, and intercellular adhesion molecule-1 (ICAM-1), are also involved in this process (Gui, Shimokado, Sun, Akasaka, & Muragaki, 2012; Libby, Ridker, & Hansson, 2011).

Simultaneously, during leukocyte recruitment, endothelial permeability increases, and changes in the composition of the extracellular matrix occur, allowing the entrance of LDL particles in the intima. Once in the arterial wall, these LDL particles are oxidized (oxidized LDL; oxLDL), triggering an inflammatory reaction within the intima. Consequently, several cytokines, such as interleukin-1β and tumor necrosis factor-α (TNF-α), are released, which in turn increase the expression of adhesion molecules in endothelial cells, enhancing the binding and recruitment of leukocytes to the arterial wall (Libby, 2006).

Once monocytes adhere to the arterial endothelium (the most numerous type of recruited leukocyte), chemokine (C-C motif) ligand 2 (CCL2) mediates cell migration into the intima, where the maturation of monocytes to macrophages occurs. The next step involves the expression

of scavenger receptors on macrophages' membrane, which mediate the uptake of various substances, including oxLDL and cholesterol-rich, ApoB-containing lipoproteins retained in the intima. As a consequence, intracellular cholesterol accumulates in lipid droplets, yielding the formation of foam cells that develop fatty streaks (Figure 3B) (Choi et al., 2016; Gui et al., 2012; Insull, 2009; Libby et al., 2011). Although foam cells are a biomarker of early-stage atherosclerosis, foam cell formation *per se* might not be atherogenic, as the intracellular storage of cholesterol reduces the build-up of extracellular cytotoxic cholesterol in the intima. The storage capacity is maintained by removing excess cholesterol through HDL biogenesis (Choi et al., 2016). When cholesterol storage is greater than its removal, cholesterol spills over into non-lipid droplet compartments and activates the inflammatory signalling pathways that promote atherogenesis.

Lesion progression consists of SMC recruitment and migration from the tunica media into the intima, where SMCs proliferate in response to mediators, such as platelet-derived growth factor. In the intima, the SMCs produce extracellular matrix macromolecules, such as collagen, elastin, and proteoglycans, which form a fibrous cap (Libby et al., 2011). In addition, macrophages and SMCs present in the intima tunica may die, releasing lipids that accumulate in the central region of the plaque, often called the lipid or necrotic core. Advancing plaques also contain cholesterol crystals and microvessels, along with calcium deposits in the wall, which initially occur as small aggregates and later appear as large nodules (Figure 3C) (Insull, 2009).

Thrombosis is the ultimate complication of atherosclerosis, and it often promotes the physical disruption of the atherosclerotic plaque. This process is mediated by the presence of T-lymphocytes, which accelerates plaque disruption by producing enzymes that degrade collagen and by generating mediators that lead to the death of SMCs, which are the main source of arterial collagen (Libby, 2013). Additionally, the macrophages present in plaques promote the thrombogenicity of the lipid core by producing the procoagulant protein tissue factor. When a plaque ruptures, blood coagulation components come into contact with the tissue factor in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it can impede blood flow (Figure 3D) (Gui et al., 2012; Libby

et al., 2011). The presence of these plaques usually causes clinical manifestations, not only by provoking thrombosis, which can interrupt blood flow, but also by producing flow-limiting stenosis, which leads to tissue ischemia (Libby et al., 2011).

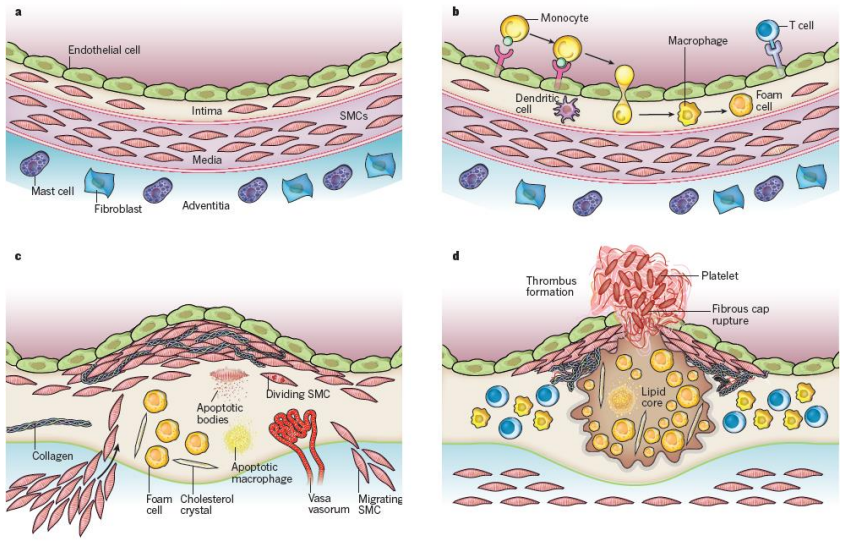

Figure 3 | Stages of the pathophysiology of atherosclerosis. A) The normal artery contains three layers: the inner layer or tunica intima, the middle layer or tunica media, and the outer layer or tunica adventitia. **B)** The initial steps of atherosclerosis include adhesion of leukocytes to the activated endothelial monolayer, migration of the bound leukocytes into the intima, maturation of monocytes into macrophages, and their uptake of lipids, yielding foam cells formation. **C)** Lesion progression involves the migration of SMCs from the media to the intima, the proliferation of SMCs, and the synthesis of extracellular matrix macromolecules. Plaque macrophages and SMCs can die in advancing lesions, and the extracellular lipid derived from dead and dying cells can accumulate in the central region of a plaque (the lipid or necrotic core). Advancing plaques also contain cholesterol crystals and microvessels. **D)** Thrombosis, the ultimate complication of atherosclerosis, often complicates a physical disruption of the atherosclerotic plaque. Shown is a fracture of the plaque's fibrous cap, which has enabled blood coagulation components to come into contact with Tissue Factor in the plaque's interior, triggering the thrombus that extends into the vessel lumen, where it impedes blood flow. Source: Libby et al., 2011.

1.3 Cardiovascular risk factors

Cardiovascular risk (CVR) is defined as the probability of suffering a CVD event in a given period of time, which is usually within 5–10 years. CVR factor is defined as a measurable trait that predicts an individual's likelihood of developing CVD and has emerged from a combination of epidemiological studies performed worldwide. For instance, the Framingham Heart Study demonstrated that CVR directly correlates with circulating levels of LDL-C (Martínez, Guisado, Ocaña, & Salgado, 2016). The comprehensive understanding of these CVR factors is a useful tool for implementing strategies to decrease CVD mortality. In this sense, the WHO launched a new initiative to tackle CVD on the margins of the United Nations General Assembly in September 2016. The initiative, called "Global Hearts," aims to push back the global threat of CVD, including heart attacks and strokes (WHO, 2016).

CVR factors can be classified into two categories: non-modifiable risk factors and modifiable risk factors. On one hand, the non-modifiable factors are comprised of ethnicity, age, gender and genetic predisposition. Male gender, together with increasing age, are major risk factors for CVD, as the risk of stroke doubles every decade after age 55. People with African or Asian ancestry are at higher risks of developing CVD than other racial groups. Moreover, there is substantial evidence of the heritability of many CVR factors (Acín et al., 2006; Perk et al., 2012; Piepoli et al., 2016). On the other hand, the modifiable factors are behavioral factors, such as tobacco use, harmful use of alcohol, unhealthy dietary habits (in particular, low fruit and vegetable intake), sedentary lifestyle and psychosocial stress. The importance of modifiable factors lies in the fact that they all can be prevented and if prevention is addressed properly, it would be possible to stave off at least 80% of CVDs (WHO, 2016). The effects of behavioral risk factors may show up in individuals with overweight and obesity, hypertension, hyperlipidemia, and diabetes, ultimately leading to the onset and development of CVD. Worldwide, these intermediate risk factors are considered the traditional or classical risk factors (Piepoli et al., 2016; Schnohr et al., 2015).

1.3.1 Classical CVR factors

a) Overweight and obesity: Overweight and obesity are defined as abnormal or excessive fat accumulation that may impair health. Both are major risk factors present in many chronic metabolic disorders and are associated with several comorbidities, such as CVD, type 2 diabetes *mellitus* (T2DM), insulin resistance, dyslipidemia, hypertension and cancer (WHO, 2016). The metabolic network involved in obesity and these medical conditions is commonly known as metabolic syndrome, which is defined as a complex disorder that directly increases the CVR (Samson & Garber, 2014). Specifically, visceral adipose tissue is a metabolically active organ capable of synthesizing an important variety of molecules and releasing them into the bloodstream, thus playing a vital role in cardiovascular homeostasis (Perk et al., 2012). The 2016 European Guidelines on CVD prevention in clinical practice state that by achieving and maintaining a healthy weight has a favorable effect on metabolic risk factors and lowers CVR (Piepoli et al., 2016).

b) Hypertension: Both systolic and diastolic blood pressure have a positive association with CVD events, and even small increases in blood pressure are associated with an increased risk of developing CVD. Hypertension is more common in patients with T2DM than in the general population. A recent systematic review and meta-analysis of randomized trials of blood pressure-lowering agents in patients with T2DM confirmed that lowering blood pressure reduces the risk of all-cause mortality, CV events, coronary artery disease (CAD) events, and stroke, among others (Piepoli et al., 2016). Reducing the incidence of hypertension by implementing population-wide policies to reduce behavioral risk factors, including a harmful use of alcohol, physical inactivity, overweight, obesity and high salt intake, is essential to attaining this target (WHO, 2016).

c) Diabetes: T2DM is a chronic metabolic disease characterized by elevated levels of glycaemia, as a result of the body's ineffective use of insulin, which leads to severe damage to the heart, blood vessels, eyes, kidneys, and nerves. T2DM is largely the result of excess body weight and physical inactivity. The risk of CVD outcomes increases progressively among normoglycemic individuals with impaired fasting glucose or

impaired glucose tolerance (Anand et al., 2012). In fact, a 1 mmol/L increase in the fasting plasma glucose level is associated with a 17% increase in the risk of future CVD events or death (Ton, Martin, Blumenthal, & Blaha, 2013).

d) Dyslipidemia: The vital role of dyslipidemia, especially hypercholesterolemia, in the development of CVD is widely acknowledged by genetic, pathology, observational and intervention studies. Lipids, such as cholesterol and triglycerides (TG), circulate bound to lipoproteins. On one hand, the main carrier of cholesterol in plasma, the LDL, is considered the greatest atherogenic factor (Besseling, Van Capelleveen, Kastelein, & Hovingh, 2013; Perk et al., 2012). In this respect, meta-analyses of many lipid-lowering drugs trials show a dose-dependent reduction in CVD with a reduction in LDL-C levels. Every 1 mmol/L reduction in LDL-C is associated with a corresponding 20-25% reduction in CVD mortality. On the other hand, hypertriglyceridemia and low HDL-C concentrations are independent CVR factors. The PROCAM study has shown that each 1 mg/dL increase in HDL-C levels was associated with a decrease in CVR by 2-3% (G Assmann, Cullen, & Schulte, 2002). The role of HDL in risk estimation is being systematically re-examined by several authors. Overall, HDL-C has a modest but useful effect in redefining risk estimation but this effect may not be seen in some low-risk populations (Piepoli et al., 2016).

1.3.2 Emerging CVR factors

Despite the well-known role of the aforementioned classical risk factors, a percentage of individuals suffering from CVD have only one or none of these risk factors, and they might even show a worse prognosis. In other words, the classical CVR factors do not fully explain CVR. There has been substantial interest in the identification of new CVR factors, also known as emerging risk factors, and their usefulness for predicting the risk of CVD events (Ge & Wang, 2012; Gómez et al., 2009).

Some of these emerging CVR factors include oxidative stress, inflammation, endothelial dysfunction, lipoprotein profile (including particle count and size of the different lipoproteins) and HDL quality or

functionality (Cole et al., 2013; Ferretti et al., 2006; Kontush & Chapman, 2006; MacKey et al., 2012; Mora, Glynn, & Ridker, 2013; Parish et al., 2012; Qi et al., 2015; Rohatgi, Shaul, States, Passages, & County, 2015; Steffen et al., 2015; T. J. Wang et al., 2006).

2. HIGH-DENSITY LIPOPROTEIN

2.1 The role of HDL in CVD

Cholesterol is needed for correct cellular function as it is involved in several cellular processes. In particular, cholesterol is an important constituent of mammalian plasma membranes and plays fundamental roles in diverse biological processes, such as membrane permeability, lateral lipid organization, membrane fluidity modulation, signal transduction via the formation of lipid rafts, and membrane trafficking. Moreover, cholesterol is also a precursor of vitamin D and steroid hormones, including adrenal gland hormones (aldosterone and cortisol) and sex hormones (progesterone, estrogens, and testosterone). Hence, its cellular and whole body metabolism and distribution are subject to complex and dynamic regulation by circulating enzymes and lipoproteins, such as HDL, very-low density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and LDL (Rosenson et al., 2011).

In regard to the role of HDL in CVD, this particle is considered the only antiatherogenic lipoprotein. An imbalance between circulating levels of the proatherogenic ApoB-containing lipoproteins (VLDL, IDL, and LDL) and the antiatherogenic ApoA-containing lipoproteins (HDL) is associated with atherogenic dyslipidemia and premature atherosclerosis (Figure 4) (Kontush & Chapman, 2006).

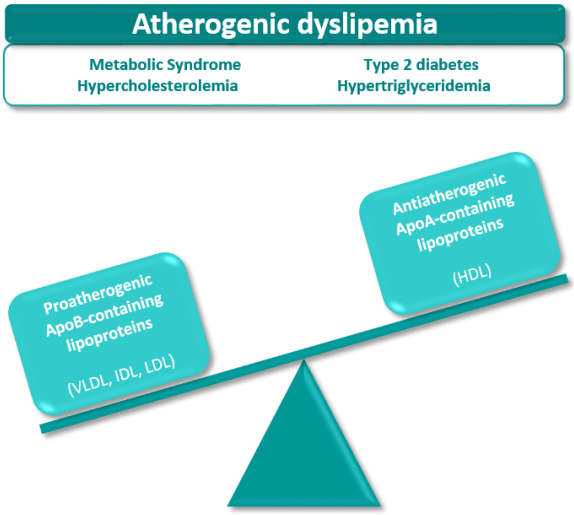

Figure 4 | Atherogenic dyslipidemia is an imbalance between circulating levels of antiatherogenic lipoproteins (HDL) and the proatherogenic lipoproteins (VLDL, IDL, LDL). Source: adapted from Kontush *et al.,* 2006.

However, the understanding of the role of HDL in CVD is currently under debate. In 1975, it was observed that plasma HDL-C levels were reduced in several conditions associated with an increased risk of future CVD (Miller & Miller, 1975).

The importance of low HDL-C as an independent CVD risk factor triggered decades of intense research to figure out the underlying mechanisms of this prognostic relationship, which would allow the development of new therapeutic strategies to raise HDL-C levels and therefore tackle CVD (Choi et al., 2016). It was soon established that the association between plasma levels of HDL-C and CVD is strong and coherent among all kinds of populations, even when corrected for confounding factors. The Framingham Heart Study showed that cardiovascular morbidity and mortality was more than eight-fold higher in individuals with HDL-C levels < 35 mg/dL compared to individuals with HDL-C levels > 65 mg/dL, and as HDL-C levels increased, there was a progressive reduction in CHD risk, irrespective of gender and LDL-C levels (T. Gordon et al., 1977). In the same cohort, a 10 mg/dL increase in HDL-C concentration was associated with

not only lower cardiovascular mortality but also lower all-cause mortality (Wilson et al., 1988a).

These data were confirmed in the PROCAM study, where a four-fold increase in CHD risk was found in men with HDL-C levels < 35 mg/dL compared with those with HDL-C levels ≥ 35 mg/dL over a period of 6 years. This prognostic association between low HDL-C levels and increased risk of CHD remained, even after adjustment for other risk factors (Gerd Assmann et al., 1996). Moreover, a meta-analysis of four prospective studies (Framingham Heart Study, Lipid Research Clinics Prevalence Mortality Follow-up Study, Coronary Primary Prevention Trial, and Multiple Risk Factor Intervention Trial) confirmed these findings by showing that there was a 2-3% increase in CVR for every 1 mg/dL decrease in plasma levels of HDL-C, independent of other CVR factors, including plasma LDL-C (Gordon et al., 1989). The strength of this relationship between low HDL-C levels and increased risk of CHD was found to be stronger in women than men, as indicated by data from both this meta-analysis (Gordon et al., 1989) and the Atherosclerosis Risk in Communities study (Sharrett et al., 2001). Furthermore, this association was even found in elderly patients (≥ 85 years) (Weverling-Rijnsburger, Jonkers, van Exel, Gussekloo, & Westendorp, 2003) (Figure 5).

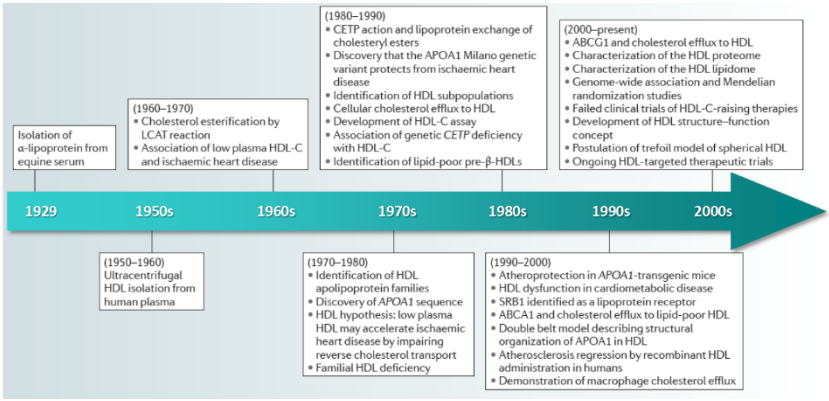

Figure 5 | Timeline of the understanding of human HDL. Several key milestones can be recognized along the timeline of developments over the past century in the knowledge of HDL biology. Source: adapted from Kingwell *et al*., 2014.

2.2 The HDL hypothesis

The strength of the evidence that low HDL-C levels are associated with CVD led to the formulation of the "HDL hypothesis": HDL plays a causal role in the pathogenesis of atherosclerotic CVD. Thus, it was assumed that interventions that increase plasma HDL-C concentrations would reduce CVR (Wilson et al., 1988a).

However, conflicting evidence has cast doubt on the HDL hypothesis, as therapeutic strategies that increase HDL-C levels in the circulation have not yielded clinical benefits in CVD (Tables 1 and 2) (J.C. Escolà-Gil et al., 2013; Salazar et al., 2015; Santos-Gallego, 2015). The failure of recent large clinical trials that aimed to improve CVD outcomes by raising HDL-C in circulation suggests that HDL-C levels might be a biomarker of cardiovascular health, rather than a mediator that protects against atherosclerosis (Kingwell et al., 2014; Lüscher et al., 2014). In this sense, pharmacological treatment with HDL-C-rising drugs, such as CETP inhibitors (Barter et al., 2007) or niacin (Boden et al., 2011; Landray et al., 2014), managed to increase such HDL-C levels, but did not succeed in demonstrating any clinical cardiovascular benefit. What is more, a clinical trial with torcetrapib (a CETP inhibitor) was called off in 2006 when phase III studies showed excessive all-cause mortality in the group receiving a combination of atorvastatin (Lipitor) and torcetrapib versus the group taking the atorvastatin alone (Barter et al., 2007; Pearson, 2006; Tall et al., 2007).

In the same vein, some hereditary syndromes characterized by low HDL-C levels have not been shown to display early atherosclerosis. For example, in the case of Tangier disease, less than 50% of Tangier patients develop CAD before age 40, yet they present virtually undetectable levels of HDL-C (Eren et al., 2012). Moreover, patients presenting the ApoA-I_{Milano} mutation present a cardioprotective phenotype, even though they have low HDL-C and high TG levels (Elshourbagy et al., 2014; Hafiane & Genest, 2015; Salazar et al., 2015). Two familial forms of LCAT deficiency (familial LCAT deficiency and fish-eye disease) present very low HDL-C levels, even lower than 0,39 mmol/L, but they present only a modest increase in the risk for premature atherosclerosis (Eren et al., 2012; Ossoli et al., 2015).

Finally, a causal relationship between HDL-C levels and CVD development has also been questioned by several Mendelian genetic studies, which failed to show associations between gene variants associated with low HDL-C and increased CVD risk, raising concerns about the use of HDL-C as a target to tackle CVD (Frikke-Schmidt, 2008; Gugliucci & Menini, 2015; Hafiane & Genest, 2015).

Table 1 | Clinical and epidemiological studies showing HDL-C as a good predictor of CVD. Source: adapted from Salazar *et al.,* 2015.

Author	Methodology	Conclusions
Barter et al.	Post hoc analysis of data from the Treating to NewTargets Study, a multicentric, randomized,double-blind study which assessed the predictivevalue of HDL-C in 9,770 subjects with established CVD on statin therapy.	Based on HDL-C quintiles, a multivariate analysis revealed individuals with HDL-C >55 mg/dL to have a lower risk of cardiovascular mortality than subjects with HDL-C <38 mg/dL (HR: 0.75; IC 95%: 0.60–0.95). In subjects on statin therapy, the best lipid predictor for CVD was HDL-C, even when LDL-C <70 mg/dL.
Castelli et al.	Multicentric case-control study with 6859 subjects ofdiverse ethnicities from the Cooperative Lipoprotein Phenotyping Study.	HDL-C concentration was significantly higher in subjects without established CVD. An inverse correlation was ascertained between these factors, without significant variation after adjustment for total cholesterol, LDL-C, and TAG levels.
Gordon et al.	Prospective report from the Framingham Heart Study, with 2,815 subjects aged 49–82 years, whoseserum lipids were quantified throughout a 4-year	142 individuals developed CVD (79 males, 63 females), with HDL-C being the best CVR predictor. These variables shared an inverse correlation in both genders,even after adjustment for multiple other risk factors.
Wilson et al.	Prospective report from the Framingham Heart Study, with 2,748 individuals aged 50–79 years after a 12-year follow-up period.	An inverse relationship was identified between HDL-C levels and coronary artery disease mortality in both genders (p> 0.05). HDL-C was found to be the best predictor of cardiovascular mortality.
Emerging Risk Factors Collaboration	302,430 subjects from the Emerging Risk Factors Collaboration Study data without history of coronary artery disease were studied to analyze the association between serum lipids and CVR.	A strong inverse association was found between risk of coronary artery disease and HDL-C levels after adjusting for nonlipid risk factors (HR: 0.71; IC 95%: 0.68–0.75) and even after adjustment for non-HDL cholesterol (HR: 0.78; IC 95%: 0.74–0.82).
Assmann et al.	The incidence of coronary artery disease was determined in 4,559 male subjects aged ≥40 years from the Prospective Cardiovascular Münster Study over a 6-year follow-up period.	Univariate analysis revealed a significant inverse relationship between CAD and HDL-C (p> 0.001), even after adjustment for several other risk factors.

Table 2 | Clinical and genetic studies suggesting HDL-C is not the best predictor of CVR in certain contexts. Source: adapted from Salazar *et al.,* 2015.

Author	Methodology	Conclusions
Barter et al.	Randomized, double-blind study on 15,607 subjects with high CVR, who received atorvastatin and torcetrapib or atorvastatin alone.	Although treatment with torcetrapib raised HDL-C 72% from the baseline (p< 0.001), it entailed an increase in cardiovascular mortality in these subjects (HR: 1.25; 95% CI: 1.09–1.44; p = 0.001).
Nissen et al.	Prospective, multicentric, randomized, double-blind study on 1,188 patients with CAD who underwent intravascular ultrasonography and received atorvastatin + torcetrapib or atorvastatin + placebo.	Subjects on atorvastatin + torcetrapib had a 61% increase in HDL-C and a 20% decrease in LDL-C levels when compared to the group on atorvastatin + placebo. However, the former also suffered a greater rise in blood pressure (21.3% versus 8.2%) and incidence of hypertensive cardiovascular events (23.7% versus 10.6%), without significant differences in progression of atherosclerosis, as evaluated by intravascular ultrasonography.
Kastelein et al.	850 heterozygotes with familial hypercholesterolemia were treated with 20, 40, or 80 mg of atorvastatin for a 4-week period, followed by atorvastatin monotherapy or atorvastatin + torcetrapib 60 mg for 24 months, and underwent ultrasonography for evaluation of intima-media thickness.	HDL-C levels were significantly higher in the atorvastatin + torcetrapib group (81.5 ±22.6 mg/dL versus 52.4 ±13.5 mg/dL; p< 0.001), who also displayed lower LDL-C and TAG concentrations. Nevertheless, those on monotherapy were found to have greater intima-media thickness in the common carotid artery.
Voight et al.	Mendelian randomization study which evaluated the association between the LIPG Asn396Ser SNP and incident myocardial infarction in 50,763 participants from six prospective cohort studies and case-control studies involving an additional 16,685 cases of myocardial infarction and 48,872 controls and proposed a genetic score combining 14 common SNP that exclusively associate with HDL cholesterol and then tested this score in up to 12,482 cases of myocardial infarction and 41,331 controls.	The LIPG Asn396Ser allele had a prevalence of 2.6% and was associated with increased HDL-C, without effect on LDL-C y TAG. In meta-analysis, carrier status for Asn396Ser was associated with an increase of roughly 0.29 SD units in HDL-C (p = 8×10−13), with no associations to other risk factors. Nevertheless, this allele was not associated with myocardial infarction (OR: 0.99; 95% CI 0.88–1.11, p= 0.85), without significant heterogeneity among the studies included (p > 0.05). Finally, a 1 SD increase in HDL-C due to genetic score was not associated with risk of myocardial infarction (OR: 0.93; 95% CI: 0.68–1.26, p = 0.63).
Haase et al.	The APOA1 gene was resequenced in 190 subjects, evaluating the effects of mutations on HDL-C levels, risk of ischemic heart disease, myocardial infarction, and mortality in 10,440 individuals from the prospective Copenhagen City Heart Study, who were followed for 31 years. Results were validated in an independent case-control study with 16,035 subjects.	The A164S mutation was found to be a predictor of ischemic heart disease (HR: 32; 95% CI: 1.6–6.5), myocardial infarction (HR: 5.5; CI 95% 2.6–11.7), and mortality (HR: 2.5; 95% CI: 1.3–4.8) in heterozygotes, in comparison to noncarriers. A164S heterozygotes also showed normal levels of Apo A-I, as well as HDL-C and other serum lipids.
Rohatgi et al.	Multiethnic, population-based cohort study on 2,416 adults free from CVD who were participants in the Dallas Heart Study, where the association between cholesterol efflux capacity and CVD incidence was assessed.	HDL-C levels were found to be unrelated to CVD incidence after adjustment for traditional cardiovascular risk factors. Cholesterol efflux capacity was associated with lower CVR, even after adjustment for HDL-C concentration, HDL particle concentration, and traditional cardiovascular risk factors (HR: 0.33; 95% CI: 0.19–0.55).
Sirtori et al.	21 subjects with the Apo A-IMilano mutation were compared with age- and sex-matched control subjects from the same kindred and with 2 series of matched subjects with primary hypoalphalipoproteinemia (HDL-C levels under the 10th percentile for their gender and age), regarding ultrasonographic findings in carotid arteries.	Subjects with hypoalphalipoproteinemia had greater intima-media thickness (0.86 ±0.25 mm) than the control group (0.64 ±0.12 mm) and subjects with the Apo A-IMilano mutation (0.63 ±0.10 mm);p < 0.005. Moreover, subjects with hypoalphalipoproteinemia had a significantly higher prevalence of atherosclerotic plaques than both of the other groups, despite the lower HDL-C levels (19.8 ±9.8 mg/dL, p< 0.05).
Schwartz et al.	Randomized, single-blind study on 15,781 subjects with recent diagnoses of acute coronary syndrome who received dalcetrapib 600 mg daily or placebo.	Subjects on dalcetrapib had a 31–40% increase in HDL-C levels, with minimal effects on LDL-C. Compared to placebo, the dalcetrapib group did not show significantly higher CVR (HR: 1.04; 95% IC: 0.93–1.15, p = 0.52).

The paradoxical association between low HDL-C levels and high CVD risk can be explained by the fact that HDL is a highly dynamic particle that undergoes constant remodeling and recycling. Rather than being homogeneous, the HDL pool encompasses a heterogeneous population of HDL particles that differ in shape, size, density, surface charge, lipid composition, and antigenicity. This heterogeneity is translated into several HDL subclasses that exhibit differences in functionality and atherogenicity irrespective of their cholesterol content (Ansell et al., 2007; Cybulska & Kłosiewicz-Latoszek, 2014).

As a consequence, the focus of HDL has now begun to change from a cholesterol-centric view to a view of HDL functionality. In other words, HDL quantity (*i.e.,* HDL-C) is not the factor that determines HDL-mediated protection from atherosclerosis, but its quality or functionality (*i.e.,* biological function) is defined by HDL heterogeneity (Akinkuolie et al., 2014; Choi et al., 2016; K. Rye & Barter, 2014; Santos-Gallego, 2015). Therefore, therapies aimed only to raise HDL-C may not affect the structure of the HDL particles to such an extent to appropriately modify HDL functionality and consequently decrease the CVR (Hafiane & Genest, 2015).

The interest in HDL quality or functionality has been growing in the last decades, as the number of related publications has been climbing exponentially during these years, as depicted in Figure 6.

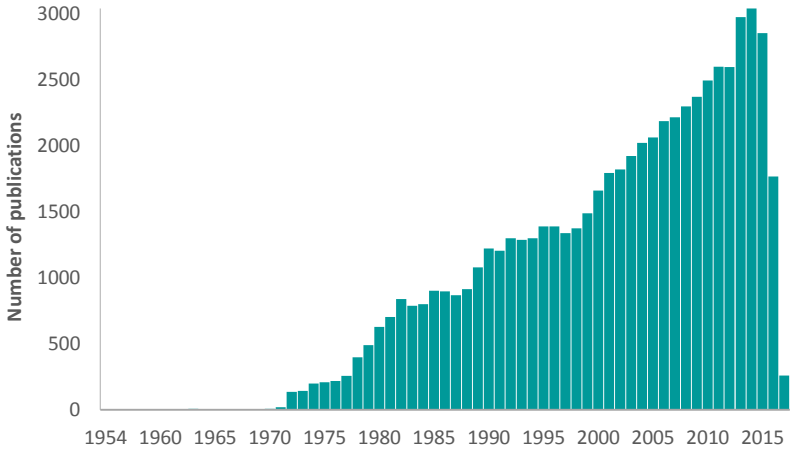

Figure 6 | Publications par year on HDL functionality. Source: data retrieved from PubMed MEDLINE on April 12th 2017.

2.3 HDL structure, composition, and heterogeneity

HDLs are the lipoproteins found in the density range of 1,063-1,210 g/mL in the ultracentrifuge, and they are the smallest and densest of all serum lipoproteins. HDL are also the most numerous lipoproteins in plasma and are present at a micromolar concentration, while LDL, VLDL, and IDL are present at nanomolar concentrations (Eren et al., 2012; Kontush & Chapman, 2006; Salazar et al., 2015).

HDL is an extremely dynamic particle that undergoes constant remodeling and recycling, therefore resulting in a heterogeneous lipoprotein in terms of not only density but also shape, size, lipid and protein composition, and surface charge (Figure 7) (Choi et al., 2016; K. Rye & Barter, 2014).

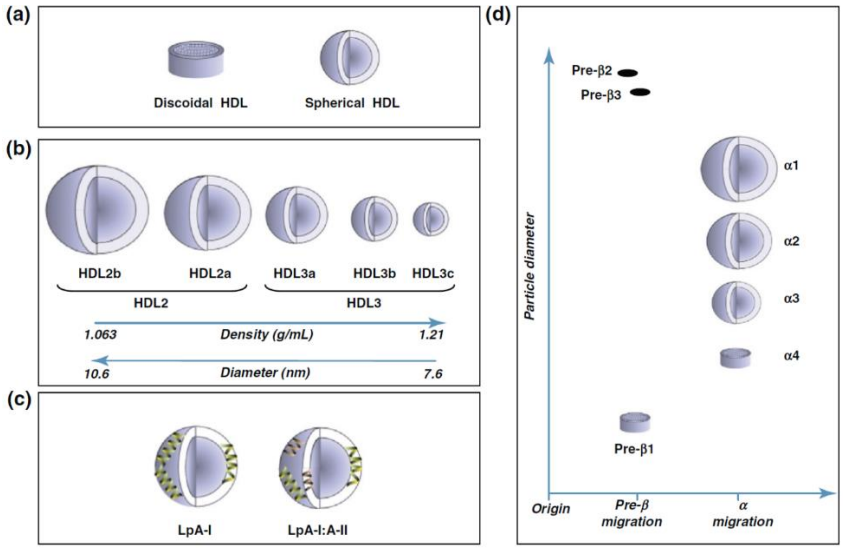

Figure 7 | HDL heterogeneity. HDL is an extremely dynamic particle that undergoes constant remodeling and recycling, therefore resulting in a heterogeneous lipoprotein in terms of shape (A), density and size (B), Apolipoprotein composition (C), and surface charge (D). Source: Camont *et al.,* 2011.

The major subfractions or subclasses of HDL are commonly grouped according to their density: the smaller and denser and HDL_3 (1,125-1,210 g/mL) and the largest, but less dense, HDL_2 (1,063-1,125 g/mL) (Kontush & Chapman, 2006). Nevertheless, total HDL particles can also be split into

several subpopulations on the basis of the aforementioned characteristics: mass (with non-denaturing polyacrylamide gradient gel electrophoresis; PAGGE), mass together with superficial charge (with 2D-PAGGE techniques), and size (with NMR technique) (Choi et al., 2016; K.-A. Rye et al., 2009) (Figure 8). Finally, it is worth mentioning that HDL protein composition is also used to define the known lipid-free and lipid-poor ApoA-I subfraction, which comprises the smallest (≤ 9 nm) and therefore the densest HDLs and corresponds to pre-β-HDL (Kontush & Chapman, 2006). The importance of this HDL subfraction lies in the fact that it is considered one of the most functional HDL, having a central role in lipid efflux mediated by ATP-binding cassette (ABC) A1 transporter (Kontush, 2014; K. Rye & Barter, 2014).

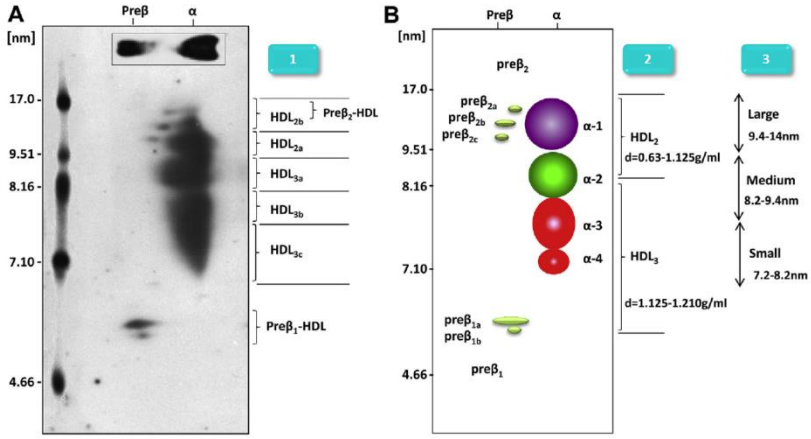

Figure 8 | HDL heterogeneity and measurement techniques. A) HDL subpopulations separated by 2D-PAGGE of a normolipidemic male subject. The plasma was subjected to 2-dimensional agarose/native PAGGE, samples were then transferred to nitrocellulose membrane, and probed for radiolabeled-I^{125} ApoA-I. B) Schematic diagram of all the HDL particles separated as indicated in panel A. Nomenclatures of HDL subclasses determined by different methods are also shown: 1) ND-PAGGE and 2D-PAGGE (mass and charge); 2) Ultracentrifugation separation (density); and 3) NMR (size). Sources: adapted from Hafiane *et al.,* 2015 and Choi *et al.,* 2016.

The use of different techniques to isolate and define HDL subclasses has led to different terms in defining HDL species. To provide guidelines for future studies and to compare and contrast published data obtained from different methods, Rosenson *et al.* proposed a new HDL nomenclature according to the density and size of HDL particles (Table 3) (Rosenson et al., 2011).

Table 3 | Classification of HDL by physical properties of HDL: very large, large, medium, small and very small HDL particles. One-dimensional electrophoresis was performed in non-denaturing gradient polyacrylamide gel (4-20%). *HDL-P, HDL particle. Source: adapted from Rosenson *et al.,* 2011.

Proposed Term	Very large HDL (HDL-VL)	Large HDL (HDL-L)	Medium HDL (HDL-M)	Small HDL (HDL-S)	Very small HDL (HDL-VS)
Density gradient ultracentrifugation	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Density range, g/mL	1,063–1,087	1,088–1,110	1,110–1,129	1,129–1,154	1,154–1,170
Gradient gel electrophoresis	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Size range, nm	12,9–9,7	9,7–8,8	8,8–8,2	8,2–7,8	7,8–7,2
2-D gel electrophoresis	α-1	α-2	α-3	α-4	Pre-β1 HDL
Size range, nm	11,2–10,8	9,4–9,0	8,5–7,5	7,5–7,0	6,0–5,0
NMR	Large HDL-P*	Medium HDL-P		Small HDL-P	
Size range, nm	12,9–9,7	9,7–8,8	8,8–8,2	8,2–7,8	7,8–7,2
Ion mobility	HDL2b	HDL2a and HDL3			
Size range, nm	14,5–10,5	10,5–7,65			

The high density of HDL particles is due to the elevated protein content, which is estimated to be approximately 35-65% by weight (Kontush & Chapman, 2006; Salazar et al., 2015). However, HDL also contains a lipid portion: a surface amphipathic monolayer of free cholesterol (FC; 2-10%) and phospholipids (PL; 26-46%) and a core of cholesteryl esters (CE; 15-30%) with a small amount of TG (3-15%; Figure 9) (Eren et al., 2012). Each of these components follows a distinct metabolic pathway, depending on the organ or tissue (Choi et al., 2016).

	HDL2b	HDL2a	HDL3a	HDL3b	HDL3c
Diameter (nm)	10,4	10,3	9,9	8,0	7,3
Density (g/mL)	1,099	1,107	1,123	1,155	1,186
M_r (kDa)	410	400	360	200	160
Plasma levels (µM)	1,7	1,8	1,9	1,3	1
Apolipoproteins (mol/mol HDL)					
ApoA-I	4-5	4	3-4	3	2-3
Others	≤ 2	≤ 2	≤ 2	1	≤ 1
Surface Lipids (mol/mol HDL)					
Phospholipids	130	140	120	45	25
Free cholesterol	70	40	25	15	10
Core Lipids (mol/mol HDL)					
Cholesteryl ester	180	160	140	70	40
Triglyceride	30	20	15	10	5

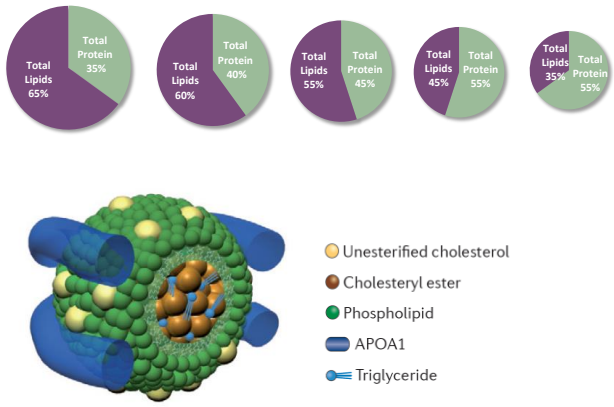

Figure 9 | Physicochemical properties and structure of HDL from healthy normolipidemic subjects. Source: adapted from Eren *et al.,* 2012.

2.3.1 HDL proteome

Up-to-date proteomic studies are providing original data about the protein cargo of HDL particles. Each particle consists of 2-4 molecules of its main protein, ApoA-I, which represents approximately 70% of the total protein moiety of HDL, while ApoA-II represents approximately 20% (Eren et al., 2012). These two proteins are related to the structural integrity of HDL and have other biological functions, as recently reviewed by Kontush *et al.,* (Kontush et al., 2015). The remaining protein components (< 10%) include minor Apo, enzymes, lipid transfer proteins, acute-phase proteins, complement components, proteinase inhibitors, and other protein

components (Table 4) (Choi et al., 2016; Eren et al., 2012; Karathanasis et al., 2017; Pedret et al., 2015; Scanu & Edelstein, 2008).

Table 4 | Major components of the HDL proteome. Only proteins detected in more than 50 % of the studies are listed, together with seven others previously known to be associated with HDL (ApoC-IV, ApoH, LCAT, PAF-AH, GSPx-3, PLTP, and CETP). CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; GSPx-3, glutathione seleno peroxidase 3; Hrp, haptoglobin-related protein; LDL-R, LDL receptor; LCAT, lecithin cholesterol acyltransferase; LPL, lipoprotein lipase; LpPLA2, lipoprotein-associated phospholipase A2; LRP, LDL receptor-related protein; MPO, myeloperoxidase; PAF-AH, platelet-activating factor acetylhydrolase; PL, phospholipid; PLTP, phospholipid transfer protein; PON1, paraoxonase 1; SAA, serum amyloid A; TG, triglyceride. Source: adapted from Kontush *et al.,* 2015.

Protein	kDa	Major function
Apolipoproteins		
ApoA-I	28	Major structural and functional apolipoprotein, LCAT activator
ApoA-II	17	Structural and functional apolipoprotein
ApoA-IV	46	Structural and functional apolipoprotein
ApoC-I	6.6	Modulator of CETP activity, LCAT activator
ApoC-II	8.8	Activator of LPL
ApoC-III	8.8	Inhibitor of LPL
ApoC-IV	11	Regulates TG metabolism
ApoD	19	Binding of small hydrophobic molecules
ApoE	34	Structural and functional apolipoprotein, ligand for LDL-R and LRP
ApoF	29	Inhibitor of CETP
ApoH	38	Binding of negatively charged molecules
ApoJ	70	Binding of hydrophobic molecules, interaction with cell receptors
ApoL-I	44/46	Trypanolytic factor of human serum
ApoM	25	Binding of small hydrophobic molecules
Enzymes		
LCAT	63	Esterification of FC to CE
PON1	43	Calcium-dependent antioxidant enzyme
PAF-AH (LpPLA2)	53	Hydrolysis of short-chain oxidised PL
MPO	84	Highly oxidative enzyme
GSPx-3	22	Reduction of hydroperoxides by glutathione

(continued)

(continued)

Protein	kDa	Major function
Lipid transfer proteins		
PLTP	78	Conversion of HDL into larger and smaller particles, transport of LPS
CETP	74	Heteroexchange of CE and TG and homoexchange of PL between HDL and ApoB-containing lipoproteins
Acute-phase proteins		
SAA1	12	Major acute-phase reactant
SAA4	15	Minor acute-phase reactant
α-2-HS-glycoprotein	39	Negative acute-phase reactant
Fibrinogen α chain	95	Precursor of fibrin, cofactor in platelet aggregation
Complement components		
C3	187	Complement activation
Proteinase inhibitors		
α-1-antitrypsin	52	Inhibitor of serine proteinases
Hrp	39	Decoy substrate to prevent proteolysis
Other proteins		
Transthyretin	55	Thyroid hormone binding and transport
Serotransferrin	75	Iron binding and transport
Vitamin D-binding protein	58	Vitamin D binding and transport
α-1-B-glycoprotein	54	Unknown
Hemopexin	52	Heme binding and transport

Although approximately 90 proteins are typically attributed to HDL in healthy individuals, recent proteomic analyses have consistently identified up to 127 proteins, employing state-of-the-art technology (Pedret et al., 2015). Furthermore, under certain pathophysiological conditions, such as metabolic stress, inflammation, acute coronary syndromes, and renal failure, the HDL proteome shifts to reflect an inflammatory status (Choi et al., 2016). These data firmly endorse the concept of HDL as a particle with pleiotropic functions, which include its role in not only the RCT pathway, antioxidant balance, and inflammation but also emerging functions. The bulk of these major and minor proteins associated with HDL are helping to identify novel biomarkers and to devise new strategies for coping with CVD.

2.3.2 HDL lipidome

The lipid content of HDL is estimated to be approximately 35-65% by weight depending on HDL subclass (Kontush et al., 2015). This lipid content is mainly located on the surface of the HDL, forming a monolayer of PL (35-50% of HDL lipids) that encloses the particle, with phosphatidylcholine as the major lipid. This monolayer also contains minor sphingolipids (which represent 5-7% of HDL total lipid mass; in particular, sphingomyelin and ceramides), along with FC and other sterols (which account for an additional 5-10%). The core of HDL is composed of CE (35-40% of HDL lipids) with few TG (which represent 2-3% of HDL lipids) (Table 5) (Choi et al., 2016; Karathanasis et al., 2017; Kontush et al., 2015).

Table 5 | Major components of the HDL lipidome. Data are shown for HDL obtained from normolipidemic healthy subjects. SPC, sphingosylphosphorylcholine; S1P, sphingosine-1-phosphate; IPGE2, isoprostaglandin E2. Source: adapted from Kontush *et al.,* 2015.

Lipid class	HDL content in mol % of total lipids
Phospholipids	**37.4–49.3**
Phosphatidylcholine	32–35
PC-plasmalogen	2.2–3.5
LysoPC	1.4–8.1
Phosphatidylethanolamine	0.70–0.87
PE-plasmalogen	0.54–0.87
Phosphatidylinositol	0.47–0.76
Cardiolipin	0.077–0.201
Phosphatidylserine	0.016–0.030
Phosphatidylglycerol	0.004–0.006
Phosphatidic acid	0.006–0.009
Sphingolipids	**5.7–6.9**
Sphingomyelin	5.6–6.6
Ceramide	0.022–0.097
Hexosyl Cer	0.075–0.123
Lactosyl Cer	0.037–0.060
S1P d18:1	0.015–0.046
S1P d18:0	0.007
SPC d18:1	0.001
Neutral lipids	**46.7–54.0**
Cholesteryl esters	35–37
Free cholesterol	8.7–13.5
Triacylglycerides	2.8–3.2
Diacylglycerides	0.17–0.28
Minor lipids	
Free fatty acids	16:0, 18:0, 18:1
Isoprostane-containing PC	ND (IPGE2/D2-PC (36:4))

2.4 HDL monolayer fluidity

HDL composition has a great impact on the physical properties of HDL, such as fluidity, molecular order and electric charges (Ferretti et al., 2006; Girona, LaVille, Solà, Motta, & Masana, 2003). In particular, the lipids on the surface determine the fluidity or viscosity of the HDL monolayer, which in turn influences ApoA-I conformation and binding to HDL:

a) Cholesterol forms complexes with PL, creating structures of low fluidity known as lipid rafts. Therefore, the presence of FC decreases the fluidity of the HDL monolayer.

b) Fluidity also depends on the length and saturation of the fatty acids (FA) present in the HDL monolayer. In this sense, short-chain and low-saturated FA (SFA) increase the fluidity of this lipoprotein (Davidson et al., 1995).

Lipid peroxidation is also known to decrease HDL fluidity too (Girona et al., 2003). Moreover, some authors have described that an increment of antioxidants in biological membranes (Suwalsky, Orellana, Avello, Villena, & Sotomayor, 2006) and in the HDL monolayer (Álvaro Hernáez et al., 2014) increases fluidity, while others have reported that antioxidants can rigidify the membrane (Pérez-Fons, Garzón, & Micol, 2010).

The importance of HDL fluidity consists in the fact that it reflects the functional behavior of HDL to such an extent that an increased fluidity has been considered an intermediate marker of enhanced HDL functionality (Bonnefont-Rousselot et al., 1995; R Solà et al., 1993). In particular, some authors have reported that the more fluid the HDL monolayer is, the greater the ChE rate from lipid-laden macrophages to HDL production is (Helal, Berrougui, Loued, & Khalil, 2013; Álvaro Hernáez et al., 2014; R Solà et al., 1993).

2.5 HDL metabolism

The metabolism of HDL is a complex process in which different Apos, enzymes, cofactors, receptors, and lipid transfer proteins function in a coordinated manner to remodel the particle as they shuttle lipids between cells, tissues, organs and other lipoproteins (Figure 10) (Karathanasis et al., 2017).

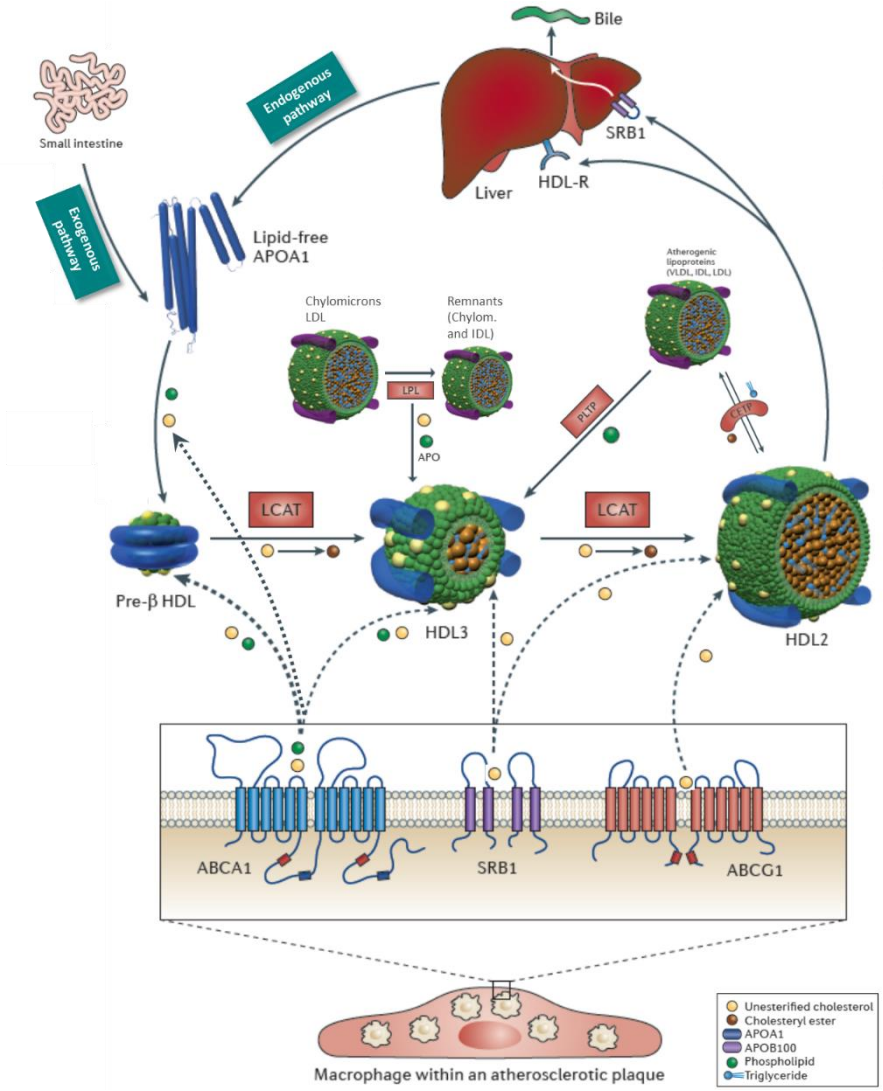

Figure 10 | Metabolism of HDL. ApoA-I is synthesized in the liver and small intestine in humans. ApoA-I associates with cholesterol and PL to form nascent HDLs. Initial lipidation of such HDL occurs via ABCA1-mediated ChE of FC from peripheral cells. LCAT associates with the discs and is activated by ApoA-1, resulting in their conversion to spheroidal particles (HDL_3 and HDL_2) with a core of CE, as the cholesterol is esterified and replaced by further FC from tissues. In addition, spherical HDL (primarily large HDL_2) can efflux cholesterol from peripheral cells via ABCG1 and SRB1. In humans, FC and CE are transferred from HDL_2 to the liver directly via binding to SRB1 in hepatocytes or indirectly via Chylomicrons, VLDL, IDL and LDL after CETP activity. After entering hepatocytes, the CE are preferentially oxidized and excreted through bile acids or used to synthesize new lipoproteins. Source: adapted from Kingwell *et al.,* 2014.

The biosynthesis of endogenous HDL begins with the production and secretion of lipid-free ApoA-I by the liver or intestine. Nascent, discoidal pre-β HDL is then formed through lipidation of ApoA-I. This HDL is cholesterol poor, but some cholesterol is spread among the PL molecules. These particles then arrive at lipid-laden macrophages and other peripheral tissues, and FC and PL are removed from them via the ABCA1 receptor. This process is known cholesterol efflux (ChE), and it is the first step of the RCT.

The next step involves the action of the LCAT enzyme. After travelling to the blood via the lymphatic system, LCAT associates with discoidal HDL and it is activated by ApoA-I. This enzyme can esterify the FC present in the HDL monolayer to CE, which can then be internalized into the core of the HDL particle, initiating its transformation from discoidal to spherical HDL, particularly the small HDL3 particles. The low cholesterol content of these lipoproteins, together with the esterification and internalization of FC, is thought to form a cholesterol gradient that enables more cholesterol binding onto the HDL3 surface to continue, collecting excess cholesterol from cell membranes as part of the ChE process.

Once the LCAT substrate is exhausted, HDL3 begins accepting PL, FC, and Apos from other lipoproteins, such as chylomicrons and LDL, which are derived from the activity of the lipoprotein lipases that form remnant lipoproteins (chylomicron remnants and IDL, respectively). Concomitantly, phospholipid transfer protein (PLTP) mediates the transfer of excess PL from the surface of TG-rich lipoproteins, particularly atherosclerotic VLDL, IDL, and LDL. As a consequence, these HDL particles become enriched in PL, which can further accept FC effluxed by ABCG1 and scavenger receptor type BI (SR-BI) to become larger, more mature, spherical HDL, or the HDL2 subfraction.

Mature HDL_2 is also an LCAT substrate, allowing further removal of FC from peripheral tissues and becoming an HDL with a CE-enriched core. At the same time, CETP mediates the transfer of such CE to TG-rich lipoproteins, which, in sequence, delivers TG in return, contributing to HDL maturation to bigger, PL-enriched HDL_2 (Eckardstein & Kardassis, 2015; Karathanasis et al., 2017; Kuai et al., 2016; Salazar et al., 2015).

The final process of RCT involves the delivery of the cholesterol cargo of HDL_2 to the liver by two main pathways: after reversibly binding to SR-BI in hepatocytes, the cargo is indirectly transferred to atherogenic lipoproteins (chylomicrons, VLDL, IDL and LDL) via CETP. Then, chylomicron remnants and LDL are taken up by the LDL receptor in hepatocytes. Approximately 2/3 of cholesterol returns to the liver via ApoB-containing particles, while approximately 1/3 of cholesterol returns to the liver via HDL (Karathanasis et al., 2017).

All this cholesterol cargo delivered to the hepatocytes is then oxidized for excretion through bile acids via the ABCG5 and ABCG8 transporters or used to synthesize new lipoproteins. It has been described that 8 g/day of cholesterol is excreted from peripheral tissues, but only 1-1,5 g/day is excreted by the liver. Thus, the efflux rate of FC from tissues is 5 to 8 times the net outflow rate of sterols from the whole body.

Within the HDL metabolism process, it is worth mentioning the phenomenon called conversion, in which mature HDL_3 are remodeled to lipid-free ApoA-I. This process is catalyzed by CETP, hepatic lipase, endothelial lipase and plasma PLTP and results in the formation of lipid-poor HDL particles and the shedding of lipid-free ApoA-I from HDL, which can interact with ABCA1 in the next lipidation cycle (Figure 10):

a) CETP: enzyme that transfers TG from VLDL and LDL and exchanges them for CE from HDL.

b) HL: enzyme that primarily hydrolyzes TG, remodeling HDL into smaller particles and dissociating ApoA-I.

c) EL: phospholipase that remodels HDL into smaller particles without mediating the dissociation of ApoA-I.

d) PLTP: a lipid transfer protein that transfers PL from TG-rich lipoproteins (LDL, VLDL) to HDL and between different HDL subpopulations. PLTP also disassociates ApoA-I from mature HDL creating lipid-free and lipid-poor ApoA-I (Karathanasis et al., 2017; Turner et al., 2012).

2.6 HDL biological activities

The tunica intima of the arterial wall is the site for HDL to exert its main antiatherogenic functions: cholesterol removal, anti-inflammatory activity, and antioxidant activity. For the intimal biogenesis of HDL, ApoA-I that is synthesized in the liver and intestine needs to pass through the endothelial cell layer that forms the barrier between the intima and the plasma, which is a process called transendothelial migration. Transendothelial transport of ApoA-I involves multiple factors that remain unknown, and multiple questions about this process remain unanswered (Choi et al., 2016). However, the existence of efficient transendothelial transport of ApoA-I is evident since the presence of ApoA-I in atherosclerotic intimal lesions has been described by several authors. In fact, ApoA-I content in atherosclerotic aortas is 100-fold higher than in normal lesions, and most of the ApoA-I in the aortic wall is functionally impaired and not associated with HDL particles (Di Donato et al., 2013). This ApoA-I that has infiltrated into the early-stage atherosclerotic intima forms HDL particles by removing excess cholesterol from foam cells, leaving the artery wall exposed to the blood circulation; however, ApoA-I in advanced-stage lesions is stranded in the intima and becomes dysfunctional. The mechanism that explains why the function of this ApoA-I is impaired remains unknown, although some authors have proposed ApoA-I oxidation within the intima as the likely mechanism (Choi et al., 2016).

HDL confers protection against atherosclerosis because HDL and ApoA-I have pleiotropic beneficial activities, which are determined, in part, by their physicochemical properties. Although the role of HDL in exerting RCT was first postulated as a major contributor to the causative association between low HDL-C levels and CVD, numerous other potential mechanisms have been discovered. These additional HDL pleiotropic activities comprise anti-inflammatory, antioxidant, antithrombotic, antiapoptotic, anti-infectious and vasodilatory activities, as well as effects on glucose metabolism (Figure 11) (Cybulska & Kłosiewicz-Latoszek, 2014; Karathanasis et al., 2017; Kingwell et al., 2014):

a) HDL can attenuate oxidative stress by protecting both lipid and protein moieties of LDL and other lipoproteins from free radical-induced oxidation.

b) HDL reduces the production of inflammatory cytokines (such as TNF-α) by macrophages and the endothelial expression of adhesion molecules (such as ICAM-1 and VCAM-1) that facilitate the penetration of monocytes and neutrophils into the arterial wall.

c) The antithrombotic effect of HDL is related to the reduced expression of tissue factor on endothelial cells and the prostaglandin PGI_2, which therefore inhibits of platelet activation. *In vitro* studies showed that HDL reduced the platelet aggregation induced by collagen, thrombin, and adenosine diphosphate. HDL may also inhibit thrombus formation by maintaining the integrity of endothelial cells.

d) HDL enhances nitric oxide production, resulting in a vasodilatory activity.

e) HDL has antiapoptotic effects by stimulating the expression of the antiapoptotic Bel-xL protein, among others, and increasing the recruitment of endothelial progenitor cells.

f) HDL holds anti-infectious properties since it inhibits bacterial antigens such as lipopolysaccharide, which is an essential component of the bacterial cell wall.

g) HDL also plays a role in glucose homeostasis, as it regulates insulin secretion and glucose uptake, and it enhances insulin sensitivity. Low HDL-C levels may increase the risk of developing T2DM and insulin resistance. Genetic defects leading to low HDL-C levels are associated with impaired insulin secretion and increased resistance to insulin.

The current interest in elucidating new therapies to boost HDL function has led to the discovery of emerging roles of HDL. Specifically, it has led to the use of HDL for delivering drugs. Endogenous HDL is reported to transport signalling lipids, proteins, and endogenous microRNAs to

recipient cells, suggesting that HDL plays a major role in complex intercellular communication. These features have inspired numerous laboratories and pharmaceutical industries to develop exogenous HDL particles as delivery vehicles for various therapeutic agents (Kuai et al., 2016).

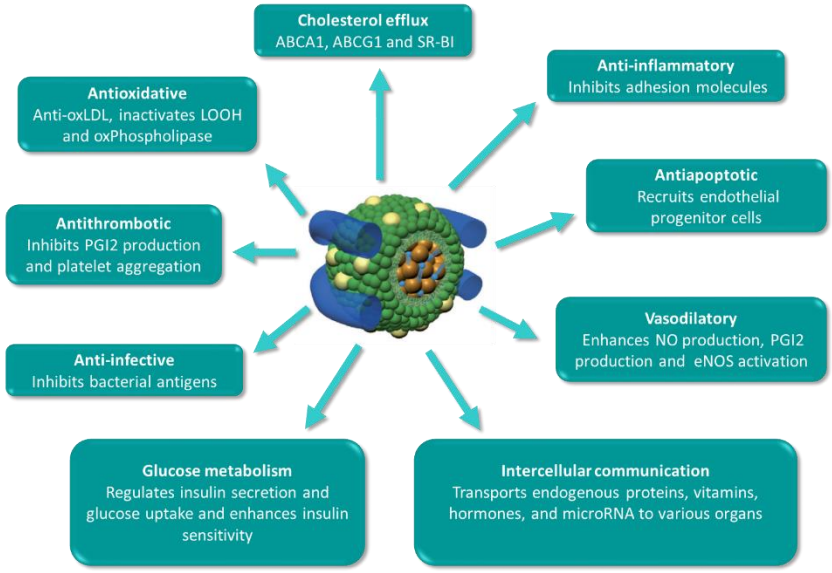

Figure 11 | Cardioprotective benefits of HDL. HDL holds pleiotropic activities in protection against arteriosclerosis. ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; eNOS, endothelial nitric oxide synthase; LOOH, lipid hydroperoxides; NO, nitric oxide; oxLDL, oxidized low-density lipoprotein; PGI2, prostaglandin I2; SR-BI, scavenger receptor class B type I. Source: adapted from Choi *et al.,* 2016.

3. REVERSE CHOLESTEROL TRANSPORT AND HDL-MEDIATED CHOLESTEROL EFFLUX

A key function of HDL is to transport and deliver excess cholesterol from the cell membrane of peripheral tissues to the liver, where it is excreted out of the body through bile or is used to synthesize new lipoproteins. Excess cholesterol is also sent to the adrenals, testes, and ovaries for steroid hormone production. This process is known as RCT. In the first step of RCT, HDL acts as an acceptor of cellular FC present in lipid-loaded cells, a process that is known as ChE (Karathanasis et al., 2017; Lüscher et al., 2014; K.-A. Rye et al., 2009).

The ChE process involves cholesterol localized on the cell membrane, which, in turn, may derive from intracellular sites, such as the late endosomal/lysosomal compartment and the Golgi apparatus (Favari et al., 2015). Given that the removal of cholesterol from lipid-laden macrophages leads to a reduction in plaque lipid content and inflammation, this flux of cholesterol out of the vascular intima could ultimately protect arteries from forming a lipid-rich necrotic core plaque.

3.1 Mechanisms involved in ChE

It was first thought that HDL particles were passive acceptors of cellular cholesterol, in which LCAT-mediated esterification prevented equilibration of cholesterol between cell membranes and HDL, thereby allowing net ChE (Glomset & Wright, 1964). However, currently additional active mechanisms involving protein transmembrane transporters are known to mediate ChE from lipid-laden macrophages and foam cells to HDL: ABCA1, ABCG1, and SR-BI.

3.1.1 Passive aqueous diffusion

In this ChE mechanism, molecules of FC desorb from the cell membrane and are then incorporated into a containing acceptor, such as the HDL monolayer (Sankaranarayanan et al., 2013). When LCAT depletes the HDL surface of FC by generating CE that move into the core of the HDL particle,

a cholesterol gradient is generated on the HDL surface. This gradient enables HDL to accept additional FC from cell membranes and other lipoproteins (Adorni et al., 2007; Jessup, Gelissen, Gaus, & Kritharides, 2006; K. Rye & Barter, 2014; Yancey et al., 2003).

3.1.2 ABCA1-mediated ChE

ABCA1 mediates the unidirectional translocation of PL and FC from the inner to outer layer of cell membranes, from where they are exported to the lipid-free and lipid-poor ApoA-I present in the extracellular space. As previously explained, progressive lipidation contributes to HDL maturation since it leads to the generation of discoidal pre-β HDL particles and HDL_3 that, in turn, are also acceptors of the cholesterol that effluxed via ABCA1. It is worth highlighting that defective ABCA1 transporter function in patients with Tangier disease has provided clear evidence that this transporter has a central role in lipid efflux mediated by lipid-poor ApoA-I (Remaley et al., 2003).

3.1.3 ABCG1-mediated ChE

ABCG1 transporter enhances unidirectional HDL lipidation and therefore maturation similarly to ABCA1: however, in this case, the efflux is mediated only to discoidal and spherical HDL, but not to lipid-free ApoA-I.

3.1.4 SR-BI-mediated ChE

SR-BI transporter mediates not only ChE but also cholesterol uptake in several cell types. This bidirectional exchange of cholesterol is carried out between cells expressing SR-BI and discoidal and spherical HDL. For instance, SR-BI present in macrophages embedded in the atherosclerotic plaque enhances ChE to HDL, while SR-BI expressed in hepatocytes is responsible for cholesterol uptake from mature HDL in the liver. This bidirectional transport is increased when HDL-C circulating levels are increased by pharmacological inhibition of CETP or by infusions of reconstituted HDL, among others.

The relative contribution of each of these ChE pathways to the net efflux is not entirely known. On one hand, for lipid-laden cells acting as foam cells embedded in atherosclerotic plaque, ChE is mediated mainly by pathways involving aqueous diffusion, ABCA1, and ABCG1, with a lesser contribution from the SR-BI transporter. This process occurs because the excess cholesterol within cells is responsible for the activation of Liver X receptor (LXR), the transcription factor controlling ABC transporter expression. On the other hand, in the absence of cholesterol enrichment, the pathway used for ChE to human sera is predominantly aqueous diffusion, with a minor contribution from the SR-BI pathway, and neither ABCA1- nor ABCG1-mediated ChE is observed (Anastasius et al., 2016). Other factors, such as HDL composition and subclass distribution, may also affect all these processes.

3.2 Role of ApoE in ChE

A relevant role in ChE from macrophages has been attributed to ApoE. This protein is synthesized by many cell types, including macrophages, as a response to different stimuli, such as lipid enrichment. Upon synthetis, ApoE is secreted from lipid-laden macrophages and this process can promote ChE in the absence of cholesterol acceptors or in presence of exogenous HDL, causing the generation of nascent HDL particles. There is evidence that ApoE enhances ChE by both ABCA1-dependent and independent mechanisms (Favari et al., 2015).

3.3 Methods for assessing RCT and ChE

As previously mentioned, HDL-C is not a good CVR predictor, as it may fall short in identifying risk groups in CVD. Therefore, the assessment of HDL functionality rather than HDL quantity is of paramount importance in evaluating the CVR of patients (Karathanasis et al., 2017). No definitive common structural feature determines HDL functionality, and the complexity and high cost of measuring all HDL functions preclude these tests from becoming routine clinical laboratory tests. Animal studies have suggested that HDL-mediated ChE through the different RCT steps is a better predictor of atherosclerosis than the quantification of circulating HDL-C (Eren et al., 2012).

Several laboratory models have been developed to monitor the effect of HDL on RCT, including assays of ChE from cholesterol-loaded cells, HDL tracer kinetics, and fecal sterol excretion (Joan Carles Escolà-Gil et al., 2015; Favari et al., 2015; Santos-Gallego, Giannarelli, & Badimón, 2011). When assays of various HDL functions have been performed on the same samples the results have been concordant, despite the varying methodologies (Ansell et al., 2007; Favari et al., 2015).

Several authors evaluate RCT by assessing HDL capacity to promote ChE in cellular models. This method requires a standardized assay that would measure the capacity of HDL to remove cholesterol from cells, without modifying or excluding subpopulations of HDL, which would allow distinguishing between the various ChE mechanisms involved (Adorni et al., 2007; Favari et al., 2015; Khera et al., 2011). The HDL cholesterol efflux capacity (CEC) measured in these *in vitro* assays has been causally and directly linked to the prevention of atherosclerosis in animal models (Rader et al., 2009) and is currently considered as both a diagnostic tool and a promising target for the treatment of atherosclerosis (DY Litvinov, Savushkin, Garaev, & Dergunov, 2016). Furthermore, strategies to measure CEC have been used successfully in clinical studies, revealing inverse correlations between CEC and prevalent CAD that are independently of the HDL-C levels. In this sense, the Dallas study demonstrated the inverse association of ChE with incident atherosclerotic CVD in a population-based cohort free from CVD at baseline. This association persisted after multivariate adjustment, suggesting that HDL function is associated with CVR by means of processes distinct from those reflected by the HDL-C levels or additional traditional CVR factors (Rohatgi et al., 2014).

Several *in vitro* models have been devised to assess ChE though there is no gold standard method for measuring CEC in humans and protocols vary considerably. However, the measurement of ChE employing *in vitro* models always requires three components: a cholesterol-loaded donor cell, a cholesterol tracer or probe, and a cholesterol acceptor.

3.3.1 Cholesterol-loaded donor cell

In the assessment of ChE *in vitro*, a cholesterol donor first needs to be loaded with a cholesterol tracer. Macrophages, such as the human macrophage cell line THP-1 and the murine macrophage cell lines J774 and RAW 264.7, are the most relevant cell types for studies of atherosclerosis, given the central role of macrophage foam cells in disorders of lipid accumulation (Joan Carles Escolà-Gil et al., 2015; Rohatgi et al., 2015). Furthermore, efflux pathways in these cells, particularly ABCA1 and ABCG1, are efficiently upregulated by exposing the cells to cAMP or to LXR agonists such as GW3965 or T0901317 (Sankaranarayanan et al., 2011).

Increasingly, ChE assays are also performed using different cell lines that do not express the ABC transporters themselves or cell lines that express a single cholesterol transporter. Such is the case of baby-hamster kidney cells or human epithelial HeLa cells, which are both stably transfected with mifepristone-inducible human ABCA1 cDNA. Fu5AH hepatoma cells and adipocytes are also used as donor cells to obtain information about the transporters involved in the ChE process (Adorni et al., 2007; Anastasius et al., 2016; Joan Carles Escolà-Gil et al., 2015; S. M. Gordon et al., 2016; Sankaranarayanan et al., 2009; Zhang et al., 2010).

Studies in humans and animals suggest that ABCA1-mediated efflux is an important mode of efflux by which the severity of atherosclerosis is modulated. Therefore, it is plausible that the monitoring of ABCA1-mediated efflux rather than ABCAG1, provides a proper method for assessing the mode of efflux of greatest relevance to evaluating the severity of atherosclerosis and its clinical consequences (Rohatgi et al., 2014).

3.3.2 Cholesterol tracer or probe

Concerning the cholesterol tracer used in the model, there is no consensus regarding a gold standard, although the typical method that is currently used is radiolabeling cells with Tritiated cholesterol (^{3}H-Cholesterol; ^{3}H-Ch). This radioactive approach for measuring ChE has provided large amounts of data on both the efficiency of various extracellular acceptors and different efflux pathways (Joan Carles Escolà-Gil et al., 2015; Favari et al., 2015). However, a protocol using radiolabeled cholesterol does not

lend itself to the development of a high-throughput assay that can efficiently screen large numbers of specimens, such as in the case of clinical trials. Sankaranarayanan *et al.* developed a cellular model to assess ChE using boron dipyrromethene difluoride (BODIPY) cholesterol (BODIPY-cholesterol; Bd-Ch), a fluorescent cholesterol probe in which C24 of the cholesterol molecule is linked directly to a BODIPY moiety (Figure 12). This group demonstrated that Bd-Ch undergoes efflux to HDL at an almost identical rate as ^{3}H-Ch in murine J774 macrophages (Sankaranarayanan et al., 2011). The use of this fluorescent probe can be useful to study ChE, thus avoiding the usage of radioactivity, even if the radioactive model involves low quantities and radioisotopes with a short half-life, such as ^{3}H-Ch.

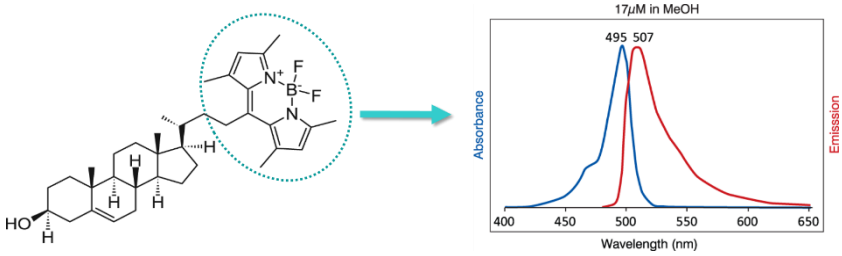

Figure 12 | BODIPY-cholesterol structure and spectroscopic data in methanol. BODIPY-cholesterol is a fluorescent cholesterol probe in which carbon 24 of the cholesterol molecule is linked directly to a BODIPY moiety, depicted with a discontinued circle in this figure. The use of this fluorescent probe can be useful to study ChE avoiding the usage of radioactivity, even if the model involves low quantities and short half-life time radioisotopes such as ^{3}H-Ch. Source: adapted from Avanti Polar Lipids, Inc.

The synthesis and development of new fluorescent cholesterol probes, with similar characteristics but different spectroscopic behaviors than Bd-Ch, could provide an interesting tool for studying and understanding cholesterol movement and metabolism within and between cells, not only for ChE but also for cholesterol distribution into the different cellular pools and the eventual uptake of cholesterol in receptor tissues. Liu *et al*. demonstrated that fluorescent red-shifted Bd-Ch analogues exhibit similar cellular localization and ChE properties to unlabelled cholesterol.

Therefore, these analogues may be useful probes for the simultaneous visualization of intracellular cholesterol pools and for monitoring ChE from cells to extracellular acceptors (Liu et al., 2014).

3.3.3 Cholesterol acceptor

Regarding the cholesterol acceptors, several specimens have been used in *in vitro* models assessing ChE. These include total serum or plasma, ApoB-depleted serum and isolated HDLs (Anastasius et al., 2016).

De la Llera *et al.* identified that most of the ChE stimulated by human serum was attributable to the non-ApoB lipoprotein-containing fraction and that this ChE could be measured simply and reliably using total plasma without ApoB (plasma from which ApoB had been precipitated) (de la Llera Moya et al., 1994). The use of ApoB-depleted plasma eliminates the role of LDL and VLDL in assessing ChE, making the assessment more specific for HDL-mediated ChE than assessments using total serum or plasma. Although it is cheap and fast to obtain this specimen, possible cholesterol acceptors other than HDL subfractions, such as albumin, are also present, and they may interfere in HDL-mediated ChE (Rohatgi, 2015; Rosenson et al., 2016). It has been demonstrated that albumin mediates the bidirectional flux of cellular FC and functions as a low affinity, high capacity cholesterol transporter (Sankaranarayanan et al., 2013).

The use of isolated HDL by ultracentrifugation also has its limitations. For instance, ultracentrifugation methods limit HDL density to 1.21 g/mL and do not accurately represent the contribution of pre-β HDL, which is found in the density range 1,21–1,25 g/mL (Miyazaki, Ogihara, Fukamachi, & Kasumi, 2014; Rosenson et al., 2016).

No data are available to suggest that the use of a particular acceptor might be more physiologically relevant or better than another for assessing cellular ChE mediated by HDL.

4. ANTIOXIDANT ACTIVITY OF HDL: THE ROLE OF PARAOXONASE ENZYME FAMILY

Oxidation in the intima of arteries is the result of an imbalance between pro-oxidants and antioxidants that is in favor of the former. Cellular oxidative systems include myeloperoxidase (MPO), nicotinamide adenine dinucleotide phosphate oxidase, NO synthase, and lipoxygenase. They produce a variety of reactive chlorine, nitrogen and oxygen species (ROS) (Karlsson et al., 2015).

HDL can attenuate oxidative stress present in the intima of the atherosclerotic lesion. This HDL antioxidant capacity is typically observed as protection of both lipid and protein moieties of LDL from free radical-induced oxidation. This fact has been reported *in vitro* on their co-incubation and *in vivo* upon HDL supplementation (Karlsson et al., 2015). Even though LDL is thought to be the major physiological target of HDL antioxidant action *in vivo*, HDL is also able to inhibit oxidant modification of other targets containing PL, such as other lipoproteins or cell membranes (Eren et al., 2012), and inhibits the generation of ROS *in vitro* and *in vivo* (Kontush & Chapman, 2006, 2010).

HDL antioxidant capacity comprises two steps. The first one involves the transfer of oxidized lipids from cells and LDL to HDL. The second step consists of the inactivation of these oxidized lipids in HDL. Depending on their structure, oxidized lipids can be inactivated via reduction by ApoA-I and other redox-active HDL components or via hydrolysis by HDL hydrolytic enzymes (Kontush & Chapman, 2010; M. Navab, 2004; Zerrad-Saadi et al., 2009).

4.1 Proteins accountable for the antioxidant activity of HDL

The antioxidant property of HDL is down to the presence of several enzymes and Apos within its proteome, as described below.

4.1.1 HDL antioxidant Apos

The HDL-associated Apos that possess antioxidant activity include ApoA-I, ApoA-II, ApoA-IV, ApoE, and ApoJ. It has been reported that the lipid hydroperoxides that are associated with HDL are reduced to less deleterious lipid hydroxides by means of a process that is accompanied by the concomitant oxidation of ApoA-I methionine residues 112 and 148. ApoA-I can also prevent and delay LDL oxidation by removing oxidized PL from LDL and from arterial wall cells (Kontush & Chapman, 2006, 2010).

Antioxidant properties have also been reported for ApoA-II (Boisfer et al., 2002). However, the capacity of ApoA-II to protect against oxidation is questionable, given that overexpression of human ApoA-II in animal models increases oxLDL accumulation in the aortic lesion. This process might be mediated by the displacements of ApoA-I and PON1 by ApoA-II from HDL particles. In fact, overexpression of human ApoA-II in animal models increases oxLDL accumulation in the aortic lesion due to such displacements (Ribas et al., 2004; Rotllan et al., 2005).

Moreover, additional HDL-associated Apos, such as ApoA-IV, ApoE, and ApoJ, also have antioxidant effects *in vitro*, although data explaining the exact mechanism are still scarce (Kontush & Chapman, 2006).

4.1.2 HDL antioxidant enzymes

The major HDL enzymes with antioxidant activity are LCAT, platelet-activating factor acetylhydrolase (PAF-AH; also called lipoprotein-associated phospholipase A2), glutathione selenoperoxidase (GSPx) and the PON family. All these enzymes have been proposed to hydrolyze oxidized PL, particularly short-chain oxidized PL from LDL particles, detoxifying them (Karlsson et al., 2015). Local arterial expression of PAF-AH has been described to reduce the accumulation of oxLDL and inhibiting inflammation, among others (Kontush & Chapman, 2006).

4.2 PON enzyme family: synthesis, body distribution, and activities

The PON enzyme family, considered to be a major contributing factor to the antioxidant potential of HDLs, is comprised of three members: PON1,

PON2, and PON3 (Figure 13). The genes encoding these proteins are clustered on the long arm of chromosome 7 (7q21.3-q22.1). All human PON genes have nine exons and share approximately 70% homology at the nucleotide level and 65% homology at the amino acid level (Schrader & Rimbach, 2011). In mammals, the similarity of these three genes is higher and reaches 81-90% and 79-90% homology at the nucleotide and amino acid levels, respectively (Chistiakov, Melnichenko, Orekhov, & Bobryshev, 2017).

Synthesized in the liver, these calcium-dependent enzymes have different activities and localizations and thus have different functions in the body. In humans, both PON1 and PON3 are mainly expressed in the liver and kidney and are expressed to a lesser extent in tissues such as brain, colon, heart, lung and small intestine. They both are secreted into the blood, where they associate with ApoA-I-containing HDL. While liver is the major tissue of PON1 gene expression, lipoprotein-assisted circulation of PON1 in plasma delivers the enzyme to multiple tissues that do not express PON1 themselves (Dmitry Litvinov, Mahini, & Garelnabi, 2012; M. Mackness & Mackness, 2015; Schrader & Rimbach, 2011). PON2 is exclusively intracellular, and it is expressed in nearly all human tissues, though it is not found in the circulation (Jordi Camps, Marsillach, & Joven, 2009; Précourt et al., 2011).

Concerning their activity, PON1 is considered as a "promiscuous" enzyme due to its ability to hydrolyze lipid peroxides and many other substrates, such as toxic organophosphorus compounds, non-phosphorous arylesters, statin adducts, glucocorticoids, the quorum-sensing factor of *Pseudomonas aeruginosa*, the atherogenic L-homocysteine thiolactone, and other lactones, with lactones being considered to be the primary substrate (Seung, Marsillach, Furlong, & Jarvik, 2013).

The name of the PON enzymes comes from the capacity of PON1 to hydrolyse the organophosphorus compound paraoxon, the toxic metabolite of parathion, as this was the first described PON1 activity. Years later, the other two members of the family were identified and were consequently termed PON2 and PON3 (Primo-Parmo, Sorenson, Teiber, & La Du, 1996). However, based on structure-reactivity studies of PON1, it is assumed that lactones are likely to be their native substrates (Chistiakov et al., 2017).

All PON enzymes have lactonase activity, and PON1 can also hydrolyze aromatic esters and certain organophosphate compounds, such as paraoxon, due to their arylesterase and paraoxonase activities (Figure 13). Therefore, PON activities can be defined as:

a) Paraoxonase activity: Hydrolysis of organophosphorus compounds, such as paraoxon, soman, sarin, and others.

b) Lactonase activity: Hydrolysis of aromatic and aliphatic lactones such as 5-thiobutyl butyrolactone (TBBL), or other lactones such as dihydrocoumarin.

c) Arylesterase activity: Hydrolysis of aromatic esters, such as phenyl acetate or 4-(p)-nitrophenyl acetate (M. Mackness & Mackness, 2015; Seung et al., 2013).

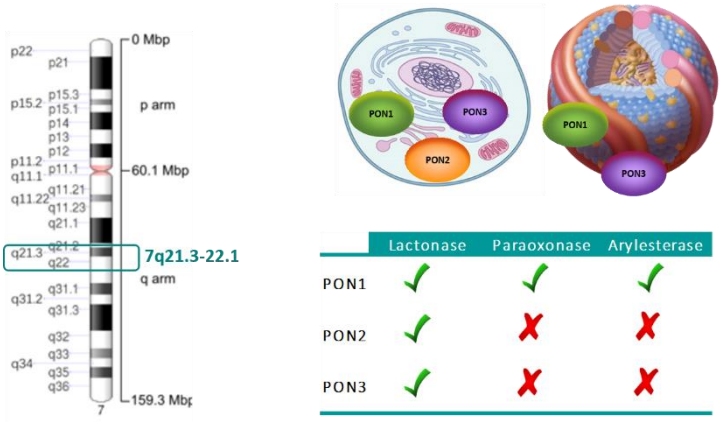

Figure 13 | PON enzyme family chromosome location, body distribution, and associated activities. The genes encoding these proteins are clustered on the long arm of chromosome 7 (7q21.3-q22.1). PON1 and PON3 are found in many tissues and in circulation almost exclusively associated with HDL-containing ApoA-I, while PON2 is exclusively intracellular. In particular, PON1 is found in liver and to a lesser extent in brain, colon, heart, kidney, lung and small intestine. PON1 is the only one holding arylesterase and paraoxonase activities whereas PON1, PON2, and PON3 share lactonase activity. Source: created by the author.

Hydrolysis of homocysteine thiolactone by PON1 is considered to be protective against CAD (Litvinov et al., 2012). In addition, down to the lactonase activity PON1 and PON3, they can also metabolize several lactone-like pharmacologic drugs such as antibiotics, glucocorticoids, and statins, bioactivating them or limiting the systemic side effects of some of these drugs (Seung et al., 2013).

According to some studies, arylesterase activity is less sensitive to modulating factors, such as genetic polymorphisms, diet, oxidative stress and acute-phase response, and it is a better representation of the amount of circulating enzyme than activity towards paraoxon (Beltowski, Wojcicka, Mydlarczyk, & Jamroz, 2002).

4.3 The role of ApoA-I and ApoA-II in PON activation

Circulating PON1 is in equilibrium between lipoprotein-bound (80%) and free forms (20%), though only the tightly associated PON1 is fully active. Under certain physiological and pathological conditions, PON dissociates from HDL and becomes dysfunctional (Gugliucci & Menini, 2015). The protein composition of HDL, in particular ApoA-I and ApoA-II, is essential for PON1 binding to HDL and is therefore essential for activation. PON binds to HDL through the interaction of its hydrophobic N-terminus to PL and through direct interactions with PON1– ApoA-I.

ApoA-I not only stabilizes PON1 binding to HDL but also directly activates PON1 (Gugliucci & Menini, 2015). Consequently, ApoA-I levels increase paraoxonase and arylesterase activities in reconstituted HDL as a result of PON1-HDL stabilization (Dmitry Litvinov et al., 2012). ApoA-I levels correlate to such an extent with PON1 activities that it was found that ApoA-I concentration is the strongest predictor of arylesterase activity, not HDL-C levels (Seung et al., 2013). Moreover, ApoA-I-containing HDL was found to significantly increase the lactonase activity of PON1, promoting the inhibition of LDL oxidation and stimulating macrophage ChE compared with ApoA-II containing HDL (Dmitry Litvinov et al., 2012). Studies with HDL isolated from ApoA-I-deficient patients revealed that PON1 even binds to HDL in the absence of ApoA-I. However, the PON1–HDL complex is less stable, and PON1 loses activity faster in this complex than in normal controls (Noto et al., 2001).

An *in vitro* study of reconstituted HDL demonstrated that ApoA-II destabilizes the HDL–PON1 complex (Gaidukov & Tawfik, 2005). Overexpression of human ApoA-II in animal models increases oxLDL accumulation in the aortic lesion due to the displacements of ApoA-I and PON1 by ApoA-II from HDL particles (Kontush & Chapman, 2010; Ribas et al., 2004; Rotllan et al., 2005).

4.4 Atheroprotective properties of PON family

PON1 and PON3 enzymes have a significant impact on the pathogenesis of CVD and atherosclerosis since this family not only prevents oxidation (M. Mackness & Mackness, 2015; Marsillach et al., 2015) but also promotes ChE (Berrougui, Loued, & Khalil, 2012; Rosenblat, Vaya, Shih, & Aviram, 2005) and prevents inflammation (Sans et al., 2012) (Figure 14):

1. Several *in vitro* and *in vivo* studies have demonstrated that PON1 and PON3 can prevent lipoprotein oxidation by metabolizing lipid peroxides formed in the process of the oxidation of LDL (M. Mackness & Mackness, 2015; Marsillach et al., 2015). PON1 is also able to hydrolyse oxidized PL and thus protect lipoproteins and membranes from oxidative modifications. That is, PON1 was also shown to protect HDL itself from oxidation (Seung et al., 2013; Zhu et al., 2014).

2. It has been found that PON1 and PON3 have an important role in promoting HDL-mediated ChE. Both PONs enhance HDL binding to the ABCA1 lipid transporter that mediates the ChE (Berrougui et al., 2012; Rosenblat et al., 2005).

3. PON1 and PON3 enzymes possess anti-inflammatory activity by inhibiting CCL-2 (Sans et al., 2012). This chemokine is expressed in endothelial cells, and it is essential in monocyte recruitment by endothelial cells, transendothelial migration into the intima layer, and the subsequent differentiation into macrophages, which are processes involved in the early stages of atherosclerosis. The mechanisms that underlie this CCL-2 inhibitory ability are closely related to the aforementioned PON capacity to protect LDL from oxidation, as unmetabolized lipid peroxides directly induce the production of CCL-2 (Chistiakov et al., 2017; B. Mackness, Hine, Liu, Mastorikou, & Mackness, 2004; M. Mackness & Mackness, 2015).

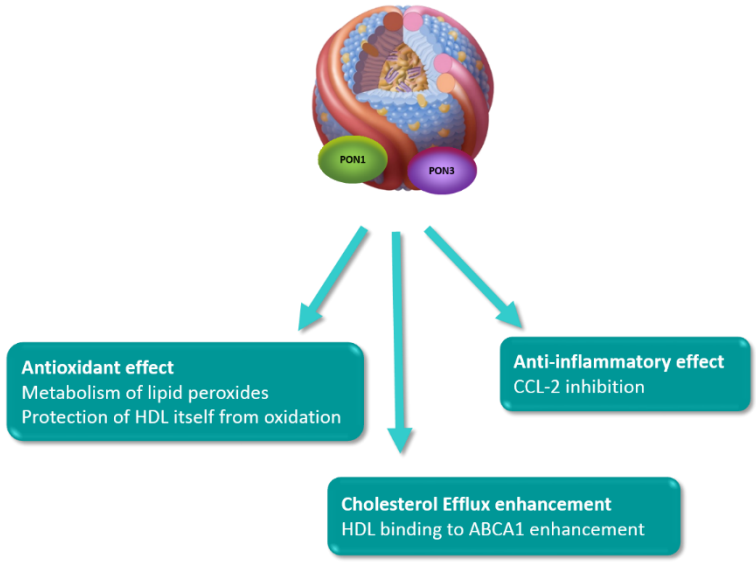

Figure 14 | Atheroprotective properties of PON family. PON enzymes exert their cardioprotective function by means of several mechanisms: preventing oxidation (Mackness *et al.,* 2015; Marsillach *et al.,* 2015), preventing inflammation (Sans *et al.,* 2012), and promoting ChE (Berrougui *et al.,* 2012; Rosenblat *et al.,* 2005). Source: created by the author.

It is important to highlight that PON1 activities positively correlate with the improvement of HDL antioxidant properties to such an extent that PON activities have been proposed as new biomarkers of HDL function and CVR (Hafiane & Genest, 2015; Tang et al., 2012).

4.5 PON protein levels and activities in pathological conditions

PON protein levels and their associated activities play a major role in the onset and development of several chronic diseases whose underlying mechanisms comprise an impairment in oxidative stress and an inflammatory component. In particular, the PON system is altered in atherosclerosis, T2DM, rheumatoid arthritis, systemic lupus erythematosus, cancer and several hepatic and renal diseases, which are characterized by having dysfunctional HDL and increased CHD rates

(Goswami, Tayal, Gupta, & Mallika, 2009; Karathanasis et al., 2017; M. Mackness & Mackness, 2015).

4.5.1 PON protein levels and activities in liver disease

Given that the liver plays a key role in the synthesis of the PON enzymes, it is safe to assume that liver diseases are characterized by an impairment in the PON system. For instance, chronic liver impairment patients present lower paraoxonase and lactonase activities than healthy subjects to such an extent that these activities represent a good measurement for the clinical evaluation of patients with liver disease (Marsillach et al., 2009). The decrease in these activities can also be due to abnormalities in HDL lipid and protein composition, including PON1 glycosylation (Karathanasis et al., 2017; Marsillach et al., 2010). Serum PON3 protein levels are also decreased in chronic hepatitis and liver cirrhosis (García-Heredia et al., 2011).

4.5.2 PON protein levels and activities in CVD

Mounting evidence from both animal and human studies links increased PON1 protein levels and decreased PON1-associated activities to an increased likelihood of developing CVD (Abelló, Sancho, Camps, & Joven, 2014). Lactonase activity is accountable for hydrolyzing L-homocysteine thiolactone, a human biomarker associated with atherosclerosis (Jakubowski, 2000). Nevertheless, paraoxonase and arylesterase activities have also been implicated in CVD, as have PON1 and PON3 protein circulating levels. Thus, the whole PON system plays a crucial role in CVD. In fact, in experimental models and therapeutic interventions in humans, PON1 activity measurement has been proposed as a novel biomarker of HDL function and CVR that is independent of HDL-C levels. For instance, PON1 activity was used in ApoA-I mimetic drug studies as an indicator of the improvement of HDL antioxidant properties (Hafiane & Genest, 2015). Overproduction of human PON3 in ApoE-deficient mice caused significant regress in plaque development compared with non-transgenic animals (Chistiakov et al., 2017).

4.5.3 PON protein levels and activities in T2DM

A decrease in serum PON1 arylesterase and PON3 lactonase activities was found in T2DM patients with CAD when compared with T2DM patients without CAD. This decrease was found to be associated with ApoA-I glycation, which indeed impairs HDL function and thus accelerates the atherosclerotic process in these patients (Shen et al., 2015).

4.5.4 PON protein levels and activities in autoimmune diseases

Similar to T2DM, elevated blood glucose (> 10 mmol/L) in type 1 diabetes (T1D) patients was associated with lower PON1 protein levels and paraoxonase and arylesterase activities when compared to patients with glucose levels below 10 mmol/L (Soran, Younis, Charlton-Menys, & Durrington, 2009), and this fact that could be explained by similar mechanisms to those described in T2DM patients.

PON3 protein levels were found to be depleted in HDL from patients with subclinical atherosclerosis and those suffering from autoimmune diseases, such as T1D or systemic lupus erythematosus (Soran et al., 2009).

In other autoimmune conditions such as rheumatoid arthritis, a decrease in paraoxonase activity compared to healthy subjects has also been reported (Tanimoto et al., 2003).

4.5.5 PON protein levels and activities in sepsis and inflammation

Other medical conditions related to sepsis and inflammation trigger changes in the PON enzyme family. An inverse PON pattern has been observed in patients with sepsis, and this pattern was found to be related to the resolution of their infection after receiving treatment. In this sense, PON1 and PON3 protein concentrations showed a decreasing trend during the first days of sepsis, while lactonase and paraoxonase further increased. With clinical improvement, PON1 and PON3 increased in these patients. This observation could be explained by the fact that during the acute-phase response, proinflammatory proteins, such as C-reactive protein, Serum Amyloid A protein, and ceruloplasmin, are carried by HDL, replacing ApoA-I and PON proteins on the surface of HDL. As a result of

this decay, these proteins turn HDL into a proinflammatory particle rather than an anti-inflammatory particle (Sans et al., 2012).

In another study carried out with patients with sepsis, lower paraoxonase and arylesterase activities were reported in these patients when compared to healthy subjects. Within the sepsis group, both of these activities were lower in the non-surviving patients than in the surviving patients, providing new insights into the prognosis of patients with sepsis (Li, Zhai, Li, Mei, & Qiu, 2013).

4.6 Factors modifying PON enzyme family

PON activities and protein concentrations show large inter-individual variability. PON1 protein concentrations can differ up to 13 times and its activity can differ up to 40 times (Rajkovic, Rumora, & Barisic, 2011). Several determinants of PON expression and activity have been described, particularly non-modifiable and modifiable factors.

4.6.1 Non-modifiable factors in PON system

The effect of age, gender and genetic background on the PON system has been broadly studied (Abelló et al., 2014; Seung et al., 2013; Soran et al., 2009).

a) Age: PON activities decline in the first stages of life and in elderly subjects due to low PON1 genetic expression in the liver at birth and the increase in oxidative status inherent to ageing.

b) Gender: female subjects show significantly higher PON activities due to hormonal factors, in particularly the inhibition of PON1 gene expression by male-pattern growth hormone, along with the stabilization of PON1 protein carried out by estrogens (Kim et al., 2013; Marsillach et al., 2015; Soran et al., 2009).

c) Genetic background: It is important to highlight the paramount importance of genetic variation of in PON in inter-individual variability, as it indeed influences its activity:

- PON1: The effects of the two major single-nucleotide polymorphisms (SNPs) present in the coding region of PON1 (L55M and Q192R) have

been extensively studied. There is complete agreement that 192R and 55L alleles increase PON1 paraoxonase and lactonase activities, while 192Q and 55M decrease such PON1 activities (Abelló et al., 2014). The mechanisms that explain these activity differences include the fact that in the 192RR genotype, PON1 protein is tightly associated with HDL and is thus active. In the192QQ and 192RQ phenotypes, serum PON1 is present as free PON1 protein, and is thus inactive, increasing the risk of CHD (Gugliucci & Menini, 2015; Lou-Bonafonte et al., 2015).

Apart from the polymorphisms located in the coding region of PON1, some prominent SNPs in the promoter region account for inter-individual differences in PON serum activity: C-107T, A-162G, and C-907G. In particular, -107C and -162A exhibit greater promoter activity than their respective variants. Contradictory results were obtained regarding enhanced activity in C-907G. Other SNPs in the promoter region, such as A-126G and A-824G, have not been found to modify PON1 activities (Schrader & Rimbach, 2011).

- PON2: The polymorphisms A148G and S311C are located in the coding region of thePON2 gene and have been found to be associated with variations in serum cholesterol and ApoA-I concentration in a genetically isolated Canadian population (Boright et al., 1998). Moreover, the SNP S311C affects CVR in Asian Indians (Sanghera, Aston, Saha, & Kamboh, 1998). Recent studies showed that these two SNPs affect their capacity to hydrolyze several lactones (Stoltz et al., 2009).

- PON3: PON3 is also a polymorphic gene, and its polymorphisms have been recently described. They are located in the promoter region, and their physiological role is still unclear (Marsillach et al., 2009).

Despite the effects of some of these polymorphisms on PON activities, characterization of these SNPs does not provide an accurate prediction of their plasmatic levels or activity, as all these SNPs account for only 20-25% of PON1 activity. This result suggests that environmental factors play an important role in PON activities and concentrations (Abelló et al., 2014; Kim et al., 2012). Moreover, several studies have failed to establish a link between PON gene polymorphisms and CVD, while PON protein and activities have been found to correlate with several CVR factors. In this

sense, the PON enzyme family may be a better biomarker for CVD risk than PON genotyping (Abelló et al., 2014; Kim et al., 2012; Robertson, Hawe, Miller, Talmud, & Humphries, 2003).

4.6.2 Modifiable factors in PON system

Several modifiable factors have been reported to influence PON expression and associated activities: alcohol consumption, smoking habit, and nutritional factors, among others.

a) Alcohol consumption: Moderate alcohol consumption (≈ 40 g/day) increases PON1 protein and activity, whereas chronic alcoholism (> 80 mg/day) produces a significant decrease in these parameters, irrespective of the type of beverage consumed (Kim et al., 2012).

b) Smoking habits: Cigarette smoking habit was found to negatively correlate with PON1 activity and expression in patients with CAD, although this effect can be rapidly reversed (James, Leviev, & Righetti, 2000).

c) Nutritional factors: An enhancement of PON1 expression and activities has been described both in animal and in human studies as a response to several nutritional factors, which mainly include dietary lipid intake, antioxidant vitamins and PC.

4.7 Nutritional and dietary determinants in PON enzyme family

4.7.1 Dietary lipid and cholesterol intake

Dietary cholesterol intake has been found to increase PON1 activity in animal (Rainwater et al., 2005) and human (Kim, Burt, Ranchalis, et al., 2012) studies, whereas the intake effects of FA on PON entirely depend on the type of FA consumed and on the way that it is consumed (Table 6).

The intake of raw fat rich in SFA resulted in an 18% increase in arylesterase activity in a postprandial state, while cooked fat rich in SFA decreased this activity by 27% in humans (Rajkovic et al., 2011; Sutherland et al., 1999).

The source of lipid intake has also been reported to affect PON activities, surely due to dissimilar FA content (Table 6). Paraoxonase activity increased in rats fed with virgin coconut oil (with 90.5% SFA, particularly

lauric and myristic acids) compared to rats fed with oils with lower SFA content: OO (with 24.8% SFA and rich in monounsaturated FA (MUFA), particularly oleic acid: 62.3%) and sunflower oil (with 32% SFA and rich in polyunsaturated FA (PUFA), particularly linoleic acid: 47.1%). This paraoxonase increase correlated with an increase in HDL-C levels and ApoA-I synthesis (Arunima & Rajamohan, 2013). When a diet rich in SFA was replaced by a diet rich in trans-unsaturated FA, PON1 paraoxonase activity was reduced by 6% in humans, regardless of gender (de Roos, Schouten, Scheek, van Tol, & Katan, 2002).

Oxidized lipid consumption decreases PON1 activity, whereas MUFAs have been reported to increase this activity by 6% in humans (Kim et al., 2012). In this sense, oleic acid (c18:1, ω-9) from OO has been reported to increase PON1 activity in mouse and human studies. This FA is known to increase HDL-C levels, together with PON1 paraoxonase activity, but only in subjects with the QR or RR PON1 genotype (Tomás et al., 2001). Moreover, oleic acid protects PON1 itself from oxidative inactivation by stabilizing this protein (Table 6) (Nguyen & Sok, 2003).

VOO consumption has been reported to be effective in increasing PON1 activity due to its low SFA content along with oleic acid enrichment of the PL present in HDL particles, which in turn favors PON1 activity in animals and humans (Lou-Bonafonte et al., 2015). In concordance, a VOO-rich diet increases the anti-inflammatory properties of HDL particles and ameliorates the age-related decrease in PON1 paraoxonase and arylesterase activities in humans by preventing oxidative stress-mediated inactivation of PON1 (Loued et al., 2013).

Table 6 | Dietary fat as a modulator of PON1 *in vivo.* Enhancement of PON1 expression and activities has been described both in animal and in human studies, as a response to several nutritional factors that mainly include dietary lipid intake. f, females; m, males; f/m, females and males. $↑, increase; ↓, decrease; ↔, no differences; #na, not analyzed; § (f), significant effects only in females. Source: adapted from Schrader *et al.,* 2011.

Dietary factor	Species	Gender	Health status	Dosage	Duration of treatment	PON1		
						mRNA	Protein	Activity
atherogenic diet	mice (C57BL/6)	f*	healthy	15.75 % fat, 1.25 % cholesterol, 0.5 % sodium-cholate	12 weeks	↑$	na#	↓$
	mice (LDLr-/-)	f	healthy	15.75 % fat, 1.25 % cholesterol, 0.5 % sodium-cholate	7 days	↔$	↓	↓
high-fat diet	rats (Wistar)	f/m	healthy	32 % fat (14 % SFA, 14.5 % MUFA, 3.4 % PUFA, 0.06 % cholesterol)	14 weeks	↔	↔	↓
high fat, hypercaloric diet	rats (Wistar)	f/m	healthy	55.2 % fat, 21602 kJ/g	14 weeks	↓(m)§	↔	↓
mediterra-nean diet	humans	m	healthy	1000 kcal, 45 % MUFA of total fat	single meal	na	na	↑
western-type diet	Mice (apoE-/-)	m	healthy	42 % fat, 0.15 % cholesterol	2 weeks	na	na	↓
	Mice (LDLr-/-)	m	healthy	42 % fat, 0.15 % cholesterol	2 weeks	na	na	↔
	humans	m	healthy	1000 kcal, 57 % SFA of total fat	single meal	na	na	↔
thermally stressed olive oil	humans	f/m	diabetes mellitus type 2	60 g oil in a milkshake	single meal	na	na	↑(f)
thermally stressed safflower oil	humans	f/m	diabetes mellitus type 2	60 g oil in a milkshake	single meal	na	na	↓(f)
ω3-PUFA-concentrate	humans	f/m	Familial combined hyper-cholesterolaemia	4 capsules/day, 1 capsule = 1g ω3-PUFA	8 weeks	na	↑	na

4.7.2 Phenolic compounds

PC, which are naturally present in fruit and vegetables, such as red fruits, black radishes, onions, coffee, cereals, spices, berries, broccoli, tomato, tea, citrus fruits, and OO (Pérez-Jiménez, Neveu, Vos, & Scalbert, 2010), modulate hepatic PON1 gene expression and hepatic and plasma activities in mice (Bayram et al., 2012; Loued et al., 2013; Rosenblat, Volkova, Coleman, Almagor, & Aviram, 2008) and humans (M Aviram et al., 2000; Rock et al., 2008).

For instance, pomegranate juice rich in flavonoids (tannins and anthocyanins) and phenolic acids (hydroxybenzoic acid) triggered an increase in arylesterase activity in mice (26-43%) (Kaplan et al., 2001) and in humans (18%) (M Aviram et al., 2000). Red wine flavonoids and stilbenes increased paraoxonase activity in hypercholesterolemic hamsters that were fed an atherogenic diet that was supplemented with two dealcoholized wines (Persimmon and Merlot) with different PCs contents and compositions (Suh et al., 2011).

Supplementation of OO with PC from other sources, especially flavonoids, has yielded promising results for the PON enzyme family. Intake of extra-VOO that was enriched or not enriched with green tea flavonoids (*i.e*., catechins, mainly epigallocatechin gallate) increased serum arylesterase activity by 10% and 6%, respectively, in ApoE-deficient mice. Impressively, the same study revealed that both extra-VOO and enriched-extra-VOO enhanced *in vitro* HDL-mediated ChE by 42% and 139%, respectively (Rosenblat et al., 2008).

Conflicting results about the effects of ingested PC isolated from a lipid matrix have been reported. In this regard, pure-HT administration to ApoE-deficient mice increased hepatic PON1 gene expression, although this increase was not accompanied by an increase in serum PON1 protein levels or arylesterase activity (Acín et al., 2006). Rats and mice fed with quercetin (a flavonoid broadly present in many fruits, vegetables, leaves, and grains) showed an increase in PON1 gene expression and activity. It has also been reported that supplementation of 320 mg/day for 24 weeks of the flavonoid anthocyanin increased HDL-C (11,39%), decreased LDL-C (9,72%), increased ChE (17,7%) and increased PON paraoxonase activity in hypercholesterolemic humans (Zhu et al., 2014).

PC from red wine, mainly quercetin and resveratrol, have also been shown to increase PON1 activity in *in vitro* experiments. Concordantly, it has been reported that there was a decrease in paraoxonase activity (by 32%) in hypercholesterolemic hamsters fed with an atherogenic diet compared with those fed with a standard diet. When the atherogenic diet was supplemented with two dealcoholized wines (Persimmon and Merlot) with different PC contents and compositions, the previously observed decrease in paraoxonase activity was reverted (Suh et al., 2011). Red wine is well-documented to be one of the most cardioprotective alcoholic beverages. However, this property is not associated with ethanol, but with the high content of antioxidant molecules, essentially flavonoids, in the wine. The search for alternative sources of natural flavonoids that do not have the deleterious effects of ethanol has resulted in several studies assessing the consumption of fruit juices, such as pomegranate juice (J Camps, Marsillach, & Joven, 2009).

The particular effects of olive oil phenolic compounds (OO-PC) have been reported to promote changes in PON1 paraoxonase activity *in vivo* and *in*

vitro, as recently reviewed by Lou-Bonafonte. In this sense, the intake of a common OO by rats with non-alcoholic fatty liver disease resulted in a decrease in hepatic PON1 paraoxonase activity. In contrast, an increase in this activity was observed in mice fed with a diet containing 10% phenol-rich OOs. The latter effect was attributed to the increase in hepatic PON1 mRNA and protein expressions induced by these PC (Lou-Bonafonte et al., 2015).

Flavonoids also help to control the extent of lipid peroxidation by chelating metal ions (*i.e.,* copper). However, this phenolic group makes a very small contribution to the total antioxidant capacity of VOO because of its low concentration (Rubió, Motilva, et al., 2012). In concordance, supplementation of OO with PC from other sources has yielded promising results on PON1 (Kim, Burt, Ranchalis, et al., 2012). In this respect, intake of extra-VOO enriched or not enriched with green tea PC (such as catechins, mainly epigallocatechin gallate epigallocatechin gallate) increased serum arylesterase activity by 10% and 6%, respectively, in ApoE-deficient mice. Impressively, both extra-VOO and enriched-extra-VOO enhanced HDL-mediated ChE *in vitro* by 42% and 139%, respectively (Rosenblat et al., 2008).

A detailed description of PC effects in PON1 is compiled in Tables 7 and 8.

Table 7 | PC and phenol-rich foods as modulators of PON1 *in vitro*. *↑ = increase; §na = not analyzed. Source: adapted from Schrader *et al.,* 2011.

Dietary factor	Cell line	Dosage	Duration of treatment	PON1			
				Promoter activation	mRNA	Protein	Activity
catechin	HuH7 hepatocytes	10 µmol/l	48 hours	↑*	↑	na§	↑
ellagic acid	HuH7 hepatocytes	17.5 – 70 µg GAE/ml	24 hours	na	↑	↑	↑
gallic acid	HuH7 hepatocytes	17.5 – 70 µg GAE/ml	24 hours	na	↑	↑	↑
isorhamnetin	HuH7 hepatocytes	25 µmol/l	48 hours	↑	na	na	na
naringenin	HuH7 hepatocytes	50 µmol/l	48 hours	↑	↑	↑	na
punicalagin	HuH7 hepatocytes	17.5 – 70 µg GAE/ml	24 hours	na	↑	↑	↑
quercetin	HuH7 hepatocytes	50 µmol/l	48 hours	na	↑	na	↑
	HuH7 hepatocytes	10 and 20 µmol/l	48 hours	na	↑	↑	↑
	HuH7 hepatocytes	25 µmol/l	48 hours	↑	na	na	na
resveratrol	HuH7 hepatocytes	10 µmol/l	48 hours	↑	↑	↑	↑
	human primary hepatocytes	10 µmol/l	48 hours	na	↑	na	na
	HC04 hepatocytes	2 - 20 µmol/l	24 hours	na	na	↑	↑
promegranate juice	HuH7 hepatocytes	17.5 – 70 µg GAE/ml	24 hours	na	↑	↑	↑

Table 8 | PC and phenol-rich foods as modulators of PON1 in vivo. **f; females; m, males; f/m, females and males. $↑ = increase; ↓= decrease; ↔ = no differences. #na = not analyzed. § ns = not specified. Source: adapted from Schrader* et al., *2011.*

Dietary factor	Species	Gender	Health status	Dosage	Duration of treatment	PON1		
						mRNA	Protein	Activity
quercetin	mice (C57BL/6)	f*	healthy	0.05 – 2 mg/g diet	6 weeks	↑$	↑	na#
	mice (LDLr-/-)	f/m	healthy	12.5/25 mg/dl liquid diet	4 and 8 weeks	↑	na	↑
	mice (apoE3/apoE4)	f	healthy	2 mg/g diet	6 weeks	↑	na	na
	mice (apoE-/-)	ns§	healthy	50 µg/day	6 weeks	na	na	↑
	rats (Wistar)	m	healthy	10 mg/l liquid diet	4 weeks	↑	na	↑
	humans	f/m	healthy	50 - 150 mg/d	2 weeks	na	na	=
catechin	mice (apoE-/-)	ns	healthy	0.5 ml/day	6 weeks	na	na	↑
resveratrol	mice (apoE-/-)	m	healthy	0.02%nd 0.06% of diet	20 weeks	na	na	↑
	mice (C57BL/6)	f	hyperhomo-cysteineaemic	0.001% in drinking water	4 weeks	↔$	na	↓$
	mice (C57BL/6)	f	hyperhomo-cysteineaemic	0.001% in drinking water + high methionine diet	4 weeks	↑	na	↓
blueberry powder	mice (apoE-/-)	f	healthy	1% of diet	20 weeks	na	na	↑
green tea	rats (Sprague Dawley)	m	healthy	2% green tea solution	3 and 6 weeks	na	na	↔
	rats (Sprague Dawley)	m	diabetic	2% green tea solution	3 weeks	na	na	↔
	rats (Sprague Dawley)	m	diabetic	2% green tea solution	6 weeks	na	na	↑
grape seed extract	rats (Sprague Dawley)	m	healthy	100 mg/kg/day	6 weeks	na	na	↔
	rats (Sprague Dawley)	m	diabetic	100 mg/kg/day	6 weeks	na	na	↑
pomegranate juice	mice (apoE-/-)	m	healthy	~ 31 µl/day	2 month	na	na	↑
	humans	m	healthy	50 ml/day	≤ 2 weeks	na	na	↑
	humans	f/m	diabetes mellitus type 2	50 ml/day	4 weeks	na	stabilization ↑, HDL-association ↑	↑
	humans	m	diabetes mellitus type 2	50 ml/day	4 weeks	na	stabilization ↑, HDL-association ↑	na
	humans	f/m	carotid artery stenosis	50 ml/day	1-3 years	na	na	↑
red wine	mice (apoE-/-)	ns	healthy	50 µg/day	6 weeks	na	na	↑
red wine polyphenolic extract	mice (C57BL/6)	ns	hyperhomo-cysteineaemic	no information	4 weeks	↑	na	↑
soy isoflavone mixture	rats	m	NASH	100 mg/kg/day	8 weeks	na	na	↑

5. HETEROGENEITY IN HDL FUNCTIONALITY

5.1 The concept of dysfunctional HDL

HDL is susceptible to undergoing dramatic modifications in structure and composition mediated by various mechanisms, including oxidation, glycation, homocysteinylation or enzymatic degradation, some of which occur as a consequence of genetic factors (Rosenson et al., 2016). These changes have been found to alter HDL biological activities and associated enzymes (Ferretti et al., 2006; Karathanasis et al., 2017).

In pathological conditions, qualitative and quantitative molecular changes occur within the HDL proteome and lipidome and are usually associated with a loss of normal physiological function and a gain of pathologic dysfunction, impairing the atheroprotective function of HDL (Eren et al., 2012; Karathanasis et al., 2017). Although these modifications lead to a "dysfunctional HDL" in most cases (Lüscher et al., 2014), such as in LCAT, CETP and PON deficiencies, a minority of modifications boost HDL functionality (Ferretti et al., 2006; Ossoli et al., 2015). That is the case of the ApoA-I_{Milano} mutation, a polymorphism that modifies ApoA-I structure, increasing its affinity for lipids and easy removal from cells, which confers a cardioprotective phenotype (Ansell et al., 2007).

Changes in the proteome to an inflammatory phenotype, for instance, are associated with a decrease in cellular ChE. Specifically, dysfunctional HDL results in an increased content of Serum Amyloid A protein, complement C3, and other inflammatory proteins. The pro-oxidant MPO mediates selective oxidation of HDL and ApoA-I in the vessel, activates nuclear factor NF-κB, and promotes arterial inflammation (Rosenson et al., 2016). Moreover, HDL and ApoA-I oxidation by MPO results in the selective inhibition of ABCA1-dependent ChE from macrophages (L. Zheng et al., 2004). Moreover, MPO oxidation weakens PON1 binding to HDL, and subsequently PON function also results impaired (Gugliucci & Menini, 2015). A loss of anti-inflammatory and antioxidant proteins, perhaps in combination with a gain in the aforementioned proinflammatory proteins, might be another important component in rendering HDL dysfunctional (Rosenson et al., 2016). Additionally, glycosylation of the HDL proteome can affect its function. That is the case for PON enzyme glycosylation,

which results in a decrease in paraoxonase and lactonase activities in patients with chronic liver impairment (Marsillach et al., 2010), T2DM (Shen et al., 2015), and T1D (Soran et al., 2009). Dysfunctional HDL are stranded within the intima, as observed in advanced-stage lesions. These retained HDL are, in turn, more susceptible to further detrimental modifications during inflammatory and oxidative stress and might eventually increase the intimal cholesterol burden because arterial tissue cannot catabolize HDL-C (Choi et al., 2016).

Considering the existence of dysfunctional HDL particles (Kontush & Chapman, 2006; Rosenson et al., 2016) stemming from their chemical and structural modifications, there is a growing scientific interest in the management of CVD using pharmacological agents that boost HDL functionality instead of raising HDL-C levels. Therapies aimed at only raising HDL-C may fail because they do not affect the structure of the HDL particles to such an extent to properly modify HDL functionality and consequently decrease CVD risk (Hafiane & Genest, 2015). Therefore, therapeutic strategies for preventing CVD should consider not only HDL quantity but also HDL quality. Some of these approaches include promoting elevations in HDL-P and potentially antiatherogenic function using ApoA-I-mimetic peptides, ApoA-I transcriptional regulators, and recombinant LCAT (J.C. Escolà-Gil et al., 2013; Kingwell et al., 2014; Mohamad Navab et al., 2006; Sethi, Amar, Shamburek, & Remaley, 2007; Sviridov et al., 2013).

5.2 HDL subpopulations functionality

In addition to the heterogeneity of dysfunctional HDL particles, the heterogeneity of "healthy" HDL has also been reported. Physicochemical heterogeneity of the HDL subclasses is intrinsically related to the heterogeneity of HDL functional quality (Karathanasis et al., 2017). In this sense, *in vivo* studies in humans and transgenic mice have suggested that HDL subclasses do not all protect against atherosclerosis equally well as subgroups of particles might hold distinct antiatherogenic benefits (Camont, Chapman, & Kontush, 2011; K.-A. Rye et al., 2009).

The assumption that HDL subpopulations are functionally distinct raises a question as to which subpopulations are more atheroprotective and are therefore better therapeutic targets to tackle CVD. Thus, recent investigations have been focused on elucidating the influence of HDL subpopulations on HDL functions.

5.2.1 Influence of HDL subpopulations on RCT and ChE

The efficacy of different HDL subpopulations in promoting ChE depends on the receptors that are involved:

a) Small HDL (lipid-free ApoA-I, lipid-poor ApoA-I, and pre-β particles) are the preferred acceptors of the cholesterol effluxed from cells that express ABCA1 in their membranes (Favari et al., 2015; Yokoyama, 2006). Indeed, plasma levels of pre-β HDL particles correlate with serum capacity to induce ABCA1-mediated ChE from J774 macrophages (Movva & Rader, 2008). ABCA1-mediated ChE also varies inversely with HDL subpopulation size (Anastasius et al., 2016).

b) Larger, spherical HDL particles are the preferred acceptors of the cholesterol released from cells via ABCG1 (N. Wang, Lan, Chen, Matsuura, & Tall, 2004) and via SR-BI (Jessup et al., 2006). SR-BI-mediated ChE to large HDL_2 is greater than that to small HDL_3 (Camont et al., 2011).

Small HDL particles play a key role in cellular ChE, which is consistent with the capacity of ABCA1 to account for the greater part of ChE from macrophages. The potent capacity of small HDL to enhance ChE can be attributed to low lipid content and high surface fluidity, which can induce conformational changes in ApoA-I, resulting in an increased capacity to acquire large amounts of PL and the enhancement of LCAT activity. In particular, the differences in these ChE properties could be mediated by HDL core and monolayer composition. On one hand, in HDL with a TG-rich core, ApoA-I is more loosely bound to the HDL surface, and consequently, this particle has less capacity to enhance ChE (Sparks, Davidson, Lundkatz, & Phillips, 1995). On the other hand, an HDL surface with a high cholesterol/PL ratio is also a characteristic related to lower CEC via the aqueous diffusion pathway, as the direction of net cholesterol mass transport is determined by the cholesterol concentration gradient (Phillips, Johnson, & Rothblat, 1987). In this sense, the Effect of Olive Oils

on Oxidative Damage in European Populations (EUROLIVE) study recently found that a decrease in the TG content of the HDL core was observed following an intervention with a phenol-rich OO, which was surely mediated by a decrease in CETP activity. Concordantly, an increase in ChE was observed after the EUROLIVE dietary intervention (Álvaro Hernáez et al., 2014) and after traditional Mediterranean diets enriched with VOO or nuts (Á Hernáez et al., 2017). Nevertheless, Yassine *et al.* found that high levels of TG in diabetes facilitated ChE via ABCA-1 in part via CETP activation as a compensatory mechanism (Yassine et al., 2015).

However, when comparing CEC of HDL subpopulations, it is essential to consider the HDL concentration basis employed for such comparison. Thus, on the basis of PL content, small, dense HDL more potently promote ChE (Camont et al., 2011; Kontush & Chapman, 2006; Sankaranarayanan et al., 2009), whereas on a particle number basis, large HDL (l-HDL) are more effective (Camont et al., 2011; Monette et al., 2016). In this sense, HDL efflux capacity is directly associated with l-HDL and medium HDL (m-HDL) particle concentrations and is inversely associated with small HDL (s-HDL) particle concentration in multivariable adjusted models. Moreover, HDL particle size is significantly associated with HDL efflux capacity. This evidence suggests that differences in HDL efflux capacity may be due to structural differences in HDL particles, as described in a cohort of asymptomatic older adults (Mutharasan et al., 2017).

5.2.2 Influence of HDL subpopulations on antioxidant defence

The ability of different HDL subpopulations to protect LDL from oxidative damage induced by one-electron oxidants, such as ROS, is not completely understood. While few investigators have found that the antioxidant capacity of small, dense HDL is impaired, at least in subjects with metabolic syndrome (Hansel et al., 2004; Kontush, Chantepie, & Chapman, 2003), the majority of authors have reported that small, dense, HDL particles act as potent protectors of LDL oxidation (Kontush & Chapman, 2010). Moreover, small, dense HDL_3 have been described as being more resistant to oxidative damage compared to large, light HDL_2. Finally, HDL_3 may be superior to HDL_2 in terms of their capacity to remove oxidized lipids from other lipoproteins and cellular membranes (Camont et al., 2011).

The underlying mechanisms for this heterogeneity in oxidation defence might be the diminished content of sphingomyelin, a structural lipid that increases monolayer rigidity and has negative impact on LCAT activity, and FC, which is equally capable of increasing PL monolayer rigidity, in small HDL. As a result of the increased fluidity in these small HDL, the incorporation of oxidized lipids in HDL is enhanced (Camont et al., 2011; Kontush & Chapman, 2010). The subsequent inactivation of oxidized lipids following their transfer to HDL occurs more rapidly in small, dense particles via different mechanisms. First, reduction of lipid hydroperoxides to hydroxides is more efficient in HDL_3 compared with HDL_2 because of the relative enrichment of small, dense HDL_3 in ApoA-I. Moreover, a distinct conformation of ApoA-I in HDL_3 might facilitate the redox reaction between the methionine residues of ApoA-I and lipid hydroperoxides. Second, hydrolysis of short-chain oxidized PL by HDL-associated hydrolytic enzymes, such as PON, PAF-AH, and LCAT, also appears to be enhanced in small, dense HDL_3 (Camont et al., 2011; K. Rye & Barter, 2014).

5.2.3 Influence of HDL subpopulations on anti-inflammatory properties

The results of *in vitro* studies have shown that HDL_3 inhibits both TNF-α-induced inflammation and VCAM-1 and ICAM-1 expression more effectively than HDL_2, which is surely due to their different PL composition rather than their Apo content (K. Rye & Barter, 2014). *In vivo* intravenous infusions of lipid-free ApoA-I inhibit the acute inflammation response (Nicholls et al., 2005). This injected lipid-free ApoA-I rapidly acquires PL and is incorporated into the endogenous HDL fraction as discoidal HDL, which inhibits endothelial inflammation *in vitro* (K. Rye & Barter, 2014).

The influence of HDL subpopulations on further HDL functions is summarized in Table 9.

Table 9 | Biological activities of HDL subpopulations. ABCA1, ATP-binding cassette A1; LCAT, lecithin cholesterol transferase; ABCG1, ATP-binding cassette G1; SR-BI, scavenger receptor class B type I; PL, phospholipids; LDL, low-density lipoprotein; LOOH, lipid hydroperoxides; SM, sphingomyelin; S1P, sphingosine-1-phosphate; TFPI, tissue factor pathway inhibitor; LPS, lipopolysaccharide; Hrp, haptoglobin-related protein; LBP, lipopolysaccharide binding protein. Source: adapted from Camont *et al.,* 2011.

Subpopulation	Feature	Role of HDL components
Cholesterol efflux		
Small, dense HDL	Potent efflux from lipid-loaded macrophages Potent efflux via ABCA1 Potent LCAT activation	Distinct ApoA-I conformation, reduced content of PL and other lipids, fluidity
Large, light HDL	Potent efflux via ABCG1 and SR-BI	Elevated content of PL
Antioxidative activity		
Small, dense HDL	Potent protection of LDL from oxidative damage Potent inactivation of LOOH and other oxidised PL	Depletion in SM, enrichment in ApoA-I and hydrolytic enzymes
Anti-inflammatory activity		
Small, dense HDL	Inhibition of endothelial cells adhesion molecules expression Immunomodulatory capacities towards neutrophils	Distinct ApoA-I conformation and composition of PL Factor H-related proteins
Cytoprotective activity		
Small, dense HDL	Potent protection of endothelial cells from oxidised LDL-induced apoptosis	Enrichment in ApoA-1 and S1P
Vasodilatory activity		
Large, light HDL	Attenuated production of thromboxane A_2	
Anti-thrombotic activity		
Small, dense HDL	Potent anticoagulant activity	Enrichment in TFPI and S1P
Large, light HDL	Inhibition of platelet aggregation	Enrichment in ApoE
Anti-infectious activity		
Dense HDL	Trypanosome lytic factor	Complex of apoL-1, Hrp and ApoA-II
Small, dense HDL	Potent integrin-mediated cell adhesion of neutrophils in response	Factor H-related proteins, ApoA-I, LBP

Taking into consideration all the data mentioned above, it seems safe to ensure that across the HDL subpopulation spectrum, small, dense, protein-rich HDLs display higher atheroprotective properties than large HDL. HDL heterogeneity in HDL functionality is intrinsically related to their diverse structure, where the proteome and lipidome of each subpopulation are the determinants (Camont et al., 2011; Salazar et al., 2015).

Born out of this heterogeneity in HDL quality, there is a growing interest in identifying the lipoprotein profile of individuals, *i.e.*, HDL subpopulation size and distribution. Emerging techniques, such as proton NMR spectroscopy, allow us to directly measure such lipoprotein profile (Anastasius et al., 2016; Eren et al., 2012).

6. LIPOPOPROTEIN PROFILE

6.1 Defining the concept and importance of lipoprotein profile

Lipoprotein profile is defined as the study of lipoprotein particle number, size, and subpopulations distribution, irrespective of the cholesterol content in these lipoproteins. In light of the functional heterogeneity of HDL subclasses, there is a growing need to evaluate the lipoprotein subclass distribution of individuals and to find, in some of these parameters, a predictive value in CVD. This result would allow the scientific society to determine new biomarkers that will better predict CVR and can be used to assess the clinical benefits of novel HDL-targeted therapies.

While the cholesterol content in LDL and HDL and the TG content in VLDL are used to approximate LDL, HDL, and VLDL circulating levels, respectively, these parameters do not fully represent the number of circulating lipoproteins, let alone the cardioprotective value attributed to each lipoprotein.

Regarding LDL, small, dense LDL particles have been more consistently related to high CVR than large LDL, irrespective of their cholesterol content. Patients with equal LDL-C values may present dissimilar CVR according to the size and density of their circulating LDL particles, as reported by several clinical studies (Cole et al., 2013; El Harchaoui et al., 2007; J. D. Otvos et al., 2006). Moreover, circulating IDL particle numbers were reported to be more associated with subclinical atherosclerosis in patients with systemic lupus erythematosus than the routinely measurement of lipid profile (Gonzalez et al., 2010).

HDL concentration can be quantified as the plasma concentration of cholesterol within this particle (HDL-C) or as the number of HDL-P in the blood circulation. Plasma concentrations of HDL-C, while being epidemiologically predictive of atherosclerotic CVD events in large populations, are not the proper parameter to measure HDL amount, let alone to capture the functional variation in HDL particles and the CVR associated with HDL (Eren et al., 2012). They are not the proper parameter because HDL-C is the cholesterol content within all of the α-HDL particles that exist in plasma; however, cholesterol accounts for only 15-20% of total weight of HDL particles, and the HDL-C concentration does account

for neither the HDL proteome nor the remaining lipidome. Furthermore, the HDL-C measurement makes no definitive statement about the total number of HDL-P, HDL size or the presence of pre-β particles. These pre-β HDL particles consist mainly of PL, ApoA-I, and little cholesterol, but no core of lipids, despite boasting high functionality. Thus, the measurement of HDL-C concentration is unlikely to reflect the amount of lipid-poor HDL and therefore does not reflect the functional quality of the whole pool of HDL particles (Komoda, 2014).

6.2 Proton NMR spectroscopy

A relatively new approach for the direct measurement of lipoprotein profile is to use proton NMR spectroscopy, which provides not only total particle counts of the major lipoprotein fractions (HDL, LDL, and VLDL) but also their mean size and subclass distribution (Jeyarajah, Cromwell, & Otvos, 2006; J. D. Otvos, 2002).

The use of proton NMR spectroscopy to measure plasma lipoprotein particle concentrations in an efficient manner was introduced in the early 1990s by researchers at North Carolina State University (J. D. Otvos, Jeyarajah, Bennett, & Krauss, 1992; J. Otvos, Jeyarajah, & Bennett, 1991). The method was further developed and refined to enable the simultaneous quantification of multiple lipoprotein subclasses (J. Otvos, 2000; J. Otvos, Jeyarajah, & Bennett, 1996). The test was commercialized for clinical research in 1997 by LipoScience, Inc. (Raleigh, North Carolina) and was then made available for patient care as the NMR LipoProfile test.

Currently, this up-to-date technology is increasingly used in clinical settings to monitor patients with moderate and high CVR. In fact, the relationships between NMR-measured LDL and HDL sizes and CHD risk have been found to be slightly higher than those obtained with PAGGE, another widely accepted method for measuring LDL and HDL particle size (Arsenault et al., 2010).

6.2.1 Principle of proton NMR spectroscopy

This NMR methodology takes advantage of the observation that the terminal lipid methyl group proton signal emitted by plasma lipoprotein

particles is modulated by lipoprotein size as a result of differences in local magnetic fields. That is, each lipoprotein subclass broadcasts a distinctive NMR signal whose amplitude can be accurately and reproducibly measured. The measured subclass signal amplitudes are directly proportional to the number of subclass particles emitting the signal, irrespective of variations in particle lipid composition. As a consequence, using the LipoProfile-3 algorithm, the average particle size and concentrations of VLDL, LDL, and HDL can be quantified. Subparticle concentrations can also be determined for three VLDL subclasses (large or chylomicrons, medium, and small), three LDL subclasses (IDL, large, and small), and three HDL subclasses (large, medium, and small), as detailed in Table 10 (Movva & Rader, 2008; Rosenson et al., 2011). Unlike other methods for analyzing HDL particles, NMR spectroscopy does not require a physical separation step because the protons within lipoprotein particles of different sizes have a natural magnetic distinctness arising from their unique physical structure (Rosenson et al., 2011).

Table 10 | Estimated diameter ranges of lipoprotein subclasses measured by proton NMR spectroscopy. Source: adapted from Jeyarajah *et al.,* 2006.

NMR lipoportein parameter	Diameter range (nm)
VLDL	
Large VLDL/chylomicrons	>60
Medium VLDL	35-60
Small VLDL	27-35
LDL	
IDL	23-27
Large LDL	21,2-23
Small LDL	18-21,2
Medium small LDL	19,8-21,2
Very small LDL	18-19,8
HDL	
Large HDL	8,8-13
Medium HDL	8,2-8,8
Small HDL	7,3-8,2

Many signals appear in the spectrum from numerous molecules, but only the signal observed at approximately 0.8 parts per million (ppm) is used for lipoprotein particle quantification. The subclass NMR signals highly overlap, making it necessary to computationally deconvolute the NMR signal envelope (0.7–0.9 ppm) to extract the amplitudes of the subclass signals that are used to calculate subclass concentrations (Rosenson et al., 2011). An example of a plasma NMR spectrum and its corresponding deconvolution is shown in Figure 15.

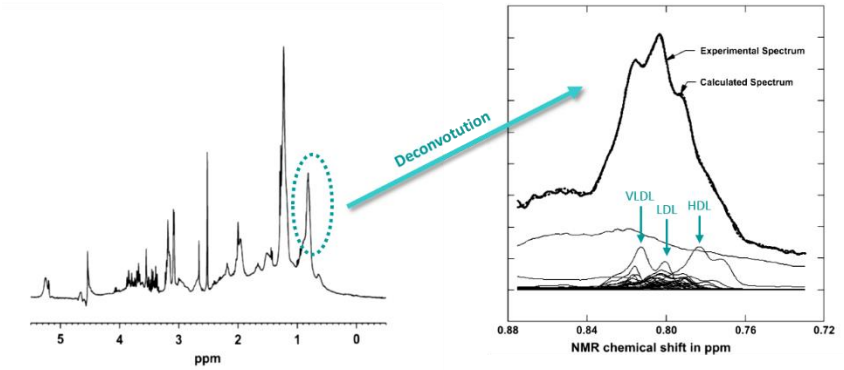

Figure 15. | Proton NMR spectrum of blood plasma and its corresponding deconvolution. A typical proton NMR spectrum of human blood plasma recorded at 47ºC on a 400-MHz NMR clinical analyzer. The signal envelope centered at approximately 0.8 ppm arises from the terminal lipid methyl group proton signals of the lipoproteins. Deconvolution of such methyl signal (0.7–0.9 ppm) results in VLDL, LDL (including IDL) and HDL signals differentiation. Source: adapted from Jeyarajah et al., 2006.

6.2.2 Insulin resistance index

In addition to lipoprotein profile, the NMR technique provides a lipoprotein particle-derived measure of insulin resistance (LP-IR), resulting from an algorithm that combines six lipoprotein parameters to produce the LP-IR index: HDL, LDL, and VLDL size, together with large VLDL, small LDL, and l-HDL particle number. This score ranges from 0 (most insulin sensitive) to 100 (most insulin resistant).

LP-IR has been reported as being directly related to the Homeostatic Model Assessment (HOMA) index and inversely related to the Glucose Disposal Rate, the gold standard for assessing insulin sensitivity, and it has

been proposed as a simple method for assessing the risk to develop a prediabetic or diabetic state (Shalaurova, Connelly, Garvey, & Otvos, 2014).

6.3 The impact of VLDL particle number and size on CVD

Dyslipidemia in individuals with T2DM is characterized not only by high TG concentrations and low HDL-C but also by decreases in LDL and HDL particle size and an increase in VLDL particle size (Garvey et al., 2003). In these individuals, VLDL particle size is inversely associated with GRD and directly associated with incident hypertension (Garvey et al., 2003; Paynter, Sesso, Conen, Otvos, & Mora, 2011). Moreover, patients with T1D present a lipoprotein profile characterized by lower small LDL, medium VLDL and large VLDL TG compared to healthy subjects (Brugnara et al., 2015).

6.4 The impact of LDL particle number and size on CVD

In several clinical studies, the measurement of total LDL particle number (LDL-P) by NMR was found to be more effective as a positive CVR factor than measurements of the cholesterol content of the LDL (LDL-C) or ApoB-100 concentrations (Cole et al., 2013). This was found to be the case in the prospective case-control studies Veterans Affairs HDL Intervention Trial (VA-HIT) (J. D. Otvos et al., 2006) and the European Prospective Investigation into Cancer and Nutrition (EPIC)-Norfolk study (El Harchaoui et al., 2007).

Small, dense LDL particles have been consistently related to high CVR. The existence of small, dense LDL particles is associated with the depletion of CE and TG enrichment of LDL particles, together with an increased density of these particles. These properties reduce LDL affinity for its receptor (Bhakdi et al., 1995). As a consequence, small, dense LDL have an increased residence time in the circulation, making them more susceptible to oxidation or glycosylation, which considerably increases their atherogenic potential (Lyons, 1993).

6.5 The impact of HDL particle number and size on CVD

Results from large observational studies, such as the Multi-Ethnic Study of Atherosclerosis (MESA) (MacKey et al., 2012), Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin (Mora et al., 2013), and Heart Protection Study (Parish et al., 2012), showed that measurement of HDL-P appeared to be better than HDL-C as a CVR marker. The same association was found in case-control studies, such as the VA-HIT (J. D. Otvos et al., 2006) and EPIC-Norfolk (Kontush, 2015) studies, and in The Multiple Risk Factor Intervention Trial, a primary prevention study (Kuller, Grandits, Cohen, Neaton, & Ronald, 2007). Furthermore, in randomized controlled trials, treatment with CETP inhibitors or niacin were found to have little effect on HDL-P number, even if HDL-C was substantially increased, thus resulting in increased cholesterol-overloaded HDL-P (Rader & Tall, 2012; Rashedi, Brennan, Kastelein, Nissen, & Nicholls, 2011).

Large studies note that the size of the HDL particle is a key determinant of its capacity to promote cellular ChE (Mutharasan et al., 2017). Although small HDL particles are considered to have greater efficiency in promoting ChE compared to larger, more mature spherical HDL particles, it would be of interest to examine how well HDL particle size distribution predicts CVD (Anastasius et al., 2016).

It is noteworthy that within the MESA study, the LDL-P/HDL-P ratio seemed to have the strongest independent association with CHD, with significant net reclassification improvements in the American College of Cardiology/American Heart Association (AHA/ACC) CHD risk scores (Qi et al., 2015; Steffen et al., 2015).

6.6 Cholesterol-overloaded HDL as a new cardiovascular biomarker

Each HDL particle is estimated to contain an average of 46 cholesterol molecules in healthy subjects. However, it has been described that the HDL-C/HDL-P ratio, which reflects the cholesterol molecules per HDL particle, can increase in atherosclerotic patients, resulting in the presence of cholesterol-overloaded HDL (Qi et al., 2015). The existence of cholesterol-overloaded particles is currently considered a potential new measure of HDL function, as they have been shown to be directly related

to atherosclerosis progression in a CVD-free population (Qi et al., 2015; Remaley, 2015; Zhao, 2015).

These particles appear to be harmful, as experimental studies have reported that cholesterol-overloaded HDL-P exert a negative impact on the cardioprotective function of HDL. The potential mechanisms of such effect, depicted in Figure 16, are as follows:

1. Given that the exchange of cholesterol between large, dense HDL and peripheral cells is bidirectional (via SR-BI), cholesterol-overloaded HDL-P may become cholesterol donors instead of acceptors (Huang et al., 2014; Lee et al., 2012; Lüscher et al., 2014; Remaley, 2015; Rosenson et al., 2013). Additionally, cholesterol-overloaded HDL-P have also been shown to be ineffective in SR-BI–mediated cholesterol uptake in the liver and filtration and clearance in the kidney. Therefore, all the cholesterol clearance results impaired, leading to cholesterol-overloaded HDL-P formation (Albers, Vuletic, & Cheung, 2012; Vergeer, Holleboom, Kastelein, & Kuivenhoven, 2010).

2. Large, less dense HDL have less oxidative capacity than smaller, denser HDL. Moreover, these particles are more prone to be oxidized by MPO (Huang et al., 2014; Qi et al., 2015).

3. HDL may become proinflammatory as they become dysfunctional since they are easily oxidized by MPO. As a result, cholesterol-overloaded HDL cannot inhibit the expression of adhesion molecules such as VCAM-1 and ICAM-1, enhancing monocyte adhesion and recruitment in the endothelial layer (Qi et al., 2015; Remaley, 2015).

4. Once oxidized, cholesterol-overloaded HDL-P are not recognized by endothelial SR-BI and therefore, the PI3K/Akt pathway, which is involved in the anti-inflammatory response, is not activated (Qi et al., 2015).

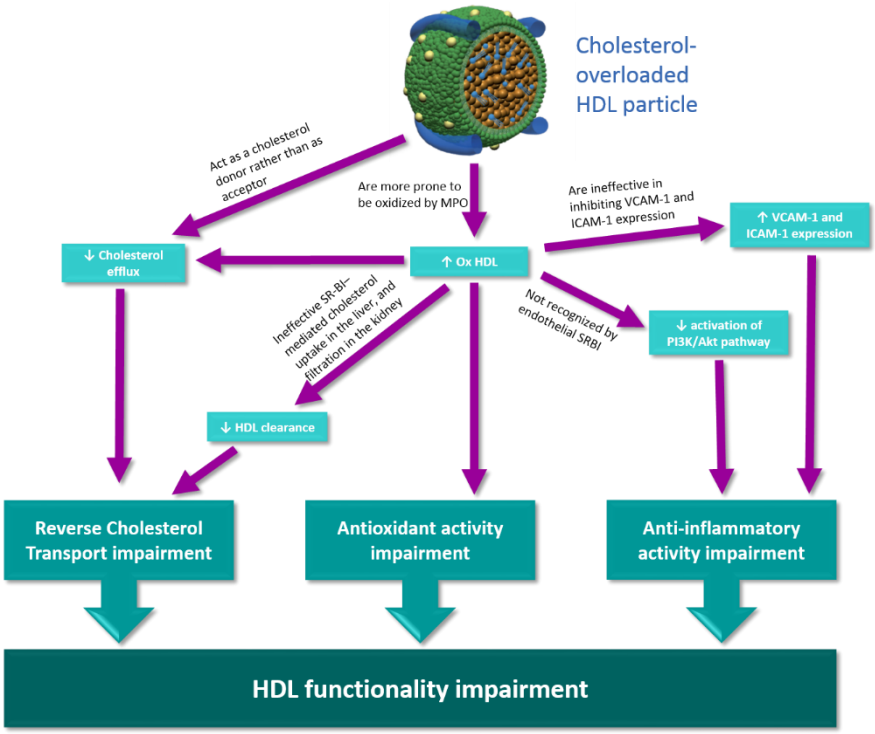

Figure 16 | Likely mechanisms that may explain the HDL functionality impairment of cholesterol-overloaded HDL-P. Firstly, RCT is impaired since cholesterol-overloaded HDL-P become cholesterol donors instead of acceptors as a consequence of their high content of cholesterol. Additionally, these HDL-P are not recognized by hepatic SR-BI, and therefore HDL clearance is diminished. Secondly, cholesterol-overloaded HDL-P are more prone to be oxidized by MPO generating dysfunctional particles with diminished antioxidant and anti-inflammatory activities. As a result, the whole HDL functionality is compromised. Source: created by the author.

7. OLIVE OIL

7.1 Mediterranean diet

The Mediterranean diet was first defined by Ancel Keys as the dietary pattern observed in Greece and Southern Italy during the 1960s; it is characterized by being low in saturated fat and high in vegetable oils. In this dietary pattern, VOO is the main source of fat consumption, assuring optimal MUFA and PUFA intake while limiting SFA intake (Martín-Peláez, Covas, Fitó, Kušar, & Pravst, 2013).

The Mediterranean diet has been broadly claimed as being partly responsible for the low incidence of CVD registered in the Mediterranean area, despite the high prevalence of the typical CVR factors (Bloomfield et al., 2015; J. López-Miranda et al., 2010; Lou-Bonafonte et al., 2015; Pauwels, 2011; Visioli et al., 2005). In this sense, the *Prevención con Dieta Mediterránea* (PREDIMED) study, the first randomized clinical trial designed to assess the beneficial effects of the Mediterranean diet on the primary prevention of CVD, has demonstrated that the Mediterranean diet supplemented with extra-VOO or nuts decreases the incidence of major CVD events in subjects with high CVR (Damasceno et al., 2013).

7.2 Major components of OO: MUFA

The major components of OO are FA, of which MUFA represents 55 to 83% of the total FA, with PUFA from 4 to 20% and SFA from 8 to 14% (María Isabel Covas et al., 2015).

The MUFA oleic acid (c18:1 ω9) has been traditionally proposed as the main factor that is responsible for the antiatherogenic benefits attributed to the Mediterranean diet, as it accounts for approximately 70-80% of the MUFA content in VOO (María Isabel Covas, 2007; Tomás et al., 2001). In fact, in November 2004, the Food and Drug Administration of the U.S.A endorsed the following qualified health claim:

> *"Scientific evidence suggests that eating about two tablespoons (23 grams) of OO daily may reduce the risk of CHD due to the monounsaturated fat in OO. To achieve this possible benefit, OO is to*

replace a similar amount of saturated fat and not increase the total number of calories you eat in a day" (US Food and Drug Administration, 2004).

Nevertheless, OO is more than a MUFA fat. It is considered a functional food that contains high levels of not only MUFA but also other minor components with biological properties (M I Covas, De la Torre, Kafatos, Lamuela-Raventos, & Osada, 2006). In fact, oleic acid is one of the predominant FA in foods of animal origin that are widely consumed in Western diets, such as poultry and pork. However, lesser cardioprotective benefits have been observed with the Western dietary pattern than those observed with OO consumption (María Isabel Covas, 2007). Thus, it is plausible that a high oleic acid intake is not the sole primary responsible agent for the healthy properties of OO.

7.3 Minor components of virgin OO: PC

VOO minor components are comprised of PCs, which represent 1-2% of the total content of OO. PC, also known as polyphenols, are naturally occurring non-nutritive plant compounds, that are synthesized as secondary metabolites of plants in response to adverse environmental factors, mainly fungal infections and extensive ultraviolet radiation. PC are abundant in the human diet, particularly in fruits, vegetables and plant-derived foods, and they are an important feature of the Mediterranean diet pattern (Spencer, Abd El Mohsen, Minihane, & Mathers, 2008).

These components are removed during the refining process of OO. As a consequence, these compounds are specific to VOO. Even though PC account for a small amount of OO weight, they have been shown to hold cardioprotective benefits (María Isabel Covas, 2007; Fitó et al., 2004; J. López-Miranda et al., 2010; Lou-Bonafonte et al., 2015).

7.3.1 PC classification

The structure of PC is characterized by the presence of at least an aromatic ring carrying one or more hydroxyl groups. Different classes of PC can be considered according to the number of phenol rings and the structural

elements that bind these rings (Figure 17) (Andrés-Lacueva et al., 2009; Manach C, Scalbert A, Morand C, 2004; Spencer et al., 2008):

a) Phenolic acids: phenolic acids are secondary aromatic plant metabolites that are present in a wide range of plants, and they have been associated with the color and sensory qualities of foods. This PC group is characterized by having only one benzene ring. VOO have been reported to contain vanillic, caffeic, protocatechuic, sinapic, *p*-hydroxybenzoic, and gallic acids; ferulic and cinnamic acids have also been quantified, but in lesser quantities (less than 1 mg analyte/kg VOO).

b) Flavonoids: this is the largest and most diversified group of PC with a basic structure of two benzene rings joined by a heterocyclic ring. Luteolin, apigenin, and their glycoside forms are present in VOO.

c) Lignans: this group encompasses (+)-pinoresinol and (+)-1-acetoxy-pinoresinol, which are both present in VOO.

d) Phenolic alcohols or simple phenols: these are compounds with a hydroxyl group attached to an aromatic hydrocarbon group. The main phenolic alcohols in VOO are 3,4-dihydroxyphenyl ethanol (also known as 3,4-DHPEA or HT) and *p*-hydroxyphenyl ethanol (also known as *p*-HPEA or tyrosol). They are also the most important physiological compounds when VOO is ingested, as they are released in large amounts after the hydrolysis of secoiridoids in the stomach and small intestine.

e) Secoiridoids: this PC group is the main PC present in VOO, and secoiridoids are characterized by the presence of either elenolic acid or elenolic acid derivatives in their molecular structure. Secoiridoids comprise ligstroside derivatives and oleuropein derivatives, such as oleuropein aglycone, also known as 3,4-DHPEA-EDA, the latter being the main PC found in VOO. They are the primary cause of the particular bitter organoleptic attribute and oxidative stability of the VOO (Andrewes, Busch, De Joode, Groenewegen, & Alexandre, 2003; Busch, Hrncirik, Bulukin, Boucon, & Mascini, 2006; Gutiérrez-Rosales, Ríos, & Gómez-Rey, 2003).

Once absorbed, secoiridoids undergo extensive first-pass intestinal/hepatic metabolism in the body, resulting in a significant increase of free HT entering the small intestine (Rubió, Macià, et al., 2014). Therefore, HT becomes the major PC absorbed from the intestinal tract,

and it is further subjected to an extensive metabolism, both in the intestinal epithelium and the liver, leading to the formation of phase II metabolites, mainly sulfate, methyl, and methyl–sulfate conjugates and glucuronides of HT (Catalán et al., 2015).

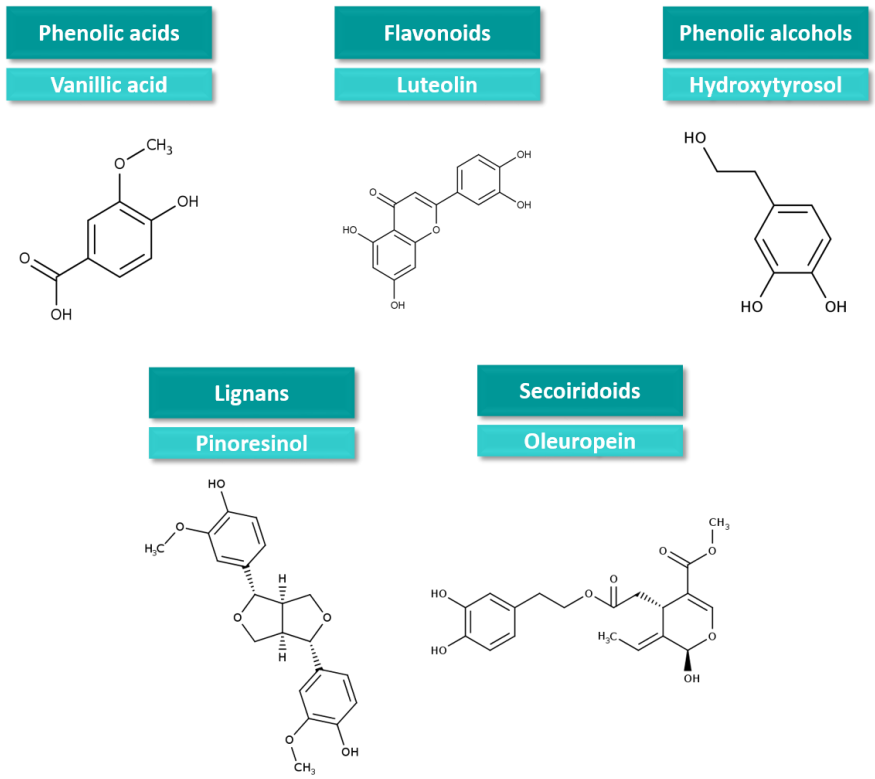

Figure 17 | Major PC present in VOO. Source: adapted from Phenol Explorer version 3.6 and Neveu et al., 2010.

7.3.2 Limited PC content in VOO

The beneficial effects of OO-PC have been extensively reported (M. I. Covas, 2008; Fitó et al., 2004; Martín-Peláez et al., 2013). Born out of this evidence, in November 2011, the EFSA released a health claim concerning the beneficial properties of OO-PC consumption on heart disease risk

factors: protection of LDL particles from oxidative damage, maintenance of normal HDL-C levels, maintenance of normal blood pressure, and anti-inflammatory properties, among others. The EFSA Panel considers that in order to support the claim, 5 mg of HT and its derivatives should be consumed daily (EFSA Panel on Dietetic Products. Nutrition and allergies & (NDA)., 2011).

The phenolic content in VOO is influenced by multiple agronomic and environmental factors, such as the cultivar, climate, and ripeness of the olives at harvesting, along with technologic aspects of oil preparation. As a result, it is difficult to ensure a fixed intake of PC through VOO consumption. Furthermore, the typical consumption of OO in the Mediterranean diet is 25 mL/day, which represents a PC consumption of 3-15 mg of total phenols/day. Thus, the phenolic concentration in most VOOs available on the market is too low to allow the consumption of 5 mg of HT and its derivatives within the context of a balanced diet (Rubió, Valls, et al., 2012). Moreover, OO cannot be consumed in large quantities, despite being recognized as a healthy food.

For these reasons, a good approach to ensure the optimal intake of PC in the context of a balanced diet is to enrich VOO with their own PC (Suárez, Romero, Ramo, Macià, & Motilva, 2009). This strategy allows increasing VOO health-promoting properties while consuming the same amount of fat.

7.4 OO and PC beneficial effects on CVD

7.4.1 OO and VOO cardioprotective effects

OO consumption has been inversely associated with CHD mortality in the EPIC study (Bendinelli et al., 2011; Buckland, Mayén, et al., 2012; Buckland, Travier, et al., 2012) and with stroke risk in women in the Three-City Study (Samieri et al., 2011). The PREDIMED study reported that VOO consumption within the framework of the Mediterranean diet reduces atrial fibrillation risk (Martínez-González et al., 2014). This study also reported that an unrestricted-energy Mediterranean diet supplemented with extra VOO or nuts reduced the incidence of major CVD outcomes, with a relative risk reduction of approximately 30% and reduced CVD mortality in people at high CVR (Estruch et al., 2013).

7.4.2 Phenol-rich foods cardioprotective effects

In experimental and human studies, phenol-rich foods have been shown to improve the lipid CVR profile, as recently reviewed (Loffredo, Perri, Nocella, & Violi, 2017). Plant extracts, such as those from red yeast rice, sugar cane-derived policosanols, and artichoke leaf, have been reported to have an LDL-C lowering effect in subjects with moderate hyperlipidemia (Ogier et al., 2013). PC from green tea and herbs have also been shown to have hypocholesterolemic effects, lowering LDL-C in human (Larsson, 2014) and animal models (Bravo et al., 2014). Thyme extracts, rich in flavonoids, decrease total cholesterol and LDL-C and increase HDL-C in animal models (Alamgeer et al., 2014) as well as reduce the susceptibility to *in vitro* LDL oxidation (Kulisić, Krisko, Dragović-Uzelac, Milos, & Pifat, 2007). Cocoa flavonols also increase HDL-C in humans (Mursu et al., 2004; Sarriá et al., 2015). PC from red wine have been shown to protect HDL and LDL from oxidation *in vitro* (Rifici, Schneider, & Khachadurian, 2002).

Among all the phenol-rich food items, OO in particular has received attention from several researchers, since its major components (such as MUFA) and minor components (mainly PC) have been shown to have cardioprotective benefits.

7.4.3 OO-PC cardioprotective effects

Particular effects of OO-PC on CVD have been reported (M. I. Covas, 2008; Fitó et al., 2004; Martín-Peláez et al., 2013). These phenols boast pleiotropic biological activities, being the anti-inflammatory and antioxidant the most consistently associated with a decreased risk of cancer, CVD and other chronic diseases (Carluccio et al., 2003; Cicerale, Lucas, & Keast, 2010; J. López-Miranda et al., 2010; Medina-Remón et al., 2017; Parkinson & Cicerale, 2016; J. Zheng et al., 2017). Despite the fact that PC represent the most abundant antioxidants in human diets (Manach C, Scalbert A, Morand C, 2004), these components exert protective effects through the antioxidant scavenging of free radicals and by modulating signal transduction, cell signaling, gene expression and cellular communications in various pathways (Farràs et al., 2013; Kang, Shin, Lee, & Lee, 2011). OO-PC ameliorate lipid profile and endothelial function, modify hemostasis, and have antithrombotic properties in

humans (M. Covas et al., 2006; Perez-Jimenez et al., 2005; Ruano et al., 2005; Rosa Solà et al., 2015; Valls et al., 2015). Data from human studies have shown that OO-PCs are protective against CVR factors, particularly in individuals with an imbalance in the oxidative status (M I Covas et al., 2006; J. López-Miranda et al., 2010; Perez-Jimenez et al., 2005).

In the EUROLIVE study, the sustained consumption of phenol-rich OO was shown to increase HDL-C, reduce TC/HDL-C and LDL-C/HDL-C ratios, decrease *in vivo* lipid oxidative markers, such as oxLDL (María Isabel Covas et al., 2006; Gómez et al., 2014), and increase HDL-mediated ChE from macrophages (Álvaro Hernáez et al., 2014). These cardioprotective effects turn out to have a direct relationship with the PC content of the administered OO. Supporting these data, a functional OO enriched with its PC has also been shown to increase the expression of ChE-related genes (Farràs et al., 2013).

7.5 OO beneficial effects on HDL function

7.5.1 Anti-inflammatory HDL function

VOO consumption has been shown to enhance HDL capacity to reduce ICAM-1 expression and therefore reduce monocyte adhesion to endothelial cells (Loued et al., 2013). It has also been reported that different OO-PC and VOO consumed within the framework of the Mediterranean diet can reduce acute-phase proteins in HDL, promoting a less proinflammatory lipoprotein status (Pedret et al., 2015).

7.5.2 HDL-mediated ChE

The consumption of different OO-PC has been reported to enhance HDL-mediated ChE in humans (Berrougui, Cloutier, Isabelle, & Khalil, 2006; Lim, Mat Junit, Abdulla, & Abdul Aziz, 2013; Uto-Kondo et al., 2010). For instance, in a 47-male subsample of the EUROLIVE study, the consumption of 25 mL of VOO for 3 weeks increased CEC. Other improvements were observed after this sustained intervention:

a) Modification of lipoprotein profile: an increase in HDL_2 levels at the expense of HDL_3 level reduction was observed

b) Increase in monolayer fluidity

c) Modification of HDL composition (decrease in TG levels in HDL core)

d) Incorporation of OO-PC biological metabolites into HDL, which could confer HDL a better antioxidant protection (Álvaro Hernáez et al., 2014).

7.5.3 Antioxidant HDL function

Concerning antioxidant HDL function, it has been demonstrated that the consumption of OO-PC was associated with a decrease in LDL oxidation status in a dose-dependent way (M. Covas et al., 2006), which is surely due to HDL antioxidant capacities. An enhancement of this antioxidant activity has been reported in ApoE-deficient mice with spontaneous atherosclerosis development after the consumption of a VOO-rich diet (Arbonés-Mainar et al., 2007).

The main proteins involved in the antioxidant function of HDL are ApoA-I and PON1. Some VOO interventions in humans have increased PON1 activity (Cherki et al., 2005) and ApoA-I concentrations (Derouiche et al., 2005; José López-Miranda et al., 2000).

8. FUNCTIONAL FOODS

8.1 Defining the concept of functional food

The definition of functional food has been changing over the years. This concept was first proposed in Japan in 1984 by scientists who were studying the relationships between nutrition, sensory satisfaction, fortification, and the modulation of physiological systems (Hosoya, 1998).

In the latter half of the 1990s, the European Commission established a science-based approach to explore the concept of functional foods. This Concerted Action, the "Functional Food Science in Europe" (FUFOSE), involved a large number of European experts in nutrition and related sciences, who produced a consensus report that has become widely used as a basis for discussion and further evolution of thinking on the topic (Diplock et al., 1998). As a result of the FUFOSE action, 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."*

FUFOSE also established that functional foods must remain foods and must demonstrate their effects when consumed on a daily basis and in quantities that can normally be expected.

In 2006, the European Union limited the use of nutrition and health claims by establishing the need for a robust substantiation of the evidence of the claimed effect (Regulation EC No. 1924/2006 on nutrition and health claims made on foods).

Currently, there is a broad range of possibilities to define the concept of functional foods. A functional food can be:

a) an unmodified natural food;

b) a food in which a component has been enhanced through special growing conditions, breeding or biotechnological means;

c) a food in which a component has been added to provide benefits;

d) a food in which a component has been removed by technological or biotechnological means so that the food produces beneficial benefits;

e) a food in which a component has been replaced by an alternative component with favorable properties;

f) a food in which a component has been modified by enzymatic, chemical or technological means to provide a benefit;

g) a food in which the bioavailability of a component has been modified;

h) a combination of any of the above situations.

Despite the variety of definitions, the main purpose of a functional food must be to improve human health and well-being. Moreover, the development of functional foods must be tested in human clinical intervention trials with an appropriate design (British Nutrition Foundation, 2017).

8.2 Phenol-enriched functional OOs

In 2011, The EFSA released a health claim concerning the beneficial properties of OO-PC consumption. The EFSA Panel considered that 5 mg of HT and its derivatives in OO should be consumed daily to support the claim (EFSA Panel on Dietetic Products. Nutrition and allergies & (NDA)., 2011). The phenolic concentration in most VOOs available on the market is too low to allow the consumption of 5 mg of HT and its derivatives within the context of a balanced diet (Rubió, Valls, et al., 2012).

For these reasons, a good approach to ensure the optimal intake of PC in the context of a balanced diet may be the enrichment of VOO with its own PC. That allows increasing VOO health-promoting properties while the same amount of fat is consumed (Suárez et al., 2009). However, high

concentrations of OO-PC result in a bitter and pungent taste due to the presence of secoiridoids (HT and its derivatives), which could lead to rejection by consumers, particularly those from non-Mediterranean countries (Rubió, Motilva, et al., 2012). Moreover, PC-enriched foods could have a dual action since antioxidants can also act as oxidants. The administration of high doses of a single type of antioxidant could even promote, rather than reduce, lipid peroxidation, and it has been shown to increase atherosclerotic areas in animal models (Acín et al., 2006). In contrast, the combination of different antioxidants has been demonstrated to be effective in reducing atherosclerosis in human trials (Salonen et al., 2000). Moreover, in terms of biological effects, synergistic effects with stronger improvements have been reported when different PCs are combined in comparison to single treatments (Herrmann & Wink, 2011).

A suitable approach might be the development of functional OO enriched with complementary PC, according to their antioxidant mechanism suppressing the oxidative stress by chelating and scavenging components. The PC present in VOO, specially secoiridoid derivatives of HT, act in similar manner as phenolic acids, inhibiting lipid oxidation by trapping free and peroxy radicals and chelating metal ions.

8.3 Flavored OOs

The enrichment of a VOO by complementing its own PC with PC from aromatic herbs has been considered by several authors to improve the nutritional profile and organoleptic characteristics of OO.

Spices and various herbs, including thyme and rosemary, are widely used as flavor enhancers of OO. Earlier studies on herbs and spices have considered their bioactive compounds from the perspective of antioxidants and anti-inflammatories, where one of the most important isolated phytochemicals includes flavonoids (luteolin, thymusin, and xanthomicrol, among others). Thyme (*Thymus zyguis*) could enhance the benefits of a phenol-enriched VOO because it is one of the richest sources of flavonoids (such as naringenin, eriodictyol, thymusin, xanthomicrol, and 7-methylsudachitin) and phenolic acids (such as rosmarinic acid, ferulic acid, and caffeic acid) (Rubió, Motilva, et al., 2012).

Consequently, the enrichment of VOOs with OO-PC and complementary PC from aromatic herbs, such as thyme, might be a good strategy to provide an optimum balance among the different kinds of flavonoids, simple phenols, monoterpenes, and phenolic acids, and therefore this strategy might enhance the beneficial properties attributed to common VOO on CVD.

Hypothesis & Objectives

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS
Sara Fernández Castillejo

Hypothesis

The primary hypothesis of the present thesis is that the sustained intake of functional VOOs enriched with their own PC, predominantly secoiridoids, such as HT and its derivatives, or with their own PC plus additional complementary PC from thyme, such as flavonoids, may modify the physicochemical properties of HDL particles towards a cardioprotective mode and may promote changes in HDL subclass distribution, leading to the consequent enhancement of HDL functionality in hypercholesterolemic subjects.

The secondary hypothesis is that several HDL physicochemical properties, such as composition, fluidity, oxidative status, and size, might be the main determinants of the capacity of HDL to promote ChE from cells.

Combining PC from different classes and sources might allow increasing phenol consumption to optimize the likely effects observed with the consumption of a single type of PC. The intake of phenol-enriched VOOs may ensure the optimal intake of PC to obtain the known VOO health-promoting properties in the context of a balanced diet, while consuming the same amount of fat.

Objectives

The primary objective of the present thesis is to assess whether the sustained intake of phenol-enriched VOOs could enhance HDL functionality in hypercholesterolemic subjects by modifying the following:

1. HDL metabolism and maturation and therefore subpopulations distribution (Study 1: Fernández-Castillejo *et al*., 2016),
2. antioxidant content (*i.e.*, fat-soluble antioxidants and phenolic metabolites) (Study 2: Farràs & Fernández-Castillejo *et al*., under review),
3. ChE and HDL characteristics influencing ChE (*i.e.*, fluidity), (Study 2: Farràs & Fernández-Castillejo *et al*., under review), and
4. PON enzyme family (Study 3: Fernández-Castillejo *et al*., 2017).

The primary objective has been complemented with the following secondary objectives:

1. To assess the effect of the sustained intake of phenol-enriched VOOs on several CVR parameters, such as VLDL and LDL subpopulation distribution, and LDL-P/HDL-P, HDL-C/HDL-P, and s-HDL/l-HDL atherogenic ratios (Study 1: Fernández-Castillejo *et al*., 2016).

2. To devise a high-throughput *in vitro* ChE assay to efficiently screen large numbers of specimens, such in the case of clinical trials, avoiding the use of radiolabeled cholesterol (Study 2: Farràs & Fernández-Castillejo *et al*., under review).

3. To assess whether the acute intake of phenol-enriched VOOs could instigate short-term modifications in PON-related variables (Study 3: Fernández-Castillejo *et al*., 2017).

4. To investigate the mechanisms of action involved in the PON system in an *in vivo* animal model (Study 3: Fernández-Castillejo *et al*., 2017).

5. To define which parameters are the best predictors for CEC and its interrelations with the fluidity of HDL monolayer after VOO intake (Study 4: Fernández-Castillejo *et al*., under review).

Methods & Results

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THE VOHF STUDY

The present doctoral thesis has been carried out within the framework of the multidisciplinary "*Virgin Olive Oil and HDL Functionality (VOHF) project: a model for tailoring functional food*" study.

The methods of the VOHF project were designed accordingy to its global aim: to assess whether phenol-enriched VOOs, enriched with either their own PC or with their own PC plus additional complementary phenols from thyme, could act as nutraceuticals in regard to the *in vivo* quantity and quality (functionality) of the HDL particle.

This aim was expected to be attained in two consecutive steps:

1. The first step was comprised of the preparation of similar phenol-enriched functional VOOs enriched with OO-PC, but differing in their total phenolic content (TPC). By the conducting an acute-intake study, the best functional VOO enriched with its own PC was selected to develop the following step.

2. The second step was aimed at assessing whether the enrichment of the functional VOO (selected in the acute-intake study) with complementary Th-PC could provide more benefits on human HDL functional quality than the functional VOO enriched only with OO-PC. To complete this second step, a sustained-intake study was conducted.

To reach these two steps some methodological aspects were covered, which are described in the following sections.

a) Phenol-enriched VOO preparation and composition

Different sets of VOOs were prepared for each of the two studies:

1. For the acute-intake study, three VOOs enriched with their own PC but differing in their total phenolic content were prepared:

    a. Low-functional VOO (L-FVOO) with 250 ppm of TPC
    b. Medium-functional VOO (M-FVOO) with 500 ppm of TPC
    c. High-functional VOO (H-FVOO) with 750 ppm of TPC.

A natural VOO with 80 ppm of TPC was used as the matrix to prepare these phenol-enriched VOOs by adding a freeze-dried olive cake extract rich in OO-PC, mainly secoiridoids or oleuropein derivatives, as the main source of HT.

2. For the sustained-intake study, the same parental VOO used for the acute-intake study was used as a control condition and as a matrix to prepare two functional VOOs, which were both enriched with an equal content of PC (500 ppm), but differed in their PC source:

   a. A functional VOO (FVOO) enriched with OO-PCs (mainly secoiridoid derivatives)

   b. A functional VOO (FVOOT) enriched with OO-PC (50%; mainly secoiridoid derivatives) plus complementary Th-PC (50%; mainly flavonoids, but also phenolic acids and monoterpenes).

These functional VOOs did not differ in fat and micronutrient composition, except for their phenolic content. The quantity of TPC was set at 500 ppm according to the results obtained after the acute-intake study, as described in section *c)*.

b) Design and study procedures

Both studies were randomized, controlled, double-blind, crossover trials.

The acute-intake study (Figure 18A) was carried out in 12 subjects in three one-day experimental sessions. Participants were considered healthy according to a physical examination and routine laboratory tests. Participants were instructed to follow a stabilization diet for two weeks before the first intervention and during 1-week washout periods between each VOO intervention to avoid any potential carry-over effect. In the interventions, participants ingested a single dose of 30 mL of raw VOO (L-FVOO, M-FVOO or H-FVOO). Fasting blood samples were collected at baseline and at different postprandial times (2 h, 4 h, and 6 h).

The sustained-intake study (Figure 18B) was conducted in 33 hypercholesterolemic subjects (TC > 200 mg/dL). Participants ingested a daily dose of 25 mL of raw VOO (control VOO, FVOO or FVOOT) for three weeks during meals, according to the assigned sequence of intervention,

preceded by 2-week washout periods with a common OO. To avoid an excessive intake of antioxidants, such as PC, during the clinical trial period, participants were advised to limit the consumption of phenol-rich food. Blood samples were collected at the beginning and end of each intervention.

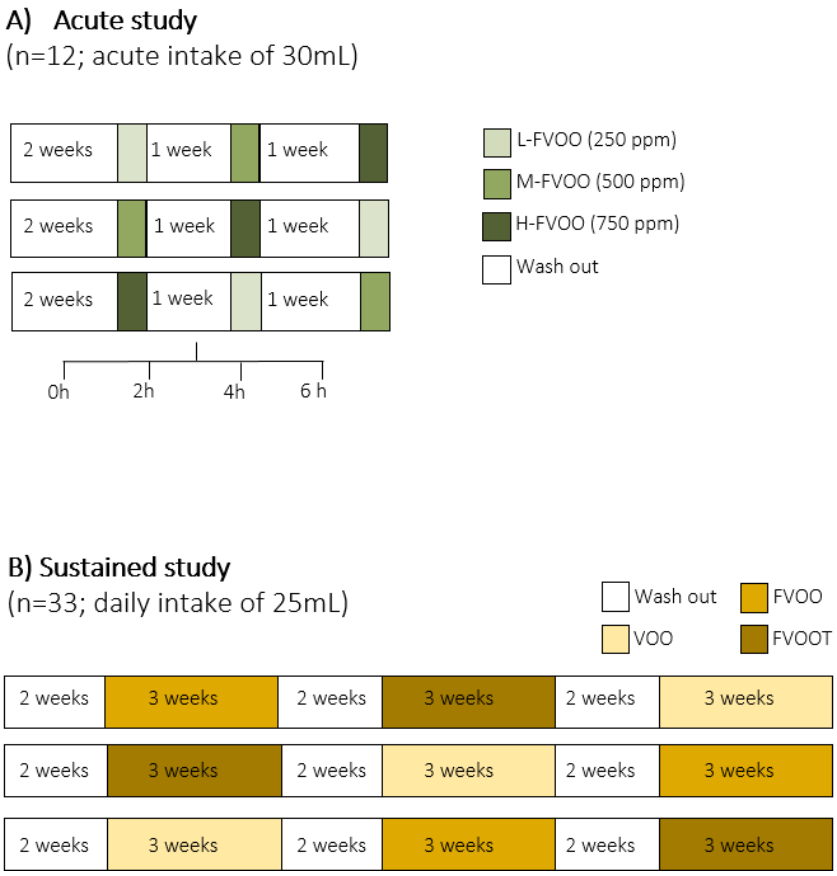

Figure 18 | VOHF study design. A) In the acute-intake study participants consumed a single dose of 30 mL of L-FVOO (250 ppm), M-FVOO (500 ppm), and H-FVOO (750 ppm). B) In the sustained-intake study participants were instructed to ingest a daily dose (3 weeks) of 25 mL of control VOO (80 ppm), FVOO (500 ppm of OO-PC 500 ppm), and FVOOT (250 ppm of OO-PC and 250 ppm of Th-PC). Source: created by the author.

The selection of the 2-week washout period was made according to turnover studies, which showed that the mean plasma residence of HDL is 4-6 days and that this turnover is not affected by diet (Zannis et al., 2015). Moreover, a 2-week washout period allows the plasma lipid profile to reach equilibrium, as longer intervention periods with fat-rich diets did not modify the lipid concentrations (Fielding et al., 1995).

The flowchart of the sustained-intake study is detailed in Figure 19.

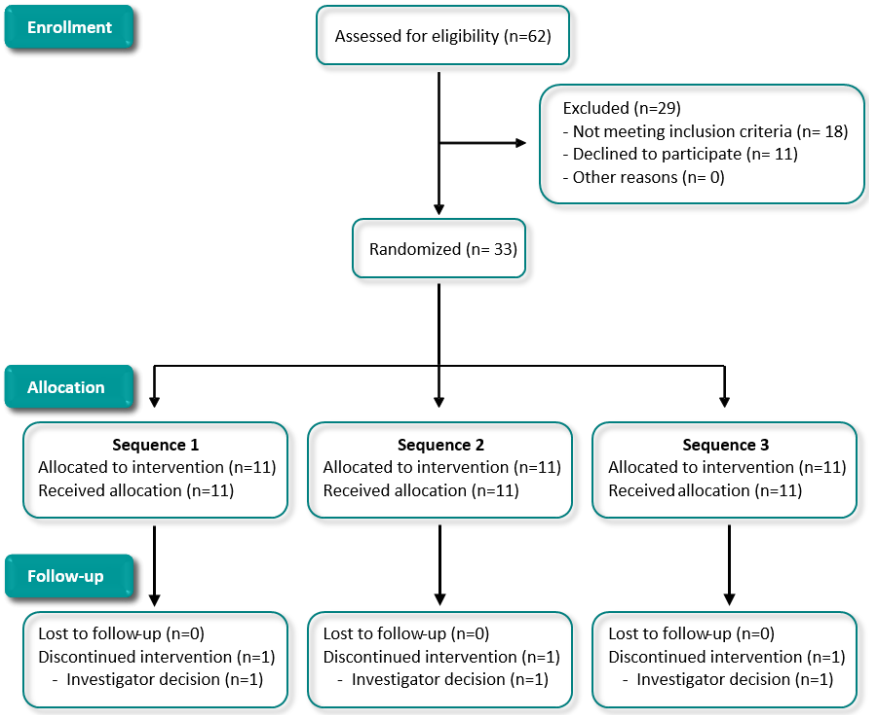

Figure 19 | Flowchart of VOHF sustained-intake study. 62 subjects were assessed for eligibility, and 33 were randomized and therefore allocated in one of the three sequences or intervention.

c) Functional VOO selection for conducting the sustained-intake study

To select the functional VOO to carry out the sustained-intake study, the acute-intake study was conducted. In this dose-response study, our group evaluated the following:

1. The bioavailability of the main phenolic biological metabolites. In particular, we evaluated whether high doses of PC could be absorbed from phenol-enriched VOOs in a dose-dependent manner in humans. This analysis of phenol metabolites revealed a dose-dependent response in the human systemic circulation. However, the pharmacokinetics of HT metabolites did not show a complete linear response after the intake of H-FVOO and M-FVOO, indicating that a threshold could exist in OO-PC absorption.

2. The effects of phenol enrichment of VOO on the endothelial dysfunction by means of ischemic reactive hyperemia (IRH) assessment. L-FVOO and M-FVOO produced significant increases in IRH values with respect to their baseline. M-FVOO was the first to show a significant increase in IRH values at 4 h, and it linearly increased until 6 h. L-FVOO only presented a significant increase at 6 h.

3. The effects of phenol enrichment of VOO on antioxidant capacity, measured by the oxygen radical absorbance capacity (ORAC) assay. A dose-dependent increase in the antioxidant capacity of the three FVOOs was observed based on an increase in phenolic content.

4. The sensorial acceptance of the three prepared FVOOs, which was scored on a 6-point acceptance scale, ranging from “I dislike it very much” (0) to “I like it very much” (6). It must be taken into account that high amounts of phenols increase the bitter and pungent sensory attributes of VOO, a fact that can reduce the consumer acceptance of enriched VOO. VOOs showed an inverse relationship between the phenol content and the acceptability level of enriched VOO. L-FVOO with the lowest phenolic enrichment did not show significant differences from the VOO control. M-FVOO and H-FVOO, with mean scores of 3,3 and 2,5 out of 6, respectively, presented lower scores, which is probably due to the increased bitter taste of these oils. The enriched VOO with the middle dose (M-FVOO) scored between “I like it a bit” and “I neither like nor dislike,” which implies a

satisfactory level of acceptance, considering that it contained 5-fold the phenol content of standard VOO.

Taking all the aforementioned data into consideration, M-FVOO (500 ppm of TPC) was selected as the functional VOO with the proper enrichment to carry out the sustained-intake study (Rubió, Valls, et al., 2012; Valls et al., 2017). This M-FVOO succeeded in achieving a correct balance between the content of VOO phenols, their absorption, and consumer acceptance.

d) Phenolic intake in the VOHF study

The phenolic intake through the ingested dose of the VOOs used in the acute- (30 mL) and sustained-intake studies (25 mL) is shown in Table 11.

Table 11 | Phenolic intake of phenol-enriched VOOs used in the acute- and in the sustained-intake studies, determined by HPLC-MS/MS. L-FVOO, Low phenolic content VOO; M-FVOO, Medium phenolic content VOO; H-FVOO, High phenolic content VOO; VOO, Virgin Olive Oil; FVOO, Functional VOO enriched with its own phenolics; FVOOT, Functional VOO enriched with both its own phenolics and phenolics from Thyme; 3,4-DHPEA-AC, 4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehyde form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycone; n.d.: not detected. † Dose-response study: mg/30mL oil; Sustained-intake study: mg/25mL oil. * Quantified with a calibration curve of hydroxytyrosol. **Quantified with a calibration curve of pinoresinol.

Compound	Acute study			Sustained study		
(mg/oil ingested dose) †	L-FVOO	M-FVOO	H-FVOO	VOO	FVOO	FVOOT
Hydroxytyrosol	0.21	0.29	0.37	0.01	0.21	0.12
3,4-DHPEA-AC	0.16	0.60	0.90	n.d.	0.84	0.39
3,4-DHPEA-EDA*	1.51	4.67	8.42	0.04	6.73	3.43
3,4-DHPEA-EA*	0.36	0.71	1.18	0.26	0.71	0.36
Total hydroxytyrosol derivatives	*2.23*	*6.27*	*10.87*	*0.30*	*8.49*	*4.30*
p-hydroxybenzoic acid	0.00	0.02	0.03	n.d.	0.02	0.06
Vanillic acid	0.02	0.08	0.10	n.d.	0.07	0.13
Caffeic acid	n.d	n.d	n.d	n.d.	0.00	0.06
Rosmarinic acid	n.d	n.d	n.d	n.d.	n.d.	0.41
Total phenolic acids	*0.02*	*0.10*	*0.13*	*-*	*0.09*	*0.65*
Pinoresinol	0.27	0.19	0.19	0.05	0.12	0.10
Acetoxipinoresinol**	6.95	5.98	5.81	2.47	3.66	3.24
Total lignans	*7.22*	*6.17*	*6.00*	*2.52*	*3.78*	*3.34*
Luteolin	0.17	0.28	0.57	0.04	0.18	0.21
Apigenin	0.06	0.08	0.10	0.02	0.06	0.10
Naringenin	n.d.	n.d.	n.d.	n.d.	n.d.	0.20
Eriodictyol	n.d.	n.d.	n.d.	n.d.	n.d.	0.17
Thymusin	n.d.	n.d.	n.d.	n.d.	n.d.	1.22
Xanthomicrol	n.d.	n.d.	n.d.	n.d.	n.d.	0.53
7-methylsudachitin	n.d.	n.d.	n.d.	n.d.	n.d.	0.53
Total flavonoids	*0.22*	*0.35*	*0.67*	*0.06*	*0.23*	*2.95*
Thymol	n.d.	n.d.	n.d.	n.d.	n.d.	0.64
Carvacrol	n.d.	n.d.	n.d.	n.d.	n.d.	0.23
Total monoterpenes	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*0.86*
Total phenols HPLC-MS/MS	**9.69**	**12.89**	**17.67**	**2.88**	**12.59**	**12.10**

Both clinical trials were conducted in accordance with the Helsinki Declaration and the Good Clinical Practice for Trials on Medical Products in the European Community and International Conference of Harmonization. The subjects gave their written informed consent before their participation. The acute-intake study was registered at ClinicalTrials.gov (Identifier: NCT01347515) (ClinicalTrials.gov, 2011). The sustained-intake study was registered at the International Standard Randomized Controlled Trial Register (Identifier: ISRCTN77500181) (International Standard Randomized Controlled Trial Register, 2012).

Study 1

Polyphenol rich olive oils improve lipoprotein particle atherogenic ratios and subclasses profile: A randomized, crossover, controlled trial

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Mol Nutr Food Res. 2016 Jul;60(7):1544-54

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1544 DOI 10.1002/mnfr.201501068 *Mol. Nutr. Food Res.* 2016, *60*, 1544–1554

RESEARCH ARTICLE

Polyphenol rich olive oils improve lipoprotein particle atherogenic ratios and subclasses profile: A randomized, crossover, controlled trial

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Received: December 28, 2015
Revised: February 12, 2016
Accepted: February 22, 2016

Scope: Lipoprotein particle measures performed by nuclear magnetic resonance (NMR), and associated ratios, may be better markers for atherosclerosis risk than conventional lipid measures. The effect of two functional olive oils, one enriched with its polyphenols (FVOO, 500 ppm), and the other (FVOOT) with them (250 ppm) and those of thyme (250 ppm), versus a standard virgin olive oil (VOO), on lipoprotein particle atherogenic ratios and subclasses profiles was assessed.

Methods and results: In a randomized, double-blind, crossover, controlled trial, 33 hypercholesterolemic individuals received 25 mL/day of VOO, FVOO, and FVOOT. Intervention periods were of 3 weeks separated by 2-week washout periods. Lipoprotein particle counts and subclasses were measured by NMR. Polyphenols from olive oil and thyme modified the lipoprotein subclasses profile and decreased the total LDL particle/total HDL particle (HDL-P), small HDL/large HDL, and HDL-cholesterol/HDL-P ratios, and decreased the lipoprotein insulin resistance index (LP-IR) ($p < 0.05$).

Conclusion: Olive oil polyphenols, and those from thyme provided benefits on lipoprotein particle atherogenic ratios and subclasses profile distribution. Polyphenol-enriched olive oil is a way of increasing the olive oil healthy properties while consuming the same amount of fat, as well as a useful and complementary tool for the management of cardiovascular risk individuals.

Keywords:
HDL-C/HDL-P and LDL-P/HDL-P ratios / Lipoprotein subclasses / Olive oil / Polyphenols / Thyme

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Abbreviations: **CHD**, coronary heart disease; **HDL-C**, high density lipoprotein cholesterol; **HPLC-ESI-MS/MS**, high performance liquid chromatography-electrospray tandem mass spectrometry; **JUPITER**, Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin; **LDL-C**, low-density lipoprotein cholesterol; **LP-IR**, Lipoprotein Insulin Resistance Index; **MLTPAQ**, Minnesota Leisure Time Physical Activity Questionnaire; **NHANES**, National Health and Nutrition Examination Survey

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1 Introduction

In experimental and human studies, polyphenol-rich foods have shown to improve the lipid cardiovascular risk profile. Thyme extracts decrease total and LDL cholesterol (LDL-C) and increase HDL cholesterol (HDL) in animal models [1], as well as reduce the susceptibility to in vitro LDL oxidation [2]. Plant extracts, such as those from red yeast rice, sugar cane-derived policosanols, and artichoke leaf, have also been shown to have an LDL-C lowering effect in subjects with moderate hyperlipidemia [3]. Cocoa flavonols increase the HDL-C in human studies [4, 5]. Data from the NHANES study showed that urinary enterolignan concentrations were positively associated with serum HDL-C and negatively associated with serum triglycerides (TG) in US adults [6]. In the EUROLIVE (The effect of olive oil on oxidative damage on European populations) study, sustained consumption of polyphenol-rich olive oils have been shown to: (i) increase HDL-C; (ii) reduce total cholesterol (TC)/HDL-C and LDL-C/HDL-C ratios; (iii) decrease in vivo lipid oxidative markers, such as oxidized LDL [7, 8]; and (iv) increase HDL cholesterol efflux from macrophages [9]. Supporting these data, a functional olive oil enriched with its polyphenols has also been shown to increase the expression of cholesterol-efflux-related genes [10].

A relatively new approach for the measurement of lipoproteins is to use nuclear magnetic resonance (NMR), which provide not only total particle counts of the major lipoprotein fractions but also their mean size and size subclass distribution [11]. In several studies, the measurement of LDL particles (LDL-P) by NMR was more effective than those of the cholesterol content of the LDL, or apolipoprotein (Apo) B100 concentrations, as a positive risk marker for CHD [12]. Results from large studies such as MESA (Multi-Ethnic Study of Atherosclerosis) [13], JUPITER [14], and HPS (Heart Protection Study) [15], also showed that measurement of HDL particles (HDL-P) appeared to be better than HDL-C as cardiovascular disease risk marker. Also within the MESA study, the LDL-P/HDL-P ratio was shown to be an independent risk factor for CHD [16].Recently, it has been described that cholesterol-overloaded particles, reflected in the HDL-C/HDL-P ratio, are independently associated with the progression of carotid atherosclerosis in a cardiovascular-disease-free population [17].

The aim of this work was to assess the effect of two functional olive oils, one enriched with its polyphenols, and the other with them and those of thyme, on the NMR lipoprotein particle profile and atherogenic ratios. We hypothesized that besides its positive effect on the standard lipid profile, the consumption of a polyphenol-rich olive oil rich diet may also improve cardiovascular risk parameters, as determined by NMR, and that this may provide an alternative way to monitor such treatment.

2 Materials and methods

2.1 Olive oil characteristics

A natural virgin olive oil (VOO, 80 ppm of phenolic compounds (PC)) was used as a control condition, and as a matrix to prepare a functional VOO (FVOO; PC = 500 ppm) by enrichment of the VOO with its own PC. A second functional olive oil was prepared by enrichment of the VOO both with its PC and those of thyme (FVOOT; PC = 500ppm (250 ppm from VOO and 250 ppm from thyme)). Olive oils did not differ in fat and micronutrient composition, with the exception of the phenolic content (Supporting Information Table 1).

2.2 Study subjects

Hypercholesterolemic (TC >200 mg/dL) individuals were recruited from newspaper and university advertisements. Volunteers were preselected when their clinical record, physical examination, and blood pressure were within a predefined normal range and the candidate was nonsmoker. Next, complete blood count, routine biochemical laboratory analyses, and urinary dipstick tests were performed. We included candidates with values, other than total and LDL-C, within the reference range for routine hematological and biochemical analyses. Exclusion criteria were the following: LDL-C ≥190 mg/dL, TG≥350 mg/dL, fasting blood glucose >126 mg/dL, plasma creatinine levels >1.4 mg/dL for women and >1.5 mg/dL for men, body mass index (BMI)>35, smokers (>1 cigarrete/day), athletes with physical activity (>3000 METS.min/day), hypertension, multiple allergies, intestinal diseases, chronic diseases (i.e. diabetes, cardiovascular, etc.), or other conditions that would impair the adherence to the study. All participants provided written informed consent, and the institutional ethic committee (CEIC-IMAS 2009/3347/I) approved the protocol.

2.3 Design and study procedure

This work was conducted in the frame of the VOHF (Virgin Olive Oil and HDL Functionality) Study. The trial was a randomized, crossover, double-blind, controlled study. We randomly assigned participants consecutively to 1 of 3 sequences of raw olive oil administration. Participants received a daily dose of 25 mL (22 g) of raw VOO, FVOO, or FVOOT. Administration sequences were: FVOO, FVOOT, VOO (sequence 1, n = 11); FVOOT, VOO, FVOO (sequence 2, n = 11); and VOO, FVOO, FVOOT (sequence 3, n = 11). Random allocation to each sequence was performed using a specific software developed at the Institut Hospital del Mar d'Investigacions Mèdiques (IMIM). Three-week interventions were preceded

by 2-week washout periods, in which participants consumed a common olive oil, a mixture of VOO and refined olive oil, with a very low phenolic content, and avoided other types of raw fats as well as olive tables consumption. We chose the 2-week washout period to reach equilibrium in the plasma lipid profile, because longer intervention periods with fat-rich diets did not modify the lipid concentrations [18]. Daily doses of 25 mL of olive oil were blindly prepared in containers and delivered to the participants at the beginning of each intervention period. Containers were assigned a code number concealed from participants and investigators, and disclosed only after statistical analyses completion. We instructed participants to return the 21 containers at the end of each intervention period so that the daily amount of unconsumed olive oil could be registered. Twenty-four hour urine and blood samples were collected at a fasting state at the start of the study and before and after each treatment. Plasma EDTA and serum samples were obtained by whole blood centrifugation and preserved at −80°C. 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 (http://ec.europa.eu/health/files/eudralex/vol-10/3cc1aen_en.pdf). The protocol is registered with the International Standard Randomized Controlled Trial register (www.controlled-trials.com:ISRCTN77500181).

2.4 Dietary adherence

We measured 24-h urinary hydroxytyrosol-sulfate and thymol-sulfate, before and after each intervention period as biomarkers of adherence to the FVOO and FVOOT interventions, respectively. Measurements were performed by HPLC-ESI-MS/MS [19]. A 3-day dietary record was administered by the participants at baseline and before and after each intervention period. Participants were asked to avoid a high intake of foods rich in antioxidants (i.e. vegetables, legumes, fruits, etc.). A nutritionist personally advised participants to replace all types of habitually consumed raw fats with the olive oils catered, and to limit their rich-polyphenol food consumption.

2.5 Outcomes and data collection

Main outcome measures were changes in lipoprotein particle atherogenic ratios and subclasses. We assessed outcome measures at the beginning of the study (baseline) and before (preintervention) and after (postintervention) each olive oil intervention period. Anthropometric variables were also recorded. Blood pressure was measured with a mercury sphygmomanometer after at least a 10-min rest in the seated position. Physical activity was recorded at baseline and at the end of the study and assessed by the MLTPAQ, which has been validated for its use in Spanish men and women [20, 21]. Plasma glucose, TC, and TG were measured using standard enzymatic automated methods and ApoAI and ApoB100 by immunoturbidimetry, in a PENTRA-400 autoanalyzer (ABX-Horiba Diagnostics, Montpellier, France). HDL-C was measured by an accelerator selective detergent method (ABX-Horiba Diagnostics, Montpellier, France). LDL-C was calculated by the Friedewald equation.

Serum samples were shipped to the National Heart, Lung and Blood Institute, National Institutes of Health (NIH; Bethesda, MD, USA). Lipoprotein subclasses measurement was performed by NMR in a Vantera clinical spectrometer, produced by LipoScience (Raleigh, NC, USA). The NMR LipoProfile test by LipoScience involves measurement of the 400 MHz proton NMR spectrum of samples and uses the characteristic signal amplitude of the lipid methyl group broadcast by every lipoprotein subfraction as the basis for quantification. NMR using the LipoProfile-3 algorithm was performed to quantify the average particle size and concentrations of VLDL, LDL, and HDL. Subparticle concentrations were determined for three VLDL subclasses (large or chylomicrons: >60 nm; medium: 35–60 nm; and small: 27–35 nm); three LDL subclasses (intermediate-density lipoprotein (IDL): 23–27 nm; large: 21.2–23 nm; and small: 18–21.2 nm); and three HDL subclasses (large: 8.8–13 nm; medium: 8.2–8.8 nm; and small: 7.3–8.2 nm) [22]. LP-IR, a lipoprotein particle-derived measure of insulin resistance [23] was also assessed.

2.6 Sample size and power analysis

Assuming a dropout rate of 15% and a Type I error of 0.05 (2-sided), a sample size of 32 individuals allows at least 80% power to detect a statistically significant difference among groups of 1.5 μmol/L in the total HDL particle number. The population standard deviation of this variable is 4.4μmol/L [17].

2.7 Statistical analyses

The normality of variables was assessed by the Kolmogorov–Smirnov test. We used the Kruskal–Wallis test or 1-factor analysis of variance (ANOVA), as appropriate, with Bonferroni correction for multiple comparisons, to determine differences in baseline characteristics. Carryover effect was discarded by testing a period-by-treatment interaction term in general linear models. Comparisons among changes were carried out by a covariance model. Age, gender, sequence of olive oil administration, LDL-C, and baseline values were the covariates. The p value for a trend among oils: from VOO to FVOO to FVOOT was assessed. Statistical significance was defined as a p value less than 0.050 for a two-sided test. We performed analyses by using SPSS for Windows, version 22 (IBM corp., Armonk, NY, USA).

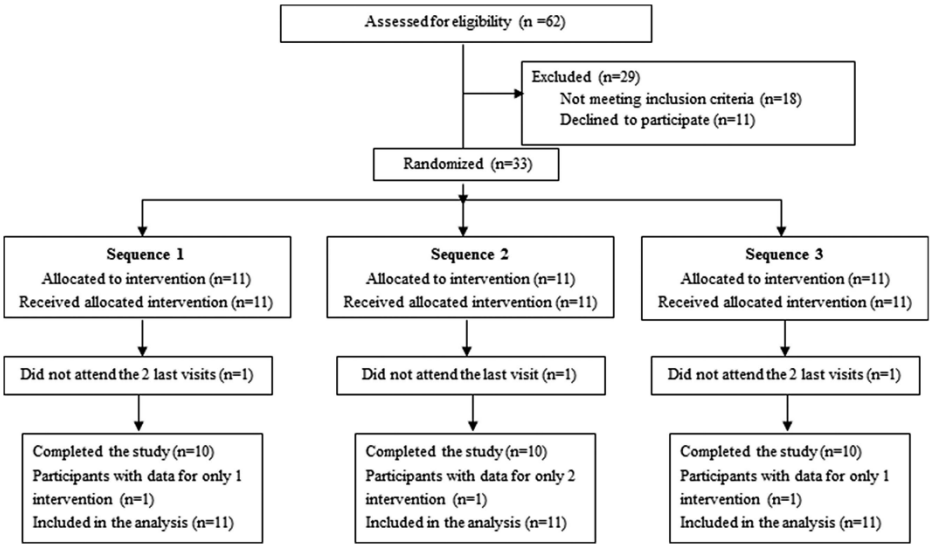

Figure 1. Flow-chart of the study.

3 Results

3.1 Characteristics of the study participants

From 62 subjects who were assessed for eligibility, 29 were excluded. Finally, 33 eligible participants (19 men, 14 women) entered the study. One participant in each sequence discontinued the intervention (Fig. 1). We could not identify any adverse effects related to the olive oils intake. No significant differences in participants' baseline characteristics were observed among sequences of olive oil administration, with exception of the small HDL particles (s-HDL) which were slightly higher in sequence 3 versus 1 (Table 1). No changes in daily energy expenditure in leisure-time physical activity were observed from the beginning to the end of the study (data not shown).

3.2 Dietary intake and adherence

Table 2 shows the daily dietary intake after intervention periods. Diet was similar in all intervention groups. Participant adherence was good, as reflected in the changes in urinary hydroxytyrosol sulfate and thymol sulfate excretion after olive oil interventions (Fig. 2). Hydroxytyrosol sulfate increased after FVOO ($p < 0.05$). Thymol sulfate increased after FVOOT, the change reaching significance versus those of the other two olive oils ($p < 0.05$).

3.3 Classical cardiovascular risk factors

Functional olive oils consumption did not change either glucose levels or the classical cardiovascular lipid profile (TC, TG, LDL-C, and HDL-C), nor ApoAI or ApoB100 concentrations versus VOO. An exception was a decrease in LDL-C after FVOO, which was significant versus changes after the other two olive oil interventions ($p < 0.05$) (Table 3). No changes were observed in blood pressure or BMI associated with the interventions.

3.4 NMR lipoprotein particle counts and subclasses

Total LDL-P, IDL-P, and total ApoB100 containing lipoproteins concentrations decreased after FVOO, the decrease reaching significance versus changes after VOO and FVOOT ($p < 0.001$). The decrease observed in small LDL particles after FVOO intervention was statistically significant ($p < 0.05$) when compared with the changes observed after FVOOT intervention. LDL particle size decreased after FVOO intervention, the decrease reaching significance versus changes after the other interventions ($p < 0.05$) (Table 3).

Comparison among changes after interventions in HDL-related measures (Table 3) showed that both FVOO and FVOOT promoted an increase in large HDL (l-HDL) particles versus VOO ($p < 0.05$). s-HDL particles decreased after FVOO intervention versus changes after VOO and FVOOT

Table 1. Baseline characteristics of the study participants

Variable	Sequence 1	Sequence 2	Sequence 3
Age (year)	54.9 ± 12.6	55.3 ± 11.9	55.5 ± 7.8
Gender (male/female)	5/6	7/4	7/4
Body mass Index (kg/m^2)	25.6 ± 3.7	26.3 ± 5.2	27.8 ± 4.7
Physical activity (METs.min/w)	3499 (1755–8092)	1189 (742–1687)	3322 (861–3664)
Systolic blood pressure (mmHg)	125 ± 18.7	128 ± 16.7	130 ± 17.9
Diastolic blood pressure (mmHg)	68.1 ± 13.5	72.3 ± 9.3	71.9 ± 13.4
Glucose (mg/dL)	88.5 ± 11.6	93.0 ± 13.3	90.9 ± 10.5
Total cholesterol (mg/dL)	228 ± 42.7	232 ± 32.7	219 ± 31.2
LDL measures			
LDL cholesterol (mg/dL)	150 ± 32.3	152 ± 28.5	142 ± 25.7
ApoB100 (mg/dL)	115 ± 0.21	117 ± 0.18	109 ± 0.16
NMR LDL particle concentration (nmol/L)			
Total	1473 ± 348	1420 ± 319	1322 ± 254
IDL	297 ± 95.1	295 ± 165	289 ± 79.4
Large	593 ± 139	515 ± 152	433 ± 227
Small	600 ± 389	560 ± 381	464 ± 199
Average NMR LDL particle size (nm)	21.2 ± 0.69	21.1 ± 0.71	21.0 ± 0.60
VLDL measures			
Triglycerides, mg/dL	94 (75–149)	119 (95–168)	117 (81–126)
NMR VLDL particle concentration (nmol/L)			
Total	54.5 ± 36.1	65.6 ± 26.6	59.4 ± 32.0
Large	3.8 ± 4.6	5.3 ± 4.2	2.8 ± 2.1
Medium	16.2 ± 11.5	20.2 ± 11.3	16.8 ± 10.7
Small	35.0 ± 26.2	39.6 ± 16.5	39.5 ± 21.9
Average NMRV VLDL particle size (nm)	45.8 ± 9.7	48.2 ± 5.5	45.3 ± 3.8
NMR ApoB100-containing particles concentration (nmol/L)	1517 ± 310	1478 ± 298	1384 ± 235
HDL measures			
HDL cholesterol (mg/dL)	52.8 ± 11.7	53.0 ± 12.8	53.4 ± 9.5
ApoAI (mg/dL)	142 ± 0.23	137 ± 0.21	147 ± 0.16
NMR HDL particle concentration (μmol/L)			
Total	35.6 ± 5.9	34.6 ± 5.3	38.0 ± 4.0
Large	7.0 ± 3.8	6.2 ± 3.4	6.6 ± 2.6
Medium	13.0 ± 6.2	9.2 ± 6.3	9.3 ± 4.7
Small	14.4 ± 6.1	18.0 ± 5.2	21.1 ± 4.9*
Average NMR HDL particle size (nm)	9.4 ± 0.63	9.3 ± 0.54	9.2 ± 0.34

Values are expressed as means ± SD or median (25th-75th percentile). Sequence 1: FVOO, FVOOT, VOO; Sequence 2: FVOOT, VOO, FVOO; Sequence 3: VOO, FVOO, FVOOT.
*$p < 0.05$ versus Sequence 1.

($p < 0.05$). Both functional olive oils increased HDL particle size when comparing with changes after the VOO intervention, the increase being higher after FVOO ($p < 0.0$). Both functional olive oils decreased medium VLDL particles versus VOO intervention, the decrease being higher after FVOO ($p < 0.05$). The average VLDL particle size decreased after FVOO intervention, reaching significance versus changes observed after the other two olive oil interventions ($p < 0.05$).

3.5 Atherogenic lipoprotein particle ratios and LP-IR

Figure 3 shows the results obtained in the atherogenic lipoprotein particle ratios and LP-IR after FVOO and FVOOT versus control (VOO). The LDL-P/HDL-P ratio decreased after FVOO ($p < 0.05$) versus VOO and FVOOT ($p < 0.05$). Both functional olive oils also decreased the HDL-C/HDL-P and the s-HDl/l-HDL ratios ($p < 0.05$). The decrease in s-HDL/l-HDL after FVOO was higher than that after FVOOT ($p < 0.05$). The LP-IR ratio also decreased after both functional olive oils ($p < 0.05$).

4 Discussion

In this study, we assessed the effects of polyphenol-rich olive oils on lipoprotein particle atherogenic ratios and subclasses distribution, as determined by NMR. From our results, polyphenols from olive oil decreased LDL-C and the LDL-P/HDL-P ratio, and improved the lipoprotein subclasses by decreasing total ApoB100 containing lipoproteins, LDL and IDL particle concentration, as well as LDL and VLDL particle size. Both, polyphenols from olive oil, and combined equally with those of thyme, decreased medium VLDL particles,

Table 2. Daily energy consumption and selected nutrient intake after olive oil interventions

	Olive oil intervention					
	VOO		FVOO		FVOOT	
	Postintervention	Change	Postintervention	Change	Postintervention	Change
Energy (kcal)	1882 (170–2057)	−92.4 (−268 to 83)	1914 (1758–2070)	−2.67 (−220 to 215)	1899 (1687–2111)	−120 (−338 to 96)
Carbohydrate (%)[a)]	42.1 (39.6–45.1)	1.24 (−1.4 to 3.9)	42.6 (40.0–45.1)	−1.32 (-4.6 to 2.0)	42.5 (39.7–45.4)	1.67 (−1.1 to 4.4)
Protein (%)[a)]	19.8 (18.8–21.4)	1.35 (−0.4 to 3.1)	19.8 (18.2–21.3)	−0.05 (-2.4 to 2.3)	19.7 (18.1–21.4)	0.13 (−2.1 to 2.4)
Total fat (%)[a)]	38.7 (35.5–41.9)	−2.44 (−5.9 to 1.1)	37.5 (34.5–40.6)	−0.73 (−5.3 to 3.8)	38.3 (35.4–41.3)	−3.20 (−6.5 to 0.1)
Monounsaturated fat (%)[a)]	16.2 (14.8–17.6)	−1.51 (−3.1 to 0.1)	16.2 (14.4–18.0)	0.08 (−2.1 to 2.3)	16.1 (14.8–17.4)	−0.47 (−2.2 to 1.3)
Polyunsaturated fat (%)[a)]	4.5 (4.21–4.9)	−0.29 (−0.7 to 0.1)	4.8 (4.3–5.2)	−0.10 (−0.7 to 0.5)	4.9 (4.4–5.4)	0.21 (−0.5 to 0.9)
Saturated fat (%)[a)]	11.3 (9.9–12.8)	−1.08 (−2.7 to 0.5)	11.6 (9.6–13.6)	0.82 (−1.0 to 2.6)	10.7 (9.0–12.4)	−1.50 (−3.3 to 0.3)
Vitamin C (g)	108 (83.8–132)	10.6 (−12.6 to 33)	117 (94.9–139)	−9.49 (−38 to 19.2)	125 (94.1–155)	12.1 (−22 to 46)
Vitamin E (mg)	5.6 (4.7–6.6)	−0.37 (−1.2 to 0.5)	5.9 (4.7–7.2)	−0.16 (−1.4 to 1.06)	6.4 (5.–7.5)	0.13 (−1.6 to 1.8)
Vitamin K (μg)	0.25 (0.00–0.5)	−0.33 (−0.8 to 0.2)	0.19 (0.05–0.3)	0.21 (0.04 to 0.4)	5.30 (-5.1 to 15.6)	4.99 (-5.4 to 15)
Alcohol (g)	7.41 (4.5–10.3)	1.43 (−0.9–3.7)	7.47 (4.9–10.1)	0.54 (−1.7 to 2.8)	9.49 (5.4–13.5)	−1.03 (−2.9 to 0.8)
Wine (g)[b)]	3.05 (1.6–4.5)	−0.87 (−2.5 to 0.8)	4.05 (2.1–6.0)	1.30 (-0.23 to 2.8)	4.63 (2.6–6.7)	0.31 (−1.0–1.6)

Values are means (95% CI) of data obtained from the 3-day dietary record obtained after each intervention period. VOO, virgin olive oil (control); FVOO, functional virgin olive oil, a VOO enriched with its polyphenols (PC) 500 mg/kg of PC from VOO); FVOOT, functional virgin olive oil, a VOO enriched with PC from VOO (250 mg/kg) and PC from thyme (250 mg/kg).
a) Expressed as percentage of total energy intake.
b) Grams of alcohol per day consumed in white and red wine.

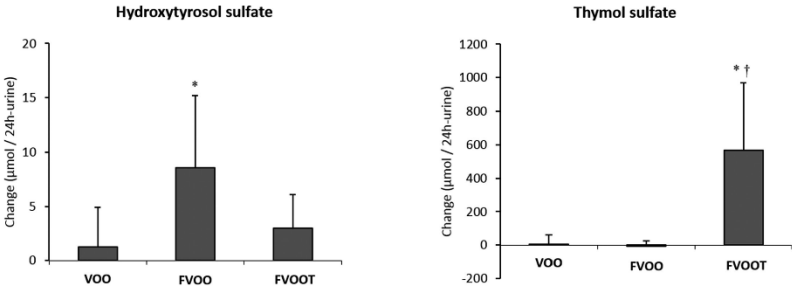

Figure 2. Changes in urinary hydroxytyrosol sulfate and thymol sulfate after olive oil interventions. VOO, virgin olive oil; FVOO, functional virgin olive oil enriched with its phenolic compounds (500 ppm); FVOOT, functional olive enriched with its phenolic compounds (250 ppm) and those from thyme (250 ppm). $^{*}p < 0.05$ versus VOO; $^{\dagger}p < 0.001$ versus FVOO.

increased large HDL particles, decreased s-HDL/l-HDL and HDL-C/HDL-P ratios, and the lipoprotein insulin resistance index (LP-IR) ($p < 0.05$).

LDL-C, LDL-P, LDL size, and the LDL-P/HDL-P ratio have all been shown to be directly associated with the risk of coronary heart disease (CHD) [24,25]. In the present work, olive oil polyphenols decreased all these cardiovascular risk biomarkers. The decrease in LDL-C observed is in agreement with that recently reported at postprandial state after extra-VOO consumption [26]. Polyphenols from green tea and from herbs have shown to have hypocholesterolemic effects lowering LDL-C in human [27] and animal models [28]. Mechanisms for explaining this effect are still unknown. The decrease in LDL-P after polyphenol-rich olive oil consumption observed in this study is also in agreement with our recent results from the EUROLIVE study with rich-polyphenol olive oil [29]. Concerning the decrease in LDL size, depletion of cholesteryl esters and triglyceride enrichment of LDL particles are associated with a decreased size, as well as an increased density of these particles, and these properties reduce their affinity for the LDL receptor [30]. As a consequence, small and dense LDLs are likely to have an increased residence time in the circulation, making them more susceptible to oxidation or glycosylation, which considerably increases their atherogenic potential [31]. Olive oil polyphenols have shown to protect human LDL in vivo from oxidation [7]. In this sense, in November 2011, the European Food Safety Authority (EFSA) released a claim concerning the benefits of polyphenol-rich olive oil consumption protecting the LDL from oxidation [32]. Of all LDL particle biomarkers tested, the ratio of LDL-P/HDL-P seems to have the strongest independent association with CHD, with significant net reclassification improvements in the AHA/ACC CHD risk scores [16].

To date the strong epidemiological association between HDL-C and CHD has failed to be translated into clinical benefit in terms of drug development. HDL, however, encompasses a heterogeneous population of lipoproteins that differ in shape, density, size, surface charge, and antigenicity that could exhibit differences in functionality irrespective of its cholesterol content. Studies reporting associations between HDL particle subclasses and CHD have been conflicting, and it is still unclear whether specific subclasses of HDL are more cardioprotective [33]. In most, but not all population studies [34], small HDL particles are considered to be more strongly associated with increased CHD risk than large HDL [35,36]. High levels of small HDL particles and/or low levels of the large HDL ones are often present in CHD, ischemic stroke, and type-II diabetes mellitus [37–39]. In our study we observed a decrease in the s-HDL/l-HDL ratio after both functional olive oils consumption. Our results agree with those previously obtained in the VOHF study, when, by using gradient electrophoresis, an increase in the large less dense HDL (HDL_2) and a decrease in the small denser HDL (HDL_3) after both functional olive oils was observed, although significance was only reached for FVOOT [40]. Differences among results could be explained by the fact that different methods (NMR versus gradient electrophoresis) and scores for HDL subclasses classification were used.

A potentially important advance in cardiovascular biomarker testing related to HDL is the HDL-C/HDL-P ratio and is considered to be a potential new measure of HDL cardioprotective function [17,41]. This ratio indicates the enrichment of the HDL particle in cholesterol, and has been shown to be directly related with the atherosclerosis progression in cardiovascular-free individuals [17]. In our study, both functional olive oils decreased the HDL-C/HDL-P ratio versus a natural VOO. Thus, olive oil rich in its polyphenols and those from thyme were able to decrease the cholesterol enrichment of HDL. The most likely mechanism proposed to explain the benefits of this decrease is that cholesterol enrichment of HDL impairs its ability to promote the efflux of cholesterol from peripheral cells, considered to be one of the main HDL anti-atherogenic functions and inversely related to CVD risk. This cholesterol efflux impairment can be explained by the fact that these cholesterol-overloaded HDLs may act as donor more than as acceptors of

Table 3. Changes (95% CI) in total lipoprotein particles and subclasses after 3 weeks of sustained consumption of virgin olive oil (VOO) and two functional olive oils, enriched with VOO phenolic content (FVOO) and with them and phenolic compounds from thyme (FVOOT)

Variable	Olive oil intervention						*p*-value for trend	Between-group differences		
	VOO		FVOO		FVOOT			FVOO vs VOO	FVOOT vs VOO	FVOO vs FVOOT
	Post-inter	Change	Post-inter	Change	Post-inter	Change				
LDL measures										
LDL cholesterol (mg/dL)	139	1.0 (−3.2 to 5.2)	140	−13 (−17 to −9.4)	139	0.24 (−3.9 to 4.4)	<0.001†	−14 (−20 to −8.6)§	−0.76 (−6.6 to 5.1)	−14 (−19 to −7.9)§
ApoB100 (mg/dL)	108	−0.5 (−5.9 to 4.8)	109	−3.8 (−10 to 2.4)	110	2.0 (−2.9 to 6.9)	ns	−3.2 (−10 to 3.9)	2.5 (−5.0 to 10)	−5. 8 (−14 to 3.0)
NMR LDL particle concentration (nmol/L)										
Total	1392	−3.2 (−40 to 34)	1353	−109 (−145 to −74)	1403	7.0 (−29 to 43)	<0.001†	−106 (−157 to −55)§	10 (−42 to 62)	−116 (−167 to −66)§
IDL	238	−13 (−40 to 14)	228	−69 (−95 to −44)	238	−13 (−40 to 13)	0.001†	−56 (−93 to −19)§	−0.39 (−38 to 37)	−56 (−93 to −19)§
Large	491	−20 (−8.1 to 48)	480	−8.8 (−34 to 16)	502	−1.6 (−28 to 25)	ns	−29 (−66 to 9.1)	−21 (−60 to 17)	−7.1 (−44 to 29)
Small	586	−2.5 (−21 to 16)	548	−6.7 (−23 to 9.7)	543	20 (3.4 to 37)	ns	−4.1 (−29 to 21)	23 (−2.5 to 48)	−27 (−50 to −3.4)§
Average NMR LDL particle size (nm)	21.2	0.02 (−0.04 to 0.1)	21.1	−0.10 (−0.16 to −0.04)	21.3	0.03 (−0.02 to 0.09)	0.001†	−0.12(−0.2 to −0.03)§	0.02 (−0.07 to 0.1)	−0.14 (−0.2 to −0.05)§
HDL measures										
HDL cholesterol (mg/dL)	52	0.14 (−2.4 to 2.7)	53	−0.73 (−3.5 to 2.1)	53	0.86 (−1.8 to 3.5)	ns	−0.91 (−4.8 to 3.0)	0.72 (−2.7 to 4.2)	−1.6 (−5.7 to 2.5)
ApoAI (mg/dL)	145	2.83 (−4.0 to 9.6)	142	−1.96 (−7.5 to 3.6)	144	0.74 (−4.2 to 5.7)	ns	−6.4 (−16 to 3.9)	−6.7 (−16 to 3.1)	0.26 (−6.1 to 6.2)
NMR HDL particle concentration (μmol/L)										
Total	36.5	−0.37 (−0.7 to −0.09)	35.8	−0.70 (−1.0 to −0.4)	36.4	−0.005 (−0.3 to 0.3)	0.003†	−0.33 (−0.7 to 0.6)	0.4 (−0.03 to 0.8)	−0.70 (−1.1 to −0.3)§
Large	7.1	−0.15(−0.3 to −0.03)	7.2	0.10 (−0.01 to 0.2)	7.1	0.06 (−0.06 to 0.2)	<0.05*†	0.25 (0.1 to 0.4)§	0.20 (0.04 to 0.4)§	0.05(−0.1 to 0.2)
Medium	9.0	−1.89 (−3 to −0.5)	10.0	−0.83 (−2 to 0.3)	8.9	−1.8 (−3 to −0.7)	ns	0.86 (−0.8 to 2.5)	−0.14 (−1.8 to 1.5)	1.0 (−0.8 to 2.6)
Small	19.3	1.53 (0.5 to 2.6)	17.2	−0.24 (−1 to 0.7)	19.5	2.0 (1 to 3)	<0.01†	−1.77 (−3 to −0.3) ‡	0.47 (−1 to 1.9)	−2.2 (−4 to −0.8)§
Average NMR HDL particle size (nm)	9.2	−0.08 (−0.1 to −0.04)	9.3	0.04 (0.01 to 0.08)	9.3	−0.01(−0.05 to 0.02)	<0.05*†	0.12 (0.07 to 0.17)§	0.06 (0.01 to 0.1)§	0.05(0.003 to 0.1)§
VLDL measures										
Triglycerides (mg/dL)	119	−7.9 (−25 to 9.1)	120	3.8 (−14 to 21)	124	3.8 (−14 to 22)	ns	11.7 (−13 to 36)	11.6 (−13 to 36)	0.06 (−25 to 25)
VLDL-Trigl (mg/L)	76	0.96 (−2.5 to 4.4)	75	−0.43 (−3.4 to 3.3)	76	−2.1 (−5.6 to 1.3)	ns	−1.0 (−5.8 to 3.8)	−3.1 (−8.0 to 1.8)	2.1 (−2.7 to 6.9)
NMR VLDL particle concentration (nmol/L)										
Total	65	1.58 (−3.4 to 8.6)	61	3.43 (−1.3 to 8.2)	64	−1.29 (−8.2 to 3.6)	ns	1.85 (−5.1 to 8.8)	−2.8 (−9.9 to 4.1)	4.75 (−2.1 to 12)
Large	3.5	0.08 (−0.2 to 0.4)	3.9	0.21 (−0.05 to 0.5)	3.5	−0.13 (−0.4 to 0.1)	ns	0.13 (−0.2 to 0.5)	−0.21 (−0.6 to 0.2)	0.34 (−0.03 to 0.7)
Medium	17.2	0.55 (−0.4 to 1.4)	15.4	−2.93 (−3.8 to −2.1)	16.8	−1.11 (−2.0 to − 0.2)	0.05*‡	−3.5 (−4.7 to − 2.2)§	−1.7 (−2.9 to −0.4)§	−1.8 (−3.1 to −0.6)§
Small	45.0	2.01 (−3.1 to 7.1)	41.8	6.1 (1.3 to 11)	43.2	−0.17 (−5.2 to 4.8)	ns	4.1 (−2.9 to 11)	−2.2 (−9.3 to 5.0)	6.3 (0.6 to 13)
Average NMR VLDL particle size (nm)	45.2	0.51 (−0.3 to 1.3)	45.2	−1.64 (−2.4 to −0.8)	45.0	−0.14 (−0.9 to 0.7)	<0.001†	−2.16 (−3.3 to −1.0)§	−0.65 (−1.8 to 0.5)	−1.51 (−2.6 to −0.4)§
ApoB100 containing lipoproteins (nmol/L)	1453	−1.77 (−34 to 31)	1414	−96 (−127 to −65)	1461	4.67 (−27 to 36)	<0.001†	−94 (−135 to −50)§	6.44 (−39 to 52)	−101 (−57 to −145)§

Postinter, Postintervention values. *p* value, *p* value for trend among oils: VOO, FVOO, FVOOT. LDL, low density lipoproteins. IDL, intermediate density lipoproteins. HDL, high density lipoproteins. VLDL, very low density lipoproteins. Adjusted by age sex, sequence, LDL-*C*, and baseline values. *p* value for trend across oils: * linear trend; † quadratic trend; ns, nonsignificant. §$p < 0.05$.

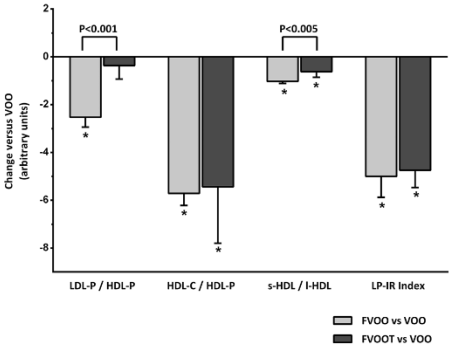

Figure 3. Changes (mean ± SEM) in atherogenic lipoprotein particle atherogenic ratios and lipoprotein insulin resistance index (LP-IR) after consumption of functional olive oils versus natural virgin olive oil (VOO). FVOO, functional virgin olive oil enriched with its phenolic compounds (500 ppm); FVOOT, functional olive enriched with its phenolic compounds (250 ppm) and those from thyme (250 ppm). LDL-P, low density lipoprotein particle-concentration; HDL-P, high density lipoprotein particle-concentration; HDL-C, HDL cholesterol; s-HDL, small HDL lipoprotein particle concentration; l-HDL, large HDL lipoprotein particle concentration. *$p < 0.001$ versus VOO. Differences between functional olive oils are indicated by square brackets with the corresponding significance.

cholesterol from peripheral tissues [42]. Moreover, HDL particles enriched in cholesterol may be less prone to be recognized by hepatic SR-BI receptors and thus, cholesterol clearance may be compromised. This fact can be explained by the assertion that myeloperoxidase enzyme oxidizes phospholipids and ApoA1 present in large, dense HDL [43]. Moreover, as reviewed by Lee *et al.*, SR-BI have a lower binding affinity for oxidized phospholipids, blocking thus cholesteryl esters transfer from oxidized HDLs to hepatic cells [44]. In addition, cholesterol-enriched HDLs become more pro-inflammatory, as well as more prone to be oxidized, which in turn impairs cholesterol efflux [17, 42]. In agreement with the results obtained in the present study, we also previously reported that olive oil polyphenols increase the efflux of cholesterol in vivo in humans [9], at least in part due via a transcriptomic effect [10]. Consumption of olive oils rich in polyphenols promoted a greater HDL stability, reflected in a triglyceride-poor core, and decreased the HDL oxidative status, through an increase in the olive oil polyphenol metabolites content in the lipoprotein [9].

Dyslipidemia among individuals with type 2 diabetes is characterized not only by high TG concentrations and low HDL-C concentrations, but also by decreases in the size of LDL and HDL particles and increases in the size of VLDL particles [45]. In individuals with insulin resistance (IR) and diabetes, VLDL particle size is inversely associated with the glucose disposal rate (GRD) [46] the gold standard for assessing insulin sensitivity, and directly associated with incident hypertension [46]. In our study, FVOO consumption reduced the VLDL particle size in dyslypemic individuals versus the other olive oils, and both functional olive oils were effective, versus VOO, on reducing medium VLDL subclass concentration. Larger VLDL particles have been associated with diabetes [45], and both large and medium VLDL concentrations with hypertension [46]. The American College of Endocrinology Task Force on the IR Syndrome states the clinical preventive value of identifying individuals with IR [47]. One measure to assess IR is the LP-IR index derived from lipoprotein NMR measurements. LP-IR is directly related with the HOMA index and inversely related with GRD, and has been proposed as a simple method for assessing the risk to develop a prediabetic or diabetic state [23]. In agreement with all the other benefits derived from the consumption of the functional olive oils on the lipoprotein subclasses, both FVOO and FVOOT also decreased the LP-IR index in our dyslypemic individuals.

In summary, sustained consumption of VOO enriched with its PC (FVOO), or equally enriched with them and those of thyme (FVOOT), promoted benefits, particularly FVOO, on lipoprotein subclasses distribution versus a natural virgin olive oil (VOO). Compared with VOO consumption, both functional olive oils decreased the LP-IR index and the atherogenic ratios: HDL-C/HDL-P ands-HDL/l-HDL. FVOO also improved the LDL-P/HDL-P ratio. To the best of our knowledge this is the first time that a decrease in these atherogenic ratios associated to a dietary intervention has been reported. Our results provide first level evidence of the benefits of olive oil polyphenols and those from thyme on lipoprotein subclasses distribution and their associated ratios. Olive oil, a recognized healthy food, cannot, however, be readily consumed in large quantities. Thus, polyphenol-enriched olive oil is a way of increasing its healthy properties while the same amount of fat is consumed. Data from this study provide further evidence to recommend polyphenol-rich olive oil consumption as a possibly useful and complementary tool for the management of cardiovascular risk individuals.

This study was supported by the Spanish Ministry of Education and Science, The VOHF Project (AGL2009-13517; DOI 10.1186/ISRCTN77500181). Work done by ATR was supported by the intramural National Heart, Lung and Blood Institute of the National Institutes of Health, in Bethesda, MD. O.C. was supported by a research contract of the ISCIII JR14/00008. We thank NUPROAS HB for their substantial contribution interpreting the data and revising the manuscript critically.

S.F.C., M.I.C., A.T.R., and R.S. have designed, analyzed, and interpreted the data, as well as draft the manuscript and revised it critically. M.F. and M.J.M. have designed, analyzed, and contributed in the data interpretation. R.M.V., O.C., L.R., U.C., A.P., A.M., and M.L.S. have contributed in the analysis of the data and also revised the manuscript critically.

There are no competing interests to declare, as there is no relationship with industry that should be disclosed.

Note: The acknowledgement was updated on July 8, 2016.

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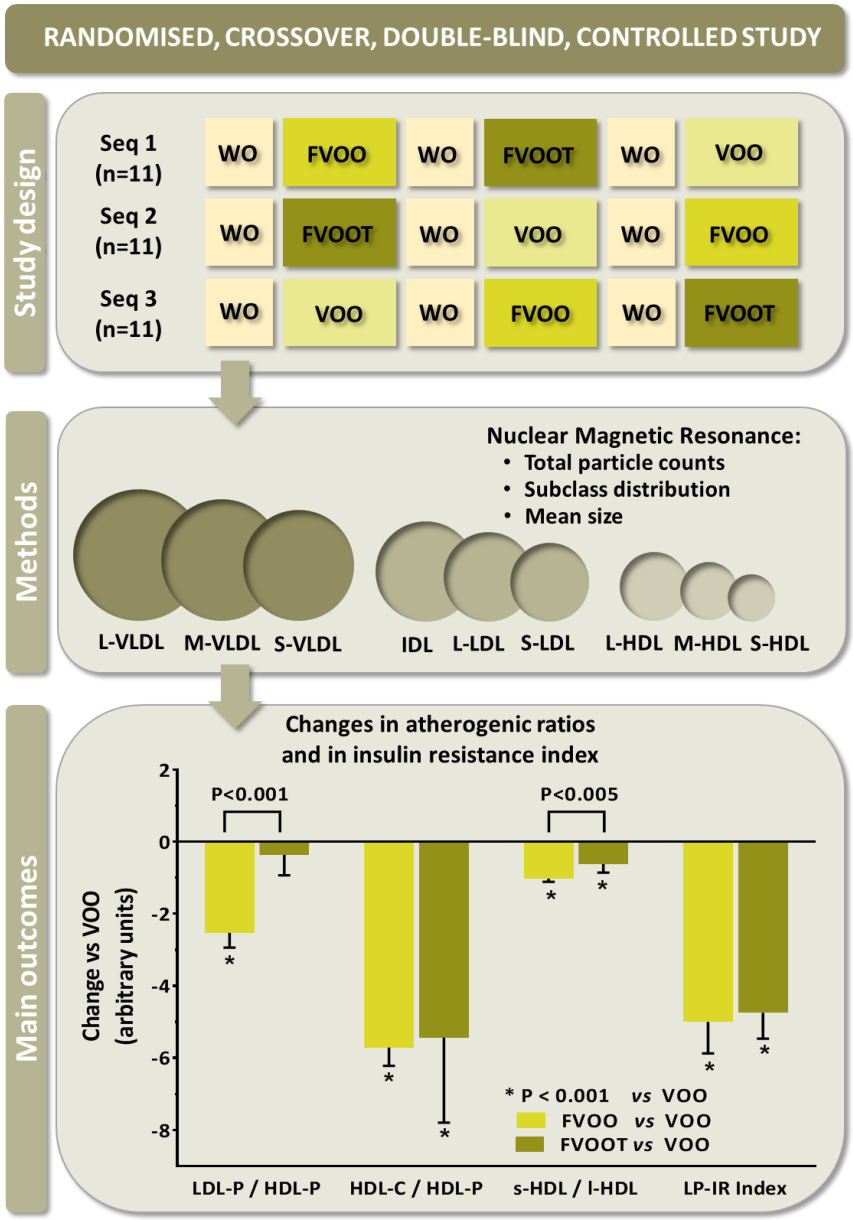
Supporting Information on line

Supporting Table 1. Olive oils composition

	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	nd	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	0.026 ± 0.04	0.71 ± 0.06	0.36 ± 0.03
Total Hydroxytyrosol Derivatives	*0.30*	*8.49*	*4.30*
p-hydroxybenzoic acid	nd	0.02 ± 0.00	0.06 ± 0.00
Vanillic acid	nd	0.07 ± 0.00	0.13 ± 0.01
Caffeic acid	nd	0.00 ± 0.00	0.06 ± 0.00
Rosmarinic acid	nd	nd	0.41 ± 0.03
Total phenolic acids	-	*0.09*	*0.65*
Thymol	nd	nd	0.64 ± 0.05
Carvacrol	nd	nd	0.02 ± 0.00
Total monoterpenes	-	-	*0.86*
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*
Luteolin	0.004 ± 0.000	0.18 ± 0.02	0.21 ±0.02
Apigenin	0.02 ± 0.000	0.06 ± 0.00	0.10 ±0.00
Naringenin	nd	nd	0.20 ± 0.02
Eriodictyol	nd	nd	0.17 ± 0.01
Thymusin	nd	nd	1.22 ± 0.09
Xanthomicrol	nd	nd	0.53 ± 0.06
7-Methylsudachitin	nd	nd	0.53 ± 0.09
Total flavonoids	*0.06*	*0.23*	*2.95*
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- cryptoxantin	0.02 ± 0.00	0.03 ± 0.00	0.05 ± 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
Araquidonic acid	0.36	0.36	0.36
Behenic acid	0.11	0.11	0.11
Total saturated fatty acids	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 fatty acids	77.71	77.80	77.72
Linoleic acid	7.43	7.36	7.43
Timmodonic acid	0.36	0.36	0.35
Linolenic acid	0.43	0.43	0.43
Total polyunsaturated fatty acids	8.22	8.15	8.22

nd, non detected; 3,4-DHPEA-AC, Hydroxytyrosol acetate; 3,4-DHPEA-EDA, Oleuropein aglycone dialdehyde; 3,4-DHPEA-EA, Oleuropein aglycone dialdehyde.

Graphical abstract



Volunteers from VOHF Study (n=33) followed a 2-week wash-out periods (WO; Olive oil with a very low phenolic content) between the three-week interventions:

- VOO: Control Virgin Olive Oil (80 ppm)
- FVOO: Functional VOO enriched with phenolic compounds from VOO (500 ppm)
- FVOOT: FVOO enriched with 250 ppm from VOO plus 250 ppm from thyme (500 ppm)

Samples were analyzed by NMR. Main outcomes include an improvement in the lipoprotein subclasses profile and a decrease in the LDL-P/HLD-P, HDL-C/HDL-P and s-HDL/l-HDL atherogenic ratios, and in the LipoProtein-Insulin Resistance Index.

Study 2

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

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***** These authors contributed equally to the study.

Manuscript under review

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS
Sara Fernández Castillejo

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

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Short title: Phenol-enriched olive oils and HDL antioxidant content

Clinical Trial Registration: ISRCTN77500181

ABBREVIATIONS

ApoA-I: apolipoprotein A-I

CoQ: coenzyme Q

EC: esterified cholesterol

FC: free cholesterol

FVOO: functional virgin olive oil

FVOOT: functional virgin olive oil with thyme

OO: olive oil

PC: phenolic compounds

PL: phospholipid

RCT: reverse cholesterol transport

TC: total-cholesterol

TG: triglyceride

VOO: virgin olive oil

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

ABSTRACT

At present, HDL-function is thought to be more relevant than HDL-cholesterol quantity. Consumption of olive oil phenolic-compounds (PC) has beneficial effects on HDL related markers. Enriched food with complementary antioxidants could be a suitable option to obtain additional protective effects. Our aim was to ascertain whether virgin olive oils (VOOs), enriched with (i) their own PC (FVOO) and (ii) with their own PC plus complementary ones from thyme (FVOOT) could improve HDL status and function.

33-hypercholesterolemic individuals ingested (25mL/day, 3 weeks) (i) VOO (80 ppm), (ii) FVOO (500 ppm), and (iii) FVOOT (500 ppm) in a randomised, double-blind, controlled, cross-over trial. A rise in HDL-antioxidant compounds was observed after both functional olive oil interventions. Nevertheless, α-tocopherol, the main HDL antioxidant, only augmented after FVOOT versus its baseline.

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

Word count: 171

1. INTRODUCTION

Olive oil (OO) phenolic compounds (PC) have been shown to prevent CHD, especially in humans with oxidative stress [1]. Due to the fact that HDL-cholesterol (HDL-C) levels are inversely and independently related to cardiovascular disease[2], pharmacological and natural product development has been oriented to the augmentation of their concentrations. Nevertheless, ineffectiveness, and even increased mortality risk of cholesteryl ester transfer protein antagonists have been reported in clinical trials [3, 4]. Such finding, combined with recent evidence that a number of genetic variables predisposing to high HDL-C levels are not associated with a lower risk of suffering a coronary event [5], have led to the consideration that future therapeutic approaches should improve HDL functionality rather than quantity [6].

Reverse cholesterol transport (RCT) is the main HDL biological function. It consists of extracting the cholesterol excess from the peripheral cells (cholesterol efflux) and taking it to the liver for further metabolism and excretion. This functional property has been tested in macrophage cell lines and shown to be inversely related to early atherosclerosis development and a high risk of experiencing a coronary event [7]. Increased HDL-C concentrations, and decreased in vivo lipid oxidative damage, in a dose-dependent manner with the PC content of the OO administered were reported in the EUROLIVE study [8]. In this regard, from a subsample of healthy humans we have, for the first time, first –level evidence that virgin olive oil (VOO) improves (i) cholesterol efflux, (ii) HDL monolayer fluidity, and (iii) HDL PC-content [9]. These characteristics can be altered by physicochemical changes and inflammatory protein binding resulting in a dysfunctional particle [10-12]. Protection against such a transformation could be provided by pharmacotherapy or functional foods oriented to improving HDL oxidative-inflammatory status.

PC-enriched foods could increase the healthy effects of some beneficial compounds without raising the fat content. However, enrichment with only a single antioxidant may produce a dual action because, depending on the dose, antioxidants could also revert to pro-oxidants [13,14]. One option to achieve greater beneficial health effects might be the development of functional foods with complementary-antioxidants, according to their

structure/activity relationship. In a randomized, double-blind, cross-over, and controlled trial our objective was to ascertain whether VOOs enriched (i) with their own PC (FVOO; 500ppm from OO) and (ii) with their own PC plus additional ones from Thyme (FVOOT; 250 ppm from OO and 250 ppm from Thyme) could enhance HDL antioxidant content.

2. MATERIALS AND METHODS

2.1. OO preparation and characteristics

The two phenol-enriched OOs (FVOO and FVOOT; 500 ppm) were prepared using a low- phenolic content VOO (80 ppm) which also served as control. For the wash-out period a common OO was used. The procedure to obtain the phenolic extracts and the enriched oils has been previously described [15]. In short, VOO with a low phenolic content was used as a control treatment and as an enrichment matrix for the preparation of both phenol –enriched olive oils. FVOO was enriched with its own PCs by adding a phenol extract obtained from freeze-dried olive cake collected from a commercial olive mill in an olive-growing area (Les Garrigues, Lleida, Catalonia, Spain). FVOOT was enriched with its own PC (50%) plus those from thyme (50%) using a phenol extract made up of a mixture of olive cake and commercially available dried thyme (Thymus zyguis). The phenolic extracts used for enrichment were obtained in the laboratory using an accelerated solvent extractor (ASE 100 Dionex, Sunnyvale, CA).

The Supplemental material Fig. 1 shows the PCs, the fatty acid, and the fat soluble micronutrient daily intake with 25mL of VOO, FVOO, and FVOOT. The VOO phenolic profile was assessed by high-performance liquid chromatography coupled to tandem mass spectrometry (HPLC/MS/MS) as described Rubió et al. [15]. Tocopherol, fatty acid, and carotenoid-contents in VOO, were analyzed using previously described methods [16,17].

2.2. Study design

Thirty-three hypercholesterolemic volunteers (total cholesterol > 200 mg/dL) participated in the VOHF study, a randomized, double-blind, crossover, controlled trial. Exclusion criteria were the following: BMI>35 Kg/m^2, smokers, athletes with high-physical activity (>3000 Kcal/day),

diabetes, multiple allergies, intestinal diseases, or any other disease or condition that would worsen adherence to the measurements or treatment.

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

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

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

2.3. Dietary adherence

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

2.4. Systemic biomarker analyses

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

2.5. HDL isolation and lipid-protein analyses

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

To assure the purity of HDL fractions, ApoB100 and albumin levels were also determined in these samples by automatic immunoturbidimetric methods (ABX-Horiba Diagnostics) (Supplemental material Table 1). The lipid and protein composition of HDL has been previously described [20].

2.6. HDL fatty acid analyses

Lipids from HDL were transesterified by incubation of 5 mg of lyophilized HDL sample in 2 mL of methanol/acetyl chloride (93:7 v/v) at 75ºC for 90 min. After methanolysis 1 mL of saturated NaCl solution was added to stop the reaction and 0.75 mL of hexane to extract the fatty acid methyl esters. After 5 min of vortex, samples were centrifuged at 2212 g for 10 min and the supernatant was injected into the chromatographic system. The analysis of fatty acids was performed by gas chromatography (GC) (Agilent 7890A Series) using a capillary SP-2330 column (30 m x 0.25 mm x 0.2 µm) (Supelco, Bellefonte, USA), coupled to a flame ionization detector (FID). The column temperature was programmed at 100ºC rising by 8ºC/min until it reached 200ºC then 3ºC/min to 225ºC (total run time 23.8

minutes). Helium was the carrier gas (2 mL/min). Injection was carried out with a split injector (1:30) at 250ºC, detector temperature was 260ºC and 1 µL of the solution was injected into the GC/FID system. The identification and the relative percentage (area %) of the fatty acids were determined, in duplicate, using a reference mixture of methyl esters of fatty acids (Sigma-Aldrich, St. Louis, MO, USA).

2.7. Analyses of HDL compounds with antioxidant properties

2.7.1. Fat-soluble antioxidants:

All sampling procedures were performed under low ambient light conditions. For sample pre-treatment, 400 µL of HDL was added to 400 µL of ethanol containing internal standard (α-tocopherol acetate 100 mg/L) and butylated hydroxytoluene (BHT) (0.063%). Hexane phases were completely evaporated to dryness at room temperature under a nitrogen stream. The residue was re-dissolved in 75 µL of methanol and the fat-soluble antioxidants (carotenoids, retinol, ubiquinol, and tocopherols) were analyzed by liquid chromatography (HPLC) the same day of extraction. The HPLC system was made up of a Waters 717 plus Autosampler, a Waters 600 pump, a Waters 996 Photodiode Array Detector, and a Waters 2475 Fluorescence Detector managed by Empower software (Waters Inc., Milford, MA). A 150x4.6 mm i.d. YMC C30 analytical column (3 µm) (Waters Inc., Milford, MA) was used for the separation of all components and HPLC analysis was performed following the procedure of Gleize et al. (2007) [21]. All compounds were identified by their retention time compared with pure standards or, when unavailable (lutein and β-crypthoxanthin), with compounds obtained and purified in the laboratory, the concentrations of which were determined by spectrophotometry using the molecular extinction coefficient (ε) of the molecule. Ubiquinol, the reduced form of Coenzyme Q (CoQ) 10 detected in HDL, was quantified with the calibration curve of ubiquinone standard (oxidized form) using a correction factor (200:1) as previously defined [22]. For the plasma quantification of each analyte, five-point standard curves were constructed with stock solutions individually prepared with appropriate solvents (correlation coefficients <0.99). They were run in duplicate.

2.7.2. Phenolic and monoterpene metabolites:

The phenolic and monoterpene biological metabolites were extracted from HDL by solid- phase extraction system using OASIS HLB 60 mg cartridges (Waters Corp., Milford, MA). Extractions were performed by loading 500 µL of HDL sample which had previously been mixed with 500 µL of distilled water and 60 µL of phosphoric acid 85% to break the bonds between the proteins and phenolic compounds, and 100 µL of catechol as internal standard. The retained phenolic compounds were eluted using 3 mL of methanol, which was evaporated to dryness under nitrogen flow. Prior to chromatographic analysis, the sample was reconstituted with 50 µL of methanol, before chromatographic analysis. The analysis of the phenolic metabolites was carried out by UPLC/MS/MS based on the method described by Rubió et al. (2012) [23]. The selected ion monitoring (SRM) transitions, cone voltage, and collision energy values were previously optimized in plasma for each phenol metabolite [18]. Only 6 were detected in HDL among all the analysed phenolic metabolites (Supplemental material. Table 2). Most of the PC (mainly the native structures present in the oils) were not found in HDL samples, thus, quantification was not undertaken. The metabolites hydroxytyrosol sulfate (sulfHT) and thymol sulfate (sulfTHY) were quantified, the rest of the metabolites, due to the lack of reference standards, were tentatively quantified with the calibration curves corresponding to their phenolic precursors or to similar metabolite compounds. In this regard, the sulfate conjugates derived from hydroxytyrosol, hydroxytyrosol acetate sulfate (sulfHTAc) and homovanillic alcohol sulfate (sulfHVAlc) were quantified with the calibration curve of sulfHT. Caffeic acid sulfate (sulfCA) and hydroxyphenylpropionic acid sulfate (sulfHPPA) were tentatively quantified by the calibration curve of caffeic acid and 3-(4-hydroxyphenyl)-propionic acid, respectively. All calibration curves were performed in HDL sample matrix. All analyses were run in duplicate.

2.8. HDL monolayer fluidity determination

The measurement of the HDL particle fluidity was based on the determination of the steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), as previously described [24]. In brief, HDL fractions were incubated with DPH 1µM for 30 minutes at room temperature in constant

agitation. After that, samples with the DPH probe were stimulated with a vertically polarized light at 360 nm. Fluorescent emission intensities were detected at 460 nm, in duplicate, in a Perkin-Elmer LS5OB spectrofluorometer (Perkin Elmer, Waltham, MA, USA), through a polarizer orientated in parallel and perpendicular to the direction of polarization of the emitted beam. Subsequently, we were able to measure the intensities of the perpendicular polarized fluorescence produced by the probe (Ip), which could vary depending on the sample fluidity. The steady-state fluorescence anisotropy (r) was calculated with these Ip values, and with the grating correction factor of the monochromator (G), using the following formula: r = (Ivv-GIvh)/(Ivv+2GIvh). The steady-state anisotropy refers to the rigidity of the sample, therefore the inverse value of this parameter (1/r) is the fluidity index.

2.9. HDL cholesterol efflux capacity determination

HDL cholesterol efflux was determined in a subsample of the study (n= 27). Murine J-774A.1 macrophages were seeded at a density of 75000 cells/cm^2 and routinely grown for 24 hours. To assess cholesterol efflux capacity, the fluorescent TopFluor-Cholesterol probe (Avanti Polar Lipids, USA), which consists of a BODIPY molecule anchored to the lipid moiety of the cholesterol molecule, was used. Confluent monolayers were labelled in DMEM containing 0.125mM total cholesterol, where the fluorescent cholesterol accounted for 20% of total cholesterol. Labelled cells were subsequently washed in the presence of the non-steroidal LXR agonist TO-901317 (3µM; Sigma-Aldrich, USA) so that ABCA1 and ABCG1 reverse cholesterol transporters expression was up-regulated. Following 18 hours of equilibration, cells were incubated with DMEM containing volunteers' HDL (100 µg/mL). All these incubations were performed in the presence of the Acyl -CoA cholesterol acyltransferase (ACAT) enzyme inhibitor Sandoz 58-035 (5µM; Sigma-Aldrich, USA). Media and cell fractions were pipetted onto a black plate, and fluorescence intensity was monitored in the multi-detection Microplate Reader Synergy HT (BioTek Instruments; USA) at λEx/Em=485/528nm. Cholesterol efflux capacity of HDL was calculated according to the following formula: [media fluorescence/(media fluorescence+cells fluorescence)] *100. Background efflux (that observed in cholesterol-loaded cells incubated without HDL) was then subtracted from cholesterol efflux values obtained in the presence of HDL. All

conditions were run in triplicate and data were pooled for each experiment.

2.10. Sample size and power analyses

The sample size of 30 individuals allows at least 80% power to detect a statistically significant difference among groups of 3 mg/dL of HDL-C, and a standard deviation of 1.9, assuming a drop out rate of 15% and a Type I error of 0.05 (2-sided).

2.11. Statistical analyses

Normality of continuous variables was evaluated by probability plots. Non-normally distributed variables were log transformed if necessary. Non-compliant participants, as defined previously, were excluded from analysis in these interventions. To compare means (for normal distributed variables) or medians (for non-normal distributed variables) among groups, analysis of variance (ANOVA) and Kruskal-Wallis tests were used, respectively; whereas $\chi 2$ and exact F-test, as appropriate, were employed to compare proportions. To assess relationships among variables Pearson and Spearman correlation analyses were performed. A general linear model for repeated measurements was employed to evaluate the intra- and inter-intervention effects. For binary variables recoded as being above or below a threshold level, a Mc Nemar test was performed to assess the statistical significance both within and between treatment effects. Presence of carry-over effect was assessed testing the period by treatment interaction significance under a mixed effects model introducing participant as a random intercept. Carry-over effect was discarded in all variables. A value of $p<0.05$ was considered significant. R2.12.0 software (R Development Core Team) and SPSS18.0 software (IBN Corp) were employed to perform the statistical analyses.

3. RESULTS

3.1. Participant characteristics, dietary adherence, and systemic biomarkers

From the sixty-two subjects evaluated, thirty-three eligible volunteers (19 men) were finally included. Supplemental Figure 2 shows the flow of

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

3.2. HDL fatty acids

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

3.3. HDL compounds with antioxidant properties

Regarding fat-soluble antioxidants, an increase of HDL ubiquinol, β-cryptoxanthin, and lutein was observed after both FVOOT and FVOO interventions from baseline ($p<0.05$). Ubiquinol and lutein were also significant after FVOO versus VOO ($p<0.05$). ß-cryptoxanthin was significant after FVOO versus VOO, and after FVOOT versus VOO ($p<0.05$). Additionally, α-tocopherol increased only after FVOOT from baseline, and retinol increased only after FVOO versus baseline and versus VOO and FVOOT interventions ($p<0.05$). Thymol sulfate, caffeic acid sulfate, and hydroxyphenylpropionic acid sulfate were the main phenolic compounds observed after FVOOT versus its baseline, and after FVOOT compared with VOO and FVOO ($p<0.05$). An increase of hydroxytyrosol acetate sulfate was found after FVOO versus its baseline ($p<0.05$) (Figure 1).

HDL antioxidant distribution showed cross-linked correlations with systemic biomarkers and with HDL composition. The HDL α-tocopherol post-value directly correlated with HDL cholesterol/protein ratio after VOO, FVOO, and FVOOT intakes ($r>0.6$; $p<0.001$). In addition, HDL α-tocopherol directly correlated with the HDL PL/protein ratio after FVOOT intake ($r=0.587$; $p=0.002$).

3.4. HDL monolayer fluidity and HDL cholesterol efflux capacity

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

4. DISCUSSION

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

The antioxidant system is a complex network of interacting molecules. When an antioxidant is oxidized it is converted into a harmful radical that needs to be turned back to its reduced form by complementary-antioxidants. It has been reported that supplementing high-risk individuals with a single type of antioxidant promoted rather than reduced lipid-peroxidation. In contrast, the combination of different antioxidants has been shown to be effective in reducing atherosclerosis in human trials [25]. All of the above suggests that the enrichment of VOO with hydroxytyrosol derivatives combined with complementary phenols from aromatic herbs, such as thyme, might be a good strategy to provide the optimum balance among the different kinds of flavonoids, simple phenols, monoterpenes, and phenolic acids [15].

A number of antioxidants associated with HDL could improve its antioxidant function and preserve its structure. The EUROLIVE study revealed that PC acquired through a high PC- VOO intervention could bind to HDL in a dose-dependent manner and thus contribute to the enhancement of its functionality [9]. In our work, after both phenol-enriched VOO interventions we found an increase in HDL from antioxidants with

various activities. Furthermore, the co-existence of lipo- and hydro-philic antioxidants linked to HDL may confer additional protection. On one hand, lipophilic antioxidants can act by scavenging aqueous peroxyl radicals at the surface of the membrane, and by scavenging lipid peroxyl radicals within it. Lipophilic chain-breaking antioxidants in lipoproteins, such as α-tocopherol, retinol, and carotenoids, may play a key role in protecting lipids and proteins from oxidative damage [26,27]. It has been reported that a physiological concentration of β-carotene and CoQ inhibits LDL and HDL oxidation in vitro [28,29]. On the other hand, hydrophilic antioxidants, such as phenols, would be more effective if free radical injury occurred at the lipid/aqueous interphase. Some in vitro studies have shown that PC do penetrate the phospholipid bilayer of the liposomes, probably as a consequence of their hydrophilic properties and their non-planar structures which confer conformational mobility [30]. In the present study, both phenol-enriched VOOs increased lipophilic and hydrophilic antioxidants in HDL, and consequently both OOs improved the antioxidant state of the HDL particle.

A major issue in lipoprotein antioxidants is the rescue of vitamin E (α, β, γ tocopherols), the major antioxidant in human plasma, which is carried by HDL and LDL. The most potent antioxidant of the tocopherol family is α-tocopherol which is the main initial chain-breaking antioxidant during lipid peroxidation. It is fully localized in the hydrophobic zone of the lipid bilayer [31]. In turn, CoQ recycles the resultant α-tocopherol phenoxyl back to its biologically active reduced form [32]. In this regard, we observed an augmentation of α-tocopherol and CoQ after the FVOOT intervention, while after FVOO only CoQ was increased. In addition, some authors have reported that a fraction of highly active phenolic acids (such as rosmarinic and caffeic ones) could regenerate α-tocopherol. Specifically, caffeic acid has been reported to protect α-tocopherol in LDL [33]. In our study, the FVOOT intervention increased rosmarinic acid biological metabolites (caffeic acid sulfate and hydroxyphenylpropionic acid sulfate), as well as α-tocopherol, which might suggest a better α-tocopherol regeneration and protection through this mechanism. Thus, the FVOOT intervention could be better at improving HDL antioxidant activity and consequently preserving the HDL protein structures. Furthermore, Peruguini et al (2000) [34] reported that HDL α-tocopherol is related to the cholesterol- and PL-

/protein ratios, correlations that were also reproduced in the present work.

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

The antioxidant properties of OOPC in vivo are well-known. The EUROLIVE study showed a decrease in vivo in lipid oxidative damage and an increase of HDL-C in a dose-dependent manner with the PC of the OO administered [8]. In concurrence, in a recent paper from the VOHF-study, an increment in HDL-C was observed in the subsample of volunteers without hypolipidemic medication [20]. The effects of PC-rich OO on protecting LDL from oxidation have been acknowledged by the European Food Safety Authority [37]. Nevertheless, in this study, although a decrease in the oxidized LDL was observed after three interventions, no significant change effect was detected. Hypercholesterolemic status and pharmacological treatment could explain such a result.

The crossover, randomized design of the study is a strength because it meant that inter-individual variability was reduced as the participants consumed all the kinds of OOs. In addition, the three OOs had a similar matrix (fat-soluble, vitamins, and fatty acids), with only their PC content varying. A further strength is the centralization of laboratory analyses and the time-series samples from the same participant being measured in the same run to minimize imprecision. The reduced sample size represents a possible limitation as it could have led to diminished statistical power in a

number of biomarkers with increased intra-individual variability. A synergistic effect on HDL-parameters from PC and other OO constituents remains to be elucidated. The inability to assess potential interactions among the OOs and other dietary components and medication is also a limitation. In this regard, medication and diet was controlled throughout the study and no changes were registered.

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

CONCLUDING REMARKS

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

HIGHLIGHTS

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

ACKNOWLEDGEMENTS

This work has been done within the context of Universitat Autònoma de Barcelona (Biochemistry, Molecular Biology, and Biomedicine PhD). We thank Xavier Mayol for his help in cell cultures and Borges Mediterranean Group for providing the common OO used in the study. It was funded by the Spanish Ministry of Economy and Competitiveness (MINECO) financing projects (AGL2009-13517-C03-01, AGL2009-13517-C03-02, AGL2009-13517-C03-03, AGL2012-40144-C03-01), and the FPI-fellowship (BES-2010-040766), by a Catalan Government-ISCIII-FEDER contract (FIS-CP06/00100), by a Sara Borrell (CD10/00151), and Joan Rodés (JR14/00008) contracts, and by grants from AGAUR (2014 SGR 240) and ISCIII FEDER (CB06/03/0028). CIBEROBN, CIBERDEM, and CIBERESP are initiatives of ISCIII.

CONFLICT OF INTEREST STATEMENT

The authors have declared that no competing interests exist.

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FIGURE LEGENDS

Figure 1. HDL compounds with antioxidant properties after the interventions. Values represent pre- and post-interventions. Values expressed as mean + SE or as median and 75th percentile.

* Intra-treatment p-value<0.05

| Inter-treatment FVOO-VOO p-value<0.05

· Inter-treatment FVOO-FVOOT p-value<0.05

Inter-treatment FVOOT-VOO p-value<0.05

Figure 2. Mean change of cholesterol efflux after the interventions. Values represent the mean differences of cholesterol efflux after the interventions. Values expressed as mean ± SE

* Inter-treatment p-value<0.05

^ Intra-treatment p-value<0.09.

Table 1. Baseline characteristics of the participants.

	Total[a] (n=33)
GENERAL	
Sex: male	19 (57.6%)
Age	55.21 ± 10.62
BMI (Kg/m^2)	26.64 ± 4.54
Hypolipidemic medication: no	19 (57.6%)
Physical activity (Kcal/week)	2423.25 (897.38;4543.75)
Diastolic blood pressure (mmHg)	70.76 ± 12.01
Systolic blood pressure (mmHg)	127.94 ± 17.37
SYSTEMIC LIPID PROFILE AND GLYCAEMIA	
Total-cholesterol (mg/dL)	226 ± 35
Triglycerides (mg/dL)	114 (85;145)
Glucose (mg/dL)	91 ± 12
HDL-cholesterol (mg/dL)	53 ± 11
LDL-cholesterol (mg/dL)	148 ± 28
ApoA-I (g/L)	1.4 ± 0.2
Apolipoprotein-B100 (g/L)	1.1 ± 0.2

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

Figure 1

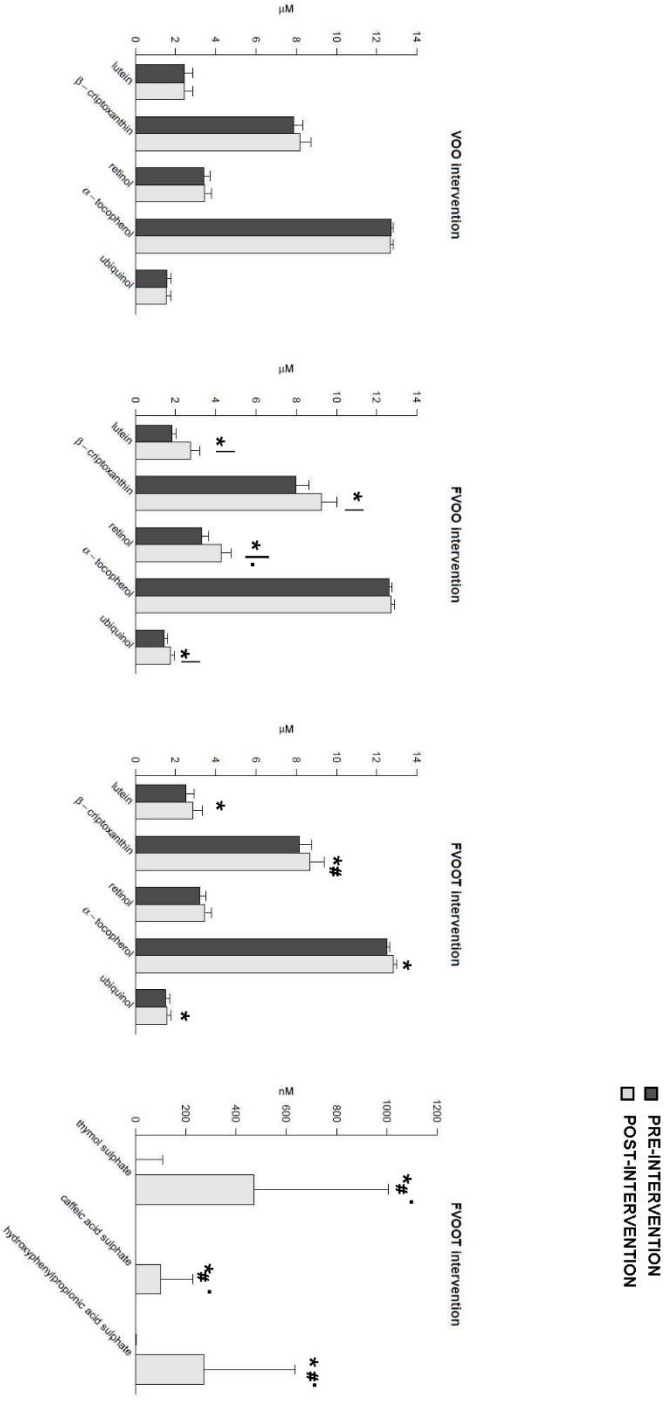

VOO intervention
FVOO intervention
FVOOT intervention
FVOOT intervention
μM
nM
lutein
β - criptoxanthin
retinol
α - tocopherol
ubiquinol
thymol sulphate
caffeic acid sulphate
hydroxyphenylpropionic acid sulphate
PRE-INTERVENTION
POST-INTERVENTION

Figure 2

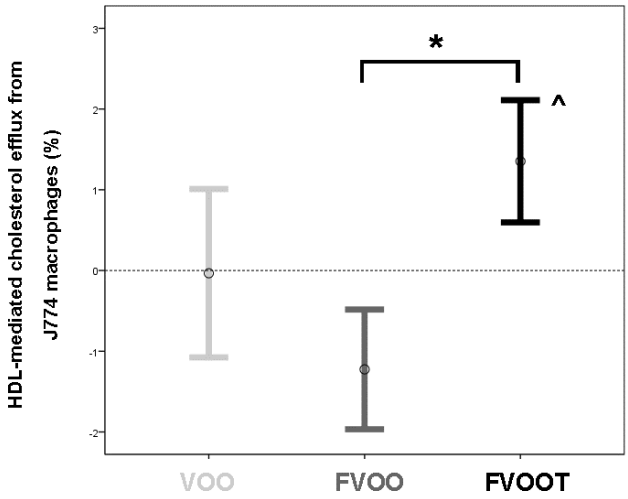

HDL-mediated cholesterol efflux from J774 macrophages (%)
3
2
1
0
-1
-2
*
^
VOO
FVOO
FVOOT

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

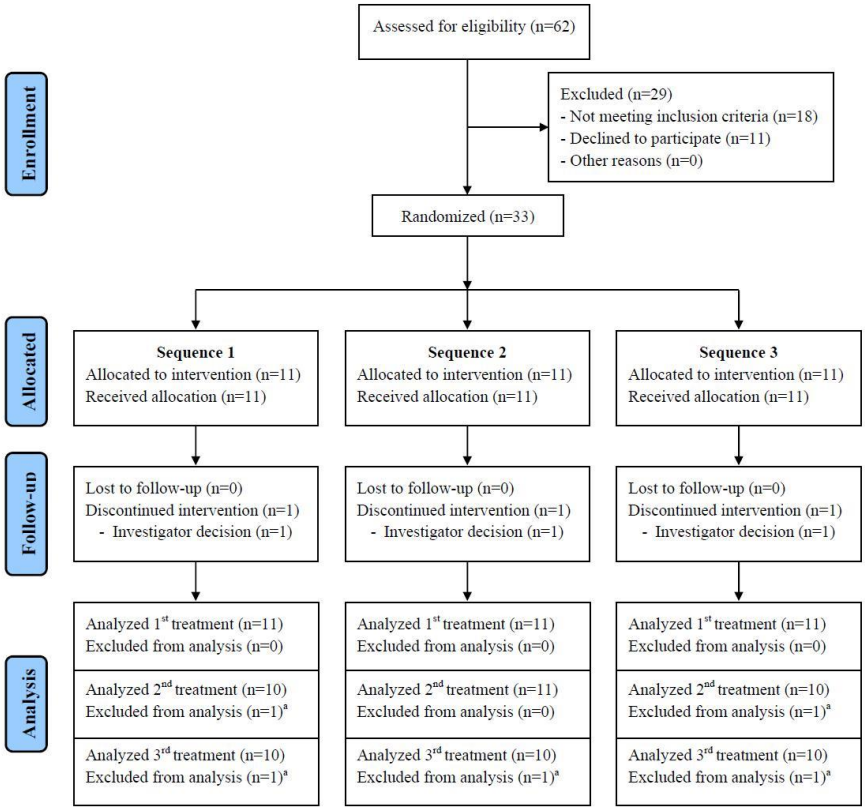
	VOO	FVOO	FVOOT
PHENOLIC COMPOUNDS (mg/25 mL/day)			
hydroxytyrosol	0.01 ± 0.00	0.21 ± 0.02	0.12 ± 0.00
3,4-DHPEA-AC	n.d.	0.84 ± 0.06	0.39 ± 0.04
3,4-DHPEA-EDA	0.04 ± 0.00	6.73 ± 0.37	3.43 ± 0.29
3,4-DHPEA-EA	0.26 ± 0.04	0.71 ± 0.06	0.36 ± 0.03
Total HT derivates	***0.30***	***8.49***	***4.30***
p-hydroxybenzoic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

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

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

Abbreviations: VOO, virgin olive oil; FVOO, functional VOO enriched with its own phenolic compounds; FVOOT, functional VOO enriched with its own phenolic-compounds plus additional complementary ones from thyme; 3,4-DHPEA-AC,4-(acetoxyethyl)-1,2-dihydroxybenzene; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA,oleuropein-aglycone.

Figure 2 Supplemental material. Flowchart of VOHF-study.



[a] Non-intervention

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

	VOO		FVOO		FVOOT		Inter-int.
	Pre-int.[a]	Post-int.[a]	Pre-int.[a]	Post-int.[a]	Pre-int.[a]	Post-int.[a]	p-value
ApoB100 (g/L)	<0.11	<0.11	<0.11	<0.11	<0.11	<0.11	- - -
Albumin (g/L)	1.32 ±0.85	1.22 ±0.74	1.11 ±0.50	1.10 ±0.67	1.20 ±0.62	1.29 ±0.78	0.711 (VOO-FVOOT) 0.195 (FVOO-FVOOT) 0.404(VOO-FVOO)

[a)] Values expressed as mean ± S.D.

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

Abbreviations: VOO, virgin olive oil; FVOO, functional VOO enriched with its own phenolic compounds; FVOOT, functional VOO enriched with its own phenolic compounds plus additional complementary ones from thyme; HDL, high density lipoprotein; LDL, low density lipoprotein.

Table 2 Supplemental material. Optimized SRM conditions used for the identification of phenolic compounds in HDL analysis.

Phenolic compound	MW (g/mol)	SRM quantification	
		Transition	Cone voltage (V) / Collision energy (eV)
Olive Oil			
3,4-DHPEA-EDA	320	319 > 195	40 / 5
3,4-DHPEA-EA	378	377 > 275	35 / 10
Acetoxypinoresinol	416	415 > 151	45 / 15
Alcohol homovanillic sulphate	248	247 > 167	40 / 15
Apigenin	270	269 > 117	60 / 25
Apigenin glucoside	432	431 > 269	45 / 25
Caffeic acid	180	179 > 135	35 / 15
Cinamic acid	148	147 > 103	20 / 10
Chlorogenic acid	354	353 > 191	30 / 10
Coumaric acid	164	163 > 119	35 / 10
Dihydroxyphenylpropionic acid	182	182 > 137	20 / 10
Elenolic acid	242	241 > 139	30 / 15
Ferulic acid	194	193 > 134	30 / 15
Homovanillic acid	182	181 > 137	25 / 10
Homovanillic acid glucuronide	358	357 > 181	40 / 20
Homovanillic acid sulphate	262	261 > 181	40 / 15
Homovanillic alcohol glucuronide	344	343 > 167	40 / 20
Homovanillic alcohol sulphate	248	247 > 167	40 / 15
Hydroxyphenylacetic acid	152	151 > 107	20 / 10
Hydroxyphenylpropionic acid sulphate	346	245 > 165	35 / 15
Hydroxyphenylpropionic acid glucuronide	342	341 > 165	35 / 15
Hydroxytyrosol	154	153 > 123	35 / 10
Hydroxytyrosol acetate	196	195 > 135	30 / 10
Hydroxytyrosol acetate sulphate	276	275 > 195	35 / 15
Hydroxytyrosol acetate glucuronide	372	371 > 195	35 / 15
Hydroxytyrosol glucuronide	330	329 > 153	40 / 20
Hydroxytyrosol sulphate	234	233 > 153	40 / 15
Ligstroside	524	523 > 361	35 / 15
Ligstroside derivate (1)	336	335 > 199	40 / 10
Ligstroside derivate (2)	394	393 > 317	40 / 15
Luteolin	286	285 > 133	55 / 25
Luteolin glucoside	448	447 > 285	50 / 25
Methyl 3,4-HPEA-EA	410	409 > 377	30 / 5
Methyl oleuropein aglycone	392	391 > 255	35 / 15
Oleuropein	540	539 > 377	35 / 15
Oleuropein derivate	366	365 > 299	35 / 10
p-HPEA-EA	362	361 > 291	30 / 10
p-HPEA-EDA	304	303 > 285	30 / 5
Pinoresinol	358	357 > 151	40 / 10
Rutin	610	609 > 300	55 / 25
Tyrosol	138	137 > 106	40 / 15
Tyrosol glucuronide	314	313 > 137	25 / 30
Tyrosol sulphate	218	217 > 137	40 / 20
Vanillic acid	168	167 > 123	30 / 10
Vanillin	152	151 > 136	20 / 10

Thyme			
Apigenin glucuronide	446	445 > 269	40 / 25
Apigenin rutinoside	578	577 > 269	35 / 15
Caffeic acid glucuronide	356	355 > 179	40 / 15
Caffeic acid sulphate	260	259 > 179	35 / 15
Carvacrol	150	149 > 134	40 / 15
Coumaric acid glucuronide	340	339 > 163	35 / 15
Coumaric acid sulphate	244	243 > 163	35 / 15
Dihidrokaempferol	288	287 > 259	45 / 10
Dihidroquercetin	304	303 > 285	40 / 10
Dihidroxanthomicol	346	345 > 301	40 / 20
Eriodictyol	288	287 > 151	40 / 15
Eriodictyol glucoside	450	449 > 287	45 / 10
Eriodictyol glucuronide	464	463 > 287	40 / 20
Eriodictiol rutinoside	596	595 > 287	40 / 20
Eriodictyol sulphate	368	367 > 287	40 / 15
Ferulic acid glucuronide	370	369 > 193	35 / 15
Ferulic acid sulphate	274	273 > 193	35 / 15
Hydroxyphenylpropionic acid	166	165 > 121	20 / 10
Hydroxyphenylpropionic acid glucuronide	342	341 > 165	40 / 25
Hydroxyphenylpropionic acid sulphate	246	245 > 165	35 / 15
Isorhamnetin glucoside	478	477 > 315	45 / 20
Isorhamnetin rutinoside	624	623 > 315	55 / 25
Kaempferol glucuronide	462	461 > 285	40 / 25
Kaempferol rhamnoside	432	431 > 285	45 / 20
Luteolin glucuronide	462	461 > 285	40 / 25
Methoxyluteolin	300	299 > 119	35 / 15
Methylsudachitin	374	373 > 358	40 / 20
Myricetin glucoside	480	479 > 317	45 / 20
Naringenin	272	271 > 151	40 / 15
Naringenin glucoside	434	433 > 271	45 / 10
Naringenin glucuronide	448	447 > 271	40 / 25
Naringenin rutinoside	580	579 > 271	40 / 20
Naringenin sulphate	352	351 > 271	40 / 20
p-cymene diol glucuronide	342	341 > 165	40 / 25
Quercetin	302	301 > 151	40 / 15
Quercetin arabinoside	434	433 > 301	45 / 20
Quercetin glucoside	464	463 > 301	45 / 25
Quercetin glucuronide	478	477 > 301	40 / 20
Quercetin rhamnoside	448	447 > 301	40 / 15
Quercetin sulphate	382	381 > 301	40 / 20
Rosmarinic acid	360	359 > 161	40 / 20
Rosmarinic acid glucuronide	536	535 > 359	40 / 20
Rosmarinic acid sulphate	440	439 > 359	40 / 20
Thymol	150	149 > 134	40 / 15
Thymol glucuronide	326	325 > 149	20 / 25
Thymol sulphate	230	229 > 149	40 / 20
Thymusin (1)	330	329 > 286	40 / 25
Thymusin (2)	330	329 > 314	40 / 25
Thymusin glucuronide	506	505 > 329	40 / 20
Thymusin sulphate	410	409 > 329	40 / 20
Xanthomicol	344	343 > 328	40 / 20

MW: Molecular weight; 3,4-DHPEA-EDA, dialdehydic form of elenolic acid linked to hydroxytyrosol; 3,4-DHPEA-EA, oleuropein aglycon; p-HPEA-EDA, dialdehydic form of elenolic acid linked to tyrosol; p-HPEA-EA, ligstroside-aglycone.

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

	VOO		FVOO		FVOOT		
	Pre-int.[a]	Post-int.[a]	Pre-int.[a]	Post-int.[a]	Pre-int.[a]	Post-int.[a]	Inter-intervention p-value
Tryglicerides (mg/dL)	113 (93.75;137.5)	116.5 (94.25;145)	110 (78.75;150.5)	122 (71.25;155.75)	113 (83;149)	111 (91;148)	0.986 (VOO-FVOOT) 0.469 (FVOO-FVOOT) 0.660 (VOO-FVOO)
Total cholesterol (mg/dL)	217.33 ± 40.48	215.70 ± 33.01	221.21 ± 33.38	218.00 ± 33.23	214.33 ± 32.46	217.96 ± 35.07	0.828 (VOO-FVOOT) 0.405 (FVOO-FVOOT) 0.488 (VOO-FVOO)
HDL-cholesterol (mg/dL)	52.75 ± 11.22	52.07 ± 11.07	53.58 ± 12.51	53.37 ± 13.54	51.43 ± 11.84	52.81 ± 12.47	0.668 (VOO-FVOOT) 0.433 (FVOO-FVOOT) 0.650 (VOO-FVOO)
LDL-cholesterol (mg/dL)	139.43 ± 33.80	139.90 ± 30.04	144.13 ± 27.00	140.54 ± 24.44	138.86 ± 29.14	140.33 ± 27.95	0.836 (VOO-FVOOT) 0.406 (FVOO-FVOOT) 0.275 (VOO-FVOO)
ApoA1 (g/L)	1.42 ± 0.24	1.45 ± 0.23	1.44 ± 0.24	1.42 ± 0.24	1.44 ± 0.25	1.44 ± 0.23	0.173 (VOO-FVOOT) 0.933 (FVOO-FVOOT) 0.211 (VOO-FVOO)
ApoB100 (g/L)	1.09 ± 0.21	1.08 ± 0.19	1.12 ± 0.19	1.09 ± 0.18	1.07 ± 0.19	1.10 ± 0.20	0.497 (VOO-FVOOT) 0.186 (FVOO-FVOOT) 0.358 (VOO-FVOO)

[a] Values expressed as mean ± S.D. or median (25th to 75th). Intra-and inter- intervention p-values were not significant.

Abbreviations: VOO, virgin olive oil; FVOO, functional VOO enriched with its own phenolic compounds; FVOOT, functional VOO enriched with its own phenolic compounds plus additional complementary ones from thyme; HDL, high density lipoprotein; LDL, low density lipoprotein.

Study 3

Phenol-enriched olive oils modify paraoxonase–related variables: a Randomized, Crossover, Controlled Trial

Sara Fernández-Castillejo, Ana-Isabel García-Heredia, Rosa Solà, Jordi Camps, Maria-Carmen López de la Hazas, Marta Farràs, Anna Pedret, Úrsula Catalán, Laura Rubió, Maria- José Motilva, Olga Castañer, María-Isabel Covas, and Rosa-Maria Valls

Manuscript accepted in Mol Nutr Food Res, on April 21st 2017

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS
Sara Fernández Castillejo

Mol. Nutr. Food Res. 0, 0, 2017, 1600932 DOI 10.1002/mnfr.201600932 (1 of 13) 1600932

RESEARCH ARTICLE

Phenol-enriched olive oils modify paraoxonase-related variables: A randomized, crossover, controlled trial

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Received: October 21, 2016
Revised: April 20, 2017
Accepted: April 21, 2017

Scope: Low paraoxonase (PON)1 activities, and high PON1 and low PON3 protein levels are characteristic of cardiovascular disease. Our aim was to assess short- and long-term effects of virgin olive oils (VOO), enriched with their own phenolic compounds (PC; FVOO) or with them plus complementary PC from thyme (FVOOT), on PON-related variables and the mechanisms involved.

Methods and results: Two randomized, controlled, double-blind, and crossover interventions were conducted. In an acute intake study, participants ingested three FVOOs differing in PC content. In a sustained intake study, participants ingested a control VOO and two different FVOOs with the same PC content but differing in PC source. Acute and sustained intake of VOO and FVOO decreased PON1 protein and increased PON1-associated specific activities, while FVOOT yielded opposite results. PON3 protein levels increased only after sustained consumption of VOO. Mechanistic studies performed in rat livers showed that intake of isolated PC from VOO and from thyme modulate mitogen-activated protein kinases and peroxisome proliferator-activated receptors regulating PON synthesis, while a combination of these PCs cancels such regulation.

Conclusion: This study reveals that the intake of phenol-enriched FVOOs modulates oxidative balance by modifying PON-related variables according to PC content and source, and this modulation can be perceived as beneficial.

Keywords:
Flavonoids / Olive oil / Phenolic compounds / PON / Secoiridoids

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Abbreviations: ***Ahr***, Aryl hydrocarbon receptor; **ANOVA**, Analysis of variance; **CCL-2**, Chemokine (C-C motif) ligand 2; **CON**, Control

1 Introduction

The paraoxonase (PON) enzyme family comprises of three members (PON1-3). In humans, PON1 and PON3 are expressed mainly in the liver and kidney, and to a lesser extent in other organs such as brain, colon, heart, kidney, lung and small intestine. They both are secreted into the blood where they associate with apolipoprotein (Apo)A-I-containing high-density lipoprotein (HDL) [1–3]. PON2 is exclusively intracellular and it is expressed in nearly all human tissues, though it is not found in circulation [4, 5]. All PON enzymes have lactonase activity, and PON1 can also hydrolyze aromatic esters and certain organophosphate compounds, such as paraoxon, having arylesterase and paraoxonase activities [2]. PON1 and PON3 exert beneficial effects on cardiovascular disease (CVD) by preventing lipoprotein oxidation, metabolizing lipid peroxides, promoting cholesterol efflux to HDL, and showing anti-inflammatory activity via inhibition of chemokine (C-C motif) ligand 2 (CCL-2) [2, 6–9]. PON1 and PON3 proteins, together with PON1-associated activities, play a crucial role in CVD and other related conditions, since decreased PON3 mass, increased PON1 mass, and decreased PON1-associated activities have been related to an increase in plaque formation and to high CVD risk [5,7,10–13].

Phenolic compounds (PC), naturally present in fruit and vegetables, modulate hepatic PON1 gene expression as well as hepatic and plasma activities in mice [14–16] and humans [17, 18]. In particular, hydroxytyrosol from olive oil (OO) and flavonoids from several sources have been extensively described as triggering PON1 activities in animals [15, 19, 20] and humans [18, 21, 22]. In 2011 the European Food Safety Authority (EFSA) released a claim concerning the effects of a daily intake of 5 mg of the PC hydroxytyrosol (a simple phenolic compound) and its derivatives on low-density lipoprotein (LDL) protection from oxidation [23]. Nevertheless, the phenolic content in most virgin OOs (VOO) available in the market is low to allow the recommended consumption of hydroxytyrosol and its derivatives within the context of a balanced diet [23]. The enrichment of VOO with its own PC, or with complementary PC from other sources, has been postulated as an interesting strategy to increase the daily PC intake without increasing caloric intake [24, 25]. Thyme (*Thymus zyguis*) is the herb selected in this study for VOO flavoring, as it is one of the richest sources of flavonoids [26]. Our previous studies have reported that PC from thyme (Th-PC) enhances the bioavailability of the PC from OO (OO-PC) [27, 28], and that a thyme-enriched OO intervention improves HDL oxidative status [29] and DNA protection against oxidation [30], and exerts a cardioprotective impact on HDL particles [31–33]. However, the prospective beneficial properties of these phenol-enriched VOOs on PON enzymes remain unknown. Furthermore, most of the studies assessing PON concentrations and activities have been undertaken after sustained interventions and thus only long-term modifications have been studied. As HDL turnover is considerable, it seems safe to predict that PON enzymes might also undergo short-term modifications. As a result, postprandial studies are needed to obtain further insights regarding PC effects on PON family. Additionally, no data concerning the transcriptional effects of OO-PC on PON1 have been published to date, as most mechanistic studies are focused on PON1 regulation carried out by drugs or flavonoids [1–3, 34].

Our hypothesis is that the consumption of OO-PC may modulate PON-related variables (i.e. PON1 and PON3 protein levels along with PON-1 lactonase and paraoxonase activities) towards a beneficial mode, and that blending OO-PC with complementary Th-PC, might instigate synergic effects. The aim of this study was to assess the short- and long-term effects of the acute and sustained intake of different VOOs, enriched with OO-PC or with them plus complementary Th-PC, on PON-related variables. A secondary objective was to investigate the mechanisms involved in the OO-PC and Th-PC effects on such variables.

2 Materials and methods

2.1 Phenol-enriched OO preparation and composition

Different set of VOOs were prepared for each of the two interventions. For the acute study, three VOOs enriched with their own PC, but differing in their total phenolic content were prepared as previously described [24]: Low-functional VOO (L-FVOO: 250 ppm), Medium-functional VOO (M-FVOO: 500 ppm) and High-functional VOO (H-FVOO: 750 ppm). A VOO (80 ppm) was used as the matrix to prepare these phenol-enriched VOOs by adding a freeze-dried olive cake extract rich in the main OO-PC, mainly secoiridoids or oleuropein derivatives as the main source of hydroxytyrosol [35]. Regarding the sustained intake study, the same parental VOO used for the acute study was used as a control condition and as a matrix to prepare two functional VOOs, enriched with equal content of PC (500 ppm) but differing in the PC source. The first one was enriched with its own PCs (FVOO) while the second one (FVOOT) was enriched with its own PC (50%; mainly secoiridoid derivatives) plus

diet; **CVD**, Cardiovascular disease; **FVOO**, Functional virgin olive oil enriched with its own phenolic compounds; **FVOOT**, Functional virgin olive oil enriched with phenolic compounds from olive oil and thyme; **HDL-C**, HDL cholesterol; **H-FVOO**, High-FVOO; **L-FVOO**, Low-FVOO; **M-FVOO**, Medium-FVOO; **MAPK**, Mitogen-activated protein kinases; **OO**, Olive oil; **OO-PC**, Olive oil phenolic compounds; **PC**, Phenolic compounds; **PON**, Paraoxonase; **Ppar**, Peroxisome proliferator-activated receptor; **SEC**, Control diet supplemented with OO-PC extract, mainly secoiridoids or hydroxytyrosol derivatives; **SEC+THY**, Diet supplemented with secoiridoids and thyme phenols extracts; **Th-PC**, Phenolic compounds from thyme; **THY**, Diet supplemented with thyme phenols extract; **TPC**, Total phenol content; **VOO**, Virgin olive oil
Colour Online: See the article online to view Fig. 1 in colour.

complementary PC from thyme (50%; mainly flavonoids, phenolic acids and monoterpenes) [26]. These three VOOs did not differ in fatty acid and micronutrient composition other than the phenolic content [29, 31]. The phenolic composition of the VOOs used in both studies was quantified by UPLC-MS/MS following the chromatographic method described previously [27]. The phenolic intake through the ingested dose of the VOOs used in the acute (30 mL) and sustained study (25 mL) is shown in Supporting Information Table S1.

2.2 Designs of the studies and participants' characteristics

Both studies were randomized, controlled, double-blind and crossover trials. In the acute study the short-term effects of VOO with different PC content on PON-related variables were determined. In the sustained study the long-term effects of phenol-enriched VOOs, with the same PC content but with PC from different sources, on PON-related variables were determined. As depicted in Fig. 1A, the acute study was carried out in 12 participants, considered healthy according to a physical examination and routine laboratory tests. Participants were instructed to follow a stabilization diet for 2 weeks before the first intervention and 1-week washout periods between each VOO intervention in order to avoid potential carryover effect. In the interventions, participants ingested a single dose of 30 mL of raw VOO (L-FVOO, M-FVOO or H-FVOO). Fasting blood samples were collected at baseline and at different postprandial times (2, 4 and 6h). The sustained study (Fig. 1B) was conducted in 33 hypercholesterolemic subjects (total cholesterol > 200 mg/dL). Participants ingested a daily dose of 25 mL of raw OO for 3 weeks, accordingly with the assigned sequence of intervention, preceded by 2-week washout periods with a common OO. To avoid an excessive intake of antioxidants during the clinical trial period, participants were instructed to limit the consumption of PC-rich foods. Blood samples were collected at the beginning and at the ending of each intervention, and sera and plasma EDTA samples were stored at –80°C until their use. Glucose and lipid profile were measured in plasma EDTA, in a Cobas-Mira Plus (Roche Diagnostic System, Spain) and ApoA-I, ApoA-II and ApoB-100 in a PENTRA-400 (ABX-Horiba Diagnostics, France) automated analyzers. The details of the participants and the protocol of both studies have been previously described [29, 33]. The present clinical trials were conducted in accordance with the Helsinki Declaration and the Good Clinical Practice for Trials on Medical Products in the European Community and International Conference of Harmonization. The subjects gave their written informed consent before their participation. Acute study was registered at ClinicalTrials.gov (Identifier: NCT01347515). Sustained study was registered at the International Standard Randomized Controlled Trial register (Identifier: ISRCTN77500181).

2.3 Experimental procedures with rats

To add insights to the underlying mechanisms involved in PON modulation, 20 male and female Wistar rats (Charles River Laboratories; Spain) weighing 300–350 g were allocated to four groups: CON, control diet; SEC, control diet supplemented with OO-PC extract, mainly secoiridoids or hydroxytyrosol derivatives; THY, diet supplemented with Th-PC extract; and SEC+THY, diet supplemented with secoiridoids and Th-PC extracts. Extracts were prepared as previously described [35]. SEC and SEC+THY extracts correspond to the ones used for FVOO and FVOOT preparation, respectively. Rats ingested 5 mg of the appropriate phenolic extract/kg rat/day for 21 days. As the matrix employed in the four groups was the same, i.e. the commercial feed pellets Teklad Global 14% Protein Rodent Maintenance Diet (Harlan Laboratories, Santa Perpètua de Mogoda, Spain), the effects observed between groups may be entirely attributed to their different phenolic content. The dose selected was extrapolated to human dose through normalization to body surface area [36]. THY group was used to investigate the effect of an equivalent phenolic dose exclusively from thyme. Details of the diets and dosage administered are explained in Supporting Information Table S2. Rats were anesthetized with isoflurane (IsoFlo, Veterinarian Esteve, Italy) and sacrificed by intracardiac puncture. After blood collection, rats were perfused with an isotonic solution of sodium chloride 0.9% to remove the remaining blood irrigating the tissues, and their livers were excised. Sera samples were obtained and stored at –80°C until their use. The procedures were conducted in accordance with the guidelines of the European Communities Directive 86/609/EEC regulating animal research and approved by the local ethical committee (CEEA-Universitat de Lleida, reference 7675). Glucose and lipid profile were measured in an autoanalyzer Cobas-Mira Plus (Roche Diagnostic System, Spain).

2.4 PON3 and PON1 protein concentration

PON3 and PON1 protein concentrations were assessed in human sera by in-house ELISA with rabbit polyclonal antibodies generated against synthetic peptides with sequences specific for mature PONs (kindly provided by Dr. Mackness, Manchester University, UK) as previously described [37].

2.5 Paraoxonase and lactonase activities

Since PON1 has esterase and lactonase activities we decided to measure the catalytic activity of PON1 using two different substrates: paraoxon (an ester) and 5-thiobutyl butyrolactone (TBBL, a synthetic lactone). Both activities were assessed in human sera as previously described [38, 39]. These activities are expressed as U/L (1U = 1μmol of substrate hydrolyzed

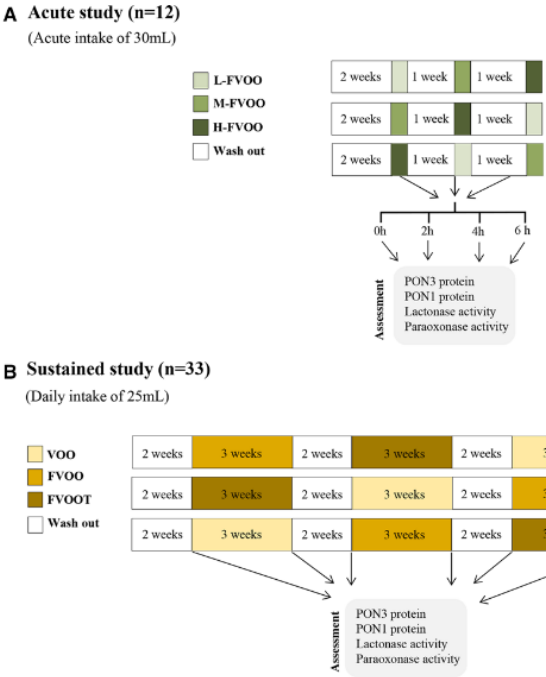

Figure 1. Human studies design. (A) In the acute study, the short-term effects of phenol-enriched VOOs differing in their PC content were assessed: L-FVOO with a low total phenol content (TPC; 250 ppm), M-FVOO with a medium TPC (500 ppm) and H-FVOO with a high TPC (750 ppm). (B) In the sustained study, the long-term effects of phenol-enriched VOOs differing in its composition were assessed: control VOO (80 ppm); FVOO, enriched with OO-PC (500 ppm); FVOOT, combining OO-PC (250 ppm; 50%) and Th-PC (250 ppm; 50%). PON1 and PON3 protein levels, and lactonase and paraoxonase activities were measured at the specified time-points.

per minute), and are referred as raw activities throughout this work. Specific activities were calculated as the ratio between the activity and the corresponding concentration.

2.6 Kinases array

A kinases proteome profiler (R&D systems, USA) was used in rat liver homogenates to assess the activation of not only the three major families of Mitogen-Activated Protein Kinases (MAPK; ERK1/2, JNK1-3 and p38α/β/δ/γ) but also other related kinases that play an important role in cellular signaling pathways. 125 mg of hepatic tissue were lysed using a commercial lysis buffer (Cayman Chemical, USA) containing DTT and proteases and phosphatases inhibitors. Finally, 200 μg of total protein assessed with Bradford assay, were employed to perform the array. Chemiluminescence signal was measured in Amersham Imager 600 reader and data were acquired using Image Quant TL program (GE Healthcare, Spain). CON values were set at 1 and SEC, THY and SEC+THY results are expressed as fold-increase or -decrease versus CON values.

2.7 Real-time quantitative PCR

Gene expression of *Pon1*, *Pon3*, *Ccl-2*, *aryl hydrocarbon receptor* (*Ahr*), *peroxisome proliferator-activated* (*Ppar*)α, δ and γ were analyzed in rat liver homogenates. 30 mg of hepatic tissue were homogenized and lysed with a rotor-stator DIAX 900 homogenizer (Heidolph, Germany). Total RNA was purified, quantified and reverse transcribed to cDNA as previously described [40]. Real-time quantitative PCR was performed in ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, USA). Data were analyzed using the $2^{-\Delta\Delta C_T}$ method with *glyceraldehyde 3-phosphate dehydrogenase* as housekeeping gene. CON values were set at 1 and SEC, THY and SEC+THY results are expressed as fold-increase or -decrease versus CON values.

2.8 Sample size and power analysis

In the acute study, a sample size of 12 individuals allows a power of at least 80% to detect a statistically significant difference between groups of 1.7 mg/dL of HDL cholesterol

(HDL-C) and a SD of 1.9. In the sustained study, a sample size of 30 individuals allows a power of at least 80% power to detect a statistically significant difference between groups of 3 mg/dL of HDL-C and a SD of 1.9. In both cases, a dropout rate of 15% and a Type I error of 0.05 (2-sided) were assumed.

2.9 Statistical analyses

Kruskal-Wallis test or 1-factor analysis of variance (ANOVA) with Bonferroni correction was used to discard differences in baseline characteristics. Carryover effect was discarded by testing a period-by-treatment interaction term in general linear models. A general linear model for repeated measures was used to assess the OOs effects within and between interventions (intra- and inter-intervention differences, respectively). In the animal studies, ANOVA with Bonferroni correction was used to assess the effects of the phenolic extracts. Pearson correlations were calculated between the variables described in results section. Statistical significance was defined as $p < 0.05$ for a 2-sided test. Analyses were performed using SPSS for Windows, version 22 (IBM corp., USA).

3 Results

3.1 Characteristics of the study participants

For the acute study 13 participants were recruited, and 12 were eligible and completed the study. For the sustained study, 62 participants were recruited and 33 were eligible to be enrolled in the study. No adverse events related to OO intake were reported. Baseline characteristics of acute and sustained study participants are described in Supporting Information Tables S3 and S4, respectively. No statistical differences were found between treatment sequences.

3.2 Acute study

3.2.1 PON3 and PON1 protein levels

All OO tested showed a similar time-course trend for PON3 characterized by a decrease at 4h-time point (4.2–11%; $p < 0.05$; Fig. 2A) versus their baseline. No statistical differences were observed among the functional VOOs tested at any time point. Regarding PON1 (Fig. 2B), L-FVOO and M-FVOO decreased this protein after 2 h of OO intake (5.1–6.4%; $p < 0.05$), while H-FVOO promoted a trend to increase PON1 at this time point versus baseline (6.8%), although without statistical significance. These different time-course trends were statistically significant at 2 h when comparing L-FVOO and M-FVOO with H-FVOO ($p < 0.05$). Detailed data of PON1-3 proteins are shown in Supporting Information Tables S5 and S6.

3.2.2 PON1 lactonase and paraoxonase activities

After all the functional VOOs tested, lactonase raw activity peaked at 2h-time point (3.3–7.1%) and then reached a steady state at 4 h (Fig. 2C). Inter-interventions changes were observed at 4h- and 6h-time points between H-FVOO and the other two functional VOO ($p < 0.05$). Concerning paraoxonase raw activity, L-FVOO and M-FVOO intake prompted a mild increase at 2h-time point followed by a decrease at 4h-time point ($p < 0.05$). In contrast, H-FVOO triggered a steady drop throughout the time-frame assessed ($p < 0.05$). Nevertheless, no inter-interventions changes were observed in the paraoxonase kinetics at any time point (Fig. 2D). Regarding lactonase and paraoxonase specific activities, both peaked 2 h after L-FVOO and M-FVOO intake, dropping to basal values at 4h-time point (Fig. 2E and F; $p < 0.05$). H-FVOO consumption followed different kinetic trend, as no peak was observed. When inter-intervention differences were addressed, H-FVOO showed lower paraoxonase and lactonase specific activities at 2 h when compared with L-FVOO and M-FVOO, respectively ($p < 0.05$). Detailed data of PON1-associated activities are shown in Supporting Information Tables S5 and S6.

3.3 Sustained study

3.3.1 PON3 and PON1 protein levels

PON3 levels increased by 5.1% after VOO intervention ($p < 0.05$; Fig. 3A). This increase was significant when compared with the changes observed after FVOO and FVOOT interventions ($p < 0.05$). PON1 levels decreased by 10.9–12.4% after VOO and FVOO respectively, whilst increased by 4.04% after FVOOT ($p < 0.05$; Fig. 3B). This increase turned out to be statistically significant when compared with the changes observed after VOO and FVOO interventions ($p < 0.05$).

3.3.2 PON1 lactonase and paraoxonase activities

FVOO decreased lactonase raw activity (3.39%; $p < 0.05$; Fig. 3C), which turned out to be significant when compared with VOO and FVOOT interventions. VOO increased and FVOOT decreased paraoxonase raw activity ($p < 0.05$; Fig. 3D). These changes were statistically different when compared with FVOO intervention ($p < 0.05$). VOO and FVOO increased lactonase and paraoxonase specific activities to similar extent (8.1–12%) whereas FVOOT promoted a trend to decrease these activities (9.3 and 13.7%, respectively) although without statistical significance (Fig. 3E and F). These decreases observed after FVOOT were statistically different when compared with the increases observed after VOO and FVOO intake ($p < 0.05$). Comprehensive data of PON levels obtained in the chronic study are shown in Supporting

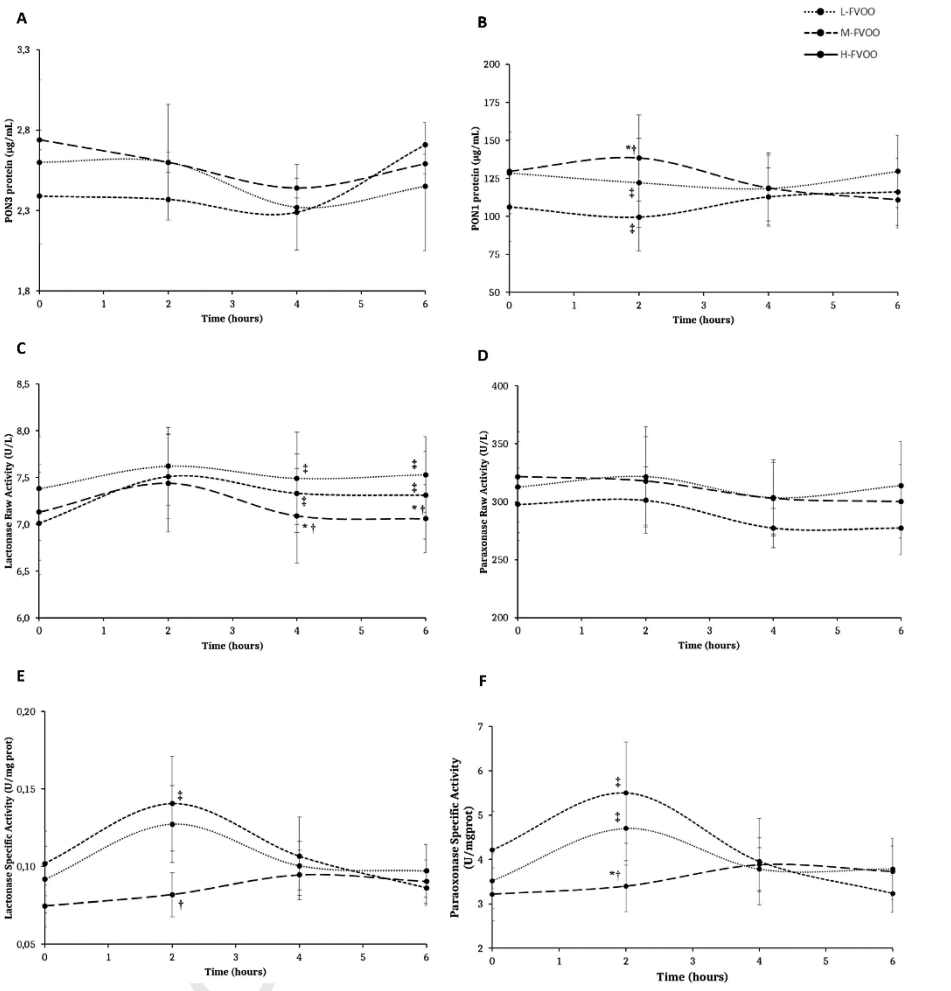

Figure 2. PON kinetics observed in the acute intake study. Participants ingested 30 mL of a phenol-enriched VOO differing in their PC content (L-FVOO with a low TPC, 250 ppm; M-FVOO with a medium TPC, 500 ppm; H-FVOO with a high TPC, 750 ppm). PON-related variables were assessed at different postprandial times. (A) PON3 protein, (B) PON1 protein, (C) Lactonase raw activity, (D) Paraoxonase raw activity, (E) Lactonase specific activity, (F) Paraoxonase specific activity. Results are expressed as mean ± SEM. $p < 0.05$ versus *L-FVOO; versus †M-FVOO; versus ‡H-FVOO.

Information Table S7. After all OO tested, strong positive correlations were observed between the activities assessed ($p < 0.05$; Supporting Information Table S8) but no between activities and PON1-3 proteins (data not shown). In addition, after VOO intake, paraoxonase specific activity directly correlated with ApoA-I determined in HDL and in plasma EDTA ($r = 0.529$ $p = 0.003$; $r = 0.481$ $p = 0.007$, respectively). Paraoxonase specific activity and ApoA-I also correlated positively after FVOO and FVOOT intake, but such correlations were weaker and statistically non-significant.

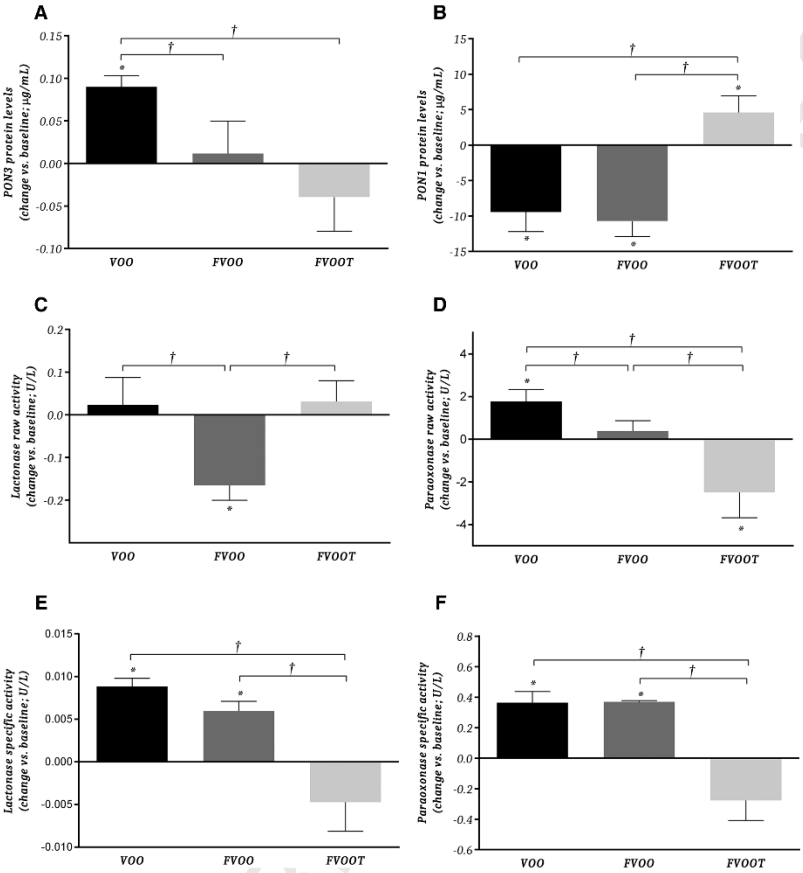

Figure 3. Changes in PON-related variables observed in the sustained study. Participants ingested 25 mL (22 g) of raw OO/day for 3 weeks: control VOO (80 ppm); FVOO enriched with its own PCs (500 ppm) or FVOOT enriched with the same content of PC but differing in their source (500 ppm; 50% OO-PC and 50% Th-PC). PON-related variables were assessed in serum samples at baseline and at the end of each intervention: (A) PON3 protein, (B) PON1 protein, (C) Lactonase raw activity, (D) Paraoxonase raw activity, (E) Lactonase specific activity, (F) Paraoxonase specific activity. Results are expressed as mean ± SEM. $^{*}p < 0.05$ versus baseline; $^{\dagger}p < 0.05$ between interventions.

3.4 Mechanistic study: Animal model

3.4.1 Glucose and plasma lipid profile

No statistical differences were found between treatment groups (CON, SEC, THY and THY+SEC) at the end of the interventions (Supporting Information Table S9).

3.4.2 Kinases activation

MAPK activation was assessed in rat hepatic tissue, as several transcription factors regulated by this kinase family modulate PON1 expression in human hepatic cells [41,42] and rat liver homogenates [43]. Activation of the vast majority of MAPK was observed after diet supplementation with SEC and THY

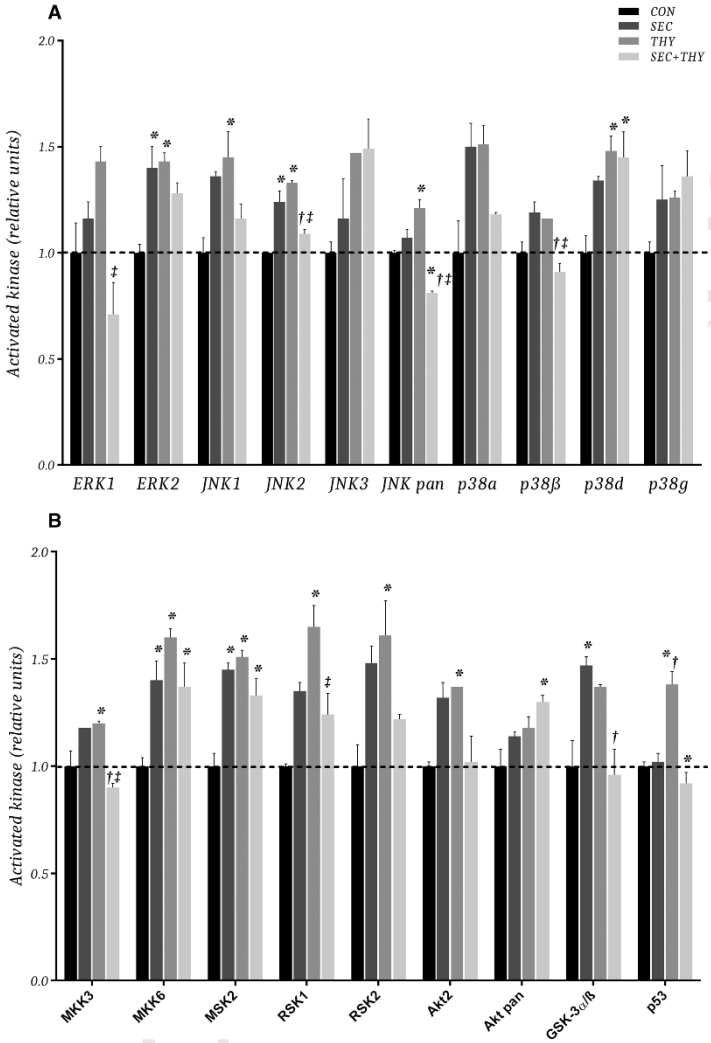

Figure 4. PC effects on (A) MAPK and (B) MAPK-related kinases activation measured in rat hepatic tissue. Rats ingested 5 mg of the appropriate phenolic extract/kg rat/day for 21 days and at the end of the study rats were anesthetized with isoflurane and sacrificed by intracardiac puncture. After blood collection, rats were perfused with an isotonic solution of sodium chloride 0.9% to remove the remaining blood irrigating the tissues, and their livers were excised. Hepatic tissue was then lysed and kinases activation was assessed in this tissue. Other kinases with no statistical changes were studied (HSP27, Akt1, Akt3, CREB, GSK-3β, and TOR; data not shown). CON values were set at 1 and SEC, THY and SEC+THY results are expressed as fold-increase or -decrease *versus* CON values. $*p < 0.05$ versus control; $\dagger p < 0.05$ versus SEC; $\ddagger p < 0.05$ versus THY.

extracts when compared with control diet (CON; $p < 0.05$; Fig. 4A). Diet supplementation with SEC+THY abolished ERK1, JNK2, JNK-PAN and p38β activation observed after SEC and THY intake ($p < 0.05$). Kinases, other than MAPK, activation was also assessed (Fig. 4B).

SEC and THY intake increased almost all these kinases activation to the same extent ($p < 0.05$), except for p53 that was activated only by THY. SEC+THY intake activated (MKK6, MSK2, Akt pan) and inhibited (p53) some kinases compared with CON ($p < 0.05$). This regulation resulted statistically significant also compared with SEC and THY (MKK3, RSK1 and GSK-3α/β; $p < 0.05$). Other kinases with no statistical changes were studied (HSP27, Akt1, Akt3, CREB, GSK-3β and TOR; data not shown).

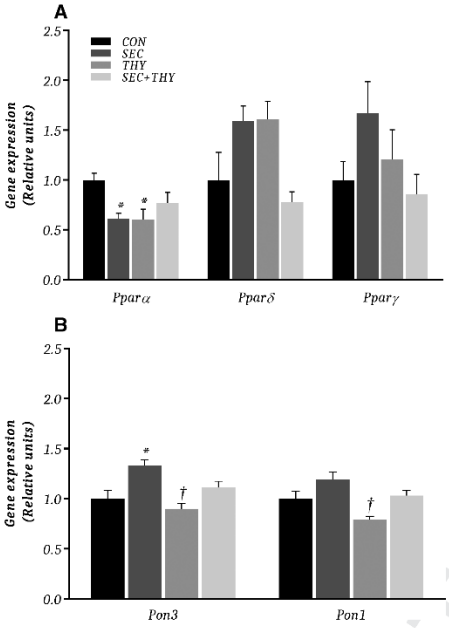

Figure 5. PC effects on gene expression in rat hepatic tissue. (A) *Pparα*, *Parδ* and *Pparγ*; (B) *Pon3* and *Pon1*. Rats ingested 5 mg of the appropriate phenolic extract/kg rat/day for 21 days and at the end of the study rats were anesthetized with isoflurane and sacrificed by intracardiac puncture. After blood collection, rats were perfused with an isotonic solution of sodium chloride 0.9% to remove the remaining blood irrigating the tissues, and their livers were excised, homogenized and lysed to perform Real-time quantitative PCR. CON values were set at 1 and SEC, THY and SEC+THY results are expressed as fold-increase or -decrease *versus* CON values. $*p < 0.05$ versus control; $\dagger p < 0.05$ versus SEC.

3.4.3 Transcription factors gene expression

SEC and THY diet supplementation decreased *Pparα* to the same extent compared to CON ($p < 0.05$), showing a tendency to increase *Pparδ* and *Pparγ*. No effects were observed after SEC+THY in any *Ppar* studied (Fig. 5A). No changes were observed on *Ahr* gene expression in response to the intake of any of the extracts studied (data not shown). However, *Ahr* gene expression positively correlated with *Pparδ* in SEC and had a tendency to correlate with *Pon1* in CON ($r = 0.953$; $p = 0.047$ and $r = 0.840$; $p = 0.075$, respectively; Supporting Information Table S10).

3.4.4 Pon1, Pon3 and Ccl-2 gene expression

SEC intake increased *Pon3* hepatic gene expression and had a tendency to increase *Pon1* versus CON group ($p < 0.05$), while THY intake decreased *Pon1* and *Pon3* versus SEC group ($p < 0.05$; Fig. 5B). *Pon1* gene expression correlated with *Pparγ* ($r = 0.966$; $p = 0.034$) and had a tendency to correlate with *Pparα* ($r = 0.932$; $p = 0.068$) in SEC group (Supporting Information Table S10). No statistical differences were shown in *Ccl-2* gene expression, surely due to the high inter-individual variability (data not shown).

4 Discussion

This study confirms the hypothesis that the acute and sustained intake of phenol-enriched VOOs modulates PON-related variables according to PC source and content. On the one hand, after the acute and sustained intake of OO-PC (via VOO and FVOO intake) a decrease in PON1 protein, together with increases in PON3 protein levels and PON1-associated specific activities are reported. On the other hand, sustained intake of a mixture of OO-PC and Th-PC (via FVOOT intake) induces opposite results rather than producing synergic effects as we had hypothesized. These differences may be due to the combination of OO-PC with Th-PC intake, rather than the sole presence of Th-PC, since mechanistic studies revealed that single-type PC modulated PON synthesis, while no effects were observed when PC were combined. To the best of our knowledge, this is the first time that a human trial has been conducted to integrate the short- and long-term effects of PC consumption on PON-related variables.

In the human acute study, the time-course trend for PON1 protein and associated specific activities turn out to be different according to the content of OO-PC of the FVOO consumed. PON1 protein reached minimum and specific activities peaked 2 h after L-FVOO and M-FVOO intake while different kinetics were observed with H-FVOO. Such observations are supported by the bioavailability of the OO-PC themselves, as OO-PC undergo a rapid absorption with T_{max} around 1–2 h after ingestion, and plasma concentrations return to baseline values 6 h after ingestion [24]. Actually, the selection of M-FVOO for the sustained-intake study was made according to previous works developed by our group. On the one hand, a pharmacokinetic study revealed that plasmatic phenolic metabolites did not show a complete linear response after 500 and 750 ppm, indicating that a threshold could exist in OO phenolic absorption [24]. On the other hand, M-FVOO provided additional benefits in the endothelial function versus L-FVOO and H-FVOO, and showed a better postprandial response on several cardiovascular risk biomarkers [44].

In the sustained intake study, this study shows that VOO and FVOO intake equally decreased PON1 protein and increased PON1-associated specific activities, irrespective of OO-PC content. Increased PON1 protein levels and decreased activities are characteristic of diseases whose underlying

mechanisms comprise an impairment of oxidative stress, in particular CVD [10,11,45,46], diabetes *mellitus* [47], inflammatory diseases [48,49], cancer, and several hepatic and renal diseases [50, 51], all characterized by having dysfunctional HDL particles and increased CVD risk [2]. Furthermore, PON1 activities positively correlate with the improvement of HDL antioxidant properties, to such an extent that PON1 activities have been proposed as new biomarkers of HDL function and CVD risk [10, 52]. Therefore, the decrease in PON1 protein and the increase in its associated activities can be perceived as being beneficial as they might be indicative of a proper oxidative balance and therefore of HDL function enhancement.

The combination of OO-PC with Th-PC via FVOOT yielded opposite effects than those observed after OO-PC intake, as FVOOT increased PON1 protein and decreased its associated specific activities. Hence, beneficial effects observed after OO-PC may be due to the secoiridoids (the main phenolic compounds of VOOs) irrespective of their content, and these effects may be reversed by Th-PC, mainly flavonoids. Our previous studies have reported that flavonoids present in FVOOT enhance cardioprotective benefits [29–33]. Similarly, pomegranate juice rich in flavonoids and phenolic acids, and extra-VOO enriched with green tea flavonoids, increase arylesterase activity in ApoE-deficient mice [16, 20] and humans [18]. Red wine flavonoids and stilbenes trigger paraoxonase activity in hypercholesterolemic hamsters [19]. The dissimilarities between the results obtained in the present work and those from other authors could be attributed to the combination of Th-PC with OO-PC, rather than the sole presence of flavonoids, as discussed further on.

PON3 protein levels increased after the sustained intake of control VOO suggesting that such VOO plays a protective role, as PON3 depletion from HDL is associated with the presence of subclinical atherosclerosis in patients with autoimmune diseases [7], chronic hepatitis and liver cirrhosis [53]. Our results partially agree with those observed in a proteomic study carried out in the HDL from the VOHF participants, where an increase in PON3 protein was reported after the sustained intake of VOO, FVOO and FVOOT [33].

The effects observed in PON-related variables in the present work can be translated in an enhancement of HDL functionally. Within the frame of the VOHF study, an improvement in HDL functionality, particularly in the HDL-mediated cholesterol efflux has been observed following phenol-enriched VOOs consumption (submitted data). Additional effects related to the enhancement of HDL function after phenol-enriched VOOs have already been published by our group such as improvement of oxidative status [29], DNA protection against oxidation [30], changes in HDL size and distribution [31] and changes in HDL proteome [33]. Moreover, PON1 activities have been proposed as promising biomarkers of HDL function and CVD risk [10,52]. Therefore, the increase in PON1-associated activities observed after the intake of phenol-enriched VOO may be indicative of HDL functionality enhancement.

In this study, mechanistic studies were conducted in rat liver homogenates to provide insight into the intracellular pathways involved in hepatic PON synthesis modulation [1–3, 34] and to disclose whether the effects observed after FVOOT were due to the Th-PC *per se* or to their combination with OO-PC. To date, this is the first time that p38 MAPK family and MAPK-related kinase effects on PON family are reported. The intake of a single-type PC (SEC or THY groups) activated the three families of MAPKs and a vast majority of MAPK-related proteins, decreased *Pparα*, and showed a tendency to increase *Pparδ* and *Pparγ* to the same extent, as similarly described by our group in human white blood cells [54]. Interestingly, these effects were not observed after the combination of PC (SEC+THY group). However, a minor impact on PON gene expression was observed. Despite showing equal effects on *Ppars* and kinases activation, SEC increased *Pon3* and showed a tendency to increase *Pon1* expression while THY decreased both *Pon1* and *Pon3. Moreover, Pon1* directly correlated with *Pparγ* and *Pparα* in the SEC group. This suggests that hepatic PON1 regulation may be mediated via *PPARγ*, which in turn is mediated via ERK1/2 activation, as previously described in glitazones, statins and pomegranate juice [34, 53, 54]. In concordance, our group has previously reported that SEC down-regulates Nuclear Factor-kB activation, a transcription factor that is inhibited by *PPARγ* [30]. THY decreased hepatic *PON1* and *PON3* expression compared to SEC, despite showing equal effects on *PPAR*, MAPK and MAPK-related proteins. These data suggest that Th-PC modulates PON hepatic synthesis by means of intracellular pathways other than the PPAR family. The combination of OO-PC with Th-PC had no impact on PON hepatic synthesis, in concordance with the lack of effects on kinases and PPARs activation observed in the current study. In general terms single-type PC, but not their combination, modulated intracellular pathways that regulate hepatic PON synthesis. These results help us to understand those obtained in the human studies. That is, the combination of OO-PC and Th-PC, rather than the sole intake of Th-PC, is accountable for the effects on PON-related variables.

Several factors other than PON hepatic synthesis regulation might explain the PC effects observed in the human studies. Those include hepatic PON1 degradation and secretion to the extracellular space [43], PON1 binding to HDL and activation by ApoA-I [3, 50, 57], and PON1 redistribution to large and mature HDL [47, 58]. Concordantly, we have previously reported that FVOO decreases the small-HDL/large-HDL ratio, increases HDL particle size [31], and increases CETP activity [29], all of them indicative of an enhancement of HDL maturation. Additionally, the EUROLIVE study showed that the presence of PC metabolites in HDL subsequent to the sustained intake of OO-PC-enriched VOOs was accompanied by the increase of HDL_2 and the decrease in HDL_3 particles [59].

Taking the previously data into consideration, the present work provides first level evidence of the modulation of PON enzymes family following the acute and sustained intake of PC-enriched VOOs, according to PC source and content. Sev-

eral mechanisms may be involved in PON enzymes family modulation, such as the activation of hepatic MAPK and *Ppars* among others. The changes in PON enzymes family observed may be translated into an improvement in the oxidative balance and therefore in the HDL functionality.

SF-C, A-IG-H, M-CLH, AP, and UC conceived, designed and performed the experiment. SF-C, A-IG-H, RS, and JC analyzed and interpreted the data. SF-C, RS, and JC drafted the paper. This paper is critically revised by RS, JC, M-CLH, MF, AP, LR, M-JM, OC, M-IC, and R-MV.

This study was supported by the Spanish Ministry of Economy (MINECO) financing the projects AGL2009-13517-457 C03-01, C03-02 and C03-3, AGL2012-40144-C01-03, C02-03 and C03-03, and the FPI-fellowship (BES-2010-040766), by a contract from the Catalan Government and the ISCIII-FEDER (FISCP06/00100), and by grants from ISCIII FEDER (CB06/03/0028), AGAUR (2014SGR240) and AGAUR (2014SGR783). AP is Torres Quevedo contract (Subprograma Estatal de Incorporación, Plan Estatal de Investigación Científica y Técnica y de Innovación). UC is a PERIS contract (Pla estratègic de recerca i innovació en salut). LR is Sara Borell contract (CD14/00275) co-financed by and IISPV. Thanks to the University of Lleida for the M-CLH PhD. grant.

CIBERDEM and CIBEROBN is an initiative of the Health Institute Carlos III.

We thank Borges Mediterranean Group for providing the common olive oil.

There are no competing interests to declare.

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Supporting Information Table S1. Phenolic intake through the ingested dose of the VOOs used in the acute (30 mL) and sustained study (25 mL), determined by HPLC-MS/MS. Values are expressed as mg compound by ingested dose.

Compound	Acute study			Sustained study		
(mg/oil ingested dose) †	L-FVOO	M-FVOO	H-FVOO	VOO	FVOO	FVOOT
Hydroxytyrosol	0.21	0.29	0.37	0.01	0.21	0.12
3,4-DHPEA-AC	0.16	0.60	0.90	n.d.	0.84	0.39
3,4-DHPEA-EDA*	1.51	4.67	8.42	0.04	6.73	3.43
3,4-DHPEA-EA*	0.36	0.71	1.18	0.26	0.71	0.36
Total hydroxytyrosol derivatives	*2.23*	*6.27*	*10.87*	*0.30*	*8.49*	*4.30*
p-hydroxybenzoic acid	0.00	0.02	0.03	n.d.	0.02	0.06
Vanillic acid	0.02	0.08	0.10	n.d.	0.07	0.13
Caffeic acid	n.d	n.d	n.d	n.d.	0.00	0.06
Rosmarinic acid	n.d	n.d	n.d	n.d.	n.d.	0.41
Total phenolic acids	*0.02*	*0.10*	*0.13*	-	*0.09*	*0.65*
Pinoresinol	0.27	0.19	0.19	0.05	0.12	0.10
Acetoxipinoresinol**	6.95	5.98	5.81	2.47	3.66	3.24
Total lignans	*7.22*	*6.17*	*6.00*	*2.52*	*3.78*	*3.34*
Luteolin	0.17	0.28	0.57	0.04	0.18	0.21
Apigenin	0.06	0.08	0.10	0.02	0.06	0.10
Naringenin	n.d.	n.d.	n.d.	n.d.	n.d.	0.20
Eriodictyol	n.d.	n.d.	n.d.	n.d.	n.d.	0.17
Thymusin	n.d.	n.d.	n.d.	n.d.	n.d.	1.22
Xanthomicrol	n.d.	n.d.	n.d.	n.d.	n.d.	0.53
7-methylsudachitin	n.d.	n.d.	n.d.	n.d.	n.d.	0.53
Total flavonoids	*0.22*	*0.35*	*0.67*	*0.06*	*0.23*	*2.95*
Thymol	n.d.	n.d.	n.d.	n.d.	n.d.	0.64
Carvacrol	n.d.	n.d.	n.d.	n.d.	n.d.	0.23
Total monoterpenes	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*n.d.*	*0.86*
Total phenols HPLC-MS/MS	9.69	12.89	17.67	2.88	12.59	12.10

† Dose-response study: mg/30mL oil; Sustained study: mg/25mL oil.

* Quantified with calibration curve of hydroxytyrosol.

**Quantified with calibration curve of pinoresinol.

Abbreviations: L-FVOO, Virgin Olive Oil enriched with a Low phenolic content (250ppm); M-FVOO, Virgin Olive Oil enriched with a Medium phenolic content (500ppm); H-FVOO, Virgin Olive Oil enriched with a High phenolic content (75ppm); VOO, Virgin Olive Oil (80ppm); FVOO,

Functional Virgin Olive Oil enriched with its own phenolics (500ppm); FVOOT, Functional Virgin Olive Oil enriched with both its own phenolics (250ppm) and phenolics from Thyme (250ppm); 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; n.d.: not detected.

Supporting Information Table S2. Diet characteristics of the animal experiment.

Group	Diet characteristics	Dose administered through feedstuff
CON: control	Not-enriched feedstuff	Not applied
SEC: Secoiridoids (Equivalent to FVOO)	Feedstuff enriched with OO-PC extract, mainly secoiridoids or hydroxytyrosol derivatives extracted from olive cake*	5 mg 3,4-DHPEA-EDA/kg weight rat
THY: Thyme PC	Feedstuff enriched with Th-PC extract, PC from thyme**	5 mg of thyme PC (rosmarinic acid + thymusin)/kg weight rat
SEC+THY: Secoiridoids + Thyme PC (Equivalent to FVOOT)	Feedstuff enriched with a combination of OO-PC extracted from olive cake* and Thy-PC extracted from thyme (*Thymus zyguis*)**	2.5 mg 3,4-DHPEA-EDA/kg weight rat + 2.5 mg thyme PC (rosmarinic acid + thymusin)/kg weight rat

* Dose prepared based in the main PC in the olive cake extract: 3,4-DHPEA-EDA (dialdehydic form of elenolic acid linked with hydroxytyrosol).

** Dose prepared based in the concentration of the main PC in the thyme extract: rosmarinic acid and the flavonoid thymusin.

Abbreviations: PC, Phenolic Compounds; OO-PC, Olive oil PCs; Th-PC, PC from Thyme; FVOO, Functional Virgin Olive Oil enriched with its own phenolics (500ppm); FVOOT, Functional Virgin Olive Oil enriched with both its own phenolic compounds (250ppm) plus phenolic compounds from Thyme (250ppm).

Supporting Information Table S3. Baseline characteristics of the acute study participants (n=12).

Variable	Mean ± SD
Age (years)	53 ± 13
Gender (male/female)*	6/6
BMI (Kg/m^2)	27.20 ± 2.77
Weight (Kg)	74.35 ± 13.88
SBP (mm Hg)	128.75 ± 21.34
DBP (mm Hg)	79.08 ± 15.91
Glucose (mg/dL)	92.44 ± 11.33
TC (mg/dL)	211.46 ± 35.34
LDL-C (mg/dL)	137.87 ± 32.39
HDL-C (mg/dL)	52.78 ± 11.75
PL (mg/dL)	209.70 ± 21.72
TG **	94(75.00; 149.00)
ApoA-I (mg/dL)	133.67 ± 18.06
ApoA-II (mg/dL)	29.53 ± 3.18
ApoB-100 (mg/dL)	104.33 ± 23.72

* Values are expressed as number of individuals.

** Values are expressed as median (25th; 75th percentiles).

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; PL, phospholipids; TG, triglycerides.

Supporting Information Table S4. Baseline characteristics of the sustained study participants (n=33).

Variable	Sequence 1	Sequence 2	Sequence 3
Age (y)	54.9 ± 12.6	55.3 ± 11.9	55.5 ± 7.8
Gender (male/female)*	5/6	7/4	7/4
BMI (kg/m^2)	25.6 ± 3.7	26.3 ± 5.2	27.8 ± 4.7
Weight (kg)	74.8 ± 16.8	74.6 ± 19.5	84.5 ± 17.7
SBP (mmHg)	125 ± 18.7	128 ± 16.7	130 ± 17.9
DBP (mmHg)	68.1 ± 13.5	72.3 ± 9.3	71.9 ± 13.4
Glucose (mg/dL)	88.6 ± 11.6	93.0 ± 13.3	90.9 ± 10.5
TC (mg/dL)	228.4 ± 42.7	231.9 ± 32.7	218.8 ± 31.2
LDL-C (mg/dL)	150.4 ± 32.3	152.1 ± 28.5	142.3 ± 25.7
HDL-C (mg/dL)	52.8 ± 11.8	53.0 ± 12.8	53.4 ± 9.6
PL (mg/dL)	231.4 ± 35.2	228.9 ± 29.1	222.0 ± 16.2
TG (mg/dL)**	94.00	119.00	117.00
ApoA-I (mg/dL)	(75.00; 149.00)	(95.00; 168.00)	(81.00; 126.00)
ApoA-II (mg/dL)	142.2 ± 22.7	136.6 ± 20.6	147.5 ± 16.3
ApoB-100 (mg/dL)	31.9 ± 4.7	30.2 ± 3.8	31.0 ± 2.8

Values are expressed as mean ± SD unless otherwise stated.
* Values are expressed as number of individuals.
** Values are expressed as median (25th; 75th percentiles).

Sequence 1: FVOO, FVOOT, VOO; Sequence 2: FVOOT, VOO, FVOO; Sequence 3: VOO, FVOO, FVOOT. No statistical differences were found between sequences.

Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-c, HDL cholesterol; LDL-c, LDL cholesterol; PL, phospholipids; TG, triglycerides.

Supporting Information Table S5. Within-intervention changes in PON-related variables observed in the acute intake study (n=12).

Variables	L-FVOO				M-FVOO				H-FVOO			
	0h	2h	4h	6h	0h	2h	4h	6h	0h	2h	4h	6h
PON3 protein (µg/mL)	2,60 ± 0,51	2,60 ± 0,36	2,32 ± 0,27 †	2,45 ± 0,40 †	2,39 ± 0,32	2,37 ± 0,31	2,29 ± 0,38	2,71 ± 0,43 *†‡	2,74 ± 0,58	2,60 ± 0,39	2,44 ± 0,38 †	2,59 ± 0,39 †‡
PON1 protein (µg/mL)	128,6 ± 27,0	122,06 ± 29,259 *	118,22 ± 23,571 *	129,56 ± 23,823 ‡	106,22 ± 22,456	99,47 ± 22,227 *	112,75 ± 19,256 †	116,13 ± 22,13 *†	129,68 ± 21,26	138,44 ± 28,249	118,72 ± 21,578 *†	110,93 ± 18,559 *†‡
Lactonase raw activity (U/L)	7,38 ± 0,55	7,62 ± 0,414	7,49 ± 0,491	7,53 ± 0,404 †	7,01 ± 0,545	7,51 ± 0,451 *	7,33 ± 0,42 *†	7,31 ± 0,468 *†	7,13 ± 0,513	7,44 ± 0,524 *	7,09 ± 0,506 *†	7,06 ± 0,363 †
Paraxonase raw activity (U/L)	312,7 ± 39,3	321,6 ± 43,2 *	303,5 ± 32,8	314,0 ± 38,1	297,7 ± 31,2	301,3 ± 28,8	277,2 ± 17	277,3 ± 23,2 *†	321,6 ± 38,7	317,9 ± 38,1 *	303,0 ± 31,0 *	300,3 ± 31,8 *†‡
Lactonase especific activity (U/mg prot)	0,092 ± 0,022	0,127 ± 0,025 *	0,101 ± 0,016 †	0,097 ± 0,017 †‡	0,102 ± 0,021	0,141 ± 0,031 *	0,107 ± 0,025 †	0,086 ± 0,011 †	0,075 ± 0,013	0,082 ± 0,014 *	0,095 ± 0,016 *†	0,090 ± 0,014 *†
Paraxonase especific activity (U/mg prot)	3,52 ± 0,63	4,70 ± 0,83 *	3,78 ± 0,48 †	3,78 ± 0,69 *†	4,21 ± 0,87	5,50 ± 1,14 *	3,95 ± 0,97 *†	3,23 ± 0,42 †	3,21 ± 0,59	3,40 ± 0,57 *	3,88 ± 0,61 *†	3,73 ± 0,57 *†‡

Values are expressed as mean ± SEM: µg/mL for protein determinations; U/L for raw activities; U/mg of protein for specific activities.

* p≤ 0.05 *vs*. 0h; † p≤ 0.05 *vs*. 2h; ‡ p≤ 0.05 *vs*. 4h.

Abbreviations: L-FVOO, Virgin Olive Oil enriched with a Low phenolic content (250ppm); M-FVOO, Virgin Olive Oil enriched with a Medium phenolic content (500ppm); H-FVOO, Virgin Olive Oil enriched with a High phenolic content (750ppm).

Supporting Information Table S6. Inter-interventions differences in PON-related variables in the acute intake study (n=12).

Variables	L-FVOO vs M-FVOO			L-FVOO vs H-FVOO			M-FVOO vs H-FVOO		
	2h	4h	6h	2h	4h	6h	2h	4h	6h
PON3 protein (µg/mL)	0,314 (-0,323 to 0,951)	0,128 (-0,684 to 0,939)	-0,175 (-0,858 to 0,508)	0,121 (-0,710 to 0,952)	0,966 (-0,818 to 0,850)	-0,024 (-0,872 to 0,525)	-0,193 (-0,952 to 0,533)	-0,111 (-0,914 to 0,691)	0,152 (-0,700 to 1,003)
PON1 protein (µg/mL)	3,733 (-9,388 to 16,855)	-9,741 (-19,736 to 0,255)	-2,019 (-14,184 to 10,147)	-25,695* (-50,773 to -0,618)	-8,343 (-27,592 to 10,906)	9,688 (-8,903 to 28,280)	-29,429* (-57,771 to -1,086)	1,398 (-19,832 to 22,627)	11,707 (-11,867 to 35,281)
Lactonase raw activity (U/L)	-0,136 (-0,307 to 0,034)	-0,164 (-0,379 to 0,052)	-0,021 (-0,216 to 0,174)	0,072 (-0,244 to 0,388)	0,213* (0,093 to 0,332)	0,334* (0,094 to 0,574)	0,208 (-0,049 to 0,465)	0,376* (0,082 to 0,670)	0,355 * (0,137 to 0,572)
Paraxonase raw activity (U/L)	-4,427 (-36,256 to 27,401)	6,687 (-27,649 to 41,023)	14,509 (-17,820 to 46,837)	-9,127 (-50,703 to 32,449)	-8,263 (-39,923 to 23,396)	1,560 (-35,562 to 38,683)	-4,700 (-44,353 to 34,953)	-14,951 (-52,731 to 22,830)	-12,948 (-46,330 to 20,433)
Lactonase especific activity (U/mg prot)	-0,010 (-0,069 to 0,049)	-0,005 (-0,052 to 0,043)	0,014 (-0,020 to 0,048)	0,049 (-0,004 to 0,101)	0,006 (-0,030 to 0,043)	0,008 (-0,029 to 0,045)	0,059* (0,008 to 0,109)	0,011 (-0,026 to 0,048)	-0,006 (-0,025 to 0,012)
Paraxonase especific activity (U/mg prot)	-0,622 (-2,765 to 1,522)	-0,093 (-1,943 to 0,757)	0,747 (-0,625 to 2,118)	1,507* (0,314 to 0,701)	-0,014 (-0,771 to 0,743)	0,214 (-0,712 to 1,140)	2,129 (-0,062 to 4,319)	0,079 (-1,762 to 1,921)	-0,533 (-1,615 to 0,548)

Values are expressed as differences mean (95% CI): µg/mL for protein determinations; U/L for raw activities; U/mg of protein for specific activities.

* p≤ 0.05 between specified interventions.

Abbreviations: L-FVOO, Virgin Olive Oil enriched with a Low phenolic content (250ppm); M-FVOO, Virgin Olive Oil enriched with a Medium phenolic content (500ppm); H-FVOO, Virgin Olive Oil enriched with a High phenolic content (750ppm).

Supporting Information Table S7. Intra- and inter-interventions differences in PON-related variables observed in the sustained intake study (n=33).

Variable	VOO			FVOO			FVOOT			p-value for trend	Between-groups differences					
	Post-Interv	Change		Post-Interv	Change		Post-Interv	Change			FVOO vs VOO		FVOOT vs VOO		FVOOT vs FVOO	
PON3 protein (µg/mL)	1,67	0,09 (0,06 to 0,12)	5,11% *	1,52	0,01 (-0,07 to 0,09)	0,72%	1,51	-0,04 (-0,12 to 0,04)	-2,72%	0,017 ‡ 0,001 §	-0,08	(-0,14 to -0,02) †	-0,13	(-0,19 to -0,07) †	-0,05	(-0,11 to 0,01)
PON1 protein (µg/mL)	96,01	-9,43 (-15,07 to -3,79)	-10,89% *	96,74	-10,70 (-15,19 to -6,22)	-12,44% *	109,45	4,61 (-0,20 to 9,43)	4,04% *	0,014 ‡	-1,28	(-5,37 to 2,82)	14,04	(10,69 to 17,39) †	15,32	(11,74 to 18,89) †
Lactonase raw activity (U/L)	5,42	0,02 (-0,11 to 0,15)	0,44%	5,06	-0,17 (-0,24 to -0,09)	-3,39% *	5,14	0,03 (-0,07 to 0,13)	0,60%	<0,001 ‡ <0,001 §	-0,20	(-0,27 to -0,11) †	0,01	(-0,06 to 0,08)	0,20	(0,15 to 0,25) †
Paraxonase raw activity (U/L)	203,38	1,78 (0,66 to 2,90)	0,87% *	203,02	0,38 (-0,62 to 1,38)	0,19%	198,52	-2,49 (-4,93 to -0,06)	-1,27% *	<0,001 ‡ <0,001 §	-1,40	(-1,64 to -1,16) †	-4,27	(-5,63 to -2,92) †	-2,87	(-4,38 to -1,37) †
Lactonase especific activity (U/mg prot)	0,07	0,01 (0,01 to 0,01)	11,28% *	0,07	0,01 (0,004 to 0,01)	8,11% *	0,06	-0,01 (-0,01 to 0,002)	-9,25%	0,001 §	0,00	(-0,01 to 0,001)	-0,01	(-0,02 to -0,01) †	-0,01	(-0,02 to -0,01) †
Paraxonase especific activity (U/mg prot)	2,67	0,36 (0,22 to 0,51)	12,01% *	2,80	0,37 (0,36 to 0,38)	11,69% *	2,30	-0,28 (-0,55 to -0,01)	-13,72%	<0,001 §	0,01	(-0,13 to 0,14)	-0,64	(-1,04 to -0,25) †	-0,65	(-0,93 to -0,37) †

Changes are expressed as mean (95%CI) and % of change *vs.* baseline: µg/mL for protein determinations; U/L for raw activities; U/mg of protein for specific activities. *p≤0.05 *vs.* baseline; † p≤0.05 between treatments. P-value for trend among oils: ‡ p-value for linear trend, § for quadratic trend.

Abbreviations: Post-Interv, post-intervention; p-value for trend, p-value for trend among oils; VOO, Virgin Olive Oil (80ppm); FVOO, Functional Virgin Olive Oil enriched with its own phenolics (500ppm); FVOOT, Functional Virgin Olive Oil enriched with both its own phenolics (250ppm) and phenolics from Thyme (250ppm).

Supporting Information Table S8. Pearson correlations between PON1-associted activities.

		Lactonase			Specific lactonase		
		VOO	**FVOO**	**FVOOT**	**VOO**	**FVOO**	**FVOOT**
Paraxonase	Pearson correl	0,753	0,695	0,675	-	-	-
	(Sig)	(<0,001)	(<0,001)	(<0,001)			
Specific paraxonase	Pearson correl	-	-	-	0,912	0,944	0,944
	(Sig)				(<0,001)	(<0,001)	(<0,001)

Results are expressed as Pearson correlation coefficient (p-value).

Abbreviations: VOO, Virgin Olive Oil (80ppm); FVOO, Functional Virgin Olive Oil enriched with its own phenolics (500ppm); FVOOT, Functional Virgin Olive Oil enriched with both its own phenolics (250ppm) and phenolics from Thyme (250ppm).

Supporting Information Table S9. Glucose and lipid profile of the animals employed for the mechanistic study.

Variables	CON	SECO	THY	SECO+THY
Weight (gr)	128,62 ± 2,91	129,92 ± 3,23	127,50 ± 2,31	129,12 ± 3,07
Glucose (mg/dL)	233,80 ± 18,78	212,80 ± 40,53	233,60 ± 23,85	223,60 ± 48,24
TC(mg/dL)	68,68 ± 7,42	59,32 ± 11,16	63,42 ± 12,90	67,75 ± 11,45
HDL-c (mg/dL)	29,78 ± 7,34	22,59 ± 4,25	26,68 ± 6,06	27,46 ± 5,59
LDL-c (mg/dL)	8,04 ± 3,81	5,87 ± 1,94	6,03 ± 2,23	6,50 ± 1,48
PL (mg/dL)	170,94 ± 33,69	144,76 ± 21,48	162,93 ± 12,30	172,33 ± 19,66
TG (mg/dL)	80,60 ± 43,34	59,88 ± 22,17	81,84 ± 58,85	109,12 ± 74,42

Results are expressed as mean ± SD.

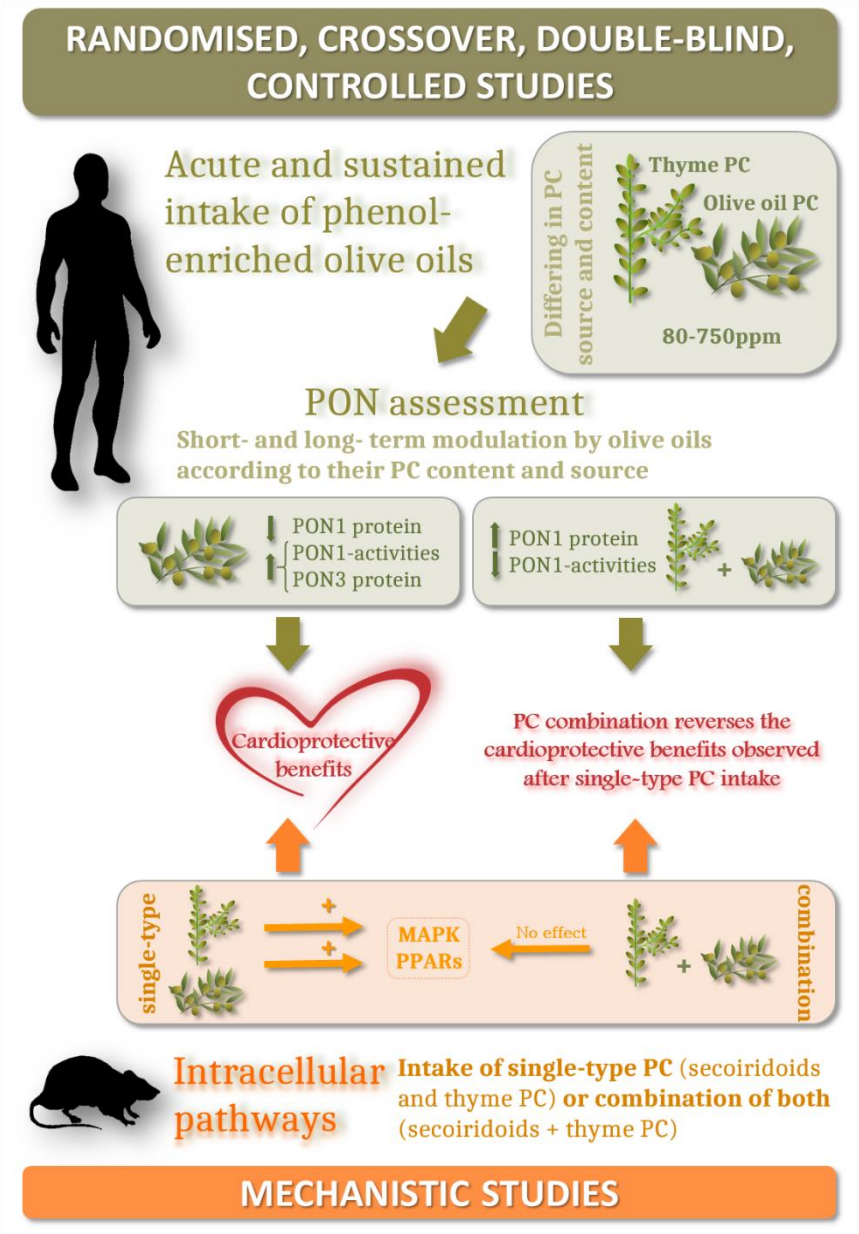
Abbreviations: PC, phenolic compounds; CON, control diet group; SECO, feedstuff enriched with secoiridoids (3.4-DHPEA-EDA) extracted from olive cake group; THY, feedstuff enriched with PC from thyme (rosmarinic acid and thymusin) group; SEC+THY, feedstuff enriched with a combination of secoiridoids extracted from olive cake and PC from thyme group; TC, total cholesterol; HDL-c, HDL cholesterol; LDL-c, LDL cholesterol; PL, phospholipids; TG, triglycerides.

Supporting Information Table S10. Pearson correlations between transcription factors and PON gene expression performed in livers of rats.

		PPARα				PPARδ				PPARγ				AHR				CCL2			
		CON	SEC	THY	SEC+THY	CON	SEC	THY	SEC+THY	CON	SEC	THY	SEC+THY	CON	SEC	THY	SEC+THY	CON	SEC	THY	SEC+THY
PON1	Pearson correl	ns	0,932	ns	ns	ns	ns	ns	ns	ns	0,966	ns	ns	0,840	ns	ns	ns	ns	ns	ns	ns
	(Sig)		0,068								0,034			0,075							
PON3	Pearson correl	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	(Sig)																				
PPARα	Pearson correl	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	(Sig)																				
PPARδ	Pearson correl	ns	ns	ns	ns	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	(Sig)																				
PPARγ	Pearson correl	ns	ns	ns	ns	ns	ns	ns	ns	-	-	-	-	-	-	-	-	-	-	-	-
	(Sig)																				
AHR	Pearson correl	ns	ns	ns	ns	ns	0,953	ns	ns	ns	ns	ns	ns	-	-	-	-	-	-	-	-
	(Sig)						0,047														
CCL2	Pearson correl	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	-	-	-	-
	(Sig)																				

Results are expressed as Pearson correlation coefficient (p-value). Abbreviations: CON, control diet group; SEC, group whose diet was supplemented with secoiridoids; THY, group whose diet was supplemented with thyme phenols; SEC+THY, group whose diet was supplemented with secoiridoids and thyme phenols; *Ppar*, peroxisome proliferator-activated receptor; *Ahr*, aryl hydrocarbon receptor; *Ccl-2*, chemokine (C-C motif) ligand 2; ns, no significant.

Graphical abstract



Two randomized, controlled, double-blind, and crossover interventions were conducted:

a) Acute study: intake of functional virgin olive oils (FVOO) differing in phenolic compounds (PC) content;

b) Sustained study: intake of different FVOOs (same PC content but different source).

Main outcomes include that the acute and sustained consumption of olive oil-PC in humans modifies PON-related variables towards a beneficial mode, increasing PON1 protein and decreasing its specific activities and PON3 protein. A combination of olive oil and thyme PC cancels such regulation.

Study 4

Determinants of HDL cholesterol efflux capacity after virgin olive oil ingestion: Interrelationships with fluidity of HDL monolayer

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Manuscript under review

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS
Sara Fernández Castillejo

Determinants of HDL cholesterol efflux capacity after virgin olive oil ingestion: Interrelationships with fluidity of HDL monolayer.

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Keywords: BODIPY-cholesterol, cholesterol efflux capacity, fluidity, HDL, Virgin Olive Oil.

ABSTRACT

Scope Cholesterol efflux capacity of HDL (CEC) is inversely associated with cardiovascular risk. High-density lipoprotein (HDL) composition, fluidity, oxidation, and size are related with CEC. We aimed at which HDL parameters were CEC determinants after virgin olive oil (VOO) ingestion.

Methods and Results Post-hoc analyses from the VOHF study, a crossover intervention with three types of VOO. We assessed the relationship of 3-week changes in HDL-related variables after intervention periods with independence of the type of VOO. After univariate analyses, mixed linear models were fitted with variables related with CEC and fluidity. Fluidity and Apolipoprotein (Apo)A-I content in HDL were directly associated, and HDL oxidative status inversely, with CEC. A reduction in free cholesterol and an increase in triglycerides in HDL, and a decrease in small HDL particle number or an increase in HDL mean size, were associated to HDL fluidity.

Conclusions HDL fluidity, ApoA-I concentration, and oxidative status are major determinants for CEC after VOO. The impact on CEC of changes in free cholesterol and triglycerides in HDL, and those of small HDL or HDL mean size, could be mechanistically linked through HDL fluidity. Our work points out novel therapeutic targets to improve HDL functionality in humans through nutritional or pharmacological interventions.

Total word count: 203

INTRODUCTION

High-density lipoprotein (HDL) cholesterol levels have been shown to be inversely related to cardiovascular disease [1]. However, data from multiple gene association [2] and intervention [3] studies lead to consider the functional quality of HDL as a more important parameter than the circulating quantity of HDL. The main functional property of the HDL particle, the reverse cholesterol transport pathway, is the capacity of HDL to remove the excess of cholesterol in peripheral cells and transport it to the liver cells to be metabolized. In particular, cholesterol efflux, the first step of the reverse cholesterol transport, from macrophages to HDL in atherosclerotic plaques is thought to be critical in the anti-atherogenic effect of HDL [4]. Cholesterol efflux capacity of HDL (CEC) has been shown to be inversely associated with prevalent coronary disease and incident atherosclerotic cardiovascular disease and is considered a useful biomarker for cardiovascular risk [5,6].

Several HDL characteristics have been related to its CEC, namely composition, fluidity of the monolayer, oxidative status, and size [7]. Interrelationships among these features have also been described. First, HDL composition deeply affects its function. The distribution of HDL lipids between surface and core is crucial for apolipoprotein (Apo)A-I stability in HDL, a key protein involved in CEC [4], although ApoA-II is also able to efficiently remove cholesterol from macrophages in vivo [8]. From our previous data, changes in HDL composition also modulate HDL fluidity [9, 10], considered an intermediate marker of enhanced HDL functionality [11]. Low levels of phospholipids, or high levels of free cholesterol (FC), on the HDL surface may contribute to a less fluid HDL, and therefore a less functional one [4]. Second, HDL oxidation also plays a pivotal role in HDL functionality. We have previously reported that oxidized HDL are less fluid, and therefore less able to perform cholesterol efflux from human THP-1 macrophages [11, 12]. This decrease in HDL functionality could be promoted through the impairment of the ApoA-I binding to cholesterol transporters due to ApoA-I/HDL oxidation [13,14]. Finally, the relationship between HDL particle size and CEC remains controversial [15,16]. On one hand, in some in vitro studies small HDL particles were the most efficient mediators of CEC [15]. On the other hand, large HDL particles were the

best promoters for CEC in human studies, being also related to coronary endothelial dysfunction [16].

From our data, and those from others, consumption of virgin olive oil (VOO) increases not only the plasma HDL cholesterol concentration levels [17], but also the CEC, and HDL fluidity [10, 18, 19], due, at least in part, to a transcriptomic effect [20]. As we previously reported in experimental and human studies, phenol-containing VOO, besides to increase HDL fluidity [10, 18], has shown to: modify HDL composition by increasing the HDL content of oleic acid [21] and ApoA-I and ApoA-II levels [22], increase the HDL antioxidant content [23], improve the HDL oxidative status [18, 20, 23], and promote a less atherogenic HDL subclasses profile [18, 24]. However, it still remains unknown how these functional enhancements are inter-related and how they all explain an improvement in HDL functionality. Due to this, in this study we examined the associations between CEC and variables related with HDL composition, fluidity, oxidative/antioxidative status, and particle size after VOO consumption. Our aim was to define which parameters can be the best predictors for CEC after a dietary intervention which enhances it.

METHODS

Subjects

Thirty-three hypercholesterolemic (total cholesterol > 200 mg/dL) individuals (19 men and 14 women) were recruited from newspaper and University advertisements. Volunteers were preselected when non-smokers, and their clinical record, physical examination, and blood pressure were within a normal range. Next, complete blood count, routine biochemical laboratory analyses, and urinary dipstick tests were performed. Candidates were included when values, other than total and low-density lipoprotein cholesterol (LDL-C), were within the reference range. Exclusion criteria were: LDL-C $\geqslant$190 mg/dL, triglycerides $\geqslant$350 mg/dL, fasting blood glucose >126 mg/dL, plasma creatinine levels >1.4 mg/dL for women and >1.5 mg/dL for men, body mass index (BMI) > 35 kg/m^2, smokers (> 1 cigarrete/day), athletes with physical activity (> 3000

METS.min/day), hypertension, multiple allergies, intestinal diseases, chronic diseases (i.e. diabetes, cardiovascular, etc.), or other conditions that would impair the adherence to the study. Participants provided written informed consent, and the local institutional ethics committee approved the protocol (CEIC-IMAS 2009/3347/I).

Design and study procedure

This work was conducted in the frame of the VOHF (Virgin Olive Oil and HDL Functionality) Study. A crossover, double-blind, controlled trial was performed with three types of olive oil: a natural VOO containing its phenolic compounds, and this VOO enriched with its phenolic compounds or with them plus those from thyme in a 1:1 ratio. The three VOOs did not differ in fat and micronutrient composition, with the exception of the phenolic content (80, 500, and 500 ppm, respectively). Three-week intervention periods (25 mL/day VOO) were preceded by two-week washout periods with refined oil. 24-hour urine and blood samples were collected at fasting state at the start of the study and before and after each treatment. A 3-day dietary record was administered at baseline and after each intervention period. Participants were asked to avoid a high intake of rich antioxidant foods (i.e. vegetables, legumes, fruits, etc.). A nutritionist advised to participants for replacing all types of habitually consumed raw fats with the olive oils catered. Plasma EDTA and serum samples were obtained by whole blood centrifugation and preserved at−80ºC. The clinical trial was conducted in accordance with the Helsinki Declaration and the Good Clinical Practice for Trials on Medical Products in the European Community (http://ec.europa.eu/health/files/eudralex/vol-10/3cc1aen_en.pdf). The protocol is registered with the International Standard Randomized Controlled Trial register (www.controlled-trials.com: ISRCTN77500181).

The present study is a post-hoc analysis with samples of the participants in the VOHF study assessing, with independence of the type of olive oil consumed, the relationship of the 3-week changes after all intervention periods among CEC, the fluidity of the HDL monolayer, and variables related to HDL composition, oxidative/antioxidative status, and size.

Data collection

Anthropometric variables were recorded. Blood pressure was measured with a mercury sphygmomanometer after at least a 10-min rest in the seated position. Physical activity, recorded at baseline and at the end of the study was assessed by the Minnesota Leisure Time Physical Activity Questionnaire which has been validated for its use in Spanish men and women [25]. Plasma glucose, total cholesterol (TC), and triglycerides (TG) were measured by standard enzymatic automated methods, plasma HDL-cholesterol by an accelerator selective detergent one (ABX-Horiba Diagnostics, Montpellier, France), and ApoA-I and ApoA-II by immunoturbidimetry, in a PENTRA-400 automated analyzer (ABX-Horiba Diagnostics, Montpellier, France). LDL cholesterol was calculated by the Friedewald equation.

HDL isolation

HDL was isolated from plasma by ultracentrifugation with a density gradient preparation method [26], using at once two solutions of different densities, 1.006 g/mL and 1.21 g/mL. To ensure the purity of the HDL fractions, ApoB100 and albumin levels were determined in the samples by automatic immunoturbidimetric methods (ABX-Horiba Diagnostics).

HDL composition

Fatty acids in HDL. HDL lipids were trans-esterified and after methanolysis, 1 mL of saturated NaCl solution was added to stop the reaction and 0.75 mL of hexane to extract the fatty acid methyl esters. Samples were centrifuged at 2212 g for 10 min and the supernatant injected into the chromatograph. The analysis was performed by gas chromatography (Agilent 7890A Series) using a capillary SP-2330 column (30 m x 0.25 mm x 0.2 µm) (Supelco, Bellefonte, USA), coupled to a flame ionization detector.

Lipids and proteins in HDL. Total and free cholesterol (FC), TG, and phospholipids in HDL were quantified by automatic enzymatic-colorimetric methods, and ApoA-I and ApoA-II by automatic immunoturbidimetric methods. Determinations were performed in a Cobas-Mira Plus automated analyzer (Roche, Basel, Switzerland) with

reagents from Spinreact (Barcelona, Spain). Esterified cholesterol (EC) was quantified as TC minus FC. The TG content of the HDL core was assessed as the ratio between TG and EC in HDL [18].

HDL oxidative/antioxidative status

HDL resistance to oxidation. It was determined by the conjugated dienes formation after copper oxidation of isolated HDL. Briefly, dialyzed HDL (10 mg/dL of HDL cholesterol) was incubated with cupric sulphate (5 µM) in phosphate buffered saline at 37ºC for 4h. Absorbance at 234 nm was continuously monitored at 3 min intervals in an INFINITE M200 reader (Tecan Group Ltd). The x-axis value corresponding to the intercept of the propagation phase tangent with the extrapolated line for the slow propagation reaction was calculated (lag time, min). Oxidation rate, derived from the slope of the propagation phase tangent, and maximum dienes formation were calculated by the maximum increase of the absorbance at 234 nm using the molar absorbance ε234nm for conjugated dienes ($29.5000 \cdot L \cdot mol\text{-}1 \cdot cm^{-1}$). Determinations were performed in duplicate. We used an HDL pool from healthy volunteers as between assay control. Data from HDL resistance to oxidation were only available from a subsample of 25 volunteers.

Antioxidant compounds in HDL. Carotenoids, retinol, ubiquinol, tocopherols, and phenolic compounds were analyzed by high-pressure liquid chromatography (HPLC) [27, 28]. Compounds were identified by their retention time compared with pure standards or, when unavailable (lutein and β-cryptoxanthin), with compounds obtained and purified in the laboratory. Determinations were run in duplicate.

NMR HDL lipoprotein particle count and subclasses

Serum samples were shipped to the National Heart Lung and Blood Institute, National Institutes of Health (NIH; Bethesda, MD, USA). Lipoprotein subclasses measurement was performed by Nuclear Magnetic Resonance (NMR) in a Vantera clinical spectrometer, produced by LipoScience (Raleigh, NC, USA). The NMR LipoProfile test by LipoScience involves measurement of the 400 MHz proton NMR spectrum of samples and uses the characteristic signal amplitude of the lipid methyl group broadcasted by every lipoprotein subfraction as the basis for

quantification. LipoProfile-3 algorithm was performed to quantify the mean particle size and concentrations of HDL lipoproteins. Subparticle concentrations were determined for three HDL subclasses (large: 8.8–13 nm; medium: 8.2– 8.8 nm; and small: 7.3–8.2 nm) [29].

HDL monolayer fluidity

HDL particle fluidity was determined by the steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) [18]. Briefly, HDL samples were incubated with DPH 1µM (30 min) and stimulated with a vertically polarized light at 360 nm. Fluorescent emission intensities were detected at 460 nm in a Perkin-Elmer LS5OB spectrofluorometer (Perkin Elmer, Waltham, MA, USA), through a polarizer orientated in parallel and perpendicular to the direction of polarization of the emitted beam. The steady-state fluorescence anisotropy (r) was calculated with the Ip values, and with the grating correction factor of the monochromator (G), using the following formula: r = (Ivv-GIvh)/(Ivv+2GIvh). The steady-state anisotropy refers to the rigidity of the sample, therefore the inverse value of this parameter (1/r) is the fluidity index. Determinations were performed in duplicate.

Cholesterol Efflux determination

Murine J-774A.1 macrophages were labeled for 1 h with TopFluor-Cholesterol, a fluorescent cholesterol probe in which the cholesterol molecule is linked to boron dipyrromethene difluoride (BODIPY) moiety (Avanti Polar Lipids, USA). The labeling of cells was performed in Dulbecco's Modified Eagle's medium (DMEM) containing 0.125mM total cholesterol, where TopFluor-Cholesterol accounted for 20% of total cholesterol. Labeled cells were incubated with TO-901317 (3µM; Sigma-Aldrich, USA) to upregulate ATP-binding cassette (ABC) transporters, in low-glucose DMEM supplemented with bovine serum albumin (BSA; 0.2%). This assay determines CEC mediated predominantly via ABCA1. Cells were then incubated with volunteers' HDL (100 µg/mL). The Acyl-CoA cholesterol acyltransferase (ACAT) inhibitor Sandoz 58-035 (5µM; Sigma-Aldrich, USA) was present during the whole experimental procedure. Cells were solubilized with cholic acid (1%) and mixed on a plate shaker for 1 h at room temperature. Fluorescence intensity of media and cells was monitored in Microplate Reader Synergy HT (BioTekInstruments; USA) at

$\lambda_{Ex/Em}$=485/528nm. CEC was calculated as: [media fluorescence/(media fluorescence + cells fluorescence)] * 100. Background efflux was subtracted from values obtained in the presence of HDL. Data were adjusted by the HDL particle number. All conditions were run in triplicate.

Statistical analyses All analyses were carried out using SPSS software version 21.0 (SPSS, Inc., Chicago, IL). Normality of continuous variables was evaluated by probability plots. Non-normally distributed variables were log transformed if necessary. Univariate associations between variables were examined by Pearson's correlation coefficients. Stepwise mixed linear models were fitted with variables significantly associated with 3-week changes in cholesterol efflux or fluidity in the univariate analyses. When collinearity between variables exists, separate models were fitted. Models were adjusted by age and sex, and individual level of test subjects as random effect. Significance was defined at the 5% level using a two-tailed test.

RESULTS

Baseline characteristics of the subjects are shown in Table 1. Subjects under antilipidemic medication did not change either their dose or type of medication through the study. No changes in physical activity were observed from the beginning to the end of the study. No changes in daily energy intake or those of micro or macronutrients were observed among intervention periods. A significant increase at post-intervention versus pre-intervention values was observed in CEC ($p=0.042$), HDL oleic acid concentration ($p=0.041$), HDL ApoA-I concentration ($p=0.014$), and antioxidants in HDL: α-tocopherol ($p=0.017$), β-cryptoxanthin ($p<0.001$), coenzyme-Q ($p=0.005$), lutein ($p<0.001$), retinol ($p=0.011$), and phenolic compounds ($p<0.001$).

Cholesterol efflux associations

Cholesterol efflux associations

Table 2 shows the Pearson's correlation coefficients for the association between changes in CEC and in HDL fluidity, and changes in variables related to HDL composition, oxidative/antioxidative status, and size. CEC

was directly related with the fluidity of the HDL (p=0.004). CEC was inversely related with concentration of linoleic acid in HDL (p=0.034) and with that of small HDL particles (p=0.001), but directly related to the concentration in HDL of stearic acid (p=0.003), ApoA-I (p=0.004), ApoA-II (p=0.001), TC (p=0.008), EC (p=0.40), TG (p=0.021), phospholipids (p<0.001), lag time of conjugated dienes formation (p=0.006), concentration in HDL of β-cryptoxanthin (p=0.008), and concentration of medium (p=0.012) and large (p=0.041) HDL particles.

Results of the stepwise mixed linear models are shown in Table 3. Given that determinations of the lag time of conjugated dienes were not available in the whole sample we performed Model 1 with all variables which had significance in the univariate analyses, but without entering the lag time in the model. Model 2 shows the results with all variables that had significance in the univariate analyses. Results of these models point out an increase in HDL fluidity, ApoA-I, and resistance against oxidation (lag time of conjugated dienes) as main determinants for CEC. Model 3 shows the results without entering HDL fluidity in the full model. In this case, small HDL particle number appear inversely and significantly related, indicating that it could be a variable which reflects the effect of fluidity on CEC. Thus, we performed the same analyses with fluidity as the dependent variable.

HDL fluidity associations

Table 2 shows the Pearson's correlation coefficients for the univariate association between changes in HDL fluidity and in CEC, and changes of those variables related to HDL composition, oxidative/antioxidative status, and size. HDL fluidity was inversely related to the concentration of albumin (p=0.038) and FC (p= 0.003) in HDL, and that of small HDL particles (p<0.001). HDL fluidity was directly related to the concentration of EC in HDL (p=0.036), total TG content (p<0.001), TG in HDL core (p=0.044), HDL mean size (p< 0.001), and with the concentration of medium HDL particles (p=0.027).

Results of the stepwise mixed linear model including all variables which reached significance in the univariate analyses showed that the content of FC and TG in HDL and the concentration of small and medium HDL particles

appeared as main determinants of the fluidity of HDL monolayer. However, the strong inverse relationship between small and medium HDL particles number ($r= -0.752$, $p<0.001$) (see Supplemental Figure 1) promotes a collinearity in the model distorting the results. Due to this, two models were fitted including or not small or medium HDL particles in them. Results of the models are shown in Table 4. An increase in TG and a decrease in FC levels in HDL, together with a decrease in small HDL particle number or an increase in the HDL mean size appear as main determinants of the fluidity of the HDL particles.

DISCUSSION

In this study, we examined the association between 3-week changes in CEC and HDL fluidity after VOO ingestion, as well as the association of the changes in both variables with those of several characteristics of the HDL. Our results point out an increase in HDL fluidity and in the concentration of ApoA-I in HDL, and a decrease in HDL oxidative status as major determinants of CEC. A reduction in FC together with an increase in TG in HDL, and a decrease in the concentration of small HDL particle number or an increase in HDL mean size appeared to be the main determinants for HDL fluidity. A schema of the interrelationships is depicted in Figure 1.

The molecular determinants of CEC are still largely unknown, and multiple pathways, which can be modulated by HDL composition and modification, are involved [30]. Mechanisms accounting for CEC include passive diffusion process as well as active pathways mediated by ABCA1, ABCG1, and the scavenger receptor class B type I (SR-BI). The posttranscriptional regulation of cholesterol efflux via ABCA1 includes mechanisms that involve the stabilization of ABCA1 protein by ApoA-I [4, 13]. In this sense, oxidation of HDL and ApoA-I results in a selective inhibition of ABCA1-dependent cholesterol efflux from macrophages [13]. The ABCA-1/ApoA-I complex absorbs antioxidants (i.e lutein, zeaxanthin, etc.) which are LDL-protecting molecules, thus contributing to the formation of nascent HDL [31]. ABCA1/ApoA-I activity is fundamental for the formation of the nascent HDL (pre-β1 fractions), which are also efficient acceptors of cell cholesterol via ABCG1 [4]. In agreement with our

past findings, HDL oxidation decreases HDL fluidity and concomitantly CEC [11, 12]. Lipoprotein oxidation promotes the linkage between fatty acids reducing the mobility of the chains which increases the rigidity of the lipid monolayer [32]. In turn, the fluidity of the HDL monolayer influences ApoA-I conformation and binding to HDL [33]. Thus, a close interrelationship exists between HDL oxidative status, ApoA-I, and HDL fluidity, the three main factors which appear related to CEC in our study.

Changes within the HDL lipid core can also promote HDL dysfunction. The main constituents of the HDL lipidome are the phospholipids, followed by EC, and by TG and FC. They are spatially organized according to their hydrophilic properties: phospholipids and FC in the external hydrophilic monolayer which encloses a hydrophobic core rich in EC and TG [34]. An upregulation of the activity of the cholesterol ester transfer protein (CETP), like occurs in insulin resistance states, alters the TG/EC ratio in HDL, the index used to assess the quantity of TG in the core of the HDL [18]. Higher TG contents in the lipid core impair transfer of EC through SR-BI, hindering CEC [35]. Despite all this referred before, data from human in vivo studies show that to maintain a critical pool size of triglyceride-rich lipoproteins, that affects cholesterol from HDL, promotes the efficiency of CEC [36]. A positive correlation between the capacity of plasma to effect cholesterol efflux and plasma levels of TG has been demonstrated in several studies [36]. Thus, the controversy concerning the role of TG on CEC lead us to hypothesize that maintaining an equilibrium in the TG content of HDL versus other lipids could be the clue for the HDL functionality. From our data, the influence of TG and FC on CEC could be mediated through their effect on the HDL fluidity. We have previously described a positive association between the content of TG, oleic acid, and phospholipids in HDL and the fluidity of the lipoprotein, FC content of the HDL being negatively associated [9]. Fluidity depends on the length and saturation of the fatty acids present in the phospholipids of the HDL monolayer. In this sense, a decrease in acyl chain length and an increase in chain unsaturation, such as in the case of oleic acid, increase the fluidity of the lipoprotein [37, 38]. In contrast, FC complexes with phospholipids form the lipid rafts which are known to harden the membrane and to decrease its fluidity [38]. An increase in TG, a well know effector for

increasing lipid membranes fluidity [39], and a decrease in FC in HDL were major determinants for the HDL fluidity in our population.

A huge body of data, somewhat contradictory, exists concerning HDL size and CEC. On the basis of the phospholipid content, small HDL more potently promote CEC [15, 40, 41], whereas on a particle number basis, large HDL are more effective [16, 40]. In this sense, recent studies in peri-menopausal women and elderly adults show CEC to be directly related to large and medium HDL particle concentration and inversely to that of small HDL particle number in multivariate adjusted models [42, 43]. In agreement with these data, in our lineal mixed model small HDL particles were inversely related with CEC, when fluidity was not present in the model. When entering the fluidity of the HDL in the model, small HDL particle number was no longer associated with CEC, but it was, however, inversely related with HDL fluidity in the mixed model when HDL fluidity was the dependent variable. In a similar way that occurred for the lipid HDL composition, in our models the size of the HDL lipoprotein seem to exert their effect on CEC through changes in the HDL monolayer fluidity, with an improvement at larger HDL particle size. The fluidity of the HDL, as well as its CEC, is considered to be inversely related to the sphingomyelin content of the HDL. The sphingomyelin content of the HDL subpopulations remains, at present, controversial [44]. The complexity of sphingomyelin species, their distribution in the different HDL particles, and their role in HDL fluidity and CEC is considered a promising target for future studies [44].

Our study has limitations. First, cholesterol efflux was measured ex vivo, but the transport of the cholesterol to the liver, the second part of the reverse cholesterol transport was not assessed. Because our CEC assay involved the use of cell lines [30] our results may not reflect the real in vivo situation. However, CEC as measured by a similar method with J744 cells, but using ApoB-depleted plasma as acceptor, has been shown to be inversely related with cardiovascular risk [6]. We have measured ApoA-I concentration as a surrogated marker for ApoA-I functionality in our study. Also, we have related 3-week changes in HDL variables with independence of the olive oil intervention period, the possible confounding effect avoided by the introduction of the individual level of test subjects as

random effect in the model. Although this approach has advantages in mitigating the effect from confounding factors, it could buffer the pre-post differences. Also, the fact that our population was hypercholesterolemic subjects, can limit the extrapolation of the results to a more general population. A major strength of this study was its crossover, double-blind, placebo controlled design, which helps to limit the effect from confounding factors such as differences in physical activity or diets in tests subjects. As essential strength of our work, it is one of the first comprehensive association studies between HDL functional characteristics (also covering an intensive lipidomic and metabolomic profile) and the most relevant HDL functionality trait, CEC.

Among factors which can modify CEC and HDL characteristics, besides VOO alone, we have reported that diets rich in VOO and antioxidants such as the Mediterranean diet have shown to increase the CEC together with improvements in HDL oxidative status, composition, ApoA-I, and HDL particle size [45–47]. Also, novel pharmaceutical approaches are being developed for improving HDL characteristics and functionality.Therapies based on the infusion of ApoA-I formulations (rHDL) in humans, increased ApoA-I levels and cholesterol esterification, and caused a transient accumulation of very small HDL species, followed by an enlargement of particles present within medium and large HDL. These changes were concomitant with an increase in the CEC [48].

In summary, by using a model as VOO consumption in which improvements in HDL characteristics and functionality have been reported [10, 17–24], we looked for the major determinants of the CEC. From our results, an increase in the fluidity of the HDL and in the concentration of ApoA-I in HDL, and a decrease in HDL oxidative status appear as major determinants of CEC. The impact of a reduction in FC, together with an increase in TG in HDL on CEC could be mediated by their effect increasing the fluidity of the HDL monolayer. The same occurs for the decrease in small HDL particle number or the increase in the HDL mean size. Our work point out several promising new therapeutic targets, besides HDL cholesterol levels, for improving HDL functionality in humans through dietary, nutraceutical, or pharmaceutical interventions.

ACKNOWLEDGEMENTS

This study was supported by the Spanish Ministry of Economy (MINECO) financing the projects AGL2009-13517-457 C03-01, C03-02 and C03-3, AGL2012-40144-C01-03, C02-03 and C03-03, and the FPI-fellowship (BES-2010-040766). LR is Sara Borell contract (CD14/00275) co-financed by and IISPV. UC is a PERIS contract (Pla estratègic de recerca i innovació en salut). AP is a Torres Quevedo contract (Subprograma Estatal de Incorporación, Plan Estatal de Investigación Científica y Técnica y de Innovación). OC was supported by a research contract of the ISCIII JR14/00008. CIBERDEM and CIBEROBN is an initiative of the Health Institute Carlos III. Work done by ATR was supported by the intramural National Heart, Lung and Blood Institute of the National Institutes of Health, in Bethesda, MD. We thank NUPROAS HB for their substantial contribution interpreting the data and revising the manuscript critically, Borges Mediterranean Group for providing the VOO, and Doctor Claude Motta for his priceless help in teaching the fluidity measurement methods.

CONFLICTS OF INTEREST

None declared

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Figure 1. A proposal model for the interrelationship among the main determinants (dense arrows) of HDL cholesterol efflux capacity in hypercholesterolemic subjects after virgin olive oil ingestion. Intermittent arrows point out the expected contribution of the main virgin olive oil components.

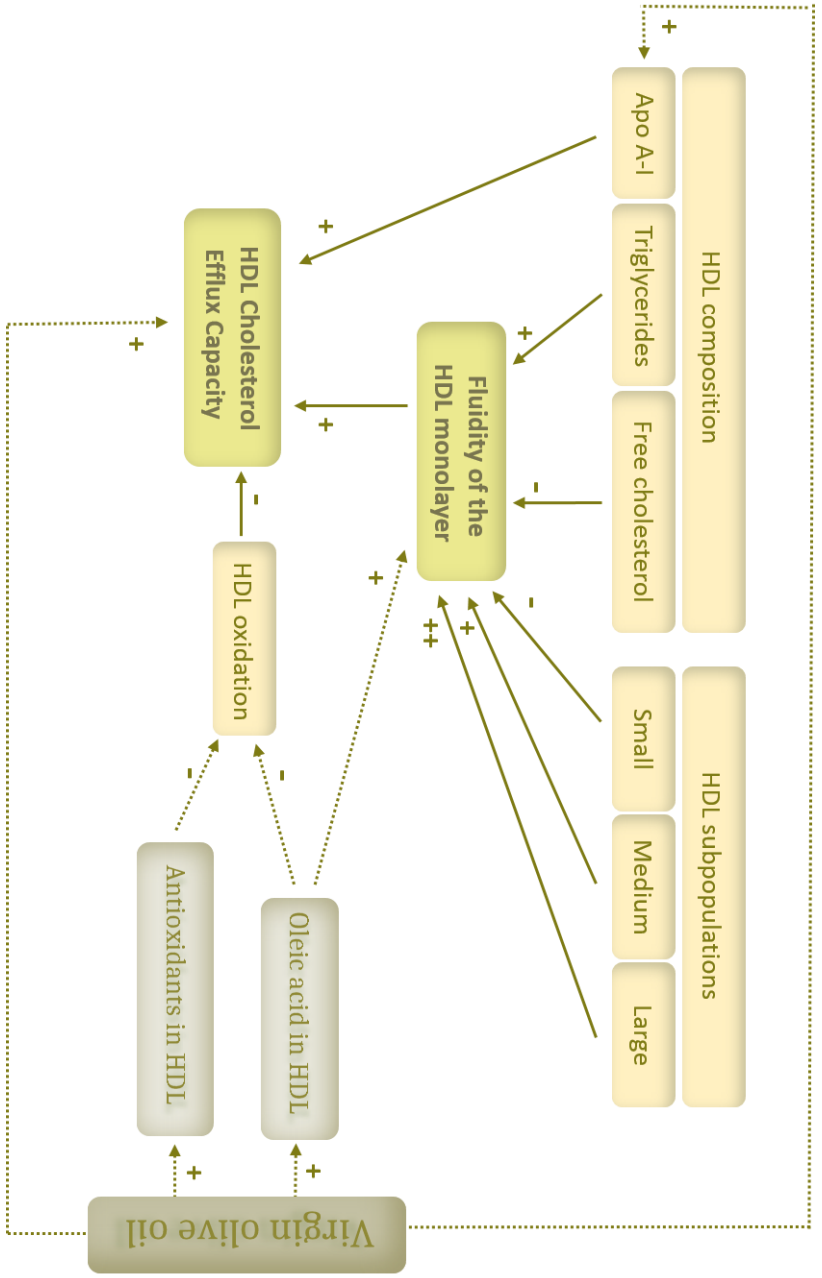


Table 1. Baseline characteristics of the participants

Variable	Mean ± SD
Age, *years*	55.6 ± 10.0
Gender, *male/female*	19/14
Body mass index, *kg/m²*	26.6 ± 4.5
Physical activity, *METs.min/w**	2423 (897-4544)
Systolic blood pressure, *mmHg*	128.2 ± 14.6
Diastolic blood pressure, *mmHg*	73.15 ± 10.0
Glucose, *pl, mg/dL*	90.8 ± 11.6
Cholesterol ,*pl*, *mg/dL*	
Total	226.4 ± 35.2
LDL	148.3 ± 28.8
HDL	53.01 ± 11.13
Triglycerides, *pl, mg/dL**	114.2 (85.5 ± 145.0)
Use of antilipemic medication, *yes/no*	14/19
HDL mediated cholesterol efflux, %	3.87 ± 1.23
Fluidity of the HDL monolayer, *1/AU*	4.96 ± 0.25
HDL composition	
Fatty acids in HDL, *%*	
Arachidonic	9.95 ± 4.96
Eicosatreanoic	1.32 ± 2.20
Linoleic	29.80 ±9.43
Oleic	19.45 ±4.09
Palmitic	25.10 ±4.78
Stearic	13.03 ±6.81
Proteins in HDL, *g/L*	
Albumin	1.23 ± 0.70
Apolipoprotein A-I	0.66 ± 0.15
Apolipoprotein A-II	0.16 ±0.04
Lipids in HDL, *mg/dL*	
Total Cholesterol	31.29 ± 10.36
Free Cholesterol	11.80 ± 5.38
Esterified cholesterol	19.48 ±8.05
Triglycerides	7.48 ± 2.44
Phospholipids	60.25 ± 17.04
Oxidative status of HDL†	
Lag time, *min**	33.11 (25.42-59.74)
Maximum absorbance, *Abs*	0.47 ± 0.08
Oxidation rate, *Abs/s*	6.91 ± 2.74
Antioxidative status of HDL, *µmols/L*	
Total phenolic content* ‡	14.41 (0-144.5)
α-tocopherol	12.44 ± 0.80
γ-tocopherol	0.04 ± 0.02
β-carotene*	1.69 (0-2.86)
β -cryptoxanthin	7.10 ± 3.00
Coenzime-Q	237.3 ± 69.05
Luteine	1.9 ± 1.06
Retinol	3.69 ± 1.48
HDL particle size, *µmol/L*	
HDL mean size¥	9.30 ± 0.51
Small HDL particles	17.86 ± 5.96
Medium HDL particles	10.52 ± 5.89
Large HDL particles	6.58 ± 3.20

SD, Standard deviation. pl, plasma. AU, arbitrary units * Values expressed as median (25th-75th percentile) †Conjugated dienes formation curve. ‡Expressed in nmol/L. ¥Expressed in nanometers

Table 2. Correlation coefficients (R) between 3-week Changes in HDL Cholesterol Efflux and Fluidity of the HDL monolayer and HDL characteristics after olive oil ingestion

	Cholesterol efflux		Fluidity	
Variable (*3-week changes*)	**R**	**P value**	**R**	**P value**
Cholesterol efflux, %	1	1	0.308	0.004
Fluidity, 1/anisotropy (AU)	0.308	0.004	1	1
HDL Composition				
Fatty acids in HDL, *%*				
Arachidonic	0.043	0.700	0.097	0.387
Eicosatreanoic	0.084	0.456	0.033	0.768
Linoleic	-0.235	0.034	0.057	0.613
Oleic	0.025	0.823	0.004	0.972
Palmitic	0.128	0.256	-0.155	0.156
Stearic	0.328	0.003	0.140	0.213
Proteins in HDL, *g/L*				
Albumin	-0.199	0.069	-0.277	0.038
Apolipoprotein A-I	0.309	0.004	0.123	0.264
Apolipoprotein A-II	0.364	0.001	0.070	0.537
Lipids in HDL, *mg/dL*				
Total Cholesterol	0.287	0.008	-0.010	0.927
Free Cholesterol	0.073	0.509	-0.322	0.003
Esterified Cholesterol	0.225	0.040	0.229	0.036
Triglycerides	0.251	0.021	0.383	<0.001
Phospholipids	0.382	<0.001	0.186	0.091
Triglycerides in HDL core*	-0.041	0.713	0.220	0.044
Oxidative/Antioxidative status of HDL				
Oxidative status†				
Lag time, *min*	0.331	0.006	-0.049	0.606
Maxim absorbance, *Abs*	0.053	0.708	0.125	0.375
Oxidation rate, *Abs/s*	-0.168	0.189	0.175	0.215
Antioxidative status of HDL, *µmol/L*				
α-tocopherol,	-0.021	0.859	-0.001	0.903
γ-tocopherol	-0.022	0.850	0.069	0.549
β-carotene	0.044	0.701	0.016	0.892
β-cryptoxanthin	0.306	0.008	0.034	0.772
Coenzime-Q	0.128	0.291	0.075	0.539
Luteine	0.211	0.066	0.065	0.575
Retinol	0.158	0.170	-0.179	0.120
Phenolic compounds‡	0.138	0.182	0.127	0.271
HDL particle size, µmol/L				
HDL mean size (total) ¥	0.076	0.494	0.379	<0.001
Small HDL particles	-0.349	0.001	-0.635	<0.001
Medium HDL particles	0.273	0.012	0.241	0.027
Large HDL particles	0.224	0.041	0.155	0.158

AU, arbitrary units. *Calculated as triglycerides/esterified cholesterol ratio. †Conjugated dienes formation curve. ‡ nmol/L. ¥nanometers

Table 3. Determinants of 3-week changes in cholesterol efflux in hypercholesterolemic subjects after virgin olive oil ingestion.

Predictor variable ***(3-week changes)***	**B coefficient**	**SE**	**T**	**P value**
Model 1*				
Fluidity of the HDL monolayer	0.731	0.279	2.62	0.010
Apolipoprotein A1	2.54	0.73	3.45	0.001
Model 2†				
Fluidity of the HDL monolayer	0.777	0.289	2.69	0.009
Apolipoprotein A1	2.47	0.892	2.77	0.007
Lag time	0.008	0.004	2.36	0.022
Model 3‡				
Apolipoprotein A1	1.92	0.94	2.04	0.046
Lag time of conjugated dienes	0.008	0.004	2.26	0.027
Small HDL particle number	-0.042	0.016	-2.68	0.009

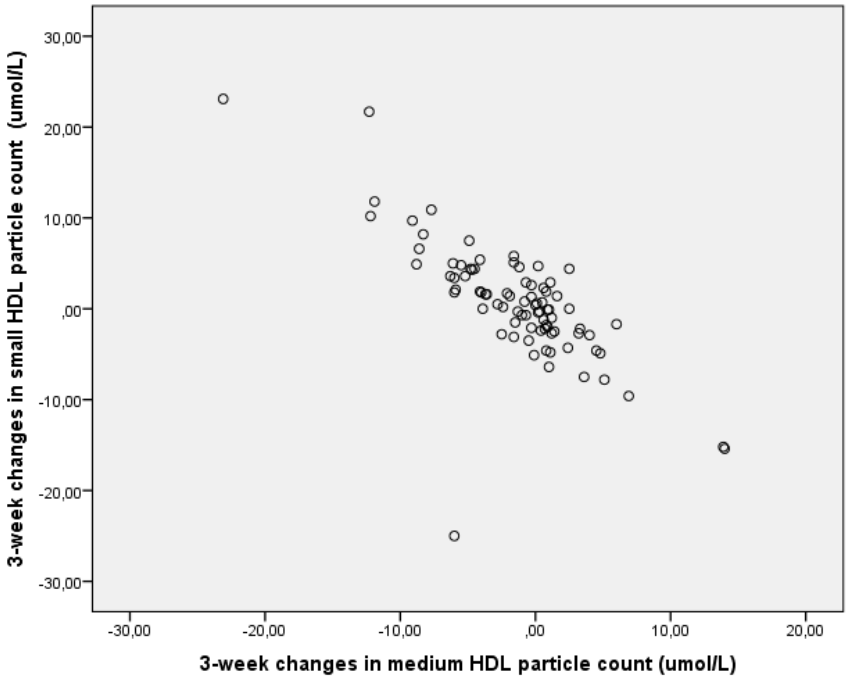
Stepwise mixed linear models adjusted for age and sex, and individual level of test subjects as a random effect. SE, standard error. *Model 1. Without the lag time of conjugated dienes formation; † Model 2, including all variables; ‡Model 3, without fluidity of the HDL monolayer

Table 4. Determinants of 3-week changes in the HDL fluidity in hypercholesterolemic subjects after virgin olive oil ingestion.

Predictor variable ***(3-week changes)***	**B coefficient**	**SE**	**T**	**P value**
Model 1*				
Free cholesterol in HDL	-0.032	0.007	-4.73	<0.001
Triglycerides in HDL	0.035	0.013	2.68	0.010
Small HDL particles	-0.025	0.004	-6.28	<0.001
Model 2†				
Free cholesterol in HDL	-0.035	0.077	-4.47	<0.001
Triglycerides in HDL	0.061	0.014	4.25	<0.001
HDL mean size	0.290	0.104	2.79	0.007

Stepwise mixed linear model adjusted for age and sex, and individual level of test subjects as a random effect. SE, standard error. *Model 1, without medium HDL particles. †Model 2, without small HDL particles.

Supplemental Figure 1. Relationship between 3-week changes in small and in medium HDL particle number after virgin olive oil ingestion. Pearson's correlation coefficient.



UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS
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Global discussion

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The present thesis confirms the hypothesis that the sustained intake of functional VOOs enriched with their own PC or with their PC plus additional complementary PC from thyme modifies the physicochemical properties of HDL particles towards a cardioprotective mode, and promotes changes in HDL subclass distribution, leading to the subsequent enhancement of HDL functionality in hypercholesterolemic subjects. These changes are performed according to phenol source and content in the tested functional VOOs. In particular, these results, which were obtained within the framework of the randomized, double-blind, crossover, controlled VOHF study, provide the first level of evidence that FVOO intake improves HDL, LDL and VLDL subclass profile and their associated atherogenic ratios towards a less atherogenic pattern, increases fat-soluble antioxidant content in HDL, and exerts a beneficial impact on the PON enzyme family towards a cardioprotective mode, indicating a proper oxidative balance. Moreover, FVOOT intake improves the HDL subclass profile and its associated atherogenic ratios, along with increasing ChE, fat-soluble antioxidants, and PC metabolite content in HDL. Additionally, we have reported that increases in the fluidity of the HDL monolayer and the concentration of ApoA-I in HDL and a decrease in HDL oxidative status are the major determinants of HDL-mediated ChE after VOO intake. In turn, monolayer fluidity is determined by changes in HDL lipid composition (in particular, a reduction in FC together with an increase in TG in HDL) and by a decrease in the concentration of small HDL particles or an increase in HDL particle size. All these results are comprehensively detailed in the articles that constitute the present thesis. Moreover, the present discussion integrates the results obtained on HDL functional quality after the intake of phenol-enriched VOOs developed in the VOHF study.

In Study 1 ("*Polyphenol-rich olive oils improve lipoprotein particle atherogenic ratios and subclasses profile: A randomized, crossover, controlled trial"*), we demonstrated that the intake of phenol-enriched VOOs, *i.e.*, FVOO and FVOOT, modifies HDL size and subclass distribution towards larger and more mature HDL particles. Both functional VOOs increased HDL size and l-HDL particle number, and decreased the s-HDL/l-HDL and HDL-C/HDL-P ratios, while FVOO also decreased s-HDL particle number. The modifications in HDL subclass profile observed in Study 1 can

be similarly translated into changes in HDL function, since each HDL subclass exhibits differences in functionality that are irrespective of its cholesterol content (HDL-C). For instance, s-HDL are more efficient in promoting ChE and inhibiting inflammation than l-HDL (Camont et al., 2011; Karathanasis et al., 2017; K.-A. Rye et al., 2009). However, in most studies, s-HDL particles are more strongly associated with increased CHD risk than l-HDL particles (Joshi & Toth, 2016; Martin et al., 2015), and high levels of s-HDL and/or low levels of l-HDL particles are often present in CHD, ischemic stroke, and T2DM (Borggreve, De Vries, & Dullaart, 2003; Sankaranarayanan et al., 2009; Zeljkovic et al., 2010). Moreover, in a cohort of asymptomatic older adults, CEC was inversely associated with s-HDL particle levels and was directly associated with l-HDL, m-HDL, and HDL size (Mutharasan et al., 2017). In concordance with this evidence, Study 4 ("*Determinants of HDL cholesterol efflux capacity after virgin olive oil ingestion: Interrelationships with fluidity of HDL monolayer*") revealed that CEC was directly related to HDL size, with s-HDL being inversely related to CEC, and that this association is carried out through changes in HDL monolayer fluidity. This paradoxical evidence on the presence of high levels of s-HDL in CVD, together with s-HDL being more functional than l-HDL, may be explained by the hypothesis that increased s-HDL particles in the serum may indicate an aberration in the maturation of s-HDL particles, therefore increasing the risk of CVD (Eren et al., 2012; Mutharasan et al., 2017).

From our results, it is noteworthy that both phenol-enriched VOOs decreased the HDL-C/HDL-P ratio versus the control VOO, as described in Study 1. This ratio indicates the enrichment of the HDL particle in cholesterol, and it is considered a new potential measure of HDL function, it is directly related to atherosclerosis progression in CVD-free individuals (Qi et al., 2015; Remaley, 2015; Zhao, 2015). Increased values of the HDL-C/HDL-P ratio reflect the presence of cholesterol-overloaded HDL-P. These particles appear to exert a negative impact on the cardioprotective function of HDL by impairing HDL ability to promote ChE from peripheral cells, decreasing HDL clearance, and impairing HDL anti-inflammatory and antioxidant properties (Huang et al., 2014; Khera et al., 2011; Qi et al., 2015; Rosenson et al., 2013). The existence of cholesterol-overloaded HDL particles might be due to an impairment in CETP activity, as hypothesized

by Qi *et al*. (Qi et al., 2015). Thus, the decrease in the HDL-C/HDL-P ratio after FVOO and FVOOT intake observed in Study 1 is indicative of the decrease in cholesterol-overloaded HDL particles and therefore the enhancement of HDL function. Accordingly, our group has previously noted a slight increase in CETP activity after the consumption of both phenol-enriched VOOs in the VOHF study (Farràs et al., 2015).

In Study 1, we also found that FVOO decreased LDL-C, LDL-P, ApoB-containing particle number, small LDL, medium VLDL, LDL size, VLDL size, LDL-P/HDL-P ratio, and the LP-IR index, while FVOOT only decreased medium-VLDL and the LP-IR index. All these parameters are commonly associated with the risk of CHD and with dyslipemia in individuals with T2DM (Bhakdi et al., 1995; Blake, Otvos, Rifai, & Ridker, 2002; El Harchaoui et al., 2007; Garvey et al., 2003; Kingwell et al., 2014; Lyons, 1993; McQueen et al., 2008; Yusuf et al., 2004). These results observed after FVOO are in agreement with the decrease in LDL-C levels after extra-VOO consumption (Violi et al., 2015) and with the decrease in LDL-P observed in the EUROLIVE study (Álvaro Hernáez et al., 2015). Both studies were conducted with VOOs with similar phenolic content (435 ppm and 366 ppm, respectively) to the FVOO employed in the VOHF study (500 ppm). Moreover, the PREDIMED study has recently demonstrated that a Mediterranean diet enriched with VOO, decreases the estimated LDL size decreasing, therefore, the LDL atherogenicity in high CVR individuals (Álvaro Hernáez et al., 2017). Of all the LDL and VLDL particle biomarkers tested, the LDL-P/HDL-P ratio seems to show the strongest independent association with CHD, as the higher the LDL-P/HDL-P ratio is, the higher the CHD risk is observed, therefore leading to significant net reclassification improvements in the AHA/ACC CHD risk scores (Steffen et al., 2015). Therefore, both phenol-enriched VOOs, namely FVOO and FVOOT, promoted cardioprotective benefits in hypercholesterolemic patients since they shifted the HDL, LDL, and VLDL subclass distribution and their associated ratios toward a less atherogenic pattern. FVOO could be better at improving lipoprotein subclass distribution than FVOOT, since FVOOT had a minor impact in HDL, LDL, and VLDL particle distribution.

The results reported in Study 1 are indicative of an enhanvement of HDL maturation, and they can be explained, in part, by the slight increases in CETP and LCAT activity that were previously reported by our group after

the consumption of both phenol-enriched VOOs in framework of the VOHF study (Farràs et al., 2015). Moreover, these changes in HDL maturation and in CETP and LCAT activities may also contribute to the increase in ChE reported in Study 2 ("*Phenol-enriched olive oils improve HDL antioxidant content in hypercholesterolemic subjects. A randomised, double-blind, cross-over, controlled trial*"). This study revealed that ChE tended to increase after FVOOT versus its baseline, and it increased versus FVOO. The LCAT enzyme is activated by ApoA-I and is involved in a key event in the RCT pathway, namely, the esterification of FC effluxed from cell. It is located in the HDL surface, thus generating a FC gradient in the HDL monolayer. Because of CE hydrophobicity, CE formed after the action of LCAT is partitioned into the core of HDL, enhancing HDL maturation from small discoidal particles to larfer, mature spherical HDL particles. L-HDL particles are further remodeled by the CETP enzyme, which transfers CE to TG-rich lipoproteins and delivers TG to HDL in return, contributing to HDL maturation to bigger HDL particles. TG-rich and EC-poor HDLs can be delipidated by the action of hepatic lipase (which hydrolyses TG) and endothelial lipase (which hydrolyses PL) and therefore converted into smaller HDLs and lipid-free ApoA-I, which are eventually reintegrated in the RTC pathway. The FC gradient resulting from LCAT activation and the conversion phenomena resulting from CETP activity contribute not only to RCT but also to HDL maturation, in concordance with the results obtained in Studies 1 and 2 of the present thesis. Similarly, the EUROLIVE and the PREDIMED studies demonstrated that the intake of phenol-rich VOOs induced the formation of larger HDL particles. These changes in HDL size and distribution were accompanied by increases in CEC, LCAT and CETP activity, antioxidant and anti-inflammatory properties, and vasodilatory capacity (Damasceno et al., 2013; Hernáez et al., 2017; Hernáez et al., 2014).

Factors other than LCAT and CETP activity may be responsible for the enhancement of ChE observed after FVOOT intake. Study 2 revealed that the antioxidant content of the HDL particle improved after phenol-enriched VOOs intake, as it increased lipophilic (retinol, ubiquinol, α-tocopherol, and carotenoids, such as lutein and β- cryptoxanthin) and hydrophilic (phenolic metabolites) antioxidants in HDL. The co-existence of these lipophilic and hydrophilic antioxidants linked to HDL may confer

additional benefits by protecting lipids and proteins from oxidative damage via different antioxidant mechanisms (Mezzetti et al., 1995; Paiva-Martins, Gordon, & Gameiro, 2003), as the antioxidant system is a complex network of interacting molecules. In response to oxidative stress, antioxidant molecules are oxidized and converted into harmful free radicals that need to be turned back to their reduced form by complementary antioxidants. The benefits of antioxidant complementarity are supported by the fact that supplementing high-risk individuals with a single type of antioxidant promotes, rather than reduces, lipid peroxidation, whereas the combination of different antioxidants has been shown to be effective in reducing atherosclerosis in human trials (Salonen et al., 2000). These data reaffirm the assertion that the enrichment of VOOs with their own PCs, mainly secoiridoids, combined with complementary PC from thyme, mainly flavonoids, provides the optimum balance among different kind of antioxidants (*i.e.*, simple phenols, phenolic acids, flavonoids, and monoterpenes). Accordingly, our group has recently reported that a combination of OO-PC with Th-PC via FVOOT intake provides major protection against oxidative DNA damage compared to an intake of the same amount of PC from only VOO (500 ppm; via FVOO) in hypercholesterolemic subjects (Romeu et al., 2016). This evidence might be due to not only a synergic effect of different types of antioxidants but also an enhancement of the bioavailability of OO-PC in the presence of Th-PC, as previously reported in mice (Rubió, Serra, et al., 2014).

It is worth highlighting that α-tocopherol is one of the main antioxidants in human plasma, and it is present in the circulation anchored to HDL and LDL. α-tocopherol is the main initial chain-breaking antioxidant during lipid peroxidation and, subsequently, the resultant α-tocopherol is recycled back to its biologically active reduced form by coenzyme Q (CoQ) (Kagan, Fabisiak, & Quinn, 2000; Laureaux et al., 1997). In addition, some active phenolic acids, such as rosmarinic and caffeic acids, can also regenerate α-tocopherol (Laranjinha, Vieira, Madeira, & Almeida, 1995). In Study 2, the FVOOT intervention increased α-tocopherol, ubiquinol (the reduced form of CoQ), caffeic acid sulfate, and hydroxyphenyl propionic acid sulfate while FVOO only increased ubiquinol but not α-tocopherol, caffeic acid sulfate, and hydroxyphenyl propionic acid sulfate. These data suggest

better α-tocopherol regeneration, leading to enhanced protection against oxidation after FVOOT intake, which is in concordance with the previous results (refrerences earlier) on DNA protection against oxidation following FVOOT (Romeu et al., 2016). Thus, the FVOOT intervention may be better at improving HDL antioxidant activity and may consequently preserve HDL protein structures.

The antioxidant content of HDL observed following the consumption of both phenol-enriched VOOs is translated into improvements in HDL physicochemical characteristics and therefore HDL functionality, as described below. In this sense, the EUROLIVE study revealed that the intake of phenol-rich VOOs resulted in PC binding to HDL, contributing to the enhancement of HDL functionality, ChE in particular, according to the phenol content of the consumed VOO (Hernáez et al., 2014). Moreover, the PREDIMED study has recently shown that a 1-year intervention with a Mediterranean diet enriched with VOO increases HDL atheroprotective functions in humans at high CVR (Hernáez et al., 2017). In the VOHF study, several HDL physicochemical characteristics, such as monolayer fluidity and lipid composition, and biological functions have been monitored. As reported in Study 2, no significant changes were observed in HDL monolayer fluidity after any intervention. However, when all VOO interventions where tested together (Study 4), the fluidity of the HDL monolayer was one of the main determinants for ChE enhancement. The importance of this HDL characteristic lies in the fact that it reflects the functional behavior of HDL to such an extent that fluidity has been considered as an intermediate marker of HDL functionality. In particular, the more fluid the HDL monolayer is, the greater ChE rate from lipid-laden macrophages to HDL is observed (Helal et al., 2013; Álvaro Hernáez et al., 2014; R Solà et al., 1993). Moreover, lipid peroxidation is known to rigidify HDL monolayer fluidity, resulting in less ChE in *in vitro-ex vivo* experiments (Bonnefont-Rousselot et al., 1995; Girona et al., 2003). In Study 4, we showed that increases in fluidity and decreases in HDL oxidative status are major determinants for CEC. When the VOOs were examined separately, FVOOT intake increased ChE in concordance with the high presence of antioxidants linked to HDL, as described in Study 2. Regarding HDL lipid composition, increased PL/FC and EC/FC ratios in HDL are indicative of ChE enhancement, as the direction of net cholesterol transport is determined

by the FC gradient between the cell membrane and the HDL monolayer (Phillips et al., 1987). In the VOHF study, we previously reported an increase in these PL/FC and EC/FC ratios after FVOOT intake versus FVOO and control VOO (Farràs et al., 2015), indicating that these particles could be more efficient in enhancing ChE. Consistent with these data, in Study 4, we reported that lipid content in HDL could be a key factor in HDL functionality, since a decrease in FC and an increase in TG were found to be major determinants of monolayer fluidity in our population, and therefore, they were also major determinants of CEC enhancement. The EUROLIVE study showed that phenol-rich VOO intake increases HDL monolayer fluidity in healthy subjects, and that this increase was accompanied by an increase in ChE, that corresponded to the phenol content of the VOOs (Álvaro Hernáez et al., 2014). The PREDIMED study recently reported that a Mediterranean diet enriched with VOO increases the PL content in the HDL monolayer. These changes resulted in the enhancement of CEC, among other functions, although HDL monolayer fluidity was not assessed in this study (Á Hernáez et al., 2017). The differences observed between these three studies may arise from the different characteristics of the studied populations, since the EUROLIVE study was carried out in healthy volunteers and the PREDIMED study was conducted in individuals at high CVR, while the VOHF study was undertaken in hypercholesterolemic subjects. Moreover, in the VOHF study, a phenol-riched VOO (80 ppm) was used as a control, while a low-fat diet and a refined OO were employed in the PREDIMED and EUROLIVE studies, respectively.

Other factors, such as ApoA-I conformation and binding to HDL, can also exert a substantial impact on HDL-mediated ChE. In particular, ApoA-I stabilizes ABCA1 and activates LCAT, promoting ChE (Choi et al., 2016; Eckardstein & Kardassis, 2015; Karathanasis et al., 2017; Salazar et al., 2015). Oxidative modifications of ApoA-I are associated with an impairment in ApoA-I binding to HDL and therefore an impairnment in ChE (Favari et al., 2015; Zheng et al., 2004). Although no changes in ApoA-I sera concentrations were observed in the VOHF study after any VOO intake (Study 1 and Study 2), the higher content of antioxidants in HDL observed in Study 2 may confer better antioxidant protection to ApoA-I, LCAT and CETP, and these increases could be responsible for the observed

enhancement of ChE. In agreement with this data, Study 4 revealed that a close interrelationship exists between HDL oxidative status and ApoA-I characteristics, along with HDL monolayer fluidity, as previously mentioned, and these are the three main factors that appear to be related to CEC in our study.

Another HDL functionality enhancement provided by the phenol-enriched VOOs in the VOHF study is the modulation of PON enzyme family towards a cardioprotective mode based on the PC content and source, as described in Study 3 ("*Phenol-enriched olive oils modify paraoxonase–related variables: a Randomized, Crossover, Controlled Trial*"). On one hand, after the acute and sustained intake of OO-PC (provided by control VOO and FVOO intake), a decrease in PON1 protein levels, together with increases in PON3 protein levels and PON1-associated specific activities, *i.e.*, lactonase and paraoxonase, are reported. On the other hand, the sustained intake of a mixture of OO-PC and Th-PC (provided by FVOOT intake) induces the opposite results.

PON1 reacts with lipid peroxides to reduce and degrade them, inactivating the enzyme. As a consequence, the total PON1 activity is decreased and the body increases PON1 protein levels as a compensatory mechanism (Michael Aviram et al., 1999). Moreover, high PON1 protein levels and low associated activities are characteristic of diseases whose underlying mechanisms comprise an impairment of oxidative stress, in particular CVD (Abelló et al., 2014; Bayrak et al., 2012; Hafiane & Genest, 2015; Tang et al., 2012), T2DM (Dullaart, Otvos, & James, 2014), inflammatory diseases (Li et al., 2013; Tanimoto et al., 2003), cancer, and several hepatic and renal diseases (Goswami et al., 2009; Gugliucci & Menini, 2015). All these medical conditions are characterized by dysfunctional HDL particles and increased CVD risk (M. Mackness & Mackness, 2015). Furthermore, it is worth mentioning that PON1 positively correlates with the improvement of HDL antioxidant properties to such an extent that PON1 activities have been proposed as new biomarkers of HDL function and CVD risk (Breton et al., 2014; Hafiane & Genest, 2015). Therefore, the decrease in PON1 protein and the increase in its associated activities observed in Study 3 following the intake of PC from VOO can be perceived as beneficial, as they might be indicative of a proper oxidative balance, supporting HDL function

enhancement. Similarly, the PREDIMED study recently reported that a Mediterranean diet enriched with VOO increased arylesterase activity, the overall antioxidant protection of HDL particles, and other related HDL functions (Hernáez et al., 2017).

Although several authors have claimed that flavonoid-rich foods have a beneficial impact by increasing PON1-associated activities (M Aviram et al., 2000; Kaplan et al., 2001; Rosenblat et al., 2008; Suh et al., 2011), in the VOHF study, FVOOT intake decreased lactonase and paraoxonase activities and increased PON1 protein circulating levels, contrary to the effects observed after control VOO and FVOO intake. These differences may be due to the combination of OO-PC with Th-PC intake, rather than the sole presence of Th-PC, since mechanistic studies revealed that single-type PC modulated PON1 synthesis, while no effects were observed when multiple types of PC were combined.

PON3 protein depletion from HDL is associated with the presence of subclinical atherosclerosis in patients with autoimmune diseases (Marsillach et al., 2015). In addition, serum PON3 protein levels were decreased in chronic hepatitis and liver cirrhosis, suggesting a hepatoprotective role of PON3 in chronic liver impairment (García-Heredia et al., 2011). Therefore, the increase of serum PON3 protein levels observed in Study 3 after the sustained consumption of VOO seems to play a cardioprotective role. Our results partially agree with those observed in a proteomic study carried out in the HDL from the VOHF participants, where an increase in PON3 protein was reported not only after the sustained intake of VOO, but also after the intake of FVOO and FVOOT (Pedret et al., 2015). The dissimilarities observe d between these two approaches can be attributed to the different samples and methodologies employed.

The changes in the PON enzyme family observed in Study 3 can be translated into an improvement in the oxidative balance, leading to enhanced HDL functionality.

Serval mechanisms may explain the modulation of PON status observed in the present study. First, PON1 synthesis and expression are regulated by different stimuli through several transcription factors, which are activated via MAPK phosphorylation (Jordi Camps et al., 2012; Garige, Gong,

Varatharajalu, & Lakshman, 2010; Khateeb, Gantman, Kreitenberg, Aviram, & Fuhrman, 2010; Dmitry Litvinov et al., 2012; M. Mackness & Mackness, 2015; Schrader & Rimbach, 2011). As the mechanistic studies carried out in Study 3 revealed, single-type PC but not their combination, modulated the intracellular pathways that regulate hepatic PON synthesis. That is, the combination of OO-PC and Th-PC, rather than the sole intake of Th-PC, is accountable for the effects observed on PON-related variables in the VOHF study.

Second, PON1 binding to HDL and activation by ApoA-I are essential for PON activation (Gugliucci & Menini, 2015; Dmitry Litvinov et al., 2012; Rock et al., 2008; Seung et al., 2013). In fact, ApoA-I levels correlate to such an extent to PON1 activities that it has found that the ApoA-I concentration is the strongest predictor of arylesterase activity (Seung et al., 2013). The higher content of antioxidants in HDL observed in Study 2 confers better antioxidant protection to ApoA-I, which could stabilize PON1 binding to HDL, and thus enhance PON1 activation.

Third, HDL maturation is a key factor in modulating the PON system, since PON1 is not evenly present across all HDL subclasses. The PON1 protein rapidly shifts between HDL subclasses as it matures, which is also accompanied by a parallel ApoA-I shift towards larger HDL particles. Moreover, it has been reported that the inhibition of HDL maturation via the inhibition of CETP and LCAT reduces PON1 activation (Dullaart, Gruppen, & Dallinga-Thie, 2015; Gugliucci & Menini, 2015). Positive correlations have been observed between arylesterase activity and HDL-P, l-HDL, and HDL size in healthy and T2DM subjects (Dullaart et al., 2015, 2014). Concordantly, in the VOHF study, phenol-enriched VOOs decreased the s-HDL/l-HDL ratio, increased l-HDL, decreased s-HDL, and increased HDL size (Study 1). These results are indicative of HDL maturation enhancement and are therefore indicative of PON1 activation.

One of the strengths of the present study is that it is a crossover, randomized, controlled design, which enables collection of the first level of scientific evidence. The crossover design, in which each subject acts as the corresponding control, minimizes inter-individual variability. In

addition, the three VOOs employed in this study had the same parental matrix, and therefore they differed only in their PC content. A common matrix enabled isolation of the effects of PC without the interference of additional nutrients. Further strengths are that the laboratory analyses were centralized and that all the time-series samples from the same volunteer were measured in the same run.

The reduced sample size represents a possible limitation, as it may have led to diminished statistical power in some biomarkers due to increased intra-individual variability. Another limitation was the inability to assess potential synergies and interactions in HDL parameters from PC and other VOO constituents. Nevertheless, the controlled diet followed throughout the trial should have limited the scope of these interactions. One potential limitation of the study was that although the trial was blinded, some participants might have identified the type of olive oil that they ingested based on its organoleptic characteristics.

A determining limitation in the VOHF study is the method used to isolate HDL to measure ChE. This method limits HDL density to 1,063-1,210 g/mL and does not accurately represent the contribution of pre-β HDL, which is found in the density range 1,210–1,250 g/mL (Miyazaki et al., 2014; Rosenson et al., 2016). An alternative to this method would have been the use of ApoB-depleted plasma, but cholesterol acceptors other than HDL subfractions, such as albumin, are also present, and they may interfere in HDL-mediated ChE assessment (Rohatgi, 2015; Rosenson et al., 2016; Sankaranarayanan et al., 2013).

Novel therapeutic strategies should focus their efforts on not only increasing HDL-C but also enhancing HDL functionality, not least its CEC, through dietary, nutraceutical, or pharmaceutical interventions. Our results point out that HDL monolayer fluidity, ApoA-I content in HDL, and HDL oxidative status are the main determinants for CEC. In turn, lipid HDL composition and particle size exert their effect on CEC through changes in monolayer fluidity. Finally, our results provide the first level of evidence that the intake of VOO enriched with its own PC or with its own PC plus complementary ones from thyme could be a good nutraceutical to enhance the functionality of HDL particles, and thus it could be a

complementary tool for the management of hypercholesterolemic individuals. More specifically, considering all the evidence presented in this thesis, it seems safe to assume that FVOO intake may be better at modulating lipoprotein subclass distribution and PON enzyme family, while FVOOT intake may be better at promoting ChE.

Conclusions

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS
Sara Fernández Castillejo

The main conclusions that can be drawn from the results of the present thesis are as follows:

1. **Sustained consumption of phenol-enriched VOOs**, namely FVOO and FVOOT, shifted subclass distribution and associated ratios of lipoproteins to a **less atherogenic pattern**:

   1.1. Both phenol-enriched VOOs enhanced HDL maturation, since they modified HDL size and subclass distribution towards larger and more mature HDL particles.

   1.2. FVOO decreased the LDL and VLDL parameters commonly associated with CHD risk and dyslipemia.

   1.3. Both phenol-enriched VOOs decreased several atherogenic ratios.

   1.4. **FVOO could be better at improving lipoprotein subclass distribution**, since FVOOT had a milder impact on HDL and VLDL subclass distribution and atherogenic ratios than FVOO, and had no impact on LDL particle distribution.

2. **Sustained consumption of both phenol-enriched VOOs improved the HDL antioxidant content**, since they increased the lipophilic and hydrophilic antioxidants in HDL. The co-existence of these antioxidants linked to HDL may confer additional benefits by protecting lipids and proteins from oxidative damage via different antioxidant mechanisms. However**, FVOOT intervention could be better at improving HDL antioxidant activity**, as it increased α-tocopherol, the major antioxidant in human plasma. Moreover, FVOOT also increased phenolic acids in HDL, which are known to regenerate α-tocopherol.

3. **Acute and sustained intake of phenol-enriched VOOs modify the PON enzyme family towards a cardioprotective mode** according to the phenolic content and source. In particular, **FVOO intake may be better at modulating the PON enzyme family**, as it promoted changes that are indicative of a **proper oxidative balance**, supporting HDL function enhancement. On the other hand, FVOOT induced opposite results due to the combination of PC from OO and thyme, in comparison to the sole intake of PC from thyme. Several mechanisms are involved in PON system

modulation, such as PON synthesis, higher content of antioxidants in HDL, and enhanced HDL maturation.

4. Decreases in HDL oxidative status and increases in HDL monolayer fluidity and ApoA-I content in HDL are the **major determinants of CEC**. In turn, monolayer fluidity is determined by HDL lipid composition (in particular, by an increase in TG and a reduction in FC), together with a decrease in the concentration of s-HDL particles or an increase in HDL mean size.

5. Sustained intake of FVOOT increased **ChE** versus FVOO intake, despite the fact that no changes were observed in HDL **monolayer fluidity**.

6. Factors other than monolayer fluidity may be responsible for the ChE enhancement observed after FVOOT. These include a) a higher presence of antioxidants in HDL, which may confer better antioxidant protection and may therefore enhance ChE, b) a decrease in FC in the HDL monolayer, and c) the modulation of HDL subclass distribution.

Global conclusion

The present thesis confirms the hypothesis that the sustained intake of functional VOOs enriched with their own PC or with their own PC plus additional complementary ones from thyme modifies the physicochemical properties of HDL particles towards a cardioprotective mode and promotes changes in HDL subclass distribution, leading to the consequent enhancement of HDL functionality in hypercholesterolemic subjects. These changes occurred based on the phenol content and source in the tested VOOs.

The enrichment of VOOs with PC is a way of increasing the healthy properties of VOO without increasing the individual's caloric intake. Therefore, the tailoring of functional VOOs is an interesting and useful strategy for enhancing the functional quality of HDL, and thus, it is a complementary tool for the management of hypercholesterolemic individuals.

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EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS
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Annexes

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Scientific contributions published during the course of the doctoral thesis

1. Aranda, N., Valls, R., Romeu, M., Sánchez-Martos, V., Albaladejo, R., Fernández-Castillejo, S., Nogués, R., Catalán, Ú., Pedret, A., Espinel, A., Delgado, MA., Arija, V., Sola, R. & Giralt, M. (2017). Consumption of seafood and its estimated heavy metals are associated with lipid profile and oxidative lipid damage on healthy adults from a Spanish Mediterranean area: a cross-sectional study. Environmental Research, 156, 644–651. https://doi.org/10.1016/j.envres.2017.04.037

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International stays

1. Erasmus Intensive Course COSPI "Combating Obesity: Strategies for Prevention and Intervention"

University of Graz, Austria; February 22nd – March 8th, 2009.

INSTITUT FÜR MOLEKULARE BIOWISSENSCHAFTEN
UNIVERSITÄT GRAZ

UNI GRAZ

Human Nutrition & Metabolism Research and Training Center Graz
Grazer Forschungs- und Weiterbildungszentrum für Humanernährung & Ernährungsmedizin
Assoz.-Prof. Dr. med. Brigitte Winklhofer-Roob
Fachärztin für Kinder- und Jugendheilkunde

hnmrc
HUMAN NUTRITION & METABOLISM RESEARCH AND TRAINING CENTER

CERTIFICATE OF ATTENDANCE

This is to verify that

SARA FERNÁNDEZ CASTILLEJO

attended the

Erasmus Intensive Course
COSPI
Combating Obesity:
Strategies for Prevention and Intervention

Grundlsee, February 22 – March 8, 2009

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Human Nutrition & Metabolism Research and Training Center
Lehrstuhl für Humanernährung und Ernährungsmedizin
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2. Predoctoral stay at the National Institutes of Health

Lipoprotein Metabolism Section; Cardio-Pulmonary Branch; National Heart, Lung and Blood Institute (NHLBI); National Institutes of Health (NIH), Bethesda, MD, USA; August-November 2013.

DEPARTMENT OF HEALTH & HUMAN SERVICES Public Health Service

Dr. Alan T. Remaley
National Institutes of Health
Bethesda, Maryland 20892
Building : 10
Room : 2C-431
Telephone : (301) 496-3386
Fax : (301) 402-1885

Name	Sara FERNANDEZ-CASTILLEJO (PhD student)
Travel Fellowship	(Institut d'Investigació Sanitària Pere VIRGILI 2013)
Group Supervisor	Dr. Alan T. REMALEY National Institutes of Health Bldg. 10, Rm. 2C-433 10 Center Dr. Bethesda, MD, USA 20892-1508 Fax: 301-402-1885 Cell: 301-219-9233 email: aremaley1@cc.nih.gov Dra. Rosa SOLÀ I ALBERICH (SPAIN)
Room/Location	Department of Public Health and Clinical Nutrition.
Start date	June
End date	August
Period	13 weeks

Dear Dr. Rosa Solà,

I am the Chief of the Lipoprotein Metabolism Laboratory at NHLBI, National Institutes of Health. I am happy to inform you that I would be pleased to accept your student Sara FERNÁNDEZ-CASTILLEJO as a visitor in my laboratory. I am looking forward to working with you and her on the the project related to cholesterol efflux and would be happy to host your student for a few months this summer to initiate our collaboration.

Sincerely,

A. Remaley

Alan T. Remaley, M.D, Ph.D.
Investigator and Chief, Lipoprotein Metabolism Section
Cardiovascular and Pulmonary Branch, NHLBI

DEPARTMENT OF HEALTH & HUMAN SERVICES
Public Health Service
Cardiovascular and Pulmonary Branch
National Heart, Lung, and Blood Institute
National Institutes of Health

November 27, 2013

FROM: Alan Remaley, M.D., Ph.D., Investigator, DLM, CC, NIH

SUBJECT: Recommendation letter for Ms. Sara Fernandez-Castillejo

I am the section chief of the Lipoprotein Metabolism Laboratory at NHLBI, National Institutes of Health. Ms. Fernandez-Castillejo, who is currently a graduate student in Spain, worked in my laboratory as a special volunteer from August 2013 through Nov. of 2013. She worked on a project related to using fluorescent cholesterol analogues for monitoring cholesterol efflux from cells. She did a superb job and is a very dedicated and talented scientist. Her work is now being prepared for publication. Please contact me if you have any questions.

Alan Remaley, M.D., Ph.D.
Investigator, CPB, NHLBI

UNIVERSITAT ROVIRA I VIRGILI
EFFECTS OF PHENOL-ENRICHED VIRGIN OLIVE OILS ON HDL FUNCTIONALITY IN HYPERCHOLESTEROLEMIC SUBJECTS
Sara Fernández Castillejo

Special mentions

1. Special mention in the Master of Clinical Nutrition and Metabolism
Universitat Rovira i Virgili; 2011; Tarragona, Spain.

UNIVERSITAT ROVIRA I VIRGILI

El rector de la Universitat Rovira i Virgili atorga el

Premi Extraordinari de Final d'Estudis

a

Sara Fernández Castillejo

del màster universitari en Nutrició i metabolisme,
vinculat a la Facultat de Química de Tarragona, corresponent a l'any acadèmic 2009-10,
segons l'acord pres pel Consell de Govern d'aquesta Universitat el dia 21 de desembre de 2010.

Tarragona, 4 de maig de 2011

EL RECTOR

Francesc Xavier Grau Vidal

EL SECRETARI GENERAL

Antoni González i Senmartí

2. International Mobility Grant; URV
Universitat Rovira i Virgili; 2013; Tarragona, Spain.

3. International Mobility Grant; Biomedicine Doctoral Program
Departament de Medicina i Cirurgia, Programa de Doctorat de Biomedicina, Universitat Rovira i Virgili; 2013; Reus; Spain.

UNIVERSITAT ROVIRA I VIRGILI
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