UNIVERSITAT DE BARCELONA

Effects of the mediterranean diet and virgin olive oil on the function of high-density lipoproteins and the atherogenicity of low-density lipoproteins in humans

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EFFECTS OF THE MEDITERRANEAN DIET AND VIRGIN OLIVE OIL ON THE FUNCTION OF HIGH-DENSITY LIPOPROTEINS AND THE ATHEROGENICITY OF LOW-DENSITY LIPOPROTEINS IN HUMANS

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[Inserte aquí una cita célebre
que le haga parecer inteligente o sensible]

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Suposo que és moment d'acomiadar-me, esperant
No haver-te emprenyat massa, no haver semblat un boig.
Que la força ens acompanyi, adéu, fins sempre, sort!

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ABSTRACT

Cardiovascular diseases are the main cause of death worldwide and a relevant source of economic cost and physical disability. The Mediterranean Diet, associated with a high intake of virgin olive oil, has been shown to be protective against the development of cardiovascular diseases. Adherence to the Mediterranean Diet and the consumption of virgin olive oil induce this protection by improving several cardiovascular risk factors, such as the lipid profile. These two dietary interventions are known to increase cholesterol levels in high-density lipoproteins (HDLs) and decrease cholesterol concentrations in low-density lipoproteins (LDLs). However, it is becoming increasingly more accepted that the information provided by HDL and LDL cholesterol levels is quite limited. On the one hand, the biological functions of HDLs may reflect the anti-atherogenic role of the lipoprotein better than HDL cholesterol levels. On the other hand, several LDL characteristics beyond LDL cholesterol levels, such as the pro-atherogenic LDL traits (LDL size, oxidation, composition, etc.), may be more informative with respect to the unexplained cardiovascular risk of an individual. Nevertheless, despite their growing relevance, very few randomized controlled trials have examined the effects of healthy lifestyle modifications on these properties.

The aim of the present thesis project was to assess whether adherence to the Mediterranean Diet or the consumption of virgin olive oil was able to increase HDL functionality and decrease LDL pro-atherogenic traits in humans.

Samples in the present project were obtained from two randomized controlled trials: the EUROLIVE Study (*Effects of Olive Oil Consumption on Oxidative Damage in European Populations*) and the PREDIMED Study (*Effects of Mediterranean Diet on the Primary Prevention of Cardiovascular Disease*). In both cases, we isolated HDLs and LDLs in different sub-samples from volunteers in order to perform a complete battery of determinations related to HDL function and LDL atherogenicity.

The consumption of virgin olive oil increased the main HDL function: its cholesterol efflux capacity. An increase in the content of olive oil phenolic compounds in HDL, as well as the enhancement of HDL composition and size, may explain the functional improvement. Regarding the Mediterranean Diet, increasing adherence to this dietary pattern improves the four main HDL functions: cholesterol efflux capacity, HDL role in other steps of reverse cholesterol transport, HDL antioxidant activities, and HDL vasodilatory capacity. An improvement in HDL oxidation, size, and composition may also justify HDL enhanced function.

In addition, the consumption of virgin olive oil decreased LDL levels and LDL atherogenicity (increasing LDL size and resistance against oxidation). Adherence to the Mediterranean Diet also improved LDL pro-atherogenic traits, by increasing LDL size and resistance against oxidation, decreasing LDL oxidation *in vivo*, improving LDL composition, and decreasing LDL cytotoxicity in macrophages.

In conclusion, the present PhD thesis project shows that the consumption of virgin olive oil and adherence to the Mediterranean Diet improved HDL function and LDL pro-atherogenic traits in humans in two randomized controlled trials. Our findings provide two novel mechanisms to explain part of the benefits of these healthy diet interventions, and support previous evidence concerning the cardioprotective role of virgin olive oil and the Mediterranean Diet in humans.

RESUMEN

Las enfermedades cardiovasculares son la principal causa de muerte en el mundo y una relevante fuente de gasto social y discapacidad física. La Dieta Mediterránea, asociada a un alto consumo de aceite de oliva virgen, ha demostrado ser protectora frente al desarrollo de enfermedades cardiovasculares. La adherencia a una Dieta Mediterránea o la ingesta de aceite de oliva virgen inducen esta protección mejorando numerosos factores de riesgo cardiovascular, como el perfil lipídico. Ambas intervenciones son capaces de incrementar los niveles de colesterol en lipoproteínas de alta densidad (HDL, en sus siglas en inglés) y de disminuir las concentraciones de colesterol en lipoproteínas de baja densidad (LDL, en sus siglas en inglés). Sin embargo, cada vez es más aceptado que la información proporcionada por los niveles de colesterol HDL y LDL es limitada. Por un lado, el análisis de las funciones biológicas de las HDLs ha demostrado reflejar el papel anti-aterogénico de la lipoproteína mejor que los niveles de colesterol HDL. Por otro lado, ciertas características de las LDL más allá de sus niveles de colesterol, como los rasgos pro-aterogénicos de las LDLs (tamaño, oxidación, composición, etc.), podrían ser más informativas del riesgo cardiovascular residual de los individuos. No obstante, y a pesar de su creciente relevancia, muy pocos ensayos clínicos aleatorizados y controlados han estudiado los efectos protectores de modificaciones dietéticas saludables sobre estas propiedades.

El objetivo del presente proyecto de tesis fue determinar si la adherencia a una Dieta Mediterránea o el consumo de aceite de oliva virgen podía incrementar la funcionalidad de las HDLs o disminuir la aterogenicidad de las LDLs en humanos.

Las muestras del presente proyecto procedieron de dos ensayos clínicos aleatorizados y controlados: el estudio EUROLIVE (*Effects of Olive Oil Consumption on Oxidative Damage in European Populations*) y el estudio PREDIMED (*Effects of Mediterranean Diet on the Primary Prevention of Cardiovascular Disease*). En ambos casos, aislamos las HDLs y LDLs de diferentes sub-muestras de voluntarios y realizamos una batería completa de determinaciones relacionadas con la función de HDL y la aterogenicidad de LDL.

El consumo de aceite de oliva virgen incrementó la principal función de HDL, la capacidad de eflujo de colesterol. Un incremento en el contenido de metabolitos de los compuestos fenólicos del aceite de oliva en las HDLs, así como una mejora de su composición y tamaño, podría justificar la anteriormente referida mejora funcional. Respecto a la Dieta Mediterránea, aumentar la adherencia a este patrón dietético mejoró las cuatro funciones principales de HDL: la capacidad de eflujo de colesterol, el rol de las HDLs en otros pasos del transporte reverso de colesterol, la capacidad antioxidante de las HDLs y la función vasoprotectora de las lipoproteínas. Una mejora en la oxidación, composición y tamaño de las HDLs también podría justificar la mejora funcional de las HDLs.

Además, el consumo de aceite de oliva virgen disminuyó los niveles de LDLs circulantes y la aterogenicidad de las mismas (aumentando el tamaño y la resistencia frente a la oxidación de las LDLs). La adherencia a la Dieta Mediterránea también mejoró las características pro-aterogénicas de las LDLs, aumentando el tamaño y la resistencia frente a la oxidación de las lipoproteínas, disminuyendo su oxidación *in vivo*, mejorando su composición y disminuyendo su citotoxicidad en macrófagos.

En conclusión, el presente proyecto de tesis demuestra que el consumo de aceite de oliva virgen o la adherencia a una Dieta Mediterránea mejora la función de las HDLs y la aterogenicidad de las LDLs en humanos en dos ensayos clínicos aleatorizados. Nuestros resultados proporcionan dos nuevos mecanismos para explicar parte de los beneficios de dichas intervenciones dietéticas saludables y apoyan las evidencias previas que indican el rol cardioprotector del aceite de oliva virgen y la Dieta Mediterránea en humanos.

RESUM

Les malalties cardiovasculars són la principal causa de mort en el món i una rellevant font de despesa social i discapacitat física. La Dieta Mediterrània, associada a un alt consum d'oli d'oliva verge, ha demostrat ser protectora davant el desenvolupament de malalties cardiovasculars. L'adherència a una Dieta Mediterrània o la ingesta d'oli d'oliva verge indueixen aquesta protecció millorant nombrosos factors de risc cardiovascular, com el perfil lipídic. Ambdues intervencions són capaces d'incrementar els nivells de colesterol en lipoproteïnes d'alta densitat (HDL, en les seves sigles en anglès) i de disminuir les concentracions de colesterol en lipoproteïnes de baixa densitat (LDL, en les seves sigles en anglès). Però, cada vegada s'accepta més que la informació proporcionada pels nivells de colesterol HDL i LDL és limitada. Per un costat, l'anàlisi de les funcions biològiques de les HDLs han reflectit el paper anti-aterogènic de la lipoproteïna millor que els nivells de colesterol HDL. Per un altre, certes característiques de les LDLs més enllà dels seus nivells de colesterol, com els trets pro-aterogènics de les LDLs (grandària, oxidació, composició, etc.), podrien ser més informatives del risc cardiovascular residual dels individus. No obstant això, i malgrat la seva creixent rellevància, molt pocs assajos clínics aleatoritzats i controlats han estudiat els efectes protectors d'intervencions dietètiques saludables sobre aquestes propietats.

L'objectiu del present projecte de tesi va ser determinar si l'adherència a una Dieta Mediterrània o el consum d'oli d'oliva verge era capaç d'incrementar la funcionalitat de les HDLs o de disminuir l'aterogenicitat de les LDLs en humans.

Les mostres del present projecte van procedir de dos assajos clínics aleatoritzats i controlats: l'estudi EUROLIVE (*Effects of Olive Oil Consumption on Oxidative Damage in European Populations*) i l'estudi PREDIMED (*Effects of Mediterranean Diet on the Primary Prevention of Cardiovascular Disease*). En ambdós casos, vam aïllar les HDLs i LDLs de diferents sub-mostres de voluntaris i vam realitzar una bateria completa de determinacions relacionades amb la funció d'HDL i l'aterogenicitat de LDL.

El consum d'oli d'oliva verge va incrementar la principal funció d'HDL, la capacitat d'eflux de colesterol. Un increment en el contingut de metabòlits dels compostos fenòlics de l'oli d'oliva a les HDLs, així com unes millors composició i grandària, podrien justificar l'anterior millora funcional. Respecte a la Dieta Mediterrània, augmentar l'adherència a aquest patró dietètic va millorar les quatre funcions principals de les HDLs: la capacitat d'eflux de colesterol, el rol de les HDLs en altres punts del transport revers de colesterol, la capacitat antioxidant de les HDLs i la funció vasoprotectora de les lipoproteïnes. Una millora a la oxidació, composició i grandària de les HDLs també podria justificar la millora funcional de les HDLs.

A més, el consum d'oli d'oliva verge va disminuir els nivells de LDL i l'aterogenicitat de les mateixes (augmentant la grandària i la resistència davant l'oxidació de les

lipoproteïnes). L'adherència a una Dieta Mediterrània també va millorar les característiques pro-aterogèniques de les lipoproteïnes, augmentant la grandària i la resistència a l'oxidació de les lipoproteïnes, disminuint la seva oxidació *in vivo*, millorant la seva composició i disminuint la seva citotoxicitat en macròfags.

En conclusió, el present projecte de tesi demostra que el consum d'oli d'oliva verge o l'adherència a una Dieta Mediterrània millora la funció de les HDLs i l'aterogenicitat de les LDLs en humans en dos assajos clínics aleatoritzats. Els nostres resultats proporcionen dos nous mecanismes per explicar part dels beneficis d'aquestes intervencions dietètiques saludables i recolzen les evidències prèvies que indiquen el rol cardioprotector de l'oli d'oliva verge i la Dieta Mediterrània en humans.

ABBREVIATIONS

ABCA1: ATP-binding cassette A1
ABCG1: ATP-binding cassette G1
ABDP: apolipoprotein B-depleted plasma
ApoA-I: apolipoprotein A-I
ApoA-II: apolipoprotein A-II
ApoB: apolipoprotein B
ApoB-100: apolipoprotein B-100
ApoC-II: apolipoprotein C-II
ApoC-III: apolipoprotein C-III
ApoE: apolipoprotein E
CETP: cholesterol ester transfer protein
CM: chylomicron
CVD: cardiovascular disease
DAF-2DA: 4,5-diaminofluorescein diacetate
DPH: 1,6-diphenyl-1,3,5-hexatriene
ELISA: enzyme-linked immunosorbent assay
eNOS: endothelial nitric oxide synthase
GPx: glutathione peroxidase
H2DCF-DA: 2',7'-dichlorodihydrofluorescein diacetate
HDL: high-density lipoprotein
HDL-C: HDL cholesterol
HPCOO: high phenolic content olive oil
HUVEC: human umbilical vein endothelial cell
IDL: intermediate-density lipoprotein
LCAT: lecithin cholesterol acyltransferase
LDL: low-density lipoprotein
LDL-C: LDL cholesterol
LDL-R: LDL receptor
LPCOO: low phenolic content olive oil
LPL: lipoprotein lipase
LpPLA2: lipoprotein-associated phospholipase A2
MDA: malondialdehyde
MPCOO: medium phenolic content olive oil
MTT: Thiazolyl Blue Tetrazolium
MUFA: monounsaturated fatty acid
NO: nitric oxide
PAF-AH: platelet activating factor acetylhydrolase
PBMCs: peripheral blood mononuclear cells

PLTP: phospholipid transfer protein
PON1: paraoxonase-1
PUFA: polyunsaturated fatty acid
S1P: sphingosine-1-phosphate
SAA: serum amyloid A
SR-BI: scavenger receptor B1
TBARS: thiobarbituric acid reactive species
TMD: Traditional Mediterranean Diet
TMD-Nuts: Traditional Mediterranean Diet supplemented with nuts
TMD-VOO: Traditional Mediterranean Diet supplemented with virgin olive oil
VLDL: very low-density lipoprotein
VOO: virgin olive oil

CONTENTS

1. Introduction

1.1. CARDIOVASCULAR DISEASES

1.1.1. DEFINITION AND EPIDEMIOLOGICAL OVERVIEW

Cardiovascular diseases (CVDs) are clinical disorders that affect the heart and blood vessels. Within this group we include: coronary heart diseases (the total or partial obstruction of coronary arteries of the heart, which may lead to different types of anginas or a myocardial infarction), cerebrovascular diseases (the total or partial obstruction of carotid arteries, which may result in ischemic injuries in the brain), and other diverse events (peripheral artery disease, deep vein thrombosis, pulmonary embolism, rheumatic heart disease, and congenital heart diseases)[1]. The most common cause for all these pathologies is atherosclerosis, the accumulation of fatty deposits in the wall of the blood vessels[2].

CVDs became the first cause of mortality in the world in the 20th century (approximately 30% of deaths worldwide) with the concurrent economic cost and physical disability[1]. This situation appeared due to population aging and an increase in unhealthy lifestyle habits (excessive calorie intake, low levels of physical activity, tobacco use, consumption of alcoholic beverages and the like). In the last 30 years, however, particularly in middle- and high-income countries, public policies have been able to decrease the incidence and the mortality of heart diseases through the promotion of healthy lifestyles and the intensive use of cardiovascular drugs in the population[3].

1.1.2. CARDIOVASCULAR RISK FACTORS

Cardiovascular risk factors are the clinical characteristics that induce the apparition of CVDs[4]. Apart from some inherent traits (age, sex, genetic background), this group includes several modifiable classical factors that have been studied since the 60s: hypertension[5], obesity[6], diabetes[7], tobacco use[8], and high levels of blood cholesterol[9]. Particularly in relation to cholesterol and blood lipids, these are transported in special particles called lipoproteins, with different functions. The delivery of lipids to peripheral cells is performed by chylomicrons, very-low density lipoproteins (VLDLs) and low-density lipoproteins (LDLs), and the uptake of the excess of cholesterol from the cells is developed by the high-density lipoproteins (HDLs). Low levels of cholesterol in HDLs (HDL-C) are independently associated with a high risk of developing a CVD[10], whilst high levels of cholesterol in LDLs (LDL-C) are linked with greater cardiovascular risk and are also one of the main therapeutic targets in cardiovascular therapy in both primary and secondary prevention[11].

Classical cardiovascular risk factors are able to explain a major part of CVD incidence, but not its totality. For instance, 15.4% of women and 19.4% of men that develop a coronary disease did not present any of the classical risk factors[12,13]. As a result, in the previous two decades, several new factors have emerged in order to

complement the information provided by the classical ones. There is not, however, clear consensus about their utility, since in some cases they rely on insufficient scientific evidence or there is a lack of standardized assays to be applied in large population studies. In addition to the classical cardiovascular risk factors, some of the most frequently studied ones are: homocysteinemia[14], microalbuminuria[15], inflammatory markers such as C-reactive protein[16], endothelial function (measured as flow mediated dilation[17]), plasminogen activator inhibitor-1[18], vitamin D deficiency[19], lipoprotein (a)[20], HDL function[21,22], and LDL characteristics[23].

Some cardiovascular risk factors are modified by lifestyle. Unhealthy lifestyle traits (tobacco use, physical inactivity, and inadequate diet) are responsible for approximately 80% of the world's CVD burden[24]. In recent decades, the modification of unhealthy lifestyle habits has proven to be preventive against the development of CVDs in the population, through the modulation of a number of cardiovascular risk factors[3]. The simultaneous correction of several unhealthy lifestyle features at the same time (for example, through the adherence to a healthy dietary pattern) is a feasible strategy for cardiovascular prevention. For instance, following a Mediterranean Diet has been shown to decrease the development of cardiovascular events in primary and secondary prevention trials[25,26].

1.1.3. PATHOPHYSIOLOGY OF CVDS: ATHEROSCLEROSIS

The main pathophysiologic process responsible for CVDs is atherosclerosis. This term refers to the accumulation of a fatty streak in the space below the endothelium of the blood vessels, accompanied by the stiffness of the arterial wall, due to a chronic local inflammatory response[2]. Atherosclerosis is a complex phenomenon in which a number of biochemical processes (LDL oxidation, inflammatory response, cell proliferation and migration, angiogenesis and the like) and several cell types (endothelial cells, macrophages, neutrophils, and vascular smooth muscle cells) are involved[2].

Endothelial dysfunction is necessary to trigger the atherosclerotic response. Endothelial dysfunction is characterized by the inability of the endothelium to avoid the transfer of different substances from the blood to the sub-endothelial space. Such a process takes place due to the attacks from different substances that may be present in the bloodstream: 1) high levels of reactive oxygen and nitrogen species, known as free radicals (due to smoking, unhealthy eating, and high pollution levels); 2) hyperglycemia; 3) hyperhomocysteinemia; 4) constant hypertensive stimulation (due to high levels of angiotensin II in the circulation); 5) infectious microorganisms (*Chlamydia pneumoniae*, herpesviruses, etc.); and 6) a combination of these factors[2].

Once the endothelium is dysfunctional, LDL particles are able to traverse the endothelial barrier and become oxidized in the sub-endothelial space (also known as the intima). Oxidized LDLs induce the release of chemokines by endothelial cells (such as the monocyte chemoattractant protein-1), as well as the expression of adhesion

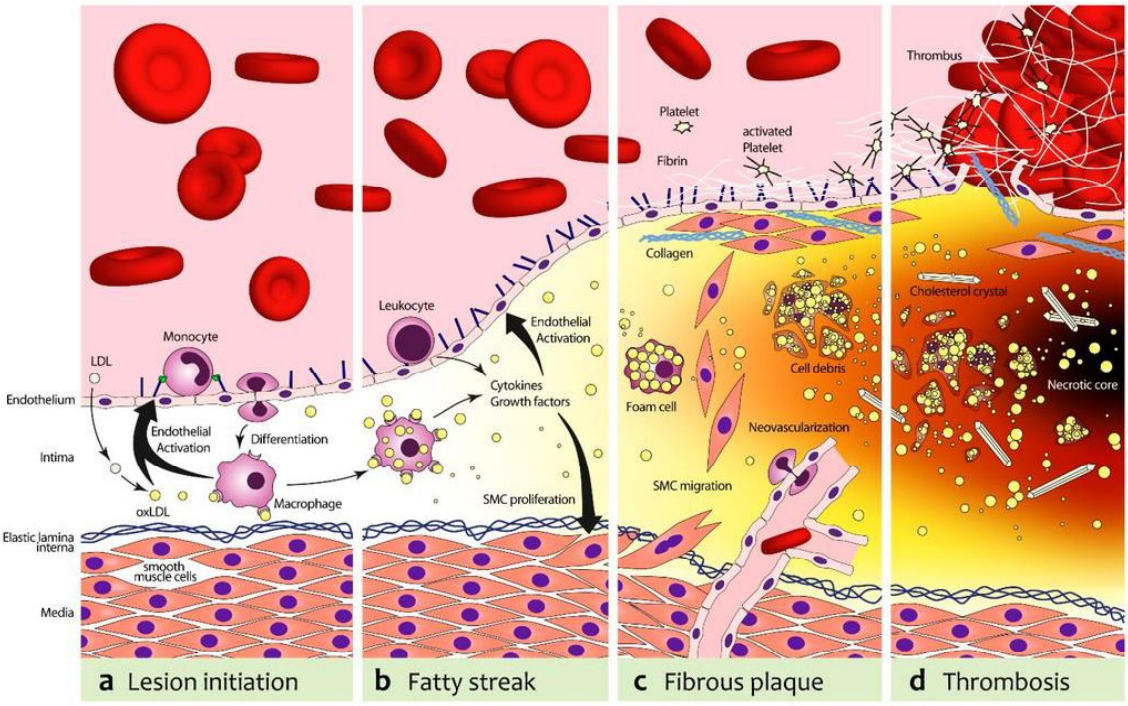
molecules in the surface of the endothelium (such as vascular cell adhesion molecule 1, intracellular adhesion molecule-1, and P-selectin)[2]. These biochemical changes induce the attraction of circulating monocytes to the region, their adhesion to the local endothelium, their transmigration to the sub-endothelial space, and finally their differentiation/activation to macrophages. At this point, active macrophages begin to express several receptors able to recognize modified and native LDLs (such as CD36, scavenger receptor A, and the classical LDL receptor **–**LDL-R**–**) and phagocyte the modified LDLs in the region[27].

Excessive phagocytosis of modified LDLs blocks the negative feedback of the uptake process in macrophages and induces a disproportionate accumulation of phagocyted lipids inside the cells. Lipid-overloaded macrophages become overexcited, release continuously different pro-inflammatory markers, and induce the local migration of other activated immune cells. Newcoming immune cells induce the arrival of more pro-inflammatory cells, worsening the endothelial dysfunction, and thus the process constantly recommences. Once the macrophages are fully lipid-loaded they become foam cells and lead to the formation of the sub-endothelial fatty streak[28].

In this pro-inflammatory environment, vascular smooth muscle cells from the media migrate to the sub-endothelial space and start to proliferate[29]. Some of these cells also begin to accumulate lipids and commence their transformation into new foam cells[30]. Moreover, activated smooth muscle cells begin to release extracellular matrix molecules and form a fibrous cap over the growing atherosclerotic plaque. In parallel, the chronic pro-inflammatory environment induces the death of foam cells, which release their cell content to the intima and contribute to the formation of the necrotic core of the plaque. Additionally, an angiogenic response is locally induced, allowing the formation of new blood vessels around the plaque[28]. The aberrant function of all the cells at this point contributes to the release of cellular calcium, leading to the progressive calcification and stiffness of the plaque. If the plaque turns too rigid, and the fibrous cap over the plaque breaks, the plaque content is released to the bloodstream and induces the formation of thrombi[2,28,31].

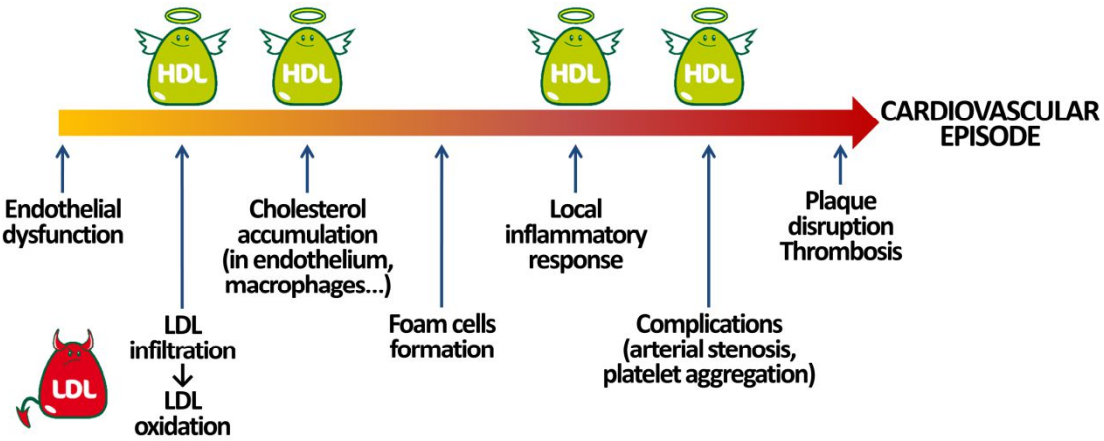
In this stage, atherosclerotic plaques progressively invade the blood vessel lumen and decrease its width. When plaques are located in coronary arteries they may lead to some clinical symptoms in the heart: 1) if the blood flow is constantly limited, stable anginas are developed; 2) if a thrombus interrupts blood flow temporarily, we find unstable anginas; and 3) if a thrombus interrupts blood flow permanently, a myocardial infarction appears[32]. In the case that plaque-induced collapses develop in carotid arteries, due to blood flow stenosis or plaque disruption, they may induce ischemic or hemorrhagic strokes, respectively[33] (**Figure 1**).

Figure 1. The development of atherosclerosis (Steinl DC, *Int J Mol Sci*, 2015)[28].



Some of the pathophysiologic steps of atherosclerosis can be modulated. On the one hand, healthy lifestyle and cardiovascular drug interventions are able to correct several of the cardiovascular risk factors involved in the atherosclerotic process[3]. On the other hand, there are a number of physiological agents that are able to ameliorate the process while it is taking place, such as endothelium-based vasodilatory responses, anti-coagulant signals, and the protective role of HDLs[2,32]. Particularly in the case of HDLs, these lipoproteins can: 1) decrease LDL oxidation in the sub-endothelial space; 2) contribute to the cholesterol removal from macrophages; 3) downregulate some of the pro-inflammatory responses of macrophages and immune cells; 4) contribute to endothelial homeostasis and vasodilation; and 5) modulate local coagulation responses[34–36] (**Figure 2**).

Figure 2. The progression of the atherosclerotic lesion, LDLs and HDLs.

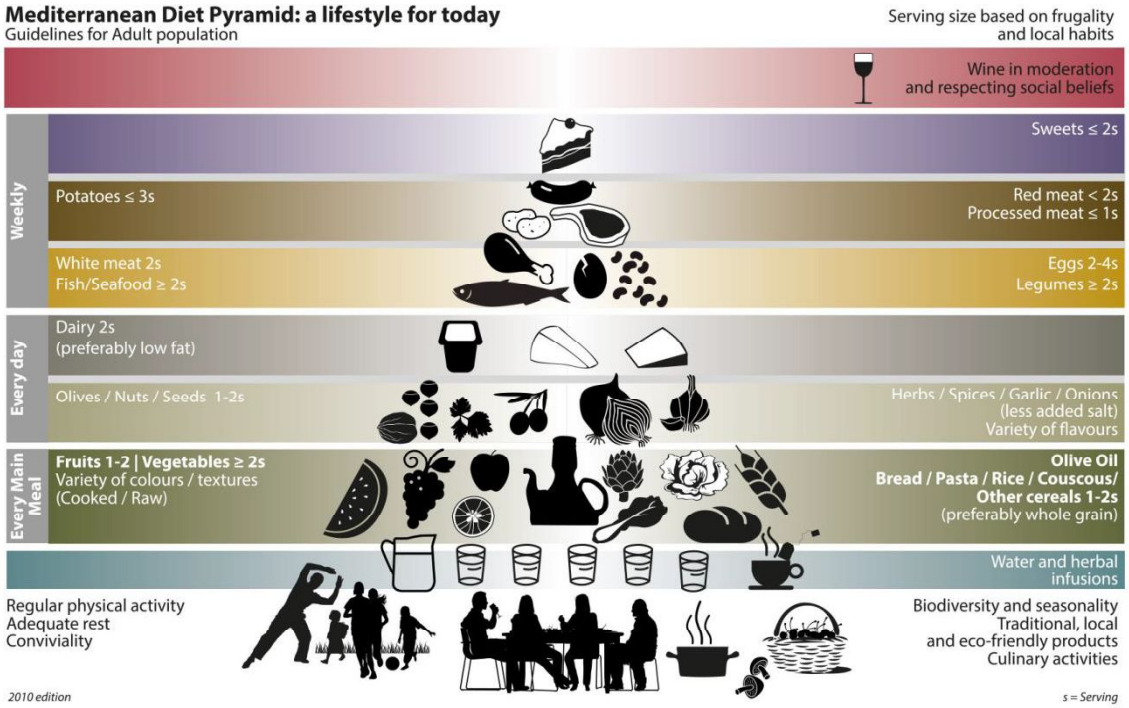


1.2. THE MEDITERRANEAN DIET, VIRGIN OLIVE OIL, AND CARDIOVASCULAR PROTECTION

1.2.1. THE MEDITERRANEAN DIET

The Traditional Mediterranean Diet (TMD) is the dietary pattern present in the cultures around the Mediterranean basin. It is characterized by: 1) olive oil, mainly the virgin one, as the main source of fat; 2) a high intake of vegetal foods (fruit, vegetables, legumes, whole grains, and nuts); 3) a moderate consumption of fish, poultry, eggs, and dairy products (mainly as dairy derivatives); 4) a moderate consumption of wine during the meals; and 5) a low intake of meat and processed meats[25] (**Figure 3**).

Figure 3. Mediterranean Diet pyramid (www.dietamediterranea.org).



The Mediterranean basin has classically been a geographic area with low CVD incidence. Back in the 50s, Professor Ancel Keys observed this fact and included some Mediterranean countries (Italy and Greece) in "The Seven Countries Study" considered to be the first epidemiological study about the effects of dietary patterns on health and in which the term "Mediterranean Diet" was coined[37]. From that moment, several epidemiological trials have revealed that the TMD is able to decrease overall mortality, CVD mortality and incidence, cancer mortality and incidence, and neurodegenerative disease incidence[38–40]. Recently, the PREDIMED Study (*Effects of Mediterranean Diet on the Primary Prevention of Cardiovascular Disease*), a long-term, randomized, controlled trial has demonstrated that adherence to a TMD (supplemented with virgin olive oil –VOO– or nuts) is able to decrease by 30% the incidence of major cardiovascular events, in primary prevention, in a population at high cardiovascular

risk[25]. The Lyon Diet Heart also showed that a modified Mediterranean Diet (enriched with α-linoleic acid, one of the most relevant lipids in walnuts) was able to decrease the incidence of secondary cardiovascular events by 47%[26].

The TMD is able to correct several cardiovascular risk factors. Within the frame of the PREDIMED Study, adherence to this dietary pattern decreased the incidence of type-2 diabetes[41] (due to improvements in dietary glycemic load and dietary glycemic index[42]), decreased blood pressure[43], improved lipid profile[25,44], and decreased abdominal obesity[45]. Regarding novel cardiovascular risk factors, following a TMD decreased the oxidation of plasma lipids, LDLs, and DNA[46,47], improved the gene expression related to CVDs[48], enhanced the plasma fatty acid composition pattern[49], and improved several pro-inflammatory biomarkers[50,51].

The TMD induces these benefits due to its richness in antioxidants and bioactive compounds. On the one hand, it is abundant in several healthy nutrients such as antioxidant vitamins[52,53], folates[54], monounsaturated fatty acids (MUFAs)[55], polyunsaturated fatty acids (PUFAs)[56], and phenolic compounds[57], which are able to induce diverse protective effects. On the other hand, the TMD provides low doses of toxic nutrients (trans-fatty acids[58]) and moderate doses of nutrients with certain toxic potential (sodium[59], ethanol[60]). This nutrient network generates a healthy dietary matrix which boosts its protective potential and leads to greater benefits than those expected for the individual consumption of each of its foods[61]. Within this synergistic protection, the use of VOO instead of other culinary fats is a major factor.

1.2.2. VIRGIN OLIVE OIL (VOO)

Olive oil is the main culinary fat of the Mediterranean Diet and the hallmark of this dietary pattern. Regarding its fatty acid content, it is composed mainly of oleic acid (55-83%, a MUFA of 18 carbon atoms), PUFAs (4-20%), and saturated fatty acids (8-14%)[62]. Besides, olives also contain several minor constituents. These components constitute 1-2% of the total oil content, and can be classified as: 1) the unsaponifiable fraction (non-polar components, such as squalene, triterpenes, phytosterols, tocopherols, and carotenoids), and 2) the soluble fraction (polar components, with the phenolic compounds being the most relevant ones)[63,64]. The soluble fraction is present in olive oil only when it is obtained by direct pressure or centrifugation (VOO), since most of olive minor components are lost in the refining process[65]. Whilst the rest of its components are present in quite stable concentrations, olive oil phenolic compounds vary in quantity (50-1000 mg/kg of oil) depending on: the olive variety, the age of the tree, the agricultural techniques used in the cultivation and harvest, the composition of the soil, the climate, the technique used to process the olives and to produce the oil, and the storage[66]. The concentration ranges of the different olive oil components are available in **Table 1**[63,64], and the different families of olive oil phenolic compounds are described in **Table 2**[67].

Table 1. Concentration ranges of olive oil components (adapted from Boskou D, *World Rev Nutr Diet*, 2007; and Guillén N et al, *Rev Esp Cardiol*, 2009)[63,64].

Fatty acid composition		Olive oil minor components	
Common name	**Concentration (%)**	**Common name**	**Concentration (mg/kg oil)**
Oleic acid	55-83	Terpenic compounds	1000-3500
Linoleic acid	3.5-21	Phytosterols	1000-2500
Palmitic acid	7.5-20	Hydrocarbons	
Stearic acid	0.5-5.0	Squalene	1500-8000
Palmitoleic acid	0.3-3.5	Carotenes	5-10
Linolenic acid	0.0-1.0	Phenolic compounds	50-1000
Arachidic acid	≤0.6	Aliphatic alcohols	100-700
Eicosanoic acid	≤0.4	Tocopherols	5-300
Heptadecanoic acid	≤0.3	Esters	100-200
Heptadecenoic acid	≤0.3	Aldehydes/ketones	40-80
Behenic acid	≤0.2	Chlorophylls	1.0-20
Lignoceric acid	≤0.2		
Myristic acid	≤0.05		

Table 2. Classification of the main olive oil phenolic compounds (adapted from Lou-Bonafonte JM et al, *Mol Nutr Food Res*, 2012)[67].

Chemical structure	Compounds
Simple phenols	Alcohols: tyrosol, hydroxytyrosol Acids: caffeic, gallic, p-coumaric, protocatechuic, sinapic, syringic, vanillic
Flavonoids	Apigenin, luteolin
Secoiridoids	Oleuropein glucoside, aglycones of oleuropein glucoside and ligstroside, and dialdehydic forms of oleuropein glucoside and ligstroside without a carboxymethyl group
Lignans	(+)-pinoresinol, (+)-1-acetoxy-pinoresinol

Three types of olive oil are present in the market: VOO, ordinary olive oil, and pomace olive oil. VOO is obtained only by direct pressing or centrifugation of the olives, and it is the richest one in phenolic compounds (150-400 mg/kg, as an average). Whenever VOO presents a high acidity (a free fatty acid content above 3.3 g/100 g of oil, or 2.0 g/100 g of oil in the European Union), the oil is submitted to a refining process to eliminate it. However, the refining process also leads to practically the total loss of the phenolic compounds, and to the partial loss of other minor compounds[68]. By mixing the resulting refined oil with VOO, ordinary olive oil is produced (containing 50-150 mg/kg of phenolic compounds). Finally, after the production of VOO, the remaining olive drupe and seeds are pressed again and the resulting oil is submitted to another refining process. This refined oil is mixed again with VOO, to obtain pomace olive oil (containing 10-70 mg/kg of phenolic compounds)[69].

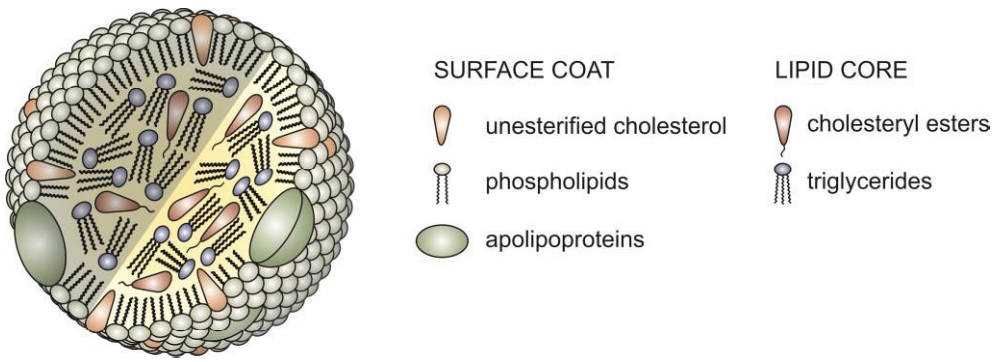
VOO can be considered a functional food since its consumption promotes several health effects in humans. Olive oil intake has been associated with a decrease in all-cause mortality, general CVD mortality and incidence, and stroke incidence[55]. To explain these effects, the consumption of MUFAs has been shown to be able to enhance HDL-C levels, triglyceride levels, blood pressure, adiposity, and glucose metabolism[70–72]. In addition, the intake of olive oil phenolic compounds is able to improve HDL-C levels, blood pressure, oxidation of plasma lipids, DNA oxidation, endothelial function, pro-thrombotic profile, and systemic pro-inflammatory markers[73–79]. As a result of this, in 2004 the United States Food and Drug Administration recognized the capacity of olive oil to reduce cardiovascular risk due to its MUFA content (substituting an equivalent amount of saturated fat for 25 mL of olive oil)[80]. In 2011, the European Food Safety Authority recognized the ability of VOO to avoid LDL oxidation *in vivo* (following the consumption of 5 mg/day of olive oil phenolic compounds)[81]. We can conclude that VOO is more than a monounsaturated fat, with some of its beneficial properties still to be revealed.

1.3. LIPID DELIVERY IN PLASMA: LIPOPROTEINS

1.3.1. DEFINITION OF PLASMA LIPOPROTEINS

Plasma lipoproteins are the particles in which lipids are transported in the bloodstream. Generally, lipids are non-polar substances, insoluble in water-based dilutions such as blood. Therefore, to be solubilized these lipids have to be packed in special structures called micelles. In these structures, polar chemical groups are exposed towards the polar medium, whilst the non-polar chemical groups are isolated on the inside, to form a stable spherical particle. Lipoproteins are a specific type of micelles in which the non-polar lipids present in plasma (triglycerides and cholesteryl esters) are placed in the hydrophobic core, and the polar plasma lipids (phospholipids and free cholesterol) are located in the hydrophilic surface. Around the classic micelle structure of the lipoprotein, we find a group of proteins that contribute to stabilizing the particle (apolipoproteins), as well as other proteins responsible for additional functions[82] (**Figure 4**).

Figure 4. Structure of lipoproteins (www.commons.wikimedia.org)



There are two lipoprotein groups: the intestinal synthesis lipoproteins (chylomicrons and their remnant particles) and the hepatic synthesis ones (very low-, low-, intermediate-, and high-density lipoproteins). The physicochemical characteristics of these six lipoproteins are available in **Table 3**.

Table 3. Physicochemical characteristics of plasma lipoproteins (adapted from Feingold KR, *Endotext*, 2015)[82].

Lipoprotein name	Density (g/mL)	Size (nm)	Principal lipids	Principal apolipoproteins
Chylomicrons (CMs)	<0.930	75-1200	Triglycerides	B-48, C-I, C-II, E, A-I, A-II
Chylomicron remnants	0.930-1.006	30-80	Triglycerides Cholesterol	B-48, E
Very low-density lipoproteins (VLDLs)	0.930-1.006	30-80	Triglycerides	B-100, E, C-I, C-II
Intermediate-density lipoproteins (IDLs)	1.006-1.019	25-35	Triglycerides Cholesterol	B-100, E, C-I, C-II
Low-density lipoproteins (LDLs)	1.019-1.063	18-25	Cholesterol	B-100
High-density lipoproteins (HDLs)	1.063-1.210	5-12	Cholesterol Phospholipids	A-I, A-II, C-II, E

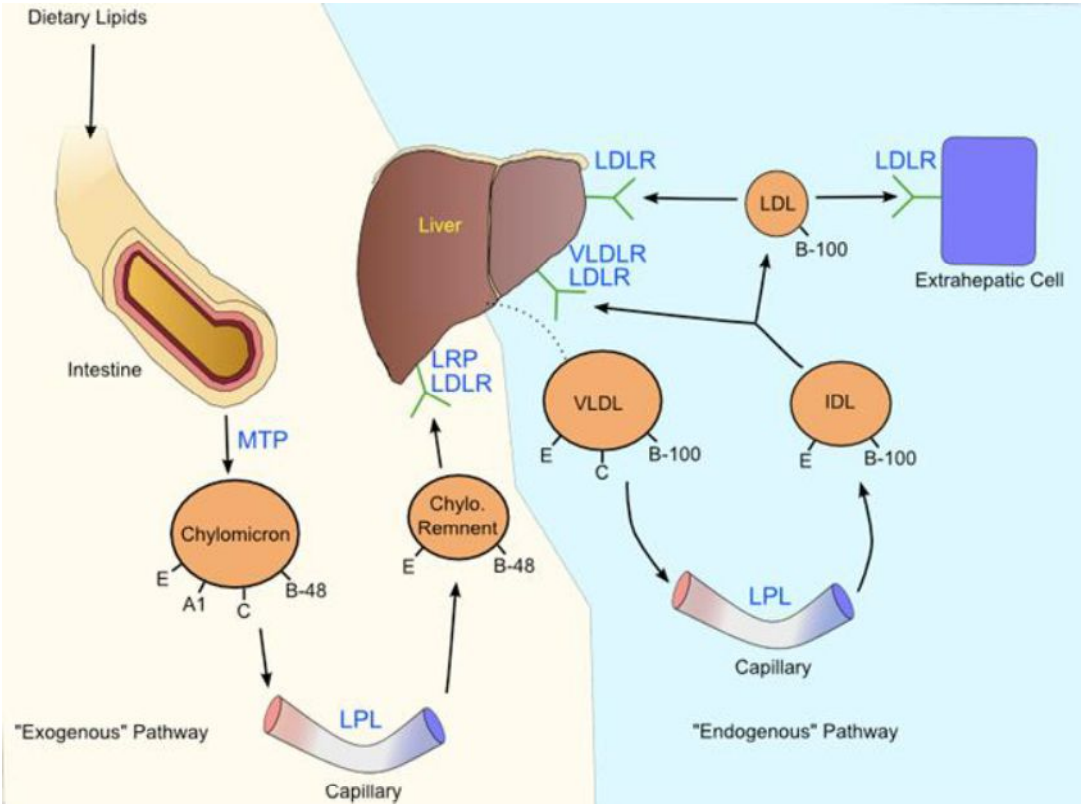
1.3.2. THE LIPOPROTEIN CYCLE

Lipoproteins are involved in two cycles of lipid distribution in plasma: the delivery of dietary lipids, and the delivery of lipids of endogenous origin. The dietary lipids are transported by the lipoproteins of intestinal synthesis (chylomicrons and their remnant particles), and the lipids of endogenous origin are transported by some hepatic lipoproteins (VLDLs, IDL, and LDLs). The uptake of lipid excess in the peripheral cells is finally performed by HDLs.

After digestion, dietary lipids are absorbed by the enterocytes. Fatty acids and monoacylglycerols are combined into triglycerides, and the free cholesterol is esterified locally. These lipids are packed with phospholipids around one unit of apolipoprotein B-48 molecule by the microsomal triglyceride transfer protein to form a chylomicron (CM). The CMs are released to the lymph and, subsequently, reach the bloodstream. CMs deliver mainly triglycerides to muscle and adipose tissue, by the function of lipoprotein lipase (LPL). When the apolipoprotein C-II (ApoC-II) in the CM recognizes the LPL, both bind and LPL catalyzes the release and hydrolysis of the triglycerides in the CM[83]. The poorer the CM is in triglycerides, the lower its content in ApoC-II particles. While they are in circulation, CMs also lose other apolipoproteins they transported in the beginning (mainly apolipoprotein As and other apolipoprotein Cs), which are directed principally to HDLs. When CMs turn triglyceride-poor, they become CM remnants and are picked up by the liver through the binding of their apolipoprotein Es (ApoEs) to the ApoE-receptors in hepatocytes[83].

The remaining cholesterol and triglycerides in CM remnants, and the triglycerides and cholesterol esters synthesized locally in the hepatocytes, are packed with phospholipids around one unit of apolipoprotein B-100 (ApoB-100), in a process catalyzed again by the microsomal triglyceride transfer protein. In this case, the resulting lipoproteins are nascent VLDLs. These particles are released to circulation and impact with HDLs. As a consequence, HDLs donate ApoC-IIs and ApoEs to nascent VLDLs, which are then considered mature VLDLs. VLDLs deliver triglycerides to tissues in a similar way to CMs: the ApoC-IIs in VLDLs bind to LPLs in the surface of peripheral cells, and catalyze the release and hydrolysis of triglycerides. When VLDLs turn triglyceride-poor they become IDLs, which are partially (50%) cleared by the liver through the binding of their ApoEs to the ApoE receptors in hepatocytes. The remaining triglycerides in IDLs are hydrolyzed by the hepatic lipase in plasma, the remaining apolipoproteins in IDLs are transferred to other lipoproteins (principally HDLs), and IDLs become LDLs. LDLs are cholesterol-rich, and deliver it to the cells through the binding of their ApoB-100 to the LDL-R in peripheral cells. Cells express LDL-R in their surface when the cholesterol levels of the cells are low, and terminate LDL-R expression when the cholesterol levels in the cell are reestablished. Remnant LDLs are picked up by the hepatocytes, following a similar mechanism[83,84] (**Figure 5**).

Figure 5. Delivery of lipids to peripheral cells (Daniels TF, *Int J Biol Sci*, 2009)[85].



IDL: **intermediate-density lipoprotein*; MTP*: microsomal triglyceride transfer protein; *LDL*: low-density lipoprotein; *LDL-R*: LDL receptor; *LPL*: lipoprotein lipase; *LRP*: LDL receptor related protein; *VLDL*: very low-density lipoprotein; *VLDL-R*: VLDL receptor**

In all these cycles, HDLs are the "regulatory lipoproteins". Since cholesterol can be toxic for the cells when it is found at high concentrations, HDLs pick up the cholesterol excess in peripheral cells in order to transport it back to the liver (for metabolization or excretion). HDLs may also accept cholesterol and phospholipids from CMs, VLDLs, and IDLs, due to the function of the phospholipid transfer protein (PLTP). Moreover, as previously commented, they contribute to carrying the excess of apolipoproteins in CMs and IDLs, and help in the maturation of VLDLs[83,84,86].

1.4. HIGH-DENSITY LIPOPROTEINS

As we have just commented, HDLs are the main regulatory particles in the lipoprotein cycle. They are the smallest and densest lipoproteins (as they contain a greater quantity of proteins in their structure). However, unlike the rest of the lipoproteins, they are highly heterogeneous in their structural and biochemical properties[82].

1.4.1. HDL BIOLOGICAL CYCLE

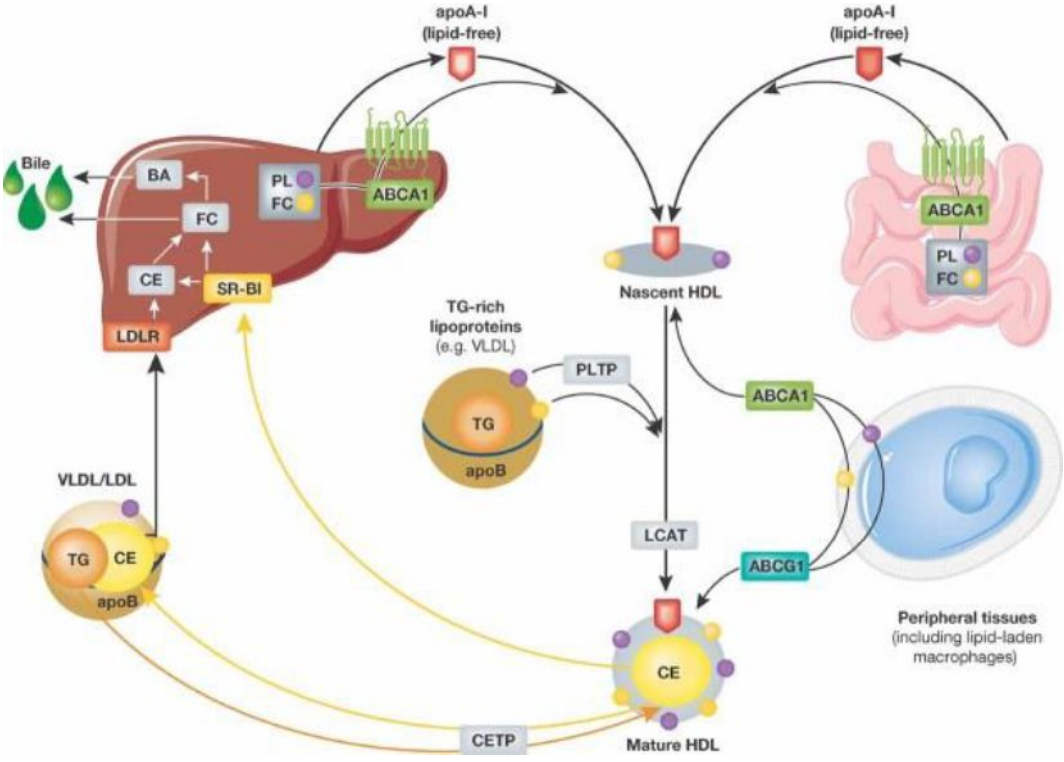
The formation of HDLs begins with the release to the bloodstream of its most relevant protein, apolipoprotein A-I (ApoA-I). ApoA-I can be biosynthesized in enterocytes and hepatocytes, or can come from the delipidation of mature HDLs due to the action of several plasma lipases (endothelial lipase, hepatic lipase)[87]. Lipid-free ApoA-Is begin to pick up cholesterol from peripheral cells by binding to several types of cholesterol transporters in the surface of cells, in a process called cholesterol efflux. Through this capacity, HDLs receive free cholesterol and some phospholipids from cells. As these two lipids are polar, their stabilization in a particle is complex: the mixture of ApoA-Is and these lipids leads to the formation of immature, discoidal HDLs[86,88].

To stabilize the particle, nascent HDLs incorporate an enzyme, lecithin-cholesterol acyltransferase (LCAT), to their structure. This enzyme contributes to the esterification of the free cholesterol molecules: LCAT catalyzes the union of an acyl group from the phospholipids in the particle to the hydroxyl group of the free cholesterol molecules in the nascent HDL. This fusion leads to the formation of esterified cholesterol molecules which are non-polar and, therefore, insoluble in the blood. Thus, esterified cholesterol molecules are internalized and the phospholipids and free cholesterol molecules surround the non-polar core, forming the HDL polar surface. HDLs then develop the micelle form, characteristic of the lipoproteins, and acquire their definitive mature structure. From now on, all the non-polar lipids they synthesize or pick up will remain in the core, and all polar lipids will be located in the surface[86,88].

Mature HDL particles perform cholesterol efflux capacity by binding to several cholesterol transporters in the surface of cells. The stabilization and internalization of

the effluxed cholesterol due to LCAT leads to the formation of bigger HDLs. However, HDLs must in some way return the cholesterol to the liver. There are two possible forms to do this: indirectly and directly. On the one hand, the indirect way (the main one) involves triglyceride-rich lipoproteins. HDLs are able to exchange non-polar lipids with these lipoproteins, through the action of the cholesterol ester transfer protein (CETP). CETP catalyzes the exchange of one molecule of esterified cholesterol from HDLs with a triglyceride molecule from triglyceride-rich lipoproteins. As this process is isomolecular, HDLs become enriched in triglycerides and triglyceride-rich lipoproteins in esterified cholesterol. When the remnants of triglyceride-rich lipoproteins are picked up by hepatocytes, the cholesterol excess will also return to the liver. On the other hand, HDLs are also able to deliver cholesterol directly to the liver. This process involves: 1) the binding of HDLs to some specific receptors in the surface of hepatocytes (for instance, HDLs can be picked up by the ApoE receptors if they have incorporated ApoE to their structure in the circulation); or 2) the transfer of a gradient of cholesterol from HDLs to hepatocytes through the scavenger receptor B1 (SR-BI). SR-BI receptor catalyzes a bidirectional cholesterol flux, following a cholesterol gradient: it is able to catalyze the exit of cholesterol from peripheral cells to HDLs (since cholesterol is more concentrated inside the cells), although it is able to catalyze the exit of cholesterol from HDLs to hepatocytes (as, in this case, cholesterol is more concentrated in the lipoproteins)[86,88].

Figure 6. HDL biological cycle (Besler C et al, *EMBO Mol Med*, 2012)[89].



***ABCA1*: ATP-binding cassette A1; *ABCG1*: ATP-binding cassette G1; *ApoA-I*: apolipoprotein A-I; *ApoB*: apolipoprotein B; *BA*: bile acid; *CE*: cholesterol ester; *CETP*: cholesterol ester transfer protein; *FC*: free cholesterol; *LCAT*: lecithin cholesterol acyltransferase; *LDLR*: LDL receptor; *PL*: phospholipid; *PLTP*: phospholipid transfer protein; *SR-BI*: scavenger receptor B1; *TG*: triglyceride.**

When HDLs become considerably lipid-laden, it may be necessary to reverse them to smaller states, in order to avoid an "infinite growth" that could lead to dysfunctional particles. On the one hand, as previously commented, they can exchange their non-polar lipids with triglyceride-rich lipoproteins through the activity of CETP. On the other hand, they can suffer the lipolytic effect of several plasma lipases such as the endothelial and hepatic ones. These lipases decrease HDL size by reducing theirlipid content, transferring the lipids to other lipoproteins or releasing them into circulation. In some cases, they may totally disaggregate HDL structure, and liberate lipid-free ApoA-Is which are able to restart the HDL biological cycle anew[86,88].

The overall action of HDLs on the metabolism of cholesterol excess in peripheral cells (uptake and delivery to the liver) is a global process called reverse cholesterol transport which constitutes the primordial function of HDL[89] (**Figure 6**).

While HDLs are in circulation they are able to bind several active proteins. HDLs can incorporate into their structure some major ones, such as apolipoproteins A-II (ApoA-II, the second most abundant in HDLs, representing approximately 20% of their protein content) and ApoE (partially responsible for their binding to hepatocytes[86,88]). HDLs may also bind other apolipoproteins that contribute to the lipid delivery process (such as apolipoproteins C-I and C-II)[82]. Moreover, HDLs can bind some proteins related to their second most relevant function, their antioxidant capacity. In addition to reverse cholesterol transport, HDLs contribute to maintaining plasma lipids non-oxidized, a key role to avoid LDL oxidation. To this purpose, they can incorporate enzymes such as paraoxonase-1 (PON1) or the lipoprotein-associated phospholipase A2 (LpPLA2, also known as platelet activating factor acetylhydrolase or PAF-AH), and other minor proteins such as apolipoprotein J, ceruloplasmin, and the like[90]. Finally, HDLs can also bind some elements related to another of their functions, their ability to facilitate endothelial protection and vasodilation: HDLs are able to protect endothelial homeostasis and nitric oxide (NO) release from endothelial cells. In this case, HDL may bind apolipoprotein M, the ligand for sphingosine-1-phosphate (S1P), a lipid component that is thought to help in HDL endothelial functions[91]. A more complete description of HDL proteome is available in **Table 4**[82,86,88,91–94].

Table 4. Main elements of HDL proteome (adapted from Kontush A et al, *Pharmacol Rev*, 2006; Kontush A et al, *Front Pharmacol*, 2015; and Feingold KR, *Endotext*, 2015)[82,86,88].

APOLIPOPROTEINS	
Apolipoprotein A-I	HDL major structural protein (≈70% of its proteome). It is principally responsible for cholesterol efflux capacity, and contributes to HDL antioxidant and endothelial properties.
Apolipoprotein A-II	HDL second most abundant protein (≈20% of its proteome). Although it is an HDL structural protein, an excessive content may displace ApoA-I from HDL structure and decrease HDL functionality

Apolipoprotein A-IV	Structural function, not well characterized yet. It is associated with full HDL function, and is diminished in high cardiovascular risk states.
Apolipoprotein C-I	Modulator of CETP activity, and LCAT activator.
Apolipoprotein C-II	Ligand and activator of LPL.
Apolipoprotein C-III	Inhibitor of LPL and blocker of the interaction of triglyceride-rich lipoproteins with other receptors.
Apolipoprotein D	Lipocalin (transporter of small lipids and hydrophobic substances). It has an unclear role in high cardiovascular risk states and some neurodegenerative diseases.
Apolipoprotein E	Principal ligand for ApoE-receptor, mediates the direct uptake of lipoproteins by the liver. It also mediates several cellular processes and plays a key role in the development of neurodegenerative diseases (mainly Alzheimer's disease).
Apolipoprotein J	Chaperone for misfolded proteins, also known as clusterin. Catalyzes the degradation of oxidized structures (contributes to HDL antioxidant capacity).
Apolipoprotein M	Binds small hydrophobic molecules. Its main ligand is S1P, an activator of the S1P3 receptor and mediator of HDL endothelial protection.
ENZYMES AND OTHER PROTEINS	
Lecithin cholesterol acyltransferase (LCAT)	The main enzyme responsible for the esterification of free cholesterol molecules. It also contributes to the hydrolysis of oxidized HDL lipids.
Cholesterol ester transfer protein (CETP)	The main enzyme responsible for the isomolecular exchange of esterified cholesterol from HDLs and triglycerides from triglyceride-rich lipoproteins.
Paraoxonase-1 (PON1)	Calcium-dependent lactonase, principally transported in plasma by HDLs, catalyzes the hydrolysis of several oxidized lipids.
Lipoprotein-bound phospholipase A2 (LpPLA2)	Also known as platelet-activating factor acetylhydrolase (PAF-AH). When bound to HDLs it is protective and catalyzes the hydrolysis of short-chain oxidized lipids. Paradoxically, when bound to LDLs, it increases cardiovascular risk.
Glutathione peroxidase (GPx)	It catalyzes the reduction of hydroxyperoxides, coupled to the oxidation of glutathione. It can also be present free in plasma.
Phospholipid transfer protein (PLTP)	It catalyzes the incorporation of free cholesterol and phospholipids from triglyceride-rich lipoproteins to HDLs. It also mediates the conversion of HDLs into smaller particles.

The avidity of HDLs to bind proteins is also the basis to explain how they can become dangerous under certain circumstances. In pathological states characterized by chronic inflammatory and oxidative responses (such as CVDs), HDLs can become enriched in acute-phase proteins, which can make HDLs lose their functions and even turn pro-inflammatory[95]. Among these acute-phase proteins, serum amyloid A-1 (SAA-

1) is especially relevant: it forces the detachment of ApoA-I from the HDL surface and induces pro-inflammatory responses. Other pro-inflammatory proteins that can be present in HDL in these pathological states are SAA-2, C3 component protein, α-2-HS-glycoprotein, myeloperoxidase, and the fibrinogen α-chain. Beyond acute-phase proteins, in high cardiovascular risk states, HDLs can also become enriched in other pro-atherogenic proteins, such as ApoC-III. These proteomic changes have been described in several pathologies with high cardiovascular risk, such as diabetes, chronic dyslipidemias, and obesity[86,88,89,95].

1.4.2. EPIDEMIOLOGICAL EVIDENCE: BEYOND HDL CHOLESTEROL LEVELS

HDLs play a central role in CVD prevention since they are able to pick up excess cholesterol from the peripheral cells and transport it back to the liver for metabolism or excretion[86]. Low levels of HDL-C are classically considered an independent cardiovascular risk factor[10]. Considering this fact, several therapeutic efforts in the previous decades have attempted to increase HDL-C levels so as to reduce cardiovascular risk[34]. However, it has been recently observed that high HDL-C levels do not always lead to a decrease in cardiovascular risk. First, the phase-III trial of torcetrapib (a cardiovascular drug able to drastically increase HDL-C) had to be halted as its use increased cardiovascular mortality[96] (although the underlying mechanism of this toxic effect was not clearly related to the effect of the drug on HDL-C[97]). Second, a recent review about the effects of the various drugs able to increase HDL-C levels (fibrates, niacin, and drugs from the torcetrapib family) when co-administered with statins, showed no protective effects against all-cause mortality, coronary heart disease mortality, and coronary heart disease and stroke incidence, despite the rises in HDL-C levels resulting from their use[98]. Finally, in a Mendelian randomization study, genetic variants that were associated with high HDL-C levels did not decrease the risk of suffering a myocardial infarction[99]. All this evidence has raised a new question among clinicians and researchers: are levels of HDL-C a right clinical target?

It is increasingly more accepted that HDL functions explain the protective role of the lipoprotein better than HDL-C levels[35]. Low HDL-C levels may be a reflection of an insufficient HDL function: if HDLs are dysfunctional they are unable to pick up cholesterol and, consequently, HDL-C levels are low. However, high cholesterol levels in dysfunctional HDLs (or contained in a low number of HDL particles) would justify the lack of utility of HDL-C levels in some pathological states. Due to these reasons, in the last two decades, several research groups have tried to reveal the functional profile of HDLs[86].

1.4.3. HDL FUNCTIONS

1.4.3.1. CHOLESTEROL EFFLUX CAPACITY

As previously defined, cholesterol efflux capacity is the ability of HDLs to pick up the excess of cholesterol from peripheral cells in order to transport it back to the liver (for metabolization or excretion)[86]. This step of reverse cholesterol transport is the most relevant phase of the process and the most studied one in humans.

To perform this function, ApoA-Is bind to several types of cell receptors and catalyze the process. Lipid-free ApoA-Is and lipid-poor HDLs are able to catalyze cholesterol efflux from cells by binding with the ATP-binding cassette A1 (ABCA1). Lipid-loaded HDLs bind preferentially with the ATP-binding cassette G1 (ABCG1) and the SR-BI receptor[100]. ABCA1 and ABCG1 catalyze a fast, unidirectional exit of cholesterol from cells, whilst SR-BI catalyzes a slow, bidirectional cholesterol transfer: SR-BI-dependent efflux is only possible when free cholesterol is more concentrated inside the cells, and therefore flows from where it is more to where it is less concentrated[100]. It has been recently revealed that other types of cholesterol receptors (such as the β-chain of the ecto-F1-ATPase, coupled to purinergic P2Y receptors) are also able to facilitate the efflux process in special cell types, such as endothelial cells[101]. In addition, all HDLs present a partial fraction of cholesterol efflux due to a free passive diffusion of cholesterol from the surface of cells[100]. Apart from these efflux processes, HDLs are also able to catalyze the exit of oxidized steroid molecules (7-oxysterols) from cells, which is a possible mechanism by which HDLs contribute to maintain the homeostasis of macrophages[102] and endothelial cells[103].

Cholesterol efflux capacity has been shown to be highly informative with respect to individual cardiovascular risk in large-scale human studies. Low values of cholesterol efflux capacity have been associated with increased cardiovascular risk levels and subclinical atherosclerosis in cardiovascular high-risk patients, and are able to predict these parameters better than HDL-C levels[104]. More recently, it has been demonstrated that low cholesterol efflux capacity values are also associated with a greater incidence of coronary heart disease[21]. As reported in some randomized controlled trials, cholesterol efflux capacity can improve after the use of a supplement of anthocyanins[105] and cardiovascular drugs such as niacin[106] and pioglitazone[104].

It is possible to simulate the process of cholesterol efflux *in vitro*, using cell line cultures and HDL isolated *in vivo* from humans. These approaches which are based on a mixture of *in vitro* cell support and *in vivo* samples are known as *ex vivo* techniques. Most of the *ex vivo* simulations of cholesterol efflux have used different kinds of macrophages[100]. The most common ones are a murine cell line of J774 macrophages (since they grow easily and fast)[21,104]. Murine models, however, present a low innate expression of ABCA1 which has to be induced by chemical agents such as cyclic adenosine monophosphate[100]. As a consequence, researchers have indicated that the most physiological imitation of the efflux process should be based on human macrophage cells. Regarding human cell lines, the most extended model is based on THP-1 monocyte-derived macrophages. The expression profile of the different cholesterol transporters in this cell model is similar to the one in human macrophages

in vivo[100]. To generate such a model, THP-1 monocytes are first grown in suspension, and finally differentiated into macrophages by a pro-inflammatory agent (such as phorbol-myristate-acetate)[107,108]. Macrophages derived from circulating human monocytes can also be extracted from blood samples and conserved as primary cell culture. However, their maintenance is more complex than the one in cell lines (for this reason they are less employed in cholesterol efflux models)[109].

1.4.3.2. HDL ROLE IN OTHER STEPS OF REVERSE CHOLESTEROL TRANSPORT

Cholesterol that has been picked up by HDLs is stabilized through the action of the LCAT enzyme[86]. Although LCAT is clearly relevant for the HDL cycle, the modulation of its activity has not been clearly related to changes in cardiovascular risk in epidemiological studies[110]. However, some healthy lifestyle interventions (such as a diet rich in fruit and vegetables, and a diet supplemented with lycopene) have increased LCAT function in humans[111,112]. As observed in these studies, LCAT activity can be measured in the serum and plasma of human volunteers and fluorimetric and colorimetric kits are now marketed to measure it in *in vivo* samples. Moreover, it is also possible to assess LCAT quantity in a sample by enzyme-linked immunosorbent assay (ELISA) kits[111]. This quantification of LCAT mass in serum and plasma samples can be used to calculate the specific activity of the enzyme (adjusting a measurement of LCAT activity by the mass of the enzyme, as performed by our team in one of the manuscripts included in the present thesis project).

Another key point of the role of HDLs in reverse cholesterol transport is CETP activity. As previously commented, CETP catalyzes the isomolecular exchange of non-polar lipids between HDLs and triglyceride-rich lipoproteins, transferring cholesterol esters from HDLs to triglyceride-rich lipoproteins, and transferring triglycerides back from these lipoproteins to HDLs. Excessive CETP activity, due to hypertriglyceridemia, is linked to an increased withdrawal of cholesterol from HDLs (which decreases HDL-C levels) and an HDL enrichment in triglycerides[113]. In parallel, low CETP activities due to genetic variants have been associated with high HDL-C concentrations and low cardiovascular risk levels[114]. In order to determine the role of the enzyme in CVDs, CETP activity and mass can be easily measured in human plasma and serum samples since several activity kits and ELISAs are available on the market[111,115].

Due to the central role it plays in HDL biology, CETP has attracted the attention of clinical researchers from a considerable time. In the past decade, the pharmaceutical industry started to test several drugs able to block the enzyme (the *trapib* family), in order to decrease cardiovascular risk by increasing HDL-C levels. Nevertheless, this therapeutic strategy has not provided very promising results to date: 1) the torcetrapib phase-III trial had to be stopped due to increased mortality risk in the intervention group[96]; 2) dalcetrapib did not reduce the risk of suffering a new cardiovascular event in individuals that had previously undergone an acute coronary syndrome[116]; and 3) the phase III trial on evacetrapib (the most potent CETP inhibitor studied to date) has

also been terminated due to lack of efficacy[117]. Only the research on anacetrapib (the last member of this drug family) is ongoing, although its early results are not especially remarkable[118]. Therefore, since an extensive blockade of CETP with pharmacological agents has not been shown to induce beneficial effects for the moment, healthy lifestyle interventions may be more useful in modulating CETP activity in a more physiological way. The decrease in CETP activity after a physical activity protocol[115], a lycopene supplement[111], and an anthocyanin supplement[105] may contribute to explain the decreases in cardiovascular risk as a result of these healthy lifestyle interventions.

1.4.3.3. HDL ANTIOXIDANT FUNCTION

HDL antioxidant capacity is the second most important function of HDLs[86]. HDL antioxidant function is especially relevant towards LDLs, as LDL oxidation is one of the first biochemical events in the development of atherosclerotic plaques[2]. This HDL function is a multifactorial process in which several components are involved: 1) PON1; 2) HDL-LpPLA2 (or PAF-AH); 3) ApoA-I; 4) HDL-bound dietary antioxidants; 5) LCAT; 6) GPx; 7) apolipoprotein J; and 8) ceruloplasmin[86,119].

Since HDL antioxidant function is a multifactorial capacity, the scientific community is interested in directly determining the entire antioxidant ability of the lipoprotein. On the one hand, we can measure the capacity of HDLs to counteract the formation of oxidized species in LDLs in a pro-oxidant environment. In this group, the most relevant techniques are the capacity of HDLs to inhibit the formation of conjugated dienes[90] and thiobarbituric acid reactive species (TBARS)[120] in LDLs. On the other hand, we can assess the capacity of HDLs to inactivate the pro-oxidant/pro-inflammatory effects of modified lipids. In this category, the most significant techniques are the cell-free assay of the HDL inflammatory index[119], the dihydrorhodamine-123 method[121], and the fluorescein-ORAC one[122]. The use of a supplement of anthocyanins[123] and statins[124] has been able to improve some of these properties in previous randomized controlled trials.

However, most studies about HDL antioxidant capacity only assess the activity of some of the enzymes involved in the process, and most especially PON1. As previously described, plasma PON1 is almost totally bound to HDLs and plays a central role in HDL antioxidant properties[125]. PON1 is able to catalyze the hydrolysis of organophosphates as paraoxon (paraoxonase activity), ester groups next to an aromatic structure (arylesterase activity), and cyclic esters (lactonase activity). These three activities are intimately associated with the antioxidant function of the enzyme and are frequently measured in human trials: high PON1 arylesterase activity is associated with a lower incidence of cardiovascular events[22] and a lower severity of coronary artery diseases[126]. Several healthy lifestyle interventions have been able to improve PON1 function in humans in randomized controlled trials: the supplementation with lycopene[111], anthocyanins[123], soy beans[127] and fish oil[128], and a physical activity program[129].

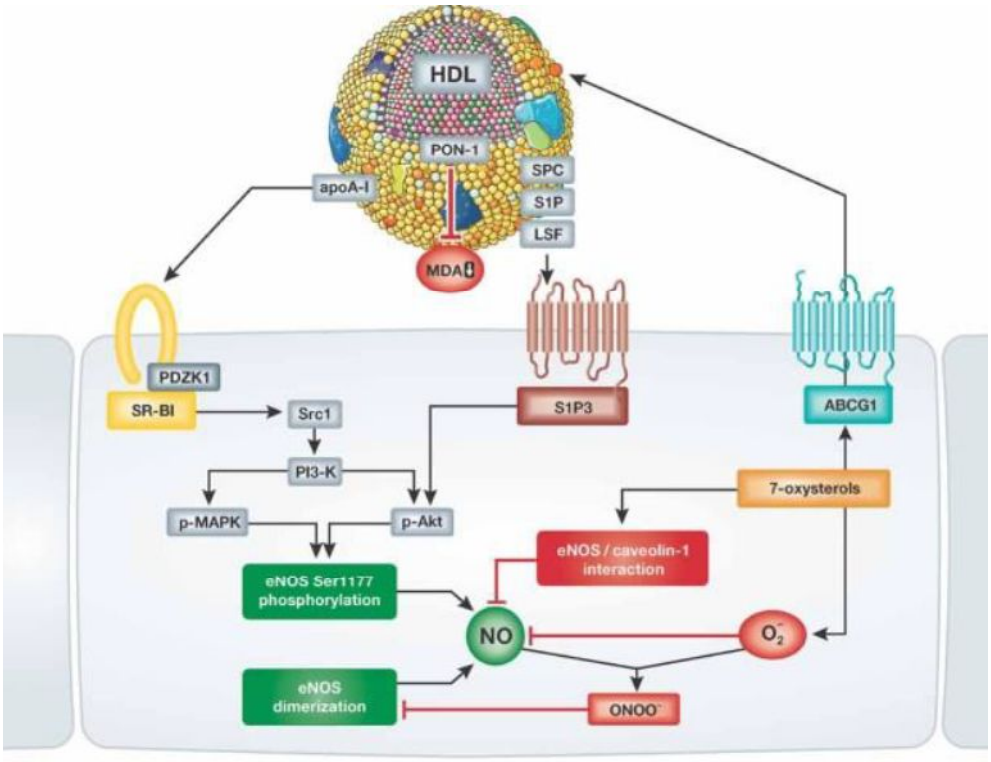
Other key enzymes for HDL antioxidant function are LpPLA2, LCAT, and ApoA-I. First, LpPLA2 is responsible for the hydrolysis of short-chain oxidized lipids but only when it is found in the surface of HDLs[130]. Second, LCAT has been shown to be responsible for counteracting the oxidation of several LDL lipids[131]. Finally, a correct ApoA-I function has also been reported to contribute to the antioxidant effect of HDLs on LDL[132]. In addition to enzymatic proteins, HDLs are able to pick up several dietary antioxidants[111,133] that may prevent HDL oxidation and also contribute to their antioxidant function[86]. Particularly, dietary antioxidants may exert a direct antioxidant protection, keep non-oxidized other antioxidant vitamins in HDL (such as vitamin E), and protect the active centers of antioxidant enzymes against oxidative attacks (in order to maintain their function).

1.4.3.4. HDL ENDOTHELIAL PROPERTIES

HDLs can modulate the biochemical response of the vascular endothelium and play a central role in preserving endothelial homeostasis[89]. HDLs are able to induce the key enzyme for endothelium-based vasodilation, endothelial NO synthase (eNOS)[134,135]; decrease the pro-inflammatory response of endothelial cells after a cytotoxic aggression[136]; and contribute to healing endothelial damage after an oxidative or pro-inflammatory attack[89]. HDLs produce these effects through several biochemical mechanisms: 1) the induction of eNOS through the stimulation of an SR-BI-dependent pathway; 2) the induction of eNOS through the stimulation of the S1P3 receptor 3 (S1P3) by different lysophospholipids carried by HDL (among which the most relevant one is S1P); and 3) the ABCG1-dependent efflux of oxysterols from the endothelial cells[89]. Since oxysterols directly inactivate eNOS and contribute to the generation of new reactive oxygen species (that may decrease the bioavailability of NO and also inactivate eNOS), the decrease of oxysterol levels in endothelial cells through this mechanism is vasoprotective (**Figure 7**).

Although these properties seem to play a central role in HDL atheroprotection, and a group of interventions have improved some of HDL endothelial characteristics in randomized controlled trials (e.g., a massive weight loss after a gastric surgery[137], a physical activity program[138], and the use of extended-release niacin[139]), the role of HDL endothelial protection in cardiovascular risk has not as yet been clearly established in epidemiological studies . Nevertheless, interest in these HDL properties is growing and several *ex vivo* tests have been developed in the last decade to measure them. The most frequent technique is the determination of the ability of HDLs to induce the secretion of NO from endothelial cells, through fluorimetric[137,140] and radioactivity-based tests[122]. It is also possible to determine the capacity of HDLs to: increase the viability of endothelial cells[129], decrease the apoptosis of endothelial cells due to inflammation[137], and increase the endothelial cell capacity to heal a mechanic injury[141].

Figure 7. HDL endothelial protection (Besler C et al, *EMBO Mol Med*, 2012)[89].



***ABCG1*: ATP-binding cassette G1; *ApoA-I*: apolipoprotein A-I; *eNOS*: endothelial nitric oxide synthase; *LSF*: lysosulphatide; *MDA*: malondialdehyde; *NO*: nitric oxide; *p-Akt*: phospho-AKT; *p-MAPK*: phospho-MAP kinase; O_2^-: superoxide anion; *ONOO*$^-$: peroxynitrite radical; *PI3K*: phosphoinositide-3 kinase; *PON1*: paraoxonase-1; *S1P*: sphingosine-1-phosphate; *S1P3*: S1P receptor 3; *SPC*: sphingosylphosphorylcholine; *SR-BI*: scavenger receptor B1.**

1.4.3.5. OTHER HDL FUNCTIONS

Beyond the classical HDL functions, several new biological activities of these lipoproteins have been described. First, HDLs moderate the pro-inflammatory responses of some immune cells[142]: they are able to decrease the capacity of monocytes to adhere and become active[143], the activation of T lymphocytes[144], and the response of neutrophils[145]. Second, HDLs have shown a relevant role in the regulation of the function of platelets and in the coagulation cascades[146]. Finally, they also play a central role in the regulation of glucose metabolism: HDLs are able to promote insulin release from beta cells, insulin-independent glucose uptake in peripheral cells, and insulin sensitivity in the glucose target cells[147].

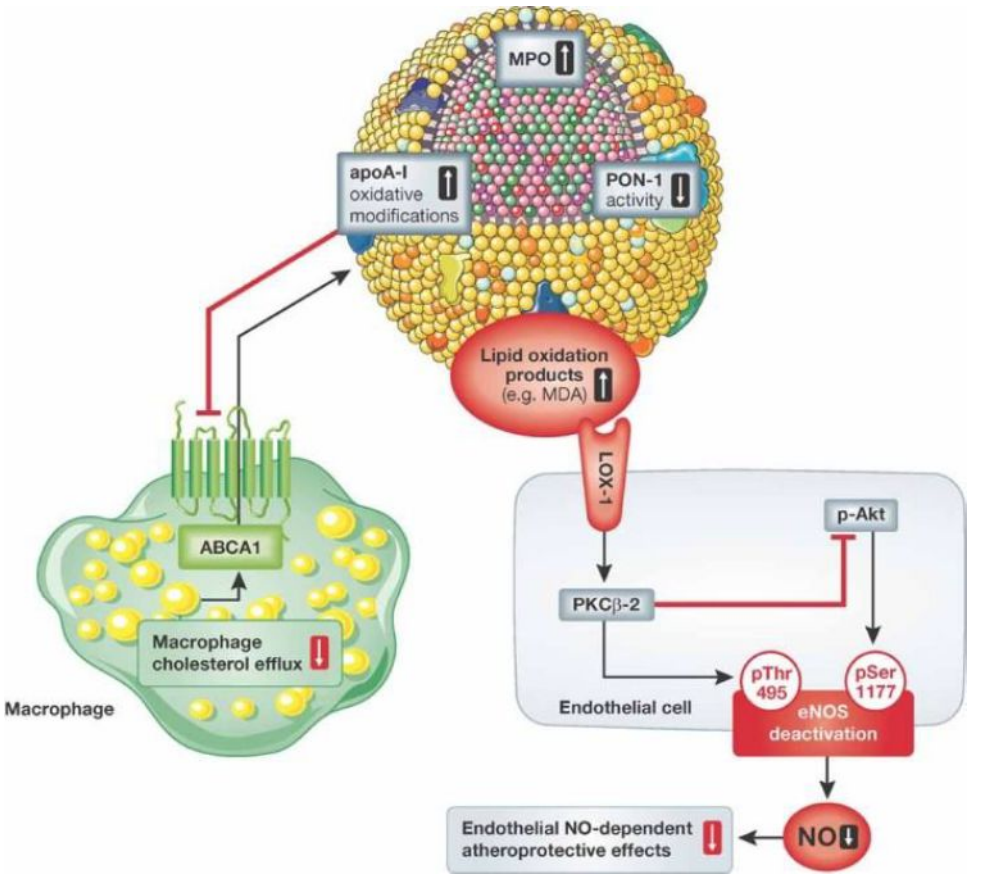
1.4.4. HDL FUNCTIONALITY-RELATED PROPERTIES

HDL functions are strongly related to the physicochemical state of the lipoprotein[86]. Several HDL quality characteristics affect the capacity of the lipoprotein to develop its functions correctly. We could group these HDL characteristics in four categories: oxidative status, composition, size distribution, and particle number.

1.4.4.1. HDL OXIDATIVE STATUS

Although HDLs can fight against lipid oxidation, they are also susceptible to becoming oxidized which is one of the main causes of their dysfunction: oxidized HDLs lose their cholesterol efflux capacity, antioxidant functions, and endothelial protection[86,88,89]. On the one hand, several active centers of HDL proteins and enzymes can become oxidized and thus lose their functions. It has been previously described that ApoA-I capacity is impaired when is oxidized: the ApoA-I binding to cholesterol transporters (ABCA1, ABCG1) is less efficient due to the oxidation of some methionine residuals in its active center, and therefore its cholesterol efflux capacity decreases[89,148]. PON1 and LCAT are also highly susceptible to partially lose their functional capacities when they suffer oxidative modifications[149,150]. On the other hand, the oxidation of HDL lipids may also mediate a decrease in the role of the lipoprotein. The oxidation of HDL lipids decreases HDL fluidity[151], and this lack of fluidity hinders the ability of the lipoprotein to adapt physically to the receptor environment, which leads to a less efficient interaction of HDLs with other proteins[152]. Moreover, the accumulation of oxidized lipids in HDLs makes the lipoproteins bind more easily to the lectin-type oxidized LDL receptor-1 receptor in endothelial cells, which leads to a partial inactivation of the eNOS[89] (**Figure 8**).

Figure 8. HDL dysfunction due to oxidation (Besler C et al, *EMBO Mol Med*, 2012)[89].



***ABCA1*: ATP-binding cassette A1; *ApoA-I*: apolipoprotein A-I; *eNOS*: endothelial nitric oxide synthase; *MDA*: malondialdehyde; *MPO*: myeloperoxidase; *NO*: nitric oxide; *p-Akt*: phospho-AKT; *PKC*: protein kinase C; *PON1*: paraoxonase-1**

Although the role of HDL oxidation in the function of the lipoprotein is clear, its ability to predict cardiovascular risk has not been assessed to date in epidemiological studies. Nevertheless, HDL *in vivo* oxidation has been studied in several randomized controlled trials, and it improved after a diet rich in PUFAs and supplemented with vitamin E[153] and the use of pioglitazone[154].

There are a number of available techniques to detect HDL oxidation. First, it is possible to measure the levels of oxidized lipids in HDL. In this group, the most relevant approach is the detection of the equivalents of malondialdehyde (MDA) by the TBARS reaction[155]. Second, it is also possible to measure the resistance of HDLs against oxidation *in vitro*. In these procedures, HDLs are incubated in a pro-oxidant environment (in the presence of Cu^{2+} or AAPH) and the formation of oxidized species is monitored: it is possible to define HDL oxidation kinetics (assessing the formation of conjugated dienes in the molecule)[154] or to determine the total amount of oxidized lipids at the end of the process (by the TBARS technique)[156]. Third, we can measure the activity of one of the main enzymes responsible for HDL oxidative modifications, myeloperoxidase[157]. In this case, we can assess its activity on HDLs or the levels of some of its reaction products (3-chlorotyrosine or 3-nitrotyrosine). Finally, we can measure the oxidative modifications of some of the proteins in HDL, such as the levels of oxidized methionine residuals in apolipoproteins A[148].

1.4.4.2. HDL COMPOSITION

HDL lipid composition deeply affects its function. In this category, the distribution of HDL lipids between its surface and its core is one of the most relevant structural traits. As previously described, due to their polarity characteristics, HDL polar lipids (phospholipids and free cholesterol) are orientated towards the exterior of the lipoprotein, whilst the non-polar lipids (triglycerides and esterified cholesterol) are kept on the inside. The distribution balance of these lipid groups affects the properties of the lipoprotein. On the one hand, whenever the core of the lipoprotein becomes triglyceride-rich, HDL molecular structure changes and the stability of ApoA-Is in the surface decreases. Consequently, ApoA-Is function deficiently, are loosely bound to HDLs, can detach, and lead to spontaneous HDL lysis[158,159]. Since HDL becomes triglyceride-rich in hypertriglyceridemia, this mechanism may explain why HDL levels can spontaneously disintegrate and lead to low HDL-C levels in hypertriglyceridemic patients[160]. Moreover, triglyceride-rich HDLs have also been shown to be more pro-inflammatory[161]. On the other hand, low levels of phospholipids (or high levels of free cholesterol) on the HDL surface may contribute to the rigidity of the lipoprotein and, therefore, to a lower HDL functionality[162].

An increased content of triglycerides in the HDL core may be considered a consequence of an exacerbated function of CETP and also an indirect marker of deficient LCAT activity (if the enzyme does not efficiently esterify free cholesterol, the content of esterified cholesterol in HDL core decreases and the relative content of

triglycerides increases). In this regard, a decreased content of phospholipids in HDL surface could also be considered a marker of a deficient function of LCAT. As a consequence, these compositional markers reflect the whole dynamic role of HDL in cholesterol metabolism and may become biomarkers of high cardiovascular risk states. These compositional biomarkers can be easily determined (we first have to isolate HDLs and then identify their composition as we would do for plasma samples). However, their predictive role has not been assessed to date in epidemiological studies.

Some particular lipids can also be measured in HDLs. We have already commented on the inductive role of S1P[89]. S1P bound to HDLs is increasingly becoming a functionality marker of the lipoprotein in human studies, since the potential functions attributed to S1P3 receptor on cardiovascular protection are constantly growing[91]. It is also possible to determine the fatty acid composition of the HDL lipids, since it can vary according to the diet and can be considered a biomarker of compliance of certain dietary interventions. Changes in the HDL acyl composition can also affect HDL fluidity (the more unsaturated the fatty acids in HDL are, the more fluid the lipoprotein)[163,164] and, indirectly, HDL functions.

Finally, the HDL proteome can also be determined[138,165–167]. HDL proteomic analyses have revealed that high cardiovascular risk patients present a decreased content of apolipoproteins A-I, A-IV, and E[168,169] and an increase in the levels of acute-phase proteins such as SAA and C3 complement[168]. Such an approach is beginning to be present in randomized controlled trials, for instance the ones with rosuvastatin[170] and functional olive oils[167].

1.4.4.3. HDL SIZE

Among HDL quality properties, size has been the most studied although it still remains controversial[171]. HDL size depends on the point of the biological cycle in which we find the lipoprotein: “young”, lipid-free or lipid-poor HDLs will be small, whilst “mature”, lipid-laden HDLs will be large. Besides, HDL total protein content also affects size: protein-rich HDLs tend to be small, whilst protein-poor HDLs (lipid-rich HDLs) are larger[86,88]. HDL size classification includes pre-beta particles (which refer almost totally to lipid-free ApoA-Is or very lipid-poor HDLs) and alpha particles, which can be sub-divided in the HDL3 (small, lipid-poor HDLs) and the HDL2 families (large HDLs)[82].

HDL size differs between healthy and high cardiovascular risk individuals. In healthy individuals, small HDLs are protective: they are protein-rich and carry the functional proteins that develop HDL functions correctly. However, in high cardiovascular risk individuals, small HDLs tend to be dysfunctional: high cardiovascular risk states present higher levels of pro-inflammatory proteins that can bind to HDLs, impair their function, and even turn them into pro-inflammatory particles. Therefore, increasing HDL size is cardioprotective in high cardiovascular risk patients[86,88]. However, the utility of HDL size in cardiovascular risk prediction is limited, since HDL size is highly correlated with

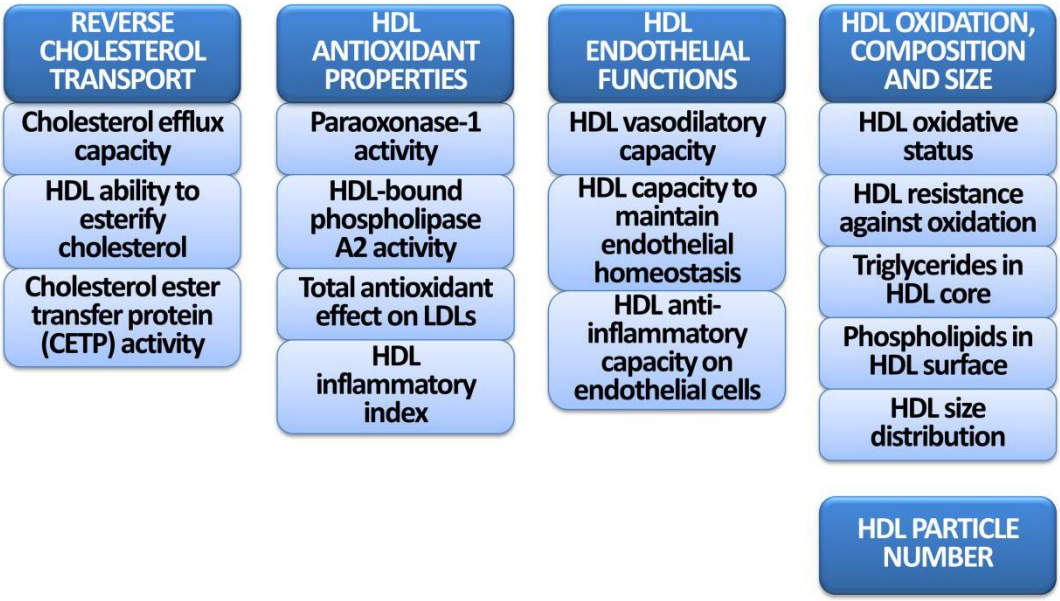
HDL-C levels and does not provide more accurate information about the cardiovascular risk of an individual than HDL-C levels[172].

Despite this controversy, HDL size has been extensively used in lipoprotein research in the past decades: high cardiovascular risk patients present low levels of large HDLs and high levels of small HDLs[86,171], a size pattern that can be corrected through several interventions (supplements of fish oil[173], a physical activity program[129], and statin therapy[174]). The techniques to determine HDL size are diverse: ultracentrifugation according to density, 1-dimension gel electrophoresis, 2-dimension gel electrophoresis, and immunoaffinity[82]. Moreover, the different size populations of HDL can also be obtained when analyzing the number of HDL particles in plasma[172].

1.4.4.4. HDL PARTICLE NUMBER

Irrespective of their characteristics, a high number of HDL particles signifies that HDL functions will be performed to a greater extent. The number of circulating HDL particles is becoming one of the most promising HDL biomarkers, since low levels of HDL particles have been associated with a greater incidence of coronary events in high cardiovascular risk individuals[172]. HDL particle number can be easily determined with nuclear magnetic resonance spectroscopy[88] and it was reported to have increased in randomized controlled trials after statin use[175] and smoking cessation[176].

Figure 9. Main HDL function and quality-related traits.



1.5. LOW-DENSITY LIPOPROTEINS

LDLs are the remnant particles of the triglyceride-rich lipoproteins synthesized by the liver (VLDLs, IDLs), and are responsible for the delivery of cholesterol to peripheral cells. As previously described, they are cholesterol-rich micelles, wrapped in an ApoB-

100 molecule[83]. LDLs are the most atherogenic lipoproteins, as they are the main lipoprotein group that can traverse the endothelial barrier, become oxidized in the sub-endothelial space, and trigger the development of atherosclerosis[2].

1.5.1. EPIDEMIOLOGICAL EVIDENCE: BEYOND LDL CHOLESTEROL LEVELS

If they become oxidized, LDLs are cytotoxic for macrophages and endothelial cells. Presenting high levels of circulating LDLs increases the probabilities of starting an atherosclerotic lesion and, consequently, cardiovascular risk. Thus, raised LDL-C levels have been associated with a greater risk of suffering a coronary event[11,177,178]. Moreover, the extent of fatty streaks in the atherosclerotic plaques is directly associated with LDL-C levels, among other cardiovascular risk factors[179]. Most lipid lowering drugs on the market are therefore aimed at decreasing LDL-C levels. These drug-induced reductions in LDL-C levels have been responsible for a decline in the incidence of major CVDs in the previous decades[180,181].

However, some individuals treated with lipid lowering drugs (with LDL-C levels below 50 mg/dL) still present a residual cardiovascular risk that we are unable to explain according to classical cardiovascular risk factors, and continue to develop CVDs[182]. Recent therapeutic approaches have tried to decrease LDL-C levels even more by adding an extra lipid lowering drug (fenofibrate, niacin, omega-3 PUFAs, and CETP inhibitors) to statin treatment, in a strategy that has not reduced the incidence of major CVDs and has increased the risk of liver injury (except in the of ezetimibe)[98,183]. Thus, following a similar approach to HDLs, the scientific community is starting to study several LDL characteristics beyond LDL-C levels in order to explain this unjustified residual cardiovascular risk.

1.5.2. LDL ATHEROGENICITY

LDLs may present physicochemical characteristics that make them more prone to traversing the endothelial barrier and inducing a cytotoxic response. Such characteristics are related to: modifications of LDL structure, LDL resistance against oxidative attacks, LDL size, LDL particle number, LDL lipid and protein composition, LDL electronegativity, and LDL direct cytotoxicity on cells.

1.5.2.1. MODIFICATIONS OF LDL STRUCTURE

While they are in the bloodstream, LDLs can suffer several structural modifications. These alterations make LDLs hard to recognize for LDL receptors and potentiate their aggregation into highly-atherogenic complexes. Therefore, modified LDLs are more likely to remain for longer in circulation and increase their atherogenic potential[184]. Oxidation is the main LDL structural modification although other transformations should also be considered.

Oxidized LDLs are known to be pro-inflammatory for monocytes and macrophages, and cytotoxic for endothelial and smooth muscle cells[184]. Circulating levels of oxidized

LDLs have been considered to reflect the degree of pro-inflammatory events in the sub-endothelial space. Thus, high levels of oxidized LDLs in plasma have been found to be predictive for coronary heart disease in some studies[185], as well as for all-cause mortality[186]. Moreover, they are associated with subclinical atherosclerosis[187], instability of the atherosclerotic plaque in coronary heart disease[188], and incidence of metabolic syndrome and its components[189]. Oxidized LDL levels in plasma can be measured easily by means of ELISA kits[73] or determining the amount of oxidized lipids (MDA, conjugated dienes) in isolated LDL samples[190]. They have been corrected in randomized controlled trials after healthy lifestyle modifications such as real-life doses of VOO[73] and supplements of vitamin E[191].

Glycation or glycosylation is another frequent type of LDL modification. Glucose in plasma can react with lysine residuals in LDL proteins (mainly ApoB-100), modifying their structure in a similar way to advanced glycation end products[192,193]. Thus, this specific LDL modification is especially present in diabetic individuals. Moreover, in a similar manner to oxidized LDLs, glycated LDLs are also hard to recognize for LDL receptors, tend to be retained for a long time in circulation, and induce similar cytotoxic effects in macrophages. Actually, glycated and oxidized LDLs are quite interrelated *in vivo*[192]. Data, however, is scarce concerning their role in cardiovascular risk in humans: a direct relationship between glycated LDLs and sub-clinical atherosclerosis has only been observed in diabetic patients[194].

Other possible modifications of LDL structure that lead to pro-atherogenic particles are: acetylated LDLs[195], LDL-containing circulating immune complexes[196], and aggregated LDLs[197]. Similarly to glycated LDLs, the role of these modified LDLs on the development of CVDs in humans remains to be revealed.

1.5.2.2. LDL RESISTANCE AGAINST OXIDATION

When LDLs are more resistant against oxidative modifications, they become less oxidized and, therefore, less cytotoxic. LDL resistance against oxidation is directly proportional to the antioxidant content of the lipoprotein[198,199], and inversely proportional to its PUFA content (since PUFAs are especially prone to suffer oxidative attacks due to their high content in double bonds)[200]. Low LDL resistance against oxidation has been associated with sub-clinical atherosclerosis[201], it is present in high cardiovascular risk patients (such as type-1 and type-2 diabetic individuals)[202], and is one of the characteristics of the pro-atherogenic LDL profile that predisposes to the development of CVDs[203]. This property has been shown to be modulated by antioxidant-rich strategies such as the use of antioxidant supplements[198,199]. However, the role of LDL resistance against oxidation on cardiovascular risk still has to be corroborated.

The measurement of this property is frequently performed as a kinetic reaction: isolated LDLs are incubated in a pro-oxidant environment (in the presence of Cu^{2+} or AAPH) and the formation of oxidized species (conjugated dienes) is monitored

spectrophotometrically. In these kinetic curves, the time when maximal oxidation starts (LDL lag time) and the peak velocity of oxidation (LDL oxidation rate) can be calculated[202,203]. LDLs are more resistant against oxidation when they present high lag time values or low oxidation rate levels.

1.5.2.3. LDL SIZE

Size greatly affects LDL atherogenicity. Small LDLs: 1) interact more poorly with LDL receptors (and therefore remain for longer in circulation, increasing LDL chances to suffer modifications); 2) are more susceptible to suffer oxidative attacks; 3) adhere more frequently to the proteoglycans in the surface of endothelial cells (leading to a greater interaction with cells); and 4) traverse more efficiently the endothelial barrier[23,203,204]. Thus, high levels of small LDLs are associated with greater cardiovascular risk and the development of early atherosclerosis[205–207], as well as with an increased incidence of cardiovascular events[208]. Small LDLs seem to be easily modulated by cardiovascular drug therapy and healthy lifestyle changes: they decrease after the use of several lipid-lowering drugs[209,210] or after adherence to a healthy dietary pattern such as a TMD[45], among others.

LDL size has been broadly determined in clinical trials through a diversity of techniques: 1) separation of LDL fractions according to density by ultracentrifugation (high density corresponds to small size) and quantification of cholesterol in each of the fractions[211]; 2) separation of small LDLs from the rest of lipoproteins through immunoprecipitation techniques and subsequent quantification[212]; 3) LDL-C/ApoB ratio in plasma (whenever there is an increase in LDL-C levels at a fixed ApoB concentration in plasma, more LDL-C has to be transported per each ApoB unit and, therefore, LDLs will become larger)[213]; 4) LDL buoyancy tests (approximation to LDL density)[207]; 5) electrophoretic separation of LDLs according to size (LDL size can be expressed as the % of small LDLs)[206]; 6) LDL average diameter[45]; and 7) direct quantification of small LDL particles in plasma by nuclear magnetic resonance spectroscopy[45].

1.5.2.4. LDL PARTICLE NUMBER

Irrespective of the atherogenicity of each LDL particle, a high number of circulating LDL particles will increase the probabilities of finding them in the sub-endothelial space. This novel biomarker is being increasingly used in clinical trials since it has been shown to be more accurate than LDL-C levels to predict the future incidence of CVDs in a high cardiovascular risk population[214–216]. LDL particle number can be measured, as in the case of HDL particle number, by nuclear magnetic resonance spectroscopy[214,217], and has been reported to be improved in randomized controlled trials after the use of statins[175] and fibrates[217] and a reduction in the consumption of trans fatty acids[218].

1.5.2.5. LDL COMPOSITION

There are several LDL traits relative to its composition that may transform the lipoprotein into becoming pro-atherogenic. Regarding LDL lipid composition, the proportion of triglycerides in the lipoprotein has been described to affect LDL atherogenicity. Triglyceride-rich LDLs tend to present ApoB-100s in an unstable conformation, which leads to an inefficient binding of the lipoprotein to LDL receptors[219,220]. LDL triglyceride levels are increased in coronary artery disease patients (relative to healthy controls), are able to discriminate between these two groups of individuals, and are also associated with chronic inflammation and vascular dysfunction markers[221]. Moreover, like HDLs, LDLs become triglyceride-rich in some high cardiovascular risk states[220]. Their role, however, in the prediction of the cardiovascular status, and the possibility to modulate these characteristics in randomized controlled trials, remains to be elucidated.

LDLs may also become enriched in several proteins that increase their atherogenicity. There is, therefore, great interest in studying LDL proteome in humans[222,223], which can change under certain pathological circumstances (such as obesity)[224]. In this regard, one of the most promising protein markers of LDL atherogenicity is ApoC-III content. ApoC-IIIs are able to increase triglyceride levels in plasma by inducing the secretion of VLDLs from the liver, and inhibiting LPL and the intestinal uptake of triglycerides, and are considered an independent cardiovascular risk factor[225], especially when they are bound to lipoproteins. In particular, a high ApoC-III content in apolipoprotein B-containing lipoproteins is related to a greater risk of suffering a cardiovascular event[226]. Its determination in plasma or lipoproteins is simple and, consequently, is becoming increasingly more studied in clinical trials. The use of some lipid-lowering drugs (such as atorvastatin) has been reported to be able to decrease ApoC-III levels in apolipoprotein-B containing lipoproteins in a randomized controlled trial[227].

Other pro-atherogenic proteins can be found in LDLs. First, LpPLA2 (or PAF-AH), whilst it is an antioxidant enzyme when found in HDLs, when bound to LDLs it is associated with an increase in the atherogenicity of the lipoprotein[130]. Second, greater SAA-1 levels in LDLs also turn them pro-atherogenic: SAA may reach the sub-endothelial space together with LDLs and induce locally harmful pro-inflammatory reactions[228]. The SAA-LDL complex is considered a novel prognostic marker for stable coronary disease[229], it is increased in high cardiovascular risk states such as metabolic syndrome and obesity[230], and its levels were modulated after the use of statins and a healthy lifestyle intervention (diet and physical exercise) in previous clinical trials[231,232]. Finally, the levels of α1-antitrypsin in LDLs have also attracted the interest of researchers. α1-antitrypsin (a proteinase whose deficiency is related to deficient pulmonary function[233]) is able to form complexes with LDLs in active macrophages[234]. These complexes are known to contribute to the pro-inflammatory activity of modified LDLs in macrophages[235]. However, little is known about the relevance of this complex *in vivo* in humans: α1-antitrypsin has only been shown to be augmented in LDLs from

obese subjects[224] and greatly correlate with tobacco use[236]. In addition to these examples, the appearance of new LDL protein biomarkers is expected in the coming years[222–224].

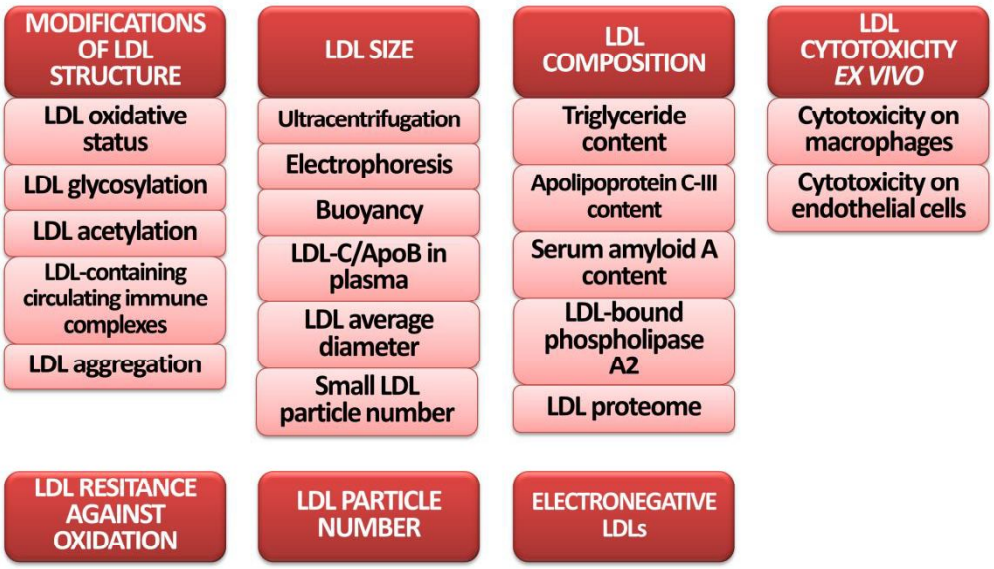
1.5.2.6. LDL ELECTRONEGATIVITY

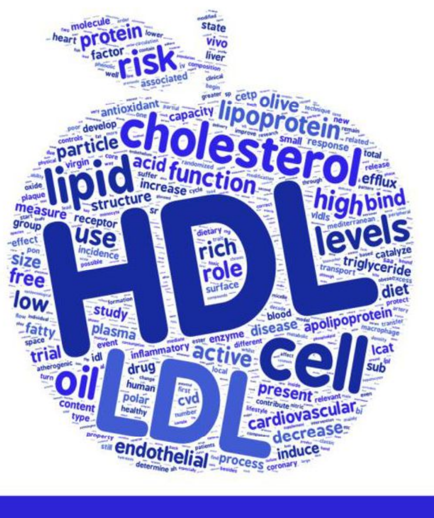
Besides LDL modifications in lipid and protein composition, atherogenic LDLs also suffer changes in their charge. In particular, an increasee in their negative charge leads to a rise in their atherogenicity, thus transforming the particles in electronegative LDLs[237]. Due to the negative charge, the structure of ApoB-100 in these LDLs is aberrant, resulting in a decreased ApoB function and a natural trend of these LDLs to cluster and induce aggregation[238]. Moreover, these LDLs bind more easily to proteoglycans (which could be linked to a greater transmigration to the sub-endothelial space[238]), and also tend to be smaller, more oxidized, more glycated, and to bind more pro-atherogenic proteins (such as LpPLA2)[239–241]. As expected, electronegative LDLs are elevated in high cardiovascular risk states (such as hypercholesterolemia, metabolic syndrome, and coronary artery disease[242,243]). These LDLs are easy to detect in plasma (by means of ELISA kits[244]) and have been shown to be decreased after the use of simvastatin and a physical activity intervention[245,246].

1.5.2.7. LDL CYTOTOXICITY *EX VIVO*

LDLs are considered pro-atherogenic as they induce cytotoxic effects in cells such as macrophages and endothelial cells[23]. However, in most of the clinical studies related to the topic, researchers have only determined the biochemical parameters that make LDLs pro-atherogenic (oxidation, composition, size, and the like), whilst they have not ascertained the direct cytotoxicity of LDLs in *ex vivo* models. As previously commented, it is possible to grow *in vitro* models of human macrophages (e.g. THP-1 monocyte derived macrophages)[107] and endothelial cells (e.g. human umbilical vein endothelial cells –HUVECs–)[137] and to incubate afterwards these cell supports with LDLs. Such experiments would provide a direct measurement (for instance, cell viability measured by the MTT technique[247]) of the effects of *in vivo* isolated LDLs from human volunteers in a more physiological model. Whenever LDLs are more oxidized, more modified, smaller, richer in triglycerides, or richer in pro-inflammatory proteins, a greater cytotoxicity in the cell model would be expected.

Figure 10. Main LDL atherogenic traits.




HDL
LDL
cholesterol
lipid
cell
lipoprotein
risk
protein
function
levels
oil
high
bind
olive
particle
antioxidant
use
rich
role
active
free
low
trial
fatty
plasma
endothelial
cardiovascular
decrease
present
apolipoprotein
triglyceride
efflux
capacity
acid
structure
increase
receptor
measure
size
study
disease
enzyme
diet
lcat
cvd
drug
human
induce
process

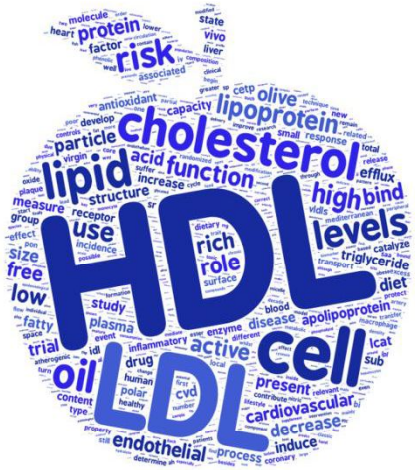
2. Rationale

In the previous decades, the effects of the TMD and VOO on cardiovascular risk factors were intensely studied in humans[25,73]. Concerning cholesterol metabolism, the consumption of VOO was shown to be able to increase HDL-C levels, due to its content in MUFAs[72] and phenolic compounds[73]. Some interventions with ordinary olive oil and VOO were also reported to induce a number of positive effects on HDL activities and functionality in non-randomized, non-controlled clinical trials[109,163,164,248]. In addition, the consumption of a phenolic compound-rich olive oil was found to improve gene expression related to HDL function and regulation[249], and several mechanisms in non-human models may support a protective effect of VOO on HDL biological activities[250]. However, no randomized controlled trial about the effects of real-life doses of VOO on HDL functionality has been performed to date. The first manuscript of the present thesis project deals with this question.

Adherence to the TMD is also expected to improve HDL function in humans. This diet has been shown to improve HDL-related lipid profile in previous clinical trials[25,44], and dietary interventions related to it (for example, a diet rich in fruit and vegetables, or supplements of carotenoids or polyphenols) have been able to ameliorate some traits associated with HDL function in humans[105,111,112,123]. However, no randomized controlled trial about the effects of an overall healthy dietary pattern (such as the TMD) on a complete profile of HDL functional characteristics has been carried out to date. Therefore, in the second manuscript of this PhD thesis we assess for the first time the effects of the consumption of a TMD on the most relevant HDL functional traits in a high cardiovascular risk population.

Regarding LDL biology, VOO and the TMD have also induced several beneficial effects. In relation to VOO, the consumption of MUFAs has been shown to be able to decrease LDL-C levels[251] and improve some LDL atherogenic properties in previous trials[252,253]. In addition, the consumption of olive oil phenolic compounds decreased oxidized LDL levels in humans[73], as well as leading to an increase in the content of vitamin E and other antioxidants in the LDL particle[254]. However, the effects of VOO consumption on other LDL atherogenic traits (total LDL particle number, small LDL particle number, LDL-related gene expression) were still unknown. The third manuscript of the present project refers to these questions.

Finally, in relation to TMD and LDL atherogenicity, adherence to this dietary pattern has led to improvements in LDL-related lipid profile and some LDL atherogenic traits (such as the number of small LDL particles in plasma and the levels of oxidized LDLs)[25,45,47]. However, TMD effects on an expanded battery of LDL-related characteristics (LDL resistance against oxidation, LDL composition, LDL cytotoxicity *ex vivo*) have not yet been revealed. Thus, we will focus on the study of the effects of a long-term adherence to a TMD on LDL atherogenicity in a high cardiovascular risk population in the fourth and last manuscript of the present thesis project.



3. Objectives

The general aim of this PhD project is to study the effects of antioxidant-rich dietary interventions on HDL functionality and LDL atherogenicity.

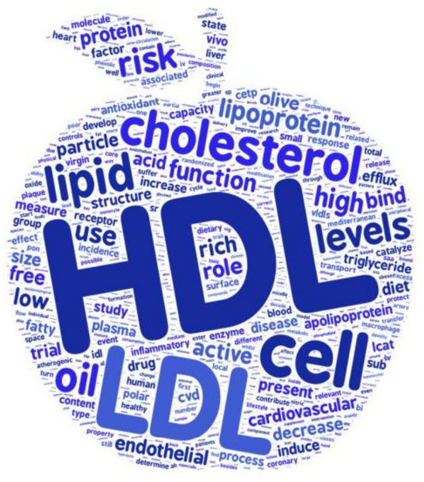
In particular, the specific aims of the present project are the following.

Regarding HDL function:

1. To assess the protective effects of the consumption of real-life doses of VOO on HDL function and HDL quality-related characteristics in healthy volunteers in a randomized controlled trial.
2. To explore the protective effects of long-term adherence to a TMD on HDL function and HDL quality-related characteristics in high cardiovascular risk individuals in a randomized controlled trial.

Regarding LDL atherogenicity:

3. To determine the protective effects of the consumption of real-life doses of VOO on LDL quantity and atherogenic characteristics in healthy volunteers in a randomized controlled trial.
4. To analyze the protective effects of long-term adherence to a TMD on LDL atherogenic characteristics in high cardiovascular risk individuals in a randomized controlled trial.


HDL
LDL
cholesterol
cell
lipid
levels
lipoprotein
risk
function
high
protein
oil
olive
particle
use
rich
role
active
apolipoprotein
cardiovascular
endothelial
decrease
present
low
free
trial
fatty
plasma
triglyceride
efflux
bind
diet
lcat
acid
structure
antioxidant

4. Methods

4.1. STUDY POPULATIONS

The present thesis includes findings from two research projects. Results about the protective effects of the consumption of phenolic compound-rich, VOO (**Manuscripts I** and **III**) belong to the EUROLIVE Study. Those concerning the protective effects of the adherence to a TMD (**Manuscripts II** and **IV**) come from the PREDIMED Study.

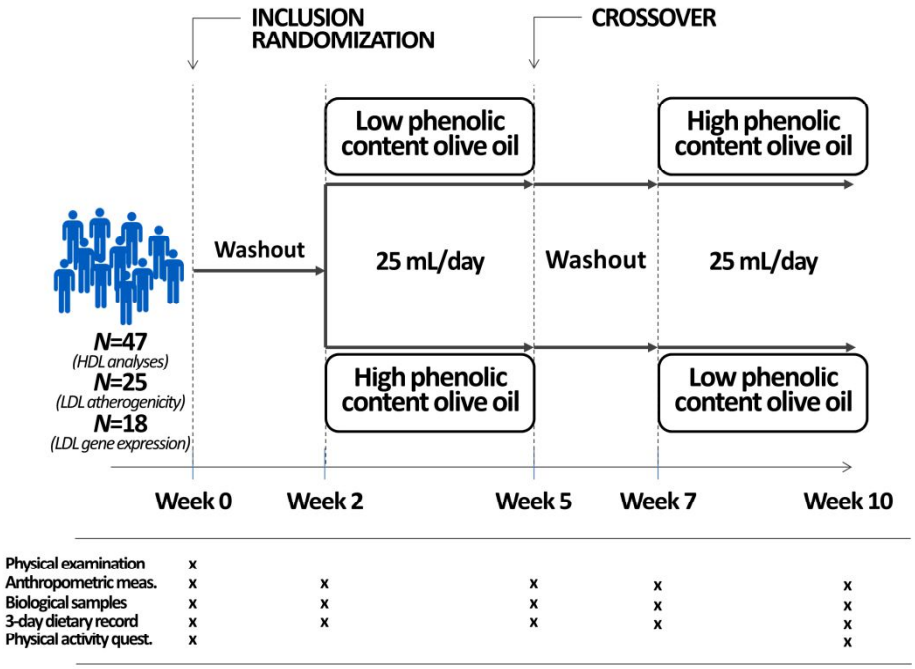
4.1.1. THE EUROLIVE STUDY: DESIGN

The EUROLIVE Study (*Effects of Olive Oil Consumption on Oxidative Damage in European Populations*) was a multicenter, crossover, randomized, controlled trial orientated to determining the effects of the sustained consumption of three olive oils (differing with respect to their content in phenolic compounds) on the lipid profile and the oxidative damage of the plasma components in a healthy population. Participants were 200 healthy men, from five different European countries (Spain, Italy, Germany, Denmark, and Finland), aged 20-60. Volunteers were randomized to three consecutive 3-week interventions in which they consumed 25 mL/day of the three olive oils with different phenolic contents: a low phenolic content olive oil (LPCOO, 2.7 mg of phenolic compounds/kg of oil), a medium phenolic content olive oil (MPCOO, 164 mg/kg), and a high phenolic content olive oil (HPCOO, 366 mg/kg). Volunteers were instructed to consume these olive oils raw (adding it to salads, bread or cold soups). Each intervention was preceded by a 2-week washout period in which the participants were requested to avoid the consumption of olive oil, olives, and other antioxidant-rich foods.

Before and after the three interventions, we collected blood samples (for biochemical analyses), and we also carried out several health questionnaires (general health status, lifestyle habits, diet, physical activity) and anthropometric measurements (height, weight, waist circumference, blood pressure, and the like). The local institutional ethics committees approved the protocol of the study, which has been previously published[73], and the volunteers gave informed written consent before joining. The protocol of the EUROLIVE Study was registered in the ISRCTN Database, with the code number ISRCTN09220811.

Within the frame of the EUROLIVE Study, in **Manuscript I** we studied the effects of the two most extreme olive oil interventions according to their content of phenolic compounds (the LPCOO and the HPCOO ones) on HDL function and HDL-related properties in a random subsample of 47 individuals. In **Manuscript III** we observed the effects of the previous two olive oil interventions on LDL quantity and some LDL atherogenic traits in a random subsample of 25 individuals. We also assessed the effects of the two olive oil interventions on LDL-related gene expression in another random subsample of 18 individuals (**Figure 11**).

Figure 11. EUROLIVE Study: analyses performed and methodological design.



4.1.2. THE PREDIMED STUDY: DESIGN

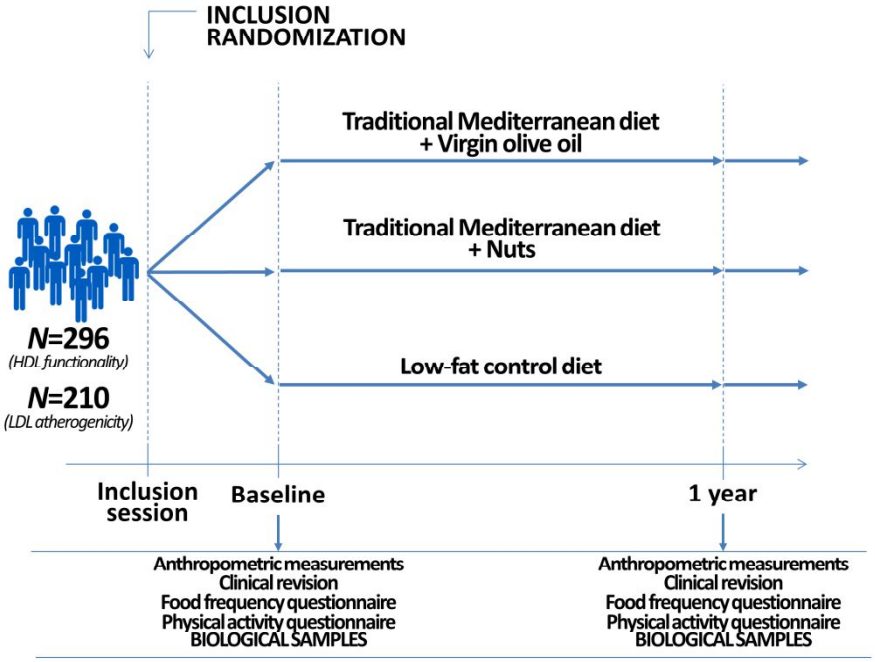
The PREDIMED Study (*Effects of Mediterranean Diet on the Primary Prevention of Cardiovascular Disease*) was a multicenter, parallel, long-term, randomized, controlled trial to determine the effects of adherence to a TMD on the primary prevention of major CVDs in high cardiovascular risk individuals. Participants were 7447 Spanish men and women (men aged 55-80, and women aged 60-80), with type-2 diabetes mellitus or at least three of the following cardiovascular risk factors: smoking habit, hypertension, LDL-C levels >160 mg/dL, HDL-C levels <40 mg/dL, overweight or obesity (BMI >25 kg/m^2), or a family history of premature coronary heart disease. History of previous CVD was an exclusion criterion. Volunteers were randomly assigned to any of these three interventions: 1) a TMD supplemented with VOO (TMD-VOO); 2) a TMD supplemented with mixed nuts (TMD-Nuts); and 3) a low-fat control diet.

Volunteers received personalized dietary advice at the beginning and throughout the study. Participants assigned to the TMDs were taught several strategies to increase their compliance to the diet: 1) to cook with olive oil in all meals; 2) to increase their intake of vegetables, fruit, nuts, and fish; 3) to eat white meat instead of red or processed meat; 4) to prepare dishes based on a homemade mix of stir-fried tomato, garlic, onion, and aromatic herbs (the traditional "sofrito"); and 5) in the case that the volunteer consumed alcohol, to moderately drink red wine (1 small glass/meal)[255]. Participants assigned to the low-fat diet were instructed to follow the instructions in the American Heart Association guidelines: 1) to decrease the use of any fat for cooking (oils, butter, and the like); 2) to increase their intake of vegetables and fruit; 3) to substitute the consumption of red meat for white meat, and to remove visible fat

from the meat before cooking it; and 4) to reduce the consumption of other fat-rich foods (processed meats, fatty fish, nuts, bakery products, processed foods, snacks, and sauces)[256]. In order to increase compliance with the interventions, participants assigned to the TMD were given 1 L/week of VOO (when assigned to the TMD-VOO group) or 210 g/week of mixed nuts (when assigned to the TMD-Nuts group). We provided additional VOO and nuts to the participants to reinforce compliance and cover family needs. Participants assigned to the low-fat diet received pecuniary gifts along the study. To reinforce compliance, participants also attended group sessions in which they received: 1) an itemized description of recommended foods (typical Mediterranean foods for TMDs, or recommendations of the American Heart Association for the low-fat diet); 2) shopping lists for every season; 3) meal plans adapted for each dietary intervention; and 4) a list of suitable recipes. Moreover, participants could contact the dietitians of the study in case of any doubt. Volunteers received no suggestions to reduce their caloric intake or increase their physical activity practice.

At baseline and after every year of intervention, blood samples were collected (for biochemical analyses), and also several health questionnaires (general health status, lifestyle habits, diet, physical activity) and anthropometric measurements (height, weight, waist circumference, blood pressure, and the like) were performed. The local institutional ethics committees approved the protocol of the study, which has been published elsewhere[61], and the volunteers provided informed written consent prior to joining. The protocol of the PREDIMED Study was registered in the ISRCTN Database, with the code number ISRCTN35739639.

Figure 12. PREDIMED Study: performed analyses and design.



Within the frame of the PREDIMED Study, in **Manuscript II** we studied the effects of a 1-year adherence to a TMD on HDL function and HDL-related properties in a random subsample of 296 individuals (*N*=100 for the TMD-VOO group, *N*=100 for the TMD-Nuts group, and *N*=96 for the low-fat control diet). In **Manuscript IV** we observed the effects of a 1-year adherence to a TMD on LDL atherogenic traits in another random subsample of 210 individuals (*N*=71 for the TMD-VOO group, *N*=68 for the TMD-Nuts group, and *N*=71 for the low-fat diet) (**Figure 12**).

4.2. MEASUREMENTS

4.2.1. HDL FUNCTIONALITY ANALYSES

In **Manuscripts I** and **II**, we collected blood samples from the volunteers at fasting state, as well as first morning spot urine, before and after the interventions. From blood samples, we obtained serum and plasma. We collected HDLs by two isolation methods: 1) density gradient ultracentrifugation of plasma (in a Beckman Coulter L-100-XP ultracentrifuge)[257]; and 2) obtaining apolipoprotein B-depleted plasma (ABDP, plasma in which all lipoproteins but HDLs are removed), by precipitating VLDLs, IDLs, and LDLs with a polyethylene-glycol 8000 20% suspension[104]. All these samples were kept at -80ºC until use. The exact biochemical specimen used for the determination of every laboratory test is available in **Table 5** (there are differences between studies due to sample availability issues and variations in the design).

Table 5. Determinations and specimens used in HDL-related manuscripts.

DETERMINATIONS	EUROLIVE Study (Manuscript I)	PREDIMED Study (Manuscript II)
Biochemical profile (glucose, triglycerides, total cholesterol, HDL-C, ApoA-I, LDL-C)	PL	PL
Biomarkers – Consumption of VOO	U	U
Biomarkers – Consumption of nuts	-	PL
Olive oil phenolic compound metabolites in HDLs	I-HDL	-
Cholesterol efflux capacity	I-HDL	ABDP
CETP activity	S	PL
LCAT activity	PL	-
HDL cholesterol esterification index	-	PL
PON1 arylesterase activity	-	S
HDL antioxidant capacity on LDLs	-	I-HDL
HDL inflammatory index	-	ABDP
HDL vasodilatory capacity	-	ABDP
HDL oxidation *in vivo*	-	ABDP
HDL resistance against oxidation	-	I-HDL

HDL composition	I-HDL	I-HDL
HDL fluidity	I-HDL	-
HDL size distribution	PL	PL
HDL particle number	PL	-

ABDP: apolipoprotein B-depleted plasma; *I-HDL*: isolated HDLs; *PL*: plasma; *S*: serum; *U*: urine

BIOCHEMICAL PROFILE. In plasma samples, we determined the volunteers' biochemical profile (glucose, total cholesterol, triglycerides, HDL cholesterol), as well as ApoA-I levels, in an ABX-Pentra 400 autoanalyzer (Horiba-ABX). LDL-C levels were calculated by the Friedewald formula, whenever triglycerides were <300 mg/dL.

VOO- AND NUT-RELATED BIOMARKERS. Regarding the biomarkers of compliance, consumption of VOO (or adherence to the TMD-VOO intervention) was determined as the excretion of total tyrosol and hydroxytyrosol in first morning urine[258], and adherence to the TMD-Nuts intervention was assessed as the increase in plasma α-linolenic content by gas chromatography-mass spectrometry[25,61]. Levels of olive oil phenolic compound metabolites in HDL (hydroxytyrosol sulfate, and homovanillic acid sulfate, and glucuronate) were determined in isolated HDLs by high performance liquid chromatography-mass spectrometry[259].

CHOLESTEROL EFFLUX CAPACITY. We determined the cholesterol efflux capacity in a model of human THP-1 monocyte-derived macrophages: 1) we grew monocytes in suspension in RPMI-1640 medium (supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin) and refreshed them every 72h; 2) we differentiated them into macrophages by incubating the cells with phorbol-myristate-acetate (Sigma) for 96h; 3) we then incubated the cells with 0.2 µCi/mL of [1,2-^{3}H(N)]-cholesterol (Perkin-Elmer) for 24h, washed them, incubated them with fresh RPMI-1640 medium (now supplemented with 1% bovine serum albumin **–**Sigma**–**, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin) for 24h, and washed them again; 4) we incubated the macrophages with the volunteers' HDLs (5 mg/dL of isolated HDLs, or 5% ABDP) for 16h; and 5) we measured radioactivity in the cells and the supernatant to calculate cholesterol efflux: (radioactivity in the supernatant*100)/radioactivity in supernatant + cells.

CETP AND LCAT-RELATED PROPERTIES. Regarding the enzymes related to HDL cholesterol metabolism, we determined CETP activity by the *CETP Assay Kit* (MBL International), LCAT activity by the *LCAT Assay Kit* (Calbiochem, Merck Millipore), and LCAT mass by *Lecithin Cholesterol Acyltransferase ELISA Kit* (Sekisui Diagnostics), all of them in an Infinite M200 reader (Tecan Ltd.). LCAT mass was used to calculate HDL ability to esterify cholesterol (percentage of esterified cholesterol in HDL/LCAT mass).

HDL ANTIOXIDANT CAPACITIES. In relation to HDL antioxidant enzymes, we measured PON1 arylesterase activity by the *Paraoxonase/Arylesterase Assay Kit* (ZeptoMetrix). We also determined direct HDL antioxidant capacity on LDLs as follows: 1) we dialyzed isolated HDLs from the volunteers and a pool of LDLs from healthy donors against PBS in *PD-Desalting Columns* (GE Healthcare); 2) we incubated the

dialyzed HDLs (concentration: 3 mg/dL HDL-C) and LDLs (concentration: 9 mg/dL LDL-C) with a pro-oxidant agent ($CuSO_4$, 5 µM) at 37ºC in an Infinite M200 reader (Tecan Ltd.) for 6h; 3) the formation of oxidized species (conjugated dienes) was monitored by measuring the absorbance at 234 nm every 3 minutes; 4) with these kinetic data we built the LDL oxidation kinetic curves in the presence and absence of HDLs; 5) in both curves, we calculated LDL lag time (the time when maximal LDL oxidation begins; low values of LDL lag time mean low resistance of LDLs against oxidation); and 6) we compared LDL lag times in the presence of absence of HDLs as follows: (LDL lag time with HDL **–** LDL lag time without HDL)*100/LDL lag time without HDL.

HDL INFLAMMATORY INDEX. HDL inflammatory index is an indirect measurement of the ability of HDLs to block pro-oxidant reactions induced by oxidized LDLs, and it was measured as follows: 1) we diluted 2'-7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Sigma) in methanol for 30 minutes to obtain the deacetylated form of the reagent (H2DCF); 2) we incubated H2DCF (concentration: 3 µg/mL) and oxidized LDLs (obtained as previously described[124], concentration: 1.5 µg/mL) with 5 µL of ABDP from the volunteers, or without it (negative control), in black polystyrene plates, at 37ºC in an Infinite M200 reader (Tecan Ltd); 3) we shook and stimulated the plate with light every 3 minutes for 60 minutes, and measured the fluorescence at the end of the incubation process (*Ex/Em*: 485/530 nm); 4) we subtracted the fluorescence blank from the samples; and 5) we calculated the HDL inflammatory index in the following way: fluorescence in the presence of ABDP **–** fluorescence without ABDP.

HDL VASODILATORY CAPACITY. We assessed HDL vasodilatory capacity as the capacity of HDLs to induce the release of NO from HUVECs. In the cell culture medium we used 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma), able to react with NO to produce a fluorescent substrate. We performed the experiment as follows: 1) we grew HUVECs in supplemented EGM-2 medium (Lonza), refreshing cells every 72h; 2) when reached confluence, we trypsinized cells and re-seeded them in 96-well plates at 80% confluence; 3) after 24h, we washed cells and incubated them in EGM-2 medium (now supplemented with 0.75% BSA, 1% fetal calf serum, and 1% penicillin-streptomycin), with the DAF-2DA (5 µM) and the volunteers' HDLs (30% ABDP) (or without HDLs, as negative control); 4) 30 and 60 minutes after, we measured fluorescence in the plate (*Ex/Em:* 485/532 nm) in an Infinite M200 reader (Tecan Ltd); 5) with these points, we calculated the velocity of NO formation in the presence and absence of HDLs; and 6) we subtracted the blank fluorescence from all the values, and calculated the increment in the NO formation velocity as indicated: (velocity in ABDP-treated cells **–** velocity in non-treated cells)*100/velocity in non-treated cells.

HDL OXIDATION AND RESISTANCE AGAINST OXIDATION. We determined HDL oxidation *in vivo* as the content of MDA equivalents in ABDP adjusted for the cholesterol content of the sample[155]. In addition, we measured HDL resistance against oxidation as follows: 1) we dialyzed isolated HDLs from the volunteers against PBS in *PD-10 Desalting Columns* (GE Healthcare); 2) we incubated HDLs (concentration: 3

mg/dL) with a pro-oxidant agent ($CuSO_4$, 5 µM) at 37ºC in an Infinite M200 reader (Tecan Ltd.) for 6h; 3) we monitored the formation of oxidized species (conjugated dienes) by measuring absorbance at 234 nm every 3 minutes; and 4) with these kinetic data, we built the HDL oxidation kinetic curves, in which we calculated HDL lag time (the time when maximal HDL oxidation begins; low values of HDL lag time mean low resistance of HDLs against oxidation).

HDL COMPOSITION. In isolated HDL samples, we determined their content in: 1) total cholesterol, free cholesterol, triglycerides and phospholipids (by enzymatic methods); and 2) ApoA-I and ApoA-II (by immunoturbidimetry), in both cases in an ABX Pentra-400 autoanalyzer (Horiba-ABX). With these values, we calculated the content of esterified cholesterol in HDLs (total cholesterol – free cholesterol), and computed two ratios: the triglycerides/esterified cholesterol ratio (the relative levels of triglycerides in HDL core), and the phospholipids/free cholesterol ratio (the relative content of phospholipids in HDL surface).

HDL FLUIDITY. Fluidity of HDL particles was calculated as the inverse value of the steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) bound to HDLs[152]. In brief, HDLs were incubated with 1 µM DPH for 30 minutes, and then the ability of the treated HDL samples to polarize fluorescent light was determined in a LS5OB spectrofluorometer (Perkin Elmer, *Em*: 430 nm). HDL capacity to polarize the light beam is related to their structural rigidity, and can be quantified from the values of the intensity of the polarized light after impacting on the HDL probes, as previously described[152].

HDL SIZE. We determined HDL size distribution in plasma samples by gel electrophoresis, in an HDL Lipoprint System (Quantimetrix)[260], as follows: 1) HDLs were seeded in the gels, forced to move through them due to an electric current, and separated according to their size; 2) gels were then scanned and, using visual software, ten bands of HDLs of different size were detected (and their HDL content quantified by densitometry); and 3) following the manufacturer's instructions, the first three bands corresponded to the large HDLs, bands 4-7 to intermediate size HDLs, and band 8-10 to small HDLs. Alternatively, HDL2 subtype corresponded to large HDLs (bands 1-3), and HDL3 subtype to the sum of bands 4 to 9[260].

HDL PARTICLE ANALYSES. HDL particle number, average diameter, and the number of small, intermediate-size and large HDL particles were determined by nuclear magnetic resonance spectroscopy in a Vantera Clinical Autoanalyzer (LipoScience Inc.), as previously described[261].

4.2.2. LDL ATHEROGENICITY ANALYSES

As described for HDL-related trials, we obtained blood samples from the volunteers at fasting state, as well as first morning spot urine, before and after the interventions. From blood samples, we obtained serum and plasma, as well as peripheral blood mononuclear cells (PBMCs) for gene expression analyses[262]. We isolated LDLs by

density gradient ultracentrifugation of plasma samples (in a Beckman Coulter L-100-XP ultracentrifuge)[257]. We also calculated some parameters related to apolipoprotein B-containing lipoproteins (such as the difference between plasma and apolipoprotein-B depleted plasma samples[104]). Urine, plasma, serum, PBMCs, and isolated LDLs were kept at -80ºC until use. The exact biochemical specimen used for the determination of every laboratory test is available in **Table 6** (again, there are differences between studies due to sample availability issues and variations in the design).

Table 6. Determinations and specimens used in LDL-related manuscripts.

DETERMINATIONS	EUROLIVE Study (Manuscript III)	PREDIMED Study (Manuscript IV)
Biochemical profile (glucose, triglycerides, total cholesterol, HDL-C, LDL-C, ApoB)	PL	PL
Biomarkers – Consumption of VOO	U	U
Biomarkers – Consumption of nuts	-	PL
LDL particle analyses	PL	-
Size of LDL and VLDL particles	PL	-
LDL-C/ApoB ratio	-	PL
Estimated VLDL-C levels	-	PL, ABDP
Oxidized LDLs	PL	-
LDL oxidation in vivo	-	I-LDL
LDL resistance against oxidation	I-LDL	I-LDL
LDL composition	-	I-LDL
ApoC-III in ApoB-containing lipoproteins	-	PL, ABDP
LDL cytotoxicity *ex vivo*	-	I-LDL
LDL-related gene expression	PBMCs	-

ABDP: apolipoprotein B-depleted plasma; *I-LDL*: isolated LDLs; *PBMCs*: peripheral blood mononuclear cells; *PL*: plasma; *S*: serum; *U*: urine

BIOCHEMICAL PROFILE. As reported for HDL-related determinations, we measured in plasma samples the volunteers' biochemical profile and ApoB-100 levels in an ABX-Pentra 400 autoanalyzer (Horiba-ABX), and calculated LDL-C levels by the Friedewald formula, whenever triglycerides were <300 mg/dL. We computed the estimated VLDL-C levels as follows: cholesterol in plasma **–** cholesterol in apolipoprotein B depleted plasma **–** LDL-C levels.

VOO- AND NUT-RELATED BIOMARKERS. The biomarker of compliance for the consumption of VOO (or adherence to the TMD-VOO intervention) was determined in first morning spot urine[258], whilst adherence to the TMD-Nuts intervention was measured in plasma, as previously described in HDL function methodology[25,61].

LDL PARTICLE AND SIZE ANALYSES. LDL particle number, and the number of small, intermediate-size, and large LDL and VLDL particles were assessed by nuclear magnetic resonance spectroscopy in a Vantera Clinical Autoanalyzer (LipoScience Inc.)[261]. As an

indirect marker of LDL size, we calculated the ratio between LDL-C and ApoB-100 levels in plasma.

LDL OXIDATION AND RESISTANCE AGAINST OXIDATION. We determined oxidized LDL levels in plasma with an ELISA kit (Mercodia). We calculated LDL oxidation degree *in vivo* in isolated LDL samples as the content of MDA equivalents in LDL samples adjusted for their cholesterol content[155]. We determined LDL resistance against oxidation as follows: 1) we dialyzed isolated LDLs from the volunteers against PBS in *PD-10 Desalting Columns* (GE Healthcare); 2) we incubated LDLs (concentration: 10 mg/dL) with a pro-oxidant agent ($CuSO_4$, 5 μM) at 37ºC in an Infinite M200 reader (Tecan Ltd.) for 4h; 3) we monitored the formation of oxidized species (conjugated dienes) by determining the absorbance at 234 nm every 3 minutes; and 4) with these kinetic data, we built the LDL oxidation kinetic curves, in which we calculated LDL lag time (the time when maximal HDL oxidation begins) and LDL oxidation rate (the maximal velocity of oxidation of the sample).

LDL COMPOSITION. In isolated LDL samples, we measured: 1) total cholesterol and triglycerides (by enzymatic methods); 2) total protein content (by the Biuret reaction); and 3) ApoB-100 (by immunoturbidimetry), in the three cases in an ABX Pentra-400 autoanalyzer (Horiba-ABX). With these values, we calculated the triglyceride/cholesterol ratio in LDLs, the content of ApoB in LDLs (adjusted for cholesterol levels), and the percentage of proteins in LDL other than ApoB in the following way: (total proteins in LDL – ApoB in LDL)*100/total proteins in LDL.

APOC-III-RELATED PROPERTIES. With respect to ApoC-III, we first determined their levels in plasma by immunoturbidimetry in an ABX Pentra-400 autoanalyzer (Horiba-ABX). To calculate the adjusted ApoC-III levels in apolipoprotein B-containing lipoproteins (VLDL+LDLs, as samples were obtained in fasting state), we determined ApoC-III in ABDP samples, we calculated the ApoC-III content in apolipoprotein B-containing lipoproteins (ApoC-III in plasma – ApoC-III in ABDP), and adjusted this value for the cholesterol content of these lipoproteins.

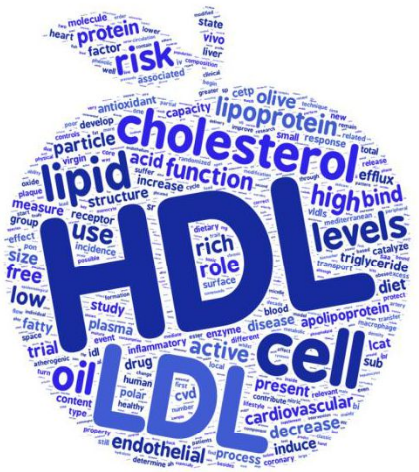
LDL CYTOTOXICITY. We also measured LDL cytotoxicity in two human cell models *ex vivo*: THP-1 monocyte-derived macrophages and HUVECs. We assessed LDL cytotoxicity as the capacity of LDLs to decrease the viability of the cells, and measured it with the MTT technique. The MTT bromide (Thiazolyl Blue Tetrazolium bromide, Sigma) is a soluble yellow pigment that cells are able to pick up and transform into insoluble, blue MTT-formazan crystals. MTT transformation into blue crystals is directly proportional to cell viability. Therefore, a decrease in the production of MTT crystals by cells after the incubation with LDLs would be associated with an increased LDL cytotoxicity. We performed the experiments as follows: 1) we grew both cell lines as previously described (see the methodology of the experiments of cholesterol efflux capacity and HDL vasodilatory capacity), and incubated them with isolated LDLs (concentration: 10 mg/dL), during 16h for macrophages and 4h for HUVECs; 2) we washed the cells and incubated them with 0.5 mg/mL soluble MTT bromide for 4h; 3)

we removed the supernatant, washed the cells again, and dissolved the cell content (and the blue MTT-formazan crystals inside the cells) with dimethyl sulfoxide (Sigma), for 1h under stirring; 4) we measured absorbance at 570 nm in an Infinite M200 reader (Tecan Ltd); 5) we subtracted the absorbance blank from all cells, and calculated LDL cytotoxicity (relative to the absence of LDLs in the culture) in the following way: (absorbance in LDL-treated cells – absorbance in non-treated cells)*100/absorbance in non-treated cells. A decreased blue color in the plate would be associated with a decreased production of MTT-formazan crystals by the cells and, therefore, with an increased LDL cytotoxicity.

LDL-RELATED GENE EXPRESSION. In the LDL-related gene expression analyses, we first isolated mRNA from PBMCs, checked its integrity and purity, transformed it into cDNA, and determined its expression by quantitative PCR as previously described[262]. We measured the expression of the lipoprotein lipase gen (*LPL*) by the $2^{-\Delta\Delta Ct}$ method (correcting its expression by the expression of the housekeeping gene and the baseline expression of the gene in the volunteers)[262].

4.2.3. QUALITY CONTROL OF THE NOVEL DETERMINATIONS

To improve inter-assay comparability, most novel techniques (cholesterol efflux capacity, CETP activity, HDL cholesterol esterification index, PON1 arylesterase activity, direct HDL antioxidant capacity, HDL inflammatory index, HDL vasodilatory capacity, HDL oxidation *in vivo*, HDL resistance against oxidation, LDL oxidation *in vivo*, LDL resistance against oxidation, and LDL cytotoxicity *ex vivo*) were performed considering three quality control precautions: 1) we analyzed all samples from the same volunteer in the same experimental run; 2) we ran determinations in duplicate (except for CETP, LCAT, and PON1 analyses), and did not allow intra-repetition coefficients of variation values > 15%; and 3) to minimize inter-assay variability, we included a sample obtained from a pool of 20 healthy volunteers in every experiment as control. The value obtained for every control was used to calculate the inter-assay coefficients of variation, and to normalize the values of the samples of the volunteers analyzed in the same experimental run (by dividing the value of every sample by the value obtained for the control). Therefore, the results of these novel techniques are expressed as normalized ratios without units.


HDL
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5. Results

Four manuscripts constitute the results of the present thesis. The specific objectives covered by each publication are summarized in **Table 7**.

Table 7. Thesis objectives and corresponding publications.

OBJECTIVE	PUBLICATION ASSIGNED
To assess the protective effects of the consumption of real-life doses of VOO in HDL function and HDL quality-related characteristics in healthy volunteers in a randomized controlled trial.	MANUSCRIPT I. Hernáez A, Fernández-Castillejo S, Farràs M, et al. **Olive oil polyphenols enhance high-density lipoprotein function in humans: a randomized controlled trial.** *Arteriosclerosis, Thrombosis, and Vascular Biology*, 2014;34(9):2115-2119.
To explore the protective effects of long-term adherence to a TMD on HDL function and HDL quality-related characteristics in high cardiovascular risk individuals in a randomized controlled trial.	MANUSCRIPT II. Hernáez A, Castañer O, Elosua R, et al. **The Mediterranean Diet improves HDL function in high cardiovascular risk individuals: a randomized controlled trial.** [Submitted]
To determine the protective effects of the consumption of real-life doses of VOO on LDL quantity and atherogenic characteristics in healthy volunteers in a randomized controlled trial.	MANUSCRIPT III. Hernáez A, Remaley AT, Farràs M, et al. **Olive oil polyphenols decrease LDL concentrations and LDL atherogenicity in men in a randomized controlled trial.** *Journal of Nutrition*, 2015;145(8):1692-1697.
To analyze the protective effects of long-term adherence to a TMD on LDL atherogenic characteristics in high cardiovascular risk individuals in a randomized controlled trial.	MANUSCRIPT IV. Hernáez A, Castañer O, Goday A, et al. **The Mediterranean Diet decreases LDL atherogenicity in high cardiovascular risk individuals: a randomized controlled trial.** [Submitted]

The main results obtained in each manuscript are the following:

MANUSCRIPT I. Olive oil polyphenols enhance high-density lipoprotein function in humans: a randomized controlled trial.

- The most important HDL function, cholesterol efflux capacity, improved after the consumption of a phenolic compound-rich olive oil (366 mg/kg olive oil phenolic compounds) in healthy, young individuals.
- The dose-dependent binding of the biological metabolites of olive oil phenolic compounds to HDLs could be responsible for the effect, due to an improvement in the oxidative status of the lipoproteins.
- Moreover, olive oil phenolic compounds induced *in vivo* a low-cardiovascular risk HDL profile in the study volunteers.
- This is the first randomized controlled trial to report an improvement in HDL function after the consumption of real-life doses of a dietary compound.

MANUSCRIPT II. The Mediterranean Diet improves HDL function in high cardiovascular risk individuals: a randomized controlled trial.

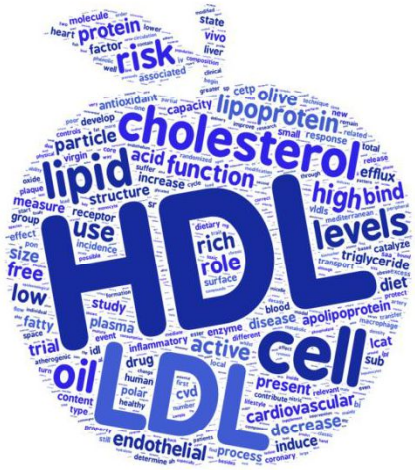
- Adherence to a TMD, especially when supplemented with VOO, improved the four most relevant HDL functions: cholesterol efflux capacity, HDL role in other steps of cholesterol metabolism, HDL antioxidant capacity, and HDL vasoprotective functions.
- The TMD could have induced these benefits through an improvement of HDL oxidative status, composition, and size distribution.
- This is the first randomized controlled trial to report an improvement in the four most relevant HDL functions, in the largest number of individuals (*N*=296) and for the longest intervention (1 year) to date.

MANUSCRIPT III. Olive oil polyphenols decrease LDL concentrations and LDL atherogenicity in men in a randomized controlled trial.

- The consumption of olive oil phenolic compounds decreased LDL concentrations, directly measured as levels of apolipoprotein B-100 or the total LDL particle number in plasma in healthy, young individuals.
- The consumption of olive oil phenolic compounds also reduced LDL atherogenicity, as observed in a decrease in the number of small LDL particle and an increase in LDL resistance against oxidation.
- An improved LDL oxidative status and an enhanced expression of the lipoprotein lipase gene (*LPL*) may contribute to explaining these changes.

MANUSCRIPT IV. The Mediterranean Diet decreases LDL atherogenicity in high cardiovascular risk individuals: a randomized controlled trial.

- Adherence to a TMD decreased LDL atherogenicity, as observed in a reduction in LDL size, an increase in LDL resistance against oxidation, improvements in LDL composition, and a decrease in LDL cytotoxicity *ex vivo* in a model of human macrophages.
- An improved LDL oxidative status may help to explain these changes.
- This randomized controlled trial is the first to study these LDL atherogenic properties together after a healthy lifestyle modification.



6. Manuscripts

MANUSCRIPT I

Olive oil polyphenols enhance high-density lipoprotein function in humans: a randomized controlled trial. *Arteriosclerosis, Thrombosis, and Vascular Biology*, 2014;34(9):2115-2119.

National Cholesterol Awareness Month Article
Clinical and Population Studies

Olive Oil Polyphenols Enhance High-Density Lipoprotein Function in Humans

A Randomized Controlled Trial

Álvaro Hernáez, Sara Fernández-Castillejo, Marta Farràs, Úrsula Catalán, Isaac Subirana, Rosa Montes, Rosa Solà, Daniel Muñoz-Aguayo, Anna Gelabert-Gorgues, Óscar Díaz-Gil, Kristiina Nyyssönen, Hans-Joachim F. Zunft, Rafael de la Torre, Sandra Martín-Peláez, Anna Pedret, Alan T. Remaley, María-Isabel Covas, Montserrat Fitó

Objective—Olive oil polyphenols have shown beneficial properties against cardiovascular risk factors. Their consumption has been associated with higher cholesterol content in high-density lipoproteins (HDL). However, data on polyphenol effects on HDL quality are scarce. We, therefore, assessed whether polyphenol-rich olive oil consumption could enhance the HDL main function, its cholesterol efflux capacity, and some of its quality-related properties, such HDL polyphenol content, size, and composition.

Approach and Results—A randomized, crossover, controlled trial with 47 healthy European male volunteers was performed. Participants ingested 25 mL/d of polyphenol-poor (2.7 mg/kg) or polyphenol-rich (366 mg/kg) raw olive oil in 3-week intervention periods, preceded by 2-week washout periods. HDL cholesterol efflux capacity significantly improved after polyphenol-rich intervention versus the polyphenol-poor one (+3.05% and −2.34%, respectively; P=0.042). Incorporation of olive oil polyphenol biological metabolites to HDL, as well as large HDL (HDL_2) levels, was higher after the polyphenol-rich olive oil intervention, compared with the polyphenol-poor one. Small HDL (HDL_3) levels decreased, the HDL core became triglyceride-poor, and HDL fluidity increased after the polyphenol-rich intervention.

Conclusions—Olive oil polyphenols promote the main HDL antiatherogenic function, its cholesterol efflux capacity. These polyphenols increased HDL size, promoted a greater HDL stability reflected as a triglyceride-poor core, and enhanced the HDL oxidative status, through an increase in the olive oil polyphenol metabolites content in the lipoprotein. Our results provide for the first time a first-level evidence of an enhancement in HDL function by polyphenol-rich olive oil. **(*Arterioscler Thromb Vasc Biol*. 2014;34:2115-2119.)**

Key Words: diet ■ high-density lipoproteins ■ olive oil

Olive oil consumption has proven to be protective against the development of cardiovascular pathologies[1] because of its monounsaturated fatty acid content[2] and to other bioactive compounds, such as polyphenols.[3,4] Previous results from our group showed that olive oil polyphenols increased dose dependently high-density lipoprotein (HDL) cholesterol levels.[3] Although it has been widely reported that low HDL cholesterol levels are strongly associated with high cardiovascular risk,[5] recent data indicate that increased HDL cholesterol levels do not imply a reduction in the risk of experiencing a myocardial infarction.[6] A key objective in cardiovascular disease prevention strategies should, therefore, be not only to increase HDL cholesterol but also to enhance its biological function.

Within this context, our group recently reported that olive oil polyphenols are able to enhance the expression of genes related to HDL metabolism and function.[7] However, no randomized controlled study in humans has been at present performed on the effects of olive oil polyphenols on HDL

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The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBAHA.114.303374/-/DC1.

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Nonstandard Abbreviations and Acronyms	
CETP	cholesterol ester transfer protein
HDL	high-density lipoprotein
HPCOO	high-polyphenol content olive oil

functionality. Because of this, our aim was to elucidate whether long-term consumption of high-polyphenol content olive oil (HPCOO) would be able to enhance HDL activity and quality characteristics.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Participants Characteristics

The crossover design of the study is described in Figure 1. As shown in Table I in the online-only Data Supplement, no significant differences in baseline parameters between our subsample and the whole EUROLIVE study population (The Effect of Olive Oil on Oxidative Damage in European Population; where our volunteers subsample was extracted from) were found. Energy expenditure in leisure time physical activity through the study and dietary patterns remained stable throughout the study. Volunteers' compliance was correct, as reflected in the biomarkers of compliance.[3] Systemic lipid profile did not change after interventions, as described in Table II in the online-only Data Supplement.

In a subsample of 19 volunteers, a favorable but nonsignificant trend toward higher values of HDL particle count was observed after the HPCOO intervention. Raw values of all relevant HDL markers, before and after both interventions, are shown in Table III in the online-only Data Supplement.

Olive Oil Polyphenols Enhance HDL Cholesterol Efflux Capacity

As we see in Figure 2A, the cholesterol efflux capacity from THP-1 macrophages of the participants' HDL fraction was significantly higher after the HPCOO intervention, compared with the low-polyphenol content olive oil one (P=0.043).

Multiple linear regression analyses showed that basal values of cholesterol efflux capacity were significantly related to the HDL particle count. For each increase in 1 μmol/L of baseline HDL particle count values, cholesterol efflux values increased by 0.06 points (P<0.05).

Olive Oil Polyphenols Bind to HDL in a Dose-Dependent Manner

As shown in Figure 2B, the main metabolites of olive oil polyphenols bound to HDL in a dose-dependent manner. This dose-dependent binding was especially relevant for hydroxytyrosol sulfate (P<0.001, when comparing the HPCOO intervention with both baseline and the low-polyphenol content olive oil one).

Increases of olive oil polyphenol metabolites in HDL were associated with enhancements of HDL function. Multiple linear regression analyses revealed that, after the HPCOO intervention, cholesterol efflux capacity values increased significantly with the increment of olive oil polyphenol metabolites in HDL. For each increase in 1 ppb of hydroxytyrosol sulfate in HDL, there was an increase of 15.6% of cholesterol efflux capacity of the volunteers (P<0.05).

Olive Oil Polyphenols Induce the Formation of Larger HDLs

As described in Figure 2C, after the HPCOO intervention, levels of large HDL (HDL_2) in plasma increased significantly compared with baseline (P=0.010) or with the low-polyphenol content olive oil one (P=0.050). In parallel, levels of small HDL (HDL_3) were significantly lower compared with baseline (P=0.039).

Olive Oil Polyphenols Enhance Some HDL Biophysical Parameters

No changes were observed in HDL cholesterol, triglycerides, phospholipids, and apolipoproteins A1 and A2 after interventions (data not shown). However, olive oil polyphenols induced some changes in biophysical parameters related to the HDL fluidity and core quality (Table IV in the online-only Data Supplement).

When evaluating the triglyceride content in the HDL core, a significant decrease was observed after the HPCOO intervention compared with baseline (P=0.049), reaching a borderline significance (P=0.057) compared with the low-polyphenol content olive oil intervention. The fluidity of the HDL particle also changed after the consumption of olive oil polyphenols, increasing significantly compared with baseline (P=0.033).

Discussion

Our results show that a 3-week consumption of olive oil polyphenols induced a significant enhancement of the main HDL biological function, its cholesterol efflux capacity. Olive oil polyphenols also induced changes in the biochemical properties of the lipoprotein, which may have contributed to the HDL function enhancement. To our knowledge, this is the first time that first-level evidence about the in vivo health effects of polyphenols on HDL function is reported in healthy volunteers.

The main biological function of the HDL is extracting the excess of cholesterol from the peripheral cells and taking it to the liver to be metabolized and excreted. This cholesterol capture is broadly known as cholesterol efflux, and the main cell types involved in it are the macrophages under the endothelium in blood vessels.[8] This HDL functional property has been broadly tested in several macrophage cell lines and has been inversely related to early atherosclerosis development and to high risk of experiencing a coronary event.[9] In our study, the consumption of olive oil polyphenols enhanced the cholesterol efflux capacity of the volunteers' HDL in a physiological model of THP-1 monocyte-derived macrophages. A similar effect has been described after extravirgin olive oil consumption in a noncontrolled, linear study,[10] after walnuts consumption[11] or a pioglitazone treatment.[9]

The HDL cholesterol efflux enhancement after the HPCOO intervention may be promoted by the observed increase in the

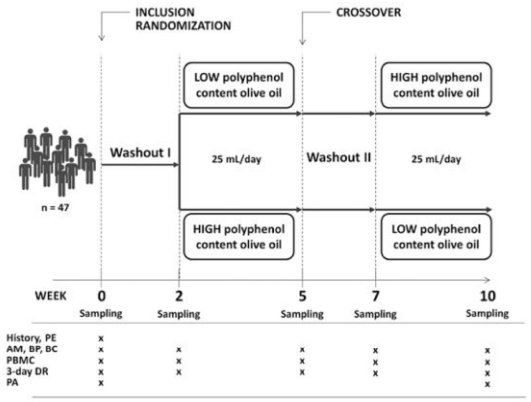

Figure 1. Study design (n=47). AM indicates anthropometric measurements; BC, blood/urine collection; BP, blood pressure measurements; DR, dietary record; PA, physical activity measurement by the Minnesota Leisure Time Physical Activity Questionnaire; PBMC, peripheral blood mononuclear cell collection for gene expression analyses; and PE, physical examination.

olive oil polyphenol metabolites bound to the lipoprotein. It has been previously described that an oxidized HDL is more rigid and presents a lower cholesterol efflux capacity.[12] Thus, a better antioxidative protection, conferred by a higher content of olive oil polyphenols in the HDL, may contribute to explain its functional enhancement. The olive oil polyphenol metabolites bound to the HDL after the HPCOO intervention have been shown to have antioxidant properties.[13] Thus, a local antioxidant effect on the HDL is expected. In parallel, in the HPCOO intervention, a less rigid HDL was observed, and an increased HDL fluidity is considered an intermediate marker of enhanced HDL functionality.[12] Considering our evidences, we can hypothesize that the binding of olive oil polyphenols to HDL would increase the fluidity of the particle, thus enhancing the HDL capacity for promoting the cholesterol efflux from cells.

Olive oil polyphenols also induced changes in HDL size distribution, which has been closely related to HDL quality.[14] Low levels of large HDL and high levels of small HDL are a pathological trait present in cardiovascular pathologies, as coronary heart disease,[15] although they have not been described as associated with incident coronary events in some prospective studies.[16] Thus, after the HPCOO intervention, large HDL (HDL_2) levels were significantly higher, whereas small HDL (HDL_3) levels were significantly lower. Our results agree with those reported after an olive oil-rich diet in healthy men[17] or after a Mediterranean diet enriched in nuts.[18]

Up to the present, the only HDL functional parameter that has been correlated to incident coronary events is the HDL particle number.[19] Thus, we assessed whether positive changes in this direction happened after the consumption of olive oil polyphenols. Although nonsignificant, we observed a trend toward higher HDL particle count values after the HPCOO intervention. Moreover, a direct relation between baseline values of this parameter and cholesterol efflux capacity has been found in the present work. However, as these results are not conclusive, and because few treatments have been able to induce an enhancement in HDL particle count values,[20] further studies in this line are priority to check this hypothesis.

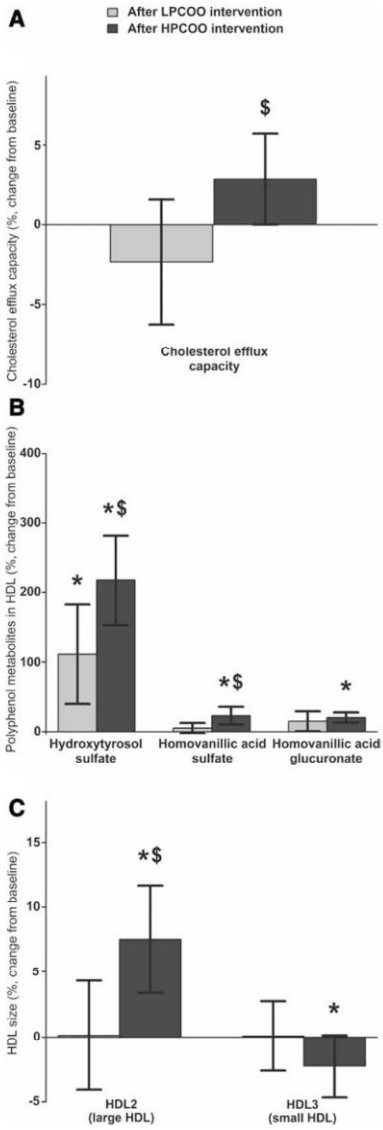

Figure 2. Main changes induced on high-density lipoproteins (HDL) by olive oil polyphenol consumption. **A**, Changes in the cholesterol efflux capacity of the HDL isolated fraction from THP-1 macrophages, expressed as the normalized ratio against the HDL control pool from each experiment. After the high-polyphenol content olive oil (HPCOO) intervention, the efflux is significantly higher, compared with the low-polyphenol content olive oil (LPCOO) one. **B**, Changes in the content of biological metabolites of olive oil polyphenols in HDL, after LPCOO and HPCOO interventions. Olive oil polyphenol metabolites bind to HDL in a dose-dependent manner. **C**, Changes in HDL size distribution show that large HDLs are significantly higher after the HPCOO intervention and that the small HDLs are moderately reduced. In all cases, changes in percentage compared with baseline values are shown, presented as mean±SEM. *P<0.05, compared with baseline. $$P$<0.05, compared with LPCOO intervention (mixed linear model).

Previous studies demonstrated that high triglyceride levels in the HDL core are present in several cardiovascular pathologies, such as coronary heart disease,[21] and they are related to a less stable conformation of apolipoprotein-A1 in HDL surface (ie, a less stable lipoprotein structure).[22,23] In our work, significantly lower triglyceride levels in the HDL core were observed. This decrease could be explained by a lower activity of the cholesterol ester transfer protein (CETP). CETP extracts esterified cholesterol from the HDL core to triglyceride-rich lipoproteins, returning triglycerides from triglyceride-rich lipoproteins to HDL.[24] We observed a trend toward a lower CETP activity after HPCOO intervention. This could have happened through a direct blockade of CETP enzyme, as it has been described for apple polyphenols,[25] or through a nutrigenomic inhibition of *CETP* gene expression, similar to the one reported after a Mediterranean diet consumption.[26] Further studies in this field are required to confirm these hypotheses.

One of the strengths of this study is its crossover design, which permitted the participants to ingest all olive oil types and also reduced possible interferences with confounding variables. As expected, changes after interventions were modest because real-life doses of a single food (which cannot be consumed in high quantities daily) were administered to healthy volunteers. A study limitation was its sample size, responsible for reduced statistical power in some biomarkers with high interindividual variability. It is also possible that an amount of polyphenols, similar to those provided by olive oil, could have come from other food types. A synergistic effect on HDL parameters between polyphenols and other olive oil biological metabolites is as yet unknown.

In conclusion, this is the first time that HDL cholesterol efflux enhancement by polyphenols has been reported in healthy, young population, in a randomized controlled trial. Apart from the enhancement in the main antiatherogenic HDL function, olive oil polyphenols induced a change in HDL size toward larger values. These, together with the enhancement of the stability and the oxidative status of the HDL particle, led to a better HDL functionality after the consumption of olive oil polyphenols. Our data are in line with the previous evidences supporting that the consumption of olive oil polyphenols helps to reduce cardiovascular risk.

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Disclosures

None.

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Significance

The cholesterol efflux capacity of high-density lipoproteins is considered their main antiatherogenic property, and, in our data, it improved after a long-term consumption of olive oil polyphenols. The dose-dependent binding of the biological metabolites of these polyphenols to the high-density lipoproteins could be responsible for the effect, through an enhanced oxidative status of the particles. Apart from this improvement, olive oil polyphenols induced in vivo a low-cardiovascular risk high-density lipoproteins profile in the volunteers of the study. This has been the first time that the previous effects have been associated to food polyphenols in healthy volunteers in a randomized, parallel, controlled trial with a robust design.

MATERIAL & METHODS

Study design

The EUROLIVE study was a parallel, crossover, randomized controlled trial performed with 180 healthy European men, from six European cities, aged 20-60 years. From the 344 people who agreed to be screened, 200 were eligible and enrolled in the study from September 2002 to June 2003. Exclusion criteria were the following: smoking; use of antioxidant supplements, aspirin or any drug with known antioxidant properties; hyperlipidemia; diabetes mellitus; hypertension; intestinal disease; or any other physiological condition or disease that could impair adherence. Women were excluded to avoid possible interferences of estrogens, due to their potential antioxidant properties. In all cases, participants provided written informed consent to join the study, and local institutional ethics committees approved the protocol, whose details have been previously published (1). This protocol is registered with the International Standard Randomized Controlled Trial Number ISRCTN09220811 (www.controlled-trials.com).

HDL function and quality analyses were performed in a random subsample of 47 EUROLIVE participants (17 from Germany, 15 from Finland, and 15 from Spain). The samples studied were taken before and after interventions with high polyphenol content olive oil (HPCOO, a natural virgin olive oil with 366 mg/kg of polyphenols), and low polyphenol content olive oil (LPCOO, 2.7 mg/kg, a refined one). Since phenolic compounds are lost during the refinement process, the refined olive oil had a reduced phenolic content. Both olive oil compositions were identical, with the exception of their polyphenol content (1). In the crossover design (**Figure 1)**, participants followed 3-week intervention periods, in which they ingested 25 mL/day of raw olive oil distributed among meals. Volunteers followed a brief training to learn to replace other dietary fats with olive oil in all meals. The intervention periods were preceded by 2-week washout periods, in which volunteers avoided olive oil and olive ingestion, as well as a high intake of antioxidants. According to the half-life of the main olive oil phenolic compounds in the body, a 2-week washout period was sufficient to guarantee a complete elimination of the antioxidants between interventions (2).

Dietary adherence and physical activity

24-hour urinary excretion of tyrosol and hydroxytyrosol (the two major phenolic compounds in olive oil, as simple forms or conjugates) were measured by gas chromatography and mass spectrometry (3), as biomarkers of adherence to the type of olive oil ingested. Taking this criterion into account, noncompliant volunteers were excluded from all the subsequent analyses.

To control their diet, participants were asked to keep a 3-day dietary record at the beginning of the study and after each intervention period, and to maintain their normal diet during the study. They also received nutritional education, learning to replace raw fats with olive oils.

Physical activity of the volunteers was measured at the beginning and the end of the study, by means of a validated Minnesota Leisure Time Physical Activity Questionnaire.

Systemic biomarkers

Plasma glucose, total and HDL cholesterol, and plasma triglycerides were measured using automatic enzymatic-colorimetric methods, in a Cobas-Mira Plus autoanalyzer (Roche, Basel, Switzerland), using specific reagents from Spinreact (Barcelona, Spain). LDL cholesterol was calculated with the Friedewald formula, whenever plasma triglycerides were below 300 mg/dL. Plasma oxidized LDL levels were measured by an ELISA (*Mercodia Oxidized LDL ELISA AB*, Mercodia, Uppsala, Sweden), in an INFINITE M200 reader (Tecan Group Ltd., Männedorf, Switzerland).

Lipoprotein extraction

Fasting human plasma from volunteers was collected in K_2-EDTA containing tubes. HDL lipoprotein fraction was extracted by ultracentrifugation, as previously described (4). HDL fraction was stored at -80ºC in a buffer containing 2.5% sucrose until the moment of use.

Measurement of HDL cholesterol efflux capacity

THP-1 monocytes were grown in DMEM medium, supplemented with 10% heat-inactivated FBS, 1% sodium pyruvate, 1% L-glutamine, and 1% penicillin-streptomycin. Culture medium was refreshed each 72h. THP-1 monocytes were differentiated into macrophages incubating the cells in the presence of 200 nM of phosbol-myristate-acetate (Sigma, St. Louis, MO, USA) for 96h.

To test HDL cholesterol efflux capacity, these monocyte-derived macrophages were then incubated in fresh culture medium with 0.2 µCi/mL of [H^3]-cholesterol for 24 hours. Afterward, macrophages were washed and incubated in DMEM supplemented with 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) for 24h for the equilibration process. Cells were then washed again and suspended in fresh DMEM medium, supplemented with 1% BSA, and with either 50 µg/mL of HDL from the volunteers or without HDL (control) for 16h. After this last incubation, the supernatant medium was extracted from the culture, the cells were lysed by freezing and the remains removed from culture plates.

The cholesterol efflux capacity of HDL fraction was determined by liquid scintillation counting. Radioactivity in both supernatant medium and cell lysate was measured with a beta scintillation counter Tri-Carb 2800TR (Perkin-Elmer, Waltham, MA, USA). Thus, the cholesterol efflux capacity was calculated using the following formula: (counts per minute in supernatant/counts per minute in supernatant + cells) x 100.

For all these determinations, samples were analyzed in triplicate, with a mean intra-plate coefficient of variation of 4.39% and a mean inter-plate coefficient of variation of 7.43%.

Determination of biological metabolites of olive oil polyphenols in HDL fraction

Determination and quantification of the biological metabolites of olive oil polyphenols in HDL were performed by means of High Performance Liquid Chromatography associated with mass spectrometry, following previously described methodology (5).

HDL size distribution

HDL size distribution was determined using the Lipoprint HDL System (Quantimetrix, Redondo Beach, CA, USA). The Lipoprint HDL System focuses on the size distribution of HDL lipoproteins and is able to detect nine size-dependent, HDL electrophoretic bands. The first band corresponds to the bigger HDLs present in the sample, the second band corresponds to slightly smaller HDLs, and so on until the ninth band. As has been previously published (6), the first three bands have been described as corresponding to the large HDL subclass (HDL_2) and, from 4^{th} to 9^{th} band, to the small HDL subclass (HDL_3).

HDL particle count analyses

HDL particle count, HDL average particle size and count of small, medium and large HDL particles were determined using NMR technology, in a Vantera Clinical Analyzer (LipoScience Inc., Raleigh, NC, USA), as previously described (7).

HDL composition and activities of HDL metabolic enzymes

In HDL lipoprotein fractions, total and free cholesterol, triglycerides and phospholipids were quantified by automatic enzymatic-colorimetric methods. Apolipoproteins A1 and A2 were also determined by automatic immunoturbidimetric methods. All these determinations were performed in a Cobas-Mira Plus autoanalyzer (Roche, Basel, Switzerland), using specific reagents from Spinreact (Barcelona, Spain) for both techniques. The triglyceride content of the HDL core was assessed as the ratio between HDL triglycerides and HDL esterified cholesterol. The activities of the main enzymes related to HDL metabolism were also analyzed. Cholesterol ester transfer protein (CETP) and lecithin:cholesterol acyl-transferase (LCAT) activities were measured, in serum and EDTA-plasma respectively, using specific commercial fluorimetric kits (*LCAT Assay Kit*, Calbiochem, Merck Millipore, Billerica, MA, USA; *CETP Assay Kit*, MBL International, Woburn, MA, USA) in an INFINITE M200 reader (Tecan Group Ltd., Männedorf, Switzerland).

HDL fluidity determination

The fluidity of the HDL particle was measured based on the determination of the steady-state anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH), as previously described (8). 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 430 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. Subsequently, we were able to measure the intensities of the perpendicular polarized fluorescence produced by the probe (I_p), which could vary depending on the sample fluidity. The steady-state fluorescence anisotropy (r) was calculated with these I_p values, and with the grating correction factor of the monochromator (G), using the following formula:
$r = (I_p - GI_p)/(Ip_+ 2GI_p)$
The steady-state anisotropy refers to the rigidity of the sample. Therefore, the inverse value of this parameter ($1/r$) is the fluidity index.

Sample size calculation

A sample size of 45 participants allowed a ≥ 80% power to detect a significant difference between olive oil interventions of 3 mg/dL of HDL cholesterol, considering a 2-sided type I error of 0.05 and a loss rate of 2%. Calculations were made from our previous data, taking into account the SD of HDL cholesterol levels in healthy volunteers (1).

Statistical analyses

Distribution of continuous variables was determined through normal probability plots and graphics and the Shapiro-Wilk test. Non-normally distributed variables were subsequently log transformed. A mixed linear model was used to determine the effect of each intervention compared to its baseline and the differences between treatments. The possible carry-over effect was determined by testing a period-by-treatment interaction. Pearson's correlation analyses were performed to determine relationships among variables. Multiple linear regression analyses were carried out, with the baseline values and changes after the HPCOO intervention, to explore whether HDL-associated variables could be related to cholesterol efflux capacity. To assess possible relations, age and those variables which presented a Pearson's correlation with cholesterol efflux (with a significance of $P<0.1$) were introduced into the model. Then, a forward stepwise analysis was applied. Variables firstly introduced in the regression models were age, HDL cholesterol, apolipoprotein A1, HDL_2/HDL_3 ratio and HDL particle count for baseline values, and age, HDL_2/HDL_3 ratio and hydroxytyrosol sulfate in HDL for intervention changes. We considered any *P* value below 0.05 significant. All statistical analyses were performed with R Software, version 2.15.2 (R Development Core Team, 2013; www.R-project.org) and with SPSS Software, version 18.0 (IBM Corp).

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SUPPLEMENTAL TABLE I
Baseline characteristics of the participants

	Baseline value	
	Study subsample (n = 47)	EUROLIVE study whole population (n = 180)
Age (years)	33.5 (10.9)[1]	33.2 (11.0)
Weight (kg)	77.8 (10.9)	76.4 (10.5)
Height (m)	1.79 (0.07)	1.79 (0.07)
Total cholesterol (mg/dL)	170 (43)	174 (41)
LDL cholesterol (mg/dL)	96 (38)	98 (36)
HDL cholesterol (mg/dL)	52 (11)	53 (12)
Tryglycerides (mg/dL)[2]	99 (70 to 129)	99 (68 to 134)

[1]: Mean (SD)
[2]: Median (1st to 3rd quartile in parentheses)

SUPPLEMENTAL TABLE II
Changes in systemic parameters after olive oil interventions

	Olive oil interventions				
	Low polyphenol content olive oil		High polyphenol content olive oil		
	Post-intervention	Change[1]	Post-intervention	Change[1]	*P* between groups[2]
Systemic lipid profile					
Total cholesterol (mg/dL)	176 (47)[3]	↑1.83% (12.2)	171 (45)	↓2.43% (10.9)	0.064
LDL cholesterol (mg/dL)	100 (42)	↑2.13% (20.5)	99 (41)	↓2.19% (16.2)	0.245
HDL cholesterol (mg/dL)	54 (12)	↑3.36% (13.2)	54 (13)	↓0.40% (11.4)	0.153
Triglycerides (mg/dL)[4]	80.5 (55.1 to 123.4)	↓5.71% (-20.7 to 24.5)	78.8 (60.4 to 109.4)	↓7.03% (-23.3 to 9.38)	0.306
Oxidized LDL (U/L)	41.9 (20.3)	↑7.59% (38.5)	40.5 (16.2)	↓6.01% (22.0)*	0.023
HDL particle count analysis (*n* = 19)					
HDL particle count (µmol/L)	32.2 ± 3.80	↑0.30% (10.5)	32.0 ± 2.99	↑3.25% (9.79)	0.429
HDL average particle size (nm)	9.42 ± 0.53	↓0.16% (3.17)	9.36 ± 0.56	↑0.84% (3.34)	0.395
Small HDL particle count (µmol/L)	15.6 ± 5.25	↑15.8% (55.0)	15.8 ± 4.62	↑1.99% (22.6)	0.766
Medium+large HDL particle count (µmol/L)	16.5 ± 4.85	↑1.50% (32.7)	16.2 ± 5.24	↑5.07% (22.5)	0.444

[1]: Change in percentage, compared to baseline, expressed as Mean (SD)
[2]: *P* for inter-group comparisons (mixed linear model)
[3]: Mean ± SD
[4]: Median (1st to 3rd quartile in parentheses)
*: *P*<0.05 after intervention, compared to baseline (mixed linear model)

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SUPPLEMENTAL TABLE III
Raw measurements of main HDL-related parameters

	Olive oil interventions						
	Low polyphenol content olive oil			High polyphenol content olive oil			
	Pre-intervention[1]	Post-intervention	Change[2]	Pre-intervention	Post-intervention	Change[2]	*P* between groups[3]
HDL cholesterol (mg/dL)	53.0 (11.6)	54.0 (11.8)	↑3.36% (13.2)	54.5 (11.9)	54.2 (12.8)	↓0.40% (11.4)	0.153
Cholesterol efflux capacity[4]	0.95 (0.18)	0.91 (0.17)	↓2.34% (13.5)	0.92 (0.20)	0.94 (0.19)	↑3.05% (9.98)	0.042
Hydroxytyrosol sulfate in HDL (ppb)	16.0 (12.2)	26.5 (18.6)	↑111% (224)*	17.2 (11.8)	46.8 (39.6)	↑215% (207)*	0.001
Homovanillic acid sulfate in HDL (ppb)	11.8 (3.76)	13.1 (4.46)	↑5.21% (12.5)	13.8 (6.13)	17.6 (9.96)	↑24.0% (22.7)*	0.028
Homovanillic acid glucuronate in HDL (ppb)	13.7 (3.64)	14.6 (5.33)	↑15.2% (30.8)	13.1 (3.12)	16.1 (5.13)	↑20.5% (15.8)*	0.163
HDL_2 particles (%)	38.1 (9.80)	38.0 (10.5)	↓0.04% (14.2)	37.5 (9.13)	40.0 (8.03)	↑7.16% (13.7)*	0.050
HDL_3 particles (%)	60.3 (9.01)	60.3 (9.82)	↑0.50% (8.57)	60.4 (8.62)	58.8 (8.12)	↓1.92% (7.79)*	0.146
HDL particle count (µmol/L)	31.6 (4.22)	32.2 (3.8)	↑0.30% (10.5)	31.1 (3.39)	32.0 (2.99)	↑3.25% (9.79)	0.429
Triglycerides in HDL core[5]	0.36 (0.23)	0.38 (0.29)	↑22.1% (86.9)	0.37 (0.23)	0.32 (0.18)	↓9.29% (29.6)*	0.057
HDL fluidity	4.99 (0.21)	5.02 (0.21)	↑0.73% (3.54)	4.93 (0.18)	4.98 (0.22)	↑1.12% (3.61)*	0.555

[1]: Mean (SD)
[2]: Change in percentage, compared to baseline, expressed as Mean (SD)
[3]: *P* for inter-group comparisons (mixed linear model)
[4]: Expressed as normalized values, respecting to control HDL
[5]: Measured as the HDL triglycerides vs. HDL esterified cholesterol ratio
*: $P<0.05$ after intervention, compared to baseline (mixed linear model)

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SUPPLEMENTAL TABLE IV
Changes in HDL metabolic enzymes activities and biophysical parameters after olive oil interventions

	Olive oil interventions				
	Low polyphenol content olive oil		High polyphenol content olive oil		
	Post-intervention	Change[1]	Post-intervention	Change[1]	*P* between groups[2]
HDL metabolism enzymes[3]					
CETP activity (U/L, *n* = 36)	496 ± 43.4[4]	↑1.52% (5.48)	489 ± 39.5	↓1.18% (7.78)	0.122
LCAT activity (U/L, *n* = 28)	0.63 ± 0.055	↑0.95% (7.03)	0.62 ± 0.051	↓0.68% (5.63)	0.390
HDL core-related parameters					
Triglycerides in HDL core[5]	0.38 ± 0.29	↑22.1% (86.9)	0.32 ± 0.18	↓9.29% (29.6)*	0.057
HDL biophysical characteristics					
HDL fluidity	5.02 ± 0.21	↑0.73% (3.54)	4.98 ± 0.22	↑1.12% (3.61)*	0.555

1: Change in percentage, compared to baseline, expressed as Mean (SD)
2: *P* for inter-group comparisons (mixed linear model)
3: CETP, cholesterol ester transfer protein; LCAT, lecithin:cholesterol acyltransferase
4: Mean ± SD
5: Measured as the HDL triglycerides vs. HDL esterified cholesterol ratio
*: $P<0.05$ after intervention, compared to baseline (mixed linear model)

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SUPPLEMENTAL TABLE V
Pearson's correlation values for baseline measurements of HDL-related parameters

	Age	HDL cholesterol	Cholesterol efflux capacity[1]	HDL2 (%)	HDL3 (%)	HDL particle count	HDL ApoA1	Triglycerides in HDL core[2]	HDL fluidity
Age	1	0.292	-0.232	-0.241	0.264	0.322	0.020	-0.009	-0.013
HDL cholesterol		1	0.496**	0.622***	-0.655***	0.238	-0.072	-0.420*	-0.119
Cholesterol efflux capacity[1]			1	0.589***	-0.598***	0.562 (*P*=0.072)	0.053	-0.202	-0.305
HDL2 (%)				1	-0.964***	0.435	0.063	-0.346 (*P*=0.083)	-0.135
HDL3 (%)					1	-0.428	-0.099	0.410*	0.133
HDL particle count						1	0.147	0.179	0.578
HDL ApoA1							1	-0.327	-0.099
Triglycerides in HDL core[2]								1	0.052
HDL fluidity									1

[1]: Expressed as normalized values, respecting to control HDL
[2]: Measured as the HDL triglycerides vs. HDL esterified cholesterol ratio
*: *P*<0.05. **: *P*<0.01. ***: *P*<0.001

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MANUSCRIPT II

The Mediterranean Diet improves HDL function in high cardiovascular risk individuals: a randomized controlled trial. [Submitted]

The Mediterranean Diet improves HDL function in high cardiovascular risk individuals: a randomized controlled trial

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Short title: **Hernáez – Mediterranean Diet improves HDL function**

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ABSTRACT

Background. The biological functions of high-density lipoproteins (HDLs) contribute to explain the cardioprotective role of the lipoprotein beyond quantitative HDL cholesterol levels. A few small-scale interventions with a single antioxidant have improved some HDL functions. However, to date, no long-term, large-scale, randomized, controlled trial has been conducted to assess the effects of an antioxidant-rich dietary pattern (such as a Traditional Mediterranean Diet, TMD) on HDL function in humans.

Methods and Results. We studied the effects of a TMD on the main HDL functions (reverse cholesterol transport, antioxidant defenses, and endothelial protection) in a random sub-sample of volunteers from the PREDIMED Study (*Prevención con Dieta Mediterránea*) (*N*=296) after a 1-year intervention. We compared two TMDs, one supplemented with virgin olive oil (TMD-VOO, *N*=100) and another with nuts (TMD-Nuts, *N*=100), with respect to a low-fat control diet (*N*=96). Both TMDs increased cholesterol efflux capacity relative to baseline (*P*=0.018 and *P*=0.013, respectively). The TMD-VOO intervention decreased the cholesterol ester transfer protein activity (relative to baseline, *P*=0.028), and increased HDL ability to esterify cholesterol, paraoxonase-1 arylesterase activity, and HDL vasodilatory capacity (relative to control, *P*=0.046, *P*=0.051, and *P*=0.020, respectively). The TMD-Nuts intervention decreased HDL inflammatory index (relative to control, *P*=0.042). Both TMDs induced these beneficial changes by improving HDL oxidative status and composition. The three diets increased the percentage of large HDL particles (relative to baseline, *P*<0.001).

Conclusions. The TMD, especially when supplemented with virgin olive oil, improved HDL atheroprotective functions in humans.

Clinical Trial Registration. ISRCTN35739639 (http://www.controlled-trials.com/ISRCTN35739639).

Key words: lipoproteins, diet, antioxidant, cholesterol, trials

INTRODUCTION

A growing and consistent body of evidence (from observational and randomized controlled trials) supports that the Traditional Mediterranean Diet (TMD) protects against the development of cardiovascular diseases[1]. The PREDIMED Study (*Prevención con Dieta Mediterránea*), a multi-center, parallel, randomized controlled trial, has been crucial to show this protection in primary cardiovascular disease prevention[2,3]. Several mechanisms may contribute to explain the protection of this traditional food pattern against atherosclerosis, such as its ability to preserve DNA and systemic lipids against oxidative modifications, its anti-inflammatory effects, its modulatory capacity on the metabolomic profile, and its capacity to modulate gene expression related to cardiovascular diseases[4–7]. The TMD has also improved the lipid profile related to high density lipoproteins (HDLs)[8,9]. However, it is becoming increasingly more accepted that HDL function may reflect the anti-atherogenic role of the lipoprotein better than HDL cholesterol (HDL-C) levels[10]. Several foods and nutrients present in the TMD have already improved a number of HDL functions in humans in previous trials[11–14]. To date, however, no evidence of the effects of the whole TMD on HDL properties has been reported.

The aim of the present study was to determine, in a random sub-sample of the PREDIMED Study, whether the long-term consumption of a TMD, supplemented with virgin olive oil or nuts, was able to improve the different HDL functional properties *in vivo* in humans.

METHODS

An extended description of methods is available in the online-only Data Supplement.

Study population

Our study population was a random sub-sample (*N*=296) of volunteers from the PREDIMED Study[2,15]. They were randomly assigned to one of the following three interventions: 1) a TMD supplemented with virgin olive oil (TMD-VOO); 2) a TMD supplemented with nuts (TMD-Nuts); and 3) a low-fat control diet. We assessed the effects of the TMD interventions on HDL functions before and after one year of dietary intervention. We also registered the changes in general clinical and sociodemographic variables, adherence to the TMD, a food frequency questionnaire of the previous year, and physical activity[15]. The local Research and Ethics Committee approved the protocol of the study. Volunteers gave informed consent before joining the trial.

HDL functions and quality-related properties

We determined the volunteers' biochemical profile (glucose, triglycerides, total cholesterol, HDL-C, apolipoprotein A-I –ApoA-I–, and apolipoprotein B –ApoB–) in an ABX-Pentra 400 autoanalyzer (Horiba ABX)[11]. We calculated LDL cholesterol levels with the Friedewald formula (whenever triglycerides were <300 mg/dL)[16], and the HDL-C/ApoA-I and ApoB/ApoA-I ratios. We isolated HDLs from the plasma of the participants by density gradient ultracentrifugation[11] or by polyethylene glycol precipitation of apolipoprotein B-containing lipoproteins[17]. We measured cholesterol efflux capacity in a model of human THP-1 monocyte-derived macrophages[11]. We

calculated the capacity of HDLs to esterify cholesterol (the HDL cholesterol esterification index) as follows: percentage of esterified cholesterol in HDL/mass of lecithin-cholesterol transfer protein (LCAT, measured in plasma as previously reported[18]). We assessed the activity of cholesterol ester transfer protein (CETP) in plasma and the arylesterase activity of paraoxonase-1 (PON1) in serum in commercial kits[11,18]. We measured HDL antioxidant capacity as the HDL ability to decrease the formation of conjugated dienes in LDLs in a pro-oxidant environment[16], in a random sub-selection of 90 volunteers. HDL inflammatory index (HII) was based on the oxidation of the fluorescent 2',7'-dichlorodihydrofluorescein[19]. We determined HDL vasodilatory capacity as the HDL-induced increase in nitric oxide production in human umbilical vein endothelial cells[20]. The degree of HDL oxidation *in vivo* (the HDL oxidation index) was measured as follows: equivalents of malondialdehyde in HDL[21]/cholesterol in HDL. We assessed the resistance of HDLs against oxidation (HDL oxidation lag time in a pro-oxidant environment) in a random sub-sample of 90 volunteers[16]. We assessed HDL composition (total cholesterol, free cholesterol, esterified cholesterol, triglycerides, phospholipids, and apolipoproteins A-I, A-II –ApoA-II– and C-III –ApoC-III–) in isolated HDL samples in an ABX-Pentra 400 autoanalyzer (Horiba ABX)[11]. From these data, we calculated the amount of each apolipoprotein per mg of cholesterol, and the following ratios: HDL triglycerides/HDL esterified cholesterol ("triglycerides in HDL core"), and HDL phospholipids/HDL free cholesterol ("phospholipids in HDL surface")[11,18]. Finally, we determined HDL size distribution by the LipoPrint technology (Quantimetrix)[11].

Sample size

Main sample. A sample size of 96 participants per group allowed ≥ 80% power to detect a significant difference of 0.025 points in normalized cholesterol efflux capacity values between pre-and post-intervention values, and of 0.035 points among the three interventions, considering a 2-sided type I error of 0.05, a loss rate of 5%, and the standard deviation of the differences in cholesterol efflux capacity (SD=0.084) after an analogous dietary intervention[11].

Sub-group for HDL antioxidant capacity and HDL resistance against oxidation. As we had no previous data available on these properties, we calculated this sample size considering the variability of the differences in the activity of the main HDL antioxidant enzyme (PON1) after an analogous dietary intervention[22]. A sample size of 28 participants per group allowed ≥ 80% power to detect a significant difference of 0.057 points in the previous normalized capacity between pre-and post-intervention, and of 0.08 points among the three interventions, considering a 2-sided type I error of 0.05, a loss rate of 5%, and the standard deviation of the differences in this property (SD=0.104)[22].

Statistical analyses

We checked the distribution of continuous variables in normality plots and by the Shapiro-Wilk test, and log-transformed the non-normally distributed ones. We looked for differences between our subsample and the whole PREDIMED population by a T-test, and for differences in the baseline values among the three intervention groups by a one-way ANOVA.

Differences between pre- and post-intervention values in every intervention were analyzed by a paired T-test. We checked the effects of the three interventions on the

changes in the variables of interest in a multivariate regression analysis (using two dummy variables, one for each intervention group), in which we included as co-variables: sex, age, participant's center of origin (*k*-1 dummy variables), baseline value of the variable, and changes in the use of lipid-lowering drugs.
Any *P*-value <0.05 was considered significant. Analyses were performed with R Software, version 3.0.2 (*R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria*).

RESULTS

Participants

Study design is available in **Supplemental Figure 1**. We found no significant differences among groups in the baseline characteristics of the volunteers (**Table 1**). When comparing the baseline characteristics between our subsample and the whole population of the PREDIMED trial (**Supplemental Table 1**), our volunteers were on average 1.1 year older and there were 6.8% more males. However, no differences in their main clinical characteristics were observed. Energy expenditure in leisure time physical activity and caloric intake did not change throughout the study, and volunteers' compliance with the interventions was correct according to biomarkers of compliance, TMD adherence scores, and the 1-year food frequency questionnaire. Increased adherence to TMD after the TMD-VOO intervention was due to: 1) increases in the consumption of virgin olive oil, legumes, and fish; 2) substitution of red/processed meat for white meat; and 3) decreases in the consumption of precooked foods and industrial sweets (*P*<0.05). Increased adherence to TMD after the TMD-Nuts intervention was due to: 1) increases in the consumption of nuts, virgin olive oil (less than in the TMD-VOO intervention), fruits, vegetables, and oily fish; and 2) decreases in the consumption of red and processed meat, precooked meals, and industrial sweets (*P*<0.05). Finally, adherence to a low-fat diet was observed as a decrease in total fat intake (particularly saturated fats), due to decreases in the consumption of high-fat dairy products, red and processed meat, precooked meals, and industrial sweets (*P*<0.05) (**Supplemental Tables 2-3**).
Regarding the biochemical profile of the participants (**Supplemental Table 4-5**), we observed an expected decline in total cholesterol levels after the low-fat control diet (*P*=0.039 and *P*=0.023, relative to baseline and the TMD-VOO intervention, respectively). This was mainly due to a decrease in LDL cholesterol levels (*P*=0.019 and *P*=0.014, relative to baseline and the TMD-VOO intervention, respectively). Regarding the HDL-related biochemical profile, we did not observe significant changes in apolipoprotein levels. However, there was a significant decrease in the HDL cholesterol/ApoA-I ratio values in plasma in the TMD interventions relative to baseline (*P*=0.031 and *P*<0.001 in the TMD-VOO and the TMD-Nuts interventions, respectively). We did not find inter-treatment changes in these parameters.

Cholesterol efflux capacity

The cholesterol efflux capacity of the volunteers augmented only after the two TMD interventions relative to baseline (*P*=0.018, and *P*=0.013, for TMD-VOO and TMD-Nuts respectively) (**Figures 1A-1B**). We did not observe significant inter-intervention changes in the multivariate models.

HDL role in other steps of reverse cholesterol transport

The ability of HDLs to esterify cholesterol (the HDL cholesterol esterification index) increased significantly after the TMD-VOO intervention relative to baseline (P=0.007) and the low-fat control intervention (P=0.046) (**Figures 1C-1D**).

Regarding CETP activity (**Figure 1E-1F**), it decreased significantly only after the TMD-VOO intervention relative to baseline (P=0.008). We observed no significant inter-treatment changes among interventions.

HDL antioxidant capacities

PON1 arylesterase activity did not change significantly relatie to baseline after any intervention. However, when compared with the low-fat diet, it tended to increase after the TMD-VOO intervention (P=0.051).

The HDL capacity to directly counteract LDL oxidation also rose after the TMD-VOO intervention, relative to baseline values (P=0.004) (**Figures 2C-2D**), and tended to increase relative to the control intervention (P=0.096).

With respect to other antioxidant/anti-inflammatory properties of HDL, the HII increased significantly, when compared with the pre-intervention values, after the low-fat control diet (P=0.025), whilst it did not rise after the TMD interventions (**Figures 2E-2F**). This led to a significant decrease in HII values after the TMD-Nuts intervention relative to the control diet (P=0.042).

HDL vasodilatory capacity

HDL ability to promote the production of nitric oxide in endothelial cells (the HDL vasodilatory capacity) did not change significantly relative to baseline after any intervention. However, it increased significantly after the TMD-VOO intervention relative to the low-fat diet (P=0.020) (**Figures 3A-3B**).

HDL oxidation

The degree of HDL oxidative modifications *in vivo* (the HDL oxidation index) decreased after the TMD-VOO and the low-fat control diet, relative to their baseline values (P=0.028 and P=0.011, respectively) (**Figures 4A-4B**).

HDL dynamic resistance against oxidation (HDL oxidation lag time) increased after the TMD-VOO intervention, relative to baseline values (P=0.006) (**Figures 4C-4D**). We observed no inter-treatment differences in this property.

HDL composition

When compared with the low-fat diet, the content of triglycerides in HDL core decreased significantly after the TMD-Nuts intervention (P=0.045), and tended to decrease after the TMD-VOO intervention (P=0.062) (**Figures 4E-4F**).

Regarding phospholipids (**Figures 4G-4H**), there was a significant increase in the content of phospholipids in the HDL surface after the TMD-VOO intervention, relative to baseline (P=0.003), and also in comparison with the low-fat control diet (P=0.048).

We found no significant changes in the levels of apolipoproteins A-I, A-II and C-III in HDL in any of the interventions.

HDL size distribution

The three interventions increased the levels of large HDLs relative to their baseline values (P<0.001 for the three interventions) (**Figures 4I-4J**). We observed no differences among the three of them in the multivariate regression model.

All values of the comparisons between post- and pre-intervention values, and between the changes in the TMD interventions relative to the low-fat diet, are available in **Supplemental Tables 6** and **7**, respectively.

DISCUSSION

Our results show that one year of intervention with a TMD improves the *in vivo* functions of HDL (cholesterol efflux capacity, cholesterol metabolism, antioxidant/anti-inflammatory properties, and vasodilatory capacity) in individuals at high cardiovascular risk. To the best of our knowledge, this is the first time that the effect of a real-life healthy lifestyle on the whole set of HDL functional properties has been studied. Moreover, our randomized controlled trial has involved, to date, the largest sample size (N=296) and the longest duration of the intervention (1.13±0.21 years, mean±SD) in an HDL functionality study in humans.

Cholesterol efflux capacity is the main biological function of HDL: HDLs extract excess cholesterol from peripheral cells to carry it to the liver for metabolization or excretion[23]. Low values of cholesterol efflux capacity are related to higher cardiovascular risk[17] and greater incidence of coronary events[24]. In the present trial, both TMD interventions increased the cholesterol efflux capacity of the HDLs of the individuals relative to baseline. An improved HDL oxidative status[25], lipid composition[12], or HDL-related gene expression[26] may contribute to explain these changes. Similar increments in cholesterol efflux capacity have been observed in clinical trials with real-life doses of virgin olive oil[11], supplements of polyphenols[27,28] or polyunsaturated fatty acids[14].

After cholesterol efflux, HDLs esterify and internalize the cholesterol they have picked up, in order to continue with the reverse cholesterol transport[29]. This property can be measured by LCAT activity[29] or the HDL cholesterol esterification index. In our study, the TMD-VOO intervention significantly increased the HDL cholesterol esterification index. As LCAT is very sensitive to oxidative attacks[30], dietary antioxidants in the TMD may contribute to maintain LCAT non-oxidized and functional. A fruit and vegetable-rich diet[31] and a supplement of the antioxidant lycopene[13] have induced similar effects in previous studies.

CETP is a key enzyme in the cholesterol transfer from HDLs to triglyceride-poor lipoproteins (and subsequently to the liver) whose activity is frequently exacerbated in high cardiovascular risk states[32]. However, the mere blockade of its function with pharmacological agents has resulted in some controversial results[32]. A more physiological modulation of the enzyme activity could, therefore, provide greater beneficial effects in cardiovascular prevention. A 1-year TMD-VOO intervention decreased CETP activity in our study, as has occurred after other antioxidant-rich interventions (such as a lycopene-rich diet[13] and an anthocyanin supplement[27]).

The second most relevant atheroprotective role of HDL is its antioxidant capacity. HDLs are able to counteract the oxidation of LDL, one of the main biochemical triggers of the development of the atherosclerotic plaque[10]. HDLs develop this protection through the coordinated action of several active proteins, among which PON1 is the

central enzyme[33]. Moreover, low PON1 arylesterase activity has been described as an independent predictor of cardiac events[34]. In the present trial, the TMD-VOO intervention increased the overall antioxidant protection of HDLs on LDLs, and also incremented PON1 arylesterase activity. The different bioactive compounds in the TMD (olive oil phenolic compounds[18], other polyphenols[28], carotenoids such as lycopene[13] and omega-3 polyunsaturated fatty acids[14]) may act synergistically and could: 1) induce local antioxidant functions; 2) preserve other dietary antioxidants in HDLs (such as vitamin E); and 3) protect PON1 against oxidative modifications and/or potentiate its function[35,36].

HDLs can also counteract the pro-inflammatory effects of some pro-oxidant substances, such as modified LDLs, due to the combined action of their active proteins[19,33]. The HII is an index that captures an indirect measurement of these properties. It was abnormally elevated in subjects with cardiovascular pathologies and could discriminate patients with acute coronary syndrome from those with stable angina or healthy controls[19]. In the present study, the TMD-Nuts intervention decreased HII values. Whilst statin therapy[19] and smoking cessation[21] have been shown to diminish HII, the TMD is the first reported diet-based intervention able to have such an effect.

Besides their classical functions, HDLs also have vasoprotective properties[37], which are lost in high cardiovascular risk states[38]. In the present trial, the TMD-VOO intervention increased the capacity of HDLs to induce the production of nitric oxide from endothelial cells, a vasodilatory trait (which can also be considered as vasoprotective). The improvement in HDL oxidative status and cholesterol efflux capacity after the TMD-VOO intervention may be responsible for this beneficial effect[37]. Comparable effects have been observed after massive weight losses due to gastric surgery[20], supplementation with omega-3 polyunsaturated fatty acids[14], and some pharmacological agents such as extended-release niacin[38]. However, TMD is the first real-life dietary modification that has been shown to be able to promote HDL vasoprotective properties.

The main mechanism by which the TMD could increase HDL functionality is through an improvement in the oxidative status of the lipoprotein. Oxidized HDLs are less functional[25], due to oxidation of their lipids and active proteins (**Figure 5**). In our trial, HDLs after the TMD-VOO intervention, and also after the low-fat diet, were less oxidized. However, the HDLs were more resistant against dynamic oxidation only after the TMD-VOO intervention. We observed no significant changes after the TMD-Nuts intervention probably due to the incorporation of polyunsaturated fatty acids to the HDL structure, which may have increased the susceptibility of these HDLs to become oxidized. Both the TMD-VOO and the low-fat intervention were rich in fruit and vegetables[15]. Some antioxidants, such as carotenoids, are highly present in both and are able to bind to HDLs and protect them locally against oxidative modifications[13]. This better oxidative status could be partially responsible for some of the benefits observed after the low-fat diet (e.g. better HDL size distribution). However, antioxidant protection could be greater in the TMD due to its particular antioxidants, such as olive oil phenolic compounds[11,36]. Moreover, these antioxidants are present in higher doses in the TMD and, therefore, could explain a number of benefits observed exclusively in the TMD intervention, especially when supplemented with virgin olive oil.

Another mechanism that could explain the benefits of the TMD on HDL function could be a better HDL composition profile. The TMD decreased the content of triglycerides in

HDL core, especially in individuals with greater cardiovascular risk at baseline. Low-fat diets are known to induce transient hypertriglyceridemias[39], which could lead to an indirect hypertriglyceridemic state in HDLs. Nevertheless, HDL hypertriglyceridemias appeared to be reversed by the TMD in our trial. In the specific case of the TMD-VOO intervention, the decreased CETP activity could help to explain the reduced triglyceride content in HDLs. In the particular case of the TMD-Nuts intervention, the nut consumption may have decreased systemic triglyceride levels[40] and, indirectly, reduced HDL triglyceride content. These decrements in the HDL triglyceride content after the TMD interventions are associated with a more stable conformation of ApoA-I in the HDLs[41] and could partially explain the improved HDL role in cholesterol efflux capacity or antioxidant functions (**Figure 5**).

Continuing with HDL composition, after the TMD-VOO intervention, HDLs had more phospholipids in their surface. The improved HDL capacity to esterify cholesterol may explain this change: when the free cholesterol content in the surface decreases, the relative content of phospholipids increases. This could be linked to a greater HDL fluidity, which has already been observed after the consumption of virgin olive oil[11,36] and could contribute to explaining an increased HDL functionality (**Figure 5**).

Some parameters related to the structure of HDL, such as the HDL-C/ApoA-I ratio in plasma, also improved after the TMD interventions. High values of the HDL-C/ApoA-I ratio have been associated with increased cardiovascular, cancer, and all-cause mortality[42]. The ratio could be considered an inverse marker of ApoA-I-rich HDLs in circulation: the lower the ratio, the higher the content of ApoA-I in HDLs. This mechanism has been hypothesized to promote the function of ApoA-I and, therefore, to promote some of the HDL functions[36] (**Figure 5**).

Finally, size is also a major but controversial trait of lipoproteins. Low concentrations of large HDLs and high concentrations of small HDLs are present in high cardiovascular risk individuals[43]. The increased consumption of bioactive compounds of the diet could help to explain the increase in the number of large HDLs in the TMD interventions[11], as previously described for TMD[44]. A better maturation (due to the modulation of the esterification and the CETP capacities of HDLs) may be responsible for this benefit (**Figure 5**).

The present study has several strengths. The first is its parallel design, which allowed us to determine the long-term effects of the TMD in a 1-year follow-up. The second is its sample size (*N*=296), large for a trial of these characteristics. The third is that we have checked almost every HDL functional property whose methodology has already been standardized and published elsewhere. Nevertheless, it also has limitations. The participants of the trial were elderly people at high cardiovascular risk which hinders the extrapolation of our results to the general population. As expected, we found only slight differences since: 1) the trial is based on modest real-life modifications of the diet; and 2) the control diet is already a well-known healthy dietary pattern. The use of cellular models, although it is a non-invasive alternative to test relevant physiological functions, may not have demonstrated the effect of contra-regulatory mechanisms, which can modify the final *in vivo* outcome in humans. Due to availability issues, in the samples from two of the study centers (*N*=67) we could not perform the analyses related to HDL main enzymatic proteins (CETP and PON1 activities, and LCAT mass). In the samples from one study center (*N*=37) we were also unable to assess HDL size. Finally, we could not perform HDL vasodilatory capacity analyses in 16% of the individuals in all the interventions due to technical issues in cell cultures.

In conclusion, we report for the first time the effects of an integral dietary intervention on HDL functionality. To date, this is the first randomized controlled trial to study simultaneously the four most important HDL functional traits (cholesterol efflux capacity, HDL cholesterol metabolism, HDL antioxidant/anti-inflammatory properties, and vasoprotective effects), in a larger sample size than the previous studies (*N*=296) and with the longest duration of the intervention. The TMD intervention, especially when supplemented with virgin olive oil, was able to increase the four main HDL functions. The TMD could have induced these benefits through improvements in HDL oxidative status, composition, and size (**Figure 5**). Our data provide a plausible additional mechanism to explain part of the benefits of the Mediterranean Diet and support previous evidence about its cardioprotective role.

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DISCLOSURES

None.

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573 Mediterranean diet supplemented with nuts reduces waist circumference and
574 shifts lipoprotein subfractions to a less atherogenic pattern in subjects at high
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576

577 **TABLES**

578

579 **TABLE 1**

580 Baseline characteristics of the volunteers in the three intervention groups of the study.

VARIABLES	TMD-VOO *N*=100	TMD-Nuts *N*=100	Low-fat diet *N*=96	*P*-value
Age (years)	66.3 (5.78)[a]	66.4 (6.93)	65.0 (6.49)	0.247
Sex (% male)	56.0%	47.0%	50.0%	0.432
Body Mass Index (kg/m^2)	30.1 (3.85)	29.0 (3.76)	29.9 (3.87)	0.087
Waist Circumference (cm)	100 (10.7)	101 (10.3)	102 (11.2)	0.469
Leisure-time physical activity (MET·min/day)	176 [69.3;284][b]	175 [68.3;408]	174 [41.3;362]	0.870
Smoking status (% of smokers)	15.0%	10.0%	12.5%	0.565
Type 2 diabetes (% of diabetic patients)	48.0%	52.0%	46.9%	0.751
Hypertension (% of hypertensive patients)	78.0%	78.0%	80.2%	0.910
Dyslipidemia (% of dyslipidemic patients)	79.0%	70.0%	83.3%	0.074
Glucose[a] (mg/dL)	110 [93.8;137]	108 [92.5;140]	108 [94.0;131]	0.982
Triglycerides[a] (mg/dL)	207 (36.8)	196 (36.2)	204 (36.8)	0.112
Total cholesterol (mg/dL)	110 [93.2;158]	100 [72.5;144]	113 [83.0;140]	0.160
HDL cholesterol (mg/dL)	50.2 (12.3)	49.8 (10.3)	49.1 (11.6)	0.777
LDL cholesterol (mg/dL)	130 (28.5)	123 (30.4)	130 (31.7)	0.181
Apolipoprotein A-I (mg/dL)	138 (23.5)	134 (20.2)	131 (19.4)	0.232
Apolipoprotein B (mg/dL)	106 (21.6)	98.2 (18.9)	103 (21.8)	0.062

581

582 [a]: Mean (SD). [b]: Median [1st-3rd quartile].

583 *MET*: metabolic equivalent of task. *TMD-VOO*: Traditional Mediterranean Diet

584 supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet

585 supplemented with mixed nuts.

586 **FIGURES**

587

588 **FIGURE 1**

589 Post- vs. pre-intervention changes (A, C, E) and inter-intervention changes in a
590 multivariate linear regression model (B, D, F) in HDL variables related to reverse
591 cholesterol transport: cholesterol efflux capacity (A-B), HDL cholesterol esterification
592 index (C-D), and activity of cholesterol ester transfer protein (CETP, E-F). Results are
593 shown as means with 95% CI. TMD-VOO: Traditional Mediterranean Diet
594 supplemented with virgin olive oil; TMD-Nuts: Traditional Mediterranean Diet
595 supplemented with nuts. *: *P*<0.05.

596

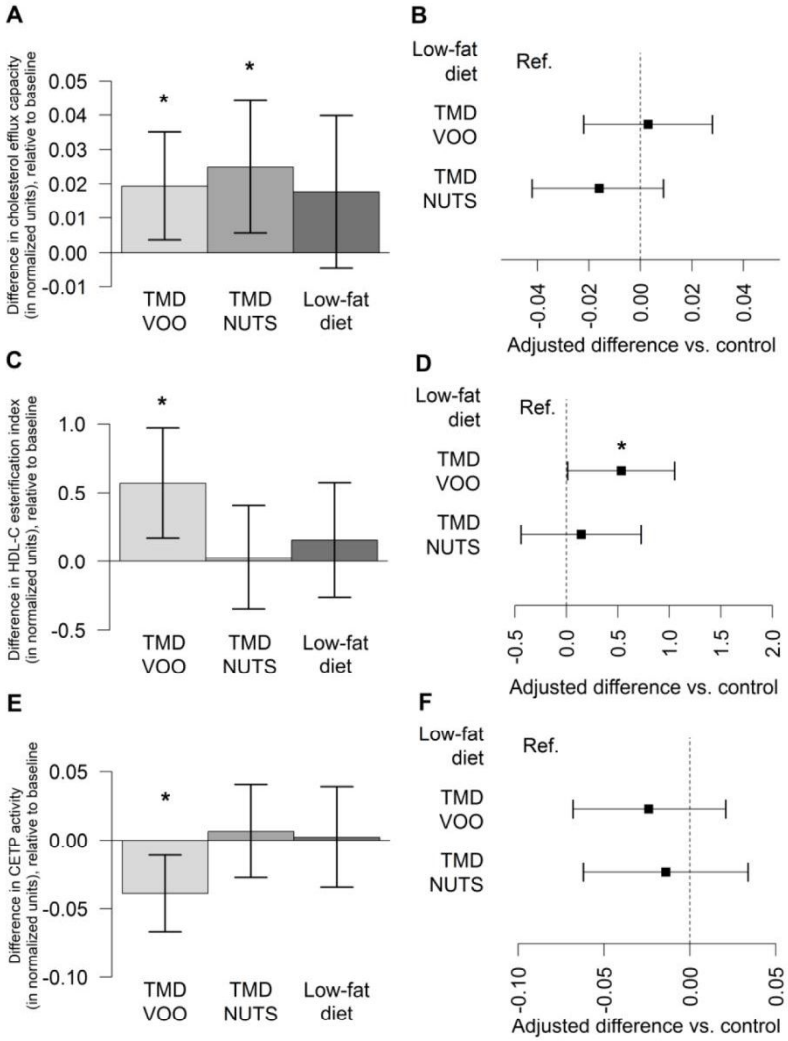
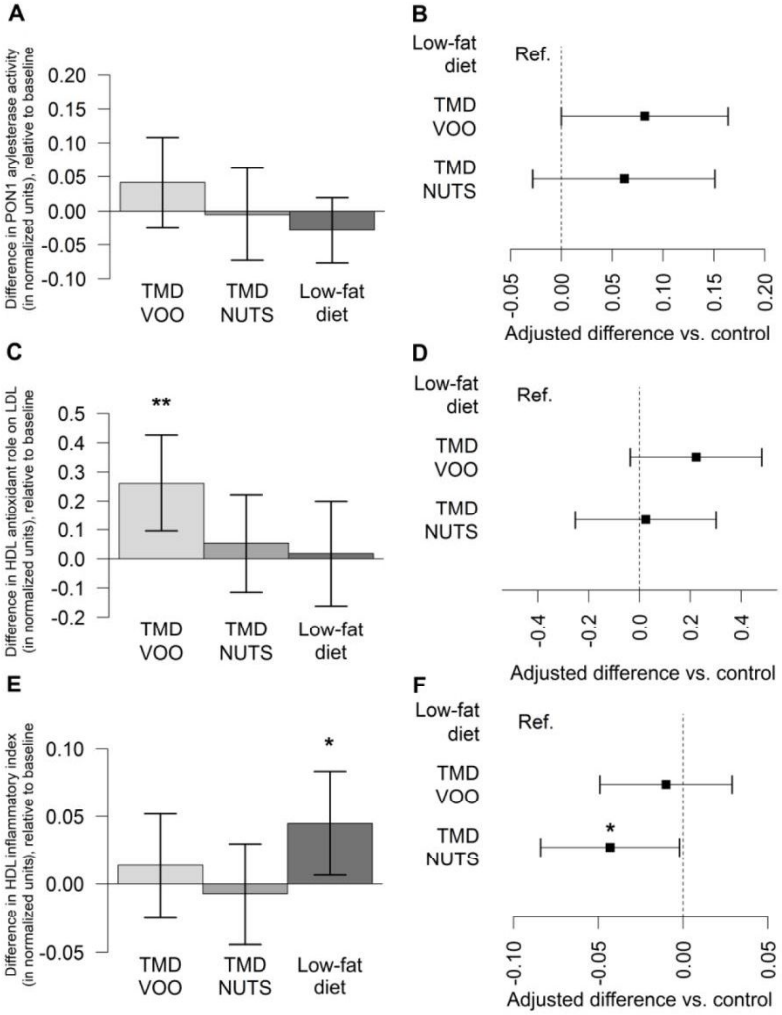


FIGURE 2

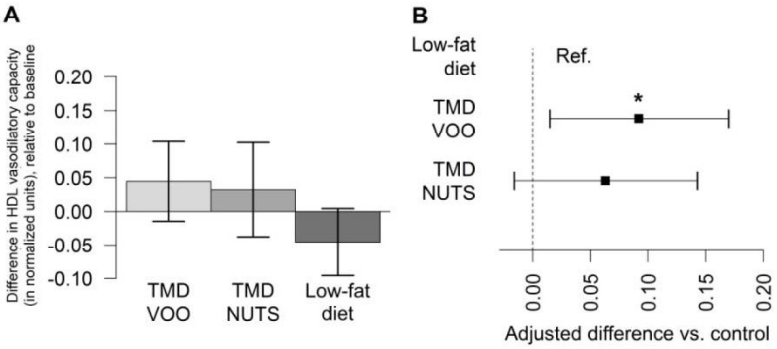
Post- vs. pre-intervention changes (A, C, E) and inter-intervention changes in a multivariate linear regression model (B, D, F) in variables related to HDL antioxidant capacity: paraoxonase-1 arylesterase activity (A-B), HDL capacity to increase LDL lag time (C-D), and HDL inflammatory index (E-F). Results are shown as means with 95% CI. TMD-VOO: Traditional Mediterranean Diet supplemented with virgin olive oil; TMD-Nuts: Traditional Mediterranean Diet supplemented with nuts.

*: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.



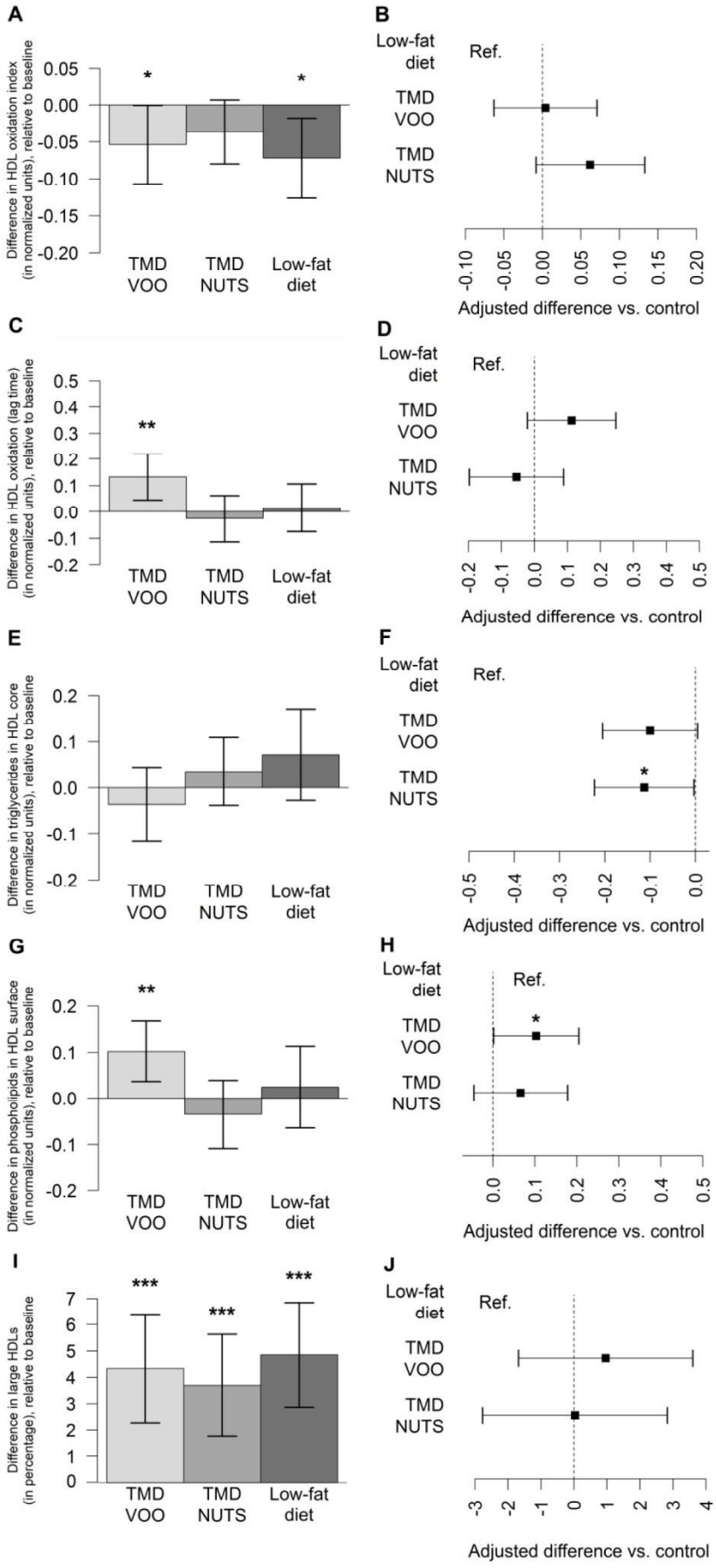
606 **FIGURE 3**

607 HDL vasodilatory capacity: post- vs. pre-intervention changes (A) and inter-intervention
608 changes in a multivariate linear regression model (B). Results are shown as means
609 with 95% CI. TMD-VOO: Traditional Mediterranean Diet supplemented with virgin olive
610 oil; TMD-Nuts: Traditional Mediterranean Diet supplemented with nuts.
611 *: *P*<0.05.
612



613 **FIGURE 4**

614



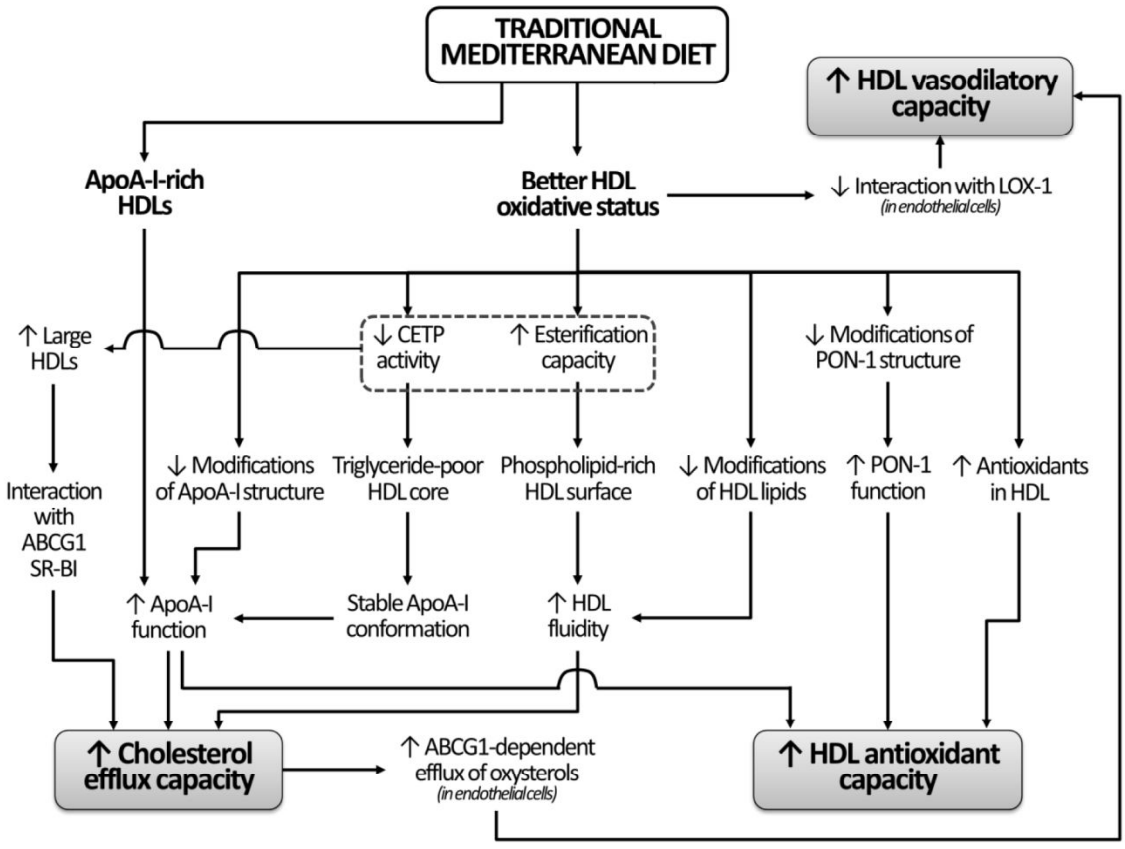
615 **FIGURE 4 – LEGEND**

616 Post- vs. pre-intervention changes (A, C, E, G, I) and inter-intervention changes in a
617 multivariate linear regression model (B, D, F, H, J) in variables related to HDL
618 oxidation, composition and size: HDL oxidation index (A-B), HDL resistance against
619 oxidation (HDL lag time) (C-D), quantity of triglycerides in HDL core (E-F), quantity of
620 phospholipids in HDL surface (G-H), and percentage of large HDLs (I-J). Results are
621 shown as means with 95% CI. TMD-VOO: Traditional Mediterranean Diet
622 supplemented with virgin olive oil; TMD-Nuts: Traditional Mediterranean Diet
623 supplemented with nuts. *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

FIGURE 5

Hypothetical mechanisms for the benefits of the traditional Mediterranean Diet on HDL function. The matrix of antioxidants and dietary bioactive compounds induce a better HDL oxidative status that is responsible for a lower level of oxidative modification of HDL proteins and lipids. Non-oxidized, ApoA-I-rich HDL proteins remain functional and are able to work normally, allowing: a full maturation of the lipoprotein, a complete interaction with peripheral cells to perform cholesterol efflux, and an adequate antioxidant/anti-inflammatory capacity. When HDLs are less oxidized and perform more efficiently some kinds of cholesterol efflux in endothelial cells, they also increase their protective capacities on the endothelium.

ApoA-I: apolipoprotein A-I; *ABCG1*: ATP-binding cassette G1; *CETP*: cholesterol ester transfer protein; *LOX-1*: lectin-like oxidized LDL receptor-1; *PON1*: paraoxonase-1; *SR-BI*: scavenger receptor B-I.



SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

STUDY DESIGN

Our study population is a subsample of the PREDIMED Study (*Prevención con Dieta Mediterránea*). The PREDIMED Study was a randomized, controlled, parallel, large-scale (*N*=7447), multicenter trial to study the long-term effects of a traditional Mediterranean Diet (TMD) in the primary prevention of cardiovascular events in a population at high cardiovascular risk[1]. Volunteers were randomly assigned to one of the following interventions: 1) TMD supplemented with virgin olive oil (TMD-VOO); 2) TMD supplemented with nuts (TMD-Nuts); and 3) a low-fat control diet, following the indications of the American Heart Association[2]. Characteristics of the participants and additional details of the study have been previously published[1]. The protocol of the study was approved by local institutional ethic committees, and all participants provided informed content before joining the trial[1]. The protocol of the PREDIMED Study has been registered with the International Standard Randomized Controlled Trial Number ISRCTN35739639 (www.controlled-trials.com).

In the present project, we assessed the effects of a TMD on the functionality of high-density lipoproteins (HDLs) in a random subsample of 296 volunteers from the PREDIMED Study, before and after one year of intervention with a TMD (*N*=100 in the TMD-VOO group, *N*=100 in the TMD-N group, and *N*=96 in the LFCD one). We obtained blood samples from fasting participants before and after the intervention, and stored them at -80ºC until the present project started. The samples suffered no thaw-freeze cycles before our experiments. The pre- and post-intervention samples of every volunteer were analyzed in the same experimental run in all biochemical and biocellular experiments.

CLINICAL EXPLORATION, DIETARY ADHERENCE, AND PHYSICAL ACTIVITY

We gathered several types of general clinical data before and after the intervention. Data were collected on sex, age, height, weight, waist circumference, systolic and diastolic blood pressure. We also performed a general clinical questionnaire (assessing health conditions, lifestyle, sociodemographic characteristics, and clinical history), a 14-item questionnaire of adherence to the TMD[3] plus two biomarkers of compliance for each of the interventions and a food frequency questionnaire of the previous year, and a Minnesota Leisure-Time Physical Activity Questionnaire, to determine the clinical features, dietary adherence, and levels of physical activity of the volunteers, respectively[1].

DIETARY INTERVENTION

From the data about the baseline adherence to a TMD, and according to the diet group they had been randomly assigned to, volunteers received personalized dietary advice at the beginning of the study. Participants assigned to the low-fat control diet were instructed to reduce the consumption of all types of fat (oils, nuts, butter, meat, and fatty fish), following the guidelines of the American Heart Association[2]. Those assigned to the TMD interventions were taught several strategies to increase their adherence to the Mediterranean interventions: 1) to use olive oil for cooking all meals; 2) to increase their consumption of vegetables, fruit, nuts, and fish; 3) to substitute red or processed meat for white meat (chicken, turkey or rabbit); 4) to prepare dishes based on the homemade mix of stir-fried tomato, garlic, onion, and aromatic herbs (the traditional

"sofrito"); and 5) when the volunteer consumed alcohol, to moderately drink red wine (1 small glass/meal)[4]. In order to increase compliance with the interventions, participants assigned to the TMD interventions received no suggestions to reduce caloric intake, and were given 1 L/week of virgin olive oil (when assigned to the TMD-VOO group) or 210 g/week of mixed nuts (when assigned to the TMD-Nuts group). We provided additional virgin olive oil and nuts to the participants to reinforce compliance and account for family needs. After the intervention assignment, participants attended group sessions in which they received: 1) a detailed description of recommended foods (typical Mediterranean foods for TMDs, or recommendations of the American Heart Association for the low-fat diet); 2) seasonal shopping lists; 3) meal plans adapted to each dietary intervention; and 4) sets of adapted recipes. Moreover, participants could contact their reference dietitian of the study whenever they needed to.

SAMPLE COLLECTION

We collected K3-EDTA plasma and serum at baseline and after the intervention. We also prepared two specific laboratory specimens from plasma for the present project. On the one hand, we obtained apolipoprotein-B depleted plasma (ABDP). ABDP is a modified plasma specimen in which all lipoproteins but HDLs are removed. To prepare it, we incubated plasma with 20% polyethylene glycol 8000 (Sigma) in a 200 mM glycine buffer pH 7.4 (Sigma), at 4ºC for 20 minutes. We centrifuged the mixture (10,000 g, 4ºC, 15 minutes), picked up the supernatants and finally stored them until use at -80ºC[5]. On the other hand, we obtained isolated HDLs by means of a density gradient ultracentrifugation, and kept them at -80ºC in 2.5% sucrose until use[6].

BIOCHEMICAL PROFILE

We performed all systemic determinations in plasma in an ABX Pentra-400 autoanalyzer (Horiba ABX). We measured glucose, triglycerides, and total cholesterol by enzymatic methods (ABX Pentra Glucose HK CP, ABX Pentra Triglycerides CP, and ABX Pentra Cholesterol CP, all from Horiba ABX). Regarding the HDL-related biochemical profile, we determined HDL cholesterol by the Accelerator Selective Detergent method (ABX Pentra HDL Direct CP, Horiba ABX), and apolipoprotein A-I (ApoA-1) and B (ApoB) by immunoturbidimetry (ABX Pentra ApoA1 and ABX Pentra ApoB, Horiba ABX). The inter-assay coefficients of variation of the prior determinations were: 1.91% for glucose, 4.07% for triglycerides, 1.24% for total cholesterol, 5.17% for creatinine, 1.79% for HDL cholesterol, 1.68% for apolipoprotein A-I, and 1.59 for apolipoprotein B.

We also calculated LDL cholesterol levels according to the Friedewald formula, whenever triglycerides were <300 mg/dL: total cholesterol - HDL cholesterol - (triglycerides/5). We also computed the following ratios: HDL cholesterol/ApoA-I (as an indirect measurement of the quantity of ApoA-I in HDLs) and ApoB/ApoA-I.

CHOLESTEROL EFFLUX CAPACITY

We grew human THP-1 monocytes in RPMI 1640 medium (supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin) and refreshed cells every 72-96h. We differentiated monocytes into macrophages incubating them with phorbol-myristate-acetate (Sigma) 200 nM, for 96h. We then incubated THP-1 monocyte-derived macrophages with 0.2 µCi/mL of [1,2-^{3}H(N)]-cholesterol (Perkin-Elmer) for 24h, washed them, incubated them for 24h in

fresh RPMI 1640 medium (in this case, supplemented with 1% bovine serum albumin – BSA–, Sigma), washed them again, and finally cultured them for 16h in fresh RPMI 1640 medium + 1% BSA in the presence of 5% ABDP of the volunteers, or without it (negative control). We collected the culture supernatants and extracted the cell culture lipids by 60-minute incubation with ice-cold isopropanol. We measured radioactivity in both supernatants and fractions of cell lipids in a beta scintillation counter Tri-Carb 2800TR (Perkin-Elmer). Finally, we calculated the cholesterol efflux capacity for each well as follows: counts per minute in supernatant*100/counts per minute in supernatant + cells. We subtracted the cholesterol efflux capacity value in the absence ABDP (blank) from all efflux values of the samples of the experiment[6].

We ran samples in duplicate. No intra-repetition coefficient of variation over 15% was allowed. To minimize inter-assay variation, we included a pool of ABDP from 20 healthy volunteers in each experiment, and then divided all cholesterol efflux values of the samples by the efflux value of the pool, to obtain normalized ratios. The global inter-assay coefficient of variation (*N*=10) was 9.61%.

HDL ROLE IN OTHER STEPS OF REVERSE CHOLESTEROL TRANSPORT

HDL cholesterol esterification index. This index measures the ability of HDLs to esterify and internalize the effluxed cholesterol. First of all, we calculated the percentage of esterified cholesterol in isolated HDLs, relative to the total HDL lipid content (see below, "HDL composition" section). To obtain the index, we divided this percentage of esterified cholesterol by the mass of the main enzyme responsible for the esterification process, the lecithin cholesterol acytransferase (LCAT). LCAT mass was determined by the *Lecithin Cholesterol Acyltransferase ELISA Kit* (Sekisui Diagnostics), in serum samples in an Infinite M200 reader (Tecan Ltd)[7]. To control inter-assay variation, we included a serum pool of 20 healthy donors in every experiment. The inter-assay coefficient of variation of the determination of the LCAT mass was 8.15%.

Activity of cholesterol ester transfer protein (CETP). We measured this activity by the *CETP Assay Kit* (MBL International, Woburn, MA, USA), in serum samples in an Infinite M200 reader (Tecan Ltd)[6]. To minimize inter-assay variation, we included a pool of serum from 20 healthy volunteers in each experiment, and then divided CETP activity values of the samples by the CETP activity of the pool, to obtain normalized, ratios without units. The inter-assay coefficient of variation of the CETP determination was 9.97%.

HDL ANTIOXIDANT/ANTI-INFLAMMATORY PROPERTIES

Paraoxonase-1 arylesterase activity. We determined this activity with the *Paraoxonase/Arylesterase Assay Kit* (ZeptoMetrix), in serum, in an Infinite M200 reader (Tecan Ltd)[7]. To minimize inter-assay variation, we included a pool of serum from 20 healthy volunteers in each experiment. We divided the values of arylesterase activity of the samples by the value of arylesterase activity of the pool, to obtain normalized ratios without units. The inter-assay coefficient of variation was 6.17%.

Direct HDL antioxidant capacity on LDL. Participants' HDLs were incubated with a pool of LDLs, isolated from the plasma of 20 healthy volunteers by density gradient ultracentrifugation[8], in the presence of an oxidizing agent ($CuSO_4$). In this context, we determined the ability of HDLs to inhibit the accumulation of Cu^{2+}-induced conjugated dienes in the LDLs. We first dialyzed lipoproteins against PBS in *PD-10 Desalting*

Columns (GE Healthcare). We simulated a 1:3 relationship between HDL-cholesterol and LDL-cholesterol, the average found in individuals with medium-high cardiovascular risk[9]. We incubated HDLs (final concentration: 3 mg cholesterol/dL) with LDLs (final concentration: 9 mg cholesterol/dL) plus $CuSO_4$ (final concentration: 5 μM) in 96-well transparent plates, at 37ºC, in an Infinite M200 reader (Tecan Ltd). We also oxidized HDLs alone (to determine HDL oxidation) and LDLs alone (to determine the oxidation of the reference LDLs) in the same plate. We measured absorbance at 234 nm each 3 minutes for 6 hours to assess the formation of conjugated dienes.

The consecutive measurements of absorbance configured the different curves of lipoprotein oxidation. We first subtracted the values of absorbance in HDL alone from the values of absorbance of HDL plus LDL, and thus we obtained the oxidation curves of the HDL-protected LDLs. In these calculated curves, and in the oxidation curves of the reference LDLs, we calculated LDL lag time (the time when maximal oxidation started)[8]. Finally, we compared the lag times of the HDL-protected LDLs with their respecting values in the reference LDLs: (kinetic parameter in HDL-protected LDL – kinetic parameter in reference LDL)/kinetic parameter in reference LDL*100. HDLs were considered antioxidant when they increased LDL lag time values.

We analyzed samples in duplicate, and allowed no coefficient of variation >15%. We included a control HDL (isolated from the plasma of 20 healthy volunteers) in each experiment to control inter-assay variation. We then divided all values of the samples by the values of the pool, to obtain normalized, unitless ratios. After this transformation, the ratios were directly associated with a higher HDL antioxidant capacity: the greater the adjusted ratio, the higher the HDL antioxidant capacity. The inter-assay coefficient of variation for this parameter was 11.2%.

HDL inflammatory index. This index consists of the capacity of HDLs to inhibit the oxidation of 2'-7'-dichlorodihydrofluorescein diacetate (H2DCF-DA, Life Technologies) in the presence of oxidized LDLs[10]. We diluted H2DCF-DA in methanol (final concentration: 2 mg/mL) for 30 minutes, to obtain its deacetylated form (H2DCF). We prepared oxidized LDLs from a pool of LDLs, isolated from the plasma of 20 healthy volunteers by density gradient ultracentrifugation[8]. We oxidized LDLs with $CuSO_4$ as previously reported[10], diluted them to 100 mg/L of LDL protein and stored the lipoproteins at -80ºC until use. Finally, we incubated H2DCF (final concentration: 3 μg/mL) and oxidized LDLs (final concentration: 1.5 μg/mL) with 5 μL of ABDP from the volunteers, or without it (negative control), in 96-well, black polystyrene plates, at 37ºC in an Infinite M200 reader (Tecan Ltd). The reader shook and stimulated the plate with light every 3 minutes for 60 minutes, and measured the fluorescence at the end of the incubation process (Ex/Em: 485/530 nm). To calculate the HDL inflammatory index, we subtracted the fluorescence values obtained in the negative control from the fluorescence values of the samples. A greater oxidation of H2DCF is associated with a greater fluorescent signal and a lower HDL antioxidant/anti-inflammatory capacity: therefore, low HII values are related to HDLs with high antioxidant/anti-inflammatory capacity.

We analyzed samples in duplicate. We did not allow intra-repetition coefficients of variation >15%. To minimize inter-assay variation, we included a pool of ABDP from 20 healthy volunteers in each experiment, and then divided all HDL inflammatory index values of the samples by the value of the pool, to obtain normalized ratios. The inter-assay coefficient of variation was 4.55%.

HDL ENDOTHELIAL PROPERTIES

HDL vasodilatory capacity. We measured the capacity of HDLs to induce the release of nitric oxide (NO) from a line of human umbilical vein endothelial cells (HUVEC; Lonza). The detection of NO is based on its capacity to react with the potential fluorogen 4,5-diaminofluorescein diacetate (DAF-2DA, Sigma), in a modification of a previously described method[11].

We cultured the HUVECs in supplemented EGM-2 medium (Lonza), and refreshed them every 48-72h. We trypsinized and seeded cells in 96-well plates to 80-90% confluence 24h before the experiments. We then washed the cells, incubated them in fresh EGM-2 medium (now supplemented with 0.75% BSA, 1% fetal calf serum, and 1% penicillin-streptomycin) with the DAF-2DA (final concentration: 5 μM) and 30% ABDP of the volunteers, or without it (negative control). 30 and 60 minutes after, we determined the fluorescent signal (Ex/Em: 485/532 nm*)* in an Infinite M200 reader (Tecan Ltd).

After the cellular experiment, we calculated the velocity of NO generation in the culture. First, we subtracted the blank fluorescence of cells from all the fluorescence measurements. We then computed the slope between 30 and 60 minutes (between these points, there was a lineal relationship between fluorescence and time). Finally, we calculated the increment in the NO production in the cells, relative to cells non-treated with ABDP: (slope in ABDP-treated cells – slope in non-treated cells)/slope in non-treated cells*100.

We studied samples in duplicate. We did not permit intra-repetition coefficients of variation >15%. To minimize inter-assay variation, we included a pool of ABDP from 20 healthy volunteers in each experiment. We divided the HDL vasodilatory index values of the samples by the value of the pool, to obtain normalized ratios without units. The inter-assay coefficient of variation (*N*=12) was 14.5%.

HDL COMPOSITION

We performed all systemic determinations in an ABX Pentra-400 autoanalyzer (Horiba ABX)[6]. In isolated HDL samples, we measured triglycerides (ABX Pentra Triglycerides CP, Horiba ABX), total cholesterol, free cholesterol and phospholipids (Cholesterol-LQ, Free Cholesterol-LQ, and Phospholipids, all from Spinreact) by enzymatic methods, and apolipoprotein A-I (ABX Pentra ApoA1, Horiba ABX) and A-II (Apolipoprotein AII, Spinreact) by immunoturbidimetry. In ABDP samples, we measured total cholesterol (Cholesterol-LQ, Spinreact) by enzymatic methods, and apolipoprotein C-III (Apolipoprotein C-III, Spinreact) by immunoturbidimetry. The inter-assay coefficients of variation of the previous determinations were: 4.62% for triglycerides, 3.86% for total cholesterol in HDL, 3.86% for total cholesterol in ABDP, 3.16% for free cholesterol, 2.61% for phospholipids, 1.68% for apolipoprotein A-I, 2.68% for apolipoprotein A-II, and 3.25% for apolipoprotein C-III.

We calculated the content of esterified cholesterol (total cholesterol in HDL - free cholesterol in HDL), and the percentage of esterified cholesterol in HDL as follows: esterified cholesterol/total cholesterol*100 (we used this percentage to calculate the HDL cholesterol esterification index, see above). We defined two compositional ratios: triglycerides in HDL core (triglycerides in HDL/esterified cholesterol in HDL), and phospholipids in HDL surface (phospholipids in HDL/free cholesterol in HDL). Finally, we calculated the quantity of the different lipoproteins, relative to cholesterol in HDLs:

apolipoprotein A-I in HDL/cholesterol in HDL, apolipoprotein A-II in HDL/cholesterol in HDL, and apolipoprotein C-III in ABDP samples/cholesterol in ABDP samples.

HDL RESISTANCE AGAINST OXIDATION

HDL oxidation index. This index represents the *in vivo* oxidation of the volunteers' HDLs. First, we determined the oxidation levels of the HDLs (the malondialdehyde equivalents in the ABDP of the volunteers) by the thiobarbituric acid reactive species technique[12]. Then, we calculated the HDL oxidation index by dividing the malondialdehyde equivalents by the cholesterol content in each ABDP sample. We ran the oxidation test in duplicate, and did not accept intra-repetition coefficients of variation >15%. To reduce inter-assay variation, we included a pool of ABDP from 20 healthy volunteers in each experiment. Finally, we divided the value of the HDL oxidation degree of each sample by the HDL oxidation degree value of the pool, to obtain normalized ratios without units. The inter-assay coefficient of variation was 1.68%.

HDL resistance against oxidation. We incubated isolated HDLs in the presence of an oxidizing agent ($CuSO_4$), to determine their resistance to accumulate Cu^{2+}-induced conjugated dienes. We dialyzed HDLs against PBS in *PD-10 Desalting Columns* (GE Healthcare) and incubated them (final concentration: 3 mg cholesterol/dL) with $CuSO_4$ (final concentration: 5 µM) in 96-well transparent plates at 37ºC in an Infinite M200 reader (Tecan Ltd). We measured absorbance at 234 nm every 3 minutes for 6 hours to assess the conjugated diene formation.

The consecutive measurements of absorbance configured the different curves of HDL oxidation. In these curves we calculated HDL lag time (the time when maximal oxidation started in HDL, in minutes)[8]. HDLs are more resistant against oxidation when their lag time values are high.

We analyzed samples in duplicate. No intra-repetition coefficient of variation >15% was permitted. We included a control HDL (isolated from the plasma of 20 healthy volunteers) in each experiment to control inter-assay variation. We then divided the values of the kinetic parameters of the samples by the values of the pool, to obtain normalized ratios without units. The inter-assay coefficient of variation for HDL lag time was 8.29%.

HDL SIZE DISTRIBUTION

We measured HDL size distribution by the Lipoprint HDL System (Quantimetrix), an electrophoretic system able to separate HDLs In ten differentiated bands according to their size[6]. The first of the bands corresponds to the largest HDL fraction, the second to a slightly smaller HDL fraction, and so on until the tenth and last band. Following the manufacturer's instructions, we grouped the electrophoretic bands as 1-3, 4-7, and 8-10 to compute the percentage of large, intermediate, and small HDLs, respectively. To minimize inter-assay variation, we included a pool of plasma from 20 healthy volunteers in each experiment. The inter-assay coefficient of variation for the percentage of large HDLs was 10.4%.

SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE 1

Comparison of the baseline characteristics of the volunteers of our study (*N*=296) and the whole population of the PREDIMED Trial (*N*=7151).

VARIABLES	Sub-sample (HDL) *N*=296	PREDIMED Study *N*=7151	*P*-value
Age (years)	65.9 (6.43)[a]	67.0 (6.19)	0.003
Sex (% male)	49.0%	42.2%	0.025
Body Mass Index (kg/m^2)	29.6 (3.85)	30.0 (3.85)	0.141
Waist Circumference (cm)	101 (10.7)	100 (10.3)	0.229
Leisure-time physical activity (MET·min/day)	175 [59.6;350][b]	175 [64.2;319]	0.814
Smoking status (% of smokers)	12.5%	14.1%	0.482
Type 2 diabetes (% of diabetic patients)	49.0%	48.8%	0.987
Hypertension (% of hypertensive patients)	78.7%	82.9%	0.072
Dyslipidemia (% of dyslipidemic patients)	77.4%	72.1%	0.055
Distribution among intervention groups:			0.953
TMD-VOO	33.8%	34.2%	
TMD-Nuts	33.8%	32.9%	
Low-fat control diet	32.4%	32.9%	

[a]: Mean (SD). [b]: Median [1st-3rd quartile]. *MET*: metabolic equivalent of task. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts

291 **SUPPLEMENTAL TABLE 2**

292 Differences between post- and pre-intervention values in the dietary profile of the volunteers in the three interventions of the study.

293

	TMD-VOO		TMD-Nuts		Low-fat control diet	
VARIABLES	**Difference**	***P*-value**	**Difference**	***P*-value**	**Difference**	***P*-value**
Adherence to TMD (score)	1.53 (1.82)[a]	<0.001	1.24 (1.91)	<0.001	-0.031 (1.99)	0.878
Total energy intake (kcal/day)	-7.95 (545)	0.884	57.6 (600)	0.344	-55.6 (661)	0.412
Carbohydrates (g/day)	-10.6 (79.3)	0.186	-6.18 (80.1)	0.447	4.95 (88.6)	0.585
Proteins (g/day)	2.01 (23.3)	0.391	-0.13 (22.0)	0.952	-4.30 (25.7)	0.105
Total fats (g/day)	3.26 (24.9)	0.194	10.0 (28.9)	<0.001	-5.83 (30.9)	0.069
MUFAs (g/day)	4.14 (13.0)	0.002	6.07 (15.3)	<0.001	-2.73 (16.6)	0.112
SFAs (g/day)	-1.58 (7.85)	0.046	-1.04 (7.63)	0.181	-3.20 (9.73)	0.002
PUFAs (g/day)	0.086 (6.96)	0.901	3.92 (7.50)	<0.001	-0.78 (7.07)	0.285
Fiber (g/day)	-0.50 (9.30)	0.591	2.40 (9.28)	0.012	0.18 (10.4)	0.863
Vegetables (g/day)	-2.06 (141)	0.885	28.1 (137)	0.045	15.1 (155)	0.346
Fruits (g/day)	-15.9 (217)	0.466	36.4 (170)	0.037	-35.9 (234)	0.138
Legumes (g/day)[b]	0.12 (0.58)	0.040	0.041 (0.49)	0.418	0.016 (0.48)	0.743
Starchy foods (g/day)	-0.060 (118)	0.996	-7.72 (117)	0.516	7.93 (128)	0.545
Whole grains (g/day)[b]	-0.19 (1.94)	0.334	-0.34 (2.13)	0.115	0.49 (2.07)	0.022
Refined grains (g/day)	0.38 (85.2)	0.964	1.79 (111)	0.874	5.55 (127)	0.671
Dairy products (g/day)	-4.83 (181)	0.790	-4.63 (179)	0.798	-62.0 (212)	0.005
Low-fat dairy products (g/day)[b]	0.24 (1.66)	0.153	-0.032 (1.98)	0.874	0.14 (1.92)	0.462
High-fat dairy products (g/day)[b]	-0.49 (1.97)	0.015	-0.36 (2.06)	0.089	-0.48 (1.84)	0.012
Meat products (g/day)	-6.02 (55.4)	0.281	-13.0 (47.9)	0.009	-20.0 (55.8)	<0.001
Red meat (g/day)	-10.1 (31.7)	0.002	-8.42 (36.1)	0.023	-14.6 (41.9)	<0.001
White meat (g/day)	10.3 (37.0)	0.007	-2.27 (36.4)	0.539	0.40 (31.2)	0.900
Processed meat (g/day)	-5.00 (16.9)	0.004	-3.23 (16.3)	0.054	-4.91 (19.2)	0.015
Fish products (g/day)	25.4 (45.8)	<0.001	-2.17 (51.9)	0.680	11.7 (53.7)	0.035
White fish and seafood (g/day)	13.4 (37.6)	<0.001	-10.4 (38.7)	0.009	9.58 (45.7)	0.044

Oily fish (g/day)	8.97 (24.5)	<0.001	6.41 (22.9)	0.007	2.80 (23.0)	0.236
Precooked meals (g/day)[b]	-0.37 (1.20)	0.003	-0.38 (1.35)	0.006	-0.32 (1.20)	0.011
Industrial sweets (g/day)[b]	-0.45 (1.73)	0.010	-0.54 (1.50)	<0.001	-0.40 (1.57)	0.015
Total olive oil (g/day)	11.0 (15.2)	<0.001	8.21 (18.1)	<0.001	1.06 (18.1)	0.566
Virgin olive oil (g/day)	32.4 (21.3)	<0.001	9.01 (22.4)	<0.001	5.37 (23.7)	0.029
Refined olive oil (g/day)	-21.4 (19.5)	<0.001	-0.76 (22.5)	0.740	-4.83 (19.9)	0.020
Nuts (g/day)[b]	0.058 (1.49)	0.697	1.44 (1.36)	<0.001	-0.11 (1.50)	0.458
Wine (mL/day)[b]	-0.14 (1.32)	0.275	-0.37 (1.54)	0.018	-0.15 (1.63)	0.379

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295 [a]: Mean (SD). [b]: Log-transformed variables.

296 *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with

297 mixed nuts

SUPPLEMENTAL TABLE 3

Differences between the Traditional Mediterranean Diet interventions and the low-fat control diet in the dietary profile of the volunteers.

	TMD-VOO vs. Low-fat control diet		TMD-Nuts vs. Low-fat control diet	
VARIABLES	**Coefficient β [CI 95%][a]**	**P-value**	**Coefficient β [CI 95%][a]**	**P-value**
Adherence to TMD (score)	1.28 [0.85;1.71]	<0.001	1.34 [0.89;1.79]	<0.001
Total energy intake (kcal/day)	-8.80 [-133;116]	0.890	-17.4 [-147;112]	0.792
Carbohydrates (g/day)	-19.2 [-37.3;-1.12]	0.038	-29.4 [-48.2;-10.6]	0.002
Proteins (g/day)	2.50 [-2.77;7.78]	0.353	1.42 [-4.04;6.88]	0.610
Total fats (g/day)	7.37 [1.55;13.2]	0.014	11.0 [4.98;17.1]	<0.001
MUFAs (g/day)	6.06 [2.95;9.18]	<0.001	5.81 [2.56;9.05]	<0.001
SFAs (g/day)	0.59 [-1.12;2.30]	0.501	1.08 [-0.71;2.86]	0.239
PUFAs (g/day)	1.02 [-0.34;2.37]	0.142	4.35 [2.94;5.76]	<0.001
Fiber (g/day)	-0.25 [-2.46;1.96]	0.822	-0.24 [-2.54;2.07]	0.840
Vegetables (g/day)	-3.12 [-37.4;31.1]	0.858	-7.52 [-42.9;27.8]	0.677
Fruits (g/day)	-3.81 [-49.5;41.9]	0.870	-3.57 [-51.2;44.0]	0.883
Legumes (g/day)[b]	0.14 [0.012;0.26]	0.032	0.020 [-0.11;0.15]	0.767
Starchy foods (g/day)	-12.8 [-37.7;12.2]	0.316	-32.5 [-58.4;-6.58]	0.015
Whole grains (g/day)[b]	-0.58 [-1.08;-0.075]	0.025	-0.61 [-1.13;-0.083]	0.024
Refined grains (g/day)	-11.6 [-35.4;12.2]	0.340	-21.7 [-46.3;3.02]	0.087
Dairy products (g/day)	29.9 [-19.1;78.9]	0.233	33.7 [-17.0;84.4]	0.194
Low-fat dairy products (g/day)[b]	0.008 [-0.42;0.44]	0.971	-0.082 [-0.53;0.37]	0.721
High-fat dairy products (g/day)[b]	0.038 [-0.43;0.51]	0.875	0.10 [-0.38;0.59]	0.673
Meat products (g/day)	5.28 [-7.37;17.9]	0.414	6.76 [-6.46;20.0]	0.317
Red meat (g/day)	1.96 [-5.72;9.64]	0.618	2.35 [-5.65;10.3]	0.566
White meat (g/day)	6.97 [-1.70;15.7]	0.116	1.29 [-7.75;10.3]	0.781
Processed meat (g/day)	-3.44 [-7.28;0.40]	0.080	1.83 [-2.18;5.83]	0.372
Fish products (g/day)	10.2 [-1.51;21.9]	0.089	-8.16 [-20.4;4.09]	0.193
White fish and seafood (g/day)	1.12 [-8.62;10.9]	0.821	-11.9 [-22.2;-1.61]	0.024

Oily fish (g/day)	7.51 [1.73;13.3]	0.011	5.24 [-0.80;11.3]	0.090
Precooked meals (g/day)[b]	-0.067 [-0.37;0.24]	0.667	0.11 [-0.21;0.43]	0.488
Industrial sweets (g/day)[b]	-0.41 [-0.80;-0.013]	0.044	-0.16 [-0.58;0.24]	0.431
Total olive oil (g/day)	10.3 [6.71;13.9]	<0.001	2.73 [-1.00;6.47]	0.153
Virgin olive oil (g/day)	26.5 [21.7;31.4]	<0.001	3.22 [-1.80;8.23]	0.210
Refined olive oil (g/day)	-15.6 [-19.6;-11.7]	<0.001	0.47 [-3.66;4.60]	0.823
Nuts (g/day)[b]	0.30 [0.014;0.59]	0.041	1.68 [1.38;1.98]	<0.001
Wine (mL/day)[b]	$1 \cdot 10^{-4}$ [-0.40;0.41]	0.999	-0.013 [-0.44;0.41]	0.952

[a]: β coefficient of the multivariate linear regression model, adjusted by: sex, age, volunteer's center of origin, baseline value of the variable, and change in the use of lipid-lowering drugs. [b]: log-transformed variables. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts

SUPPLEMENTAL TABLE 4

Differences between post- and pre-intervention values in the biochemical profile of the volunteers in the three interventions of the study.

	TMD-VOO		TMD-Nuts		Low-fat control diet	
VARIABLES	**Difference**	***P*-value**	**Difference**	***P*-value**	**Difference**	***P*-value**
Glucose[a] (mg/dL)	-0.037 (0.22)[b]	0.092	-0.003 (0.23)	0.916	-0.013 (0.24)	0.616
Triglycerides[a] (mg/dL)	0.011 (0.35)	0.762	0.047 (0.35)	0.193	0.045 (0.41)	0.297
Total cholesterol (mg/dL)	-0.35 (25.7)	0.896	-0.46 (26.5)	0.865	-7.72 (35.6)	0.039
HDL cholesterol (mg/dL)	-1.12 (6.58)	0.092	-0.99 (6.95)	0.169	-0.18 (8.88)	0.845
LDL cholesterol (mg/dL)	2.45 (20.9)	0.275	-0.24 (21.5)	0.916	-7.22 (28.7)	0.019
Apolipoprotein A-I (mg/dL)	0.38 (12.7)	0.814	1.88 (12.9)	0.196	2.08 (11.9)	0.135
Apolipoprotein B (mg/dL)	-0.78 (14.5)	0.669	0.98 (14.5)	0.550	-0.66 (18.2)	0.748
HDL cholesterol/Apolipoprotein A-I (unitless ratio)	-0.006 (0.020)	0.031	-0.010 (0.025)	<0.001	-0.005 (0.026)	0.129
Apolipoprotein B/Apolipoprotein A-I (unitless ratio)	-0.007 (0.13)	0.668	-0.004 (0.10)	0.745	-0.017 (0.15)	0.345

[a]: Log-transformed variables. [b]: Mean (SD). *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts

SUPPLEMENTAL TABLE 5

Differences between the Traditional Mediterranean Diet interventions and the low-fat control diet in the biochemical profile of the volunteers.

	TMD-VOO vs. Low-fat control diet		TMD-Nuts vs. Low-fat control diet	
VARIABLES	**Coefficient β [CI 95%][a]**	**P-value**	**Coefficient β [CI 95%]**	**P-value**
Glucose[b] (mg/dL)	-0.032 [-0.091;0.027]	0.283	0.019 [-0.043;0.081]	0.551
Triglycerides[b] (mg/dL)	0.001 [-0.09;0.092]	0.982	-0.071 [-0.17;0.025]	0.149
Total cholesterol (mg/dL)	8.83 [1.25;16.4]	0.023	0.27 [-7.71;8.25]	0.948
HDL cholesterol (mg/dL)	0.52 [-1.35;2.38]	0.586	-1.07 [-3.05;0.91]	0.292
LDL cholesterol (mg/dL)	8.06 [1.70;14.4]	0.014	1.43 [-5.28;8.14]	0.676
Apolipoprotein A-I (mg/dL)	-0.041 [-4.03;3.95]	0.984	-0.21 [-4.28;3.86]	0.920
Apolipoprotein B (mg/dL)	1.29 [-3.35;5.93]	0.586	-1.00 [-5.73;3.73]	0.679
HDL cholesterol/Apolipoprotein A-I (unitless ratio)	0.001 [-0.007;0.008]	0.821	-0.005 [-0.013;0.002]	0.181
Apolipoprotein B/Apolipoprotein A-I (unitless ratio)	0.005 [-0.034;0.044]	0.808	-0.01 [-0.05;0.031]	0.642

[a]: β coefficient of the multivariate linear regression model, adjusted by: sex, age, volunteer's center of origin, baseline value of the variable, and change in the use of lipid-lowering drugs. [b]: log-transformed variables. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts

SUPPLEMENTAL TABLE 6

Differences between post- and pre-intervention values in HDL functional properties in the three interventions of the study

	TMD-VOO		TMD-Nuts		Low-fat control diet	
VARIABLES	**Difference**	***P*-value**	**Difference**	***P*-value**	**Difference**	***P*-value**
Cholesterol efflux capacity (unitless ratio)	0.019 (0.074)[a]	0.018	0.025 (0.095)	0.013	0.018 (0.104)	0.119
HDL cholesterol esterification index (unitless ratio)	0.57 (1.59)	0.007	0.028 (1.32)	0.883	0.156 (1.8)	0.467
Cholesterol ester transfer protein activity (unitless ratio)	-0.039 (0.11)	0.008	0.007 (0.12)	0.706	-0.010 (0.19)	0.667
Paraoxonase-1 arylesterase activity (unitless ratio)	0.042 (0.26)	0.227	-0.005 (0.24)	0.887	-0.029 (0.21)	0.249
HDL antioxidant capacity (on LDL lag time) (unitless ratio)	0.26 (0.46)	0.004	0.054 (0.43)	0.539	0.018 (0.51)	0.844
HDL inflammatory index (unitless ratio)	0.014 (0.19)	0.494	-0.007 (0.19)	0.705	0.045 (0.19)	0.025
HDL vasodilatory capacity (unitless ratio)	0.045 (0.27)	0.143	0.032 (0.32)	0.368	-0.046 (0.22)	0.078
HDL oxidation index[b] (unitless ratio)	-0.067 (0.29)	0.028	-0.037 (0.21)	0.101	-0.072 (0.26)	0.011
HDL lag time (unitless ratio)	0.13 (0.24)	0.006	-0.025 (0.23)	0.581	0.016 (0.25)	0.731
Triglycerides in HDL core[b] (unitless ratio)	-0.036 (0.41)	0.385	0.036 (0.36)	0.346	0.072 (0.49)	0.158
Phospholipids in HDL surface[b] (unitless ratio)	0.102 (0.33)	0.003	-0.035 (0.36)	0.359	0.025 (0.44)	0.587
Apolipoprotein A-I in isolated HDLs (adjusted) (unitless ratio)	-0.047 (0.31)	0.136	0.001 (0.32)	0.985	0.010 (0.22)	0.677
Apolipoprotein A-II in isolated HDLs (adjusted) (unitless ratio)	-0.007 (0.086)	0.442	0 (0.093)	0.977	-0.006 (0.073)	0.460
Apolipoprotein C-III in in isolated HDLs (adjusted) (unitless ratio)	-0.005 (0.048)	0.367	0 (0.039)	0.944	0.002 (0.052)	0.670
Large HDLs (%)	4.34 (9.32)	<0.001	3.70 (9.03)	<0.001	4.85 (9.32)	<0.001
Intermediate HDLs (%)	0.34 (6.55)	0.643	-0.38 (5.53)	0.530	0.19 (6.46)	0.792
Small HDLs (%)	0.46 (5.66)	0.475	0.45 (4.58)	0.372	0.074 (4.91)	0.891

[a]: Mean (SD). [b]: Log-transformed variables. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil; *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts

SUPPLEMENTAL TABLE 7

Differences between the Traditional Mediterranean Diet interventions and the low-fat control diet in HDL functional properties

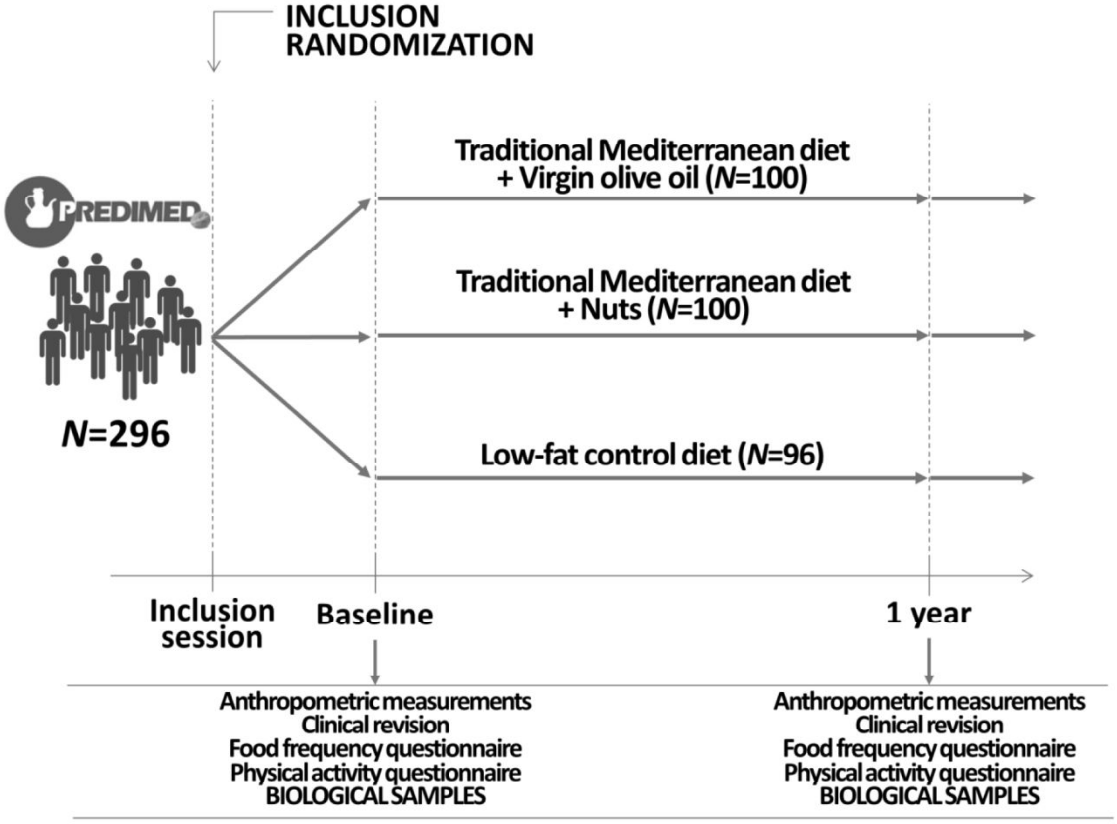
	TMD-VOO vs. Low-fat control diet		TMD-Nuts vs. Low-fat control diet	
VARIABLES	**Coefficient β [CI 95%]**	**P-value**	**Coefficient β [CI 95%]**	**P-value**
Cholesterol efflux capacity (unitless ratio)	0.003 [-0.022;0.028][a]	0.806	-0.016[-0.042;0.009]	0.209
HDL cholesterol esterification index (unitless ratio)	0.53 [0.014;1.05]	0.046	0.144[-0.439;0.727]	0.629
Cholesterol ester transfer protein activity (unitless ratio)	-0.024 [-0.068;0.021]	0.298	-0.014[-0.062;0.034]	0.579
Paraoxonase-1 arylesterase activity (unitless ratio)	0.082 [0;0.16]	0.051	0.062[-0.028;0.151]	0.179
HDL antioxidant capacity (on LDL lag time) (unitless ratio)	0.22 [-0.036;0.48]	0.096	0.025[-0.252;0.302]	0.858
HDL inflammatory index (unitless ratio)	-0.01 [-0.049;0.029]	0.618	-0.043[-0.084;-0.002]	0.042
HDL vasodilatory capacity (unitless ratio)	0.092 [0.015;0.17]	0.020	0.063[-0.016;0.143]	0.121
HDL oxidation index[b] (unitless ratio)	0.004 [-0.063;0.071]	0.904	0.062[-0.008;0.133]	0.084
HDL lag time (unitless ratio)	0.11 [-0.021;0.25]	0.104	-0.054[-0.197;0.089]	0.463
Triglycerides in HDL core[b] (unitless ratio)	-0.1 [-0.21;0.005]	0.062	-0.113[-0.223;-0.003]	0.045
Phospholipids in HDL surface[b] (unitless ratio)	0.10 [0.002;0.21]	0.048	0.066[-0.045;0.178]	0.243
Apolipoprotein A-I in isolated HDLs (adjusted) (unitless ratio)	-0.037 [-0.11;0.039]	0.340	-0.004[-0.085;0.076]	0.915
Apolipoprotein A-II in isolated HDLs (adjusted) (unitless ratio)	-0.006 [-0.027;0.016]	0.609	-0.006[-0.028;0.017]	0.622
Apolipoprotein C-III in in isolated HDLs (adjusted) (unitless ratio)	-0.005 [-0.019;0.009]	0.477	-0.003[-0.018;0.011]	0.659
Large HDLs (%)	0.96 [-1.68;3.60]	0.476	0.033[-2.765;2.831]	0.982
Intermediate HDLs (%)	-0.27 [-2.10;1.55]	0.771	-0.469[-2.397;1.459]	0.634
Small HDLs (%)	0.38 [-1.14;1.91]	0.623	-0.059[-1.655;1.537]	0.942

[a]: β coefficient of the multivariate linear regression model, adjusted by: sex, age, center of origin of the volunteer, baseline value of the variable, and change in the use of lipid-lowering drugs. [b]: log-transformed variables. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts

SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE 1

Design of the long-term, large-scale, parallel, randomized controlled trial.



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MANUSCRIPT III

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Olive Oil Polyphenols Decrease LDL Concentrations and LDL Atherogenicity in Men in a Randomized Controlled Trial[1–3]

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Abstract

Background: Olive oil polyphenols have shown protective effects on cardiovascular risk factors. Their consumption decreased oxidative stress biomarkers and improved some features of the lipid profile. However, their effects on LDL concentrations in plasma and LDL atherogenicity have not yet been elucidated.

Objective: Our objective was to assess whether the consumption of olive oil polyphenols could decrease LDL concentrations [measured as apolipoprotein B-100 (apo B-100) concentrations and the total number of LDL particles] and atherogenicity (the number of small LDL particles and LDL oxidizability) in humans.

Methods: The study was a randomized, cross-over controlled trial in 25 healthy European men, aged 20–59 y, in the context of the EUROLIVE (Effect of Olive Oil Consumption on Oxidative Damage in European Populations) study. Volunteers ingested 25 mL/d raw low-polyphenol-content olive oil (LPCOO; 366 mg/kg) or high-polyphenol-content olive oil (HPCOO; 2.7 mg/kg) for 3 wk. Interventions were preceded by 2-wk washout periods. Effects of olive oil polyphenols on plasma LDL concentrations and atherogenicity were determined in the sample of 25 men. Effects on lipoprotein lipase (*LPL*) gene expression were assessed in another sample of 18 men from the EUROLIVE study.

Results: Plasma apo B-100 concentrations and the number of total and small LDL particles decreased (mean ± SD: by 5.94% ± 16.6%, 11.9% ± 12.0%, and 15.3% ± 35.1%, respectively) from baseline after the HPCOO intervention. These changes differed significantly from those after the LPCOO intervention, which showed significant increases of 6.39% ± 16.6%, 4.73% ± 22.0%, and 13.6% ± 36.4% from baseline ($P < 0.03$). LDL oxidation lag time increased by 5.0% ± 10.3% from baseline after the HPCOO intervention, which was significant only relative to preintervention values ($P = 0.038$). *LPL* gene expression tended to increase by 26% from baseline after the HPCOO intervention ($P = 0.08$) and did not change after the LPCOO intervention.

Conclusion: The consumption of olive oil polyphenols decreased plasma LDL concentrations and LDL atherogenicity in healthy young men. This trial was registered at www.controlled-trials.com as ISRCTN09220811. *J Nutr* doi: 10.3945/jn.115.211557.

Keywords: olive oil polyphenols, randomized clinical trial, low-density lipoproteins, apolipoprotein B-100, LDL particle number, small LDL particles, LDL oxidation, lipoprotein lipase, healthy individuals

Introduction

Virgin olive oil consumption protects against the development of cardiovascular diseases (1) due to its content of MUFAs (2) and polyphenols (3). To assess the beneficial properties of olive oil polyphenols on lipid profile and oxidation, the Effect of Olive Oil Consumption on Oxidative Damage in European Populations (EUROLIVE)[18] Study was conducted. This project showed that the consumption of polyphenol-rich olive oil was beneficial

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for the oxidative status of LDLs. Olive oil polyphenols dose-dependently decreased circulating concentrations of oxidized LDLs, C18 hydroxy-FAs, and uninduced conjugated dienes (4). In a previous study, our group also observed that olive oil polyphenols induced changes in LDL composition, increasing the LDL content of oleic acid, vitamin E, and olive oil phenolic compounds (5).

However, the effects of olive oil polyphenols on LDL concentrations, LDL proatherogenic properties, such as LDL size, and the expression of some key genes related to LDL concentrations, such as lipoprotein lipase (*LPL*), have not been determined in vivo in humans. Thus, our objective was to determine whether the consumption of a polyphenol-rich olive oil would improve all of these properties.

Methods

Study participants

Our study population consisted of a subsample of the EUROLIVE study, a parallel, crossover, randomized controlled trial conducted in 180 healthy men, aged 20–59 y, from 6 European cities. The purpose of the study was to determine the effects of olive oil polyphenols on lipid profile and oxidative stress biomarkers. Local institutional ethics committees approved the protocol of the study, the details of which have been previously described (4). In all cases, written informed consent was provided by the participants before joining the trial. The protocol of the EUROLIVE study is registered as ISRCTN09220811 (www.controlled-trials.com).

We studied the effects of olive oil polyphenols on lipid profile, apo B-100 concentrations, LDL particle distribution, and LDL oxidizability ex vivo in a random subsample of 25 EUROLIVE volunteers from 3 centers (9 from Potsdam, Germany; 9 from Kuopio, Finland; and 7 from Barcelona, Spain). We assessed the effects of polyphenols on the expression of *LPL* in another random subsample of 18 volunteers of the EUROLIVE study, 8 of whom were also present in the first subsample of individuals. Blood samples were taken from fasting participants before and after dietary interventions with high-polyphenol-content olive oil (HPCOO; a natural virgin olive oil with 366 mg/kg polyphenols) and low-polyphenol-content olive oil (LPCOO; a refined olive oil with 2.7 mg/kg polyphenols). Polyphenols become degraded during the refinement process and thus the refined olive oil had a lower phenolic content. The composition of both olive oils was identical, except for their polyphenol content (4). Samples were stored at −80°C until the present experiments commenced. No thaw-freeze cycles were applied to the samples before the present work.

As shown in the crossover design (**Supplemental Figure 1**), volunteers were assigned to 3-wk intervention periods in which they ingested 25 mL/d raw olive oil distributed among meals. Participants were instructed to replace other dietary fats with olive oil. Intervention periods were preceded by a 2-wk washout period, during which olive oil, olives, and antioxidant-rich foods were avoided. A 2-wk washout period was sufficient to eliminate olive oil polyphenols between interventions, considering the half-life of the sum of the main olive oil phenolic compounds (8 h) (6). The washout period was also sufficient if the half-life of the LDL particle is considered (3 d) (7). A more detailed description of the diet of the participants was previously published (4, 8).

Study measurements

Dietary adherence, physical activity, and oxidative status of volunteers. Dietary adherence was determined through 24-h urinary excretion of tyrosol and hydroxytyrosol, which are the 2 main phenolic compounds in olive oil and are considered as biomarkers of olive oil consumption. These compounds were determined by GC and MS, as previously described (6). Participants' diet was controlled through a 3-d dietary record. This dietary control was performed at the beginning of the study and after each dietary intervention. Volunteers were asked to maintain their usual diet during the whole study. The physical activity level of participants was also measured and was calculated at the beginning and the end of the study by using the validated Minnesota Leisure Time Physical Activity Questionnaire (4). The oxidative status of the volunteers was also assessed by means of the determination of different oxidative biomarkers (oxidized LDLs, C18 hydroxy-FAs, and reduced ascorbic acid and dehydroascorbic acid), as previously reported (4).

Lipid profile and apo B-100 determination. We performed lipid profile and apo B-100 analyses with the use of an ABX Pentra 400 autoanalyzer (Horiba Diagnostics). TGs and total cholesterol were measured by using enzymatic methods (ABX Pentra Triglycerides CP and ABX Pentra Cholesterol CP, respectively; Horiba Diagnostics). HDL cholesterol was determined by the Accelerator Selective Detergent method (ABX Pentra HDL Direct CP; Horiba Diagnostics), and apo B-100 concentrations were measured by immunoturbidimetry (ABX Pentra Apo B; Horiba Diagnostics). Interassay CVs of the previous determinations were as follows: 1.48% for TGs, 1.54% for total cholesterol, 3.34% for HDL cholesterol, and 1.95% for apo B. We also calculated LDL-cholesterol concentrations by the Friedewald formula whenever TGs were <300 mg/dL.

LDL particle analyses. We determined LDL-cholesterol concentrations (directly measured) and the number of total LDL particles, total VLDL particles, small LDL particles, large LDL particles, small VLDL particles, and medium+large VLDL particles by NMR spectroscopy. Values were calculated from the measured amplitudes of the NMR signals of the lipid methyl groups in the samples (9). These analyses were performed by using a Vantera Clinical Analyzer (LipoScience) and could only be performed in the available samples of 20 of the volunteers. This technique showed an interassay CV of 5.30% for the determination of total LDL particle number for the range of low values (typical in healthy individuals), under the most unfavorable conditions.

Analyses of LDL resistance against oxidation. Before analysis, LDLs were isolated from K_2-EDTA plasma of the volunteers by density gradient ultracentrifugation (10) and stored at −80°C in 2.5% sucrose until the analyses. In the isolated LDL samples, we determined LDL resistance against oxidation by measuring the accumulation of Cu^{2+}-induced conjugated dienes in the lipoprotein. First, we dialyzed the isolated LDLs with PBS to discard possible contaminants. We then incubated the dialyzed LDLs (at a final concentration of 10 mg/dL LDL cholesterol) in the presence of $CuSO_4$ to induce the oxidation reaction (at a final concentration of 5 μM $CuSO_4$) at 37°C for 4 h. During incubation, absorbance at 234 nm was determined every 3 min by using an INFINITE M200 reader (Tecan Group Ltd.).

Consecutive measurements of absorbance defined the LDL oxidation kinetic curves. For each of these curves, we calculated the following: *1*) lag time (the time when maximal oxidation started, in minutes) and *2*) oxidation rate [the slope of the kinetic curve at peak velocity, expressed as the increase in the concentration (mM) of conjugated dienes per minute and milligrams of LDL cholesterol], as previously described (*5*). All determinations were performed in duplicate. We used an LDL pool

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[3] Supplemental Figure 1 and Supplemental Table 1 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
[18] Abbreviations used: EUROLIVE, Effect of Olive Oil Consumption on Oxidative Damage in European Populations; HPCOO, high-polyphenol content olive oil; *IFNG*, interferon γ; LPCOO, low-polyphenol content olive oil; *LPL*, lipoprotein lipase.
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from healthy volunteers as an interassay control. The interassay CVs of the previous determinations were as follows: 2.89% for lag time and 4.77% for oxidation rate.

LPL gene expression analyses. First, we isolated total RNA from peripheral blood mononuclear cells by means of a liquid-liquid method. We then checked RNA purity and integrity and converted RNA to cDNA. Afterward, *LPL* gene expression was quantified by using a real-time PCR in TaqMan Low Density microfluidic cards (Applied Biosystems, Life Technologies). Four replicates of each RNA sample were used in the experiments. Data were analyzed by using the Sequence Detection System software (SDS 2.1.; Applied Biosystems, Life Technologies), following the manufacturer's instructions. *LPL* gene expression was finally calculated by the relative quantification method (using the $2^{-\Delta\Delta Ct}$ formula). This technique presents an interassay CV of 0.98%, calculated in the control pool for the housekeeping gene (*GAPDH*).

Sample size calculation

Biochemical determinations. A sample size of 25 individuals allowed ≥80% power to detect significant differences of 4 mg/dL apo B-100 concentrations between both olive oil interventions, considering a 2-sided type I error of 0.05. Calculations were made by using previous data on our group, considering the SD of apo B-100 concentrations in healthy volunteers.

LPL gene expression. A sample size of 18 individuals allowed ≥80% power to detect significant differences of 0.5 units of $\log_2$ ratio relative quantification in the expression of a reference gene [human interferon γ (*IFNG*)] between both olive oil interventions, considering a 2-sided type I error of 0.05. Calculations were made by using previous data on our group, considering the SD of *IFNG* gene expression in healthy volunteers (8).

Statistical analyses

We confirmed the normal distribution of the continuous variables by normal probability plots and the Shapiro-Wilk test. To take into consideration interindividual variability in the variables studied, we investigated possible differences in baseline values between the 2 interventions by a paired *t* test, and we expressed the differences between baseline and postintervention as percentage changes. These percentage changes were calculated as follows: (postintervention value − baseline value)/baseline value × 100.

We evaluated the effect of olive oil interventions compared with baseline, and the differences between treatments, in a mixed linear model. We considered the interaction between treatment (LPCOO or HPCOO) and the pre-post intervention differences as the term of interest and included the following variables as adjustment variables: study period, age, and country of origin of the volunteers. Moreover, taking into consideration the fact that we performed repeated measurements in the study due to the study design (crossover), we used "individual" as a factor of random effect in the model. We checked the period-by-treatment interactions to discard possible carry-over effects. We tested the relations between variables through Pearson's correlation analyses.

In all cases, we considered significant any *P* value <0.05. All of the previous analyses were performed with R software, version 3.0.2 (R Foundation for Statistical Computing) and with SPSS software, version 18.0 (IBM Corporation). Mixed models were adjusted by using the lme4 package in R (11).

Results

General characteristics of the participants. Supplemental Figure 1 shows the design of the study. No significant differences in baseline values were found between our subsamples and the total EUROLIVE study population (**Table 1**). Dietary patterns and energy expenditure in leisure-time physical activity also did not change. As previously reported (4), participants' compliance was correct, because urinary excretions of tyrosol and hydroxytyrosol were 9-fold and 18-fold higher, respectively, after the HPCOO intervention relative to baseline. These increases were significantly greater than those after the LPCOO intervention, which were 0.3-fold and 0.7-fold higher, respectively, and were significant ($P < 0.001$ in both cases). The lipid profile of the volunteers (TGs, total cholesterol, HDL cholesterol, and LDL cholesterol calculated by the Friedewald formula) did not differ between the intervention periods (data not shown).

The consumption of olive oil polyphenols improved the oxidative status of the volunteers. We observed significant decreases in concentrations of oxidized LDL and C18 hydroxy-FAs, equivalent to those previously reported (4). The ratio between the reduced and the oxidized forms of ascorbic acid increased significantly after the HPCOO intervention when compared with baseline values ($P = 0.018$). Data for these and other variables are shown in **Supplemental Table 1**.

Olive oil polyphenols decreased LDL concentrations. We directly determined LDL concentrations by 3 different approaches: directly measured LDL cholesterol, apo B-100 concentrations, and the number of total LDL particles. As shown in **Figure 1**A, after the HPCOO intervention, apo B-100 concentrations and the number of total LDL particles decreased by 5.9% ± 16.6% and 11.9% ± 12.0%, respectively, relative to baseline values. These variables increased by 6.4% ± 16.6% and 4.7% ± 22.0%, respectively, after the LPCOO intervention. Both decreases in apo B-100 concentrations and the number of

TABLE 1 Baseline characteristics and plasma lipid profile of participants in the 2 subsamples of the study compared with the whole EUROLIVE study population[1]

	Subsample		
	Biochemical analyses (n = 25)	Gene expression (n = 18)	EUROLIVE study population (n = 180)
Age, y	32.3 ± 11.2	36.9 ± 12.3	33.2 ± 11.0
Weight, kg	78.2 ± 10.9	78.1 ± 10.9	76.4 ± 10.5
Height, m	1.79 ± 0.08	1.79 ± 0.08	1.79 ± 0.07
Total cholesterol, mmol/L	4.5 ± 1.2	4.8 ± 0.9	4.5 ± 1.1
LDL cholesterol, mmol/L	2.6 ± 1.0	2.8 ± 0.9	2.5 ± 0.9
HDL cholesterol, mmol/L	1.3 ± 0.3	1.4 ± 0.3	1.4 ± 0.3
TGs,[2] mmol/L	1.1 (0.8, 1.4)	1.2 (0.9, 1.5)	1.1 (0.8, 1.5)

[1] Values are means ± SDs unless otherwise indicated. EUROLIVE, Effect of Olive Oil Consumption on Oxidative Damage in European Populations.
[2] Values are medians (first quartile, third quartile).

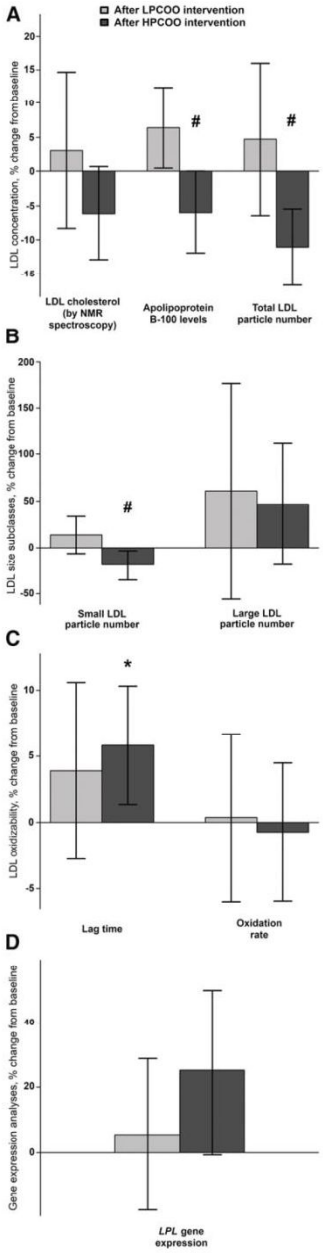

FIGURE 1 Changes from baseline values of directly measured LDL concentrations (determined as LDL cholesterol, apo B-100 concentrations and total LDL particle number) (A), LDL size distribution (B), LDL oxidizability (C), and *LPL* gene expression (D) in healthy European men, aged 20–59 y, after 3-wk consumption of LPCOO or HPCOO. Values are means ± SEMs of percentage changes relative to baseline values; $n = 25$ (for panels A, B, and C) and $n = 18$ (for panel D). *Different from baseline, $P < 0.05$. #Different from LPCOO intervention, $P < 0.05$. HPCOO, high-polyphenol-content olive oil; LPCOO, low-polyphenol-content olive oil; *LPL*, lipoprotein lipase.

total LDL particles after the HPCOO intervention were significant compared with the LPCOO intervention ($P = 0.004$ and $P = 0.013$, respectively).

Olive oil polyphenols decreased the number of small LDL particles. As shown in Figure 1B, the number of small LDL particles decreased by 15.3% ± 35.1% after the HPCOO intervention relative to baseline values. However, after the LPCOO intervention, this value increased by 13.6% ± 36.4% relative to baseline. The decrease in the number of small LDL particles after the HPCOO intervention was significant when compared with the LPCOO intervention ($P = 0.029$). No significant changes in the number of large LDL particles were found after either intervention. High adherence to the consumption of olive oil polyphenols (reflected as an increase in urinary tyrosol excretion) and improvements in oxidative status after the HPCOO intervention (reflected as an increase in the reduced/oxidized ascorbic acid ratio) correlated with greater decreases in small LDL particle numbers ($P = 0.042$, $r = -0.53$, and $P = 0.005$, $r = -0.66$, respectively).

Olive oil polyphenols increased the resistance of LDL against oxidation. As shown in Figure 1C, the LDL oxidation lag time increased by 5.01% ± 10.3% after the HPCOO intervention and by 3.17% ± 19.1% after the LPCOO intervention. After the HPCOO intervention, lag-time values were significantly higher only compared with baseline ($P = 0.038$). Changes in lag time did not differ between interventions. LDL oxidation rate did not change significantly after either intervention.

Changes in LPL gene expression. *LPL* gene expression tended to increase by 26%, relative to baseline, after the HPCOO intervention ($P = 0.08$) (Figure 1D). It did not change significantly after the LPCOO intervention. Changes in this variable did not differ between the interventions.

Discussion

The present work shows that 3-wk consumption of olive oil polyphenols decreased LDL concentrations and LDL atherogenicity in vivo and reveals, to date, some of the most considerable decreases in the number of total and small LDL particles that have been reported in humans due to dietary bioactive compounds.

LDL-cholesterol concentrations are directly and strongly associated with coronary artery disease risk (12). This association justifies their determination in most epidemiologic and interventional cardiovascular studies. Although the direct quantification of LDL cholesterol is possible, in several studies LDL-cholesterol concentrations were calculated by using indirect equations such as the Friedewald formula (an approximation based on TGs and total and HDL cholesterol) (13). These formulas may underestimate LDL-cholesterol concentrations compared with direct measurements, particularly in nonpathologic ranges of TG values. Thus, direct and more precise determinations of apo B-100 concentrations or the total number of LDL particles are recommended (13). Moreover, apo B-100 concentrations and the total number of LDL particles are more accurate than LDL cholesterol to quantify cardiovascular risk in high-risk patients (e.g., individuals who have suffered premature coronary events or with metabolic syndrome) (14) and both are directly related to a greater incidence of cardiovascular events (15, 16).

In this context, we directly assessed the effects of olive oil polyphenols on LDL concentrations. The consumption of olive oil polyphenols was significantly associated with a decrease in apo B-100 concentrations and total number of LDL particles (5.9% and 11.9%, respectively). Similar decreases have been reported after other antioxidant-rich dietary approaches. Concentrations of apo B-100 decreased after consumption of a hazelnut-enriched diet (17) and concentrated red grape juice (18). The number of LDL particles also decreased after long-term consumption of a high-fiber oat cereal (19).

The decrease in LDL concentrations may be explained through an improvement in the systemic oxidative status or by an increase in the gene expression of *LPL*, as observed in our study. Three different mechanisms may be involved in this hypothesis. First, oxidative stress states are associated with increased LDL concentrations, especially due to an increased number of small LDL particles (20). An improved oxidative status due to the consumption of olive oil polyphenols may counteract increases in LDL concentrations by decreasing the number of small LDL particles, as we reported. Second, increases in the expression of *LPL* may help the organism to decrease concentrations of TG-rich lipoproteins (e.g., LDL), because LPL is the main enzyme involved in the removal of TGs from the blood and presents some LDL receptor activity (21). Finally, improvements in general oxidative status have been associated with a better activity of LPL (22, 23).

LDLs are more atherogenic when they are small and dense because *1*) LPL does not recognize them properly, *2*) they easily traverse the endothelial barrier, and *3*) they are easily oxidized in the subendothelial space (24). They are thus associated with early atherosclerosis and high cardiovascular risk (25, 26) and were directly related to a greater incidence of cardiovascular events in some studies (16). In our data, the number of small LDL particles decreased by 15.3% after the consumption of olive oil polyphenols. This decrease was greater when there was a higher adherence to the HPCOO intervention.

The decrease in the number of small LDL particles may be explained by an improvement in oxidative status. As we previously commented, a better oxidative status may result in a lower production of small LDLs, because the number of small LDL particles increases when the levels of oxidative stress are higher (20). This hypothesis concurs with the significant correlation between the decrease in the number of small LDL particles and the increase in the ratio between reduced and oxidized ascorbic acid in our data. In addition, similar effects were also observed after other antioxidant-rich dietary interventions, such as a Mediterranean diet supplemented with nuts (27) and the consumption of a polyphenol-rich supplement made from freeze-dried strawberries (28).

LDL oxidation is considered to be a trigger for the biochemical processes that take place in the subendothelial space and leads to the formation of an atherosclerotic plaque (29). In particular, LDL resistance against oxidation ex vivo predicts artery dysfunction, even when adjusted for other cardiovascular risk factors (30). In our study, olive oil polyphenols increased LDL resistance against oxidation. Increases in LDL antioxidant defenses after the consumption of olive oil polyphenols explain this beneficial effect (5). Our results confirm the decrease in this LDL atherogenic trait after consumption of virgin olive oil (31) and after consumption of an antioxidant-rich vegetarian diet (32).

One of the strengths of the present study is its crossover design, which reduced interference from confounding variables. We administered real-life doses of a food that cannot be consumed in great quantities. Thus, some of the changes observed were modest. However, the LDL-related traits that we describe in this work help to explain residual cardiovascular risk (33) and are directly related to a greater incidence of cardiovascular diseases (15, 16). Therefore, even modest decreases in these variables may be protective against the development of cardiovascular events. A possible limitation of our work was that we performed systemic and gene expression analyses in 2 different subsamples of individuals. However, both subgroups did not present significantly different baseline characteristics and were representative of the whole EUROLIVE population. Other limitations of the study are that the amount of polyphenols equivalent to that provided by the HPCOO intervention could have come from other food types or that synergistic effects between olive oil polyphenols and other olive oil components on LDL biology have not yet been identified.

In conclusion, the consumption of olive oil polyphenols decreased LDL concentrations directly measured as concentrations of apo B-100 and the total number of LDL particles. The consumption of olive oil polyphenols also decreased LDL atherogenicity, as reflected in the smaller number of small LDL particles and enhanced LDL resistance against oxidation. An improved oxidative status and an increased gene expression of *LPL* may contribute to explain these changes. These data support previous evidence indicating that olive oil polyphenols can contribute highly to the control of cardiovascular risk.

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MANUSCRIPT IV

The Mediterranean Diet decreases LDL atherogenicity in high cardiovascular risk individuals: a randomized controlled trial. [Submitted]

The Mediterranean Diet decreases LDL atherogenicity in high cardiovascular risk individuals: a randomized controlled trial

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Running title: **The Mediterranean Diet reduces LDL atherogenicity**

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ABSTRACT

Objective. The Traditional Mediterranean Diet (TMD) protects against cardiovascular disease through several mechanisms such as decreasing low-density lipoprotein (LDL) cholesterol. However, evidence regarding the effects of the TMD on LDL characteristics that make them more atherogenic (resistance against oxidation, composition, size, cytotoxicity) is scarce.

Approach and Results. We assessed the effects of a 1-year intervention with TMD on LDL atherogenic traits in a random sub-sample of individuals from the PREDIMED Study (*N*=210). Two TMDs were compared: one enriched with virgin olive oil (*N*=71) and another with nuts (*N*=68), with respect to a low-fat diet (*N*=71). When analyzing both Mediterranean interventions together, the TMD increased LDL resistance against oxidation (LDL lag time), decreased LDL oxidation *in vivo* (equivalents of malondialdehyde in LDLs), and raised LDL average size (the LDL cholesterol/apolipoprotein B ratio) (all $P<0.05$, relative to control). After both TMDs and the low-fat diet, the content of LDL proteins other than apolipoprotein B decreased ($P<0.001$, in all diet interventions, relative to baseline). After the three interventions we also observed an increase in the content of apolipoprotein C-III in apolipoprotein B-containing lipoproteins relative to baseline ($P<0.001$, in all cases). However, the TMD significantly moderated this increment relative to the low-fat diet ($P=0.031$, when both TMD interventions were analyzed together). Finally, LDL cytotoxicity on macrophages decreased after the TMD intervention supplemented with virgin olive oil ($P=0.019$ and $P=0.052$, relative to baseline and the low-fat diet, respectively).

Conclusions. The TMD improved several traits related to LDL atherogenicity in high cardiovascular risk individuals.

ABBREVIATIONS

ApoB: apolipoprotein B
ApoC-III: apolipoprotein C-III
LDL: low-density lipoprotein
LDL-C: low-density lipoprotein cholesterol
TMD: Traditional Mediterranean Diet
TMD-VOO: TMD intervention supplemented with virgin olive oil
TMD-Nuts: TMD intervention supplemented with nuts
VLDL: very low-density lipoprotein

INTRODUCTION

A consistent body of evidence coming from observational and randomized controlled trial supports that the consumption of a Traditional Mediterranean Diet (TMD) protects against the development of cardiovascular diseases[1]. The PREDIMED Study (*Prevención con Dieta Mediterránea*), a multi-center, parallel, randomized controlled trial, has demonstrated with high degree scientific evidence that a TMD has protective effects on primary cardiovascular disease prevention[2,3]. Due to its richness in dietary antioxidants, and other bioactive molecules, the TMD protects against atherosclerosis by improving blood lipid levels, the oxidative/inflammatory status, the metabolomic profile, and the gene expression associated with the development of cardiovascular diseases[4–8]. The TMD has also been shown to enhance some characteristics related to low-density lipoproteins (LDLs), such as the levels of total LDL particles and oxidized LDLs[9,10]. Besides these properties, there are other characteristics that make an LDL especially atherogenic, including: 1) LDL resistance against oxidative modifications; 2) LDL content of triglycerides, apolipoprotein C-III –ApoC-III– and other proteins; 3) LDL cytotoxic potential on macrophages and endothelial cells; or 4) LDL ability to transfer cholesterol to hepatocytes. Our group has previously studied the effects of a typical TMD food, virgin olive oil, on some of these atherogenic traits[11]. To date, however, the effects of the whole TMD on LDL atherogenicity remain to be fully elucidated.
The aim of the present study was to assess in a random sub-sample of the PREDIMED trial (*N*=210) whether a long-term consumption of a TMD, enriched with virgin olive oil (TMD-VOO) or nuts (TMD-Nuts), could improve a combination of pro-atherogenic LDL traits *in vivo* in humans.

MATERIALS AND METHODS

Materials and Methods are available in the online-only Data Supplement.

RESULTS

Participants

Study design is available in **Supplemental Figure I**. No differences in the baseline characteristics were found among the three groups in our subsample (**Table 1**). With respect to the whole PREDIMED Study population, our volunteers were on average 1.6 years younger, with 9.2% more males, and 6.9% more dyslipidemic individuals at baseline ($P<0.05$) (**Supplemental Table I**). We found no differences in energy expenditure in leisure-time physical activity between interventions. Compliance of the volunteers with the interventions was correct, as observed in the TMD adherence markers and the food frequency questionnaire administered to the participants. The augmented TMD adherence after the TMD-VOO intervention was observed as: 1) increments in the consumption of virgin olive oil, legumes, and fish; and 2) decreases in the intake of red and processed meat, refined olive oil, and precooked meals ($P<0.05$). The augmented TMD adherence after the TMD-VOO intervention was due to: 1) increases in the intake of nuts, virgin olive oil (although less than in the TMD-VOO intervention), and canned and oily fish; and 2) decrements in the consumption of

meat, refined olive oil, precooked meals, and industrial sweets (P<0.05). Finally, adherence to the low-fat diet was reflected as a reduction in the intake of saturated fats, due to decreases in the consumption of whole-fat dairy products, meat (particularly red and processed), processed meals, and industrial sweets (P<0.05) (**Supplemental Tables II-III**).

The changes in the biochemical profile of the volunteers are resumed in **Supplemental Tables IV-V**. As expected, we observed a decrease in the levels of total cholesterol after the low-fat diet (P=0.023 and P=0.014, relative to baseline values and the TMD-VOO intervention). The reduction happened essentially through a decline in LDL cholesterol (LDL-C) levels (P=0.007 and P=0.008, when compared to baseline and the TMD-VOO intervention, respectively). However, the calculated levels of cholesterol in very-low density lipoproteins (VLDLs) lessened after both TMD interventions relative to baseline (P=0.042 and P=0.048, after the TMD-VOO and the TMD-Nuts interventions, respectively). The triglyceride levels decreased after the TMD-Nuts interventions when compared to the low-fat diet (P=0.022).

With respect to apolipoprotein levels in plasma, despite the changes in LDL and VLDL cholesterol levels, the apolipoprotein B (ApoB) levels remained unchanged after the three interventions.

LDL oxidation

LDL resistance against oxidation increased after both TMD interventions. After the TMD-VOO intervention, LDL lag time increased relative to baseline and the low-fat diet (P<0.001 and P=0.004, respectively), and after the TMD-Nuts intervention, it increased significantly relative to baseline and tended to increase with respect to the low-fat diet (P=0.002 and P=0.076, respectively). When analyzing the two TMD interventions together, the increase in lag time was both significant relative to baseline and the low-fat diet (P<0.001 and P=0.006, respectively) (**Figure 1A-1B**).

LDL oxidation *in vivo* (assessed as malondialdehyde equivalents in LDL, adjusted for the cholesterol content in isolated LDLs) was only reduced after the TMD-VOO intervention relative to the low-fat diet (P=0.028). When we analyzed both TMD interventions together, LDL oxidation in vivo decreased significantly relative to baseline and the low-fat diet (P=0.010 and P=0.047, respectively) (**Figure 1C-1D**).

LDL size

The LDL-C/ApoB ratio, an indirect measurement of LDL size, diminished after the low-fat diet relative to baseline (P<0.001). As a result, we observed two borderline increases in LDL size after both TMD interventions relative to the low-fat one (P=0.053 and P=0.090, for the TMD-VOO and the TMD-Nuts interventions, respectively). When studying the two aggregated TMD interventions, we observed a significant increase in LDL size after the TMD group relative to the low-fat diet (P=0.030) (**Figure 2A-2B**).

LDL composition

The ApoB content in isolated LDLs only tended to decrease after the TMD-Nuts intervention relative to the low-fat diet (P=0.059). However, when we assessed the effect of both TMD interventions together, the ApoB content in LDLs declined significantly with respect to the low-fat diet (P=0.048) (**Figure 3A-3B**).

The content of LDL proteins other than ApoB decreased relative to baseline after the three dietary interventions (P=0.003, P=0.004, and P=0.020 for the TMD-VOO, the

TMD-Nuts, and the low-fat diet, respectively) (**Figure 3C-3D**). We found no statistically significant differences among the three interventions.
The ratio between triglycerides and cholesterol in isolated LDLs only tended to decrease after the TMD-Nuts intervention relative to the low-fat diet (P=0.080) (data not shown).
Finally, ApoC-III content in VLDL+LDLs increased relative to baseline after the three dietary interventions (P<0.001, for all of them). However, when comparing the TMD interventions versus the low-fat diet, the TMD tended to moderate these increases (P=0.081 and P=0.059, for the TMD-VOO and the TMD-Nuts interventions, respectively) (**Table 2**). This counteraction was statistically significant when both TMD interventions were analyzed together (P=0.031). The variables related to ApoC-III content in VLDL+LDLs (ApoC-III in plasma, triglycerides and estimated VLDL-C levels) are also available in **Table 2** for the three interventions. ApoC-III levels in plasma increased after the low-fat diet relative to baseline (P=0.010).

LDL cytotoxicity

After the TMD-VOO intervention, the toxicity of LDLs in human macrophages lessened relative to baseline (P=0.019) and was marginally reduced with respect to the low-fat diet (P=0.052) (**Figure 4A-4B**). We found no effects after the TMD-Nuts intervention. Regarding toxicity on endothelial cells, it tended to decrease after the TMD-Nuts intervention with respect to the low-fat diet (P=0.080) (**Figure 4C-4D**). We observed no changes after the TMD-VOO intervention.

Supplemental Table VI contains information about the comparisons between the post- versus pre-intervention values for the three interventions. **Supplemental Table VII** describes the comparisons between the TMD interventions and the low-fat control diet. **Supplemental Table VIII** summarizes the effect of both TMD interventions analyzed together (the post- versus pre-intervention comparisons, and the differences between the TMD and the low-fat diet).

DISCUSSION

Our results indicate that a 1-year intervention with the TMD improves several LDL traits related to LDL atherogenicity (resistance against oxidation, size, composition, and cytotoxicity on cells) in high cardiovascular risk individuals. To the best of our knowledge, this is the first time that the effect of a healthy lifestyle on a battery of novel biomarkers of LDL atherogenicity has been studied in humans.
LDL oxidation is one of the most relevant biochemical events that lead to the formation of an atherosclerotic plaque[12]. Oxidized LDLs are avidly phagocytized by macrophages which leads to their transformation to foam cells[13] , and they also induce cytotoxic responses in endothelial cells[14]. Increased oxidized LDL levels and high susceptibility of LDLs to oxidation have been associated with greater cardiovascular risk[15,16]. In our trial, the TMD (especially when supplemented with virgin olive oil) augmented LDL resistance against oxidation and decreased LDL oxidation *in vivo*. Some of these effects have previously been observed after a TMD intervention[5], real-life doses of virgin olive oil[11], and supplements of antioxidant vitamins[17]. In the case of the TMD-Nuts intervention, the antioxidant protection was potent enough to counteract the

deleterious effects of a high intake of polyunsaturated fatty acids on LDL oxidation[18]. TMD dietary antioxidants may bind to LDLs or preserve other dietary antioxidants in the lipoprotein (such as vitamin E) in a non-oxidized state, in order to increase the resistance of the lipoprotein against oxidative attacks[19] (**Figure 5**).

Reduced size in LDL is another trait that makes it more atherogenic[20]. Small LDLs remain longer in circulation (they interact more poorly with LDL receptors), are more easily oxidized, and traverse the endothelial barrier with less difficulty than large ones[21]. Therefore, high concentrations of small LDLs have been associated with a greater incidence of coronary heart disease[22]. In our trial, with respect to the low-fat diet, the TMD was able to increase LDL size (measured as an indirect marker, the LDL-C/ApoB ratio[23]) and reduce ApoB levels in isolated LDLs. If the ApoB levels decrease and the cholesterol in LDLs remains constant, more cholesterol will be carried by each ApoB unit and LDLs will become larger[24]. In agreement with this, a real-life increase in LDL size through the consumption of virgin olive oil[11] and a TMD supplemented with nuts[9] , but not with a low-fat diet [25], has been observed in several studies. Since oxidative states are linked to an increased number of small LDL particles in circulation[26], the protection due to TMD antioxidants could contribute to reverse this situation.

LDL composition affects the atherogenicity of the particle. On the one hand, protein-rich LDLs are more prone to carry proteins related to inflammation and thrombotic responses, especially in states associated with high cardiovascular risk[27]. Moreover, the most atherogenic LDLs (small, dense, electronegative ones) are protein-rich[27]. On the other hand, triglyceride-rich LDLs are present in high cardiovascular risk states[28] and have been linked to changes in ApoB conformation that hinder its binding to LDL receptors[29]. According to our data, the content of LDL proteins other than than ApoB decreased after the TMD interventions and the low-fat diet. Moreover, LDLs tended to become triglyceride-poor after the TMD-Nuts intervention with respect to the low-fat diet. These changes may reflect the fact that LDLs are less prone to carry pro-inflammatory/pro-thrombotic proteins, less toxic for macrophages, and more likely to bind to LDL receptors[27,29].

Regarding minor LDL proteins, one of the most relevant ones is ApoC-III. ApoC-IIIs increase triglyceride levels in plasma by inducing the release of VLDLs by the liver and the intestinal uptake of triglycerides, and inhibiting the function of lipoprotein lipase. Therefore, ApoC-III is considered an independent cardiovascular risk factor[30], particularly when it is bound to lipoproteins. High levels of ApoC-III bound to ApoB-containing lipoproteins is associated with a greater risk of coronary heart disease[31]. In our trial, the TMD interventions were able to partially reverse the increase in ApoC-III in these lipoproteins relative to the low-fat diet (although after all diets ApoC-III levels in ApoB-100-containing lipoproteins increased). As a possible explanation, ApoC-III levels in plasma rose after the low-fat diet and did not change after the TMD interventions. However, since the volunteers in our study were high cardiovascular risk individuals, some LDL atherogenic traits were likely to worsen after one year.

Atherogenic LDLs are toxic for several cell types. When macrophages phagocyte modified LDLs they begin to release pro-inflammatory signals and finally become foam cells[13]. When modified LDLs access endothelial cells, they induce local oxidative stress and the expression of pro-adhesive signals[12,14]. In the present trial, on the one hand, the TMD-VOO intervention decreased LDL cytotoxicity on human macrophages. In this regard, an *in vitro* treatment with a flavonoid-rich extract has been previously reported

to decrease the cytotoxic response induced by oxidized LDLs on macrophages[32]. On the other hand, the TMD-Nuts intervention marginally decreased the cytotoxic potential of LDLs on endothelial cells. In this respect, an *in vitro* treatment with flavonoids has also been reported to decrease the cytotoxicity of oxidized LDLs on endothelial cells[33]. To the best of our knowledge, however, this is the first time that an intervention in humans has been able to decrease *ex vivo* LDLs cytotoxicity. The improved oxidative status, size, and composition of LDLs after the TMD interventions could help to explain this decrease in LDL cytotoxicity[12].

Besides the changes related to LDL atherogenicity, we observed differences in the cholesterol content of ApoB-100-containing lipoproteins. In the present trial, the TMD interventions decreased the estimated VLDL cholesterol levels of the volunteers and the low-fat diet reduced LDL cholesterol levels. In agreement with this, in previous trials the TMD has shown to be able to decrease VLDL cholesterol levels[34] and a low-fat diet lessened LDL cholesterol concentrations[25]. Considering these effects, and the reduction in triglycerides after the TMD-Nuts intervention, we could hypothesize that a TMD, especially when supplemented with nuts, may promote VLDL conversion into LDL by increasing the VLDL delivery of lipids to peripheral cells. This effect has been reported after other interventions, such as a polyunsaturated fatty acid supplement[35], and could occur through the improved expression of LDL-related receptors (lipoprotein lipase, LDL receptor-related protein 1) due to the joint action of all TMD bioactive components[11,36,37]. The decrease in VLDL levels exclusively after the TMD interventions could also be partially justified by the significant increase in ApoC-III only after the low-fat diet. As previously commented, ApoC-III induces the secretion of VLDLs from the liver and inhibits the activity of lipoprotein lipase[30].

Our study has various strengths. First, its parallel design permitted a long-term follow-up. Second, it combined classic LDL parameters and some novel cell-based techniques that had not been previously performed in a randomized controlled trial of these characteristics. Nevertheless, the study has also limitations. The volunteers were elderly people with high cardiovascular risk values; hence the extrapolation of our results to general population is complex. The results obtained are modest because: 1) the dietary intervention in our trial is based on discreet lifestyle changes; and 2) the low-fat control intervention is a well-known healthy diet. Finally, although the sample selection was random, and the baseline characteristics of the three groups were comparable, they differed modestly from the baseline characteristics of the whole PREDIMED Study population. Differences between the changes observed in our results and other PREDIMED Study sub-samples, particularly relative to the lipid profile, could be due to the longer duration of the intervention in our sub-group (lipid profile changes have been essentially assessed in other sub-samples after a 3-month adherence to a TMD), and the different proportion of patients with dyslipidemia or treated with lipid-lowering drugs. Nevertheless, to take into consideration all the possible confounders, we included age, sex, center, and the classical cardiovascular risk factors in our multivariate linear regression analyses.

In conclusion, the TMD decreased LDL atherogenicity (ameliorating LDL characteristics related to oxidation, size, and composition) and LDL *ex vivo* cytotoxicity. A better oxidative/inflammatory status due to the TMD may have contributed to this general improvement[5,6]. Moreover, the enhanced function of high-density lipoproteins due to the TMD (or some of its typical foods[38]) may also support these benefits *in vivo* (**Figure 6**). This is the first study to report such a set of effects after a healthy, antioxidant-rich

dietary intervention. Our data present an LDL-based mechanism to explain some of the benefits of the Mediterranean Diet and reinforce the previous evidence regarding the cardioprotective effects of this dietary pattern.

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DISCLOSURES

None.

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427

HIGHLIGHTS

- The Traditional Mediterranean Diet increased the resistance of LDLs against oxidation and decreased LDL oxidation *in vivo*.
- The Traditional Mediterranean Diet increased the average LDL size.
- The Traditional Mediterranean Diet and the low-fat diet decreased the LDL content in proteins different than apolipoprotein B (e.g., pro-inflammatory and pro-thrombotic proteins).
- The improvements in LDL size, oxidation and composition due to the Traditional Mediterranean Diet may justify a decreased LDL cytotoxicity on macrophages.
- This work provides an additional mechanism to explain the anti-atherogenic potential of the Traditional Mediterranean Diet.

TABLES

TABLE 1

Baseline features of the volunteers of the study in the three intervention groups.

VARIABLES	TMD-VOO *N*=71	TMD-Nuts *N*=68	Low-fat diet *N*=71	*P*-value
Age (years)	66.5 (6.34)[a]	65.1 (6.85)	64.7 (6.58)	0.270
Sex (% male)	0.45 (0.50)	0.62 (0.49)	0.48 (0.50)	0.111
Body Mass Index (kg/m^2)	30.2 (3.96)	29.2 (3.92)	29.7 (3.98)	0.386
Waist Circumference (cm)	99.8 (10.7)	102 (10.2)	101 (11.5)	0.489
Leisure-time physical activity (MET·min/day)	156 [67.5;247][b]	169 [59.1;323]	150 [15.5;332]	0.782
Smoking status (% of smokers)	16.9%	11.8%	12.7%	0.642
Type 2 diabetes (% of diabetic patients)	76.1%	76.5%	84.5%	0.380
Hypertension (% of hypertensive patients)	47.9%	55.9%	38.0%	0.107
Dyslipidemia (% of dyslipidemic patients)	83.1%	77.9%	85.9%	0.458
Glucose[a] (mg/dL)	105 [92.5;127]	118 [96.0;140]	105 [94.0;128]	0.470
Triglycerides[a] (mg/dL)	108 [90.7;157]	105 [73.0;147]	115 [97.0;140]	0.610
Total cholesterol (mg/dL)	206 (39.1)	198 (35.9)	210 (38.4)	0.231
HDL cholesterol (mg/dL)	49.8 (11.8)	49.2 (10.8)	49.2 (10.6)	0.932
LDL cholesterol (mg/dL)	129 (30.0)	125 (30.1)	135 (33.0)	0.190
Apolipoprotein B (mg/dL)	104 (22.0)	97.6 (17.1)	105 (22.7)	0.121

[a]: Mean (SD). [b]: Median [1st-3rd quartile].

MET: metabolic equivalent of task. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil.

TABLE 2

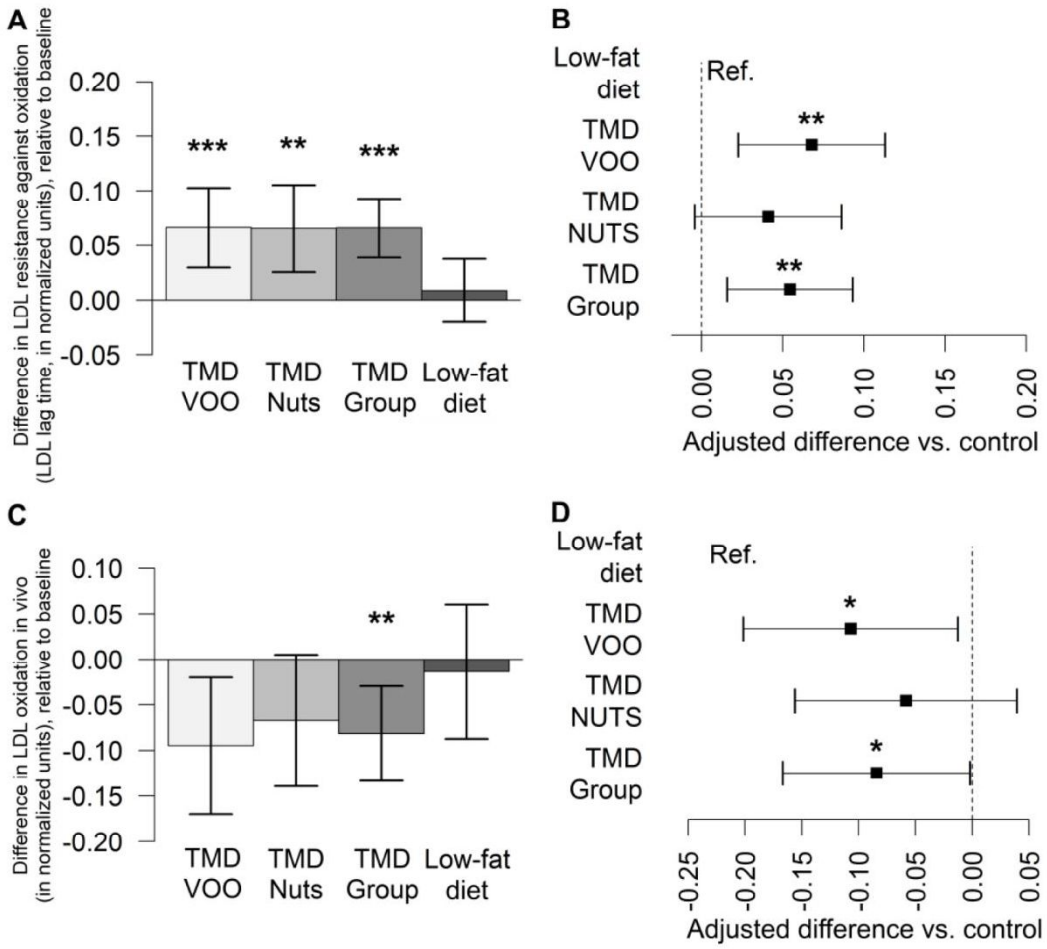
Changes in the characteristics related to apolipoprotein C-III: differences between post- and pre-intervention values after the dietary interventions (paired T-test), and changes after the TMD interventions relative to the low-fat diet (multivariate linear regression model, adjusted for age, sex, participant's center of origin, baseline value of the parameter, and presence of: dyslipidemia, diabetes, hypertension, and tobacco use).

	TMD-VOO		TMD-Nuts		TMD-Group		Low-fat diet		TMD-VOO vs. Low-fat diet		TMD-Nuts vs. Low-fat diet		TMD-Group vs. Low-fat diet	
VARIABLES	**Diff.**	***P*-value**	**Diff.**	***P*-value**	**Diff.**	***P*-value**	**Diff.**	***P*-value**	**Coef.**	***P*-value**	**Coef.**	***P*-value**	**Coef.**	***P*-value**
ApoC-III in ApoB-containing lipoproteins (unitless ratio)	0.006 (0.011)[a]	<0.001	0.007 (0.013)	<0.001	0.006 (0.012)	<0.001	0.010 (0.015)	<0.001	-0.004 [-0.009;5·10^{-4}]	0.081	-0.005 [-0.010;1·10^{-4}]	0.059	-0.005 [-0.009;-5·10^{-4}]	0.031
ApoC-III in plasma (mg/dL)	0.77 (4.17)	0.181	0.48 (3.67)	0.355	0.63 (3.92)	0.104	1.36 (4.07)	0.010	-0.28 [-1.65;1.10]	0.695	-1.25 [-2.66;0.17]	0.087	-0.74 [-1.91;0.44]	0.220
Triglycerides[b] (mg/dL)	0.055 (0.33)	0.174	-0.02 (0.29)	0.572	0.019 (0.31)	0.494	0.071 (0.38)	0.128	-0.001 [-0.11;0.11]	0.979	-0.14 [-0.25;-0.024]	0.018	-0.063 [-0.16;0.032]	0.199
Estimated VLDL cholesterol (mg/dL)	-3.08 (10.6)	0.042	-2.58 (8.90)	0.048	-2.84 (9.79)	0.004	0.66 (11.1)	0.642	-2.65 [-6.41;1.11]	0.169	-3.78 [-7.80;0.24]	0.068	-3.15 [-6.42;0.12]	0.061

[a]: Mean (SD). [b]: log-transformed variables. *ApoB*: apolipoprotein B. *ApoC-III*: apolipoprotein C-III. *Coef.*: Coefficient of the multivariate linear regression, with its 95% confidence interval. *Diff.*: Difference between post- and pre-intervention values. *TMD-Group*: Traditional Mediterranean Diets, supplemented with virgin olive oil and nuts, analyzed together. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil.

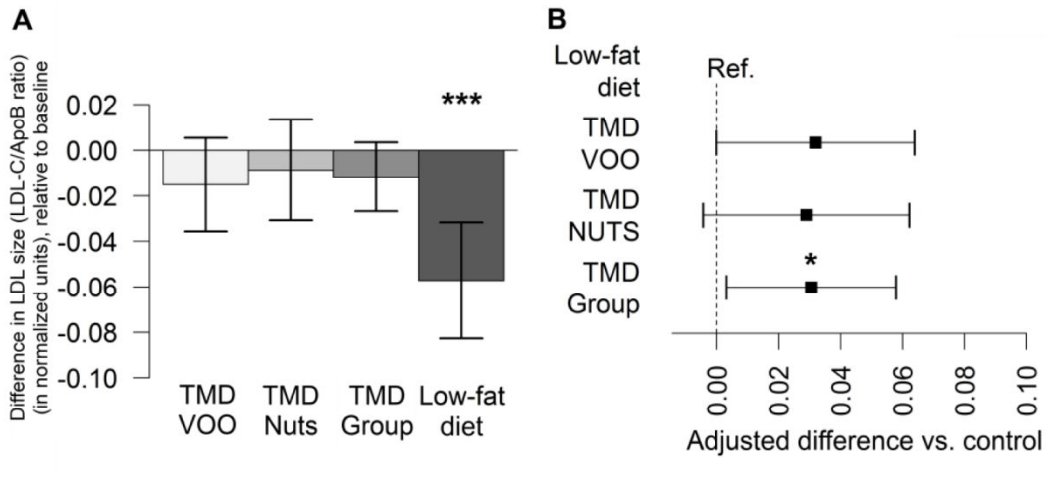
FIGURES

FIGURE 1.



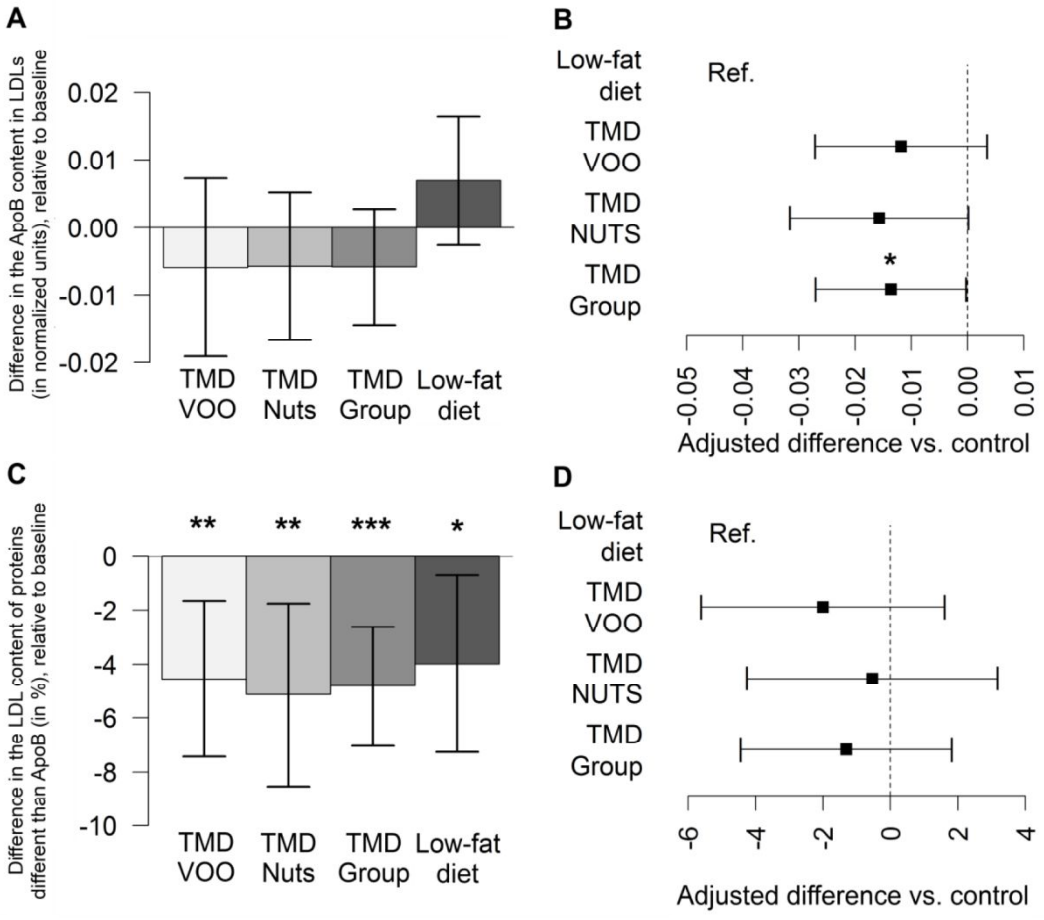
Effects of the Traditional Mediterranean Diet (TMD), supplemented with virgin olive oil (TMD-VOO), nuts (TMD-Nuts), or when both TMD interventions were analyzed together (TMD-Group), relative to a low-fat diet, on the resistance of LDLs against oxidation (LDL lag time) (**A-B**) and the LDL *in vivo* oxidation (**C-D**). **A,C**. Post- vs. pre-intervention changes (mean ± SEM). **B,D**. Inter-treatment differences in a multivariate linear regression model, adjusted for age, sex, center of origin of the volunteer, baseline value of the variable, and baseline presence of: dyslipidemia, diabetes, hypertension, and tobacco use (adjusted coefficient, with IC95%). *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

FIGURE 2.



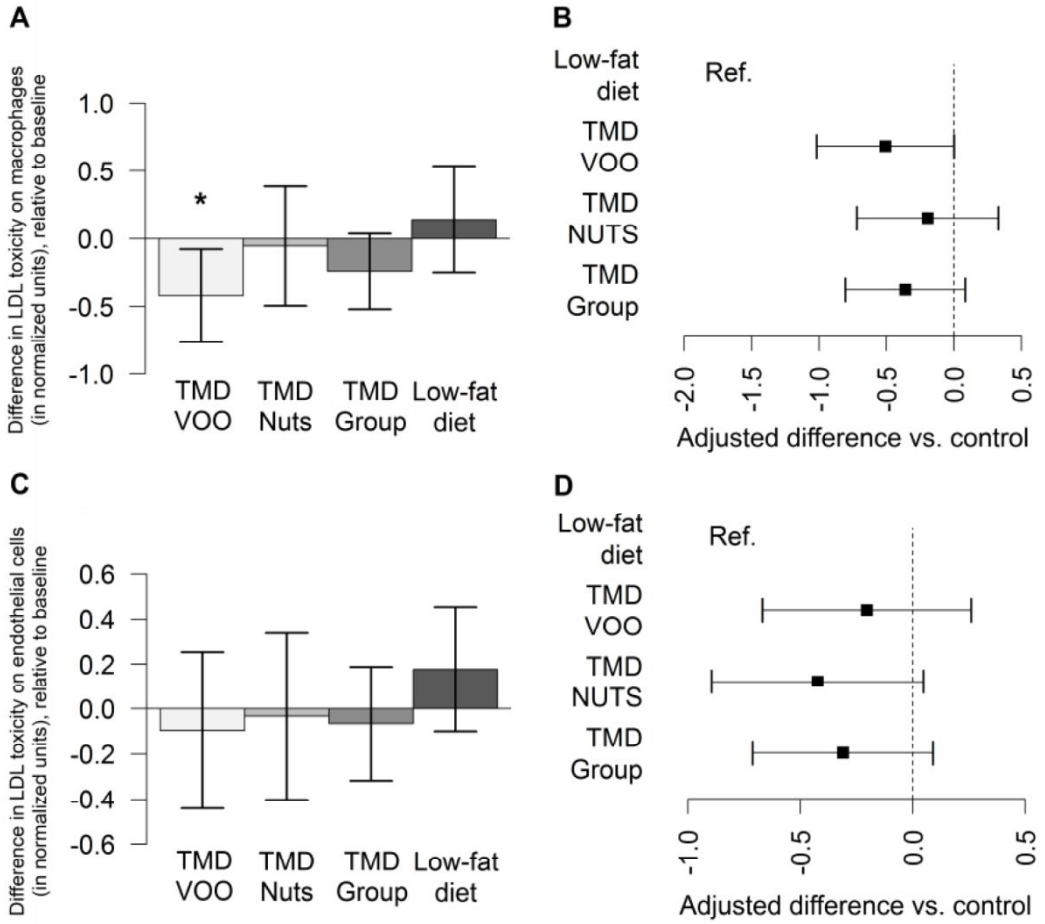
Effects of the Traditional Mediterranean Diet, supplemented with virgin olive oil (TMD-VOO), nuts (TMD-Nuts), or when both TMD interventions were analyzed together (TMD-Group), relative to a low-fat diet, on LDL size (the LDL cholesterol/apolipoprotein B ratio) (**A-B**). **A**. Post- vs. pre-intervention changes (mean ± SEM). **B**. Inter-treatment differences in a multivariate linear regression model, adjusted for age, sex, center of origin of the volunteer, baseline value of the variable, and baseline presence of: dyslipidemia, diabetes, hypertension, and tobacco use (adjusted coefficient, with IC95%). *: $P<0.05$; ***: $P<0.001$.

FIGURE 3.



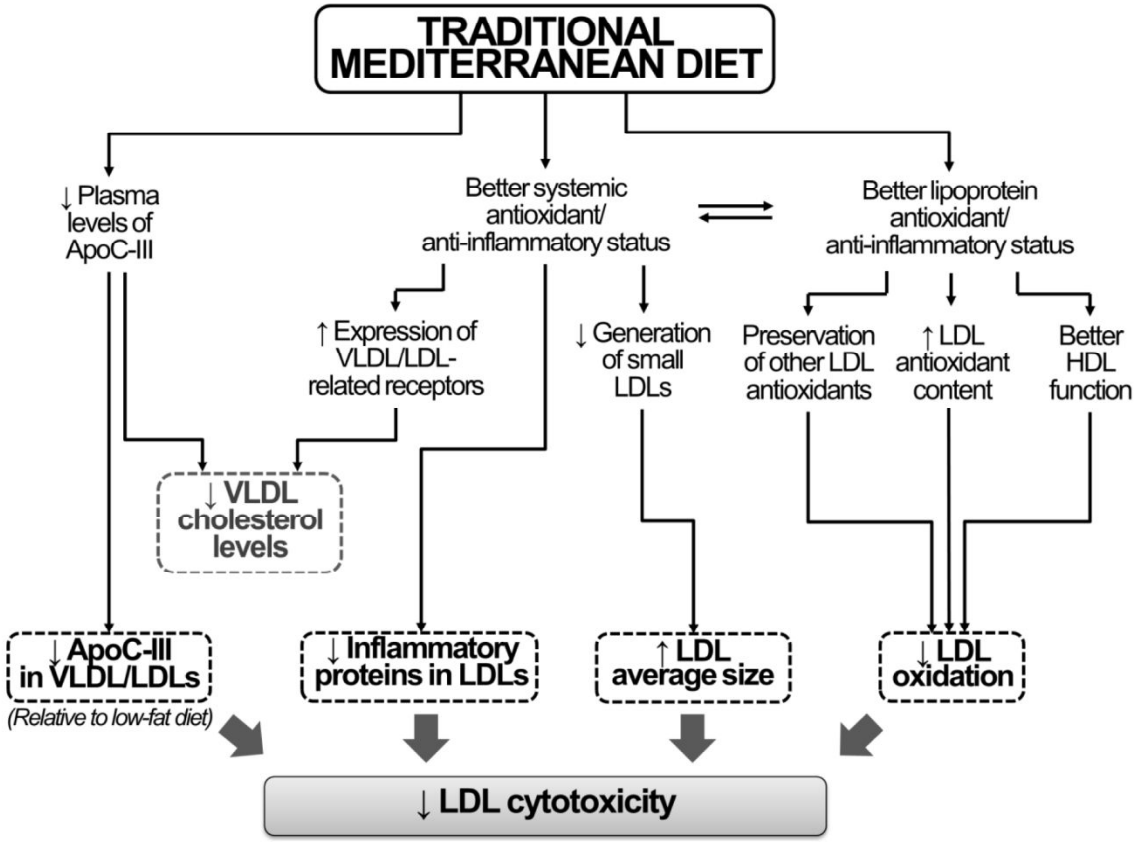
Effects of the Traditional Mediterranean Diet, supplemented with virgin olive oil (TMD-VOO), nuts (TMD-Nuts), or when both TMD interventions were analyzed together (TMD-Group), relative to a low-fat diet, on the content of apolipoprotein B in LDLs (**A-B**), and the percentage of LDL proteins different than apolipoprotein B (**C-D**). **A,C**. Post- vs. pre-intervention changes (mean ± SEM). **B,D**. Inter-treatment differences in a multivariate linear regression model, adjusted for age, sex, center of origin of the volunteer, baseline value of the variable, and baseline presence of: dyslipidemia, diabetes, hypertension, and tobacco use (adjusted coefficient, with IC95%). *: *P*<0.05; **: *P*<0.01; ***: *P*<0.001.

FIGURE 4.



Effects of the Traditional Mediterranean Diet, supplemented with virgin olive oil (TMD-VOO), nuts (TMD-Nuts), or when both TMD interventions were analyzed together (TMD-Group), relative to a low-fat diet, on the cytotoxicity of LDLs on macrophages (**A-B**), and the cytotoxicity of LDLs on endothelial cells (**C-D**). **A,C**. Post- vs. pre-intervention changes (mean ± SEM). **B,D**. Inter-treatment differences in a multivariate linear regression model, adjusted for age, sex, center of origin of the volunteer, baseline value of the variable, and baseline presence of: dyslipidemia, diabetes, hypertension, and tobacco use (adjusted coefficient, with IC95%). *: *P*<0.05.

FIGURE 5.



Hypothetical mechanisms for a decreased LDL atherogenicity after a Traditional Mediterranean Diet. The adherence to a Traditional Mediterranean Diet improved the antioxidant/anti-inflammatory status in the systemic biochemical profile and also in lipoproteins. These changes led to a general improvement in several LDL characteristics (size, oxidation, composition), that induced a decrease of LDL cytotoxic response.

SUPPLEMENTAL MATERIAL

DETAILED METHODS

STUDY DESIGN

Our trial studied a random subsample of volunteers from the PREDIMED Study (*Prevención con Dieta Mediterránea*). The PREDIMED Study was a randomized, controlled, large-scale (*N*=7447), parallel, multicenter trial that assessed the long-term effects of a Traditional Mediterranean Diet (TMD) on the primary prevention of cardiovascular events in a high cardiovascular risk population[1]. Participants were randomly allocated to one of the following intervention groups: 1) a TMD enriched with virgin olive oil (TMD-VOO); 2) a TMD enriched with nuts (TMD-Nuts); and 3) a low-fat control diet, following the indications of the American Heart Association[2]. Local institutional ethic committees approved the protocol of the study, and all volunteers provided a signed informed consent before entering the trial. Further details of the study have been previously published[1]. The PREDIMED Study protocol was registered with the International Standard Randomized Controlled Trial Number ISRCTN35739639 (www.controlled-trials.com).

In this trial, we studied the effects of a TMD on the characteristics related to the atherogenicity of low-density lipoproteins (LDL) in a random subsample of 210 volunteers from the PREDIMED Study, before and after one year of intervention (*N*=71 for the TMD-VOO group, *N*=68 for the TMD-Nuts group, and *N*=71 for the low-fat diet control group). Blood samples from the volunteers were collected in fasting state before and after the intervention, stored at -80ºC and kept frozen until the project started. In all the biochemical and cellular determinations, pre- and post-interventions samples were studied in the same analysis run.

CLINICAL DATA, DIETARY ADHERENCE, AND PHYSICAL ACTIVITY

At baseline and post-intervention, information was gathered regarding: 1) the general clinical status of the volunteers (sex, age, weight, waist circumference height, blood pressure); 2) their adherence to the TMD (through a 14-item short questionnaire of adherence, a long food frequency questionnaire of the previous year, and specific compliance biomarkers for each TMD intervention); and 3) their levels of physical activity (through a validated Minnesota Leisure-Time Physical Activity questionnaire)[1,3].

DIETARY INTERVENTION

Taking into consideration the baseline adherence of the volunteers to the TMD, and the dietary intervention they had been assigned to, the participants received personalized dietary assistance in the first session of the trial. Those allocated to the low-fat control group were instructed to moderate their consumption of all sources of fat (all type of oils, butter, nuts, fatty meat, fatty fish)[2]. Volunteers assigned to the TMD interventions were instructed to maintain their habitual caloric intake and to increase their adherence to some classical features of the TMD through several dietary strategies: 1) to use olive oil as main culinary fat in all their meals; 2) to increase their general consumption of vegetal foods in all their meals (vegetables, fruit, legumes, nuts); 3) to substitute red or processed meat for fish or white meat; 4) to prepare more homemade dishes with a basis of stir-fried onion, garlic, tomato, and aromatic herbs; and 5) to drink red wine moderately at meals (1 small glass) whenever the volunteers habitually consumed alcohol[4]. To reinforce their adherence to the dietary interventions, participants in the TMD-VOO group received without charge 1 L/week of virgin olive oil, those in the TMD-

Nuts group received 210 g/week of mixed nuts, and those in the low-fat control group received small pecuniary presents. Moreover, participants could contact the team of dietitians of the study at any moment and were invited to group sessions to receive: 1) a specific description of the recommended foods for each dietary group (typical foods of the Mediterranean dietary pattern for the TMD groups, or recommendations according to the American Heart Association for the low-fat control group); 2) shopping lists and seasonal suggestions; 3) adapted meal programs, personalized for each dietary group; and 4) specific recipe suggestions.

SAMPLE OBTENTION

K3-EDTA plasma samples were obtained from the blood collected from the participants before and post-intervention. The samples did not suffer any thaw-freeze cycles before our experiments. We isolated LDLs from a plasma aliquot by means of a density gradient ultracentrifugation[5]. Apolipoprotein-B depleted plasma samples from the volunteers were also available[6]. Both sample types were stored at -80ºC until required.

BIOCHEMICAL PROFILE

We performed all systemic determinations in plasma samples in an ABX Pentra-400 autoanalyzer (Horiba-ABX). We determined the levels of glucose, triglycerides, and total cholesterol by enzymatic methods (ABX Pentra Glucose HK CP, ABX Pentra Triglycerides CP, and ABX Pentra Cholesterol CP, all from Horiba Diagnostics), the levels of HDL cholesterol by the Accelerator Selective Detergent method (ABX Pentra HDL Direct CP, Horiba Diagnostics), and the levels of apolipoprotein B (ApoB) (ABX Pentra Apo B, Horiba Diagnostics) and apolipoprotein C-III (ApoC-III) (Apolipoprotein C-III, Spinreact) by immunoturbidimetry. The inter-assay coefficients of variation (CVs) of these determinations were: 1.91% for glucose, 4.07% for triglycerides, 1.24% for total cholesterol, 1.79% for HDL cholesterol, 1.59% for ApoB and 3.25% for ApoC-III. We also calculated LDL cholesterol levels (according to the Friedewald formula, whenever triglycerides were <300 mg/dL).

In ApoB-depleted plasma samples (plasma in which all lipoproteins but HDL have been precipitated[6]) we determined the levels of cholesterol (Cholesterol-LQ, Spinreact), again in an ABX Pentra-400 autoanalyzer (Horiba-ABX). With these values, we calculated the amount of estimated cholesterol in VLDLs (total cholesterol – cholesterol in ApoB-depleted plasma – calculated LDL cholesterol levels).

LDL OXIDATION

LDL *in vitro* resistance against oxidation. We incubated isolated LDLs with an oxidizing agent ($CuSO_4$) to assess their resistance to accumulate Cu^{2+}-induced conjugated dienes. We dialyzed LDLs against PBS in *PD-10 Desalting Columns* (GE Healthcare) and incubated them (final concentration: 10 mg cholesterol/dL) with $CuSO_4$ (final concentration: 5 µM) in 96-well transparent plates at 37ºC in an Infinite M200 reader (Tecan Ltd). We measured absorbance at 234 nm every 3 minutes for 4 hours to obtain the curves of LDL oxidation. In the curves, we calculated the lag time (the time when maximal oxidation started, in minutes)[5]. High lag time values are associated with LDLs that are more resistant to oxidation. We ran samples in duplicate, and allowed no intra-repetition CVs over 15%. To minimize inter-assay variability, we included in every experiment a pool of LDLs (isolated from a pool of plasma of 20 healthy donors). We divided all LDL lag time values of the samples by the LDL lag time

value of the pool, to obtain normalized ratios without units. The inter-assay CV was 12.4%.

LDL oxidation degree *in vivo*. First, we measured the oxidation levels of the LDLs (the malondialdehyde equivalents in the ABDP of the volunteers) by the thiobarbituric reactive acid species technique in isolated LDL samples[7]. Then, we divided the malondialdehyde equivalents by the cholesterol content in each LDL sample (see below, "LDL composition").

We ran the oxidation test in duplicate, and did not permit intra-repetition coefficients of variation over 15%. To reduce inter-assay variation, we included a pool of LDL from 20 healthy volunteers in each experiment. Finally, we divided the value of the LDL oxidation degree of each sample by the value in the pool, to obtain unitless, normalized ratios. The inter-assay coefficient of variation was 9.21%.

LDL AVERAGE SIZE

From the data of the plasma lipid profile of the volunteers, we calculated an approximation of LDL average size, the LDL cholesterol/ApoB ratio, without units. Low ratio values are associated with small LDL average size[8,9].

LDL COMPOSITION

We analyzed the composition of isolated LDLs in an ABX Pentra-400 autoanalyzer (Horiba-ABX). We measured the levels of triglycerides (ABX Pentra Triglycerides CP, Horiba Diagnostics) and cholesterol (Cholesterol-LQ, Spinreact) by enzymatic methods, total protein (ABX Pentra Total Protein CP, Horiba Diagnostics) by the Biuret reaction, and ApoB (ABX Pentra Apo B, Horiba Diagnostics) by immunoturbidimetry. The inter-assay CVs of these measurements were: 4.62% for triglycerides, 3.86% for cholesterol, 2.47% for total protein, and 1.59% for ApoB.

From these values, we calculated the triglyceride/cholesterol ratio and the ApoB content in isolated LDLs (adjusted for cholesterol in isolated LDLs), and the percentage of LDL proteins other than ApoB, as follows: (total protein in LDL – ApoB in LDL)/total protein in LDL x100.

In parallel, we determined some parameters related to ApoC-III in ApoB-containing lipoproteins (VLDL+LDLs). We first measured the ApoC-III levels in ApoB-depleted plasma (HDL) in an ABX Pentra-400 autoanalyzer, using the specific reagent from Spinreact (inter-assay CV: 3.25 %). We then calculated the concentration of ApoC-III in VLDL+LDLs (ApoC-III levels in plasma – ApoC-III levels in ApoB-depleted plasma), and adjusted the values for the quantity of cholesterol in ApoB-containing lipoproteins (cholesterol in plasma – cholesterol in ApoB-depleted plasma).

LDL CYTOTOXICITY ON MACROPHAGES

Human THP-1 were grown in RPMI-1640 medium (complemented with 10% fetal bovine serum, 1% penicillin-streptomycin, 1% L-glutamine, and 1% sodium pyruvate), refreshed every 72h, and differentiated into macrophages (by incubating them with 200 nM phorbol-myristate-acetate –Sigma–, for 96h). Next, we washed the macrophages and incubated them with isolated LDLs (concentration: 10 mg/dL LDL cholesterol) or without LDLs (as negative control), for 16h. After the incubation, we washed the cells and incubated them with 0.5 mg/mL soluble MTT bromide (Thiazolyl Blue Tetrazolium bromide, Sigma), during 4h. Then, we removed the supernatant, washed the cells again, and dissolved the cell content (and the MTT-formazan crystals inside the cells)

with DMSO (Sigma), for 1h under stirring. Finally, we measured absorbance at 570 nm in an Infinite M200 reader (Tecan Ltd). If the viability of the cells is high, they transform the soluble MTT pigment more rapidly into insoluble MTT-formazan crystals, and the absorbance of the DMSO-dissolved cell content is higher. Therefore, high LDL cytotoxicity is related to low MTT-absorbance values.

To calculate the index of LDL cytotoxicity in macrophages, we subtracted the blank (absorbance of the cells non-treated with MTT) from all absorbance values, and calculated the difference in the MTT-absorbance in the LDL-treated cells versus the untreated cells (the negative control): (MTT-absorbance in LDL-treated cells – MTT-absorbance in untreated cells)/MTT-absorbance in untreated cells*100.

We analyzed samples in duplicate, and did not allow intra-repetition CVs over 15%. To minimize inter-assay variability, we included an LDL pool (isolated from the plasma of 20 healthy volunteers) in each experiment. We divided LDL cytotoxicity values of the samples by the values of the pool, to obtain normalized ratios without units. The inter-assay CV of the experiment (*N*=7) was 35.5%.

LDL CYTOTOXICITY ON ENDOTHELIAL CELLS

We cultured human umbilical vein endothelial cells (HUVECs) in supplemented EGM-2 medium (Lonza). We refreshed the cells each 48-72h and seeded them to 80-90% confluence 24h before the experiments. We then washed the cells and incubated them in fresh EGM-2 medium (in this case, supplemented with 0.75% bovine serum albumin, 1% fetal bovine serum, and 1% penicillin-streptomycin), with isolated LDLs (concentration: 10 mg/dL LDL cholesterol) or without LDLs (as negative control), for 4h. After the incubation, we washed the cells, and measured cell viability by the MTT technique (as described in the "LDL cytotoxicity in macrophages" section). As previously described, if the MTT-absorbance was high after the incubations, then the LDL cytotoxicity on endothelial cells was low.

We ran samples in duplicate and no intra-repetition of CVs over 15% was allowed. To minimize inter-assay variability, we included an LDL pool (isolated from the plasma of 20 healthy volunteers) in each experiment. We divided LDL cytotoxicity values of the samples by the values of the pool, to obtain unitless, normalized ratios. The inter-assay CV of the experiment (*N*=9) was 27.5%.

SAMPLE SIZE CALCULATION

A sample size of 68 participants per group allowed ≥ 80% power to detect a significant difference of 0.05 points in LDL lag time values (expressed as normalized units) between pre-and post-intervention values, and of 0.07 points among the three interventions, considering a 2-sided type I error of 0.05, a loss rate of 1%, and the standard deviation of the differences in normalized LDL lag time values (SD=0.144) after an analogous dietary intervention[5].

STATISTICAL ANALYSES

First, we examined the distribution of continuous variables in normality plots and the Shapiro-Wilk test, and log-transformed the non-normally distributed variables. To look for possible differences in the baseline characteristics of our subsample and the whole PREDIMED population, we performed a T-test. To investigate possible differences in baseline values among the three intervention groups (confounders), we carried out a one-way ANOVA.

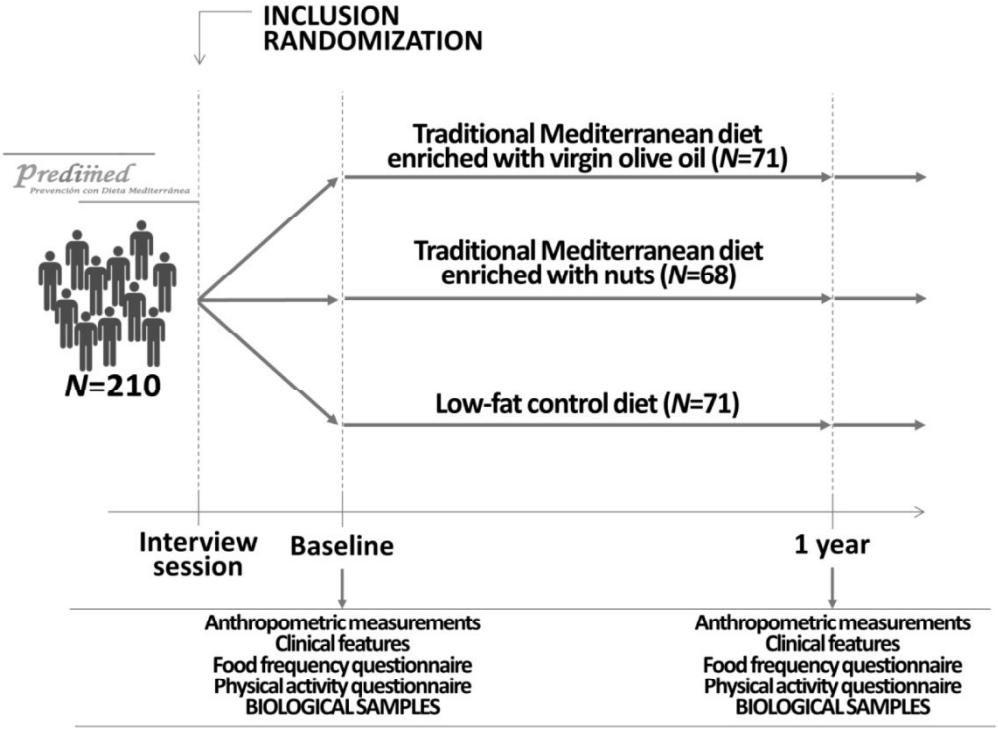
We studied the differences between pre- and post-intervention values after the three interventions in a paired T-test. We also analyzed the effects of the TMD interventions (relative to the low-fat diet) on the changes in the variables of interest in a multivariate regression analysis (using two dummy variables, one for each intervention group) adjusted for: sex, age, center of origin of the volunteer (*k*-1 dummy variables), baseline value of the variable, presence of hypercholesterolemia, diabetes, and hypertension at baseline, and smoking status at baseline.

In parallel, we grouped all the individuals in the TMD interventions to assess the behavior of the TMD intervention as a whole. Again, we assessed the difference between pre- and post-intervention values in a paired T-test, and the effect of the TMD relative to the low-fat diet on the changes in the variables of interest in a multivariate linear regression analysis, adjusted for the same variables as previously described.

We accepted any *P*-value <0.05 as significant. We performed the previous analyses in R Software, version 3.0.2 (*R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria*).

SUPPLEMENTAL FIGURES

SUPPLEMENTAL FIGURE I. Study design (*N*=210).



SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE I

Baseline characteristics of the study volunteers (*N*=296), and comparison with the baseline characteristics of the whole population of the PREDIMED Study (*N*=7237).

	PREDIMED Study	Sample (LDL study)	
VARIABLES	***N*=7237**	***N*=210**	***P*-value**
Age (years)	67.0 (6.19)	65.4 (6.60)	0.001
Sex (% male)	3057 (42.2%)	108 (51.4%)	0.010
Body Mass Index (kg/m^2)	30.0 (3.85)	29.7 (3.95)	0.349
Waist Circumference (cm)	100 (10.3)	101 (10.8)	0.401
Leisure-time physical activity (MET·min/day)	175 [65.1;319]	156 [47.8;319]	0.172
Smoking status (% of smokers)	1018 (14.1%)	29 (13.8%)	0.996
Type 2 diabetes (% of diabetic patients)	3533 (48.8%)	99 (47.1%)	0.683
Hypertension (% of hypertensive patients)	5990 (82.8%)	173 (82.4%)	0.957
Dyslipidemia (% of dyslipidemic patients)	5218 (72.1%)	166 (79.0%)	0.032
Distribution among intervention groups:			0.959
TMD-VOO	2472 (34.2%)	71 (33.8%)	
TMD-Nuts	2386 (33.0%)	68 (32.4%)	
Low-fat control diet	2379 (32.9%)	71 (33.8%)	

[a]: Mean (SD). [b]: Median [1st-3rd quartile]. *MET*: metabolic equivalent of task. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts.

SUPPLEMENTAL TABLE II

Differences between post- and pre-intervention values in the dietary profile of the volunteers in the three interventions of the study.

	TMD-VOO		TMD-Nuts		Low-fat control diet	
VARIABLES	**Difference**	***P*-value**	**Difference**	***P*-value**	**Difference**	***P*-value**
Adherence to TMD (score)	1.49 (1.86)	<0.001	1.49 (2.04)	<0.001	0.18 (2.03)	0.450
Total energy intake (kcal/day)	79.7 (523)	0.204	46.8 (622)	0.537	-33.6 (647)	0.662
Carbohydrates (g/day)	1.60 (73.8)	0.855	-9.34 (79.9)	0.339	8.19 (90.7)	0.449
Proteins (g/day)	4.42 (20.7)	0.076	-0.95 (23.3)	0.738	-4.66 (24.6)	0.115
Total fats (g/day)	5.84 (25.5)	0.058	10.6 (29.9)	0.005	-5.20 (29.8)	0.145
MUFAs (g/day)	5.22 (13.2)	0.001	7.10 (15.5)	<0.001	-1.64 (15.5)	0.377
SFAs (g/day)	-0.89 (7.46)	0.321	-1.15 (8.22)	0.253	-3.27 (9.62)	0.005
PUFAs (g/day)	0.81 (6.43)	0.292	3.68 (7.81)	<0.001	-0.16 (6.63)	0.836
Fiber (g/day)	0.43 (8.02)	0.650	1.89 (10.4)	0.137	0.34 (10.3)	0.783
Vegetables (g/day)	-17.9 (138)	0.286	23.5 (142)	0.176	10.5 (157)	0.576
Fruits (g/day)	9.59 (200)	0.687	1.96 (162)	0.921	-44.7 (234)	0.115
Legumes (g/day)[b]	0.17 (0.60)	0.019	0.081 (0.52)	0.211	-0.032 (0.47)	0.571
Starchy foods (g/day)	12.2 (119)	0.390	-5.05 (114)	0.717	25.4 (129)	0.101
Whole grains (g/day)[b]	-0.26 (1.91)	0.256	-0.18 (2.24)	0.500	0.49 (2.19)	0.065
Refined grains (g/day)	7.26 (83.6)	0.470	4.40 (102)	0.726	21.5 (132)	0.175
Dairy products (g/day)	8.14 (181)	0.706	-16.6 (171)	0.427	-60.3 (220)	0.024
Low-fat dairy products (g/day)[b]	0.36 (1.87)	0.116	0.046 (1.65)	0.817	0.05 (1.75)	0.811
High-fat dairy products (g/day)[b]	-0.44 (1.87)	0.049	-0.44 (1.95)	0.071	-0.45 (1.87)	0.047
Meat products (g/day)	-9.62 (50.1)	0.110	-15.8 (49.3)	0.011	-30.2 (55.9)	<0.001
Red meat (g/day)	-10.5 (31.8)	0.007	-8.64 (38.0)	0.065	-17.4 (42.2)	<0.001
White meat (g/day)	6.61 (34.8)	0.114	-5.49 (34.7)	0.197	-4.34 (29.6)	0.221
Processed meat (g/day)	-4.03 (15.6)	0.033	-3.13 (16.7)	0.128	-7.11 (19.2)	0.003
Fish products (g/day)	26.4 (47.4)	<0.001	1.96 (52.9)	0.761	12.6 (51.7)	0.044
White fish and seafood (g/day)	12.7 (39.6)	0.009	-7.84 (36.3)	0.082	6.46 (44.0)	0.220
Canned fish (g/day)	2.52 (10.2)	0.042	3.59 (11.9)	0.015	0.47 (12.1)	0.746

Oily fish (g/day)	10.6 (25.4)	<0.001	7.86 (25.1)	0.012	5.37 (21.3)	0.037
Precooked meals (g/day)[b]	-0.35 (1.19)	0.016	-0.38 (1.26)	0.016	-0.37 (1.19)	0.010
Industrial sweets (g/day)[b]	-0.29 (1.76)	0.172	-0.58 (1.38)	<0.001	-0.50 (1.61)	0.010
Total olive oil (g/day)	12.1 (15.1)	<0.001	10.3 (17.6)	<0.001	2.71 (17.3)	0.191
Virgin olive oil (g/day)	33.3 (19.3)	<0.001	15.8 (21.0)	<0.001	5.86 (24.2)	0.046
Refined olive oil (g/day)	-21.2 (19.7)	<0.001	-5.50 (21.5)	0.039	-3.15 (18.9)	0.164
Nuts (g/day)[b]	0.21 (1.36)	0.202	1.26 (1.28)	<0.001	-0.014 (1.49)	0.937
Wine (mL/day)[b]	-0.049 (1.30)	0.750	-0.39 (1.55)	0.040	-0.28 (1.63)	0.153
Beer (mL/day)[b]	-0.10 (1.33)	0.516	-0.85 (1.98)	<0.001	-0.42 (1.67)	0.037

[a]: Mean (SD). [b]: Log-transformed variables.

TMD-VOO: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts.

232 **SUPPLEMENTAL TABLE III**

233 Differences between the Traditional Mediterranean Diet interventions and the low-fat control diet in the dietary profile of the volunteers.

	TMD-VOO vs. Low-fat control diet		TMD-Nuts vs. Low-fat control diet	
VARIABLES	**Coefficient β [CI 95%][a]**	**P-value**	**Coefficient β [CI 95%][a]**	**P-value**
Adherence to TMD (score)	0.98 [0.45;1.51]	<0.001	1.27 [0.72;1.81]	<0.001
Total energy intake (kcal/day)	73.1 [-74.1;220]	0.332	110 [-40.4;261]	0.153
Carbohydrates (g/day)	-8.65 [-30.5;13.2]	0.439	-17.8 [-40.2;4.54]	0.120
Proteins (g/day)	5.50 [-0.79;11.8]	0.088	5.31 [-1.12;11.7]	0.107
Total fats (g/day)	9.84 [3.08;16.6]	0.005	17.5 [10.6;24.5]	<0.001
MUFAs (g/day)	6.79 [3.13;10.4]	<0.001	8.78 [5.02;12.5]	<0.001
SFAs (g/day)	1.28 [-0.69;3.25]	0.203	2.57 [0.54;4.60]	0.014
PUFAs (g/day)	1.58 [-0.01;3.18]	0.053	5.43 [3.77;7.08]	<0.001
Fiber (g/day)	0.31 [-2.48;3.10]	0.830	1.31 [-1.57;4.19]	0.374
Vegetables (g/day)	-13.2 [-56.5;30.2]	0.553	0.81 [-43.1;44.7]	0.971
Fruits (g/day)	17.1 [-38.4;72.6]	0.546	14.8 [-42.2;71.7]	0.612
Legumes (g/day)[b]	0.21 [0.054;0.37]	0.009	0.16 [-0.001;0.32]	0.053
Starchy foods (g/day)	-12.0 [-42.9;18.9]	0.447	-31.0 [-62.8;0.74]	0.057
Whole grains (g/day)[b]	-0.64 [-1.26;-0.023]	0.043	-0.34 [-0.98;0.30]	0.294
Refined grains (g/day)	-14.4 [-44.1;15.3]	0.343	-26.0 [-56.5;4.48]	0.096
Dairy products (g/day)	36.3 [-22.6;95.2]	0.229	24.2 [-36.2;84.6]	0.432
Low-fat dairy products (g/day)[b]	0.032 [-0.46;0.53]	0.899	-0.049 [-0.56;0.46]	0.851
High-fat dairy products (g/day)[b]	0.18 [-0.38;0.73]	0.532	0.32 [-0.25;0.89]	0.276
Meat products (g/day)	6.17 [-8.67;21.0]	0.416	9.49 [-5.89;24.9]	0.228
Red meat (g/day)	1.96 [-7.32;11.2]	0.679	3.38 [-6.18;12.9]	0.489
White meat (g/day)	5.14 [-4.67;14.9]	0.306	-0.097 [-10.2;10.0]	0.985
Processed meat (g/day)	-2.34 [-7.15;2.46]	0.341	3.38 [-1.56;8.32]	0.182
Fish products (g/day)	13.7 [-0.21;27.5]	0.055	0.48 [-14.0;15.0]	0.948
White fish and seafood (g/day)	1.49 [-10.3;13.2]	0.804	-9.51 [-21.7;2.72]	0.129

Oily fish (g/day)	0.70 [-2.42;3.83]	0.659	2.06 [-1.12;5.24]	0.205
Canned fish (g/day)	10.6 [3.47;17.8]	0.004	9.30 [1.90;16.7]	0.015
Precooked meals (g/day)[b]	-0.013 [-0.38;0.35]	0.944	0.17 [-0.21;0.55]	0.381
Industrial sweets (g/day)[b]	-0.27 [-0.76;0.22]	0.276	-0.13 [-0.64;0.37]	0.606
Total olive oil (g/day)	10.1 [5.84;14.4]	<0.001	5.09 [0.67;9.50]	0.025
Virgin olive oil (g/day)	28.2 [22.5;34.0]	<0.001	10.3 [4.35;16.2]	<0.001
Refined olive oil (g/day)	-17.9 [-22.8;-12.9]	<0.001	-4.99 [-10.1;0.064]	0.054
Nuts (g/day)[b]	0.32 [-0.027;0.66]	0.073	1.60 [1.24;1.96]	<0.001
Wine (mL/day)[b]	0.20 [-0.30;0.69]	0.432	0.051 [-0.46;0.56]	0.845
Beer (mL/day)[b]	-0.026 [-0.53;0.48]	0.919	-0.20 [-0.73;0.32]	0.444

[a]: β coefficient of the multivariate linear regression model, adjusted by: sex, age, volunteer's center of origin, baseline value of the variable, and change in the use of lipid-lowering drugs. [b]: log-transformed variables. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts.

SUPPLEMENTAL TABLE IV

Differences between post- and pre-intervention values in the biochemical profile of the volunteers in the three study interventions.

	TMD-VOO		TMD-Nuts		Low-fat control diet	
VARIABLES	**Difference**	***P*-value**	**Difference**	***P*-value**	**Difference**	***P*-value**
Glucose[a] (mg/dL)	-0.026 (0.21)[b]	0.314	0.013 (0.22)	0.642	0.021 (0.20)	0.377
Triglycerides[a] (mg/dL)	0.055 (0.33)	0.174	-0.02 (0.29)	0.572	0.071 (0.38)	0.128
Total cholesterol (mg/dL)	-2.12 (27.4)	0.532	-1.25 (25.0)	0.692	-10.9 (38.7)	0.023
HDL cholesterol (mg/dL)	-1.53 (6.25)	0.042	-0.95 (7.23)	0.296	-0.61 (8.29)	0.542
LDL cholesterol (mg/dL)	1.35 (22.6)	0.641	0.56 (20.4)	0.827	-10.5 (30.8)	0.007
Estimated VLDL cholesterol (mg/dL)	-3.08 (10.6)	0.042	-2.58 (8.90)	0.048	0.66 (11.1)	0.642
Apolipoprotein B (mg/dL)	0.37 (14.6)	0.868	1.22 (14.0)	0.545	-1.42 (19.7)	0.579
Apolipoprotein C-III (mg/dL)	0.77 (4.17)	0.181	0.48 (3.67)	0.355	1.36 (4.07)	0.010

[a]: Log-transformed variables. [b]: Mean (SD). *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts.

SUPPLEMENTAL TABLE V

Differences between the TMD interventions and the low-fat control diet in the biochemical profile of the volunteers (in a multivariate linear regression model, adjusted for age, sex, center of origin of the volunteer, baseline value of the parameter, presence of dyslipidemia, diabetes, hypertension, and tobacco use).

	TMD-VOO vs. Low-fat control diet		TMD-Nuts vs. Low-fat control diet	
VARIABLES	**Coefficient [CI 95%]**	***P*-value**	**Coefficient [CI 95%]**	***P*-value**
Glucose[a] (mg/dL)	-0.050 [-0.12;0.018][b]	0.149	-0.022 [-0.094;0.050]	0.549
Triglycerides[a] (mg/dL)	-0.001 [-0.11;0.11]	0.979	-0.14 [-0.25;-0.024]	0.018
Total cholesterol (mg/dL)	12.2 [2.53;22.0]	0.014	6.38 [-3.81;16.6]	0.221
HDL cholesterol (mg/dL)	1.46 [-0.70;3.62]	0.187	0.72 [-1.56;3.00]	0.538
LDL cholesterol (mg/dL)	11.1 [2.77;19.5]	0.010	7.34 [-1.47;16.2]	0.104
Estimated VLDL cholesterol (mg/dL)	-2.65 [-6.41;1.11]	0.169	-3.78 [-7.80;0.24]	0.068
Apolipoprotein B (mg/dL)	2.57 [-3.60;8.73]	0.416	-0.42 [-6.79;5.95]	0.897
Apolipoprotein C-III (mg/dL)	-0.28 [-1.65;1.10]	0.695	-1.25 [-2.66;0.17]	0.087

[a]: log-transformed variables. [b]: Coefficient of the multivariate linear regression, with its 95% confidence interval. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts.

SUPPLEMENTAL TABLE VI

Differences between post- and pre-intervention values in the properties related to LDL atherogenicity in the three study interventions.

	TMD-VOO		TMD-Nuts		Low-fat control diet	
VARIABLES	**Difference**	***P*-value**	**Difference**	***P*-value**	**Difference**	***P*-value**
LDL resistance against oxidation (Lag time) (normalized ratio)	0.066 (0.15)	<0.001	0.065 (0.15)	0.002	0.009 (0.12)	0.561
LDL oxidation in vivo (normalized ratio)	-0.078 (0.34)	0.065	-0.053 (0.31)	0.185	-0.014 (0.31)	0.719
LDL size (LDL cholesterol/Apolipoprotein B) (normalized ratio)	-0.015 (0.068)	0.157	-0.009 (0.078)	0.447	-0.057 (0.1)	<0.001
Apolipoprotein B in LDLs (normalized ratio)	-0.006 (0.057)	0.383	-0.006 (0.046)	0.307	0.007 (0.041)	0.158
LDL proteins other than apolipoprotein B (%)	-4.54 (12.3)	0.003	-5.15 (13.7)	0.004	-3.99 (14.0)	0.020
Apolipoprotein C-III in VLDL+LDLs (normalized ratio)	0.006 (0.011)	<0.001	0.007 (0.013)	<0.001	0.01 (0.015)	<0.001
Triglycerides/cholesterol ratio (in LDLs)[b] (normalized ratio)	-0.002 (0.25)	0.955	-0.017 (0.22)	0.526	0.043 (0.22)	0.106
LDL cytotoxicity on human macrophages (normalized ratio)	-0.42 (1.46)	0.019	-0.054 (1.84)	0.813	0.14 (1.69)	0.478
LDL cytotoxicity on human endothelial cells (normalized ratio)	-0.095 (1.47)	0.593	0.079 (1.74)	0.716	0.18 (1.18)	0.213

[a]: Mean (SD). [b]: Log-transformed variables. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil; *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts.

SUPPLEMENTAL TABLE VII

Differences between the TMD interventions and the low-fat control diet in the properties related to LDL atherogenicity (in a multivariate linear regression model, adjusted for age, sex, center of origin of the volunteer, baseline value of the parameter, presence of dyslipidemia, diabetes, hypertension, and tobacco use).

	TMD-VOO vs. Low-fat control diet		TMD-Nuts vs. Low-fat control diet	
VARIABLES	**Coefficient [CI 95%]**	***P*-value**	**Coefficient [CI 95%]**	***P*-value**
LDL resistance against oxidation (Lag time) (normalized ratio)	0.068 [0.023;0.11]	0.004	0.041 [-0.004;0.086]	0.076
LDL oxidation in vivo (normalized ratio)	-0.11 [-0.20;-0.012]	0.028	-0.058 [-0.16;0.039]	0.244
LDL size (LDL cholesterol/Apolipoprotein B) (normalized ratio)	0.032 [$-7\cdot10^{-5}$;0.064]	0.053	0.029 [-0.004;0.062]	0.090
Apolipoprotein B in LDLs (normalized ratio)	-0.012 [-0.027;0.003]	0.132	-0.016 [$-0.032;2\cdot10^{-4}$]	0.054
LDL proteins other than apolipoprotein B (%)	-2.00 [-5.61;1.61]	0.279	-0.54 [-4.25;3.18]	0.778
Apolipoprotein C-III in VLDL+LDLs (normalized ratio)	-0.004 [$-0.009;5\cdot10^{-4}$]	0.081	-0.005 [$-0.010;1\cdot10^{-4}$]	0.059
Triglycerides/cholesterol ratio (in LDLs)[b] (normalized ratio)	-0.026 [-0.095;0.043]	0.454	-0.063 [-0.13;0.009]	0.086
LDL cytotoxicity on human macrophages (normalized ratio)	-0.51 [-1.02;0.002]	0.052	-0.19 [-0.72;0.33]	0.471
LDL cytotoxicity on human endothelial cells (normalized ratio)	-0.20 [-0.67;0.26]	0.390	-0.42 [-0.89;0.048]	0.080

[a]: Coefficient of the multivariate linear regression, with its 95% confidence interval. [b]: log-transformed variables. *TMD-VOO*: Traditional Mediterranean Diet supplemented with virgin olive oil. *TMD-Nuts*: Traditional Mediterranean Diet supplemented with mixed nuts.

SUPPLEMENTAL TABLE VIII

Differences in the properties related to LDL atherogenicity after the TMD interventions (when analyzing both TMD interventions together): differences between post- and pre-intervention values, and changes relative to the low-fat control diet (in a multivariate linear regression model, adjusted for age, sex, center of origin of the volunteer, baseline value of the parameter, presence of: dyslipidemia, diabetes, hypertension, and tobacco use).

	Post- vs. pre-intervention change		TMD vs. Low-fat control diet	
VARIABLES	**Difference (SD)**	***P*-value**	**Coefficient [CI 95%]**	***P*-value**
Glucose[a] (mg/dL)	-0.007 (0.21)[b]	0.688	-0.038 [-0.098;0.022][c]	0.222
Triglycerides[a] (mg/dL)	0.019 (0.31)	0.494	-0.063 [-0.16;0.032]	0.199
Total cholesterol (mg/dL)	-1.69 (26.1)	0.463	9.58 [1.04;18.1]	0.029
HDL cholesterol (mg/dL)	-1.26 (6.71)	0.031	1.13 [-0.78;3.04]	0.248
LDL cholesterol (mg/dL)	0.95 (21.4)	0.621	9.42 [2.08;16.8]	0.013
Estimated VLDL cholesterol (mg/dL)	-2.84 (9.79)	0.004	-3.15 [-6.42;0.12]	0.061
Apolipoprotein B (mg/dL)	0.83 (14.2)	0.579	1.16 [-4.10;6.42]	0.667
Apolipoprotein C-III (mg/dL)	0.63 (3.92)	0.104	-0.74 [-1.91;0.44]	0.220
LDL resistance against oxidation (Lag time) (normalized ratio)	0.066 (0.15)	<0.001	0.054 [0.016;0.093]	0.006
LDL oxidation in vivo[a] (normalized ratio)	-0.073 (0.32)	0.010	-0.084 [-0.17;-0.002]	0.047
LDL size (LDL cholesterol/Apolipoprotein B) (normalized ratio)	-0.012 (0.073)	0.134	0.031 [0.003;0.058]	0.030
Apolipoprotein B in LDLs (normalized ratio)	-0.006 (0.051)	0.186	-0.014 [-0.027;$-2\cdot10^{-4}$]	0.048
LDL proteins other than apolipoprotein B (%)	-4.83 (12.9)	<0.001	-1.31 [-4.44;1.82]	0.414
Apolipoprotein C-III in VLDL+LDLs (normalized ratio)	0.006 (0.012)	<0.001	-0.005 [-0.009;$-5\cdot10^{-4}$]	0.031
Triglycerides/cholesterol ratio (in LDLs)[a] (normalized ratio)	-0.009 (0.23)	0.647	-0.043 [-0.10;0.017]	0.161
LDL cytotoxicity on human macrophages (normalized ratio)	-0.24 (1.66)	0.093	-0.36 [-0.80;0.086]	0.115
LDL cytotoxicity on human endothelial cells (normalized ratio)	-0.010 (1.60)	0.942	-0.31 [-0.71;0.091]	0.131

[a]: log-transformed variables. [b]: Mean (SD). [c]: Coefficient of the multivariate linear regression, with its 95% confidence interval. *TMD*: Traditional Mediterranean Diet.

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SUPPLEMENTAL MANUSCRIPT I

Olive oil phenolic compounds and high-density lipoprotein function. *Current Opinion in Lipidology*, 2015;27(1):47-53.

REVIEW

Olive oil phenolic compounds and high-density lipoprotein function

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Purpose of review
The functional capacities of high-density lipoproteins (HDLs) reflect the physiological role of the particle better than the quantity of HDL cholesterol. Owing to its phenolic compounds, the consumption of virgin olive oil has emerged as a promising therapy to promote these capacities. This review highlights the human studies that explain these benefits and explores some possible mechanisms.

Recent findings
The consumption of olive oil phenolic compounds increased the ability of HDLs to pick up cholesterol excess in peripheral cells (the cholesterol efflux capacity). Olive oil phenolic compounds have also been shown to improve HDL antioxidant capacities and some anti-inflammatory traits. These changes respond to an improvement of HDL oxidative status and composition.

Summary
Novel strategies to increase HDL functional capacities are in demand from clinicians. The attainment of a fully-functional HDL through dietary or lifestyle changes is a priority in cardiovascular research. Within this context, the consumption of virgin olive oil, because of its phenolic compounds, may be a relevant protective approach. Further studies in large-scale, randomized controlled trials are, however, required to confirm these effects in HDL functionality.

Keywords
cholesterol efflux capacity, high-density lipoprotein antioxidant capacity, high-density lipoprotein function, olive oil phenolic compounds

INTRODUCTION

High-density lipoprotein (HDL) cholesterol (HDL-C) levels are inversely and independently associated with cardiovascular disease (CVD) [1]. Several interventions, based on pharmacological and natural products, have been able to increase HDL-C concentrations although high levels have not always been associated with low cardiovascular risk [2]. Moreover, pharmacologically raised HDL-C levels have not always led to a decrease in cardiovascular risk and some studies have even reported an increased mortality risk [3]. The physiological role of HDL seems, therefore, to be better reflected by its function than HDL-C quantity.

HDLs play a central role in reverse cholesterol transport. They remove excess cholesterol from peripheral cells [the cholesterol efflux capacity (CEC)] and transport it to the liver for further metabolism and excretion [4]. CEC has been shown to predict coronary event incidence [5] and be inversely related to the development of early atherosclerosis [6]. HDLs present other atheroprotective capacities: they counteract the oxidation of low-density lipoproteins (LDLs), present anti-inflammatory functions, and help preserve endothelium integrity [7].

Olive oil, the main source of fat in the Mediterranean diet, is considered to play a major role in its protective effects on CVD [8]. In observational studies adherence to the Mediterranean diet decreased the development of chronic CVD [9] while intervention studies demonstrated the beneficial effect of this diet on CVD primary and

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KEY POINTS

- HDL function reflects the physiological role of the lipoprotein better than HDL-C.
- Lifestyle changes that may increase HDL functional capacities *in vivo* are in high demand from clinicians.
- The phenolic compounds in VOO are able to improve HDL-C efflux capacity.
- Olive oil phenolic compounds also increase HDL antioxidant capacities.
- Large-scale, long-term randomized controlled trials with virgin olive-rich dietary interventions are required to confirm the protective effects of olive oil phenolic compounds on HDL function.

secondary prevention [10,11]. Our group reported that the consumption of virgin olive oil (VOO) increased HDL-C and decreased in-vivo lipid oxidative damage. This effect was dose-dependently associated with the olive oil phenolic compound content [12]. Taking into account the rise in HDL-C quantity, an increase in the functional capacities of HDL because of the consumption of VOO could be expected. The whole matrix of phenolics in olive oil is complex and diverse (Table 1) [13] and would be responsible for these potentially beneficial effects.

Our aim is to review the reported benefits of the consumption of phenolic compounds in VOO on the functionality of HDLs in humans. Further discussion of the possible related mechanisms is also provided.

HIGH-DENSITY LIPOPROTEIN CHOLESTEROL EFFLUX CAPACITY

The consumption of phenolic compounds has increased CEC in some in-vivo studies [14]. With respect to olive oil phenolic compounds, our group provided first-level evidence for the first time in humans of an increase in CEC after the consumption of a real-life dose of VOO (366 mg/kg) [15▪▪]. To explain this change, we observed, on the one hand, an increase in the biological metabolites of olive oil phenolic compounds bound to the HDLs (hydroxytyrosol sulfate, and homovanillic acid sulfate, and glucuronate). These compounds could exert a local antioxidant protection in HDLs [16,17,18▪] that may prevent oxidative modifications of the apolipoprotein A-I (ApoA-I), the main HDL protein involved in CEC [4], and of other HDL proteins. Such protection would also avoid oxidative modifications of HDL lipids, making the lipoprotein more fluid and thus more functional [19]. Improvements in CEC and HDL fluidity have also been described in a noncontrolled trial with VOO in healthy volunteers [20]. On the other, we found a decrease in the relative content of triglycerides in the HDL core. A triglyceride-poor HDL core is related to a more stable conformation of ApoA-I in the lipoprotein [21] which could lead to better HDL capacity to perform CEC.

Functional VOOs (FVOOs) are also a promising therapy to improve CEC. Our group recently developed a randomized, controlled trial in hypercholesterolemic individuals to test the effects on the HDL characteristics related to CEC of two FVOOs: one enriched with olive oil phenolic compounds (500 mg/kg) and another with phenolic compounds from olive (250 mg/kg) plus additional complementary phenolic compounds from thyme (250 mg/kg) (total: 500 mg/kg), compared with a standard VOO (80 mg/kg) [22▪]. The functional oils increased the quantity and bioavailability of the phenolic compounds in the real food matrix without raising the final fat intake [23]. Three main effects were observed. First, an increase in the lecithin-cholesterol acyltransferase (LCAT) mass in plasma after the FVOO supplemented with olive and thyme phenols versus the control VOO was reported. LCAT mediates the esterification of free cholesterol and its migration into the particle core. This process leads to the conversion of the immature HDLs into small particles and, finally, into larger ones [4]. The increase in LCAT mass could, therefore, be linked to a decrease in the relative content of free

Table 1. Classification of the main olive oil phenolic compounds

Chemical structure	Compounds
Simple phenols	Alcohols: hydroxytyrosol, tyrosol
	Acids: caffeic, gallic, p-coumaric, protocatechuic, sinapic, syringic, vanillic
Flavonoids	Apigenin, luteolin
Secoiridoids	Oleuropein glucoside, aglycones of oleuropein glucoside and ligstroside, and dialdehydic forms of oleuropein glucoside and ligstroside without a carboxymethyl group
Lignans	(+)-pinoresinol, (+)-1-acetoxy-pinoresinol

cholesterol and an increase in the relative content of phospholipids in the HDL surface, both observed after the FVOO supplemented with olive and thyme phenols versus the control VOO [22▪]. Such changes may result in more fluid HDLs, indirectly associated with a greater CEC [19]. Second, in a pooling sample proteomic approach, we observed an increase in the ApoA-I content in HDL after the consumption of VOO and both functional VOOs [24▪]. ApoA-I-rich HDLs may be more efficient in exerting CEC as ApoA-I is the main HDL protein involved in the process. Finally, in the same proteomic approach, we also found an increase in the content of affamin in HDL after the FVOO supplemented with olive and thyme phenols [24▪]. Affamin is a transporter of α-tocopherol and, as a result, we would expect an increased α-tocopherol content in HDL which could complement the local antioxidant protection of the phenolic compounds.

CEC can also be modulated by the direct effect of olive oil phenolic compounds on CEC-related gene expression. In a postprandial study with pre/hypertensive individuals, a real-life dose of phenolic compound-enriched olive oil (961 mg/kg) enhanced the gene expression of transmembrane cholesterol transporters [ATP-binding cassette transporter A1 (*ABCA1*), scavenger receptor B1 (*SCARB1*)], and some transcription factors related to the peroxisome proliferator-activated receptors (*PPARA*, *PPARG*, *PPARD*, and *MED1*), when compared with a control VOO (289 mg/kg) [25]. The improvement in *ABCA1* and *SCARB1* expression could be mediated through the upregulation of the *PPAR*-dependent pathway [26]. We additionally reported an augmentation in the *PPAR*-related gene expression in a previous postprandial study with an acute dose of 50 ml of VOO [27]. With respect to long-term studies, similar increases in the expression of some transmembrane cholesterol transporters [*ABCA1*, ATP-binding cassette transporter G1 (*ABCG1*)] in macrophages were described in a nonrandomized trial with VOO in healthy volunteers [20], although no significant changes in *ABCA1* expression were reported after the consumption of a VOO-rich Mediterranean diet in healthy individuals [28].

HIGH-DENSITY LIPOPROTEIN ANTIOXIDANT ACTIVITY

HDLs protect LDLs against oxidative modifications, a relevant property, as oxidized LDLs are a key trigger for the onset of atherosclerotic plaque [29]. The consumption of VOO has been dose-dependently associated *in vivo* with a decrease in oxidized LDLs [12], part of this protection could take place through an induction of the HDL antioxidant capacities. After the consumption of a VOO-rich diet an increase in HDL antioxidant activity was observed in *ApoE*-deficient mice [29], a finding that could be expected to occur in humans. The main agents involved in HDL antioxidant capacity are ApoA-I and paraoxonase-1 (PON1). Nevertheless, other proteins, such as the LCAT and the platelet-activating factor acetylhydrolase (PAF-AH), and the indirect protection exerted by HDL antioxidant content, may also collaborate [4].

As previously commented, the consumption of olive oil phenolic compounds may enhance the ApoA-I functionality in HDL particles, preventing oxidative modifications of the protein and leading to a more stable conformation of ApoA-I in HDLs [15▪▪]. These characteristics might also improve HDL capacity to pick up oxidized lipids [4].

In parallel, olive oil phenolic compounds could enhance the function of some HDL-related antioxidant enzymes, such as PON1. PON1 is present in the circulation mainly linked to HDLs and is one of the principal agents involved in the hydrolysis of oxidized lipids in plasma [30]. PON1 antioxidant activity was enhanced in healthy humans after a 3-week VOO-rich intervention [17] and also significantly upgraded after the consumption of the previously described FVOO supplemented with olive and thyme phenols [22▪]. These improvements in PON1 function are in line with others found after supplementation with VOO in several murine models [31–33]. Three possible mechanisms may explain this increment in PON1 function. First, olive oil phenolics could increase the biosynthesis of PON1, as the consumption of VOO induced a rise in the PON1 plasma concentrations of healthy study participants [18▪]. The capacity of these phenolic compounds to stimulate the gene expression dependent on the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) could indirectly enhance PON1 production [33]. Second, HDLs may become rich in PON1, in a similar way to their becoming abundant in paraoxonase-3 (a PON1 genotypic isoform), as observed after the consumption of VOO and the previously described FVOO supplemented with olive and thyme phenols [24▪]. Finally, the consumption of VOO increased the HDL content of olive oil phenolic compound metabolites which may protect PON1 structure and function as reported for ApoA-I [15▪▪,17,18▪].

With respect to other enzymes related to HDL antioxidant capacity, we have previously commented on an increment of LCAT mass after the consumption of the FVOO supplemented with olive and thyme phenols when compared to a control VOO [22▪]. In the case of the PAF-AH, no significant

changes in PAF-AH quantity or activity in HDLs because of VOO have as yet been reported.

HIGH-DENSITY LIPOPROTEIN ANTI-INFLAMMATORY AND VASOPROTECTIVE EFFECTS

In addition to their CEC and antioxidant properties, HDLs are also considered to be relevant anti-inflammatory and vasoprotective agents. Endothelial dysfunction and the inflammatory responses of macrophages and endothelial cells, key factors for the perpetuation of atherosclerosis [29], all seem to be counteracted by HDLs [7]. VOO consumption has been shown to be highly protective for vascular response and endothelial integrity, as observed in a number of VOO-rich interventions in humans [34–36]. HDLs could act as transporters of several derivatives of olive oil phenolic compounds to the endothelial cells where they may prevent oxidative damage in cell mitochondria and preserve the production of nitric oxide, as described *in vitro* [35,37].

Regarding HDL anti-inflammatory capacity, the consumption of VOO increased the ability of HDLs to block the secretion of intracellular adhesion molecule-1 and the adhesion of monocytes to endothelial cells in healthy humans [18▪]. Such an enhancement could be because of two hypothetical mechanisms. First, the improvement in HDL antioxidant function caused by olive oil phenolic compounds may partially explain these benefits. HDL antioxidant and anti-inflammatory functions are intimately related [7] and PON1 is one of the principal agents involved [7,30]. In agreement with this, the anti-inflammatory properties of PON1 activity increased significantly after the consumption of VOO in healthy study participants [18▪]. Second, the consumption of VOO may enhance HDL functionality by decreasing the levels of acute-phase proteins in the lipoprotein. Inflammatory states increase the content of acute-phase proteins in HDLs, transforming the lipoproteins into proinflammatory, dysfunctional particles [4]. The consumption of different VOOs [24▪] and a VOO-rich Mediterranean diet [38] has been reported to decrease the content of acute-phase proteins in HDLs and may, therefore, promote a less proinflammatory state of the particles.

OTHER HIGH-DENSITY LIPOPROTEIN-RELATED PROPERTIES

In addition to the functional properties of HDLs there are other novel characteristics related to their quantity and quality. The number of HDL particles in circulation, determined by NMR-spectroscopy, has shown to be a promising biomarker of HDL concentration, for instance in the prediction of CVD incidence [39]. A short-term consumption of VOO in healthy individuals induced a nonsignificant increasing trend in HDL particle number [15▪▪]. This protective effect was significantly confirmed after a long-term consumption of a Mediterranean diet supplemented with nuts [40]. A rise in systemic ApoA-I levels, such as those reported after some VOO-based dietary interventions in humans [41,42], may justify these changes. Mechanistically, a regular intake of VOO may enhance the intestinal expression of *Apoa1* gene, as observed in a rat model [43]. Olive oil phenolic compounds may also decrease ApoA-I degradation, as described in individuals whose HDL-C concentrations increased after a short-term consumption of a Mediterranean diet [44].

High-CVD risk patients usually present a profile characterized by low levels of large HDLs, high levels of small HDLs, and variable values of lipid-poor/lipid-free HDLs (the preβ fraction, the most effective one for CEC) [45]. The consumption of VOO has shown to increase large HDLs and decrease small ones [15▪▪]. The change toward greater HDL size has been confirmed after the consumption of a FVOO supplemented with olive and thyme phenols [22▪], a VOO-rich Mediterranean diet [40,46], and in a rat model after supplementation with VOO [47]. There is, however, some controversy with respect to this issue. Some authors consider that the preβ HDL and lipid-free ApoA-I are the more functional particles [48]. In contrast, a number of in-vitro studies indicate that small HDLs have similar effects to the large ones [49]. Furthermore, increased levels of small HDLs in plasma may indicate an aberration in HDL maturation and decreased reverse cholesterol transport [4]. Large HDLs also bind better to the ABCG1 and scavenger receptor B1 cholesterol transporters, promoting cholesterol efflux via these receptors [4]. Moreover, some HDL physicochemical modifications (such as the ones in inflammatory states) can transform the lipoprotein into a small, dysfunctional particle [4]. As a result, the interpretation of HDL size without taking into account the overall biochemical context is controversial.

Finally, some VOO-based interventions, particularly the FVOO supplemented with olive and thyme phenols, have increased the content of other active proteins in HDLs, such as that of apolipoprotein A-IV [24▪]. This effect has also been observed in ApoE-deficient mice after the consumption of a VOO-rich diet [31]. A rise in HDL apolipoprotein A-IV content may be cardioprotective as decreased apolipoprotein A-IV content in HDLs appears in patients with stable or acute coronary syndrome [50].

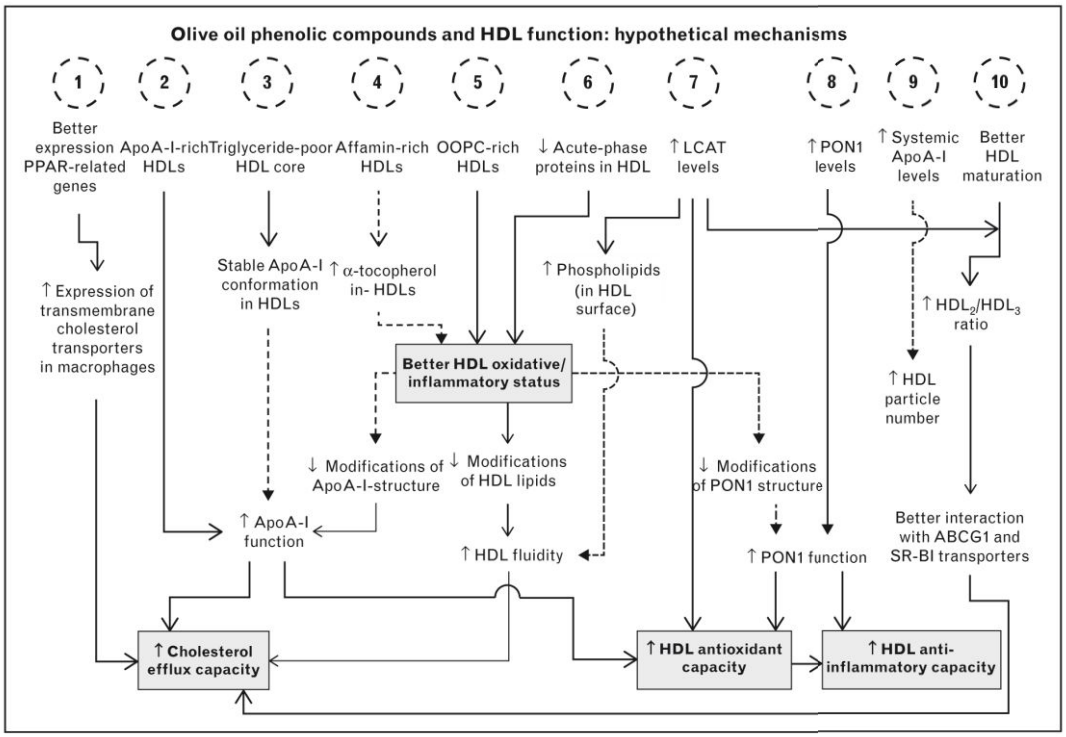

FIGURE 1. Olive oil phenolic compounds and HDL function: hypothetical mechanisms. The consumption of olive oil phenolic compounds enhances HDL cholesterol efflux capacity, HDL antioxidant capacities and some HDL anti-inflammatory properties. Olive oil phenolic compounds increase cholesterol efflux capacity by improving HDL size distribution, increasing the gene expression of cholesterol transporters, and enhancing ApoA-I function and HDL fluidity (in both cases because of a better HDL oxidative/inflammatory status and changes in HDL composition). HDL antioxidant capacity is incremented after the consumption of virgin olive oil through the preservation of ApoA-I and PON1 function, and an increase in lecithin-cholesterol acyltransferase levels. Finally, HDL anti-inflammatory properties are also augmented, mainly because of the increase in HDL antioxidant function. Continuous lines reflect well established relations between variables, and discontinuous lines hypothetical associations. ApoA-I, apolipoprotein A-I.

CONCLUSION

HDL functions reflect the physiological role of the lipoprotein better than HDL-C quantity. As indicated in Fig. 1, the intake of olive oil phenolic compounds resulted in an improvement in CEC, HDL antioxidant defenses, HDL size distribution, and other characteristics related to HDL quality. Olive oil phenolic compounds bound to HDLs, or surrounding the lipoprotein, improve their oxidative/inflammatory status which may justify an increase in HDL functionality. Modifications in HDL composition because of the consumption of VOO might also explain these changes. However, large-scale, randomized controlled trials with VOO-rich dietary interventions are required to definitively confirm the protective role of olive oil phenolic compounds in HDL biological functions.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

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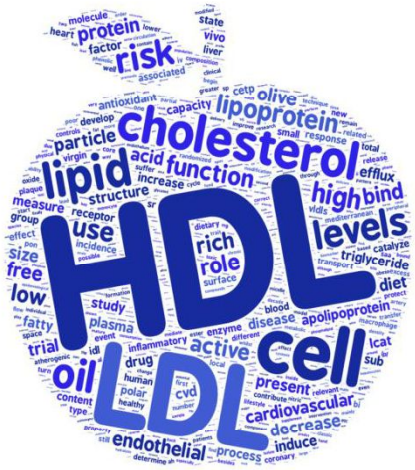


7. Discussion

This thesis research project presents four manuscripts about the effects of the consumption of dietary antioxidants on the functions of HDLs and the atherogenicity of LDLs. Research was based on data from two randomized controlled trials: the EUROLIVE Study and the PREDIMED Study. In this discussion section, we will consider the results of the manuscripts included in the project from a general point of view, provide an update on current research on the topic, and present a broader interpretation of the main results.

In this thesis research, we first studied the effects of an antioxidant-rich food item (VOO) on HDL function and quality-related properties in a healthy population. Second, we examined the effects of an antioxidant-rich dietary pattern (TMD) on these HDL functionality characteristics. Third, we explored the effects of VOO on LDL quantity and atherogenic traits. Finally, we assessed the effects of TMD on LDL atherogenicity.

7.1. UPDATE ON RECENT RESEARCH IN THIS AREA

7.1.1. IMPROVEMENTS IN HDL FUNCTION

HDL function is thought to represent the role of the lipoprotein in the prevention of CVDs more accurately than HDL-C levels[21]. Among HDL functions, the most relevant one for cardiovascular prevention is cholesterol efflux capacity[100], it is also the most studied in humans. As a result, researchers and clinicians are highly interested in finding protective strategies to increase cholesterol efflux capacity as a way to decrease cardiovascular events in the population. In this regard, as reported in this thesis project, the consumption of an antioxidant-rich food, VOO, in dietary doses was the first real-life healthy intervention able to increase cholesterol efflux capacity in humans. Likewise, adherence to a TMD (both TMDs supplemented with VOO or nuts) also enhanced cholesterol efflux capacity in high cardiovascular risk individuals. Dietary antioxidants may have improved HDL oxidative/inflammatory status and, therefore, induced an increase in its function[152]. An enhancement in the expression of the genes related to cholesterol efflux capacity and HDL metabolism after the consumption of olive oil phenolic compounds could also have contributed to the effect[249]. Other antioxidant-rich interventions have induced similar effects, such as a supplement of anthocyanins[123]. High intakes of polyunsaturated fatty acids have also increased cholesterol efflux capacity, as observed after a PUFA-rich diet[263] and a supplement of eicosapentaenoic acid[141]. Moreover, a number of pharmacological agents such as niacin[106], pioglitazone[104], and two inhibitors of the CETP activity (the *trapib* family)[106,264] additionally increased this HDL function.

A key point following cholesterol efflux capacity is the esterification of the effluxed cholesterol by LCAT in HDLs, in order to be able to transport it in a stable manner to the core of the lipoprotein[265]. From our data, the ability to esterify cholesterol by HDLs rose after the consumption of a TMD enriched in VOO. Since LCAT is very sensitive to

oxidative modifications[150], the protection of its active center against oxidative attacks by dietary antioxidants is a plausible mechanism to justify our findings. This increase in our data is in agreement with others described after the consumption of antioxidant-rich diets, such as a vegetable and fruit-rich diet[112] and a lycopene-supplemented one[111].

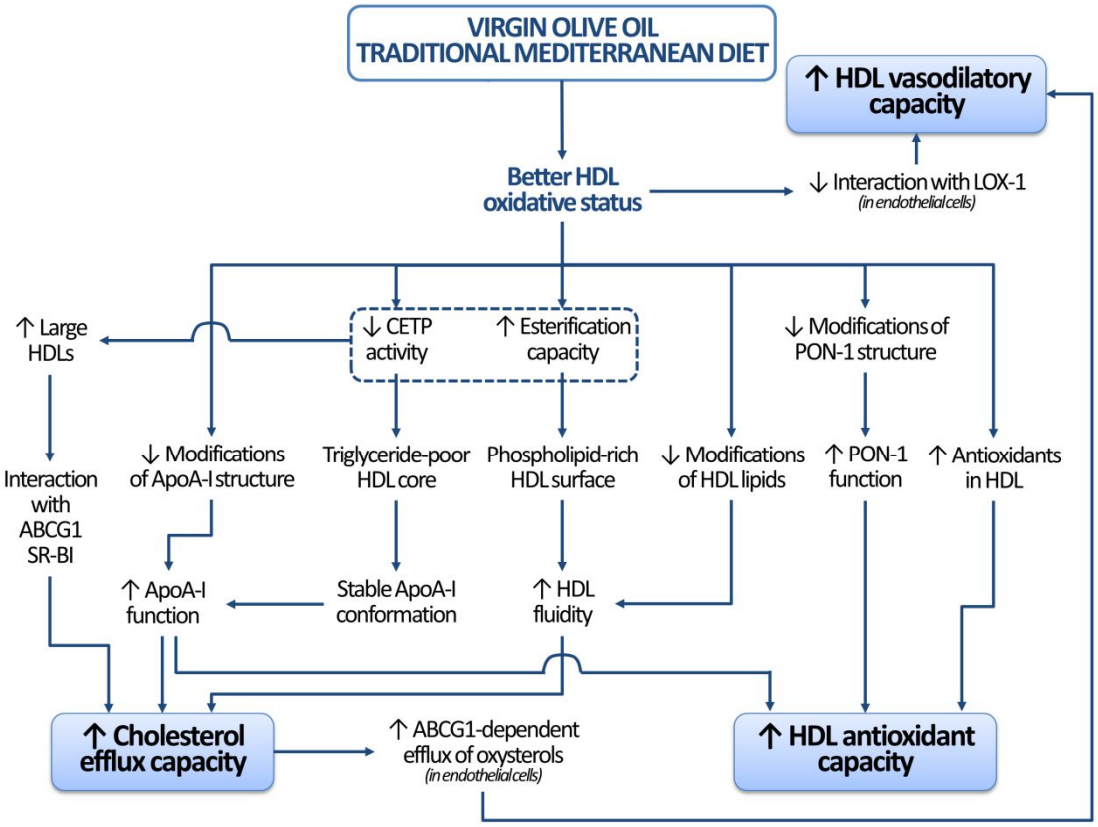
A third key point in the HDL cholesterol metabolism is its transfer to triglyceride-rich lipoproteins due to the action of the CETP enzyme[113]. As previously commented, in high cardiovascular risk states (especially hypertriglyceridemia) CETP activity is exacerbated and leads to the formation of triglyceride-rich, dysfunctional HDLs[114,158,159]. However, since a large blockade of the CETP function has provided several controversial results[96,116–118], a modulation of the enzyme with dietary compounds has arisen as a more physiological strategy to reestablish its normal activity. In this project, the adherence to a TMD supplemented with VOO decreased CETP function. However, the expected reduction in the enzyme function was not totally observed after the consumption of VOO (P=0.122), perhaps due to a lack of statistical power. As possible mechanisms, we could think of a direct modulation of the enzyme due to antioxidants[105,266] or changes in its gene expression caused by the modulation of regulatory pathways involved in the transcription of the *CETP* gene[267,268]. Our findings support previous results reported after the consumption of dietary antioxidants including apple polyphenols[266], anthocyanins[105], and carotenoids such as lycopene[111].

The second most relevant HDL function is its antioxidant capacity[86]. The coordination of the activities of several antioxidant proteins carried by HDLs (PON1, ApoA-I, LCAT, LpPLA2 and the like)[90] and HDL-bound antioxidant compounds (vitamin E)[90,153] contributes to hindering LDL oxidation and, therefore, the triggering of oxidative events leading to the formation of atherosclerotic plaques[2]. As previously commented, HDL antioxidant capacity is crucial for cardiovascular protection, since low PON1 arylesterase activity is associated with greater incidence of cardiovascular episodes[22]. In our data, adherence to a TMD (particularly when supplemented with VOO) increased HDL antioxidant capacity on LDLs, and also PON1 arylesterase function. Regarding VOO, as the content of olive oil phenolic compound metabolites bound to HDL increased after the intervention, these compounds may have kept PON1, LCAT, and ApoA1 non-oxidized and functional[148–150], acted as chemical scavengers of reactive oxygen species[269], and helped to preserve other antioxidants in HDLs, such as vitamin E. We hypothesize that this protection is also present after the TMD interventions, due to olive oil phenolic compounds and other antioxidants. Our results concur with other antioxidant-rich strategies, such as the use of polyphenol-enriched functional VOOs[270], anthocyanin supplements[123], and lycopene supplementation[111].

As previously described, HDLs are also able to maintain endothelial homeostasis and facilitate its functions[89], a number of which are lost in some high cardiovascular

risk states[139]. Adherence to a TMD supplemented in VOO increased HDL vasoprotective functions in our data. A greater ability to perform cholesterol efflux may also lead to an enhanced capacity to pick up oxysterols from endothelial cells and, therefore, preserve endothelial integrity[89]. Moreover, non-oxidized HDLs (as observed after the TMD-VOO intervention) are also less toxic for endothelial cells[89]. To the best of our knowledge, this is the first real-life dietary modification that has increased HDL endothelial protection, and it is consistent with other cardioprotective interventions such as a massive weight loss due to gastric surgery[137] or the use of extended-release niacin[139].

Figure 13. Possible mechanisms for the effects of VOO and TMD on HDL functions.



As possible mechanisms to explain these functional improvements (**Figure 13**), we have considered four critical points: HDL oxidation, composition, fluidity, and size. First, regarding HDL oxidation, we have already commented that olive oil phenolic compound metabolites bound to HDLs increased after the consumption of VOO in one of the trials, which may explain several of its benefits. In addition, adherence to a TMD supplemented with VOO improved HDL oxidative status and resistance against oxidation. A lower degree of oxidative modifications of HDL proteins and lipids may partially explain the benefits described after the interventions[148–150,152]. Second, HDL cores became triglyceride-poor after the consumption of VOO and adherence to a TMD. This trait is associated with an increased ApoA-I stability on HDLs and, therefore,

a more complete ApoA-I function[158]. The partial blockade of CETP function (after the interventions rich in VOO) or the triglyceride-lowering capacity of nuts (after the TMD intervention supplemented with nuts)[271] may contribute to this effect. Third, HDL fluidity increased after the consumption of olive oil phenolic compounds, and we hypothesize a similar effect after the adherence to a TMD. Since more fluid HDLs would interact more efficiently with cholesterol transporters and HDL receptors[86], the increase of HDL functionality may have been partially due to this improvement. As previously commented, the increment of the phospholipid content on the HDL surface and the ameliorated HDL oxidative status may explain greater HDL fluidity[151,152,162]. Finally, there was an increase in the proportion of large HDLs after all the interventions studied in this project. This effect seems atheroprotective, since large HDLs are decreased in high cardiovascular risk states[171]. An increased ability of HDL to fully mature, due to an improved HDL oxidative status, is a possible explanation[86]. Since large HDLs tend to interact efficiently with ABCG1 and SRBI receptors[100], greater HDL size may also contribute to explaining an improved cholesterol efflux capacity through these pathways after our interventions.

7.1.2. IMPROVEMENTS IN LDL ATHEROGENICITY

LDL characteristics have attracted much attention from the scientific community in recent years as a possible approach to explain residual cardiovascular risk in individuals with low LDL-C levels[182]. Among these strategies, we should highlight LDL oxidative modifications and LDL resistance to undergoing oxidative alterations. Levels of oxidized LDLs in circulation have been considered a biomarker of potential LDL oxidation in the sub-endothelial space, and have been associated with the incidence of cardiovascular events[185] and the presence of subclinical atherosclerosis[187]. As expected, low LDL resistance against oxidation is linked with greater LDL oxidation. It is also associated with subclinical atherosclerosis[201] and is present in some high cardiovascular risk states[202]. Our group previously determined that the consumption of VOO, and adherence to a TMD, decreased the levels of oxidized LDLs in the bloodstream[47,73], findings we have confirmed in the present project. These two interventions also increased LDL resistance against oxidation (measured as LDL lag time). This effect was especially remarkable in the TMD intervention supplemented with nuts: a high consumption of PUFAs makes LDL more prone to becoming oxidized, as they present a greater number of double bonds, the position in which lipid oxidation usually occurs[200]. Nevertheless, the high content of dietary antioxidants within the context of the TMD was able to reverse this deleterious effect, and even increase LDL resistance against oxidation to a similar level as the TMD-VOO intervention. The consumption of other antioxidant-rich food items (green tea)[272] and the use of antioxidant supplements[198,199] has also been shown to be able to raise LDL resistance against oxidation in previous trials. The increased content of olive oil phenolic compound metabolites in LDLs after the intake of VOO may directly counteract oxidative attacks. It could also contribute to

enhancing the bioavailability of vitamin E in the lipoproteins, making LDLs more resistant against oxidation[254,273]. The present mechanism may be extrapolated to other dietary antioxidants in the TMD.

LDL size is another major atherogenic trait. As previously explained, small LDLs are more pro-atherogenic since they remain longer in circulation (and therefore tend to suffer more structural modifications), interact poorly with LDL receptors, adhere more frequently to endothelium proteoglycans, and are more prone to traversing the endothelial barrier[23,203,204]. Therefore, high concentrations of small LDLs are associated with a greater incidence of cardiovascular events[208]. From our data, the consumption of VOO decreased the number of circulating small LDL particles, whilst the TMD increased LDL average size (as an increment is an indirect indicator of LDL size, the LDL-C/ApoB ratio in plasma). As possible mechanisms to explain these changes, we hypothesize that: 1) an improvement in general oxidative status may be associated with a reduction in the production of small LDLs, as elevated small LDL levels are present in high oxidative stress states[274]; and 2) an improved expression of the receptors responsible for the uptake of triglyceride-rich lipoproteins (LPL) would help to decrease LDL circulating levels, especially affecting the levels of small LDLs. In this regard, we observed a borderline reduction in *LPL* gene expression after the consumption of VOO, as well as a significant decrease in total LDL particle number due to fewer small LDL particles. A reduction in small LDLs had already been reported in the PREDIMED Study[45] and after the intake of other dietary antioxidants, such as strawberry polyphenols[275].

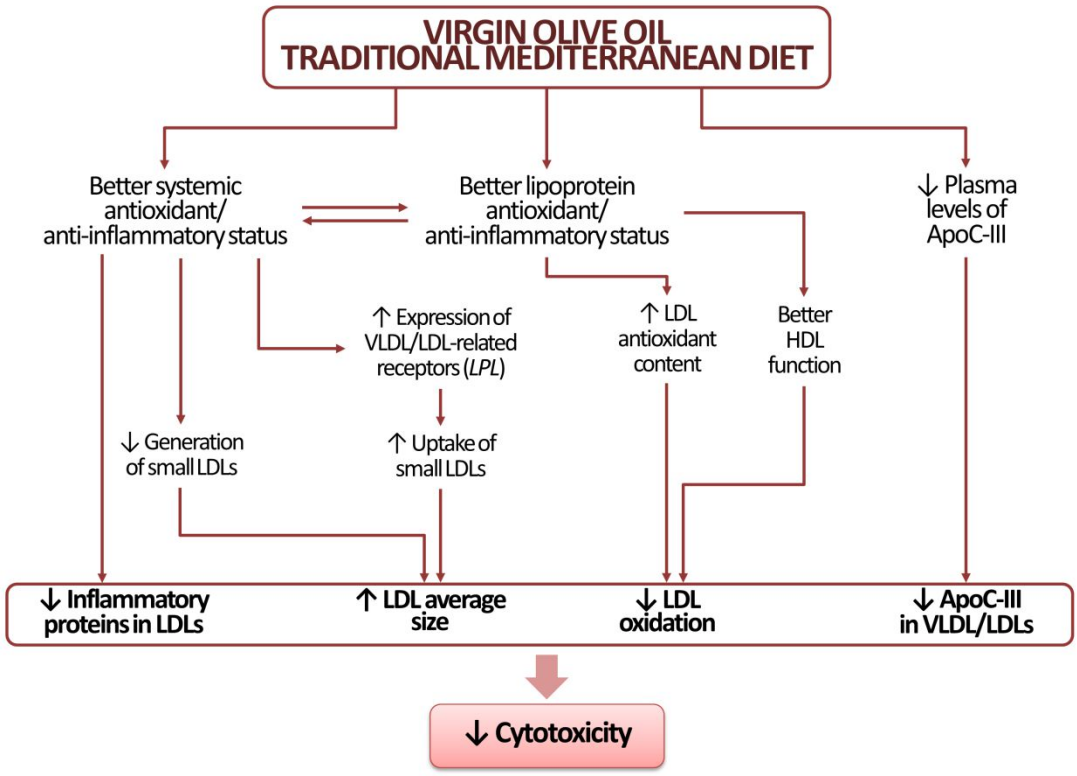
LDL composition may also condition LDL atherogenicity. After the TMD interventions, LDLs presented a decreased content in proteins other than ApoB. Moreover, although apoB-containing lipoproteins increased their ApoC-III content after the TMD interventions and the control diet, the TMD interventions were able to partially reverse this rise relative to the low-fat intervention. A decreased LDL protein content makes the lipoproteins less prone to carry pro-inflammatory, pro-atherogenic proteins[276]. As adherence to a TMD reduces the levels of circulating pro-inflammatory proteins in the bloodstream[46,47,50,277], LDLs would be less likely to bind to them and, therefore, a decreased content of pro-inflammatory proteins (and other proteins) in LDL would be expected. Regarding ApoC-III levels in apoB-containing lipoproteins, although the TMD was able to partially reverse the increase observed after the low-fat diet, deterioration in some LDL properties after one year in high cardiovascular risk patients was still present. As commented in **Manuscript IV**, ApoC-III levels in plasma only augmented significantly in the low-fat diet, and this may have induced a special impairment in the ApoC-III-related properties after the control intervention. However, further research in the field is required to fully understand the mechanisms by which a change of this nature took place.

LDLs prone to suffer oxidative modifications, with small size and an aberrant composition, are likely to induce cytotoxic responses in macrophages and endothelial

cells[2,23,184,203,204,224]. However, no randomized controlled trials have assessed the *ex vivo* cytotoxicity of LDLs in human macrophages and endothelial cell lines. The TMD, especially when supplemented with VOO, decreased LDL cytotoxicity in macrophages, and the TMD supplemented with nuts tended to reduce it in endothelial cells. These cell-based methodologies provide a physiological overview of LDL effects on biological systems, and could be useful in the future to reflect whether the changes in the classic LDL pro-atherogenic traits could be associated with benefits in an *in vivo* system. However, such strategies should be checked and optimized carefully in order to control for possible biases (such as high variability).

Associations among LDL atherogenic traits and the hypothetical mechanisms to justify their modulation are available **in Figure 14**.

Figure 14. Possible mechanisms for the effects of VOO and the TMD on LDL atherogenic traits

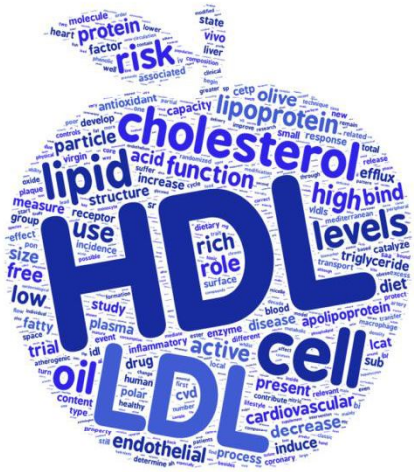


7.2. STRENGTHS AND LIMITATIONS

In relation to **Manuscripts I** and **III** (sub-studies of the EUROLIVE Trial), their main strength was the cross-over design which allowed the entire number of participants to ingest all types of olive oil and, therefore, reduced interferences from confounding variables. Nevertheless, these two studies also presented limitations. First, the observed changes were modest, since we administered real-life doses of a single food.

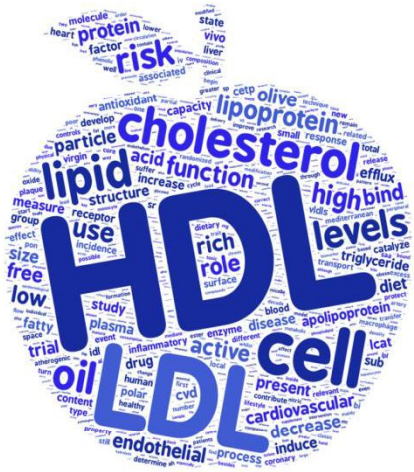
Nevertheless, the enhanced variables (cholesterol efflux capacity and small LDL levels) play a crucial role in cardiovascular protection. Second, it was possible that some phenolic compounds could have come from other food types. The consumption of antioxidant-rich foods was, however, controlled throughout the study, and no significant changes were found. Finally, the sample sizes were not large (*N*=47 and *N*=25, for HDL and LDL analyses, respectively) and could be responsible for a lack of significant results in parameters with high variability.

In relation to **Manuscripts II** and **IV** (sub-studies of the PREDIMED Trial), their main strengths are: 1) their parallel design, which permitted us to measure the long-term effects of adherence to a TMD; 2) the large sample size (particularly for HDL analyses, *N*=296), the greatest for a trial of these characteristics; and 3) the joint study of almost all the lipoprotein properties that have already been standardized and previously published. Nevertheless, these studies also presented limitations. First, as in the results related to the consumption of VOO, we reported modest changes due to the fact that they took place after real-life modifications of diet. Nevertheless, since the affected variables are critical in lipoprotein biology, the cardiovascular protection obtained could be relevant. Moreover, the control intervention (low-fat diet) is already a well-known healthy dietary pattern and its differences with respect to the TMD interventions may not be very remarkable. Second, our volunteers were elderly, high cardiovascular risk individuals; this makes it difficult to extrapolate our findings to the general population. Finally, cell-based *ex vivo* models, although they are a non-invasive alternative to assess several physiological functions, may not take into consideration the presence of contra-regulatory mechanisms able to modify the final outcome *in vivo* in humans.



8. Conclusions

1. The consumption of olive oil phenolic compounds (contained in VOO) is able to increase the most relevant HDL functional ability, cholesterol efflux capacity, in healthy individuals. Improvements in HDL oxidative status, composition, fluidity, and size due to olive oil phenolic compounds could explain the previous HDL functional improvement.
2. A long-term adherence to a TMD, especially when supplemented with VOO, in high cardiovascular risk individuals, is able to enhance the four most relevant HDL functions: cholesterol efflux capacity, HDL roles in other phases of the reverse cholesterol transport, HDL antioxidant capacity, and HDL vasodilatory ability. Improvements in HDL oxidative status, composition, and size distribution may explain the previous HDL functional enhancement.
3. The consumption of olive oil phenolic compounds decreased LDL concentrations. VOO intake also improves LDL atherogenicity: it increases LDL resistance against oxidation, and decreases the circulating number of small LDL particles, in healthy individuals.
4. A long-term adherence to a TMD is able to reduce LDL atherogenicity in high cardiovascular risk individuals: it increases LDL resistance against oxidation, decreases LDL size, improves LDL composition, and lowers LDL cytotoxicity in macrophages.
5. Our findings provide two inter-related novel mechanisms to explain part of the benefits of the consumption of VOO and adherence to a TMD. They also support previous evidence concerning the cardioprotective role of the two dietary interventions.



9. Future perspectives

9.1. WHAT'S NEXT?

Lipoproteins are one of the crucial agents in the development of atherosclerosis, and clinicians have recently realized that the information they provide goes far beyond HDL-C and LDL-C levels. The study of lipoprotein capacities and functional phenotypes in large human populations is a way to establish the real role of lipoproteins in atherosclerosis, in order to demonstrate a clear mechanism to explain the pleiotropic process of the development of CVDs. However, data in the field are scarce and to date have only been obtained in small-scale studies. Therefore, the development of a multilevel analysis of classical cardiovascular phenotypes (BMI, abdominal obesity, blood pressure, cholesterol levels, glucose metabolism status) with novel biomarkers (HDL functionality, LDL atherogenicity, novel inflammatory/oxidative biomarkers), together with the analysis of omics data[99,278], in larger study populations, may contribute to the unraveling of these complex processes.

The understanding of the intricate mechanisms of lipoprotein biology could also provide new therapeutic agents for the prevention of CVDs. The present work has revealed several novel mechanisms by which dietary antioxidants (or antioxidant-rich dietary patterns) contribute to atheroprotection, and a number of other bioactive agents may induce a similar set of effects through common mechanisms. The determination of the critical points in which antioxidants affect these mechanisms could contribute to discovering new therapeutic targets. Considering the recent therapeutic failures regarding HDL-C[96,98,116,117] and LDL-C levels[98,183], studying novel strategies to increase HDL function, or decrease LDL atherogenicity, may improve our capacity to prevent the development of atherosclerotic diseases. These agents may be novel functional foods[270], nutraceuticals[105,123], or new drug families[279,280].

9.2. FUTURE RESEARCH LINES IN THE GROUP

Since cholesterol efflux capacity and PON1 arylesterase activity have been shown to be associated with the incidence of cardiovascular events, at present we are studying the effects of the remaining HDL functional properties on cardiovascular risk values, as well as their relationship with the classical cardiovascular risk factors (diabetes, dyslipidemia, hypertension, obesity, and smoking habit). Moreover, our group will assess in the near future whether other HDL functionality properties (HDL vasoprotective capacity, HDL anti-inflammatory capacities in endothelial cells and macrophages, HDL oxidation degree, HDL-bound LpPLA2, HDL levels of SAA-1 and S1P) can also be predictive of incident cardiovascular episodes in large-scale prospective trials. Finally, regarding the mechanistic aspects of HDL functionality, our group is also involved in a study about the influence of epigenetic mechanisms on HDL function, which will be published shortly.

Given the ability of antioxidant-rich dietary interventions to improve HDL functional profile, our group will expand this research line in the subsequent years. Following the encouraging outcomes in HDL functionality after massive weight losses in obese patients[137], we will assess in an ongoing project the effects of a hypocaloric Mediterranean Diet (accompanied by physical activity and behavioral support) on HDL functions and quality-related profile. We are also open to initiating new collaborations to explore the effects of other healthy lifestyles on HDL functionality. In addition, we will perform a transcriptomic analysis in a subset of volunteers from the PREDIMED Study, in order to establish whether changes in HDL-related gene expression could explain any of the improvements in HDL functional characteristics observed after the consumption of a Traditional Mediterranean Diet. Finally, we are currently working on a systematic review about the different therapies that have improved HDL functions in previous clinical trials, in order to clarify the real capacity of diet to modulate the novel properties.

Regarding LDL atherogenicity, we are at present studying the main effects of LDL properties on cardiovascular risk scores, and how they are modified in high cardiovascular risk states. We are additionally exploring the relationship between the whole set of HDL functional capacities and LDL pro-atherogenic traits. A potential future research line may be to test the ability of the most novel LDL-related parameters (LDL cytotoxicity, LDL glycosylation, LDL electronegativity, determination of LDL-bound proteins –SAA, ApoC-III, LpPLA2–) to predict future cardiovascular events, as well as the ability of healthy lifestyle changes to modulate these characteristics. Finally, we are considering the possibility of examining the associations between LDL atherogenicity and transcriptomic data in a sub-sample of the volunteers from the PREDIMED Study, in order to establish whether changes in lifestyle habits may modulate LDL-related gene expression.


HDL
LDL
cholesterol
lipid
cell
lipoprotein
risk
function
levels
high
oil
protein
cardiovascular
endothelial

Appendix

SUMMARY

Álvaro Hernáez finished his Bachelor in Pharmacy in the *Universidad de Salamanca* in 2011. He obtained his Master Degree in Nutrition and Metabolism in the *Universitat de Barcelona* in 2012. After obtaining a FPU pre-doctoral fellowship (FPU2012/01318), he continued his research career in the PhD program of Food Science and Nutrition of the *Universitat de Barcelona*, and joined the Cardiovascular Risk and Nutrition Research Group of the *Institut Hospital del Mar d'Investigacions Mèdiques* in Barcelona. During his doctoral research period, he collaborated as undergraduate lecturer in the subject of "Nutrition" of the Bachelors of Human Biology and Medicine of the *Universitat Pompeu Fabra* (from 2013), and in the subject of "Nutrigenetics and Nutrigenomics" in the Master of Food Research, Development and Innovation of the *Universitat de Barcelona* (from 2016). He also participated as teacher in the "Nutrigenetics and Nutrigenomics" and "Nutrition and Cardiovascular Risk" subjects of the distance learning IL3 program (*Universitat de Barcelona*, from 2016). He has tutored four Bachelor and Master research works (from bachelor students of *Universitat Autònoma de Barcelona* and *Universitat de Barcelona*, and master students of *Universitat Pompeu Fabra* and *ETH-Zürich*). Besides which he has participated as speaker in several popular science programs during his pre-doctoral training (Barcelona and Madrid Science Weeks 2012, 2013 and 2015; and "*Camins Infinits*" popular science program in high schools, between 2014 and 2016).

ADDITIONAL PUBLICATIONS RELATED TO THE THESIS

- Sayols-Baixeras S, **Hernáez A**, Subirana I, Lluis-Ganella C, Fitó C, Marrugat J, Elosua R. *DNA methylation modulates HDL functionality: the REGICOR Study*. [Submitted].
- Farràs M, Castañer O, Martín-Peláez S, **Hernáez A**, Schröder H, Subirana I, Muñoz-Aguayo D, Gaixas S, Torre Rde L, Farré M, Rubió L, Díaz Ó, Fernández-Castillejo S, Solà R, Motilva MJ, Fitó M. *Complementary phenol-enriched olive oil improves HDL characteristics in hypercholesterolemic subjects. A randomized, double-blind, crossover, controlled trial. The VOHF study.* Mol Nutr Food Res. 2015 Sep;59(9):1758-1770.
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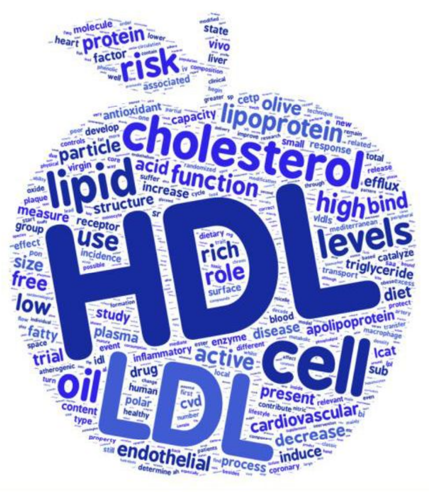
HDL
LDL
cholesterol
cell
lipid
lipoprotein
risk
levels
function
oil

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