Prospective association of HDL functions and coronary artery disease in the general population. Lifestyle interventions as a modulatory factor

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“Hay que ver, menudo lío"

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Albert Sanllorente

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Abbreviations and acronyms

ApoA-I: Apolipoprotein A-I

ApoA-IV: Apolipoprotein A-IV

ApoB-100: Apolipoprotein B-100

ApoC-II: Apolipoprotein C-II

ApoC-III: Apolipoprotein C-III

ApoE: Apolipoprotein E

BMI: Body mass index

C3: Complement component 3

CAD: Coronary artery disease

CEC: Cholesterol efflux capacity

CETP: Cholesteryl ester transfer protein

CVD: Cardiovascular disease

HDL: High density lipoprotein

HDL-C: HDL cholesterol

HOII: HDL oxidative/inflammatory index

IDL: Intermediate density lipoprotein

LCAT: Lecithin cholesterol acyltransferase

LDL: Low-density lipoproteins

LDL-C: LDL cholesterol

MedDiet: Mediterranean diet

MET: metabolic equivalents of task

PON1: Paraoxonase 1

ROS: Reactive oxygen spices

SAA: Serum amyloid A

S1P: Sphingosine-1-phosphate

VLDL: Very low-density lipoprotein

Abstract

Background

High-density lipoprotein (HDL) functions have arisen as alternative biomarkers to better explain HDL atheroprotective capacity rather than HDL cholesterol levels.

Aims

To study the association between HDL functions and the incidence of coronary artery disease, and assess the capacity of lifestyle interventions to improve HDL functions.

Methods

We studied several HDL functional determinations in three population-based studies. First, we analyzed the association between HDL functions and the incidence of coronary artery disease in a general population (REGICOR study). Second, we evaluated the association between changes in the consumption of key Mediterranean diet food groups and HDL functions in individuals at high cardiovascular risk (PREDIMED study). Third, we examined the effect of an intervention with an energy-restricted Mediterranean diet and physical activity on HDL functions in a population with metabolic syndrome (PREDIMED-plus study). Finally, we performed a systematic review of randomized controlled trials evaluating the effects of lifestyle modifications on HDL functions.

Results

High HDL levels of complement component 3 and low concentrations of apolipoprotein A-I and sphingosine-1-phosphate in HDL particles were associated with a greater incidence of coronary artery disease.

Regular consumption of virgin olive oil, nuts, legumes, whole grains, and fish was associated with improved HDL function. In addition, the lifestyle intervention with an energy-restricted Mediterranean diet and physical activity enhanced the HDL role in triglyceride metabolism. Finally, evidence summarized in the systematic review suggested that dietary interventions with polyunsaturated fatty acids and dietary antioxidants improve HDL functions.

Conclusion

Dysfunctional HDLs are a risk factor for coronary artery disease in the general population. Several lifestyle modifications are capable of increasing HDL functionality in individuals at high cardiovascular risk.

Resum

Antecedents

Les funcionalitats biològiques de les lipoproteïnes d'alta densitat (HDL) han sorgit com a marcadors alternatius als nivells de colesterol continguts en les HDL per explicar les capacitats protectores contra l'aterosclerosi d'aquesta lipoproteïna.

Objectius

Els objectius principals de la tesi són estudiar l'associació entre les funcionalitats HDL i la malaltia coronaria i analitzar la capacitat moduladora de les intervencions d'estil de vida en la funcionalitat HDL.

Mètodes

Per abordar aquests objectius, es van analitzar una àmplia bateria de determinacions de les funcionalitats de les HDL en tres estudis poblacionals. Primer, es va analitzar l'associació entre les funcionalitats HDL i la incidència en la malaltia coronària en mostres de la cohort REGICOR. Segon, es va estudiar l'associació entre els canvis de consumició en els grups d'aliments més importants de la dieta mediterrània i els canvis en la funcionalitat de les HDL en mostres de l'estudi PREDIMED. Tercer, es va analitzar l'efecte d'una intervenció amb una dieta Mediterrània amb restricció calòrica i promoció d'activitat física en la funcionalitat de les HDL en una mostra de l'estudi PREDIMED-plus. Finalment, es va realitzar una revisió sistemàtica per resumir l'evidència científica sobre l'efecte de

intervencions aleatoritzades d'estil de vida en la funcionalitat de les HDL.

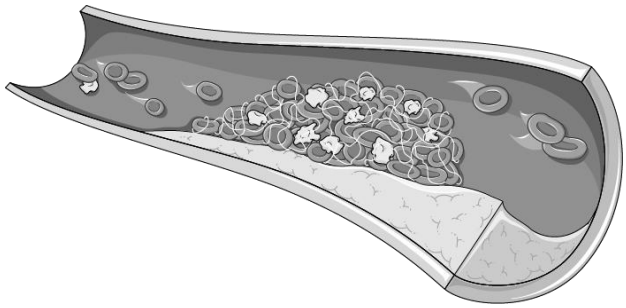
Resultats

Els nivells en HDL de component 3 del complement, de la apolipoproteïna A-I i de esfingosina 1 fosfat es van associar amb la incidència de malaltia coronaria en població general.

Respecte a les intervencions d'estil de vida, la consumició regular de oli d'oliva verge, productes integrals, fruits secs, llegums i peix s'han associat a millores de diverses funcionalitats de les HDL. A més a més, una intervenció amb dieta Mediterrània amb restricció calòrica i promoció d'activitat física és capaç de millorar sobretot el metabolisme de triglicèrids de les HDL. Finalment, la revisió sistemàtica subratlla la capacitat de les intervencions riques en àcids grassos poliinsaturats i antioxidants de millorar les funcionalitats de les HDL.

Conclusions

La disfuncionalitat de les HDL es un factor de risc per la malaltia coronaria en població general. Varies intervencions d'estil de vida son capaces de millorar diverses funcionalitats HDL en persones amb un alt risc cardiovascular.

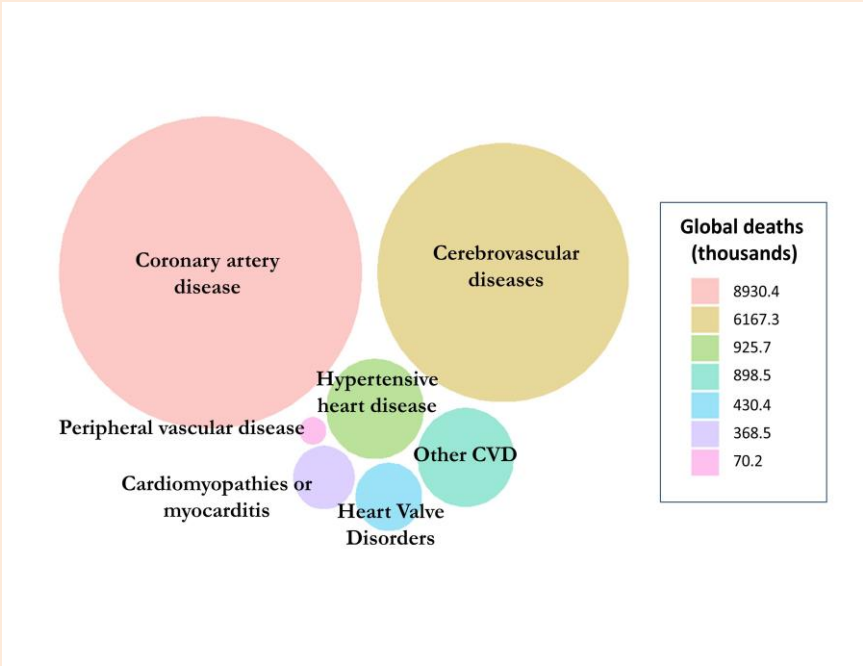


1. Introduction

1.1. Epidemiology of cardiovascular disease

Cardiovascular disease (CVD) is a term used to encompass a group of disorders affecting the heart and blood vessels. It includes: 1) coronary artery disease (CAD) (also known as coronary heart disease or ischemic heart disease) which is based in the accumulation of an atherosclerotic plaque in the arteries supplying the heart; 2) cerebrovascular diseases, when the plaques affect the blood vessels of the brain; 3) peripheral arterial disease, when the vessels supplying extremities are affected; 4) hypertensive heart diseases; 5) heart valve disorders (including rheumatic heart diseases); 6) cardiomyopathies

Figure I. Cardiovascular diseases classified by absolute mortality



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and myocarditis (affections and inflammatory disorders of the heart muscle); and 7) other cardiovascular diseases from different etiologies, such as congenital heart diseases, aneurysms, vein thrombosis, and pulmonary embolism (**Figure I**).

During the past three decades, CVDs have been the leading cause of death and disability (1). The Global Burden of Disease project has published the most recent and comprehensive data regarding mortality, premature death, and years lived with disability. This project estimated that CVDs were responsible for 17.8 million of deaths worldwide, representing approximately one third of global deaths in 2017. In addition, CVDs also are the principal contributor to reduced quality of life. They represent 330 million years lost due to premature deaths, 35.6 million years lived with disability (2), and 366 million of disability-adjusted life years. The sum of years of life lost and years lived is used as an indicator of the absolute burden of this disease. In this regard, among CVD, CAD is the major contributor to mortality and burden of disease (3) (**Figure I**).

Observing CVD progression from 2005, the global number of deaths is increasing (4), although such situation is not equally distributed. The major burden of the disease happens in low- and middle-income countries, where populations in economic transition are progressively growing and, consequently, ageing (3). Additionally, age-specific death rates have been globally decreasing, meaning that cardiovascular mortality is appearing at older ages (5). The scenario in high-income countries, with better health systems and public policies, is, however, quite different. For example, in most European countries mortality

rates of coronary artery disease and stroke progressively decreased from 1980 to 2015 and resulted in the most important reductions in age-specific death rates (6). In 2017, age-adjusted CVD mortality was 3-fold lower in high-income countries relative to low- or middle-income ones (7). Such a gap could be explained by differing preventive interventions to control cardiovascular risk factors. In wealthier countries, there has been a greater management of dyslipidemia, high blood pressure, diabetes, and acute CVD due to stronger health systems, more availability of CVD medication, and better public policies (7). Even so, CVD and CAD are still the leading cause of death. Spain is a region with a low incidence of CVD, mainly due to the Mediterranean diet (MedDiet) (8). Nonetheless, CAD is still the first cause of death, representing 14.6% of total mortality (followed by Alzheimer and other dementias (13.6%)) (9). Since 1990, CVD incidence has augmented by nearly 30%. Following the same tendency as high-income countries, age-standardized mortality rate and disability-adjusted life years have decreased, indicating that new cases are older and with fewer years lived with disability (6,9).

High rates of mortality and years lived with disability represent a major economic burden for the health systems. In 2015, CVD generated a cost of €210 billion in European Union countries, and $351.3 billion in the United States. 60% of the costs were attributable to direct health care cost (primary, emergency, hospital inpatient and outpatient care, and medication expenditure), and the rest were due to productivity losses and informal care (6,10).

In 2015, the United Nations and the World Health Organization launched a global challenge with the objective of reducing CVD mortality by 25% before 2025 (11). This plan, commonly named 25 by 25, highlights the importance of improving lifestyle factors. Specific goals include enhancing diet quality, increasing physical activity, and decreasing tobacco use. Such preventive strategies reduce risk factors for CVD in its early stages (12).

1.2. Atherosclerosis

Atherosclerosis is the main subclinical outcome leading to CAD development (13). It consists of the accumulation of lipids, pro-inflammatory cellular waste products, calcium, fibrin, and collagen-rich proteins within the intima layer of artery walls. This, in turn, forms a plaque and stiffens the artery wall (13).

The pathophysiology of atherosclerosis is complex and involves many different processes (**Figure II**) (14). The earliest step is endothelial dysfunction, a malfunction of the endothelial cells, the first cell layer of the blood vessels in direct contact with blood. This endothelial layer is a key regulator of vascular tone, cellular adhesion, thrombosis, proliferation of smooth muscle cells, and inflammation (15). Endothelial dysfunction disrupts all the protective functions of endothelium, which leads to an increase in layer permeability, reduced vasodilation, and the promotion of a pro-inflammatory and a pro-thrombotic response (16). The possible cause of endothelial damage is considered to be prolonged exposure to pathological conditions such

as high level blood pressure, triglycerides, glucose, homocystein, free radicals, and certain infectious microorganisms (16,17). A more permeable endothelium allows circulating low-density lipoproteins (LDLs) (which are cholesterol-rich) to leave the bloodstream and reach the intima (also known as the subendothelial layer). Here, LDLs are retained and accumulated by the union of apolipoprotein B-100 (ApoB-100, LDL main protein) with proteoglycans of the extracellular matrix (18). In parallel, endothelial dysfunction is also linked to the activation of an immune response. Damaged endothelium releases cytokines (interleukin 1, interleukin 6, tumor necrosis factor γ), chemokines (monocyte chemoattractant protein 1, interleukin 8), and adhesion molecules (vascular cell adhesion protein 1, endothelial cell adhesion molecule-1, endothelial cell adhesion molecule-1, E-selectin), which in turn attract monocytes and T-lymphocytes into the sub-endothelium space (19). The local microenvironment becomes richer in reactive oxygen species (ROS). The higher presence of oxidative substances induces the oxidation of LDL (modifying its proteins and lipids) and turns LDLs into pro-inflammatory oxidized ones. These modified lipoproteins display a substantial chemotactic activity and attract more monocytes (20). The pro-oxidant, pro-inflammatory microenvironment contributes to the differentiation of local monocytes into active macrophages. Macrophages are able to engulf oxidized LDL through scavenger receptors (cluster of differentiation 36, scavenger receptor-AI/II) (21). Under normal conditions, macrophages can properly metabolize and eliminate the cholesterol content in phagocyted LDLs by transferring it to high-density lipoproteins (HDL). These particular lipoproteins can finally transport cholesterol to the liver to be transformed or excreted as bile acids

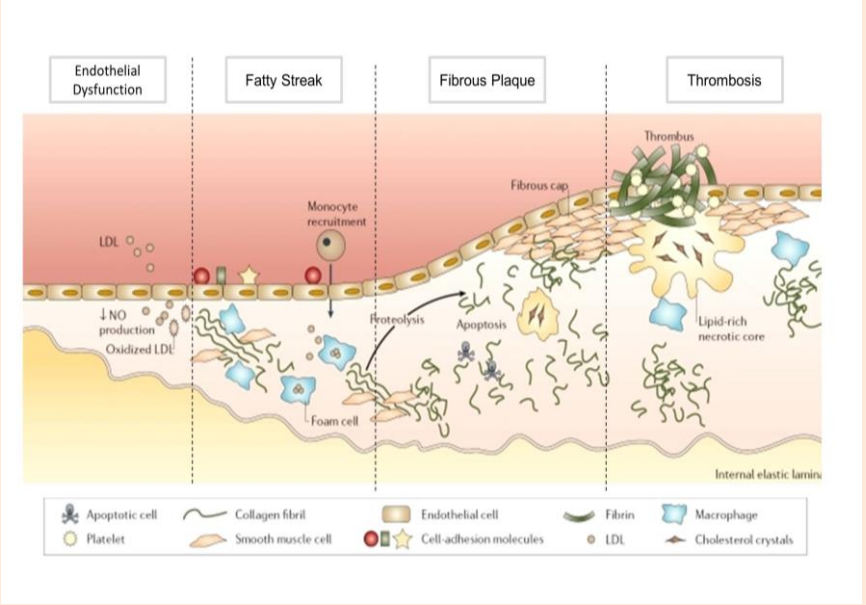
(22,23). However, during atherosclerosis, the excessive phagocytosis of oxidized LDLs disrupts the capacity of macrophages to properly metabolize cholesterol. The excessive cholesterol uptake is disproportionatedly accumulated in macrophages, which transforms them into hyperreactive, pro-inflammatory foam cells, one of the hallmarks of plaque formation. Foam cells worsen the pro-inflammatory process by releasing more chemokines and cytokines (24) which enter into a positive feedback loop. Foam cells also contribute to the production of more ROS, leading to an increased oxidation of the LDL particle.

The formation and accumulation of foam cells is crucial for the development of the plaque and the narrowing of the blood vessel diameter. Foam cells, other immune cells, and endothelial cells secrete growth factors, which induce the proliferation and migration of vascular smooth muscle cells to the subendothelium. One of the major functions of these cells within the fatty streak is the production of extracellular matrix (proteoglycans, type I and III collagen, and fibronectin). The extracellular proteins and vascular smooth muscle cells will progressively form a fibrous plaque encapsulating the fatty streak to contain its progression (13,25). In parallel, foam cells overloaded with cholesterol eventually undergo necrosis. Necrotic cells break their plasma membrane and release their lipid content, DNA, calcium, and other cellular waste products, which constitute the main components of the necrotic core. At first, the fibrous plaque is stable and can contain the atherosclerotic lesion asymptomatically. Progressive calcification, however, and growth of the necrotic core makes it more rigid and fragile which results in a vulnerable or

unstable plaque with a tendency to collapse (26). Plaque disruption produces the release of the necrotic core, which is very thrombogenic and causes the formation of a blood clot.

Atherosclerosis progression can lead to a considerable number of severe clinical consequences. The process is initiated at a young age over an extensive period without any clinical symptoms. Nevertheless, over the years the plaque formation could: a) progressively narrow the artery lumen and limit blood flow; and b) promote embolisms which can also disturb the blood flow. The continued narrowing of the artery lumen will eventually decrease the blood flow to the adjacent tissues

Figure II. Atherosclerosis progression



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(ischemia). In addition, a plaque rupture might release its thrombogenic content into the blood stream and induce the generation of a thrombus (27).

Atherosclerosis can be considered as the result of a misbalance between pro-inflammatory and inflammation-resolving mechanisms (28). A combination of a proper lifestyle intervention with lipid-lowering medications could delay, reduce, or even reverse its progression at initial stages. Complete regression, however, of the atheroma plaque remains a challenging issue and further research is warrented (29). Contrary to LDL, HDL is an extremely pleiotropic lipoprotein which has shown a wide variety of anti-atherosclerotic properties (30). HDLs are able to reduce foam cell formation due their capacity to pick up cholesterol excess from macrophages. They are also able to counteract oxidation of LDLs, neutralize ROS generated in the subendothelial space, reduce inflammatory responses, and contribute to normal endothelial function. The study of HDL anti-atherosclerotic functions has arisen as a promising area to better understand atherosclerosis and improve its treatment.

1.3. Cardiovascular risk factors

One of the most relevant characteristics of CVD is that in most cases there is a total absence of symptoms prior to a heart attack or other severe complication (12). It is, therefore, crucial to identify early medical conditions that help predict CVD incidence. These intermediate markers are known as cardiovascular risk factors, a term

coined in the 1950s by investigators from the Framingham Heart Study, one of the first long-term, comprehensive, epidemiological studies (31). Since then, several risk factors have been associated with CVD. The most predominant are age, sex, family history of CVD, dyslipidemia, hypertension, smoking, diabetes mellitus, and obesity (32). Clearly, some such as age, sex, genetic heritage, and ethnicity cannot be modified. Other factors, however, can be influenced by lifestyle and pharmacological interventions, and controlling them may prevent up to 80% of premature CVD deaths (33). In this section, we will describe the main cardiovascular risk factors.

1.3.1 Age and sex

Ageing, an inevitable part of life that represents numerous changes in physiological and molecular mechanisms, has been established as the main contributor to cardiovascular risk (34). The risk of presenting CVD before the age of 40 is very low, nevertheless, CVD morbidity and mortality increase exponentially afterwards. The major burden of disease occurs in individuals over 65 years old and accounts for 40% of all causes of death (35). Such an increase can be partially explained by the acquisition (or worsening) of other traditional cardiovascular risk factors over the years. Yet in multilinear regression models age still appears as an independent risk factor (36). This fact could suggest that age englobes other physiological modifications unexplained by traditional risk factors.

Sex represents the second major non-modifiable cardiovascular risk factor. Among men and women CAD is the leading cause of death (37,38), however, there are marked differences between the sexes. Men have a higher risk of developing CAD (and at younger ages) than women (39). In contrast, women have a greater risk of suffering a stroke (39). The contribution of some cardiovascular risk factors may additionally differ between sexes. For example, women tend to present lower levels of blood pressure and a better lipid profile (40,41). Outcomes that have sustained the belief that CVD is more associated with men, despite it being also the major cause of mortality and disability in women (37). As a consequence, women have been underrepresented in clinical trials (42) and are less likely to receive proper therapy (43).

1.3.2 Hypertension

Hypertension is a chronic medical condition that occurs when levels of blood pressure are elevated. It affects approximately 30% of the total adult population and is considered the leading modifiable cardiovascular risk factor (44).

Blood pressure, the pressure produced by circulating blood on the artery walls, is measured in millimeters of mercury (mmHg) using sphygmomanometers. It is divided into two measurements: 1) systolic, blood pressure, the levels of blood pressure occurring after the systole; and 2) diastolic blood pressure, the levels of blood pressure occurring during diastole. Greater levels of systolic or diastolic blood pressure

are directly associated with a higher risk of CVD following a linear trend (45,46). Every 10 mmHg reduction in systolic blood pressure is related with a 17% decrease of CAD risk (46). The most common thresholds used in Europe to diagnose hypertension are resting systolic blood pressure ≥ 140 mmHg, and diastolic blood pressure ≥90 mmHg (45). In general, patients with 130-139 mmHg and 85-89 mmHg, for systolic and diastolic blood pressure, respectively, are periodically controlled and provided with life-style recommendations. North American guidelines, however, have recently revised the cutoff points as 130 mmHg for systolic blood pressure and 80 mmHg for diastolic blood pressure (47). Proper control of hypertension is crucial to reduce the burden of CVD (45). Nevertheless, a considerable proportion of population remains underdiagnosed due to the symptomless character of this condition (48).

1.3.3 Diabetes and hyperglycemia

Diabetes is a chronic metabolic disease characterized by elevated levels of circulating glucose (hyperglycemia). There are different types of diabetes classified by etiology, the most common being diabetes type 1, diabetes type 2, and gestational diabetes. Type 1 diabetes is an autoimmune disease caused by the destruction of the insulin-producing beta cells leading to an insulin deficiency. It represents about 5-10% of diabetes cases and mainly develops during childhood (49). Type 2 diabetes, however, is a consequence of insulin resistance. It appears when muscle, adipose, or hepatic tissues do not properly respond to insulin and beta pancreatic cells cannot produce enough

insulin to compensate this situation. Finally, gestational diabetes has an etiology similar to type-2 diabetes, is usually transient, and reverts after delivery under proper control. Among diabetes types, type 2 diabetes accounts for approximately 90% of cases and is the most analyzed in epidemiological studies (50). According to European guidelines, diabetes is diagnosed when levels of fasting glucose are ≥6.99 mmol/L (≥126 mg/dL) or levels of glycated hemoglobin are 48 mmol/mol (51). Since 1980, the global prevalence of diabetes has been continuously rising. In 2019, it affected 463 million individuals, a figure that is estimated to increase to 700 million by 2045 (50,52). Lifestyle interventions inducing weight loss are effective strategies for the prevention and management of diabetes (51,53,54) in a dose-dependent manner (55).

Diabetes has been consistently established as a CVD risk factor. A meta-analysis of 102 prospective studies reported that presenting diabetes increases by two-fold the risk of having a CAD event (51,56). In addition, CAD risk was higher in participants with long-standing diabetes. Most individuals with type 2 diabetes also present other CVD risk factors such as hypertension, dyslipidemia, and obesity (57,58). Increases in fasting glucose levels are also associated with higher CVD risk. Glucose >100 and >126 mg/dL increases CAD risk by 11% and 78%, respectively (56).

1.3.4 Excess weight

Excess weight is a chronic condition defined as the excessive accumulation of body fat. The most common method to measure and

classify body weight and obesity is the body mass index (BMI). It is calculated as the individual's weight in kilograms divided by the square of the height in meters. Several categories according to BMI values have been defined: underweight (<18.5 kg/m^2), normal weight (18.5-24.9 kg/m^2), overweight (25.0.-29.9 kg/m^2), and obesity (≥30.0 kg/m^2). Obesity can be further categorized in class I (30.0-34.9 kg/m^2), class II (35.0-39.9 kg/m^2), and class III or severe obesity (≥ 40.0 kg/m^2) (59). Besides BMI, waist circumference measure is a complementary method that evaluates abdominal fat. It is measured using an anthropometric tape as the measure in cm midway between the lowest rib and the iliac crest. Both measurements have been consistently associated with CVD (60,61).

Obesity affects nearly one third of the world's population (62). Obese individuals are at risk of presenting several co-morbidities (CVD, type 2 diabetes) leading to further mortality and disability (63). This disease also has adverse effects on other cardiovascular risk factors. It is associated with dyslipidemia (high levels of LDLs and triglycerides, and low levels of HDL), hypertension, and hyperglycemia (64).

As a rapidly growing tendency obesity has come to represent a pandemic. From 1990 to 2017, the global number of deaths attributed to this condition doubled, reaching a total of 4.7 million (65). This trend is also present in populations with low cardiovascular risk such as Spain. Since 1987, overweight and obesity have increased 0.28% every year (66).

1.3.5 Smoking

Smoking, responsible for approximately 20% of CVD deaths, is one of the most important modifiable risk factors (67). The risk of presenting an acute CAD event in smokers is double compared to non-smokers (68). Smoking increases the risk of CAD events and mortality in a dose-response manner (68). Even low doses and second-hand smoke are a strong cardiovascular risk factors (69,70).

Smoking is involved in the initiation and development of atherosclerosis. Cigarette smoke contains thousands of chemical products which are responsible for the formation of ROS and other mediators of oxidative stress (71). Oxidative stress is a key inductor of endothelial dysfunction, the first step of atherosclerosis (16,17). In addition, it contributes to lipid oxidation of LDLs, chronic inflammation, and platelet activation (71).

Smoking cessation is crucial for CVD prevention and treatment (72). Quitting smoking reduces cardiovascular risk and mortality over time (68). Nevertheless, cardiovascular risk remains elevated for several years after quitting, especially during the first 5 years (68,73). General public policies are effective strategies to reduce tobacco use and its subsequent CVD incidence. The Spanish 2006 law, which banned smoking in the workplace and several public and private spaces, was able to reduce acute myocardial infarction incidence and hospitalizations by 11% (74).

1.3.6. Dyslipidemia

The general disorder in the levels of lipids and/or lipoproteins is defined as dyslipidemia. It can be characterized by the presence of high levels of triglycerides (hypertriglyceridemia) and LDL cholesterol (LDL-C), and low levels of HDL cholesterol (HDL-C) (75,76). Clinical management of blood triglycerides and cholesterol, through the modification of the levels of their main carriers (LDL, very-low density lipoprotein [VLDL], and HDL) is one of the most successful strategies for CVD prevention (77).

- **Hypertriglyceridemia**

Hypertriglyceridemia is defined as plasma triglyceride levels > 150 mg/dL (1.7 mmol/L) (78). These levels reflect the quantity of triglycerides transported by the VLDLs, chylomicrons and their remnants (also known as triglyceride-rich lipoproteins). Epidemiological studies have described high triglyceride levels as an independent cardiovascular risk factor (79–81). A meta-analysis reported 14% and 37% increases in CAD risk in men and women, respectively, per each 1 mmol/L increase in blood triglycerides (82). Its causal association with CVD has been confirmed by Mendelian randomization studies (genetic predisposition to presenting high triglyceride levels throughout life is linked to greater risk of CVD) (81,83).

The reduction of triglyceride levels is associated with decreases in cardiovascular risk. Each 1 mmol/L (88.57 mg/dL) fall in triglyceride levels through pharmacological interventions (statins, fibrates, niacin) led to 16% less risk of major CVD (84). Triglyceride management can be improved by lifestyle interventions. Weight loss interventions with diet and exercise in obese/overweight individuals reduced triglyceride levels (85–87). Alcohol consumption, however, raises triglyceride concentrations and should be avoided (88).

- **High LDL cholesterol concentrations**

In clinical practice, the level of cholesterol transported by LDL lipoproteins (LDL-C) is usually recommended to be below 2.59 mmol/L (100 mg/dL). For patients at high cardiovascular risk, however, the therapeutic goal is to achieve LDL-C reductions of ≥50% from baseline and levels below 1.81 mmol/L (70 mg/dL) (87). In a large number of epidemiological studies LDL-C has been consistently established as a cardiovascular risk factor. Indeed, high levels of LDL-C are log-linearly associated with higher CAD risk (87,89). In a general population the risk of myocardial infarction and CVD incremented by 34% and 16% per each 1 mmol/L increase in LDL-C, respectively (90). The causal association between high LDL-C levels and CVD has been proven in multiple Mendelian randomization studies (genetic predisposition to high LDL-C concentrations throughout life is related to increased CVD risk) (83,89,91,92).

The clinical benefits of LDL-C lowering therapies on cardiovascular health are also clear. Statins are first line drugs recommended to decrease LDL-C concentration, and are effective in reducing the risk of major CVD with a good safety profile and low cost (87,93). For each 1 mmol/L decrease in LDL-C concentrations achieved by statins, CVD risk diminished by 23% (93). Even populations at low cardiovascular risk presented equivalent reductions in the risk of CVD after statin use (94). In addition to these drugs, there are other second line treatments to lower LDL-C such as ezetimibe, cholestyramine, and proprotein convertase subtilisin/kexin type 9 inhibitors (evolocumab, alirocumab) (93). The combination of these drugs with statins is recommended in patients at high cardiovascular risk. In particular, evolocumab and alirocumab present the highest capacity to reduce LDL-C levels (~60% relative to baseline levels) alone or in combination with statins (87,95,96). Changes in LDL-C levels through diet or weight loss are usually modest. Nevertheless, diets promoting the substitution of saturated and trans fats for polyunsaturated and monounsaturated ones have shown reductions in LDL-C (-8 to -15 mg/dL) (97). A 1-year intervention with the MedDiet, and dietary interventions in obese individuals, have has also been linked to modest decreases in LDL-C (98,99).

- **HDL-C levels**

The association between HDL-C and cardiovascular risk is more controversial. HDL-C concentrations below 1 mmol/L (40 mg/dL) in men, or below 1.3 mmol/L (50 mg/dL) in women, have been

associated with increased CVD risk in classic epidemiological studies (100). The first evidence of an inverse association between HDL-C concentration and CVD was published in 1977 in the Framingham and the Tromsø Heart Studies (101,102). Since then, other studies have validated this relationship (103,104). This inverse association suggested a possible protective capacity of high HDL-C levels on CVD and encouraged the scientific community to search for HDL-C raising therapies. Recent evidence, however, has questioned the role of HDL-C as a clinical target.

Several clinical trials have evaluated the use of HDL-C raising pharmacological agents and CVD outcomes. Cholesteryl ester transfer protein (CETP) inhibitors are the drugs displaying the highest capacity to increase HDL-C levels (31-138% relative to baseline levels). However, no CETP inhibitor (anacetrapib, dalcetrapib, torcetrapib) has decreased CAD mortality, incidence of non-fatal myocardial infarction or stroke (103,105,106). The use of torcetrapib was even linked wth increases in cardiovascular risk in high cardiovascular risk participants in the ILLUMINATE Study (107) and patients with familial hypercholesterolemia (108). Neither did niacin, another drug capable of incrementing HDL-C levels by 20%, decrease CVD mortality and major CVD incidence in high cardiovascular risk patients (109,110). Finally, an extensive meta-analysis summarized the effects of the most relevant HDL-C raising therapies (CETP inhibitors, niacin, and fibrates) on CV outcomes. It included a total of 39 randomised controlled trials with 117,411 participants. The study concluded that CETP inhibitors, niacin, and fibrates did not reduce all-cause mortality, CAD mortality, and CAD events after increasing

HDL-C levels (105). In addition, Mendelian randomization studies failed to find a causal role between high HDL-C levels and lesser incidence of CVD (111,112).

The failure of the HDL-C raising therapies, and the lack of association between genetic predisposition to high HDL-C concentrations throughout life and CVD risk, have led to a change of paradigm in the research field. Scientists are starting to assess HDL functional properties besides HDL-C levels. HDL particles have been associated with a wide range of atheroprotective functions, such as their ability to pick up cholesterol excess from peripheral cells, antioxidant capacities, and protective functions of the endothelium (113). Such properties may be linked to the future development of CVD risk better than mere HDL-C concentrations (30). The relationship between HDL functions and CVD will be discussed in depth in the following section after a description of the different HDL functions.

1.4. HDL function and cardiovascular disease

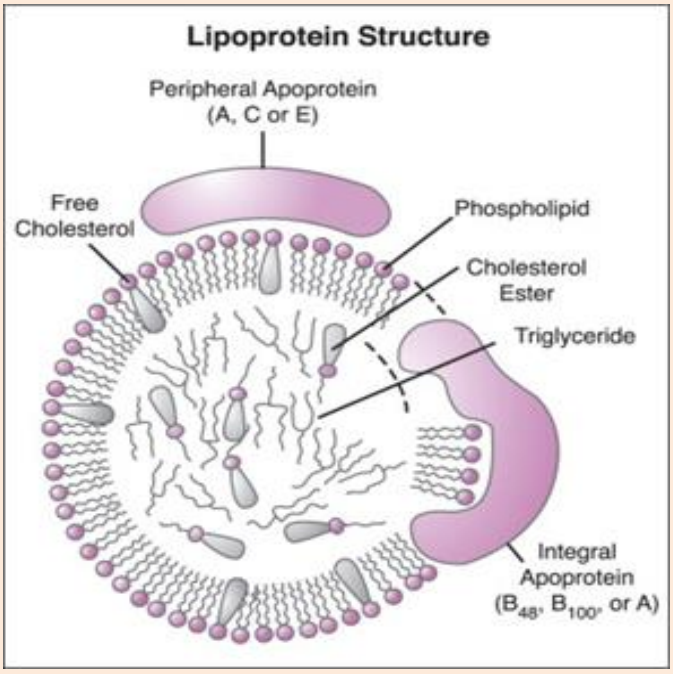
To understand the complexity of HDL functions, we need to describe first the whole cycle in which they are involved.

1.4.1 The biological cycle of lipoproteins

Lipoproteins are complex particles whose main function is to transport lipids in blood, together with some enzymes and proteins.

They are formed by a hydrophilic monolayer (phospholipids, free cholesterol, other polar lipids, proteins) in contact with blood and a hydrophobic core (triglycerides, cholesterol esters, other non-polar lipids). (**Figure III**) (114,115). There are 5 main plasma lipoproteins (chylomicrons, VLDLs, intermediate density lipoproteins [IDLs], LDLs and HDLs), which vary according to size, density, lipid composition, major apolipoproteins, and function. HDLs are the smallest (8-10 nm) and most dense (1.063–1.21 g/ml) due to their high protein content (116).

Figure III. Lipoprotein structure



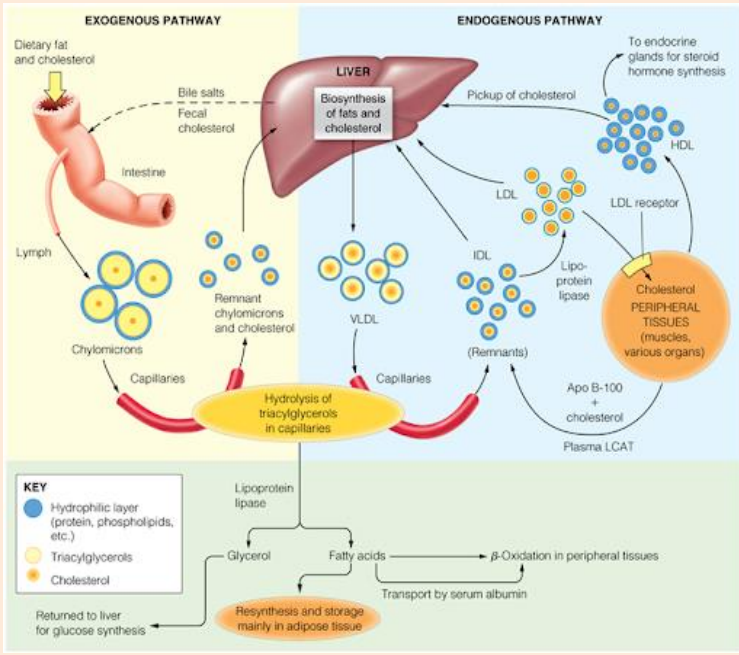
Obtained from [115]

Lipoproteins are responsible for lipid distribution in our body (**Figure IV**). The cycle can be divided in two main parts; 1) the exogenous pathway, which explains the distribution of dietary lipids by chylomicrons and chylomicron remnants; and 2) the endogenous pathway, which describes the distribution of lipids synthesized in the liver by VLDLs, IDLs and LDLs and their removal from peripheral tissues by HDLs.

- **Exogenous pathway**

The absorption of dietary triglycerides, free cholesterol, and cholesteryl esters occurs in the small intestine. The presence of fats stimulates the secretions of bile acids which emulsify lipids to facilitate their mixture into the intestinal fluid. Inside enterocytes, triglyceride droplets are coated with cholesterol, phospholipids, and apolipoprotein B-48 (ApoB-48) forming nascent chylomicrons (114,117). These then diffuse into the lymph and arrive into the blood stream through the thoracic duct. At that point, nascent chylomicrons obtain two apolipoproteins from HDLs, apolipoprotein E (ApoE) and C-II (ApoC-II) (118,119). After this process, nascent chylomicrons turn into mature chylomicrons which are able to transfer triglycerides to muscles and adipocytes. This transference is mediated by the lipolytic action of lipoprotein lipase, activated after union with Apo C-II (120). Triglyceride-poor chylomicrons (chylomicron remnants) return ApoC-II to HDLs to be cleared in the liver in a process mediated by the binding of ApoE to the ApoE receptors (the so-called LDL receptors and LDL receptor-related proteins). The

Figure IV. Lipoproteins biological cycle



Obtained from [http://anitapopescu.me/projects/Notes/12-Lipoprotein-Metabolism.html]

remaining cholesterol and triglycerides in chylomicron remnants endocyted by the liver are used for storage, the formation of bile (in the case of cholesterol), or later distribution to peripheral tissues packed in VLDLs (114).

- **Endogenous pathway**

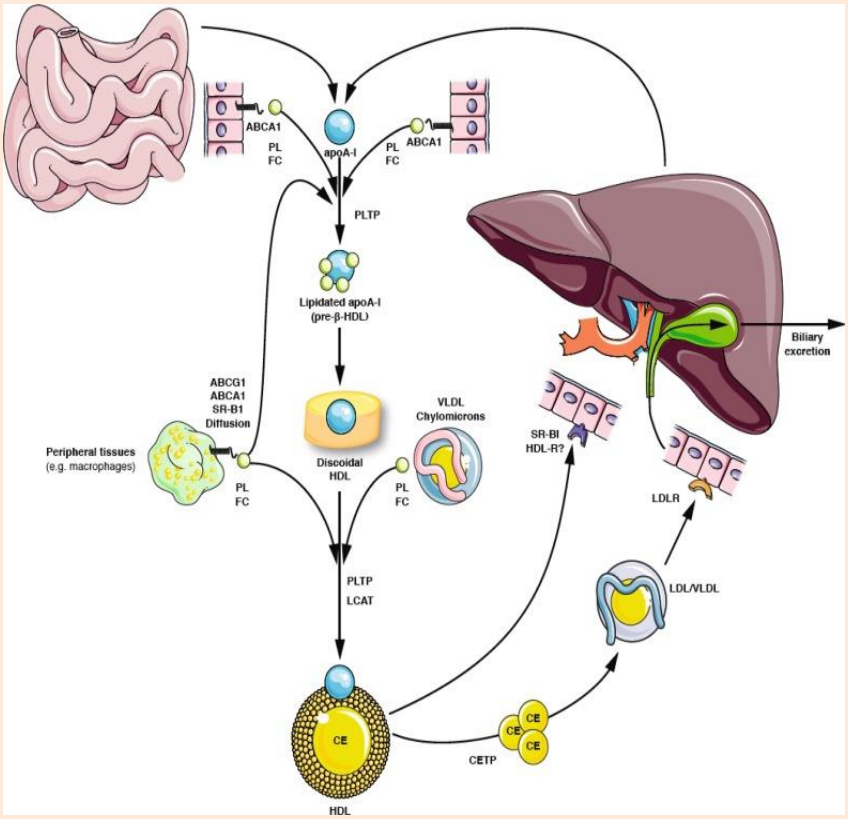
Dietary cholesterol and triglycerides, and those coming from the de novo synthesis in the liver, are recirculated into the bloodstream by packing them together with ApoB-100 (similar to ApoB-48, encoded

by the same gene but differently spliced) into VLDLs (121). This lipoprotein, very rich in triglycerides, presents a very similar function to chylomicrons. VLDL obtains ApoC-II and ApoE from HDLs and allows the transfer of triglycerides to peripheral cells by binding to lipoprotein lipase. After losing most of their triglycerides, VLDLs return ApoC-II to HDLs and become VLDL remnants, also known as IDLs (114,122). About 50% of IDLs are cleared by the liver through the interaction of ApoE with ApoE receptors. The rest have their triglycerides hydrolyzed by hepatic and endothelial lipases, lose their ApoEs, and become LDLs (123). The function of LDL is to transport cholesterol to peripheral tissues. It is present in cell membranes and necessary for the synthesis of sexual hormones in sexual organs, glucocorticoids and mineralocorticoids in the adrenal cortex, and cholecalciferol in the epithelial cells of the skin. ApoB-100 interacts with ApoE receptors on the cell surface, LDLs are endocyted, and are finally degraded. Circulating LDLs are collected by the liver through its ApoE receptors. Under normal conditions, most LDLs are recycled (about 70%) (114,122). However, excessive LDL levels in circulation increase the chances of developing atherosclerosis. As previously described in section 1.2, LDLs can randomly cross the endothelial barrier, become oxidized in the subendothelial space (especially in regions with endothelial dysfunction), and trigger the development of atherosclerosis (21).

- **HDL biological cycle**

HDLs are mainly responsible for the reverse cholesterol transport from peripheral tissues to the liver (**Figure V**). The majority of

Figure V. HDL biological cycle



ABCA1: ATP-binding cassette transporter A1. ABCG1: ATP-binding cassette transporter G1. CE: cholesterol esters. CETP: Cholesteryl ester protein. FC: free cholesterol. HDL: High density lipoprotein LCAT: Lecithin cholesterol acyltransferase. LDL: Low density lipoprotein. PLTP: Phospholipid transfer protein. VLDL: Very low density lipoprotein.

Obtained from [125]

peripheral tissues are not capable of catabolizing cholesterol. Its excess is especially critical in macrophages, in which accumulation leads to conversion into foam cells and the development of atherosclerosis. Thus, the capacity of HDLs to remove cholesterol contained in cells such macrophages is crucial for maintaining cholesterol homeostasis. This capacity is named cholesterol efflux capacity (CEC) and is one of the most relevant HDL functions (124).

HDLs originate from apolipoprotein A-I (ApoA-I), their main protein. This is mainly synthesized in enterocytes and hepatocytes, but can also come from the disintegration of mature HDL (125). ApoA-I is secreted as a lipid-free monomer which forms homodimers in circulation. Once in circulation, ApoA-I can incorporate small amounts of phospholipids through the action of the phospholipid transfer protein, an enzyme which transfers phospholipids from VLDLs to HDLs. Phospholipid incorporation creates an immature form of HDL known as lipid-poor ApoA-I or pre-β HDL (126). Lipid-free ApoA-I and immature HDLs are capable of interacting with peripheral cells to collect their cholesterol excess. In particular, lipid-free ApoA-I and immature HDLs bind ATP-binding cassette A1 transporters in the cell surface. Through this channel, free cholesterol and phospholipids are transferred from cells (127). Free cholesterol is transformed into cholesterol esters by the function of the lecithin cholesterol acyltransferase (LCAT) enzyme and internalized to the core of the lipoprotein. This process allows the maturation of HDLs into fully functional spherical particles (127–129). Mature HDLs will continue to pick up cholesterol interacting with other cholesterol membrane transporters such as the ATP-binding cassette G1 and the

scavenger receptor class B type I (125,130). The former allows active cholesterol transfer from cells to HDLs (independent from the cholesterol concentration in HDL particles), whilst the latter only permits a downward gradient flow (from higher to lower concentrations) (125,130).

This cholesterol will be eventually eliminated. There are two pathways to return cholesterol back to the liver, a direct and an indirect one. Direct transfer occurs when HDLs interacts with the membrane receptors of hepatocytes such ApoE receptors (by endocytosis of HDL containing ApoE) or by union with scavenger receptor class B type I (by selective cholesterol ester uptake and bi-directional diffusion of free cholesterol) (130,131). The indirect pathway, which is the main one, requires the intervention of apoB–containing lipoproteins (mainly VLDL). HDL transfers cholesterol esters to apoB–containing lipoproteins in exchange for an equal content of triglycerides. During this process, mediated by the CETP enzyme, cholesterol is recirculated from where it is very dangerous (macrophages) and transported back to the liver when VLDLs become LDLs and are captured by the liver (132).

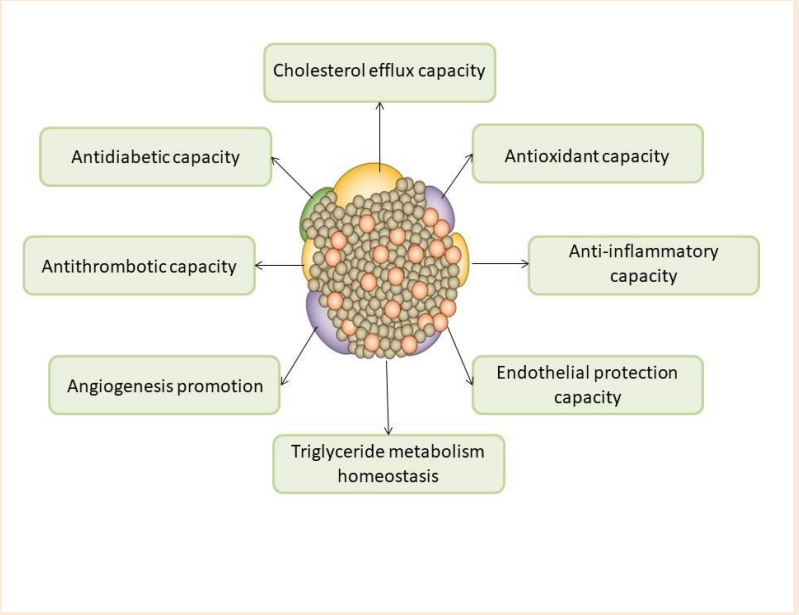
HDLs also are key regulators of triglyceride metabolism. They obtain triglycerides in exchange for cholesteryl esters through the action of the CETP enzyme. Triglyceride-rich HDLs (depleted from cholesterol) can be cleared by the liver whenever they are internalized by hepatocytes after binding to ApoE receptors (132). In addition, HDLs are involved in the biological cycle of various apolipoproteins strictly related with triglyceride metabolism. As previously explained,

HDLs collect and transfer ApoC-II from and to VLDLs and chylomicrons. This apolipoprotein is the ligand for lipoprotein lipase in triglyceride-rich lipoproteins, and thus its levels are inversely related with triglyceride concentrations (118,119). HDLs can also carry apolipoprotein C-III (ApoC-III) on their surface, a known inhibitor of lipoprotein lipase (133) which delays triglyceride lipolysis and, therefore, promotes triglyceride accumulation in lipoprotein (133–135). ApoC-III additionally disrupts the binding of ApoB and ApoE to hepatic receptors, in turn interrupting lipoprotein clearance (133). Both apolipoproteins can be found in HDLs, LDLs, VLDLs, and chylomicrons and are continuously exchanged and shared among them (136). Finally, apolipoprotein C-I seems to play a role in the complex regulation of the activity of enzymes and receptors involved in the metabolism of VLDL and HDL.

1.4.2 HDL function and dysfunction

HDL presents a wide set of atheroprotective functions. To date, the most studied are CEC, antioxidant capacities, role on inflammation, ability to protect endothelial cells, and contribution to triglyceride metabolism (137) (**Figure VI**). Such functions are partly due to the bioactive constituents of HDL which include apolipoproteins, enzymes, and active lipid components (116,138). Under chronic pro-inflammatory responses, however, HDLs might lose their anti-atherosclerotic functions and turn into pro-inflammatory lipoproteins (139,140). Dysfunctional HDLs are characterized by changes in their proteome (141) which reveal increased levels of serum amyloid A

Figure VI. HDL functions



Modified from [137]

(SAA) and complement C3 (C3) (142), both capable of displacing ApoA-I (143). A second mechanism involved in dysfunction is the oxidation of HDL proteins such ApoA-I and paraoxonase (144–147). The most important bioactive components of HDL are summarized in **Table 1**.

Table 1. Major HDL proteins

Name	Synthesis	Function and properties
Apolipoproteins		
Apolipoprotein A-I	Liver and intestine	Major HDL protein (approximately 70% of total protein content). Structural protein involved in HDL stability. Mediator of the interaction with peripheral cell receptors. Major functional protein which mediates CEC and antioxidant capacity.
Apolipoprotein A-II	Liver	Second most abundant (15-20% of total protein content). Structural protein involved in HDL stability. Functional role is still unclear.
Apolipoprotein A-IV	Intestine	Structural protein. Involved in antioxidant and CEC functional capacities.
Apolipoprotein C-I	Liver	Modulator of lipid metabolism. Inhibitor of CETP and hepatic lipase. Activator of LCAT.

Name	Synthesis	Function and properties
Apolipoprotein C-II	Liver	Modulator of lipid metabolism. Activates lipoprotein lipase stimulating triglyceride hydrolysis.
Apolipoprotein C-III	Liver	Modulator of lipid metabolism. Inhibitor of lipoprotein lipase. Blocks interaction between ApoB and ApoE with hepatic lipase reducing liver uptake of lipoproteins.
Apolipoprotein C-IV	Liver	Modulator of triglyceride metabolism.
Apolipoprotein D	Liver	Binding of small hydrophobic molecules.
Apolipoprotein E	Liver	Involved in HDL stability. Ligand of LDL receptor and LDL receptor related proteins. Regulates lipoprotein hepatic clearance. Possibly related with CEC through interaction with ATP-binding cassette transporter A1.

Name	Synthesis	Function and properties
Apolipoprotein F	Liver	Inhibitor of CETP.
Apolipoprotein H	Liver	Binding of negatively charged molecules.
Apolipoprotein J	Liver	Binding of hydrophobic molecules and interaction with cell receptors.
Apolipoprotein M	Liver	Principal carrier of Sphingosine-1-phosphate and involved in HDL endothelial protection function.
Enzymes		
Lecithin cholesterol acyltransferase	Liver, Brain	Regulator of lipoprotein metabolism. Esterification of free cholesterol to cholesteryl esters. Involved in HDL CEC and antioxidant capacity.
Cholesteryl ester transfer protein	Liver	Regulator of lipoprotein metabolism. Exchange of CE and triglycerides, and exchange of PL between HDL and apoB-containing lipoproteins. Involved in HDL CEC and antioxidant capacity.

Name	Synthesis	Function and properties
Paraoxonase 1	Liver	Calcium-dependant enzyme with lactonase, arylesterase, and paroxonase activity. Major enzyme involved in HDL antioxidant capacity.
Paraoxonase 3	Liver	Calcium-dependant enzyme. Potent lactonase and limited arylesterase activity.
Platelet-activating factor-acetyl-hydrolase	Macrophage	Hydrolysis of short-chain oxidized phospholipids. Involved in HDL antioxidant capacity.
Phospholipid transfer protein	Widely expressed in different tissues	Phospholipid transfer to HDL particles. Regulator of HDL size. Possibly involved in HDL antioxidant capacity.

Name	Synthesis	Function and properties
Sphingolipids		
Sphingosine-1-phosphate	Secreted from blood platelets and erythrocytes	Stimulates nitric oxide production of endothelial cells. Preserves endothelial cell homeostasis. Associated in HDL with apolipoprotein M.
Acute-phase proteins & complement proteins		
Serum amyloid A	Liver	Major acute-phase reactant protein associated with HDLs. Found mainly under chronic inflammatory responses. Pro-inflammatory protein involved in HDL dysfunction.
Complement component 3	Liver	Complement activation. Found mainly under chronic inflammatory responses. Pro-inflammatory protein involved in HDL dysfunction.

Name	Synthesis	Function and properties
Alpha-1-antitrypsin	Ubiquitous	Inhibitor of elastase and other proteases secreted by macrophages. Involved in HDL anti-inflammatory capacity.

Modified from Kontush A, Handbook of Experimental Pharmacology, 2014.

In the following sections, we will summarize the evidence of the most widely studied HDL functions and their main implications for cardiovascular health to date.

- **Cholesterol efflux capacity**

CEC, which quantifies the capacity of HDLs to remove cholesterol from cells, is the most studied HDL function. It can be measured by in vitro assays in macrophage-derived cell cultures treated with fluorescent-labelled or radio-labeled cholesterol. HDL particle number has been related to CECin some studies, although not in all (148,149). ApoA-I modifications (oxidation, glycation) are linked to decreased CEC values in individuals with previous CVD or type-2 diabetes (145,150). The presence of other minor apolipoproteins such as apolipoprotein A-II, apolipoprotein A-IV (ApoA-IV), and ApoE has also been shown to be associated with increased CEC (151–153), as well as a reduced HDL content of CETP (154,155).

CEC is associated with subclinical atherosclerosis and incident CVD. In the high impact journal, New England Journal of Medicine, CEC was linked with subclinical atherosclerosis and incident CVD by using ex vivo cell experiments (156,157). An association, independent of HDL-C concentrations, which has been confirmed in some prospective studies in high cardiovascular risk individuals (158) and a general population (159), although not by all authors (113). A recent meta-analysis estimated that each 1-SD increase in CEC values was linked to 14% and 23% less CAD and CVD mortality, respectively (113).

- **HDL antioxidant capacity**

HDLs are also capable of preventing LDL oxidation (160). They carry several antioxidant enzymes and proteins such as paraoxonase, which is capable of degrading oxidized lipids in lipoproteins (161–164). Paraoxonase in HDL can be found in two isoforms, paraoxonase-1 (PON1, the most common) and paraoxonase-3 (165). It presents three types of hydrolytic activities (lactonase, arylesterase, and paroxonase activities) to metabolize ozidized lipids (166). ApoA-I also exhibits key antioxidant capacities (185,186) and is crucial in the stabilization of antioxidant enzymes such as paraoxonase (167). HDLs are also able to directly uptake oxidized lipids from other lipoproteins and therefore improve their oxidative state (168,169).

There are various in vitro techniques to evaluate HDL antioxidant capacity. The HDL oxidative/inflammatory index (HOII) is a cell-free assay that estimates the global HDL capacity to prevent the oxidation of a fluorescent lipid marker by oxidized LDLs. Decreased values of HOII are related with greater antioxidant capacity, whilst increased ones are indicative of a dysfunctional, pro-oxidant, pro-inflammatory lipoprotein (170). We can also evaluate the oxidative status of HDL itself by measuring its content of malondialdehyde (a lipid peroxide) (141,143,144,171).

Regarding HDL antioxidant capacity, the activity of PON1 enzyme has been inversely associated with incident CAD events in some epidemiological studies (172,173). A meta-analysis estimated 19% lower PON1 activity in CAD patients compared to controls (173). These results agree with studies evaluating HOII. Three different observational studies reported increased levels of HOII in participants with acute coronary syndrome (174–176).

- **HDL anti-inflammatory capacity**

HDL has been shown to modulate inflammatory responses in in vitro and animal models (177,178). HDLs can inhibit the transmigration of monocytes treated with oxidized LDLs in cell models (179). This reduced migration is accompanied by a reduction in the expression of endothelial adhesion proteins (vascular cell adhesion protein 1, endothelial cell adhesion molecule-1, E-selectin) (180,181) and chemokines (such monocyte chemoattractant protein 1) (179).

Dysfunctional HDLs are, however, also known to present an increased levels of acute phase proteins such as SAA or C3 (182), which displace active proteins such as ApoA-I and paraoxonase from the HDL surface. HDL-bound SAA has been reported to be able to promote the production of monocyte chemoattractant protein 1 in vascular smooth muscle cells (183).

A relationship between HDL-related inflammatory markers and CAD has been suggested. An HDL proteomic analysis revealed that the levels of SAA and C3 in HDLs were higher in individuals with acute coronary syndrome compared to matched controls (142).

- **HDL role on endothelial protection**

There is also growing evidence indicating a relevant role of HDL in endothelial homeostasis. A healthy endothelium maintains proper permeability and regulates vascular tone, cellular adhesion, thromboresistance, and proliferation of smooth muscle cells (15). In this regard, HDLs facilitate the release of nitric oxide from endothelial cells. Nitric oxide is the main physiological vasodilator in blood vessels and its production is decreased during atherosclerosis (184). HDLs binding to endothelial cells through SR-BI receptors is one of the mechanisms postulated for the activation of endothelial nitric oxide synthase, the enzyme responsible of nitric oxide production (185,186). Whilst most of this evidence comes from in vitro studies (186–188), it has also been observed in vivo in hypercholesterolemic men (189). Another bioactive agent intimately involved in the promotion of

endothelial function is a phospholipid, sphingosine-1-phosphate (S1P). More than 70% of S1P in circulation is transported in HDLs, bound to apolipoprotein M (190). There, S1P can bind S1P receptors, which in turn promote the function of endothelial nitric oxide synthase (186,191,192), the synthesis of other vasodilators such as cyclooxygenase-2 and prostaglandin I-2 (both eicosanoids) (193), and the homeostasis of the endothelial barrier by enhancing the synthesis of tight junction proteins (138,194).

Although very few epidemiological studies have assessed the atheroprotective role of HDL-bound S1P it has been reported that individuals with CAD history had lower levels of S1P in HDL compared to healthier controls (138,195). In addition, low levels of S1P in HDLs have been linked to an increased incidence of acute coronary syndrome in individuals at high cardiovascular risk (158).

- **HDL role on triglyceride metabolism**

Whilst most references are focused on reverse cholesterol transport, the role of HDL in triglyceride transport and metabolism has been barely studied. We have already highlighted that HDLs are involved in the metabolism of triglyceride-rich lipoproteins (132) and can carry apolipoproteins directly involved in the regulation of some key elements in triglyceride metabolism such as lipoprotein lipase (136). In addition, triglyceride-rich HDLs are related to an impaired HDL functionality due to changes in the lipoprotein structure that lead to an unstable ApoA-I conformation (196,197). Excessive CETP activity,

which results in an increase in HDL triglyceride content (132), is increased in hypertriglyceridemia, and this greater triglyceride accumulation in HDLs may also be linked to a poorer function (198).

Two markers related with HDL triglyceride metabolism have been linked with CVD in epidemiological studies. High triglyceride levels in HDL have been causally associated with a greater risk of CAD in a Mendelian randomization study (199). In addition, elevated ApoC-III levels in HDLs have been recently associated with greater CAD incidence in a meta-analysis of two large cohorts with adults free from previous cardiovascular diseases (200).

- **Other HDL functions**

In addition to the previously mentioned functions, there are other HDL atheroprotective capacities described in the literature. On the one hand, HDLs are known to have an antithrombotic capacity, reducing platelet aggregation and inducing fibrinolysis. In animal studies, HDLs have been related with a reduced expression of selectins, thrombin, platelet-activating factors, and pro-aggregant eicosanoids such as thromboxane A2, and an increased expression of antithrombotic elements such as plasminogen activator inhibitor-I (201). On the other hand, HDL may also improve glucose metabolism. In this regard, animal studies have described the capacity of HDLs to promote glucose uptake in skeletal muscle cells (202).

1.5. Lifestyle interventions, cardiovascular health, and HDL functions

The most important strategy for primary CVD prevention is the motivation of a healthy lifestyle due to its capacity to improve all modifiable cardiovascular risk factors. The main lifestyle interventions currently proposed in North American and European guidelines are: 1) the promotion of a healthy diet such the MedDiet; 2) the maintenance of adequate levels of physical activity; 3) weight loss in overweight and obese individuals; 4) smoking cessation (72,203). We summarize the most recent evidence of their protective effects on cardiovascular health in general and HDL functions in particular.

1.5.1 Mediterranean diet

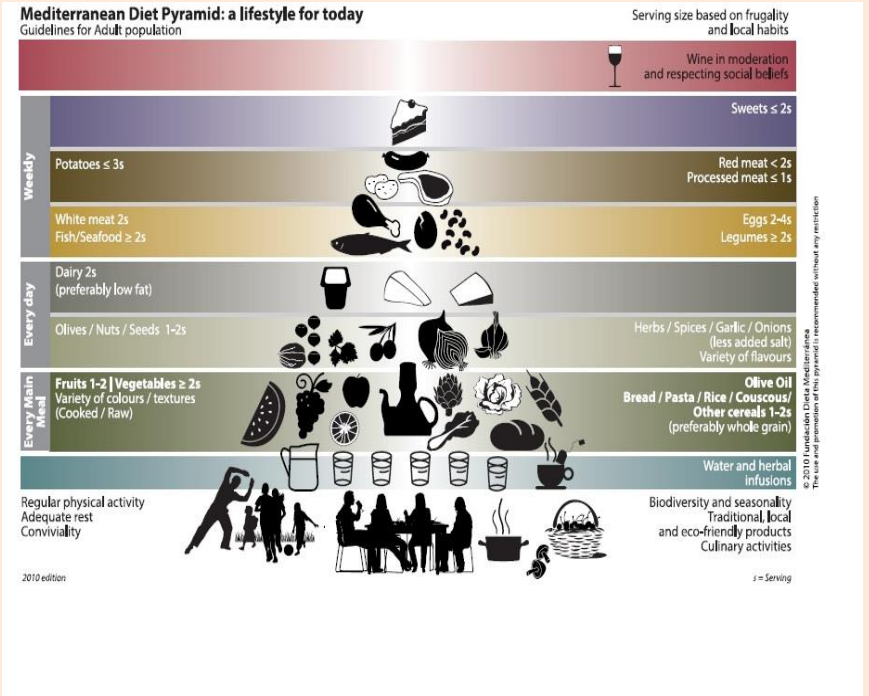
The MedDiet is a dietary pattern based on the traditional culinary habits of countries bordering the Mediterranean Sea (Spain, Italy, Greece, northern African countries, etc.). It is also a valuable cultural heritage representing customs, elaborated cooking styles and recipes, and implying crucial social interaction during meals. In 2010 it was declared as intangible heritage by the UNESCO. The MedDiet is characterized by: 1) a high intake of fruit, vegetables, nuts, legumes, cereals, and whole-grain products; 2) the use of olive oil as the main culinary fat; 3) a moderate consumption of fish, poultry, and dairy products; and 4) a reduced intake of processed foods, red or processed

meat, free sugars (soft drinks, pastries, cakes) and fat spreads (butter, margarine) (**Figure VII**).

- **Mediterranean diet and cardiovascular disease**

The Traditional MedDiet is capable of preventing the development of primary and secondary CVD (204). The Seven Countries Study led by Ancel Keys in 1958 was the first epidemiological approach to identify an association between MedDiet and lower CVD incidence (205,206). From the Seven Countries Study several prospective studies have

Figure VII. Mediterranean diet Pyramid



Modified from [https://dietamediterranea.com/ca/nutricio-saludable/]

supported the protective effect on CVD (207). To date, the PREDIMED Study (Prevención con Dieta Mediterránea) has provided scientific evidence of the highest quality on the effects of the MedDiet on CVD prevention. A 5-year, large-scale, randomized, controlled, parallel-group, multicentric trial with 7,447 older adults at high cardiovascular risk, the PREDIMED compared three different diets; 1) a traditional MedDiet enriched with extra-virgin olive oil; 2) a traditional MedDiet enriched with nuts; 3) a low-fat control diet following American Heart Association dietary recommendations at the time of trial commencement (2003). Both MedDiet groups decreased the incidence of a number of CVD outcomes (myocardial infarction, stroke, and death from cardiovascular causes) by 30% (208). The MedDiet also presents beneficial effects on the secondary prevention of CVD. In this respect, the Lyon Diet Heart study, a 46-month, randomized, controlled trial with 605 patients who had survived a myocardial infarction, assessed the effect of a MedDiet enriched with α-linoleic acid on the incidence of new cardiovascular events compared to a control group following a western-diet. A 50% reduction in cardiovascular recurrence (including fatal and nonfatal myocardial infarction, unstable angina, stroke, heart failure, pulmonary or peripheral embolism, and minor hospital admission) was reported (209).

The MedDiet effects on CVD incidence can be explained by changes in cardiometabolic risk factors. According to various meta-analyses, following a MedDiet increases HDL-C levels, lowers the concentrations of total cholesterol, triglycerides, fasting glucose, and glycated hemoglobin, and normalizes blood pressure. It also decreases

the levels of low-grade inflammation biomarkers (such as tumor necrosis factor alpha, interleukin-6, and C reactive protein), indicators of oxidative stress, and linked to a decreased incidence of clinical outcomes that increase cardiovascular risk such as type 2 diabetes (204,210–212).

The MedDiet is abundant in several food components that may explain improvements in CVD incidence and cardiovascular risk factors (213). First, it is very rich in antioxidant compounds such as antioxidant vitamins (vitamin C and E), phenolic compounds (monophenols, flavonoids, and other types of polyphenols), and carotenoids (coming from extra-virgin olive oil, fruit, vegetables, nuts, and legumes) (214–217). Antioxidants are able to reduce oxidative stress (they neutralize ROS), and in turn decrease low-grade inflammation (218), counteract LDL oxidation (219), and prevent endothelial dysfunction (220). In addition, some phenolic compounds are able to induce AMP-activated protein kinase, a key regulator of cell metabolism. The induction of AMP-activated protein kinase: 1) activates the response of peroxisome-proliferator activated receptor alpha in the liver, which decreases the de novo synthesis of triglycerides and boosts the production of ApoA-I (in turn decreasing triglyceride and increasing HDL-C levels) (221,222) and the synthesis of acute phase proteins such as SAA, C reactive protein, and fibrinogen (223); 2) optimizes glucose metabolism in insulin-dependent cells, which decreases glucose levels (224,225); 3) promotes the function of endothelial nitric oxde synthase, which favours vasodilation and an antihypertensive effect; and 4) decreases the low-grade inflammation responses in several immune cells (226). Second,

the MedDiet is very rich in unsaturated fatty acids such as monounsaturated fats from olive oil (oleic acid), and omega-3 polyunsaturated fats from nuts (alpha linolenic acid) and fish (eicosapentaenoic and docosahexaenoic acids). Unsaturated fats have been consistently associated with decreases in CAD incidence and mortality (227–230). They are inductors of the response of peroxisome proliferator activated receptor alpha (which increases HDL-C and decreases the levels of triglycerides and acuite phase proteins, as previously described), peroxisome proliferator activated receptor gamma (which increases insulin sensitivity), and facilitators in the decrease of pro-inflammatory responses in immune cells such as macrophages. Third, vegetables, fruit, legumes, and whole grains are a source of dietary fibre, whose consumption has been associated with lower CAD incidence and decreased levels of total cholesterol, LDL-C, triglycerides, and glucose (231). Dietary fibre delays and diminishes the absorption of dietary glucose and cholesterol, and adsorbs dietary cholesterol and sterol-related compounds such as bile acids. This helps explain its capacity to decrease glycemia and cholesterolemia. In parallel, the metabolism of dietary fibre by intestinal probiotic bacteria produces short-chain fatty acids (such as butyric, propionic, and acetic acids). Short-chain fatty acids have been related with some cardioprotective mechanisms. They are capable of stimulating AMP-activated protein kinase, which improves lipid profile and low-grade inflammation as previously explained (232–234), and contribute to a more efficient secretion of insulin and its reduced consumption of red or processed meat, processed foods, and carbohydrates of high glycaemic index, which are associated with greater CVD incidence and mortality (235–237).

- **The Mediterranean diet and HDL functions**

The MedDiet is able to improve a wide variety of HDL functions, as observed in a large sub-sample of high cardiovascular risk participants in the PREDIMED study. A one-year intervention with a MedDiet rich in extra-virgin olive oil in high cardiovascular risk participants was able to promote the function of some HDL antioxidant enzymes (e.g. PON1) and increase the capacity of HDLs to induce the release of nitric oxide in cultured human endothelial cells relative to the low-fat control diet. It was also linked to increases in CEC, and total HDL antioxidant capacity, and decreases in HDL oxidation and CETP activity relative to baseline levels (238). These effects could be explained by wide variety of MedDiet food components. For example, the consumption of virgin olive oil enriched with anthocyanins and carotenes has been individually associated with greater CEC values (239–241) and improved HDL antioxidant and anti-inflammatory properties (242,243). Nevertheless, most studies evaluating the association between the cardioprotective food components of the MedDiet and HDL functions have presented limited statistical power and short follow-ups.

1.5.2 Physical activity

Physical activity is the generic term referring to any movement of the body produced by skeletal muscle that requires energy expenditure. It includes all movements performed during the day (at work, home, leisure time movements, etc.). When physical activity is planned and

presents structured movements it is known as exercise (e.g. walking, swimming, housework, etc.) (244,245). According to its intensity, it can be differentiated into light, moderate, or vigorous. Physical activity intensity is measured in metabolic equivalents of task (METs). One MET is equivalent to the energy consumed while sitting at rest and corresponds to 1.0 kcal/kg·h. More clearly, METs express the energy cost of exercise as a multiple of resting metabolic rate (246). Light intensity physical activity corresponds to activities of less than 3 METs (e.g. walking), moderate intensity to activities between 3 to 5.9 METs (e.g. walking at a brisk pace), and vigorous activity to ≥6 METs (e.g. team sports, running). Two other characteristics that influence the effects of physical activity are frequency (the number of sessions per day or per week) and duration. Finally, physical activity can be also classified by the nature of the exercise performed: 1) aerobic or anaerobic; 2) strength or resistance training; and 3) balance or stability training (244).

It is estimated that almost 1 out of 4 adults present a sedentary lifestyle (characterized by an excessive number of hours/day sitting or lying) and this proportion increases in older ages (247). For older adults it is crucial to follow moderate and regular levels of physical activity to maintain an adequate quality of life (248). Ageing and the marked presence of co-morbidities can, however, hinder this population's capacity to undertake physical activity. The World Health Organization has recommended a minimum of 150 minutes per week of moderate-intensity physical activity (replaceable by 75 minutes of vigorous activity) and two sessions of muscle strengthening. Once this minimum has been reached it is recommended to gradually increase

physical activity (intensity, duration, and frequency) according to the individual's possibilities (245,248).

- **Physical activity and cardiovascular disease**

Sedentarism or physical inactivity has a major impact on cardiovascular health. The Framingham Study in the 1960s first reported an increased risk of presenting myocardial infraction in participants with a sedentary lifestyle (249). Since then, several epidemiological studies have shown associations between physical inactivity and CVD (250–254), and quantified that up to 6% of total CVD is due to this risk factor (255). For this reason, physical activity promotion is a first line strategy for primary CVD prevention (256). Augmenting physical activity leads to reductions in mortality and incidence of CVD, hypertension, type-2 diabetes, weight loss, and raised HDL-C concentrations (247,257–260). Even modest increases in physical activity are beneficial for CVD prevention, and increases in intensity, frequency, and duration add extra-beneficial effects to CVD health (261,262). A recent meta-analysis has reported that changing from a sedentary lifestyle to meeting physical activity recommendations (150 min/week of moderate-intensity leisure-time physical activity) decreases CAD risk by 14%. Additionally, doubling the amount of physical activity resulted in a CAD reduction of 20% (263).

The cardioprotective mechanisms of physical activity are complex and not yet fully understood. Physical activity improves cardiovascular risk

factors such as obesity, and induces activation of the AMP-activated protein kinase (see previous comments for a more thorough description of the latter mechanism). Taken together, the two lead to decreases in glucose levels, insulin resistance, blood pressure, triglycerides, and LDL-C, and boost HDL-C concentrations (264,265). The generation of ROS and low-grade inflammation responses are also counteracted (266).

- **Physical activity and HDL functions**

Physical activity has been also related with improvements in HDL functions in some prospective trials. Non-randomized controlled trials with moderate-intensity interventions on high cardiovascular risk individuals have shown improvements in HDL antioxidant and anti-inflammatory properties. They reported increments in PON1 activity, decreases in HDL oxidation, and a promotion of HDL capacity to decrease the secretion of pro-inflammatory cytokines (such as monocyte chemoattractant protein 1) and the expression of vascular cell adhesion protein 1 in endothelial cell cultures (267–269). Some randomized controlled trials have, however, observed contradictory effects (270). The role of physical activity promotion on CEC is less clear. Whilst high-intensity exercise increased CEC values in overweight participants (271), interventions with lower intensities failed to modify CEC in individuals with obesity (270), cardiovascular risk factors (267), and history of peripheral artery disease (272). Physical activity promotion represents a potential intervention to improve HDL functions, nevertheless, the heterogeneity and small-

scale nature of the studies performed to date warrant new high-quality evidence to confirm such results.

1.5.3 Healthy dietary patterns combined with weight loss

Considering the positive effects of the MedDiet and physical activity separately, their combination may result in greater improvements in cardiovascular health and HDL functions. Such interventions could be particularly beneficial for individuals with obesity, overweight, type 2 diabetes, or metabolic syndrome, for whom the current recommendations are mainly focused on intensive lifestyle interventions with weight loss goals (273). The two main approaches to achieve weight loss are calorie restrictions and the promotion of physical activity. Calorie restriction is based on low-fat or low-carbohydrate diets, which display similar capacities to reduce weight (274). Although these dietary patterns tend to reduce weight in short-term studies they tend to lose efficacy after 12 months (275), and their overall nutritional quality is not always high. Therefore, the use of another dietary pattern with a better nutritional composition, such as the MedDiet (rich in fat vegetal products and with a high palatability and adherence), may lead to weight loss maintenance and extend its cardiovascular benefits due to the biological properties of its bioactive compounds (276).

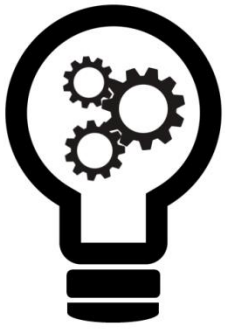
- **Healthy dietary patterns combined with weight loss and cardiovascular diseases**

Sustained weight loss interventions are usually recommended to reduce cardiovascular risk factors in obese individuals and metabolic syndrome patients. To date, however, no long-term, randomized, controlled trial has demonstrated any effect on CVD incidence and mortality. The Look AHEAD trial evaluated the effect of a long-term, weight loss intensive intervention based on dietary recommendations and physical activity in 5,145 overweight or obese participants with type 2 diabetes. After a median follow-up of 9.6 years, they reported no effects on CVD incidence (277). Similar results have also been found in a meta-analysis assessing the effects of different kinds of weight loss interventions based on lifestyle modification on CVD outcomes (278). Weight loss improves cardiovascular risk factors. Even moderate decreases in body weight (5-10%) and short-term interventions are linked to improvements in fasting glucose, blood pressure, triglycerides, and LDL-C/HDL-C levels (85,279,280). The PREDIMED-plus study (Prevención con dieta Mediterránea-Plus) is an ongoing, large-scale, randomized, controlled trial. It will evaluate the effect of a 6-year intervention with an intensive lifestyle intervention (based on the combination of calorie restriction, MedDiet, and physical activity for specific weight loss goals) relative to a non-restricted MedDiet intervention without physical activity on the primary incidence of CVD in 6,874 elderly Spanish participants with metabolic syndrome. Although the trial has not yet finished, it has presented some preliminary results after 1 year of intervention. The

intensive intervention decreased weight, waist circumference, fasting glucose, insulin resistance, and triglycerides, and increased HDL-C levels (280).

- **Healthy dietary patterns combined with weight loss and HDL functions**

The evidence regarding combined lifestyle modifications on HDL functions is very scarce and comes from modest clinical trials. A 3-month intervention with a calorie-restricted DASH diet and physical activity promotion in 53 metabolic syndrome participants was associated with an increase of CEC values and a decrease of CETP activity relative to baseline (281). Another randomized, controlled trial with 16 obese adolescents in a 10-month intervention with calorie restriction and physical activity increased CEC values and HDL capacity to restore endothelial function compared with control participants under usual care (282). Additionally, some non-controlled prospective trials with similar interventions reported increments in HDL antioxidant capacity (283,284). To date, no long-term, large-scale, randomized controlled trials with such interventions have assessed their effects on HDL functionality.



2. Hypothesis

Rationale

The lack of a causal relationship between HDL-C and CAD has placed HDL functions as better biomarkers to explain the HDL atheroprotective role.

CEC, the most studied HDL function, has been inversely associated with subclinical atherosclerosis (156) and incidence of cardiovascular events in a general population (157) and a high cardiovascular risk one (158). Evidence of associations between other HDL functions and CVD incidence in the general population is, however, scarce.

Lifestyle interventions such as the Mediterranean diet and physical activity have been shown to be able to improve HDL functions, especially in high cardiovascular risk patients (268,285). Nevertheless, it is yet to be established which food groups in this diet are particularly linked to improvements in HDL functions, or whether a combination of a healthy diet and physical activity may further improve HDL biological capacities.

Considering this background, we established the following hypotheses.

- **Hypothesis 1**

A comprehensive set of HDL functions (CEC, HDL antioxidant capacity, HDL role on endothelial homeostasis…) may be associated with the incidence of cardiovascular events in the general population.

- **Hypothesis 2**

Cardioprotective food groups from the Mediterranean diet (virgin olive oil, nuts, legumes, fish, whole grains) could be linked to improved HDL functions in high cardiovascular risk individuals.

- **Hypothesis 3**

An intervention with a combination of a calorie-restricted Mediterranean diet and physical activity may improve HDL functions in older adults at high cardiovascular risk.

3. Objectives

Objective 1

To establish whether HDL functions are associated with CAD incidence at 5 years in a sample of Spanish adults, representative of the general population.

Objective 2

To evaluate whether increases in the consumption of cardioprotective food groups are related to improvements in HDL functions in older adults at high cardiovascular risk.

Objective 3

To assess whether an intensive life-style intervention with a calorie-restricted Mediterranean Diet and physical activity, versus a traditional Mediterranean diet pattern, improves HDL functions in older adults at high cardiovascular risk.

Objective 4

To summarize the results of all randomized controlled trials to date that study the effect of lifestyle modifications on HDL functions.

4. Methods

4.1 Study populations

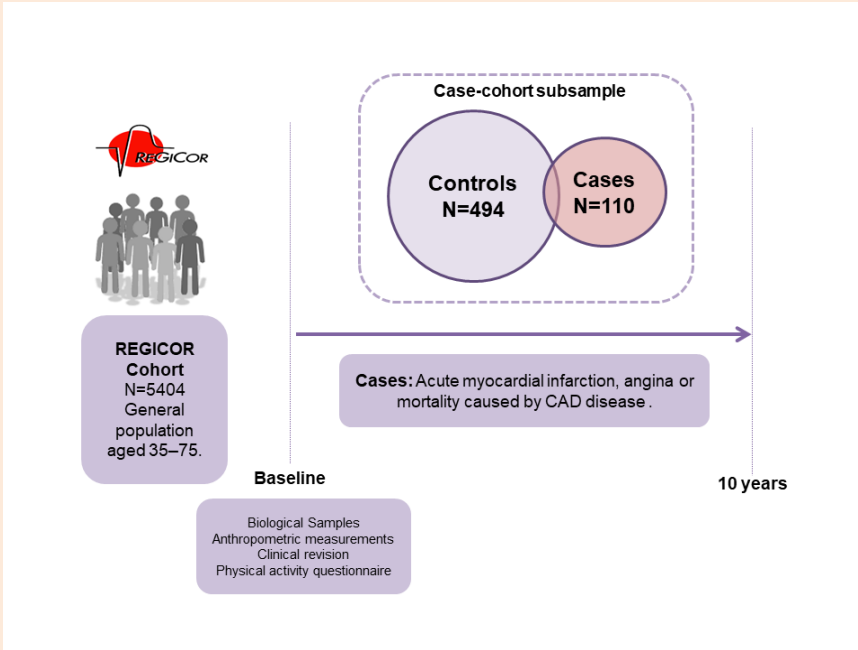
The present thesis includes four manuscripts. The first three were designed within the framework of three different study populations. The fourth is a systematic review of 118 randomized controlled trials. **Manuscript I** analyzed the association between HDL functions and CAD in a general population from the REGICOR study. **Manuscript II** studied the associations between the consumption of cardioprotective food groups and HDL functional improvements in a high cardiovascular risk population from the PREDIMED study. **Manuscript III** examined the capacity of an intensivelifestyle intervention to improve HDL functions in high cardiovascular risk individuals from the PREDIMED-plus trial.

4.1.1 The REGICOR study

The REGICOR study (in Catalan: Registre Gironí del Cor) is a long-term population-based cohort conducted in the province of Girona (northeastern Spain) (https://regicor.cat/es/). The primary objective of the study was to assess the prognosis and evolution of CAD and its related risk factors at a population scale. The REGICOR study started in 1978 and, from then on, three different cohorts have been performed (in 1995, 2000, and 2005). From the 2005-REGICOR cohort, 5,404 participants were included. The participants were non-institutionalized adults aged 35-75 years old without history of any CVD at the beginning of the study. To verify the apparition of CAD

events, participants had a ten-year follow-up (mean time: 6.15 years) with standardized telephone interviews. The primary outcome was a composite endpoint including fatal and non-fatal first occurrence of acute myocardial infarction, angina, or mortality caused by CAD. Events were evaluated by an expert committee that revised participants' interviews and medical records. Fatal CAD events were further verified through the revision of the Official Catalan Death Registry and autopsies. Outcomes corresponded to the International Classification of Diseases ICD-9 codes: 410, 411.0, 411.1, 412, 414; or to the International Classification of Diseases ICD-10: I21, I22, I24, and I25. All participants signed the informed consent. The study

Figure VIII. Case-cohort subsample design from the 2005 REGICOR cohort.



protocol was conducted under the ethical principles of the declaration of Helsinki and was approved by an Institutional Review Board. Further details of study protocol have been published elsewhere (286).

In **Manuscript I** we used a sub-sample from the 2005-REGICOR cohort to analyze the association between HDL functions and CAD. We designed a case-cohort study with 110 CAD cases and 494 non-cases. From the 5,404 participants, 117 developed a CAD event after the follow-up period. The case-cohort participants were selected as follows. We randomly selected a sub-sample of 518 participants (20 CAD cases and 498 non-cases). Eleven biological samples were lost before sample processing. The randomly selected cohort included 494 non-cases and 13 incident CAD cases. The 97 remaining CAD cases were non-randomly included making up a total of 110 CAD cases.

At the study baseline, trained nurses and interviewers collected general and clinical data, anthropometric measurements, physical activity questionnaires validated for Spanish adult population (the Minnesota leisure-time physical activity questionnaire), and biological samples.

4.1.2. The PREDIMED study

The PREDIMED study (in Spanish: Prevención con dieta Mediterránea) is a long-term, multi-center, parallel, randomized controlled trial, which evaluated the effects of a traditional MedDiet high in extra-virgin olive oil or nuts on the primary prevention of

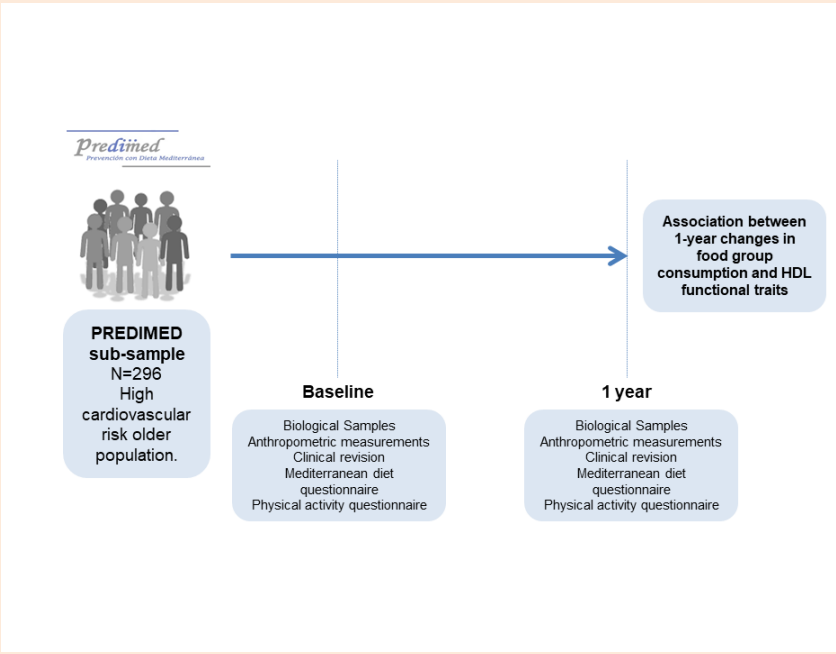
CVD relative to a low-fat control one (http://www.predimed.es/). The study recruited a total of 7,447 high cardiovascular risk older Spanish adults (women aged 60-80, and men 55-80) free from previous CVD. Inclusion criteria were to present type 2 diabetes or at least 3 of the following cardiovascular risk factors: 1) smoking habit (≥ 1 cigarrette/day during the previous month); 2) hypertension (systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg or being treated with antihypertensive drugs); 3) LDL-C < 160 mg/dL or a treatment with lipid-lowering drugs; 4) HDL-C levels < 50 mg/dL for women and 40mg/dL for men; 5) overweight or obesity (BMI ≥ 25 kg/m^2), and 6) a family history of premature CV deaths. All participants signed the informed consent before trial commencement. The study protocol was approved by an institutional ethical committee and registered with the International Standard Randomized Controlled Trial Number ISRCTN35739639. Full study protocol has been published elsewhere (287).

The PREDIMED participants were randomly allocated to one of three dietary interventions: 1) a traditional MedDiet enriched with extra-virgin olive oil (participants were given 1 liter per week for cooking and dressing to take into account the needs of the whole family); 2) a traditional MedDiet enriched with mixed nuts (15 g walnuts, 7.5 g hazelnuts, and 7.5 g almonds per day); and 3) a control group with low-fat diet counseling. Further extra-virgin olive oil or nuts were provided to participants to increase intervention compliance. Dietary interventions consisted of personalized educational interviews performed by trained dietitians at baseline and repeatedly throughout the study. Both MedDiet groups were

instructed to: 1) increase their consumption of vegetables, fruit, nuts, and fish; 2) use olive oil as the main culinary fat; 3) decrease their intake of red or processed meat; 4) prepare homemade traditional foods based on "sofrito" (a mixture of stir-fried tomato, onions, garlic, and aromatic herbs); 5) in participants who reported drinking alcohol, moderate consumption of red wine (limited to 300 ml/day) with meals was recommended (288,289). MedDiet groups also received meal plans, shopping lists, dietary recipes, and detailed descriptions of recommended foods. The control group was encouraged to follow a low-fat diet according to the American Heart Association guidelines in effect at the beginning of the study (2003) (290). The main recommendations were: 1) to increase consumption of fruit, vegetables, and legumes; 2) to decrease consumption of red, processed meats, and other fat-rich products (confectionery, processed foods, snacks, nuts, fatty fish, and sauces); 3) to reduce the use of cooking fats; and 4) to select non-fatty alternatives of standard food items and remove visible fat from meat products.

In **Manuscript II** we evaluated the association between 1-year changes in food group consumption and HDL functional traits in a randomly selected, sub-sample of 296 volunteers from the PREDIMED study (291). Information about the different cardioprotective food groups were obtained from the validated 137-item food frequency questionnaires of the study. The MedDiet cardioprotective food groups analyzed were: 1) virgin olive oil; 2) nuts (walnuts, hazelnuts, almonds, pistachios, and pine nuts); 3) fruit and vegetables (green leafy vegetables, tomatoes, carrots, cruciferous plants, beans, peppers, allium plants, cucurbits, asparagus, other

Figure IX. PREDIMED sub-study design.



vegetables, all fruit from rosacea and citrus families, bananas, berries, melons, watermelons, pineapples, kiwis, grapes, and others); 4) legumes (chickpeas, lentils, beans, and peas); 5) whole grains (breads, biscuits, and other cereals); 6) fish (lean and fatty fish); and 7) wine (red, rosé, white, sparkling, and sweet). At baseline and after one year of intervention, we collected anthropometric measurements (height, weight, waist circumference, and blood pressure), plasma samples (for biochemical analyses), and health questionnaires (the 14-item questionnaire for adherence to the MedDiet, the Minnesota leisure time physical activity questionnaire, and other general health questionnaires).

4.1.3 The PREDIMED-plus study

The PREDIMED-plus trial (in Spanish: Prevención con dieta Mediterránea-Plus) is a long-term, multi-center, parallel, randomized controlled trial with 6,874 high cardiovascular risk participants from Spain. The main objective of the trial is to evaluate the effect of an intensive lifestyle intervention consisting of a calorie-restricted MedDiet and physical activity on the primary prevention of CVD. The study is conducted in older adults (range age: 60-75 years for women and 55-75 for men) with a BMI between 27 and 40 kg/m^2 and who meet at least three components for metabolic syndrome diagnosis. Metabolic syndrome is a cluster of cardiovascular risk factors, which are: 1) triglyceride levels ≥150 mg/dL or receiving triglyceride-lowering medication; 2) fasting glucose levels of ≥100 mg/dL or being treated with glucose-lowering drugs; 3) blood pressure below 130/85 mmHg or treatment with antihypertensive medication; 4) HDL-C levels <50 mg/dL for women and <40 mg/dL for men; and 5) waist circumference ≥80 cm in women and ≥ 94 cm in men (292). Study protocol is available in the study website (https://www.predimedplus.com/en/project). It was designed under the principles of the declaration of Helsinki and approved by institutional review boards of all participating study centers. The International Standard Randomized Controlled Number is ISRCTN89898870.

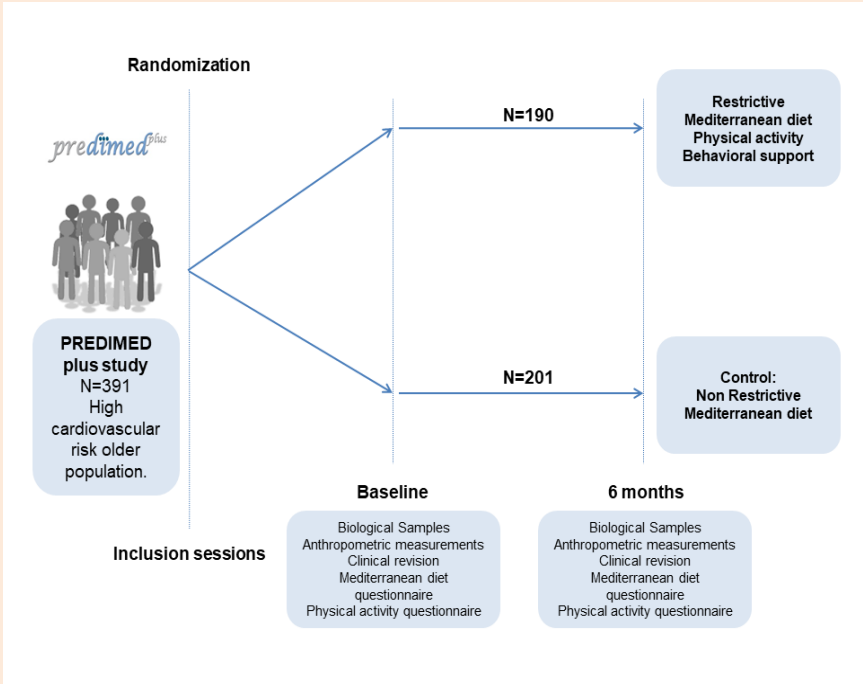
Candidates were randomly allocated to either receive a calorie-restricted MedDiet combined with physical activity promotion (intensive lifestyle group) or to a non-restrictive MedDiet (control

group). Participants allocated in the control group received individual and group educational sessions with trained dietitians to follow a traditional MedDiet with spontaneous caloric intake and no changes in physical activity. The dietary intervention was the same as previously described for the PREDIMED study (289). Participants in the intensive lifestyle group received individual and group educational sessions with the specific purpose of achieving weight reductions of about 8% of their initial weight and 5% decreases in waist circumference during the first six months of the study. After that, the objective was to maintain these reductions until the end of the study. Subjects were encouraged to combine a daily aerobic activity of moderate intensity (a minimum of 45 min/day of activities such as brisk walking, swimming, cycling, etc.) with 2 sessions a week of resistance, balance, or flexibility exercises (30-40 min/day). All recommendations were adapted to an older population to gradually achieve physical activity goals. Moreover, dietitians modified the previously described MedDiet recommendations to progressively reach a 30% decrease in energy requirement according to each participant's basal metabolic rate (a reduction of about 600 kcal/day) (280). Calorie restriction was promoted by decreasing the consumption of red meat, processed meats, processed foods, sugary drinks, and spread fats and the substitution of refined carbohydrates with whole grains (293).

Manuscript III was carried out with a sub-sample of 391 participants from one of the PREDIMED-plus recruitment centers (Hospital del Mar Medical Research Institute in Barcelona, Spain). All available plasma samples collected at baseline and after 6 months of

intervention were selected (201 samples from the control group and 190 from the intensive lifestyle one). The objective of the study was to determine whether an intensive lifestyle intervention (relative to a non-restrictive MedDiet with no recommendations on physical activity) was able to improve a wide set of HDL functions. Plasma samples, anthropometric measurements, and questionnaires were obtained at baseline and after 6-months of intervention. Physical activity was evaluated by the Minnesota-REGICOR leisure-time physical activity questionnaire validated for the Spanish population (294). Total energy intake and adherence to a calorie-restricted MedDiet were estimated using a 143-item semi-quantitative food frequency questionnaire and a 17-item questionnaire, respectively (293,295).

Figure X. PREDIMED-plus sub-study design.



4.2 Laboratory determinations

The study of HDL functions and HDL components with functional connotations is the link among the four manuscripts of the thesis. A summary of all determinations performed in each study is available in **Table 2**.

Fasting plasma or serum samples were obtained from study participants, aliquoted and stored at -80ºC prior to use in all cases. HDL functional analyses were mainly determined in ApoB-depleted plasma or ApoB-depleted serum. ApoB-depleted samples are specimens in which all lipoproteins but HDLs have been removed by precipitation. Briefly, plasma or serum specimens were mixed with a 20% polyethylene glycol 8000 suspension (Sigma-Aldrich, Madrid, Spain), incubated 20 minutes at 4ºC, and centrifuged at 10,000g during 15 minutes at 4ºC. Then the supernatant (the ApoB-depleted specimen) was collected, aliquoted, and stored at -80ºC (156).

Table 2. Laboratory determinations and specimens used in each study

Determinations	REGICOR study (Manuscript I)	PREDIMED study (Manuscript II)	PREDIMED -plus study (Manuscript III)
Biochemical profile	Serum	Plasma	Plasma
Cholesterol efflux capacity	ApoB-depleted serum	ApoB-depleted plasma	ApoB-depleted plasma
HDL oxidative/inflammatory index	ApoB-depleted serum	-	ApoB-depleted plasma
HDL oxidation	-	-	ApoB-depleted plasma
HDL Endothelial protection capacity	-	ApoB-depleted plasma	-
PON1 arylesterase activity	ApoB-depleted serum	Serum	-
Cholesteryl ester transfer protein activity	-	Serum	-
HDL capacity to esterify cholesterol	-	HDLs isolated by sequential ultracentrifugation	-
Lecithin cholesterol	-	Serum	-

acyltransferase mass			
Serum amyloid A	-	-	ApoB-depleted plasma
Complement component 3	ApoB-depleted serum	-	ApoB-depleted plasma
Sphingosine-1-phosphate	ApoB-depleted serum	-	ApoB-depleted plasma
Triglycerides	-	-	ApoB-depleted plasma
Apolipoprotein A-I	ApoB-depleted serum	-	ApoB-depleted plasma
Apolipoprotein A-IV	-	-	ApoB-depleted plasma
Apolipoprotein C-III	-	-	ApoB-depleted plasma
Apolipoprotein E	-	-	ApoB-depleted plasma

4.2.1 Biochemical profile

All systemic determinations in serum (for **Manuscript I**) and plasma (for **Manuscripts II-III**) were performed in an ABX Pentra autoanalyzer (Horiba ABX). Levels of glucose (Glucose HK CP, Horiba ABX, Madrid, Spain), triglycerides (Triglycerides CP, Horiba ABX, Madrid, Spain), and total cholesterol (Cholesterol CP, Horiba ABX, Madrid, Spain) were measured by enzymatic methods. HDL-C

levels were determined by a selective accelerator detergent methodology (HDL Direct CP, Horiba ABX, Madrid, Spain). Finally, LDL-C was estimated with the Friedewald Equation whenever triglyceride levels were <300 mg/dL.

4.2.2 Cholesterol efflux capacity

The determination of CEC was performed in a human THP-1 monocyte-derived macrophages cell line. First, THP-1 monocytes were grown in suspension at 37ºC and 5% CO2 with RPMI-1640 medium (supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin). The cell medium was refreshed every 72h. After reaching confluence, monocytes were differentiated into macrophages incubating cells with 200 nM of phorbol-myristate-acetate (Sigma-Aldrich, Madrid, Spain) for 96h. Once differentiated, macrophages were incubated for 24h with 0.2 μCi/mL of [1,2- 3 94 H(N)]-cholesterol (Perkin-Elmer) (in **Manuscript II**) or with 0.025 mM fluorescent 23-(dipyrromethene boron difluoride)-24-norcholesterol (Avanti Polar Lipids Alabaster, AL, USA) (in **Manuscripts I and III**). Then, the medium with the radiolabeled or the fluorescent-labeled cholesterol analog was washed and substituted for RPMI-1640 (with 1% of bovine serum albumin, 1% L-glutamine, 1% sodium pyruvate, 1% penicillin-streptomycin, and without supplementation of fetal bovine serum) for 24h. Finally, macrophages were washed again and incubated with participants' samples of ApoB-depleted plasma or ApoB-depleted serum at 5% for 16h. Culture supernatant was

collected, and the remaining cell content treated with Triton X-100 1%, 4ºC for 1h, homogenized, and collected for its evaluation. The radioactivity response was read in a beta scintillation counter Tri-Carb 101 2800TR (Perkin-Elmer) and the fluorescent response in Infinite 200 reader (Tecan Ltd). Cholesterol efflux was calculated as the ratio between the radioactive or fluorescent response in the supernatant × 100 divided by response in the homogenized cells + response in the supernatant. The reading of samples without incubation with ApoB-depleted plasma or ApoB-depleted serum samples was used as a blank.

4.2.3 HDL antioxidant capacities and HDL oxidative status

The global antioxidant capacity was measured with the HDL oxidative/inflammatory index (HOII). It evaluates the capacity of HDL to protect a fluorescent marker (2'-7'dichlorohydrofluorescein – Life Technologies, USA) from becoming oxidized by oxidized LDLs. The steps required were the following: 1 we diluted the marker 2'-7'-dichlorodihydrofluorescein diacetate in methanol for 30 minutes to obtain the fluorescent metabolite (2'-7'dichlorohydrofluorescein); 2) we mixed 3 μg/mL of 2'-7'dichlorohydrofluorescein with 5μL of volunteers' ApoB-depleted plasma or ApoB-depleted serum and with 1.5 μg/mL of oxidized LDLs (Cu^{2+}oxidized) in black polystyrene plates in an Infinite M200 reader (Tecan Ltd); 3) we added the fluorescent marker to the mix and measured fluorescence (Ex/Em: 485/530 nm) every 3 minutes at 37ºC during 60 minutes in an Infinite 200 reader (Tecan Ltd); 4) in all the results, we subtracted the florescent blank, and then, the negative control values (wells without

ApoB-depleted plasma or ApoB-depleted serum). The index was calculated as the slope of the linear regression with the values read between 15 and 60 minutes (158).

In some of the studies we assessed the PON1 arylesterase activity in serum samples using a commercial enzymatic activity kit (Arylesterase/Paraoxonase Assay Kit, Zeptometrix, USA).

The oxidative status of HDL was assessed as the content of malondialdehyde equivalents per unit of protein (an indicator of oxidative stress produced from lipid peroxidation) in ApoB-depleted plasma samples (291).

4.2.4 HDL pro-inflammatory markers

We determined the HDL content of C3 by immunoturbidimetry in an ABX Pentra autoanalyzer (C3; Horiba ABX, Madrid, Spain) and SAA with a sandwich ELISA kit (Human SAA ELISA Kit, Life Technologies, USA).

4.2.5 HDL endothelial properties

In some of the articles, we measured the HDL capacity to promote the endothelial release of nitric oxide in human umbilical vein endothelial cells (HUVEC, Lonza). Briefly, the protocol proceeds as follows: 1) we grew human umbilical endothelial cells at 37ºC and 5%

CO2 with EGM-2 medium (Lonza). Cell media were refreshed every 48-72h until reaching 80% confluence; 2) cells were trypsinized, transferred to 96-well plates, and incubated during 24h; 3) cells were incubated with EGM-2 medium (supplemented with 0.75% bovine serum albumin, 1% fetal calf serum, and 1% penicillin-streptomycin) with the fluorescent marker 4,5-diaminofluorescein diacetate at final concentration: 5 μM (DAF-2DA, Sigma) and with 30% of participants' ApoB-depleted plasma (or a negative control without ApoB-depleted plasma); and 4) the fluorescent response was read after 30 and 60 minutes in an Infinite M200 reader (Tecan Ltd) (Ex/Em: 485/532 nm). We measured the velocity of nitric oxide formation in the endothelial cell culture. First, we calculated the slope of the linear regression between fluorescent response at 30 and 60 minutes. Second, we subtracted the blank signal from all the values. Finally, we calculated the velocity of nitric oxide formation with the following formula: (slope of cells treated with ApoB-depleted plasma - slope of negative control × 100) / slope of negative control.

Regarding HDL components that are actively involved in HDL protective properties in endothelial cells, we quantified the levels of HDL-bound S1P in ApoB-depleted samples with a competitive ELISA kit (Sphingosine 1 Phosphate BioAssay ELISA Kit, US Biological, USA).

4.2.6 CETP and LCAT determinations

We measured CETP activity in serum using the CETP Assay Kit (MBL International, USA). LCAT concentration was assessed with the Lecithin Cholesterol Acyltransferase ELISA Kit (Sekisui Diagnostics, USA).

The HDL capacity to esterify cholesterol was calculated as the percentage of esterified cholesterol in HDL divided by LCAT mass. To evaluate the percentage of esterified HDL, we first measured the quantity of total cholesterol and free cholesterol in HDL isolated by ultracentrifugation in an ABX Pentra autoanalyzer (Cholesterol-LQ, Free Cholesterol-LQ, Spinreac). Second, we estimated the content of esterified cholesterol (total cholesterol in HDL - free cholesterol in HDL). Finally, the percentage of esterified cholesterol was calculated as: content of esterified cholesterol × 100 / total cholesterol in HDL.

4.2.7 HDL components

In some of the thesis articles, we determined the levels of ApoA-I, ApoC-III, ApoE, triglycerides, and total protein content in ApoB-depleted samples in an ABX Pentra autoanalyzer (ApoA1, Triglycerides CP, and Total Protein CP; Horiba ABX, Spain; ApoC-III, and ApoE, Spinreact, Spain). ApoA-IV levels were measured with a sandwich ELISA kit (Human Apolipoprotein A-IV ELISA Kit, UK).

4.2.8 Quality control of HDL functional determinations

Complex laboratory determinations with biological samples, cellular models, and multiple assay runs increase the risk of presenting high variability. In order to reduce the batch effect in different experimental runs: 1) we examined all the study samples in a random order; 2) we analyzed samples from the same participant in the same experimental run (e.g. samples from baseline and from follow-ups of the same volunteer); 3) we included a sample pool collected from 20 healthy volunteers in each experimental run to calculate intra-assay and inter-assay coefficients of variation; 4) we performed HDL functions (CEC, HOII, HDL oxidation, PON1 activity, and HDL capacity to promote endothelial release of nitric oxide) in duplicate and removed results with coefficients of variations >15%; and 5) we normalized the results from non-automatized assays with the sample pool included in each experiment, obtaining unitless ratios.

5. Results

Manuscript I

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RESEARCH LETTER

High-density lipoprotein functional traits and coronary artery disease in a general population: a case–cohort study

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Low levels of high-density lipoprotein cholesterol (HDL-C) levels are associated with higher incidence of coronary disease in epidemiological studies. However, this association has not been shown to be causal according to pharmacological interventions and Mendelian randomization studies.[1] Thus, HDL atheroprotective role could lie in its functional properties, particularly in individuals at high cardiovascular risk.[2,3] The most studied HDL functions are (i) cholesterol efflux capacity (CEC, the ability of HDL to pick up the cholesterol excess from peripheral cells, such as macrophages[1]); (ii) HDL antioxidant capacity [measurable as the HDL oxidative/inflammatory index (HOII) or the activity of some HDL antioxidant enzymes—paraoxonase-1 (PON1)[1,4]]; (iii) HDL role on inflammation [HDLs can change from anti-atherogenic into pro-inflammatory particles when they lose functional proteins, such as apolipoprotein A-I (ApoA-I), and bind acute phase elements, such as complement component 3 (C3)[1,5]]; and (iv) HDL endothelial protection [proportional to the levels of bioactive lipids like sphingosine-1-phosphate (S1P)[6]]. Only inconsistent and scarce information is available on the association of HDL function with respect to coronary artery disease (CAD) incidence in the general population.[1] Our aim was to determine whether a hypothesis-driven set of HDL functional traits (namely CEC, HOII, PON1 arylesterase activity, and HDL levels of ApoA-I, C3, and S1P) were associated with 10-year incidence of CAD in a population-based cohort.

For this purpose, we designed a case–cohort study within the REGICOR (*Registre Gironí del Cor*) cohort. A sample of 110 incident CAD cases and 494 non-cases was considered[7] (Supplementary material online, *Figure S1*). We studied HDL functional traits in serum samples in which HDLs were the only lipoproteins present (apolipoprotein B-depleted serum).[8] We measured CEC in THP-1 monocyte-derived human macrophages incubated with fluorescent cholesterol.[8] High-density lipoprotein oxidative/inflammatory index was defined as the ability of HDL to avoid the oxidation of 2'-7'dichlorohydrofluorescein.[9,10] We assessed PON1 arylesterase activity with an enzymatic activity kit.[9,10] We quantified ApoA-I and C3 by immunoturbidimetry and S1P by an ELISA kit.[8]

We evaluated the association between HDL functional traits and CAD risk using multivariate Cox proportional hazards regression models weighted by the Lin–Ying method.[7] The outcome was CAD incidence and follow-up time was defined as the period between the date of enrolment and the event (cases) or censoring at 10 years (non-cases). We standardized raw HDL functional traits as *z*-scores to assess the change in CAD risk for 1-standard deviation increase. Models were adjusted for age, sex, HDL-C levels, diabetes, hypercholesterolaemia, triglyceride levels, hypertension, smoking habit, body mass index, and leisure-time physical activity. These analyses were additionally stratified by HDL-C level categories (low—<40/<50 mg/dL in men/women—and desirable levels) to study their potential confusing effect on the associations. All variables whose association with CAD risk was suggested were considered for a final, mutually adjusted analysis. Cox models were fitted using the '*cch*' function of the 'survival' package[7] in R Software (R Core Team,

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Table I Associations between high-density lipoprotein functional traits and incidence of coronary artery disease

	Main analyses, HR (95% CI)	Stratified analyses by HDL-C levels			Mutually adjusted analyses, HR, (95% CI)
		Low HDL-C levels (<40/<50 mg/dL –men/women–)	Desirable HDL-C levels (≥40/≥50 mg/dL –men/women–)	*P*-value for interaction	
HDL cholesterol levels (+ 1-SD)	0.78 (0.58–1.05)	—	—	—	—
Cholesterol efflux capacity (+ 1-SD)	1.04 (0.80–1.35)	0.81 (0.53–1.22)	1.36 (0.95–1.93)	0.247	—
Apolipoprotein A-I in apolipoprotein B-depleted serum (+ 1-SD)	0.85 (0.59–1.21)	0.45 (0.23–0.87)	1.03 (0.70–1.51)	0.028	**0.65 (0.44–0.98)**
Sphingosine-1-phosphate in apolipoprotein B-depleted serum (+ 1-SD)	0.84 (0.66–1.08)	0.93 (0.65–1.35)	0.70 (0.49–0.999)	0.051	**0.80 (0.62–1.03)**
Complement component 3 in apolipoprotein B-depleted serum (+ 1-SD)	1.32 (1.08–1.60)	1.40 (1.08–1.82)	1.40 (0.97–2.02)	0.005	**1.42 (1.16–1.75)**
Paraoxonase-1 arylesterase activity in apolipoprotein B-depleted serum (+ 1-SD)	0.82 (0.63–1.06)	0.71 (0.44–1.14)	0.82 (0.59–1.14)	0.089	—
HDL inflammatory index (+ 1-SD)	1.00 (0.75–1.32)	1.06 (0.70–1.61)	0.85 (0.56–1.29)	0.522	—

Main analyses were adjusted for age, sex, HDL-C levels, diabetes, hypercholesterolaemia, triglyceride levels, hypertension, smoking habit, body mass index, and leisure-time physical activity. Stratified analyses were adjusted for the same covariates but HDL-C concentrations. Mutually adjusted analyses were adjusted for main analyses covariates, apolipoprotein A-I, sphingosine-1-phosphate, and complement component 3 levels in apolipoprotein B-depleted serum.

2014). Detailed methodology is available in the Supplementary material online, *Methods*.

Coronary artery disease cases were older and more likely to be men; have type 2 diabetes, hypercholesterolaemia, and hypertension; be current smokers; and present lower HDL-C and higher triglyceride levels (*P*-value ≤ 0.001, Supplementary material online, *Table S2*). As observed in *Table 1*, high C3 levels in apolipoprotein B-depleted serum were associated with 32% greater CAD incidence [hazard ratio (HR): 1.32 (1.08–1.60)]. High S1P concentrations were only associated with decreased CAD risk in individuals with desirable HDL-C levels [$HR_{Low\ HDL\text{-}C}$: 0.93 (0.65–1.35); $HR_{Desirable\ HDL\text{-}C}$: 0.70 (0.48–0.99); *P*-interaction: 0.051]. Greater ApoA-I levels were only associated with reduced CAD risk in individuals with low HDL-C levels [$HR_{Low\ HDL\text{-}C}$: 0.45 (0.23–0.87); $HR_{Desirable\ HDL\text{-}C}$: 1.03 (0.65–1.51); *P*-interaction: 0.028]. Finally, in the mutually adjusted analysis, associations of C3 and ApoA-I in apolipoprotein B-depleted serum with CAD risk remained present and their magnitude became greater [HR_{C3}: 1.42 (1.16–1.75); $HR_{ApoA\text{-}I}$: 0.65 (0.44–0.98)].

Our results reveal an independent association between C3 in apolipoprotein B-depleted serum (an indicator of pro-inflammatory HDL particles[1]) and incident CAD events. C3 concentrations have been reported to be elevated in apolipoprotein B-depleted plasma (although non-significantly) in individuals with high unstable angina odds.[8] Second, ApoA-I levels in apolipoprotein B-depleted serum were inversely associated with CAD in our work. This association relied particularly on individuals with low HDL-C concentrations (in which increments in ApoA-I levels could be due to a greater number of ApoA-I per HDL particle, HDL particles or a lower lipid content in HDLs, all cardioprotective[1]) and agrees with previous evidence.[8] Finally, an association between increased S1P levels in apolipoprotein B-depleted serum (essential in HDL endothelial protection[6]) and decreased CAD risk is suggested among individuals with desirable HDL-C concentrations, in agreement with previous evidence.[8] The lack of association in individuals with low HDL-C levels could be due to the fact that S1P may need a specific concentration of HDL particles or a synergism with other HDL components to be fully functional.[6] Sex-stratified analyses are presented in Supplementary material online, *Results* and *Discussion*.

Our work had some limitations. We based our analyses on a limited number of CAD events ($n = 110$), limiting our statistical power.

Seven incident cases of the cohort could not be included due to lack of availability of biological samples. Our conclusions only apply to a general population. Finally, HOII presented 15% missing values (rest of determinations: <2%) and this may have hindered our capacity to detect associations with CAD risk.

In conclusion, in the first study to assess the relationship between a hypothesis-driven set of HDL functional traits and CAD incidence in general population, high levels of C3 and low concentrations of ApoA-I in apolipoprotein B-depleted serum were associated with decreased CAD incidence, and high concentrations of S1P were linked to decreased CAD risk in participants with desirable HDL-C values. Our findings support the hypothesis that, together with HDL-C levels, HDL function could contribute to explaining its atheroprotective role in cardiovascular disease.

Supplementary material

Supplementary material is available at *European Journal of Preventive Cardiology* online.

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Conflict of interest: none declared.

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SUPPLEMENTAL DATA

SUPPLEMENTAL METHODS

Study design

We designed a case-cohort study to determine associations between HDL functionality biomarkers and coronary artery disease (CAD). We used a subsample of participants from the 2005 REGICOR (*Registre Gironí del Cor*) cohort situated in the Girona province (Catalonia, Spain). Eligible participants were community-dwelling adults aged 35-75 years old free from any previous cardiovascular disease at study entry. The study protocol complied with the Declaration of Helsinki and was approved by an Institutional Review Board. Participants provided written informed consent before joining. The study protocol, recruiting methods, further information on inclusion and exclusion criteria, and data collection processes have been described elsewhere[1].

From 5,404 participants, all 10-year incident CAD cases from the REGICOR population cohort of 2005 (*n*=117), and a random subsample of the cohort at ratio 1:4 were initially considered[2]. The random sub-cohort included 14 of the 117 cases. Due to sample availability issues, a final sample of 110 incident CAD cases and 494 non-cases were included in the analyses (**Supplemental Figure 1**). The STROBE checklist for our study is available in **Supplemental Table 1**.

Outcomes

As previously described[2], the composite endpoint was defined as fatal or non-fatal first occurrence of acute myocardial infarction, angina or mortality caused by CAD, verified by an expert clinical committee. Non-fatal outcomes (International Classification of Diseases ICD-9 codes: 410, 411.0, 411.1, 412, 414; International Classification of Diseases ICD-10: I21, I22, I24, and I25, including subtypes) were ascertained by regular standardized telephone interviews for up to 10 years (mean follow-up time: 6.15 years), and a revision of medical records. Fatal events (with the same ICD-9 and ICD-10 codes) were ascertained through linkage with the Official Catalan Death Registry, and the revision of autopsies and medical records.

Laboratory determinations

We collected fasting serum samples at the time of inclusion in the study and aliquoted and stored them at -80ºC until time of use. We measured glucose, triglycerides, and total cholesterol levels using enzymatic methods in an ABX Pentra autoanalyzer (*Glucose HK CP*, *Triglycerides CP*, *Cholesterol CP*; Horiba ABX, Madrid, Spain). We determined HDL-C concentrations by selective accelerator detergent methodology (*HDL Direct CP*, Horiba ABX, Madrid, Spain). Finally, we calculated LDL cholesterol levels by the Friedewald method whenever triglycerides were <300 mg/dL.

We studied HDL functionality-related properties in serum samples in which HDLs were the only lipoproteins present (apolipoprotein B-depleted serum). We prepared these samples by precipitating apolipoprotein B-containing lipoproteins using 20% polyethylene glycol 8000 (Sigma-Aldrich, Madrid, Spain)[3]. We measured CEC in a THP-1 monocyte-derived human macrophage cell line incubated with 0.025 mM fluorescent 23-(dipyrrometheneboron difluoride)-24-norcholesterol (Avanti Polar Lipids, Alabaster, AL, USA)[4]. HOII was defined as the ability of HDL to avoid the oxidation of

2'-7'dichlorohydrofluorescein induced by oxidized low-density lipoproteins[4]. We assessed PON1 arylesterase activity with an enzymatic activity kit (*Arylesterase/Paraoxonase Assay Kit*, Zeptometrix, Buffalo, NY, USA)[3]. Finally, we quantified ApoA-I and C3 by immunoturbidimetry in an ABX Pentra autoanalyzer (*Apo A1* and *C3*; Horiba ABX, Madrid, Spain) and S1P by ELISA kit (*Sphingosine 1 Phosphate BioAssay ELISA Kit*, US Biological, Salem, MA, USA).

We minimized inter-assay variability by: 1) assaying samples in random order; 2) running functional assays in duplicate and excluding values with a coefficient of variation >10%; and 3) normalizing the values obtained in non-automatized techniques against a pool obtained from 20 healthy volunteers of apolipoprotein B-depleted serum that was included in all experimental runs. Inter-assay coefficients of variation were 2.73% for glucose, 4.45% for triglycerides, 2.64% for total cholesterol, 3.47% for HDL-C, 5.75% for CEC, 11.7% for HOII, 7.91% for PON1 activity, 2.93% for ApoA-I, 4.93% for C3, and 11.8% for S1P. Functional tests presented some missing values (15.3% for HOII, 2.11% for CEC, and 1.14% for S1P). Due to the order in sample testing, we can assume that these data were missing completely at random.

Covariates

Trained nurses and interviewers collected information on anthropometric, clinical, and lifestyle variables as previously described[1]. Type-2 diabetes was defined as presenting fasting glucose levels >126 mg/dL or being treated with insulin or glucose-lowering medications. Hypercholesterolemia was defined as presenting total cholesterol levels >200 mg/dL or being user of cholesterol-lowering medications. Hypertension was defined as systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg, or being user of antihypertensive medication. Participants were classified as never smokers, former smokers (only when they quitted smoking 1 year or more ago), and current smokers (including former smokers of less than 1 year). Body mass index was calculated as weight in kilograms divided by squared height in meters. Finally, leisure-time physical activity was estimated (in metabolic equivalents of task-minute per day) by a Minnesota leisure-time physical activity questionnaire previously validated in Spanish adult population[5].

Sample size

A sample size of 604 individuals (from all the cohort of 5404 participants) allowed us to detect, with ≥80% power, hazard ratios of ≥1.60 per each increment in 1 standard deviation of the exposure variable. We considered a 2-sided type-I error of 5% and a 10-year incidence of the outcome of 2.70%. We calculated the sample size using the "ccsize" function of the "gap" package (version 1.2.2) in R Software[6].

Statistical analyses

We assessed differences in baseline characteristics of cases and non-cases by chi-squared test for categorical variables, t-test for continuous normally distributed ones, and MannWhitney U-tests for continuous non-normally distributed ones.

The first objective was to evaluate the association between HDL functional traits and CAD risk. Taking into account the case-cohort design, we used multivariate Cox proportion hazard regression models weighted by the Lin-Ying method[7]. The outcome was CAD incidence and follow-up time was defined as the period between the date of enrolment and that of the event (cases) or censoring at 10 years (non-cases, as there

was complete follow-up for these individuals for 10 years). We standardized raw HDL functional traits as z-scores to assess the change in CAD risk for 1-standard deviation increase in each biomarker level. Main analyses were adjusted for age (continuous) and sex, HDL-C levels (continuous), diabetes (yes/no), hypercholesterolemia (yes/no), triglyceride levels (continuous), hypertension (yes/no), smoking habit (current/former/never smoker), body mass index (continuous), and leisure-time physical activity (continuous). Due to the potentially confusing effect of HDL-C levels on the associations between HDL functional traits and CAD risk, we assessed whether there was a significant interaction between HDL-C level categories (low –<40 mg/dL in men, <50 mg/dL in women–and desirable levels) and the biomarkers. We fitted weighted Cox models where the outcome was CAD, included an interaction product-term "HDL functional trait x HDL-C level category", and applied a Wald test between the models with and without it. As there were nominally significant interactions (*P*-value for interaction <0.1) between HDL-C concentrations and three HDL functional traits (ApoA-I, S1P, and C3), we stratified these analyses by HDL-C level categories. Stratified analyses were adjusted for all previous covariates but HDL-C concentrations. Additional analyses stratified by sex were also performed. Finally, all variables whose association with CAD risk was suggested in any of the previous analyses were considered for a final, mutually adjusted analysis (adjusted for the covariates in main analyses together with the levels of each of these biomarkers).

Cox models were fitted using the "cch" function of the "survival" package in R Software[2]. We performed all analyses in R Software version 3.6.1[8].

SUPPLEMENTARY RESULTS AND DISCUSSION

Sex-stratified analyses on C3 levels

We observed a significant interaction between C3 levels in apolipoprotein B-depleted plasma and sex on CAD risk: the positive association between C3 levels and CAD risk relied particularly on male subjects (HR_{men}: 1.74 [1.28-2.36]; HR_{women}: 1.20 [0.82-1.76]; *P*-interaction: 0.004). As a potential explanation to this fact, men are classically related to greater levels of low-grade inflammation and circulating concentrations of pro-inflammatory cytokines, and are more clearly linked to the morbidity and mortality of inflammation-based pathologies when compared to women[9]. This situation may also apply to CAD, which may contribute to explaining the clearer association between increments in inflammation-related biomarkers and greater cardiovascular risk.

SUPPLEMENTAL TABLES

Supplemental Table 1. STROBE checklist.

	Item No.	Recommendation	Page No.	Relevant text from manuscript
Title and abstract				
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title/abstract	1	Title
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	-	Not applicable
Introduction				
Background/ rationale	2	Explain the scientific background and rationale for the investigation being reported	3	Paragraph 1
Objectives	3	State specific objectives, including any pre-specified hypotheses	3	Paragraph 1
Methods				
Study design	4	Present key elements of study design early in the paper	3	Paragraph 2
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	3	Paragraph 2; Supplemental Methods
Participants	6	Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls.	3	Paragraph 2; Supplemental Methods
		For matched studies, give matching criteria and the number of controls per case	-	This is a case-cohort study with no matching
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	3	Paragraph 2; Supplemental Methods
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	3	Paragraph 2; Supplemental Methods
Bias	9	Describe any efforts to address potential sources of bias	3-4	Case-cohort design minimized

					selection bias. Data gathering by trained nurses and interviewers using standardized questionnaires minimized information bias. Intensive adjustment for covariates minimized confusion bias. Finally, our study was hypothesis-driven and all parameters tested are shown, minimising publication bias
Study size	10	Explain how the study size was arrived at.	-	Supplemental Methods
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	4	Paragraph 3; Supplemental Methods
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	4	Paragraph 3; Supplemental Methods
		(b) Describe any methods used to examine subgroups and interactions	4	Paragraph 3; Supplemental Methods
		(c) Explain how missing data were addressed	4	Paragraph 3; Supplemental Methods
		(d) *Cohort study*—If applicable, explain how loss to follow-up was addressed *Case-control study*—If applicable, explain how matching of cases and controls was addressed; *Cross-sectional study*—If applicable, describe analytical methods taking account of sampling strategy	-	This is a case-cohort study with no matching
		(e) Describe any sensitivity analyses	4	Paragraph 3; Supplemental Methods
Results				
Participants	13*	(a) Report numbers of individuals at each stage of study— e.g., numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	3	Supplemental Figure 1 (flow diagram)
		(b) Give reasons for non-participation at each stage	3	Supplemental Figure 1 (flow diagram)
		(c) Consider use of a flow diagram	3	Supplemental Figure 1 (flow

5

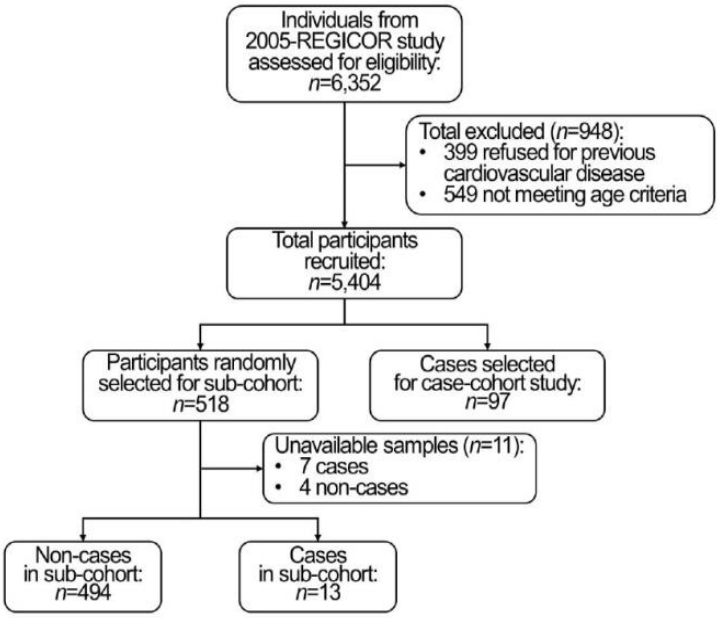
				diagram)
Descriptive data	14*	(a) Give characteristics of study participants (e.g., demographic, clinical, social) and information on exposures and potential confounders	4	Paragraph 4; Supplemental Table 2
		(b) Indicate number of participants with missing data for each variable of interest	-	Supplemental Methods
		(c) Summarize follow-up time (e.g., average and total amount)	3	Supplemental Methods
Outcome data	15*	Report numbers in each exposure category, or summary measures of exposure	-	Supplemental Table 1
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g., 95% confidence interval). Make clear which confounders were adjusted for and why they were included	4	Paragraph 4; Table 1
		(*b*) Report category boundaries when continuous variables were categorized	4	Paragraph 4; Table 1
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	-	-
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	4	Paragraph 4; Table 1
Discussion				
Key results	18	Summarize key results with reference to study objectives	5	Paragraph 7
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	5	Paragraph 6
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	5	Paragraph 5
Generalizability	21	Discuss the generalizability (external validity) of the study results	5	Paragraph 6
Other information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	6	"Funding" section

Supplemental Table 2. Baseline characteristics of our population

	All (*n*=604)	Non-cases (*n*=494)	Cases (*n*=110)	*P*-value (non-cases vs. cases)
Age (years), mean ± SD	55.4 ± 10.9	54.3 ± 10.9	60.4 ± 9.55	<0.001
Female sex, *n* (%)	293 (48.5)	262 (53.0)	31 (28.2)	<0.001
Diabetes, *n* (%)	72 (11.9)	48 (9.72)	24 (21.8)	0.001
Hypercholesterolemia, *n* (%)	387 (64.1)	299 (60.5)	88 (80.0)	<0.001
HDL cholesterol, mg/dL, mean ± SD	51.2 (14.0)	52.3 (13.6)	46.4 (14.9)	<0.001
Triglycerides, mg/dL, median (1^{st}-3^{rd} quartile)	100 (74.0-130)	95.0 (71.0-124)	120 (93.0-174)	<0.001
Hypertension, *n* (%)	233 (38.6)	168 (34.0)	65 (59.1)	<0.001
Smoking status:				<0.001
Never smokers, *n* (%)	301 (49.8)	265 (53.6)	36 (32.7)	
Current smokers, *n* (%)	147 (24.3)	103 (20.9)	44 (40.0)	
Former smokers, *n* (%)	156 (25.8)	126 (25.5)	30 (27.3)	
Body mass index (kg/m^2), mean ± SD	27.8 ± 4.63	27.7 ± 4.61	28.4 ± 4.65	0.133
Leisure-time physical activity, metabolic equivalents of task·min/day, median (1^{st}-3^{rd} quartile)	235 (113-396)	235 (111-388)	237 (125-442)	0.454
Cholesterol efflux capacity, ratio, mean ± SD	1.09 ± 0.14	1.08 ± 0.14	1.09 ± 0.14	0.699
Apolipoprotein A-I, g/L, mean ± SD	0.94 ± 0.17	0.95 ± 0.17	0.89 ± 0.17	0.004
Sphingosine-1-phosphate, ng/dL, mean ± SD	31.2 ± 10.8	31.1 ± 11.1	31.2 ± 9.54	0.962
Complement component 3, mg/dL, median (1^{st}-3^{rd} quartile)	18.0 (13.3-25.5)	17.8 (13.1-24.8)	20.1 (14.7-28.0)	0.009
Paraoxonase-1 arylesterase activity, normalized ratio, mean ± SD	0.82 ± 0.22	0.83 ± 0.22	0.77 ± 0.21	0.017
HDL oxidative/inflammatory index, ratio, mean ± SD	1.19 ± 0.36	1.19 ± 0.35	1.18 ± 0.39	0.684

SUPPLEMENTAL FIGURES

Supplemental Figure 1. Study flowchart



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Manuscript II

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RESEARCH ARTICLE

High-Density Lipoprotein

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Increased Consumption of Virgin Olive Oil, Nuts, Legumes, Whole Grains, and Fish Promotes HDL Functions in Humans

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Scope: To evaluate whether increases in the consumption of cardioprotective food groups (virgin olive oil, nuts, fruits/vegetables, legumes, whole grains, fish, and wine) are associated with improvements in high-density lipoprotein (HDL) functions in high cardiovascular risk subjects.

Methods and Results: The association between 1-year changes in food group consumption and HDL functionality traits in 296 high cardiovascular risk subjects is assessed. Increases in virgin olive oil (10 g d^{-1}) and whole grain consumption (25 g d^{-1}) are associated with increments in cholesterol efflux capacity (+0.7%, $P = 0.026$, and +0.6%, $P = 0.017$, respectively). Increases in nut (30 g d^{-1}) and legume intake (25 g d^{-1}) are linked to increments in paraoxonase-1 activity (+12.2%, $P = 0.049$, and +11.7%, $P = 0.043$, respectively). Legume intake increases are also related to decreases in cholesteryl ester transfer protein activity (–4.8%, $P = 0.028$). Fish consumption increments (25 g d^{-1}) are associated with increases in paraoxonase-1 activity (+3.9%, $P = 0.030$) and declines in cholesteryl ester transfer protein activity (–1.6%, $P = 0.021$), HDL cholesterol concentrations (–1.1%, $P = 0.039$), and functions related to HDL levels (cholesterol efflux capacity, –1.1%, $P = 0.010$).

Conclusion: Increases in the consumption of virgin olive oil, nuts, legumes, whole grains, and fish (achievable through a regular diet) were associated with improvements in HDL functions in high cardiovascular risk subjects.

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1. Introduction

Few real-life dietary modifications have been shown to be able to improve the biological functions of high-density lipoproteins (HDLs) in humans. Only increases in the intake of polyphenol-rich virgin olive oil (25 mL d^{-1}),[1] a lycopene-rich diet,[2] and a traditional Mediterranean diet[3] have been reported to enhance HDL functions in clinical trials. Regarding the Mediterranean diet, our research group has demonstrated that adherence to this dietary pattern (associated with a high intake of virgin olive oil, nuts, fruit, vegetables, legumes, and whole grains, and a moderate consumption of fish and wine with meals)[4] improved several HDL functions: it promoted cholesterol efflux capacity (their capacity to pick up cholesterol), HDL ability to esterify cholesterol (necessary for the effective transport of cholesterol in these lipoproteins), paraoxonase-1 activity (PON1, a key HDL-bound antioxidant enzyme), and HDL capacity to promote endothelial release of nitric oxide, and decreased the activity of the cholesteryl ester transfer protein (CETP, pro-atherogenic when excessively active).[3] The food items individually responsible for such effects (within the context of a healthy dietary pattern such as this or others), however, remain to be elucidated.

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Our aim was to determine whether real-life increases in the intake of cardioprotective food groups (virgin olive oil, nuts, fruit and vegetables, legumes, whole grains, fish, and wine) for 1 year were linked to improvements in HDL biological functions in high cardiovascular risk subjects.

2. Experimental Section

2.1. Study Population

The analyses were performed in a random sub-sample of 296 volunteers from the PREDIMED trial (*PREvención con DIeta MEDiterránea*)[4,5] in which HDL functions were previously assessed.[3] The following information[5] was collected: 1) clinical variables (age, sex, weight, height, blood pressure, and biochemical profile); 2) use of cardiovascular drugs; 3) consumption of 137 foods by a validated food frequency questionnaire; 4) adherence to a traditional Mediterranean diet by a validated 14-item score; 5) levels of physical activity with a validated Minnesota Leisure Time Physical Activity questionnaire; and 6) smoking habit. Body mass index was calculated as the ratio between weight (kg) and the height squared (m^2), and three categories were established: normoweight (18.5–24.9 kg m^{-2}), overweight (25.0–29.9 kg m^{-2}), and obesity (≥30.0 kg m^{-2}). Hypercholesterolemia was defined as the presence of total cholesterol ≥200 mg dL^{-1} or the use of statins; hypertriglyceridemia as the presence of triglycerides ≥150 mg dL^{-1} and/or the use of fibrates or pharmacological doses of omega-3 PUFAs; type-II diabetes mellitus as the presence of an altered glucose metabolism or the use of antidiabetic drugs; and hypertension as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or the use of antihypertensive agents.[5] Finally, the consumption of food groups was computed from the results of the food frequency questionnaire as follows: 1) "virgin olive oil" as the sum of all virgin and extra virgin olive oils consumed; 2) "nuts" as the sum of the intake of walnuts, almonds, pistachios, hazelnuts, and pine nuts; 3) "fruit and vegetables" as the sum of the consumption of green leafy vegetables, tomatoes and tomato soup (gazpacho), peppers, carrots, allium plants, cucurbits, cruciferous plants, green beans, asparagus, other vegetables, fruits of the Rosacea and Citrus families, berries, bananas, melons, watermelons, pineapples, kiwis, grapes, and other minor fruits; 4) "legumes" as the sum of consumed lentils, chickpeas, beans, and peas; 5) "whole grains" as the sum of the intake of whole grain bread and biscuits; 6) "fish" as the sum of the consumption of all lean and fatty fish (fresh or preserved naturally or in oil); and 7) "wine" as the sum of all red, rosé, white, sparkling, and sweet wine consumed.[5] Average intake of all food items was expressed as g d^{-1} (excepting wine, expressed as mL d^{-1}).

Study participants provided written informed consent before joining the trial. The study protocol was approved by local Research and Ethics Committees and registered with the International Standard Randomized Controlled Trial Number ISRCTN35739639. Details have been published elsewhere.[4,5]

2.2. HDL Functionality Determinations

HDL particles were first isolated from volunteers' plasma samples by density gradient ultracentrifugation (isolated HDL

fraction)[1,3] and polyethylene glycol-induced precipitation of apolipoprotein B-containing lipoproteins (apolipoprotein B-depleted plasma),[3] and the samples were stored at –80 °C until use. The participants' HDL cholesterol levels were analyzed in an ABX-Pentra 400 autoanalyzer (Horiba ABX, Montpellier, France).[3] The following were determined: 1) cholesterol efflux capacity in a model of human THP-1 monocyte-derived macrophages with ^{3}H-cholesterol treated with 5% apolipoprotein B-depleted plasma samples as previously described;[3] 2) the ability of HDLs to esterify cholesterol as the ratio between the percentage of esterified cholesterol (in isolated HDL samples obtained by ultracentrifugation) and lecithin cholesterol acyltransferase concentration in serum samples;[3] 3) the activities of CETP and PON1 enzymes in plasma and serum samples, respectively, by commercial kits;[1,3] and 4) HDL capacity to promote endothelial release of nitric oxide in vitro in a human umbilical vein endothelial cell model treated with apolipoprotein B-depleted plasma samples.[3]

2.3. Sample Size

Accepting a type-I error of 0.05, a type-II error of 0.2, and a 1% loss rate in a two-sided test, a sample size of 196 subjects provided sufficient statistical power to determine that Pearson's correlation coefficients ≥0.2 were significantly different from zero. Sample size was incremented by 50% (up to 294 volunteers) to allow adjustments for different covariates.

2.4. Statistical Analyses

The 1-year differences in HDL functionality variables were computed as percentage changes to simplify data interpretation ((1-year value – baseline value)/baseline value × 100), the 1-year differences in dietary variables as linear differences (1-year value – baseline value), and the distribution of continuous variables was assessed using normality plots and histograms. The association between the changes in consumption of food groups and changes in HDL functions by multivariate linear regression models was determined, without any adjustment and adjusted for: age (continuous); gender; study site; study intervention group (three categories[4,5]); adherence to a traditional Mediterranean diet (continuous); 1-year changes in the status of dyslipidemia (hypercholesterolemia + hypertriglyceridemia), type-II diabetes, hypertension, and tobacco use; body mass index category at baseline, energy intake at baseline (continuous), and physical activity at baseline (tertiles). These analyses were repeated after stratifying subjects in quartiles according to baseline HDL cholesterol levels, and whether there were linear trends in the association coefficients when increasing along quartiles by Pearson's tests was assessed.

Any two-sided *P*-value <0.05 was accepted as significant and the previous analyses in R Software, version 3.4.1, using the "lme4" package were executed.[6,7]

3. Results

Characteristics of study participants are available in Table S1, Supporting Information.

Associations between 1-year changes in food intake and variations in HDL function are depicted in **Table 1**. Regarding the fully adjusted model, increases in the daily consumption of a serving of virgin olive oil (10 g, one spoonful) and whole grains (25 g) were independently associated with increments in cholesterol efflux capacity of 0.7% ($p = 0.026$) and 0.6% ($p = 0.017$), respectively. Increases in the consumption of nuts in 30 g d^{-1} (a fistful) and legumes in 25 g d^{-1} (≈2 servings per week) was independently linked to increments of 12.2% ($p = 0.049$) and 11.7% ($p = 0.043$) in PON1 antioxidant activity, respectively. The increase in legume intake was also linked to a 2.6% rise in HDL cholesterol levels ($p = 0.036$) and a 4.8% decrease in CETP activity ($p = 0.028$). Increments of fish consumption in 25 g d^{-1} (≈2 servings per week) were associated with a 3.9% promotion of PON1 antioxidant activity ($p = 0.030$) and a protective 1.6% decline in CETP activity ($p = 0.021$), together with a 1.1% decrease in HDL cholesterol concentrations ($p = 0.039$), and in those functions possibly related to HDL levels (such as cholesterol efflux capacity, –1.1%, $p = 0.010$). When studying fish subtypes, only augmentations in fresh fatty fish consumption (25 g d^{-1}) were linked to a greater decrease in CETP activity (–2.3%, $p = 0.043$). Finally, when stratifying the previous analyses according to baseline HDL cholesterol concentrations, we observed that the associations of increasing nuts or fish consumption with increments in PON1 antioxidant activity were particularly present in those subjects with high HDL cholesterol levels (Table S2, Supporting Information).

No significant differences in HDL properties were associated with changes in the consumption of fruit/vegetables and wine. Raw baseline data have already been described in a previous publication.[3]

4. Discussion

Our results show that 1-year increases in the consumption of virgin olive oil, nuts, legumes, whole grains (and fish, in a more ambiguous way) were associated with improvements in HDL functions in high cardiovascular risk individuals. Such enhancements were unrelated to other lifestyle- and cardiovascular-related variables.

Specifically, we have confirmed the protective capacity of increasing the consumption of virgin olive oil on cholesterol efflux capacity,[1,3] an essential measurement of HDL function that is inversely related to the incidence of coronary events,[8] and observed that incrementing the consumption of a serving of whole grains (25 g d^{-1}, a slice of whole bread) induces a similar protective effect. Such foods are a key source of fiber, polyphenols, and other bioactive components that could contribute to explaining our results.[9,10] To the best of our knowledge, this is the first time that the effect of increasing whole grain consumption on HDL function has been reported in humans.

Our data also showed that legumes strongly modulated HDL functional traits in our data: they promoted HDL antioxidant function and moderated CETP function. The enhancement of PON1 activity after increasing the consumption of 25 g d^{-1} of legumes (≈2 servings per week; +11.7%) was similar to that achieved after incrementing the consumption of nuts to a portion per day (+12.2%). The richness in fiber and antioxidants of these

Table 1. Association between increases in the consumption of different food items and changes in HDL-related traits (in %).

Variables[a)]	↑ 10 g d^{-1} of virgin olive oil		↑ 30 g d^{-1} of nuts		↑ 25 g d^{-1} of legumes		↑ 25 g d^{-1} of whole grains		↑ 25 g d^{-1} of fish	
	Raw model	Adjusted model	Raw model	Adjusted model	Raw model	Adjusted model	Raw model	Adjusted model	Raw model	Adjusted model
Change in HDL cholesterol concentrations (%)	−0.057 [−0.70; 0.59]	0.005 [−0.76; 0.77]	1.43 [−1.03; 3.90]	1.66 [−1.31; 4.62]	3.13* [0.70; 5.58]	2.60* [0.18; 5.03]	0.25 [−0.40; 0.90]	0.26 [−0.39; 0.91]	−1.17* [−2.20; −0.15]	−1.14* [−2.21; −0.065]
Change in cholesterol efflux capacity (%)	0.54* [0.036; 1.03]	0.68* [0.084; 1.27]	2.03 [−0.043; 4.11]	1.36 [−1.32; 4.05]	0.59 [−1.50; 2.65]	0.82 [−1.31; 2.95]	0.53* [0.018; 1.05]	0.64* [0.12; 1.16]	−0.93* [−1.73; −0.12]	−1.11* [−1.96; −0.27]
Change in HDL capacity to esterify cholesterol (%)	0.33 [−1.01; 1.67]	−0.068 [−1.70; 1.57]	−3.90 [−9.84; 2.04]	−2.03 [−9.93; 5.85]	−0.13 [−7.00; 6.75]	0.78 [−6.55; 8.13]	−0.49 [−1.72; 0.74]	−0.35 [−1.63; 0.93]	−0.46 [−2.65; 1.73]	−0.36 [−2.75; 2.04]
Change in cholesteryl ester transfer protein activity (%)	0.003 [−0.76; 0.76]	0.54 [−0.40; 1.48]	0.63 [−2.75; 4.02]	0.37 [−4.29; 5.01]	−3.35 [−7.25; 0.53]	−4.80* [−9.03; −0.57]	0.26 [−0.45; 0.97]	0.24 [−0.52; 0.99]	−1.41* [−2.63; −0.18]	−1.63* [−3.00; −0.27]
Change in paraoxonase-1 antioxidant activity (%)	2.56* [0.62; 4.51]	2.09 [−0.33; 4.51]	3.48 [−5.37; 12.4]	12.2* [0.13; 24.2]	14.6* [4.25; 24.9]	11.7* [0.44; 22.8]	0.17 [−1.67; 2.01]	−0.13 [−2.08; 1.82]	3.18* [−0.003; 6.33]	3.93* [0.40; 7.45]
Change in HDL capacity to promote endothelial release of nitric oxide (%)	0.26 [−0.99; 1.51]	−0.28 [−1.79; 1.23]	2.07 [−2.69; 6.81]	−1.79 [−7.80; 4.20]	1.37 [−3.53; 6.25]	2.02 [−2.93; 6.95]	0.064 [−1.27; 1.40]	−0.28 [−1.65; 1.08]	1.29 [−0.70; 3.28]	1.88 [−0.19; 3.95]

[a)]Adjusted models have been adjusted for: age; sex; study site; PREDIMED intervention group; changes in the status of type-II diabetes, dyslipidemia (hypercholesterolemia and hypertriglyceridemia), hypertension, and tobacco use; and baseline values of body mass index category, adherence to a Mediterranean diet, and physical activity (tertiles); *$p < 0.05$.

food items may account for their cardiovascular benefits.[10,11] To date, no association between legume consumption and HDL functionality has been reported.

Finally, the effects of increasing fish consumption on HDL functions were more ambiguous in our work. We observed an association between a 25 g d^{-1} increase in fish intake with higher PON1 activity (+3.9%) and lower CETP function (–1.6%), in parallel to lower concentrations of HDL cholesterol (–1.1%) and some HDL functional properties that could be possibly related to HDL quantity (such as cholesterol efflux capacity, which decreased to the same extent as HDL cholesterol, –1.1%). In this regard, the relationship between omega-3 PUFAs in fish and HDL is still controversial. Some authors have reported that their consumption promotes the cholesterol content of large HDLs only, others indicate that they may increase the catabolic rate of apolipoprotein A-I, and according to a comprehensive review of the topic there is as yet no consensus on their effects.[12] Nevertheless, we observed that the decrease in CETP activity was the only significant association with increases in the consumption of fresh fatty fish. In addition, increases in the intake of the other omega-3-rich food item (nuts) were also related with increments in PON1 antioxidant activity, being these associations particularly present whenever subjects presented high HDL cholesterol levels at baseline. We hypothesize that, whether HDL cholesterol concentrations of these subjects are higher, they may also present greater levels of the enzyme in circulation and be particularly sensitive to potential functional benefits. Finally, the improvements in PON1 and CETP activities that we have reported were of greater magnitude than the decline in HDL cholesterol levels, and concur with earlier evidence indicating that omega-3 PUFAs may promote PON1 function and decrease CETP activity.[13,14]

This study has strengths and limitations. Regarding our strengths, we have reported associations between prospective data (changes in food consumption and the promotion of HDL functions), provided a quantitative measurement of beneficial effects (percentage changes in HDL functions), and used standardized protocols in a large sample size to comprehensively study key HDL functions. However, it also presents limitations. First, this was a prospective change analysis in a high cardiovascular risk population and our conclusions should be confirmed in randomized controlled trials and could only be extrapolated to high cardiovascular risk subjects. To increase the generalizability of our conclusions, our regression models have been fully adjusted for several co-variates that may affect HDL function (such as age, sex, cardiovascular risk factors, energy intake, and physical activity) and are independent from the effect of the diet as a whole (our results are also adjusted for the allocation of the volunteers to Mediterranean diets or a low-fat one and their adherence to a traditional Mediterranean diet). Second, as expected, the changes observed in this work were modest because they were associated with moderate real-life diet modifications. Third, several HDL functions were determined in cellular models that, while noninvasive, may not reflect possible counter-regulatory mechanisms affecting the final outcome. Finally, CETP and PON1 activities, and HDL capacity to promote endothelial release of nitric oxide could not be measured due to sample availability and technical issues in 67 and 50 volunteers, respectively.

In conclusion, we report that real-life increases in the 1-year consumption of virgin olive oil, nuts, legumes, whole grains, and fish may lead to relevant improvements in HDL functions in high cardiovascular risk subjects. This study describes for the first time an association between incrementing the consumption of

legumes and whole grains and enhancements in HDL function. It also confirms the beneficial effects of virgin olive oil, nuts, and fish on these properties, and reinforces the idea that a healthy diet may promote HDL functionality. Further randomized controlled trials are warranted to investigate whether these dietary modifications may contribute to promoting HDL function in humans.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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A.H. and M.Fitó designed the study. A.H. acquired the data. M.A.M.-G., E.R., X.P., R.E., J.S.-S., D.C., A.M.A.G., L.S.-M., M.Fiol, J.L., R.M.L.-R., and M.Fitó contributed with biological samples and/or participated in the design and development of the clinical trial. A.H. and A.S. wrote the manuscript that was critically reviewed by O.C., M.A.M.-G., E.R., X.P., R.E., J.S.-S., D.C., A.M.A.G., L.S.-M., M.Fiol, J.L., R.T, R.M.L.-R., and M.Fitó. All authors approved the final version of the manuscript. The authors thank Daniel Muñoz-Aguayo, Gemma Blanchart, and Sonia Gaixas for their technical assistance, and Stephanie Lonsdale for her help in editing the English text. The CIBER de Fisiopatología de la Obesidad y Nutrición (CIBEROBN) is an initiative of the Instituto de Salud Carlos III, Spain. This work was supported by: Agència de Gestió d'Ajuts Universitaris i de Recerca (2014-SGR-240) and the Instituto de Salud Carlos III (CB06/03/0028, CD17/00122, CES12/025, JR14/00008, OBN17PI02, PI11/01647, and PI15/00047).

Conflict of Interest

Emilio Ros reports receiving grants/research support through his Institution from the California Walnut Commission, being a nonpaid member of its Scientific Advisory Committee, and receiving lecture fees from Nuts for Life and Danone. Ramón Estruch reports serving on the board of and receiving lecture fees from the Research Foundation on Wine and Nutrition (FIVIN), serving on the boards of the Beer and Health Foundation and the European Foundation for Alcohol Research (ERAB), and receiving lecture fees from Cerveceros de España. Jordi Salas-Salvadó reports receiving grants/research support through his Institution from the International Nut and Dried Fruit Foundation (in whose Scientific Committee he is a nonpaid member) and the American Pistachio Growers, receiving honoraria from Nuts for Life, Danone and Eroski, and being a member of the executive committee of the Instituto Danone Spain. Rosa-María Lamuela-Raventós reports serving on the board of and receiving lecture fees from FIVIN, receiving lecture fees from Cerveceros de España, and receiving lecture fees and travel support from PepsiCo. No other potential conflict of interest relevant to this article has been reported.

Keywords

fish, high-density lipoprotein functionality, legumes and grains, nuts, virgin olive oil

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SUPPLEMENTAL TABLES

SUPPLEMENTAL TABLE 1. Characteristics of study population

Age (years)	65.9 (6.43)*
Sex (% male)	49%
Type-II diabetes mellitus (% of diabetic volunteers)	49%
Dyslipidemia (% of dyslipidemic volunteers)	77%
Hypertension (% of hypertensive volunteers)	79%
Obesity (% of obese volunteers)	45%
Tobacco use (% of smokers)	12%
Adherence to a Mediterranean Diet (score)	8.91 (1.77)
Leisure-time physical activity (METs·min/day)	175 [59.6;350]†

*: Mean (SD). †: Median [1st-3rd quartile]. MET: metabolic equivalent of task.

SUPPLEMENTAL TABLES 2. Stratified analyses of the associations between increases in food group intake and changes in HDL functional capacities in quartiles according to baseline HDL cholesterol levels.

Supplemental Table 2.1. Associations with increases in virgin olive oil intake.

	↑ 10 g/day of virgin olive oil					
Variables	*Quartile 1*	*Quartile 2*	*Quartile 3*	*Quartile 4*	*p-value (linear trend)*	*All subjects*
Change in HDL cholesterol concentrations (%)	-0.70 [-2.59; 1.20]	-0.050 [-1.79; 1.68]	0.18 [-1.20; 1.56]	0.70 [-0.70; 2.09]	0.014	0.005 [-0.76; 0.77]
Change in cholesterol efflux capacity (%)	0.77 [-0.88; 2.40]	1.77* [0.45; 3.10]	0.37 [-1.28; 2.01]	0.10 [-1.02; 1.22]	0.401	0.68* [0.084; 1.27]
Change in HDL capacity to esterify cholesterol (%)	-0.35 [-2.58; 1.87]	3.67 [-1.42; 8.76]	-0.90 [-4.77; 2.97]	-1.92 [-6.49; 2.65]	0.512	-0.068 [-1.70; 1.57]
Change in cholesteryl ester transfer protein activity (%)	-1.04 [-3.21; 1.13]	3.70** [1.22; 6.17]	1.88 [-0.56; 4.32]	0.49 [-0.96; 1.94]	0.822	0.54 [-0.40; 1.48]
Change in paraoxonase-1 antioxidant activity (%)	5.56* [0.81; 10.3]	-0.89 [-6.47; 4.69]	2.29 [-5.77; 10.3]	3.45 [-3.86; 10.8]	0.849	2.09 [-0.33; 4.51]
Change in HDL capacity to promote endothelial release of nitric oxide (%)	-1.60 [-5.67; 2.46]	2.19 [-2.06; 6.44]	1.10 [-2.72; 4.91]	1.13 [-2.12; 4.38]	0.434	-0.28 [-1.79; 1.23]

Supplemental Table 2.2. Associations with increases in nut intake.

	↑ 30 g/day of nuts					
Variables	*Quartile 1*	*Quartile 2*	*Quartile 3*	*Quartile 4*	*p-value (linear trend)*	*All subjects*
Change in HDL cholesterol concentrations (%)	4.44 [-1.59; 10.4]	2.69 [-4.41; 9.81]	-2.65 [-9.45; 4.17]	-1.36 [-6.75; 4.02]	0.120	1.66 [-1.31; 4.62]
Change in cholesterol efflux capacity (%)	2.06 [-3.36; 7.47]	-1.34 [-7.80; 5.13]	5.16 [-3.60; 14.0]	1.93 [-3.54; 7.41]	0.702	1.36 [-1.32; 4.05]
Change in HDL capacity to esterify cholesterol (%)	-2.76 [-11.0; 5.46]	-28.9* [-51.3; -6.54]	13.8 [-1.80; 29.3]	6.1 [-28.9; 41.1]	0.518	-2.03 [-9.93; 5.85]
Change in cholesteryl ester transfer protein activity (%)	5.46 [-2.56; 13.5]	-5.16 [-19.2; 8.88]	-9.60 [-20.5; 1.28]	-2.08 [-12.3; 8.10]	0.449	0.37 [-4.29; 5.01]
Change in paraoxonase-1 antioxidant activity (%)	10.2 [-9.36; 29.9]	16.5 [-14.1; 47.1]	23.3 [-12.3; 58.8]	25.2 [-25.4; 75.9]	0.022	12.2* [0.13; 24.2]
Change in HDL capacity to promote endothelial release of nitric oxide (%)	1.53 [-12.7; 15.8]	-11.1 [-25.3; 3.10]	2.06 [-15.6; 19.7]	-5.16 [-19.4; 9.12]	0.856	-1.79 [-7.80; 4.20]

Supplemental Table 2.3. Associations with increases in legume intake.

	↑ 25 g/day of legumes					
Variables	*Quartile 1*	*Quartile 2*	*Quartile 3*	*Quartile 4*	*p-value (linear trend)*	*All subjects*
Change in HDL cholesterol concentrations (%)	-1.21 [-11.5; 9.05]	3.55 [-1.02; 8.10]	2.48 [-2.26; 7.23]	1.69 [-3.78; 7.15]	0.516	2.60* [0.18; 5.03]
Change in cholesterol efflux capacity (%)	-8.48 [-17.6; 0.58]	1.13 [-2.8; 5.08]	4.53 [-0.95; 10.0]	-2.28 [-6.83; 2.25]	0.489	0.82 [-1.31; 2.95]

Change in HDL capacity to esterify cholesterol (%)	12.1 [-1.44; 25.8]	-8.85 [-30.5; 12.8]	8.55 [-3.78; 20.9]	5.15 [-25.0; 35.5]	0.950	0.78 [-6.55; 8.13]
Change in cholesteryl ester transfer protein activity (%)	-4.83 [-19.3; 9.65]	-1.51 [-13.8; 10.8]	-5.85 [-14.2; 2.49]	-6.95 [-16.0; 2.11]	0.411	-4.80* [-9.03; -0.57]
Change in paraoxonase-1 antioxidant activity (%)	-6.93 [-42.0; 28.0]	7.43 [-16.2; 31.0]	16.9 [-9.80; 43.8]	-19.9 [-77.8; 37.8]	0.765	11.7* [0.44; 22.8]
Change in HDL capacity to promote endothelial release of nitric oxide (%)	-3.03 [-24.2; 18.1]	4.58 [-4.35; 13.5]	0.013 [-14.5; 14.6]	2.12 [-10.2; 14.4]	0.565	2.02 [-2.93; 6.95]

Supplemental Table 2.4. Associations with increases in whole grain intake.

	↑ 25 g/day of whole grains					
Variables	*Quartile 1*	*Quartile 2*	*Quartile 3*	*Quartile 4*	*p-value (linear trend)*	*All subjects*
Change in HDL cholesterol concentrations (%)	-0.47 [-2.49; 1.54]	-0.30 [-1.54; 0.95]	0.43 [-0.58; 1.43]	0.87 [-0.69; 2.43]	0.022	0.26 [-0.39; 0.91]
Change in cholesterol efflux capacity (%)	-0.99 [-2.88; 0.90]	1.01* [0.076; 1.94]	1.34* [0.10; 2.58]	0.98 [-0.32; 2.27]	0.242	0.64* [0.12; 1.16]
Change in HDL capacity to esterify cholesterol (%)	-1.87 [-4.90; 1.17]	-0.49 [-4.00; 3.05]	0.3 [-1.84; 2.43]	-2.21 [-7.90; 3.50]	0.974	-0.35 [-1.63; 0.93]
Change in cholesteryl ester transfer protein activity (%)	-0.65 [-3.78; 2.47]	0.87 [-0.94; 2.68]	-0.47 [-1.90; 0.96]	-0.73 [-2.50; 1.04]	0.728	0.24 [-0.52; 0.99]
Change in paraoxonase-1 antioxidant activity (%)	2.19 [-5.28; 9.65]	-0.24 [-3.83; 3.33]	1.30 [-3.25; 5.88]	1.05 [-8.15; 10.3]	0.757	-0.13 [-2.08; 1.82]
Change in HDL capacity to promote endothelial release of nitric oxide (%)	-1.72 [-6.50; 3.05]	-0.31 [-2.78; 2.16]	0.032 [-2.95; 3.03]	-1.29 [-4.93; 2.37]	0.740	-0.28 [-1.65; 1.08]

Supplemental Table 2.5. Associations with increases in fish intake.

	↑ 25 g/day of fish					
Variables	*Quartile 1*	*Quartile 2*	*Quartile 3*	*Quartile 4*	*p-value (linear trend)*	*All subjects*
Change in HDL cholesterol concentrations (%)	-0.39 [-3.33; 2.58]	-0.16 [-2.55; 2.21]	-0.85 [-2.58; 0.89]	-0.91 [-3.10; 1.30]	0.195	-1.14* [-2.21; -0.065]
Change in cholesterol efflux capacity (%)	0.32 [-2.10; 2.75]	-0.45 [-2.41; 1.52]	-2.75 [-4.80; -0.72]	-1.34 [-3.08; 0.39]	0.287	-1.11* [-1.96; -0.27]
Change in HDL capacity to esterify cholesterol (%)	3.08 [-0.43; 6.60]	-3.30 [-10.5; 3.85]	-2.95 [-7.40; 1.50]	0.70 [-6.90; 8.30]	0.713	-0.36 [-2.75; 2.04]
Change in cholesteryl ester transfer protein activity (%)	-0.50 [-4.28; 3.28]	-3.68 [-7.30; -0.062]	-0.63 [-3.83; 2.58]	-0.37 [-2.78; 2.04]	0.723	-1.63* [-3.00; -0.27]
Change in paraoxonase-1 antioxidant activity (%)	-2.63 [-11.6; 6.35]	-2.15 [-9.68; 5.38]	5.28 [-4.73; 15.3]	13.4* [2.31; 24.5]	0.049	3.93* [0.40; 7.45]
Change in HDL capacity to promote endothelial release of nitric oxide (%)	8.58** [3.28; 13.9]	-1.68 [-6.23; 2.88]	3.33 [-1.31; 7.95]	-5.83* [-11.2; -0.48]	0.209	1.88 [-0.19; 3.95]

Manuscript III

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Original Research Communications

A lifestyle intervention with an energy-restricted Mediterranean diet and physical activity enhances HDL function: a substudy of the PREDIMED-Plus randomized controlled trial

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ABSTRACT

Background: Consumption of a Mediterranean diet, adequate levels of physical activity, and energy-restricted lifestyle interventions have been individually associated with improvements in HDL functions. Evidence of intensive interventions with calorie restriction and physical activity is, however, scarce.

Objectives: To determine whether an intensive lifestyle intervention with an energy-restricted Mediterranean diet plus physical activity enhanced HDL function compared to a non-hypocaloric Mediterranean eating pattern without physical activity.

Methods: In 391 older adults with metabolic syndrome (mean age, 65 years; mean BMI, 33.3 kg/m^2) from 1 of the Prevención con Dieta Mediterránea-Plus trial centers, we evaluated the impact of a 6-month intervention with an energy-restricted Mediterranean diet plus physical activity (intensive lifestyle; $n = 190$) relative to a nonrestrictive Mediterranean diet without physical activity (control; $n = 201$) on a set of HDL functional traits. These included cholesterol efflux capacity, HDL oxidative/inflammatory index, HDL oxidation, and levels of complement component 3, serum amyloid A, sphingosine-1-phosphate, triglycerides, and apolipoproteins A-I, A-IV, C-III, and E in apoB-depleted plasma.

Results: The intensive-lifestyle intervention participants displayed greater 6-month weight reductions (−3.83 kg; 95% CI: −4.57 to −3.09 kg) but no changes in HDL cholesterol compared with control-diet participants. Regarding HDL functional traits, the intensive lifestyle decreased triglyceride levels (−0.15 mg/g protein; 95% CI: −0.29 to −0.014 mg/g protein) and apoC-III (−0.11 mg/g protein; 95% CI: −0.18 to −0.026 mg/g protein) compared to the control diet, with weight loss being the essential mediator (proportions of mediation were 77.4% and 72.1% for triglycerides and apoC-III levels in HDL, respectively).

Conclusions: In older adults with metabolic syndrome, an energy-restricted Mediterranean diet plus physical activity improved the HDL triglyceride metabolism compared with a nonrestrictive Mediterranean diet without physical activity. This trial is registered at isrctn.com as ISRCTN89898870. *Am J Clin Nutr* 2021;00:1–9.

Keywords: high-density lipoprotein, physical activity, calorie restriction, Mediterranean diet, randomized controlled trial

Introduction

Raised levels of HDL cholesterol have been associated with lower risks of cardiovascular disease (1). Pharmacological interventions and Mendelian randomization studies have, however, questioned the causal association between increased HDL cholesterol concentrations and lower cardiovascular risks (2, 3). Thus, HDL functional traits merit further investigation as to their possible roles in modifying such a risk (2). These include: *1)* cholesterol efflux capacity (CEC), the ability of HDLs to pick up cholesterol excess from cells, such as macrophages; *2)* HDL antioxidant/anti-inflammatory properties [HDL oxidative/inflammatory index (HOII); HDL oxidation status; HDL levels of acute-phase proteins, such as complement component 3 (C3c) and serum amyloid A (SAA); etc.]; *3)* HDL

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endothelial protection [related to their sphingosine-1-phosphate (S1P) content]; *4*) HDL role on triglyceride metabolism; and *5*) HDL-bound apolipoprotein concentrations (4–6). Reduced CEC values, pro-oxidative/pro-inflammatory HDLs (with increased HOII values and elevated levels of C3c), S1P-poor HDLs, dysfunctional HDLs on the triglyceride metabolism (enriched in disruptors of the triglyceride metabolism, such as apoC-III), and HDLs with impaired levels of apolipoproteins such as apoA-I, have been associated with greater cardiovascular risks in several cohorts (7–10). In addition, a recent Mendelian randomization study established a potentially causal relationship between HDL quality characteristics beyond HDL cholesterol levels and coronary artery disease (11), suggesting that HDL functional/quality characteristics could act as potential therapeutic targets for cardiovascular disease.

Adequate levels of physical activity are key in the prevention of cardiovascular disease (12). Additional benefits to cardiovascular risks can be achieved when this lifestyle modification is accompanied by energy restriction, leading to sustained weight reduction (13). Regarding HDL functions, several short-term, small-scale, randomized controlled studies and noncontrolled trials have assessed the individual associations among physical activity, weight loss, and HDL functionality. In most cases, results were inconsistent or of lesser scientific quality. The relationship between physical activity and CEC has been shown to be controversial (14–18). Calorie restriction has been linked to decreases in CEC values in 2 noncontrolled studies (19, 20), and studies combining both have also reported conflicting or uncontrolled findings (21–23). HDL antioxidant capacities and HDL oxidation have only been studied in noncontrolled trials, although enhancements in both have been associated with physical activity (18, 23–28). Physical activity has also been linked to improvements in HDL anti-inflammatory properties in further noncontrolled studies (18, 28), although findings were inconsistent in a randomized controlled trial (15). Finally, the associations between physical activity and HDL proteome (HDL levels of acute-phase proteins such as C3c, SAA, apoA-I, apoA-IV, apoC-III, and apoE, among many others) have also been investigated in 2 observational studies (23, 29). Testing the effects of promoting physical activity and calorie restriction within the frame of a Mediterranean diet (MedDiet) would therefore be a logical next step. This dietary pattern, and some of its key foods, have been associated with improvements in CEC, the HDL cholesterol metabolism, HDL antioxidant properties, HDL oxidation, HDL-bound levels of acute-phase proteins, HDL endothelial protection, HDL's role in the triglyceride metabolism, and HDL levels of certain apolipoproteins, such as apoA-I (30–33).

The aim of this study was to determine whether a lifestyle intervention consisting of an energy-restricted MedDiet and physical activity improved HDL functional traits in individuals with metabolic syndrome, compared to a MedDiet with spontaneous caloric intake and no changes in physical activity.

Methods

Participants

Our study population is a subsample of 391 volunteers from the Prevención con Dieta Mediterránea-Plus (PREDIMED-Plus) study. The subjects were recruited in the Hospital del Mar Medical Research Institute (Barcelona, Spain) and provided plasma samples at baseline and after 6 months of intervention. The PREDIMED-Plus study is a multicenter, parallel, randomized controlled trial that aims to evaluate the effect of a lifestyle intervention with an energy-restricted MedDiet combined with physical activity and behavioral support relative to a MedDiet with spontaneous caloric intake and without physical activity (control group) on the primary incidence of cardiovascular disease (13, 34). Participants were community-dwelling males (aged 55–75 years) and females (aged 60–75 years) with a BMI between 27 and 40 kg/m^2. They presented at least 3 criteria for metabolic syndrome: *1*) triglycerides ≥150 mg/dL or triglyceride-lowering medication; *2*) fasting glucose ≥100 mg/dL or glucose-lowering medication; *3*) systolic/diastolic blood pressure ≥130/85 mmHg or antihypertensive medication; *4*) HDL cholesterol levels <40 mg/dL in males and <50 mg/dL in females; and/or *5*) waist circumference ≥94 cm in males and ≥80 cm in females (35). The study protocol complied with the Declaration of Helsinki and is registered at the International Standard Randomized Controlled Number Registry as ISRCTN89898870. It has also been published elsewhere (35) and is available on the PREDIMED-Plus study website (https://www.predimedplus.com/en/project). This particular sub-project was approved by the Parc de Salut Mar Clinical Research Ethics Committee. All participants provided written informed consent at the beginning of the study. The study flowchart is depicted in **Figure 1**.

Exposure: lifestyle intervention

Participants were randomly allocated to a 1:1 ratio of either intensive-lifestyle or nonrestrictive MedDiet intervention groups by a centrally controlled, computer-generated, random-numbered, internet-based system with stratification by center, age, and sex, as previously described (13).

Participants allocated to the control group were instructed by trained dieticians to follow a traditional MedDiet without caloric intake restrictions (the dietary intervention described in the PREDIMED study) (36). We encouraged: *1*) the consumption

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Supplemental Tables 1–7 and Supplemental Figures 1 and 2 are available from the "Supplemental data" link in the online posting of the article at https://academic.oup.com/ajcn/.

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Abbreviations used: C3c, complement component 3; CEC, cholesterol efflux capacity; HOII, HDL oxidative/inflammatory index; MedDiet, Mediterranean diet; MET, metabolic equivalents of task; PREDIMED, Prevención con Dieta Mediterránea; S1P, sphingosine-1-phosphate; SAA, serum amyloid A.

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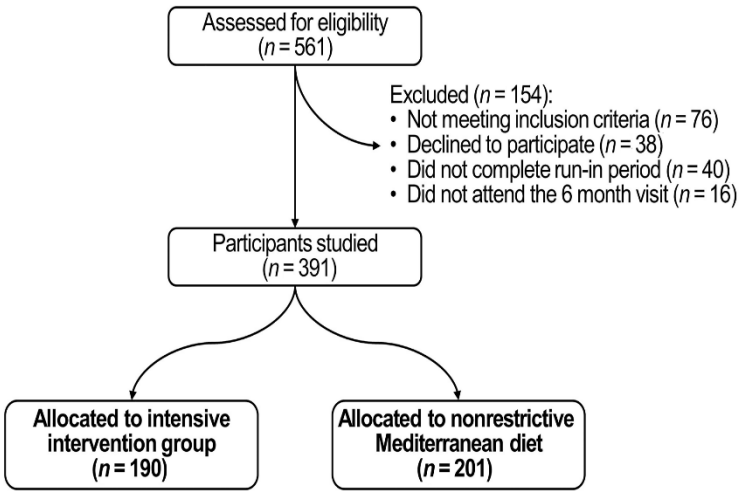

FIGURE 1 Study flowchart.

of fruit, vegetables, legumes, nuts, and fish; *2*) the use of extra-virgin olive oil as the main culinary fat and for traditional food preparation techniques such as "sofrito"; and *3*) a reduction in the intakes of red/processed meats (by replacing them with poultry), sugary drinks, pastries, confectionery, sweets, and fatty spreads (36). Participants in the control group did not receive recommendations to increase their levels of physical activity or lose weight. The follow-up in this group consisted of an individual on-site interview at the beginning of the study and after each 6 months (35).

Participants allocated to the intensive-intervention group were instructed to follow an energy-restricted MedDiet, together with physical activity recommendations, with the purpose of achieving specific weight loss goals (we aimed at an 8% weight reduction or ≥5% decrease in waist circumference). Regarding physical activity, subjects were encouraged to perform at least 45 minutes per day of moderate-intensity aerobic activity (such as brisk walking, cycling, and swimming) and carry out resistance, balance, or flexibility training. They were additionally advised to perform different exercises to develop the strength of the main muscles for at least 2 days/week (duration: 30–40 minutes/day), as well as directed balanced activities (e.g., yoga, tai chi) if they felt motivated and had access to these activities. Dietitians adapted the previous recommendations to gradually achieve physical activity goals, considering the participants' preferences. In addition, the energy-restricted MedDiet intervention was aimed at a long-term, progressive, sustained caloric decrease of approximately 30% of estimated energy requirements (about 600 kcal/day), according to each participant's basal metabolic rate and physical activity levels, following the Institute of Medicine equations (13). This calorie restriction was recommended within the context of the previously described traditional MedDiet pattern, with some particularities: *1*) there were more restrictive limits for the consumption of red/processed meats, fatty spreads, and sugary drinks; and 2) there were greater limitations regarding the intake of refined carbohydrates (such as added sugar in beverages, white bread, and refined cereals) and a promotion of whole-grain consumption (35). To accomplish such goals, this intensive-intervention group followed a more thorough visit plan (1 face-to-face individual interview, 1 group session, and 1 phone call every month) (13, 34, 35).

Dietary quality, physical activity levels, and energy intake were evaluated in all participants using 3 questionnaires. Adherence to the energy-restricted MedDiet pattern was assessed by a 17-item questionnaire, with scores ranging from 0 (null adherence) to 17 (full adherence) (35). We measured the total energy expenditure from physical activity with the Minnesota-REGICOR (REgistre GIroní del COR) leisure-time physical activity questionnaire (37). It was estimated in metabolic equivalents of task (METs) minutes per week by multiplying the METs linked to each activity collected in the questionnaires with the mean duration in minutes/week reported by the participants. Finally, we measured the intake of total energy (kcal/day) using the information gathered in a 143-item, semi-quantitative FFQ validated in an adult Spanish population (38).

Outcomes: HDL functional traits

We collected fasting EDTA plasma samples at baseline and after 6 months of the intervention and stored them at −80°C until use. In these samples, we measured levels of glucose (Glucose HK CP, Horiba ABX), total cholesterol (Cholesterol CP, Horiba ABX), triglycerides (Triglycerides CP, Horiba ABX), and HDL cholesterol (HDL Direct CP, Horiba ABX) in an autoanalyzer ABX Pentra. LDL cholesterol was calculated using the Friedewald equation when triglycerides were <300 mg/dL.

We determined all HDL functional traits in apoB-depleted plasma, a modified preparation in which all lipoproteins except HDL are eliminated (low- and very low–density lipoproteins) by precipitation with 20% polyethylene glycol 8000

(Sigma-Aldrich) (31). CEC was measured in a human THP-1 monocyte-derived macrophage cell line incubated with 0.025 mM fluorescent 23-(dipyrromethemeboron difluoride)-24-norcholesterol (Avanti Polar Lipids) (7). The antioxidant/anti-inflammatory capacity of HDL was estimated by the HOII technique [the HDL capacity to prevent the oxidation of the fluorescent marker 2'-7'dichlorohydrofluorescein (Life Technologies) by oxidized LDLs] (7, 31). HDL oxidation status [HDL content of oxidized lipids (malondialdehyde equivalents) per unit of protein] was measured by the thiobarbituric acid reactive substances assay as previously described (31). ELISA kits were used to determine levels of SAA (Human SAA ELISA Kit, Life Technologies), S1P (Sphingosine 1 Phosphate BioAssay ELISA Kit, US Biological), and apoA-IV (Human Apolipoprotein A-IV ELISA Kit) (7). Finally, in an ABX Pentra autoanalyzer we determined the levels of C3c, triglycerides, apoA-I, apoC-III, apoE, and total protein content in apoB-depleted plasma samples [ApoA1, Triglycerides CP, and Total Protein CP (Horiba ABX); Complement C3, ApoC-III, and ApoE (Spinreact)] (7, 31). Levels of C3c, SAA, S1P, triglycerides, apoA-I, apoA-IV, apoC-III, and apoE in apoB-depleted plasma were normalized against the total protein concentration in these samples.

Interassay variability was minimized by: *1*) examining the pre- and postintervention samples from the same participant in the same experimental run; *2*) analyzing the pair of samples from a participant of the intervention group followed by the samples of a participant of the control arm, according to a random sequence established prior to analyses; and *3*) including in each experiment a sample pool (isolated from 20 healthy volunteers) used to calculate interassay CVs. Regarding functional tests (CEC and HOII): *1*) both were assayed in duplicate and values with CVs ≥15% were eliminated; and 2) interassay variability was minimized by dividing CEC and HOII values of samples by those obtained for the control pool, providing normalized ratios without units as results (7, 31). Interassay CVs and the number of missing values for all determinations are available in **Supplemental Table 1**.

Covariates and other variables

Trained staff collected data on the following variables at the baseline visit: age; sex; educational level; glucose-lowering, cholesterol-lowering, and antihypertensive drug use; and smoking habit. Qualified health-care providers measured weight and height using calibrated weight scales and stadiometers, and waist circumference (midway between the lowest rib and the iliac crest) using an anthropometric tape. BMI was calculated as weight divided by height squared (kg/m^2). Blood pressure was measured using a calibrated automated oscillometer (35). Type 2 diabetes was defined as described in the PREDIMED-Plus protocol (35); hypercholesterolemia was described as presenting with total cholesterol levels ≥200 mg/dL or using cholesterol-lowering mediation; and hypertension was described as presenting with systolic blood pressure ≥140 mmHg, presenting with diastolic blood pressure ≥90 mmHg, or using antihypertensive drugs.

Sample size

A sample size of 190 participants per group allowed ≥80% power to detect differences of 0.019 units in normalized CEC between pre- and postintervention values and of 0.026 units between the 2 interventions, considering a 2-sided type 1 error of 0.05, a loss rate of 5%, and the SD of the differences in CEC reported after an analogous dietary intervention in individuals at high cardiovascular risk (SD, 0.089) (31).

Statistical analyses

We described normally distributed continuous variables by means and SDs, nonnormally distributed continuous variables by medians (1st to 3rd quartile), and categorical variables by proportions.

As main analyses, we assessed whether there were differences in the postintervention values in lifestyle variables, continuous cardiovascular risk factors, and HDL functional traits in the energy-restricted MedDiet + physical activity group relative to the nonrestrictive MedDiet arm by multivariable linear regressions adjusted for: baseline levels of each outcome parameter (continuous), age (continuous), sex, educational level (primary/secondary/greater/unavailable), HDL cholesterol (continuous), triglycerides (continuous), prevalence of type 2 diabetes mellitus (yes/no), hypercholesterolemia (yes/no), hypertension (yes/no), smoking habit (current/former/never smoker), BMI (continuous), physical activity (continuous), and total energy intake (continuous). Multicollinearity among covariates was ruled out by checking their variance inflation factor values in all regression models, and normal distribution of all model residuals was confirmed by their quantile-quantile (Q-Q) plots. Models were fitted using the "lme4" package in R Software (R Foundation for Statistical Computing) (39). We also calculated the mediating effect of the 6-month weight loss on the associations between the intervention and the changes in HDL functionality traits using the "mediation" package in R Software (40). The proportion of mediation was calculated as the ratio between the effect size of the association through the 6-month BMI changes and the total effect size. Finally, as exploratory analyses, we assessed the average change across groups relative to preintervention values. We analyzed whether there were differences relative to baseline in all study participants by paired *t*-tests in normally distributed continuous variables and Wilcoxon signed-rank tests in nonnormally distributed variables. These analyses were also performed within groups when the intergroup differences were significant. We did not perform any multiple testing adjustment because our analyses were hypothesis driven and the phenotypes of interest were correlated and not independent (**Supplemental Figures 1** and **2**).

Analyses were performed using R Software version 3.6.1 (41).

Results

Study participants

Participants were 391 older adults (mean age, 65.5 ± 4.64 years; 52% women) with excess body weight (19% of the population presented BMI values of 27.0–29.9 kg/m^2, and the remaining 81% presented values of 30.0–40.0 kg/m^2) and a high prevalence of cardiovascular risk factors (85% hypertension, 69% hypercholesterolemia, 35% diabetes, 9% current smokers). No differences at baseline between intervention

and control groups were found for these characteristics, adherence to the MedDiet, and leisure-time physical activity levels (**Table 1**).

Lifestyle modifications

All participants increased their estimated total energy expenditure in physical activity and decreased their calorie intake relative to baseline values. However, participants in the intensive-lifestyle intervention displayed a greater increase (relative to the control arm) in physical activity (+726 METs-min/week; 95% CI: 294–1160 METs-min/week) and a modest but greater decrease in energy intake (−75.8 kcal/day; 95% CI: −147 to −4.5 kcal/day; **Supplemental Table 2**). Both intervention arms, based on MedDiets, were associated relative to baseline with increases in the consumption of virgin olive oil, vegetables, legumes, nuts, whole grains, poultry, and white and fatty fish and decreases in the intakes of refined grains, red meat, processed meat, and alcoholic beverages (all P values < 0.001). Adherence to the energy-restricted MedDiet pattern was, however, greater in the intensive-lifestyle intervention group (+1.43 score points; 95% CI: 0.93–1.93 score points). This intervention arm presented higher increases in the consumption of legumes, nuts, and poultry and decreases in the intake of refined grains (a marginal reduction in the consumption of red meat was also suggested; Supplemental Table 2). No changes in smoking status were observed (**Supplemental Table 3**).

Changes in continuous cardiovascular risk factors

Irrespective of the study group, relative to baseline all participants had decreases in fasting glucose values, total and LDL cholesterol levels, systolic and diastolic blood pressure, body weight, BMI, and waist circumference and increases in HDL cholesterol concentrations. However, those allocated to the intensive-lifestyle group, compared to the control group, experienced greater 6-month reductions in fasting glucose (−4.71 mg/dL; 95% CI: −9.06 to −0.35 mg/dL), triglycerides (−21.1 mg/dL; 95% CI: −30.5 to −11.6 mg/dL), systolic blood pressure (−4.36 mmHg; 95% CI: −6.87 to −1.84 mmHg), diastolic blood pressure (−3.57 mmHg; 95% CI: −5.26 to −1.89 mmHg), body weight (−3.83 kg; 95% CI: −4.57 to −3.09 kg), BMI (−1.43 kg/m^2; 95% CI: −1.71 to −1.16 kg/m^2), and waist circumference (−3.44 cm; 95% CI: −4.28 to −2.61 cm). No intergroup differences in total, HDL, and LDL cholesterol levels were observed (**Supplemental Table 4**).

Changes in HDL functional traits

Compared to participants in the control group, those in the intensive-intervention group had greater 6-month reductions in levels of triglycerides (−0.15 mg/g protein; 95% CI: -0.29 to -0.014 mg/g protein) and apoC-III (−0.11 mg/g protein; 95% CI: −0.18 to −0.026 mg/g protein) in apoB-depleted plasma (**Table 2**). Intergroup differences in both parameters were substantially mediated by 6-month weight changes [triglycerides: proportion of mediation = 77.4% (95% CI: 22.3%–382%; P value = 0.016); apoC-III: proportion of mediation = 72.1% (95% CI: 30.3%–265%; P value = 0.006); **Supplemental Table 5**]. Intergroup differences, stratified by sex and baseline prevalence of diabetes, are available in **Supplemental Tables 6** and **7**. No intergroup differences in 6-month changes were detected in the remaining HDL functional traits. Nevertheless, we observed decreases in the HDL oxidative/inflammatory potential, HDL oxidation, and concentrations of C3c, SAA, and S1P and increases in apoA-I relative to baseline values across groups (Table 2).

Discussion

An intervention with an energy-restricted MedDiet plus physical activity improved HDL functionality on the triglyceride metabolism in older adults with metabolic syndrome compared with a nonrestrictive MedDiet without physical activity.

HDLs are intimately related to the triglyceride metabolism. High triglyceride levels in HDLs destabilize their structure and function (42) and, in turn, have been causally linked to greater coronary artery disease (11). Moreover, HDLs carry lipoproteins involved in the triglyceride metabolism, such as apoC-III, which inhibits lipoprotein lipase activity and the hepatic clearance of triglyceride-rich lipoproteins (43) and is directly linked to coronary heart disease risks (44). In our study, intervention with an energy-restricted MedDiet plus physical activity was able to decrease both apoC-III and the triglyceride content of HDLs, mainly through the associated weight loss. This factor could partially explain decreases in these parameters, as obesity is related to greater HDL content of apoC-III and triglycerides (42, 45). ApoC-III synthesis is also exacerbated in impaired glucose metabolism states (46), which may diminish after weight loss. Finally, the molecular effects of physical activity and energy restriction may additionally contribute to decreasing triglyceride levels. Aerobic physical activity and caloric restriction have been shown to be able to stimulate AMP-activated protein kinase, which, in turn, decreases the activation of lipogenic transcription factors involved in triglyceride synthesis in the liver (47). A synergistic effect between these lifestyle modifications and some MedDiet bioactive compounds could additionally be present. Phenolic compounds and SCFAs derived from the bacterial metabolism of dietary fiber in the intestine have been reported to be able to boost AMP-activated protein kinase through alternative metabolic pathways (48, 49).

Contrary to what was observed for HDL's role in the triglyceride metabolism, we did not observe any intergroup differences in HDL properties related to oxidative status and low-grade inflammation, because there was a decrease in these properties relative to baseline in both study arms. Both were based in antioxidant-rich dietary patterns (50), and previous human studies have indicated that dietary antioxidants are able to bind to HDLs and possibly induce a local antioxidant effect (30, 31, 51). In addition, a MedDiet has been shown to decrease the levels of circulating pro-inflammatory cytokines (52), probably due to the ability of dietary antioxidants to modulate various transcriptomic mechanisms (53), which in turn could be associated with reduced adhesion of these molecules to the surface of HDL. These findings agree with previous evidence, since an improvement in HDL antioxidant/anti-inflammatory properties has been reported after a 1-year intervention with a MedDiet in individuals with a high cardiovascular risk (31, 33). Finally, the 2 intervention arms failed to increase CEC.

TABLE 1 Baseline characteristics of participants

	All participants, $n = 391$	Control group, $n = 201$	Intensive intervention, $n = 190$
Age, years, mean ± SD	65.5 ± 4.64	65.3 ± 4.61	65.7 ± 4.68
Female sex, *n* (%)	204 (52.2)	105 (52.2)	99 (52.1)
Type 2 diabetes, *n* (%)	137 (35.0)	72 (35.8)	65 (34.2)
Glucose-lowering medication, *n* (%)	89 (22.8)	53 (26.4)	36 (18.9)
Hypercholesterolemia, *n* (%)	270 (69.4)	142 (70.6)	128 (68.1)
Cholesterol-lowering medication, *n* (%)	182 (46.5)	95 (47.3)	87 (45.8)
Hypertension, *n* (%)	334 (85.4)	170 (84.6)	164 (86.3)
Antihypertensive medication, *n* (%)	299 (76.5)	152 (75.6)	147 (77.4)
Status according to BMI			
BMI between 27.0–29.9 kg/m^2, *n* (%)	76 (19.4)	32 (15.9)	44 (23.2)
BMI between 30.0–40.0 kg/m^2, *n* (%)	315 (80.6)	169 (84.1)	146 (76.8)
Abdominal obesity, *n* (%)	377 (97.2)	196 (98.5)	181 (95.8)
Smoking status			
Never smokers, *n* (%)	193 (49.4)	91 (45.3)	102 (53.7)
Current smokers, *n* (%)	36 (9.21)	16 (7.96)	20 (10.5)
Former smokers, *n* (%)	162 (41.4)	94 (46.8)	68 (35.8)
Educational level			
Elementary school, *n* (%)	163 (41.6)	86 (42.8)	77 (40.5)
High school, *n* (%)	137 (35.0)	73 (36.3)	64 (33.7)
Undergraduate education, *n* (%)	40 (10.2)	15 (7.46)	25 (13.2)
Graduate or postgraduate, *n* (%)	48 (12.3)	27 (13.4)	21 (11.1)
Unavailable information, *n* (%)	3 (0.77)	0 (0)	3 (1.58)
Adherence to the MedDiet, score, mean ± SD	7.32 ± 2.46	7.17 ± 2.38	7.48 ± 2.55
Leisure-time physical activity, metabolic equivalents of task-minute/week, median (1st to 3rd quartile)	1958 (895–3413)	1,734 (895–3413)	2,168 (899–3378)

In a prior study comparing a traditional MedDiet intervention with a low-fat diet, no intergroup difference was observed in CEC values, although they increased in the MedDiet intervention groups relative to baseline (31). A weight-loss intervention based on a healthy dietary pattern [Dietary Approaches to Stop Hypertension (DASH) diet] plus physical activity was also linked to increased CEC levels in an observational study (29). Such divergent findings might be due to: *1*) differing proportions of individuals prone to lower CEC values (likely to benefit from the intervention), such as participants with type 2 diabetes or excess weight (54); *2*) distinct intervention lengths (6 months in the present study, 12 months in our prior work, and 3 months for the DASH diet); *3*) different magnitudes of weight loss among studies; and *4*) the techniques used to quantify CEC (in the present study we worked with a fluorescent-labeled cholesterol probe, whilst in the others a radiolabeled cholesterol analog was used).

Our study has some strengths. As far as we know, this is the largest study to address the effect of a whole-lifestyle intervention on a comprehensive, hypothesis-driven set of HDL functional traits. Its sample size, together with its randomized design, provide high-quality evidence and minimize the influence of confounding and bias. There are, however, a number of limitations. First, results were obtained in older adults with metabolic syndrome and excess body weight, and cannot therefore be extrapolated to other populations. Second, as expected, we only found moderate differences between intervention arms, given that we used an active comparator as a control group (a healthy, traditional MedDiet) and the intensive intervention consisted of real-life changes of diet and physical activity, adapted to the participants' clinical conditions. Third, whilst a substantial increase in the physical activity levels of the participants in the intensive-lifestyle intervention arm was observed, the intergroup differences in energy intake were of a lower magnitude. Nevertheless, the aimed decrease in energy consumption is ambitious and intended to be achieved throughout the whole study. Currently, we are only considering the 6 first months of the intervention. Fourth, 16 participants from the 407 recruited individuals in our center were lost to follow-up after 6 months of the study. This may represent a potential source of bias in our analyses. Fifth, our study design compares an intensive intervention based on the combination of calorie restriction, physical activity, and a Mediterranean dietary pattern relative to a control arm based on a non-hypocaloric Mediterranean diet exempt of physical activity recommendations. Our design does not allow us to discriminate between the individual effects of calorie restriction or physical activity, nor to examine their interactions. Possible synergistic or additive effects should be further explored in more specific designs. Sixth, our study is based on a hypothesis-driven approach and investigates secondary outcomes of the PREDIMED-Plus study (which are correlated and not independent). Thus, we did not correct our results according to multiple testing, and the *P* values reported in our findings should be interpreted with caution. Finally, the results of the mediation analyses presented wide CIs due to the limited sample size and should also be interpreted carefully.

In conclusion, in older adults with metabolic syndrome, an intensive-lifestyle intervention with an energy-restricted MedDiet and physical activity improved HDL functions on the

TABLE 2 Differences in 6-month changes in HDL functionality traits between control and intervention groups

	Nonrestrictive MedDiet, control group		Energy-restricted MedDiet + physical activity		Average change across groups *P* value	Intergroup difference	
	Preintervention values	Postintervention values	Preintervention values	Postintervention values		Adjusted difference (95% CI)	*P* value
Cholesterol efflux capacity, ratio	1.08 ± 0.17	1.09 ± 0.17	1.05 ± 0.15	1.05 ± 0.15	0.372	−0.006 (−0.028 to 0.016)	0.616
HDL oxidative/inflammatory index, ratio	0.89 ± 0.20	0.83 ± 0.19	0.91 ± 0.17	0.87 ± 0.18	<0.001	0.022 (−0.005 to 0.048)	0.107
HDL oxidation, μg malondialdehyde (MDA)/g protein	10.1 ± 2.24	9.92 ± 2.33	10.3 ± 2.34	9.92 ± 2.20	<0.001	−0.095 (−0.31 to 0.12)	0.386
Complement component 3 in apoB-depleted plasma, mg/g protein	4.15 ± 1.18	3.94 ± 1.15	3.92 ± 1.18	3.75 ± 1.02	<0.001	−0.040 (−0.21 to 0.13)	0.646
Serum amyloid A in apoB-depleted plasma, μg/g protein	477 (249–993)	398 (212–883)	437 (264–870)	347 (208–893)	<0.001	76.5 (−76.4 to 229)	0.327
Sphingosine-1-phosphate in apoB-depleted plasma, μg/g protein	3.65 ± 1.20	3.63 ± 1.21	4.00 ± 1.35	3.76 ± 1.27	0.028	−0.11 (−0.32 to 0.11)	0.327
Triglycerides in apoB-depleted plasma, mg/g protein	3.85 ± 0.96	3.67 ± 0.90[1]	3.83 ± 1.02	3.54 ± 0.97[1]	<0.001	−0.15 (−0.29 to -0.014)	0.032
ApoA-I in apoB-depleted plasma, mg/g protein	26.6 ± 4.10	26.8 ± 4.17	25.8 ± 3.91	26.3 ± 4.17	0.009	0.15 (−0.32 to 0.62)	0.531
ApoA-IV in apoB-depleted plasma, μg/g protein	139 (97.3–208)	134 (99.5–192)	130 (101–171)	129 (94.6–175)	0.187	2.74 (−8.75 to 14.2)	0.641
Apo C-III in apoB-depleted plasma, mg/g protein	1.00 (0.66–1.50)	1.02 (0.62–1.40)	0.98 (0.61–1.42)	0.82 (0.55–1.31)[1]	<0.001	−0.11 (−0.18 to -0.026)	0.009
Apo E in apoB-depleted plasma, mg/g protein	0.31 ± 0.16	0.31 ± 0.17	0.29 ± 0.16	0.30 ± 0.16	0.699	0.010 (−0.008 to 0.028)	0.277

Pre- and post-intervention values are presented as means ± SDs for normally distributed variables or medians (1st to 3rd quartile) for nonnormally distributed variables. Intergroup comparisons in postintervention values were assessed by multivariable linear regression models adjusted for: baseline levels of the parameter, age, sex, educational level, HDL cholesterol, triglycerides, type 2 diabetes, hypercholesterolemia, hypertension, smoking, BMI, physical activity, and total energy intake. Average change across groups was assessed in the whole study population by paired *t*-tests in normally distributed variables and Wilcoxon signed-rank test in nonnormally distributed variables.

[1] *P* value < 0.05 (post- compared with preintervention values).

triglyceride metabolism relative to a nonrestrictive MedDiet control group. Our findings suggest that a healthy lifestyle may have a positive impact on HDL functionality. Further prospective studies examining whether these improvements mediate the cardiovascular benefits of the lifestyle modifications investigated in our work are warranted.

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The authors' responsibilities were as follows—AH and MF: designed the research; AS: conducted the research; MAM-G, JS-S, DC, RE, FJT, ER, and MF: conducted the clinical trial and provided study databases; AS, IS, and AH: analyzed data; AS, AH, and MF: wrote the manuscript draft; MTS-F, OC, IS, CL, MAM-G, JS-S, DC, RE, FJT, and ER: reviewed and edited the text; AH and MF: have primary responsibility for the final content; and all authors: read and approved the final manuscript.

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Data Availability

The data sets generated and analyzed in the current study are not expected to be made available outside the core research group, as neither the participants' consent forms nor ethics approval included permission for open access. We do, however, follow a controlled data-sharing collaboration model, as in the informed consent forms participants agreed to a controlled collaboration with other investigators for research related to the project's aims. Data described in the manuscript, codebook, and analytic code will be made available upon request pending application and approval by the PREDIMED-Plus Steering Committee. Investigators who are interested in this study can contact the Committee by sending a request letter (predimed_plus_scommittee@googlegroups.com). A data-sharing agreement indicating the characteristics of the collaboration and data management will be completed for the proposals that are approved.

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ON-LINE SUPPLEMENTARY MATERIAL

A lifestyle intervention with an energy-restricted Mediterranean diet and physical activity enhances HDL function: a sub-study of the PREDIMED-plus randomized controlled trial

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Supplemental Table 1. Inter-assay coefficients of variability and number of missing values

	Inter-group coefficient of variation (%)	Missing data, *n* (%)
Determinations in plasma		
Glucose	2.73%	0 (0%)
Total cholesterol	2.64%	0 (0%)
HDL cholesterol	3.47%	0 (0%)
Triglycerides	4.45%	0 (0%)
Determinations in apolipoprotein B-depleted plasma		
Cholesterol efflux capacity	7.21%	19 (2.43%)
HDL oxidative/inflammatory index	5.65%	2 (0.26%)
HDL oxidation	4.24%	12 (1.53%)
Complement component 3	4.08%	7 (0.90%)
Serum amyloid A	16.0%	6 (0.77%)
Sphingosine-1-phosphate	10.9%	16 (2.04%)
Triglycerides	1.65%	3 (0.38%)
Apolipoprotein A-I	1.78%	9 (1.15%)
Apolipoprotein A-IV	11.3%	4 (0.51%)
Apolipoprotein C-III	6.15%	4 (0.51%)
Apolipoprotein E	3.23%	6 (0.77%)

Supplemental Table 2. Differences in 6-month changes in lifestyle and dietary parameters between control and intensive intervention groups

	Non-restrictive MedDiet, control group		Energy-restricted MedDiet + physical activity		Average change across groups	Inter-group difference	
	Pre-interv. Values	Post-interv. values	Pre-interv. values	Post-interv. values	*P*-value	Adjusted diff. [95% CI]	*P*-value
Adherence to an energy-restricted MedDiet (score points)	7.17 ± 2.38	10.3 ± 2.64[1]	7.48 ± 2.55	11.7 ± 2.40[1]	<0.001	1.43 [0.93; 1.93]	<0.001
Leisure-time physical activity (METs-min/week)	1730 (895-3410)	2240 (1120-3480)	2170 (899-3380)	2970 (1710-5010)[1]	<0.001	726 [294; 1160]	0.001
Energy intake (kcal/day)	2420 (2110-2730)	2300 (2070-2570)[1]	2300 (2050-2620)	2190 (2020-2440)[1]	<0.001	-75.8 [-147; -4.48]	0.038
Carbohydrates (g/day)	225 (188-256)	199 (172-241)[1]	209 (179-250)	190 (172-210)[1]	<0.001	-11.0 [-20.2; -1.77]	0.020
Proteins (g/day)	105 (91.9-118)	107 (97.8-117)	101 (87.9-113)	108 (97.1-117)	<0.001	1.45 [-1.69; 4.58]	0.367
Total fat (g/day)	115 (99.4-135)	116 (100-128)	110 (96.1-131)	110 (98.2-123)	0.251	-2.51 [-6.51; 1.49]	0.220
Saturated fatty acids (g/day)	29.4 (24.6-35.1)	24.1 (20.6-29.1)[1]	28.5 (22.9-33.8)	22.3 (19.7-25.8)[1]	<0.001	-2.19 [-3.35; -1.04]	<0.001
Monounsaturated fatty acids (g/day)	59.6 (51.9-69.2)	62.4 (52.4-72.5)	57.3 (50.0-67.2)	63.1 (53.2-72.6)	<0.001	1.06 [-1.86; 3.98]	0.478
Polyunsaturated fatty acids (g/day)	17.0 (13.8-21.8)	22.6 (17.4-25.2)	16.0 (13.4-20.7)	22.6 (18.9-25.0)	<0.001	0.72 [-0.29; 1.73]	0.162
Omega-3 polyunsaturated fatty acids (g/day)	0.92 (0.71-1.55)	1.49 (0.80-1.65)	0.91 (0.69-1.55)	1.51 (0.85-1.66)	<0.001	0.064 [-0.032; 0.16]	0.193
Dietary fiber (g/day)	24.0 (20.3-29.0)	30.3 (25.4-35.1)[1]	24.4 (19.9-29.5)	32.2 (26.8-36.7)[1]	<0.001	1.51 [0.088; 2.93]	0.038
Alcohol (g/day)	4.98 (1.37-12.7)	2.17 (0.69-10.3)[1]	3.50 (0.69-11.9)	1.46 (0.00-5.14)[1]	<0.001	-1.84 [-3.18; -0.50]	0.007
Virgin olive oil (g/day)	25.0 (10.0-50.0)	50.0 (50.0-50.0)	25.0 (10.0-50.0)	50.0 (50.0-50.0)	<0.001	0.20 [-2.18; 2.57]	0.872

Vegetables (g/day)	322 (244-414)	376 (300-460)	331 (274-413)	382 (313-482)	<0.001	13.7 [-12.8; 40.1]	0.312
Fruits (g/day)	341 (240-460)	335 (250-439)	339 (218-441)	352 (288-429)	0.233	15.8 [-14.2; 45.8]	0.304
Legumes (g/day)	20.6 (12.6-25.1)	25.1 (20.6-29.7)[1]	17.1 (12.0-25.1)	25.7 (21.1-29.7)[1]	<0.001	2.67 [0.76; 4.58]	0.006
Nuts (g/day)	12.6 (2.00-25.7)	32.0 (25.6-49.1)[1]	9.42 (4.00-25.7)	38.6 (30.0-53.6)[1]	<0.001	7.08 [2.70; 11.4]	0.002
Refined grains (g/day)	109 (72.3-157)	44.5 (20.5-99.9)[1]	99.7 (57.2-139)	42.3 (20.5-57.8)[1]	<0.001	-11.4 [-21.2; -1.55]	0.024
Whole grains (g/day)	8.33 (0.00-75.0)	75.0 (32.1-82.2)	8.33 (0.00-75.0)	75.0 (58.9-82.1)	<0.001	1.48 [-8.68; 11.6]	0.775
Dairy products (g/day)	346 (257-452)	346 (275-538)	308 (233-412)	336 (268-404)	0.028	-8.85 [-44.9; 27.2]	0.630
Eggs (g/day)	25.7 (25.7-25.7)	25.7 (25.7-25.7)	25.7 (12.9-25.7)	25.7 (25.7-25.7)	0.113	0.20 [-1.26; 1.66]	0.787
Poultry and rabbit (g/day)	64.3 (42.8-85.7)	74.3 (64.3-85.7)[1]	74.3 (52.8-85.7)	85.7 (74.3-85.7)[1]	<0.001	10.2 [4.21; 16.2]	<0.001
Red meat (g/day)	64.3 (31.4-85.7)	41.4 (31.1-64.3)	52.8 (31.4-84.3)	31.4 (21.4-42.8)	<0.001	-4.66 [-10.1; 0.81]	0.096
Processed meat (g/day)	36.2 (26.7-47.1)	30.3 (24.2-39.5)	35.5 (26.4-41.8)	29.0 (22.0-35.0)	<0.001	-2.18 [-4.93; 0.58]	0.122
White fish (g/day)	64.3 (25.4-68.3)	64.3 (25.4-68.3)	64.3 (25.4-68.3)	64.3 (30.0-68.3)	<0.001	0.88 [-3.43; 5.18]	0.691
Fatty fish (g/day)	32.8 (21.9-62.8)	59.0 (25.7-62.8)	30.1 (21.9-62.7)	59.0 (25.7-62.8)	<0.001	2.18 [-2.33; 6.68]	0.345
Seafood (g/day)	30.6 (26.6-45.9)	30.6 (30.1-45.9)	30.6 (21.9-45.9)	30.6 (26.6-45.9)	0.809	1.01 [-2.84; 4.87]	0.606
Wine (mL/day)	20.0 (6.66-70.4)	13.3 (2.50-44.5)[1]	14.3 (6.66-54.5)	6.66 (0.00-42.8)[1]	<0.001	-9.22 [-18.2; -0.22]	0.045
Beer (mL/day)	22.0 (0.00-141)	22.0 (0.00-47.1)[1]	22.0 (0.00-47.1)	0.00 (0.00-22.0)[1]	<0.001	-36.3 [-55.6; -17.0]	<0.001

[1]: *P*-value <0.05 (post- versus pre-intervention values: paired t-test for normally distributed variables, Wilcoxon signed-rank test for non-normally distributed variables)

Pre- and post-intervention values are presented as means ± standard deviations for normally distributed variables or medians (1^{st}-3^{rd} quartile) for non-normally distributed variables. Inter-group comparisons in post-intervention values were assessed by multivariable linear regression models adjusted for: baseline levels of the parameter, age, sex, educational level, HDL-C, triglycerides, type-II diabetes, hypercholesterolemia, hypertension, smoking, body mass index, physical activity, and total energy intake. Average change across groups was assessed in the whole study population by paired t-tests in normally distributed variables and Wilcoxon signed-rank test in non-normally distributed variables.

Supplemental Table 3. Changes in the proportion of non-smokers or ever smokers after 6 months of intervention

Non-smokers vs. smokers				
		Post-intervention		McNemar's test *P*-value
		Non-smokers	Smokers	
Pre-intervention	Non-smokers	350 (89.5%)	5 (1.28%)	0.724
	Smokers	3 (0.77%)	33 (8.44%)	
Never smokers vs. ever smokers				
		Post-intervention		McNemar's test *P*-value
		Never smokers	Ever smokers	
Pre-intervention	Never smokers	181 (46.3%)	12 (3.07%)	0.361
	Ever smokers	18 (4.60%)	180 (46.0%)	

Supplemental Table 4. Differences in 6-month changes in clinical parameters between control and intensive intervention groups

	Non-restrictive MedDiet, control group		Energy-restricted MedDiet + physical activity		Average change across groups	Inter-group difference	
	Pre-interv. Values	Post-interv. values	Pre-interv. values	Post-interv. values	*P*-value	Adjusted diff. [95% CI]	*P*-value
Glucose, mg/dL	110 (100-132)	106 (97-124)	110 (102-130)	106 (97-118)[1]	<0.001	-4.71 [-9.06; -0.35]	0.035
Total cholesterol, mg/dL	218 ± 41.4	215 ± 40.9	222 ± 40.7	217 ± 37.2	0.012	0.20 [-5.51; 5.91]	0.946
HDL cholesterol, mg/dL	54.4 ± 11.1	55.4 ± 11.9	52.5 ± 11.2	55 ± 12.5	<0.001	1.10 [-0.34; 2.54]	0.136
LDL cholesterol, mg/dL	134 ± 35.3	129 ± 33.7	139 ± 37.1	136 ± 33.3	0.010	2.61 [-2.26; 7.48]	0.294
Triglycerides, mg/dL	144 (107-187)	136 (99-183)[1]	131 (103-179)	118 (91-155)[1]	<0.001	-21.1 [-30.5; -11.6]	<0.001
Systolic blood pressure, mmHg	140 ± 12.5	138 ± 14.5[1]	141 ± 12.0	135 ± 14.1[1]	<0.001	-4.36 [-6.87; -1.84]	<0.001
Diastolic blood pressure, mmHg	75 ± 10.2	74 ± 9.96	76 ± 8.84	72 ± 9.73[1]	<0.001	-3.57 [-5.26; -1.89]	<0.001
Body weight, kg	89.0 ± 13.8	86.3 ± 13.7[1]	87.4 ± 14.0	81.0 ± 12.8[1]	<0.001	-3.83 [-4.57; -3.09]	<0.001
Body mass index, kg/m^2	33.6 ± 3.49	32.6 ± 3.61[1]	33.1 ± 3.5	30.7 ± 3.42[1]	<0.001	-1.43 [-1.71; -1.16]	<0.001
Waist circumference, cm	111 ± 9.59	109 ± 9.70[1]	110 ± 9.71	104 ± 9.30[1]	<0.001	-3.44 [-4.28; -2.61]	<0.001

[1]: *P*-value <0.05 (post- versus pre-intervention values: paired t-test for normally distributed variables, Wilcoxon signed-rank test for non-normally distributed variables)
Pre- and post-intervention values are presented as means ± standard deviations for normally distributed variables or medians (1^{st}-3^{rd} quartile) for non-normally distributed variables. Inter-group comparisons in post-intervention values were assessed by multivariable linear regression models adjusted for: baseline levels of the parameter, age, sex, educational level, HDL-C, triglycerides, type-II diabetes, hypercholesterolemia, hypertension, smoking, body mass index, physical activity, and total energy intake. Average change across groups was assessed in the whole study population by paired t-tests in normally distributed variables and Wilcoxon signed-rank test in non-normally distributed variables

Supplemental Table 5. Proportion of inter-change differences mediated by 6-month decreases in body mass index

	Proportion of mediation [95% CI]
Cholesterol efflux capacity, ratio	1.52 [-582; 454]
HDL oxidative/inflammatory index, ratio	5.67 [-182; 235]
HDL oxidation, µg MDA/g protein	48.5 [-761; 704]
Complement component 3 in apoB-depleted plasma, mg/g protein	137 [-2350; 2900]
Serum amyloid A in apoB-depleted plasma, µg/g protein	-29.6 [-594; 375]
Sphingosine-1-phosphate in apoB-depleted plasma, µg/g protein	127 [-1450; 1140]
Triglycerides in apoB-depleted plasma, mg/g protein	77.4 [22.3; 382]
Apolipoprotein A-I in apoB-depleted plasma, mg/g protein	52.5 [-761; 1330]
Apolipoprotein A-IV in apoB-depleted plasma, µg/g protein	8.69 [-547; 566]
Apolipoprotein C-III in apoB-depleted plasma, mg/g protein	72.1 [30.3; 265]
Apolipoprotein E in apoB-depleted plasma, mg/g protein	61.4 [-687; 637]

Analyses were adjusted for baseline levels of the parameter, age, sex, educational level, HDL-C, triglycerides, type-II diabetes, hypercholesterolemia, hypertension, smoking, body mass index, physical activity, and total energy intake.

Supplemental Table 6. Sex-stratified inter-group analyses

	Women		Men		Interaction (*P*-value)
	Inter-group diff. [95% CI]	*P*-value	Inter-group diff. [95% CI]	*P*-value	
Cholesterol efflux capacity, ratio	0.005 [-0.024; 0.033]	0.753	-0.009 [-0.045; 0.026]	0.600	0.547
HDL oxidative/inflammatory index, ratio	0.004 [-0.033; 0.042]	0.824	0.036 [-0.003; 0.074]	0.073	0.330
HDL oxidation, µg MDA/g protein	-0.013 [-0.31; 0.28]	0.934	-0.13 [-0.44; 0.19]	0.422	0.606
Serum amyloid A in apoB-depleted plasma, µg/g protein	116 [-114; 347]	0.323	-25.7 [-224; 173]	0.800	0.369
Complement component 3 in apoB-depleted plasma, mg/g protein	-0.007 [-0.26; 0.25]	0.958	-0.097 [-0.34; 0.15]	0.434	0.484
Sphingosine-1-phosphate in apoB-depleted plasma, µg/g protein	-0.065 [-0.37; 0.24]	0.683	-0.15 [-0.45; 0.15]	0.330	0.419
Triglycerides in apoB-depleted plasma, mg/g protein	-0.13 [-0.35; 0.085]	0.237	-0.20 [-0.37; -0.026]	0.025	0.749
Apolipoprotein A-I in apoB-depleted plasma, mg/g protein	0.35 [-0.35; 1.05]	0.330	-0.11 [-0.75; 0.52]	0.726	0.622
Apolipoprotein A-IV in apoB-depleted plasma, µg/g protein	4.18 [-13.2; 21.5]	0.638	1.31 [-13.8; 16.4]	0.865	0.804
Apolipoprotein C-III in apoB-depleted plasma, mg/g protein	-0.073 [-0.18; 0.030]	0.165	-0.15 [-0.27; -0.026]	0.019	0.367
Apolipoprotein E in apoB-depleted plasma, mg/g protein	0.002 [-0.024; 0.028]	0.858	0.019 [-0.006; 0.044]	0.133	0.410

Inter-group comparisons in post-intervention values were assessed by multivariable linear regression models adjusted for: baseline levels of the parameter, age, educational level, HDL-C, triglycerides, type-II diabetes, hypercholesterolemia, hypertension, smoking, body mass index, physical activity, and total energy intake. We tested whether there was a significant association between the intervention group and sex on post-intervention HDL functional properties by applying a likelihood ratio test between the regression models with and without the interaction product-term “intervention group x sex”.

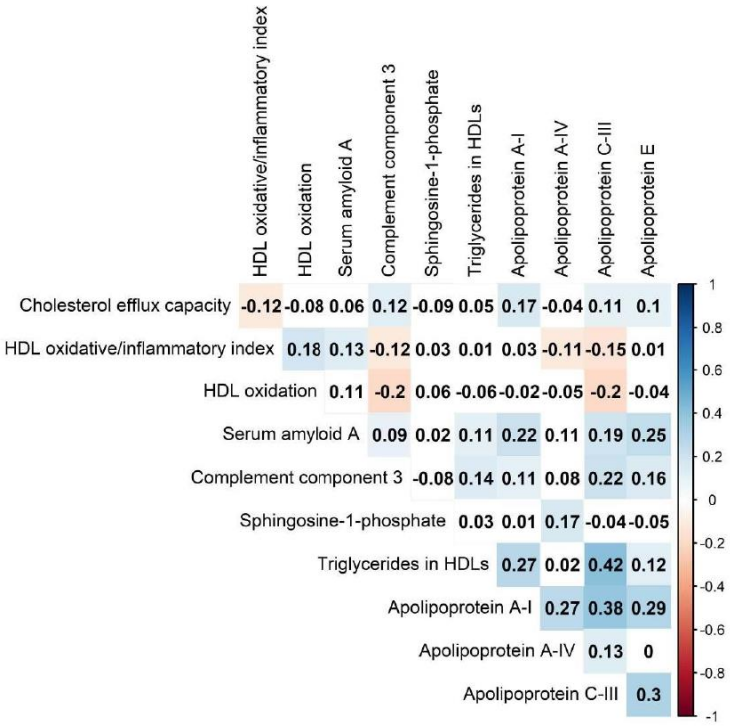
Supplemental Table 7. Diabetes-stratified inter-group analyses

	Non-diabetic		Diabetic		Interaction (*P*-value)
	Inter-group diff. [95% CI]	*P*-value	Inter-group diff. [95% CI]	*P*-value	
Cholesterol efflux capacity, ratio	0.012 [-0.013; 0.036]	0.356	-0.051 [-0.119; 0.018]	0.145	0.119
HDL oxidative/inflammatory index, ratio	0.017 [-0.014; 0.048]	0.273	0.019 [-0.032; 0.07]	0.472	0.894
HDL oxidation, µg MDA/g protein	-0.13 [-0.36; 0.090]	0.242	-0.010 [-0.45; 0.43]	0.963	0.675
Serum amyloid A in apoB-depleted plasma, µg/g protein	20.1 [-176; 216]	0.841	103 [-167; 373]	0.454	0.455
Complement component 3 in apoB-depleted plasma, mg/g protein	0.011 [-0.20; 0.22]	0.917	-0.12 [-0.43; 0.20]	0.473	0.273
Sphingosine-1-phosphate in apoB-depleted plasma, µg/g protein	-0.12 [-0.40; 0.16]	0.401	-0.076 [-0.43; 0.28]	0.677	0.590
Triglycerides in apoB-depleted plasma, mg/g protein	-0.18 [-0.36; 0.005]	0.057	-0.087 [-0.32; 0.14]	0.457	0.660
Apolipoprotein A-I in apoB-depleted plasma, mg/g protein	-0.12 [-0.68; 0.45]	0.679	0.65 [-0.23; 1.53]	0.149	0.115
Apolipoprotein A-IV in apoB-depleted plasma, µg/g protein	-11.1 [-26.7; 4.39]	0.161	19.3 [1.70; 36.8]	0.033	0.021
Apolipoprotein C-III in apoB-depleted plasma, mg/g protein	-0.16 [-0.27; -0.061]	0.002	-0.011 [-0.14; 0.12]	0.873	0.031
Apolipoprotein E in apoB-depleted plasma, mg/g protein	0.002 [-0.023; 0.026]	0.882	0.026 [$-4 \cdot 10^{-4}$; 0.052]	0.056	0.088

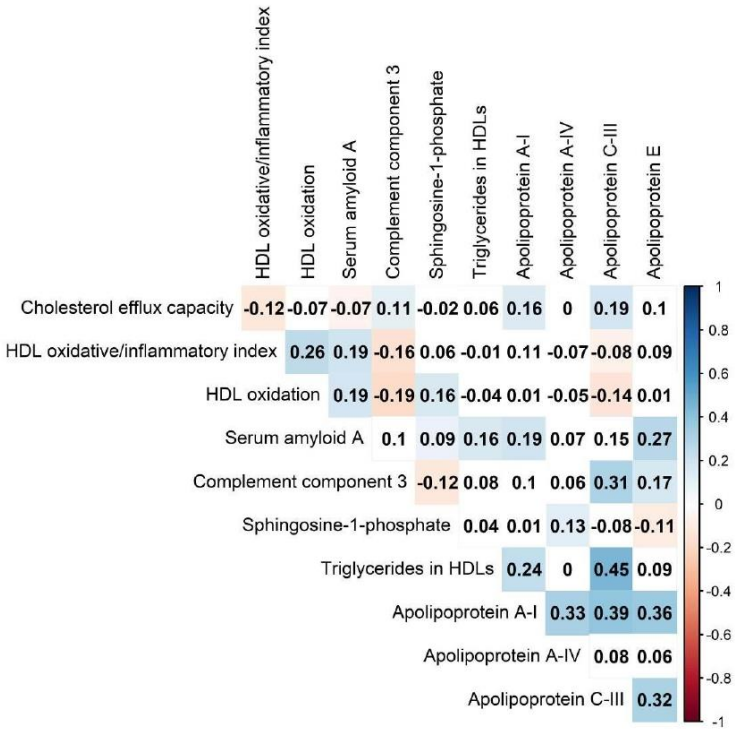
Inter-group comparisons in post-intervention values were assessed by multivariable linear regression models adjusted for: baseline levels of the parameter, age, sex, educational level, HDL-C, triglycerides, hypercholesterolemia, hypertension, smoking, body mass index, physical activity, and total energy intake. We tested whether there was a significant association between the intervention group and diabetes on post-intervention HDL functional properties by applying a likelihood ratio test between the regression models with and without the interaction product-term “intervention group x prevalence of diabetes at baseline”.

SUPPLEMENTAL FIGURES

Supplemental Figure 1. Correlation matrix among baseline HDL functionality parameters.



Supplemental Figure 2. Correlation matrix among post-intervention HDL functionality parameters.



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Modification of high-density lipoprotein functions by diet and other lifestyle changes: a systematic review of randomized controlled trials

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ABSTRACT (200 words, unstructured)

High-density lipoprotein (HDL) functional traits have emerged as relevant elements that can explain HDL antiatherogenic capacity better than HDL cholesterol levels. These properties have been improved in several lifestyle intervention trials. The aim of this systematic review is to summarize the results of such trials on the most commonly used dietary modifications (fatty acids, cholesterol, antioxidants, alcohol, and calorie restriction) and physical activity. Articles were screened from the Medline database until March 2021, and 118 randomized controlled trials were selected. Results from HDL functions including cholesterol efflux capacity, cholesteryl ester transfer protein, lecithin-cholesterol acyltransferase, HDL antioxidant capacity, HDL oxidation status, paraoxonase-1 activity, HDL anti-inflammatory and endothelial protection capacity, HDL-associated phospholipase A2, HDL-associated serum amyloid A, and HDL-alpha-1-antitrypsin were extracted.

In mainly short-term clinical trials, the consumption of monounsaturated and polyunsaturated fatty acids (particularly omega-3 ones in fish), and dietary antioxidants, showed benefits on HDL functionality specially in subjects with cardiovascular risk factors. In this regard, antioxidant-rich dietary patterns were able to improve HDL function in both healthy individuals and subjects at high cardiovascular risk. In addition, in randomised trial assays performed mainly in healthy individuals, reverse cholesterol transport with ethanol in moderate quantities enhanced HDL function.

Nevertheless, the evidence summarised was of unclear quality and short-term nature, and presented heterogeneity in lifestyle modifications, trial designs, and biochemical techniques for the assessment of HDL functions. Interpretation of such findings should therefore be made with caution. Large-scale, long-term, randomized, controlled trials in different populations and individuals with diverse pathologies are warranted.

72 **KEYWORDS**

73 High-density lipoprotein, fatty acids, antioxidants, ethanol, physical activity, trials

74 **Introduction**

75 Low concentrations of high density lipoprotein (HDL) cholesterol (HDL-C) have
76 been linked with greater incidence of coronary heart disease in epidemiological studies
77 (Boekholdt et al. 2013; Di Angelantonio et al. 2009). Nevertheless, experimental and
78 genetic studies have questioned the therapeutic utility of raising HDL-C levels.
79 Pharmacological interventions aiming at increasing HDL-C concentrations (fibrates,
80 niacin, statins, inhibitors of the cholesteryl ester transfer protein –CETP–) have failed to
81 reduce the incidence of cardiovascular disease (CVD) (Keene et al. 2014; Boekholdt et
82 al. 2013). In addition, Mendelian randomization studies have reported that presenting
83 genetic predisposition to high levels of HDL-C is not linked to lower CVD risk (Holmes
84 et al. 2015; Voight et al. 2012), although recent results show a likely causal association
85 with medium size HDL-C (Prats-Uribe et al. 2020). On the other hand, HDL functional
86 characteristics have been shown to be independently associated with lower CVD
87 incidence (Soria-Florido et al. 2020) and stand as promising biomarkers to explain the
88 HDL athero-protective role. The most widely studied HDL functions are: 1) cholesterol
89 efflux capacity (CEC, the ability of HDLs to remove cholesterol excess from cells)
90 (Talbot et al. 2018); 2) antioxidant properties (measured as HDL capacity to directly
91 decrease the oxidation of low-density lipoproteins –LDLs–, the activity of HDL
92 antioxidant enzymes such as paraoxonase-1 –PON1– and HDL-bound phospholipase
93 A2 –HDL-LpPLA2–, and HDL oxidative/inflammatory index –HOII–) (Brites et al. 2017);
94 3) anti-inflammatory properties (which can be assessed by the presence of pro-
95 inflammatory proteins in HDLs such as serum amyloid A –SAA– and alpha-1-
96 antitrypsin) (Birner-Gruenberger et al. 2014); and 4) endothelial protection (measured
97 as HDL ability to induce the endothelial production of nitric oxide, its capacity to
98 decrease the endothelial release of adhesion molecules and cytokines) (Besler et al.
99 2012).

100 In a recent meta-analysis, high CEC values have been inversely related to
101 CVD incidence (Soria-Florido et al. 2020). Furthermore, CVD incidence has been

linked to HDL antioxidant/anti-inflammatory properties and HDL-related endothelial protection in individual prospective studies in a general population and one at elevated cardiovascular disease risk (Soria-Florido et al. 2020; Khera et al. 2017; Sanllorente et al. 2021). There is a wide range of lifestyle intervention studies regarding other HDL functions. They include interventions on several HDL functions with different dietary modifications (intake of monounsaturated, polyunsaturated, saturated, and trans fatty acids –MUFAs, PUFAs, SFAs, and TFAs, respectively–, cholesterol, antioxidant vitamins, phenolic compounds and other minor compounds, ethanol, and calorie restrictions) and physical activity. The aim of this systematic review is to summarize all the evidence of dietary modifications and physical activity on HDL functions.

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Methods

2.1. Search Strategy

To identify relevant trials, we searched the electronic database PubMed, looking for articles published until 10th of March, 2021. We performed twelve searches, one for each HDL function-related term; 1) CEC activity; 2) CETP activity; 3) lecithin cholesterol acyl transferase (LCAT) activity; 4) HDL antioxidant capacity; 5) HDL oxidation status; 6) PON1 activity; 7) HDL anti-inflammatory and endothelial protection properties; 8) HDL-associated phospholipase A2; 9) HDL-associated with SAA; 10) HDL sphingosine-1-phosphate content; 11) HDL-alpha-1-antitrypsin; and 12) HDL-associated complement proteins. The exact search terms in each case are displayed in the **Supplementary Material (Appendix 1)**.

The literature search was developed by two authors (A.S. and A.H.) with a standardized strategy. The articles were first filtered according to title, then to abstract, and finally to full text content. The bibliography of each selected article was also reviewed to find extra references. Any discrepancy between the authors was scheduled to be solved by a third (M.F.).

2.2. Study Selection, Inclusion and Exclusion Criteria

Studies included were randomized controlled trials (RCTs) with any lifestyle intervention in humans including whole diets, individual dietary components or near-dietary dose supplements, and physical activity which modified HDL functional traits. Post-prandial studies, interventions of less than a week or fewer than ten participants, and studies which used pharmacological therapies or high dose supplements were discarded. The search was limited to articles written in English.

2.3. Data Extraction

The following information was extracted from each article: author, year, country, number of participants and characteristics (basal disease), type of intervention (randomization, control group(?), dose, duration), parameter measured, and study outcomes. The study outcomes were quite heterogeneous due to the diversity of methodologies employed to measure HDL functions. All methodologies are stated in the beginning of each section of the review.

To facilitate analyses, lifestyle interventions were sub-grouped into five categories: dietary lipid interventions, antioxidant-rich interventions, ethanol, physical activity and calory restriction interventions, and other lifestyle interventions.

2.4. Risk of Bias Assessment

The risk of bias of each RCT was assessed with the Cochrane Collaboration Risk of Bias Tool (Higgins et al. 2011) by two authors (A.S. and A.H.). This instrument includes seven domains: 1) random sequence generation; 2) allocation concealment; 3) blinding of participants and personnel; 4) blinding of outcome assessment; 5) incomplete outcome data; 6) selective reporting; and 7) other potential biases. We individually assessed and classified each domain as low, high, or unclear risk of bias when the information provided was insufficiently clear. The overall risk of an article was evaluated as low when the majority of the domains corresponded to low risk and no

key domain was high risk; as high if one or more key domains were classified as high risk; and unclear when the majority of the key domains were categorized as unclear risk of bias.

Results

Study selection and description

We obtained 12,796 results when the twelve searches were combined. After screening the primary search and excluding duplicates, 815 were selected. Following screening by abstract, 263 were considered adequate for full-text review. Finally, after examining the full-text, 88 articles were excluded for not following a randomized design, 18 for using pharmacological therapies in combination with lifestyle interventions, 24 did not study any pre-specified HDL function technique, 8 employed vitamins at considerably high dosage, and 7 were post-prandial interventions or shorter than a week or including fewer than 10 participants. Finally, a total of 118 RCTs were selected for a qualitative synthesis (**Figure 1**).

Cholesterol efflux was studied in 37 of the trials, HDL CETP activity or mass in 53, LCAT activity in 32, antioxidant properties in 6, HDL oxidation in 6, PON1 activity in 45, HDL anti-inflammatory and endothelial protection properties in 9, HDL-associated phospholipase A2 in 1 article, HDL-bound SAA in 2 articles, and HDL-alpha-1-antitrypsin in 1 article. No results were found for HDL sphingosne-1-phosphate and HDL-associated complement proteins.

Characteristics of studies included

All studies were RCT (with a parallel group or cross-over design) and included a total of 5,645 participants. Most were short-term interventions ranging from 7 days to 6 months. Only 10 RCTs (8.4% of the total) (Albaghdadi et al. 2017; Hernáez et al. 2017; Hernáez et al. 2020; Tiainen et al. 2016; Wesnigk et al. 2016; Wu et al. 2015; Zhu et al. 2014; Arnett et al. 2019; Williams et al. 1990; Damasceno et al. 2013; Sarzynski et al.

2018) lasted from 6 to 12 months. In addition, sample sizes were generally modest. With respect to number of participants, 73.4% of the studies included 50 or fewer subjects, 17.8% had between 51 and 100, and 8.4% (ten studies) more than 100 (Liu et al. 2018; Homma et al. 2003; Qin et al. 2009; Zhu et al. 2014; Balsan et al. 2019; Tiainen et al. 2016; Damasceno et al. 2013; Sarzynski et al. 2018; Hernáez et al. 2017; Hernáez et al. 2020). Due to the nature of lifestyle interventions most of the studies were not blinded to participants.

Quality assessment

From 118 RCTs 28.0% presented a low risk of bias, 17.8% a high risk, and 54.2% an unclear risk (**Figure 2**). Unclear risk was mainly caused by the absence of detailed information in the study protocols. Individual bias evaluation of the articles is available in **supplemental table 1.**

Dietary lipids and HDL function

Monounsaturated fatty acids (MUFA): oleic acid-rich oils

To determine the effect of MUFA interventions on HDL functions 9 studies were selected (**Table 1**). All of them assessed the effect of MUFAs from olive oil, canola oil, and peanut oil compared to saturated fats and PUFAs.

In a 4-week RCT, MUFA intake (olive oil) was associated with a 4.7% increase in CEC values compared to an equivalent fat-calorie intake from cheese (Brassard et al. 2018). Similarly, an RCT with a 4-week consumption of canola oil (59% MUFAs) or a high-oleic canola oil (72% MUFAs) increased CEC by 39.1% and 33.6%, respectively, relative to baseline levels, but not when compared to three interventions rich in PUFAs (Liu et al. 2018). In contrast, an 8-week oleic acid-rich diet did not improve CEC relative to a linoleic acid-rich one (Solà et al. 1997).

213 In one RCT MUFA intake was associated with decreased CETP activity relative
214 to SFA-rich and TFA-rich diets of 6-weeks (Lagrost et al. 1999). No significant effects
215 were reported, however, in another RCT comparing a 35-day diet rich in canola oil
216 (49% MUFAs) compared to diets rich in palm oil (50% SFAs), soybean oil (44%
217 PUFAs), and partially hydrogenated soybean oil (13% TFAs) (Vega-López et al. 2006).
218 A MUFA-rich 6-week RCT based on the consumption of peanut oil increased
219 serum LCAT activity relative to diets rich in rapeseed oil (also MUFA-rich) and dairy
220 fats (from butter and cream, SFA-rich) (Baudet et al. 1988). In contrast, LCAT activity
221 was not modified by a MUFA-rich dietary intervention compared to two interventions
222 rich in PUFA oils during 2-weeks (Singer et al. 1990). HDL particles did not present
223 higher levels of LCAT on their surface after a 32-day MUFA-rich diet intervention
224 compared to a high carbohydrate diet (Andraski et al. 2019).
225 Finally, in relation to oxidative stress-related properties, a MUFA-based
226 supplemented intervention was not linked to changes in PON1 paraoxonase activity
227 compared to supplements of EPA and DHA (2g/day) during 6-weeks (Stirban et al.
228 2014). Nevertheless, overall HDL oxidation was decreased relative to an 8-week
229 linoleic-rich diet (Solà et al. 1997).

From the nine studies regarding MUFA intervention, five reported no effect on HDL function biomarkers at all (four of them, however, were compared with PUFA interventions), two observed some effect in healthy volunteers, and two more found some effect in obese and metabolic syndrome patients.

230

231 *Polyunsaturated fatty acids: vegetable oils and nuts*

232 Twenty-one RCTs evaluated the effect of vegetable oils, linoleic-rich nuts, and
233 linolenic fatty acids on HDL functions (**Table 2**). Seven assessed the effects of
234 vegetable PUFAs against SFA and TFA interventions, 6 against other PUFA types and
235 MUFAs, and seven compared them to low fat diets and PUFA-free ones.

236 Regarding cholesterol metabolism, the consumption of almonds (43 g/day for 6
237 weeks) increased non-ABCA1 CEC compared to an isocaloric muffin without PUFAs in
238 normal weight participants with non-significant results for global and ABCA1 CEC
239 (Berryman et al. 2017). A 4-week intervention with corn and safflower oils (both rich in
240 linoleic acid), and an intervention with flaxseed oil (rich in linolenic acid) incremented
241 CEC values relative to baseline (Liu et al. 2018). Another RCT with 2 servings/day of
242 pistachios for 4 weeks was associated with CEC improvements relative to a subgroup
243 of participants with low C reactive protein levels consuming 1 serving/day (Holligan et
244 al. 2014). However, another four RCTs assessing vegetal PUFA intake did not report
245 associations with changes in CEC relative to MUFA-rich diets (Solà et al. 1997; Tindall
246 et al. 2020) or SFA or TFA ones (Buonacorso et al. 2007; Králová Lesná et al. 2008).
247 Regarding CETP, a flaxseed oil intervention (α-linolenic acid, 5.5 g/day for 12
248 weeks) decreased CETP activity relative to the control diet with corn oil (Kawakami et
249 al. 2015). A safflower oil-rich diet for 6 weeks (50% of total dietary fat, rich in linoleic
250 acid) decreased CETP from HDLs to apolipoprotein B-containing lipoproteins in relation
251 to a butter-rich diet (Cox et al. 1995). A 17-day linoleic acid-rich diet (8% of total
252 energy) decreased CETP activity relative to a TFA-rich one (van Tol et al. 1995). And
253 an 8-week baru almond-enriched diet (30.13% of fat as linoleic acid) decreased plasma
254 CETP levels (de Souza et al. 2018). Other trials, however, did not report any changes
255 in CETP activity or concentrations after an intervention with pistachios (Gebauer et al.
256 2008), and two PUFA-rich diets relative to SFA-rich dietary arms (Chung et al. 2004;
257 Lottenberg et al. 1996).
258 LCAT activity increased after a 6-week diet complemented with 60 ml/day of
259 sunflower oil (rich in linoleic acid) compared to a low erucic acid rapeseed oil diet (rich
260 in MUFAs) and a diet rich in dairy fats (SFAs) (Baudet et al. 1988). Nevertheless, LCAT
261 activity in plasma was decreased in relation to baseline values after 2 weeks of 60
262 g/day flaxseed oil (rich in α-linolenic acid) (Singer et al. 1990), although no changes
263 were reported in a similar trial (Abbey et al. 1989).

264 Finally, when assessing HDL antioxidant properties, the consumption of a
265 walnut paste-enriched meat (20% walnut paste, 750 g/week) increased PON1
266 paraoxonase activity relative to the control intervention with a low-fat diet (Sánchez-
267 Muniz et al. 2012), as well as relative to baseline in two similar RCTs with the same
268 walnut intake (Canales et al. 2007; Canales et al. 2011). Intake of linoleic acid-rich
269 safflower oil (4.5g/day for 4-weeks) was also associated with increases in PON
270 arylesterase activity relative to trans conjugated linoleic acid intervention in an RCT
271 (Pfeuffer et al. 2011).

Briefly, in healthy individuals no effect of a PUFA intervention (vegetable oils and nuts) on HDL function was observed in four out of seven studies while in three studies some effect was reported. From 14 studies in subjects with cardiovascular risk-related pathologies, 10 demonstrated some effect while in four no benefit was reported.

272

273 *Polyunsaturated fatty acids: fish, eicosapentaenoic and docosahexaenoic acids (EPA,*
274 *DHA)*

275 The effect of fish fatty acids (eicosapentaenoic and docosahexaenoic acids
276 (EPA, DHA)) on HDL functions was evaluated in eleven RCTs (**Table 3**). Three
277 interventions made comparisons with other PUFAs, and eight were supplements
278 compared with placebos or non-supplemented interventions.

279 An intervention with an DHA-enriched canola oil smoothie (5.8% DHA content)
280 was associated with a 55% increment in CEC values relative to baseline in metabolic
281 syndrome patients (Liu et al. 2018). A trial, however, studying the effect of dietary fatty
282 fish (1g/day EPA+DHA) compared to lean fish or with camelina oil (high in linolenic) did
283 not show effects on CEC (Manninen et al. 2019). Additionally, higher doses of EPA +
284 DHA in supplements (4 capsules of 1.88g EPA + 1.48 g of DHA per day) did not result
285 in effects on CEC compared to a placebo(Calabresi et al. 2004).

286 Cholesteryl ester transference from HDLs to apolipoprotein B-containing
287 lipoproteins decreased relative to baseline after an EPA + DHA supplement (4g/day)

(Pownall et al. 1999). In contrast, CETP levels did not change in an RCT with a EPA+DHA supplement (4 capsules 1.88g EPA + 1.48g DHA per day) (Calabresi et al. 2004). LCAT activity was also decreased relative to baseline values in an RCT after a fish oil supplement (3.8g/day) (Abbey et al. 1989) whilst the circulating levels of the enzyme did not change in RCTs assessing an EPA + DHA supplement (Calabresi et al. 2004), and in lower doses in an EPA/DHA-enriched milk (131.25mg EPA and 243.75mg DHA) (Lambert et al. 2017).

With respect to HDL antioxidant properties, PON1 activity was significantly increased relative to a placebo control in RCTs with fish oil supplements (1 g/day) (Ghorbanihaghjo et al. 2012) and EPA (2 g/day). PON1 circulating levels also incremented in RCTs with 2 g/day EPA supplements (Golzari et al. 2017) compared to control, and with EPA/DHA-enriched milk (relative to baseline) (Lambert et al. 2017). No significant differences on PON1 levels were found, however, in an RCT studying a low-dose supplement of DHA (500 mg/day) (Shidfar et al. 2016), and after the consumption of 2 g/day purified EPA and DHA relative to olive oil (Stirban et al. 2014). The global antioxidant capacity of HDL was evaluated in an intervention with EPA + DHA supplementation (4g/day) which was associated with a deleterious effect on HDL inflammatory index in heart failure patients compared to lesser doses of EPA + DHA (1g/day) and placebo treatments (Wurm et al. 2018).

From the 11 studies in patients with cardiovascular risk-related pathologies, rheumatoid arthritis or heart failure, seven showed some effect on HDL function after a fish, EPA, or DHA intervention whilst four reported no effects.

Saturated (SFA) and trans fatty acids (TFA)

Sixteen RCTs evaluated the effect of SFAs and TFAs on HDL functions (**Table 4**). Among them, twelve were compared with PUFAs and MUFAs.

Regarding CEC, a diet containing 12.5% SFAs from butter improved CEC values (+4.3%) relative to a cheese-rich isocaloric diet or a carbohydrate rich one

316 (Brassard et al. 2018), whilst no difference relative to baseline were observed after a 4-
317 week intervention with palm oil-rich and TFA-rich (hydrogenated soybean oil) diets in
318 healthy individuals (Buonacorso et al. 2007).

319 Increases in CETP activity and mass were reported in an RCT with a palmitic
320 acid-enriched diet (45% total fat content) (Lagrost et al. 1999) compared with MUFA-
321 rich dietary patterns. Rises in CETP activity were also found in RCTs comparing a
322 butter-rich diet with a linoleic acid-rich intervention (Cox et al. 1995) and a lauric acid-
323 rich diet (relative to baseline) (Schwab et al. 1995). In contrast, no effects were
324 observed in two short-term RCTs after consumption of palm oil-rich diets relative to two
325 PUFA-rich ones (Vega-López et al. 2006; Chung et al. 2004), and in a general
326 saturated fat-rich diet relative to a PUFA-rich one (Lottenberg et al. 1996). Another
327 RCT comparing the effect of 70 g/day saturated medium-chain fatty acids (caprylic and
328 capric acids) with a high MUFA intervention (70 g/day) did not report changes in CETP
329 activity (Tholstrup et al. 2004).

330 Intake of TFAs was associated with an increase in CETP activity when a 17-day
331 TFA-diet rich intervention (8% trans fats) was compared with stearic and linoleic acid-
332 rich diets (van Tol et al. 1995). In a similar manner, a 5-week margarine-rich diet also
333 incremented it relative to butter-rich and semiliquid margarine-rich ones (Lichtenstein et
334 al. 2001), and a marginally significant increase was reported after a 5-week high-TFA
335 margarine consumption in older hypercholesterolemic women (Matthan et al. 2001). In
336 comparison, no effects on CETP were observed after a 5-week consumption of
337 partially-hydrogenated soybean oil in hypercholesterolemic individuals (Vega-López et
338 al. 2006; Vega-López et al. 2009), and another TFA-rich intervention (Chardigny et al.
339 2008).

340 Regarding LCAT activity, 6-week SFA-rich diets (from butter and cream)
341 decreased LCAT activity relative to sunflower and peanut oils (rich in linoleic and oleic
342 acids) (Baudet et al. 1988). No effects, however, were reported after a 5-week TFA-rich

dietary modification (based on partially-hydrogenated soybean oil with 3% TFAs) (Vega-López et al. 2009).

Finally, when referring to HDL antioxidant properties, a 4-weekTFA-rich intervention (9.3% total fat in TFA from margarine) decreased PON1 paraoxonase activity relative to a SFA-rich one (De Roos et al. 2002). No effects were reported, however, after a 5-week consumption of a partially-hydrogenated soybean oil (13% total fats as TFAs) or palm oil (50% total fats as SFAs) (Vega-López et al. 2006).

From 16 studies with SFA and TFA intervention, eight were conducted with healthy volunteers and some detrimental effect on HDL function was reported in four of them. In addition, in subjects with dyslipidemia or obesity some effect was observed in four studies.

Dietary cholesterol

The effects of dietary cholesterol, provided mainly by egg intake, on HDL functions were studied in 15 RCTs (**Table 5**).

Consuming 2 eggs/day incremented significantly CEC relative to control interventions (a low-fat diet with the same weight of yolk-free eggs) after 24 days and 4 weeks, respectively (Blanco-Molina et al. 1998; Sawrey-Kubicek et al. 2019), and a similar effect relative to baseline values was described after a 12-week intervention with 3 eggs/day (Andersen et al. 2013).

Three RCTs increased CETP activity after the consumption of 1 egg/day (64mg/day) per 1 month in cholesterol-sensitive participants (individuals whose plasma total- and HDL-C levels increased after egg consumption) in relation to non-egg consumption (Herron et al. 2002; Herron et al. 2003) and relative to baseline levels (Herron et al. 2004). Increases in CETP mass (also parallel to increases in HDL-C levels) were observed in a high-cholesterol diet (320 mg/1000 kcal) relative to a low-cholesterol arm (80 mg/1000 kcal) (Martin et al. 1993), and after increasing egg consumption in men (Ginsberg et al. 1994), but not in women (Ginsberg et al. 1995).

371 Conversely, no effects were reported in four RCTs based on the consumption of 3
372 eggs/day during 12-weeks in metabolic syndrome patients (Blesso et al. 2013), after 2
373 eggs/day for 4 weeks in overweight postmenopausal women (Sawrey-Kubicek et al.
374 2019), in heathy young participants (Missimer et al. 2018), and after a 1 egg/day
375 intervention during 4-weeks in postmenopausal women (Waters et al. 2007) .
376 LCAT activity incremented parallel to increases in cholesterol intake. In one
377 RCT, it augmented in interventions based on 1 and 2 eggs/day for a month (relative to
378 lower egg intake) in healthy young individuals (Vorster et al. 1992), 3 eggs/day for 12
379 weeks in carbohydrate-restricted diets in metabolic syndrome and excess weight
380 patients (relative to baseline levels) (Mutungi et al. 2010; Blesso et al. 2013), and 1
381 egg/day for 1 month in dietary cholesterol-sensitive individuals (Herron et al. 2003;
382 Herron et al. 2004). No effects were found in a 4-week 2-egg intervention (Sawrey-
383 Kubicek et al. 2019; Missimer et al. 2018) and in a 1-egg intervention after a month
384 (Waters et al. 2007).
385 Finally, in some RCTs the consumption of eggs did not modify HDL antioxidant
386 capacity or PON1 circulating activity (Morgantini et al. 2018; Sawrey-Kubicek et al.
387 2019). It was, however, associated with HDLs with greater lipid hydroperoxide content
388 (more oxidized) and pro-inflammatory proteins (serum amyloid A) (Morgantini et al.
389 2018).
390 Briefly, from the 11 studies in healthy subjects, eight of them reported some
391 effect. Four more studies were performed with subjects with some pathology
392 (overweight/obese, metabolic syndrome) and they all observed some effect on HDL
393 function after an egg intake intervention.
394
395 **Antioxidants and HDL function**
396
397 *Antioxidant nutrients and antioxidant-rich foods*

398 The capacity of polyphenols, carotenoids, and antioxidant vitamins in
399 foods and supplements to improve HDL functions was assessed in 26 RCTs (**Table 6**).
400 A 3-week intervention with a virgin olive oil naturally rich in phenolic compounds
401 (25 mL/day, raw) increased CEC by 3% relative to the control intervention (a refined
402 olive oil) (Hernáez et al. 2014), and a functional virgin olive oil, further enriched with
403 thyme polyphenols (25 mL/day, consumed raw), also augmented CEC relative to a
404 standard virgin olive oil after 3-weeks (Farràs et al. 2018). Supplements of
405 anthocyanins (320 mg/day) for 12 and 24 weeks were associated with significant
406 increases in CEC (by 18-20%) relative to placebo (Zhu et al. 2014; Qin et al. 2009). A
407 3-week intervention based on an anthocyanin-rich grape powder (60 g/day) did not,
408 however, lead to changes in metabolic syndrome patients (Millar et al. 2018).
409 CETP activity was also investigated in several short-term antioxidant-based RCTs.
410 Lycopene supplements (70 mg/week) for 12 weeks were associated with decreased
411 CETP activity relative to placebo (McEneny et al. 2013). A similar effect was described
412 after 12-week anthocyanin supplements (320 mg/day) (Qin et al. 2009) and the
413 consumption of a lyophilized grape powder (6 g) for 4 weeks (Zern et al. 2005). No
414 effects were observed, however, after a 6-week intake of one cup of raisins per day
415 (Puglisi et al. 2009). The use of French press coffee (high in diterpenes such cafestol
416 and kahweol) increased CETP activity compared to a control filtered coffee (lesser
417 content in diterpenes) (De Roos et al. 2000). Only two studies, one assessing the
418 effects of phenol-enriched functional virgin olive oils and the other a dietary intervention
419 with vegetables, berries, and apples, reported no changes in CETP activity (Farràs et
420 al. 2015; Freese et al. 2002).
421 Finally, LCAT activity also appears to improve after antioxidant-rich
422 interventions. High intake of lycopene (such as a lycopene-rich diet –224 to 350
423 mg/week– or a supplement –70 mg/week– for 12 weeks) was associated with
424 increased LCAT activity relative to baseline (McEneny et al. 2013). A phenolic
425 compound-rich, functional virgin olive oil also augmented LCAT mass relative to the

426 control intervention (with standard virgin olive oil). It induced the changes in HDL
427 composition that are usually associated with LCAT function (relative decreases in free
428 cholesterol in the lipoprotein) (Farràs et al. 2015). Two interventions with diterpenes
429 from coffee (cafestol and kahweol) showed decreased LCAT activity relative to
430 baseline levels (De Roos et al. 2000; van Tol et al. 1997). In contrast, an anthocyanin
431 supplement and a dietary intervention with vegetables, berries, and apples were not
432 related to improvements in the function of this enzyme (Qin et al. 2009; Freese et al.
433 2002).

434 In controlled trials, dietary antioxidants have also been associated with
435 beneficial effects on HDL by incrementing content and making HDL more oxidation-
436 resistant. Two interventions with natural and functional virgin olive oils also increased
437 HDL content of dietary antioxidants (olive oil phenolic compound metabolites, β-
438 cryptoxanthin, and lutein) relative to the control interventions (Hernáez et al. 2014;
439 Farràs et al. 2018). Indeed, as many as 17 clinical trials have focused on the promotion
440 of PON1 antioxidant activities. Regarding carotenoids, high intake of lycopene
441 (lycopene-rich diet –224 to 350 mg/week– or a supplement –70 mg/week–) increased
442 PON1 arylesterase activity relative to control (McEneny et al. 2013), and a supplement
443 of astaxanthin (4 mg) for 3 months increased PON1 diazoxonase activity relative to
444 baseline (Baralic et al. 2013). Two trials with tomato juice (lycopene rich –37-47
445 mg/day–) for 2 and 8 weeks did not, however, show differences relative to control
446 interventions (water and carrot juice) (Bub et al. 2002; Bub et al. 2005). In relation to
447 phenolic compounds, two anthocyanin-based interventions (an anthocyanin
448 supplement –320 mg/d for 24 weeks–, barberry juice –200 mL/day for 8 weeks–)
449 increased PON1 paraoxonase activity and concentration, respectively (Lazavi et al.
450 2018; Zhu et al. 2014), and a 6-month intervention with a pomegranate extract (1g/day)
451 incremented PON1 lactonase activity relative to placebo (Wu et al. 2015). In contrast, a
452 3-week intervention with grape powder (60 g/day) did not result in significant
453 differences in PON1 activity (Millar et al. 2018). In three 3-week RCTs, two phenolic

compound-rich oils (virgin olive and argan oils) significantly increased PON1 paraoxonase and arylesterase activities (Cherki et al. 2005). Moreover, two virgin olive oils (one natural and the other enriched with olive phenolic compounds) promoted PON1 paraoxonase and lactonase specific activities (Fernández-Castillejo et al. 2017), and another virgin olive oil enriched with thyme polyphenols boosted arylesterase activity (Farràs et al. 2015), all relative to baseline values. With regard to other phenolic compound-rich interventions in RCTs, powdered ginger (3 g/day for 3 month) increased PON1 arylesterase activity relative to placebo (Shidfar et al. 2015), an extract of Turkish oregano boosted PON1 paraoxonase and arylesterase activities relative to control after 3 months (Ozdemir et al. 2008), and a 2-month red sage extract incremented paraoxonase activity when compared to baseline (Qian et al. 2012). An intervention trial with yerba mate tea (1000 mL/day) increased PON1 circulating levels relative to control (Balsan et al. 2019), another intervention (1000 mL/day for 3 months), however, did not modify PON1 arylesterase activity (Boaventura et al. 2012). Null changes were reported in three RCTs studying a polyphenol-enriched tomato juice, an oatmeal porridge enriched with sea buckthorn flavonols, and dietary doses of vitamin E (15 mg/day) (Michaličková et al. 2019; Suomela et al. 2006; Dalgård et al. 2007). Finally, regarding other HDL properties potentially related to their antioxidant/anti-inflammatory potential, an increased consumption of lycopene decreased the levels of HDL-bound acute-phase cytokines such as serum amyloid A (McEneny et al. 2013).

From the total of 26 studies with antioxidants, some effect on HDL function was reported in 5 out of 11 with healthy individuals, and 11 out of 15 in subjects with cardiovascular risk-related pathologies. A considerable part of the effects described in subjects with pathologies employed PON methodologies.

Antioxidant-rich dietary patterns

481 Five human trials assessed the overall effect of an increase in the intake of
482 dietary antioxidants through healthy dietary patterns on HDL functions (**Table 7**).
483 Two interventions in a long-term RCT (1 year) with Mediterranean diets
484 enriched with virgin olive oil or mixed nuts were linked to increases in CEC values
485 relative to baseline (Hernáez et al. 2017). The same intervention with a Mediterranean
486 diet enriched with virgin olive oil was also associated with decreased CETP activity
487 relative to baseline, and an improvement in an indirect indicator of LCAT function
488 (Hernáez et al. 2017). CETP activity also decreased in another 1 year intervention with
489 an olive oil-enriched Mediterranean diet relative to baseline (Damasceno et al. 2013). A
490 diet rich in fruit and vegetables (6 portions/day of fruit and vegetables for 8 weeks) was
491 related to an increase in LCAT activity relative to baseline (Daniels et al. 2014). In
492 contrast, a diet rich in vegetables, berries, and apples presented no changes in LCAT
493 activity at 6 weeks (Freese et al. 2002).
494 Regarding the relationship between HDL and oxidative stress in RCTs, HDLs
495 became more resistant against oxidation after the intervention with a Mediterranean
496 diet enriched with virgin olive oil relative to baseline (Hernáez et al. 2017). They also
497 increased their content of carotenoids (α-carotene, β-cryptoxanthin, lutein, and
498 lycopene) after an intervention with a diet rich in fruit and vegetables relative to one
499 poor in plant-based foods (Daniels et al. 2014). In addition, HDL capacity to directly
500 decrease LDL oxidation improved after the intervention with a virgin olive oil-rich
501 Mediterranean diet (Hernáez et al. 2017). Following this same Mediterranean diet and
502 one rich in fruit and vegetables also increased PON1 arylesterase activity when
503 compared to control diets in two large RCTs (Hernáez et al. 2017; Daniels et al. 2014).
504 Conversely, a 5-week high vegetable diet decreased PON1 activity relative to a low
505 vegetable one (Rantala et al. 2002), and a second trial based on a 6-week high intake
506 of vegetables, berries, and apples decreased PON1 activity relative to baseline (Freese
507 et al. 2002). Antioxidant-rich diets have additionally been shown to improve the role of
508 HDL in low-grade inflammation. In an RCT, a Mediterranean diet enriched with virgin

olive oil decreased the concentrations of HDL-bound cytokines (α_1-antitrypsin) relative to control interventions (Hernáez et al. 2020). Finally, in two RCTs, following a virgin olive oil-rich Mediterranean diet has been observed to increase the activity of another antioxidant/antithrombotic enzyme carried by HDLs (HDL-bound phospholipase A2, also known as platelet activating factor acetylhydrolase) and HDL capacity to promote the endothelial release of nitric oxide in vitro (Hernáez et al. 2020; Hernáez et al. 2017), in both cases relative to the control intervention.

Briefly, while one study with an antioxidant-rich dietary pattern was performed in healthy subjects without benefits on HDL function, four more were conducted in subjects at high cardiovascular risk with benefits reported in all of them.

Ethanol and HDL function

Moderate consumption of alcohol was analysed in 8 studies comparing the intake of wine, beer, gin, and whisky (from 15g/day to 40g/ day) with non-alcoholic beverages (**Table 8**).

Ethanol intake increased CEC in healthy populations comparing: red wine (30 g alcohol/day for 2 weeks) with alcohol-free wine (Senault et al. 2000); red wine, beer, and gin (40 g alcohol/day for 3 weeks) with an equivalent volume of carbonated water (van der Gaag et al. 2001); white wine with grape juice (24 g alcohol/day for 3 weeks) (Sierksma et al. 2004); whisky (40 g alcohol/day for 17 days) with an equivalent volume of water (Beulens et al. 2004); and beer (30 g/day alcohol –men–, 15 g/day –women– for 4 weeks) with alcohol-free beer (Padro et al. 2018). A marginally significant increase was also observed in a trial comparing beer (1 L/day –36 g ethanol/day– for 4 weeks) with an equivalent volume of non-alcoholic beverages (Královà Lesná et al. 2010). In contrast, alcohol intake did not modify CETP activity in two RCTs comparing red wine with alcohol-free wine (30 g alcohol/day for 2 weeks) (Senault et al. 2000) and white wine with grape juice (24g alcohol/day for 3 weeks) (Sierksma et al. 2004).

536 Finally, alcohol intake promoted PON1 paraoxonase activity in an RCT after a
537 3-week consumption of 40 g alcohol/day (as red wine, beer, or gin) relative to
538 carbonated water (van der Gaag et al. 1999), and beer (40 g alcohol/day –men–, 30
539 g/day –women– for 3 weeks) relative to alcohol-free beer (Sierksma et al. 2002). These
540 results agree with the increase in HDL antioxidant capacity on LDLs observed after the
541 consumption of beer during 4 weeks relative to baseline (30g alcohol/day for men and
542 15g/day for women) (Padro et al. 2018).
543 From the eight studies with ethanol intake (seven in healthy individuals) benefits
544 in CEC were mainly observed in four with healthy subjects and in one with
545 overweight/obese participants. Two studies with healthy individuals showed
546 improvements in PON1 activity.
547
548 **Physical activity, calorie restriction, and HDL function**
549 Physical activity, caloric restriction, and their combinations were studied as
550 possible promoters of HDL function in thirteen RCTs (**Table 9**).
551 Regarding CEC, the effect of a high amount of vigorous intensity activity (16
552 Kcal/kg/week at 75% $\dot{V}O_2$ reserve) during 6 months increased radio-labeled CEC
553 compared with two groups with different amounts of moderate intensity physical activity
554 and a group combining moderate intensity exercise with a low fat diet (Sarzynski et al.
555 2018). A 6-month high level endurance training (with a caloric goal of 20 Kcal/kg/week
556 at 65-85% $\dot{V}O_2$) increased only the non-ABCA1 cholesterol efflux relative to a control
557 group without exercise (Sarzynski et al. 2018). In the previous two studies the same
558 interventions evaluated with fluorescent-labeled CEC technique, did not find any
559 changes in CEC. Moreover, an RCT based on aerobic/resistance training (3
560 times/week for 24 weeks) also reported no effects (Albaghdadi et al. 2017). In a similar
561 manner, another intervention with aerobic/resistance training (4 times/week for 12
562 weeks) did not report changes in CEC (Woudberg et al. 2018). Neither did a very low

563 calorie restriction (500 Kcal/day for 6 weeks) accompanied with an average weight loss
564 of 10 kg lead to changes in CEC (Talbot et al. 2018).
565 The combination of physical activity with a hypocaloric diet was associated with
566 improvements in CEC relative to usual care in a small group of obese adolescents with
567 a diet of 1500-1800 kcal combined with supervised exercise sessions for 10 months
568 (aerobic/ resistance training 3 times/week and 2h/day of lifestyle activities) (Wesnigk et
569 al. 2016). A second trial reported improvements of CEC relative to baseline levels after
570 a combination of aerobic training (4 times/week) and a DASH diet (with reductions of
571 600 kcal/day) for 12 weeks in a small group of metabolic syndrome participants relative
572 to baseline (Khan et al. 2018). No differences were found, however, in a trial combining
573 calorie restriction counselling and aerobic exercise (mainly brisk walking 5 times per
574 week) (Dokras et al. 2017).
575 Regarding CETP, 6 months of aerobic training (4 times/week) did not modify its
576 activity (Tiainen et al. 2016). Nevertheless, a 6-week endurance training (3-5
577 times/week) decreased CETP activity relative to an untrained control (Vislocky et al.
578 2009). Two weight loss interventions did not modify CETP in an intervention with the
579 National Cholesterol Education Program Step I, and neither in a 4-week diet (Miida et
580 al. 1998) or a very low calorie one (500kcal/ day for 6 weeks) (Talbot et al. 2018).
581 LCAT activity and plasma concentrations were unaffected by aerobic activity (ranging
582 from 11 weeks to 1 year) in three RCTs performed some years ago (Thomas et al.
583 1985; Rönnemaa et al. 1988; Williams et al. 1990).
584 A study with an aerobic and resistance exercise program for 12 weeks in obese
585 women decreased serum PON1 activity relative to a control without exercise, however
586 PON1 expression in isolated HDLs remained unaltered (Woudberg et al. 2018). The
587 same study also found no changes in the expression of the isolated HDLs of the
588 antioxidant/antithrombotic enzyme phospholipase A2 bound in HDLs (Woudberg et al.
589 2018).

590 Finally, regarding HDL endothelial properties, an intervention with a healthy diet
591 combined with exercise (1500-1800kcal/day) and aerobic/resistance training improved
592 the HDL role on the activation of endothelial nitric oxide synthase compared to a usual
593 care intervention (Wesnigk et al. 2016). In contrast, an aerobic and resistance exercise
594 program for 12 weeks in obese women did not modify HDL anti-inflammatory capacity
595 on endothelial cells in an RCT (Woudberg et al. 2018).
596 In summary, there were six studies in healthy subjects with physical activity
597 and/or calorie restriction, and in one of them a benefit in CETP was described. From 8
598 papers with participants presenting cardiovascular risk-related pathologies, some
599 effect, mainly on CEC, was reported in three of them.
600
601 **Other lifestyle interventions**
602 Very few studies have evaluated dietary interventions unrelated to fats,
603 antioxidants, ethanol, and physical activity/calorie intake (**Table 10**).
604 The effect of a prebiotic and probiotic enriched pasta (with β-glucans -2.3 g/100
605 g- and Bacillus coagulans) was evaluated in a 12-week intervention. This study
606 increased ABCG1 mediated CEC relative to a control pasta (Favari et al. 2020).
607 Only one RCT has evaluated the effects of the isocaloric exchange between
608 high and low glycaemic index carbohydrates on HDL function, and found no effects on
609 CEC (Meng et al. 2018).
610 Interventions with soy protein reported no changes in CEC after 6 weeks of 25-
611 50g soy protein and a control (Richter et al. 2017), no changes in CETP mass and
612 LCAT activity after a 4-week 20g/day intake of soy protein (Higashi et al. 2001), and an
613 increase in PON1 activity in a trial of 50g/soy protein relative to a placebo (Shidfar et al.
614 2009).
615 One RCT evaluated the association of carbohydrate restriction with HDL
616 functions and found it was related to decreased LCAT activity relative to baseline, but
617 no changes in CETP function (Wood et al. 2006).

618 A whole dietary intervention with TLC/Step 2 diet for 32 days, a dietary pattern
619 low in fats, high in vegetables, carbohydrates and fibre, did not change CETP activity
620 (Hernáez et al. 2002)
621 Two RCTs investigated the effects of a supplement of psyllium fibre (one of
622 them also included plant sterols) and reported a decrease in CETP activity relative to
623 placebo pills, but no changes in LCAT function (Shrestha et al. 2007; Vega-López et al.
624 2001).
625 Finally, plant sterols were also studied in two RCTs and were associated with
626 decreases in CETP mass relative to a control after 4 weeks of margarine with
627 phytosterols (1.68g/day) (Lottenberg et al. 2003), and relative to baseline values after 4
628 weeks of 2-3g/day plant stanol (Homma et al. 2003). Soy intervention did not modify
629 LCAT activity (Lottenberg et al. 2003)
630 There were 11 studies with other interventions than those previously referred to,
631 five papers (some effect on HDL function in three) in healthy subjects and six studies
632 (some effect in four) with cardiovascular risk-related pathologies.
633
634 **Discussion**
635 In this systematic review we have summarized the existing evidence regarding
636 the effect of lifestyle changes on HDL functional traits. The short-term consumption of
637 dietary antioxidants and alcohol was more clearly related with HDL functional
638 improvements in subjects with cardiovascular risk and healthy individuals, respectively,
639 especially regarding CEC and HDL antioxidant properties. Additionally, in subjects at
640 cardiovascular risk, an effect on HDL functions was suggested after the intake of
641 MUFAs and long-chain PUFAs whilst an antioxidant-rich dietary pattern was able to
642 improve HDL function in both groups, healthy individuals and subjects at high
643 cardiovascular risk.
644 MUFA and long-chain PUFA intake has been analysed in a considerable
645 number of studies with controversial results. Such diverse findings can be partially

646 explained by the high heterogeneity of the study designs. For instance, the different
647 types of fatty acids used in diets/supplements and control arms, and the wide range of
648 doses employed. The clearest improvements in HDL functions were observed when
649 MUFAs and PUFAs were compared to low fat, SFA, and TFA interventions. The
650 comparisons between MUFAs and PUFAs, or between different types of PUFAs
651 (linoleic acid, linolenic acid, EPA, and DHA) did not, however, show differences
652 regarding HDL functionalities. MUFA interventions reported increased levels of CEC,
653 CETP, and LCAT only when compared with SFAs and TFAs. Such results are
654 consistent with previous non-randomized studies in humans in which increases in CEC
655 (Sola et al. 1993), CETP (Jansen et al. 2000; Abbey et al. 1994), and LCAT activity
656 have also been reported (Pieke et al. 2000). All long-chain PUFA studies consistently
657 improved HDL antioxidant capacity through augmented PON1 activity. Antioxidant and
658 anti-inflammatory capacities were also described in non-randomized trials in which
659 doses of EPA-DHA (1.8-2 g/day) increased PON1 mass and arylesterase activity, and
660 decreased the expression/secretion of pro-inflammatory proteins such as VCAM-1,
661 alpha-1-antitrypsin, and complement proteins (Tanaka et al. 2014; Burillo et al. 2012).
662 Long-chain fatty acid intake also suggested improvements in CEC (when not compared
663 to other unsaturated fatty acid interventions) accompanied by improvements in CETP
664 and LCAT activities. Changes of CEC could be caused by increments in apolipoprotein
665 A-I, the major apolipoprotein in HDL involved in CEC, as observed after the
666 consumption of EPA+DHA (Lambert et al. 2017; Burillo et al. 2012). On the other hand,
667 a detrimental effect of higher doses of EPA and DHA (>3g/day) on LCAT and HDL
668 inflammatory indices, with no changes in CEC, has been proposed. Hypotheses
669 describing the potential beneficial effects of MUFAs and PUFAs on HDL function are
670 diverse. A first explanation lies in the capacity of these fatty acids to increase HDL
671 particle fluidity. HDLs, which are more fluid, are thought to have a greater capacity to
672 adapt to the shape of cholesterol transporters in cells, and allow the export of
673 cholesterol excess (Gillotte et al. 1998; Sola et al. 1993; Harayama et al. 2020).

674 Second, omega-3 PUFAs are known ligands for peroxisome-proliferator activated
675 receptor α (PPARα). This cell receptor, whose activation leads to an increased
676 production of apolipoprotein A-I (the HDL main active protein), may improve HDL
677 function beyond an increase in HDL-C levels (Bragt et al. 2008; Duval et al. 2007). An
678 increase in apolipoprotein A-I levels could also mediate a greater stability and
679 antioxidant function of PON1 (Pizzini et al. 2017), beyond the intrinsic potential
680 capacity of PUFAs to increase hepatic synthesis and its release (Ferretti et al. 2012).
681 Third, omega-3 PUFAs are able to decrease low-grade inflammation. This may lead to
682 a reduction in the circulating levels of cytokines and acute-phase proteins, thus
683 favouring a lower binding of these molecules to HDL particles (Ichimura et al. 2014).
684 Moreover, most of these interventions are potentially rich in dietary antioxidants, which
685 may additionally contribute to the effects observed (Hernáez et al. 2016) .Finally, we
686 cannot exclude the fact that the decrease in triglyceride concentrations due to PUFAs
687 may play a role in explaining some of the benefits on HDL cholesterol metabolism.
688 PUFAs can reduce hepatic synthesis of triglycerides and very low-density lipoproteins
689 (VLDL) activating PPAR receptors (G et al. 2009). Considering the close relationship
690 between both lipids, decreases in triglycerides and VLDL could decrease CETP
691 enzymatic activity and improve HDL cholesterol metabolism (Lamarche et al. 1999).
692 Saturated and trans fats presented opposite effects to MUFAs and PUFAs with
693 lower CEC and LCAT and increased CETP. Nevertheless, an article which studied
694 different sources of saturated fats (from cheese and butter) described contraposed
695 effects in CEC (Brassard et al. 2018). Findings that could be due to the fact that the
696 toxic effect of long-chain SFAs, such as palmitic acid (Sun et al. 2015), is not present in
697 shorter chain species (Karupaiah et al. 2011; Senanayake et al. 2020). A few studies
698 have also suggested a diminished HDL profile (worse PON1 and CETP activities) after
699 TFAs when compared with SFA-rich diets (De Roos et al. 2002; van Tol et al. 1995;
700 Lichtenstein et al. 2001). Probably, impairment in HDL functions is secondary to the

increment of total cholesterol and fractions induced by SFAs and TFAs (Dhaka et al. 2011; Sun et al. 2015).

Dietary cholesterol intake promotes increases in CEC, CETP, and LCAT activities. Augmented intake may increment the pool of cholesterol in circulation (in both LDLs and HDLs), which could be related to raised cholesterol levels in the organism. Consequently, leading to a greater uptake of cholesterol from peripheral cells (CEC), and a stronger necessity to metabolize this cholesterol by CETP and LCAT to transfer it back to the liver. Increases of CEC caused by cholesterol intake could not, however, reflect a better athero-protective capacity of HDL. Cholesterol intake showed increases of large HDL particles and HDL diameter (Blesso et al. 2013; Mutungi et al. 2010) which are associated with greater cardiovascular risk (Prats-Uribe et al. 2020). In addition, HDLs may be more oxidized and contain higher levels of acute-phase proteins, such as SAA, suggesting increased levels of oxidative stress and low-grade inflammation.

Under chronic oxidative stress and inflammation, HDL lipoprotein loses its athero-protective capacity and becomes dysfunctional (Rosenson et al. 2016). Thus, antioxidant-rich dietary patterns are promising interventions to preserve HDL athero-protective capacity. As described in this review, olive oil enriched with phenolic compounds, anthocyanins, carotene extracts, and supplements is clearly associated with improvements in HDL functionalities. The Mediterranean diet, which includes all the previously mentioned beneficial antioxidants, presents a positive effect on a wide battery of functions (CEC, antioxidant, anti-inflammatory, and endothelial protection capacity of HDLs) (Hernáez et al. 2017; Hernáez et al. 2020; Damasceno et al. 2013). In contrast, the few interventions which only increased dietary vegetable content failed to improve the CETP, LCAT, and PON1 activities of HDL (Freese et al. 2002; Rantala et al. 2002). Possibly, changes in vegetable quantity without other beneficial compounds (such as olive oil rich in polyphenols, nuts, and other sources of omega-3 fatty acids) are not enough to change HDL functions in a whole dietary pattern. Several

729 intertwined molecular mechanisms have been suggested as hypothetical explanations.
730 First, a number of HDL proteins involved in reverse cholesterol transport
731 (apolipoprotein A-I, LCAT) and antioxidant capacity (PON1) have been described as
732 having their functional capacity decreased if they become oxidized (Shao 2012; Wang
733 et al. 2000; Aviram et al. 1999). Thus, antioxidants may keep these proteins non-
734 oxidized and functional. Second, oxidized lipids are also known to become less fluid,
735 therefore antioxidants may enhance HDL lipid fluidity (Bonnefont-Rousselot et al.
736 1995). Third, some phenolic compounds may induce a slight boost of AMP-activated
737 protein kinase (AMPK), a cellular metabolic regulator capable of activating PPARα and
738 the subsequent synthesis of apolipoprotein A-I (Herzig et al. 2018; Hernáez et al.
739 2016). Fourth, antioxidant-rich HDLs are hypothesized as employing the antioxidant
740 compounds they carry to counteract the oxidation of other lipids. Fifth, regarding HDL
741 anti-inflammatory potential, the decrease in reactive oxygen species due to dietary
742 antioxidants may moderate the activation of the nuclear factor-kappa beta (NF-κβ), a
743 pivotal regulator of inflammatory responses (Zhang et al. 2016). This, in turn, can
744 reduce the concentrations of cytokines/acute-phase proteins that bind to HDLs. Some
745 particular antioxidants, such as flavonoids, have been reported to be able to directly
746 downregulate NF-κβ activation (Mansuri et al. 2014). In addition, several phenolic
747 compounds are also able to promote an AMPK-mediated decrease in the production of
748 low-grade inflammation signals (Madeo et al. 2019; Salt et al. 2017). Finally, the
749 capacity of some phenolic antioxidants to decrease hepatic liver synthesis through an
750 AMPK-dependent mechanism could also lead to a decrease in circulating levels of
751 triglycerides (Herzig et al. 2018). This could be linked to an enhancement in HDL
752 cholesterol metabolism due to the close relationship between HDL-C and triglycerides
753 (Lamarche et al. 1999).
754 Alcohol consumption is clearly associated with greater levels of HDL-C
755 accompanied by increments of apolipoprotein A-I (Brien et al. 2011). In the same
756 manner, moderate ethanol consumption has presented consistent increases in CEC

and PON1 activity. Although two RCTs did not demonstrate changes in CETP, some non-randomized trials have reported improvements in CETP and LCAT activities (Hagiage et al. 1992; Serdyuk et al. 2000) and detrimental effects in CETP after alcohol withdrawal (M.J. et al. 1990; M. et al. 1993). The main hypothesis to explain the potentially beneficial effects of alcohol on HDL metabolism is related to the transient increase in acetate, the main ethanol metabolite after its ingestion (Rosales et al. 2020). Acetate is a short-chain fatty acid known to be capable of decreasing lipolysis (Ge et al. 2008) which lowers the levels of non-esterified fatty acids released into circulation. These fatty acids are physiologically transformed into triglycerides in the liver, and subsequently packed in VLDLs. A transient decrease in VLDLs is therefore expected after alcohol intake (Rosales et al. 2020). VLDLs are the main destination of the cholesterol esters collected by HDL particles in CEC. Thus, if transiently low VLDL levels are present, CETP activity is halted and HDL-C concentrations increase due to greater cholesterol content per HDL particle (larger HDLs) (Dai et al. 1985; Pownall et al. 1999). Finally, an increment in CEC directed to these large HDLs, mediated by specific cholesterol transporters such as ABCG1 and SR-BI, could be expected, and a subsequent esterification of the collected cholesterol by LCAT takes place (Rosales et al. 2020). In addition, the promotion in PON1 antioxidant function could be partially justified by the high content of dietary antioxidants in some alcoholic beverages (Gutiérrez-Escobar et al. 2021; Martinez-Gomez et al. 2020).

The effect of physical activity on HDL function is still unclear. Regarding CEC, only large amounts of vigorous exercise were associated with improvements (Sarzynski et al. 2018). Moderate intensity activities did not show any changes in CEC, CETP or LCAT activities, suggesting a possible dose-dependent relationship. Further evidence was found in non-randomized trials which also reported the absence of effect of moderate physical activity (Rönnemaa et al. 1988; Sang et al. 2015). In addition, a very low calorific diet changed neither CEC nor CETP activity (Talbot et al. 2018). In fact, non-randomized studies even found negative effects on CEC (Vasudevan et al.

785 2011; Aicher et al. 2012). Nevertheless, interventions combining physical activity with
786 modest calorie restriction suggested increases in CEC and decreases in CETP activity
787 (Khan et al. 2018; Wesnigk et al. 2016) in non-randomized trials (Mathew et al. 2018;
788 Boyer et al. 2018). Physical activity and/or calorie restriction are known to promote the
789 activation of AMPK (Richter et al. 2009), a mechanism that seems essential in the
790 hypothetical explanation of the molecular effects of these two lifestyle modifications on
791 HDL function (Hernáez et al. 2020). First, as already commented, AMPK is capable of
792 stimulating PPARα, a transcription factor that promotes the hepatic synthesis of
793 apolipoprotein A-I and the release of cholesterol transporters in peripheral cells such as
794 macrophages, leading to increases in HDL-C circulating levels and potential
795 improvements in CEC values (Duval et al. 2007). In parallel, the beneficial decrease in
796 CETP activity could be secondary to the reduction in triglyceride plasma levels due to
797 the ability of AMPK to lower the production of triglyceride synthesis enzymes in the liver
798 (Barter et al. 1994). Second, AMPK simulation has also been linked to a greater
799 production of several antioxidant enzymes (Greer et al. 2009; Klotz et al. 2015; Mo et
800 al. 2014). This antioxidant protection could be related to a stronger preservation of HDL
801 proteins such as apolipoprotein A-I and PON1 in a non-oxidized, functional state,
802 boosting CEC and HDL antioxidant capacities (Shao et al. 2012; Aviram et al. 1999).
803 Finally, AMPK stimulation may counteract low-grade inflammation, which in turn may
804 be related to decreased levels of cytokines and acute-phase proteins bound to HDLs
805 and a reduction in the pro-inflammatory potential of the lipoprotein (Madeo et al. 2019;
806 Salt et al. 2017).
807 This systematic review has some limitations to be considered. First, the high
808 percentage of studies classified as unclear risk could conceal the presence of bias
809 questioning their quality. Second, there is a marked heterogeneity in the laboratory
810 procedures to evaluate HDL functions. For example, to evaluate CEC the analyzed
811 studies employed 6 different types of cell cultures (J774, THP-1, Fu5AH, CHO,
812 RAW264.7, and PBMCs cells) and three different types of labeled cholesterol (2 radio-

813 labeled and 1 fluorescent). Third, there was heterogeneity in the lifestyle modifications
814 and trial designs. Finally, most of the studies evaluated were short-term interventions
815 with modest sample sizes. For all these reasons, results need to be interpreted with
816 caution.
817 As strengths, we have reviewed publications including an exhaustive wide
818 range of both HDL functional abilities and lifestyle interventions.
819 **Conclusions**

820 Given that healthy diet and lifestyle are consistently related to decreased
821 cardiovascular disease risk, their link to improved HDL function has been widely
822 investigated in human trials. In brief, beyond an improvement in HDL-C levels, mainly
823 in short-term clinical trials, the consumption of MUFAs, PUFAs (particularly long-chain,
824 omega-3 ones in fish), and dietary antioxidants such as phenolic compounds (in dietary
825 or near-dietary doses) showed benefits on HDL functionality mainly in subjects with
826 cardiovascular risk factors. In this regard, antioxidant-rich dietary patterns were able to
827 improve HDL function in both healthy individuals and subjects at high cardiovascular
828 risk. In addition, reverse cholesterol transport with ethanol at moderate quantities, in
829 studies mainly performed in healthy individuals, was able to enhance HDL function.
830 Finally, cholesterol dietary interventions with eggs increase circulant cholesterol
831 fractions and, in concordance, HDL function.
832 Such findings suggest the capacity of dietary and lifestyle modifications to
833 modulate cardiovascular risk factors. Nevertheless, given the marked heterogeneity in
834 study design and procedures to assess HDL functions, more homogeneous, large-
835 scale, long-term, randomized, controlled trials are required to confirm these results.
836 Moreover, they should be performed over different periods, in varying populations, and
837 with individuals presenting diverse pathologies.
838

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Figure 1. Flow diagram of study selection

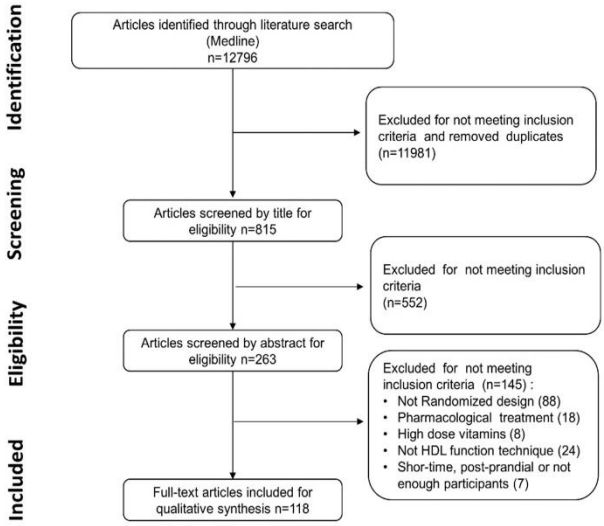

Figure 2. Overall risk of bias across studies

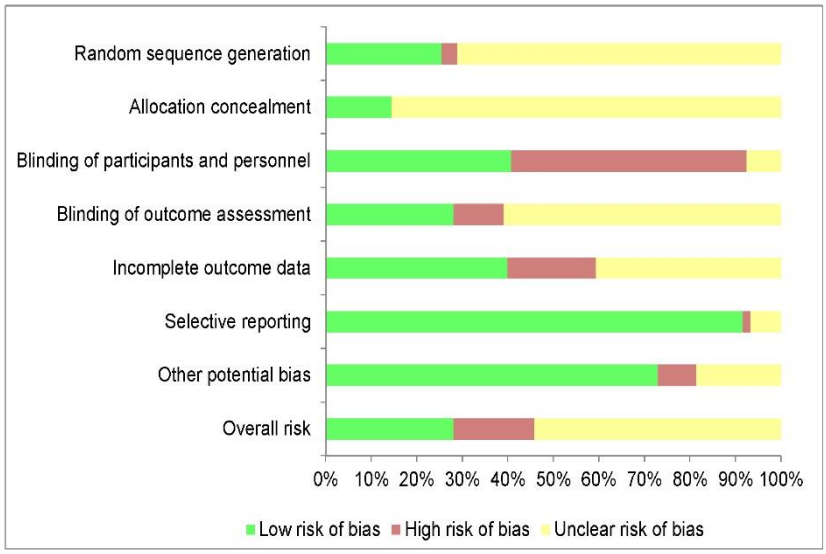

Table 1. Studies with monounsaturated fatty acids (MUFA): oleic acid rich oils

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Andraski, (2020)	USA	12 overweight participants (5 men and 9 women) Mean age: 42.16 years	Cross-over diets with: 1) High MUFA diet (23% of total calorie content). 2) High carbohydrate diet (65% of total calorie content).	32 days each intervention	Plasma LCAT activity	No effect
Baudet MF, (1988)	France	20 healthy women Mean age ± SD: 39 ± 9 years	Cross-over intervention diets with 15.6% of total calories from: 1) Low erucic acid rapeseed oil. 2) Sunflower oil. 3) Peanut oil. 4) Milk fats (butter or cream).	6 week each intervention	Serum LCAT activity	Increase of LCAT activity in peanut group relative to milk fats diets or low erucic rapeseed oil.
Brassard D, (2018)	Canada	77 Abdominal obesity patients (21 men and 25 women) Mean age: 41.4 ± 14.2 years	Cross-over intervention with: 1) Cheese rich diet. 2) Butter rich diet. 3) Olive oil diet. 4) Corn oil. 5) Carbohydrate rich diet.	5 weeks each intervention	^{3}H CEC in J774 cells in ABDP samples	Increase in olive oil intervention relative to cheese and carbohydrate diet.
Lagrost L, (1999)	France	32 healthy participants (14 men and 18 women) Age range: 20-60 years	Cross-over diets enriched with: 1) Palmitic acid (45% total fats). 2) Lauric acid (44% total fats). 3) Oleic acid (62% total fats).	6 weeks	Serum CETP activity and mass	Decreased CETP activity and mass in oleic acid diet relative to palmitic and lauric diet.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Liu X, (2018)	Canada/ USA	101 Metabolic syndrome participants (50 men and 51 women) Mean age: 49.5 years	Cross-over design with 5 isocaloric diets supplemented with 60g of: 1) Canola oil. 2) Canola oil high oleic acid content. 3) Canola oil high in DHA and oleic acid. 4) Corn oil combined with safflower oil. 5) Safflower oil combined with flax oil.	4 weeks each intervention	3-NBD CEC in THP-1 in serum samples	Increase by 39.1% canola oil group and 33.6% in canola oil rich in oleic acid and 55.3% in canola oil rich in DHA and oleic acid relative to baseline levels.
Singer P, (1990)	Germany	40 men with mild essential hypertension	Parallel diets with 60 ml/day of: 1) Olive oil. 2) Sunflower oil. 3) Linseed oils.	2 weeks	LCAT activity	No effect.
Stirban A, (2014)	Germany	34 participants with type 2 diabetes Mean age: 56.8 years	Parallel intervention with: 1) 2g of EPA and DHA. 2) Olive oil placebo.	6 weeks	Serum Paraoxonase-1 activity	No effect.
Solà R, (1997)	Spain	22 healthy men Mean age ± SD: 49.7± 0.6 years	Cross-over intervention with isocaloric diets with 15.6% of: 1) Sunflower oil rich in oleic acid. 2) Sunflower oil rich in linoleic acid.	8 weeks each intervention	^{3}H CEC in primary macrophages cells in isolated HDL3	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					HDL3 Oxidation status by TBARS assay	Decrease of malondialdehyde production in oleic rich diet compared to linoleic rich diet.
Vega-López S, (2006)	USA	15 participants with high levels of LDL cholesterol (5 men and 15 women) Mean age ± SD: 63.9 ± 5.7 years	Cross-over intervention with four diets with 20% fat content provided by: 1) Partially hydrogenated soybean oil (13% TFAs). 2) Soybean oil (44% PUFAs). 3) Palm oil (50% SFAs). 4) Canola oil (49% MUFAs).	5 week each intervention	Plasma CETP activity	No effect.

SFA: Saturated fatty acids. TFA: Trans fatty acids. MUFA: Monounsaturated fatty acids. PUFA: Polyunsaturated fatty acids. DHA: Docosahexaenoic acid. EPA: Eicosapentaenoic acid. CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. LCAT: Lecithin–cholesterol acyltransferase. ABDP: Apolipoprotein B-depleted plasma TBARS: Thiobarbituric acid reactive substance.

Table 2. Studies with polyunsaturated fatty acids (PUFA): vegetable oils and nuts

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Abbey M, (1990)	Australia	33 hypercholesterolemic men Mean age ± SD: 47.4±2.5 years	Parallel intervention with supplements of: 1) 14g/day linoleic acid (safflower oil). 2) 9g α-linolenic acid (linseed oil). 3) 3.8 g of n-3 FA (fish oil).	6 weeks	Plasma LCAT activity	No effect
Baudet MF, (1988)	France	20 healthy women Mean age ± SD: 39 ± 9 years	Cross-over intervention diets with 15.6% total calories from: 1) Low erucic acid rapeseed oil. 2) Sunflower oil. 3) Peanut oil. 4) Milk fats (butter or cream).	6 week each intervention	Serum LCAT activity	Increase of LCAT activity in sunflower oil group relative to milk fats diets or low erucic rapeseed oil.
Berryman CE, (2017)	USA	48 participants with high LDL cholesterol (22 men and 26 women) Mean age ± SD:50 ± 9 years	Cross-over intervention with: 1) 43 g almonds/day. 2) Control diet (isocaloric cholesterol-lowering diet (without almonds)).	6 weeks each intervention	^{3}H CEC in J774 cells in ABDS samples	Increase in non-ABCA1 CEC relative to control diet
Buonacorso V, (2007)	Brazil	30 healthy participants (9 men and 21 women) Mean age ± SD: 35,3 ± 10 years	Parallel intervention with diets enriched with: 1) TFAs (8.3% total energy). 2) PUFAs (14.6% total energy). 3) SFAs (13.2% total fats).	4 weeks	^{3}H CEC in primary macrophages cells in isolated HDL3 and HDL2	No effect

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Canales A, (2011)	Spain	22 participants at high cardiovascular risk (12 men and 10 women) Mean age: 54.8 years	Cross-over intervention with: 1) 300 g/week of walnut enriched meat (20% walnut paste). 2) Control low fat diet.	5 weeks each intervention	Serum Paraoxonase 1 activity	Increased PON1 activity inn walnut meat group relative to control diet
Canales A, (2007)	Spain	22 participants at high cardiovascular risk (12 men and 10 women) Mean age: 54.8 ± 8.3 years	Cross-over intervention with; 1) 600 g/week of walnut enriched meat (20% walnut paste). 2) Control low fat diet.	5 weeks each intervention	Serum Paraoxonase 1 activity	Increased PON1 activity inn walnut meat group relative to baseline.
Chung BH, (2004)	USA	16 healthy participants (8 men and 8 women) Mean age men ± SD: 35.3±4.5 years Mean age women ± SD: 51.9± 6.6 years	Cross-over diet: 1) A PUFA-rich diet. 2) SFA-rich diet.	16 days each intervention	Plasma CETP mass	No effect
Cox C, (1995)	New Zealand	28 hypercholesterlemic participants (13 men and 15 women) Age range: 26-64 years	Cross-over isocaloric diets: 1) Safflower (10% energy from PUFAs) oil diet. 2) Coconut oil diet (20% energy from SFA). 3) Butter diet (20% energy	6 weeks each intervention	Cholesteryl ester transfer activity (CETA)	Decreased CETA activity in Safflower oil group relative to butter intervention

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
			from SFA).			
De Souza, (2018)	Brazil	46 overweight or obese women Age range: 20-59y	Two parallel isocaloric diets: 1) 20 g/day of baru almonds. 2) Control diet with 800 maltodextrin supplement.	8 weeks	Plasma CETP mass	Decrease in baru almond diet relative to control diet
Gebauer SK, (2008)	USA	28 hypercholesterolemic patients (10 men and 18 women) Mean age ± SD:48 ± 1.5 years	Cross-over intervention with: 1) 10% energy from pistachios. 2) 20% from pistachios. 3) Control low fat diet.	4 weeks	Serum CETP mass	No effect
Holligan SD, (2014)	USA	28 participants with high LDL cholesterol (10 men and 18 women) Mean age ± SD:48.0 ± 1.5 years	Cross-over intervention with: 1) 10% energy from pistachios. 2) 20% from pistachios. 3) A control low fat diet.	4 weeks each intervention	^{3}H CEC in J774 cells in ABDS samples	Increase ABCA1 CEC in 20% pistachio diet relative to10% pistachio diet in participants with low-CRP levels.
Kawakami Y, (2015)	Japan	26 healthy men Mean age ± SD: 44.5 ± 3.1 years	Cross-over diet interventions: 1) 10g of flaxseed oil (5.49g of α-Linolenic acid). 2) 10g of corn oil (0.09g of α-Linolenic acid).	12 weeks each intervention	CETP mass	Flaxseed oil decreased CETP mass compared to corn oil

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Kralova-Lesna I, (2008)	Czech Republic	14 healthy men Age range: 18-55 years	Cross-over intervention two diets with diets containing a 40% from fats: 1) A high SFA diet (52% SFA). 2) A high-PUFA diet (41% PUFA).	4 weeks each intervention	^{14}C CEC in THP-1 cells in serum	No effect
Liu X, (2018)	Canada/ USA	101 Metabolic syndrome participants (50 males and 51 females) Mean age: 49.5 years	Cross-over design with 5 isocaloric diets with 60g of: 1) Canola oil. 2) Canola oil high oleic acid content. 3) Canola oil high in DHA and oleic acid. 4) Corn oil combined with safflower oil. 5) Safflower oil combined with flax oil.	4 weeks each intervention	3-NBD CEC in THP-1 in serum samples	Increase of 49.2% in corn oil + safflower oil, and 50.7% in safflower oil combined with flax oil relative to baseline levels
Lottenberg AM, (1996)	Canada	19 hypercholesterolemic women Mean age ± SD: 51.3 + 12.7 years	Cross-over diet: 1) High SFA diet (45% total fat from SFA oil). 2) High PUFA diet (50% total fat from PUFA oil).	3 weeks each intervention	Plasma CETP activity and mass	No effect
Pfeuffer M, (2011)	Germany	85 obese men Age range: 45-68 years	Intervention with supplements of: 1) 4.5g/d conjugated linoleic acid. 2) Safflower oil.	4 weeks	Paraoxonase 1 and arylesterase activity	Increase in arylesterase activity in both safflower oil compared to a conjugated linoleic

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
			3) Heated safflower oil. 4) Control olive oil.			acid group.
Sánchez-Muniz FJ, (2012)	Spain	22 participants at high cardiovascular risk (12 men and 10 women) Mean age: 54.8 years	Cross-over intervention with; 1) 750 g/week of walnut enriched meat (20% walnut paste). 2) Control low fat diet.	4 to 6 weeks each intervention	Paraoxonase 1 activity	Increased PON1 activity inn walnut meat group relative to control diet.
Singer P, (1990)	Germany	40 males with mild essential hypertension	Parallel diets with 60 ml/day of: 1) Olive oil. 2) Sunflower oil. 3) Linseed oils.	2 weeks	LCAT activity	Decrease of LCAT activity relative to baseline intervention.
Solà R, (1997)	Spain	22 healthy men Mean age ± SD: 49.7± 0.6 years	Cross-over intervention with isocaloric diets with 15.6% of: 1) Sunflower oil rich in oleic acid. 2) Sunflower oil rich in linoleic acid.	8 weeks each intervention	^{3}H CEC in primary macrophages cells in isolated HDL3	No effect.
					Oxidation status of HDL3 by TBARS assay	Increase in of malondialdehyde production in linoleic rich diet compared to oleic rich diet.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Tindall AM, (2020)	USA	34 High cardiovascular risk participants (21 men and 13 women) Mean age ± SD: 44 ± 10 years	Cross-over diet interventions: 1) Walnut diet (57–99 g/d walnut, 16% PUFAs). 2) Walnut fatty acid–matched diet (linolenic acid matched (16% PUFAs)). 3) High oleic diet (12% MUFAs).	6 weeks each intervention	CEC in J774 cells in ABDS	No effect.
Van Tol A, (1995)	The Netherlands	55 healthy participants (25 men and 30 women) Age range:19-49 years	Parallel isocaloric diets with 8% of energy from: 1) Linoleic acid. 2) Stearic acid. 3) Trans fatty acid.	17 days	ABDP CETP activity	Decrease in linoleic rich diet relative to trans fatty acids diet.

SFA: Saturated fatty acids. TFA: Trans fatty acids. MUFA: Monounsaturated fatty acids. PUFA: Polyunsaturated fatty acids. LTP: Lipid transfer protein. PON1: Paraoxonase-1 activity. DHA: Docosahexaenoic acid. EPA: Eicosapentaenoic acid. CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. CETA: Cholesteryl ester transfer activity. LCAT: Lecithin–cholesterol acyltransferase. PON1: Paraoxonase-1. ABDP: Apolipoprotein B-depleted plasma. ABDS: Apolipoprotein B-depleted serum. TBARS: Thiobarbituric acid reactive substance.

Table 3. Studies with polyunsaturated fatty acids (PUFA): fish, eicosapentaenoic and docosahexaenoic acids (EPA, DHA)

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Abbey M, (1990)	Australia	33 hypercholesterolemic men Mean age (SD): 47.4±2.5 years	Supplement with: 1) 14g/day linoleic acid (safflower oil). 2) 9g α-linolenic acid (linseed oil). 3) 3.8 g fish oil (EPA +DHA).	6 weeks	Plasma LCAT activity	Decrease in fish oil 21% relative to baseline.
Calabresi, (2004)	Italy	14 participants with familial hypercholesterolemia	Cross-over design with capsules of: 1) 4g (EPA + DHA) and 4mgα-tocopherol. 2) Placebo.	4 weeks each intervention	^{3}H CEC in Fu5AH cells in plasma	No effect.
					Plasma CETP mass	No effect.
					Plasma Paraoxonase-1 mass	Higher PON1 mass in omega 3 relative to placebo.
Ghorbanihaghjo A, (2012)	Iran	83 Rheumatoid arthritis women Mean age (SD): 50 (18–74) years	Parallel intervention with capsules of: 1) Fish oil (1 g/day). 2) Placebo.	12 weeks	Paraoxonase-1 mass in HDL	Higher content in PON1 in omega 3 group compared to phytosterol supplemented.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Golzari MH, (2017)	Iran	36 patients with type 2 diabetes Age range: 35-50 years	Parallel intervention with capsules of: 1) Fish oil (EPA 2 g/day). 2) Placebo.	8 weeks	Serum Paraoxonase-1 activity	Increase in EPA group compared t placebo.
Lambert C, (2017)	Spain	32 overweight or obese participants 13 men and 19 women Mean age (SD): 50.5 ± 1.6 years	Cross-over design with: 1) Omega 3 supplemented milk (131.25 mg EPA + 243.75 mg DHA/250mL). 2) Phytosterol supplemented milk (1.6 g of plant sterols/250 mL).	4 weeks each intervention	Serum LCAT mass	No effect.
Liu X, (2018)	Canada/ USA	101 Metabolic syndrome participants 50 males and 51 females Mean age: 49.5	Cross-over design with 5 isocaloric diets with 60g of: 1) Canola oil. 2) Canola oil high oleic acid content. 3) Canola oil high in DHA and oleic acid. 4) Corn oil combined with safflower oil. 5) Safflower oil combined with flax oil.	4 weeks each intervention	3-NBD CEC in THP-1 in serum samples	Increase by 55.3% in canola oil rich in DHA and oleic acid relative to baseline levels.
Manninen, (2019)	Finland	79 participants with impaired glucose metabolism Mean age(SD): 58.9±6.5 years	Four parallel isocaloric diets with: 1) 27 g/day camelina oil (10g ALA). 2) Fatty fish (1 g /day	12 weeks	^{3}H CEC in primary macrophage cells in isolated HDL	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
			DHA +EPA). 3) Lean fish; 4) control diet group.			
Pownall HJ, (1999)	USA	56 participants (40 with hypertriglyceridemia and 16 healthy) 24 men and 17 women Mean age (SD): 51.4±1.9 years	Two parallel interventions with capsules of: 1) 4g fish oil (EPA + DHA) with 4 mg α-tocopherol. 2) Placebo.	6 weeks	Serum cholesteryl ester transfer activity (CETA)	20 % of decrease in fish oil group relative to baseline levels.
Shidfar F, (2015)	Iran	76 women with iron deficiency Mean age (SD): 33.03± 8.73 years	Parallel intervention with capsules of: 1) 500 mg of DHA+ iron supplement 2) Placebo + iron supplement	12 weeks	Serum Paraoxonase-1 mass	No effects.
Stirban A, (2014)	Germany	34 patients with type 2 diabetes	Parallel intervention with capsules of: 1) 2 g EPA+ DHA supplement 2) Placebo supplement of olive oil.	6 weeks	serum Paraoxonase-1 activity	No effects.
Wurm R	Austria	40 advanced heart failure participants (34 men and 6 women)	Parallel intervention with capsules of: 1) 1 g EPA+ DHA. 2) 4 g EPA+ DHA. 3) Placebo.	12 weeks	HDL oxidative/inflammatory index (HOII) in ABDP	Increase in HOII after 4g fish oil per day relative to 1 g and placebo group.

LTP: Lipid transfer protein. ALA: Alpha-linolenic acid. DHA: Docosahexaenoic acid. EPA: Eicosapentaenoic acid. CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. CETA: Cholesteryl ester transfer activity. LCAT: Lecithin–cholesterol acyltransferase. ABDP: Apolipoprotein B-depleted plasma. ABDS: Apolipoprotein B-depleted serum. PON1: Paraoxonase-1. TBARS: Thiobarbituric acid reactive substance. HOII: HDL oxidative/inflammatory index.

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Table 4. Studies with saturated (SFA) and trans fatty acids (TFA)

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Baudet MF, (1988)	France	20 healthy women Mean age ± SD: 39 ± 9 years	Cross-over intervention diets with 15.6% of total calories from: 1) Low erucic acid rapeseed oil. 2) Sunflower oil. 3) Peanut oil. 4) Milk fats (butter or cream).	6 weeks each intervention	Serum LCAT activity	Decrease of LCAT activity of milk fats relative to peanut oil group.
Brassard D, (2018)	Canada	77 Abdominal obesity patients (21 men and 25 women) Mean age ± SD: 41.4 ± 14.2 years	Cross-over intervention with : 1) Cheese-rich diet. 2) Butter-rich diet. 3) Olive oil diet. 4) Corn oil. 5) Carbohydrate-rich diet.	5 weeks each intervention	^{3}H CEC in J774 cells in ABDP samples	Increase in butter intervention compared to cheese or carbohydrate diets.
Buonacorso V, (2007)	Brazil	30 healthy participants 9 men and 21 women Mean age ± SD: 35,3 ± 10 years	Parallel intervention with diets enriched with: 1) TFAs (8.3% total energy). 2) PUFAs (14.6% total energy). 3) SFAs (13.2% total fats).	4 weeks	^{3}H CEC in primary macrophages cells in isolated HDL3 and HDL2	No effect.
Chardigny JM, (2008)	France	40 healthy participants 19 men and 21 women Mean age ± SD: 27.6 ± 7.1 years	A cross-over intervention with food items containing: 1) TFAs from natural sources.	3 weeks each intervention	Plasma CETP activity	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
			2) TFAs from industrial sources.			
Chung BH, (2004)	USA	16 healthy participants (8 men and 8 women) Mean age men ± SD: 35.3±4.5 years Mean age women ± SD: 51.9± 6.6 years	Cross-over diet; 1) a PUFA-rich diet. 2) SFA-rich diet.	16 days each intervention	Plasma CETP mass	No effect.
Cox C, (1995)	New Zealand	28 hypercholesteremic participants (13 men and 15 women) Age range: 26-64 years	Cross-over isocaloric diets: 1) Safflower (10% energy from PUFAs) oil diet. 2) Coconut oil diet (20% energy from SFA). 3) Butter diet (20% energy from SFA).	6 weeks each intervention	Cholesteryl ester transfer activity (CETA)	Increase in CETA activity in butter intervention group relative to safflower intervention.
de Roos NM, (2002)	Netherlands	32 healthy participants (11 men and 21 women) Age range: 18-69 years	A cross-over intervention diets with: 1) SFA-rich margarine (0.3% TFAs). 2) trans FA-rich margarine (9.3% TFAs).	4 weeks each intervention	Paraoxonase-1 activity	TFA group decreased by 6% PON1 activity compared to SFA group.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Lagrost L, (1999)	France	32 healthy participants (14 men and 18 women) Age range:20-60 years	Cross-over diets enriched: 1) Palmitic acid (45% total FA). 2) Lauric acid (44% total FA). 3) Oleic acid (62% total FA).	6 weeks	Serum CETP activity and mass	Higher CETP activity and mass in palmitic and lauric acid groups relative to oleic acid group.
Lichtenstein AH, (2001)	USA	36 participants with high LDL cholesterol (18 men and 18 women) Mean age ± SD: 63 ± 6 years	A cross-over intervention with 20% calories from: 1) Semiliquid margarine. 2) Stick margarine. 3) Butter.	5 weeks each intervention	Plasma CETP activity	Increase in CETP activity in stick margarine group relative to butter or semiliquid margarine.
Lottenberg AM, (1996)	Canada	19 hypercholesterolemic women Mean age ± SD: 51.3 + 12.7 years	Cross-over diet: 1) High SFA diet (45% total fat from SFA oil) 2) High PUFA diet (50% total fat from PUFA oil).	3 weeks each intervention	Plasma CETP activity and mass	No effect.
Matthan NR, (2001)	USA	14 women with high LDL cholesterol Age range: 65-71 years	A cross-over intervention with 20% calories from: 1)Soybean oil (0.6% TFAs). 2) Low trans squeeze (0.9% TFA). 3) Medium trans tub (3.3% TFAs). 4) High trans stick (6,7% TFAs) margarines.	5 weeks each intervention	Plasma CETP activity	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Schwab US, (1995)	Finland	15 healthy women Age range: 19-34 years	Two parallel diets with 36% fats from: 1) Palmitic enriched diet (22-33 g palm oil). 2) Lauric acid enriched diet (16-26 g coconut oil).	5 weeks	Plasma CETP activity	Increase in CETP activity in lauric acid group relative to baseline.
Tholstrup T, (2004)	Denmark	17 healthy men Mean age ± SD: 23.4 ± 2.2 years	A cross-over intervention with 70 g fats containing: 1) Medium chain fatty acids (65% caprylic acid and 33% capric acid). 2) High oleic sunflower oil.	3 weeks each intervention	Plasma CETP activity	No effect
Van Tol A, (1995)	The Netherlands	55 healthy participants (25 men and 30 women) Age range:19-49 years	Isocaloric parallel diets with 8% energy from: 1) Linoleic acid. 2) Stearic acid. 3) Trans fatty acid.	17 days	ABDP CETP activity	Increase in trans fatty acids diet relative to linoleic rich diet.
Vega-López S, (2006)	USA	15 participants with high levels of LDL cholesterol (5 men and 15 women) Mean age ± SD: 63.9 ± 5.7 years	Cross-over intervention with four diets with 20% fat content provided by: 1) Partially containing hydrogenated soybean oil (13% trans fats). 2) Soybean oil (44% PUFAs). 3) Palm oil (50% saturated fats).	5 weeks each intervention	Plasma CETP activity	No effect

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
			4) Canola oil (49% MUFAs).			
					Paraoxonase activity	No effect.
Vega-López S, (2009)	USA	30 post-menopausal women with moderate hypercholesterolemia Mean age ± SD: 64.2 ± 7.5 years	A cross-over intervention with 20% calories from: 1) Corn oil 2) Partially hydrogenated soybean oil	5 weeks each intervention	Plasma LCAT activity	No effect.

SFA: Saturated fatty acids. TFA: Trans fatty acids. MUFA: Monounsaturated fatty acids. PUFA: Polyunsaturated fatty acids. DHA: Docosahexaenoic acid. EPA: Eicosapentaenoic acid. CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. CETA: Cholesteryl ester transfer activity. LCAT: Lecithin–cholesterol acyltransferase. ABDP: Apolipoprotein B-depleted plasma. ABDS: Apolipoprotein B-depleted serum. PON1: Paraoxonase-1. TBARS: Thiobarbituric acid reactive substance. HOII: HDL oxidative/inflammatory index.

Table 5. Studies with dietary cholesterol

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Andersen CJ, (2013)	USA	37 Metabolic syndrome (12 men and 25 women) Age range: 30-70 years	Two parallel diet interventions with: 1) 3 whole eggs (534 mg cholesterol). 2) Equivalent egg substitute (without cholesterol).	12 weeks	^{3}H CEC in RAW 264.7 cells in isolated HDLs	Increase of 2.4% in egg group relative to baseline.
Blanco-Molina A, (1998)	Spain	15 healthy men Mean age ± SD: 23.4 ± 5.6 years	Cross-over diet with: 1) Low-fat NCEP-Step I diet supplemented with 2 eggs. 2) Low-fat NCEP-Step I diet without eggs. 3) MUFA-rich diet supplemented with 2 eggs 4) MUFA-rich diet without eggs.	24 days each intervention	^{3}H CEC in Fu5AH cells in serum	Increase in low fat diet enriched with eggs compared to the low fat diet without eggs.
Blesso CN, (2013)	USA	37 Metabolic syndrome patients (12 men and 25 women) Mean age ± SD: 51.9 ± 7.7 years	Parallel carbohydrate restricted diet interventions with: 1) 3 whole eggs/day (534 mg cholesterol). 2) Yolk free eggs.	12 weeks	Plasma CETP activity	No effect.
					Plasma LCAT activity	Increase in LCAT in whole egg group relative to baseline.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Ginsberg HN, (1995)	USA	13 healthy women Mean age ± SD: 23.5±1.9 years	Cross-over diets with: 1) 1 egg. 2) 2 eggs. 3) 3 eggs.	8 weeks each intervention	Plasma CETP mass	No effect.
Ginsberg HN, (1994)	USA	20 healthy men Mean age ± SD: 24.4 ± 2.7 years	Cross-over low-fat diets with: 1) 0 egg. 2) 1 egg. 3) 2 eggs. 4) 4 eggs.	8 weeks each intervention	Plasma CETP mass	4 eggs/day increased CETP levels by 6% compared to other diet interventions.
Herron KL, (2004)	USA	52 healthy participants (25 men and 27 women) Age range: 18-50 years	Cross-over diets with: 1) Eggs (640 mg/day cholesterol). 2) Placebo egg substitute.	1 month each intervention	Plasma CETP activity	Increased CETP activity in egg group compared to control in a subgroup of hyper-responders to dietary cholesterol.
					Plasma LCAT activity	Increased LCAT activity in egg group compared to baseline in a subgroup of hyper-responders to dietary cholesterol.
Herron KL, (2003)	USA	40 normolipidemic men Age range: 20-50 years	Cross-over diets with: 1) Eggs (640 mg/day cholesterol). 2) Placebo egg substitute.	1 month each intervention	Plasma CETP activity	Increased CETP activity in egg group compared to control in a subgroup of

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
						hyper-responders to dietary cholesterol.
					Plasma LCAT activity	Increased LCAT activity in egg group compared to control in a subgroup of hyper-responders to dietary cholesterol.
Herron KL, (2002)	USA	51 premenopausal women Age range: 19-49 years	Cross-over diets with: 1) Eggs (640 mg/day cholesterol). 2) Placebo egg substitute.	1 month each intervention	Plasma CETP activity	Increased CETP activity in egg group compared to control in a subgroup of hyper-responders to dietary cholesterol.
Martin LJ, (1993)	USA	30 healthy men Mean age ± SD: 23.0 ± 2.6 years	Cross-over diets with: 1) Low cholesterol diet (80 mg/1000Kcal). 2) High cholesterol diet (320mg/1000Kcal).	35 days each intervention	Plasma CETP mass	Increased levels in high cholesterol diet compared to low cholesterol diet.
Missimer, (2018)	USA	50 healthy young participants (24 men and 26 women) Mean age ± SD: 23.3 ± 3.1 years	Cross-over diets with: 1) Two large eggs/day (370 mg cholesterol). 2) Oatmeal (384 g/day).	4 weeks each intervention	Plasma CETP activity	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Morgantini, (2018)	Italy	14 healthy participants Mean age ± SD: 25.0±2.3 years	Cross-over diets with: 1) Low fat and low cholesterol diet (100-150 mg/day;5-10% SFA). 2) High fat and high. cholesterol diet (250-300 mg/day;15-20% SFA).	2 weeks each intervention	Paraoxonase activity	No effect.
					HDL hydroperoxides content	Increase in hydroperoxide content compared to low fat and low cholesterol diet.
					HDL associated SAA	Increase in SAA content in HDL compared to low fat and low cholesterol diet.
Mutungi G, (2010)	USA	31 overweight or obese men Age range: 40-70 years	Parallel carbohydrate restricted diets with: 1) 3 liquid eggs. 2) Substitute egg placebo.	12 weeks	LCAT activity	Increase in egg group relative to control.
Sawrey-Kubicek, (2019)	USA	20 overweight women Mean age ± SD: 57.7 ± 5.3 years	Cross-over diet with: 1) 2 whole eggs per day (100 g/egg). 2) 2 yolk free eggs per day (100 g/egg).	4 weeks each intervention	BODIPY cholesterol marked CEC in J774 cells in ABDP samples	Increase of 5.69% in whole egg group compared to control.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					Plasma CETP activity	No effect.
					Plasma LCAT activity	No effect.
					Plasma paraoxonase -1 activity	No effect.
Vorster HH (1992)	South Africa	70 young healthy men Age range: 18-19 years	Parallel diet interventions with: 1) 3 eggs/ week. 2) 7 eggs/week. 3) 14 eggs/ week.	Measurements at 1,5, 7 months	Plasma LCAT activity	Increased LCAT activity in 14 eggs/week group relative to 3 eggs/week group after 1 month (but not after 5 or 7 months).
Waters D, (2007)	USA	22 postmenopausal women Age range: 50-77 years	Cross-over diets with: 1) Eggs (640 mg/day cholesterol and 600 µg of lutein+zeaxanthin). 2) Placebo egg substitute.	4 weeks each intervention	Plasma CETP activity	No effect.

MUFA: Monounsaturated fatty acids. CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. LCAT: Lecithin–cholesterol acyltransferase. ABDP: Apolipoprotein B-depleted plasma. SAA: Serum amyloid A.

Table 6. Studies with antioxidant nutrients and antioxidant-rich foods

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Balsan, (2019)	Brazil	142 overweight or obese participants (55 men and 87 women) Mean age ± SD: 50.2 ± 6.4 years	Parallel interventions with 1 L: 1) Mate tea. 2) Green tea. 3) Apple tea (control).	8 weeks	Serum PON1 mass	Higher levels of PON1 in Mate group compared to green tea and apple tea.
Baralic I, (2012)	Serbia	40 male soccer players Mean age ± SD: 17.91 ± 0.16 years	Parallel intervention with supplements of: 1) 4 mg of Astaxanthin. 2) Placebo.	3 months	Plasma PON1 paraoxonase and diazonase activity	Increase in diazonase activity relative to baseline levels.
Boaventura, (2012)	Brazil	74 dyslipidemic participants (17 men and 57 women) Mean age ± SD: 48.5 ± 11.6 years	Parallel interventions with 1 L: 1) Mate tea (1 L/day) 2) Low fat and vegetable rich diet. 3) Diet and mate tea.	3 month	Serum Arylesterase activity	No effect.
Bub A, (2005)	Germany	22 healthy young participants Mean age ± SD: 29±6 years	Cross-over intervention with 330 ml/day: 1) Tomato juice. 2) Carrot juice.	2 weeks each intervention	Serum Arylesterase activity	No effect.
Bub A, (2002)	Germany	50 elderly participants (18 men and 32 women) Mean age ± SD: 70 ± 6 years	Cross-over intervention with 330 ml/day: 1) Tomato juice. 2) Water.	8 weeks	Serum Arylesterase activity	Both interventions increased relative to baseline levels.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Cherki M, (2005)	Morocco	60 healthy men Mean age ± SD: 23.4 ± 3.85 years	Parallel intervention with two oils rich in phenolic compounds: 1) Virgin argan oil. 2) Virgin olive oil.	3 weeks	Paraoxonase and arylesterase activity	Both interventions increased relative to baseline levels.
Dalgård C (2007)	Denmark	48 participants with peripheral artery disease (35 men and 13 women) Mean age ± SD:61 ± 6 years	Parallel interventions with: 1) Vitamin E (15 mg/day) combined with orange and blackcurrant juice. 2) Placebo and combined with orange and blackcurrant juice. 3) Vitamin E combined with control juice 4) Placebo and control juice	4 weeks	Paraoxonase1 activity and mass	No effect.
De Roos B, (2000)	The Netherlands	46 healthy participants (23 men and 23 women) Mean age ± SD: 29.5 ±2 years	Parallel interventions with 0.9 L/day: 1) French-press coffee. 2) Filtered coffee.	24 weeks	Serum CETP activity	French-press coffee increased CETP activity relative to filtered coffee.
Farràs M, (2018)	Spain	33 hypercholesterolemic participants (19 men and 14 women) Mean age ± SD: 55.2 ± 10.6 years	Cross-over intervention with 25 ml of virgin olive oil per day: 1) Enriched with olive oil phenolic components (500 ppm) (FVOOT). 2) Enriched with olive oil	3 weeks each intervention	^{3}H CEC in J774 cells in isolated HDL samples	Increase in CEC in FVOOT relative to FVOO.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
			phenolic components and other phenolic components from thyme (500 ppm in the aggregate) (FVOO). 3) Not enriched (VOO).			
					HDL antioxidant compounds	Increase in β-criptoxanthin and lutein in both enriched olive oils relative to baseline.
Farràs M, (2015)	Spain	33 hypercholesterolemic participants (19 men and 14 women) Mean age ± SD: 55.2 ± 10.6 years	Cross-over intervention with 25 ml of virgin olive oil per day: 1) Enriched with olive oil phenolic components (500 ppm) (FVOOT). 2) Enriched with olive oil phenolic components and other phenolic components from thyme (500 ppm in the aggregate) (FVOO). 3) Not enriched (VOO).	3 weeks each intervention	Plasma CETP activity	No effect.
					Plasma LCAT mass	Increase in mass in FVOOT relative to VOO.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					Plasma PON1 arylesterase activity	Increase in FVOOT relative to VOO.
Fernández-Castillejo S, (2017)	Spain	33 hypercholesterolemic participants (19 men and 14 women) Mean age ± SD: 55.2 ± 10.6 years	Cross-over intervention with 25 ml of virgin olive oil per day: 1) Enriched with olive oil phenolic components (500 ppm) (FVOOT). 2) Enriched with olive oil phenolic components and other phenolic components from thyme (500 ppm in the aggregate) (FVOO). 3) Not enriched (VOO).	3 weeks each intervention	Serum PON1 and PON3 mass and paraoxonase-1 and lactonase specific activity	FVOOT increase PON1 levels relative to baseline. FVOO increase paraoxonase-1 and lactonase activity relative to baseline levels. VOO increase PON3 mass relative to FVOO and FVOOT.
Freese R, (2002)	Finland	77 healthy participants Mean age (range age): 25.1 (19-52) years	Parallel dietary intervention: 1) Low vegetable diet with high linoleic acid content. 2) High vegetable and apple diet with high linoleic content. 3) Low vegetable diet with high oleic acid content. 4) High vegetable and apple diet with high oleic acid content.	6 weeks	Plasma CETP activity	Increased CETP activity in high vegetables and linoleic group relative to baseline.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					Plasma LCAT activity	No effect.
Hernáez A, (2014)	Spain	47 healthy men Mean age ± SD: 33.5 ± 10.9 years	Cross-over intervention with 25 ml raw olive oil per day containing: 1) Polyphenol-rich oil (366 mg/kg polyphenols). 2) Polyphenol-poor oil (2.7 mg/kg).	3 weeks each intervention	^{3}H CEC in THP-1 cells in ABDS samples	Increase of 3.04 ±9.98% relative to polyphenol-poor group.
					Polyphenol metabolites in HDL	Increased content of polyphenol metabolites in intervention group. compared to baseline.
Lazavi F, (2018)	Iran	42 diabetes type 2 participants (15 men and 27 women) Mean age ± SD: 56.86 ± 8.47 years	Parallel interventions with 200 ml/day: 1) Barberry Juice 2) Control.	8 weeks	Plasma PON1 concentration	Increase relative to control group.
McEneny J, (2013)	UK	54 moderate overweight participants Mean age ± SD: 50.4 ± 3.0 years	Parallel interventions with: 1) Lycopene-rich diet (224-350 mg/d). 2) Lycopene supplements (70 mg/d). 3) Control (placebo).	12 weeks	Serum CETP activity	Decrease in Lycopene supplement relative to lycopene diet and control.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					Serum LCAT activity	Increase in both lycopene interventions relative to baseline.
					PON1 arylesterase activity	Increase in both lycopene interventions relative to control. Increase in Lycopene supplement relative to lycopene diet.
					SAA mass in isolated HDL 2 and HDL3	Decrease in both lycopene interventions relative to control.
Michaličková, (2019)	Czech Republic	26 hypertensive participants (7 men and 19 women) Mean age: 47 years	Parallel interventions with 200 g tomato juice: 1) Lycopene and polyphenol rich. 2) Control juice.	4 weeks	Serum paraoxonase-1 activity	No effect.
Millar (2018),	USA	20 Metabolic syndrome participants (12 men and 8 women) Mean age ± SD: 53.5±10.1 years	Cross-over intervention with: 1) 60 g/day freeze-dried grape powder. 2) Placebo.	3 weeks each intervention	PON1 arylesterase activity	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Ozdemir B, (2008)	Turkey	48 participants with hyperlipidemia (15 men and 33 women) Age range: 25-60 years	Parallel interventions: 1) Origanum onites aqueous distillate (75 ml/day). 2) Low fat diet.	3 months	Paraoxonase and arylesterase activity	Increase relative to control.
Puglisi MJ, (2009)	USA	34 healthy participants (17 men and 17 women) Age range: 50-70 years	Parallel interventions: 1) Origanum onites aqueous distillate (75 ml/day) 2) Low fat diet.	6 weeks	Plasma CETP activity	No effect.
Qian Q, (2012)	China	54 type 2 diabetes participants with chronic heart disease Mean age ± SD: 59.8 ± 8.7 years	Parallel interventions: 1) Salvia hydrophilic extract (10 g/d) with diet and hypoglycemic drugs. 2) Diet and hypoglycemic drugs (control).	2 months	Paraoxonase activity	9 % increase compared to baseline levels.
Qin Y, (2009)	China	120 participants with dyslipidemia (42 men and 78 women) Age range: 40-65 years	Parallel interventions with supplements: 1) Anthocyanins (320 mg/d). 2) Placebo.	12 weeks	^{3}H CEC in J774 cells in serum	Anthocyanin group increased 20% relative to placebo.
					Plasma CETP activity and mass	Decrease mass and activity relative to placebo.
					Plasma LCAT activity and mass	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Shidfar F, (2015)	Iran	50 diabetes type 2 participants Mean age ± SD: 45.2 ± 7.64 years	Parallel interventions with supplements: 1) 3 g of powdered ginger capsules daily. 2) Placebo.	3 months	Paraoxonase-1 activity	Increase relative to control.
Suomela JP, (2006)	Finland	14 healthy men Mean age ± SD: 47.2 ± 9.7 years	Cross-over interventions with supplements: 1) 185 g Sea buckthorn flavonol-enriched oatmeal porridge (78 mg flavonol). 2) Control porridge.	4 weeks	Paraoxonase-1 activity	No effect.
Van Tol A, (1997)	The Netherlands	10 healthy males Mean age ± SD: 24±4 years	Cross-over interventions with coffe extracts supplements: 1) 64 mg cafestol + 1 mg kahweol per day. 2) 60 mg cafestol + 54 mg kahweol per day.	4 weeks each intervention	Serum LCAT activity	Decrease of 11±12% in cafestol + kahweol relative to baseline.
Zern TL, (2005)	USA	44 premenopausal or postmenopausal women Mean age ± SD: 39.7± 8.5 (premenopausal); 58.5 ± 7.5 (postmenopausal)	Cross-over interventions with supplements: 1) 6 g/day grape powder 2) Placebo	4 weeks each intervention	Plasma CETP activity	Decrease relative to baseline levels (9% in premenopausal women and 29% in postmenopausal).

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Zhu Y, (2013)	China	122 Hypercholesterolemic participants (50 men and 72 women) Age range: 40-65 years	Parallel interventions with supplements: 1) Anthocyanins (320 mg/d) 2) Placebo	24 weeks	^{3}H CEC in J774 cells in isolated HDL	Anthocyanin group increased 17.7% relative to placebo.
					Paraoxonase-1 activity	Anthocyanin group increased 17.4% relative to placebo.

CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. LCAT: Lecithin–cholesterol acyltransferase. ABDP: Apolipoprotein B-depleted plasma. SAA: Serum amyloid A. PON1: Paraoxonase-1. PON3: Paraoxonase-3.

Table 7. Studies with antioxidant-rich dietary patterns

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Damasceno NR, (2013)	Spain	169 participants at high cardiovascular risk (74 men and 95 women) Mean age: 67 years	Three parallel whole diet interventions: 1) Traditional Mediterranean diet enriched with extra virgin olive oil (1L/week). 2) Traditional Mediterranean diet enriched with nuts (30 g/day of mixed nuts). 3) Low fat diets.	1 year	Serum CETP activity	A traditional Mediterranean diet enriched with olive oil and a low fat diet decreased CETP levels compared to baseline.
Daniels JA, (2014)	UK	74 type 2 diabetes obese participants (52 men and 22 women) Age range: 40-70 years	Two parallel whole diet interventions: 1) Low fruit and vegetable intake (80 g/d). 2) High fruit and vegetable intake (400 g/d).	8 weeks	Serum LCAT activity	Increase in high fruit and vegetable relative to baseline
					PON1 arylesterase activity in Serum and in HDL2 and HDL3	Increase in serum activity in high vegetable and fruit diet relative to baseline. Increase in HDL3 in high vegetable group relative to low vegetable group.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					SAA content in HDL2 and HDL3	No effect
					HDL2 and HDL3 content in carotenoids	High vegetable group increased HDL3 α-carotene, β-cryptoxanthin, lutein, and lycopene compared to low vegetable intake. In HDL2 intervention increased β-cryptoxanthin compared to control, and lutein relative to baseline.
Hernáez Á, (2020)	Spain	358 participants at high cardiovascular risk (131 men and 227 women) Mean age: 67 years	Three parallel whole diet interventions: 1) Traditional Mediterranean diet enriched with extra virgin olive oil (1L/week). 2) Traditional Mediterranean diet enriched with nuts (30 g/day of mixed nuts). 3) Low fat diets.	1 year	ABDP HDL-alpha-1-antitrypsin	Decrease in Mediterranean diet with olive oil compared to baseline.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					Nitric oxide production in HUVEC cells after ABDP.	Increase in Mediterranean diet with virgin olive oil compared to low fat diet.
Hernáez Á, (2017)	Spain	296 participants at high cardiovascular risk (151 men and 145 women) Mean age ± SD: 65.9 ± 6.43 years	Three parallel whole diet interventions: 1) Traditional Mediterranean diet enriched with extra virgin olive oil (1L/week). 2) Traditional Mediterranean diet enriched with nuts (30 g/day of mixed nuts). 3) Low fat diet.	1 year	^{3}H CEC in THP-1 cells in ABDP	Both Mediterranean diets increased CEC relative to baseline levels.
					Plasma CETP activity	Mediterranean diet with virgin olive oil decreased CETP activity relative to baseline
					Direct HDL antioxidant capacity on LDL	Increased antioxidant capacity after a Mediterranean diet with olive oil relative to baseline.
					HDL oxidation status by TBARS assay	Decreased oxidation status relative to baseline levels in Mediterranean diet with olive oil and in

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
						low fat diet.
					HDL oxidative//inflammatory index (HOII)	The control low fat diet increased HOII relative to baseline levels.
					Serum PON1 arylesterase activity	Mediterranean diet with virgin olive oil increased PON1 activity relative to low fat diet
					Nitric oxide production in HUVEC cells after ABDP.	Increase in Mediterranean diet with virgin olive oil compared to low fat diet.
Rantala M, (2002)	Finland	37 healthy women Mean age ± SD: 42.6 ± 10.1 years	Two parallel whole diet interventions: 1) Low vegetable diet (1 serving/day) 2) High vegetable diet (430 mg of vitamin C, 18 mg of carotenoids, 17 mg of vitamin E and 600 g of folate.)	5 weeks	Paraoxonase-1 activity	The high vegetable diet decreased PON activity compared to low vegetable diet.

CEC: cholesterol efflux capacity. CETP: cholesteryl ester transfer protein. LCAT: Lecithin–cholesterol acyltransferase. ABDP: Apolipoprotein B-depleted plasma. SAA: serum amyloid A. PON1: paraoxonase-1. HOIi: HDL oxidative/inflammatory index.

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Table 8. Studies with ethanol and HDL function

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Beulens JW, (2004)	The Netherlands	24 healthy men Mean age ± SD: 52 ± 5 years	Cross-over intervention with: 1) 40 g/day ethanol (whisky). 2) Water control.	17 days each intervention	^{3}H CEC in J774 and Fu5AH cells in serum	CEC increased in both cellular models relative to control water group.
Králová Lesná I, (2010)	Cezch Rep.	13 healthy men Mean age ± SD: 32.31±5.9 years	Cross-over intervention with: 1) 36 g alcohol/day (1L beer). 2) Control abstinence period.	4 weeks each intervention	^{14}C CEC in THP-1 cells in plasma	No effect.
Padro T, (2018)	Spain	36 overweight or Obese I, regular moderate alcohol consumers (21 men and 15 women) Mean age ± SD: 48.3 ± 5.4 years	Cross-over intervention with: 1) Beer (2 cans men 1 women 15g/can ethanol and 604 mg polyphenols/can). 2) Non-alcoholic beer (414 mg polyphenols/can).	4 weeks each intervention	^{3}H CEC in J774 cells in ABDS	Increase in alcoholic beer group relative to baseline levels.
					HDL Antioxidant Potential assessed by TRAP test	Both groups increased antioxidant capacity of HDLs relative to baseline.
Senault C, (2000)	France	56 healthy young men	Three parallel interventions with: 1) Red wine (30 g alcohol/day). 2) A solution with the same	2 weeks	^{3}H CEC in Fu5AH cells in serum	Increase by 7% relative to baseline.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
			degree of alcohol (30 g alcohol/day). 3) Control alcohol free red wine.			
					Plasma CETP activity	No effect.
Sierksma A, (2004)	The Netherlands	18 healthy women Mean age ± SD: 57 ± 5 years	Cross-over intervention with: 1) 24 g/day ethanol (white wine). 2) Grape juice control.	3 weeks each intervention	^{3}H CEC in Fu5AH cells in plasma	Increase of 3.4% relative to control.
					Plasma Cholesteryl ester transferactivity	No effect.
Sierksma A, (2002)	The Netherlands	19 healthy participants (10 men and 9 women). Age range:45-64 years	Cross-over intervention with: 1) 30-40 g/day ethanol (beer). 2) No alcohol control.		Serum PON paraoxonase-1 activity and PON mass	Increase of PON-1 activity and mass with beer group relative to control.
Van der Gaag MS, (2001)	The Netherlands	11 healthy men Age range: 45-60 years	Cross-over intervention with 40 g/day ethanol: 1) Red wine. 2) Beer. 3) Spirits (Dutch gin). 4) Water control.	3 weeks each intervention	^{3}H CEC in Fu5AH cells in plasma	Red wine increased CEC by 5%, beer 6.9% and gin 6% all relative to control.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Van der Gaag MS, (1999)	The Netherlands	11 healthy men Age range:45-60 years	Cross-over intervention with 40 g/day ethanol: 4) Red wine. 5) Beer. 6) Spirits (Dutch gin). 4) Water control.	3 weeks each intervention	Paraoxonase-1 activity	Red wine increased PON-1 activity by 6.9%, beer 7.4% and gin 9.3% all relative to control.

CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. LCAT: Lecithin–cholesterol acyltransferase. ABDS: Apolipoprotein B-depleted serum. PON1: Paraoxonase-1. HOII: HDL oxidative/inflammatory index. TRAP: Total radical-trapping antioxidative potential.

Table 9. Studies with physical activity, calorie restriction, and HDL function

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Albaghdadi MS, (2017)	USA	88 participants with peripheral artery disease (41 men and 47 women) Mean age ± SD: 70.85 ± 1.72 years	Parallel interventions with: 1) Endurance (treadmill) (3 times/w). 2) Strength (lower extremity resistance training group) (3 times/w). 3) Control diet group.	24 weeks	^{3}H CEC in J774 cells in ABDS	No effect.
Dokras A, (2018)	USA	87 overweight or obese women with Polycystic Ovary Syndrome Age range: 18-40 years	Parallel interventions with: 1) Oral contraceptive pills. 2) Recommendations for calorie restriction of 500 calorie deficit+brisk walking 5 times week). 3) Combined treatment.	16 weeks	^{3}H CEC in J774 cells in ABDS	No effect.
Khan AA, (2018)	Australia	53 Metabolic syndrome patients (30 men and 23 women) Mean age ± SD: 55 ±6 years	Parallel interventions with: 1) Exercise aerobic training 4 times per week of 30-40 min combined with diet (DASH) (to reduce by 600kcal/day). 2) DASH dietary intervention. 3) Control group without intervention.	12 weeks	^{3}H CEC in THP-1 cells in ABDS	DASH diet combined with exercise increased by 25% CEC relative to baseline.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					Plasma CETP activity	DASH diet combined with exercise decreased relative to baseline.
Miida T, (1998)	Japan	24 hypercholesterolemic and 12 normolipidemic (9 men and 27 women) Mean age ± SD: 57.93 ± 8.37 years	Parallel interventions with: 1) Low calorie diet NCEP Step I. 2) Probucol 500mg /day. 3) Probucol 1000mg/day. 4) Control with normolipidemic patients	4 weeks	ABDP CETP mass	No effect.
Rönnemaa T, (1988)	Finland	25 diabetic participants Mean age: 52.7 years	Parallel interventions with: 1) Aerobic exercise 5-7 days/week, 45 min/session, 70% of VO2 Max.). 2) No training group	4 months	Serum LCAT activity	No effect
Sarzynski, (2018)	USA	Participants from STRRIDE-PD trial: 106 overweight sedentary (23 men and 67 women) Age range:18-65 years	Parallel interventions with endurance training: 1) Low amount of moderate intensity exercise. 2) High amount of moderate intensity exercise. 3) High amount of vigorous exercise. 4) Low fat diet combined with moderate intensity exercise.	6 months	^{3}H and BODIPY CEC in J774 cells in ABDP	Increase of ^{3}H CEC in high amount of exercise compared to the other three interventions. No effect with BODIPY marked CEC.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
		Participants from E-MECHANIC trial: 90 overweight sedentary (39 men and 67 women) Age range: 45-75 years	Parallel weight-loss interventions with: 1) Low amount of moderate intensity exercise (to reduce 8 kcal/kg weeks) 2) High amount of exercise (to reduce 20 kcal/kg weeks) 3) No exercise group (control)	6 months	^{3}H and BODIPY CEC in J774 cells in ABDP	Increase of ^{3}H non-ABCA1 CEC in high amount exercise compared to control group. No effect with BODIPY marked CEC.
Talbot, (2018)	The Netherlands	77 overweight obese participants Age range: 18-65 years	Parallel interventions with: 1) Very-low-calorie diet (500 kcal) 2) Control group without weight loss	6 weeks	BODIPY CEC in J774 cells in ABDP Cholesterol ester transfer from radio-labeled HDL to ApoB lipoproteins.	No effect. No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Thomas TR, (1985)	USA	36 young healthy men Age range:18-25 years	Parallel interventions with: 1) 3 times/week 5 miles continuous exercise with 4 minutes intervals (1:1, work:rest) 2) 3 times/week 5 miles continuous exercise with 2 minutes intervals (1:1-1/2, work:rest) 3) No training group	11 weeks	LCAT levels	No effect.
Tiainen S, (2016)	Finland	161 sedentary women Age range: 43-63 years	Parallel interventions with: 1) Aerobic training four times week 2) Control without exercise	6 months	CETP activity	No effect.
Vislocky LM, (2007)	USA	12 healthy unfit participants (7 men and 5 women) Age range:18-30 years	Parallel interventions with: 1) 12 eggs week 2) No eggs 3) Endurance training 30-45m 3-5d week 4) No training group	8 weeks	Plasma CETP activity	32 % decrease in trained relative to untrained participants.
Wesnigk J, (2016)	Belgium	16 obese adolescents Mean age ± SD: 15.1 ± 2.5 years	Parallel interventions with: 1) Dietary restriction 1500–1800 kcal/day combined with intensive supervised exercise (2h/day of lifestyle activities+ 3 times/week 40' aerobic and resistance training) and	10 months	^{3}H CEC in J774 cells in ABDS	Increase relative to usual care group.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
			psychological support from experts 2) Usual care group			
					eNOS phosphorilation mediated by HDL in HAECs cells	Increase relative to usual care group.
Williams PT, (1990)	USA	77 healthy sedentary men Age range:30-55 years	Parallel interventions with: 1) Running group (12.7 km/week in treadmill) 2) No training group	1 year	Plasma LCAT mass	No effect.
Woudberg NJ, (2018)	South Africa	35 obese black women Mean age ± SD: 24.5 ± 0.9 years	Parallel intervention with: 1) Exercise (aerobic and resistance exercise combined 40-60 m, 4 days per week). 2) No exercise group (control)	12 weeks	^{3}H CEC in RAW264.7 cells in isolated HDL	No effect.
					serum PON1 activity	Decrease relative to control group.
					HDL-bound phospholipase A2 expression in HDLs	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					VCAM expression in isolated HDL	No effect.

CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. LCAT: Lecithin–cholesterol acyltransferase. ABDP: Apolipoprotein B-depleted plasma. ABDS: Apolipoprotein B-depleted serum. SAA: Serum amyloid A. PON1: Paraoxonase-1. TBARS: Thiobarbituric acid reactive substance. eNOS: Endothelial nitric oxide synthase. PAF-AH: Platelet-activating factor acetylhydrolase. VCAM: Vascular cell adhesion protein.

Table 10. Other lifestyle interventions

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Favari E, (2020)	Italy	41 overweight participants Age range: 30-65 years	Two parallel interventions with whole wheat pasta enriched with phenolic acids (50.3mg/100g)+ fiber(12.5g/100g): 1) Enriched with β-glucans (2.3 g/100 g) and Bacillus coagulans. 2) Non-enriched pasta.	12 weeks	^{3}H CEC in CHO cells in serum	Increase in enriched group relative to control.
Higashi K, (2001)	Japan	14 healthy men Mean age ± SD: 31±4 years	Cross-over intervention with supplements: 1) 20 g per day of soy protein. 2) Placebo.	4 weeks each intervention	CETP mass	No effect.
					LCAT activity	No effect.
Homma Y, (2003)	Japan	105 healthy participants (38 men and 67 women) Mean age ± SD: 47 ± 13 years	Two parallel interventions with: 1) 2g/plant stanol. 2) 3g/plant stanol. 3) Placebo.	4 weeks	Plasma CETP mass	Decrease of 6.1% after 2g stanol/day and 3.3% in 3g/day relative to baseline.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Lichtenstein AH, (2002)	USA	36 participants with high levels of LDL-C (18 men and 18 women) Age range: 55-74 years	Cross-over intervention with supplements: 1) TLC/Step 2 diet (low saturated fats and high in PUFA and fibre) 2) Western diet (high fat diet).	4.5 weeks each intervention	ABDP CETP activity	No effect.
Lottenberg AM, (2003)	Brazil	60 moderate hypercholesterolemic participants (10 men and 50 women) Age range: 20-60 years	Cross-over intervention with margarine (20g/day): 1) Enriched with plant sterol ester (2.8 g/day equal to 1.68 g/d phytosterols) 2) Non-enriched (control).	4 weeks each intervention	Plasma CETP mass	Decrease relative to placebo.
					Plasma LCAT activity	No effect.
Meng, (2018)	USA	11 healthy participants (7 men and 4 women) Mean age ± SD: 65 ± 8 years	Cross-over intervention with foods containing: 1) Simple carbohydrates 2) Refined carbohydrates 3) Unrefined carbohydrates	4.5 weeks	^{3}H CEC in PBMCs cells in isolated HDLs	No effect.
Richter CK, (2018)	USA	20 moderate hypertension participants (9 men and 20 women) Mean age ± SD: 51,6 ± 6,6 years	Cross-over intervention with soya protein: 1) 50 g/day 2) 25 g/day 3) No soya group (control)	6 weeks each intervention	^{3}H CEC in J774 cells in ABDP.	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
Shidfar F, (2009)	Iran	52 hypercholesterolemic postmenopausal women Age range: 49-61 years	Two parallel interventions with: 1) Soy protein (50 g/day (164 mg isoflavones)). 2) Placebo	10 weeks	Plasma Paraoxonase-1 activity	Increase in fiber group relative to control.
Shresth S, (2007)	USA	33 healthy participants (11 men and 22 women) Age range: 35-65 years	Cross-over intervention with supplements: 1) 10 g Psyllium yielding 7.68 g/d soluble fiber and 2.6 g/d plant sterols. 2) Placebo.	1 month each intervention	Plasma CETP activity	Supplemented group presented 11% lower CETP activity relative to placebo.
Vega-López S, (2001)	USA	68 healthy participants (24 men and 23 premenopausal and 21 postmenopausal) Mean age ± SD: 43.7 ± 13.2 years	Cross-over intervention with supplements: 1) 15 g/day of Psyllium fiber. 2) Placebo.	1 month each intervention	Plasma CETP activity	Decrease relative to placebo intervention.
Wood RJ, (2006)	USA	30 overweight men Age range: 20-69 years	Two parallel interventions with carbohydrate restriction diets: 1) Supplemented with fiber (3 g/day of Konjac-mannan fiber). 2) Non-supplemented control.	12 weeks	Plasma CETP activity	No effect.

First author, (year)	Location	Study participants	Intervention	Duration	HDL function analyzed	Results
					Plasma LCAT activity	Increase in fiber group relative to baseline.

CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. LCAT: Lecithin–cholesterol acyltransferase. ABDP: Apolipoprotein B-depleted plasma.

Supplementary material

Supplemental table 1. Risk of bias evaluation

First author (year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other potential bias	Overall risk of bias
Thomas TR (1985)	?	?	?	?	?	?	?	?
Baudet MF (1988)	?	?	?	?	?	?	?	-
Rönnemaa T (1988)	?	?	?	?	?	?	?	?
Abbey M (1990)	?	?	?	?	+	+	?	?
Singer P (1990)	?	?	?	?	?	?	?	?
Williams PT (1990)	?	+	-	?	+	+	+	+
Vorster HH (1992)	-	?	-	+	?	+	+	-
Martin LJ (1993)	?	?	-	?	?	+	+	?
Cox C (1994)	?	?	-	?	+	?	+	?
Ginsberg HN (1994)	?	?	+	+	-	+	+	-
Cox C (1995)	?	?	-	?	+	+	+	?
Ginsberg HN (1995)	?	?	+	+	-	+	+	-
Schwab US (1995)	?	?	-	?	?	+	-	-
van Tol A	-	?	-	?	?	+	?	-

First author (year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other potential bias	Overall risk of bias
(1995)								
Lottenberg AM (1996)	?	?	+	-	?	+	+	?
Solà R (1997)	?	?	-	?	?	+	+	?
Blanco-Molina A (1998)	?	?	-	+	+	+	+	?
Miida T (1998)	?	?	-	?	?	+	+	-
Lagrost L (1999)	?	?	+	?	-	+	+	?
Pownall HJ (1999)	?	?	+	?	?	+	+	?
Van der Gaag MS (1999)	?	?	-	?	-	+	?	?
Senault C (2000)	?	?	?	?	?	?	?	?
Higashi K (2001)	?	?	-	?	-	-	?	?
Lichtenstein AH (2001)	?	?	+	+	?	+	+	?
Matthan NR (2001)	?	?	+	+	-	+	+	?
Van der Gaag MS (2001)	?	?	-	?	-	+	?	?
Vega-López S (2001)	?	?	?	?	+	+	+	?
Bub A (2002)	?	?	-	?	+	+	?	?
de Roos NM (2002)	?	?	-	+	+	+	-	-

First author (year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other potential bias	Overall risk of bias
Freese R (2002)	?	?	+	+	+	+	+	+
Herron KL (2002)	?	?	-	?	?	+	+	?
Lichtenstein AH (2002)	?	?	+	+	?	+	+	?
Rantala M (2002)	?	?	-	?	?	+	+	?
Sierksma A (2002)	?	?	-	-	+	+	+	-
Herron KL (2003)	?	?	-	?	?	+	+	?
Homma Y (2003)	?	+	+	+	?	+	+	?
Lottenberg AM (2003)	?	?	+	?	?	+	-	-
Beulens JW (2004)	?	?	-	-	+	+	?	?
Calabresi (2004)	?	?	+	?	?	+	?	?
Chung BH (2004)	?	?	-	?	?	+	+	?
Herron KL (2004)	?	?	-	?	?	+	+	?
Sierksma A (2004)	?	?	-	-	+	+	+	-
Tholstrup T (2004)	?	?	+	?	+	+	+	?
Bub A (2005)	?	?	-	?	+	+	?	?
Cherki M	?	?	-	?	+	+	+	?

First author (year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other potential bias	Overall risk of bias
(2005)								
Zern TL (2005)	?	?	+	?	?	+	+	?
Suomela JP (2006)	?	?	+	?	-	+	+	?
Vega-López S (2006)	?	?	?	?	?	+	+	?
Wood RJ (2006)	?	?	+	?	+	+	+	+
Buonacorso V (2007)	?	?	-	?	?	+	?	?
Canales A (2007)	?	?	-	?	?	+	+	?
Dalgård C (2007)	?	?	-	+	-	+	-	-
Shrestha S (2007)	?	?	+	?	+	+	+	+
Waters D (2007)	?	?	?	?	?	-	+	?
Chardigny JM (2008)	+	?	+	?	-	+	+	+
Gebauer SK (2008)	?	?	+	+	+	+	+	+
Kralova-Lesna I (2008)	?	?	-	?	?	+	+	-
Ozdemir B (2008)	?	?	-	?	?	+	-	-
Puglisi MJ (2009)	+	?	-	?	?	+	+	?
Qin Y (2009)	?	?	+	?	+	+	+	+

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First author (year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other potential bias	Overall risk of bias
Shidfar F (2009)	+	?	+	?	?	+	+	?
Vega-López S (2009)	?	?	-	?	-	+	+	?
Vislocky LM (2009)	?	?	-	?	+	+	+	?
Králová Lesná I (2010)	?	?	-	?	?	+	-	-
Mutungi G (2010)	?	?	+	-	+	+	+	?
Canales A (2011)	?	?	-	?	-	+	+	?
Pfeuffer M (2011)	?	?	+	?	?	+	+	?
Ghorbanihaghjo A (2012)	+	?	+	?	+	+	+	+
Qian Q (2012)	?	+	+	?	-	+	+	+
Sánchez-Muniz FJ (2012)	?	?	-	?	?	+	+	?
Andersen CJ (2013)	?	?	+	?	?	+	?	?
Baralic I (2013)	?	?	+	?	?	+	?	?
Blesso CN (2013)	?	?	+	?	?	-	?	?
Damasceno NR (2013)	+	+	-	+	?	+	+	+
McEneny J (2013)	+	?	+	-	+	+	+	+
Zhu Y	?	+	+	?	+	+	+	+

First author (year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other potential bias	Overall risk of bias
(2013)								
Daniels JA (2014)	?	?	-	+	-	+	?	?
Hernáez A (2014)	+	?	-	+	+	+	+	+
Holligan SD (2014)	?	?	-	+	+	+	+	+
Stirban A (2014)	?	?	+	?	?	+	+	?
Farràs M (2015)	+	?	+	+	+	+	+	+
Kawakami Y (2015)	?	?	+	?	-	+	+	?
Shidfar F (2015)	+	?	+	+	+	+	-	+
Shidfar F (2015)	+	?	+	+	+	+	-	+
Wu PT (2015)	?	?	+	+	-	+	+	+
Tiainen S (2016)	+	+	-	+	+	+	+	+
Wesnigk J (2016)	-	?	-	?	-	+	?	-
Albaghdadi MS (2017)	+	?	-	+	+	+	+	+
Berryman CE (2017)	+	?	-	-	-	+	?	-
Dokras A (2017)	+	+	-	-	+	+	-	?
Fernández-Castillejo S	+	?	+	+	+	+	+	+

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First author (year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other potential bias	Overall risk of bias
(2017)								
Golzari MH (2017)	+	?	+	?	?	+	?	?
Hernaez A (2017)	+	+	-	+	+	+	+	+
Lambert C (2017)	?	?	+	?	?	+	+	?
Brassard (2018)	?	?	+	+	-	+	+	?
de Souza (2018)	-	+	-	+	-	+	+	-
Farràs M (2018)	+	?	+	+	+	+	+	+
Khan AA (2018)	?	?	-	?	?	+	+	?
Lazavi F (2018)	+	+	-	?	+	+	+	+
Liu X (2018)	+	+	+	?	-	+	+	+
Meng (2018)	?	?	-	+	?	+	+	?
Millar (2018)	?	?	+	+	?	?	+	?
Missimer (2018)	?	?	-	+	+	+	+	?
Morgantini (2018)	+	?	-	-	+	+	+	+
Padro T (2018)	?	?	-	-	+	+	+	?
Richter CK (2018)	?	+	+	+	+	+	+	+

First author (year)	Random sequence generation	Allocation concealment	Blinding of participants and personnel	Blinding of outcome assessment	Incomplete outcome data	Selective reporting	Other potential bias	Overall risk of bias
Sarzynski (2018)	+	+	-	-	-	+	+	-
Talbot (2018)	?	?	-	?	+	+	+	?
Woudberg NJ (2018)	+	+	-	?	-	+	+	+
Wurm R (2018)	+	+	+	?	+	+	+	+
Balsan (2019)	+	+	-	+	?	+	+	+
Manninen (2019)	+	?	-	-	+	+	-	-
Michaličková (2019)	?	?	+	?	?	+	+	?
Sawrey-Kubicek (2019)	+	?	+	-	+	+	+	+
Andraski (2020)	?	?	-	?	+	+	+	?
Favari E (2020)	+	?	+	?	+	+	+	+
Hernaez A (2020)	+	+	-	+	+	+	+	+
Tindall AM (2020)	+	?	-	?	-	+	+	-

+): Low risk of bias. (-): High risk of bias. (?): Unclear risk of bias.

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6. Discussion

6.1 General overview

This thesis presents four manuscripts exploring the association of HDL functions with cardiovascular risk and how they are modulated by lifestyle changes such as diet or physical activity. We worked within the framework of three different population-based studies: a representative cohort of adult participants from the Spanish population (the REGICOR study) and two randomized controlled trials based on lifestyle interventions (the PREDIMED and the PREDIMED-plus studies). We first analyzed the association between HDL functions and CAD in a general population within the REGICOR study (**Manuscript I**). Second, we investigated the associations between the consumption of cardioprotective food groups and improvements in HDL function in the PREDIMED study population (**Manuscript II**). Third, we examined the capacity of an intensive life-style intervention with a calorie-restricted Mediterranean Diet and physical activity to improve HDL functions in high cardiovascular risk participants from the PREDIMED-plus trial (**Manuscript III**). Finally, we summarized the results from randomized controlled trials studying the effects of dietary and physical activity modifications on HDL functions in a systematic review (**Manuscript IV**). The main characteristics of the manuscripts are summarized in **Table 3**.

The discussion will be focused on the interpretation and integration of the findings from the four texts.

Table 3. Summary of manuscripts

Objective (Manuscript)	Design /outcomes	Main results
Objective 1 (Manuscript 1)	**Design**: Case-cohort study with 10-year follow-up of CAD incidence. **Study:** REGICOR (general population). **Outcome:** Association between HDL functional traits and CAD	CAD is associated with: 1) High levels of C3 in ABDS. 2) Low levels of ApoA-I n ABDS. 3) High levels of S1P in ABDS in participants with desirable HDL-C values.
Objective 2 (Manuscript 2)	**Design:** Randomized controlled trial with three parallel interventions of 1 year: 1) Traditional MedDiet enriched with extra virgin olive oil (1L/week). 2) Traditional MedDiet enriched with nuts (30 g/day of mixed nuts). 3) Low fat diet. **Study:** PREDIMED (Metabolic syndrome participants) **Outcome:** Association between MedDiet food groups and HDL functions	HDL functions are associated with increases in consumption of: 1) Virgin olive oil. 2) Nuts. 3) Legumes. 4) Whole grains.´ 5) Fish.
Objective 3 (Manuscript 3)	**Design:** Randomized controlled trial with two parallel interventions of 6 months: 1) Energy-restricted MedDiet, together with	Intervention group presented lower levels of: 1) ApoC-III in ABDP. 2) Triglycerides in

	physical activity promotion. 2) Traditional MedDiet without calorie restriction. **Study:** PREDIMED-plus (Metabolic syndrome participants) **Outcome:** Changes on HDL functions	ABDP.
Objective 4 (Manuscript 4)	**Design:** Systematic review of dietary (fatty acids, cholesterol, antioxidants, alcohol, and calorie restriction) and physical activity randomized controlled trials. **Outcome:** Changes on HDL functions	MUFAs, PUFAs, dietary antioxidants, dietary rich patterns, ethanol, and dietary cholesterol improved HDL functions.

ABDS: Apolipoprotein B-depleted serum, ABDP: Apolipoprotein B-depleted plasma ApoA-I: Apolipoprotein A-I, ApoC-III: Apolipoprotein C-III, CAD: Coronary artery disease, C3: Complement component 3, HDL-C: HDL colesterol, MedDiet: Mediterranean diet, MUFA: Monounsaturated fatty acids, PUFA: Polyunsaturated fatty acids.

6.2 Association between HDL functions and CAD in a general population

CAD is highly prevalent and one of the leading causes of mortality (3). Nevertheless, many issues related to this condition in population studies remain unclear, thus research into new biomarkers potentially involved in CAD pathophysiology is warranted (38). Among such biomarkers, CEC, HDL antioxidant/anti-inflammatory potential, HDL content of ApoA-I, and HDL components involved in endothelial protection including S1P, have been associated with cardiovascular disease incidence in diverse populations (156–158). In **Manuscript I**, we evaluated the association between these biomarkers and some other HDL functional traits with CAD in a sample of adults representative of the Spanish general population. In particular, we reported an independent association between high C3 levels and low ApoA-I concentrations and increased CAD risk. In addition, augmented S1P levels were linked to lower CAD incidence among participants with desirable HDL-C concentrations.

HDL plays a key role on inflammatory responses in atherogenesis (296). Under certain circumstances, HDLs can become dysfunctional, pro-inflammatory particles when they incorporate acute phase proteins such as C3 into their structure (182). We report for the first time an independent association between C3 in apoB-depleted serum and incident CAD in a general population. C3 concentrations in plasma have been associated with CAD (297), and their levels in HDL particles have been related to acute coronary syndrome occurrence

(142). Moreover, concentrations have been reported to be elevated (although nonsignificantly) in individuals with high unstable angina odds (158,298). In agreement with our results, one anti-inflammatory capacity of HDL, measured as the ability of HDLs to inhibit the secrtetion of vascular cell adhesion molecule-1 from endothelial cells, has been inversely related with CAD incidence in a general population in a recent study (299).

ApoA-I, the major protein in HDLs, is crucial in several HDL functions such as CEC and its antioxidant capacity (145,149,167,300). Plasma levels of this apolipoprotein have been inversely associated with CAD by some authors (104,301,302) but not by others (303). Despite these associations in cohort studies, the causal role of systemic ApoA-I levels is currently questioned, in a similar manner to HDL-C concentrations (149). Such a lack of association with CAD of both HDL-C and ApoA-I concetration could be due to conditions intimately related to low HDL-C. In this respect, low HDL-C and (159)apoA-I levels in circulation are present when triglyceride levels are high (304) and/or HDL functionality is decreased (305). The association between HDL-C levels and cardiovascular risk may also be dose-dependent, since a null association between high HDL-C concentrations and cardiovascular protection beyond a certain threshold level have been reported in intervention trials (306). We cannot rule out that this controversy may be also extended to certain HDL functional properties. In the present work, ApoA-I levels in apoB-depleted serum were inversely associated with CAD when adjusting for HDL functional traits, and this association was stronger in individuals with low HDL-C concentrations. Our results agree with

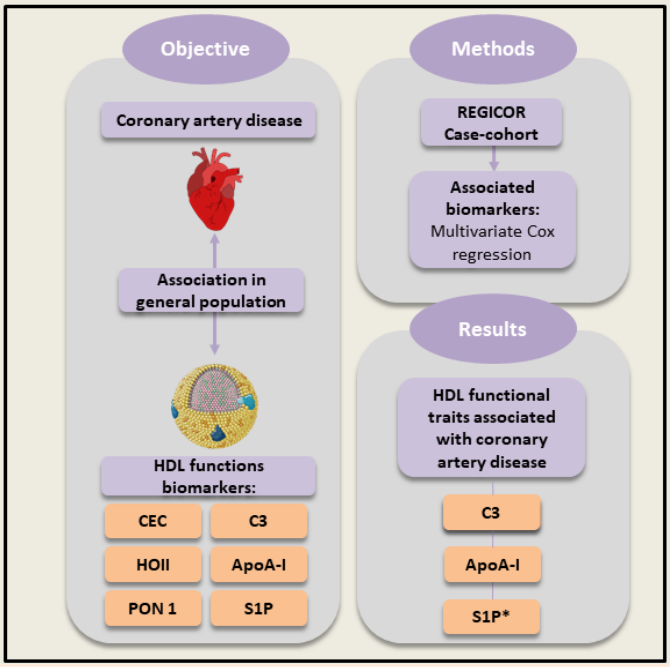
previous findings, such as the association between ApoA-I levels in apoB-depleted serum and greater CAD incidence in older adults at high cardiovascular risk (158). In this regard, the number of ApoA-I molecules bound to HDL particles could be a determinant for particle functionality especially when HDL-C levels are low. Thus, our findings suggest that greater ApoA-I levels could be particularly atheroprotective in high cardiovascular risk populations.

HDLs are also able to protect endothelial cells and promote their functionality (307). One of the essential HDL components in this function is S1P, a bioactive phospholipid involved in endothelial homeostasis (296). According to our findings, increased S1P levels in apoB-depleted serum were associated with decreased CAD risk individuals with optimal HDL-C concentrations. Our results agree with previous evidence, since S1P levels in HDL have been inversely associated with acute coronary syndrome incidence in high risk individuals (158) and the extent of atherosclerotic lesions in stable patients with CAD (308).

In contrast to other studies, we did not find an association between CEC values and incident CAD cases (157,159). Soria-Florido MT et al reported that cholesterol efflux capacity was associated with increased odds of acute coronary syndrome in high cardiovascular risk subjects (158), the article of the present thesis and two other studies were, however, conducted in a general population (157,159). The divergence could be due to several reasons. First, our CEC methodology is slightly different from previous studies. In one of them, the authors used a cell line of J774.1 murine macrophages and only measured

ABCA1-dependent efflux (157), whilst in the other one they used a radiolabeled cholesterol probe instead of a fluorescent one (159). Second, median HDL-C values in these studies were also lower (47 mg/dL (157) and 45 mg/dL (159)) in relation to our population (51 mg/dL). Third, our population is essentially Mediterranean whilst previous studies have either focused on subjects from the United

Figure XI. Graphical abstract of the association between HDL functions and CAD.



ApoA-I: Apolipoprotein A-I. C3: Complement component 3. CEC: Cholesterol efflux capacity. HDL: High density lipoprotein. HOII: HDL oxidative/inflammatory index. PON1: Paraoxonase 1. S1P: Sphingosine-1-phosphate.
*S1P is only associated in participants with desirable HDL cholesterol values (≥40/≥50mg/dL in men/women).

States, with a high prevalence of Afro-Americans (50%) (157), or northern European individuals (159). Finally, the healthy diet in the Mediterranean countries may play a role in decreased CAD incidence in these populations in relation to those in northern Europe or the United States (309).

6.3 Changes in HDL functions after lifestyle interventions

Lifestyle interventions such as following a healthy diet and physical activity are first line strategies for the prevention of primary and secondary CAD (10,256). Some of these, for instance following a MedDiet, improved novel cardiovascular risk factors such as HDL functions The most comprehensive study to date of the effects of a lifestyle intervention on HDL functions was based on a MedDiet rich in extra-virgin olive oil in high cardiovascular risk individuals from the PREDIMED study (285). Weight loss interventions based on the promotion of physical activity have also improved some HDL properties such as antioxidant and anti-inflammatory functions (267,270). In addition, the combination of physical activity with calorie restriction has been associated with improvements in CEC and HDL antioxidant/anti-inflammatory capacities in short-term, non-randomized trials (284,310). Thus, we tried to expand current knowledge by: 1) assessing the relationship between the consumption of cardioprotective food groups from the MedDiet (virgin olive oil, nuts, legumes, fruit/vegetables, whole grains, fish, and wine) and HDL

function (in **Manuscript II**); 2) studying the effects of an intervention with a calorie-restricted Mediterranean Diet and physical activity on HDL functionality (**Manuscript III**); and 3) summarizing in a systematic review the findings of all the randomized, controlled trials to date that investigated the effects of lifestyle modifications on HDL functions (**Manuscript IV**).

Increases in the consumption of virgin olive oil and whole grains were related with increments in CEC values. These results agree with those observed in the systematic review. First, the intake of phenolic compound-rich virgin olive oil has been associated with augmented CEC in two randomized controlled trials in hypercholesterolemic (240) and healthy individuals (239). Such improvements could be in part explained by an increased HDL monolayer fluidity and an increment in HDL antioxidant content. The incorporation of monounsaturated fatty acids from virgin olive oil in HDLs has been related with increments in HDL particle fluidity, a greater flexibility to bind cholesterol transporters (311–313), and finally increased CEC values (314). In addition, the high content in antioxidants in virgin olive oil could counteract the oxidation of HDL functional elements such as proteins (ApoA-I, LCAT (145,147)) and lipids (315). Finally, olive oil phenolic compounds have been shown to be able to induce AMP-activated protein kinase, a metabolic regulator that can trigger hepatic ApoA-I synthesis through the activation of the peroxisome proliferator actvated receptor α and subsequently enhance HDL function (316)(233,317). In relation to whole grains, evidence of their role on HDL function is scarce. Whole grain consumption has not been related with changes in HDL-C and ApoA-I levels (318,319).

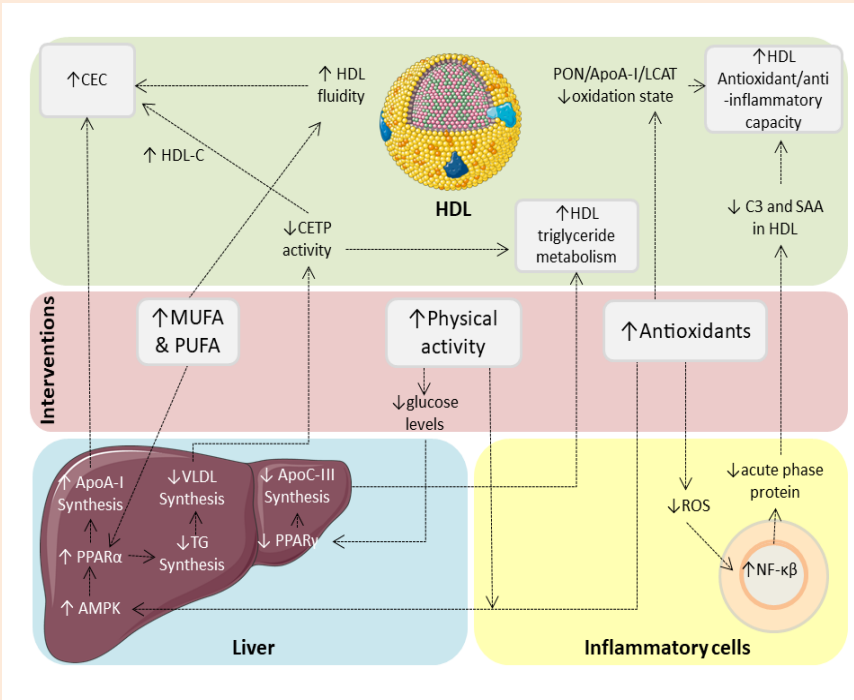
Nevertheless, interventions with fiber have been observed to decrease CETP and increase LCAT activities which could possibly mediate an increment in CEC values (320,321). Besides, dietary cholesterol and ethanol have also been shown to increase CEC, levels of total cholesterol, LDL-C, and HDL-C after the intake of dietary cholesterol (322). Increments of circulating pool cholesterol could result in a higher necessity to return it back to the liver, therefore a greater activity of the enzymes involved in the metabolism of cholesterol would be expected (LCAT, CETP) and augmented CEC (323–327). Concerning ethanol, its consumption has been consistently associated with increases in HDL-C and ApoA-I levels (328), and it is likely that these changes are linked to an improved HDL function. The main molecular hypothesis relies on the capacity of acetate, the principal metabolite of ethanol, to reduce cholesterol ester exchange between HDL and VLDLs through CETP, thus increasing the circulating levels of HDL-C. Acetate is also capable of decreasing lipolysis in adipocytes, subsequently linked to a reduction in the circulating levels of triglycerides in VLDLs, which may contribute to decreasing CETP activity (329). Finally, with respect to more general lifestyle modifications, an intensive lifestyle intervention with an energy restricted MedDiet and physical activity promotion was not able to increase CEC in high cardiovascular risk patients in the PREDIMED-plus study. These results differ from those observed after an intervention with a non-restrictive MedDiet in the PREDIMED study. Such divergence can be explained as follows. First, in the PREDIMED sub-study there were more type 2 diabetic participants (50%) relative to the PREDIMED-plus (35%), these individuals presented particularly low CEC values and could obtain major benefits

from a healthy lifestyle intervention (330). Second, the intervention in the PREDIMED-plus study was shorter and led to a substantial weight loss, which may also have affected CEC values. Finally, the techniques used to evaluate CEC in both studies are not directly comparable. The methodology employed in the PREDIMED-plus samples to evaluate CEC was adapted to a new fluorescent-labelled cholesterol marker instead of the one previously used in the PREDIMED study, radiolabelled cholesterol analog, in order to reduce radiolabelled waste products and facilitate the procedure.

HDL antioxidant and anti-inflammatory capacities are consistently enhanced by antioxidant-rich lifestyle interventions such as the MedDiet. Our results highlight the capacity of the MedDiet, and its cardioprotective food components, to improve such properties. MedDiet antioxidants, which are known to decrease oxidative stress and inflammation (331,332), can be incorporated into the HDL structure and induce a local antioxidant/anti-inflammatory effect (240,333,334). We reported an association between 1-year increases in the consumption of nuts, legumes, and fish and increments in PON1 activity. In addition, HOII, HDL oxidative status, and the circulating levels of C3 and SAA (pro-inflammatory proteins) showed decreased values relative to baseline in all the participants following a MedDiet (both study arms in the PREDIMED-plus sub-study). Our results agree with previous evidence: an intervention with a MedDiet enriched with virgin olive oil was associated with decreased HOII and increased PON1 activity relative to baseline (238). The capacity of antioxidant-rich foods to improve HDL antioxidant/anti-inflammatory capacity was reflected in the systematic review. Lifestyle

interventions with nuts, phenol-rich virgin olive oil, anthocyanins, carotenes, and a whole MedDiet pattern have been shown to be able to increase PON1 activity, decrease HOII, and reduce the levels of pro-inflammatory proteins in HDL (SAA and alpha-1-antitrypsin). In contrast, to the best of our knowledge, the association between legume consumption and higher PON1 activity has not been previously reported. An improvement in HDL antioxidant/anti-inflammatory capacities can be explained as follows. Antioxidants could prevent the oxidation of HDL proteins (145,146) and lipids (315). In addition, through a number of mechanisms antioxidants also lead to decreased low-grade inflammation (218,226,234,335). This is linked to lower levels of pro-inflammatory molecules in circulation, which in turn bind less to HDL particles. Fish intake has also been associated with greater PON1 activity in our data, although some results have proven to be controversial. Interventions with fish-derived omega 3 fatty acid supplements have been reported to increase PON1 activity compared to placebo in participants with high cardiovascular risk (336–338), whilst no differences were found in other studies in women (339) and type 2 diabetes individuals (340). Moreover, elevated doses of omega 3 fatty acids were shown to induce a detrimental effect on HOII in heart failure patients, attributed to a dose-dependent effect (341). Further research is therefore warranted.

Figure XII. Possible mechanisms for the effect of lifestyle interventions on HDL functions



AMPK: AMP-activated protein kinase. ApoA-I: Apolipoprotein A-I. ApoC-III: Apolipoprotein C-III. C3: Complement component 3. CEC: Cholesterol efflux capacity. CETP: Cholesteryl ester transfer protein. HDL: High density lipoprotein. HDL-C: HDL cholesterol. LCAT: Lecithin cholesterol acyltransferase. MUFA: Monounsaturated fatty acids. NF-κβ: Nuclear factor kappa B. PPARα: Peroxisome proliferator activated receptor alpha. PPARγ: Peroxisome-proliferator activated receptor gamma. PON: Paraoxonase. PUFA: Polyunsaturated Fatty Acids. ROS: Reactive oxygen spices. TG: Triglycerides. SAA: Serum amyloid A. VLDL: Very low-density lipoprotein.

The contribution of HDLs to triglyceride metabolism has been usually overlooked in favour of their role in cholesterol transport. Nevertheless, in recent years it has been increasingly studied. First,

triglyceride-rich HDLs have been causally associated with CAD (199). Triglyceride accumulation in the HDL core disturbs its structure and results in an impaired HDL function (196). In addition, HDL can carry ApoC-III, a key regulator of triglyceride metabolism and an emergent risk factor associated with CAD in plasma (342). ApoC-III content in HDLs has been directly associated with CAD (343,344). In our data, an intervention with an energy-restricted MedDiet and physical activity decreased the levels of triglycerides and ApoC-III in HDL particles compared to the group following a non-restrictive MedDiet. Weight loss was shown to be the main mediator of this change. As far as we know, this is the first report of such an effect after an intensive lifestyle intervention with weight loss goals. There are some plausible hypotheses to explain how weight loss could modify levels of triglycerides and ApoC-III in HDLs. Aerobic exercise can decrease triglyceride synthesis in the liver by the stimulation of AMP-activated protein kinase (233). The hepatic synthesis of ApoC-III is in turn stimulated by high levels of circulating glucose (capable of activating the carbohydrate response element-binding protein) and a high dietary intake of saturated fatty acids (activators of PPARγ). (345,346). The intensive lifestyle intervention decreased glucose levels, and the dietary intake of saturated fatty acids was moderated when compared to the control arm, both changes may thus decrease the levels of ApoC-III in plasma and subsequently in HDL particles.

Finally, lifestyle interventions have also been reported to improve the role of HDLs on endothelial integrity. As mentioned in the systematic review, a 1 year intervention with a traditional MedDiet enriched with extra-virgin olive oil increased the capacity of HDL to promote the

release of nitric oxide from endothelial cells compared to a low fat one (291). Our results, however, showed a decrease of S1P in HDL levels relative to baseline (although non-significant compared to the control group). Weight loss in the intensive intervention group could have been responsible for the reduced plasma concentrations of S1P and the subsequent lower levels in HDLs. Obesity is linked to hyperactivated adipose tissue (related to greater lipolysis) which leads to increased non-esterified fatty acids in circulation (347). These fatty acids can be transformed in the liver into ceramides such as S1P (348) thus explaining a decrease in S1P levels in HDLs after weight loss (349).

6.4 Strengths and limitations

The present thesis has several general strengths and limitations to consider. Strengths: First, a comprehensive profile of the HDL functions has been analyzed across all the studies. Second, all the population-based studies presented a large sample size, which increases the statistical power of the analysis. Third, we used standardized protocols to evaluate all the HDL functions and components, together with pools from healthy volunteers to reduce inter-assay variability and assure a proper quality control. Finally, all the results obtained from the lifestyle intervention trial in **Manuscript III** are complemented and better contextualized with the evidence summarized in the systematic review of 118 randomized controlled trials. Limitations: Some HDL functions have not been tested in all the studies. For example, PON1 activity could not be measured in the

PREDIMED-plus because only plasma treated with ethylenediaminetetraacetic acid was available (it chelates calcium, which is necessary for the activity of PON1, and therefore cannot be measured). In addition, the use of complex in-vitro techniques may not reflect the physiological presence of counter-regulatory mechanisms and the complexity of the final effect of the intervention in humans.

Due to the different nature of the three population-based studies analyzed, there are also specific strengths and limitations of each of the articles included in this thesis.

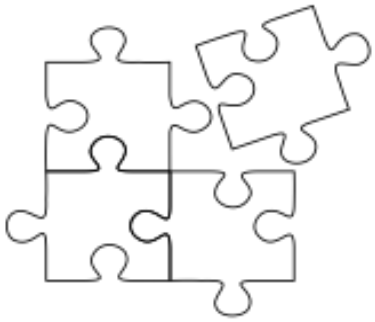
Manuscript I (case-cohort study within the REGICOR study). Strengths: As far as we know, it was the first study to evaluate the relationship between a comprehensive, hypothesis-driven range of HDL functionality traits and CAD incidence in a general population. It was a well-characterized population sample with data on several health outcomes. Limitations: First, the number of CAD events from our population was limited and may have affected the statistical power of the study. The reduced number of CAD events could be in part explained by the low incidence of CAD found in Mediterranean countries (350). Second, our results could only be generalized to a general population. Finally, HOII presented 15% missing values due to the complexity of the laboratory process (for the rest of the determinations this proportion was <2%) which may have hindered its association with CAD.

Manuscript II (cohort study within the context of the PREDIMED controlled trial). Strengths: We provided a quantitative measurement of the favorable effects of food groups on HDL functions (as percentage changes). In addition, prospective data were obtained from a large sample size using standardized protocols to evaluate HDL functions. Limitations: First, this was a prospective analysis, thus our conclusions should be confirmed in randomized controlled trials. Second, our results were obtained in a population at high cardiovascular risk and cannot be extrapolated to other populations. Finally, the intervention was based on real-life dietary modifications that, as expected, could be linked to modest changes on HDL functions.

Manuscript III (randomized controlled trial with a sub-sample of PREDIMED-plus participants). Strengths: Its main strength was the large sample size and its randomized design, which reduces the presence of bias and confounding factors. To the best of our knowledge, this has been the largest study to date to evaluate the effect of an intensive lifestyle intervention with specific weight loss goals in a set of HDL functions (hypothesis-driven selected). Limitations: First, in a similar manner to the second manuscript, results were obtained from elder participants with metabolic syndrome and cannot be generalized to other populations. Second, both groups followed a real lifestyle intervention adapted to an elder population (with a control group designed as an active comparator) and their diet patterns were Mediterranean-like, one restrictive and the other non-restrictive. Consequently, as expected, the variations between groups were modest. Finally, differences among groups regarding energy

restriction were modest compared to those from physical activity. Nevertheless, the energy restriction goals were planned with a view to maintain long-term weight loss and our analyses only showed changes for the first 6 months of the PREDIMED-plus study.

Manuscript IV (systematic review of randomized controlled trials). Strengths: It is at present the most comprehensive review summarizing the results of randomized controlled trials concerning the effects of lifestyle interventions on HDL functions. Limitations: First, a considerable number of the trials were short-term interventions with small sample sizes. Second, a high proportion of the studies included (54.2%) were attributed an unclear risk of bias according to the Cochrane tool. For that reason, presence of bias cannot be dismissed. Finally, the considerable heterogeneity in the description of exposures and outcomes prevented us from meta-analyzing the results which need to be interpreted with caution.



7. Conclusions

7.1. Association between HDL functions and CAD (Manuscript I)

General conclusion:

- There is an association between CAD and HDL functional traits in a general population.

Specific conclusions:

1) High levels of C3 and low concentrations of ApoA-I in apoB-depleted serum were associated with decreased CAD incidence.

2) High concentrations of S1P in HDL were linked to decreased CAD risk in participants with desirable HDL-C values.

7.2. Effects of lifestyle interventions on HDL functions (Manuscripts II, III, IV)

General conclusion:

- Healthy lifestyle interventions (such as a MedDiet and its cardioprotective food groups, or the combination of calorie

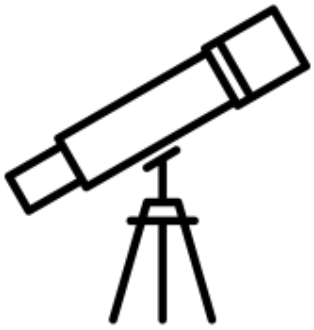
restriction and physical activity) are relevant strategies to improve a wide variety of HDL functions.

Specific conclusions:

1) Increases in the consumption of virgin olive oil, nuts, legumes, whole grains, and fish were associated with improvements in HDL functions (CEC, CETP activity, PON1 activity, and HDL capacity to promote the release of nitric oxide from endothelial cells).

2) A lifestyle intervention with a calorie-restricted MedDiet and physical activity improved the role of HDLs in triglyceride metabolism relative to a non-restrictive MedDiet control group in older adults with metabolic syndrome. Both intervention arms (based on a MedDiet pattern) were associated with improvements in HDL antioxidant/anti-inflammatory properties relative to baseline.

3) According to the literature, dietary interventions with monounsaturated and polyunsaturated fatty acids (particularly long-chain, omega-3 fatty acids in fish) and dietary antioxidants such as phenolic compounds can enhance HDL functions, particularly in individuals with cardiovascular risk factors.

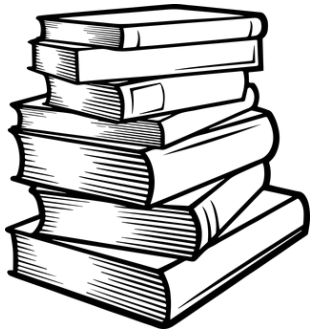


8. Future perspectives

This thesis reports an association between certain HDL functional traits and CAD in the general population. This result opens up several opportunities for future research projects. First, the next step could be to evaluate the causal nature of the association between HDL functions and CVD. A possible technique to study this question would be Mendedelian randomization. To perform this technique, we would search for genetic variants independently associated with the different HDL functions, and would then assess whether they are linked to CAD or other CVD. Second, to ensure reproducibility, our results should be replicated in larger prospective trials and in different populations. Third, there are other less studied HDLs functions that might be also associated with CVD, such as HDL antithrombotic capacity and HDL ability to improve glucose metabolism. To this purpose, suitable in vitro techniques should be standardized in the context of the REGICOR study to evaluate the association of these properties with CAD. Finally, our group is also focused on the identification of other biological determinants of HDL function. For example, it would be interesting to identify which microRNAs are associated with HDL functions (an ongoing project led is currently studying this question in biological samples of the REGICOR and the PREDIMED-plus studies).

The present thesis has also described how lifestyle interventions could modify HDL functions. In the PREDIMED-plus trial, we have shown improvements in HDL functions after 6-months of weight loss within a healthy lifestyle intervention. However, the effect of longer-term weight reductions on HDL functions is still unclear. It would also be pertinent to evaluate whether these effects are also observed in other

populations with different ethnicity, or after other weight loss interventions (e.g. intermittent fasting). In addition, other healthy dietary patterns may also enhance HDLs functions. For example, there is an increasing interest in vegetarian and vegan lifestyles and in dietary patterns that restrict meat intake. Therefore, assessing the effects of these diets on HDL functions (compared, for example, to a traditional MedDiet) would contribute to the advance of this discipline.



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