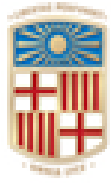# UNIVERSITAT DE BARCELONA

Role and predictive value of the HDL function for cardiovascular disease in a high risk population. HDL function-linked markers and mechanisms

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CONTENT

ABBREVIATIONS

ABDP: Apolipoprotein B-depleted plasma

ACS: Acute coronary syndrome

AMI: Acute myocardial infarction

Apo: Apolipoprotein

BMI: Body mass index

CEC: Cholesterol efflux capacity

CETP: Cholesterol ester transfer protein

CHD: Coronary heart disease

CI: Confidence intervals

CVD: Cardiovascular disease

CVR: Cardiovascular risk

CVRFs: Cardiovascular risk factors

HDL: High-density lipoprotein

HDL-C: High-density lipoprotein cholesterol

HOII: HDL oxidative-inflammatory index

HZ: Hazard ratio

LDL: Low-density lipoprotein

LDL-C: Low-density lipoprotein cholesterol

OR: Odds ratio

PAF-AH: Platelet activating factor acetylhydrolase

RR: Risk ratio

S1P: Sphingosine-1-phosphate

SA: Stable angina

SAA: Serum amyloid A

SD: Standard deviation

UA: Unstable angina

ABSTRACT

Cardiovascular disease (CVD) remains the first cause of death worldwide. The Framingham Heart Study reported that low concentration of high density lipoprotein (HDL) cholesterol (HDL-C) was an independent predictor of heart disease, evidence later confirmed by several epidemiologic studies. Pharmacological trials and Mendelian randomization studies have, however, failed to unequivocally prove an inverse association between HDL-C levels and the risk of cardiac outcomes. Interest is thus now focused on the atheroprotective functions of the HDL particle rather than the mere content of cholesterol transported by the lipoprotein.

The overall aim of the present thesis was to evaluate the predictive role for cardiovascular diseases of a battery of HDL properties and components. Moreover, we evaluated the independent relationships among major CVD risk factors, 10-year CVD risk, and HDL-functionality and low-density lipoprotein (LDL) atherogenicity as subjacent mechanisms of cardiovascular risk.

Due to the novel character of this approach, we conducted a meta-analysis to obtain an updated record of the available published literature in this field. For the meta-analysis, we selected observational studies showing the potential role of HDL function in predicting cardiovascular outcomes and all-cause mortality. We presented results as a combination of odds and hazard ratios for the highest functionality values versus the lowest and for 1-SD change together.

To assess the prognostic value for CVD of a set of novel surrogates of HDL-functionality and composition, we analyzed cholesterol efflux capacity (CEC), HDL oxidative-inflammatory index (HOII), platelet activating factor acetylhydrolase (PAF-AH) activity bound to HDL, and the concentrations of apolipoprotein (Apo) A-I and ApoA-IV, serum amyloid A (SAA), component Complement C3, and sphingosine-1-

phosphate (S1P) in samples of ApoB depleted plasma. To this purpose, we designed a nested case-control study with volunteers at high cardiovascular risk from the PreDiMed Study (*Effects of Mediterranean Diet on the Primary Prevention of Cardiovascular Disease*). Samples were matched in a 1:2 fashion by age, sex, body mass index, intervention group, and follow-up time in the study at event occurrence.

Finally, to gain deeper knowledge of the mechanisms involved in the protective role played by HDL, we cross-sectionally analyzed the relationships among HDL function, LDL atherogenicity, and the risk for cardiovascular disease measured by the FRAMINGHAM-REGICOR risk score, and classical risk factors appraised individually. For the analysis, we used data from two representative subsamples of volunteers from the PreDiMed trial.

Results from the meta-analysis support the role of HDL functionality as a predictor of cardiovascular disease. Analysis of data from the 25 studies included revealed an inverse association for cholesterol efflux and antioxidant/anti-inflammatory capacities with the risk of major adverse cardiac outcomes. This inverse association was also confirmed for the risk of all-cause mortality in the case of cholesterol efflux and antioxidant capacity.

Findings from the case-control study are in agreement with those of the meta-analysis, and display HDL-related function and its main protein, ApoA-I, as promising biomarkers of certain cardiovascular outcomes. Higher CEC, HOII, and ApoA-I levels are strong, independent surrogates of acute coronary syndrome (ACS). Moreover, S1P almost reached statistical significance, with a p-value=0.056, in the model including HDL-C as confounder. CEC and ApoA-I were both associated with a greater risk for acute myocardial infarction (AMI) independent of classical cardiovascular risk factors. Finally, higher HOII and lower ApoA-I (irrespective of type 2 diabetes mellitus, hypercholesterolemia,

hypertension, and smoking habit) increased the risk of unstable angina (UA).

High cardiovascular risk according to the FRAMINGHAM-REGICOR score was related to low HLD-C and ApoA-I, poor ability of HDL to efflux cholesterol from macrophages, and a dysfunctional profile of the lipoprotein (higher content in triglyceride, more oxidized and smaller in size) and with high levels of ApoB and pro-atherogenic LDL (smaller and less resistant to oxidation). Relationships among each classical risk factor and the characteristics and state of the lipoproteins, individually assessed in fully adjusted multivariate linear regression models, disclosed that volunteers with type-2 diabetes had low levels of HDL-C, ApoA-I, low-density lipoprotein cholesterol (LDL-C), and ApoB, and smaller, more oxidized LDL. Systemic hypercholesterolemia was related to greater HDL-C, LDL-C, ApoA-I, and ApoB levels and to small HDL size, higher CETP activity, and lower HDL capacity to esterify cholesterol. In addition, each increase of 1 kg/m^2 in body mass index was associated with low HDL-C, lower size of both HDL and LDL, and decreased HDL capacity to esterify cholesterol. Finally, men presented lower HDL-C and ApoA-I levels, greater HDL oxidation and HDL impaired vasodilatory capacity, and higher cholesterol content in LDL particles. Age was related to greater triglyceride content in the HDL core.

In summary, the meta-analysis study showed that CEC, and the anti-inflammatory and antioxidant capacities of HDL, are inverse predictors of CVD. The association persisted with CEC and antioxidant capacity for all-cause mortality risk. These results were further confirmed in the case-cohort study nested to a cohort at high risk for cardiovascular disease, in which CEC at baseline showed a strong and independent inverse association with the risk of ACS and AMI. In concordance, ApoA-I had the ability to predict ACS, AMI, and US. Furthermore, HOII proved to be a predictive index for ACS and UA. Finally, as expected in light of these findings, high cardiovascular risk scores and classical risk

factors for cardiovascular disease were associated with impaired HDL regarding its components, particle size, and function, and with a more pro-atherogenic LDL.

RESUMEN

Las enfermedades cardiovasculares (CVD, por sus siglas en inglés) son la primera causa de muerte en el mundo. El estudio Framingham demostró que una elevada concentración de colesterol asociado a la lipoproteína de baja densidad (HDL, por sus siglas en inglés) era un predictor independiente de enfermedades del corazón, evidencia posteriormente confirmada por varios estudios epidemiológicos. Sin embargo, ensayos farmacológicos y estudios de randomización Mendeliana no han podido probar inequívocamente la relación inversa entre los niveles de colesterol en HDL (HDL-C, por sus siglas en inglés) y el riesgo de evento cardiaco. Por lo tanto, actualmente el interés se centra en las funciones ateroprotectoras de la partícula de HDL y no solo en el contenido de colesterol transportado por la lipoproteína.

El objetivo general de la presente tesis fue evaluar el papel predictivo de las enfermedades cardiovasculares de una batería de propiedades y componentes HDL. Además, evaluamos las relaciones independientes entre los principales factores de riesgo cardiovascular, el riesgo de CVD a 10 años y la funcionalidad de HDL y la aterogenicidad de las lipoproteínas de baja densidad (LDL, por sus siglas en inglés), como mecanismos subyacentes de riesgo cardiovascular.

Debido al carácter novedoso de este enfoque, realizamos un meta-análisis para tener un registro actualizado de la literatura disponible en este campo de estudio. Para el meta-análisis, seleccionamos estudios observacionales sobre el papel de la función de HDL en la predicción de los resultados cardiovasculares y la mortalidad por todas las causas. Presentamos los resultados como una combinación de odds ratio y hazard ratio para los valores de funcionalidad más altos frente a los más bajos y para el cambio de 1-SD conjuntamente.

Para evaluar el valor pronóstico de enfermedades cardiovasculares de un conjunto de marcadores novedosos de la funcionalidad y

composición de HDL, analizamos la capacidad de eflujo de colesterol (CEC, por sus siglas en inglés), el índice oxidativo-inflamatorio de HDL (HOII, por sus siglas en inglés), la actividad acetilhidrolasa del factor activador de plaquetas (PAF-AH, por sus siglas en inglés) unido a HDL y la concentración de apolipoproteína (Apo) A-I y A-IV, proteína amiloide A sérica (SAA, por sus siglas en inglés), componente C3 del complemento y esfingosina-1-fosfato (S1P, por sus siglas en inglés) en muestras de plasma depletadas en ApoB. Con este fin, diseñamos un estudio anidado de casos y controles con voluntarios con alto riesgo cardiovascular del Estudio PreDiMed (Efectos de la Dieta Mediterránea en la Prevención Primaria de Enfermedades Cardiovasculares). Las muestras se combinaron de forma 1: 2 por edad, sexo, índice de masa corporal, grupo de intervención y tiempo de seguimiento en el estudio en el momento en que se produjo el evento.

Finalmente, para obtener un conocimiento más profundo de los mecanismos implicados en el papel protector del HDL, analizamos de forma transversal las relaciones entre la función del HDL, la aterogenicidad del LDL y el riesgo de enfermedad cardiovascular medido por el score de riesgo FRAMINGHAM-REGICOR y los factores de riesgo clásicos evaluados individualmente. Para el análisis, utilizamos datos de dos submuestras representativas de voluntarios del ensayo PreDiMed.

Los resultados del meta-análisis respaldan el papel de la funcionalidad HDL como predictor de enfermedad cardiovascular. El análisis de los datos que comprende 25 estudios incluidos, reveló una asociación inversa para la capacidad de eflujo de colesterol y la capacidad antioxidante y antiinflamatoria de HDL con el riesgo de eventos cardiacos adversos. Esta asociación inversa también se confirmó para el riesgo de mortalidad por cualquier causa en el caso de la capacidad de eflujo de colesterol y la capacidad antioxidante.

Los resultados del estudio de casos y controles concuerdan con los del meta-análisis y muestran la funcionalidad asociada a HDL y su principal proteína, la ApoA-I, como prometedores biomarcadores de determinados eventos cardiovasculares. Niveles más altos de CEC, HOII y ApoA-I son fuertes e independientes predictores del síndrome coronario agudo (ACS, por sus siglas en inglés). Además, S1P casi alcanzó significación estadística, con un valor p = 0.056, en el modelo que incluye HDL-C como factor de confusión. La CEC y ApoA-I se asociaron a un mayor riesgo de infarto agudo de miocardio (AMI, por sus siglas en inglés), ambos independientemente de los factores de riesgo cardiovascular clásicos. Finalmente, un HOII más alto y una ApoA-I más baja (independientemente de diabetes mellitus tipo 2, hipercolesterolemia, hipertensión y tabaquismo) aumentaron el riesgo de angina inestable (UA, por sus siglas en inglés).

Un alto riesgo cardiovascular según el score FRAMINGHAM-REGICOR se relacionó con niveles bajos de HLD-C y ApoA-I, con una reducida capacidad de HDL para extraer el colesterol de los macrófagos y un perfil disfuncional de la lipoproteína (mayor contenido en triglicéridos, más oxidada y más pequeña) y con altos niveles de ApoB y con una LDL proaterogénica (más pequeña y menos resistente a la oxidación). Las relaciones entre cada factor de riesgo clásico y las características de las lipoproteínas se evaluaron en modelos de regresión lineal múltiple totalmente ajustados. El análisis reveló que los voluntarios con diabetes tipo 2 tenían niveles bajos de HDL-C, ApoA-I, colesterol en lipoproteínas de baja densidad (LDL-C) y niveles de ApoB y LDL más pequeñas y más oxidadas; la hipercolesterolemia sistémica se relacionó con mayores niveles de HDL-C, LDL-C, ApoA-I y ApoB y con HDL de pequeño tamaño, mayor actividad de CETP y menor capacidad de HDL para esterificar el colesterol, y cada aumento en 1 kg / m2 se asoció con niveles bajos de HDL-C, HDL y LDL de menor tamaño y una menor capacidad de HDL para esterificar el colesterol. Finalmente, los hombres presentaron niveles más bajos de HDL-C y ApoA-I, una mayor

oxidación de HDL y una capacidad vasodilatadora disminuida de HDL y un mayor contenido de colesterol en partículas de LDL, y ser mayor se relacionó con un mayor contenido de triglicéridos en el núcleo de HDL.

En resumen, el meta-análisis mostró que la CEC y las capacidades antiinflamatorias y antioxidantes del HDL son predictores inversos de enfermedades cardiovasculares. La asociación se mantuvo para el riesgo de mortalidad por cualquier causa con la CEC y capacidad antioxidante de HDL. Estos resultados se confirmaron en el estudio de caso-cohorte anidado en una población con alto riesgo de enfermedad cardiovascular, en la que la CEC al inicio mostró una asociación inversa fuerte e independiente con el riesgo de ACS e IAM. En concordancia, ApoA-I presentó capacidad de predecir ACS, AMI y UA. Además, el HOII demostró ser un índice predictivo para ACS y UA. Finalmente, como se esperaba a la luz de estos resultados, altos score de riesgo cardiovascular y los factores de riesgo clásicos para la enfermedad cardiovascular se asociaron con una HDL alterada en relación a composición, tamaño de partícula y función, y con una LDL más proaterogénica.

1. Introduction

1.1. CARDIOVASCULAR DISEASES

Cardiovascular Disease (CVD), which affects the heart and blood vessels, encompasses (i) coronary heart disease (CHD) as well-known as coronary artery disease, caused by the total or partial obstruction of the arteries that supply blood to the heart; (ii) peripheral artery disease, characterized by stenosis and/or occlusion of large and medium-sized arteries, and (iii) cerebrovascular disease, in which blood vessels that supply blood to the brain are affected (1). Acute coronary syndrome (ACS) is the most common presentation of CHD and refers to any group of symptoms compatible with acute myocardial ischemia. The syndrome includes clinical conditions ranging from unstable angina to non-ST-segment elevation myocardial infarction (AMI) and ST-segment elevation myocardial infarction (2).

1.1.1.   Epidemiology of cardiovascular diseases

Nowadays, CVD remains the worldwide leading cause of mortality. Is responsible for 31.5% of deaths each year despite substantial decreases in mortality and improvements in survival rates with ischemic heart diseases and cerebrovascular diseases accounting for the largest percentage (3).

Nearly 20% of CVD deaths affect subjects under the age of 65 (4). Considering that the lifespan of the Spanish population in 2017 was 83 years according to the National Institute of Statistics, (http://www.ine.es/jaxiT3/Datos.htm?t=1414), CVD is a primary source of loss potential lifeyears. Furthermore, CVD is not only a cause of death, but also a loss of productivity and disability rise by 150 million in 2020 (5).

Although age-standardized rates of mortality attributable to CVD dropped from 1990 to 2013, the global number of deaths increased over the same period, which is associated with an aging population (3,6).

The World Health Organisation has concluded that around 75% of premature CVD is preventable (7) trough the adoption of a healthy lifestyle including smoking cessation, weight loss, healthy diet, and regular exercise (8,9), and The United Nations member states have set a target of a 25% reduction in premature death attributable to non-communicable heart diseases, including CVD by the year 2025.

1.2. ATHEROSCLEROSIS

Atherosclerosis is the underlying cause of CVD. It is associated with acute coronary syndrome (10,11) and although it has been well documented as beginning in early childhood (12) it may remain silent until adult life (13).

Atherosclerosis is an asymmetrical focal thickening of the inner artery layer leading to the narrowing of the arterial lumen. Progression of atherosclerotic plaque may decrease or eventually block the blood flow to the heart (14), resulting in a broad spectrum of clinical disease. It is the consequence of the combination of multiple processes and a cluster of components belonging to the local arterial microenvironment (15) including lipid disturbances, cell activation, inflammation, oxidative stress and cell apoptosis (16).

1.2.1. Physiopathology of atherosclerosis

a. Initiation of atherosclerosis process

There are several theories aiming at explaining the commencement of atherosclerosis. The "response-to-retention" and "response-to-injury" theories (17,18) which offer a sensitive explanation of the mechanisms behind have grown in importance over the years.

According to the "response-to-injury" theory, endothelial cells become injured and dysfunctional as result of the action of several factors. Among these, turbulent blood flow on the arterial wall has lead to

considerable research on the role of hypertension in the development of atherosclerosis as a risk factor. This could lead to the detachment of endothelial cells, allowing the platelet to aggregate, and to infiltrate the intima layer along with a range of plasma constituents, infiltrate the intima layer. Such a scenario triggers the migration and proliferation of smooth muscle cells from the media. Endothelial cells being deposited at the focal site to regenerate the injured tissue could pose a greater risk, contributing to the progression of atherosclerosis.

These stimuli, jointly contribute to endothelial dysfunction and are in line with the "response-to-retention" theory. In short, this postulates that the earliest change in the arterial wall contributing to the establishment of the atheroma plaque is the retention of apolipoprotein (Apo) B-containing lipoproteins, mainly low-density lipoproteins (LDL), in the sub-endothelial space. The artery wall comprises three layers. The tunica intima is a monolayer of endothelial cells, called endothelium, separated by the outer layers by the basal lamina. The tunica media, with many layers of smooth muscle cells and an elastic membrane adjacent to the tunica externa, rich in connective tissue (19). The endothelium responds to both humoral and mechanical stimuli of maintaining vascular homeostasis.

Nevertheless, the factors responsible for the initial retention of lipoproteins in the intima and lesion initiation are still unclear, even thought it has been demonstrated that once LDLs get trapped, they are susceptible to undergo several modifications, of which oxidation is of particular importance (18). Proteoglycans of the extracellular matrix and lipolytic enzymes released by resident cells, such as lipoprotein lipase and sphingomyelinase, enhance the adherence of LDL to the matrix surrounding the endothelial and smooth muscle cells. Furthermore, it has been documented that the binding of native LDL to proteoglycans increases the susceptibility of these lipoprotein to undergo oxidation (20).

b.   Progression of atherosclerosis

The second relevant step in the development of atherosclerosis is the activation of endothelial cells by oxidized LDL, via lectin-like oxidized LDL receptor-1, which is present not only in endothelial cells, but also in macrophages and smooth muscle cells (21–23). Activated endothelial cells release ^{chemoattractant} proteins, namely, vascular adhesion molecule-1, intracellular adhesion molecule-1, P-selecting (24) and endothelial leukocyte adhesion molecule-1 (25) which attract and direct the migration of leukocytes and monocytes into the intima layer of the artery.

Among the large number of cells that have been described as playing a role in the development and progression of atherosclerosis, macrophages emerge as key cells in the process. Once localized into the innermost layer of the artery, monocyte differentiates into macrophage. These cells engulf modified lipoproteins in a unlimited manner to become load-lipid cells, known as "foam cells" (26).

Cells involved in the initiation and progression of atherosclerosis produce a wide range of broadly connected pro-inflammatory substances. Interleukin-1 and tumor necrosis factor-α directly upregulate iInterleukin-6 synthesis, the latter being involved in the synthesis of C-reactive protein in the liver (27). Macrophages and smooth muscle cells are additionally involved in the secretion of chemokines, contributing to the maintenance of pro-inflammatory environment (28,29). C-reactive protein is implicated in the activation of complementary system leading to the production of pro-inflammatory mediators and to platelet adhesion to the endothelium (30). Moreover, macrophage foam cells promote proliferation and migration of smooth muscle cells into the intima contributing to the formation of a vulnerable fibrous cap(31).

c.   Resolution of atherosclerosis: plaque rupture

The stability of the cap depends on the presence of connective tissue matrix proteins, mainly, collagen, elastin and proteoglycans. Plaque breakdown occurs where the fibrous cap is thin and partly destroyed. In these specific sites, a high concentrations of T-lymphocytes and macrophages impairs the synthesis of collagen by smooth muscle cells, diminishes cell proliferation and increases apoptosis, compromising the restoration of partly destroyed atherosclerotic plaque (32). Apoptosis of foam cell death in plaques leads to the spill of lipids which expand the lipid core (33), and exert mechanical stress in the increasingly thinner plaque. In addition, metalloproteinases released mainly by macrophages, degrade the extracellular matrix reducing fibrous cap thickness and collagen content. They can also promote the flux of macrophages into the cap, encourage smooth muscle cells migration and proliferation, and angiogenesis, which could precipitate plaque rupture (34).

In most cases, plaque disruption leads to nonlethal coronary thrombosis (35). However, it could result in coronary thrombosis, present in 60-70% percent of cases of AMI, which is the most prevalent cause of premature death (36,37).

1.3. RISK FACTORS OF HEART DISEASE

Currently, the most widely used tool for the evaluation of future heart disease risk is the Framingham Risk Score. The model was built by Wilson et al in 1998 (38), and has been proven as an independent, reproducible instrument to calculate the risk of an individual of developing a coronary event within 10 years. Furthermore, it has been validated not only in the American population but in other communities such as the Spanish one (39).

The score includes a cluster of risk factors commonly described as "traditional": age, gender, blood pressure, smoking habit, hypercholesterolemia, diabetes mellitus and obesity (40–46). Globally, all the risk factors included in the score have been largely related to the risk and extent of CVDs (47,48). In this regard, dyslipidemia is one the most prevalent classical risk factors among individuals experiencing an acute coronary event (49,50).

Despite progress made on prevention and early diagnosis of CVD, incidence rates highlight the great challenge and burden they pose for public health. Several studies have reported that around 10-20% of patients presenting a CVD are free from major traditional risk factors (51,52). Moreover, around 55-80% of AMIs are manifested in individuals at moderate risk (53,54), in whom silent atherosclerosis is present as sudden death or a disabling heart attack. Akosah et at. reported that almost 70% of patients with a first AMI were classified as low risk (55). Additionally, several studies have demonstrated limitations of the score with underestimation of risk, particularly in young individuals and women (56), and overestimation in Japanese American men, Hispanic men, and Native American men and women (57).

In this scenario, a considerable effort has been devoted in identifying new markers that, along with traditional risk factors, could help to better predict the development of events in populations at moderate risk (58,59).

1.3.1. Dyslipidemia and cardiovascular disease

Dyslipidemia is a lipid profile disorder characterized by high levels of low-density lipoprotein cholesterol (LDL-C), high levels of systemic triglycerides and low levels of high-density lipoprotein cholesterol (HDL-C) (50,60).

Large epidemiologic studies have identified a high risk for CVD associated with high levels of LDL-C (61,62). In 2010, a meta-analysis compiling data from 26 trials, each comprising of at least 1000 participants and at least two years of follow-up, showed that reductions of 1mmol/L of LDL-C achieved a decrease of 28% in mayor vascular events (63). In addition lower LDL-C levels were associated with a 3-fold greater reduction in the risk of CVD for each lower unit of LDL-C compared to the risk reduction associated with statin treatment, reinforcing the role of early detection in preventing the outset of disease (64).

Similar results from trials displayed triglyceride circulating concentration as independent predictors for risk of ischemic heart disease (65) as reliable as LDL-C (66). However, independence of triglyceride levels from HDL-C on the prediction of risk has been under evaluation for some time (67) and several studies have stated that the concurrence of hypertriglyceridemia and low HDL-C levels in the same patient could be a better predictor than levels of triglycerides alone (68). In this regard, atherogenic dyslipidemia, which confers a lipid-associated CVD residual risk, is characterized by an excess of serum triglycerides, low HDL-C and ApoA-I levels, and small, dense LDL molecule, all associated with cardiovascular risk despite optimal concentrations of total and LDL cholesterol (69).

The present thesis is focused on the study of the potential role of high-density lipoprotein (HDL) characteristics as markers of heart disease, which will be discussed in detail hereafter.

1.4. HIGH DENSITY LIPOPROTEIN CHOLESTEROL LEVELS AND CARDIOVASCULAR DISEASE

Early in the 1970s, a number of studies revealed the impact of HDL-C levels on a range of CVD. In 1977, Gordon et al outlined HDL as an inverse predictor stronger than LDL-C levels and triglycerides for CVD (70). The same year, researchers from The Tromsø heart-study drew similar conclusions regarding the independent predictive value of HDL-C on heart diseases (71). Since then, many clinical and epidemiological studies have supported the impact of circulating levels of cholesterol in HDL and onset of cardiovascular outcomes (38,72). All of which laid the way for a large body of research aimed at describing the mechanisms and biological explanation behind this association, and opened lines of research focused on new therapeutic approaches (73,74).

The vast majority of pharmacological studies focused on increased circulating HDL-C to reduce the incidence of CVD are based on the use of niacin, cholesteryl ester transfer protein (CETP) inhibitors and fibric acid derivatives (75).

The AIM-HIGH trial, an intervention trial with niacin in patients with established cardiovascular disease and LDL-C levels lower than 70 mg/dL, failed to demonstrate reductions in the risk of death from CVD (including nonfatal AMI) despite significant improves in HDL-C (76). These results were in line with findings from the HPS-2/THRIVE trial, whose primary goal was to assess the effects of raising HDL cholesterol on the risk of heart attack or coronary death, stroke, or revascularization in individuals with a history of circulatory problems. Allocation to niacin group, had no beneficial effect on the incidence of major vascular events after a median follow-up of 3.9 years (77).

Regarding CETP in the management of cardiac residual risk, the study conducted by Barter et al, published in the New England Journal

Medicine, might be the most revealing to date. This large study involved 15067 patients with prevalent CHD to test the hypothesis whether torcetrapib, an inhibitor of CETP, could reduce the risk of CHD by increasing HDL-C. After a 12-week intervention, the increment in HDL-C levels (72.1%) was parallel to a higher risk of mortality (both of cardiovascular and non-cardiovascular causes) and major cardiovascular events. These findings led investigators to speculate on the possibility that torcetrapib could turn HDL into a dysfunctional lipoprotein, or even to a proatherogenic particle (78). Nevertheless, it must also be considered that a recently reported change after a torcetrapib treatment in 200 serum proteins (belonging to immune, inflammatory and aldosterone functions, and glycemic control) could explain secondary pharmacological effects (79). Finally, researchers from the ACCORD Study Group did not find any additional benefit in the treatment of type 2 diabetic patients with a combination of fenofibrate and simvastatin in contrast with simvastatin alone to reduce the rate of fatal cardiovascular events, nonfatal AMI, or nonfatal stroke (80).

All these interventions were summarized in a meta-analysis evaluating the effects of raising HDL-C through pharmacological interventions on cardiovascular outcomes. The study concluded that neither niacin, CETP inhibitors nor fibrates were effective in the reduction of all cause mortality, CHD mortality, AMI, or stroke (81). Furthermore, findings from a Mendelian Analysis released in 2016 failed to infer a clear causal role of HDL-C on prevalent or incident CHD (82).

In light of these controversial findings, investigators now consider that the initial benefit displayed by epidemiological studies might lie in HDL quality and function instead of only in the quantity of cholesterol carried by the lipoprotein in the circulation.

1.5. LIPOPROTEINS METABOLISM

Class	Function	Location of synthesis	Composition ofthe core	Apolipoprotein on surface
Chylomicron	Transportation of dietary cholesterol from the intestine to the liver	Intestinal mucosal cells	Triglycerides from dietary fat	Apo A-I, A-IV, A-V, B-48, Apo C-II, C-III, E
Very-low-density lipoprotein	Transports triglycerides, phospholipids, cholesterol and cholesterol esters	Liver	Primarily triglycerides with a small amount of cholesterol	Apo B, A-V, Apo C, Apo C-II, C-III, E
Low-density lipoprotein	Primary cholesterol-carrying lipoproteins	Derived from VLDL in the circulation	Lipid core containing cholesterol esters	Apo B
High-density lipoprotein	Reverse cholesterol transport	Liver, intestine	Primarily cholesterol esters	Apo A-I, A-IV, A-V, B-48, Apo C-II, C-III, E

Table1. Major lipoprotein subclasses (Adapted from Imes CC, Biol Res Nurs, 2013)[83]

Due to their hydrophobic nature, lipid compounds are transported in circulation inside complex particles called lipoproteins. Although varied in nature, all lipoproteins share the same structure: a hydrophobic core rich in phospholipids, fat soluble antioxidants, vitamins, and cholesteryl esters, surfaced by free cholesterol, phospholipids, and apolipoproteins (84).

Plasma lipoproteins fall into five major classes, based on composition: chylomicrons, very low-density lipoproteins, LDL and HDL (Table1). They differ in lipid and protein content and, therefore, in size and density (Figure1). Such variations greatly determine the role they play in lipid metabolism and in the development of cardiovascular diseases.
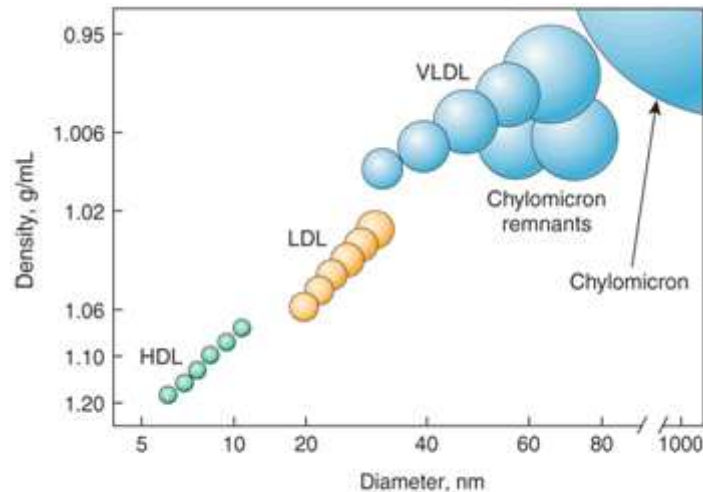
Figure 1. Major lipoprotein classes (https://thoracickey.com/disorders-of-lipoprotein-metabolism)

Circulating lipoproteins participate in three cycles of lipid distribution: the exogenous (dietary origin), the endogenous (endogenous origin) and the reverse cholesterol pathway. Dietary lipids are transported and delivered by chylomicrons and their remnant particles (which are synthesized in the intestine) whereas lipids of endogenous origin are delivered by a number of hepatic lipoproteins (very low-density lipoproteins, intermediate-density lipoprotein, and LDLs). The cholesterol reserve transport to the liver is played by HDL, with the ultimate goal of maintaining cholesterol levels inside peripheral cells within a non-toxic concentration (Figure 2).

1.5.1. Exogenous lipoprotein pathway

Triglycerides constitute roughly 95% of dietary lipids. Before absorption in the duodenum and proximal jejunum, they are hydrolyzed to free fatty acids and monoacylglycerols. Once inside the enterocyte, they are reassembled into triglycerides. Dietary cholesterol is absorbed as free cholesterol, and fatty acids released from esterified cholesterol.

Inside the enterocyte, dietetic cholesterol and triglycerides are packed into nascent chylomicrons together with ApoA-I, A-IV and B-48, the

latter being the limiting factor for the synthesis of the lipoprotein particle. In the bloodstream, they will further acquire other apolipoproteins, such as ApoC-II, C-III, and E, and undergo hydrolysis by the activity of lipoprotein lipase. The presence of ApoE in the surface of the remnant chylomicrons facilitates their clearance by LDL receptor and LDL-receptor related protein 1 in the surface of hepatocytes (85).

1.5.2.   Endogenous lipoprotein pathway

De-novo cholesterol synthesis takes place in the liver, and is packaged with triglycerides and a set of apolipoproteins, including the ApoB and others (C-II, C-III and E) to constitute very low-density lipoproteins. In circulation, they are subjected to the activity of lipoprotein lipase and cholesterol ester transfer protein. On one hand, lipoprotein lipase hydrolyzes free fatty acids from triglycerides. On the other hand, the CETP facilitates very low-density lipoproteins enrichment in ApoC-II, C-III, and E, and cholesterol from HDL. CETP causes HDL to transfer one molecule of cholesterol ester to any other circulating lipoprotein class. In turn, HDL will accept one triglyceride moiety, becoming bigger and less dense, whereas phospholipid transfer protein participates in the transference of phospholipids among subpopulations of HDLs. This is for the synthesis of discoidal HDLs or, with the intervention of hepatic lipase, smaller, denser and richer cholesterol HDLs (86).

The resulting very low-density lipoproteins remnants (83) either go to the liver or remain in circulation, and, by the action of hepatic lipase, become LDLs. LDLs are responsible for delivering free fatty acids and cholesterol to extra-hepatic tissues through interaction with LDL receptors. The synthesis of LDL receptors is regulated by the content of cholesterol in the cells thanks to which LDL in the cell is maintained at optimal concentration (83).
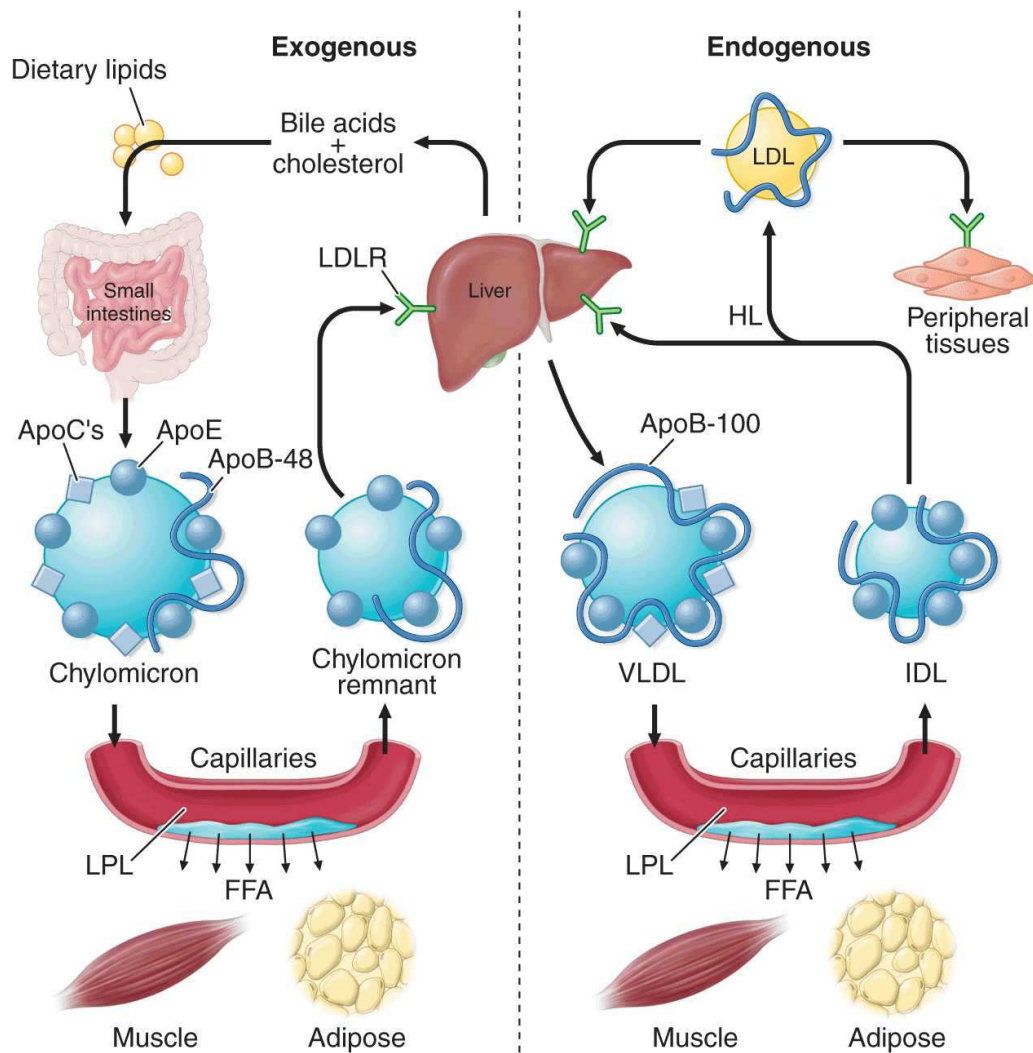
Figure2. **Exogenous** **and** **endogenous** **lipoprotein** **pathways** (https://thoracickey.com/disorders-of-lipoprotein-metabolism/)

1.5.3.   Reverse cholesterol transport

HDL is synthesized either in the form of nascent lipid-free/lipid-poor apolipoproteins, or as discoidal particles in the liver and, to a lesser extent, in the intestine (Figure3). One of the main roles of HDL is to upload the excess of cholesterol stored within the cells and take it back to the liver, where it is incorporated into nascent very low-density lipoproteins or used for any other metabolic purposes.

Lipid free/lipid poor ApoA-I accept free-cholesterol and phospholipids from peripheral cells through the interaction with ATP-binding cassette

transporter A1, or from triglyceride rich lipoprotein, which undergoes triglyceride hydrolysis by lipoprotein lipase. However, HDL may be generated by other biological pathways. ApoA-II, synthesized in the liver, joins with phospholipids and cholesterol to be exported as discoidal HDL. ApoA-IV from chylomicrons is detached by the hydrolysis of triglyceride by lipoprotein lipase and lipidated with free-cholesterol and phospholipids to form immature HDLs or to incorporate to already existent HDLs. Similarly, ApoE, initially bound to very low-density lipoproteins is dissociated from this lipoprotein by lipoprotein lipase activity and transferred to HDLs (87).

Activation of lecithin cholesterol acyltransferase by ApoA-I is critical for the maturation of HDL. This enzyme transfers fatty acids from the 2-sn position of phospholipids to esterify free cholesterol, which then translocates into the core of HDL, forcing the lipoprotein to become spherical (88). ABCG1 is of importance in the maturation of HDLs. The transporter promotes efflux of a wide range of sterols, not only free cholesterol, to spherical HDLs, LDLs and other acceptors, although it seems that cholesterol efflux to smaller HDLs is more efficient (89).

Once spherical, mature HDL is packed with cholesterol, it goes to the liver to be removed from circulation by the interaction with scavenger receptor type B1 or HDL-receptors.
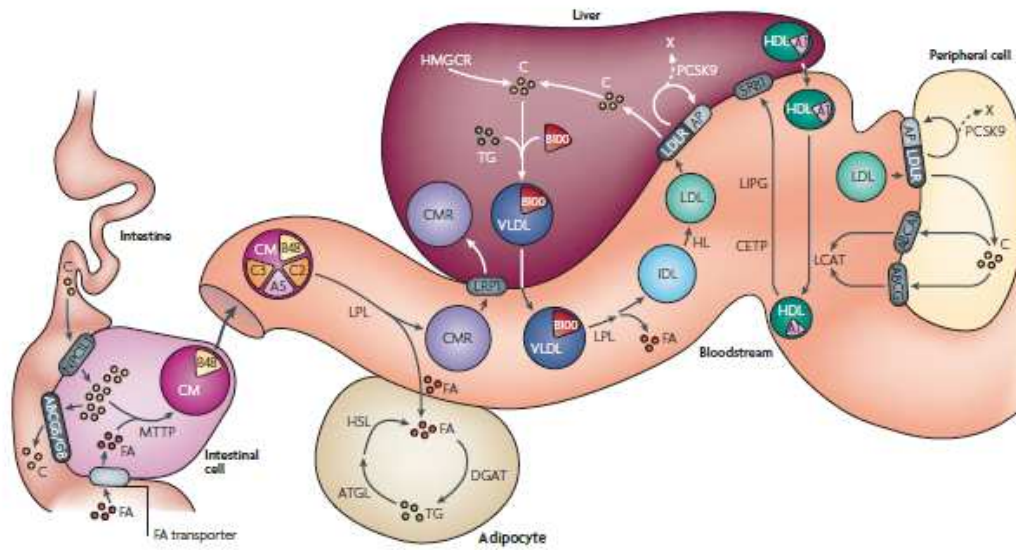
Figure3. Lipoprotein metabolism (Hegele RA, Nat Rev Genet, 2009)[84]

1.6. HDL PLEIOTROPIC FUNCTIONALITY

1.6.1. Lipoprotein characterization of the HDL particle

As can be inferred from the complex pathways to which HDL metabolism is subjected, these particles are highly heterogeneous in composition, size and conformation, having a strong impact on lipid HDL metabolism (90) and functionality (91,92).

A body of studies has confirmed the abundance and diverse presence of protein compounds in HDL, including multiple Apos (AI, AII, AIV, B, (a), CI, CII, CIII, CIV, D, E, F, H, J, L1, and M), enzymes, lipid transfer proteins, protease inhibitors, proteins of the complement, and other HDL-associated proteins which might not only play a key role in the prevention of heart diseases, but also become a biomarker of future cardiac outcomes (93–95)(Figure4). Moreover, technological improvements in analytical techniques have disclosed the complex nature of lipid compounds making up HDL, thus reinforcing the theory that HDL composition is a major determinant of its biological activity (92,96).
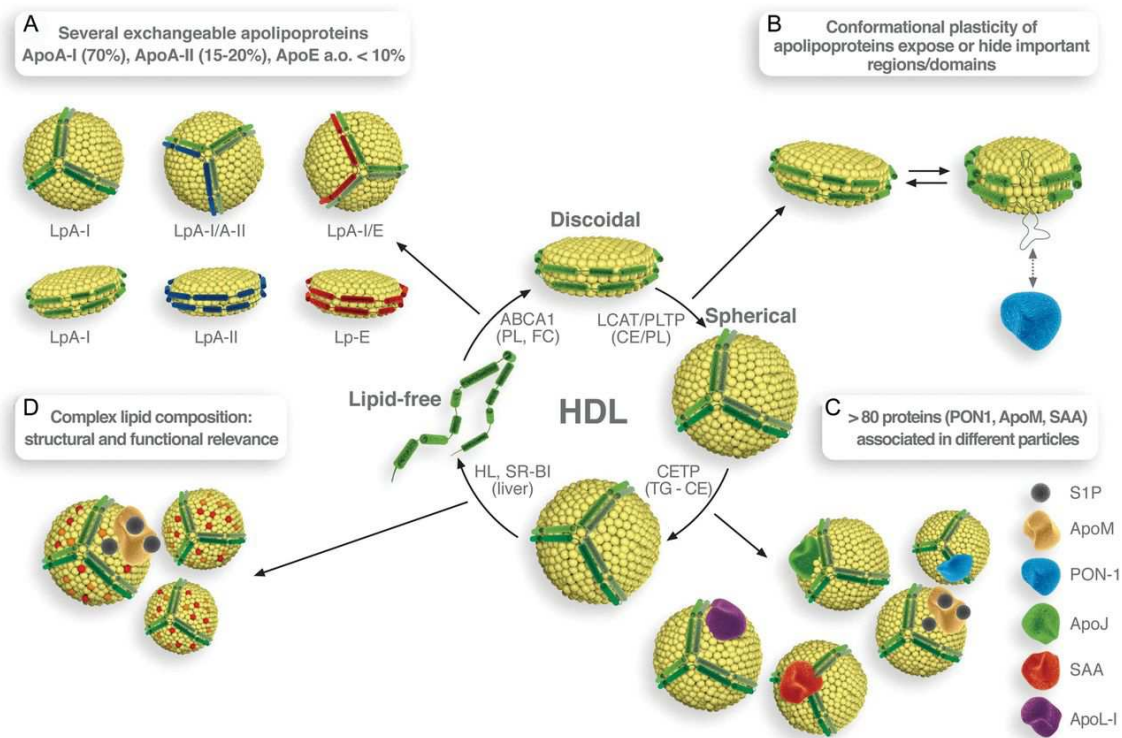
Figure4. HDL composition (Kratzer A, Cardiovasc Res, 2014)[97]

ApoA-I, represents the main protein of HDL and plays a central role in protection exerted by HDL against cardiovascular diseases. Interestingly, levels of ApoA-I are inversely associated with a 21% reduction in the incidence of cardiovascular diseases (98). ApoA-I is involved in the reverse cholesterol pathway, via enhancement of cholesterol efflux from macrophages (99) and activation of lecithin cholesterol acyltransferase (100). Furthermore, ApoA-I contributes to HDL-antioxidant activity. ApoA-I binds to, and further inactivates, lipid hydroperoxides (101) and is able to remove hydroperoxide from LDL(102).

ApoA-IV is one of the apolipoproteins present in HDL, mainly in HDL3 sub particles (103). ApoA-IV is primary synthesized in the intestine and assembled into chylomicrons. During hydrolysis of triglycerides by lipoprotein lipase, approximately 25% is transferred to HDL, and the

remaining majority is found in its free state (104). The role of ApoA-IV in the development of atherosclerosis is not fully understood. ApoA-IV has anti-oxidant, anti-inflammatory characteristics, and promotes reverse cholesterol transport (105). Conversely, ApoA-IV in HDL has been identified as an acute-phase protein (106,107) and its ability to activate lecithin cholesterol acyltransferase is diminished in a lipid-bound state. Furthermore, lipid-rich ApoA-IV is susceptible to structural and functional modification by the action of glycation end products (108), which are particularly high in plasma of diabetic patients (109). Glycated ApoA-IV has been recently revealed as a strong independent predictor of coronary artery disease in T2DM patients. This is explained by its ability to stimulate expression of pro-inflammatory cytokines, monocyte adhesion and enhance interaction of monocytes and leukocytes to endothelial cell. Similarly, glycated ApoA-IV has been independently linked to the extent and severity of heart disease (110).

Finally, ApoC-III, the last component of the ApoAI-CIII-AIV gene cluster, is another critical participant in development of atherosclerosis. ApoC-III, delays clearance of triglyceride rich lipoproteins remnants and enhance the synthesis of very low-density lipoproteins (111). But, beyond its contribution in the maintenance elevated of plasma triglycerides, ApoC-III might promote atherosclerosis promoting the of monocytes with endothelial cells and proliferation of smooth muscle cells (112). Association of ApoC-III to HDL might render the lipoprotein dysfunctional (113). Actually, HDL-bind ApoC-III is associated with a greater risk of CHD (114). Nevertheless, as summarized in a meta-analysis of observational studies, ApoC-III in LDL is an stronger predictor of a cardiovascular events than in HDL (115).

1.6.2.   HDL-Cholesterol efflux

The most widely studied function in relation to HDL-protection against CVD is its ability to remove excess cholesterol loaded in peripheral cells.

The movement of cholesterol from extra hepatic cells to HDL is called "cholesterol efflux" and constitutes the first step in "reverse cholesterol transport" to the liver (either to be excreted or recycled). Free cholesterol is initially taken up from peripheral cells by lipid-free ApoA-I through interaction with adenosine triphosphate binding cassette A1 receptors. Consequently, ApoA-I becomes discoidal pre-β1 HDL (116,117), which is particularly efficient in removing cholesterol from uploaded foam cells. The action of lecithin cholesterol acyltransferase on pre-β1 HDL promotes esterification of free cholesterol bound to the hydrophilic surface and its translocation into the core of the particle, leading to the generation of spherical mature HDL (118). In a similar manner, lipid-free and lipid-poor HDL, and mature HDL (HDL2, HDL3), react to a greater extent with ABCG1 transporter and SR-BI receptor (119).

HDL may become dysfunctional in disease states, compromising its atheroprotective role against heart diseases. Oxidative modifications of ApoA-I driven by enzymes such as myeloperoxidase could result in dysfunctional HDLs. These particles, beyond exhibiting an atheroprotective behaviour, might impair the mechanisms involved in protection against CVD (120).This hypothesis is supported by a range of studies in which cholesterol efflux promoted by HDL was reduced in the presence of pathologic states, such as AMI or coronary artery vasculopathy, compared to individuals free from disease (121,122). Furthermore, the ability to efflux cholesterol from peripheral cells, mostly macrophages, is able to predict the risk for CVD and mortality, irrespective of HDL-C concentration (123,124).

1.6.3. HDL Anti-oxidant capacity

HDL protects LDL, triglyceride-rich remnant particles and endothelial cells against oxidation. In atherosclerosis, radical oxygen species and oxidized LDL amplify the oxidative cascade by modifying endothelial and other vascular cells thus causing further atherosclerotic modifications.

It is believed that the anti-oxidant properties of HDL are conferred by its content in apolipoproteins and enzymes, mainly paraoxonase 1, platelet-activating factor-acetyl hydrolase (PAF-AH), lecithin cholesterol acyltransferase, and glutathione peroxidase. ApoA-I plays a part in the anti-oxidant protection of HDL against atherosclerosis diseases. ApoA-I activates paraoxonase 1 and lecithin cholesterol acyltransferase, and participates in the ability of HDL to neutralize free-radical oxidants in order to remove lipid hydroperoxides from LDL and protect them from oxidation (104).

Several observational studies have found an inverse association between the anti-oxidant protection exerted by HDL and risk for coronary diseases (121,125,126).

The superfamily of phospholipase A2 enzymes deserves special mention, specifically, the role of PAF-AH, a well-known lipoprotein associated with phosphohlipase A$_2$, in atherosclerosis and coronary artery diseases. Platelet activating factor, a potent pro-inflammatory mediator (127) and oxidized phospholipids are increased in CVD (128,129). In this regard, PAF-AH hydrolyzes the acetyl group from the *sn*-2 position of platelet activating factor and long-chain oxidized lipids (up to 9 carbons long) in the *sn*-2 position of phospholipids in HDL and LDL (130), which confers the enzyme with a potent anti-inflammatory and anti-atherogenic activity. HDL-associated PAF-AH concentration is positively associated with levels of plasma ApoA-I, and negatively with the presence of hyperlipidemia and diabetes mellitus (131), both well-known factors predisposed to develop CVD (132–134). Furthermore, lower activity levels of PAF-AH have been reported in heart disease patients. It must be pointed out that Tsironis et al. demonstrated that higher levels of Apo(a) (the key apolipoprotein of lipoprotein (a)), an LDL-like particle, were also found in heart disease patients in comparison with a control group (135). This might support the hypothesis of a pro-inflammatory PAF-AH in certain pathologic states.

1.6.4.   **HDL inflammatory ability**

Atherosclerosis is a chronic inflammatory disorder. The expression of several pro-inflammatory mediators, including vascular adhesion molecule-1, intracellular adhesion molecule-1, and E-selectin, has a deleterious effect on the endothelium and the expression of acute phase proteins such as C-reactive protein and Serum Amyloid A (SAA) leading to the amplification of the inflammatory response (136). Therefore, the ability of HDL to reduce the expression of cytokines by endothelial cells and leukocytes is a cornerstone of its atheroprotective character (137). HDL anti-inflammatory capacity has been inversely associated with the risk of CVD, including AMI, independent of HDL-C and other risk factors, by several authors (121,138).

As previously stated, SAA comprises a family of acute-phase proteins which are secreted in the liver. Although high levels of SAA are reliable indicators of systemic inflammation (139), the role of SAA in the development of atherosclerotic pathology and on HDL-functionality is still under debate. On one hand, the detrimental action of SAA in atherosclerosis is based on its ability to displace ApoA-I from HDL (140). During the systemic inflammatory processes, SAA, among other acute reactant proteins, replaces protein components such as ApoA-I. These changes in HDL components are associated with a smaller and more pro-inflammatory HDL (141). On the other hand, it has been described that the addition of SAA to HDL triggers the synthesis of new pre-b HDL in a dose-dependent manner, probably due to the ability of displaced ApoA-I to become associated with phospholipids to produce pre-b HDL particles (142). This finding may help to explain the synergistic enhancement of adenosine triphosphate binding cassette A1-dependent efflux by SAA bound to HDL particles (143). ApoA-IV, as previously explained in further detail, is involved in the modulation of inflammatory responses, showing anti-inflammatory properties as native apolipoprotein (105). However, glycation of the apolipoprotein in

certain states at high risk for cardiovascular disease has been proven to be a predictor of coronary artery disease (110).

Another classical indicator of systemic inflammation is the component complement C3, a key player of the complement system. The principal function of the system is to provide protection against infection and inflammation(144). Circulating C3 protein levels have been associated with cardiac outcomes. In this regard, the odds ratio for AMI in men, independent of age, was 10.7 (95% CI, 2.3-49.0) (145), and in women with pre-existing severe coronary artery disease, higher levels of serum C3 were associated with major complications of atherosclerosis (OR=4.1; 95% CI, 1.23-13.61) independent of other risk factors for atherosclerosis (146). In the present work, we set the goal to study the role of the C3 complement protein, as acute phase reactant, when linked to the HDL particle.

1.6.5.   HDL and endothelial protection

Endothelial cells lining the artery wall are crucial for its preservation: they regulate vascular tone and control smooth muscle cell proliferation and platelet aggregation. Therefore, endothelial dysfunction is regarded as a decisive event in the development of atherosclerotic diseases

HDL and its protein constituents exhibit relevant vasoprotective properties. It is believed that part of the HDL atheroprotective effect might be mediated by S1P endothelial protective properties. Roughly 60% of circulating S1P binds to HDL particles, mainly to small HDL3. This sphingolipid exerts a number of actions such as vascular tone modulation via nitric oxide pathway, maintenance of vascular permeability, and inhibition of the expression of adhesion molecules (137). Currently, data on the possible role of S1P in atherosclerotic cardiac disorders remains insufficient. Results from studies confirm that levels of HDL-bound S1P are lower in patients with established CAD (147), and more specifically in those suffering an AMI, when

compared to controls (148). Recently, HDL-associated S1P has been pointed out as an strong inverse predictor for coronary in-stent re-stenosis, independent of HDL-C, gender, age (149), and of the severity of coronary artery disease (147). However, larger studies are needed to confirm whether S1P could become a surrogate for heart disease.

1.7. DIET, HDL-FUNCTIONALITY AND CARDIOVASCULAR DISEASE

The effect of single foods on CVD has been studied in depth. Daily intake of more than five servings of fruit and vegetables has been associated with lower risk of stroke (150); greater consumption of fish per week provides a reduction in mortality due to CHD (151) and lower incidence of ACS (152); in a linear-dose response meta-analysis, whole-grain cereals and nuts were inversely associated with CHD (153);a 10% lower risk of CHD and CVD reduction was observed among individuals eating larger amount of legumes (154). Whilst no clear association has been found between CHD and egg (155,156) and milk consumption (157), higher risk of CHD has been reported for greater processed meat consumption(158).

Further steps on the prevention of acute and chronic events have led to the evaluation of whole dietary patterns and cardiovascular outcomes. Recently, adherence to the DASH diet, rich in fruit, vegetables, low-fat dairy products, and whole-grain foods, and low in saturated fat and refined sugar, has been inversely associated with better left-ventricular function, a clinical marker for heart performance (159). Assessment of the relationship between new cases of stroke during 13.5years of follow up revealed an inverse association with the Healthy Nordic diet among participants within the Danish Diet, Cancer and Health cohort (160).

However, no other eating pattern has caused so much attention as the Mediterranean diet. It is rich in extra virgin olive oil, vegetables, fruit, whole-grain cereals, nuts, and legumes; with moderate amounts of fish, eggs, white meat and dairy products; and low presence of red meat and

sweets (161). A considerable body of studies have associated adherence to a Mediterranean diet with lower risk of a cardiac event (162,163), including AMI and heart failure (164), which has been further confirmed by several meta-analyses (165,166).

In 2013, in a large multicenter study set in Spain with individuals at high-risk for heart disease researchers related a Mediterranean diet, supplemented with extra-virgin olive oil or nuts, with a reduction in the incidence of major cardiovascular events of up to 30% approximately (167). These findings brought together results from many years of research and legitimized the value of a Mediterranean diet in the prevention of major heart events.

As previously mentioned, the adoption of healthy eating behaviours is one of the main goals to achieve in the reduction of the risk of a cardiovascular event, in both healthy individuals and high risk populations. However, so far not many studies have evaluated the impact of diet on HDL-functionality. A small study was conducted with fourteen adults allocated to either a diet supplemented with saturated or poly-unsaturated fatty acids. It disclosed an impairment on HDL anti-inflammatory ability after ingestion of the saturated-fat supplemented diet along with an improvement of this functionality after the diet supplemented with polyunsaturated fatty acids (168). A prospective analysis performed in a subsample of the PreDiMed cohort concluded that the Mediterranean diet improves the ability of HDL to accept cholesterol from macrophages (169).

1.8. PHYSICAL ACTIVITY, HDL-FUNCTIONALITY AND CARDIOVASCULAR DISEASE

Physical activity promotion is associated with a decrease in the incidence of certain chronic diseases, such as heart and lung diseases, type 2 diabetes, and obesity in the general population (170) and in older adults (171,172). It has been suggested that these protective

relationships can be partially provided by an anti-inflammatory effect of physical activity (173,174).

Regarding physical activity benefits, its increasing effect on ApoA-I concentration has been established for some time (175). In addition, several undersized studies with hypocaloric diets or physical exercise programs have been shown to improve the property of effluxing cholesterol to the HDL particle (176), the anti-oxidant capacity of HDL (177–179), its anti-inflammatory capacity (180,181), and endothelial protective capacity (182,183).

2.Hypothesis

1.   New predictors related to HDL function for coronary heart disease and all-cause mortality will be established.

2.   A number of biomarkers related to HDL functionality and composition will have a predictive and prognostic value for coronary heart disease, independent of major cardiovascular risk factors, including HDL-cholesterol.

3.   A number of biomarkers related to HDL functionality and composition will have a prognostic value for coronary heart disease regarding the severity of the cardiac pathology. In this regard, it is expected acute myocardial infarction be predicted by a broader battery of markers than unstable and even stable angina.

4.   Given that traditional risk factors are closely related to the physiopathology of the atherosclerotic disease, we expect to find similar relationships among the HDL-function and LDL-atherogenic traits, 10-year risk for coronary artery disease and the classical risk factors.

3.Objectives

The general purpose of the present doctoral thesis was to study the role of HDL function in the development of future cardiovascular events.

a.　　To conduct a meta-analysis to summarize the evidence supporting the role of HDL-functionality as a predictor of cardiovascular disease events and mortality.

b.　　To assess the predictive value for coronary syndrome of a set of novel surrogates of HDL-functionality at baseline, in a case-control study performed in a subsample from a cohort of high cardiovascular risk individuals.

c.　　To uncover potential associations among HDL-functionality and LDL-pro-atherogenic traits with the risk of 10-year cardiovascular events assessed by the FRAMINGHAM-REGICOR score and with classical cardiovascular risk factors individually evaluated

4.Methods

4.1. MANUSCRIPT 1: HIGH DENSITY LIPOPROTEIN FUNCTIONALITY AND CARDIOVASCULAR EVENTS AND MORTALITY: A SYSTEMATIC REVIEW AND META-ANALYSIS.

To perform this meta-analysis, we followed the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement (184) and registered full details in the PROSPERO International Prospective Register of Systematic Reviews under the title "High-density lipoprotein functionality, cardiovascular outcomes and all-cause mortality: A Meta-Analysis on prospective studies" with the identifier CRD42017065857 (www.crd.york.ac.uk/PROSPERO).

4.1.1. Search strategy

Studies included in the meta-analysis were looked for in Medline and Embase up to 31st May 2018 by two independent investigators (CL and MSF). Any disagreement was resolved by consensus between the authors involved.

The keywords, index terms, and Boolean operators used for finding the articles are available in the supplementary material. We did not consider manuscripts published in any language other than English, and excluded conference abstracts, reports, and grey literature.

4.1.2. Study selection

The main goal of the meta-analysis was to highlight associations between HDL functionality and the risk of any cardiovascular outcome or all-cause mortality. As a result, we only included observational (case-control or cohort) studies, those performed in adult populations (inpatients, community dwellers, free-living individuals), and in which the HDL function was performed with apoB-depleted serum or apoB-depleted plasma (ABDP) or HDL obtained by other procedures. The outcome of interest was limited to a combination of major adverse cardiovascular events, including CVD mortality or all-cause mortality.

Moreover, the manuscript was required to provide the risk estimate (hazard ratio (HR), odds ratio (OR), relative risk (RR)), and associated 95% confidence intervals (95% CI). When the study did not meet the inclusion criteria, it was not considered for analysis.

4.1.3. Data extraction and quality assessment

The information recorded for each study included in the analysis comprised: first author's name, journal and year published, geographical location, eligibility criteria, outcome under investigation, biological sample (HDL, apoB depleted serum or ABDP), HDL functionality assay performed, study design (case-control or cohort), follow-up time, sample size, number of cases, sex and mean age of participants, method to model the association, adjusted factors in multivariate models, odds ratios, hazard ratio or risk ratios and their standard errors / confidence intervals. We contacted the authors when the manuscript was lacking any necessary data.

For the analysis, we chose the model adjusted for the greater number of confounders, including HDL-C when available. The Newcastle-Ottawa Scale (185) was used to evaluate the quality of the studies included. This scale has three categories (selection, comparability, and exposure) which are scored from 1 to a maximum of 9 stars. A minimum of 5 stars was set in order to be included in the meta-analysis

4.1.4. Statistical analysis

We pooled HR and OR (with corresponding 95% CI) with a random effect meta-analysis. For each functionality test and for each outcome separately, we combined the highest versus the lowest values of the HDL-function together with 1-standard deviation (SD) change.

We expressed the risk for the outcome under assessment associated with a protective functionality, and inverted the ratios provided when the relationship with the outcome was a poorer functionality.

Therefore, a pooled estimate lower than 1 indicates an inverse relationship between greater functionality and risk for the outcomes, whereas an estimate greater than 1 means that greater function is associated with higher risk.

True heterogeneity was evaluated with Q statistic and the I^2 statistic test (186). I^2 statistics provides an intuitive scale to classify heterogeneity as low (0-40%), moderate (30-60%), substantial (50-90%), and high(187) (75-100%). Furthermore, a chi-square test was used to quantitatively assess presence of heterogeneity among studies (alpha=0.05).

Finally, publication bias was visually diagnosed with funnel plots and quantified with Egger's test (188) for which a two-sided p-value lower than 0.05 was considered significant. Stata version 14 (StataCorp, TX, USA) was used for the statistical analyses.

4.2. MANUSCRIPT 2: PREDICTIVE VALUE OF HDL FUNCTION RELATED BIOMARKERS FOR ACUTE CORONARY SYNDROME OUTCOME IN PATIENTS AT HIGH CARDIOVASCULAR RISK

4.2.1. Participants and study design

This study was performed within the context of the PreDiMed Study population (*Prevención con Dieta Mediterránea*). The PreDiMed project is a multicentre, randomized, controlled diet intervention. Participants were randomly assigned to: 1) a traditional Mediterranean diet supplemented with virgin olive oil; 2) a traditional Mediterranean diet supplemented with nuts; and 3) a low-fat control diet following the American Heart Association recommendations. Eligible participants were community-dwelling men, 55 to 80 years of age, and women, 60 to 80 years of age, who fulfilled at least 1 of 2 criteria: 1) type 2 diabetes or 2) 3 or more coronary heart disease risk factors: current smoking, hypertension (blood pressure >140/90mm Hg or antihypertensive drugs), LDL-C level >160 mg/dL (or hypolipidemic drugs), HDL-C level

<40 mg/dL, body mass index (BMI) >25 kg/m², or a family history of premature coronary heart disease. The exclusion criteria included history of CVD, drug or alcohol addiction, history of allergy or intolerance to olive oil or nuts, or any severe chronic illness or condition (189).

Between October 2003 and June 2009, 8,713 candidates free from cardiovascular disease were screened for eligibility. 7,447 were randomized in the trial assay and their follow-up finished in December 2011. After a median follow-up of 4.8 years, 172 acute coronary syndrome (ACS) outcomes [98 acute myocardial infarction (AMI) and 74 unstable anginas (UA)) and 35 stable anginas (SA) occurred. Complete protocol details have been published elsewhere (190). The trial was approved by the institutional review boards and registered with the number ISRCTN35739639 in **www.controlled-trials.com**. Participants were provided with informed consent before joining the trial (http://www.predimed.es).

The nested case-control (1:2) design was matched by age, sex, BMI, intervention group, and follow-up time in the study at event occurrence.

4.2.2. Outcome ascertainment and follow-up for coronary syndrome cases

ACS was studied as the major cardiovascular events: a) dead/alive individual who suffered an AMI; and/or b) dead/alive individual who suffered an UA (189). We used four sources of information to identify end points: repeated contacts with participants; family physicians; yearly review of medical records; and consultation of the National Death Index. All medical records related to end points were examined by the end point adjudication committee, whose members were blinded to treatment allocation. Only end points that were confirmed by the adjudication committee, and that had occurred between Oct 1, 2003, and Dec 1, 2010, would be included in the analyses.

4.2.3.   Sample size calculation

A sample size of 172 cases and 344 controls allowed ≥80% power to detect an odds ratio of at least 1.9, considering a 2-sided type I error of 0.05, a loss rate of 1%, and assuming the exposed rate among controls would be 50%.

4.2.4.   General data, diet, and physical activity information

The baseline examination included a 47-item questionnaire about education, lifestyle, history of illnesses, and medication use. Assessment of Mediterranean diet adherence, by means of the 14 item-Mediterranean Diet Score, was performed (191). Physical activity practice was registered by means of a Minnesota Leisure Time physical Activity questionnaire, validated in a Spanish population (192). Height and weight were recorded at baseline and BMI was calculated as weight (kg) divided by height (m) squared. As the intervention did not target medication it was prescribed following the participants' regular medical care.

4.2.5.   Biological sample collection

Biological samples were collected at a fasting state of at least10 hours; they were coded, shipped to a central laboratory, and frozen at -80 °C until use. The apoB depleted fraction from K3-EDTA baseline-plasma samples (169) was obtained. Plasma was incubated for 20 minutes with 20% polyethylene glycol 8000 (Sigma) in a 200 mM 65 glycine buffer (pH 7.4, Sigma) at 4°C. After centrifugation (10,000 g, 4°C, 15 minutes) and removal of the lipoproteins containing apoB, we discarded the pellet and collected the supernatant containing HDL. The samples did not suffer any thaw-freeze cycles before the experiments.

4.2.6.   Quality control of the laboratory analyses

The same pre-analytical procedures were applied to all samples and all matched samples were analyzed in the same experimental run in all

determinations. All determinations were run in singles except for cholesterol efflux capacity, in which all samples were analyzed in duplicate.

Each analytical plate, for every determination included a pool of ABDP from healthy volunteers, as an internal control, to assess inter-assay variability among plates. For CEC, HDL oxidative-inflammatory index (HOII), ApoA-IV, S1P, PAF-AH activity, and SAA analyses, we included a second pool of ABDP from another group of volunteers to normalize results and minimize intra-assay variability. No intra-repetition coefficient of variation >20% was allowed.

4.2.7.   Biochemical analysis

a.   Systemic lipid profile and glycaemia

In plasma, we determined systemic concentrations of glucose, total cholesterol, triglycerides, and HDL-C in an ABX-Pentra 400 autoanalyzer (Horiba-ABX). LDL-C levels were calculated by the Friedewald formula, whenever triglycerides were <300 mg/dL.

b.   HDL composition

We measured ApoA-I, ApoC-III, and component complement C3 in ABDP fraction by immunoturbidimetry in an ABX Pentra-400 autoanalyzer (Horiba-ABX, Montpellier, France). Determination of ApoA-IV, SAA, and S1P in ABDP samples was carried out with commercial Elysa kits according to the manufacturer's instructions (Abcam, Invitrogen, and Bioassay, respectively) with previous standardization of protocols for ABDP samples. All the samples were analyzed individually. Intra and inter-assay coefficients of variation were 6.04% and 11.6% for ApoA-IV, 3.54% and 11.8% for SAA, and 7.86% and 13.18% for S1P.

c.   *HDL antioxidant capacities*

We determined PAF-AH activity in HDL. We adapted the protocol provided by the manufacturer to the ABDP samples (PAF Aceltylhydrolase Assay Kit, Cayman). The intra assay coefficient was 8.69% and the inter-assay one was 15.38%.

HOII, an indirect measurement of the antioxidant/antiinflammatory capacity of HDL, expressed as its ability to inhibit LDL oxidation, was performed as follows. We placed 2'-7'-dichloro fluorescein diacetate (Sigma) in methanol for 30 minutes to obtain the fluorescent deacetylated dichloro fluorescein. Afterwards, the mixture was incubated at a final concentration of 3 µg/mL, in the presence of pre-oxidized LDL (final concentration: 1.5 µg/mL)(169) with 5 µL of ABDP from the volunteers, or with the same volume of phosphate buffered saline (Sigma) (negative control), in black polystyrene plates. We measured the fluorescence in Infinite M200 reader (Tecan Ltd) every 3 minutes for 60 minutes at 37°C, after shaking the plate (*Ex/Em*: 485/530 nm). To calculate the index, we subtracted the fluorescence of the blank from the fluorescence of the volunteers´ samples and the following formula was used:

HOII=fluorescence in the presence of ABDP – fluorescence without ABDP

A high value of the index reflects a greater dichloro fluorescein oxidation, which is an inverse measure of LDL oxidation. Therefore, high levels of HOII are indicative of poor antioxidant/antiinflammatory HDL-protection.

We ran all the samples, blank (only media) and control negative in single. The intra and inter-assay coefficient of variation were 5.74% and 7.16%, respectively.

d.   Cholesterol efflux capacity

We suspended human THP-1 monocytes in RPMI 1640 medium (supplemented with 10% heat-inactivated fetal calf serum, 1% L-glutamine, 1% sodium pyruvate, and 1% 90 penicillin-streptomycin) in T75 culture flasks (2500cells/ml) and changed media every 72-96h. When cells reached confluence, we differentiated monocytes into macrophages by incubating them with phorbol-myristate-acetate (Sigma, 200nM) for 24h in transparent 96-well plates at 350,000cell/ml. Once differentiated and attached to the bottom of the wells, we incubated macrophages with cholesterol BODIPY (0.025mM) for 24h, washed them once, incubated them for 24h in fresh free-serum medium (RPMI 1640 only supplemented with 1% bovine serum albumin, Sigma), washed them with phosphate buffered saline again, and finally incubated them for 16h in fresh free-serum RPMI 1640 medium + 1% bovine serum albumin (we prepared the total amount for both incubations the same day) in the presence of 6.6% ABDP of the volunteers or without ABDP, but with the same amount of phosphate buffered saline (negative control). We collected 100uL of the supernatants, transferred them into a 96-well transparent half-area plate, and read the fluorescence (485/535 Ex/Em) in an Infinite M200 reader (Tecan Ltd). Immediately afterwards, we removed the remaining supernatant from the plate containing the attached cells and added 150µL de Triton X-100 (1%) previously refrigerated and placed the plate in cold (4ºC) for 60 minutes. Then, we collected 100uL of the mixture and read the fluorescence in cells as in the supernatants.

We subtracted the fluorescence of the blank to each well value with or without ABDP and calculated CEC for each well as shown:

Fluorescence in supernatant

CEC=---

Fluorescence in supernatant+Fluorescence in cell

Then, we subtracted the efflux of negative control to the efflux of every ABDP sample to obtain the adjusted efflux. The global intra-assay coefficient of variation was 2.5% and the inter-assay one 6.8%

4.2.8.   Statistical Analysis

We visually tested normal distribution for continuous variables with histograms. Means with corresponding standard deviation were calculated for normally distributed variables, and median and interquartile ranges for skewed variables. Categorical variables are presented as frequencies.

Pearson's chi-squared test was used to assess differences in frequencies among groups. Student t-test and Mann Whitney U-test was used to assess differences between the two groups for continuous normal and non-normal distributed variables, respectively. Comparison of more than two groups was performed by ANOVA or Kruskall-Wallis test, for continuous normal and non-normal distributed variables, respectively, and Pearson's chi-squared test for categorical variables. In nested case-control studies with risk-set sampling and time-to-event matching, odds ratios derived from conditional logistic regression is considered to be unbiased estimates of hazard ratios (HRs). Therefore, conditional logistic regression analyses (OR with 95% CI) were performed to model the associations of HDL-related characteristics and outcome of interest. In this regard, we constructed three models to assess the association of biomarkers with coronary event as binary outcome variable: (i) model 1, without any adjustment; (ii) model 2, consisting of the combination of age, intervention group, and HDL-C; and (iii) model 3, including the effect of age, intervention group, and the group of cardiovascular risk factors (CVRF) (presence of type 2 diabetes mellitus, hypercholesterolemia, hypertension, and smoking habit). Furthermore, a *P* for trend was computed to calculate distribution of disease across quartiles of each surrogate.

We accepted a two-sided p-value<0.05 to be significant. The statistical analyses were performed by R version R, version-3.5.0 (R Core Team (2018). R: language and environment for the statistical calculation R Foundation for Statistical Computing, Vienna Austria URL https://www.R-projectd.org/).

4.3. MANUSCRIPT 3: ROLE OF HDL FUNCTION AND LDL ATHEROGENICITY ON CARDIOVASCULAR RISK: A COMPREHENSIVE EXAMINATION

4.3.1. Study population

This cross-sectional analysis included two representative sub-groups of volunteers from the PreDiMed Study (189). The sample for the study of HDL-function (*N*=296)(169) comprised the one in which LDL atherogenic traits were examined (*N*=210)(193).

In both populations the following variables were selected(190,192): 1) general clinical variables (age, sex, body weight, height, blood pressure, and biochemical profile); 2) drug use; 3) adherence to a Mediterranean diet, rated with the Mediterranean Diet Score; 4) levels of physical activity; and 5) smoking habit. The overall risk for a 10-year coronary event was calculated as cardiovascular risk (CVR) scores according to the Framingham-REGICOR equation validated for the Spanish population (considering age and sex, presence of diabetes and smoking habit, total and HDL-C levels, and blood pressure)(194).Definition criteria for type-II diabetes mellitus, hypercholesterolemia, hypertension, and BMI have been previously defined in the present chapter and published in detail elsewhere (189,190).

4.3.2. HDL functionality determinations

Isolated HDL particles were obtained from plasma by ultracentrifugation (169,195) and ABDP by precipitation as previously

explained. Plasma, serum, isolated HDL, and ABDP samples were stored at -80ºC until use.

We analyzed the lipid profile of participants including triglycerides, cholesterol, HDL-C, and ApoA-I (169).

The CEC from macrophages mediated by HDL was determined in ABDP samples (169). The ability of HDL lipoproteins to esterify cholesterol was calculated as the percentage of esterified cholesterol in isolated HDL particles/lecithin cholesterol acyltransferase mass in plasma (169). CETP activity in plasma and arylesterase activity of paraoxonase-1 in serum were determined with commercial kits (169). HDL vasodilatory capacity was evaluated by measuring the increment in the production of nitric oxide by human endothelial cell cultures in the presence of ABDP samples from volunteers (169). The oxidation of HDL particles was expressed as equivalents of malondialdehyde per mg/dL of cholesterol in ABDP samples (169). We computed the triglyceride/esterified cholesterol ratio in HDL particles as a measurement of the content of triglycerides in the HDL core (169). Finally, we calculated the HDL2/HDL3 ratio in plasma (169,195).

4.3.3. LDL atherogenic traits

We first isolated LDL lipoproteins from plasma samples by density gradient ultracentrifugation (193,196) and stored them at -80ºC until use. From the values of the participants' lipid profile, we calculated LDL-C levels according to the Friedewald formula (whenever triglycerides were <300 mg/dL) (193,196). We quantified ApoB in an ABXPentra 400 autoanalyzer (Horiba ABX) in plasma (193,196). We measured LDL resistance against oxidation (LDL lag time) from the kinetics of formation of conjugated dienes (oxidized lipid forms) in isolated LDL samples in a pro-oxidant environment (193,196). We assessed the oxidation of LDL lipoproteins as the equivalents of malondialdehyde per mg/dL of cholesterol in isolated LDL samples (193). From the lipid profile values, we calculated an approximation of

LDL average size (the LDL-C/ApoB ratio)(193). We determined the lipid composition of isolated LDL particles in an ABX-Pentra 400 autoanalyzer (Horiba ABX) and, from these data, we calculated the triglyceride/total cholesterol ratio in isolated LDL samples. Finally, we assessed LDL *ex vivo* cytotoxicity in a THP-1 monocyte-derived macrophage model as previously described (193).

4.3.4.   Sample size

A sample size of 196 and 140 participants permitted Pearson's correlation coefficients of ≥0.2 and ≥0.237 (for HDL- and LDL-related variables, respectively) assuming a type I error of 0.05, a type II error of 0.2, and a 1% loss rate in a two-sided contrast.

4.3.5.   Statistical analyses

Distribution of variables was tested with plots and histograms. The association between lipoprotein related traits and groups of CVR (low risk –CVR score <5–, moderate risk –CVR score ≥5 and <10–, and high risk –CVR score ≥10–) was evaluated with one-way ANOVA for normally-distributed variables and Kruskal-Wallis test for non-normally distributed ones. Linear associations between both HDL-function and LDL-atherogenic traits and CVR categories were assessed with Pearson's and Spearman's tests to calculate *P*-trend values.

Linear regression allowed us to model the relationship of lipoprotein characteristics and classical CVRF. These included presence of diabetes, hypercholesterolemia, hypertension, and tobacco use (categorical variables), greater values of BMI (continuous variable), and sex and age (continuous variable). Model 1 was non-adjusted; model 2 was adjusted for the rest of the previous factors (study site, adherence to the Mediterranean diet, and levels of physical activity); and model 3 was adjusted for variables included in model 2 plus HDL-C and LDL-C levels.

A two-sided *P*-value <0.05 was accepted as significant and we performed all the analyses in R Software, version 3.4.1 (*R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria*).

5. Results

A summary of the major findings from the three chapters comprising scientific production as the main result of the present thesis work are summarized below.

MANUSCRIPT 1 (READY TO BE SUBMITTED): HIGH DENSITY LIPOPROTEIN FUNCTIONALITY AND CARDIOVASCULAR EVENTS AND MORTALITY: A SYSTEMATIC REVIEW AND META-ANALYSIS.

María Trinidad Soria Florido, Montserrat Fitó, Helmut Schröder, María Grau, Camille Lassale.

- CEC, the main vasculoprotective role of HDL, was inversely associated with the risk of cardiovascular disease and of all-cause mortality, in multivariate adjusted models, including age, sex, and cardiovascular risk factors (pooled estimate: 0.52, 95% IC: 0.38-0.70 for CVD, and 0.62, 95% IC: 0.45-0.93 for all-cause mortality).

- Higher antioxidant protection exerted by HDL was associated with lower risk of CVD with a combined estimate of 0.67 (95% IC: 0.53-0.84) and of all-cause mortality (combined estimate: 0.74, 95% IC: 0.57-0.96).

- A stronger ability of HDL to inhibit the expression of inflammatory mediators was indicative of lower risk for cardiac outcomes (combined estimate: 0.36, 95% IC: 0.12-1.03), although the association with all-cause mortality could not be assessed due to lack of publications.

- The meta-analysis represents a comprehensive summary of the state-of-the-art regarding HDL function and its utility as predictor of cardiovascular diseases and overall mortality. The mayor limitation of our findings is the considerable variability in methodology of the manuscripts included, regarding study design, population characteristics, outcomes under evaluation, and confounders the models were adjusted for. Larger studies designed to reveal specific cardiovascular outcomes, instead of combined endpoints, will minimize the variability observed.

- In summary, higher levels of CEC and antioxidant/anti-inflammatory actions promoted by the HDL particle were associated with lower risk of ischemic cardiovascular disease. A similar association was observed for the risk of all-cause mortality with cholesterol efflux and antioxidant activity. Whilst results are encouraging further research is required to fully confirm our findings.

MANUSCRIPT 2: PREDICTIVE VALUE OF HDL FUNCTION RELATED BIOMARKERS FOR ACUTE CORONARY SYNDROME OUTCOME IN PATIENTS AT HIGH CARDIOVASCULAR RISK

- Lower CEC and ApoA-I levels and greater HOII values predict ACS irrespective of CVRF, including HDL-C (OR=$9*10^{-3}$, 95% CI: $1*10^{-4}$-0.59, p=0.028 for CEC, OR=0.13, 95% CI: 0.032-0.51, p=$3*10^{-3}$ for ApoA-I, and OR=3.3, 95% CI: 1.35-8.08, p=$9*10^{-6}$ for HOII).

- Greater CEC is inversely associated with the risk of AMI in multivariate models adjusted for CVRF (OR=$5*10^{-4}$, 95% CI:$1*10^{-6}$-0.18, p=0.012). Also lower ApoA-I levels were inversely related to a greater risk of AMI in the fully adjusting model (OR=0.14, 95% CI:0.021-0.88, p=0.036)

- Unstable angina is predicted by lower ApoA-I and greater HOII in fully adjusted model (OR=0.1, 95% CI: 0.012-0.87, p=0.037 and OR=5.3, 95% CI: 1.27-22.1, p=0.022, respectively).

- In summary, our results point to HDL-functionality as a promising tool to assess residual risk for acute coronary syndrome, acute myocardial infarction, and unstable angina.

MANUSCRIPT 3 (READY TO BE SUBMITTED): ROLE OF HDL FUNCTION AND LDL ATHEROGENICITY ON CARDIOVASCULAR RISK: A COMPREHENSIVE EXAMINATION.

Álvaro Hernáez, María T Soria-Florido, Helmut Schröder, Emilio Ros, Xavier Pintó, Ramón Estruch, Jordi Salas-Salvadó, Dolores Corella, Fernando Arós, Lluis Serra-Majem, Miguel A Martínez-González, Miquel

Fiol, José Lapetra, Roberto Elosua, Rosa M Lamuela-Raventós, Montserrat Fitó.

- With regard the analysis of HDL and LDL-functional traits in relation with the risk of 10-year risk for coronary events (according to the FRAMINGHAM-REGICOR Risk Score), the group at high CVR compared to the one at less risk showed HDL with impaired capacity to efflux cholesterol from macrophages, more oxidized, smaller and richer in triglyceride.

- Furthermore, levels of HDL-C and ApoA-I in plasma were lower (*P*<0.001 for all traits except p=0.002 for particle size). In relation to LDL-atherogenic traits, the group at high CVR presented elevated ApoB levels in plasma (p=0.025) and smaller LDL (p=0.001) less resistant to oxidation (p=0.019).

- Being diabetic was independently associated with low concentrations of HDL-C and ApoA-I in plasma (*P*<0.001, p=0.004, respectively) and high ones of LDL-C and ApoB (p<0.001 and p=0.033, respectively). Moreover, greater LDL oxidation levels and reduced LDL size proved to be present even when adjusting for all classical CVRF and LDL-C levels (p=0.022 and p=0.026, respectively).

- Hypercholesterolemia state was related to greater HDL-C, LDL-C, ApoA-I, and ApoB levels (*P*<0.05, all). High CETP activity, low HDL capacity to esterify cholesterol, and smaller HDL size remained significant (p=0.035, p=0.031, and p=0.021) in a multivariate model including CVRF and HDL-C levels).

- Higher BMI was associated with low HDL-C levels, low HDL capacity to esterify cholesterol, small HDL size,and raised concentrations of triglyceride in the HDL core when adjusting for all classical CVRF and HDL-C concentrations (p=0.037, p=0.012, and p=0.087, respectively). Similarly, higher risk was linked to smaller size LDL after adjusting for all CVRF and LDL-C levels (p=0.007).

- Hypertension state was related (p=0.036) to a greater LDL oxidation when adjusting for CVRF and LDL-C levels

- Males had lower HDL-C and ApoA-I (p=<0.001) concentrations, and greater HDL oxidation with disrupted vasodilatory capacity (p=0.026, p=0.046, respectively) after adjusting for all CVRF and HDL-C concentration. Regarding LDL characteristic and composition, after adjusting for the effect of CVRF and LDL-C levels, the only remaining associations were lower LDL size and greater cholesterol content (p=0.079 and *P*<0.001, respectively).

- Greater age was only associated with higher LDL-C (*p*=0.001). No other atherogenic trait of the particle remained significant when CVRF and LDL-C levels were included in the models. Similarly, the only single relationship which persisted after adjusting for all CVRF and HDL-C was a larger triglyceride content in the HDL core (p=0.033).

- In summary, traits related to dysfunctional HDL and atherogenic LDL particles were present in high cardiovascular risk patients. Specifically, hypercholesterolemia and male gender were predominantly linked to HDL dysfunction, whilst diabetes and advanced age were associated with LDL atherogenicity. Higher BMI appeared to be equally related to impaired HDL and atherogenic LDL.

6.Manuscripts

MANUSCRIPT 1 (READY TO BE SUBMITTED): HIGH DENSITY LIPOPROTEIN FUNCTIONALITY AND CARDIOVASCULAR EVENTS AND MORTALITY: A SYSTEMATIC REVIEW AND META-ANALYSIS.

María Trinidad Soria Florido, Montserrat Fitó, Helmut Schröder, María Grau, Camille Lassale.

High density lipoprotein functionality and cardiovascular events and mortality: a systematic review and meta-analysis

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ABSTRACT

Background: Recent clinical trials and Mendelian Randomization studies have failed to find a causal link between high density lipoprotein cholesterol (HDL-C) levels and risk of cardiovascular disease (CVD). Therefore, the observed lower CVD risk for people with higher HDL-C levels may be explained by other features than the cholesterol content of the lipoprotein but to date the available evidence has never been summarized. The aim of this systematic review was to synthesize the studies linking different HDL-related functionalities and CVD or mortality risk.

Methods: We searched Medline and Embase up to 31st May 2018 for studies where HDL functionality was used as exposure and fatal or non-fatal CVD and all-cause mortality as an outcome. We used a random-effect meta-analysis model to combine hazard ratios and odds ratios (with correspondent 95% CIs), separately for each function assayed and each outcome.

Results: A total of 25 studies were included; 17 investigated cholesterol efflux capacity, 10 antioxidant capacity and only 2 measured the anti-inflammatory capacity of HDL in relation to CVD or all-cause mortality. Greater HDL-related cholesterol efflux is inversely associated with CVD outcomes (pooled risk ratio: 0.52, 95% CI 0.38-0.70) and all-cause mortality (pooled ratio: 0.62, 95% CI, 0.45-0.93). Similarly, better antioxidant capacity is associated with a lower risk of CVD (0.67, 95% CI 0.53-0.84) and all-cause mortality (0.74, 95% CI 0.57-0.96). Finally, higher anti-inflammatory ability was associated with lower CVD risk (0.36; 0.12-1.03) but there was no evidence of an association with all-cause mortality.

Conclusions: There is evidence that better HDL-related cholesterol efflux, antioxidant and anti-inflammatory capacities are associated with a lower risk of CVD and mortality. However, the large heterogeneity between studies and evidence of publication bias warrant a cautious interpretation of these results and the need for larger studies with more specific outcomes to better evaluate the value of HDL function for the prediction of cardiac outcomes.

This systematic review was registered in the PROSPERO International Prospective Register of Systematic Reviews under the number CRD42017065857 (www.crd.york.ac.uk/PROSPERO)

INTRODUCTION

Since in 1977 the Framingham Heart Study identified low levels of high density lipoprotein cholesterol (HDL-C) as the major, independent lipid risk factor for heart disease (1), many clinical and epidemiological studies have confirmed the role of HDL-C in the development of coronary events (2,3). A 1 mg/dl increase of HDL-C concentration has been associated with a decrease of 2–3% in risk of cardiovascular disease (CVD) (4).

In light of this evidence, a number of therapeutic strategies have been proposed to increase circulating levels of HDL-C as a promising tool to improve primary and secondary prevention. Unfortunately, the inconsistency of results from pharmacological interventions aiming at raising HDL-C to reduce the number of adverse coronary outcomes(5–7); has led to consider that the protective role of high density lipoprotein might lie in the functional traits of the lipoprotein rather than its cholesterol content.

HDL is a highly heterogeneous particle exhibiting a variety of functions that may contribute to its cardiovascular protective profile (8). This lipoprotein plays a key role in the removal of excess cholesterol from peripheral cells to the liver for disposal(9), which is considered a primary mechanism in the development of atherosclerotic lesions (10). Furthermore, HDL particles display a cluster of anti-inflammatory, anti-oxidative, anti-apoptotic, anti-thrombotic and vasodilatory properties which potentially contribute to its atheroprotective nature (11). This array of properties is referred to as 'HDL functionality' thereafter. Although the contribution of HDL functionality to atherosclerosis progression has been examined in many mechanical studies, mostly in vitro and animal studies, the number of studies that assessed the association between HDL functionality and CVD outcomes is still scarce, and the available evidence on the various functions of HDL has never been systematically summarized.

Therefore, our aim was to conduct a systematic review of observational studies that assessed one or various aspects of HDL functionality in relation to CVD events and mortality, and to perform a meta-analysis of the retrieved estimates.

METHODS

This meta-analysis was conducted following the Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) statement (12) and was registered in PROSPERO International Prospective Register of Systematic Reviews under the title "High-density lipoprotein functionality, cardiovascular outcomes and all-cause mortality: A Meta-Analysis on prospective studies" with the identifier CRD42017065857 (www.crd.york.ac.uk/PROSPERO).

Search strategy

Two investigators (CL and MSF) independently searched for the relevant studies on Medline and Embase up to 31stMay2018. Disagreements were discussed and resolved by consensus.

The keywords, index terms and Boolean operators used for the literature search are described in supplemental material. The search was limited to articles published in the English language and to full-text journal articles. Therefore, we excluded conference abstracts, reports and grey literature.

Study selection

To be included in this systematic review, articles had to meet the following criteria: 1) Exposure: Functionality tests performed on HDL, apolipoprotein-B (apoB) depleted serum (ABDS) or apoB-depleted plasma (ABDP); 2) Outcome: (i) A composite of major adverse cardiovascular events (MACE), including CVD mortality, or (ii) all-cause mortality ; 3) Design: observational study (cohort, case-control); 4) Population: inpatients or community dwelling, free-living setting, adults (aged ≥18 years); 5) Association estimate: available information on multivariate or univariate model with risk estimate (hazard ratio (HR), odds ratio (OR), relative risk (RR)) and associated 95%confidence intervals (95% CI). If the study had any of the following characteristics, it was excluded: 1) Exposure: measurement carried on any other biological specimen than HDL or

ABDS or ABDP; 2) Outcome: studies assessing the relationship between HDL functionality and the progression of a previous cardiovascular condition; 3) Design: retrospective study, reviews or meta-analysis; 4) Population: pregnant or lactating women, children aged under 18y

Data extraction and quality assessment

After study selection, the following information was extracted from each article: first author's name, journal and year published, geographical location, eligibility criteria, outcome under investigation, biological sample on which the functionality tests were performed (HDL, ABDS or ABDP), HDL functionality assay, study design, follow-up time if applicable, sample size, number of cases, sex and mean age, statistical modelling method, confounders adjusted for, main findings including odds ratios or hazard / risk ratios and their standard errors / confidence intervals. All the data were recorded in a standardized extraction form. When the study authors built more than one model, we kept the estimates adjusted for a greater number of factors. When the same model was adjusted for a set of factor plus HDL-C or plus apolipoprotein A-I (Apo-AI), we only kept the one including HDL-C.

Titles and abstracts were screened and articles were excluded if they did not meet the eligibility criteria. Full texts were retrieved for the articles considered eligible, and a second evaluation was carried out. When relevant information was missed or we could not access the article, we contacted the corresponding author by e-mail.

The quality of the included articles was assessed by two independent investigators (CL and MSF) using the Newcastle-Ottawa Scale (13). This star-awarding system rates the quality of the methodology over three categories; selection, comparability and exposure. Each study can be scored a maximum of 9 stars; 4 for selection, 2 for comparability and 3 for exposure. To be included in the meta-analysis, a study had to meet our quality criteria of rating 5 stars or more.

Statistical analysis

For each HDL functionality separately and for each main outcome (CVD event or all-cause mortality), we used random-effect meta-analysis (14) to combine odds ratios (OR), or hazard ratios (HR) with corresponding 95% CI and provide an overall estimate of the association between HDL functionality and the prevalence or incidence of CV events or all-cause mortality. We pooled the risk estimate for the highest versus the lowest category and for a 1-SD change together.

To keep a consistent interpretation across studies, we express all estimates associated with a greater functionality performance in relation to CVD risk or mortality. When the published results are expressed as the association between worse functionality and disease, we inverted the ORs/HRs. An estimate lower than 1 means that greater HDL-related function is associated with a lower risk of outcome, whereas an estimate greater than 1 means that greater function is associated with higher risk.

We assessed between-study heterogeneity using the Q statistic and the I^2 statistic method (15). A value of I^2 of 0-40% was regarded as low heterogeneity, 30-60% as moderate heterogeneity, 50-90% as substantial heterogeneity, and 75-100% as high heterogeneity (16). Beyond the qualitative assessment of the I^2, presence of heterogeneity was formally tested by a chi-square test. A p-value <0.10 and high estimates of I2 indicated substantial and significant heterogeneity (16).

Any evidence of publication bias was visually diagnosed using a funnel plot and quantified with Egger's test (17). We considered the publication bias statistically significant if the two-sided p-value of Egger's test was lower than 0.05. The commands metan, confunnel and metabias in Stata version 14 (StataCorp, TX, USA) were used for the statistical analyses.

RESULTS

Search results and study characteristics

The detailed workflow of the search process and study selection is shown in Figure 1. The primary search yielded 4174 after exclusion of duplicates, which were screened on title and abstract. Of these, 2326were excluded because the exposure and/or the outcome were irrelevant and 1698 did not meet our inclusion criteria regarding language or study design.

The full text of the 150 potential studies were retrieved for further evaluation and after a detailed examination, 26 met the inclusion criteria to be included in the present meta-analysis. We present the results separately for the three main HDL functionalities that were evaluated in the studies retrieved: cholesterol efflux capacity (CEC), anti-inflammatory and anti-oxidant properties. The characteristics of these studies are shown in table 1, 2, and 3 respectively.

Data not shown in three manuscripts were kindly provided by the authors under request(18–20).

Cholesterol efflux capacity

We identified 17 manuscripts appraising the incidence and/or prevalence of a cardiovascular outcome or of all-cause mortality in relation with the capacity of HDL to remove lipids from cells and in one case from a lipid matrix. A total of 17,158 participants, aged 42-75.2y was included, of which 58% were men. The 17 articles comprised 10 longitudinal cohorts (20–28) and 9 case-control studies (19,29–34). Of these, 5 cohorts (20,24–26) and 1 case-contol study (30) reported on the risk of death of all-cause. All of them provided either hazard ratio, odds ratio or both. Only in one paper (33) the β-coefficient was provided but without a standard error or confidence interval, given that we could not obtain this information we did not include these data in the analysis. In 15 articles, the authors included HDL-C as adjusting factor(19–29,32–35).

With regards to the main technical characteristics of the assays used, we highlight (i) the donor cell line, (ii) the lipid tracer and (iii) the lipid acceptor.

In 15 out of the 17 articles analyzed, the assay was performed on differentiated-macrophage cell lines, including J774 (19,20,22–24,26–32,34), J7774(30) and J774A.1 or RAW264.7(19). In three studies, THP-1 monocyte were differentiated in situ into macrophages (21,25,36). The preferred tracer of choice was tritium-labeled cholesterol, and in one report the authors used ^{14}C-cholesterol as well (19)). Only in three articles, the assay was performed with BODIPY-cholesterol (26–28). Finally, we included one paper (33) describing the ability of HDL to accept cholesterol, phospholipids and triglycerides from a lipid emulsion in a cell-free assay. Regarding the lipid acceptor, all the studies used ABDS(19,22–24,26,29,31) or ABDP(28,30,33,35,36). Higher values indicated higher cholesterol efflux action. Cholesterol efflux was expressed as the normalized value to a control pool of serum, plasma or ApoB depleted serum or plasma except for two manuscripts(19,34)The percentage of variation explained by heterogeneity rather than chance across studies included in the analysis of the risk of mortality and of a cardiac event was high (I^2 87.1% and 87.8% respectively) and Cochrane Q statistic was significant for both groups (p<0.001), witnessing high inconsistency between studies.

Visual evaluation of funnel plots (Supplemental figure 1.a and 1.b) and Egger´s test (p=0.06 for all-cause mortality and p=0.003 for MACE) suggests limited publication bias for all-cause mortality, but evidence of publication bias for MACE outcomes.

In terms of study quality, two cohort studies (20,24)were rated only 5 stars out of 9 on the Newcastle Ottawa scale, two cohort and three case-control studies were granted 6 points(19,22,23,34) and the remaining studies achieved a punctuation of 7 or more (Supplemental material tables 1 and 2).

A random-effect model was performed to meta-analyze data from selected studies. The pooled all-cause mortality estimate associated with higher versus lower CEC was 0.62 (95% CI, 0.45 to 0.93), and for CVD the overall effect was 0.52 (95% CI, 0.38 to 0.70) (Figure 2).

Anti-inflammatory capacity

There were one cohort (37) and one case-control (36) study meeting our inclusion criteria that assessed the anti-inflammatory potential of HDL in relation to CVD events. No study was found that used all-cause mortality as an outcome. The two studies comprise of 204 participants (67% men) in total, aged 63-67y. The case-control study focused on prevalent myocardial infarction, whereas the cohort study cross-sectionally compared patients with myocardial infarction to patients with non-cardiac chest pain, as well as assessed the incidence of new MACE over 3 years of follow-up. Both the case-control and cohort studies included HDL-C to model the association in multivariate adjusted models (36,37).

The main technical characteristics of the protocols evaluating the anti-inflammatory potential of HDL included (i) the cell line,(ii) the pro-inflammatory stimuli, (iii) HDL, ABDS or ABDP and (iv) the marker of inflammation.

In both papers the technique was based on an *in–vitro* system: after the incubation of human umbilical endothelial cell lines with ABDP, the cultured cells were challenged with tumor necrosis factor-α and total RNA was isolated to further measure VCAM-1 mRNA expression levels. Lower values indicated better HDL anti-inflammatory capacity. Results were normalized to control values or baseline levels in the case-control and cohort study respectively, allowing it for comparison of results.

Heterogeneity between the two studies was significant and deemed substantial (I^2=70.5% and Q-statistic p=0.07). The forest plot for the interventions included is shown in Figure 3. Presence of publication bias was revealed by the lack of studies at the right of the funnel plot (supplemental figure 1.c). Both studies were considered of high quality with 7 stars on the Newcastle Ottawa scale (Supplemental material tables 1 and 2).

Overall, individuals presenting the highest anti-inflammatory HDL capacity had a lower risk (borderline significant) of CVD compared to individuals with low anti-inflammatory capacity: pooled OR 0.36 (0.12 to 1.03).

Anti-oxidant capacity

Ten studies were included that evaluated the anti-oxidative capacity of HDL and the risk of CVD or all-cause mortality: 4 case-control studies (31,36,38,39) and 6 cohorts (18,40–44). Overall, these studies included a total of 2563 participants aged 35 to 67y, of which 65.7% were men. The four case-control studies (31,36,38,39) and two cohorts (42,43) evaluated the association of an HDL profile prone to protect low-density lipoprotein (LDL)and other compounds from oxidation with heart disease and CVD mortality. Only in one case-control and two cohort studies, the models for risk assessment were adjusted for HDL-C(31,35,39,43,44).

Two major protocols were used and we recorded the following technical details: (i) HDL, ABDP or ABDS, (ii) native or previously oxidized LDL, (iii) a marker of oxidative degradation.

In six studies, the authors assessed the anti-oxidant action of ABDS samples on pre-oxidized LDL (18,31,38–40,43) or on native LDL (41) in presence of the non fluorescent dichlorofluorescein diacetate (DCF-DA). The conversion of DCF-DA to its fluorescent form reflects the oxidation of LDL, and therefore, higher values obtained in the test were considered to be indicative of poorer protection against oxidation. A variant of this procedure was found in one study(44). No native or pre-oxidized LDL was involved in the technique and the marker of fluorescence was dihydrorhodamine (DHR). A greater percentage of reduction in oxidation reflects a better protective action of HDL.

The other protocol, used in two studies(35,42),is based on the production of thiobarbituric acid reactive substances (TBARS) as a measure of the oxidative modification of LDL. Here, HDL antioxidative capacity is calculated as the percent reduction in TBARS formation obtained relative to a negative control, and lowervalues indicate better antioxidative protection.

I^2 and Q statistics show very high heterogeneity between studies for both cardiovascular outcomes (I^2=93%, chi-squared p<0.001) and all-cause mortality (I^2=82.2%, chi-squared p<0.001). Funnel plot (supplemental figure 1.e) for interventions evaluating the risk of all-cause mortality shows asymmetry and

Egger´s test was significant (p=0.002), indicating that small studies with non significant findings are likely not published. There was also some evidence of publication bias for studies with cardiovascular outcomes by inspecting the funnel plot (supplemental figure 1.d) and the Egger´s t test for significant (p= 0.03)

In terms of methodology quality, two cohorts were rated only 5 stars(40)and another one cohort 6 stars (43,44) while the other seven manuscripts scored 7 or more stars(Supplemental material tables 1 and 2)

Higher antioxidant protection exerted by HDL was associated with lower risk of CVD with a combined estimate of 0.67, 0.53-0.84 and of all-cause mortality (combined HR = 0.74 (0.57-0.96))(Figure 4).

DISCUSSION

To our knowledge, this is the first comprehensive meta-analysis exploring the association between different functional properties of HDL and the risk of cardiovascular events and of all-cause mortality.

We have identified three main domains of functionality encountered in the literature: cholesterol efflux capacity, anti-inflammatory and anti-oxidant capacity. In summary, higher levels of cholesterol efflux capacity, and greater anti-inflammatory and antioxidant capacities exerted by HDL were associated with lower risk of ischemic cardiovascular disease. A similar association was observed for the risk of all-cause mortality with CEC and antioxidant activity, but no study was available on anti-inflammatory capacity and mortality.

Atherosclerosis, the underlying cause of coronary heart diseases (45), occurs when the lumen of the blood vessel becomes narrower (46) due to deposition of lipids and cellular detritus in the tunica intima. Infiltration of LDL particles into the media layer and further oxidation of the particles (47) triggers the expression of proinflammatory molecules and facilitate the attachment of monocytes to endothelium surface and translocation to the intima media where they mature to macrophages. Uncontrolled cholesterol uptake by monocyte-

derived macrophages leads them to become "foam cells" which contribute to the formation of the necrotic core and its instability (48).

HDL is under constant remodeling in the plasma (49), yielding a number of subparticles differing in size, density and composition that might influence its functionality (50,51). The variability in HDL particles and therefore in HDL functionality may help to better elucidate why not always high levels of HDL-C predict CHD risk (5,52,53) despite the strong inverse associations found in large epidemiological studies (1,54).

Cholesterol efflux capacity

The ability to remove cholesterol from macrophages is considered the main HDL-related protective function in relation to atherosclerotic disease (55). Since Bailey and collaborators described the ability of these lipoproteins to behave as acceptors of cellular cholesterol (56), the role of cholesterol efflux from macrophages in the physio-pathology of a range of diseases such as polycystic ovary syndrome, human immunodeficiency virus infection, rheumatoid arthritis and systemic lupus erythematosus has been studied (57–59). However, the mechanisms behind the probable protective effect of cholesterol efflux by HDL and its pathological determinants remain to be fully understood (60,61).

In our meta-analysis, we found that higher cholesterol removal from macrophages prompted by ABDS or ABDP was inversely associated with cardiovascular events and all-cause mortality. Although results from the majority of the studies are consistent, one study found opposite results. In the study by Li et al (19), out of 1150 participants, 871 had prevalent CAD and over a median 3 years of follow-up, 113 new major adverse cardiac event were registered. The hazard ratio for MACE for people in the highest versus lowest tertile of cholesterol efflux was 1.85; 95% CI, 1.11–3.06.In the study, levels of Apo A-I were lower and levels of apolipoprotein A-II (ApoA-II) higher in patients with CAD. Moreover, there were a much higher proportion of participants on statin treatment in the CAD group compared to the non CAD group.

Presence of ApoA-II in HDL has been reported to increase macrophage-efflux cholesterol(62) and use of statins have a positive impact on HDL functions(63). These might be two reasons why patients with CAD express larger cholesterol efflux under ABDS activity. We hypothesized that further adjustment of multivariate models would be desirable and might have an impact on the direction of results.

Anti-inflammatory and Antioxidant functions

Pooled data from selected studies identified an inverse association between a better protection against oxidation and inflammation mediated by HDL and decreased risk of coronary event. These results are consistent with current knowledge on the atherosclerosis etiology .Accumulation of oxidized LDL in the innermost layer of the artery wall is a key step in the initiation atherosclerotic-related processes(64), and the development of the atherosclerotic plaque is largely driven by persistent low-grade inflammation(65). Accordingly, several studies have identified impaired HDL antioxidant and anti-inflammatory capacities as a potential biomarker of the atherosclerosis processes (66,67).

In addition, oxidation and inflammation state of the HDL particle have a deleterious effect on its cholesterol efflux capacity among other HDL actions (60,68). Lipopolysaccharide-induced low-grade inflammation impairs HDL-cholesterol efflux from J774 macrophages(69)and cholesterol efflux has been inversely associated with antioxidant capacity of HDL in a study of patients with rheumatoid arthritis (70). Moreover, pre-oxidized HDL particles show a poorer ability to remove cholesterol from cells and a greater percentage of cholesteryl esters remaining within the cells compared with non-oxidized HDL(68). Our meta-analysis identified 9 articles on the antioxidant capacity and 2 articles on the anti-inflammatory ability of HDL. There is a need for larger longitudinal studies with standardized designs to deeper understand the potential role of HDL functions other than to its ability to efflux cholesterol from cells in relation to cardiovascular diseases and mortality.

Limitations

Some issues were identified in the retrieved articles and we advocate that they need to be addressed in future studies in order to advance the knowledge of the relationship between HDL related functions and CVD risk. This is essential before HDL functionality markers can be considered as novel CVD predictors.

Though this meta-analysis was primarily intended for heart and coronary diseases, we also included broader outcomes such as cardiovascular mortality, stroke or other CVD, because in most studies, the endpoint under evaluation was composite. There is therefore a lack of specificity and our meta-analysis is not able to disentangle the specific effects of HDL on different clinical manifestations and progression of atherosclerotic diseases.

Additionally, baseline characteristics of patients vary across studies and have to be taken into consideration. Age, sex and prevalent pathologies have an extensive impact on the development of cardiovascular diseases. The majority of studies report a HR and/or OR adjusted by age, but not all of them considered the effect of sex on results, even when analysis of baseline characteristics reveal significant inconsistency between the groups under comparison (24). Moreover, some of the analyses included here were not initially intended in the design of the studies. Consequently, data on risk factors and related markers was not necessarily reported and varied greatly between studies.

Differences in experimental approaches can also have a great impact on clinical results. Methods to isolate HDLs, ABDP or ABDS might have consequences on the nature of HDL subparticles, content in apolipoproteins and HDL functionality. Extraction by precipitation with polyethylene glycol tends to show better results, but is often accompanied by a considerable decrease in specimen size(71–73). Furthermore, different acceptors are involved in different pathways of cholesterol efflux(74), so consistency among studies could be gained by the adoption of a common biological sample and a standardized method when describing a specific pathway involved in the cholesterol efflux process (75).

In relation to cholesterol efflux, the use of radio labeled cholesterol still offers some advantages over fluorescent probes, in terms of sensitivity and

accuracy(76). In our meta-analysis, only a minority of studies used BODIPY-cholesterol instead of the traditional radio labeled lipid, despite many logistic and cost advantages. Although some controversy remains on the degree of correlation between results obtained with both probes(71),inter-run variability of the assay with BODIPY-cholesterol has been shown high reproducibility, thus switching to fluorescent cholesterol represents many advantages, including safety and efficiency (77,78).

CONCLUSIONS

The present meta-analysis represents a valuable comprehensive summary of the state-of-the-art regarding HDL functionality and its utility as predictor of cardiovascular diseases and overall mortality. Larger studies designed to reveal specific cardiovascular outcomes, instead of combined endpoints, will greatly minimize the variability observed and will help to obtain consistent results on the value of HDL function for the prediction of cardiac outcomes. Higher levels of cholesterol efflux capacity and anti-oxidant/anti-inflammatory capacities promoted by the HDL particle were associated with lower risk of ischemic cardiovascular disease. A similar association was observed for the risk of all-cause mortality with cholesterol efflux and antioxidant activity. Results are encouraging but our major concern is the lack of a body of studies, particularly on the anti-inflammatory role of this lipoprotein, adequate enough to draw unequivocal conclusions.

TABLES AND FIGURES

Figure1. Workflow of the search process and study selection

| Articles identified through literature search (Medlineand Embase) after removal of duplicates n=4174 |

| Records excluded - not meeting criteria for:
• Exposure/outcome: 2326
• Study design/language: 1698 |

| Studies scrutinized for full evaluation: n=150 |

| Articles further excluded - not meeting criteria for:
• Exposure / outcome: 124 |

| Full-text articles included: n=26 |
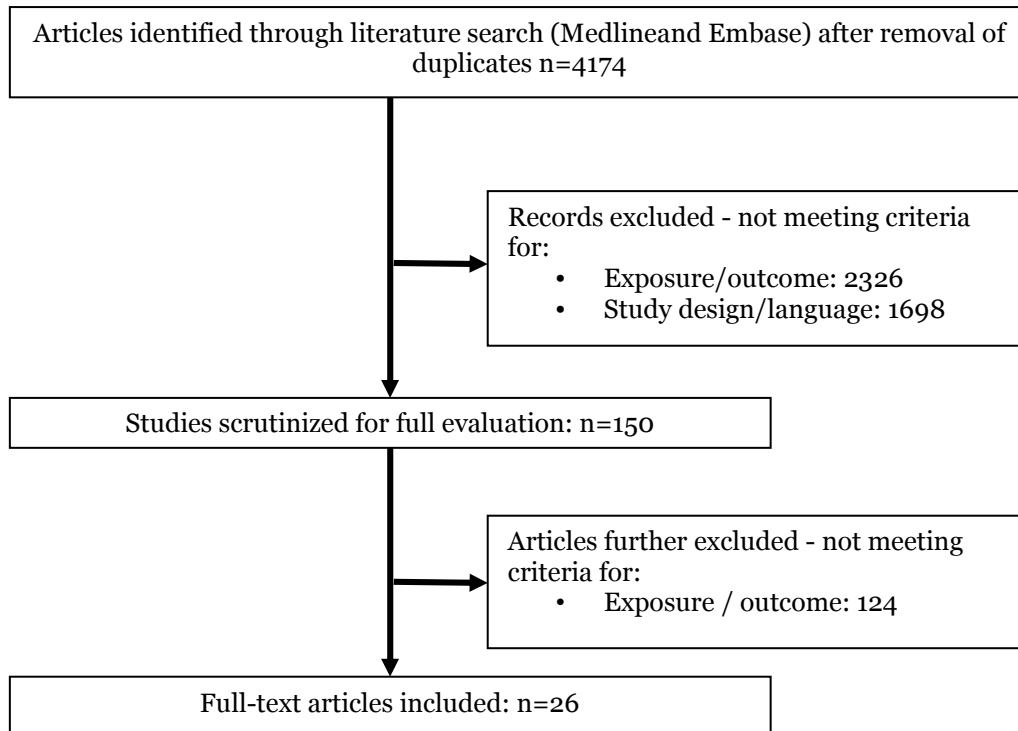
Figure2. Meta-analysis of studies investigating the association between HDL cholesterol efflux capacity and major adverse cardiac events (MACE) and all-cause mortality risk. Estimates are ORs or HRs for MACE (panel A) or all-cause mortality (panel B) for a better compared to poorer CEC function (1SD increase or categories such as quantiles, as specified)
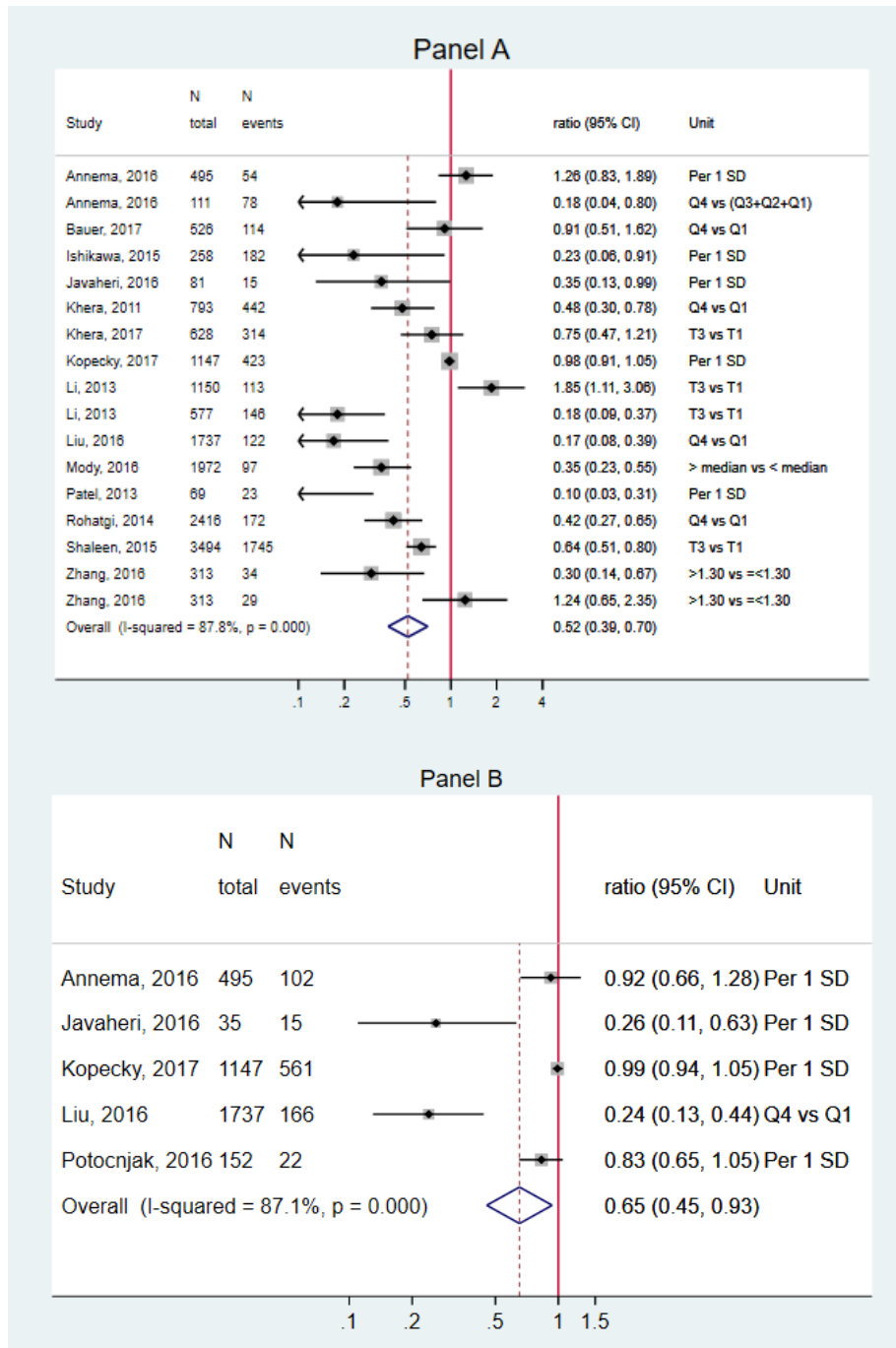
Figure3. Meta-analysis of studies investigating the association between HDL anti-inflammatory capacity and major adverse cardiac event risk. Estimates are ORs or HRs for MACE for a better compared to poorer anti-inflammatory function (1SD increase or categories such as quantiles, as specified)
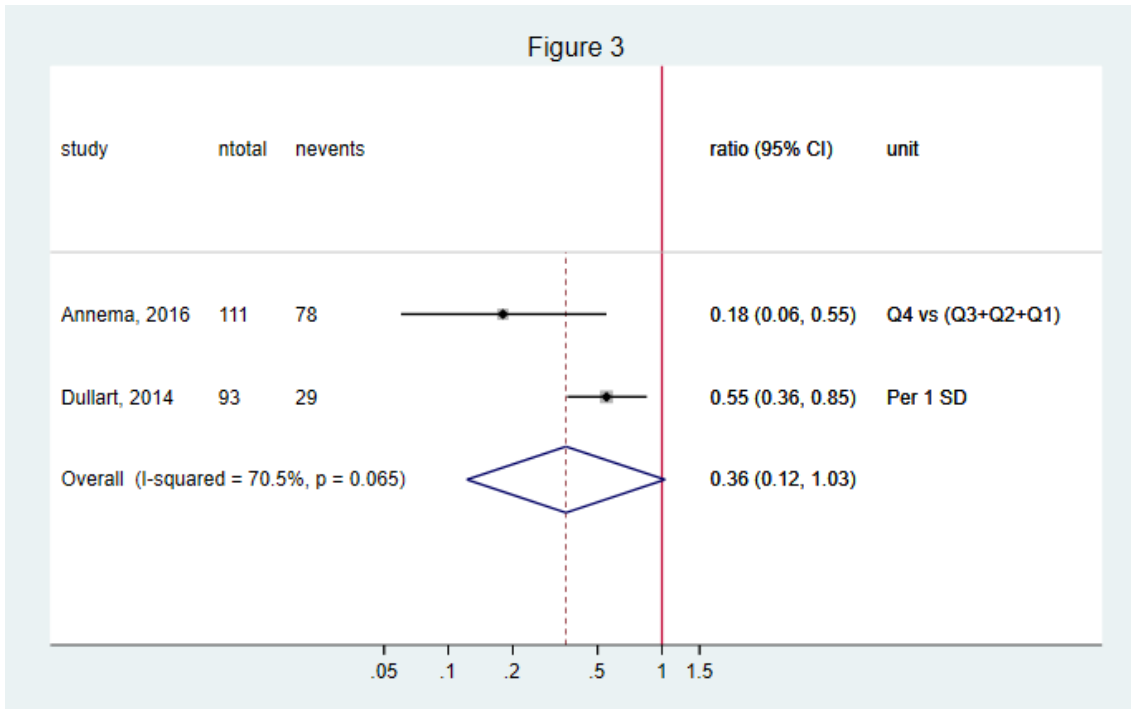
Figure4. Meta-analysis of studies investigating the association between HDL anti-antioxidant capacity and major adverse cardiac events (MACE) and all-cause mortality risk. Estimates are ORs or HRs for MACE (panel A) or all-cause mortality (panel B) for a better compared to poorer anti-oxidant function (1SD increase or categories such as quantiles, as specified)
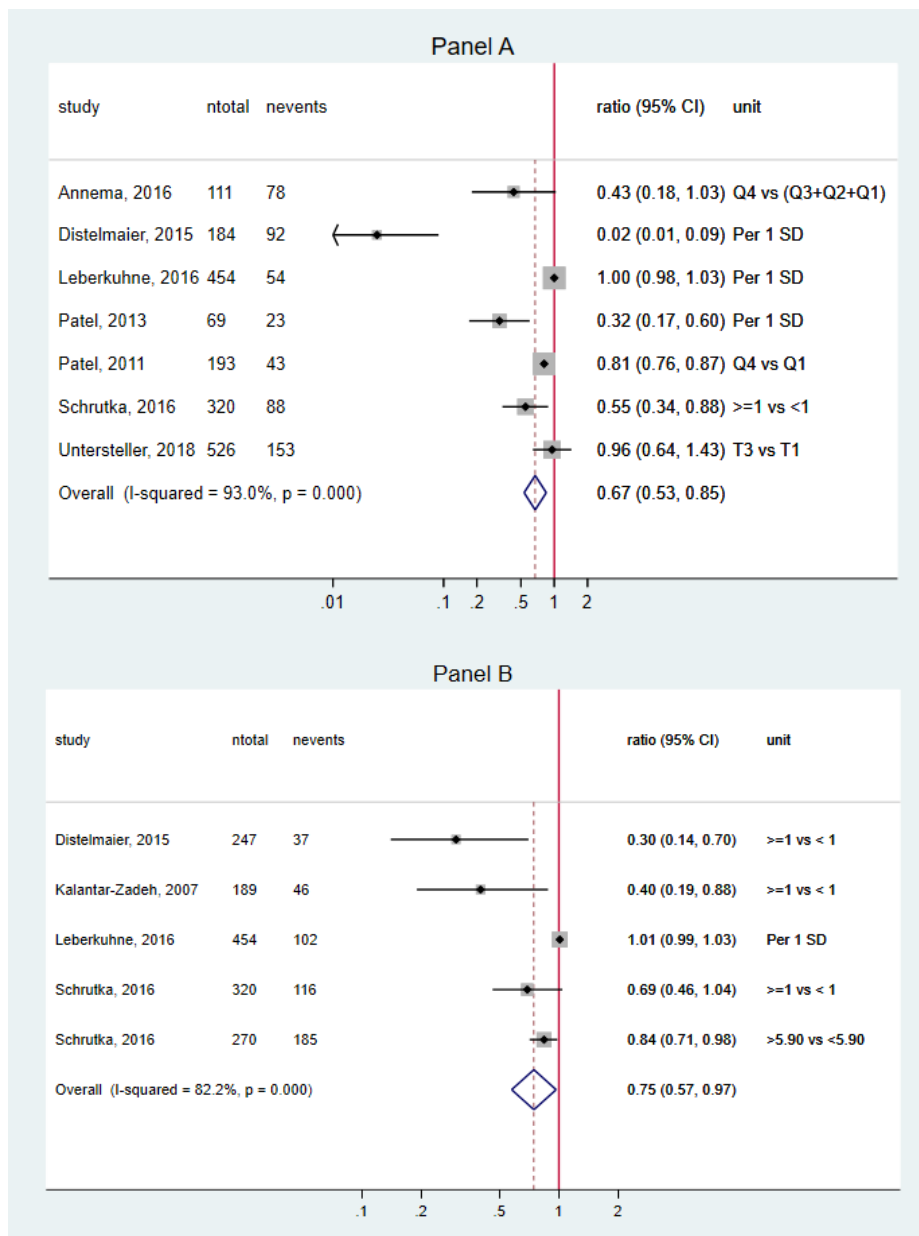
Table1. Characteristics of Studies assessing the relationship between Cholesterol Efflux Capacity and cardiovascular outcomes and all-cause mortality

Author Year Country	Design Follow up	Population, N Age, y Male, %	Outcome and number of cases	Assay	CE computation	Statistical model	HR/OR (95% CI) for greater CEC function	Adjustment factors
Annema 2016 The Netherlands	Cohort 7.0y	N=495 51.6y 54% Renal transplant recipients	102 All-cause mortality	Cell line, THP-1 derived macrophages ^{3}H-Cholesterol. ApoB-depleted plasma	CE = [(radioactivity in medium /radioactivity total)*100]-[CE without ApoB-depleted] CE normalized to an ApoB-depleted pool Higher values indicate better protection	Cox proportional hazards model	For 1 SD increase HR=0.92 (0.66–1.28)	Sex, age, HDL-C
			54 CVD mortality				For 1 SD increase HR=1.26 (0.83 to 1.89)	
Annema 2016 The Netherlands	Case/control	N=111 63-67y 67%	78 MI	Cell line, THP-1 derived-macrophages ^{3}H-Cholesterol. ApoB-depleted plasma	CE = [(radioactivity in medium /radioactivity total)*100]-[CE without ApoB-depleted] CE normalized by dividing each value by the corresponding theoretical	Logistic regression	(Q3+Q2+Q1) vs Q4 Inverted OR=0.18 (0.04-0.8)	Age, HDL-C (matched controls)

					value from pooled plasma Lower values indicate better protection			
			37 STEMI				(Q3+Q2+Q1) vs Q4 Inverted OR=0.17 (0.04-0.77)	
			41 Non-STEMI				(Q3+Q2+Q1) vs Q4 Inverted OR=0.15 (0.019-1.25) Non-STEMI vs STEMI (Q3+Q2+Q1) vs Q4 Inverted OR=0.2 (0.06-0.72)	

Bauer 2017 Austria	CARE FOR HOMeCohort 4.6y	N=526 65y 59% CKD patients with Cronic Kidney Disease	142 Primary endpoint: composite of atherosclerotic CVD (MI, coronary artery or lower limb artery angioplasty/stenting/bypass surgery, major stroke, carotid endarterectomy/stenting, or nontraumatic lower extremity amputation) and all-cause mortality	Cell line, J774 macrophages ^{3}H-Cholesterol. ApoB-depleted serum	CE= [(radioactivity in medium with ApoB-depleted-radioactivity serum-free medium /radioactivity in cells before the efflux step)*100] CE normalized to a serum pool Higher values indicate better protection	Cox proportional hazards model	Q4 vs Q1 HR=0.94(0.55-1.59)	Age, sex, BMI, mean BP, smoking status, estimated GFR, log-albuminuria, TC, HDL-C
			114 Secondary endpoint (CVD and CVD mortality)				Q4 vs Q1 HR=0.91 (0.51-1.62)	
Ishikawa 2015 Japan	Cohort	N=258 66.2-64.3y 76.7% Patients with and without CAD	182 CAD	Cell line, J774 macrophages ^{3}H-Cholesterol. ApoB-depleted serum	CE= [(radioactivity in medium with ApoB-depleted-radioactivity serum-free medium /radioactivity in cells before the efflux step)*100] CE normalized to a serum pool Higher values indicate better protection	Logistic regression	For 1 SD increase OR=0.23 (0.06-0.91)	Age, sex, HT, dyslipidemia, DM, smoking, family history of CAD, BMI, TC, TG, HDL-C, statin administration, apoA-I, HbA1c, hematocrit, creatinine, NT-proBNP

Javaheri 2016 US	Cohortsurvivors 931d (2.55y) Non-survivors 630d (1.72y)	N=35 42.8-46.5y 85.7% CAV patients	15 All-cause mortality	Cell line, J774 macrophages ³H-Cholesterol. ApoB-depleted serum	CE= [(radioactivity in medium /radioactivity total)*100] CE normalized to a plasma or serum pool Higher values indicate better protection	Cox proportional hazards model	For 1 SD increase HR=0.26 (0.11-0.63)	Age, history of ischemic cardiomyopathy, HDL-C, LDL-C
Javaheri 2016 US	CAV Cohort study 1y	N=81 78% Patients after First Cardiac Transplant	15 CAV			Logistic regression	For 1 SD increase OR=0.35 (0.13-0.99)	LDL-C, HDL-C, DM
Khera 2011 US	Case/control	N=793 57-62y 59.6%	442 CAD	Cell line, J774 macrophages ³H-Cholesterol. ApoB-depleted serum	CE= [(radioactivity in medium with ApoB-depleted-radioactivity serum-free medium /radioactivity in cells before the efflux step)*100] CE normalized to a serum pool Higher values indicate better protection	Logistic regression	Q4 vs Q1 OR=0.48 (0.30−0.78) For 1SD increase OR=0.75 (0.63-0.90)	Age, sex, smoking, DM, HT, LDL-C, HDL-C

Khera 2017 US	Case/control	N=628 69y 72.6%	314 Primary endpoint (MI, hospitalization for unstable angina, arterial revascularization, stroke, CVD mortality)	Cell line, J7774 macrophages ^{3}H-Cholesterol. ApoB-depleted plasma	CE= [(radioactivity in medium with ApoB-depleted-radioactivity serum-free medium /radioactivity in cells before the efflux step)*100] CE normalized to a serum pool Higher values indicate better protection	Logistic regression	T3 vs T1 OR=0.75 (0.47-1.21) For 1SD increase OR=0.89 (0.72-1.10)	Age, sex (matched controls), race, randomized treatment group, smoking, systolic BP, BMI, fasting glucose, LDL-C, log-TG, family history of premature CAD
	Case/control	N=1050 69-70y 71.6%	525 Secondary extended endpoint (primary endpoint + all-cause mortality)				T3 vs T1 OR=0.69 (0.48-1.01) For 1SD increase OR=0.85 (0.73-1.00)	
Kopecky 2017 The Netherlands, Austria, Germany, Brasil	Cohort 4.1y	N=1147 65.7y 54% Type 2 DM patients in hemodialysis	423 Combined primary end point (CVD mortality, non-fatal MI, and stroke)	Cell line,THP-1 derived-macrophages ^{3}H-Cholesterol. ApoB-depleted plasma	CE = [(radioactivity in medium /radioactivity total)*100]-[CE without ApoB-depleted] CE normalized to a plasma pool Higher values indicate better protection	Cox proportional hazards model	For 1SD increase HR=0.98 (0.91-1.05)	Age, sex, CAD, arrhythmia, transitory ischemic attack, congestive HF, peripheral vascular disease, smoking, SBP/DBP, BMI, albumin, phosphate, hemoglobin, HbA1c, duration of dialysis, LDL-C, HDL-C, Apo A-I, CRP
			410 Cardiac events (CVD mortality and non-fatal MI)				For 1SD increase HR=0.94 (0.85-1.04)	
			561 All-cause mortality				For 1SD increase HR=0.99 (0.94-1.05)	

Li 2013 US	Case/control 3y	N=1150 61-72y 64% Patients with and without CAD (Angiographic cohort)	871 CAD	Cell lines, RAW264.7 and J774A.1 macrophages ^{3}H-Cholesterol ^{14}C-Cholesterol ApoB-depleted serum	CE= [(radioactivity in medium /radioactivity total)*100] Higher values indicate better protection	Logistic Regression	T3 vs T1 OR=1.24 (0.77-2.02)*	Age, sex, smoking , DM, HT, HDL-C, LDL-C
			113 Major Adverse Coronary Event (MACE): death, non-fatal MI, non-fatal stroke			Cox proportional hazards model	T3 vs T1 HR=1.85 (1.11–3.06)	
			58 MI/stroke				T3 vs T1 HR=2.19 (1.02–4.74)	
Li 2013 US	Case/control	N=577 53-59y 43.8% Patients with and without CAD (Outpatient cohort)	146 CAD			Logistic Regression	T3 vs T1 OR=0.18 (0.09-0.37)*	Age, sex, smoking , DM, HT, HDL-C, LDL-C
Liu 2016 China	Cohort 3.8y	N=1737 65-61.7y 65.17% Patients with	166 All-cause mortality	Cell line, J774 macrophages BODIPY-Cholesterol ApoB-depleted	CE = [(fluorescence in medium / initial cell content of BODIPY cholesterol)]	Cox proportional hazards model	Q4 vs Q1 HR=0.24 (0.13-0.44) For 1 SD increase HR=0.10	Age, sex, BMI, smoking, alcohol drinking, prevalence of HT, DM, dyslipidemia,

				serum	CE normalized to an ApoB-depleted pool Higher values indicate better protection		(0.02-0.61)	use lipid-lowering drugs, TC, log-TG, LDL-C, HDL-C, Apo A-I
			CAD					
			122 CVD mortality				Q4 vs Q1 HR=0.17 (0.08-0.39) For 1 SD increase HR=0.08 (0.01-0.68)	
Mody 2016 US	Cohort 9.4y	N=1972 44.9y 44% Patients with and without CAD	97 Composite of atherosclerotic CVD: first non-fatal MI, non-fatal stroke, coronary revascularization (percutaneous coronary intervention, coronary artery bypass grafting), CVD mortality	Cell line, J774 macrophages BODIPY-Cholesterol ApoB-depleted plasma	CE = [(fluorescence in medium / initial cell content of BODIPY cholesterol)] CE normalized to an ApoB-depleted pool Higher values indicate better protection	Cox proportional hazards model	CEC > median vs CEC < median HR=0.35 (0.23-0.55)	Age, sex, race, DM, SBP, smoking, BMI, TC, HDL-C, history of anti-hypertensive medication use, statin use, prevalent CAC, family history of MI, elevated high sensity-CRP.
Patel 2013 US	Case/control	N=69 57.8-58.2y 58%	23 Ischaemic HF	Cell line, J774 macrophages ³H-Cholesterol ApoB-depleted serum	CE= [(radioactivity in medium with ApoB-depleted-radioactivity serum-free medium /radioactivity in cells before	Logistic Regression	For 1 SD increase Inverted OR=0.10 (0.03-0.31)	Age, BP, creatinine, TC, LDL-C, HDL mass

					the efflux step)*100] CE normalized to a control sample Lower values indicate better protection			
Potocnjak, 2016 Croatia	Cohort	N=152 75.2y 48% Acute HF patients	22 Hospital mortality	Cell line, J774 macrophages ^{3}H-Cholesterol ApoB-depleted serum	CE= [(radioactivity in medium /radioactivity total)*100]	Logistic regression	For 1 SD increase OR=0.83 (0.65-1.07) *	Age, sex, BMI, LDL-C, HDL-C, log-TG, mean BP
Rohatgi 2014 US	Cohort 9.4y	N=2416 42y 43% Free of CVD	132 Atherosclerotic CVD: non-fatal MI, non-fatal stroke, coronary revascularization – percutaneous coronary intervention or coronary artery bypass grafting- or CVD mortality 172 Total CVD: composite of atherosclerotic CVD and peripheral revascularization or hospitalization for HF or atrial fibrillation 84 Hard atherosclerotic CVD: composite of fatal or non-fatal MI or stroke	Cell line, J774 macrophages BODIPY-Cholesterol ApoB-depleted plasma	CE=[(fluorescence in medium / initial cell content of BODIPY cholesterol)] CE normalized to an ApoB-depleted plasma pool Higher values indicate better protection	Cox proportional hazards model	Q4 vs Q1 HR=0.33 (0.19–0.55) For 1SD increase HR=0.68 (0.55-0.84) Q4 vs Q1 HR=0.42 (0.27–0.65) For 1 SD increase HR=0.79 (0.67-0.94) Q4 vs Q1 HR=0.40 (0.21–0.74)	Age, sex, race, DM, HT, smoking, BMI, TC, log-TG, statin use, HDL-C, HDL particle concentration

			30 MI				Q4 vs Q1 HR=0.44 (0.17–1.18)	
			37 Stroke				Q4 vs Q1 HR=0.11 (0.02–0.47)	
			48 Coronary revascularization				Q4 vs Q1 HR=0.19 (0.08–0.45)	
			46 CVD mortality				Q4 vs Q1 HR=0.94 (0.37–2.37)	
Saleheen 2015 UK	Case/control	N=3494 65-66.1y 64.5%	1745 CHD: unstable angina, stable angina, and MI -fatal or not	Cell line,J774 macrophages ^{3}H-Cholesterol ApoB-depleted serum	CE= [(radioactivity in medium with ApoB-depleted-radioactivity serum-free medium /radioactivity in cells before the efflux step)*100] CE normalized to a control sample Higher values indicate better protection	Logistic regression	T3 vs T1 OR=0.64 (0.51–0.80) For 1 SD increase OR=0.80 (0.70–0.90)	Age, sex (matched controls), DM, HT, cigarette use, alcohol use, waist:hip ratio, BMI, LDL-C, log-TG, HDL-C

Sprandel 2015 Brazil	Case/control	N=154 63y 48.7%	79 CAD	Lipid emulsion: ^{3}H-Cholesteryl ester, ^{14}C-Phospholipid, ^{14}C-Unesterified cholesterol, ^{3}H-Triglyceride ApoB-depleted plasma	CE= % of the total incubated radioactivity found in the HDL-containing supernatant.	Logistic regression	Unesterified cholesterol transfer β= -0.416. SE not provided	Age, sex, BMI (matched controls), HDL-C, TG, TG/HDL-C, % UC, TC /UC, Apo A-I, ApoB, CETP concentration, LCAT activity, cholesteryl ester transfer, phospholipid transfer, TG transfer, UC in HDL
							Cholesteryl ester transfer β = -0.211. SE not provided	Age, sex, BMI (matched controls), HDL-C, LDL-C , non HDL-C , Apo A-I , CETP concentration, LCAT activity, UC transfer, TG transfer, TG in HDL.

							Triglyceride transfer β = -0.139. SE not provided	Age, sex, BMI (matched controls), HDL-C, LDL-C, non HDL-C, Apo A-I, CETP concentration, LCAT activity, cholesteryl ester transfer, phospholipid transfer, UC transfer, UN in HDL, TG in HDL
Zhang 2016 China	Case/control 3y	N=313 66-67y 75.7%	34 Atherosclerotic CVD: non-fatal MI, 10 non-fatal stroke, 22 CVD mortality	Cell line, J774 macrophages ^{3}H-Cholesterol ApoB-depleted serum	CE = [(radioactivity in medium /radioactivity total)*100] CE normalized to a serum pool Higher values indicate better protection	Cox proportional hazards model	CEC≤ 1.30 vs>1.30 HR=0.30 (0.14-0.67)	Age, sex, HT, DM, smoking, serum LDL-C, HDL-C
							CEC≤ 1.30 vs> 1.30 HR=0.91 (0.24-3.42)	
							CEC≤ 1.30 vs>1.30 HR=0.23 (0.08-0.68)	
			29 Secondary endpoints: 19 revascularization, 20HF				CEC≤ 1.30 vs CEC1.30 HR=1.24 (0.65-2.35)	
			19 revascularization				CEC≤ 1.30 vs>1.30	

						HR=0.96 (0.39-2.38)	
			20 HF			CEC≤ 1.30 vs>1.30 HR=1.79 (0.72-4.44)	
			214 Acute artery syndrome			Logistic regression	CEC≤ 1.30 vs CEC>1.30 OR=0.25 (0.14-0.46)	

Foot note list abbreviations: CVD indicates cardiovascular disease; HDL-C, High density lipoprotein-cholesterol; MI, Myocardial infarction; STEMI, ST-elevation Myocardial infarction; CKD, Cronic Kidney Disease; BMI, Body Mass Index; BP, Blood-pressure; GFR, Glomerular Filtration Rate; TC, Total Cholesterol; CAD, Coronary Artery Disease; HT, Hypertension; DM, Diabetes Mellitus; TG, Tryclicerides; ApoA-1, Apolipoprotein A-1; HbA1c, Hemoglobin A1C; NT-proBNP, N-terminal pro-B-type natriuretic peptide; CAV, Cardiac Allograft Vasculopathy; LDL-C, Low density lipoprotein-Cholesterol; SBP, Systolic Blood Pressure; DBP, Diastolic Blood Pressure; CRP, C-reactive protein; CAC, Caronary Artery Calcium; CHD, Coronary Heart Disease; UC, Unesterified Cholesterol; ApoB, Apolipoprotein B; CETP, Cholesteryl ester transfer protein; LCAT, lecithin cholesterol acyltransferase; OR, odds ratio; HR, hazard ratio; HUVEC, Human umbilical vascular endothelial cell; TNF-α, Tumor necrosis factor-α; VCAM-1, Vascular cell adhesion molecule-1.

 * Data provided by the author.

Table2. Characteristics of Studies assessing the relationship between anti-inflammatory capacity and cardiovascular outcomes and all-cause mortality

Author Year Country	Design Follow up	Population, N Age, y Male, %	Number cases, outcome	Assay	Anti-inflammatory activity computation	Model	HR/OR (95% CI)	Adjustment factors
Annema 2016 The Netherlands	Case control	N=111 63-67y 67%	78 MI	Human umbilical vascular endothelial cell (HUVEC) ApoB-depleted plasma Tumor necrosis factor-α (TNF-α)	Vascular cell adhesion molecule-1 (VCAM-1) mRNA expression, relative to the housekeeping gene cyclophilin Results relative to the expression of controls Lower values express a better anti-inflammatory capacity	Logistic regression	(Q3+Q2+Q1)vsQ4 Inverted OR=0.18 (0.06-0.55)	Age, HDL-C (matched controls)
			37 STEMI				(Q3+Q2+Q1)vsQ4 Inverted OR=0.06 (0.007-0.41) +	
			41 Non-STEMI				(Q3+Q2+Q1)vsQ4 Inverted OR=0.43 (0.14-1.51)	

Dullaart 2014 The Netherlands	Cross-sectional analysis	N=93 63-68y 62.3% Patients with acute chest pain	65 Acute MI	HUVEC ApoB-depleted plasma TNF-α	VCAM-1 mRNA expression, relative to the housekeeping gene cyclophilin Results relative to the expression of baseline	Logistic regression	For 1 SD: Inverted OR=0.18 (0.06-0.52)	Age, sex, previous MI, DM, smoking, treatment with statins, anti-hypertensive and anticoagulants
	Cohort 1210d(3.3y)		29 MACE: CVD mortality, MI, percutaneous coronary intervention and/or coronary artery bypass grafting	HUVEC ApoB-depleted plasma TNF-α	VCAM-1 mRNA expression, relative to the housekeeping gene cyclophilin Results relative to the expression of baseline Lower values express a better anti-inflammatory capacity	Cox proportional hazards model	For 1 SD Inverted HR=0.55 (0.36-0.85)	Age, sex, HDL-C, Apo A-I

Foot note list abbreviations: CVD indicates cardiovascular disease; MACE, major adverse cardiovascular events; OR, odds ratio; HR, hazard ratio; HDL-C, High density lipoprotein-cholesterol; MI, Myocardial infarction; STEMI, ST-elevation Myocardial infarction; DM, Diabetes Mellitus; ApoA-1, Apolipoprotein A-1; ApoB , Apolipoprotein B; HUVEC, Human umbilical vascular endothelial cell; TNF-α, Tumor necrosis factor-α; VCAM-1, Vascular cell adhesion molecule-1.

Table3. Characteristics of Studies assessing the relationship between Anti-oxidant Capacity and cardiovascular outcomes and all-cause mortality

Author Year Country	Design Follow up	Population, N Age, y Male, %	No. Case	Assay	Anti-oxidant activity computation	Model	HR/OR (95% CI)	Adjustment factors
Annema 2016 The Netherlands	Case/control	N=111 63-67y 67%	78 MI	ApoB-depleted plasma LDL Fluorescence TBARS	Result = % reduction in TBARS formation with sample relative to a TBARS formation without sample Higher values indicate better protection against LDL oxidation	Logistic regression	Q4 vs (Q3+Q2+Q1) OR=0.43 (0.18-1.03)	Age, HDL-C (matched controls)
			37 STEMI				Q4 vs (Q3+Q2+Q1) OR=0.48 (0.18-1.28)	
			41 Non-STEMI				Q4 vs (Q3+Q2+Q1) OR=0.38 (1.21-0.12)	
Distelmaier 2015 Austria	Case/Control 1y	N=184 35-37y 86%	92 Acute MI	ApoB-depleted serum Oxidized LDL DCF Fluorescence	Result = [fluorescence sample-fluorescence DCF] Log-transformed Normalized to a pool serum control Lower values indicate better protection	Logistic regression	For 1SD decrease Inverted OR=0.45 (0.26-0.78)	Age, sex (matched controls), BMI, HT, DM, smoking, TC

					against LDL oxidation			
Distelmaier 2015 Austria	Cohort 23m (1.91y)	N=247 60y 76% ST-Elevation Acute Coronary Síndrome patients	37 All-cause mortality	ApoB-depleted serum Oxidized LDL DCF Fluorescence	Result = [fluorescence sample/fluorescence negative control] Log-transformed Normalized to a pool control Values < 1 indicate better protection against LDL oxidation	Cox proportional hazards model	HOI< 1 vs HOI≥1 Inverted HR=0.30 (0.14-0.7)	Thrombolysis in MI (TIMI) risk score (age, DM/HT/angina, BP, heart rate, Killip class, body weight, anterior ST-elevation or left bundle branch block and time delay to treatment), history of MI, GFR, TG, neutrophil counts
Kalantar-Zadeh 2007 US	Cohort 30m	N=189 54-56y 54.5% Patients in hemodialysis	46 All-cause mortality	ApoB-depleted serum LDL DCF Fluorescence	Result = [fluorescence sample-fluorescence without sample] Normalized to a negative control Values < 1 indicate better protection against LDL oxidation	Cox proportional hazards model	HII< 1vsHII≥1 Inverted HR=0.40 (0.19-0.88)	Age, race, ethnicity (Blacks, Asians, and Hispanics), DM, Charlson comorbidity score, dialysis vintage, dialysis dose (Kt/V), body fat % according to the NIR measurements, serum albumin (<3.8 vs ≥3.8), CRP (≥10 versus less), IL-6 (≥10 versus less)
Leberkühne 2016 The Netherlands	Cohort 7y	N=454 51.3y 54.8% Adult Renal Transplant Recipients	54 CVD mortality / 102 All-cause mortality	ApoB-depleted plasma LDL Fluorescence TBARS	Result = % reduction in TBARS formation with sample relative to a TBARS formation	Cox proportional hazards model	For 1 SD increase HR=1.00 (0.98-1.03) / For 1 SD increase HR=1.01 (0.99-1.03)	Age, sex, high sensitivity CRP

					without sample Higher values indicate better protection against LDL oxidation			
Patel 2013 US	Case/Control	N=69 57.8-58.2y 57.9%	23 Ischaemic HF	ApoB-depleted serum Oxidized LDL DCF Fluorescence	Result = [fluorescence sample/fluoresc ence negative control] Lower values indicate better protection against LDL oxidation	Logistic regression	For 1 SD decrease Inverted OR=0.32 (0.17-0.6)	Age, BP, creatinine, TC, LDL-C, HDL mass
Patel 2011 US	Case/control	N=193 55.9-61.8y 67.3%	43 CAD	ApoB-depleted serum Oxidized LDL DCF Fluorescence	Result = [fluorescence sample-fluorescence DCF] Normalized to a serum pool Lower values indicate better protection gainst LDL oxidation	Logistic regression	Q1 vs Q4 Inverted OR=0.81 (0.76-0.87) ACS vs. Chronic CAD Q1 vs Q4 Inverted OR= 0.85 (0.82-0.08)	Age, smoking, DM, HT, LDL level, HDL mass
Schrutka 2016 Austria	Cohort 2.8y	N=320 65y 81% Chronic HF patients	116 All-cause mortality 88 MACE (CVD mortality and heart	ApoB-depleted serum Oxidized LDL DCF Fluorescence	Result = [fluorescence sample/fluoresc ence LDL] Log-transformed Normalized to a serum pool Values ≥ 1	Cox proportional hazards model	HOI <1 vs HOI≥1 Inverted HR=0.69 (0.46-1.04) HOI <1 vs HOI≥1 Inverted HR=0.55 (0.34-0.88)	Age, sex, New York Heart Association functional classification (NYHA class,), NT-proBNP, LVEF, estimated GFR, BMI, HDL-C, CRP, IL-6, PON-1 activity, type 2 DM, atrial

			transplantati on)		indicate pro-oxidant HDL, values < 1 indicate anti-oxidant HDL			fibrillation
Schrutka 2016 Austria	Cohort 9.8y	N=270 65y 69% Patients admitted to the intensive care unit (ICU) of the Department of Cardiology of the Vienna General Hospital	49 Short-term all-cause mortality (30-day)*	ApoB-depleted serum Oxidized LDL DCF Fluorescence	Result = [fluorescence sample-fluorescence DCF] Log-transformed Normalized to a serum pool Lower values indicate better protection against LDL oxidation	Cox proportional hazards model	Short-term all-cause mortality HOI<5.90 vs HOL≥ 5.90 Inverted HR=0.6 (0.45-0.82)	Simplified Acute Physiology Score (SAPS) II , age, sex, extracorporeal therapy, TC, GFR, use of catecholamines
			185 Long-term all-cause mortality (10-year)*				Long-term all-cause mortality HOI<5.90 vs HOL≥ 5.90 Inverted HR=0.84 (0.71-0.98)	
Unterstelle r 2018 Germany	CARE FOR HOMe Cohort 5.1y	526 65.1y 59% Patients with Cronic	153 Primary atherosclerot ic CVD endpoint (first of the following: MI, coronary artery	ApoB-depleted serum DHR	Values are expressed as Inhibition of oxidation in %. Higher values indicate better protection against	Cox proportional hazards model	T3 vs T1 HR=0.96 (0.64-1.43)	Age, sex, BMI, BP, smoking, DM, estimated GFR, and log-albuminuria, TC, HDL-C, CRP

		Kidney Disease	angioplasty / stenting / bypass surgery, major stroke, carotid endarterecto my / stenting, non-traumatic lower extremity amputation and / or lower limb artery angioplasty / stenting / bypass surgery, all-cause mortality)		oxidation			

Foot note list abbreviations: CVD indicates cardiovascular disease; HDL-C, High density lipoprotein-cholesterol; MI, Myocardial infarction; STEMI, ST-elevation Myocardial infarction; BP, Blood-pressure; GFR, Glomerular Filtration Rate; TC, Total Cholesterol; CAD, Coronary Artery Disease; HT, Hypertension; DM, Diabetes Mellitus; TG, Tryclicerides; NT-proBNP, N-terminal pro-B-type natriuretic peptide; LDL-C, Low density lipoprotein-Cholesterol; CRP, C-reactive protein; ApoB, Apolipoprotein B; HUVEC, Human umbilical vascular endothelial cell; TNF-α, Tumor necrosis factor-α; VCAM-1, Vascular cell adhesion molecule-1; OR, odds ratio; HR, hazard ratio; DCF, dichlorofluorescein.

* Data provided by the author

SUPPLEMENTAL MATERIAL

Supplemental figure 1.a. Contour enhanced funnel plot of association between of all-cause mortality and cholesterol efflux capacity; figure 1.b. Contour enhanced funnel plot of association between major adverse cardiac events and cholesterol efflux capacity; figure 1.c. Contour enhanced funnel plot of association between mayor adverse cardiac events and anti-inflammatory capacity; figure 1.d. Contour enhanced funnel plot of association between mayor adverse cardiac events and anti-oxidant capacity; figure 1.e. Contour enhanced funnel plot of association between all-cause mortality and anti-oxidant capacity
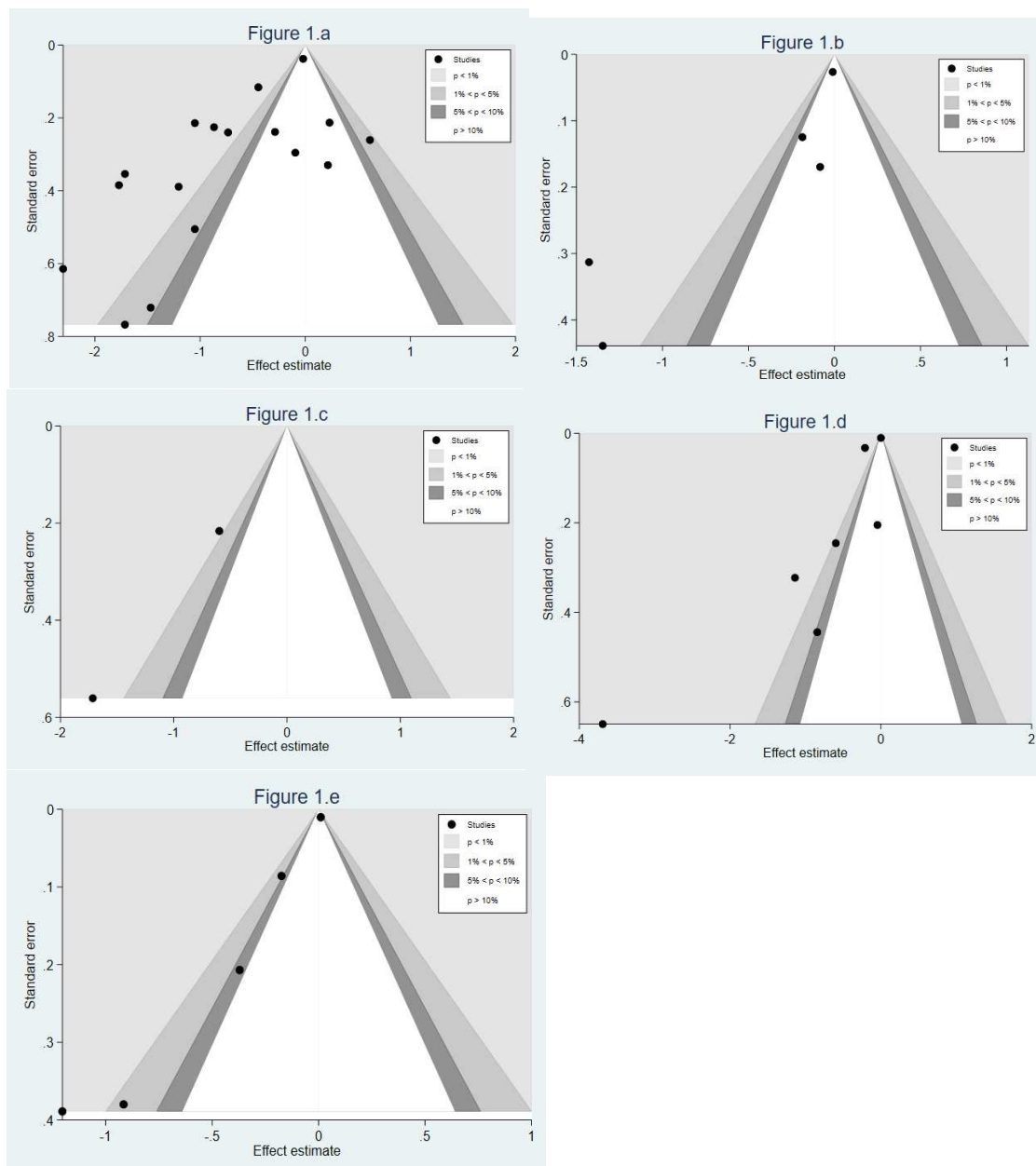
Supplemental material table 1. Quality assessment for cohort studies with Newcastle Ottawa Scale

Author	Year	Title	Quality Score
Annema	2016	HDL Cholesterol Efflux Predicts Graft Failure in Renal Transplant Recipients	7
Bauer	2017	HDL Cholesterol Efflux Capacity and Cardiovascular Events in Patients With Chronic Kidney Disease	6
Distelmaier	2015	Pro-oxidant HDL predicts poor outcome in patients with ST-elevation acute coronary syndrome	5
Dullaart	2014	The HDL anti-inflammatory function is impaired inmyocardial infarction and may predict new cardiac events independent of HDL cholesterol	7
Ishikawa	2015	High-density lipoprotein cholesterol efflux capacity as a relevant predictor of atherosclerotic coronary disease	6
Javaheri, CAVCohort	2016	Cholesterol efflux capacity of high-density lipoprotein correlates with survival and allograft vasculopathy in cardiac transplant recipients	7
Javaheri, CohortStudy	2016	Cholesterol efflux capacity of high-density lipoprotein correlates with survival and allograft vasculopathy in cardiac transplant recipients	5
Kalantar-Kadeh	2007	HDL-inflammatory index correlates with poor outcome in hemodialysis patients	7
Kopecky	2017	HDL Cholesterol Efflux Does Not Predict Cardiovascular Risk in Hemodialysis Patients	9
Leberkühne	2016	The predictive value of the antioxidative function of HDL for cardiovascular disease and graft failure in renal transplant recipients	7
Liu	2016	Cholesterol efflux capacity is an independent predictor of all-cause and cardiovascular mortality in patients with coronary artery disease: A prospective cohort study	7
Mody	2016	Beyond Coronary Calcification, Family History, and C-reactive Protein: Cholesterol Efflux Capacity and Cardiovascular Risk Prediction	7
Potočnjak	2016	Metrics of high-density lipoprotein function and hospital mortality in acute heart failure patients	5
Rohatgi	2014	HDL Cholesterol Efflux Capacity and Incident Cardiovascular Events	7
Schrutka	2016	Impaired High-Density Lipoprotein Anti-Oxidative Function Is Associated With Outcome in Patients With Chronic Heart Failure	6
Schrutka	2016	Impaired High-Density Lipoprotein Anti-Oxidant Function Predicts Poor Outcome in Critically Ill Patients	7
Untersteller	2018	HDL functionality and cardiovascular outcome among non-dialysis chronic kidney disease patients	6

Supplemental material table 2. Quality assessment for case-control studies with Newcastle Ottawa Scale

Author	Year	Title	Quality Score
Annema	2016	HDL function is impaired in acute myocardial infarction independent of plasma HDL cholesterol levels	8
Distelmaier	2015	Impaired antioxidant HDL function is associated with premature myocardial infarction	8
Khera	2011	Cholesterol Efflux Capacity, High-Density Lipoprotein Function, and Atherosclerosis	8
Khera	2017	Cholesterol Efflux Capacity, HDL Particle Number, and Incident Cardiovascular Events. An Analysis from the JUPITER Trial (Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin)	8
Li,	2013	Paradoxical Association of Enhanced Cholesterol Efflux With Increased Incident Cardiovascular Risks (CohortGeneBank)	6
Li,	2013	Paradoxical Association of Enhanced Cholesterol Efflux With Increased Incident Cardiovascular Risks (Outpatient Cohort)	6
Patel	2011	The Anti-Oxidative Capacity of High-Density Lipoprotein Is Reduced in Acute Coronary Syndrome But Not in Stable Coronary Artery Disease	8
Patel	2013	Anti-oxidative and cholesterol efflux capacities of high-density lipoprotein are reduced in ischaemic cardiomyopathy	8
Saleheen	2015	Association of HDL cholesterol efflux capacity with incident coronary heart disease events: a prospective case-control study	8
Sprandel	2015	Alterations in lipid transfers to HDL associated with the presence of coronary artery disease in patients with type 2 diabetes mellitus	7
Zhang	2016	Prognostic Usefulness of Serum Cholesterol Efflux Capacity in Patients With Coronary Artery Disease	6

Search Strategy.

Cardiovascular Diseases/ or Coronary Disease/ or Cardiovascular disease$.mp. or Coronary disease$.mp. or Coronary syndrome$.mp. orexp Myocardial Ischemia/ or Heart disease$.mp. or Heart Attack$.mp. or Myocardial Infarct$.mp. or Heart failure$.mp. or Stable Angina$.mp. or Unstable Angina$.mp. or Angina Pectori$.mp. or Stroke/ or Stroke$.mp. or Cerebrovascular Stroke$.mp. or Acute Stroke$.mp. or Cerebrovascular Accident$.mp. or Acute Cerebrovascular Accident$.mp. or CVA$.mp. or Mortality/ or Mortalit$.mp. or Death$.mp. or Cardiovascular mortality$.mp.) and (ATP Binding Cassette Transporter 1/ or Cholesterol efflux$.mp. or Reverse cholesterol transport$.mp. or Antioxidants/ or Oxidants/ or Antioxidant capacity$.mp. or Oxidation$.mp. or Antioxidant activity$.mp. or Inflammation/ or Anti-inflammatory activity$.mp. or Vasodilation/ or Vasodilation$.mp. orVasodilatory capacity$.mp. or Vasorelaxation$.mp. or Nitric oxide$.mp. orexp Cell death/ or Cell Survival/ or Cell viability assay$.mp. or Cell viability$.mp. or Cytotox$.mp. or Cell$ Cytotox$.mp. orCytotox$ assay$.mp. or Endothelial Cells/ or Endothelial function$.mp. or Endothelial protection$.mp. orexp Endothelium/ or Monocytes/ or Macrophages/ or Muscle, Smooth, Vascular/ or Endothelium, Vascular/ or Atherosclerosis/ or Atheroprotect$.mp. or Anti-atherogenic$.mp.) and (Apolipoprotein A-I/ or Apolipoprotein A-II/ or Apolipoprotein CIII/ or Apo?AI.mp. or Apo?A1.mp. or Apo?A2.mp. or Apo?CIII.mp. orApolipoproteins A/ or Apolipoprotein?A1.mp. or Apolipoprotein?AI.mp. or Apolipoprotein?A2.mp. or Apolipoprotein?AII.mp. orApolipoprotein$?A4.mp. or apolipoprotein?A?IV.mp. orApolipoprotein$?CIII.mp. or Cholesterol Ester Transfer Proteins/ or Cholester?l Ester Exchange Protein$.mp. orCholester?l Ester Transfer Protein$.mp. or CETP$.mp. orPhosphatidylcholine-Sterol O-Acyltrasnferase/ or Lecithin Cholesterol Acyltrasnferase$.mp. or LCAT$.mp. orAryldialkylphosphatase/ or PON$ 1.mp. orParaoxonase$ 1.mp. or 1-Alkyl-2-acetylglycerophosphocholine Esterase/ or PAF Acetylhydrolase$ II.mp. or PAF 2-Acylhydrolase$.mp. or Lp?PLA?2?.mp. or Lipoprotein$ Associated Phospholipase$A2.mp. or Platelet Activating Factor Hydrolaswe$.mp. or Serum Amyloid A Protein/ or Serum Amyloid$ A.mp. or SAA.mp.

orSphingolipids/ or Sphingosine 1-phosphate$.mp. or S?1?P.mp. or Complement C3/ or Complement$ C3.mp. or Cholesterol, HDL/ or Lipoproteins, HDL/ or HDL$.mp. or High density lipoprotein$.mp.) and (Cohort Studies/ or Cohort stud$.mp. or Cohort$.mp. or Prospective Studies/ or Prospective$.mp. or Longitudinal Studies/ or Longitudinal Stud$.mp. or Follow-Up Studies/ or Follow?up$ stud$.mp. or Case-Control Studies/ or Case-Control Stud$.mp. or Nested case?control$ stud$.mp. or Matched Case-Control Stud$.mp. or case-cohort stud$.mp.)

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MANUSCRIPT 2: PREDICTIVE VALUE OF HDL FUNCTION RELATED BIOMARKERS FOR ACUTE CORONARY SYNDROME OUTCOME IN PATIENTS AT HIGH CARDIOVASCULAR RISK

Predictive value of HDL function related biomarkers for acute coronary syndrome outcome in patients at high cardiovascular risk

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Running title: Cholesterol efflux capacity and risk of cardiovascular disease

Abbreviations: High-density lipoprotein; Cholesterol efflux capacity; oxidation; inflammation; apolipoprotein A-I; sphingosine-1-phosphate; risk; logistic regression

ABSTRACT

Background: Epidemiological studies have identified low plasma levels of high-density lipoprotein-cholesterol (HDL-C) as an independent biomarker for coronary heart disease. The association has, however, been challenged, with emphasis on the atheroprotective role of the lipoprotein rather than its cholesterol content. To date, few data are available on whether HDL-mediated functions might be a useful predictor for cardiac events.

Methods: A case-control study nested within the PreDiMed (Prevención con Dieta Mediterránea) cohort. 172 and 35 cases of acute coronary syndrome and stable angina, respectively, were matched (1:2) with controls by sex, age, intervention group, body mass index, and time of permanence in the study to the cardiac event. We assessed the predictive role of cholesterol efflux capacity, HDL oxidative-inflammatory index, and cholesterol, apolipoproteinA-I, and sphingosine-1-phosphate content in samples of apolipoproteinB-depleted plasma.

Results: Cholesterol efflux capacity independently predicted acute coronary syndrome and acute myocardial infarction. ApolipoproteinA-I was associated with acute coronary syndrome, acute myocardial infarction, and unstable angina irrespective of cardiovascular risk factors. In addition, HDL oxidative-inflammatory index was associated with to higher risk of acute coronary syndrome and unstable angina in a logistic regression adjusted for the same risk factors.

Conclusions: Cholesterol efflux capacity promoted by HDL, its apolipoproetinA-I content, and the HDL oxidative-inflammatory index are useful surrogates for future acute coronary syndrome, irrespective of traditional cardiovascular risk factors, in population of high cardiovascular risk.

INTRODUCTION

Cardiovascular diseases (CVD) remain the leading cause of death worldwide (1). Levels of high density lipoprotein cholesterol (HDL-C) were first identified as an independent predictor of CVD (2). However, pharmacological studies with raised HDL-C did not find a clear relationship between higher levels and lower CVD incidence (3,4). Moreover, the lack of consistency among results from a meta-analysis on the association of higher HDL-C with the risk of cardiovascular outcomes (5–7), and Mendelian randomization studies reporting that genetic variants associated with high HDL-C are not associated with acute myocardial infarction (AMI) risk, leads to the consideration that an increase in HDL function can be more relevant than a mere rise in HDL-C concentrations (8).

Apolipoprotein (Apo) A-I is the most abundant apolipoprotein in HDL, its levels are an inverse predictor of major adverse cardiovascular events in patients on statins. A relative reduction of 21% in the risk of a new cardiovascular event for every 1-standard deviation (SD) increase in ApoA-I levels has been reported (9). The role of ApoA-IV in CVD has been recently investigated although it has not yet been fully clarified. Lipid-free ApoA-IV has been shown to present antioxidant capacity (10) and to promote cholesterol efflux (11). Nevertheless, glycation of ApoA-IV can transform the HDL particle into a pro-atherogenic lipoprotein (12).

Conversion of macrophages into foam cells by the unlimited uptake of oxidized low-density lipoprotein (LDL) is a critical step in atherosclerosis, a common cause of CVD. Cholesterol efflux capacity (CEC) from macrophages is thus considered the most relevant function of HDL in heart disease prevention. Despite cholesterol efflux representing only the first step in reverse cholesterol transport to the liver, several studies have inversely associated HDL-mediated cholesterol efflux with the risk of a range of heart diseases, including AMI (13,14). Moreover, the use of cholesterol CEC to assess the risk of adverse cardiac outcomes has been recently summarized in a meta-analysis comprising 15 observational studies (15). The authors concluded that higher values of CEC

were inversely associated with decreased cardiovascular risk (RR=0.56, 95% CI: 0.37-0.85)

In addition to CEC, HDL exerts a range of pleiotropic properties. Its capacity to inhibit LDL oxidation is inversely related to a lower risk of heart disease (16–19). The exact mechanisms by which HDL protects against oxidation remain to be fully explained, however, several constituents, such ApoA-I and A-IV and a range of enzymes, seem to play a central role (10,20).

Oxidized low-density lipoprotein cholesterol (LDL-C) increases the expression of adhesion molecules by endothelial cells (21) which initiates an inflammatory cascade characteristic of atherosclerosis (22). Modulation of the expression of pro-inflammatory mediators, mainly endothelial adhesion molecules, by HDL (23) has been inversely associated with the risk of AMI independent of sex, age, and CVD risk factors (16,24). The role of the platelet activating factor acetylhydrolase (PAF-AH) in the modulation of inflammatory processes representative of ahterosclerotic CVD is convoluted. This enzyme is both pro-inflammatory due to the production of bioactive oxidized lipids (25), and anti-inflammatory resulting from the inactivation of platelet activating factor [a potent lipid mediator in inflammation (26)], and protection of LDL against oxidation by neutralizing oxidized lipids in phospholipids (27). Whilst plasma concentrations of PAF-AH have been shown to be useful in the risk evaluation of adverse coronary outcomes (28), assessment of its hydrolytic activity as a risk predictor requires further investigation (29). Furthermore, most of the studies regarding PAF-AH involvement in cardiovascular disease have been performed in plasma samples; approximately 70% of the enzyme binds to LDL in plasma and only a small fraction is found in HDL

The reduction in nitric oxide production leads to endothelium impairment which further favours the pro-inflammatory environment and increased permeability to oxidized LDL (30). Endothelial dysfunction independently predicts adverse cardiac outcomes including myocardial infarction and coronary syndrome (31). During recent years, a range of atheroprotective actions linked to the presence of sphingosine-1-phosphate (S1P) in HDL has been identified. The binding of S1P to HDL is related to an increment of nitric oxide via

endothelial nitric oxide synthase activation (32). Lately, studies have demonstrated that S1P concentration is reduced in coronary artery disease patients whose HDL is more dysfunctional than healthy controls (33). Even though the role of SIP in the prediction of adverse cardiovascular outcomes requires further clarification, some authors have already claimed it is related to both CVD occurrence and extent (34,35).

In certain diseases the HDL proteome may be greatly modified towards a pro-atherogenic profile. Levels of serum amyloid A (SAA), an acute-phase protein found in HDL during different inflammatory processes, has been recently proposed as a potential predictor of CVD and mortality (36,37). It is believed that the central mechanism behind the pro-atherogenic role of SAA in HDL is the ability of this acute phase protein to displace ApoA-I from HDL (38). In this regard, higher quantities of HDL-associated SAA predict cardiovascular outcomes although not independently from C-reactive protein levels (39). The C3 protein of the complement system, a complex mechanism activated under the influence of inflammatory and immunological triggers, may play a role as a marker (40) and predictor of CVD (41).

Our objective was to discern the predictive value for CVD of an emergent battery of biomarkers related to HDL function, in a high cardiovascular risk population.

METHODS

Participants and study design

The study was performed with a subsample from the PREDIMED (*Prevención con Dieta Mediterránea*) study, a multicentre, randomized, controlled intervention in which participants were randomly allocated to (1) a traditional Mediterranean diet supplemented with virgin olive oil, (2) a traditional Mediterranean diet supplemented with nuts, and (3) a low-fat control diet following the American Heart Association recommendations. Eligible participants were community-dwelling men, 55-80 years of age, and women, 60-80 years of age, who fulfilled at least 1 of 2 criteria: (1) type 2 diabetes mellitus or (2) 3 or more coronary heart disease risk factors: current smoking, hypertension (blood pressure >140/90mm Hg or antihypertensive drugs), LDL

cholesterol level >160 mg/dL (or hypolipidemic drugs), HDL cholesterol level <40 mg/dL, body mass index>25 kg/m², or a family history of premature coronary heart disease (42).

Between October 2003 and June 2009, 7,447 candidates were randomized into one of the 3 intervention groups. After a median follow-up of 4.8 years, 172 acute coronary syndrome (ACS) outcomes (98 acute myocardial infarctions and 74 unstable anginas (UA)), and 35 stable anginas (SA) occurred. The nested case-control (1:2) design was matched in the study by age, sex, body mass index, intervention group, and follow-up time at occurrence of the event.

Full protocol details have been published elsewhere (43). The trial was approved by the institutional review boards and registered with the number ISRCTN35739639 in www.controlled-trials.com Participants provided informed consent before joining the trial (http://www.predimed.es).

Outcome ascertainment and follow-up for coronary syndrome cases

Coronary syndrome was studied as the major cardiovascular event: a) dead/alive individual who suffered a myocardial infarction; and/or b) dead/alive individual who suffered a UA (42). We used four sources of information to identify end points: repeated contacts with participants; family physicians; yearly review of medical records; and consultation of the National Death Index. All medical records related to end points were examined by the end point adjudication committee, whose members were blinded to treatment allocation. Only end points that had been confirmed by the Adjudication Committee, and that had occurred between Oct 1, 2003, and Dec 1, 2010, were included in the analyses.

Sample size calculation

A sample size of 172 cases and 344 controls allowed ≥80% power to detect an odds ratio of at least 1.9, considering a 2-sided type I error of 0.05, a loss rate of 1%, and an exposed rate among controls of 50%.

General diet, and physical activity information

The baseline examination included a 47-item questionnaire about education, lifestyle, history of illnesses, and medication use. Assessment of Mediterranean diet adherence, by means of a 14 item-Mediterranean diet score, was performed (44). Physical activity practice was registered by means of a Minnesota Leisure Time Physical Activity Questionnaire, validated in a Spanish population (45). Height and weight were recorded at baseline, and body mass index was calculated as weight (kg) divided by height (m) squared. As the intervention did not target medication, it was prescribed following the participants' regular medical care.

Biological sample collection

Fasting plasma samples were collected and stored at -80 °C until use. The ABDP specimen was obtained by centrifugation after incubation of plasma samples with polyethylene glycol 8000 in a glycine buffer at 4°C (46). The resulting supernatant containing HDL was collected for later use. The samples did not suffer any thaw-freeze cycles before the experiments.

Biochemical analysis

Systemic lipid profile and glycaemia

In plasma we determined: glucose, total cholesterol, triglycerides, and HDL cholesterol in an ABX-Pentra 400 autoanalyzer (Horiba-ABX). LDL-C levels were calculated by the Friedewald formula whenever triglycerides were <300 mg/dL.

HDL composition in ABDP samples

We measured Apo A-I, and component complement C3, by immunoturbidimetry in an ABX Pentra-400 autoanalyzer (Horiba-ABX, Montpellier, France). The content in Apo A-IV, Serum Aamyloid A and Sphingosine-1-Phosphate were evaluated with commercial Elysa kits (Abcam,

Invitrogen and Bioassay respectively) Intra and inter-assay coefficients of variation were 6.04% and 11.6% for Apo A-IV, 3.54% and 11.8% for Serum Amyloid A, and 7.86% and 13.18% for Sphingosine-1-Phosphate.

HDL antioxidant capacities

We determined activity of platelet-activating factor acetylhydrolase with the PAF Aceltylhydrolase Assay Kit (Cayman). The intra assay coefficient was 8.69% and the inter-assay one was 15.38%.

The HDL oxidative-inflammatory index (HOII) was performed as previously published(46). Briefly, we incubated 2'-7'-dichlorodihydrofluorescein diacetate with methanol for 30 minutes to obtain the fluorescent metabolite dichlorodihydrofluorescein (H2DCF). Next, H2DCF (final concentration of 3 µg/mL) was placed with pre-oxidized LDLs (final concentration of 1.5 µg/mL) and 5 µL of ABDP from the volunteers, or 5 µL of phosphate buffered saline (negative control), in black polystyrene plates. We measured the fluorescence (Infinite M200 reader, Tecan Ltd) every 3 minutes for 60 minutes at 37ºC (*Ex/Em*: 485/530 nm). To calculate the index, we subtracted the fluorescence of the blank from the fluorescence of the samples and the following formula was used:

HOII=fluorescence in the presence of ABDP – fluorescence without ABDP

High levels of HOII are indicative of greater LDL oxidation and, therefore, poor antioxidant HDL-protection. The intra and inter-assay coefficients of variation were 5.74% and 7.16%, respectively

Cholesterol efflux capacity

We suspended human THP-1 monocytes on RPMI 1640 medium (supplemented as previously described (46)) in T75 culture flasks (2500cells/ml) and changed media every 72-96h, until confluence. We incubated the cells with phorbol-myristate-acetate (200 nM, Sigma) for 24h in 96-well transparent plates at 350,000cell/ml to differentiate monocytes into macrophages. Afterwards, cells were labeled with cholesterol BODIPY 0.025 mM for 24h, washed twice with *phosphate buffered* saline and incubated for further 24h in fresh free-serum

RPMI 1640 medium (supplemented only with 1% bovine serum albumin, Sigma). Finally, we washed the cells once again with *phosphate buffered* saline, and subjected them to a final incubation for 16h in fresh free-serum RPMI 1640 medium + 1% b*ovine serum albumin* in the presence of 6.6% ABDP from the volunteers. For the negative control, an equal amount of *phosphate buffered* saline was added instead of ABDP. To measure the fluorescence in supernatant (485/535 Ex/Em, Infinite M200 reader, Tecan Ltd), we transferred 100uL into a transparent half-area 96-well plate. Afterwards, we removed the remaining supernatant from the original plate and added 150µL of Triton X-100 (1%) at 4ºC to each well and kept the plate in refrigeration for 60 minutes. To read the fluorescence, we proceeded as in supernatant.

We subtracted the average fluorescence of the blank in supernatants and cells to each well value with or without ABDP and calculated CEC for each well as shown:

$$CEC = \frac{(fluorescence\ in\ supernatant)}{(fluorescence\ in\ supernatant + fluorescence\ in\ cell)}$$

Then, we subtracted the efflux of negative control from the efflux of each volunteer to obtain the adjusted efflux. The global intra-assay coefficient of variation was 2.5% and the inter-assay was 6.8%

Quality control of the laboratory analyses

The same pre-analytical procedures were applied to all samples. Matched samples were analyzed in the same experimental run in all determinations. In each analytical plate, for every determination, we included a pool from the volunteers, as an internal control, to assess the inter-assay variability among plates. For cholesterol efflux capacity, HDL oxidative-inflammatory index, apoliporotein A-IV, sphingosine-1-phosphate, platelet activating factor acetylhydrolase activity and serum amyloid A analyses, we included a second pool from another group of volunteers to minimize the intra-assay variability. No intra-repetition coefficient of variation over 20% was allowed for duplicates in the cholesterol efflux capacity experiment.

Statistical Analysis

We used histograms to investigate the distribution of probability of each variable. Pearson´s chi-squared test was employed to assess differences in frequencies between categorical variables. Student´s t-test or Mann Whitney U-test, respectively, allowed us to compare means (with standard deviation) of groups for continuous variables with normal or non-normal distribution respectively. When we attempted to contrast more than two groups, we performed an ANOVA or the Kruskall-Wallis test for normal and non-normal continuous variables, respectively, and Chi square Pearson´s test for categorical variables. We assessed the association between biomarkers with binary cardiovascular outcomes with conditional logistic regression to specifically compare each case with the corresponding two matched controls. In nested case-control studies with risk-set sampling and time-to-event matching, odds ratios derived from conditional logistic regression is considered to be unbiased estimates of hazard ratios (HRs)(47). Odds ratios (95% confidence interval-CI-) are reported for a 1-SD increase in the unadjusted model (model 1); in the multivariate adjusted model for age, intervention group and HDL-C (model 2); and in the model in which we replaced HDL-C by the cluster of cardiovascular risk factors (CVRFs) including type 2 diabetes mellitus, hypercholesterolemia, hypertension, and smoking habit (model 3). We evaluated whether there was a trend towards higher quartiles of each surrogate and presence of disease. Two-sided p-value<0.05 was accepted to be significant. The statistical analyses were performed in R, version-3.5.0 (R Core Team (2018). R: language and environment for the statistical calculation R Foundation for Statistical Computing, Vienna, Austria URL https://www.R-projectd.org/).

RESULTS

Baseline characteristics

The exploratory analysis of traditional risk factors between cases and controls revealed that ACS and AMI patients presented a higher percentage of diabetes (p=0.003 and 0.004, respectively) but lower hypercholesterolemia (p=0.048

and 0.024, respectively) and dyslipidemia (p=0.025 and 0.006, respectively) (Table 1). Furthermore, in the group of ACS the percentage of smokers was higher compared to controls (p=0.038) and the group of AMI had reduced values of physical activity engagement (p=0.028) (data not shown). Concerning the subjects who were later diagnosed with UA, we found more smoker subjects at the beginning of the study (p=0.048) (data not shown). Any difference was found between the group of SA patients and controls.

Bivariate associations between HDL function-related biomarkers and ACS, AMI, UA and SA

The bivariate association among the HDL function-related biomarkers assessed as continuous variables and coronary syndrome outcomes are described in detail in Table 1. Distribution across the quartiles of CEC, ApoA-I, HOII, HDL-C and SIP and comparison between the first and fourth quartile, stratified by the occurrence of ACS, AMI, and UA are depicted in Tables 2 and 3, respectively. Data about SA present no significative change in any of the comparisons.

HDL-cholesterol

HDL-C levels did not differ for ACS (p=0.077)(Table 1), AMI (p=0.167), and UA (p=0.259) compared to controls. Distribution across the quartiles showed increasing HDL-C levels for AMI patients (p=0.036) which were not confirmed for ACS and UA (Table 2). Furthermore, the distribution between first and fourth quartile was significant for AMI (22.8% in Q1 versus 11.4% in Q4, p=0.032), but not for ACS and UA (Table 3).

Cholesterol efflux capacity

The CEC value was lower in the group of subjects who later developed an AMI than in the controls (mean±standard deviation (SD): 1.00±0.07, mean±SD: 0.98±0.08, p=0.041). Furthermore, a p trend of 0.036 for increasing cases of AMI across increasing quartiles of CEC was observed (Table 2). This relationship persisted when comparing the highest versus the lowest quartile of CEC (p=0.008) (Table 3).

HDL antioxidant protection

HOII as continuous variable was higher in UA patients than in controls (mean±SD:1.09±0.23, mean±SD:1.17±0.28, p=0.043), and UA cases tended to increase across quartiles of HOII (p=0.042) (Table 2). We also observed a borderline significance in the group of ACS in contrast to the disease-free group (p=0.060) (Table 1). In addition, a trend to increase ACS cases across quartiles of HOII was observed. Regarding activity of PAF-AH, no difference was revealed when we compared cases and controls of ACS, MI, and UA (data not shown).

Endothelial protection

Only in the group of ACS was there S1P borderline significance toward higher concentration in controls than in cases (p=0.090). A trend of decreasing levels of SIP in ACS across quartiles was observed (0.098).

HDL protein content

Levels of apolipoprotein A-I were higher in matched controls than in ACS (p=0.004)(Table1) and AMI (mean±SD: 0.82±0.17, mean±SD: 0.76±0.17, p=0.016) and showed a borderline significance in UA (mean±SD: 0.82±0.19, mean±SD: 0.77±0.17, p=0.095). Increasing quartiles of ApoA-I concentrations were inversely related to the presence of ACS, AMI, and UA ($P_{for\ trend} < 0.02$ for all). Comparison of the highest quartile of ApoA-I versus the lowest was highly significant for ACS and AMI (p<0.001 and p=0.009, respectively) and reached borderline significance for UA (p=0.059).

The analysis of serum amyloid A, C3 protein complement, and apolipoprotein A-IV did not vary between cases and controls in ACS, AMI, and UA pathologies, regardless of whether we assessed the association for continuous variables or across quartiles. Similarly, no trend was depicted for the distribution of disease across increasing quartiles of any of the latter markers.

Relationship of HDL function-related biomarkers among stable angina and severity of coronary syndrome

CEC differed between AMI and UA cases (mean±SD: 0.98±0.08 vs 1.02±0.09, respectively; p-value for the comparison=0.018) and also between AMI and SA cases (mean±SD: 0.98±0.08 vs 1.02±0.08, respectively; p-value for the comparison=0.038) (Figure 1). No other relationship between HDL function-related biomarkers and coronary heart outcomes reached statistical significance.

Risk prediction of Acute Coronary Syndrome

A strong inverse association was found for the risk of ACS and ApoA-I and CEC in the unadjusted model (OR=0.094, 95% CI:0.024-0.6, p=<0.001 and OR=$4*10^{-3}$, 95% CI: $7*10^{-5}$-0.23, p=0.007 , respectively), in model 2 (OR for ApoA-I=0.12, 95% CI:0.02-0.8, p=0.028 and OR for CEC=$5*10^{-3}$, 95% CI:$7*10^{-5}$-0.37, p=0.016 , respectively), and in model 3 plus CVRFs (OR for ApoA-I=0.13, 95% CI:0.032-0.51, p=0.003 and OR for CEC=$9*10^{-3}$, 95% CI: $1*10^{-4}$-0.59, p=0.028, respectively).

The OR of ACS for a 1-SD increase in HOII in the unadjusted model was 2.67 (95% CI:1.13-6.31, p=0.025), and in the multivariate adjusted models 2 and 3 it was 3.22 (95% CI:1.27-8.18, p=0.014) and 3.3 (95% CI:1.35-8.08, p=0.009), respectively.

ORs for a 1-SD increase in S1P concentration in HDL showed a nearly significant association with the risk of ACS in the unadjusted model (OR=0.52, 95% CI: 0.27-1, p=0.051), and in model 2 (OR=0.5, 95% CI:0.25-1.02, p=0.056) although significance was lost after adjusting for the presence of CVRF (OR=0.6, 95% CI: 0.3-1.17, p=0.132).

Risk evaluation for a 1-SD increase of HDL-C was only borderline significant for ACS in the unadjusted model (p=0.089). An association that became weaker when the model was adjusted for age and intervention group (p=0.093) and was fully lost after including diabetes, hypertension, hypercholesterolemia, and smoking habit (p=0.292) (Table 4).

No significant association for PAF-AH activity, ApoA-IV, SAA, and C3 complement protein in HDL was observed, in either raw or adjusted multivariate models.

Risk prediction of Myocardial Infarction

With regard to ApoA-I in HDL, the association with AMI was significant in the raw model (OR= 0.079, 95% CI: 0.013-0.47, p=0.005) and in that including CVRF (OR=0.14, 95% CI:0.021-0.88, p=0.036). Remarkably, a strong inverse association was noted between CEC and the risk of AMI in model 1, 2, and 3 (OR=$1*10^{-4}$, 95% CI:$4*10^{-7}$-0.028, p=0.001; OR=$2*10^{-4}$, 95% CI: $4*10^{-7}$-0.067, p=0.004; and OR=$5*10^{-4}$, 95% CI:$1*10^{-6}$-0.18, p=0.012, respectively) (Table 5).

Risk prediction of Unstable Angina

In the sub-analysis of cases of unstable angina, ApoA-I was inversely associated with higher risk in the raw model and model 3 (OR=0.12, 95% CI: 0.015-0.93, p=0.042 for model 1 and OR=0.1, 95% CI: 0.012-0.87, p=0.037 for model 3). Regarding HOII, the index displayed a strong inverse independent association in the three models (model 1: OR= 5.24, 95% CI: 1.32-20.8, p=0.019; model 2: OR=5.15, 95% CI:1.17-22.7, p=0.03, and model 3: OR=5.3, 1.27-22.1, p=0.022)(Table 6).

Risk prediction of Stable Angina

In the sub-analysis of cases of stable angina, HOII was directly related to higher risk in the fully adjusted model although statistical significance was not reached.

DISCUSSION

A disrupted cholesterol efflux from macrophages promoted by HDL, and its Apo-AI content, are independent predictive biomarkers for ACS and AMI, in a high cardiovascular risk population. This HDL function could be altered due to the marked oxidation/inflammation state of the particle, reflected by an HDL antioxidant/anti-inflammatory index with predictive value for ACS and UA.

Atherosclerosis, the physiopathological subjacent process of the coronary syndrome, is a chronic inflammatory disease with a complex pathogenesis. It involves oxidized lipid accumulation, cytokine release, and the production and degradation of the extracellular matrix in the arterial wall. Besides HDL cholesterol concentration, traditionally established as an independent risk factor for CVD (48,49), the anti-atherogenic capacity of the HDL particle is emerging as a relevant factor in the onset and development of the atherosclerotic plaque. The capacity to remove excess cholesterol from peripheral cells is the most well-known biological function of the HDL particle (50). Cholesterol efflux to HDL prevents the accumulation of foamy macrophages in the sub-endothelial space. It represents the first step in the reverse cholesterol transport to the liver, contributing to corporal cholesterol catabolism. In addition, HDL directly protects the vascular wall through pleiotropic mechanisms including antioxidant and anti-inflammatory ones (51).

ApoA-I constitutes about 70% of the apolipoprotein content of HDL particles (52), and actively participates in their anti-atherosclerotic action. The acquisition of cellular cholesterol starts with ABCA1-mediated cholesterol efflux to ApoA-I, generating pre-β1 HDL (53), the first step of the HDL cycle. Lipid-poor ApoA-I participates in a similar way to lipid-free ApoA1 in the ABCA1 reaction although increasing lipidation reduces this ABCA1 ability (54). Once HDL become mature, they react to a greater extent with ABCG1 transporter and SR-BI receptor (55). In addition, ApoA-I prevents LDL oxidation by contributing to inactivation (56) and subsequent transfer of lipoperoxide from oxidised LDL to HDL (57).

Proteins such as Apo-A1, paraoxonase, and platelet-activating factor acetylhydrolase act in a coordinated fashion to transfer lipid peroxides from oxidised LDL to HDL (57). In addition, certain HDL anti-inflammatory properties are mediated by lipids such as non-esterified cholesterol and sphingosin-1 phosphate. In this regard, sphingosin-1 phosphate plays a relevant role in endothelial barrier function (58).

Decreased levels of the most relevant HDL action, its macrophage-specific cholesterol efflux property, have been described as being inversely related in

general populations to a high incidence of sub-clinical atherosclerosis (59) and coronary events (13). With respect to the antioxidant function of HDL, the arylesterase activity of paraoxonase enzyme has also been shown in the general population to be associated with a lower incidence of systolic heart failure (60). Low levels of HDL-bound S1P, a bioactive lipid which participates in HDL endothelial protection and anti-inflammatory activities, were observed to be predictive for the extent of stable coronary artery disease in a group of patients at baseline(35). Regarding other functional actions of HDL, although they are disrupted in subjects with a number of cardiometabolic pathologies (61–63), they have not been related to prediction of incidence of cardiovascular events.

The present study has shown a predictive property of Apo-AI and CEC for ACS irrespective of HDL-C concentrations, in high cardiovascular risk population. In addition, the predictive value of HDL-C reached not statistical significance in any of the assessed models. Our results are in line with the already described cholesterol uptake capacity of free Apo-AI and HDL particle. The association of HDL dysfunction with coronary heart disease in high risk individuals indicates that among general populations, those with a low-moderate cardiovascular risk score could also display a set of biomarkers belonging to the HDL function battery.

The systemic circulating concentration of ApoA-I has been associated with cardiovascular disease and is considered to be even more sensitive in predicting cardiac outcomes than HDL-C (18). In the present work, however, the Apo-AI content was measured in HDL and is thus not exactly routinely analyzed systemic ApoA-I since it is also transported in other lipoproteins than HDL ones. Our work thus provides evidence that the benefits from circulating APO-AI can be attributed, at least partially, to its content in the HDL fraction.

There is an association between the morphology of the atherosclerotic plaque and the type of ensuing coronary heart syndrome. The extremes of the spectrum with respect to the combination of cap thickness and atheroma size seem to result in different clinical outcome (65). Whereas stable lesions involve fibrous plaques with small or nil extracellular lipid, vulnerable plaques leading to an acute event are made up of a large amount of lipids and a thin or virtually

absent fibrous layer. An abundant infiltration of macrophages at the immediate site of erosion or rupture has been described in most AMI cases (66). In this regard, we observed that ACS and AMI, but not UA, were independently predicted by CEC promoted by HDL.

Although the overall morphology of the ruptured lesions is heterogeneous regarding plaque architecture, the immediate site of erosion or rupture is mainly characterized by a deep infiltration of macrophages and activated T-cells (66). Several inflammation mediators released by these cells promote destabilizing effects in the plaque (67). It must be taken into consideration that, in addition to inflammation and wall repair process, many other factors are involved in plaque evolution such as apoptosis, platelet thrombi, local flow disturbances, vasospasms, dilation, and shrinkage. Thus when our results were stratified by outcome, a predictive value of HOII for UA, but not AMI, was observed in the raw and fully adjusted models. Complexity in the structure of the atherosclerotic plaque or presenting an advanced stage, especially in AMI cases, along with the reduced subsample when stratifying, could explain the lack of predictive value.

Strengths and limitations

To the best of our knowledge, this is the first approach to comprehensively analyze the usefulness of a set of HDL function-related biomarkers, beyond HDL-C levels, in the prediction of coronary heart disease in a high risk population.

One limitation was that our study sample was made up of individuals at high cardiovascular risk with an elevated number of ACS cases (n=172) which hinders the extrapolation of results to a general population. Nevertheless, case-control studies require less time and are more economical than cohort ones. Additionally, they are particularly useful for strategic approaches to discern potential causal factors. The main limitation of our work, however, is that its design of cases and controls does not allow causality to be inferred. Moreover, after stratifying the major outcome into its individual entities (AMI, UA, and SA) the sample size may have been too small to uncover relevant associations.

CONCLUSIONS

In summary, CEC and Apo-AI inversely and independently predict ACS and AMI in a high cardiovascular risk population. In addition, low Apo-AI levels are associated with higher risk for UA, irrespective of classical risk factors for cardiovascular disease. The HDL antioxidant/anti-inflammatory index is a marker for future ACS and UA cases with multivariate CVRFs model. These results concur with recent findings supporting the idea that, beyond HDL-C concentration, the pleiotropic function of HDL may explain its atheroprotective role in cardiovascular diseases.

TABLES AND FIGURES

Table1. Baseline characteristics of acute coronary syndrome patients and controls.

	Controls (N=345)	**ACS** (N=172)	*P* Value
Age, y	67.5(6.27)*	67.7 (6.53)*	0.789
Male sex, %	33.6	33.7	1.000
Type 2 diabetes mellitus, %	43.2	57.6	**0.003**
Hypercholesterolemia, %	73.9	65.1	**0.048**
Hypertension, %	95.1	97.1	0.400
Smoking status, %	13.9	21.5	**0.038**
Body mass index, kg/m²	29.3 (3.20)	29.3 (3.23)	0.871
Leisure-time physical activity, METs*min/dd	212 [89.1-436] †	200 [84.0-356] †	0.158
Adherence to TMD (score)	8.48 (1.89)	8.60 (1.89)	0.516
Total cholesterol, mg/dL	204 (38.5)	202 (34.5)	0.519
HDL-C, mg/dL	50.2 (11.3)	48.5 (9.24)	0.077
Triglycerides, mg/dL	113 [86.0-150]	116 [92.9-169]	0.155
Glucose, mg/dL	124 (35.4)	127 (42.1)	0.364
CEC (unitless ratio)	1.01 (0.07)	1.00 (0.09)	0.131
HOII (unitless ratio)	1.10 (0.24)	1.16 (0.30)	0.060
PAF-AH activity (unitless ratio)	1.74 (0.52)	1.69 (0.54)	0.403
Sphingosine-1-phosphate (unitless ratio)	1.03 (0.37)	0.97 (0.36)	0.090

ApoA-IV (unitless ratio)	1.65 (1.52)	1.78 (1.68)	0.416
ApoA-I, mg/dL	0.82 (0.17)	0.77 (0.17)	**0.004**
SAA (unitless ratio)	2.00 (2.97)	2.22 (4.24)	0.552
C3 complement, mg/dL	10.6 (5.23)	10.9 (5.62)	0.522

HDL-C denotes high-density lipoprotein cholesterol; CEC, cholesterol efflux capacity; HOII, HDL oxidative-inflammatory index; PAF-AH, platelet activating factor acetylhydrolase; S1P, sphingosine-1-phosphate; ApoA-IV, ApoA-IV; ApoA-I, apolipoproteinA-I;SAA, serum amyolid A; MET, metabolic equivalent of task; ACS, acute coronary syndrome.

*mean (SD); †Median [first-third quartiles].

Table 2. Distribution of ACS, AMI, and UA cases across quartiles of ApoA-I, CEC, HOII, SIP and HDL-C.

	N	Control	ACS	p-trend	Control	AMI	p-trend	Control	UA	p-trend
ApoA-I, mg/dL				**<0.001**			**0.014**			**0.019**
(0.210-0.700	119	57.1 %	42.9 %		76.5 %	23.5 %		77.3 %	22.7 %	
(0.700-0.805	132	65.2 %	34.8 %		81.1 %	18.9 %		80.3 %	19.7 %	
(0.805-0.910	117	66.7 %	33.3 %		79.5 %	20.5 %		86.3 %	13.7 %	
(0.910-1.700]	126	79.4 %	20.6 %		89.7 %	10.3 %		87.3 %	12.7 %	
CEC (unitless ratio)				0.371			**0.036**			0.626
(0.670-0.964)	125	64.0 %	36.0 %		77.6 %	22.4 %		82.4 %	17.6 %	
(0.964-1.009)	138	67.4 %	32.6 %		81.2 %	18.8 %		82.6 %	17.4 %	
(1.009-1.057)	124	67.7 %	32.3 %		75.8 %	24.2 %		90.3 %	9.68 %	
(1.057-1.273]	112	69.6 %	30.4 %		91.1 %	8.93 %		76.8 %	23.2 %	
HOII (unitless ratio)				0.082			0.753			**0.042**
(0.512-0.955)	111	70.3 %	29.7 %		80.2 %	19.8 %		87.4 %	12.6 %	
(0.955-1.068)	122	70.5 %	29.5 %		82.8 %	17.2 %		85.2 %	14.8 %	
(1.068-1.236)	112	64.3 %	35.7 %		82.1 %	17.9 %		78.6 %	21.4 %	
(1.236-2.596]	118	61.0 %	39.0 %		78.8 %	21.2 %		78.8 %	21.2 %	
S1P(unitless ratio)				0.098			0.114			0.107
(0.160-0.776)	121	64.5 %	35.5 %		79.3 %	20.7 %		77.7 %	22.3%	
(0.776-0.981)	112	61.6 %	38.4 %		75.9 %	24.1 %		84.8 %	15.2 %	
(0.981-1.230)	119	72.3 %	27.7 %		84.9 %	15.1 %		84.9 %	15.1 %	
(1.230-2.545]	113	71.7 %	28.3 %		85.0 %	15.0 %		85.8 %	14.2 %	
HDL-C, mg/dL				0.089			**0.036**			0.821
(27.0-41.4)	123	65.0 %	35.0 %		77.2 %	22.8 %		86.2 %	13.8 %	
(41.4-48.5)	121	62.8 %	37.2 %		81.0 %	19.0 %		78.5 %	21.5 %	
(48.5-55.5)	124	65.3 %	34.7 %		80.6 %	19.4 %		81.5 %	18.5 %	
(55.5-157.0]	114	75.4 %	24.6 %		88.6 %	11.4 %		84.2 %	15.8 %	

HDL-C denotes high-density lipoprotein cholesterol; CEC, cholesterol efflux capacity; ApoA-I, apolipoproteinA-I; HOII, HDL oxidative-inflammatory index; S1P, sphingosine-1-phosphate; ACS, acute coronary syndrome; AMI, acute myocardial infarction; UA, unstable angina.

Table3. Distribution of ACS, AMI and UA cases between first and fourth quartile of ApoA-I, CEC, HOII, SIP and HDL-C.

	N	Control	**ACS**	p-value	Control	**AMI**	p-value	Control	**UA**	p-value
ApoA-I, mg/dL				**<0.001**			**0.009**			0.059
(0.210-0.700)	119	57.1 %	42.9 %		76.5 %	23.5 %		77.3 %	22.7 %	
(0.910-1.700)	126	79.4 %	20.6 %		89.7 %	10.3 %		87.3 %	12.7 %	
CEC (unitless ratio)				0.434			**0.008**			0.362
(0.670-0.964)	125	64.0 %	36.0 %		77.6 %	28 22.4 %		82.4 %	17.6 %	
(1.057-1.273)	112	69.6 %	30.4 %		91.1 %	8.93 %		76.8 %	23.2 %	
HOII (unitless ratio)				0.183			0.927			0.121
(0.512-0.955)	111	70.3 %	29.7 %		80.2 %	19.8 %		87.4 %	12.6 %	
(1.236-2.596)	118	61.0 %	39.0 %		78.8 %	21.2 %		78.8 %	21.2 %	
S1P (unitless ratio)				0.297			0.343			0.15
(0.160-0.776)	121	64.5 %	35.5 %		79.3 %	20.7 %		77.7 %	22.3 %	
(1.230-2.545)	113	71.7 %	28.3 %		85.0 %	15.0 %		85.8 %	14.2 %	
HDL-C, mg/dL				0.109			**0.032**			0.808
(27.0-41.4)	123	65.0 %	35.0 %		77.2 %	22.8 %		86.2 %	13.8 %	
(55.5-157.0)	114	75.4 %	24.6 %		88.6 %	11.4 %		84.2 %	15.8 %	

HDL-C denotes high-density lipoprotein cholesterol; CEC, cholesterol efflux capacity; ApoA-I, apolipoproteinA-I; HOII, HDL oxidative-inflammatory index; S1P, sphingosine-1-phosphate; ACS, acute coronary syndrome; AMI, acute myocardial infarction; UA, unstable angina.

Table4. Logistic regression analysis for acute coronary syndrome and levels of ApoA-I, CEC, HOII, S1P and HDL-C.

Model	Odds Ratio (95% CI)	*P* Value
ApoA-I, mg/dL		
Model 1	0.094 (0.024-0.36)	**<0.001**
Model 2	0.12 (0.02-0.8)	**0.028**
Model 3	0.13 (0.032-0.51)	**0.003**
CEC (unitless ratio)		
Model 1	$4*10^{-3}$ ($7*10^{-5}$-0.23)	**0.007**
Model 2	$5*10^{-3}$ ($7*10^{-5}$-0.37)	**0.016**
Model 3	$9*10^{-3}$ ($1*10^{-4}$-0.59)	**0.028**
HOII (unitless ratio)		
Model 1	2.67 (1.13-6.31)	**0.025**
Model 2	3.22 (1.27-8.18)	**0.014**
Model 3	3.3 (1.35-8.08)	**0.009**
S1P		
Model 1	0.52 (0.27-1)	0.051
Model 2	0.5 (0.25-1.02)	0.056
Model 3	0.6 (0.3-1.17)	0.132
HDL-C		
Model 1	0.98 (0.96-1)	0.089
Model 2	0.98 (0.96-1)	0.093
Model 3	0.99 (0.97-1.01)	0.292

HDL-C denotes high-density lipoprotein cholesterol; CEC, cholesterol efflux capacity; ApoA-I, apolipoproteinA-I; HOII, HDL oxidative-inflammatory index; S1P, sphingosine-1-phosphate.

Model 1: unadjusted; model 2, adjusted for age, intervention group, and HDL-C; model 3, adjusted for age, intervention group, hypercholesterolemia, hypertension, type 2 diabetes mellitus, and smoking habit.

Table5. Logistic regression analysis for myocardial infarction and levels of ApoA-I, CEC, HOII, S1P and HDL-C.

Model	Odds Ratio (95% CI)	*P* Value
ApoA-I, mg/dL		
Model 1	0.079 (0.013-0.47)	**0.005**
Model 2	0.19 (0.017-2.26)	0.191
Model 3	0.14 (0.021-0.88)	**0.036**
CEC (unitless ratio)		
Model 1	$1*10^{-4}$($4*10^{-7}$-0.028)	**0.001**
Model 2	$2*10^{-4}$($4*10^{-7}$-0.067)	**0.004**
Model 3	$5*10^{-4}$($1*10^{-6}$-0.18)	**0.012**

HOII (unitless ratio)		
Model 1	1.63 (0.53-5)	0.396
Model 2	2.14 (0.63-7.33)	0.224
Model 3	2.12 (0.63-7.13)	0.224
S1P		
Model 1	0.5 (0.22-1.17)	0.11
Model 2	0.48 (0.19-1.2)	0.118
Model 3	0.64 (0.26-1.54)	0.315
HDL-C		
Model 1	0.99 (0.96-1.01)	0.262
Model 2	0.99 (0.96-1.01)	0.258
Model 3	0.99 (0.97-1.02)	0.596

HDL-C denotes high-density lipoprotein cholesterol; CEC, cholesterol efflux capacity; ApoA-I, apolipoproteinA-I; HOII, HDL oxidative-inflammatory index; S1P, sphingosine-1-phosphate.

Model 1: unadjusted; model 2, adjusted for age, intervention group and HDL-C; model 3, adjusted for age, intervention group, hypercholesterolemia, hypertension, type 2 diabetes mellitus and smoking habit.

Table 6. Logistic regression analysis for unstable angina and levels of ApoA-I, CEC, HOII, S1P and HDL-C.

Model	Odds Ratio (95% CI)	*P* Value
ApoA-I, mg/dL		
Model 1	0.12 (0.015-0.93)	**0.042**
Model 2	0.076 (0.004-1.3)	0.075
Model 3	0.1 (0.012-0.87)	**0.037**
CEC (unitless ratio)		
Model 1	0.65 ($1*10^{-3}$-343)	0.894
Model 2	0.66 ($1*10^{-3}$-434)	0.9
Model 3	0.39 ($6*10^{-4}$-265)	0.779
HOII (unitless ratio)		
Model 1	5.24 (1.32-20.8)	**0.019**
Model 2	5.15 (1.17-22.7)	**0.03**
Model 3	5.3 (1.27-22.1)	**0.022**
S1P		
Model 1	0.55 (0.19-1.55)	0.259
Model 2	0.5 (0.16-1.55)	0.231
Model 1	0.49 (0.16-1.52)	0.216
HDL-C		
Model 1	0.98 (0.95-1.01)	0.184
Model 2	0.98 (0.94-1.01)	0.158
Model 1	0.98 (0.95-1.02)	0.301

HDL-C denotes high-density lipoprotein cholesterol; CEC, cholesterol efflux capacity; ApoA-I, apolipoproteinA-I; HOII, HDL oxidative-inflammatory index; S1P, sphingosine-1-phosphate.

Model 1: unadjusted; model 2, adjusted for age, intervention group and HDL-C; model 3, adjusted for age, intervention group, hypercholesterolemia, hypertension, type 2 diabetes mellitus and smoking habit.
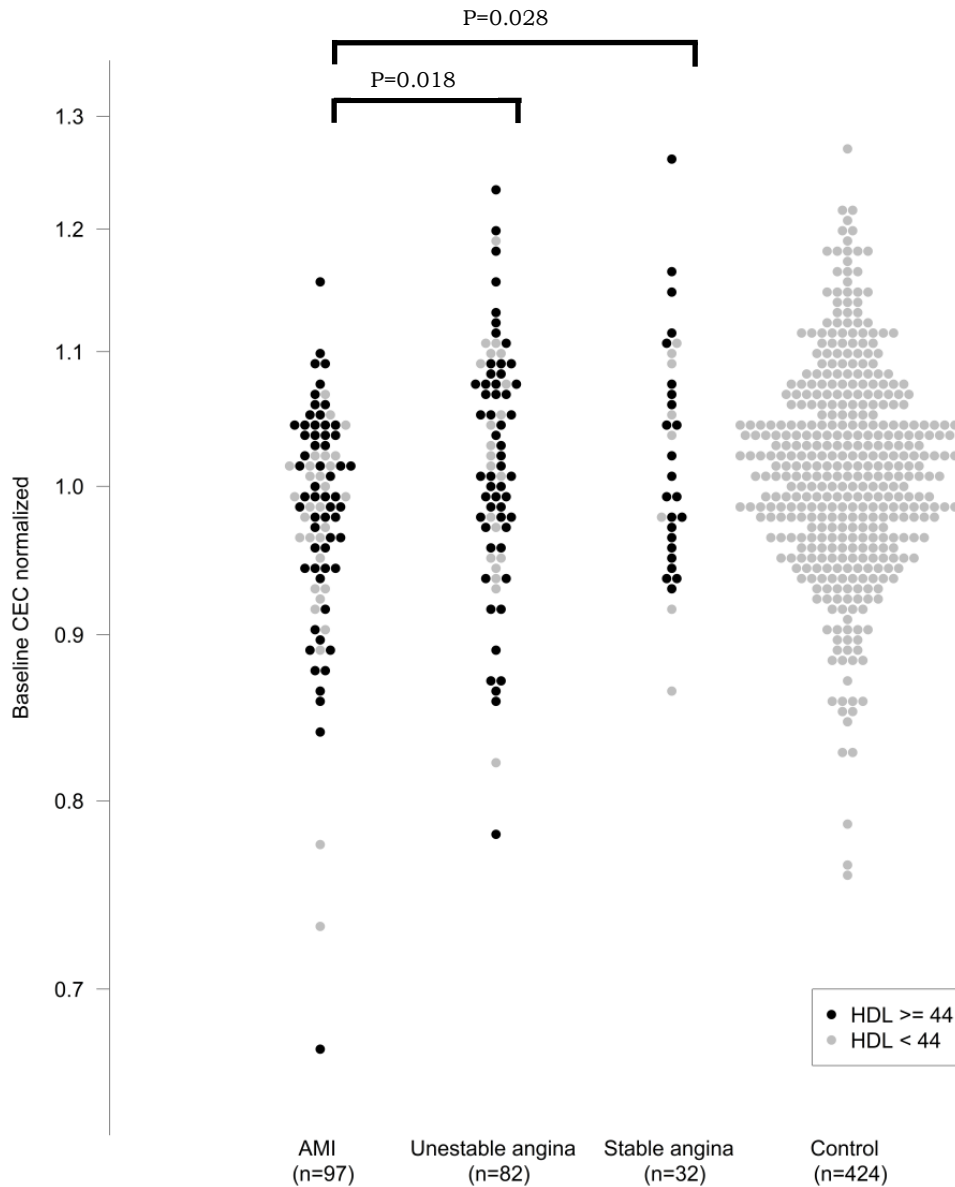
Figure1. Relationship of HDL function-related biomarkers among stable angina and severity of coronary syndrome.

Graph showing CEC values in patients with AMI, UA, and SA. Dark circles show patients with high levels of HDL-C; light circles, patients without high levels of HDL-C. n indicates the number of patients and controls analyzed

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MANUSCRIPT 3 (READY TO BE SUBMITTED): ROLE OF HDL FUNCTION AND LDL ATHEROGENICITY ON CARDIOVASCULAR RISK: A COMPREHENSIVE EXAMINATION.

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Role of HDL function and LDL atherogenicity on cardiovascular risk: a comprehensive examination

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ABSTRACT

Background. High-density lipoprotein (HDL) functionality and low-density lipoprotein (LDL) atherogenic traits describe the role of both particles on cardiovascular diseases more accurately than HDL- or LDL-cholesterol levels. However, it is unclear how these lipoprotein properties are particularly affected by different cardiovascular risk factors.

Objective: To determine which lipoprotein properties are associated with greater cardiovascular risk scores and each cardiovascular risk factor.

Methods: In two cross-sectional baseline samples of PREDIMED trial volunteers, we assessed the associations of HDL functionality (N=296) and LDL atherogenicity traits (N=210) with: 1) the 10-year predicted coronary risk (according to the Framingham-REGICOR score), and 2) classical cardiovascular risk factors.

Results: Greater cardiovascular risk scores were associated with low cholesterol efflux values; oxidized, triglyceride-rich, small HDL particles; and small LDLs with low resistance against oxidation (P-trend<0.05, all). After adjusting for the rest of risk factors; 1) type-2 diabetic individuals presented smaller and more oxidized LDLs (P<0.026, all); 2) hypercholesterolemic participants had smaller HDLs with an impaired capacity to metabolize cholesterol (P<0.035, all); 3) high body mass index values were associated to lower HDL and LDL size and a lower HDL capacity to esterify cholesterol (P<0.037, all); 4) men presented a greater HDL oxidation and lower HDL vasodilatory capacity (P<0.046, all); and 5) greater ages were related to small, oxidized, cytotoxic LDL particles (P<0.037, all).

Conclusions: Dysfunctional HDL and atherogenic LDL particles are present in high cardiovascular risk patients. Hypercholesterolemia and male sex are predominantly linked to HDL dysfunctionality, whilst diabetes and advanced age are associated with LDL atherogenicity.

INTRODUCTION

Low levels of high-density lipoprotein (HDL) cholesterol (HDL-C) and high concentrations of low-density lipoprotein (LDL) cholesterol (LDL-C) are traditionally related to a greater risk of suffering a cardiovascular event[1]. However, HDL functions may reflect the protective role of the lipoprotein better than HDL-C levels[2], and LDL characteristics provide further information on the residual atherogenic risk of these particles beyond mere LDL-C concentrations[3,4].

Both lipoprotein traits have been shown to be associated with high cardiovascular risk states in very diverse ways. On the one hand, regarding HDL functions: 1) cholesterol efflux capacity (HDL capacity to pick up cholesterol from peripheral cells) has demonstrated to be inversely related with the incidence of cardiovascular events (and shown to predict these outcomes more accurately than HDL-C concentrations)[5]; 2) deficiencies in the biological function of two enzymes related to the metabolism of cholesterol in HDLs, lecithin-cholesterol acyltransferase (LCAT, responsible for the esterification and internalization of free cholesterol after cholesterol efflux) and cholesteryl ester transfer protein (CETP, responsible for the exchange of cholesterol from HDLs to other lipoproteins), have shown to be linked to modest increments (LCAT) or decrements (CETP) in the incidence of cardiovascular events[6,7], although the effect of modifying these activities in other studies has not been shown to be conclusive[8]; 3) the activity of paraoxonase-1 (PON1, an essential antioxidant HDL enzyme) has been inversely associated with cardiovascular diseases incidence in some works[9] but not in others[10]; 4) HDLs are also thought to promote endothelial protection and are linked to a greater release of nitric oxide from endothelial cells[11], being this property transiently impaired in acute coronary events[12]; finally, 5) HDL oxidation and its global lipid composition, although being related to several aspects of a dysfunctional lipoprotein profile (a decreased capacity to perform HDL biological functions or a decreased HDL stability)[13–16], have not been associated with high cardiovascular risk (CVR) states as clearly as other HDL functional traits. On the other hand, regarding LDL atherogenic characteristics: 1) circulating levels of oxidized LDLs are directly related with incidence of coronary diseases and all-cause mortality[4,17],

whilst low LDL resistance against oxidative modifications of the particle has been linked with subclinical atherosclerosis and is present in high CVR subjects[18,19]; 2) small LDL particles (a characteristic deeply interrelated with a pro-atherogenic LDL profile, which can be indirectly measured by the ratio between LDL-C and apolipoprotein B –ApoB– levels in circulation[20]), have been associated with a greater incidence of cardiovascular events[21]; and 3) compositional changes of LDL particles such as increases in their remnant triglyceride content tend to increase ApoB-100 instability on LDL surface (which may lead to an inefficient binding to LDL receptors) and have shown to be increased in coronary artery disease patients[22,23]. Despite these associations, characteristics of HDL dysfunctionality and LDL atherogenicity are usually present simultaneously in the different high CVR states (diabetes, hypercholesterolemia, excess weight, etc.). Thus, it is still unclear which CVR factors (CVRFs) are particularly responsible for an impaired HDL functionality or an increased LDL atherogenicity (namely, which associations between a CVRF and a lipoprotein property are still significant when the confounder effect of the rest of the CVRFs is excluded).

The aim of this study was to determine the independent associations of HDL functionality and LDL atherogenic characteristics with: 1) the 10-year predicted risk of suffering a coronary event (the Framingham-REGICOR CVR score), and 2) the most prevalent CVRFs (diabetes, hypercholesterolemia, excess body weight, hypertension, and smoking habit), age, and sex, in high CVR individuals.

MATERIALS AND METHODS

Study population

This study was a cross-sectional analysis in two sub-samples of volunteers from the PREDIMED Study[24,25] at the baseline visit: one sample for the evaluation of HDL-related variables (N=296)[26] and another for the assessment of LDL atherogenic traits (N=210)[27]. The sample for the study of HDL-related parameters included the one in which the LDL-related characteristics were assessed. In these populations, we registered the values of: 1) general clinical variables (age, sex, body weight, height, blood pressure, and biochemical

profile); 2) drug use; 3) adherence to a Mediterranean Diet, by means of the Mediterranean diet Score; 4) levels of physical activity according to the Minnesota Leisure Time physical Activity questionnaire, validated in Spanish population; and 5) smoking habit[24,28]. In individuals aged 35-74, we calculated 10-year predicted risk of developing a future coronary event as the CVR scores according to the Framingham-REGICOR equation validated for the Spanish population (considering age and sex, presence of diabetes and tobacco habit, total and HDL-C levels, and blood pressure)[29]. Type-II diabetes mellitus was defined as the presence of an abnormal glucose metabolism or use of anti-diabetic drugs. Hypercholesterolemia was defined as the presence of total cholesterol levels ≥200 mg/dL or use of statins. Hypertension was defined as the presence of systolic blood pressure levels ≥140 mmHg, diastolic blood pressure levels ≥90 mmHg, or use of anti-hypertensive drugs. Body mass index (BMI) was calculated as the ratio between weight (kg) and height squared (m^2)[24].

Volunteers provided written informed consent before entering the trial. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki, was approved by the local Research and Ethics Committee, and was registered with the International Standard Randomized Controlled Trial Number ISRCTN35739639. Its details have been previously published[24,25].

HDL functionality determinations

We first isolated HDL particles from plasma by density gradient ultracentrifugation (isolated HDL fraction)[26,30] and polyethylene glycol-induced precipitation of apolipoprotein B (ApoB)-containing lipoproteins (ApoB-depleted plasma samples)[26]. Plasma, serum, isolated HDL, and ApoB-depleted plasma samples were stored at -80ºC until use. We analyzed the participants' lipid profile (triglycerides, cholesterol, HDL-C, and apolipoprotein A-I –ApoA-I–) in an ABX-Pentra 400 autoanalyzer (Horiba ABX)[26]. We determined cholesterol efflux capacity (HDL ability to pick up the cholesterol excess from cells) in a model of human THP-1 monocyte-derived macrophages treated with ApoB-depleted plasma samples[26]. We computed the ability of HDL lipoproteins to esterify cholesterol as the percentage of esterified cholesterol in isolated HDL

particles/lecithin cholesterol acyltransferase quantity in plasma[26]. We determined the function of cholesteryl ester transfer protein (CETP) in plasma[26,30] and the arylesterase activity of paraoxonase-1 (PON1) in serum[26] by commercial kits. We assessed HDL vasodilatory capacity as the HDL-induced increment in the production of nitric oxide in a human umbilical vein endothelial cell model treated with ApoB-depleted plasma samples[26]. We determined the oxidation of HDL particles as the equivalents of malondialdehyde per mg/dL of cholesterol in ApoB-depleted plasma samples[26]. We examined the lipid composition of the isolated HDL fraction in an ABX-Pentra 400 autoanalyzer (Horiba ABX) and, from these data, we calculated the triglyceride/esterified cholesterol ratio in HDL particles ("triglycerides in HDL core")[26,30]. Finally, we assessed HDL size distribution by LipoPrint technology (Quantimetrix) in plasma[26,30]. With the percentages of large and small HDL particles (HDL2 and HDL3, respectively), we calculated the HDL2/HDL3 ratio.

LDL atherogenic traits

We first isolated LDL lipoproteins from plasma samples by density gradient ultracentrifugation[27,31] and stored them at -80°C until use. From the values of the participants' lipid profile, we calculated LDL-C levels according to the Friedewald formula (whenever triglycerides were <300 mg/dL)[27,31]. We quantified ApoB in an ABX-Pentra 400 autoanalyzer (Horiba ABX) in plasma[27,31]. We measured LDL resistance against oxidation (LDL lag time) from the kinetics of formation of conjugated dienes (oxidized lipid forms) in isolated LDL samples in a pro-oxidant environment[27,31]. We assessed the oxidation of LDL lipoproteins as the equivalents of malondialdehyde per mg/dL of cholesterol in isolated LDL samples[27]. From the lipid profile values, we calculated an approximation to LDL average size (the LDL-C/ApoB ratio)[27]. We determined the lipid composition of isolated LDL particles in an ABX-Pentra 400 autoanalyzer (Horiba ABX) and, from these data, we calculated the triglyceride/total cholesterol ratio in isolated LDL samples. Finally, we assessed LDL *ex vivo* cytotoxicity in a THP-1 monocyte-derived macrophage model as previously described[27].

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 contrast, sample sizes of 196 and 140 participants provide sufficient statistical power to determine that Pearson's correlation coefficients ≥0.2 and ≥0.237 (for HDL- and LDL-related variables, respectively) were significantly different from zero. Sample sizes were increased by 50%, up to 294 and 210 subjects, to allow adjustments for different covariates.

Statistical analyses

We first assessed the distribution of continuous variables using normality plots and histograms.

To study the association between lipoprotein traits and CVR, we first compared the means of HDL- and LDL-related variables among the CVR score groups (low risk −CVR score <5−, moderate risk −CVR score ≥5 and <10−, and high risk − CVR score ≥10−) using a one-way ANOVA for normally-distributed variables and a Kruskal-Wallis test for non-normally distributed ones. To determine possible linear associations between the CVR score group and the means or medians of lipoprotein-related variables, we performed Pearson's or Spearman's tests, respectively, to calculate *P*-trend values.

We assessed the differences in the values of lipoprotein characteristics due to classical CVRFs (presence of diabetes, hypercholesterolemia, hypertension, and tobacco use −categorical variables−; and greater values of BMI −continuous variable−), sex, and age (continuous variable), in three multivariate linear regression models. Model 1 was non-adjusted. To determine the independent effect of each of these traits on lipoprotein characteristics, model 2 was adjusted for the rest of the previous factors, study site, adherence to the Mediterranean diet, and levels of physical activity. Finally, model e included HDL-C or LDL-C levels as an extra co-variate, in order to exclude the effect of lipoprotein cholesterol from the previous associations.

We accepted any two-sided *P*-value <0.05 as significant. We executed the previously described analyses in R Software, version 3.4.1 (*R: A language and*

environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria).

RESULTS

Participants

In accordance to the high CVR profile of the volunteers in the HDL functionality and LDL atherogenicity subsamples, subjects were 65.9 and 65.4 years old, 49.0 and 51.4% of the participants were male, 49.0 and 47.1% were diabetic, 77.4 and 79.0% were dyslipidemic, 78.7 and 82.4% were hypertensive, 44.9 and 44.8% were obese, and 12.5 and 13.8% were smokers, respectively.

HDL functionality, LDL atherogenicity, and CVR categories

Regarding HDL-related traits, high CVR was associated with low HDL-C and ApoA-I levels, low cholesterol efflux values, high HDL oxidation, high content of triglycerides in HDL core ($P<0.001$ in the five previous cases), and low values of the HDL2/HDL3 ratio (smaller HDL size) ($P=0.002$) (**Figures 1A-1J**).

Regarding LDL-related variables, high CVR was associated with high ApoB levels ($P=0.025$) (but not with significant differences in LDL-C levels, $P>0.05$), low LDL resistance against oxidation (low LDL lag time values) ($P=0.019$), and low estimated LDL size ($P=0.001$), and with a borderline significant trend towards high LDL oxidation ($P=0.085$) (**Figures 2A-2G**).

Figure 1. Values of HDL functionality variables in the three groups according to CVR scores: low CVR (score<5, *N*=77), moderate CVR (score ≥5 and <10, *N*=115), and high CVR (score ≥10, *N*=52)
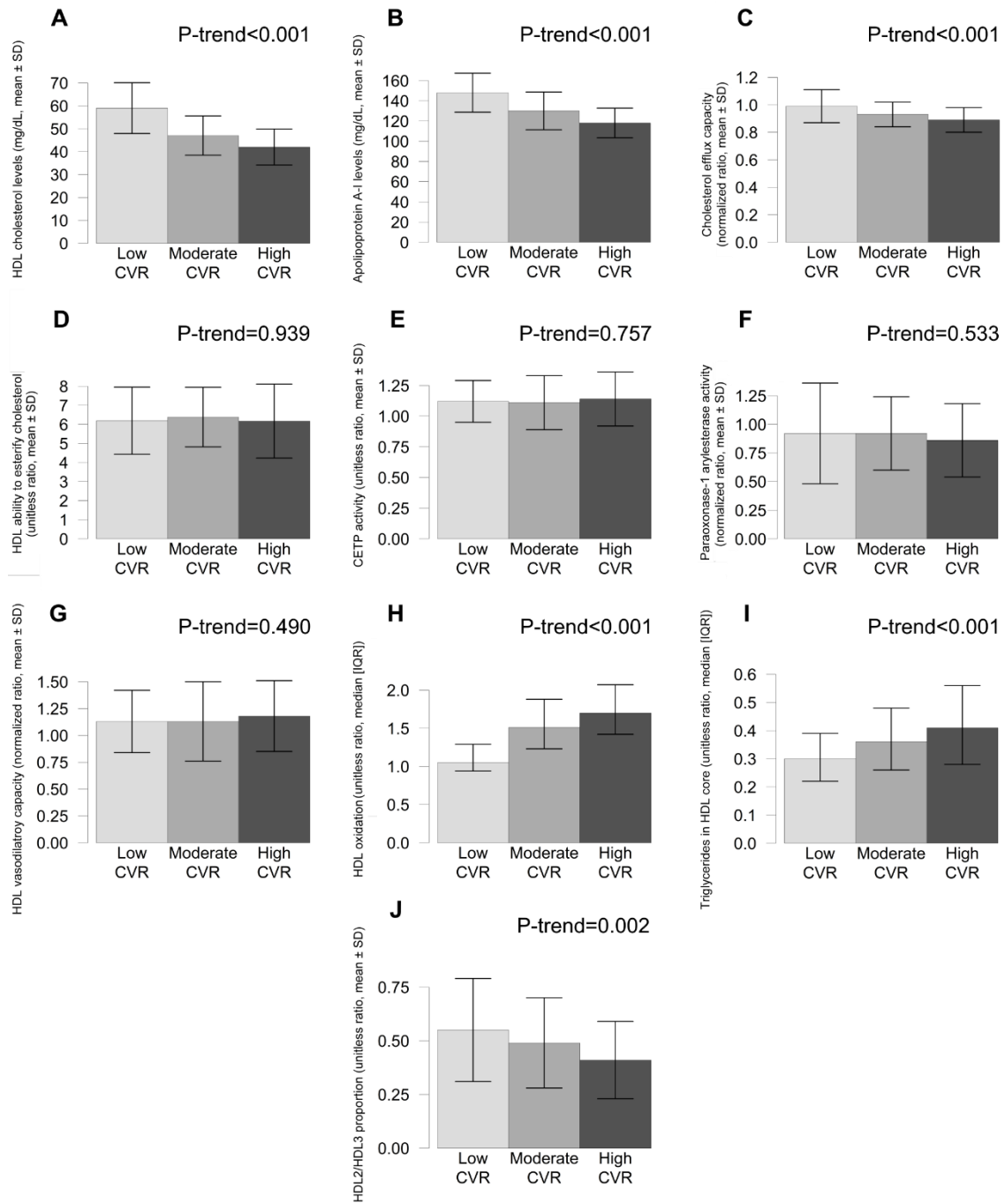
CVR, cardiovascular risk; IQR, interquartile range; SD, standard deviation.

Figure 2. Values of LDL functionality variables in the three groups according to CVR scores: low CVR (score<5, *N*=48), moderate CVR (score ≥5 and <10, *N*=88), and high CVR (score ≥10, *N*=38)
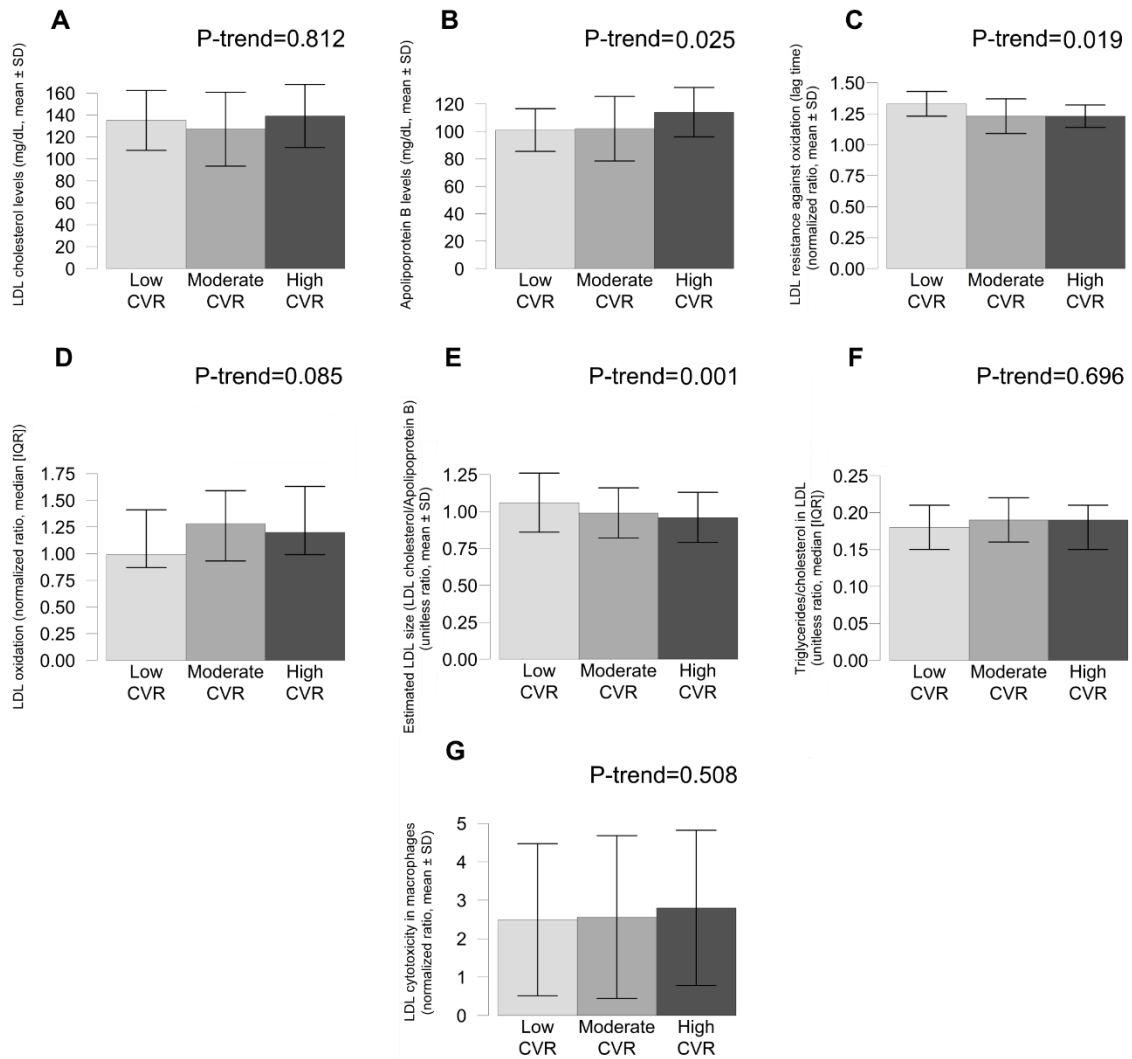
CVR, cardiovascular risk; IQR, interquartile range; SD, standard deviation.

Individual effects of CVRFs on lipoprotein traits in high CVR subjects

As observed in **Table 1**, presence of type-II diabetes was associated with low HDL-C (P<0.001) and ApoA-I levels (P=0.004), low cholesterol efflux capacity values (P=0.001), and high HDL oxidation (P=0.030). These last two associations were lost when adjusting for HDL-C levels (P>0.05). Regarding LDL properties, diabetes was related to low LDL-C (P<0.001) and ApoB levels (P=0.033), greater LDL oxidation (P<0.001) and lower LDL resistance against oxidation (P=0.007), lower estimated LDL size (P<0.001), a greater LDL triglyceride content (P=0.032), and higher LDL cytotoxicity in macrophages (P=0.025). LDL oxidation and estimated LDL size remained significantly altered even when adjusting for all classical CVRFs and LDL-C levels (P=0.022 and P=0.026, respectively).

Hypercholesterolemia was related to greater HDL-C, LDL-C, ApoA-I, and ApoB levels (P<0.05, all), high cholesterol efflux capacity values (P<0.001), high CETP activity (P=0.017) and low HDL capacity to esterify cholesterol (P=0.015). When adjusting for all classical CVRFs and HDL-C levels, cholesterol efflux capacity values were no longer significantly greater (P>0.05), but high CETP activity values, low HDL capacity to esterify cholesterol, and smaller HDL average size remained significant (P=0.035, P=0.031, and P=0.021).

High BMI values were associated with low HDL-C levels (P=0.006), low HDL capacity to esterify cholesterol (P=0.008), smaller HDL size (P<0.001), and a greater triglyceride content in the HDL core (P=0.014). These three associations were still present when adjusting for all classical CVRFs and HDL-C levels (P=0.037, P=0.012, and P=0.087, respectively). High BMI values were also linked to a lower estimated LDL size after adjusting for all CVRFs and LDL-C levels (P=0.004 and P=0.007, respectively).

Hypertension was not particularly associated with an abnormal lipoprotein profile (data not shown), although it was independently linked to a borderline significant trend towards low cholesterol efflux capacity values when adjusted for all classical CVRFs (P=0.090). When adjusting for CVRFs and LDL-C levels,

it was related to a greater LDL oxidation (*P*=0.036). Finally, being smoker was also unrelated to differences in any lipoprotein characteristic (data not shown).

Table 1. Independent associations between type-II diabetes, hypercholesterolemia, increases in 1 kg/m² of body mass index, and hypertension, and HDL- and LDL-related variables.

	Type-II diabetes			Hypercholesterolemia			Increases in 1 kg/m² of BMI		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
HDL-C levels (mg/dL)	-6.52*** [-9.05;-3.99]	-4.93*** [-7.50;-2.36]	-	6.05*** [2.95;9.14]	3.37* [0.41;6.33]	-	-0.48** [-0.82;-0.14]	-0.53** [-0.84;-0.21]	-
ApoA-I levels (mg/dL)	-8.08** [-13.6;-2.61]	-6.90* [-12.3;-1.49]	-	15.6*** [9.43;21.9]	9.10** [3.15;15.1]	-	-0.63 [-1.35;0.090]	-0.62 [-1.26;0.026]	-
Cholesterol efflux capacity (normalized ratio)	-0.041** [-0.065;-0.017]	-0.027* [-0.052;-0.003]	-0.010 [-0.033;0.013]	0.059*** [0.031;0.088]	0.042** [0.014;0.070]	0.022 [-0.004;0.049]	-0.001 [-0.004;0.002]	-0.001 [-0.004;0.002]	$7 \cdot 10^{-4}$ [-0.002;0.003]

HDL cholesterol esterification Index (unitless ratio)	-0.002 [-0.49;0.49]	-0.18 [-0.72;0.35]	-0.14 [-0.68;0.41]	-0.73* [-1.31;-0.15]	-0.58 [-1.17;0.001]	-0.67* [-1.27;-0.066]	-0.084** [-0.15;-0.022]	-0.066* [-0.13;-0.004]	-0.068* [-0.13;-0.005]
CETP activity (normalized ratio)	-0.065* [-0.12;-0.008]	-0.003 [-0.067;0.062]	-0.004 [-0.070;0.062]	0.085* [0.016;0.16]	0.069 [-0.003;0.14]	0.080* [0.006;0.15]	0.001 [-0.006;0.008]	$-7 \cdot 10^{-4}$ [-0.008;0.007]	$-3 \cdot 10^{-4}$ [-0.008;0.007]
PON1 arylesterase activity (normalized ratio)	-0.051 [-0.16;0.052]	-0.034 [-0.15;0.082]	-0.026 [-0.14;0.093]	0.074 [-0.050;0.20]	0.057 [-0.071;0.19]	0.030 [-0.10;0.16]	-0.007 [-0.020;0.006]	-0.009 [-0.022;0.005]	-0.007 [-0.021;0.007]
HDL vasodilatory capacity (normalized ratio)	0.027 [-0.056;0.11]	0.022 [-0.064;0.11]	0.006 [-0.081;0.094]	0.050 [-0.050;0.15]	0.009 [-0.089;0.11]	0.021 [-0.079;0.12]	0.004 [-0.007;0.015]	0.002 [-0.009;0.012]	$-4 \cdot 10^{-4}$ [-0.011;0.010]

HDL oxidation (normalized ratio)	0.46* [0.047;0.87]	0.086 [-0.36;0.54]	0.095 [-0.38;0.57]	0.12 [-0.37;0.62]	0.14 [-0.37;0.65]	0.15 [-0.39;0.69]	0.017 [-0.037;0.072]	0.018 [-0.037;0.073]	0.019 [-0.038;0.076]
Triglycerides in HDL core (unitless ratio)	0.032 [-0.010;0.074]	0.036 [-0.010;0.083]	0.009 [-0.033;0.052]	$4 \cdot 10^{-4}$ [-0.049;0.050]	-0.008 [-0.061;0.045]	0.022 [-0.026;0.071]	0.007* [0.001;0.012]	0.007* [0.002;0.013]	0.004 [$-6 \cdot 10^{-4}$;0.010]
HDL$_2$/HDL$_3$ (unitless ratio)	0.018 [-0.036;0.073]	-0.013 [-0.071;0.044]	0.020 [-0.035;0.075]	-0.022 [-0.088;0.043]	-0.049 [-0.11;0.016]	-0.073* [-0.14;-0.011]	-0.014*** [-0.021;-0.007]	-0.012*** [-0.019;-0.006]	-0.008* [-0.015;-0.002]
LDL-C levels (mg/dL)	-15.4*** [-23.9;-6.87]	-13.8** [-22.1;-5.47]	-	29.9*** [20.1;39.6]	28.1*** [18.5;37.7]	-	-0.14 [-1.26;0.97]	-0.70 [-1.72;0.32]	-

ApoB (mg/dL)	-7.27* [-13.9;-0.66]	-5.93 [-12.8;0.99]	-	19.8*** [12.3;27.3]	18.0*** [10.2;25.8]	-	0.49 [-0.38;1.36]	0.46 [-0.39;1.30]	-
LDL size (LDL-C/ApoB) (unitless ratio)	-0.088*** [-0.13;-0.050]	-0.072*** [-0.11;-0.030]	-0.046* [-0.084;-0.007]	0.051* [0.002;0.10]	0.032 [-0.015;0.079]	-0.017 [-0.062;0.029]	-0.005 [-0.01;$7 \cdot 10^{-4}$]	-0.007** [-0.012;-0.002]	-0.007** [-0.012;-0.002]
LDL oxidation (normalized ratio)	0.26*** [0.13;0.39]	0.21** [0.080;0.34]	0.12* [0.016;0.23]	-0.11 [-0.27;0.050]	-0.090 [-0.24;0.057]	0.084 [-0.050;0.22]	0.006 [-0.012;0.023]	0.006 [-0.010;0.021]	-0.004 [-0.017;0.009]
LDL lag time (normalized ratio)	-0.070** [-0.12;-0.019]	-0.061 [-0.12;-0.007]	-0.047 [-0.10;0.010]	0.019 [-0.042;0.080]	0.026 [-0.038;0.089]	$3 \cdot 10^{-4}$ [-0.072;0.072]	0.001 [-0.005;0.008]	0.002 [-0.005;0.009]	0.003 [-0.004;0.010]

Triglycerides/cholesterol in LDLs (unitless ratio)	0.018* [0.002;0.034]	0.018* [0.002;0.035]	0.012 [-0.004;0.028]	0.010 [-0.009;0.029]	0.001 [-0.018;0.020]	0.021* [0.002;0.040]	0.002 [-4·10^-4;0.004]	0.001 [-8·10^-4;0.003]	6·10^-4 [-0.001;0.002]
LDL cytotoxicity in macrophages (normalized ratio)	0.65* [0.084;1.21]	0.49 [-0.10;1.08]	0.36 [-0.24;0.96]	-0.16 [-0.84;0.53]	-0.096 [-0.77;0.57]	0.38 [-0.35;1.10]	-0.032 [-0.10;0.040]	-0.032 [-0.10;0.039]	-0.045 [-0.12;0.026]

ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; BMI, body mass index; CETP, cholesteryl ester transfer protein; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; PON1, paraoxonase-1.

Data are beta coefficients [95% CI] obtained in multivariate linear regression analysis, non-adjusted (Model 1), adjusted for the rest of cardiovascular risk factors, study site, adherence to a Mediterranean Diet, and levels of physical activity (Model 2), and for all the previous factors plus HDL-C or LDL-C levels (in HDL- or LDL-related variables, respectively) (Model 3). *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$.

Effects of sex and age on lipoprotein traits in high CVR patients

Male sex was associated with a highly dysfunctional HDL profile (**Table 2**): after adjusting for all classical CVRFs, men had lower HDL-C and ApoA-I levels (*P*<0.001, both), lower cholesterol efflux capacity values (*P*=0.003), lower CETP activities (*P*=0.033), greater HDL oxidation (*P*=0.018), and smaller HDL particles (*P*=0.004). When adjusting for HDL-C levels, the associations with lower cholesterol efflux capacity, CETP activity, and HDL size were no longer significant (*P*>0.05), while the one with a greater HDL oxidation remained significant (*P*=0.026) and a significant relationship with a decreased HDL vasodilatory capacity appeared (*P*=0.046). Regarding LDLs, after adjusting for CVRFs, male sex was linked to lower estimated LDL size (*P*=0.028), and to greater LDL oxidation (*P*=0.044), cholesterol content (*P*=0.009), and cytotoxicity (*P*=0.040). After discarding the effect of LDL-C levels, only the associations with estimated LDL size and cholesterol content were still present (*P*=0.079 and *P*<0.001, respectively).

Despite greater age being linked to low LDL-C concentrations (*P*<0.001), it was also independently associated with a greater LDL oxidation (*P*=0.009), a greater LDL triglyceride content (*P*=0.037), and greater LDL cytotoxic potential (*P*=0.030) when adjusting for all classical CVRFs. None of these associations remained significant when adjusting for LDL-C levels (*P*>0.05). Finally, regarding HDL functionality, greater ages were related to decreased PON-1 arylesterase activity (*P*=0.035) and larger HDL particles (*P*<0.001), but after adjusting for all classical CVRFs and HDL-C levels, we only observed a significant association with a greater triglyceride content in the HDL core (*P*=0.033).

Table 2. Independent associations between male sex and increasing 1 year of age and HDL- and LDL-related variables.

	Male sex			Increases in 1 year of age		
	Model 1	Model 2	Model 3	Model 1	Model 2	Model 3
HDL-C levels (mg/dL)	-9.24*** [-11.7;-6.83]	-7.80*** [-10.3;-5.27]	-	0.19 [-0.015;0.39]	0.084 [-0.11;0.27]	-
ApoA-I levels (mg/dL)	-19.5*** [-24.4;-14.5]	-15.5*** [-20.7;-10.2]	-	0.37 [-0.036;0.78]	0.072 [-0.30;0.45]	-
Cholesterol efflux capacity (normalized ratio)	-0.056*** [-0.080;-0.033]	-0.038** [-0.062;-0.014]	-0.010 [-0.034;0.013]	0.001 [-7·10⁻⁴;0.003]	8·10⁻⁴ [-0.001;0.003]	4·10⁻⁴ [-0.001;0.002]
HDL cholesterol esterification Index (unitless ratio)	0.59* [0.10;1.08]	0.36 [-0.15;0.87]	0.39 [-0.17;0.94]	0.014 [-0.024;0.052]	0.028 [-0.013;0.068]	0.024 [-0.017;0.064]
CETP activity (normalized ratio)	-0.081** [-0.14;-0.023]	-0.068* [-0.13;-0.006]	-0.066 [-0.13;6·10⁻⁴]	-0.004 [-0.008;7·10⁻⁴]	-0.004 [-0.009;0.001]	-0.003 [-0.008;0.002]
PON1 arylesterase activity (normalized ratio)	-0.058 [-0.16;0.045]	-0.061 [-0.17;0.051]	-0.032 [-0.15;0.088]	-0.009* [-0.016;-7·10⁻⁴]	-0.008 [-0.017;6·10⁻⁴]	-0.009 [-0.017;2·10⁻⁴]
HDL vasodilatory capacity (normalized ratio)	-0.014 [-0.096;0.069]	-0.038 [-0.12;0.048]	-0.094* [-0.19;-0.002]	0.002 [-0.004;0.008]	0.004 [-0.003;0.010]	0.004 [-0.002;0.011]

HDL oxidation (normalized ratio)	0.48* [0.063;0.89]	0.55* [0.098;0.99]	0.56* [0.071;1.05]	0.002 [-0.030;0.034]	-0.001 [-0.034;0.032]	-0.001 [-0.035;0.033]
Triglycerides in HDL core (unitless ratio)	0.018 [-0.024;0.060]	0.026 [-0.020;0.071]	-0.039 [-0.082;0.005]	0.002 [-0.001;0.005]	0.002 [-0.001;0.006]	0.003* [$3 \cdot 10^{-4}$;0.006]
HDL_2/HDL_3 (unitless ratio)	-0.068* [-0.12;-0.015]	-0.083** [-0.14;-0.027]	-0.028 [-0.084;0.029]	0.007*** [0.003;0.012]	0.004* [$1 \cdot 10^{-4}$;0.008]	0.004 [$-4 \cdot 10^{-5}$;0.008]
LDL-C levels (mg/dL)	-14.8*** [-23.3;-6.23]	-6.44 [-14.7;1.79]	-	-0.87** [-1.52;-0.22]	-1.13*** [-1.77;-0.49]	-
ApoB (mg/dL)	-6.16 [-12.8;0.52]	-2.03 [-8.73;4.66]	-	-0.51* [-0.99;-0.041]	-0.64* [-1.14;-0.14]	-
LDL size (LDL-C/ApoB) (unitless ratio)	-0.062** [-0.10;-0.022]	-0.046* [-0.086;-0.006]	-0.033 [-0.070;0.004]	-0.002 [-0.005;0.001]	-0.002 [-0.005;0.001]	$-3 \cdot 10^{-4}$ [-0.003;0.003]
LDL oxidation (normalized ratio)	0.085 [-0.051;0.22]	0.13* [0.005;0.26]	0.028 [-0.079;0.13]	0.013* [0.003;0.024]	0.013** [0.003;0.023]	0.003 [-0.005;0.012]
LDL lag time (normalized ratio)	-0.017 [-0.068;0.035]	-0.001 [-0.056;0.053]	0.006 [-0.050;0.063]	0.002 [-0.001;0.006]	0.003 [-0.002;0.007]	0.003 [-0.001;0.008]
Triglycerides/cholesterol	-0.026**	-0.022**	-0.027*	0.002*	0.001*	$6 \cdot 10^{-4}$

in LDLs (unitless ratio)	[-0.042;-0.010]	[-0.038;-0.006]	[-0.042;-0.012]	[$3 \cdot 10^{-4}$;0.003]	[$9 \cdot 10^{-5}$;0.003]	[$-6 \cdot 10^{-4}$;0.002]
LDL cytotoxicity in macrophages (normalized ratio)	0.41 [-0.16;0.98]	0.61* [0.033;1.19]	0.38 [-0.20;0.96]	0.043 [$-3 \cdot 10^{-4}$;0.086]	0.050* [0.005;0.095]	0.030 [-0.016;0.075]

ApoA-I, apolipoprotein A-I; ApoB, apolipoprotein B; BMI, body mass index; CETP, cholesteryl ester transfer protein; HDL-C, HDL cholesterol; LDL-C, LDL cholesterol; PON1, paraoxonase-1.

Data are beta coefficients [95% CI] obtained in multivariate linear regression analysis, non-adjusted (Model 1), adjusted for the rest of cardiovascular risk factors, study site, adherence to a Mediterranean Diet, and levels of physical activity (Model 2), and for all the previous factors plus HDL-C or LDL-C levels (in HDL- or LDL-related variables, respectively) (Model 3). *: $P<0.05$; **: $P<0.01$; ***: $P<0.001$

DISCUSSION

Our data show that dysfunctional HDL and atherogenic LDL particles are associated with greater CVR scores and particularly impaired in certain high CVR subjects (diabetic, hypercholesterolemic, with excess weight, male, and older) in the first systematic, comprehensive association analysis performed to date.

HDL functions are intimately related to CVR according to previous human studies. In our dataset, we have observed an association between high CVR and low cholesterol efflux, high HDL oxidation, high triglyceride content in HDL core, and smaller HDL size. Cholesterol efflux capacity has already shown to be related to high subclinical atherosclerosis and incidence of cardiovascular diseases[5,32]. Regarding HDL oxidation, it has been previously associated with high CVR states[13] as well as with decreased cholesterol efflux capacity values[14]. A high triglyceride content in the HDL core has been shown to contribute to HDL instability (it leads to an imbalance in the electrostatic relationships of the lipoprotein, promoting the detachment of ApoA-I from the HDL surface[15]), and this could be associated with an impaired HDL function. Finally, our data also agree with previous reports of low levels of large HDLs in high CVR states[33].

Regarding LDL atherogenicity properties, LDL particles with smaller estimated size and more prone to become oxidized were associated with greater CVR

scores. This concurs with previous evidence: small and oxidized LDL particles have been related to a greater coronary risk[3,4]. A lower LDL resistance against oxidation (present in coronary disease patients[19]) could facilitate LDL oxidation. Otherwise, ApoB levels, but not LDL-C concentrations, appeared to be significantly increased in high CVR states. This fact agrees with the hypothesis that alternative measurements of LDL quantity in circulation (such as ApoB levels or LDL particle number) could be more accurate and reflect better the CVR derived from these atherogenic lipoproteins[34].

Diabetes was strongly associated with dysfunctional lipoprotein characteristics in our cohort: it was associated with oxidized, small, triglyceride-rich LDL particles and with impaired cholesterol efflux capacity (although this association was lost when adjusting for HDL-C levels). Diabetes is strongly related to a suboptimal lipid profile[35] and a pro-oxidant, pro-inflammatory status that could contribute to promoting HDL dysfunctionality[36] and LDL atherogenicity[37]. The fact that there was 5% fewer hypercholesterolemic patients and 9.3% more individuals treated with statins in the group of diabetic individuals could explain their unexpectedly lower HDL-C and LDL-C levels in our study.

The association of hypercholesterolemia with lipoprotein characteristics was more complex than expected due to the relationship of some lipoprotein traits with HDL-C or LDL-C levels (both greatly increased). Once adjusted for the effect of HDL-C concentrations, being hypercholesterolemic was independently associated with greater CETP activity, lower HDL capacity to esterify cholesterol, and smaller HDL size, in agreement with previous work[38]. Hypercholesterolemia was also independently linked to LDL particles richer in triglycerides. Some authors consider this fact may be linked to a subtype of triglyceride-rich remnant lipoproteins, markedly pro-atherogenic[39].

Other classical CVRFs were shown to impair lipoprotein characteristics in our dataset. On the one hand, increased BMI values were independently associated with lower HDL-C levels, lower HDL ability to esterify cholesterol, and triglyceride-rich, small HDL particles, as well as with low estimated LDL size. Some of these lipoprotein characteristics had already been associated with excess body weight[40]. In addition, hypertriglyceridemic states in overweight or

obesity could facilitate the accumulation of triglycerides in HDL particles[40], possibly leading to the formation of more dysfunctional lipoproteins[15]. On the other hand, although not fully significantly, our results also suggest hypertension could be related to a lower cholesterol efflux capacity and to greater LDL oxidation, potential mechanisms to be addressed in future trials.

Men are known to be more strongly affected by cardiovascular diseases than women[41], hence a potentially deleterious effect of male sex on lipoprotein traits could be expected. In our data, being male was independently associated with low HDL-C levels and, once the confounding effect of HDL-C concentrations was considered, it was also linked to greater HDL oxidation and a reduced HDL capacity to promote the endothelial release of nitric oxide, pointing to two potential novel contributors for the increased CVR in men that should be checked in further studied. In addition, male sex was linked to high concentrations of oxidized, small, cytotoxic LDL lipoproteins, but the significant of these associations was blunted when adjusting for LDL-C levels. These data agree with previous works reporting increased levels of small[42] and oxidized[43] LDL particles in men.

Aging has been traditionally associated with lower cholesterol levels, particularly in LDL, in parallel with a time-dependent increase in CVR[41,44]. Our data suggest that despite this cholesterol decrease, greater age is independently associated with a highly atherogenic LDL profile (with oxidized, small, triglyceride-rich, cytotoxic LDL particles). The possible conversion of LDL into pro-atherogenic particles could explain why CVR keeps increasing throughout life. However, this hypothesis should also be further investigated.

The main strength of the present study is that it has comprehensively assessed the associations of HDL functionality and LDL atherogenicity characteristics with HDL-C and LDL-C levels, and the main factors modulating CVR. Moreover, all the relationships described in our regression models have been adjusted for the effect of the rest of CVRFs and modulators. However, there are also limitations to our study. First, its design was cross-sectional, it did not allow us to infer causality and we could only establish associations between lipoprotein characteristics and CVRFs and modulators that should be addressed

in future prospective studies. Second, our study subjects were older and at high CVR, therefore results cannot be extrapolated to the general population. To partially correct this limitation, we considered these factors as covariates in the linear regression analyses. Third, we could not perform the association analyses between CVR scores and HDL- and LDL-related characteristics in individuals aged ≥75 since the Framingham-REGICOR equation only allows the calculation of CVR scores in subjects 35 to 74 years old. Fourth, due to availability and technical issues, we were unable to analyze the HDL ability to esterify cholesterol and CETP and PON1 activities, HDL size, and HDL vasodilatory capacity in 67, 37, and 60 volunteers. Finally, we could not detect powerful associations between hypertension or smoking and lipoprotein properties since only a small proportion of our volunteers was non-hypertensive (17.6-21.3%) or a smoker (12.5-13.8%).

CONCLUSIONS

High CVR scores were associated with low cholesterol efflux capacity values, high HDL oxidation, triglyceride-rich HDL cores, small HDL size, small estimated LDL size, and low LDL resistance against oxidation. Among high CVR subjects, being hypercholesterolemic and male were preferentially associated with a dysfunctional HDL profile, while being diabetic and older was specially related to pro-atherogenic LDL particles. To date, this is the first study to comprehensively analyze the independent associations between CVR and HDL- and LDL-related variables in humans. Our data reflect the pertinence of assessing HDL function and LDL atherogenicity in clinical studies, since much more information can be provided by lipoproteins beyond HDL-C and LDL-C levels.

CONFLICT OF INTEREST

None.

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AUTHOR CONTRIBUTIONS

A.H. and M.Fitó designed the study. A.H. acquired the data. E.R., X.P., R.Estruch, J.S.-S., D.C., F.A., L.S.-M., M.A.M.-G., 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 M.T.S.-F. wrote the manuscript which was critically reviewed by H.S., E.R., X.P., R.Estruch, J.S.-S., D.C., F.A., L.S.-M., M.A.M.-G., M.Fiol, J.L., R.Elosua, R.M.L.-R., and M.Fitó. All authors approved the final version of the manuscript.

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7. Discussion

Atherosclerosis, or hardening of the arteries, is a common cause of cardiovascular disease. It begins in childhood with the deposition of lipid-laden fatty streaks, mainly in the intima of medium and large sized arteries, especially where the vessels divide (197). Endothelial dysfunction, the triggering event preceding the development of atherosclerosis (198), promotes the acquisition of a proinflammatory phenotype by endothelial cells. As a consequence, the endothelium becomes highly permeable to circulating LDL. Binding LDL to glycosaminoglycans of the extracellular matrix promotes oxidative modifications of the lipoprotein, which will be taken up by macrophages, fuelling an immune response (199).

Oxidation and inflammation, considered an intertwined process, sustained for a long period of time are especially relevant for the progression of disease. Oxidized LDLs increase circulating levels of pro-inflammatory molecules, perpetuating the inflammatory response in the sub-endothelial space. However, aside from oxidation and inflammation, the disease involves the interplay of several interrelated processes: enhanced leukocyte and platelet adhesion to endothelial cells, vascular smooth cell activation and migration into the atherosclerotic lesion and a combination of increasing matrix degradation and decreased component-synthesis of the atherosclerotic cap, and genetic factors (Figure5).
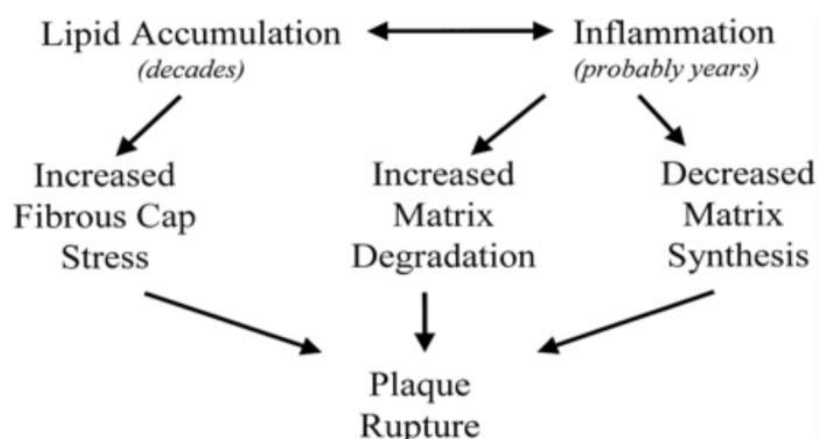
Figure5. Cascade of events leading to plaque rupture (Arroyo LH, Cardiovasc Res. 1999)[200]

The core of the atheroma plaque is made up of a large deposit of lipids, mainly oxidized cholesterol and cellular debris. In this context, cellular interactions in the artery intima layer among resident cells (smooth muscle cells and endothelial cells) and those of the immune system (leukocytes) will determine the evolution of atherosclerotic plaque to life-threatening outcomes, such as acute coronary syndromes (Table 2).

Lesion name	Lesion Description by Histopathology	Thrombosis
Non atherosclerotic intimal lesions		
1. Intimal thickening	The normal accumulation of SMCs in the intima with the absence of lipid or macrophage foam cells	Thrombus is absent
2. Intimal xanthoma or fatty streaks	Subendothelial accumulation of foam cells in intima without necrotic core or fibrous cap; animal and human data show that such lesions usually regress	Thrombus is absent
Progressive atherosclerotic lesions		
1a. Pathologic intimal thickening	SMCs in a proteoglycan-rich matrix with areas of extracellular lipid accumulation without necrosis	Thrombus is absent
1b. With erosion	Luminal thrombosis; plaque same as above	Thrombus most often mural and infrequent

		occlusive
2a. Fibrous cap atheroma	Well-formed necrotic core with overlying fibrous cap	Thrombus is absent
2b. With erosion	Luminal thrombosis; plaque same as above, no communication of thrombus with necrotic core	Thrombus most often mural and infrequent occlusive
3. TCFA	A thin fibrous cap infiltrated with macrophages	Absent, with intraplaque
a. With rupture	Fibroatheroma with cap disruption; luminal thrombus communicates with underlying necrotic core	Thrombus usually occlusive
4. Calcified nodule	Eruptive nodular calcification with underlying fibrocalcific plaque	Thrombus usually nonocclusive
5. Fibrocalcific plaque	Collagen-rich plaque usually with significant stenosis; contains large areas of calcification with few inflammatory cells; necrotic core may be present	Thrombus is absent

SMC denotes smooth muscle cell; TCFA, thin-cap fibroatheroma.

Table2. Major types of lesions in atherosclerosis (Adapted from Insull Jr., Am J Med, 2009)[201]

Considering the high prevalence of CVD, and that it remains the leading cause of death worldwide, a wide range of lines of research have emerged with respect to new markers for adverse cardiac outcomes. In this regard, HDL-C concentration is considered an inverse predictor for CVD. However, a consensus has yet to be reached on whether the concentration of cholesterol in lipoprotein alone can explain the associated risk reduction associated, or whether its atheroprotective role should be attributed in part to the function of HDL in several key steps in atherosclerotic disease.

In the present thesis, we performed a meta-analysis to obtain a preliminary approach of the state-of-the-art on HDL atheroprotective functions and the risk estimation for CVD and all-cause mortality. In brief, we found that cholesterol efflux, the most widely studied HDL-function, and antioxidant and anti-inflammatory protection mediated

by HDL, are inverse predictors of CVD in multivariate regression analysis. Furthermore, higher cholesterol efflux from macrophages to HDL and higher HDL-antioxidant capacity were inversely associated with a lower risk of all-cause mortality. However, several matters of concern arose when we performed our meta-analysis. On the one hand, the number of studies retrieved was small: 17 for cholesterol efflux, 10 for antioxidant capacity, and only 2 for anti-inflammatory property. In this regard, true heterogeneity of studies was statistically disclosed, which must be taken into consideration given that the direction of the association between HDL-function and outcome may be strongly affected by baseline characteristics of the population and design of studies(202). On the other hand, it has been previously shown that techniques to isolate HDL particle have a deep impact on the final quality of the specimen in terms of ApoB content. Considering that HDL and triglyceride-rich lipoprotein contain a number of shared constituents, this circumstance could lead to false estimation of the true effect mediated by HDL (203). In addition, comparative studies to assess the impact of using cell lines with different phenotypes in protocols to estimate HDL-functionality, result in greatly heterogeneous results(204)(205). Therefore, adoption of common methodological approaches and conducting large and specifically designed studies would improve comparability of studies. . Publication bias, an indicator of missing studies with no significant results was also identified. Publication bias could be easily addressed by the publication of results from studies with negative results, which is of paramount importance, for the inclusion of unfavourable finding could modulate or change the association under evaluation. Nevertheless, these results are highly encouraging and justify evaluating the risk protection derived from these HDL anti-atherogenic roles for major adverse cardiac events.

The pivotal work of this thesis was to experimentally appraise the suitability of an array of functions associated with the protective role

of HDL in the development of coronary heart disease as novel markers of ACS, AMI, and UA. 172 ACS cases from the cohort study at high CVD risk at baseline and 344 matched controls (1:2) were included. Stratification of ACS events yielded 98 AMI and UA. Moreover, we included 35 SA cases with 70 matched controls. Cholesterol efflux capacity (CEC) in adjusted conditional logistic regression proved to be a predictor for ACS and AMI independent of intervention group, age, time of permanence in the study, HDL-C, and cardiovascular risk factors (CVRF) (diabetes, hypercholesterolemia, hypertension, and smoking status).

Plaque rupture is the most common mechanism for the onset of ACS. There is a link between the structure of the culprit plaques and types of heart coronary diseases. Whereas stable lesions involve fibrous plaques rich in smooth muscle cell and collagen and small or any extracellular lipid, typically vulnerable plaques leading to an acute event are made up of a large amount of lipids and a thin or virtually absent fibrous layer (Figure 6). An extensive infiltration of macrophages at the immediate site of erosion or rupture has been described for AMI (206). In this regard, ACS and AMI, but not UA, were independently predicted by CEC promoted by HDL. The soft core of the atheroma plaque mostly comprises oxidized-LDL cholesterol, released from cellular death (mainly macrophage) (207) or from direct infiltration favoured by an impaired endothelium (208). Atherosclerotic plaque containing large amounts of lipids pose a great burden for plaque rupture (209). Plaque architecture and composition are markedly heterogeneous, however, the lesion site is characterized by a pronounced macrophage infiltration. Macrophage pose a great risk for plaque rupture through the release of metalloproteinases, which are responsible for the degradation of collagen and extracellular matrix, and therefore, weaken the plaque (210). The HDL ability to reduce the cholesterol content in the macrophage could thus avoid or delay macrophage transformation into pro-atherogenic foam cells. Moreover,

we found a relationship between lower cholesterol efflux capacity mediated by HDL and greater severity of disease, what might explain the lack of prognostic role of CEC for UA. Nevertheless, the sample size of UA included 74 cases and perhaps a larger sample size may be needed to detect the differences observed in AMI pathology.

As stated before, the overall morphology of the ruptured lesions is greatly heterogeneous regarding plaque structure and cell composition (206). In this regard, several mediators released by macrophages and activated T-cells in plaques can promote destabilizing effects (211). The HDL oxidative-inflammatory index is an indirect measure of the capacity of HDL to protect several components of the atherosclerotic plaque against oxidation. Amongst these, protection against oxidative modification is particularly relevant. In this line, we have demonstrated that the HDL oxidative-inflammatory index independently predicts ACS and UA in fully adjusted models irrespective of HDL-C and CVRF. Nevertheless the index's lack of prediction for AMI is surprising. It could be hypothesised that the complexity of coronary plaque in AMI masks the protective role of HDL and thus the predictive capacity might not be strong enough. The inherent complexity of the overall process may explain our results (212). In this regard, the complex cellular relationships, specific plaque structure, and/or an advanced stage, especially in AMI cases may explain the lack of predictive value for AMI of this antioxidant property of HDL. Furthermore, as we initially speculated, larger samples could lead to striking results.
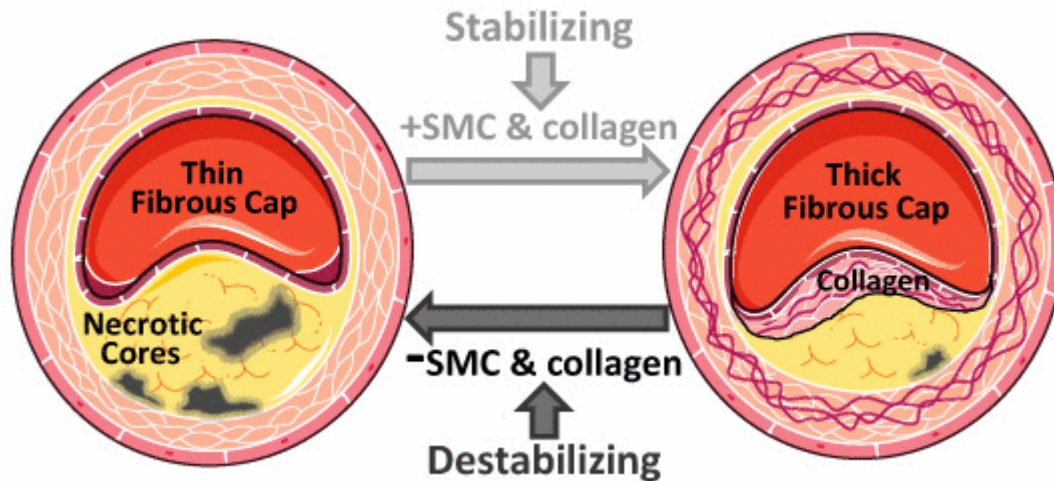
Figure6. Mechanisms contributing to plaque rupture (Ulrich V, Ulrich, Victoria, 2016)[213]

Finally, ACS, AMI, and UA were predicted by ApoA-I concentration in HDL in unadjusted and adjusted models including CVRF. It is relevant to highlight that ApoA-I showed a predictive value for ACS independent of HDL-C concentrations. ApoA-I, the main protein in the HDL particle, exerts multiple protective vascular functions participating in reverse cholesterol transport by stimulating cholesterol efflux from peripheral cells, mainly through ATP-binding cassette transporter A1 (99). Furthermore, ApoA-I contributes to antioxidant protection mediated by HDL (214) and to facilitating the HDL metabolism via activation of lecithin cholesterol acyltransferase (100). Several studies have shown that systemic circulating concentrations of ApoA-I are impaired in cardiovascular disease, and it has been suggested to be more sensitive in predicting cardiac outcomes than HDL-C (215). Nevertheless, in the present work, the Apo-AI content was determined in HDL and is thus not exactly routinely measured systemic ApoA-I since it is also transported in other lipoproteins than HDL. Our work thus provides evidence that the benefits from circulating ApoA-I can be attributed, at least partially, to its content in the HDL fraction.

The risk assessment for future coronary events in the clinical setting is performed with Framingham Risk Score, which has been adapted for

the Spanish population (39). It is not surprising that risk factors integrating the score, mainly, age, gender, blood pressure, smoking, LDL-C, HDL-C, diabetes mellitus, and prevalence of obesity, are highly related to the current knowledge of the pathology of coronary disease (40–46).

Finally, we analyzed the relationships among each HDL and LDL-property and the 10-year coronary risk score according to FRAMINGHAM-REGICOR and each risk factor independently. On the one hand, small, cholesterol-rich LDL are more prone to undergo oxidation (216), a hallmark of atherosclerosis. And on the other hand, it is not surprising that impaired HDL-function and composition are related to high CVR score. HDL enrichment in triglyceride and also oxidized/pro-inflammatory HDL impair cholesterol exchange among lipoproteins (217,218).

Our data shows a greater presence of small HDL particles in the group of high cardiovascular risk by using the electrophoresis methodology. In a prospective case-control study within the EPIC-Norfolk cohort performed in healthy participants, also small HDL particles, measured by gradient gel electrophoresis, are associated with an unfavorable cardiometabolic risk profile(219); Nevertheless, although small HDL particle size are also related to an increased coronary heart disease risk it is largely explained by the traditional risk factors(3). In this regard, Guey et al. reported that Preβ-1 HDL was directly associated with coronary heart disease and AMI even after adjustment for established risk factors, by using ultrafiltration-isotope dilution, in subjects controlled for risk-factor management (220). As opposite, results of a secondary analysis of the AIM-HIGH Study indicate that cholesterol determined in ultracentrifugated small size HDLs (HDL3) are protective against CVD whereas cholesterol linked to HDL2 are not. Thus, the authors suggest that the HDL3 subclass is primarily responsible for the indirect association of HDL-C and CVD in individuals with established CVD(221). Despite small HDL particles depict greater ability to efflux

cholesterol and protect against inflammation than larger ones(8) might be specially affected by the pro-inflammatory state. In addition, the HDL particle becomes smaller when reactant phase proteins bind onto HDL surface. This fact can add confusion when the HDL size is measured in samples of healthy subjects or under a permanent pro-inflammatory state, in the last the pro-inflammatory particles might be confused by pre-beta1 HDL. The presence of traditional risk factors has been shown to impair HDL functionality(222) (223) partly explain by inflammatory remodelation of the protein cargo in HDL (140).

Diabetic state was related to oxidized, small size, triglyceride-rich LDL particles. Patients with diabetes present a high risk for CVD, explained in part, by a large prevalence of dyslipidemia, characterized by high triglyceride and small LDL levels (224), specially oxidized LDLs, a hallmark of atherosclerosis. Lower concentrations of HDL-C and LDL-C were reported in diabetic patients. This can be explained by a greater percentage of these individuals on-statin treatment linked to a lower presence of hypercholesterolemia. As expected, HDL-C and LDL-C concentrations were high in the group of hypercholesterolemic patients. Furthermore, associations of hypercholesterolemic state and greater CETP activity, lower HDL capacity to esterify cholesterol, and smaller HDL size have been previously confirmed (225–227). Interestingly, hypercholesterolemia state was independently associated with triglyceride rich-LDL, a sub-fraction of remnant lipoprotein highly pro-atherogenic (228). Increasing BMI values were independently associated with lower levels of HDL-C (229), HDL-particle size, and ability to esterify cholesterol. Hypertriglyceridemic states in overweight or obese patients might trigger HDL enrichment in triglycerides (230), thus impairing HDL-function. Finally, smaller LDL size has already been correlated with hypertriglyceridemia in both men and women (231).

Regarding the associations of gender and age with HDL function and LDL atherogenicity, our data demonstrate that being male is related to a dysfunctional HDL while aging reflects a more atherogenic LDL. Men are

strongly affected by coronary heart disease (175). Therefore, the relationship we observed of being male with low HDL-C, greater HDL-oxidation, and reduced capacity to protect the endothelium is not surprising. Nevertheless, it requires further investigation. On the other hand, LDL proved to be more atherogenic with age in terms of oxidation, small size, triglyceride richness, and cytotoxic particle. This fact may help to explain the increase in cardiovascular risk during an individual's lifespan.

8. Conclusions

MANUSCRIPT 1: HIGH DENSITY LIPOPROTEIN FUNCTIONALITY AND CARDIOVASCULAR EVENTS AND MORTALITY: A SYSTEMATIC REVIEW AND META-ANALYSIS.

1.   A comprehensive meta-analysis exploring the association among a number of functional actions of HDL and the risk of cardiovascular events and all-cause mortality, suggested that higher levels of cholesterol efflux capacity and HDL anti-oxidant/anti-inflammatory capacities are inverse predictors of cardiovascular disease

2.   Similarly, lower cholesterol efflux and antioxidant capacities were risk factors for all-cause mortality.

3.   There is a lack of large studies regarding the role of the HDL particle on cardiovascular protection, particularly its anti-inflammatory ability.

4.   The use of a common biological sample and standardized methodology would add consistency among studies, and probably gain comparability.

MANUSCRIPT 2: PREDICTIVE VALUE OF HDL FUNCTION RELATED BIOMARKERS FOR ACUTE CORONARY SYNDROME OUTCOME IN PATIENTS AT HIGH CARDIOVASCULAR RISK

1.   In a matched case-control nested within cohort of subjects at high cardiovascular risk, low CEC and Apo-AI have a predictive value for ACS and AMI. In addition, Apo-AI predicts UA events.

2.   Elevated values of HOII are associated with a greater risk for ACS and UA, in a high cardiovascular risk population.

MANUSCRIPT 3: ROLE OF HDL FUNCTION AND LDL ATHEROGENICITY ON CARDIOVASCULAR RISK: A COMPREHENSIVE EXAMINATION

1.   In a cross-sectional analysis at baseline in a cohort of subjects with high risk for cardiovascular diseases, high risk according to the Framingham-Regicor score, is related to an impaired HDL function

characterized by low cholesterol efflux capacity and oxidized, smaller, triglyceride-rich particles.

2. High risk according to the Framingham-Regicor score is related to pro-atherogenic LDL characterized by a smaller particle size more susceptible to oxidation.

3. Type-II diabetes mellitus and obesity particularly contributed to the atherogenic LDL profile whereas hypercholesterolmic state was related to a disturbed HDL one.

4. Finally, older people and males also presented more dysfunctional HDL particles and pro-atherogenic LDL traits.

9.Future perspectives

The present work has proven that certain HDL functions are solid surrogates for future cardiovascular event, in particular coronary heart diseases. Despite case-control studies don't allow inferring causality, the results reported in this thesis regarding the matched case-control are highly valuable given that the design is a case-control nested to a cohort of high cardiovascular risk factors. Nevertheless, the next step must be to replicate results of the predictive HDL function-related biomarkers in general population in the population of REGICOR Study, also in Spain. In this regard, the association, irrespective of the classical cardiovascular risk factors, of HDL dysfunction with coronary heart disease in high risk individuals could indicate that among general population, those with a low-moderate cardiovascular risk score might also display altered biomarkers belonging to the HDL function battery. More research must be developed in this regard.

In addition, our aim is to analyse the predictive value for acute coronary syndrome of 1 year-changes of HDL function-biomarkers to determine the effect of the diet intervention on these analytes and their interaction with the event occurrence.

Furthermore, to explore the causality of the reported association we will perform Mendelian randomization analyses. We will select a number of polymorphisms with already established association with the HDL cholesterol concentration and its functionality and assess their association with coronary artery disease risk.

Our group has recently shown that both adherence to a Mediterranean Diet (supplemented either with virgin olive oil or nuts) and a regular and moderated virgin olive oil intake improve a range of HDL-functionalities in high cardiovascular risk population and general population respectively. This fact has been regarded as a valuable tool in the prevention of atherosclerotic diseases through life-style, specifically by means of a traditional Mediterranean diet pattern and its main fat source which is olive oil. several investigators of our research

unit are now replicating our results in participants from the PreDiMed-Plus study, a life-style intervention trial also performed in a population at high cardiovascular risk (obese or overweight volunteers with metabolic syndrome). The PreDiMed Plus project involves physical activity recommendation and motivational support in addition to a restrictive Mediterranean diet, this fact will allow us to evaluate the benefits on HDL function of a multifactorial profile of healthy lifestyle.

Finally, to discern molecular subjacent mechanisms, we will further investigate the gene expression changes linked to the HDL function improvements after two life-style interventions; a Mediterranean diet and a restrictive one plus physical activity recommendations, in high cardiovascular risk individuals.

10. References

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