Dysfunctional HDLs are Associated with a Greater Incidence of Acute Coronary Syndrome in a Population at High Cardiovascular Risk: A Nested-Case Control Study

Running Title: *Maria Trinidad Soria-Florido, et al HDL Function and Coronary Syndrome*

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Abstract

Background: Studies have failed to establish a clear link between high-density lipoprotein (HDL) cholesterol and cardiovascular disease, leading to the hypothesis that HDL atheroprotective role lies in its biological activity rather than in its cholesterol content. However, to date, the association between HDL functional characteristics and acute coronary syndrome has not been comprehensively investigated.

Methods: We conducted a case-control study nested within the PREDIMED (*Prevención con Dieta Mediterránea*) cohort, originally a randomized trial where participants followed a Mediterranean or low-fat diet. Incident acute coronary syndrome cases (*N*=167) were individually matched (1:2) to controls by sex, age, intervention group, body mass index, and follow-up time. We investigated its two individual manifestations (myocardial infarction, unstable angina) as secondary outcomes. We measured the following functional characteristics: HDL cholesterol concentration (in plasma); cholesterol efflux capacity; antioxidant ability measured by the HDL oxidative-inflammatory index; phospholipase A2 activity; and sphingosine-1-phosphate, apolipoproteins A-I and A-IV, serum amyloid A, and complement 3 protein (in apolipoprotein B-depleted plasma). We used conditional logistic regression models adjusted for HDL cholesterol levels and cardiovascular risk factors to estimate odds ratios (ORs) between one standard deviation increments in HDL functional characteristics and clinical outcomes.

Results: Low values of cholesterol efflux capacity (OR$_{1SD}$: 0.58, 95% CI: 0.40-0.83), and levels of sphingosine-1-phosphate (OR$_{1SD}$: 0.70, 95% CI: 0.52-0.92), and apolipoprotein A-I (OR$_{1SD}$: 0.58, 95% CI: 0.42-0.79) were associated with higher odds of acute coronary syndrome. Higher HDL oxidative inflammatory index values were marginally linked to acute coronary syndrome risk (OR$_{1SD}$: 1.27, 95% CI: 0.99-1.63). Low values of cholesterol efflux capacity (OR$_{1SD}$: 0.33, 95% CI: 0.18-0.61), sphingosine-1-phosphate (OR$_{1SD}$: 0.60, 95% CI: 0.40-0.89) and apolipoprotein A-I (OR$_{1SD}$: 0.59, 95% CI: 0.37-0.93) were particularly linked to myocardial infarction, whereas high HDL oxidative-inflammatory index values (OR$_{1SD}$: 1.53, 95% CI: 1.01-2.33) and low apolipoprotein A-I levels (OR$_{1SD}$: 0.52, 95% CI: 0.31-0.88) were associated with unstable angina.

Conclusions: Low cholesterol efflux capacity values, pro-oxidant/pro-inflammatory HDL particles, and low HDL levels of sphingosine-1-phosphate and apolipoprotein A-I were associated with increased odds of acute coronary syndrome and its manifestations in high cardiovascular risk subjects.

Clinical Trial Registration: URL: http://www.controlled-trials.com Unique identifier: ISRCTN35739639

Key Words: high-density lipoprotein; acute coronary syndrome; cholesterol efflux capacity; sphingosine-1-phosphate; HDL inflammatory index

Non-Standard Abbreviations and Acronyms
ACS: acute coronary syndrome
ApoA-I: apolipoprotein A-I
ApoA-IV: apolipoprotein A-IV
CEC: cholesterol efflux capacity
C3: complement 3 protein

HDL: high-density lipoprotein
HDL-C: high-density lipoprotein cholesterol
HDL-LpPLA2: HDL-bound phospholipase A2
HOII: HDL oxidative-inflammatory index
MI: myocardial infarction
PREDIMED: *PREvención con DIeta MEDiterránea*
SAA: serum amyloid A
S1P: sphingosine-1-phosphate
TMD: traditional Mediterranean Diet
UA: unstable angina

Clinical Perspective

What is new?

- Low values of cholesterol efflux capacity and levels of sphingosine-1-phosphate and apolipoprotein A-I in HDL were associated with a higher risk of acute coronary syndrome in high cardiovascular risk individuals, irrespective of HDL cholesterol levels and other classical cardiovascular risk factors.

- Low cholesterol efflux capacity values and sphingosine-1-phosphate levels were particularly associated with an increased risk of myocardial infarction, whereas HDL antioxidant/anti-inflammatory capacity was inversely related to unstable angina.

- This is the first longitudinal study to comprehensively examine the association of several HDL function-related biomarkers with incident acute coronary syndrome beyond HDL-C levels in a high cardiovascular risk population.

What are the clinical implications?

- Several HDL functionality measurements (related to HDL roles on cholesterol metabolism, endothelial protection, and antioxidant/anti-inflammatory defense) were associated with the incidence of acute coronary syndromes whilst HDL cholesterol levels were not

- The present work could contribute to the discovery of novel prognostic biomarkers or potential therapeutic targets of cardiovascular disease related to HDL function

Introduction

There is compelling evidence from multiple epidemiological studies that high-density lipoprotein (HDL) cholesterol (HDL-C) concentration is independently and inversely associated with atherosclerotic cardiovascular disease[1]. However, inconsistent results from meta-analyses of pharmacological interventions[2,3] and Mendelian randomization studies[4,5] have challenged the notion that increasing HDL-C levels reduces the risk of incident cardiovascular disease. This has led to the hypothesis that improving HDL function can be more relevant for cardiovascular prevention than raising HDL-C concentrations.

The most studied HDL function is cholesterol efflux capacity (CEC), the ability of HDL to remove excess cholesterol from peripheral cells, mainly macrophages[6]. Low CEC values characterize dysfunctional HDLs[7] and have been associated with a greater risk of atherosclerotic cardiovascular disease in the majority of studies[8–13], although various authors failed to find an association with incident cardiovascular disease[14–16] or found a positive association[17]. The existing studies vary widely in their experimental approaches regarding the isolation of HDL particles or apolipoprotein-depleted plasma or serum, choice of lipid acceptors, and use of cholesterol probes (radiolabeled or fluorescent cholesterol). Therefore, data are not directly comparable.

Regarding other HDL functional capacities, HDL antioxidant function appears to be highly dependent on the activity of some enzymes. In this regard, although the relationship between the activity of paraoxonase-1 and cardiovascular outcomes has been shown to be inconsistent in a recent meta-analysis[18], the association of the activity of other enzymes such as HDL-bound phospholipase A2 (HDL-LpPLA2) with cardiovascular risk remains controversial. HDL-LpPLA2 activity has been associated with lower cardiovascular risk in pre-clinical[19] and

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clinical studies[20], although the pharmacological inhibition of the overall LpPLA2 activity has failed to reduce incidence of major cardiovascular disease in two clinical trials[21,22]. It is hypothesized that the LDL-bound enzyme fraction may be responsible for this deleterious effect. In addition, the relative content in other proteins and some particular lipid species can modify HDL functional characteristics. First, HDL-bound sphingosine-1-phosphate (S1P), a modified phospholipid, is thought to be one of the key mediators of HDL protection on endothelial cells in pre-clinical models[23] and has been associated with the extent of atherosclerotic lesions in stable coronary artery disease patients[24]. Second, relative levels of apolipoproteins A-I (ApoA-I) and A-IV (ApoA-IV) in HDL particles have been shown to be involved in HDL functional properties in structural analyses[25] and impaired in high cardiovascular risk subjects[26]. Finally, HDL enrichment in certain pro-inflammatory proteins, such as serum amyloid A (SAA) and the complement 3 protein (C3)[15,17], may impair the anti-oxidant/anti-inflammatory abilities of the lipoprotein[25]. However, the association of some of these HDL biological activities with the onset of acute coronary syndrome (ACS) has not been comprehensively described in any prospective human study to date.

The aim of the present study was to assess whether a range of HDL-related properties (HDL-C levels, CEC, HDL oxidative-inflammatory index (HOII), HDL-LpPLA2 activity, and levels of S1P, ApoA-I, ApoA-IV, SAA, and C3 related to HDL particles) are associated with the development of future ACS and its two main manifestations, myocardial infarction (MI) and unstable angina (UA), in a high cardiovascular risk population.

Methods

Because of the sensitive nature of the data collected in this study, requests to access the dataset

from qualified researchers trained in human subject confidentiality protocols should be addressed

to the steering committee of the *PREvención con DIeta MEDiterránea* (PREDIMED) Study at

predimed-steering-committe@googlegroups.com.

An extended description of methods is available in the online-only Supplemental Material.

Participants

This study was performed in a subset of participants from the PREDIMED study. It was a large-scale, randomized, controlled, parallel, multicenter, intervention trial aiming to assess the long-term effects of following a traditional Mediterranean Diet (TMD) on the primary prevention of cardiovascular outcomes in a population at high cardiovascular risk. Eligible participants were community-dwelling men (55-80 years) and women (60-80 years) who fulfilled at least one out of the following two criteria: 1) type 2 diabetes mellitus or 2) three or more cardiovascular risk factors: current smoking, hypertension (blood pressure ≥140/90 mmHg or use of antihypertensive drugs), low-density lipoprotein cholesterol levels ≥160 mg/dL (or use of lipid-lowering drugs), HDL-C levels ≤40 mg/dL, body mass index ≥25 kg/m^2, or a family history of premature coronary heart disease[27]. Complete protocol details have been published elsewhere[28]. The trial was approved by the institutional review boards and registered with the number ISRCTN35739639 in www.controlled-trials.com. All participants provided written informed consent before joining the trial (http://www.predimed.es).

Covariates and biological samples

Baseline examination included questions about education, lifestyle, history of illnesses, medication use, and nurse's visit. Detailed information is available in **Supplemental Methods**.

Outcome ascertainment and sample size

The main outcome was ACS, defined as fatal or non-fatal MI[29] and/or fatal or non-fatal UA[30].

We used four sources of information to identify endpoints: repeated contacts with participants; family physicians; yearly review of medical records; and consultation of the National Death Index. An adjudication committee whose members were blinded to treatment allocation reviewed all endpoints[31].

We identified a total of 222 incident ACS cases but only 167 (93 MIs and 74 UAs, 75% of the total sample) had available plasma samples at baseline. The study flow chart is available in **Supplemental Figure 1**, and a description of the differences between included and non-included cases in **Supplemental Table 1**. Additionally, each study center quantified HDL-C levels in their subjects by enzymatic methods and collected information on age, sex, body mass index, and the presence of cardiovascular risk factors (type-II diabetes mellitus, hypertension, tobacco use)[28]. For the present analysis, we implemented a nested case-control (1:2) design, wherein incident ACS cases were matched to two controls by age (± 5 years), sex, body mass index (± 3 kg/m^2), intervention group, and time to event. The 1:2 ratio of cases to controls was based on the following sample size calculation: to detect an odds ratio of 0.75 or less for the increase in 1 standard deviation (SD) –as observed with cholesterol efflux capacity in previous studies[8,9]– with a power of 80%, a total sample size of at least 453 subjects was needed.

HDL functions

We first obtained apolipoprotein B-depleted plasma samples (an easy, reproducible procedure to obtain a laboratory specimen in which the only lipoprotein present is HDL[32]) from initial plasma aliquots, and stored them at -80ºC until use. In these samples, we measured: 1) CEC in a model of human THP-1 monocyte-derived macrophages[32] treated with fluorescent 23-(dipyrrometheneboron difluoride)-24-norcholesterol (Avanti Polar Lipids); 2) HOII (inversely proportional to HDL antioxidant ability) as the capacity of the lipoprotein to avoid the oxidation

of 2'-7'dichlorohydrofluorescein in the presence of oxidized low-density lipoproteins as pro-

oxidative stimuli[32]; 3) LpPLA2 activity with commercial *PAF Aceltylhydrolase Assay Kits*

(Cayman Chemical); and 4) levels of ApoA-I and C3 in HDL particles by immunoturbidimetry

in an ABX Pentra-400 autoanalyzer (Horiba-ABX) and those of S1P, SAA, and ApoA-IV by

ELISA kits (*Sphingosine 1 Phosphate BioAssay^{TM} ELISA Kit* –United States Biological–, *SAA

Human ELISA Kit* –Invitrogen– and *Human Apolipoprotein A-IV SimpleStep ELISA Kit –*

Abcam–, respectively). Extensive description of the assays can be found in **Supplemental

Methods**.

To decrease the variability of the laboratory measurements: 1) we analyzed matched

samples in the same experimental run in all determinations; 2) in each experimental run of non-

automatized techniques (all but the quantification of ApoA-I and C3), we included two

apolipoprotein B-depleted plasma pools (isolated from the plasma of 20 healthy volunteers): one

to assess inter-assay variability, and the other to minimize intra- and inter-assay variability (we

divided the values of HDL properties in the samples by those obtained in the apolipoprotein B-

depleted plasma pool, obtaining normalized ratios without units)[32]; and 3) we run cellular

techniques in duplicate, not allowing intra-repetition coefficients of variation >20%. Inter-assay

coefficients of variation are available in **Supplemental Table 2**. In addition, to minimize the

inter-plate batch effect, the order in which biological samples were analyzed was randomly

assigned before the determinations. In particular, a sample of cases was analyzed first, followed

by two controls; this process was randomly repeated 167 times to obtain the analytical sequence

to be used in the experiments.

Statistical analyses

We assessed univariate associations between baseline characteristics of the volunteers and ACS

by conditional logistic regression models to take into account the matched design. We compared the characteristics of included (*N*=167) and non-included ACS cases (*N*=55) by chi square tests for categorical variables, t-tests for normally distributed continuous variables, and Mann Whitney U-tests for non-normally distributed variables.

Multivariable associations between each biomarker and ACS and its clinical secondary outcomes were also modelled with conditional logistic regression. The linear nature of the relationship between each biomarker and each outcome was evaluated by modelling the biomarkers in quartiles. The shape of the association was visually tested by using floating variance[33]. Furthermore, linear and quadratic contrasts were used to assess the *P* value for trend across quartiles. Biomarkers were also modelled as standardized continuous variables to obtain odds ratios (ORs) and 95% confidence intervals (95% CI) associated with a 1SD increase in the biomarker. When a non-linear trend was detected, restricted cubic splines were fitted to better visualize the dose-response relationship. For each biomarker and for each outcome a set of two models was used: unadjusted (model 1), and further adjusted for age, body mass index, fasting glucose levels, use of glucose-lowering drugs, total and HDL cholesterol concentrations, triglyceride levels, use of lipid-lowering drugs, systolic blood pressure, use of antihypertensive drugs, smoking status, leisure-time physical activity levels, and ethanol consumption (model 2). Age and body mass index were included as covariates to correct the uncaptured variability regarding these variables in the matching process. We assessed the correlations between biomarkers by Spearman's correlation coefficients.

Finally, we conducted an exploratory secondary analysis stratified according to: 1) sex (male/female); 2) age (below vs. over the median –67 years–); 3) fasting glucose levels (<126 mg/dL vs. ≥126 mg/dL); 4) use of glucose-lowering drugs (yes/no); 5) total cholesterol levels

(<200 mg/dL vs. ≥200 mg/dL); 6) triglyceride levels (<150 mg/dL vs. ≥150 mg/dL); 7) use of lipid-lowering drugs (yes/no); 8) obesity (<30 kg/m^2 vs. ≥30 kg/m^2); 9) cumulative adherence to a traditional Mediterranean diet along the PREDIMED study follow ups (below vs. over the median); and 10) leisure-time physical activity at baseline (below vs. over the median). Presence of an interaction was tested with a likelihood ratio test between the conditional logistic models with and without the interaction term. We only interpreted interactions in which: 1) the *P* value for the linear interaction was <0.10[34], and 2) the association with ACS odds was significant in one of the strata and not in the other.

All tests were two-sided with an alpha level of 0.05. Statistical analyses were performed using R, version-3.4.1 (R Core Team, 2018) and Stata 14 (StataCorp. 2017. *Stata Statistical Software: Release 15*. College Station, TX: StataCorp LLC).

Results

Baseline characteristics

Compared to controls, participants with ACS were more likely to use glucose-lowering drug users (*p*<0.001) and be current smokers (*p*=0.013) and had greater systolic blood pressure levels (*p*<0.001). A similar trend was observed comparing MI cases and controls, but there were less users of lipid lowering (*p*=0.006) and antihypertensive drugs (*p*=0.019) in MI cases than controls. Participants with UA displayed greater adherence to the Mediterranean diet (*p*=0.031) compared to controls (**Table 1**). The 167 included ACS cases with plasma samples at baseline did not differ from the 55 non-included ones with the exception of the proportion of subjects under glucose-lowering therapies (there were 17% more glucose-lowering therapy users in the group of selected ACS cases) (χ^2 –df=220–, *p*=0.041) (**Supplemental Table 1**).

HDL function and acute coronary syndrome

All HDL function variables correlated weakly with each other, except for levels of ApoA-I in HDL and plasma HDL-C concentrations (ρ=0.68) (**Supplemental Figure 2**), therefore they were all investigated separately.

The relationships between HDL-related biomarkers and the odds of ACS, MI, and UA were nearly linear for all parameters except for ApoA-IV (**Figure 1**). Despite HDL-C levels not being significantly linearly associated with ACS (OR_{1SD}=1.03, 95% CI: 0.81-1.31), the odds of ACS were lower at higher levels of CEC (OR_{1SD}=0.58, 95% CI: 0.40-0.83), S1P (OR_{1SD}=0.70, 95% CI: 0.52-0.92), and ApoA-I levels in apolipoprotein B-depleted plasma (OR_{1SD}=0.58, 95% CI: 0.42-0.79) and marginally higher at higher HOII values (OR_{1SD}=1.27, 95% CI: 0.99-1.63) in the fully adjusted model (**Figure 2**). The relationship between HDL-C and ACS appeared non-linear but no clear trend was shown in the restricted cubic splines analysis (only the model with 6 knots had a significantly better fit than the linear model, **Supplemental Figure 3**). Similarly, the association between ApoA-IV and ACS appeared non-linear as evidenced by the spline (with 4 knots) analysis, although the trend was broadly negative (**Supplemental Figure 4**).

Regarding the secondary outcomes, ApoA-I levels in apolipoprotein B-depleted plasma was inversely related to both MI (OR_{1SD}=0.59, 95% CI: 0.37-0.93) and UA (OR_{1SD}=0.52, 95% CI: 0.31-0.88). Moreover, high MI odds were strongly associated with low CEC values (OR_{1SD}=0.33, 95% CI: 0.18-0.61) and low S1P concentrations in apolipoprotein B-depleted plasma (OR_{1SD}=0.60, 95% CI: 0.40-0.89) but not with UA, whereas higher values of HOII were associated with greater odds of UA (OR_{1SD}=1.53, 95% CI: 1.01-2.33) but not with MI. A marginally significant, direct association of SAA levels in apolipoprotein B-depleted plasma was

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observed with UA odds (OR_{1SD}=1.31, 95% CI: 0.95-1.80) but not with MI. Exact ORs and 95%

CIs for all outcomes, HDL functional properties, and statistical models are available in

Supplemental Table 3.

Exploratory secondary analysis

High HOII values were associated with significant increments in ACS odds in men but not in

women ($P_{interaction}$=0.079), and in individuals aged <67 but not in older ones ($P_{interaction}$=0.046).

High CEC values and ApoA-I levels in apolipoprotein B-depleted plasma were linked to lower

ACS odds in subjects with triglycerides <150 mg/dL but not in those with higher levels

($P_{interaction}$=0.072 and $P_{interaction}$=0.068, respectively). High C3 concentrations in apolipoprotein B-

depleted plasma were associated with greater ACS odds essentially in non-obese subjects

($P_{interaction}$=0.028). Finally, high S1P levels were related to lower ACS odds only in the

individuals with a cumulative adherence to a Mediterranean diet over the median

($P_{interaction}$=0.031). Exact ORs and 95% CIs for this analysis are available in **Supplemental Table

4**.

Discussion

Our study comprehensively investigated a wide range of markers of HDL functionality in

relation with incident ACS in a population at high cardiovascular risk. We found that impaired

HDL function, reflected by low cholesterol efflux capacity and low levels of S1P and ApoA-I in

apolipoprotein B-depleted plasma was associated with greater risk of ACS, irrespective of HDL-

C concentrations and traditional risk factors.

CEC has been consistently linked to overall atheroprotection in human trials[35]. Our data

confirm that higher CEC values are associated with a lower ACS risk (each 1SD increase in CEC

related to a 42% odds decrease of ACS incidence), and more specifically with a lower risk of MI, which concurs with prior evidence[36]. Our results highlight an interaction between CEC and triglyceride levels (as well as with ApoA-I concentrations in apolipoprotein B-depleted plasma), with greater CEC values only associated with lower ACS risk in normotriglyceridemic subjects. Whilst the relationship between high triglyceride and low HDL-C levels has been extensively described[37], our data suggest that hypertriglyceridemia could attenuate the relationship between HDL functional trait and risk of ACS. Finally, our results are also of particular interest from a technical perspective. We used: 1) a human THP-1 macrophage model, which is more akin to human biology than non-human cell lines, such as the classic murine J774 ones (J774 cells require a chemical upregulation of cholesterol transporters before the experiments in order to reach the physiological expression levels present in human macrophages[38]); and 2) non-radiolabeled fluorescent cholesterol, which makes the technique more amenable to use in standard cell culture facilities and large sample studies, favoring its precision and comparability[39]. Other cells such as human fibroblasts could also be used as cholesterol acceptors. However, until now macrophages have been generally considered the "gold standard" cell line for this technique and have been broadly used for CEC determination in almost all previous human studies[35]. In this regard, macrophages are directly affected by cholesterol excess and are a key cell in the development of atherosclerosis[40].

A complementary HDL functional capacity is its ability to counteract lipid oxidation, particularly of those in low-density lipoproteins. This property can be regarded pivotal in cardioprotection, since low-density lipoprotein oxidation is considered the primary trigger for the development of atherosclerotic plaques and a key promoter of pro-inflammatory responses in the sub-endothelial space[41]. In line with previous findings[36], we observed that a 1SD increment in

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HOII values (namely, the pro-oxidative/pro-inflammatory status of HDL particles) were marginally linked to high ACS odds (particularly due to a 53% increase in the UA odds). In this regard, the function of HDL enzymes seems essential. Besides the controversial role of paraoxonase-1 activity on cardiovascular prevention (refuted in a recent meta-analysis involving 15,064 participants and 2,958 incident cardiovascular outcomes)[18], other proteins such as LpPLA2 may be involved in this phenomenon. The activity of this enzyme in apolipoprotein B-depleted plasma has been inversely, but non-significantly, related to ACS incidence in our study and associated with lower risk of cardiovascular mortality and ACS in a population with established heart disease in other publications[20]. Further studies with larger sample sizes are required to test this hypothetical explanation for an improvement in HOII values, as well as others that have been described in the literature (e.g., the relative HDL richness in chemical antioxidants or pro-inflammatory proteins[23]).

Endothelial protection is the third vertex of the potential triangle of HDL atheroprotective function[42]. Increasing evidence supports the beneficial effects linked to the presence of bioactive lipids within the HDL particle. In particular, HDL-bound S1P has been related to an increment in nitric oxide production in endothelial cells via nitric oxide synthase activation[43]. Moreover, defective vasodilatory activity of HDL from patients with coronary heart disease is restored by uploading HDL with S1P, suggesting that infusions of this sphingolipid may contribute to re-establish the functionality of the lipoprotein[44]. In our data, a 1SD increment in S1P levels in apolipoprotein B-depleted plasma was associated with a 30% decrease in ACS risk (particularly due to an association with 40% less odds of MI). HDL-bound S1P had already been reported to predict the extent of atherosclerotic lesions in a group of stable coronary artery disease patients[24] but our study shows for the first time that S1P levels in apolipoprotein B-depleted plasma are

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inversely related to the development of ACS and MI in high cardiovascular risk individuals. The association between S1P and lower ACS odds was more powerful in those individuals with a strong adherence to a Mediterranean diet throughout the study follow-up. This result, if replicated in future studies, suggests a potentially additive effect of a healthy diet and more functional HDL for cardiovascular prevention.

HDL richness in ApoA-I could be another possible mediator of cardiovascular protection. Our data indicate that an increase by 1SD in the levels of ApoA-I in apolipoprotein B-depleted plasma were associated with almost halving the risk of suffering ACS. ApoA-I constitutes about 70% of the apolipoprotein content of HDL particles[25] and actively participates in the anti-atherosclerotic action of HDL. The acquisition of cellular cholesterol starts with ATP-binding cassette transporter A1-mediated cholesterol efflux to ApoA-I[45] and, once HDLs become mature, ApoA-I is also able to mediate efflux via other transporters[46]. Moreover, ApoA-I prevents LDL oxidation by contributing to inactivation and subsequent transfer of lipoperoxides[47]. Therefore, a relative increase in the ApoA-I levels related to HDL particles may explain the potential improvements of the overall lipoprotein functionality irrespective of the amount of cholesterol transported by the HDL.

When stratifying the analyses by subtype of ACS, we observed a particularly potent association between CEC and S1P levels and MI risk and a more potent association between pro-oxidative/pro-inflammatory HDL particles and UA. This differential behavior could be explained by the fact that different degrees of cap thickness and atheroma size can result in different atherosclerotic manifestations[48]. Whereas stable lesions involve fibrous plaques with small or nil extracellular lipid content, vulnerable plaques leading to acute events contain a large amount of lipids, a thin or virtually absent fibrous cap, and abundant infiltration of macrophages at the site

of erosion[49]. In this regard, MI, but not UA, may be more easily predicted by CEC. However, further research is needed to clarify the greater association of endothelium-related HDL functional properties and MI and this two-way mechanism taking into consideration other factors involved in plaque progression, such as infiltration of inflammatory cells, fibrosis, local flow disturbances, and vasospasm.

To date, although HDL function is a line of research with relevant potential for the clinical management of CVD risk, HDL-C concentrations are still a valuable and straightforward indicator of cardiovascular risk, with diagnostic utility for metabolic syndrome and atherogenic dyslipidemia[37]. Further studies are required to corroborate whether the lack of association between HDL-C levels and HDL functionality observed in the high cardiovascular risk population studied in our work, could also be present in general populations from both Mediterranean and non-Mediterranean areas. Nevertheless, knowledge concerning HDL functional properties could help further stratify individuals at high CVD risk and guide clinical management. Our proposal is to incorporate the measurement of those biomarkers for which routine standardized and affordable assays can be performed (e.g., those related to HDL composition such as the levels of ApoA-I or S1P in apolipoprotein B-depleted plasma samples) in patients presenting a high risk of cardiovascular disease. .

Our study has several strengths. To the best of our knowledge, this is the first longitudinal study to comprehensively examine the association of HDL function-related biomarkers with incident coronary heart disease beyond HDL-C levels in a high cardiovascular risk population. Second, the nested case-control design presents considerable logistic and economic advantages as it uses existing cohort data and has access to prospectively collected information (as opposed to standard case-control studies that collect data retrospectively). Finally, data quality is high

since it presents information available from a considerable number of ACS cases (*N*=167, all verified by a clinical adjudication committee) within the context of a well-characterized population sample with data on several health outcomes coming from in-person visits. However, our study also has limitations. First, our sample consists of individuals at high cardiovascular risk and our conclusions cannot be generalized to a healthier general population. Second, one of the cases was matched with a single control (we were unable to find two individuals following the matching requirements). Third, samples were stored for a median of 8.8 years, which could have influenced the functional determinations of our study. However, all samples were stored at -80ºC in a biobank with 24h-surveillance, presented no freeze/thaw cycles before analyses, and followed the same pre-analytical procedure prior to the laboratory assays, therefore limiting the risk of this affecting the quality of our results. Fourth, the analyses of MI and UA, conducted with 93 and 74 cases, respectively, were underpowered to detect associations of small-to-medium effect size. Finally, some missing values were present in our results due to the elaborate nature of our laboratory procedures: ≤5% of total samples for HDL-C, CEC, ApoA-I, SAA, and C3 determinations; 10% of HOII, S1P, and ApoA-IV values; and 21.6% of LpPLA2 activity (**Supplemental Table 3**). However, these missing values occurred at random and affected cases and controls in the same manner because the order in which samples were assessed was randomly assigned prior to the laboratory determinations.

Conclusions

In summary, low CEC values, and S1P and ApoA-I levels in apolipoprotein B-depleted plasma samples were associated with a higher risk of ACS in a high cardiovascular risk population, irrespective of HDL cholesterol levels and the presence of other classic cardiovascular risk factors. CEC and S1P were particularly associated with MI, whereas HDL antioxidant/anti-

inflammatory capacity was mostly associated with UA, being ApoA-I levels in apolipoprotein B-depleted plasma associated with both MI and UA. These results are in line with recent findings and support the notion that the pleiotropic function of HDL may explain its atheroprotective role in CVD beyond HDL-C concentrations.

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Table 1. Baseline characteristics of acute coronary syndrome, myocardial infarction, and unstable angina cases and controls.

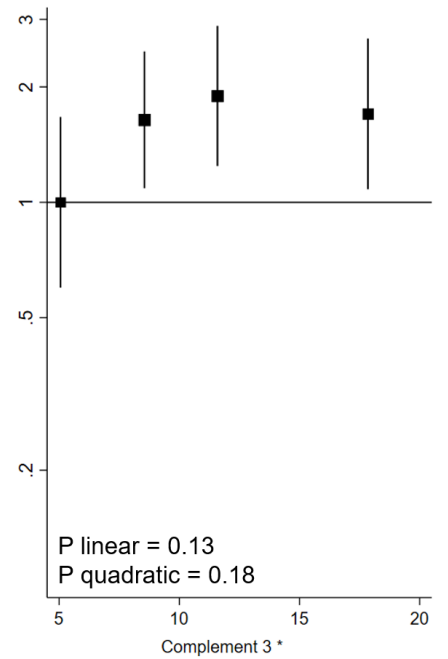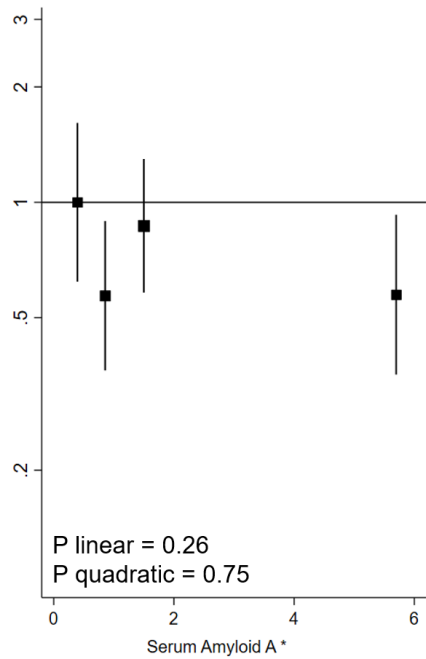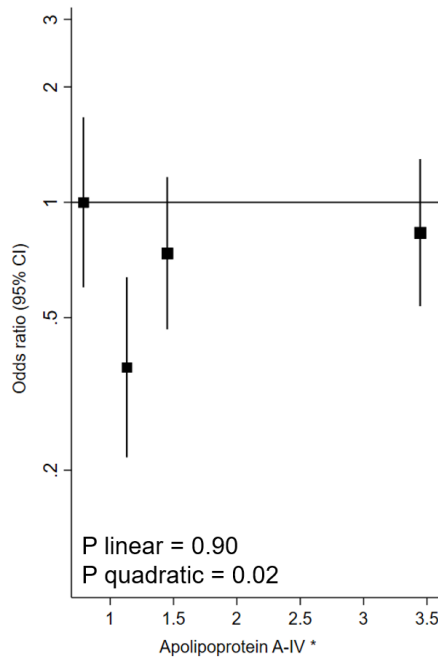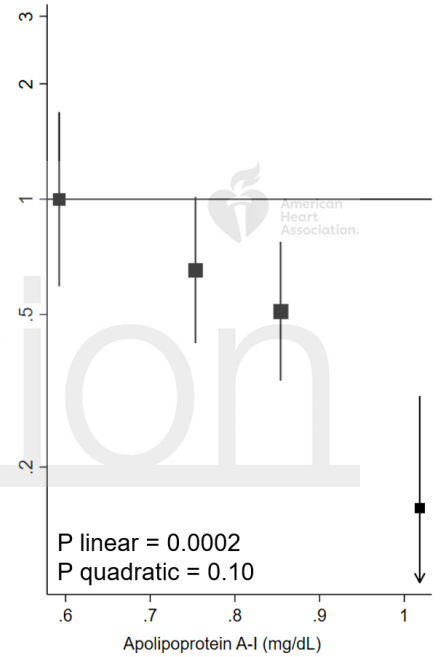|	Acute coronary syndrome		Only myocardial infarction		Only unstable angina	
	Cases (N=167)	Controls (N=333)	Cases (N=93)	Controls (N=185)	Cases (N=74)	Controls (N=148)
Age (years)	67.5 (6.49)*	67.3 (6.23)	67.5 (6.62)	67.5 (6.25)	67.4 (6.36)	67.2 (6.22)
Female sex (n, %)	55 (32.9%)	110 (33.0%)	28 (30.1%)	56 (30.3%)	27 (36.5%)	54 (36.5%)
Fasting glucose (mg/dL)	129 (43.7)	124 (36.8)	131 (48.4)	121 (31.7)‡	126 (37.0)	127 (42.1)
Glucose-lowering drug users (n, %)	80 (47.9%)	107 (32.1%)‡	47 (50.5%)	56 (30.3%)‡	33 (44.6%)	51 (34.5%)
Total cholesterol (mg/dL)	205 (34.8)	204 (35.9)	205 (34.4)	202 (35.8)	204 (35.4)	207 (36.0)
HDL cholesterol (mg/dL)	49.0 (11.1)	49.0 (10.0)	49.5 (12.9)	48.7 (10.3)	48.4 (8.32)	49.3 (9.70)
LDL cholesterol (mg/dL)	128 (31.8)	130 (32.4)	129 (32.0)	128 (32.8)	128 (31.7)	132 (31.7)
Triglycerides (mg/dL)	120 [95.7; 172]†	113 [86.6; 151]	122 [96.6; 173]	118 [92.6; 152]	120 [93.8; 170]	109 [84.5; 150]
Lipid-lowering drug users (n, %)	63 (37.7%)	143 (42.9%)	25 (26.9%)	78 (42.2%)‡	38 (51.4%)	65 (43.9%)
Systolic blood pressure (mmHg)	160 (21.3)	153 (18.5)‡	162 (22.3)	153 (19.3)‡	157 (19.9)	152 (17.5)
Antihypertensive drug users (n, %)	114 (68.3%)	236 (70.9%)	58 (62.4%)	140 (75.7%)‡	56 (75.7%)	96 (64.9%)
Body mass index (kg/m²)	29.3 (3.18)	29.4 (3.15)	29.7 (3.16)	29.7 (3.21)	28.9 (3.17)	29.1 (3.05)
Waist circumference (cm)	102 (8.41)	101 (7.96)	103 (8.22)	103 (7.50)	100 (8.49)	100 (8.33)
Current smokers (n, %)	37 (22.2%)	45 (13.5%)‡	21 (22.6%)	30 (16.2%)†	16 (21.6%)	15 (10.1%)‡
Mediterranean diet adherence (score)	8.60 (1.88)	8.46 (1.89)	8.31 (2.06)	8.49 (1.91)	8.97 (1.56)	8.41 (1.87)‡
PREDIMED Intervention group:						
Mediterranean Diet enriched with virgin olive oil (n, %)	51 (30.5%)	102 (30.6%)	27 (29.0%)	54 (29.2%)	24 (32.4%)	48 (32.4%)
Mediterranean Diet enriched with nuts (n, %)	62 (37.1%)	124 (37.2%)	32 (34.4%)	64 (34.6%)	30 (40.5%)	60 (40.5%)
Low-fat control diet (n, %)	54 (32.3%)	107 (32.1%)	34 (36.6%)	67 (36.2%)	20 (27.0%)	40 (27.0%)
Leisure-time physical activity (METs·min/day)	205 [85.2; 362]	212 [89.1; 432]	186 [80.0; 382]	247 [110; 444]	213 [106; 334]	181 [68.4; 430]
Alcohol intake (g/day)	3.18 [0.00; 11.9]	5.14 [0.00; 14.8]	3.36 [0.00; 12.3]	6.52 [0.00; 15.6]	2.27 [0.00; 11.8]	4.37 [0.00; 12.3]

*: Mean (standard deviation)
†: Median [1st quartile; 3rd quartile].
‡: p-value <0.05 (comparison of case vs. control values, according to univariate conditional logistic regression models).

Figure Legends

Figure 1. Multivariable odds ratios for acute coronary syndrome across quartiles of HDL function markers. HDL indicates high-density lipoprotein; HDL-LpPLA2, phospholipase A2 activity. All determinations but HDL cholesterol levels were measured in apolipoprotein B-depleted plasma samples. ORs and 95% confidence intervals were estimated from conditional logistic regression with floating variances (allowing a confidence interval to be attributed to the reference category) adjusted for age, HDL cholesterol levels (except when they were the exposure of interest), fasting glucose levels, use of glucose-lowering drugs, total cholesterol concentrations, triglyceride levels, use of lipid-lowering drugs, systolic blood pressure, use of antihypertensive drugs, smoking status, body mass index, leisure-time physical activity levels, and ethanol consumption.

*: normalized units.

Figure 2. Forest plots of odds ratios (95% CI) for one standard deviation increases in HDL function markers for acute coronary syndrome, myocardial infarction, and unstable angina. HDL indicates 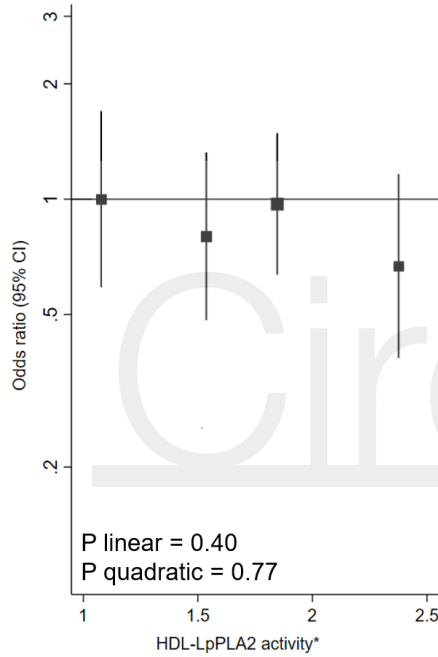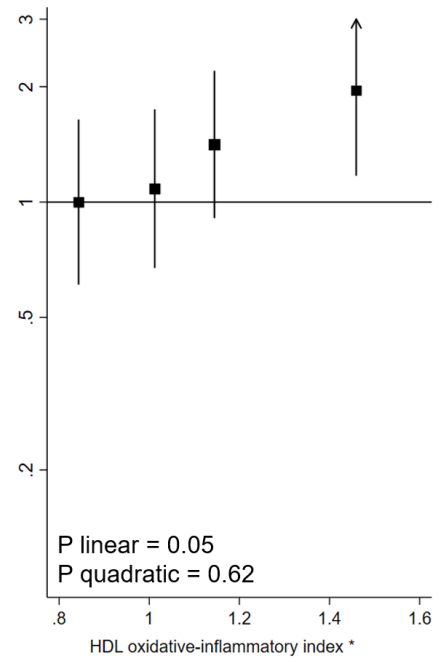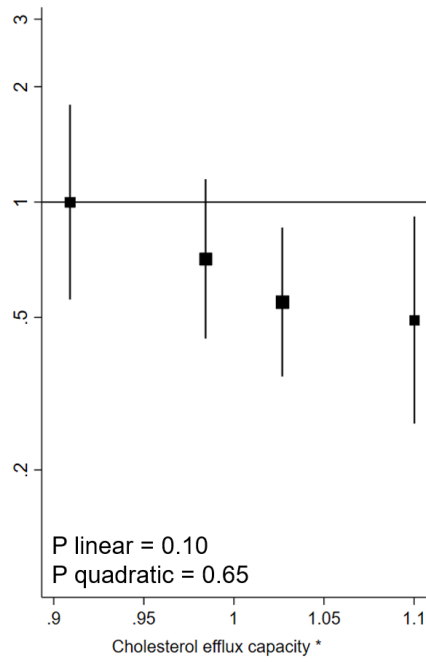high-density lipoprotein; HDL-LpPLA2, HDL phospholipase A2 activity. All determinations but HDL cholesterol levels were measured in apolipoprotein B-depleted plasma samples. Model 1, unadjusted; Model 2, adjusted for age, HDL cholesterol (except when HDL cholesterol is the exposure), fasting glucose levels, use of glucose-lowering drugs, total cholesterol concentrations, triglyceride levels, use of lipid-lowering drugs, systolic blood pressure, use of antihypertensive drugs, smoking status, body mass index, leisure-time physical activity levels, and ethanol consumption.

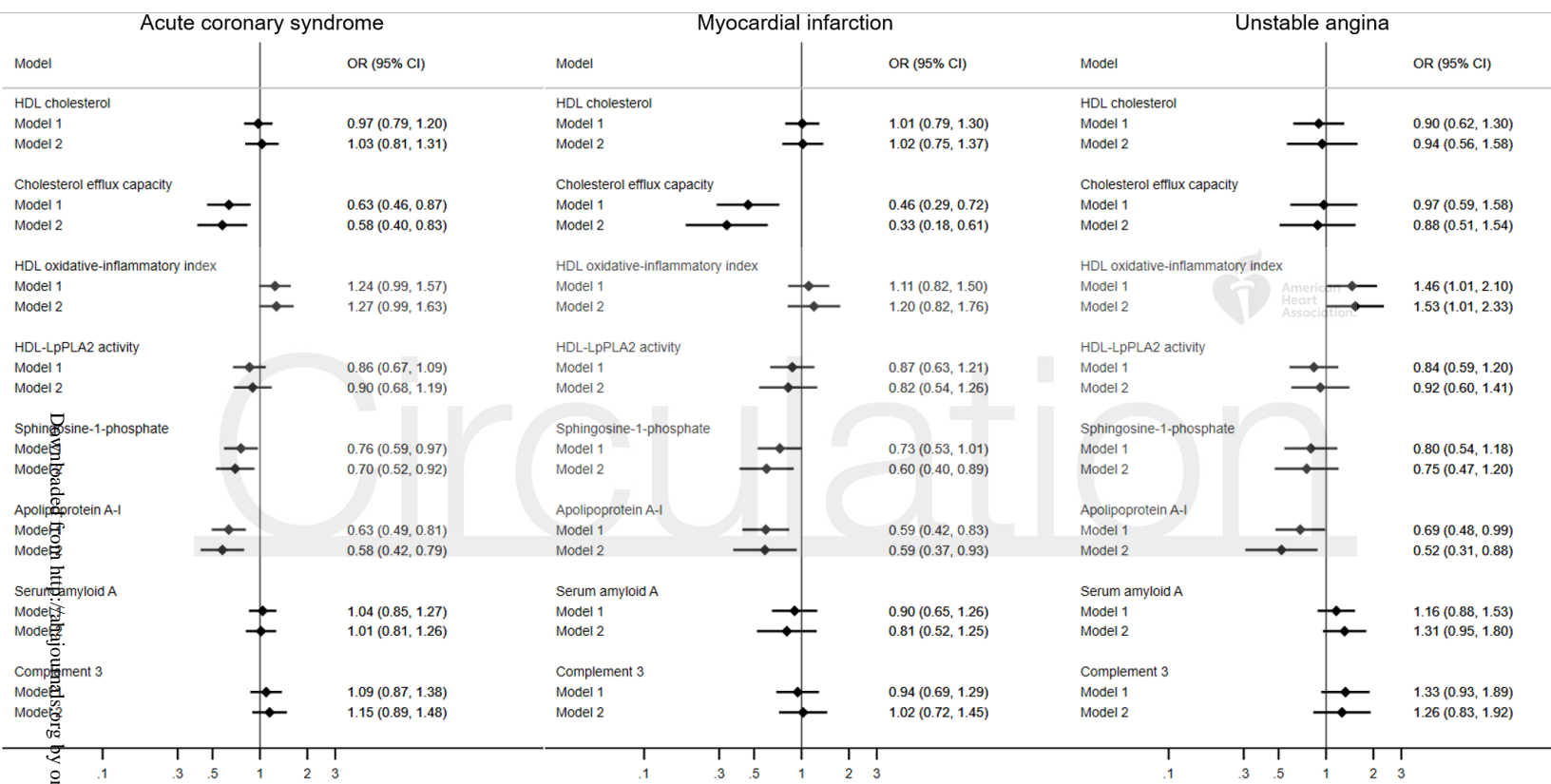| Acute coronary syndrome			Myocardial infarction			Unstable angina		
Model		OR (95% CI)	Model		OR (95% CI)	Model		OR (95% CI)
HDL cholesterol			HDL cholesterol			HDL cholesterol		
Model 1		0.97 (0.79, 1.20)	Model 1		1.01 (0.79, 1.30)	Model 1		0.90 (0.62, 1.30)
Model 2		1.03 (0.81, 1.31)	Model 2		1.02 (0.75, 1.37)	Model 2		0.94 (0.56, 1.58)
Cholesterol efflux capacity			Cholesterol efflux capacity			Cholesterol efflux capacity		
Model 1		0.63 (0.46, 0.87)	Model 1		0.46 (0.29, 0.72)	Model 1		0.97 (0.59, 1.58)
Model 2		0.58 (0.40, 0.83)	Model 2		0.33 (0.18, 0.61)	Model 2		0.88 (0.51, 1.54)
HDL oxidative-inflammatory index			HDL oxidative-inflammatory index			HDL oxidative-inflammatory index		
Model 1		1.24 (0.99, 1.57)	Model 1		1.11 (0.82, 1.50)	Model 1		1.46 (1.01, 2.10)
Model 2		1.27 (0.99, 1.63)	Model 2		1.20 (0.82, 1.76)	Model 2		1.53 (1.01, 2.33)
HDL-LpPLA2 activity			HDL-LpPLA2 activity			HDL-LpPLA2 activity		
Model 1		0.86 (0.67, 1.09)	Model 1		0.87 (0.63, 1.21)	Model 1		0.84 (0.59, 1.20)
Model 2		0.90 (0.68, 1.19)	Model 2		0.82 (0.54, 1.26)	Model 2		0.92 (0.60, 1.41)
Sphingosine-1-phosphate			Sphingosine-1-phosphate			Sphingosine-1-phosphate		
Model 1		0.76 (0.59, 0.97)	Model 1		0.73 (0.53, 1.01)	Model 1		0.80 (0.54, 1.18)
Model 2		0.70 (0.52, 0.92)	Model 2		0.60 (0.40, 0.89)	Model 2		0.75 (0.47, 1.20)
Apolipoprotein A-I			Apolipoprotein A-I			Apolipoprotein A-I		
Model 1		0.63 (0.49, 0.81)	Model 1		0.59 (0.42, 0.83)	Model 1		0.69 (0.48, 0.99)
Model 2		0.58 (0.42, 0.79)	Model 2		0.59 (0.37, 0.93)	Model 2		0.52 (0.31, 0.88)
Serum amyloid A			Serum amyloid A			Serum amyloid A		
Model 1		1.04 (0.85, 1.27)	Model 1		0.90 (0.65, 1.26)	Model 1		1.16 (0.88, 1.53)
Model 2		1.01 (0.81, 1.26)	Model 2		0.81 (0.52, 1.25)	Model 2		1.31 (0.95, 1.80)
Complement 3			Complement 3			Complement 3		
Model 1		1.09 (0.87, 1.38)	Model 1		0.94 (0.69, 1.29)	Model 1		1.33 (0.93, 1.89)
Model 2		1.15 (0.89, 1.48)	Model 2		1.02 (0.72, 1.45)	Model 2		1.26 (0.83, 1.92)