Circulation

ORIGINAL RESEARCH ARTICLE

# Dysfunctional High-Density Lipoproteins Are Associated With a Greater Incidence of Acute Coronary Syndrome in a Population at High Cardiovascular Risk

## A Nested Case–Control Study

**BACKGROUND:** Studies have failed to establish a clear link between high-density lipoprotein (HDL) cholesterol and cardiovascular disease, leading to the hypothesis that the atheroprotective role of HDL 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 investigated comprehensively.

**METHODS:** We conducted a case-control study nested within the PREDIMED (Prevención con Dieta Mediterránea) cohort, originally a randomized trial in which participants followed a Mediterranean or low-fat diet. Incident acute coronary syndrome cases (N=167) were individually matched (1:2) to control patients by sex, age, intervention group, body mass index, and follow-up time. We investigated 2 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 1-SD 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 low 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/proinflammatory 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 individuals at high cardiovascular risk.

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## Clinical Perspective

### What Is New?

- Low values of cholesterol efflux capacity and levels of sphingosine-1-phosphate and apolipoprotein A-I in high-density lipoprotein (HDL) were associated with a higher risk of acute coronary syndrome in individuals at high cardiovascular risk, irrespective of HDL cholesterol levels and other classic 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 cholesterol levels in a high cardiovascular risk population.

### What Are the Clinical Implications?

- Several HDL functionality measurements (related to HDL roles in cholesterol metabolism, endothelial protection, and antioxidant/anti-inflammatory defense) were associated with the incidence of acute coronary syndromes, whereas 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.

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 (CVD).[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 CVD. 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 CVD in the majority of studies,[8–13] although various authors failed to find an association with incident CVD[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-LpPLA2 (HDL-bound phospholipase A2) with cardiovascular risk remains controversial. HDL-LpPLA2 activity has been associated with lower cardiovascular risk in preclinical[19] and clinical[20] studies, although the pharmacological inhibition of the overall LpPLA2 activity failed to reduce incidence of major CVD in 2 clinical trials.[21,22] It is hypothesized that the low-density lipoprotein (LDL)–bound enzyme fraction might 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 S1P (sphingosine-1-phosphate), a modified phospholipid, is thought to be one of the key mediators of HDL protection on endothelial cells in preclinical models[23] and has been associated with the extent of atherosclerotic lesions in patients with stable coronary artery disease.[24] Second, relative levels of ApoA-I (apolipoprotein A-I) and ApoA-IV (apolipoprotein A-IV) in HDL particles have been shown to be involved in HDL functional properties in structural analyses[25] and impaired in subjects at high cardiovascular risk.[26] Finally, HDL enrichment in certain proinflammatory proteins, such as serum amyloid A (SAA) and C3 (complement 3 protein)[15,17], could impair the antioxidant/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 2 main manifestations, myocardial infarction (MI) and unstable angina (UA), in a population at high cardiovascular risk.

## 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 PREDIMED Study (Prevención con Dieta Mediterránea) via email at predimed-steering-committe@googlegroups.com.

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

## 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 on the primary prevention of cardiovascular outcomes in a population at high cardiovascular risk. Eligible participants were community-dwelling men (55–80 years old) and women (60–80 years old) who fulfilled at least 1 of the following 2 criteria: (1) type 2 diabetes mellitus or (2) 3 or more cardiovascular risk factors: current smoking, hypertension (blood pressure ≥140/90 mm Hg or use of antihypertensive drugs), LDL 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 in the ISRCTN Registry (ISRCTN35739639; www.controlled-trials.com). All participants provided written informed consent before joining the trial.

## Covariates and Biological Samples

Baseline examination included questions about education, lifestyle, history of illnesses, medication use, and nurse visits. Detailed information is available in the online-only Data Supplement.

## Outcome Ascertainment and Sample Size

The main outcome was ACS, defined as fatal or nonfatal MI[29] and fatal or nonfatal UA.[30] We used 4 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. An adjudication committee whose members were blinded to treatment allocation reviewed all end points.[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 Figure I in the online-only Data Supplement, and a description of the differences between included and nonincluded cases can be found in Table I in the online-only Data Supplement. 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 2 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 2 control subjects by age (±5 years), sex, body mass index (±3 kg/m$^2$), intervention group, and time to event. The 1:2 ratio of case subjects to control subjects was based on the following sample size calculation: to detect an odds ratio (OR) of 0.75 or less for the increase in 1 SD, as observed with CEC 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 −80°C until use. In these samples, we measured the following: (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 LDL as pro-oxidative stimuli[32]; (3) LpPLA2 activity with commercial platelet-activating factor acetylhydrolase 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 (S1P bioassay 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 the Methods in the online-only Data Supplement.

To decrease the variability of the laboratory measurements, we analyzed matched samples in the same experimental run in all determinations. In each experimental run of nonautomatized techniques (all but the quantification of ApoA-I and C3), we included 2 apolipoprotein B–depleted plasma pools (isolated from the plasma of 20 healthy volunteers), one to assess interassay variability and the other to minimize intra-assay and interassay 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] Last, we ran cellular techniques in duplicate, not allowing intrarepetition coefficients of variation >20%. Interassay coefficients of variation are available in Table II in the online-only Data Supplement. In addition, to minimize the interplate 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 2 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 nonincluded ACS cases (N=55) by $\chi^2$ tests for categorical variables, Student *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 modeled with conditional logistic regression. The linear nature of the relationship between each biomarker and each outcome was evaluated by modeling the biomarkers in quartiles. The shape of the association was tested visually by using floating variance.[33] Furthermore, linear and quadratic contrasts were used to assess the *P* value for trend across quartiles. Biomarkers were also modeled as standardized continuous variables to obtain ORs and 95% CIs associated with a 1-SD increase in the biomarker. When a nonlinear 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 2 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 correlation coefficients.

Finally, we conducted an exploratory secondary analysis stratified according to the following: (1) sex (male/female); (2) age (below versus over the median [67 years]); (3) fasting glucose levels (<126 mg/dL versus ≥126 mg/dL); (4) use of glucose-lowering drugs (yes/no); (5) total cholesterol levels (<200 mg/dL versus ≥200 mg/dL); (6) triglyceride levels (<150 mg/dL versus ≥150 mg/dL); (7) use of lipid-lowering drugs (yes/no); (8) obesity (body mass index <30 kg/m$^2$ versus ≥30 kg/m$^2$); (9) cumulative adherence to a traditional Mediterranean diet along the PREDIMED study follow-ups (below versus over the median); and (10) leisure-time physical activity at baseline (below versus 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 the *P* value for the linear interaction was <0.10,[34] and the association with ACS odds was significant in one of the strata and not in the other.

All tests were 2-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 (Stata Statistical Software: release 15, StataCorp LLC).

# RESULTS

## Baseline Characteristics

Compared with control subjects, participants with ACS were more likely to be glucose-lowering drug users (*P*<0.001) and to be current smokers (*P*=0.013), and they had greater systolic blood pressure levels (*P*<0.001). A similar trend was observed comparing MI case and control subjects, but there were fewer users of lipid-lowering (*P*=0.006) and antihypertensive (*P*=0.019) drugs among MI case subjects than control subjects. Participants with UA displayed greater adherence to the Mediterranean diet (*P*=0.031) than control subjects (Table). The 167 included ACS case subjects with plasma samples at baseline did not differ from the 55 nonincluded ones with the exception of the proportion of subjects under treatment with glucose-lowering therapies (there were 17% more glucose-lowering therapy users in the group of selected ACS case subjects; *P*=0.041; Table I in the online-only Data Supplement).

## HDL Function and ACS

All HDL function variables correlated weakly with each other, except for levels of ApoA-I in HDL and plasma HDL-C concentrations ($\rho$=0.68; Figure II in the online-only Data Supplement); 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 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 nonlinear, 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; Figure III in the online-only Data Supplement). Similarly, the association between ApoA-IV and ACS appeared nonlinear, as evidenced by the spline (with 4 knots) analysis, although the trend was broadly negative (Figure IV in the online-only Data Supplement).

Regarding the secondary outcomes, ApoA-I levels in apolipoprotein B–depleted plasma were 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 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 Table III in the online-only Data Supplement.

## 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 <67 years old but not in older people ($P_{interaction}$=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 nonobese 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

**Table. Baseline Characteristics of Acute Coronary Syndrome, Myocardial Infarction, and Unstable Angina Case Subjects and Control Subjects**

| | Acute Coronary Syndrome | | Only Myocardial Infarction | | Only Unstable Angina | |
|---|---|---|---|---|---|---|
| | Case Subjects (N=167) | Control Subjects (N=333) | Case Subjects (N=93) | Control Subjects (N=185) | Case Subjects (N=74) | Control Subjects (N=148) |
| Age, mean (SD), y | 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, mean (SD) 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, mean (SD), mg/dL | 205 (34.8) | 204 (35.9) | 205 (34.4) | 202 (35.8) | 204 (35.4) | 207 (36.0) |
| HDL cholesterol, mean (SD), 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, mean (SD), mg/dL | 128 (31.8) | 130 (32.4) | 129 (32.0) | 128 (32.8) | 128 (31.7) | 132 (31.7) |
| Triglycerides, median (Q1, Q3), 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, mean (SD), mm Hg | 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, mean (SD), kg/m$^2$ | 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, mean (SD), 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, mean (SD), 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, median (Q1, Q3), METs·min/d | 205 (85.2, 362) | 212 (89.1, 432) | 186 (80.0, 382) | 247 (110, 444) | 213 (106, 334) | 181 (68.4, 430) |
| Alcohol intake, median (Q1, Q3), g/d | 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) |

HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; PREDIMED, Prevención con Dieta Mediterránea; Q1, first quartile; and Q3, third quartile.
*$P$<0.05 (comparison of case vs control values, according to univariate conditional logistic regression models).

and 95% CIs for this analysis are available in Table IV in the online-only Data Supplement.

## DISCUSSION

The present study comprehensively investigated a wide range of markers of HDL functionality in relation to incident ACS in a population at high cardiovascular risk. We found that impaired HDL function, reflected by low CEC 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 consistently has been linked to overall atheroprotection in human trials.[35] The present data confirm that higher CEC values are associated with a lower ACS risk (each 1-SD increase in CEC related to a 42% odds decrease of ACS incidence), and more specifically with a lower risk of MI, which concurs with previous 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. Although the relationship between high triglyceride and low HDL-C levels has been described extensively,[37] our data suggest that hypertriglyceridemia could attenuate the relationship between HDL functional trait and risk of ACS. Finally, the present results are also of particular interest from a technical perspective. We used both a human THP-1 macrophage model, which is more akin to human biology than nonhuman cell lines, such as the classic murine J774 ones (J774 cells require a chemical upregulation of cholesterol transporters before the experiments to reach the physiological expression levels present in human macrophages[38]), and nonradiolabeled 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

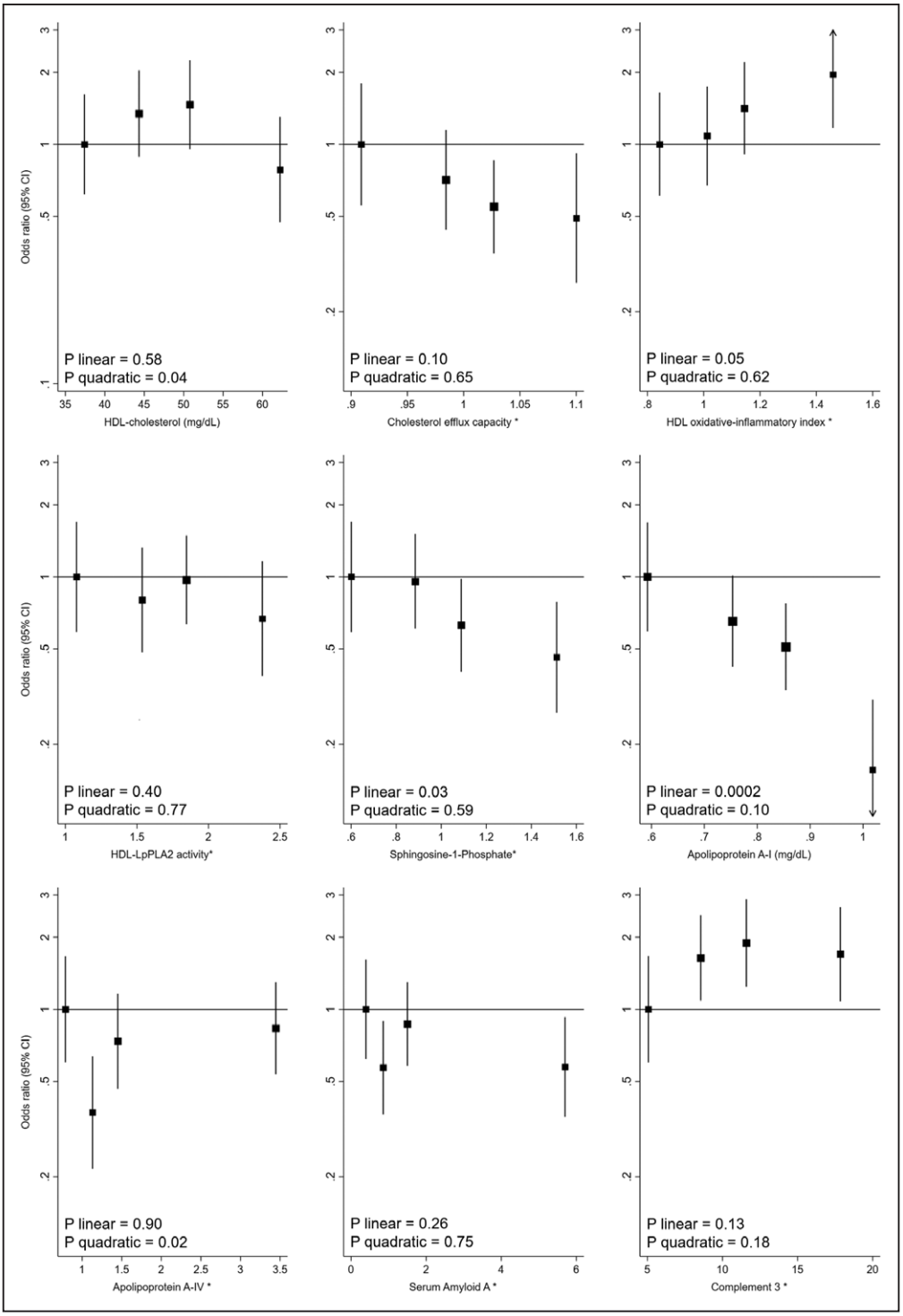


**Figure 1. Multivariable odds ratios for acute coronary syndrome across quartiles of HDL function markers.**
All determinations but HDL cholesterol levels were measured in apolipoprotein B–depleted plasma samples. Odds ratios and 95% CIs were estimated from conditional logistic regression with floating variances (allowing a CI 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. HDL indicates high-density lipoprotein; and HDL-LpPLA2, phospholipase A2 activity. *Normalized units.

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 LDL. This property can be regarded as pivotal in cardioprotection, because LDL oxidation is considered the primary trigger for the development of atherosclerotic

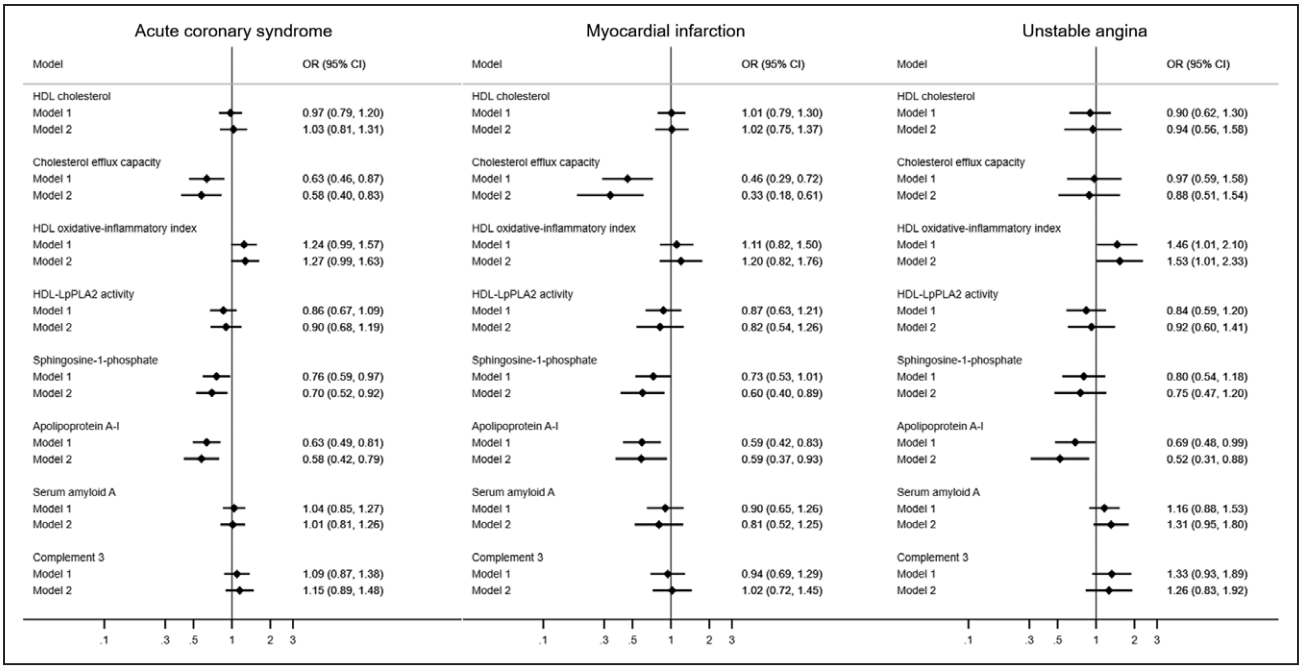


**Figure 2. Forest plots of odds ratios (95% CI) for 1-SD increases in HDL function markers for acute coronary syndrome, myocardial infarction, and unstable angina.**

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. HDL indicates high-density lipoprotein; HDL-LpPLA2, HDL phospholipase A2 activity; and OR, odds ratio



plaques and a key promoter of proinflammatory responses in the subendothelial space.[41] In line with previous findings,[36] we observed that a 1-SD increment in HOII values (namely, the pro-oxidative/proinflammatory status of HDL particles) was marginally linked to high ACS odds (particularly because of a 53% increase in 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 2958 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 was inversely, but nonsignificantly, related to ACS incidence in the present study and has been 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 (eg, the relative HDL richness in chemical antioxidants or proinflammatory 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, which suggests that infusions of this sphingolipid could contribute to re-establish the functionality of the lipoprotein.[44] In the present data, a 1-SD increment in S1P levels in apolipoprotein B–depleted plasma was associated with a 30% decrease in ACS risk (particularly because of an association with 40% lower odds of MI). HDL-bound S1P had already been reported to predict the extent of atherosclerotic lesions in a group of patients with stable coronary artery disease,[24] but the present study shows for the first time that S1P levels in apolipoprotein B–depleted plasma are inversely related to the development of ACS and MI in individuals at high cardiovascular risk. 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 1 SD in the level of ApoA-I in apolipoprotein B–depleted plasma was associated with almost halving the risk of having ACS. ApoA-I constitutes ≈70% of the apolipoprotein content of HDL particles[25] and actively participates in the antiatherosclerotic 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 might explain the potential improvements of 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/proinflammatory 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 2-way mechanism, taking into consideration other factors involved in plaque progression, such as infiltration of inflammatory cells, fibrosis, local flow disturbances, and vasospasm.

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 (eg, those related to HDL composition, such as levels of ApoA-I or S1P in apolipoprotein B–depleted plasma samples) in patients with a high risk of CVD.

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 population at high cardiovascular risk. Second, the nested case-control design presents considerable logistic and economic advantages because it uses existing cohort data and provides access to prospectively collected information (as opposed to standard case-control studies that collect data retrospectively). Finally, data quality is high, because the study presents information available from a considerable number of ACS case subjects (N=167, all verified by a clinical adjudication committee) within the context of a well-characterized population sample with data on several health outcomes obtained 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 case subjects was matched with a single control subject (we were unable to find 2 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 24-hour surveillance, had no freeze/thaw cycles before analyses, and followed the same preanalytical procedure before 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 because of 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 (online-only Data Supplement Table 3). However, these missing values occurred at random and affected case and control subjects in the same manner, because the order in which samples were assessed was randomly assigned before 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 population at high cardiovascular risk, irrespective of HDL-C 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, with ApoA-I levels in apolipoprotein B–depleted plasma being associated with both MI and UA. These results are in line with recent findings and support the notion that the pleiotropic function of HDL could explain its atheroprotective role in CVD beyond HDL-C concentrations.

## ARTICLE INFORMATION

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ORIGINAL RESEARCH ARTICLE


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