HDL (High-Density Lipoprotein) Cholesterol Efflux Capacity Is Associated With Incident Cardiovascular Disease in the General Population A Case-Control Study From the PREVEND Cohort

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CLINICAL AND POPULATION STUDIES

HDL (High-Density Lipoprotein) Cholesterol Efflux Capacity Is Associated With Incident Cardiovascular Disease in the General Population

A Case-Control Study From the PREVEND Cohort

Sanam Ebtehaj,* Eke G. Gruppen,* Stephan J.L. Bakker, Robin P.F. Dullaart,* Uwe J.F. Tietge*

OBJECTIVE: Focus is shifting from HDL-C (high-density lipoprotein cholesterol) as predictive biomarker for cardiovascular disease (CVD) towards antiatherogenic HDL functionalities. Still, limited data exist on the prospective association of HDL function metrics with CVD events. The current work aimed to determine, if baseline HDL-C efflux capacity (CEC) is associated with future CVD events in the general population.

APPROACH AND RESULTS: We performed a prospective study among participants of the PREVEND (Prevention of Renal and Vascular End-stage Disease) cohort (follow-up, 12 years). From the overall n=8592 subjects 325 with previous CVD events were excluded; of the remaining 8267 eligible participants all subjects with new CVD events during follow-up were selected and individually matched to controls for age, sex, smoking status, and HDL-C levels. CEC at baseline was quantified using human THP-1-derived macrophage foam cells and apolipoprotein B-depleted plasma. Despite identical HDL-C and apoA (apolipoprotein)-I levels between cases (n=351) and controls (n=354) CEC was significantly lower in cases (0.93±0.29 versus 1.01±0.24 arbitrary units; $P<0.001$). In all subjects combined, CEC correlated positively with HDL-C and apoA-I and negatively with body mass index, hsCRP (high-sensitivity C-reactive protein), and urinary albumin excretion. CEC was inversely associated with incident CVD events, both expressed per quartile and per 1 SD change (odds ratio, 0.73; 95% CI, 0.62–0.86; $P<0.001$); this association remained significant after adjustments for HDL-C, hsCRP, kidney function, and several other clinical covariates.

CONCLUSIONS: Combined these data demonstrate that in the general population baseline CEC is significantly associated with the future development of CVD events independent of HDL-C and apoA-I plasma levels.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: albumins ■ atherosclerosis ■ biomarker ■ cardiovascular disease ■ foam cells ■ urinary albumin excretion

Large cohort studies reproducibly showed a strong and inverse relationship of HDL-C (high-density lipoprotein cholesterol) levels with the risk of incident atherosclerotic cardiovascular disease (CVD) independent of other lipids.[1–4] However, the causal relationship of HDL-C with CVD and its usefulness as a drug target for decreasing CVD risk have been disputed by results from genetic studies as well by pharmacological intervention trials. Lifelong genetically determined HDL-C levels due to variation at the, for example, LCAT (lecithin–cholesterol acyltransferase) or ABCA1 (ATP-binding cassette transporter A1) locus did not associate with the future risk of CVD events.[5–7] Further, drugs which raise HDL-C, such as CETP (cholesteryl ester transfer protein) inhibitors or niacin, failed to produce a concomitant protection against CVD events.[8–10] Such considerations have contributed to

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Nonstandard Abbreviations and Acronyms

ABCA1	ATP-binding cassette transporter A1
apoA	apolipoprotein A
BMI	body mass index
CEC	cholesterol efflux capacity
CETP	cholesteryl ester transfer protein
CVD	cardiovascular disease
eGFR	estimated glomerular filtration rate
HDL-C	high-density lipoprotein cholesterol
hsCRP	high-sensitivity C-reactive protein
LCAT	lecithin–cholesterol acyltransferase
OR	odds ratio
PREVEND	Prevention of Renal and Vascular End-Stage Disease
SR-BI	scavenger receptor BI
UAE	urinary albumin excretion

Highlights

- Prospective case-control study among participants of the PREVEND cohort (Prevention of Renal and Vascular End stage Disease; follow-up,12 years).
- Cases with new cardiovascular disease event during follow-up (n=351) matched with controls for age, sex, and HDL-C (high-density lipoprotein cholesterol; n=354).
- Cholesterol efflux capacity at baseline quantified using human THP-1 macrophage foam cells with apoB (apolipoprotein)-depleted plasma.
- Impaired cholesterol efflux capacity is associated with incident cardiovascular disease events in the general population independent of HDL-C or apoA-I.

the emerging concept that determining the functionality of HDL particles with dynamic assays might be more relevant for CVD risk prediction than static HDL-C levels per se. Among a range of atheroprotective functions of HDL, promoting cholesterol efflux from macrophage foam cells, the first step in the reverse cholesterol transport pathway represents a key metric.[1,11–13]

Thus far, 2 studies tested if the HDL-mediated cholesterol efflux capacity (CEC) is associated with incident cardiovascular events in the general population and found that increased CEC is associated with lower CVD risk independent of the HDL-C concentration.[14,15] Both studies used for efflux assays murine cAMP (cyclic adenosine monophosphate)-stimulated J774 cells equilibrated with cholesterol, in which cholesterol efflux is largely dominated by the contribution of ABCA1. We have previously reported that the cholesterol efflux assay system that we use, that is, human THP-1-derived macrophage foam cells loaded with cholesterol, did not predict incident CVD in large cohorts of patients with reduced kidney function[16] or end-stage renal disease.[17] It is thus far unclear, if these apparent discrepancies could be ascribed to the patient populations studied, namely renal dysfunction as such, or could be due to methodological differences in CEC determinations. We chose to employ THP-1-derived macrophage foam cells for CEC measurements in prospective human cohorts, since these cells are of human origin, have the various active efflux pathways present (ABCA1; ABCG1; scavenger receptor BI, SR-BI) and macrophages loaded with modified LDL-derived cholesterol resemble in our view more closely the actual situation in atherosclerotic lesions in the vessel wall. We consider that for broader implications of CEC in cardiovascular risk prediction, consistent conclusions reached with different assay systems over a variety of populations with different characteristics would be valuable. Moreover, it is yet unknown whether any association of impaired CEC with incident CVD varies across HDL-C concentrations. Therefore, the present work was designed to test the extent to which CEC is associated with incident CVD events, in a case-control set-up, in which cases and controls were also matched for HDL-C. The study was performed in a prospective fashion using samples from men and women participating in the PREVEND study (Prevention of Renal and Vascular End-Stage Disease), a large and well-characterized cohort from the North of the Netherlands.

PATIENTS AND METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Study Population and Design

We performed a prospective case-control study among participants of PREVEND. This study investigates vascular and renal damage among inhabitants of the city of Groningen in the north of the Netherlands in a predominantly white population. Details of the study have been described elsewhere.[18,19] In summary, in 1997 to 1998, all inhabitants of the city of Groningen, aged 28 to 75 years, were sent a short questionnaire on demographics and cardiovascular morbidity and a vial to collect an early morning urine sample. Altogether, 40 856 subjects responded (47.8%). Pregnant women and diabetic subjects using insulin were excluded. All participants with urinary albumin concentration ≥10 mg/L were invited to our clinic together with randomly selected subjects with a urinary albumin concentration <10 mg/L. The study population comprised 8592 subjects who completed the total screening program. The study was approved by the medical ethics Committee of the University Medical Center Groningen. All participants gave written informed consent.

Outcome Assessment

The combined end point of our study was incident CVD, defined as death from CVD, hospitalization for myocardial infarction, percutaneous transluminal coronary angioplasty, or coronary artery bypass graft. From the time of inclusion in the study, the vital status of the participants was checked through the municipal register. The cause of death was obtained by linking the number of death certificates to the primary cause of death as coded by the Central Bureau of Statistics (Voorburg/Heerlen, the Netherlands). Causes of death were coded according to the *International Classification of Diseases, Ninth Revision*. Information on myocardial infarction, percutaneous transluminal coronary angioplasty, and coronary artery bypass graft was obtained from the national hospital information system (Prismant, Utrecht, the Netherlands).

Study Design

For the present study, 325 subjects who had experienced a cardiovascular event before the baseline evaluation were excluded, leaving 8267 potentially eligible participants. We first identified all men and women who had experienced a new cardiovascular event (cases) until end of follow-up which was January 1, 2009. The census date was the date on which the information was obtained from the municipal registry (January 1, 2009) or the date of death. If a person had moved to an unknown destination, the date on which the person was dropped from the municipal registry was used as the census date. We then divided the cases by quartiles of HDL-C, stratified according to sex and current smoking behavior at the baseline evaluation. Subsequently, the cases identified in each sex-specific and smoking status stratified HDL-C quartile were individually matched to a control subject of the same sex and with the same smoking status in a one to one ratio. Cases and control subjects were also matched for age (within 5 years). To ensure similar HDL-C values between cases and control subjects within in each HDL quartile we also matched each case with each control subject with respect to their HDL-C level applying a maximal difference of 0.10 mmol/L. The flow scheme of participant selection is shown in Figure I in the online-only Data Supplement. Of the 8267 eligible participants, 369 cases and 369 controls were initially selected. However, blood samples were not available in 33 subjects, leaving 705 subjects (351 cases and 354 controls) for the present analysis.

Clinical Measures, Procedures, and Definitions

Body mass index (BMI) was calculated as the ratio between weight and height squared (in kg/m^2). Blood pressure was measured using an automatic Dinamap XL model 9300 series device (Johnson-Johnson Medical, Tampa, FL). Hypertension was defined as systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg or the use of antihypertensive drugs. Microalbuminuria was defined as urinary albumin excretion (UAE) between 30 and 300 mg/24 h based on two 24 hour urine collections. Type 2 diabetes mellitus was defined as a fasting glucose ≥7.0 mmol/L, a random glucose ≥11.1 mmol/L, self-report of a physician diagnosis or the use of glucose-lowering drugs. Alcohol consumption was recorded assuming one drink to contain 10 g of alcohol. Smoking was categorized into current, former, and never. Estimated glomerular filtration rate (eGFR) was calculated applying the combined creatinine-cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation.[20] Information on medication use was combined with information from a pharmacy-dispensing registry, which has complete information on drug use of >95% of subjects in the PREVEND study. The participants were instructed so that venous blood samples were drawn after an overnight fast.

Laboratory Measurements

Fasting venous blood samples were obtained after 15 minutes rest. Plasma glucose was measured directly after blood sampling. EDTA plasma was obtained by centrifugation at 4°C, and the samples were stored at −80°C until analysis. Plasma total cholesterol and glucose were assessed using Kodak Ektachem dry chemistry (Eastman Kodak, Rochester, NY). Triglycerides were measured enzymatically. HDL-C was measured with a homogeneous method (direct HDL, AEROSETTM System, Abbott Laboratories, Abbott Park).[19] Non-HDL-C was calculated as the difference between total cholesterol and HDL-C. LDL-C (low-density lipoprotein cholesterol) was calculated by the Friedewald formula if triglycerides were <4.5 mmol/L, and are not given, if triglycerides were higher. apoA (apolipoprotein)-I and apoB were determined by nephelometry with reagents for Dade Behring nephelometer systems (BN II, Dade Behring Marburg, Germany).[19] hsCRP (high-sensitivity C-reactive protein) was assayed by nephelometry with a lower limit of 0.175 mg/L (BNII N; Dade Behring, Marburg, Germany). Serum creatinine was measured by an enzymatic method on a RocheModular analyzer (Roche Diagnostics, Mannheim, Germany). Serum cystatin C was measured by Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) on a Modular analyzer (Roche Diagnostics). UAE was measured by nephelometry with a threshold of 2.3 mg/L (Dade Behring Diagnostic, Marburg, Germany).

CEC was measured exactly as described[17] using human THP-1 monocytes (ATTC via LCG Promochem, Teddington, UK) differentiated into macrophages by the addition of 100 nmol/L PMA (phorbol 12-myristate 13-acetate; Sigma-Aldrich) to the cell culture medium (RPMI 1640 Glutamax medium containing 10% fetal bovine serum and penicillin [100 U/mL]/streptomycin

[100 µg/mL]) for 24 hours. Macrophage foam cells were then generated by loading them with acetylated LDL (50 µg protein/ mL) and 1 µCi/mL ^{3}H-cholesterol (PerkinElmer, Boston, MA) for 24 hours followed by equilibration for 24 hours in medium containing 2% BSA (Sigma-Aldrich).[16,21] Thereafter, cells were washed with PBS and 2% apoB-depleted plasma, generated as detailed previously,[14–17] was added in RPMI 1640 Glutamax medium containing penicillin (100 U/mL)/ streptomycin (100 µg/mL). Samples were numbered consecutively, and the person performing the efflux assays was completely blinded to the clinical outcome data. After 5 hours of efflux the medium was collected and centrifuged in a table-top centrifuge for 5 minutes at 10 000 rpm to pellet cellular debris. Effluxed cholesterol label in the medium was quantitated by liquid scintillation counting (Packard 1600CA Tri-Carb, Packard, Meriden, CT) as was the radioactivity remaining within the cells following an incubation for at least 30 minutes with 0.1 mol/L NaOH at room temperature. Efflux per well is expressed as percentage of counts released into the medium related to the total dose of radioactivity initially present (counts recovered within the medium added to the counts recovered from the cells). Values obtained from control cells without added HDL preparations were subtracted to correct for unspecific efflux (on average 1.55%). CEC measurements were performed in all respective patient samples in duplicate at the same time to limit potential variation due to different assay conditions. Individual efflux values were normalized to pooled apoB-depleted control plasma (activity, 7.56%CEC/5h) included on each plate and are expressed in arbitrary units (AU). Validation experiments showed that almost 90% of the CEC of apoB-depleted plasma is explained by the presence of HDL.[21]

Statistical Analysis

Statistical software SPSS version 23.0 (SPSS Inc, Armonk, NY) and STATA version 13.1 (StataCorp, College Station, TX: StataCorp LP) were used to perform statistical analyses. Normally distributed data were expressed as mean±SD and skewed data as median (interquartile range). Differences between subjects who experienced a cardiovascular event during follow-up (cases) and subjects who remained free of an event (controls) were compared with Student *t* test and by Mann–Whitney analyses, where appropriate. The χ^2 tests and Fisher exact tests were used to compare frequencies between groups. For correlation analysis and logistic regression analysis skewed data were $\log_e$ logarithmically transformed to achieve approximately normal distributions, which was the case for triglycerides, hsCRP, and UAE. Univariate as well as age- and sex-adjusted Pearson correlation coefficients were calculated to explore relationships between CEC, clinical variables, and laboratory parameters. Given the nested case-control design we used (multivariable) logistic regression analysis to determine the association of CEC with cardiovascular outcomes with results being expressed in odds ratios (ORs and 95% CI). ORs were determined according to quartiles of CEC, as well as per 1 SD increment. Tests of trend across quartiles of CEC were conducted by assigning the median value for each quartile as its value and treating this as a continuous variable. In addition, we compared CEC between cases and controls in each sex-stratified HDL quartile and performed subgroup analyses using interaction tests to assess statistical evidence of any differences in ORs across categories of prespecified subject characteristics, including sex (men versus women), age (<60.7 and ≥60.7, ie, the median value), current smoking (yes versus no), alcohol consumption (<10 or ≥10 g/d), diabetes mellitus (yes versus no), BMI (<30 versus ≥30 kg/m^2), hypertension (yes versus no), blood pressure (diastolic <78 and ≥78 mm Hg; systolic <138 and ≥138 mm Hg), total cholesterol (<6.0 and ≥6.0 mmol/L, ie, the median value), triglycerides (<1.39 and ≥1.39 mmol/L, ie, the median value), hsCRP <1.82 and ≥1.82 mg/L ie, the median value), eGFR (<86.0 and ≥86.0 mL/min per 1.73 m^2, ie, the median value), and albuminuria (≥30 versus <30 mg/24 h). Two-sided *P* values <0.05 were considered to be statistically significant except for CEC comparison in each separate HDL-C quartile, where we used a *P* value <0.05/4=0.0125 based on the Bonferroni method to correct for multiple comparisons.

RESULTS

There were 130 cases and 138 controls in the lowest HDL-C quartile (n=268; 171 men; Q1), 84 cases and 82 controls in the second HDL-C quartile (n=166; 123 men; Q2), 77 cases and 75 controls in the third HDL-C quartile (n=152; 114 men; Q3), and 60 cases and 59 controls in the upper HDL-C quartile (n=119; 94 men; Q4). The median follow-up time up was 10.5 (9.9–10.8) years in cases and 10.4 (9.9–10.8) years in controls. As shown in Table 1, there were no differences in age, sex distribution, smoking status, and alcohol consumption between cases and controls, but cases had a higher BMI and were more likely to be diagnosed with hypertension, to use antihypertensive medication, lipid-lowering drugs, or glucose-lowering drugs. Diabetes mellitus prevalence was not significantly different between the groups. Plasma glucose, UAE, and eGFR were not different between cases and controls, but hsCRP was higher in cases. Total cholesterol, non-HDL-C, apoB, and triglycerides were also higher in cases. As a result of the matching procedure, HDL-C and apoA-I levels were very similar in cases and controls. Notably, despite very similar

Table 1. Characteristics of the 705 Study Participants According to Case-Control Status at End of Follow-Up

	All Participants	Controls	Cases	*P* Value
N	705	354	351	
Age, y	59.0±10.8	59.1±10.7	59.0±10.9	0.90
Male sex, n (%)	502 (71.2)	253 (71.5)	249 (70.9)	0.88
BMI, kg/m^2	27.2±4.2	26.9±4.3	27.5±4.2	0.045
Smoking, n (%)				
Current	304 (43.1)	152 (42.9)	152 (43.3)	0.45
Alcohol intake, n (%)				0.60
<10 g/d	518 (73.5)	257 (72.8)	261 (74.6)	
≥10 g/d	185 (26.2)	96 (27.2)	89 (25.4)	
Hypertension, n (%)	385 (54.6)	173 (48.9)	212 (60.4)	0.002
Diabetes mellitus, n (%)	40 (5.7)	16 (4.5)	24 (6.8)	0.18
Lipid-lowering drug use, n (%)	29 (4.1)	9 (2.5)	20 (5.7)	0.035
Antihypertensive medication use, n (%)	174 (24.7)	73 (20.6)	101 (28.8)	0.008
Glucose-lowering drug use, n (%)	19 (2.7)	6 (1.7)	13 (3.7)	<0.001
Fasting glucose, mmol/L	5.12±1.35	5.11±1.15	5.17±1.48	0.55
hsCRP, mg/L	1.81 (0.88–4.00)	1.58 (0.80–3.43)	2.09 (0.98–4.48)	0.005
eGFR, ml/min per 1.73 m^2	85.6±16.7	86.4±16.9	84.9±16.5	0.64
UAE, mg/24 h	12.5 (5.5–26.7)	11.7 (7.0–26.2)	13.4 (7.7–31.0)	0.08
Total cholesterol, mmol/L	6.05±1.15	5.84±1.12	6.18±1.07	<0.001
LDL cholesterol, mmol/L	4.14±1.05	3.98±1.07	4.27±0.99	0.39
Non-HDL cholesterol	4.84±1.16	4.70±1.18	5.08±1.19	0.051
HDL cholesterol, mmol/L	1.16±0.35	1.17±0.35	1.16±0.34	0.87
Triglycerides, mmol/L	1.38 (1.00–1.94)	1.35 (0.99–1.83)	1.42 (1.02–2.03)	0.048
ApoA1, g/L	1.32±0.26	1.33±0.27	1.31±0.26	0.21
ApoB, g/L	1.16±0.32	1.14±0.33	1.20±0.30	0.012
Cholesterol efflux capacity, AU	0.98±0.27	1.01±0.24	0.93±0.29	<0.001

Data are means±SD or medians (interquartile) ranges. LDL cholesterol was calculated in 339 (96.6%) cases and 347 (98.0%) controls. Apo indicates apolipoprotein; AU, arbitrary units; BMI, body mass index; eGFR, estimated glomerular filtration rate based on the creatinine-cystatin C equation; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoproteins; and UAE, urinary albumin excretion.

HDL-C levels, CEC was on average 9% lower in cases compared with controls (*P*<0.001).

In all subjects combined as well as in the case and control groups separately, CEC was lower in men than in women (all subjects: men, 0.93±0.23 AU versus women: 1.08±0.33 AU; *P*<0.001; cases: men: 0.84±0.16 AU versus women: 1.18±0.38 AU; *P*<0.001; controls: men: 1.03±0.25 AU versus women: 0.99±0.23 AU; *P*=0.20). In univariate analysis, CEC was inversely associated with BMI, blood pressure, hsCRP, UAE, and triglycerides in all subjects combined, while being positively related to total cholesterol (Table 2). Furthermore, CEC was strongly related to HDL-C and apoA-I. These relationships did not materially change after adjustment for age and sex, and were not substantially different between the respective case and control groups as well as between men and women (Table IA and IB in the online-only Data Supplement). Of note, after additional adjustment for HDL-C, CEC was still inversely associated with BMI and positively with total cholesterol and apoA-I, but the relationship with hsCRP, UAE, and triglycerides lost significance.

As shown in Table 3, CEC was inversely associated with incident cardiovascular events in univariate analysis; both expressed per quartile of CEC and per 1 SD change in CEC. This significant inverse association of CEC remained essentially unaltered after adjustment for clinical covariates, total cholesterol, and triglycerides (models 1–3), as well as after additional adjustment for HDL-C or alternatively for apoA-I (models 4). The inverse association of CEC with incident cardiovascular events was also unaltered after further adjustment for hsCRP (models 5) and after adjustment for UAE and eGFR (model 6).

As shown in Figure, the association of CEC with incident cardiovascular events was not different between subjects with higher versus lower age, with a higher versus a lower BMI, nor according to diabetes mellitus status, presence of hypertension, higher versus lower blood pressure, higher versus lower hsCRP, higher versus lower eGFR, higher versus lower total cholesterol or triglycerides.

Table 2. Correlation Coefficients of Cholesterol Efflux Capacity With Clinical and Laboratory Variables in 705 Cases and Control Subjects Combined

	Cholesterol Efflux Capacity		
	Crude	Age and Sex-Adjusted	Age, Sex, and HDL-C Adjusted
Age	−0.013	…	…
BMI	−0.162*	−0.182*	−0.076†
Glucose	−0.045	−0.044	0.025
Systolic blood pressure	−0.097‡	−0.088†	−0.071
Diastolic blood pressure	−0.123‡	−0.070	−0.061
hsCRP	−0.129‡	−0.154*	−0.059
UAE	−0.123‡	−0.094†	−0.063
eGFR	0.063	0.080†	0.045
Total cholesterol	0.077†	0.103	0.097†
LDL cholesterol	0.040	0.045	0.142*
Non-HDL cholesterol	−0.053	−0.040	0.101‡
HDL cholesterol	0.439*	0.411*	…
Triglycerides	−0.223*	−0.207*	−0.019
ApoB	−0.062	−0.055	0.070
ApoA1	0.399*	0.376*	0.161*

Pearson correlation coefficients are shown. hsCRP, triglycerides and UAE were $\log_e$ transformed before analysis. ApoA1 indicates apolipoprotein A1; ApoB, apolipoprotein B; BMI, body mass index; eGFR, estimated glomerular filtration rate based on the creatinine-cystatin C equation; HDL, high-density lipoproteins; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; and UAE, urinary albumin excretion.

*P<0.001.

†P<0.05.

‡P<0.01.

Notably, however, there was a strong interaction with sex, with CEC being inversely associated with outcome in men and positively in women (interaction, P<0.001). In addition, there were also interactions with smoking (interaction, P=0.02), alcohol consumption (interaction, P=0.007), and microalbuminuria (interaction, P=0.007) with the inverse association of CEC with incident CVD being stronger in smokers, subjects who consumed <10 g of alcohol per day and subjects with microalbuminuria.

When evaluating the difference in CEC between cases and control subjects in each sex-stratified HDL-C quartile separately, CEC was consistently lower in cases versus control subjects in each HDL-C quartile, reaching significance in Q1, Q3, and Q4 (Table 4). This analysis did not reveal relevant differences in clinical and laboratory characteristics between cases and control subjects in each HDL-C quartile separately, except for levels of total cholesterol, non-HDL-C and LDL cholesterol which were higher in cases from the first and third HDL-C quartile (Q1 and Q3), and for triglycerides which were higher in cases from the second HDL-C quartile (Q2; Table 4).

Finally, in view of the enrichment of the PREVEND cohort with subjects with elevated UAE we performed a sensitivity analysis after excluding subjects with elevated UAE (Table II in the online-only Data Supplement). In these additional analyses including 545 subjects the main conclusions reached from the whole group of studied participants were essentially confirmed, particularly that adjustment for HDL-C and apoA-I did not change the negative association between baseline CEC and future CVD events.

DISCUSSION

The results of this prospective general population-based study demonstrate that baseline CEC is inversely associated with incident CVD events. Notably, this association occurred independent of a number of established CVD risk factors and most importantly also independent of circulating HDL-C or apoA-I levels. A novel finding of the current study is that CEC was impaired across matched groups of subjects with initially lower and higher HDL-C concentrations. In addition, our data indicate that CEC relates negatively to parameters associated with the metabolic syndrome such as obesity, blood pressure, hsCRP, and triglycerides. Remarkably, the association of CEC with CVD events was different depending on sex, alcohol consumption, smoking, and UAE. Combined these results contribute to a better understanding of the clinical significance of CEC measurements, and might help to advance the use of CEC as potential biologically meaningful, HDL-based biomarker for CVD risk prediction.

Thus far, only a few prospective studies evaluating the association of CEC with future CVD events are available. Using the same cholesterol efflux assay set-up as employed in the current work, we reported earlier in patients with end-stage renal disease on hemodialysis from the 4D study[17] as well as in kidney transplant recipients with an on average reduced kidney function[16] that CEC did not predict incident CVD events. These results contrasted with 2 previous publications using samples from general population studies, which both concluded that CEC is prospectively associated with CVD events. One reported data from the Dallas Heart Study,[14] which was multi-ethnic, included younger participants, and consequently had a relatively low number of events during follow-up (n=132). The other was a case-control study from the EPIC-Norfolk cohort[15] that investigated CEC in 1745 patients developing CVD events during follow-up and matched controls. Of note, matching in EPIC-Norfolk was done according to age and sex only, translating into the expected substantial differences in HDL-C between cases and controls. In contrast, in our study, matching included HDL-C with the rationale to be better able to detect differences in HDL function that occur independent of mass HDL-C levels. However, when comparing different studies also technical aspects of the assays should be taken into account. Both previous studies[14,15] used an efflux protocol based on a different macrophage cell type, namely the murine J774 cell line that grows in suspension culture and requires cAMP stimulation to induce ABCA1 expression. In these, efflux is then largely

Table 3. Association of CEC With Incident Cardiovascular Events in 705 Subjects (351 cases and 354 control subjects) According to CEC Quartiles (Q1-Q4) and Expressed per 1 SD Increase in Cholesterol Efflux Capacity

Cholesterol Efflux Capacity, AU	Q1	Q2	*P* Value	Q3	*P* Value	Q4	*P* Value	*P* for Trend	Per SD Increase (1 SD=0.27 Arbitrary Units)	*P* Value
	<0.79	≥0.79		≥0.93		≥1.12				
Participants, n	176	177		176		176				
Cases, n (%)	118 (67)	97 (55)		67 (38)		69 (39)				
Univariate	Ref	0.60 [0.39–0.92]	0.02	0.30 [0.20–0.47]	<0.001	0.32 [0.21–0.49]	<0.001	<0.001	0.74 [0.63–0.86]	<0.001
Model 1	Ref	0.60 [0.39–0.93]	0.023	0.294 [0.19–0.46]	<0.001	0.32 [0.20–0.50]	<0.001	<0.001	0.73 [0.62–0.86]	<0.001
Model 2	Ref	0.62 [0.40–0.97]	0.034	0.30 [0.19–0.47]	<0.001	0.32 [0.20–0.51]	<0.001	<0.001	0.74 [0.63–0.87]	<0.001
Model 3	Ref	0.56 [0.36–0.88]	0.01	0.27 [0.17–0.43]	<0.001	0.26 [0.16–0.42]	<0.001	<0.001	0.69 [0.58–0.82]	<0.001
Model 4a	Ref	0.51 [0.32–0.81]	0.004	0.23 [0.14–0.37]	<0.001	0.20 [0.12–0.34]	<0.001	<0.001	0.65 [0.54–0.78]	<0.001
Model 4b	Ref	0.56 [0.35–0.88]	0.01	0.27 [0.16–0.43]	<0.001	0.25 [0.15–0.43]	<0.001	<0.001	0.70 [0.59–0.84]	<0.001
Model 5a	Ref	0.51 [0.32–0.80]	0.004	0.23 [0.14–0.38]	<0.001	0.20 [0.12–0.34]	<0.001	<0.001	0.65 [0.54–0.79]	<0.001
Model 5b	Ref	0.55 [0.35–0.88]	0.011	0.27 [0.17–0.44]	<0.001	0.26 [0.15–0.44]	<0.001	<0.001	0.71 [0.59–0.86]	<0.001
Model 6	Ref	0.56 [0.36–0.88]	0.01	0.27 [0.17–0.43]	<0.001	0.26 [0.16–0.42]	<0.001	<0.001	0.70 [0.58–0.84]	<0.001

Data are ORs (95% CI) for incident cardiovascular disease events obtained with multivariable logistic regression models. Triglycerides and hsCRP values were $\log_e$ transformed. The use of glucose-lowering drugs and antihypertensive medication is included in the definition of diabetes mellitus and hypertension, respectively. Model 1: Univariate+age, sex, body mass index, alcohol intake (<10 g per day or ≥10 g per day), and smoking status (never, former, and current). Model 2: Model 1+diabetes mellitus status+hypertension and use of lipid-lowering drugs. Model 3: Model 2+total cholesterol and triglycerides. Model 4a: Model 3+high-density lipoprotein cholesterol. Model 4b: Model 3+apolipoprotein A-1. Model 5a: Model 4a+high-sensitivity C-reactive protein. Model 5b: Model 4b+high-sensitivity C-reactive protein. Model 6: Model 3+urinary albumin excretion+estimated glomerular filtration rate. AU indicates arbitrary units; CEC, cholesterol efflux capacity; and OR, odds ratio.

driven by ABCA1, while in THP-1-derived macrophage foam cells all 3 active efflux pathways are functional, namely ABCA1 (47% contribution, determined by addition of probucol), SR-BI (19%, determined by addition of BLT-1), and ABCG1 (30% contribution, determined by the addition of probucol and BLT-1; unpublished data). Further, in these assays cells were equilibrated with cholesterol, labeled in one case with tritium[14] and in the other fluorescently with BODIPY.[15] In contrast, our protocol employed human macrophage foam cells generated by loading with modified LDL. Other differences among studies are related to the starting sample material used for HDL isolations. While we used EDTA plasma obtained in the fasting state, exactly as in the Dallas Heart Study,[14] in EPIC-Norfolk serum was obtained in the nonfasting state for HDL isolations.[15] Serum has the disadvantage over plasma that for its formation a 20 to 30 minutes incubation at room temperature is required, a time window during which still rather extensive HDL remodeling and proteolytic degradation especially of pre β-HDL can occur.[1,22,23] However, and this is in our view reassuring news for the HDL function concept, despite different protocols for sample generation and efflux assay conditions, CEC robustly associated with future CVD independent of HDL-C in our present as well as in the 2 other studies conducted in general population cohorts.[14,15] On the contrary, these considerations indicate that the reduced kidney function probably offset the predictive power of CEC for CVD in our previous reports.[16,17] In other studies not using initially healthy individuals, variable conclusions for the predictive power of CEC for CVD events were reached. While one publication found a positive association of CEC with future CVD events in a coronary heart disease population recruited at the time of coronary angiography,[24] another report detected a significant inverse association using a similar setting, also in patients with preexisting coronary artery disease.[25] More work seems required to not only delineate why these discrepancies arise but also, in the same line, which factors are rendering HDL dysfunctional to accept cellular cholesterol in general, but also specifically in the setting of different disease states. Previous cross-sectional work indicated that among subjects with very high HDL-C levels (mean, 86 mg/dL), those with premature CAD had significantly lower CEC together with a decreased HDL phospholipid content compared with controls matched for race, sex, and HDL-C levels.[26] The identification of such factors could offer a chance to generate biomarkers that are easier to use in clinical routine than the relatively complex dynamic measurements of HDL functionalities. In this regard, it is relevant that impaired CEC was also associated with incident CVD

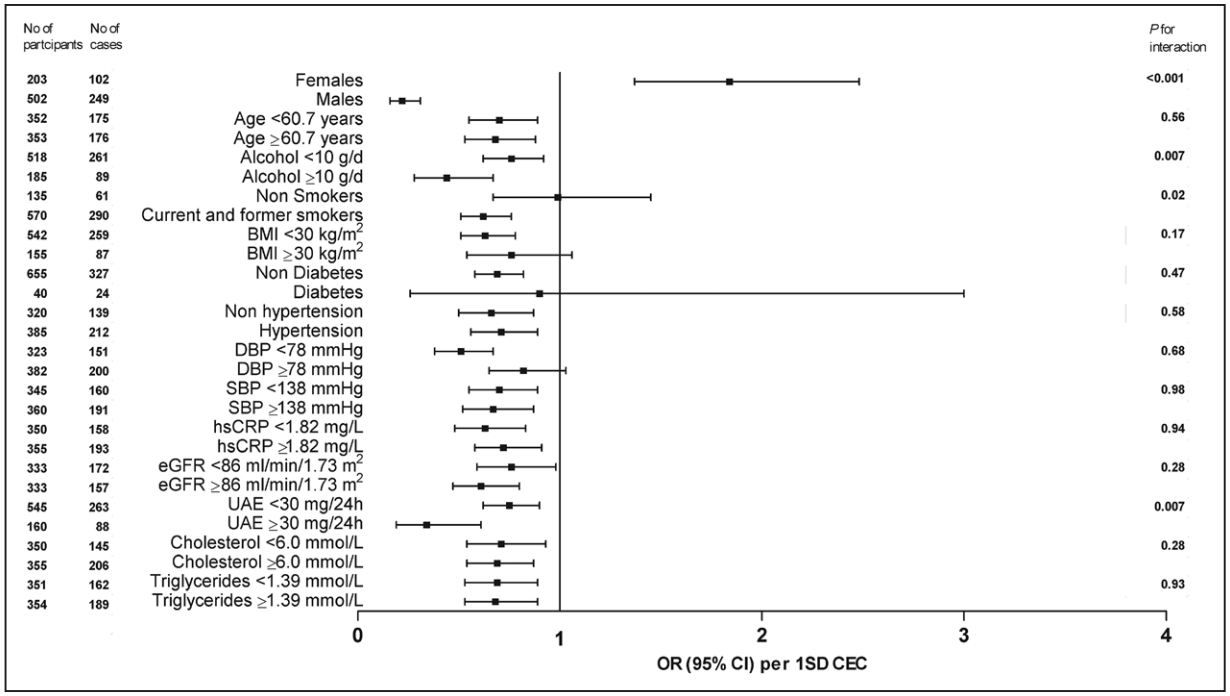

Figure. Odds ratios for incident cardiovascular disease per 1 SD increase in cholesterol efflux capacity, by several participant level characteristics.

Cut offs used for age, diastolic blood pressure, systolic blood pressure, cholesterol, triglycerides, hsCRP, eGFR, and UAE are median values. BMI indicates body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate based on the creatinine-cystatin C equation; HDL-C, high density lipoprotein cholesterol; hsCRP; high-sensitivity C-reactive protein; OR, odds ratio; SBP, systolic blood pressure; and UAE, urinary albumin excretion.

in subjects with the highest HDL-C concentrations at baseline in conjunction with higher hsCRP levels. This underscores that HDL can be dysfunctional per se even at high HDL-C concentrations.

Another aspect of the present study worth mentioning was the difference in CEC and CVD outcome by sex. Sex differences in efflux, with higher CEC in females compared with males, have been noticed before in some[15,27,28] but not all[14] studies. Particularly, higher efflux via ABCA1 and SR-BI in females has been held responsible for these sexual dimorphic effects.[29] However, the interaction with sex on outcomes noticed in the present study has, to the best of our knowledge, not been reported before, largely due to the fact that previous prospective studies did not focus on this aspect of CEC. More work seems required to determine if these sex effects are generalizable.

Limitations of the present study and potential reasons for caution should be pointed out. First, only samples from a single center were used, and PREVEND participants have a predominantly white ethnicity. Next, no efflux measurements were performed in repeat samples, since these were not available. This aspect would be interesting when it comes to investigating the robustness of CEC as a biomarker. Also, from other cohort studies such data have not been made available, so more work appears to be required to address this point in the future. In addition, next to cholesterol efflux also cellular cholesterol uptake can occur, even when apoB-depleted starting material is used for efflux assays.[30] None of the efflux assays currently employed in large cohorts, however, takes this aspect into account. In our protocol, we aimed to minimize the impact of influx by using a 5 hour efflux period in macrophage foam cells loaded with cholesterol, since it is known that cellular cholesterol loading decreases the inward cholesterol flux.[30] Another potential limitation of our work can be seen in the fact that we did not also perform CEC determinations using J774 cells, which had been the model of choice in other prospective studies that determined the impact of CEC on incident CVD events in general population cohorts.[14,15] In the future, such direct comparisons between different CEC assay protocols appear desirable. Further, due to the set-up of PREVEND there is an enrichment in participants with increased UAE; we observed an inverse correlation between CEC and UAE, that, however, disappeared after adjustment for HDL-C levels. In addition, carrying out sensitivity analyses only in subjects without an elevated UAE resulted in the same conclusions as obtained from the whole cohort. Therefore, it is unlikely that preferential inclusion of participants with higher UAE substantially impacts the generalizability of our conclusions. On the contrary, the present study is the first to suggest that the association of CEC with incident CVD events may vary according to UAE.

In general, causality is difficult to imply in studies such as the present one. For a more solid validation of CEC as clinical biomarker future studies seem required

Table 4. Characteristics According to Quartiles of HDL Cholesterol (Q1-Q4) and Case-Control Status

	Q1			Q2			Q3			Q4		
	Control	Case	*P* Value	Control	Case	*P* Value	Control	Case	*P* Value	Control	Case	*P* Value
Number	138	130		82	84		75	77		59	60	
Age, y	57.4±11.6	57.8±11.4	0.77	59.3±11.2	59.6±11.0	0.87	58.8±10.5	59.1±10.5	0.85	62.2±8.5	61.4±9.3	0.65
Male sex, n (%)	90 (65.2)	81 (62.3)	0.62	60 (73.2)	63 (75.0)	0.79	56 (74.7)	58 (75.3)	0.93	47 (79.7)	47 (78.3)	0.86
BMI	27.7±4.3	28.6±4.1	0.083	27.0±4.8	27.9±3.8	0.20	26.6±4.1	27.2±4.1	0.33	25.3±3.5	25.2±3.6	0.90
Smoking, n (%)												
Current	71 (51.4)	66 (51.2)	0.94	35 (42.7)	39 (46.9)	0.90	25 (33.3)	26 (33.8)	0.36	21 (35.6)	21 (35)	0.58
Alcohol intake, n (%)			0.90			0.68			0.36			0.58
<10 g/d	112 (81.8)	103 (79.2)		60 (73.2)	63 (75.9)		53 (70.7)	59 (76.6)		32 (54.2)	36 (60.0)	
≥10 g/d	25 (18.2)	27 (20.8)		22 (26.8)	20 (24.1)		22 (29.3)	18 (23.4)		27 (45.8)	24 (40.0)	
Hypertensio, n (%)	65 (47.1)	74 (56.9)	0.11	42 (51.2)	52 (61.9)	0.17	36 (48.0)	52 (67.5)	0.015	30 (50.8)	34 (56.7)	0.58
Diabetes mellitus, n (%)	10 (7.2)	14 (10.8)	0.21	4 (4.9)	3 (3.6)	0.49*	1 (1.3)	6 (7.8)	0.06*	1 (1.7)	1 (1.7)	0.75*
hsCRP, mg/L	2.13 [1.12–4.33]	2.91 [1.40–6.72]	0.045	1.68 [1.02–3.52]	1.97 [0.91–3.33]	0.83	1.21 [0.64–2.02]	1.65 [0.79–3.63]	0.139	1.24 [0.42–2.50]	1.34 [0.76–3.75]	0.028
UAE, mg/24 h	14.11 [7.34–29.50]	15.42 [7.76–39.61]	0.25	10.83 [5.69–24.63]	11.56 [7.28–33.11]	0.70	11.22 [5.96–26.26]	13.96 [8.15–56.28]	0.09	11.16 [7.79–18.74]	12.39 [7.25–20.14]	0.90
eGFR, mL/min per 1.73 m^2	84.8±17.9	86.3±16.2	0.47	88.3±15.7	82.4±16.6	0.026	88.1±15.3	85.5±15.9	0.31	85.4±18.2	84.1±17.5	0.70
Lipid-lowering drug use, n (%)	7 (5.1)	7 (5.1)	0.91	1 (1.2)	4 (4.8)	0.19*	0 (0.0)	5 (6.5)	0.031*	1 (1.7)	4 (6.7)	0.19*
Total cholesterol, mmol/L	5.96±1.31	6.35±1.12	0.011	5.86±1.16	6.24±1.29	0.047	5.59±0.84	6.20±1.07	<0.001	6.00±0.98	6.07±0.93	0.74
LDL cholesterol, mmol/L	4.19±1.23	4.55±1.01	0.013	4.07±1.09	4.28±0.98	0.19	3.72±0.83	4.22±0.94	0.001	3.81±0.91	3.92±0.88	0.52
Non-HDL cholesterol, mmol/L	5.1±1.3	5.3±1.1	0.009	4.7±1.2	5.1±1.3	0.043	4.3±0.8	4.9±1.1	<0.001	4.2±1.0	4.4±1.0	0.57
HDL cholesterol, mmol/L	0.88±0.15	0.88±0.16	0.71	1.12±0.15	1.11±0.14	0.80	1.31±0.18	1.30±0.18	0.81	1.72±0.31	1.68±0.28	0.43
Triglycerides, mmol/L	1.68 [1.30–2.36]	1.89 [1.33–2.44]	0.47	1.31 [0.97–1.86]	1.54 [1.17–2.10]	0.019	1.12 [0.89–1.50]	1.26 [0.97–1.75]	0.06	1.00 [0.77–1.35]	0.97 [0.80–1.20]	0.92
ApoB, g/L	1.23±0.37	1.29±0.33	0.15	1.15±0.33	1.20±0.28	0.31	1.02±0.27	1.16±0.28	<0.001	1.05±0.24	1.04±0.22	0.87
ApoA1, g/L	1.20±0.19	1.16±0.21	0.09	1.31±0.21	1.27±0.19	0.19	1.38±0.23	1.40±0.19	0.43	1.61±0.29	1.56±0.28	0.31
Cholesterol efflux capacity, AU	0.92 [0.79–1.13]	0.79 [0.70–0.94]	0.004	0.91 [0.81–1.03]	0.81 [0.72–0.97]	0.20	1.07 [1.00–1.16]	0.90 [0.82–1.01]	<0.001	1.17 [1.02–1.37]	1.01 [0.93–1.21]	0.008

Data are means±SD or medians (interquartile) ranges. Continuous variables were compared by Student *t* tests. Categorical variables were analyzed by χ^2 test. LDL cholesterol was calculated in 339 (96.6%) cases and 347 (98.0%) controls. Apo indicates apolipoprotein; AU, arbitrary units; BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL, high-density lipoproteins; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoproteins; and UAE, urinary albumin excretion.

*Categorical variables were analyzed by Fisher exact test where appropriate.

investigating if therapeutic modulation of HDL function translates into clinical benefits independent of changes in HDL-C. Ideally, also Mendelian randomization studies would be helpful to attempt to establish causality between reduced efflux and increased CVD risk. However, to initiate such studies more detailed insight is needed on the factors that either improve or decrease the capacity of the complex HDL particle to accept cellular cholesterol. Finally, a fully functional reverse cholesterol transport pathway might also be required to exploit CEC for the full clinical benefit of patients.

In summary, our present data demonstrate that impaired CEC is associated with incident CVD events in the general population independent of HDL-C or apoA-I. This study thus supports the current discussion of advancing the concept of HDL function by indicating that CEC per se is a relatively robust predictor of CVD, at least in initially healthy individuals without clinically manifest CVD.

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Disclosures

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