The Clinical Value of HDL Function Measurements

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CHAPTER 5

HDL function measurements provide clinical information independent of established cardiovascular risk metrics - a cross-sectional study from the PROCAM-CT cohort

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Manuscript in preparation

Abstract

The current concept in high density lipoprotein (HDL) research is shifting from determining HDL cholesterol (HDL-C) levels towards assaying HDL function. However, information regarding the interrelationship between various HDL functionalities as well as their association with classical risk metrics is still limited. Therefore, in the present cross-sectional study three distinct HDL functions (cholesterol efflux, anti-inflammatory and anti-oxidative function) were determined in 162 male participants of the PROCAM-CT study, in which next to the PROCAM risk score also intima media thickness (IMT) and the extent of coronary artery calcification (Agatston score) were available. As expected, HDL cholesterol efflux correlated positively with plasma total cholesterol, HDL-C and apoA1 levels (each $p<0.01$) and negatively with highly sensitive CRP ($p<0.05$). Otherwise, no further associations of HDL function indicators with circulating lipids or inflammatory markers was observed. Importantly, none of the HDL functionalities was significantly associated with the PROCAM cardiovascular risk score, Agatston score or IMT. In this sample of subjects, the different HDL functionalities did not correlate with each other. Combining the individual HDL functions into an overall HDL function score (z-score) did not reveal additional insights. In conclusion, the present study demonstrates that HDL function measurement may provide clinical information independent of a number of classical CVD risk metrics.

Introduction

Atherosclerotic cardiovascular disease (CVD) is the leading cause of morbidity and mortality in developed countries [1, 2]. Although large observational studies repeatedly demonstrated a strong inverse correlation between plasma levels of high density lipoprotein cholesterol (HDL-C) and cardiovascular risk in the general population [1, 3], genetically determined lifelong high or low HDL-C levels did not translate into the expected respective decreased or increased risk for CVD [3-5]. In addition, drugs that substantially increase HDL-C, such as fibrates, niacin or CETP inhibitors, failed to decrease incident CVD events , when used in combination with statin treatment [1, 6]. These results cast doubt on the concept of low HDL-C as a causal cardiovascular risk factor and subsequently shifted the research focus from determining static HDL-C levels towards the assessment of HDL functions [2, 7].

HDL particles combine several functional properties to counter the process of atherogenesis [2, 7, 8]. The most prominently researched of these include cholesterol efflux, the first step in reverse cholesterol transport, as well as anti-oxidative and anti-inflammatory functions. Some [9-11] but not all [12-14] of the initial clinical studies addressing the concept of HDL function indicated that determining cholesterol efflux has the potential to predict incident CVD events independent of HDL-C levels. However, only limited data are available on the interrelationship between various HDL functionalities measured in the same subjects [15-17]. In addition, it remains obscure, how each of the purportedly protective HDL functions relates to other cardiovascular risk markers.

Therefore, the aim of this cross-sectional study was to determine three key functional properties of HDL in selected patients with high estimated risk of CVD, and assess, if HDL function correlates with the PROCAM-risk score, intima media thickness, and the Agatston score as established metrics of cardiovascular risk.

Patients and methods

Study Design and Study Participants

The study protocol was approved by the medical ethics committee of the Physician Chamber of Westphalia-Lippe. Study subjects were recruited from patients attending the outpatient lipid clinic of the University Hospital Münster. They participated after written informed consent had been obtained. The study included 162 male participants, who were extensively characterized with regard to their health status, family history and lifestyle using a standardized questionnaire and underwent physical examination as well as electrocardiography. Anthropometric parameters and blood pressure were recorded in all participants. Study subjects were classified according to their cardiovascular risk calculated using the PROCAM algorithm into the low risk (<10%), medium risk (10% – 20%) and high risk (>20%) groups [18]. Intima-media thickness was assessed in all three risk groups. Only subjects in the high-risk group were admitted to the CT angiography. The exclusion criteria included symptomatic coronary heart disease (angina pectoris or status following heart attack, angioplasty or coronary bypass), AV block, kidney disease (creatinine > 1.2 mg/dL), bronchial asthma, psoriasis or lipid-lowering and anti-hypertensive therapies.

Laboratory measurements

Blood samples were collected from patients by venipuncture; for insertion of the needle, the arm was compressed, after that the compression was removed. Blood samples were centrifuged immediately after blood drawing. Plasma was aliquoted, stored at −70°C and analyzed in a routine hospital laboratory (Center for Laboratory Medicine, University Hospital Münster) using commercially available automated assays. The analytical quality was constantly monitored according to regulations of the German Chamber of Physicians (RILIBÄCK), and the laboratory took part in the external quality assessment schemes. Blood concentrations of total cholesterol and triglycerides were measured using photometric assays, HDL-C and LDL-C were determined with homogenous colorimetric assays and apoA-I and apoB concentrations were measured by turbidimetry. All measurements were performed on the ADVIA 1800 automated random-access chemistry analyzer (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany). The quantification of hsCRP and SAA was performed using enhanced nephelometric assay on a BN-II automated analyzer (Siemens). The measuring ranges for hsCRP and SAA were between 0,016 mg/dL and 12,38 mg/dL and 3,6 mg/L and 256 mg/L, respectively. The intra-assay and interassay variabilities were <4.0% and <5.8% for hsCRP and <5.4% and <6.4% for SAA, respectively. The quantification of IL-6 was done using a chemiluminescence immunoassay (CLIA) on an Immulite 2000 XP automated analyzer (Siemens). The lower limit of detection was 1.0 pg/mL and the intra-assay and interassay variabilities were <4.6% and <7.2%, respectively.

Multislice computed tomography (MSCT)

In order to quantify the total calcium burden of the coronary tree, the helical CT studies were performed in the Institute of Clinical Radiology at the University Hospital Münster using a 4-slice spiral CT scanner (Somatom Volume Zoom, Siemens, Forchheim, Germany). For calcium scanning, a standardized protocol was applied. Four axial slices with 2.5 mm collimation and 0.5 s rotation time were acquired simultaneously (tube voltage: 120 kV, effective tube current: 133 mAS), which allows coverage of the whole heart in 12 s to 15 s in one single breath-hold. Images were reconstructed with the adaptive cardio volume (AVC) algorithm in diastole with 3 mm slice width (increment = 1.5 mm). To achieve optimum image quality retrospective ECG-gating was used and the heart rate of the patient was reduced below 70/min with metoprolol perfusion. With this technique the optimal reconstruction position for every coronary artery with least motion could be achieved. The scans were scored by one experienced reader using the Agaston score, which is a measure of calcium on a coronary CT calcium scan. The score was calculated using a weighted value assigned to the highest density of calcification in a given coronary artery. For identifying calcific lesions a minimum of two 1.03 mm3 with a CT threshold >130 Hounsfield units was used. This weighted score was then multiplied by the area (in square millimeters) of the coronary calcification. The calcium score of every calcification in each coronary artery for all of the tomographic slices was then summed up to give the total coronary artery calcium score.

Determination of the carotid Intima-Media-Thickness (cIMT)

Carotid artery imaging was acquired following a standardized protocol using duplex ultrasound to enable measurement of carotid intima-medial thickness (cIMT) of the common carotid and

bulb segments of the right and left carotid arteries. Images were stored digitally and analyzed in the Institute of Clinical Radiology by trained personnel, who were blinded to disease status of participants, for measurement of far wall cIMT using auto-edge detection software. The distances between the lumen-intima interface and the media-adventitia interface were measured at the bulb and distal common carotid artery.

HDL isolation

Plasma samples were collected from patients and kept unthawed at -80^{oC} until analysis. As previously described [9, 10, 12, 13, 15, 19], apolipoprotein B-containing lipoproteins were precipitated by adding polyethylene glycol (PEG 6000, Sigma, St. Louis, MO, USA) in 10 mM HEPES (pH = 8.0) to plasma (1:2 ratio), followed by a 30 min incubation on ice. After 30 min centrifugation at 2200 g, HDL-containing supernatant was collected, kept on ice, and used directly for all HDL functionality assays. HDL function measurements were carried out in all samples at the same time to limit potential variations due to different assay conditions. All analyses were carried out in duplicates.

HDL-cholesterol efflux capacity

HDL-mediated cholesterol efflux capacity was determined using differentiated macrophages derived from THP-1 human monocytes (ATCC via LGC Promochem, Teddington, UK) exactly as previously published [12, 13, 15]. Briefly, twenty-four hours after differentiation into macrophages by the addition of 100 nM phorbol myristate acetate, macrophages were loaded with 50 µg/mL acetylated LDL and 1 µCi/mL 3H-cholesterol (Perkin Elmer, Boston, MA, USA) for an additional 24 h followed by equilibration for 18 h in RPMI 1640 medium containing 2% bovine serum albumin. Subsequently, 2% individual HDL preparations were added. After 5h, an aliquot of medium was collected, centrifuged to pellet cellular debris, and the effluxed cholesterol label was determined by liquid scintillation counting (Packard 1600CA Tri-Carb, Packard, Meriden, CT, USA). Subsequently, 0.1 M NaOH was added to the cells for 30 min at room temperature, after which the remaining radioactivity within the cells was also measured by liquid scintillation counting. Efflux per well was calculated as the 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 were subtracted to correct for nonspecific, non-HDL-mediated efflux. Results were further normalized to a pool of HDL present on each plate.

HDL anti-inflammatory capacity

The anti-inflammatory capacity of HDL was assessed in vitro using human umbilical vein endothelial cells (HUVEC, provided by the Endothelial Cell Core Facility of the UMCG) isolated and cultured essentially as described previously [15, 17, 19]. Briefly, HUVECs were pre-incubated for 30 min with either 2% HDL or with equal amounts of PBS as a control. Afterwards, 10 ng/mL tumor necrosis factor α (TNF-α, R&D systems, Abingdon, UK) or PBS as control were added. After a 5h incubation, total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA) and quantified with a NanoDrop ND-100 UV-Vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). One µg of total RNA was used for cDNA synthesis with reagents from Invitrogen. Vascular cell adhesion molecule-1 (VCAM-1) mRNA expression levels were

calculated using cyclophilin as housekeeping gene. Results were further normalized to the relative VCAM-1 expression obtained from a pool of healthy control HDL present on each plate [19]. Individual values thus represent fold induction over control values, whereby higher values indicate less suppression of VCAM-1 induction i.e. lower anti-inflammatory capacity.

HDL anti-oxidative capacity

The anti-oxidative function of HDL was assessed by measuring the capacity of individual HDL preparations to inhibit the oxidation of native LDL using a previously published method [15, 20, 21]. Briefly, native LDL was isolated from plasma of a fasted healthy male donor by density gradient ultracentrifugation (1.019<d<1.063). LDL (100 mg/ml protein concentration) was oxidized with 2.5 mM AAPH (2, 2'-azobis [2-amidinopropane] dihydrochloride) in the presence of 2% individual HDL preparations or PBS as control for 10 h at 37 °C. Then thiobarbituric acid reactive substances (TBARS) were measured and used to determine the degree of LDL oxidation as described previously [15, 20, 21)]. The anti-oxidative capacity of HDL was calculated as the difference between the maximally measured TBARS values obtained in the reactions without added HDL and the respective individual values obtained with patient HDL preparations. Values are expressed as percent reduction; higher values indicate better protection of HDL against LDL oxidation.

Statistical analysis

Continuous variables are presented as means and standard error of the mean (SEM), and categorical variables are presented as percentage. Continuous variables with skewed distributions are presented as median [interquartile range] and their log-transformed values were used for analysis. Group comparisons were made using analysis of variance (ANOVA) for continuous variables, and chi-square for categorical variables. Post-hoc analysis for ANOVA was performed using Bonferroni correction. Bivariate Pearson correlation and linear regression were used to analyze associations. To calculate the z-score for the separate HDL functionalities, each individual's measure was subtracted from the mean and then divided by the standard deviation of that variable. (z-score= individual's measure-mean/SD). The composite HDL functionality score was calculated by adding the three z-scores of the three functions. Data analysis was performed using SPSS software (Statistics software version 22, IBM Corp, NY, USA). All reported P values are two-tailed and P<0.05 was considered as statistically significant.

Results

This study determined three major HDL functionalities in 162 male high-risk patients (mean age 57.1 ± 5.08) of PROCAM-CT. First, patient characteristics were stratified according to tertiles of the different HDL functions. Patients that had higher cholesterol efflux (Table 1) showed a significant increase of total cholesterol, LDL-C, apoB, HDL-C, and apoA1. Inflammation markers as well as smoking, diabetes and family history of CVD were comparable among the tertiles of HDL efflux function. Importantly, also calculated PROCAM risk, although taking account of HDL-C, the Agatston score and IMT measurements did not differ according to efflux capacity.

Table 1. Baseline characteristics of the study population stratified by HDL cholesterol efflux.

	1st Tertile (n= 54)	2nd Tertile (n= 53)	3rd Tertile (n= 55)	*P-value*
Age (years)	57.75 ± 0.63	55.91 ± 0.78	57.64 ± 0.65	0.11
10-year PROCAM risk (%)	14.81 ± 1.34	13.94 ± 1.44	12.85 ±0.93	0.54
Agatston score	244.12 ± 121.48	188.46 ± 63.37	424.93 ± 159.06	0.37
IMT (mm)	0.95 ± 0.043	0.88 ± 0.036	0.91 ± 0.038	0.55
IL6 (pg/ml)	2.33 ± 0.36	2.01 ± 0.20	2.34 ± 0.56	0.81
Lp-plA2 (ng/ml)	485.98 ± 13.38	483.31± 14.17	480.85 ± 10.84	0.96
Cholesterol (mg/dl)	219.65 ± 5.80	243.78 ± 4.79*	255.61 ± 5.81 *	*<0.0001*
Triglyceride (mg/dl)	155.37 ± 9.18	186.63 ± 14.25	151.96 ± 10.84	0.09
LDL-C (mg/dl)	143.96 ± 5.42	160.06 ± 5.64	163.85 ± 5.60*	*<0.05*
HDL-C (mg/dl)	45.56 ± 1.32	48.18 ± 1.48	56.37 ± 1.40*†	*<0.0001*
ApoA1 (mg/dl)	135.88 ± 2.57	142.07 ± 2.61	152.7 ± 2.18*†	*<0.0001*
ApoB (mg/dl)	109.08 ± 3.47	124.59 ± 2.95*	125.17 ± 2.96*	*<0.0001*
hsCRP (mg/dl)	0.28 ± 0.03	0.23 ± 0.03	0.31 ± 0.08	0.55
SAA (mg/dl)	9.45 ± 2.15	6.62 ± 1.21	7.82 ± 1.67	0.51
Smoking (%)	31	23	22	0.52
Diabetes (%)	19	15	15	0.78
Family history (%)	29	32	30	0.96
HDL cholesterol efflux	0.96 ± 0.022	1.12 ± 0.004	1.26 ± 0.010	*<0.0001*

Abbreviations: IMT, Intima media thickness; IL6, Interleukin 6; LpplA2, Lipoprotein-associated phospholipase A2; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; ApoA1, Apolipoprotein A1; ApoB, Apolipoprotein B; hsCRP, high-sensitivity C-reactive protein; SAA, Serum amyloid A.

Data are presented as Mean ± Standard Error of Mean (SEM). Differences were tested with one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc analysis. Categorical data are presented as percentage.

* P<0.05, compared to the first tertile.

† P<0.05, compared to the second tertile.

With respect to the anti-inflammatory capacity of HDL (Table 2) none of the determined parameters were altered over the different tertiles of HDL function, including circulating lipids, lipoproteins and apolipoproteins, as well as inflammatory markers or recognized CVD risk factors. Again, neither PROCAM risk score, Agatston score or IMT showed differences among the tertiles of the anti-inflammatory function of HDL.

Table 2. Baseline characteristics of the study population stratified by HDL anti-inflammatory capacity.

	1st Tertile (n= 54)	2nd Tertile (n= 53)	3rd Tertile (n= 55)	*P-value*
Age (years)	56.98 ± 0.69	57.66 ± 0.71	56.68 ± 0.70	0.64
10-year PROCAM risk (%)	14.80 ± 1.36	14.18 ± 1.36	12.76 ± 1.01	0.50
Agatston score	337.19 ± 116.87	315.43 ± 157.59	203.65 ± 83.78	0.73
IMT (mm)	0.95 ± 0.048	0.89 ± 0.038	0.90 ± 0.029	0.48
IL6 (pg/ml)	2.09 ± 0.33	2.80 ± 0.59	1.83 ± 0.21	0.23
Lp-plA2 (ng/ml)	493.67 ± 12.55	487.33 ± 11.34	468.07 ± 14.44	0.34
Cholesterol (mg/dl)	246.36 ± 5.74	239.13 ± 6.09	235.04 ± 5.67	0.38
Triglyceride (mg/dl)	158.47 ± 10.21	180.77 ± 15.64	156.55 ± 10.32	0.31
LDL-C (mg/dl)	164.30 ± 5.21	151.64 ± 6.62	152.94 ± 4.98	0.22
HDL-C (mg/dl)	50.04 ± 1.59	50.32 ± 1.54	49.75 ± 1.52	0.97
ApoA1 (mg/dl)	142.43 ± 2.64	146.30 ± 2.77	142.06 ± 2.50	0.45
ApoB (mg/dl)	125.62 ± 3.29	116.55 ± 3.23	117.75 ± 3.21	0.10
hsCRP (mg/dl)	0.25 ± 0.04	0.32 ± 0.08	0.24 ± 0.04	0.55
SAA (mg/dl)	10.87 ± 2.22	6.08 ± 1.4 5	6.95 ± 1.24	0.11
Smoking (%)	30	25	21	0.56
Diabetes (%)	19	15	15	0.83
Family history (%)	34	34	23	0.34
HDL Anti-inflammatory Capacity (fold increase in VCAM-1 mRNA expression)	0.46 ± 0.01	0.79 ± 0.02	1.35 ± 0.03	*<0.0001*

Abbreviations: IMT, Intima-media thickness; IL6, Interleukin 6; LpplA2, Lipoprotein-associated phospholipase A2; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; ApoA1, Apolipoprotein A1; ApoB, Apolipoprotein B; hsCRP, high-sensitivity C-reactive protein; SAA, Serum amyloid A.

Data are presented as Mean ± Standard Error of Mean (SEM). Differences were tested with one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc analysis. Categorical data are presented as percentage.

* P<0.05, compared to the first tertile.

† P<0.05, compared to the second tertile.

Regarding the anti-oxidative capacity of HDL (Table 3), also none of the lipids, inflammation and clinical risk parameters changed over the different tertiles of this HDL function. The same was true for Agatston score and IMT. For the PROCAM risk score a just significant overall trend was observed, however, not with a consistent direction, reducing the potential clinical meaning of this finding. In addition, we also chose to combine the different HDL functionalities and build an overall HDL function composite score. When using this HDL function score to stratify patients (supplemental table 1), higher total cholesterol and much stronger even higher HDL-C and apoA1 were noted with increasing HDL functionality over the different tertiles. Again, none of the other determined parameters, including PROCAM risk and Agatston score as well as IMT, showed differences over the tertiles of the HDL function score. None of the above described associations and conclusions changed, when patients where stratified into two groups according to the mean of the respective HDL functionalities (data not shown).

Table 3. Baseline characteristics of the study population stratified by HDL anti-oxidative capacity.

	1st Tertile (n= 54)	2nd Tertile (n=53)	3rd Tertile (n= 55)	*P-value*
Age (years)	55.81 ± 0.67	58.02 ± 0.71 *	57.64 ± 0.64	*<0.05*
10-year PROCAM risk (%)	13.32 ± 1.01	11.64 ± 1.07	16.04 ± 1.46 †	*<0.05*
Agatston score	202.83 ± 80.02	417.85 ± 147.20	269.85 ± 135.01	0.49
IMT (mm)	0.87 ± 0.035	0.90 ± 0.037	0.96 ± 0.041	0.21
IL6 (pg/ml)	1.94 ± 0.23	1.78 ± 0.20	2.85 ± 0.58	0.13
Lp-plA2 (ng/ml)	474.26 ± 12.40	475.95 ± 13.95	497.68 ± 12.30	0.34
Cholesterol (mg/dl)	242.40 ± 4.87	231.35 ± 5.67	244.03 ± 6.51	0.26
Triglyceride (mg/dl)	175.60 ± 14.42	139.15 ± 9.82	176.47 ± 11.66	0.06
LDL-C (mg/dl)	159.17 ± 5.99	151.90 ± 4.99	155.95 ± 5.80	0.68
HDL-C (mg/dl)	48.96 ± 1.40	52.17 ± 1.52	49.47 ± 1.64	0.31
ApoA1 (mg/dl)	143.06 ± 2.80	145.81 ± 2.55	142.66 ± 2.56	0.67
ApoB (mg/dl)	123.48 ± 3.20	114.69 ± 3.41	119.76 ± 3.12	0.18
hsCRP (mg/dl)	0.26 ± 0.04	0.22 ± 0.33	0.32 ± 0.73	0.38
SAA (mg/dl)	9.47 ± 1.78	8.59 ± 1.99	6.04 ± 1.38	0.32
Smoking (%)	21	23	31	0.44
Diabetes (%)	21	17	10	0.28
Family history (%)	31	30	29	0.97
HDL Anti-oxidative Capacity (%)	29.19 ± 1.14	41.91 ± 0.28	51.06 ± 0.60	*<0.0001*

Abbreviations: IMT, Intima-media thickness; IL6, Interleukin 6; LpplA2, Lipoprotein-associated phospholipase A2; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; ApoA1, Apolipoprotein A1; ApoB, Apolipoprotein B; hsCRP, high-sensitivity C-reactive protein; SAA, Serum amyloid A.

Data are presented as Mean ± Standard Error of Mean (SEM). Differences were tested with one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc analysis. Categorical data are presented as percentage.

* P<0.05, compared to the first tertile.

† P<0.05, compared to the second tertile

We also analyzed the correlations among HDL functionalities (anti-oxidative, anti-inflammatory and HDL cholesterol efflux capacity) as well as the composite score with measures of lipid profile and inflammatory markers (Table 4). There were significant correlations between HDL cholesterol efflux and total cholesterol (r=0.235, p<0.01), and as expected with HDL-C (r=0.321, p<0.01) and apoA1 levels (r=309, p<0.01). We found no significant correlations between the anti-oxidant and anti-inflammatory functions of HDL with lipid profile, while the HDL function composite score was also significantly correlated with plasma total cholesterol (r=0.236, p<0.005), HDL-C (r=0.203, p<0.05) and apoA-1 levels (r=0.198, p<0.05). With respect to inflammatory markers, neither individual nor composite HDL functionalities were correlated with interleukin-6 (IL6) or Lp-PLA2. Cholesterol efflux was inversely associated with hs-CRP and a negative correlation was found for the anti-oxidative function of HDL with SAA, but none of the other functions showed respective significant associations. Furthermore, since we previously reported that in patients with an acute MI impaired cholesterol efflux correlated with a diminished anti-inflammatory HDL function [15], we also assessed potential relationships of the different HDL functions with each other in the current study. However, no such correlation was detected among the HDL functionalities in the currently investigated patient population (data not shown). In addition, a linear regression analysis was performed to assess if individual HDL functionalities or HDL composite score are related to the established risk markers, PROCAM score, Agatston score, or IMT (Table 5). However, no significant associations

were detected. Also, when patients were stratified by tertiles of 10-year PROCAM estimated risk, Agatston score and IMT, no differences in either individual HDL functionalities or HDL function composite score were found among the respective tertiles (data not shown).
Finally, the cohort was also stratified by HDL-C levels. As expected, cholesterol efflux capacity significantly increased over increasing tertiles of HDL-C (supplemental table 2), while for the other HDL function parameters and the HDL function composite score no significant associations were found. When looking at the main CVD risk metrics determined in this study (supplemental table 3), PROCAM risk score decreased substantially with increasing HDL-C levels, reflecting the inclusion of HDL-C in the calculatation of that score [18]. IMT on the other hand showed no association with HDL-C. Surprisingly, the Agatston score, reflecting prevalent coronary calcification, was significantly increasing with increasing tertiles of HDL-C.

Table 4. Correlation between HDL functions and the HDL function composite score and plasma lipid profiles as well as inflammatory markers

	Cholesterol		Triglyceride		LDL-C		HDL-C		Apo A1		Apo B		IL6		Lp-plA2		hsCRP		SAA	
	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*	r	*p*
Anti-oxidative	-0.008	0.92	0.75	0.34	-0.081	0.31	-0.038	0.63	-0.041	0.61	-0.099	0.21	0.142	0.07	0.084	0.29	0.8	0.31	-0.15	0.05
Anti-inflammatory	-0.102	0.20	-0.029	0.72	-0.081	0.30	-0.035	0.68	-0.059	0.46	-0.104	0.19	-0.050	0.53	-0.086	0.28	-0.04	0.53	-0.03	0.63
HDL cholesterol efflux	0.235**	*<0.01*	0.009	0.90	0.103	0.19	0.321**	*<0.01*	0.309**	*<0.01*	0.136	0.08	-0.044	0.57	-0.045	0.56	-0.17*	*<0.05*	-0.06	0.45
HDL composite score	0.236***	*<0.005*	0.057	0.48	0.103	0.20	0.203*	*<0.05*	0.198*	*<0.05*	0.148	0.06	0.087	0.28	0.065	0.41	0.058	0.46	-0.091	0.26

Abbreviations: LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; apoA-1, Apolipoprotein A1; ApoB, Apolipoprotein B.
*p-value <0.05
**p-value <0.01
***p-value <0.005

Table 5. Linear regression analysis of HDL functions and composite score with estimated 10-year PROCAM risk, IMT and Agatston score.

	10-year PROCAM risk		IMT		Agatston score	
	β	*p-value*	β	*p-value*	β	*p-value*
Anti-oxidative	0.112	0.099	0.003	0.222	0.019	0.196
Anti-inflammatory	-1.687	0.345	-0.060	0.299	-0.562	0.243
HDL cholesterol efflux	-4.606	0.385	-0.063	0.706	-0.221	0.851
HDL composite score	1.143	0.351	0.044	0.272	0.416	0.181

Abbreviations: β, Regression coefficient; IMT, Intima media thickness.

Discussion

The results of this cross-sectional study demonstrate that three different, pathophysiologically relevant anti-atherosclerotic functions of HDL particles are not associated with other established cardiovascular risk metrics in a high CVD risk patient cohort. We interpret these data to mean that HDL function assessment has the potential to provide clinical information independent of classical cardiovascular biomarkers or scores. Clinical trials attempting to raise HDL-C with the intention to lower incident CVD events as well as genetic association studies failed to provide consistent evidence for a causal association of HDL-C with future incident CVD [1-3, 8]. Due to such disappointing results the concept in cardiovascular research shifted from looking merely at static HDL-C levels to measuring potentially anti-atherosclerotic functions of HDL (2, 7, 8). Although with respect to cholesterol efflux some [9-11] but not all [12-14] studies indicated a prospective value of this HDL function metric over HDL-C determinations, data on other HDL functionalities is still rather limited. With respect to the anti-oxidative function of HDL a previous study from our group indicated that this HDL function metric is not useful to predict cardiovascular or all-cause mortality in renal transplant recipients [21]. On the other hand, it was reported that an impaired anti-oxidative activity of HDL predicts cardiovascular events in patients with heart failure [22]. Regarding the anti-inflammatory capacity of HDL, only one smaller scale study is available from our group suggesting that in patients with an acute MI, this HDL function could be useful in the prediction of recurrent major adverse cardiovascular events (MACEs) [17]. The present study could not detect an association between HDL function and other metrics of CVD risk. Coronary artery calcification (CAC) and the Agatston score as its quantitative measure as well as intima-media thickness (IMT) are both associated with an increased risk of CVD [23, 24]. Several studies suggested that the CAC score can be useful to predict incident MI and cardiovascular death and such has an added value beyond traditional risk factors for risk estimation purposes [25-27]. Also in PROCAM, it was shown that the assessment of CAC potentially provides further information additive to traditional risk factors [28]. Based on such data CAC is included in clinical decision making with respect to statin eligibility in the 2013 American College of Cardiology/American Heart Association guidelines on risk assessment [29)]. Recent results from the Dallas Heart Study indicated that adding cholesterol efflux to the CAC score improves risk prediction [30]. In our cross-sectional study there was no association between the Agatston score and cholesterol efflux as well as other HDL function parameters. These data support in principle the concept that HDL function measurements provide information independent of established risk assessment tools. More prospective studies would be required

to define the quality and the added value of such determinations. On the other hand, the clinical usefulness of IMT in predicting cardiovascular disease appears less certain and meta-analyses indicated that adding IMT measures alone only minimally improves risk stratification [31]. These observations might be due to the relatively limited possibility to standardize the measurement procedure that largely depends on the observer, choice of anatomical location for the measurement and technical equipment [32]. It was e.g. shown that IMT did not correlate with the PROCAM risk score [33]. This finding together with the lack of association between HDL function metrics and IMT in our current study would again support our interpretation that HDL function measurements provide additional clinical information independent of classical cardiovascular risk assessment tools. However, a previous cross-sectional study noticed a significant negative correlation between cholesterol efflux capacity and IMT [34]. The reason for this discrepancy is currently unclear, more work, especially prospective studies, seem warranted to come to improved risk assessment algorithms. We consider a strength of our work that only few studies until now investigated more than one HDL function in a clinical setting together in the same patients [15-17]. Moreover, a large number of clinically established risk metrics were included in the comparative analysis along with HDL function metrics. Also, potential limitations of the present study merit consideration. This was a cross-sectional study in a somewhat limited number of male patients with a high PROCAM risk score. Therefore, the extrapolation of the present findings to females [35] or the general population is not straightforward. In addition, our results do not allow conclusions on the causative association of HDL function with atherosclerotioc CVD. Prospective outcome studies comparing the clinical validity of different risk markers, including HDL function, would be warranted to address this specific question. A precedence of such an approach has recently been reported for the JUPITER trial [36], where a comparison of different biomarkers revealed that the strongest predictive factor for incident CVD was the HDL particle number and not the cholesterol efflux as the only included HDL function metric. Some methodological issues should also be pointed out. In general, dynamic HDL function tests and the interpretation of their results strongly depend on the specific assay conditions applied and such assays are not yet standardized in the same rigorous fashion as routine clinical chemistry determinations. In the case of HDL cholesterol efflux, for instance, the cell system used in the experiment (human vs. murine cells, cells equilibrated with label vs. macrophage foam cells), and the isolation method of HDL may affect the results [2]. Especially, the HDL isolation method should be taken into account. In the present study, HDL was isolated using ApoB-depleted plasma, an established, widely used procedure [9, 10, 12, 13, 34], which is faster than sequential ultracentrifugation and has the advantage of preserving preβ-HDL but potentially includes other factors in the HDL preparations. However, for the assays used in the present work, we have previously documented that a large fraction of the biological activity is attributable to the presence of HDL in the samples [15]. In summary, the findings of the present study are consistent with the notion that results from HDL function assays provide independent clinical information in addition to a number of established CVD risk metrics, including the PROCAM score, IMT and measures of coronary calcification. Further prospective studies seem warranted to establish the clinical value of the respective different HDL functions in comparison to a number of other, classical risk markers for the prediction of incident CVD in the general population.

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Supplement

Supplemental table 1. Baseline characteristics of study population stratified by HDL composite score.

	1st Tertile (n= 54)	2nd Tertile (n=53)	3rd Tertile (n= 55)	*P-value*
Age (years)	56.53 ± 0.70	56.87 ± 0.75	58.02 ± 0.63	0.282
10-year PROCAM risk (%)	12.85 ± 1.05	14.73 ± 1.39	14.12 ± 1.33	0.565
Agatston score	188.50 ± 79.98	287.80 ± 108.72	390.81 ± 170.08	0.532
IMT (mm)	0.91 ± 0.03	0.89 ± 0.04	0.94 ± 0.04	0.647
IL6 (pg/ml)	1.98 ± 0.22	2.38 ± 0.36	2.34 ± 0.57	0.749
Lp-plA2 (ng/ml)	476.34 ± 14.06	496.19 ± 13.75	477.06 ± 10.86	0.472
Cholesterol (mg/dl)	231.62 ± 4.71	235.27 ± 5.87	252.44 ± 6.51*†	*<0.05*
Triglyceride (mg/dl)	149.77 ± 9.23	176.53 ± 14.80	170.28 ± 12.67	0.284
LDL-C (mg/dl)	154.75 ± 4.67	153.20 ± 6.15	159.78 ± 6.17	0.694
HDL-C (mg/dl)	47.91 ± 1.33	48.08 ± 1.73	54.07 ± 1.44 *†	*<0.005*
ApoA1 (mg/dl)	139.60 ± 2.53	140.80 ± 2.96	150.43 ± 2.23 *†	*<0.01*
ApoB (mg/dl)	117.94 ± 3.10	117.78 ± 3.47	123.17 ± 3.18	0.409
hsCRP (mg/dl)	0.28 ± 0.04	0.24 ± 0.04	0.30 ± 0.08	0.699
SAA (mg/dl)	8.13 ± 1.76	8.32 ± 1.73	7.52 ± 1.69	0.943
Smoking (%)	28	40	33	0.407
Diabetes (%)	28	44	28	0.393
Family history (%)	32	28	40	0.533
HDL composite score	-0.6789 ± 0.057	0.0812 ± 0.016*	0.5816 ± 0.049*†	*<0.0001*

Abbreviations: IMT, Intima-media thickness; IL6, Interleukin 6; Lp-plA2, Lipoprotein-associated phospholipase A2; LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; ApoA1, Apolipoprotein A1; ApoB, Apolipoprotein B; hsCRP, high-sensitivity C-reactive protein; SAA, Serum amyloid A.
Data are presented as Mean ± Standard Error of Mean (SEM). Categorical data are presented as percentage. Differences were tested with one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc analysis, or chi square.
* P<0.05, compared to the first tertile. † P<0.05, compared to the second tertile.

Supplemental table 2. HDL functionalities among tertiles of HDL-cholesterol.

	1st Tertile	2nd Tertile	3rd Tertile	*p-value*
Anti-oxidative	42.01 ± 1.63	41.11 ± 1.36	40.40 ± 1.37	0.746
Anti-inflammatory	0.87 ± 0.06	0.85 ± 0.05	0.88 ± 0.06	0.938
HDL cholesterol efflux	1.05 ± 1.02	1.14 ± 1.10*	1.17 ± 1.14*	*<0.0001*
HDL composite score	-0.15 ± 0.07	0.05 ± 0.08	0.09 ± 0.10	0.082

Data are presented as Mean ± Standard Error of Mean (SEM). Differences were tested with one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc analysis.
* P<0.05, compared to the first tertile.
† P<0.05, compared to the second tertile.

Supplemental table 3. Cardiovascular risk metrics among tertiles of HDL-cholesterol.

	1st tertile	2nd tertile	3rd tertile	*p-value*
10-Year PROCAM risk	17.90 ± 1.23	14.65 ± 1.25	8.55 ± 0.82*†	*<0.0001*
IMT	0.89 ± 0.04	0.88 ± 0.03	0.99 ± 0.05	0.120
Agatston	84.08 ± 32.82	273.32 ± 74.9	771.30±329.85*†	*<0.005*

Data are presented as Mean ± Standard Error of Mean (SEM). Differences were tested with one-way analysis of variance (ANOVA), followed by Bonferroni post-hoc analysis.
* P<0.05, compared to the first tertile.
† P<0.05, compared to the second tertile.

Abbreviations: LDL-C, Low-density lipoprotein cholesterol; HDL-C, High-density lipoprotein cholesterol; apoA-1, Apolipoprotein A1; ApoB, Apolipoprotein B.
*p-value <0.05
**p-value <0.01
***p-value <0.005