Published in final edited form as:

Atherosclerosis. 2018 November ; 278: 278–285. doi:10.1016/j.atherosclerosis.2018.09.032.

High density lipoprotein proteome is associated with cardiovascular risk factors and atherosclerosis burden as evaluated by coronary CT angiography

Scott M. Gordon^{1,*}, Jonathan H. Chung², Martin P. Playford², Amit K. Dey², Denis Sviridov¹, Fayaz Seifuddin³, Yun-Ching Chen³, Mehdi Pirooznia³, Marcus Y. Chen⁴, Nehal N. Mehta², and Alan T. Remaley^{1,*}

¹Lipoprotein Metabolism Section, National Heart, Lung, and Blood Institute, Bethesda, Maryland, USA.

²Section of Inflammation and Cardiometabolic Diseases, National Heart, Lung, and Blood Institute, Bethesda, Maryland, USA.

³Bioinformatics and Computational Biology Core Facility, National Heart, Lung, and Blood Institute, Bethesda, Maryland, USA.

⁴Advanced Cardiovascular Imaging Laboratory, National Heart, Lung, and Blood Institute, Bethesda, Maryland, USA.

Abstract

Background and aims: High density lipoprotein cholesterol (HDL-C) is associated with risk of cardiovascular disease (CVD); however, therapeutic manipulations of HDL-C have failed to reduce CVD events. This suggests that HDL-C and the atheroprotective capacity of HDL are not directly linked. The goal of this study was to evaluate the relationships between HDL-bound proteins and measures of atherosclerosis burden and HDL function.

Methods: The HDL proteome was analyzed using mass spectrometry in 126 human subjects, who had undergone coronary computed tomography angiography (CCTA) to quantify calcified (CB) and non-calcified (NCB) atherosclerosis burden. Partial least squares regression analysis was used to evaluate associations between HDL-bound proteins and CB, NCB, or cholesterol efflux capacity (CEC).

To whom correspondence should be addressed: Scott M. Gordon, National Heart, Lung, and Blood Institute, National Institutes of Health, 10 Center Drive, Building 10 Rm. 5D09, Bethesda, MD 20892, Phone: 301-496-5114, scott.gordon@uky.edu.

Author contributions: S.M.G and A.T.R conceived and designed the study. S.M.G, D.S., and M.P.P performed experiments. J.H.C. and A.K.D. performed non-calcified plaque burden analysis. F.S., Y.C., and M.P. performed statistical analyses. N.N.M. and M.Y.C. performed participant recruitment and CT scan acquisition. S.M.G. and A.T.R wrote the manuscript. All authors have approved the final article.

Conflict of Interest: The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Results: Significant overlap was found among proteins associated with NCB and CEC. Proteins that were associated with NCB displayed an inverse relationship with CEC, supporting a link between this protective function of HDL and clinical plaque burden. CB was associated with a set of proteins mostly distinct from NCB and CEC. When CVD risk factors were evaluated, BMI had a stronger influence on important HDL proteins than gender, age, or HDL-C. Most HDL proteins associated with function or atherosclerosis burden were not significantly correlated with HDL-C.

Conclusions: These findings indicate that the HDL proteome contains information not captured by HDL-C and, therefore, has potential for future development as a biomarker for CVD risk. Additionally, the proteome effects detected in this study may provide HDL compositional goals for evaluating new and existing HDL-modification therapies.

Keywords

High Density Lipoprotein; Cholesterol efflux; Computed tomography angiography; Proteomics; Atherosclerosis

Introduction

Atherosclerosis is a progressive process of deposition of low density lipoproteins (LDL) and the subsequent inflammatory response, which eventually results in the formation of complex lesions that are the culprit of many cardiovascular events [1]. The cholesterol content of high density lipoprotein (HDL-C) exhibits a strong inverse relationship with cardiovascular events in large population studies; however, HDL-C is not necessarily directly related to the capacity of HDL to attenuate this process. This is evident from the failure of many HDL-C raising therapies to prevent cardiovascular disease [2] and the lack of association between HDL-C modulating genes and cardiovascular outcomes in Mendelian randomization studies [3]. HDL transports over 90 different proteins [4] that are potentially involved in a variety of disease processes relevant to atherosclerosis, but besides apoA-I, the most abundant protein component of HDL [5], these other HDL-associated proteins have not been systematically examined as biomarkers for cardiovascular disease [6].

Evaluation of atherosclerotic lesions in high-risk patients is routinely performed by coronary computed tomography angiography (CCTA) [7, 8]. Total atherosclerosis burden is comprised of calcified and non-calcified plaque. Calcified plaque burden (CB) has well-established prognostic value for predicting cardiovascular outcomes [9]. Although not clinically measured because of technical difficulty, non-calcified plaque burden (NCB) is considered to be more strongly linked to acute events [10], due to a higher likelihood for rupture and thrombosis compared to calcified plaque. NCB may also represent a reversible stage in the atherosclerotic process. Regression of non-calcified lesions has been demonstrated by suppression of systemic inflammation [11] or HDL infusion [12–14].

HDL-mediated cholesterol efflux capacity (CEC) has been associated with carotid intima-media thickness, angiographically confirmed coronary artery disease, and cardiovascular outcomes, and has been shown to be superior to HDL-C for predicting cardiovascular events [15, 16]. This functional assay of HDL, which measures the capacity of HDL to remove excess cellular cholesterol, is difficult to perform and to standardize as a routine diagnostic

test, because it involves the use of cell lines and typically radioisotopes. Additionally, multiple distinct atheroprotective functions, besides cholesterol efflux, have been described for HDL and are likely mediated or at least modulated by HDL-bound proteins [17]. For the most part, the relationships between HDL-bound proteins and HDL function are not well understood. Ultimately, HDL function must be derived from its composition; therefore, the HDL proteome may provide an integrated view of the functional potential of HDL, which should be more readily translatable into a routine diagnostic test than a cell-based functional assay.

The goal of this study was to identify components of the HDL proteome that are likely to be important for HDL function and relevant to cardiovascular disease risk. We analyzed the HDL proteome in 126 subjects with varying degrees of atherosclerosis burden, calcified and non-calcified, and used partial least squares regression analysis to rank HDL-bound proteins based on the strength of their association with burden measures or HDL function. Furthermore, we evaluated the influence of traditional cardiovascular risk factors on the HDL proteome.

Materials and methods

Study design and subjects

One hundred and twenty six subjects were included in this study. These included both males and females that were at least 18 years of age and with clinical indication for a coronary CT angiography. There were no additional inclusion criteria. Criteria for exclusion were current pregnancy and poor renal function. The study protocol was approved by the National Heart, Lung, and Blood Institute's Institutional Review Board and all subjects provided informed consent at enrollment. [ClinicalTrials.gov](https://clinicaltrials.gov/ct2/show/study/NCT01621594) identifier: NCT01621594.

Atherosclerosis burden assessment

Coronary CT scans were performed in a 320-detector row Aquilion ONE ViSION system (Toshiba). Coronary artery calcification was quantified, using the Agatston approach and Society of Cardiovascular Computed Tomography (SCCT) methodology [7, 18, 19]. Non-calcified coronary plaque was quantified in the left anterior descending, left circumflex, and right coronary arteries, by a single blinded reader, using QAngio CT (Medis). These measurements were averaged to determine average non-calcified burden.

HDL proteome analysis

Blood was collected from subjects at the time of atherosclerosis burden assessment. Blood was drawn into serum collection tubes and allowed to clot for 30 minutes at room temperature prior to centrifugation at 1,750g for 15 minutes. Serum aliquots were stored at -80°C until processing. HDL was isolated by size-exclusion chromatography followed by purification on lipid binding resin and proteomics analysis based on previously published methods [20]. For each individual, serum (370 uL) was applied to two Superose 6 columns arranged in series on an Akta Pure chromatography system (GE Healthcare) at a flow rate of 0.5 mL/min in Tris buffer (10 mM Tris-HCl; 150 mM NaCl; 0.5 mM EDTA; 0.01% sodium azide; pH = 7.4). Eluate was collected as 0.5 mL fractions and HDL

distribution profile was determined by measuring phospholipid content with an enzymatic assay. Fractions containing HDL were pooled and phospholipid, total cholesterol, free cholesterol, and triglyceride content were quantified by enzymatic assays. For proteomics, pooled HDL fractions were applied to a phospholipid binding resin and washed with 25 mM ammonium bicarbonate to remove contaminating proteins that are not lipid-associated. HDL-bound proteins were digested with sequencing grade trypsin (1.5 ug; Promega) overnight at 37°C. Peptides were chemically reduced by addition of dithiothreitol and carbamidomethylated by addition of iodoacetamide then desalted, using C₁₈ ZipTip (Millipore) prior to analysis by mass spectrometry. Peptides were re-suspended in 0.1% formic acid and analyzed on an Orbitrap Elite mass spectrometer (Thermo Scientific). Samples underwent on-line reverse phase separation over a 65 minute gradient. Blank runs were performed between every sample to prevent carryover. MaxQuant software was used to facilitate protein identification and label-free quantification of precursor ion intensities for detected proteins. The Andromeda search engine was used to search a custom HDL proteome database (95 commonly identified HDL proteins). First and Main search peptide tolerances were set at 20 and 4.5 ppm, respectively. Methionine oxidation was set as variable modification and cysteine carbamidomethylation was a fixed modification. Maximum missed trypsin cleavage sites was set at 2. False discovery rates at both peptide and protein level were set to 1%. These settings resulted in the identification and label-free quantification of 72 proteins.

Measurement of cholesterol efflux capacity

HDL cholesterol efflux capacity assays were performed based on published methods, using J774 cells derived from a murine macrophage cell line [21]. Briefly, the J774 cells were plated and radiolabeled with 2 µCi of ³H-cholesterol/mL. ATP-binding cassette transporter A1 (ABCA1) was up-regulated by means of a 16-hour incubation with 0.3 mmol/L 8-(4-chlorophenylthio)-cAMP. Plasma was depleted of apoB containing lipoproteins by polyethylene glycol (PEG) precipitation [22]. 20µl PEG solution (20% PEG 8000 in 200 mM glycine buffer pH=7.4) was incubated with 50 µl serum for 20 minutes at room temperature. The mixture was centrifuged for 30 minutes at 4 C; the HDL containing supernatant was collected. PEG precipitated plasma was added to the efflux medium (final concentration 2.8%) for 4 hours. To quantify the efflux of radioactive cholesterol from the cells, we used liquid scintillation counting. Efflux was calculated by using the following formula: (µCi of ³H-cholesterol in subject's plasma-containing media - µCi of ³H-cholesterol in plasma-free media / µCi of ³H-cholesterol in media containing reference plasma pool - µCi of ³H-cholesterol in plasma-free media). Adjustment of cholesterol efflux percentage to a healthy reference pool has been previously described [15, 16]. The pooled plasma was obtained from five healthy volunteers. All assays were performed in duplicate.

Partial least squares (PLS) regression analysis

Partial Least Squares Regression (PLSR) analysis was performed using the pls package in R [<https://cran.r-project.org/web/packages/pls/vignettes/pls-manual.pdf>]. Three models were constructed to include: all proteins + Log₁₀(CB), all proteins + NCB, and all proteins + CEC. All the scaled proteins (intensities) were used as training data in the partial least

squares regression model. Coefficients for each protein were extracted from the pls training model. Variable Importance scores were calculated using the same model.

Results

Cardiovascular risk factors and plaque burden measures

This study included 126 subjects referred for CCTA based on suspected coronary artery disease. Clinical characteristics and atherosclerosis burden results are presented in Table 1. This cohort is 44% female with an average age of 61 years and is generally normolipidemic or receiving statin treatment (43% on statin). CCTA revealed that 70% of subjects had CB greater than 0 (average Agatston score: 281.3 ± 616.5). Linear regression analysis was used to evaluate the relationships between burden measures and cardiovascular risk factors. In bivariate analyses, comparing burden with individual clinical variables, age, gender, and statin use were significantly associated with CB (Table 2A). The average NCB was $0.0109 \pm 0.0042 \text{ mm}^2$. NCB was significantly associated with BMI, gender, HDL-C, and age (in order of effect size) but was not associated with LDL-C or triglyceride, possibly due to the strong influence of statins on these variables (Table 2A). In a multivariate model for NCB, associations with age and HDL-C are no longer present and an effect of statin use emerges (Table 2B). The relatively large effect of HDL-C observed in the bivariate analysis was likely lost due to the strong relationship between BMI and HDL-C ($\beta = -0.34$, $p < 0.0001$). Additional demographic and atherosclerosis burden distribution data are available in the supplemental material (Supplementary Fig. 1 A-C).

HDL proteome analysis

Proteomics analysis identified a total of 72 HDL-associated proteins that were detected in at least 75% of subjects (Supplementary Table 1). To identify HDL-proteome components associated with atherosclerosis burden or HDL function, we used partial least squares (PLS) regression analysis. This statistical approach performs well with high-dimension datasets containing large numbers of variables and relatively few observations, such as those from proteomics and genomics studies [23]. Proteins were ranked according to their variable importance projection (VIP) scores (Supplementary Table 2).

HDL proteins associated with calcified plaque burden.

PLS analysis identified 13 proteins that are significantly associated with CB (Fig. 1A). Most were negative, or inverse, relationships (9 of 13). The highest ranked protein was cathelicidin antimicrobial peptide (CAMP). Although this protein is not commonly discussed with regard to HDL, plasma levels of CAMP have been demonstrated to correlate with HDL-C [24] and atherosclerosis [25]. The next highest ranking proteins with negative association were gelsolin (GELS), kininogen-1 (KNG1), and paraoxonase 1 (PON1). Proteins positively associated with CB included: apolipoprotein A-IV (APOA4), vitamin D binding protein (VTDB), alpha-2- macroglobulin (A2MG), and apolipoprotein C-II (APOC2).

HDL proteins associated with non-calcified plaque burden.

NCB was significantly associated with 15 proteins by PLS analysis (Fig. 1B). For NCB, most proteins were negatively correlated (9 of 15). The top-ranked negatively associated proteins included: apolipoprotein A-I (APOA1), apolipoprotein F (APOF), antithrombin III (ANT3), and apolipoprotein C-I (APOC1). Positively associated proteins were serum amyloid A1 (SAA), immunoglobulin heavy constant alpha 1 (IGHA1), and four proteins of the complement cascade: complement factor B (CFAB), complement C2 (CO2), complement C3 (CO3), and complement C1s subcomponent (C1S).

HDL protein composition is associated with cholesterol efflux.

CEC was associated with 16 proteins (Fig. 1C) and, in contrast to atherosclerosis burden measures, most were positive in direction (10 of 16). Proteins that were associated with increased efflux included many of the apolipoproteins: A-I, C-III, A-II, A-IV, and C-I. The top-ranking negatively associated proteins were immunoglobulin heavy constant gamma 1 (IGHG1), complement C4-A (CO4A), CO2, and CO9.

It is noteworthy that many of the proteins correlated with CEC were also correlated with NCB, but not with CB (Fig. 1D). When comparing the directionality of these relationships, we found that, in every case (7/7 proteins; $p = 0.008$), proteins that were positively associated with NCB were negatively associated with CEC and *vice versa* (Table 3). This observation supports a possible relationship between HDL protein composition and particle function.

Cardiovascular risk factors and the HDL proteome

The factors which drive HDL protein composition are not well understood. We hypothesized that traditional clinical risk factors for cardiovascular disease can influence HDL composition. This would suggest that the composition of HDL, and subsequent functional modulation, is a mechanistic effector of commonly used biometric biomarkers. We, therefore, evaluated the relationships between HDL-bound proteins and gender, age, BMI, HDL-C, and statin use in a multivariate regression model. Among proteins that were significantly associated with CB, NCB, or CEC (total = 34): 4 were associated with gender, 6 with age, 15 with BMI, and 9 with HDL-C, and 5 with statin use (Table 3 and Supplementary Table 3). Female gender was most strongly associated with decreased RET4 ($\beta = -0.31$, $p = 0.0009$). Age was most strongly associated with decreases in CAMP ($\beta = -0.27$, $p = 0.0026$) and the antioxidant protein PON1 ($\beta = -0.27$, $p = 0.003$). BMI was most strongly associated with elevated SAA ($\beta = 0.41$, $p < 0.0001$). As expected, the strongest association with HDL-C was APOA1 ($\beta = 0.44$, $p < 0.0001$). Overall, this analysis suggests that, among the evaluated risk factors, BMI has the greatest overall impact on the protein composition of HDL (Fig. 2). It is worth noting that HDL-C is correlated with surprisingly few of these proteins, supporting the hypothesis that HDL functions relevant to CVD protection may not be directly related to HDL-C.

Discussion

The results presented here provide evidence that the protein composition of HDL is associated with atherosclerosis burden and has the potential to be developed as a marker for evaluating novel HDL- targeting therapies and for predicting cardiovascular disease risk. An association between HDL protein composition and cholesterol efflux was also observed and proteins were identified that were associated with either suppression or enhancement of cholesterol efflux by HDL. These results support our overall hypothesis that the functionality of HDL, such as CEC, is associated with protein content and that this may translate to an effect on atherosclerosis burden. Finally, we show that common cardiovascular risk factors can influence the HDL proteome, particularly BMI. It seems likely that traditional risk factors (i.e. gender, age, BMI) are the impetus for physiological effects, including modification of the HDL proteome, that play mechanistic roles in atherosclerosis development (Fig. 2).

CB is a well documented independent predictor of cardiovascular disease risk and is strongly associated with age and gender [7]. NCB is an emerging risk factor whose relationship with traditional risk factors is less well understood. It is interesting that LDL-C and TG were not associated with NCB, even after adjustment for statin use, considering that these lipids are thought to directly contribute to fatty streak and plaque formation. Although statin use itself was associated with both CB and NCB in multivariate analyses. Statin use was positively associated with CB and negatively associated with NCB, findings consistent with the literature [26, 27]. The association between HDL-C and NCB is lost after adjustment for BMI. This supports a known, but not well understood, relationship between BMI and HDL [28, 29] and is consistent with our observed influence of BMI on HDL proteome.

Of the 72 measured proteins on HDL, two were significantly associated with CB, NCB, and CEC. APOA4, is positively associated with CEC and negatively with NCB, implying a protective impact by improving function and reducing burden. However, APOA4 also showed the strongest positive association with CB. This is consistent with the notion that NCB and CB occur by different mechanistic processes, however, this result poses uncertainty as to whether APOA4 should be considered as a protective or proatherogenic apolipoprotein. On the other hand, GELS was all-around “protective” by association with higher CEC and lower burden measures. Plasma GELS has recently been described as an independent factor associated with decreased risk of aortic arch calcification [30].

PON1 is a commonly studied protein on HDL and is positively associated with CEC, in this study. One possible mechanism for this could be by prevention of oxidative modification to APOA1, which has been demonstrated to reduce efflux capacity [31] and activation of lecithin cholesterol acyl transferase (LCAT) [32]. PON1 is also associated with decreased CB, likely by reducing inflammation and oxidative stress in vessel wall [33–35].

A significant overlap was found in proteins associated with NCB and CEC. In addition to APOA4 and GELS, these included several apolipoproteins, namely APOA1, APOA2, and APOC1, as well as the serine protease inhibitor ANT3 and the complement protein CO2. All

of these proteins displayed opposite associations with CEC and NCB (i.e. if positively associated with one, then negatively associated with the other). This is strongly supportive of a mechanistic link between CEC and NCB. A concept easily rationalized by the fact that CEC is an activity that involves removal of lipids from the site of plaque formation. SAA is known to significantly influence HDL function, including cholesterol efflux and its anti-inflammatory functions [36–38]. SAA was significantly associated with NCB; however, it slightly missed our statistical criteria for significance with CEC (Fig. 1C, **pink symbol**).

The mechanism by which many of these proteins modulate CEC is not clear. One possibility is that, by binding to HDL, they influence the affinity of the particle for cell membranes or cholesterol efflux transporters (e.g. ABCA1, ABCG1, or SR-B1). This could occur as a result of direct influence or indirectly perhaps by blocking important sites on apoA-I. Some HDL associated proteins found by our analysis were associated with NCB but not with CEC, indicating that they may be involved in alternate atheroprotective functions of HDL, such as anti-inflammatory and anti-oxidative activities, or preservation of normal endothelial function [6].

Most proteins that were associated with CB were not associated with NCB or CEC. This suggests that the pathophysiology of coronary artery calcification is distinct from that of soft plaque accumulation. Additionally, the functions of HDL, which influence CB, may be different from those that influence NCB. CAMP showed a strong negative association with CB. This is an antimicrobial peptide expressed by various cell types and is upregulated during an immune response. It has been detected in human atherosclerotic plaques [25] and its possible relevance to atherosclerosis may lie in the ability of this peptide to inhibit macrophage activation and modulate the immune response through a variety of regulatory activities [39]. VTDB shows the second strongest positive association with CB (second to APOA4 discussed above). This protein binds 1,25-hydroxyvitamin D (1,25[OH]D, the active form) and facilitates binding to the vitamin D receptor. It is unknown whether HDL-bound VTDB is carrying vitamin D or not. Vitamin D regulates blood calcium levels and deficiency is correlated with cardiovascular disease and arterial calcification [40, 41]. In the Multi-Ethnic Study of Atherosclerosis (MESA), VTDB was associated with cardiovascular events [42].

Vaisar et al. have previously reported a comparison of HDL proteomes in healthy controls versus subjects with stable coronary artery disease (CAD) defined as clinical angina + q waves on ECG or coronary angiography documentation of at least 1 stenotic lesion at >50% [43]. Although a direct comparison with our data is not possible due to differences in the definition of disease, some findings were consistent between this study and ours. Their study found several proteins that were enriched in CAD, including apoE, CO3, apoC-IV, PON1, and APOA4. We found that CO3 is associated with NCB and APOA4 is associated with CB. A study by Alwaili et al. found increased CO3 but decreased APOA-IV in subjects with acute coronary syndrome [44]. This study also found increased SAA in ACS subjects, consistent with our findings for NCB. Overall, these findings raise the interesting possibility that the various HDL-associated proteins are contributing to the different stages of atherosclerosis progression. Another study by Riwanto et al. reported a proteome analysis of HDL comparing healthy and CAD subjects [45]. Although the focus of this report was

primarily on antiapoptotic function of HDL, which they convincingly link to the content of clusterin (apoJ) and apoC-III, their data also show that CAMP and APOA1 were higher in healthy subjects while APOA4 and SAA were higher in CAD subjects. These findings support our top ranking proteins for both CB and NCB and in both directions.

The proteins discussed above may prove useful in the development of biomarkers for HDL function and prediction of atherosclerosis risk. Many other potentially interesting protein associations found in this study are not discussed here, but shown in the Figures and Supplemental Data. All of these associations represent opportunities for future examination of novel mechanisms for involvement of HDL-bound proteins in atherosclerosis. Given that, many are well-characterized plasma proteins with known functions, it is relatively easy to develop and test hypotheses on the putative roles of these proteins in atherosclerosis. Future efforts should also be aimed at developing high-throughput techniques for reliable quantification of HDL-bound proteins. These techniques could then be applied to formally evaluate the HDL proteome as a biomarker for cardiovascular risk in larger studies that are representative of the general population.

One limitation of this study is that an in-depth lipidomics analysis was not performed. HDL is equally diverse in the types of lipids it transports and many are biologically active signaling molecules, such as sphingosine-1-phosphate [46]. Combined studies on the relationship between the HDL lipidome and proteome may reveal additional markers that could perhaps be combined for predicting cardiovascular risk. An index based on protein and lipid composition of HDL may improve cardiovascular risk prediction and the evaluation of novel HDL modifying drugs. Additionally, because it is more difficult to measure NCB than CB, this CCTA parameter is not frequently evaluated, but based our findings, future studies comparing NCB versus CB for cardiovascular events is warranted. An advantage of this study over previous HDL proteome and CAD studies is that the application of this analysis to a population of subjects with a range of disease severities, using linear measures of burden, allowed for improved sensitivity compared to discriminant analyses. It is important to note that the proteins associated with HDL can vary, depending on the isolation procedure [43, 47]. The FPLC isolation procedures used in our study produces HDL with minimal perturbation compared to centrifugation techniques, and is not usually contaminated with Lp(a) like in ultracentrifugation methods. The method we used, however, may cause enrichment of proteins with low affinity for HDL or contaminating plasma proteins, and does not allow for the isolation of pre-beta HDL. Similarly, HDL isolation procedure can also affect functional assays, such as cholesterol efflux [48], although the PEG- precipitation method we used in our cholesterol efflux assay is the most commonly used procedure for this assay and has been shown yield results that correlate with cardiovascular endpoints [16]. Finally, the associations that we found in this study do not prove that they are mechanistically linked to the pathogenesis of cardiovascular disease and their value as a cardiovascular biomarker has to be validated in separate much larger studies, with likely more high throughput and less costly methods than the LC- MS method we used in our study.

In summary, we found that the HDL proteome is associated with cholesterol efflux capacity of HDL and with different measures of atherosclerosis burden. These findings support the

future development of HDL-based biomarkers and targets for drug development for cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

We thank the National Heart, Lung, and Blood Institute Proteomics Core Facility for advice and access to the mass spectrometer. We also thank Maureen Sampson for creation of artwork in Fig. 2.

Financial Support: All funding for this study was provided by the National Heart, Lung, and Blood Institute Intramural Research Program.

References

- [1]. Libby P, Ridker PM, Hansson GKK. Progress and challenges in translating the biology of atherosclerosis. *Nature*. 2011; 473(7347):317–25. [PubMed: 21593864]
- [2]. Nicholls SJ. The ACCELERATE trial: impact of the cholesteryl ester transfer protein inhibitor evacetrapib on cardiovascular outcome. Presented at the 65th Annual Scientific Session and Expo of the American College of Cardiology 2016.
- [3]. Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, et al. Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet* (London, England). 2012; 380(9841):572–80.
- [4]. Davidson WS. The HDL Proteome Watch 2015 [Available from: <http://homepages.uc.edu/~davidswm/HDLproteome.html>].
- [5]. Walldius G, Jungner I. Apolipoprotein A-I versus HDL cholesterol in the prediction of risk for myocardial infarction and stroke. *Current opinion in cardiology*. 2007; 22(4):359–67. [PubMed: 17556890]
- [6]. Gordon SM, Hofmann S, Askew DS, Davidson WS. High density lipoprotein: it's not just about lipid transport anymore. *Trends Endocrinol Metab*. 2011; 22(1):9–15. [PubMed: 21067941]
- [7]. Agatston AS, Janowitz WR, Hildner FJ, Zusmer NR, Viamonte M, Detrano R. Quantification of coronary artery calcium using ultrafast computed tomography. *Journal of the American College of Cardiology*. 1990; 15(4):827–32. [PubMed: 2407762]
- [8]. Pletcher MJ, Tice JA, Pignone M, Browner WS. Using the coronary artery calcium score to predict coronary heart disease events: a systematic review and meta-analysis. *Archives of internal medicine*. 2004; 164(12):1285–92. [PubMed: 15226161]
- [9]. Detrano R, Guerci AD, Carr JJ, Bild DE, Burke G, Folsom AR, et al. Coronary calcium as a predictor of coronary events in four racial or ethnic groups. *The New England journal of medicine*. 2008; 358(13):1336–45. [PubMed: 18367736]
- [10]. Motoyama S, Ito H, Sarai M, Kondo T, Kawai H, Nagahara Y, et al. Plaque Characterization by Coronary Computed Tomography Angiography and the Likelihood of Acute Coronary Events in Mid-Term Follow-Up. *Journal of the American College of Cardiology*. 2015; 66(4):337–46. [PubMed: 26205589]
- [11]. Lerman JB, Joshi AA, Chaturvedi A, Abera TM, Dey AK, Rodante JA, et al. Coronary Plaque Characterization in Psoriasis Reveals High Risk Features Which Improve Following Treatment in a Prospective Observational Study. *Circulation*. 2017.
- [12]. Tardif J-CC, Grégoire J, L'Allier PL, Ibrahim R, Lespérance J, Heinonen TM, et al. Effects of reconstituted high-density lipoprotein infusions on coronary atherosclerosis: a randomized controlled trial. *JAMA*. 2007; 297(15):1675–82. [PubMed: 17387133]
- [13]. Parolini C, Marchesi M, Lorenzon P, Castano M, Balconi E, Miragoli L, et al. Dose-related effects of repeated ETC-216 (recombinant apolipoprotein A-I Milano/1-palmitoyl-2-oleoyl phosphatidylcholine complexes) administrations on rabbit lipid-rich soft plaques: in vivo

- assessment by intravascular ultrasound and magnetic resonance imaging. *Journal of the American College of Cardiology*. 2008; 51(11):1098–103. [PubMed: 18342229]
- [14]. Ibanez B, Vilahur G, Cimmino G, Speidl WS, Pinero A, Choi BG, et al. Rapid change in plaque size, composition, and molecular footprint after recombinant apolipoprotein A-I Milano (ETC-216) administration: magnetic resonance imaging study in an experimental model of atherosclerosis. *Journal of the American College of Cardiology*. 2008; 51(11):1104–9. [PubMed: 18342230]
- [15]. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *The New England journal of medicine*. 2011; 364(2):127–35. [PubMed: 21226578]
- [16]. Rohatgi A, Khera A, Berry JD, Givens EG, Ayers CR, Wedin KE, et al. HDL cholesterol efflux capacity and incident cardiovascular events. *N Engl J Med*. 2014; 371(25):2383–93. [PubMed: 25404125]
- [17]. Shah AS, Tan L, Long JL, Davidson WS. Proteomic diversity of high density lipoproteins: our emerging understanding of its importance in lipid transport and beyond. *Journal of lipid research*. 2013; 54(10):2575–85. [PubMed: 23434634]
- [18]. Voros S, Rivera JJ, Berman DS, Blankstein R, Budoff MJ, Cury RC, et al. Guideline for minimizing radiation exposure during acquisition of coronary artery calcium scans with the use of multidetector computed tomography: a report by the Society for Atherosclerosis Imaging and Prevention Tomographic Imaging and Prevention Councils in collaboration with the Society of Cardiovascular Computed Tomography. *Journal of cardiovascular computed tomography*. 2011; 5(2):75–83. [PubMed: 21398199]
- [19]. Halliburton SS, Abbata S, Chen MY, Gentry R, Mahesh M, Raff GL, et al. SCCT guidelines on radiation dose and dose-optimization strategies in cardiovascular CT. *Journal of cardiovascular computed tomography*. 2011; 5(4):198–224. [PubMed: 21723512]
- [20]. Gordon SM, Deng J, Lu LJ, Davidson WS. Proteomic characterization of human plasma high density lipoprotein fractionated by gel filtration chromatography. *J Proteome Res*. 2010; 9(10):5239–49. [PubMed: 20718489]
- [21]. Mehta NN, Li R, Krishnamoorthy P, Yu Y, Farver W, Rodrigues A, et al. Abnormal lipoprotein particles and cholesterol efflux capacity in patients with psoriasis. *Atherosclerosis*. 2012; 224(1):218–21. [PubMed: 22858285]
- [22]. Hafiane A, Genest J. HDL-Mediated Cellular Cholesterol Efflux Assay Method. *Annals of clinical and laboratory science*. 2015; 45(6):659–68. [PubMed: 26663796]
- [23]. Boulesteix A-LL, Strimmer K. Partial least squares: a versatile tool for the analysis of high-dimensional genomic data. *Briefings in bioinformatics*. 2007; 8(1):32–44. [PubMed: 16772269]
- [24]. Benachour H, Zaiou M, Samara A, Herbeth B, Pfister M, Lambert D, et al. Association of human cathelicidin (hCAP-18/LL-37) gene expression with cardiovascular disease risk factors. *Nutr Metab Cardiovasc Dis*. 2009; 19(10):720–8. [PubMed: 19346112]
- [25]. Edfeldt K, Agerberth B, Rottenberg ME, Gudmundsson GH, Wang X-BB, Mandal K, et al. Involvement of the antimicrobial peptide LL-37 in human atherosclerosis. *Arteriosclerosis, thrombosis, and vascular biology*. 2006; 26(7):1551–7.
- [26]. Nicholls SJ, Ballantyne CM, Barter PJ, Chapman JM, Erbel RM, Libby P, et al. Effect of Two Intensive Statin Regimens on Progression of Coronary Disease. *The New England Journal of Medicine*. 2011; 365(22):2078–87. [PubMed: 22085316]
- [27]. Puri R, Nicholls SJ, Shao M, Kataoka Y, Uno K, Kapadia SR, et al. Impact of Statins on Serial Coronary Calcification During Atheroma Progression and Regression. *Journal of the American College of Cardiology*. 2015; 65(13).
- [28]. Glueck CJ, Taylor HL, Jacobs D, Morrison JA, Beaglehole R, Williams OD. Plasma high-density lipoprotein cholesterol: association with measurements of body mass. *The Lipid Research Clinics Program Prevalence Study*. *Circulation*. 1980; 62(4 Pt 2).
- [29]. Davidson WS, Heink A, Sexmith H, Dolan LM, Gordon SM, Otvos JD, et al. Obesity is associated with an altered HDL subspecies profile among adolescents with metabolic disease. *Journal of lipid research*. 2017; 58(9):1916–23. [PubMed: 28743729]

- [30]. Chiou TT, Liao S-CC, Kao Y-YY, Lee W-CC, Lee Y-TT, Ng H-YY, et al. Gelsolin and Progression of Aortic Arch Calcification in Chronic Hemodialysis Patients. *International journal of medical sciences*. 2016; 13(2):92–8. [PubMed: 26941566]
- [31]. Shao B, Tang C, Sinha A, Mayer PS, Davenport GD, Brot N, et al. Humans with atherosclerosis have impaired ABCA1 cholesterol efflux and enhanced high-density lipoprotein oxidation by myeloperoxidase. *Circulation research*. 2014; 114(11):1733–42. [PubMed: 24647144]
- [32]. Shao B, Cavigliolo G, Brot N, Oda MN, Heinecke JW. Methionine oxidation impairs reverse cholesterol transport by apolipoprotein A-I. *Proceedings of the National Academy of Sciences of the United States of America*. 2008; 105(34):12224–9. [PubMed: 18719109]
- [33]. Mackness B, Quarck R, Verreth W, Mackness M, Holvoet P. Human Paraoxonase-1 Overexpression Inhibits Atherosclerosis in a Mouse Model of Metabolic Syndrome. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 2006; 26(7):1545–50.
- [34]. Mackness B, Mackness M. Anti-inflammatory properties of paraoxonase-1 in atherosclerosis. *Advances in experimental medicine and biology*. 2010; 660:143–51. [PubMed: 20221877]
- [35]. Mackness B, Mackness M. The antioxidant properties of high-density lipoproteins in atherosclerosis. *Panminerva medica*. 2012; 54(2):83–90.
- [36]. Artl A, Marsche G, Lestavel S, Sattler W, Malle E. Role of serum amyloid A during metabolism of acute- phase HDL by macrophages. *Arteriosclerosis, thrombosis, and vascular biology*. 2000; 20(3):763–72.
- [37]. Vaisar T, Tang C, Babenko I, Hutchins P, Wimberger J, Suffredini AF, et al. Inflammatory remodeling of the HDL proteome impairs cholesterol efflux capacity. *Journal of lipid research*. 2015; 56(8):1519–30. [PubMed: 25995210]
- [38]. Han C, Tang C, Guevara ME, Wei H, Wietecha T, Shao B, et al. Serum amyloid A impairs the antiinflammatory properties of HDL. *Journal of Clinical Investigation*. 2016; 126(2):796-. [PubMed: 26829628]
- [39]. Scott MG, Davidson DJ, Gold MR, Bowdish D, Hancock RE. The human antimicrobial peptide LL-37 is a multifunctional modulator of innate immune responses. *Journal of immunology* (Baltimore, Md : 1950). 2002; 169(7):3883–91.
- [40]. Norman PE, Powell JT. Vitamin D and cardiovascular disease. *Circulation research*. 2014; 114(2):379–93. [PubMed: 24436433]
- [41]. Young KA, Snell-Bergeon JK, Naik RG, Hokanson JE, Tarullo D, Gottlieb PA, et al. Vitamin D deficiency and coronary artery calcification in subjects with type 1 diabetes. *Diabetes care*. 2011; 34(2):454–8. [PubMed: 20978098]
- [42]. Robinson-Cohen C, Zelnick LR, Hoofnagle AN, Lutsey PL, Burke G, Michos ED, et al. Associations of Vitamin D-Binding Globulin and Bioavailable Vitamin D Concentrations With Coronary Heart Disease Events: The Multi-Ethnic Study of Atherosclerosis (MESA). *The Journal of clinical endocrinology and metabolism*. 2017; 102(8):3075–84. [PubMed: 28472285]
- [43]. Vaisar T, Pennathur S, Green PS, Gharib SA, Hoofnagle AN, Cheung MC, et al. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *J Clin Invest*. 2007; 117(3):746–56. [PubMed: 17332893]
- [44]. Alwaili K, Bailey D, Awan Z, Bailey SD, Ruel I, Hafiane A, et al. The HDL proteome in acute coronary syndromes shifts to an inflammatory profile. *Biochimica et biophysica acta*. 2012; 1821(3):405–15. [PubMed: 21840418]
- [45]. Riwanto M, Rohrer L, Roschitzki B, Besler C, Mocharla P, Mueller M, et al. Altered Activation of Endothelial Anti- and Proapoptotic Pathways by High-Density Lipoprotein from Patients with Coronary Artery Disease Role of High-Density Lipoprotein–Proteome Remodeling. *Circulation*. 2013; 127(8):891–904. [PubMed: 23349247]
- [46]. Sattler K, Levkau B. Sphingosine-1-phosphate as a mediator of high-density lipoprotein effects in cardiovascular protection. *Cardiovascular Research*. 2009; 82(2):201–11. [PubMed: 19233866]
- [47]. Gordon SM, Deng J, Lu JL, Davidson SW. Proteomic Characterization of Human Plasma High Density Lipoprotein Fractionated by Gel Filtration Chromatography. *Journal of Proteome Research*. 2010; 9(10):5239–49. [PubMed: 20718489]

- [48]. Davidson WS, Heink A, Sexmith H, Melchior JT, Gordon SM, Kuklenyik Z, et al. The effects of apolipoprotein B depletion on HDL subspecies composition and function. *Journal of lipid research*. 2016; 57(4):674–86. [PubMed: 26908829]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Highlights

- The human HDL proteome is associated with HDL function and atherosclerosis burden.
- Traditional CVD risk factors influence the HDL proteome, especially BMI.
- HDL proteins that influence cholesterol efflux also associate with soft plaque.
- Calcified plaque is associated with a unique HDL proteome signature.

Fig. 1. Identification of HDL proteins with influence on HDL function and atherosclerosis burden. Partial least squares regression analysis was used to identify relationships between individual protein components of HDL and calcified plaque burden (A), non-calcified plaque burden (B), and cholesterol efflux capacity (C). Proteins with the strongest relationships (VIP cutoff = 1.3) are colored green or red for positive and negative associations, respectively. In panel C, serum amyloid A (SAA, colored pink) is known to negatively influence CEC. A Venn diagram displays high-influence proteins that are common among burden measures and CEC (D). VIP, variable importance for projection.

Fig. 2.
The HDL proteome may provide a mechanistic link between traditional cardiovascular risk factors and atherosclerosis burden.

Table 1.

Clinical characteristics of study subjects.

	Cohort
n	126
Gender (% female)	43.7
Age (years)	61.3 ± 8.7
BMI	29.8 ± 7.5
Systolic BP (mmHg)	116.1 ± 14.9
Diastolic BP (mmHg)	61.8 ± 10.7
Statins (%)	42.9
Total cholesterol (mg/dL)	180.4 ± 34.8
LDL cholesterol (mg/dL)	96 ± 31.9
HDL cholesterol (mg/dL)	58.3 ± 17.6
Triglyceride (mg/dL)	136.1 ± 95.4
HDL particle number (μmol/L)	35.2 ± 5.9
HDL size (nm)	9.4 ± 0.6
LDL particle number (nmol/L)	1183.8 ± 437.6
LDL size (nm)	20.5 ± 0.6
VLDL particle number (nmol/L)	62.3 ± 47.9
VLDL size (nm)	49.9 ± 7.4
Calcified burden > 0 (%)	69.8
Calcified burden (Agatston score)	281.3 ± 616.5
Non-calcified burden (mm ²)	0.0109 ± 0.0042

Continuous variables are expressed as mean ± standard deviation.

Table 2A.

Bivariate analyses of clinical measures vs. atherosclerosis burden.

	Variable	β	p
CB	Gender (F)	−0.25	0.0052
	Age	0.36	<.0001
	BMI	0.02	0.85
	LDL-C	−0.06	0.48
	HDL-C	−0.10	0.27
	Triglyceride	−0.05	0.56
	Statin (Y)	0.18	0.048
NCB	Gender (F)	−0.42	<.0001
	Age	−0.22	0.015
	BMI	0.74	<.0001
	LDL-C	0.03	0.76
	HDL-C	−0.40	<.0001
	Triglyceride	0.06	0.52
	Statin (Y)	−0.09	0.33

Each variable was evaluated individually in a linear regression model for association with either calcified (CB) or non-calcified burden (NCB). CB values were log₁₀ transformed prior to analysis.

Table 2B.

Multivariate analyses of clinical measures vs. atherosclerosis burden.

	Variable	β	p
CB	Gender (F)	−0.30	0.001
	Age	0.42	<.0001
	BMI	0.05	0.57
	LDL-C	−0.03	0.73
	HDL-C	−0.10	0.32
	Triglyceride	0.05	0.58
	Statin (Y)	0.26	0.007
NCB	Gender (F)	−0.36	<.0001
	Age	−0.04	0.5408
	BMI	0.70	<.0001
	LDL-C	−0.07	0.21
	HDL-C	−0.03	0.63
	Triglyceride	−0.05	0.39
	Statin (Y)	−0.12	0.047

All variables were included in multivariate linear regression models for association with calcified (CB) or non-calcified burden (NCB). CB values were log₁₀ transformed prior to analysis.

Table 3.

Multivariate regression model with traditional risk factors and statin use.

Protein	CEC	NCB	CB	Gender (F)	Age	BMI	HDL-C	Statin (Y)
APOA4	↑	↓	↑	−0.012	0.072	−0.152	0.097	0.173
GELS	↑	↓	↓	0.015	−0.024	−0.150	0.277	−0.053
ANT3	↑	↓		−0.060	0.192	−0.354	−0.052	0.062
APOA1	↑	↓		0.116	−0.015	−0.243	0.435	0.186
APOA2	↑	↓		0.024	−0.119	−0.275	0.120	0.187
APOC1	↑	↓		0.036	−0.098	−0.300	0.305	0.000
CO2	↓	↑		−0.050	−0.047	0.292	0.120	0.055
PON1	↑		↓	−0.149	−0.272	−0.074	0.254	0.006
A2AP	↑			−0.044	0.013	−0.101	0.051	−0.045
APOC3	↑			0.109	−0.077	−0.041	0.240	0.186
RET4	↑			−0.305	−0.143	−0.298	0.129	0.077
CERU	↓			−0.020	−0.205	−0.060	0.075	−0.077
CO4A	↓			−0.182	−0.150	0.164	0.165	−0.026
CO9	↓			0.132	0.105	0.250	−0.051	−0.075
IGHG1	↓			−0.039	−0.060	−0.250	−0.215	−0.063
KLKB1	↓			0.023	−0.238	0.001	0.030	0.000
ANGT		↓		0.023	−0.012	−0.211	0.034	0.058
APOF		↓		−0.140	−0.029	−0.286	0.266	0.118
KAIN		↓		0.002	−0.059	−0.362	−0.073	0.172
C1S		↑		−0.172	0.010	0.089	0.031	−0.088
CFAB		↑		0.244	−0.030	0.290	−0.117	−0.066
CO3		↑		0.140	0.066	0.292	−0.268	0.138
IGHA1		↑		−0.158	−0.089	0.109	0.012	0.063
SAA		↑		0.256	0.033	0.409	−0.017	−0.045
A2MG			↑	0.074	0.264	−0.020	0.030	0.008
APOC2			↑	0.115	0.062	−0.081	−0.080	0.166
VTDB			↑	−0.046	0.100	−0.219	−0.016	0.094
A1BG			↓	0.274	−0.011	0.051	0.001	0.101
CAMP			↓	0.044	−0.274	0.043	0.248	−0.168
CFAH			↓	−0.019	−0.178	0.121	−0.088	−0.141
FINC			↓	0.035	−0.158	0.045	0.119	0.010
HRG			↓	0.029	0.024	−0.068	0.029	−0.192
KNG1			↓	0.038	−0.111	−0.118	0.076	0.015
PON3			↓	−0.053	−0.059	0.178	−0.043	−0.091
Totals:				4	6	15	9	5

Arrows indicate directionality of association between proteins and CEC or burden measures.

Upward arrows = positive association and downward = negative association.

Linear regression model for each protein includes all clinical variables.

• Indicates association with CEC or burden measure with VIP score > 1.3

Numerical values are standardized beta coefficient. Shading indicates level of statistical significance. No fill (white) is not statistically significant.

$p < 0.05$ $p < 0.01$ $p < 0.001$ $p < 0.0001$

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript