Albuminuria, the High-Density Lipoprotein Proteome, and Coronary Artery Calcification in Type 1 Diabetes Mellitus

The DCCT/EDIC Study

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Objective—Albuminuria is an important risk factor for cardiovascular disease in diabetes mellitus. We determined whether albuminuria associates with alterations in the proteome of HDL (high-density lipoprotein) of subjects with type 1 diabetes mellitus and whether those alterations associated with coronary artery calcification.

Approach and Results—In a cross-sectional study of 191 subjects enrolled in the DCCT (Diabetes Control and Complications Trial)/EDIC study (Epidemiology of Diabetes Interventions and Complications), we used isotope dilution tandem mass spectrometry to quantify 46 proteins in HDL. Stringent statistical analysis demonstrated that 8 proteins associated with albuminuria. Two of those proteins, AMBP (α1-microglobulin/bikunin precursor) and PTGDS (prostaglandin-H2 D-isomerase), strongly and positively associated with the albumin excretion rate ($P<10^{-6}$). Furthermore, PON (paraoxonase) 1 and PON3 levels in HDL strongly and negatively associated with the presence of coronary artery calcium, with odds ratios per 1-SD difference of 0.63 (95% CI, 0.43–0.92; $P=0.018$) for PON1 and 0.59 (95% CI, 0.40–0.87; $P=0.0079$) for PON3. Only 1 protein, PON1, associated with both albumin excretion rate and coronary artery calcification.

Conclusions—Our observations indicate that the HDL proteome is remodeled in type 1 diabetes mellitus subjects with albuminuria. Moreover, low concentrations of the antiatherosclerotic protein PON1 in HDL associated with both albuminuria and coronary artery calcification, raising the possibility that alterations in HDL protein cargo mediate, in part, the known association of albuminuria with cardiovascular risk in type 1 diabetes mellitus.

Visual Overview—An online visual overview is available for this article. **(*Arterioscler Thromb Vasc Biol*. 2019;39:1483-1491. DOI: 10.1161/ATVBAHA.119.312556.)**

Key Words: albuminuria ■ atherosclerosis ■ kidney diseases ■ lipoproteins ■ proteomics ■ triglycerides

Albuminuria is an independent risk factor for cardiovascular disease (CVD) in people with type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus, and in the general population.[1–4] Albuminuria also increases the risk of progressing to end-stage renal disease (ESRD).[5,6] Albuminuria may contribute to CVD risk in type 2 diabetes mellitus subjects by promoting an atherogenic lipid profile characterized by elevated triglycerides, low HDL (high-density lipoprotein) cholesterol (HDL-C), and a shift in LDL (low-density lipoprotein) to small, dense particles.[7] However, HDL-C levels are not consistently low in T1DM subjects despite their increase in CVD risk.[8–10]

Clinical and epidemiological studies show a robust, inverse association of HDL-C levels with CVD risk.[11,12] However, several lines of evidence suggest that the association between HDL-C levels and CVD status is not a causal relationship and that elevating HDL-C is not necessarily therapeutic.[13,14] For example, a genetic mutation in SR-BI (*SCARB1*)—a protein critically involved in HDL metabolism in the liver—greatly increases both HDL-C levels and atherosclerosis.[15,16] Collectively, these observations indicate that HDL-C levels do not necessarily reflect the cardioprotective effects of HDL.

HDL metrics other than HDL-C are likely to mediate the cardioprotective effects of HDL. HDL carries a wide array of proteins linked to lipoprotein metabolism, inflammation, protease inhibition, and complement regulation, suggesting that this cargo contributes to anti-inflammatory and antiatherogenic properties of HDL.[17] We found that the HDL proteome is dramatically remodeled in patients with ESRD.[18] It is unclear whether albuminuria in T1DM affects HDL's protein cargo or whether alterations in HDL's proteins associate with CVD in T1DM.

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Nonstandard Abbreviations and Acronyms	
AER	albumin excretion rate
AMBP	α1-microglobulin/bikunin precursor
APOA1	apolipoprotein AI
CAC	coronary artery calcium
CACTI	Coronary Artery Calcification in Type 1 Diabetes
CVD	cardiovascular disease
DCCT	Diabetes Control and Complications Trial
EDIC	Epidemiology of Diabetes Interventions and Complications Study
eGFR	estimated glomerular filtration rate
ESRD	end-stage renal disease
HbA1c	hemoglobin A1c
HDL	high-density lipoprotein
HDL-C	high-density lipoprotein cholesterol
LDL	low-density lipoprotein
PON	paraoxonase
PTGDS	prostaglandin-H2 D-isomerase
T1DM	type 1 diabetes mellitus

In the current study, we used targeted mass spectrometric proteomics to test the hypotheses that alternations in the HDL proteome associate with albuminuria in T1DM subjects. Because albuminuria is a risk factor for CVD, we also determined whether changes in the abundance of proteins in the HDL proteome associate with coronary artery calcification (CAC), which strongly predicts incident CVD risk.[19] Our observations support the proposal that certain HDL proteins may be markers, and perhaps mediators, of albuminuria and the increased cardiovascular risk associated with T1DM.

Materials and Methods

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

Subjects and Experimental Design

The EDIC study (Epidemiology of Diabetes Interventions and Complications)[20] was an observational cohort study of T1DM subjects that followed the DCCT (Diabetes Control and Complications Trial). All subjects in our study were enrolled in an ancillary EDIC study of lipoproteins[21] that involved subjects from all 28 clinical sites in the United States and Canada.[21] For this study, extra plasma was obtained every other year during EDIC years 3 to 12 (1997–2006). After an overnight fast, blood was collected into ice-cold tubes containing EDTA (6 mmol/L final concentration). Plasma was prepared immediately by centrifugation (2500*g* for 15 minutes) and frozen at −80°C until analysis.[22] All studies were approved by the Human Studies Committee at the University of Washington, which coordinated the lipoprotein ancillary study, and by the EDIC clinical centers.

We designed a cross-sectional study based on urinary albumin excretion, with oversampling of participants with microalbuminuria and macroalbuminuria. Urinary albumin excretion had been quantified every other year as albumin excretion rate (AER). Fasting blood and AER had been collected in the alternate years. We first selected all the stored plasma samples from annual visits at which the ancillary EDIC subjects had demonstrated macroalbuminuria (AER, ≥300 mg/dL) at that visit, as well as 1 year earlier and 1 year later. We then randomly sampled similar numbers of participants who demonstrated microalbuminuria (AER, 30–299 mg/dL) or normoalbuminuria (AER, <30 mg/dL)[23] during the annual visit and 1 year earlier and later. Subjects were not selected on the basis of sex, HbA1c (hemoglobin A1c), or other clinical criteria. When multiple time points were available for a given participant, we used the time point closest to EDIC year 7 or 8 (calendar year 2000–2002), when EDIC measured CAC.[24] For each selected subject, we used the clinical characteristics obtained at the same visit as the plasma sample. The mean duration of diabetes mellitus at the time of sampling was >20 years in each group.

HDL Isolation

HDL (density, 1.063–1.210 g/mL) was isolated by sequential ultracentrifugation from rapidly thawed plasma,[25] using buffers supplemented with 100 μM diethylenetriaminepentaacetic acid and a protease inhibitor cocktail (Sigma, St. Louis, MO). The protein concentration of HDL was determined using the Lowry assay (BioRad), with albumin as the standard. All samples were deidentified and analyzed in a blinded manner in HDL isolation, HDL proteomics, and statistical analysis.

Targeted Quantification of HDL Proteins by Selected Reaction Monitoring With ^{15}N-Labeled APOA1 as the Internal Standard

Human [^{15}N]APOA1 (apolipoprotein AI; >99% purity after isolation) was produced by a bacterial expression system.[26] After the addition of freshly prepared methionine (10 mmol/L final concentration), HDL proteins (10 μg total proteins) and [^{15}N]APOA1 (0.5 μg, as the internal standard) were reduced with dithiothreitol and alkylated with iodoacetamide. Tryptic digests of HDL were analyzed with a nano–LC-MS/MS Thermo TSQ Vantage coupled to a Waters nanoACQUITY UltraPerformance liquid chromatography system.[18,25] Resolution for Q1 and Q3 was 0.7 Da (full width at half maximum), and analyses were performed using a scheduled transition list generated by Skyline[27]—an open-source program for quantitative data processing and proteomic analysis. At least 2 peptides per protein and 3 or 4 selective reaction monitoring transitions of each peptide were used for the Skyline analysis.[18,25] The results were expressed as the ratio of the peak areas of each peptide and the [^{15}N]APOA1 peptide [^{15}N]THLAPYSDELR. To calculate the relative levels of each peptide, we set the average ratio of the peptide to the internal standard peptide in normoalbuminuric subjects to an arbitrary unit of 1.

Statistical Analysis

Differences in relative protein expression by albuminuria status determined by selective reaction monitoring analysis were evaluated by ANOVA of the group means. We also performed multiple linear regression analyses for percentage changes of protein levels in HDL per doubling in AER with or without adjustment for clinical characteristics including age, sex, DCCT treatment group, use of renin-angiotensin-aldosterone system inhibitors, duration of diabetes mellitus, use of lipid-lowering medications, smoking, body mass index, and mean percentage of HbA1c (HbA1c%). To account for multiple comparison testing of HDL proteins, we used the Benjamini-Hochberg method with a 10% false discovery rate, that is, based on the corrected or adjusted P (Q values); only proteins with a Q value <0.10 were considered significant. We tested associations of HDL proteins with the presence of CAC by using logistic regression analysis. The logistic models were adjusted for potential confounders as described above and also for log(AER). Odds ratios are reported per analyte SD. Correlation analysis with continuous variables used Pearson coefficient. All statistical analyses were performed in R—a free software environment for statistical computing and graphics.

Results

The clinical characteristics of the 191 subjects we studied are listed in Table 1. The 3 groups (normoalbuminuria, microalbuminuria, and macroalbuminuria) had similar mean ages, percentages of smokers, body mass indexes, and HDL-C levels. LDL-C levels were slightly lower and the duration of diabetes

Table 1. Clinical Characteristics of Subjects With Type 1 Diabetes Mellitus by AER Status

Covariate	Normal AER (<30 mg/d)	Microalbuminuria (30 to <300 mg/d)	Macroalbuminuria (≥300 mg/d)	*P* Value
No. of subjects	67	64	60	
Age, y	42.0 (37.0–48.0)	44.0 (35.0–48.2)	43.5 (36.2–49.0)	0.45
Sex (female)	39 (58)	16 (25)	10 (17)	<0.0001
White	67 (100)	64 (100)	60 (100)	
DCCT treatment				
Conventional glucose control	39 (58)	41 (64)	48 (80)	0.01
Intensive glucose control	28 (42)	23 (36)	12 (20)	0.01
BMI, kg/m^2	27.6 (24.6–30.7)	27.0 (24.8–29.8)	28.3 (25.2–31.2)	0.34
Smoker	17 (25)	12 (19)	21 (35)	0.24
Blood pressure medications	41 (61)	44 (69)	48 (80)	
Lipid-lowering medications	46 (69)	49 (77)	53 (88)	0.008
Statin	18 (27)	3 (5)	18 (30)	0.0006
RAASi				
ACE inhibitor	42 (63)	49 (77)	53 (88)	0.001
ARB	10 (15)	0 (0)	9 (15)	
Duration of DM, y	26.1 (22.1–30.0)	20.0 (17.3–24.3)	22.0 (18.4–24.3)	0.04
eGFR (CKD-EPI), mL/min per 1.73 m^2	106.0 (93.8–113.4)	106.5 (95.3–114.5)	84.1 (57.1–106.5)	<0.0001
HbA1c, % and mmoL/moL	7.7 (7.0–8.8)% 61 (54–73) mmoL/moL	8.4 (7.7–9.4)% 68 (61–79) mmoL/moL	8.6 (7.8–9.6)% 70 (62–82) mmoL/moL	0.006 0.006
SBP, mm Hg	117.0 (111.5–124.8)	124.0 (114.5–132.5)	132.0 (125.0–144.0)	0.0001
DBP, mm Hg	73.0 (69.0–78.5)	78.0 (72.0–82.5)	80.0 (71.5–86.5)	0.02
HDL-C, mg/dL	50.0 (43.0–62.0)	49.0 (41.8–59.0)	50.0 (42.0–59.5)	0.55
LDL-C, mg/dL	103.0 (86.0–118.0)	110.0 (86.2–134.2)	110.0 (84.0–135.5)	0.03
Total cholesterol, mg/dL	173.0 (157.0–188.0)	183.5 (153.8–203.0)	187.0 (154.0–221.5)	0.004
Triglycerides, mg/dL	69.0 (53.0–117.0)	85.0 (54.8–111.0)	117.0 (70.0–180.5)	0.002
AER	12.0 (9.3–16.4)	69.5 (50.7–97.0)	758.8 (559.4–1497.2)	<0.0001

Entries are median (interquartile range) for continuous covariates and n (%) for categorical covariates. *P* values were obtained by ANOVA analysis of the differences among group means. ACE indicates angiotensin-converting enzyme; AER, albumin excretion rate; ARB, angiotensin II receptor blocker; BMI, body mass index; CKD-EPI, chronic kidney disease-epidemiology collaboration; DBP, diastolic blood pressure; DCCT, Diabetes Control and Complications Trial; DM, diabetes mellitus; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; RAASi, renin-angiotensin-aldosterone system inhibitor; and SBP, systolic blood pressure.

mellitus was slightly longer in the normoalbuminuria group. In contrast, the groups differed significantly in sex distribution. Also, median levels of triglycerides differed significantly among the 3 groups (higher in the macroalbuminuria group), as did estimated glomerular filtration rates (eGFRs; lower in macroalbuminuria subjects) and HbA1c levels (lower in the normoalbuminuria subjects). There were no differences in HbA1c, albuminuria, or coronary artery calcium between the EDIC subjects, whose glucose levels had been tightly controlled, and the DCCT subjects, whose levels had been controlled less tightly.

Selective Reaction Monitoring Analysis Revealed Marked Differences in the Abundance of Multiple HDL Proteins in T1DM Subjects With Albuminuria

To test whether albuminuria and CVD associate with abnormalities in HDL protein cargo in T1DM, we used tandem mass spectrometric analysis with targeted selective reaction monitoring[18] and isotope dilution to quantify relative levels of 46 proteins in HDL from the 3 groups of subjects. We selected these proteins because they were reproducibly detected in preliminary shotgun proteomics analysis[17,18] of T1DM subjects with normoalbuminuria, microalbuminuria, and macroalbuminuria (10 in each group, data not shown). All 46 proteins were detected in HDL isolated by ultracentrifugation from each of the 191 subjects in this study. Differences in protein expression were initially evaluated by a test for trend without adjustment for clinical characteristics but with adjustment for multiple comparison (Benjamin-Hochberg adjusted $P<0.10$). This analysis revealed that 14 proteins were differentially expressed in subjects with microalbuminuria or macroalbuminuria, as compared with subjects with normoalbuminuria (Table I in the online-only Data Supplement).

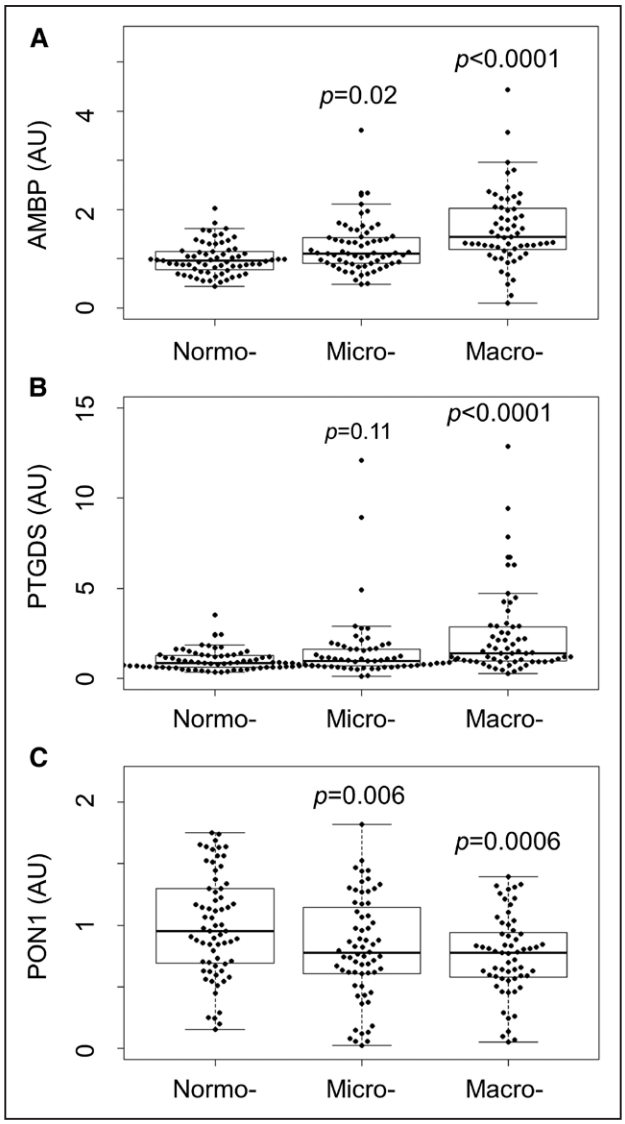

Figure 1. Selective reaction monitoring analysis of HDL (high-density lipoprotein) proteins in normoalbuminuria, microalbuminuria, and macroalbuminuria subjects. Box plots of AMBP (α1-microglobulin/bikunin precursor; **A**), PTGDS (prostaglandin-H2 D-isomerase; **B**), and PON (paraoxonase)1 (**C**) in HDL isolated from normoalbuminuria, microalbuminuria, and macroalbuminuria subjects. Peptides were quantified as the integrated peak area relative to that of [^{15}N]APOA1 (apolipoprotein AI). The box plots show the distribution of the data (median, interquartile ranges), while the dots represent individual data points. *P* values are relative to the normoalbuminuria group.

Seven proteins (AMBP [α1-microglobulin/bikunin precursor], B2M [beta-2-microglobulin], CST3 [cystatin-C], LPA [lipoprotein(a)], PSAP [prosaposin], PTGDS [prostaglandin-H2 D-isomerase], and RBP4 [retinol-binding protein 4]) were significantly increased in HDL isolated from subjects with microalbuminuria and macroalbuminuria, as compared with those with normoalbuminuria. It is noteworthy that 5 of the 7 proteins, AMBP, B2M, CST3, PTGDS, and RBP4, were increased markedly in HDL of ESRD subjects in a previous study.[18] In contrast, 7 proteins (APOA1, APOA2 [apolipoprotein A2], APOE [apolipoprotein E], PCYOX1 [prenylcysteine oxidase 1], PON [paraoxonase] 1, PON3, and SAA4 [serum amyloid A-4 protein]) were significantly reduced in HDL isolated from subjects with microalbuminuria and macroalbuminuria compared with those with normoalbuminuria. Representative examples of proteins that were markedly altered in the HDL of subjects with albuminuria are shown in Figure 1. These observations demonstrated that albuminuria in T1DM subjects is associated with abnormalities in HDL protein composition.

Table 2. Adjusted Associations of HDL Proteins With Log(AER)

Protein	Percentage Difference per Doubling in AER (95% CI)	*P* Value
AMBP	10 (5 to 15)	9E-5
PTGDS	30 (14 to 45)	0.00015
APOA2	−2 (−3 to −1)	0.0023
APOE	−4 (−7 to −1)	0.0092
CFD	−3 (−6 to −1)	0.0099
PSAP	4 (1 to 7)	0.014
SAA4	−2 (−4 to 0)	0.016
PON1	−3 (−6 to −1)	0.017

P values are from a linear regression model that adjusts for age, sex, DCCT treatment group, use of RAASi, duration of DM, use of lipid-lowering medications, smoking, BMI, and HbA1c. HDL proteins that are significant when controlling the Benjamini-Hochberg false discovery rate (*Q* values) at 10% are shown (Table II in the online-only Data Supplement). AER indicates albumin excretion rate; AMBP, α1-microglobulin/bikunin precursor; APOA2, apolipoprotein A2; APOE, apolipoprotein E; BMI, body mass index; CFD, complement factor D; DCCT, Diabetes Control and Complications Trial; DM, diabetes mellitus; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; PON, paraoxonase; PSAP, prosaposin; PTGDS, prostaglandin-H2 D-isomerase; RAASi, renin-angiotensin-aldosterone system inhibitor; SAA4, serum amyloid A-4 protein.

HDL Proteins Associate With Continuous AER

We used the absolute values of AER (ie, the continuous AER) instead of albuminuria status (ie, normoalbuminuria, microalbuminuria, and macroalbuminuria) to investigate further the relationship between the levels of HDL proteins and albuminuria. We first used a linear regression model to test the association between HDL proteins and continuous AER without adjustment for clinical characteristics. When we adjusted for multiple comparison with the Benjamini-Hochberg method, 13 proteins remained significantly associated with continuous AER. Next, we tested the association between HDL proteins and continuous AER with a multivariable linear regression model with adjustment for clinical characteristics (Table II in the online-only Data Supplement). After adjusting for differences in sex distribution and other potential confounders and controlling for multiple comparisons, 8 proteins were associated with continuous AER (Table 2). Two of those proteins, AMBP and PTGDS (Figure 2), exhibited large percentage differences per doubling of the continuous AER (≥10%) that were highly significant ($P<0.0002$). Taken together, these observations indicate that the relative abundance of certain proteins in HDL of subjects with albuminuria differed markedly from that of subjects with normoalbuminuria.

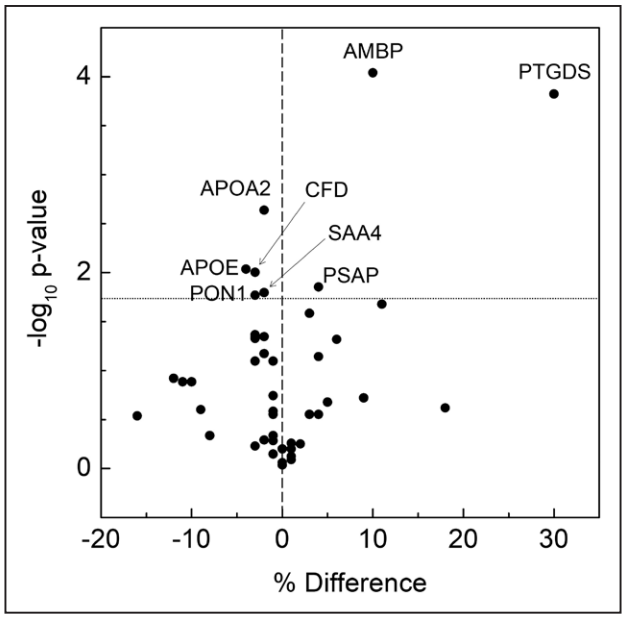

Figure 2. Volcano plot of adjusted associations of continuous log(albumin excretion rate [AER]) with each HDL (high-density lipoprotein) protein (percentage difference per doubling in AER). For each protein, *P* values are plotted vs the percent change in HDL protein levels per doubling in AER from a model that adjusts for age, sex, DCCT (Diabetes Control and Complications Trial) treatment group, use of renin-angiotensin-aldosterone system inhibitor, duration of diabetes mellitus, use of lipid-lowering medications, smoking, body mass index, and HbA1c (hemoglobin A1c). Proteins that were increased with the doubling in AER are displayed to the right of the value 0 on the *x* axis (the vertical dashed line), whereas decreased proteins are to the left. The horizontal dotted line indicates the significance threshold using Benjamini-Hochberg false discovery rate of 10%. AMBP indicates α1-microglobulin/bikunin precursor; APOA2, apolipoprotein A2; APOE, apolipoprotein E; CFD, complement factor D; PON, paraoxonase; PSAP, prosaposin; PTGDS, prostaglandin-H2 D-isomerase; and SAA4, serum amyloid A-4 protein.

Certain HDL Proteins Correlate Highly With Both eGFR and Albuminuria, Whereas Others Correlate Only With Albuminuria

We next determined the correlation between HDL proteins, albuminuria, and GFR, quantified as log-transformed AER and eGFR, respectively (Table III in the online-only Data Supplement). AMBP, PSAP, and PTGDS strongly and negatively correlated with eGFR (Table 3; r=–0.47, –0.29, and –0.43, respectively). All 3 proteins also correlated strongly and positively with log[AER] (Table 3; r=0.46, 0.22, and 0.36, respectively). In contrast, APOE, SAA4, and PON1 exhibited significant negative correlations with log[AER] (r=–0.28, –0.20, and –0.22, respectively) but did not correlate significantly with eGFR (Table 3; r<0.12). These observations suggest that certain HDL proteins (AMBP, PSAP, and PTGDS) are markers for both eGFR and albuminuria. In contrast, other proteins (APOE, SAA4, and PON1) correlate only with albumin excretion.

PON1 and PON3 in HDL Strongly and Negatively Associate With CAC

Using a logistic regression model, we next determined whether levels of HDL proteins associated with CAC. In this model, in addition to age, sex, DCCT treatment group, use of renin-angiotensin-aldosterone system inhibitors, duration of diabetes mellitus, use of lipid-lowering medications, smoking, body mass index, and HbA1c, we also adjusted for log(AER). This analysis revealed that 3 HDL proteins, PON1, PON3, and LCAT (lecithin-cholesterol acyltransferase), were negatively associated with CVD, defined as CAC >0 (Table IV in the online-only Data Supplement). For PON1 and PON3, the odds ratios per 1-SD change were 0.63 and 0.59 with a *P* of 0.018 and 0.0079, respectively (Figure 3). The mean levels of PON1 and PON3 in HDL isolated from subjects with CAC were 18% and 24% lower, respectively, than those from subjects without calcification. The significant differences for PON1 and PON3 persisted after controlling for levels of HDL-C, LDL-C, and triglycerides. Moreover, LDL-C, HDL-C, and triglycerides did not associate with CAC in the fully adjusted model (Figure 3). In contrast, the difference in LCAT levels was no longer significant after controlling for levels of HDL-C, LDL-C, and triglycerides. Although AMBP and PTGDS were strongly and positively associated with albuminuria, these 2 proteins were not associated with CAC >0 (Figure 3).

To determine whether PON1 enzymatic activity correlates with PON1 mass (assessed by isotope dilution tandem mass spectrometric analysis), we quantified PON1 activity with 2 substrates, phenyl acetate and 4-(chloromethyl)phenyl acetate,[28] in 238 subjects participating in the CACTI study (Coronary Artery Calcification in Type 1 Diabetes).[29] We found a positive correlation between PON1 mass and PON1 activity with each assay (phenyl acetate: r=0.47, $P<10^{-13}$; 4-[chloromethyl]phenyl acetate: r=0.31, $P<10^{-6}$). These observations

Table 3. Correlations (r) of HDL Proteins With Log(AER) and eGFR

Protein	R Log(AER)	*P* Value	Protein	R eGFR	*P* Value
AMBP	0.46	1E-11	AMBP	–0.47	6E-12
PTGDS	0.36	3E-07	PTGDS	–0.43	9E-10
APOE	–0.28	0.0001	APOE	0.06	0.41
PSAP	0.22	0.0024	PSAP	–0.29	6E-05
SAA4	–0.20	0.0059	SAA4	0.11	0.15
PON1	–0.22	0.0024	PON1	0.01	0.88

Pearson coefficients (r) and *P* values are from correlation analysis between the levels of HDL proteins and log(AER) or eGFR. Only HDL proteins that are significant when controlling the Benjamini-Hochberg false discovery rate (*Q* values) at 10% are shown (Table II in the online-only Data Supplement). AER indicates albumin excretion rate; AMBP, α1-microglobulin/bikunin precursor; APOE, apolipoprotein E; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; PON, paraoxonase; PSAP, prosaposin; PTGDS, prostaglandin-H2 D-isomerase; and SAA4, serum amyloid A-4 protein.

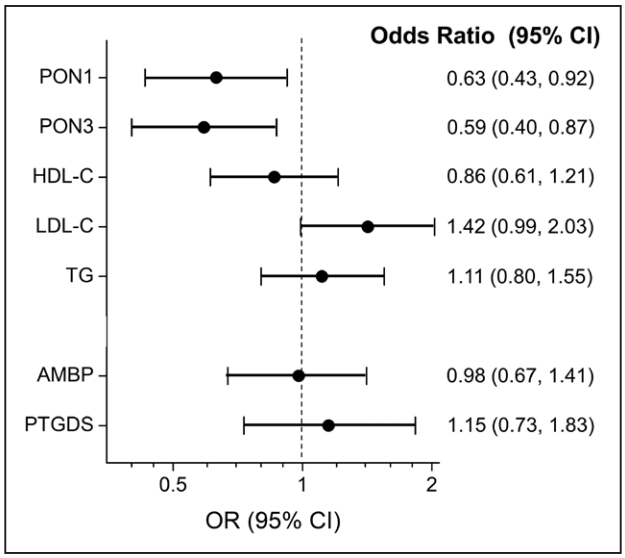

Figure 3. PON (paraoxonase) 1 and PON3 in HDL (high-density lipoprotein) associate with the presence of coronary artery calcium in a multiple regression model. Odds ratios are per analyte SD. Estimates are from a multiple regression model that additionally adjusts for age, sex, DCCT (Diabetes Control and Complications Trial) treatment group, use of renin-angiotensin-aldosterone system inhibitor, duration of diabetes mellitus, use of lipid-lowering medications, smoking, body mass index, HbA1c (hemoglobin A1c), and continuous log(albumin excretion rate). AMBP indicates α1-microglobulin/bikunin precursor; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; OR, odds ratio; PTGDS, prostaglandin-H2 D-isomerase; and TG, triglyceride.

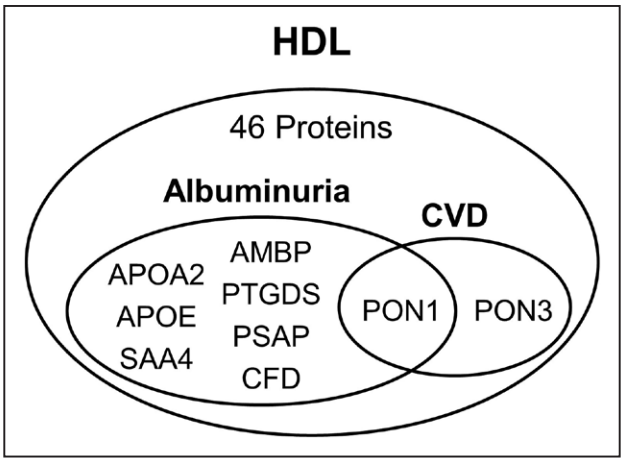

Figure 4. Venn diagram of HDL (high-density lipoprotein) proteins, albuminuria, and cardiovascular disease (CVD). Of the 46 proteins quantified in HDL isolated from type 1 diabetes mellitus subjects with or without albuminuria, 8 proteins were significantly associated with albuminuria with adjustment for clinical characteristics and controlling the Benjamini-Hochberg false discovery rate at 10%. Two of those HDL proteins were negatively associated with logit (coronary artery calcium, >0) with adjustment for clinical characteristics and controlling the HDL cholesterol, LDL cholesterol, and triglycerides levels. AMBP indicates α1-microglobulin/bikunin precursor; APOA2, apolipoprotein A2; APOE, apolipoprotein E; CFD, complement factor D; PON, paraoxonase; PSAP, prosaposin; and PTGDS, prostaglandin-H2 D-isomerase.

support the proposal that PON1 mass correlates strongly with PON1 enzymatic activity. In contrast, we found no correlation of PON1 mass or activity with cholesterol efflux capacity (measured with the Rothblatt/Rader method[30]), suggesting that PON1 mass or activity are not major modulators of HDL's ability to promote cholesterol efflux from macrophages.

Discussion

In the current study, we used targeted tandem mass spectrometric analysis with isotope dilution to quantify 46 proteins in HDL isolated from subjects in the DCCT/EDIC study to determine whether albuminuria associated with alterations in HDL protein cargo. The relative levels of 8 proteins in HDL associated significantly with continuous AER in a model corrected for multiple comparisons and clinically relevant characteristics (Figure 4). Two of the proteins, AMBP and PTGDS, were strongly and positively associated with albuminuria. Because albuminuria is an independent risk factor for CVD in both diabetic and nondiabetic subjects,[4,31] we also determined whether changes in HDL protein levels associate with CAC (>0)—a strong predictor of CVD risk.[19] We identified 2 proteins, PON1 and PON3, that strongly and negatively associated with CAC (Figure 4). Only 1 protein, PON1, associated with both AER and CVD. Taken together, our observations indicate that the HDL proteome is remodeled in albuminuria, that low levels of PON1 and PON3 associate with CAC, and that increased CVD risk in T1DM subjects with albuminuria may be mediated, in part, by decreased PON1 levels in HDL.

The PON gene family has 3 members, PON1, PON2, and PON3, that share 80% to 90% sequence identity at the amino acid level among mammalian species.[32] PON1 and PON3 are exclusively carried by circulating HDL,[33–35] whereas PON2 is an intracellular protein that localizes in the membrane fraction of cells.[36] Importantly, PON1-deficient mice and mice deficient in both PON1 and APOE developed atherosclerosis faster than control mice fed with atherogenic diet.[37,38] Overexpression of PON1 protected APOE-deficient mice from atherosclerosis.[39,40] Because PON1 is strongly antiatherogenic in mouse models of hypercholesterolemia,[37,40] and the levels of PON1 activity in HDL associate negatively with CVD in multiple human studies,[41–45] these observations suggest that albuminuria might promote proatherogenic changes in the HDL of T1DM subjects.

PON1 polymorphisms have been widely investigated for their possible involvement in atherosclerosis,[46] and epidemiological studies indicate genetic associations between PON1 and the risk of CVD in some populations of subjects.[47,48] However, the role of *PON1* genetic polymorphisms in the development of CVD remains controversial.[46,49] In contrast, levels of PON1 activity associate with CVD in nondiabetic populations in multiple studies.[41–45] We found that PON1 mass as assessed by tandem mass spectrometric analysis correlated strongly with PON1 enzymatic activity in a second cohort of T1DM subjects enrolled in the CACTI study. In contrast, PON1 mass and activity did not correlate with macrophage cholesterol efflux capacity. A previous study of T1DM subjects suggested that low levels of PON3, but not PON1, associate with established atherosclerosis.[21] However, the small number of subjects in that study (28 cases and 28 controls) may have limited the ability to detect a significant association of PON1 with atherosclerosis. Together with our observations, these findings suggest that PON1 or PON3 concentration in HDL are more predictive of the risk of atherosclerotic coronary artery disease than are *PON1* genotypes. It will be

important to quantify PON1 mass and activity and other proposed metrics of HDL's cardioprotective effects in future studies of incident CVD risk in T1DM subjects.

Previous studies demonstrated that APOE associates positively with CVD risk in women with high levels of HDL-C and C-reactive protein but not in men.[50] In an analysis adjusted for multiple comparisons, we also found that APOE associated with CAC in women but not in men in our cohort. However, that association lost significance after further adjustment for clinical variables, perhaps because there were relatively few women in the groups of albuminuric subjects. It is important to note that albuminuria is more common in men than in women with T1DM.[51]

We used a logistic regression model to determine whether any of the proteins whose HDL levels were abnormal in the T1DM subjects with albuminuria associated with prevalent coronary artery disease as assessed by CAC >0. After adjustment for clinical covariates that associate with coronary artery disease, only 1 protein, PON1, associated strongly and negatively both with CAC >0 and with log(AER). In contrast, PON3 associated strongly and negatively with calcification but not with log(AER). Importantly, these observations were independent of HDL-C, LDL-C, and triglycerides, demonstrating that alterations in HDL protein cargo can be dissociated from traditional lipid risk factors for atherosclerosis.

We also found that LCAT associated significantly with CAC in the model that adjusted for multiple clinically relevant covariates. However, this association lost significance when we also controlled for LDL-C, HDL-C, and triglyceride levels. These observations suggest that the association of LCAT with CAC reflects lipid abnormalities in T1DM subjects.

We previously demonstrated that AMBP and PTGDS are markedly elevated in HDL of patients with ESRD on dialysis.[18] In the current study, AMBP and PTGDS strongly and positively correlated/associated with AER and strongly and negatively correlated/associated with eGFR, which is consistent with the observation that EDIC subjects' albuminuria were more likely to exhibit a decrease in eGFR.[24] Proteolytic processing of AMBP generates α1-microglobulin and bikunin. α1-microglobulin is a member of the superfamily of lipocalin transport proteins that are implicated in the regulation of inflammatory pathways.[52] PTGDS is a glutathione-independent prostaglandin D synthase. It catalyzes the conversion of prostaglandin-H2 to prostaglandin D2, which regulates smooth muscle contraction and platelet aggregation. Inflammation, altered smooth muscle contraction, and platelet aggregation are implicated in renal injury, raising the possibility that the HDL-associated AMBP and PTGDS contribute to the pathogenesis of renal disease. In future studies, it will be important to determine whether elevated levels of AMBP and PTGDS in HDL predict incident albuminuria and progressive renal disease in T1DM subjects.

Previous studies have shown that elevated plasma levels of inflammatory makers predict an increased risk of progressive nephropathy in T1DM.[53] Moreover, levels of the acute-phase proteins SAA1 and SAA2 are elevated in HDL from subjects with ESRD,[18,54,55] and elevated levels of SAA1/2 during inflammation impair HDL's cholesterol efflux capacity in both humans and mice.[56] However, we found that levels of SAA1 and SAA2 in HDL were similar in subjects with normoalbuminuria and albuminuria and that they failed to associate with CAC, suggesting that inflammation-mediated changes in HDL protein cargo were not major contributors to altered HDL protein levels, kidney disease, or atherosclerosis in the T1DM subjects in our study.

Strengths of our study include a well-validated targeted proteomic approach to quantifying HDL proteins, the large number of T1DM subjects with varying degrees of albuminuria, stringent statistical analysis, and the similar clinical characteristics of the case and control subjects. A limitation is our study's cross-sectional design, which could not reveal whether the associations of the HDL proteome with CAC and albuminuria were causal. Our study also contained a relatively small number of females with albuminuria, which might have limited our ability to identify sex-dependent markers of CVD. It will, therefore, be critical to determine whether alterations to the HDL proteome predict the future onset of CVD and kidney disease in T1DM subjects.

In conclusion, we demonstrated that multiple HDL proteins are altered in T1DM subjects with albuminuria. We also found that 2 HDL proteins associate with prevalent CVD as assessed by the presence of CAC and that this association was independent of lipid levels. However, only PON1 associated with both albuminuria and CVD, raising the possibility that increased CVD risk in T1DM subjects with albuminuria is mediated, in part, by alterations in HDL's content of PON1. Our observations support the proposal that HDL protein cargo can serve as a marker, and perhaps mediator, of kidney disease and risk of atherosclerosis.

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Disclosures

J. Himmelfarb has served as a consultant to Gilead and Medikine. K.E. Bornfeldt has received research support from Novo Nordisk A/S on a different project. J.W. Heinecke is named as a coinventor on patents for the use of oxidation and protein markers to predict the risk of cardiovascular disease. The other authors report no conflicts.

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Highlights

- The HDL (high-density lipoprotein) proteome is remodeled in type 1 diabetes mellitus subjects with albuminuria.
- AMBP (α1-microglobulin/bikunin precursor), PSAP (prosaposin), and PTGDS (prostaglandin-H2 D-isomerase) in HDL all strongly and positively correlated with log(albumin excretion rate); they also strongly and negatively correlated with estimated glomerular filtration rate.
- PON (paraoxonase) 1 and PON3 levels in HDL strongly and negatively associate with atherosclerosis in type 1 diabetes mellitus, but only PON1 associates with both atherosclerosis and renal function.
- Alterations in HDL's protein cargo might mediate, in part, the known association of albuminuria with cardiovascular risk in type 1 diabetes mellitus.