The Effects of Type 2 Diabetes on Lipoprotein Composition and Arterial Stiffness in Male Youth

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Recent studies suggest HDL exists as numerous subpopulations with distinct protein/lipid compositions that are not reflected in the HDL cholesterol (HDL-C) number. In this study, we sought to evaluate HDL subpopulations in adolescents with type 2 diabetes (T2D) to determine if changes in HDL composition are associated with early vascular disease. T2D ($n = 10$), lean ($n = 9$), and obese ($n = 11$) youth were recruited. Plasma was fractionated using gel-filtration chromatography, and lipid-associated proteins were identified using mass spectrometry. Concurrently, vascular stiffness was assessed using pulse wave velocity (PWV). We found youth with T2D exhibited decreased phospholipid content in fractions containing large HDL particles that was inversely associated with PWV ($P < 0.001$). No association was noted between HDL-C and PWV. Proteomic analysis revealed changes in 7 of 45 identified proteins in the T2D group, including apolipoprotein (apo) A-II, apoE, and paraoxonase-1 ($P < 0.05$). Our data demonstrate early changes in the lipid and protein compositions of specific HDL subspecies in adolescents with T2D that are related to early markers of arterial disease. These findings suggest that analyzing the composition of HDL, rather than HDL-C, may be useful in assessing cardiovascular risk in this population. *Diabetes* **62:2958–2967, 2013**

Type 2 diabetes (T2D) is a major risk factor for cardiovascular disease (CVD) (1), as ~70% of adults with T2D die of cardiovascular-related complications (2). A key feature linking diabetes to CVD is the presence of an atherogenic dyslipidemia characterized by reduced levels of HDL cholesterol (HDL-C) and increased concentrations of very LDLs (VLDLs) (3). While LDL cholesterol (LDL-C)–lowering therapy has proven effective for reducing CVD risk in adults with T2D, a higher than expected residual incidence of CVD remains in this group (4).

Recent therapies aimed at reducing this residual CVD risk have focused on HDL because of its antiatherogenic properties. HDL is best recognized for its ability to shuttle excess cholesterol from peripheral tissues to the liver for excretion in the process of reverse cholesterol transport, likely contributing to the well-known inverse relationship between HDL-C and CVD. Additional cardioprotective roles for HDL have been identified that include anti-inflammatory, antioxidative, and antiapoptotic properties (5).

Recent work suggests that the total pool of HDL within a given individual is actually composed of numerous HDL particle subpopulations with distinct protein and lipid compositions that are not fully represented by the HDL-C number. Proteomics studies consistently identify >50 different proteins associated with HDL, suggesting each HDL particle may have a unique makeup that directs its specific function (6). These findings suggest that individuals possess unique HDL portfolios that, when considered in total, may establish an individual's risk for CVD.

Previously, our group has used gel filtration chromatography to fractionate plasma and mass spectrometry (MS) to identify protein components of HDL subfractions. These results demonstrate HDL proteins exhibited distinct distribution patterns suggestive of proteomically distinct lipoprotein subspecies in healthy male adults (7).

In this study, we examined the effects of T2D on the lipid and protein compositions of HDL subfractions in male youth. Additionally, we sought to relate compositional differences to early markers of vascular damage, carotid intima media thickness (IMT) and pulse wave velocity (PWV), that are known to predict cardiovascular events (8,9). We hypothesized that HDL lipid and protein composition would be altered in T2D and that these changes would be associated with early markers of atherosclerosis.

RESEARCH DESIGN AND METHODS

Postpubertal adolescents and young adults ages 16–23 years old were recruited and seen at an in-person study visit at Cincinnati Children's Hospital. Only males were considered to eliminate known differences in lipoproteins among sexes (10,11). Participants with T2D ($n = 10$) were classified as such based on the American Diabetes Association criteria (12), which included a fasting plasma glucose ≥126 mg/dL, a 2-h plasma glucose >200 mg/dL during an oral glucose tolerance test, or the classic symptoms of hyperglycemia (polyuria and polydipsia) and a random plasma glucose >200 mg/dL. Individuals with T2D were negative for GAD, islet cell autoantigen-512, and insulin autoantibodies (Barbara Davis Center, University of Colorado, Aurora, CO), indicating the absence of type 1 diabetes. Average duration of diabetes for the T2D group was 4 years 9 months. Antihyperglycemic therapies included metformin ($n = 5$) and insulin ($n = 5$).

Two control groups were included to separate the effects of obesity from the effects of diabetes. Lean control participants ($n = 9$) lacked evidence of any chronic disease and either had a BMI less than the 85th percentile or <25 if ≥20 years old. Obese control participants ($n = 11$) had a BMI greater than the 95th percentile or >30 if ≥20 years old. All obese participants underwent a 2-h oral glucose tolerance test to ensure they had no evidence of T2D (12). No participants were on lipid-lowering, antihypertensive therapy or had a history of smoking. All were normotensive. Written informed consent was obtained from participants ≥18 years old or the parent or guardian with written assent for participants <18 years old.

After a 10-h fast, blood was drawn. One aliquot was used for a fasting lipid panel and hemoglobin A1C. Measurements of total cholesterol (TC), HDL-C, and triglycerides (TGs) were performed enzymatically using a Roche Modular P analyzer (Roche). Determination of HDL-C was performed after precipitation of

See accompanying commentary, p. 2662.

apolipoprotein (apo) B containing particles by dextran sulfate magnesium. LDL-C was calculated using the Friedewald equation or if TGs were >400 mg/dL, LDL-C was measured by direct assay. Hemoglobin A1C was measured in erythrocytes by using high-performance liquid chromatography.

A second aliquot was collected in a BD Vacutainer (BD Biosciences) using citrate as anticoagulant. Plasma was isolated by centrifugation and applied to three Superdex 200 gel filtration columns (GE Healthcare) arranged in series as previously described (7). Eighteen collected fractions were analyzed for choline-containing phospholipid (which is >90% of the phospholipid species in HDL (13) (Wako) and cholesterol (Pointe Scientific) content using colorimetric assays. Plasma was never frozen.

Detailed proteomic methods have been previously published (7). In brief, equal volumes of fraction were applied to a calcium silica hydrate phospholipid binding resin (Lipid Removal Agent; Supelco) to isolate lipoprotein particles. Protein components were digested with trypsin while in contact with the Lipid Removal Agent resin, and then peptide fragments were collected, reduced, and carbamidomethylated. Peptides were stored at −20°C until MS analysis. Samples were injected onto a C-18 reverse-phase column (Grace 218MS 150 × 0.500 mm, 5 μm) on line with an electrospray ionization time-of-flight mass spectrometer (QStar XL; Applied Biosystems). Acquired mass spectra data, a surrogate for protein abundance (14), was scanned against the UniProtKB/Swiss-Prot Protein Knowledgebase (release 57.0, 03/2009) using both Mascot (version 2.1) and XTandem (version 2007.01.01.1) search engines to identify proteins within the 18 fractions.

Positive protein identification required 90% confidence by Protein Prophet Algorithm (15). Peptide counting was used to semiquantitate differences in proteins across fractions between groups (16). MS peptide counts were validated to quantitative biochemical techniques: ELISA and Western blotting for the common HDL proteins apoA-I and Western blot for apoE (Supplementary Figs. 1 and 4). In no instance were peptide counts used to draw conclusions about the absolute quantity or concentration of a protein.

Following the blood draw, each participant underwent noninvasive arterial vessel imaging. Arterial thickness was evaluated in the carotid arteries using high-resolution B-mode ultrasonography (GE Vivid 7; GE Healthcare) using a 7.5-MHz linear array transducer. For each participant, the right and left common carotid IMT from the far wall arteries were measured in millimeters and averaged. Images were read offline (Camtronic Medical System) using a trace technique. Coefficients of variation for repeat readings were 4.1–5.3%.

Arterial stiffness was assessed by PWV using a SphygmoCor SCOR-PVx System (Atcor Medical, Sydney, Australia). The distance from a proximal artery (carotid) to distal artery recording site (femoral) was measured to the nearest 0.1 cm twice and averaged. A tonometer was used to collect proximal and distal arterial waveforms gated by the R-wave on a simultaneously recorded electrocardiogram. PWV was then calculated as the distance from the carotid to femoral arteries divided by the time delay measured between the feet of the two waveforms reported in meters per second (8). Carotid to femoral PWV represents aortic stiffness and is an index of arterial distensibility, and an increase in PWV is associated with a future risk to develop coronary artery disease and stroke (8). Three PWV recordings were obtained and averaged. Coefficient of variation for repeat measures is <7% (17).

All data presented are mean ± SE. Three-way group differences were compared using one-way ANOVA. For proteomic data for which only two groups were compared, two-tailed Student t test with unequal variance was used. Bonferroni corrections were used to correct phospholipid, cholesterol, and proteomic data for multiple comparisons. A P value <0.05 was considered statistically significant.

In general, there were lower peptide counts for most of the detected proteins in the T2D versus lean group. Therefore, we devised a peptide count adjustment procedure to normalize the bulk of peptide counts across all groups. For each protein, an adjustment factor was calculated by dividing the T2D peptide counts by the lean counts. The averaged adjustment factor (1.42) from all identified proteins was multiplied by the raw T2D counts in each fraction to derive an adjusted peptide count. An example of this adjustment is shown in Supplementary Fig. 2. Then a two-tailed t test with Bonferroni corrections was performed to identify differences that were beyond those resulting from a general decrease in T2D plasma proteins.

RESULTS

Study population. The clinical characteristics of the study cohort are listed in Table 1. The three groups were similar in age with no significant differences in blood pressure or LDL-C. Significant differences were seen across the groups in BMI, TC, TGs, and HDL-C ($P < 0.05$). Compared with lean and obese youth, T2D youth had the most deleterious clinical lipid profiles with higher TC and TGs and lower HDL-C, consistent with previous reports in T2D youth (17,18). PWV was statistically different across groups, with the T2D group having the highest PWV, indicating increased arterial stiffness, findings similar to those seen in larger studies (17). There were no differences in the common carotid IMT. Lack of carotid IMT differences are likely due to a small number of participants or because changes in carotid IMT may reflect more advanced arterial pathology compared with changes in PWV (19). Due to the lack of carotid IMT differences between groups, no further analyses were conducted using this outcome.

Lipoprotein profiles. The phospholipid and cholesterol distributions across the 18 lipid-containing plasma gel filtration fractions are shown in Fig. 1. In this technique, LDL/VLDL-sized particles migrate together in peak 1 (fractions 14–18), and HDL particles are broadly distributed across peak 2 (fractions 19–30), as the technique is optimized to spread particles in the HDL size range (7). There were no differences in the LDL/VLDL fractions. Youth with T2D exhibited the lowest phospholipid content in the larger HDL containing fractions 22 to 23, and this was statistically lower compared with both lean and obese

TABLE 1
Study cohort

Variable	Lean	Obese	T2D	P value
n	9	11	10	—
Age (years)	21.8 ± 0.9	22.2 ± 0.8	21.5 ± 0.8	0.831
BMI (mg/kg^2)	23.8 ± 1.7	35.4 ± 1.5	37.8 ± 1.6	<0.001
TC (mg/dL)	157 ± 14	187 ± 13	211 ± 13	0.031
TGs (mg/dL)	78 ± 63	105 ± 57	286 ± 59	0.042
HDL-C (mg/dL)	50 ± 3	44 ± 3	33 ± 3	0.001
LDL-C (mg/dL)	90 ± 13	122 ± 12	124 ± 13	0.128
Systolic blood pressure (mmHg)	116 ± 4	120 ± 3	119 ± 4	0.741
Diastolic blood pressure (mmHg)	72 ± 3	77 ± 2	73 ± 2	0.335
PWV (m/s)	5.81 ± 0.33	5.81 ± 0.14	7.16 ± 0.33	0.002
Carotid IMT (mm)	0.48 ± 0.03	0.56 ± 0.02	0.53 ± 0.03	0.102
Hemoglobin A1C (%)	NA	5.0 ± 0.11	9.5 ± 1.6	0.046
Hemoglobin A1C (mmol/mol)		31 ± 3.2	80 ± 17.5	

Data are mean and SE. P value indicates differences across the three groups using ANOVA, except for hemoglobin A1C, where t test was used. NA, not available.
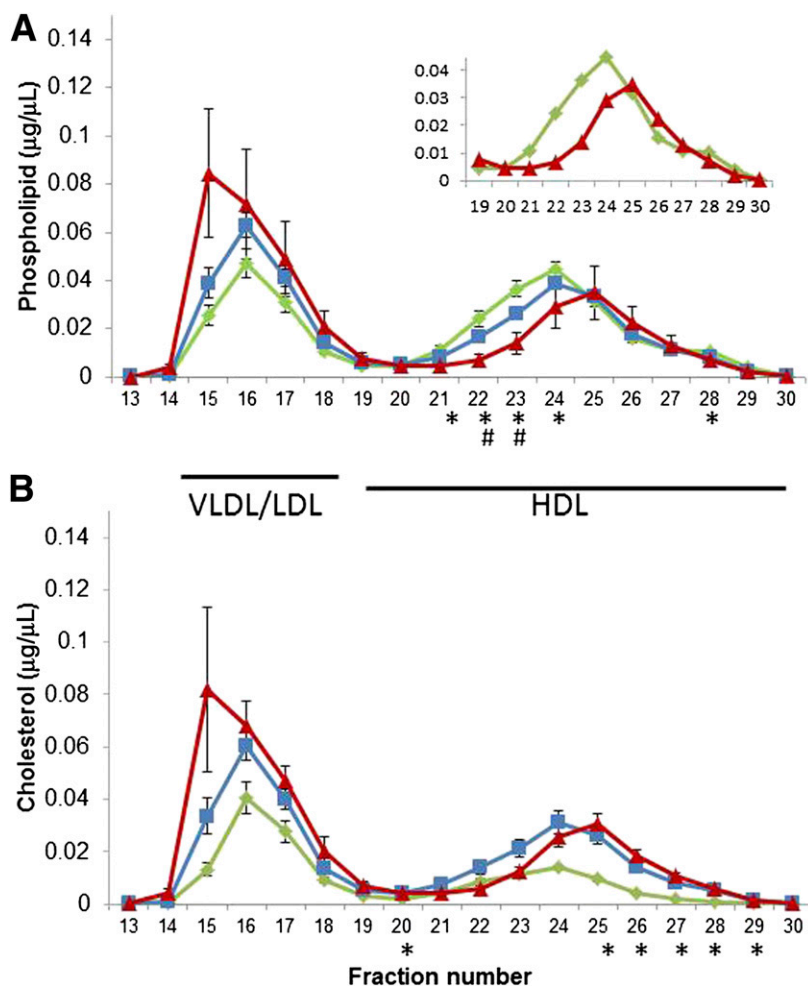
FIG. 1. Lipid distribution profiles for all lean (green), obese (blue), and T2D (red) participants. Panel *A* displays the phospholipid content across the multiple size-based plasma fractions. Panel *B* displays the cholesterol content across the multiple size-based plasma fractions. Data are mean and SE. *$P < 0.05$ between lean and T2D groups; #$P < 0.05$ between obese and T2D groups after corrections for multiple comparisons.

groups ($P < 0.05$). The obese group tended to have the highest cholesterol content in the large HDL fractions (though not statistically different), while the T2D group had the highest cholesterol content in the fractions containing small HDL, fractions 25–29 ($P < 0.05$ compared with lean group). These results are surprising given that the clinical measure of HDL-C (Table 1) indicated the highest cholesterol content in the lean group, followed by the obese and the lowest content in the T2D group. The disparity likely involves inherent differences between the gel-filtration separation and the clinical apoB precipitation methodologies for assessment of HDL-C. Taken together, the phospholipid and cholesterol distributions indicate that individuals with obesity and T2D generally have smaller HDL particles that are enriched in cholesterol relative to phospholipid, while lean adolescents exhibit a population of larger, more phospholipid-rich HDL particles, particularly in fractions 21–24.

Clinical lipids and arterial stiffness. We first determined if commonly used clinical lipid measures were associated with arterial stiffness measurements in this cohort. Univariate correlations between PWV and TC, LDL-C, and HDL-C are shown in Fig. 2. While a weak correlation was found between TC and PWV (Fig. 2A), there was no clear association between the clinical lipid measures of LDL-C or HDL-C and PWV (Fig. 2B and C).

Lipoproteins and arterial stiffness. Next, we sought to determine if lipoprotein size subfractions from our gel filtration analysis were associated with PWV. Figure 3 shows the correlation coefficients of PWV versus phospholipid content for each individual fraction with statistically significant correlation values ($P < 0.05$) falling outside the dotted lines. Combining all patients ($n = 30$), Fig. 3A shows that the phospholipid content of LDL/VLDL fractions 14, 15, and 16 and smaller HDL fractions 25, 26, and 27 were significantly and positively correlated with PWV, while larger HDL fractions 21, 22, and 23 were negatively correlated ($P < 0.05$). Similar relationships were identified for the cholesterol content (Supplementary Fig. 3), though the correlations with phospholipid content were stronger. When the same analysis was performed on data from the lean participants only (Fig. 3B), a similar pattern of correlations was apparent. However, in the obese and T2D groups (Fig. 3C and D), the correlations in the large phospholipid-rich HDL fractions 21–24 disappear. This was particularly striking in the T2D group because these participants effectively lack these particles.

The univariate correlations for the most significant fractions in each of the regions identified in Fig. 3A are shown in a scatterplot in Fig. 3E and G. The phospholipid concentrations among the lean, obese, and T2D group overlap significantly in the VLDL/LDL (fraction 15, Fig. 3E)
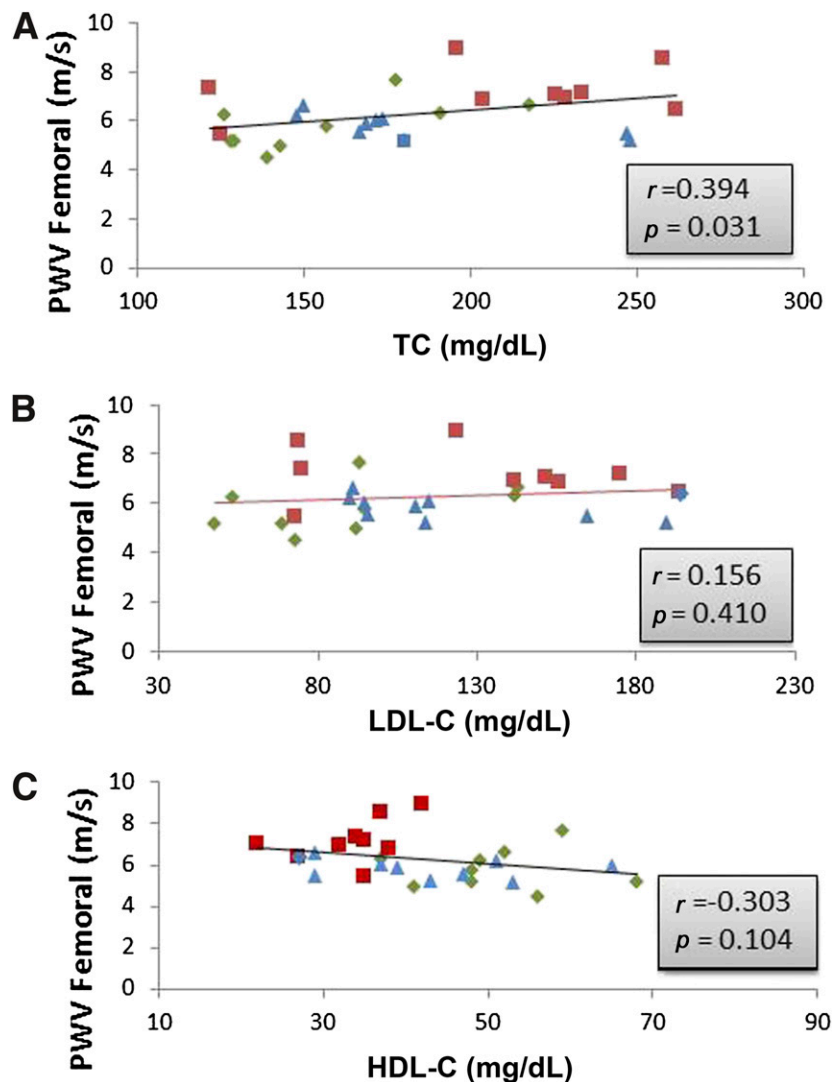
FIG. 2. Correlations of standard clinical lipid measures with PWV. TC (*A*), LDL-C (*B*), and HDL-C (*C*). Green diamonds, lean; blue triangles, obese; red squares, T2D.

and smaller HDL fractions (fraction 26, Fig. 3*G*). However, in the large HDL fraction (fraction 22, Fig. 3*B*), there is a well-defined breakpoint. All T2D participants exhibited <0.01 μg/μL phospholipid in this fraction. By contrast, all lean individuals and most in the obese group were above this threshold. In summary, the above indicate that: *1*) the phospholipid content of fractions better predicted increased PWV compared with the clinical lipid measurements; and *2*) the strongest association appears to be the inverse relationship between larger HDL fractions and PWV. **Proteomics.** We next set out to determine the proteomic makeup of the fractionated lipoproteins. Given the labor intensity associated with conducting proteomic studies, only the lean and T2D groups were analyzed. Across all fractions and all participants, we identified 45 lipid-associated proteins using our previous criteria (7). Nearly all proteins were detected in both groups, with only two that were unique to the lean group, serum amyloid A (SAA) and serotransferrin. Figure 4 shows that, compared with lean participants, youth with T2D exhibited decreased peptide counts for most of the identified proteins, including the major HDL protein apoA-I. This is consistent with the overall lower amount of phospholipid that we detected in

the HDL fractions of T2D versus lean groups (Fig. 1*A*) and the lower plasma HDL-C measured in the clinical assay (Table 1).

Figure 5*A–C* shows several examples of clear protein abundance differences between the T2D and lean groups among the HDL fractions, while Fig. 5*D–F* shows distribution profiles for some examples of proteins that were clearly similar. One of the most striking differences was apoE, which was found in larger HDL species in lean individuals, but its peptide counts were reduced nearly fivefold and shifted toward smaller HDL particles in the T2D group. Confirmation of this finding by Western blot is shown in Supplementary Fig. 4. ApoA-II and paraoxonase-1 (PON1) also showed an overall lower peptide count in the T2D group.

We attempted to correlate PON1 by MS with plasma PON1 activity. However, the activity level in our fractions was below the limit of detection of the assay (Invitrogen). Therefore, we compared whole plasma PON activity in the lean and T2D groups. We found no significant difference in whole plasma PON activity between groups (lean, 22.5 ± 4.96; T2D, 23.0 ± 5.80 U/mL; $P = 0.86$). Potential explanations for these results include: *1*) youth with T2D have
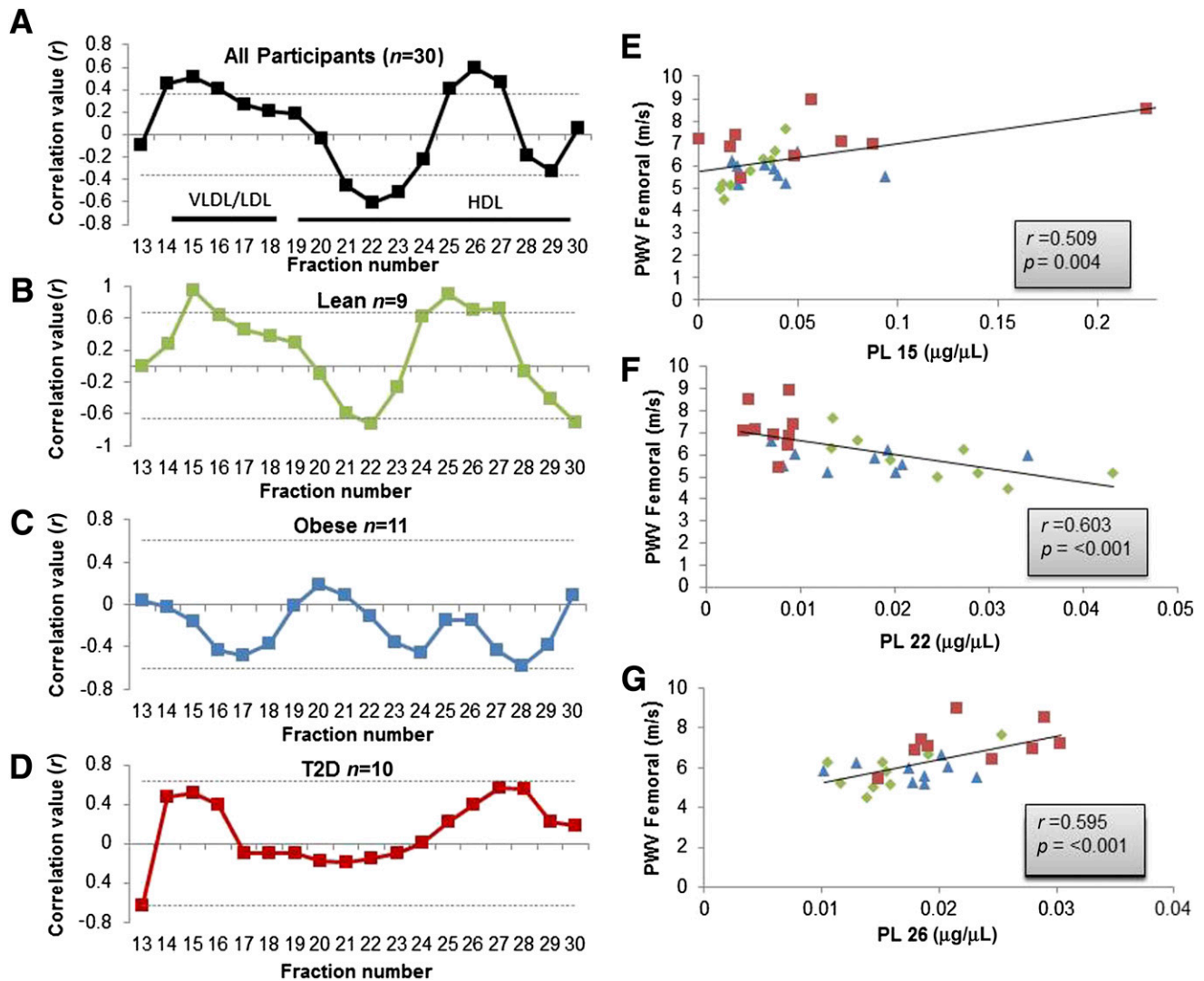
FIG. 3. Correlations of phospholipid fractions with PWV. *A* displays the correlation value (*r*) for all participants (black), *B* for lean (green), *C* for obese (blue), and *D* for T2D (red). Outer dotted lines indicate *r* values of *P* < 0.05. Panels *E*–*G* display individual fractions with the strongest correlations with PWV: fraction 16 (*E*), fraction 22 (*F*), and fraction 26 (*G*).

similar amounts of PON1, but the enzyme undergoes a shift in its HDL size distribution in diabetes; or *2*) PON1 undergoes a posttranslational modification in diabetes (i.e., glycation that reduces its detection by MS).

We noted no protein differences within the LDL/VLDL containing fractions (data not shown). We caution that our techniques are not optimized for LDL/VLDL analysis. Figure 6 shows the *P* values resulting from a *t* test comparison of the total peptide counts measured in lean participants versus the adjusted values from the T2D group. Statistically significant differences are noted in gray after stringent Bonferroni corrections. Differences without Bonferroni corrections are shown in Supplementary Fig. 5. Across all fractions, we noted significant differences in seven proteins in the T2D versus the lean group. Interestingly, a majority of these changes occurred in fraction 22, the same fraction that exhibited significant reductions in phospholipid in the T2D group (and was inversely correlated with PWV).

DISCUSSION

The hypothesis driving this study states that specific HDL subpopulations may contribute to early atherosclerosis in

the setting of T2D. If this idea is correct, then identification of specific altered lipoprotein subpopulations could lead to better, early biomarkers for assessing CVD risk in T2D. Using a plasma fractionation technique previously described by our laboratory (7), we correlated lipoprotein subfractions with early arterial dysfunction in youth with T2D.

Before discussing the implications of our findings, it is worthwhile to clarify some issues surrounding lipoprotein nomenclature. Lipoproteins are most commonly isolated by density ultracentrifugation, and the major classes, HDL and LDL, have been historically defined by their density (20). We elected to analyze lipoproteins by size through gel-filtration chromatography because we and others have noted fewer alterations in particle proteome compared with ultracentrifugation (6,7). Although lipoprotein size and density are roughly inversely correlated, there are exceptions to this rule (21). Thus, in order to relate our gel-filtration results to traditional density-centric definitions, we use the presence of apoB, the core constituent of LDL, as the key distinguisher. Therefore, the VLDL/LDL range is defined as fractions 14–18 due to the presence of apoB. We assigned the remaining fractions 19–30 as the HDL range

Comparison of phospholipid-associated proteins between groups

Protein	Count	Avg. Spectral Counts	
		Healthy	T2DM
Alpha-1-antitrypsin	9	3.7	0.8
Alpha-1B-glycoprotein	13	4.7	1.8
Alpha-2-antiplasmin	11	2.4	0.5
Alpha-2-HS-glycoprotein	14	17.3	4.7
AMBP protein	12	2	2.2
Angiotensinogen	9	2.7	0.5
Antithrombin-III	13	11.6	6.2
apo A-I	15	145	86.2
apo A-II	15	13.3	6.3
apo A-IV	15	58.1	27
apo B-100	13	36.7	6.5
apo C-I	14	8.2	3
apo C-II	13	1.6	0.8
apo C-III	14	8.6	5.3
apo E	13	22	9.8
apo M	13	4	1.2
apo-L1	10	2	1.8
Beta-2-glycoprotein 1	15	9.6	4.8
C4b-binding protein alpha chain	13	7.7	2.8
Clusterin	15	23.9	15.7
Complement C1s subcomponent	13	6.7	2.3
Complement C3	15	131.7	80.5
Complement C4-B	15	74.3	35.7
Complement factor B	15	35.9	19.5
Fibrinogen alpha chain	15	54.9	22.7
Haptoglobin-related protein	12	10	4.7
Hemopexin	15	18.9	11.2
Heparin cofactor 2	15	14.1	10.8
Insulin-like growth factor-binding protein ALS	12	10.7	4.3
Inter-alpha-trypsin inhibitor heavy chain H2	15	24.3	10
Inter-alpha-trypsin inhibitor heavy chain H4	15	31	8
Kininogen-1	14	13	7.7
Lipopolysaccharide-binding protein	6	1.1	0.5
Pigment epithelium-derived factor	15	15.4	8.2
Plasma protease C1 inhibitor	7	2.3	0.5
Prothrombin	15	15.3	9
Retinol-binding protein 4	9	2.3	0.3
Serotransferrin	3	2.8	0
Serum albumin	15	142.6	70.3
Serum amyloid A	3	1.2	0
Serum amyloid A-4	7	1.4	0.3
Serum paraoxonase/arylesterase 1	12	15.8	2.7
Transthyretin	9	4.1	1.2
Vitamin D-binding protein	15	14	3.2
Vitronectin	15	16.7	11.7

Restricted to only proteins found in 3 prior HDL studies.

Count is the number of subjects with > 0 peptides for given protein.

FIG. 4. The average phospholipid-associated proteins in the lean and T2D groups.

because their diameters are consistent with measurements for density-isolated HDL and because of the abundance of the major HDL protein apoA-I.

In this study, we noted a lack of correlation between the classical measures of TC, LDL-C, or HDL-C and PWV. This lack of association has also been noted in larger studies (17) and suggests that these measures are not robust enough to predict CVD risk in individuals. However, our results clearly identify specific lipoprotein size populations that strongly correlate with early vascular stiffness, despite
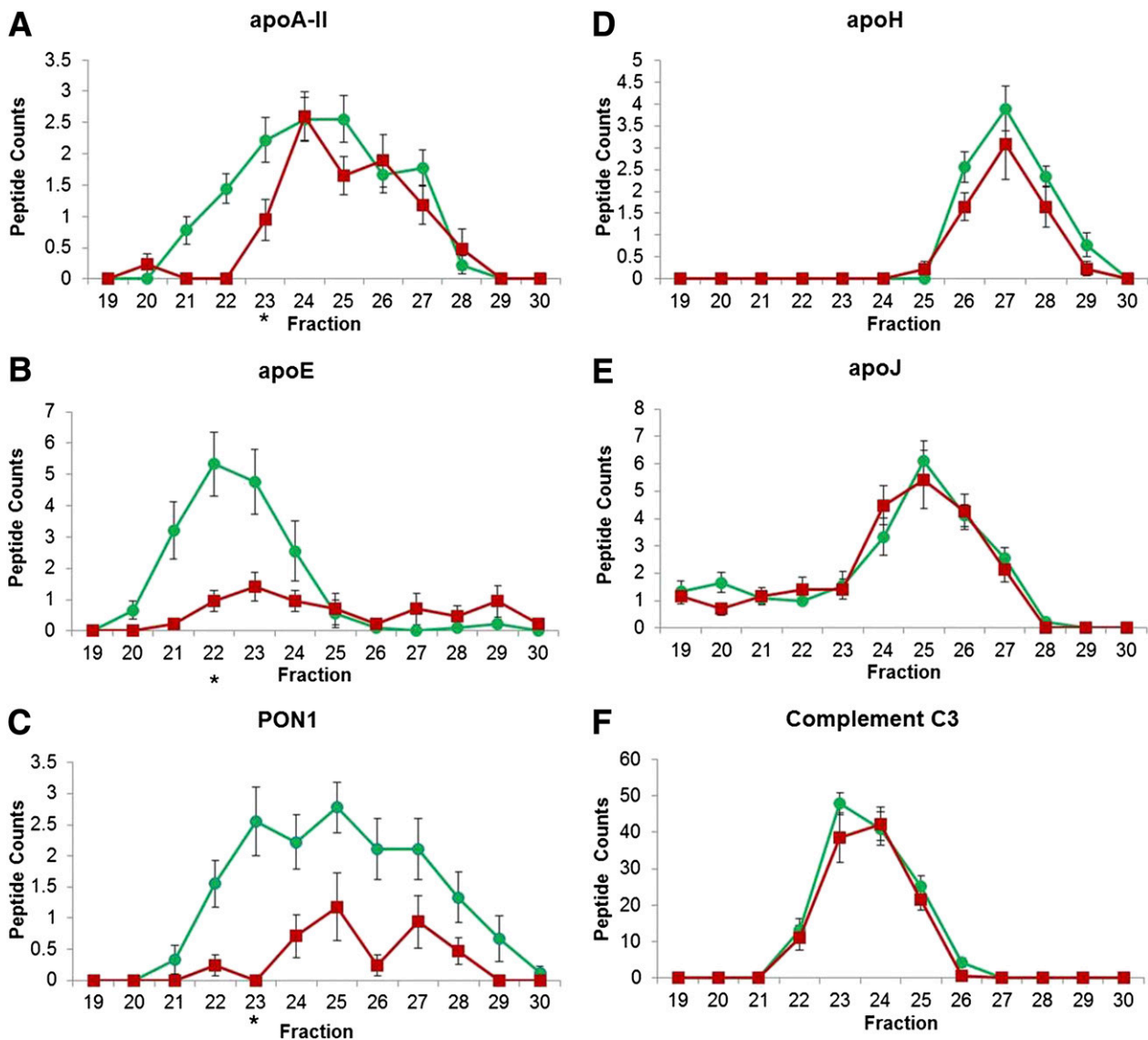
FIG. 5. Distribution patterns of phospholipid-associated proteins are altered in T2D. Adjusted distribution profiles of proteins across the collected fractions are shown for lean (green circles) and T2D (red squares): apoA-II (A), apoE (B), PON1 (C), apoH (D), apoJ (Clusterin) (E), and complement C3 (F). Data are mean and SE. *P < 0.05.

the small number of participants in this study. In the HDL size range, we noted two regions that tightly associated with PWV. Fractions 21–23, representing larger particles, were strongly inversely correlated (i.e., associated with protection), while fractions 25–27, representing smaller particles, were strongly positively correlated. Therefore, depending on the subpopulation distribution in a given individual, it is possible that the balance between these fractions may result in greater or less atheroprotection. The differential association of HDL fractions with arterial stiffness supports the idea that all HDL may not be protective, but specific HDL subpopulations may be associated with protection or pathology in the vessel wall. We were particularly intrigued by the fact that all of the T2D participants exhibited <0.01 μg/μL of phospholipid in fraction 22, whereas none of the lean individuals and only three of the obese individuals were below this threshold. Thus, particles within fraction 22 may prove useful as biomarkers for early onset of vascular disease in T2D. Studies documenting temporal changes in these fractions

over the disease course and changes as a result of therapy are needed.

With regard to the smaller fractions of HDL (25–27) that were positively associated with PWV, we noted relatively few changes in protein abundance in these fractions, and it is not obvious how reductions in these proteins might affect vascular function. However, we did find it interesting that these particles tended to be highly cholesterol enriched relative to phospholipid in the T2D population. Prior work in adults with insulin resistance and T2D has shown an increase in small cholesterol-rich HDL particles is associated with impaired reverse cholesterol transport (22) and an increase in coronary heart disease risk in adults (23). Thus, it is possible that these cholesterol-laden particles become dysfunctional in the setting of T2D and alter the atheroprotective properties of HDL.

Our proteomics analyses were consistent with our previous studies in healthy adult males in whom HDL-associated proteins distributed in distinct patterns across the size gradient (7). However, it is unclear why there were

Protein differences among lean and T2D youth		HDL Fraction Number											
		19	20	21	22	23	24	25	26	27	28	29	30
Alpha-1-antitrypsin	A1AT	1	1							1	0.9084	1	1*
Alpha-1B-glycoprotein	A1BG							1	0.7065	1			
Alpha-2-antiplasmin	A2AP							0.0255	1	0.242			
Alpha-2-HS-glycoprotein	FETUA							0.845	0.5775	0.022	0.1515	0.3215	
AMBP protein	AMBP		1	1	1								1
Angiotensinogen	ANGT	1	1						0.2118	0.212	0.1332	0.8952	
Antithrombin-III	ANT3									0.891	1	1	1
apo A-I	APOA1	1	1	0.241	0.2028	0.1152	1	1	1	1	1	1	1
apo A-II	APOA2		1	0.073	0.0027	0.4482	1	1	1	1	1		
apo A-IV	APOA4			1	0.531	1	1	0.47	1	1	1	1	1
apo B-100	APOB												
apo C-I	APOC1	1	1	1	0.5467	0.2893	1	1	1	0.628	1	1	
apo C-II	APOC2				1	0.1765	1	0.845	1				
apo C-III	APOC3	1	1	1	1	1	1	1	1	1	1	1	
apo E	APOE		0.5467	0.123	0.0264	0.3366	1	1	1	1	1	1	1
apo M	APOM			0.975	0.046	1	1	1					
apo -L1	APOL1	1	1	1	1	1	1						
Beta-2-glycoprotein 1	APOH							1	0.617	1	1	0.96	
C4b-binding protein alpha chain	C4BPA	0.3466											
Clusterin (apoJ)	CLUS	1	0.929	1	1	1	1	1	1	1	1		
Complement C1s subcomponent	C1S				0.2036	0.1884	1	0.4684					
Complement C3	CO3			1	1	1	1	1	0.2856	1		1	
Complement C4-B	CO4B	1	1	0.228	1	1	1	1					
Complement factor B	CFAB							0.4515	1	1	0.537	1	
Fibrinogen alpha chain	FIBA	1	0.6912	1	1				1	1	1	1	
Haptoglobin-related protein	HPTR	1	0.4405	1	1			1					
Hemopexin	HEMO							1	1	1	1	0.345	1
Heparin cofactor 2	HEP2							1	1	1	1	0.0665	
Insulin-like growth factor-binding protein ALS	ALS				1	1	1	0.7525	1				
Inter-alpha-trypsin inhibitor heavy chain H2	ITIH2		1	1	0.0485	0.006	0.845						
Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4			1	1	0.3861	0.087	0.2898	0.0729	0.725		1	1
Kininogen-1	KNG1			1	1	1	1	1	1	1	1	1	
Lipopolysaccharide-binding protein	LBP										1	1	1
Pigment epithelium-derived factor	PEDF								0.78	0.62	1	1	
Plasma protease C1 inhibitor	IC1			0.363	0.5152								
Prothrombin	THRB							1	1	1	0.3672	1	1
Retinol-binding protein 4	RET4									1	0.1347	0.1527	
Serotransferrin	TRFE								0.3138	0.675	0.2415		
Serum albumin	ALBU	1	1	0.788	1	1	0.853	0.3924	0.4128	1	1	1	1
Serum amyloid A protein	SAA				1	0.4152	0.415	0.676					
Serum amyloid A-4 protein	SAA4				1	1	1	0.4025	1				
Serum paraoxonase/arylesterase 1	PON1			1	0.214	0.018	0.402	0.653	0.107	1	1	1*	1
Transthyretin	TTHY		1						1	1	1	1	1
Vitamin D-binding protein	VTDB							1	1	1	0.0864	0.3006	1
Vitronectin	VTNC				1	1		1	1	1	1	0.0931	

FIG. 6. Comparison of adjusted total peptide counts in the lean versus T2D groups across the HDL size distribution. Significant protein differences of $P < 0.05$ (after Bonferroni corrections) are shaded in gray.

overall reduced levels of nearly all proteins detected in the T2D group. One possible explanation is that there is an enrichment of unstable TG-rich HDL particles the setting of T2D (24), which resulted in decreased recovery of proteins from the calcium silica hydrate phospholipid-binding resin. Another possibility is that there are post-translational modifications of plasma proteins in persons with diabetes that result in fewer peptide counts. Using Mascot and XTandem, we searched for known oxidation, carbamylation, phosphorylation, and glycosyl modifications, and none were detected. This does not eliminate the possibility of nonenzymatic glycation, nitration, or chlorination modifications of proteins as a result of diabetes (25). Because we cannot be sure of the reason for the lower peptide counts, we normalized based on peptide counts taken from all detected proteins, rather than normalizing to one protein such as apoA-I. While this rather conservative approach likely reduced our ability to detect some protein differences between groups, the likelihood that detected differences were real is increased, particularly since Bonferroni corrections were taken into consideration in the statistics. We identified

seven proteins that underwent abundance or size distribution changes. Interestingly, most of these changes corresponded with the fractions 22 to 23 that were negatively associated with PWV. One of the most striking was apoE, which underwent a nearly fivefold reduction in peptide counts.

ApoE is widely distributed on VLDLs, LDLs, and HDLs in human plasma and is known to associate with larger HDL particles. Genetic ablation of apoE in mice is well-known to cause a variety of vascular phenotypes including atherosclerosis (26), endothelial dysfunction (27), and vascular remodeling and restenosis (28). Recent work demonstrates that large apoE-rich HDL may interfere with LDL binding to proteoglycans (PGs) in the vessel wall in humans (29). Entrapment of LDL by PG triggers inflammation, causing atherosclerosis (30). Decreased content of large HDL particles in adults with T2D is thought to facilitate LDL–PG binding and contribute to increased atherosclerosis in T2D. Our results are consistent with this idea.

Another protein that exhibited decreases in the HDL particle size range was PON1. This is a serum esterase that

hydrolyzes organophosphates and has been shown by numerous studies to play an important role in the prevention and scavenging of lipid peroxidation in LDLs (31). PON1 has been implicated in the severity of atherosclerosis in T2D adults (32) and may protect against diabetes via antioxidative effects and/or through effects on insulin secretion (33). Its activity is reduced in T2D, though this may be due to reductions in specific activity rather than lower serum concentrations (34).

ApoM was also lower in the larger HDL-sized particles in the T2D group, consistent with whole plasma reductions of ~10% noted by others (35). Karuna et al. (36) recently found that apoM mediates the enrichment of sphingosine-1-phosphate content in HDLs, which is known to promote arterial vasodilation by stimulating endothelial nitric oxide production and inhibiting monocyte recruitment into the intima. Additionally, transgenic overexpression of apoM in mice leads to reduced atherosclerotic lesions, possibly by facilitating HDL remodeling, increased cholesterol efflux (37), or generating atheroprotective pre-β forms of HDL (35).

Two proteins were found in only our lean group, SAA and serotransferrin. While chronically elevated levels of SAA are associated with an increased risk for CVD (38), low levels are present in healthy individuals (7). Thus, the low SAA presence in two lean individuals may reflect mild nonobesity or diabetes-related inflammation. Serotransferrin is a major iron-binding protein that is known to associate with HDL (39). HDL containing serotransferrin inhibits in vitro oxidation of LDL by copper, suggesting an atheroprotective role for the protein (40), which explains its presence in lean persons.

While it is tempting to speculate that the lipoprotein size fractions identified in this study may be directly involved in either vascular protection or pathology, we caution that our data are purely correlative at this point. We do not know if the identified particle populations are causative or are byproducts. Nevertheless, the strong correlations of the size subpopulations with arterial stiffness are clear, and we believe that our data strongly justify efforts to biochemically identify the specific particles that are changing in the early stages of diabetes.

ACKNOWLEDGMENTS

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