

# Combined High-Density Lipoprotein Proteomic and Glycomic Profiles in Patients at Risk for Coronary Artery Disease

Sridevi Krishnan,<sup>†</sup> Jincui Huang,<sup>‡</sup> Hyeyoung Lee,<sup>§</sup> Andres Guerrero,<sup>‡</sup> Lars Berglund,<sup>||,⊥</sup>  
Erdembileg Anuurad,<sup>||</sup> Carlito B. Lebrilla,<sup>‡</sup> and Angela M. Zivkovic<sup>\*,†,‡,#</sup>

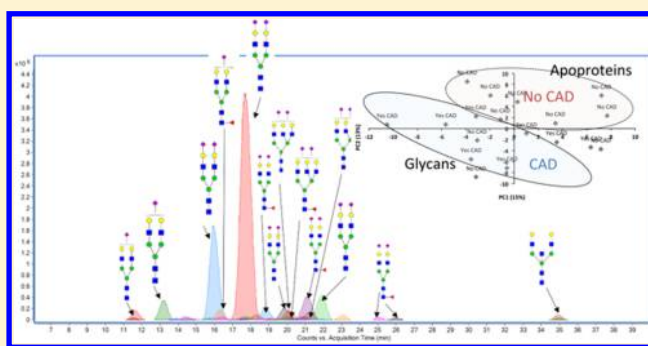
<sup>†</sup>Department of Nutrition, <sup>‡</sup>Department of Chemistry, <sup>§</sup>Department of Food Science and Technology, <sup>||</sup>Department of Internal Medicine, and <sup>#</sup>Foods for Health Institute, University of California Davis, One Shields Avenue, Davis, California 95616, United States

<sup>⊥</sup>Department of Veterans Affairs, Northern California Health Care System, Sacramento, California 95655, United States

## S Supporting Information

**ABSTRACT:** **Objectives:** To test whether recently developed methods for comprehensive profiling of the high-density lipoprotein (HDL) glycome combined with the HDL proteome can distinguish individuals with coronary artery disease (CAD) from those without. **Methods:** Twenty subjects at risk for CAD, who underwent diagnostic coronary arteriography, were analyzed. Ten subjects had CAD, and ten did not. HDL was extracted from fasting plasma samples by ultracentrifugation, followed by shotgun proteomic, glycomic, and ganglioside analyses using LC-MS. CAD vs non-CAD subjects' data were compared using univariate and multivariate statistics. **Results:** Principal components analysis showed a clear separation of CAD and non-CAD subjects, confirming that combined HDL proteomic and glycomic profiles distinguished at-risk subjects with atherosclerosis from those without. CAD patients had lower HDL apolipoprotein content (specifically ApoA-I, A-II, and E,  $p < 0.05$ ), and lower serum amyloid A2 (SAA2,  $p = 0.020$ ) and SAA4 ( $p = 0.007$ ) but higher sialylated glycans ( $p < 0.05$ ). **Conclusion:** Combined proteomic and glycomic profiling of isolated HDL was tested as a novel analytical approach for developing biomarkers of disease. In this pilot study we found that HDL proteome and glycome distinguished between individuals who had CAD from those who did not within a group of individuals equally at risk for heart disease.

**KEYWORDS:** atherosclerosis, cardiovascular disease, coronary artery disease, HDL, proteomics, glycomics, glycolipids, apolipoproteins



## INTRODUCTION

Coronary artery disease (CAD), the cause for one in four deaths in the US according to the Centers for Disease Control and Prevention (CDC), accounts for ~600,000 deaths each year.<sup>1</sup> Despite ample evidence indicating that high-density lipoprotein cholesterol (HDL-C) levels are protective against CAD across populations, many pharmaceutical interventions to increase HDL-C have failed to demonstrate benefit in cardiovascular end points.<sup>2</sup> HDL particles are heterogeneous, with multiple subclasses and biologic functions, and undergo significant remodeling in vivo.<sup>3</sup> Chemical, compositional, and structural changes transform atheroprotective HDL into less protective, pro-inflammatory particles.<sup>4</sup> The mechanisms behind these transformations are unclear. These “dysfunctional HDL” particles have a reduced capacity for reverse cholesterol transport, have a reduced ability to provide antioxidant and anti-inflammatory functions, and are associated with altered composition.<sup>4</sup> HDL particles from patients with cardiovascular disorders are compositionally different from those of healthy individuals: HDL from cardiovascular disease patients contain oxidized apolipoprotein A-I (ApoA-I) and higher levels of

apolipoprotein C-III (ApoC-III) and the complement activation system protein C3;<sup>5</sup> HDL from patients with metabolic syndrome (MetS) is enriched in serum amyloid A (SAA) and has decreased paraoxonase (PON) activity.<sup>6</sup> Remodeling of the HDL associated proteome in CAD has been linked to changes in HDL functionality,<sup>7</sup> and has also been found to be more inflammatory compared to non-CAD HDL.<sup>8</sup>

Another compositional aspect of HDL that may be altered in disease is the glycome. Protein glycosylation is a post-translational modification during which sugar residues are added to specific peptides of the protein to produce glycans that range in size and composition. The process of protein glycosylation begins in the endoplasmic reticulum, where an oligosaccharide or glycan containing N-acetylglucosamine, mannose, and glucose is transferred to the nascent protein, and this post-translational modification guides protein folding.<sup>9</sup> Trimming of the glycan structure also occurs in the endoplasmic reticulum, after which the newly glycosylated protein is

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Table 1. Baseline Characteristics of Study Volunteers, Comparing CAD vs Non-CAD Subjects<sup>a</sup>

Anthropometric and fasting clinical parameters	CAD ( <i>n</i> = 10) ( <i>n</i> = 5 male, <i>n</i> = 5 female)	no CAD ( <i>n</i> = 10) ( <i>n</i> = 5 male, <i>n</i> = 5 female)	<i>p</i> value for difference between CAD vs non-CAD	Clinically recommended range
	Mean ± SD	Mean ± SD		
Age (y)	57.2 ± 8.1	50.3 ± 7.8	<b>0.032</b>	
BMI (kg/m <sup>2</sup> )	26.3 ± 2.1	30.6 ± 3.3	<b>0.001</b>	18–25
Systolic Blood pressure (mmHg)	137.1 ± 10.4	134.4 ± 8.7	0.297	≤110
Diastolic Blood pressure (mmHg)	83.5 ± 8.8	80.6 ± 9.9	0.279	≤70
Total cholesterol (mg/dL)	207.4 ± 35.0	210.1 ± 31.7	0.429	<200
LDL-cholesterol (mg/dL)	130.8 ± 35.1	143.2 ± 26.5	0.193	<100
HDL-cholesterol (mg/dL)	37.6 ± 3.3	37.8 ± 5.3	0.460	<40
Triglycerides (mg/dL)	195.3 ± 56.0	146.2 ± 42.7	<b>0.021</b>	<150
Waist-hip ratio	0.9 ± 0.0	0.9 ± 0.1	0.151	<0.8
Glucose (mg/dL)	157.5 ± 69.2	128.4 ± 61.7	0.167	<110
Insulin (mmol/L)	23.4 ± 12.8	31.4 ± 44.8	0.298	
HOMA-IR	3.4 ± 2.0	4.1 ± 5.4	0.366	>3.0
CRP (mg/L)	4.4 ± 3.4	6.5 ± 6.4	0.196	<1.0
Composite cardiovascular score [15]	25.2 ± 10.7	4.5 ± 4.7	<b>0.000</b>	≤0

<sup>a</sup>Bolded, italicized *p* values are significant differences. LDL – low density lipoprotein, HDL – high density lipoprotein, HOMA-IR – homeostatic assessment of insulin resistance, CRP – C-reactive protein.

transported to the Golgi complex.<sup>9</sup> In the Golgi, the precursor high mannose glycans can be modified by glycosyltransferases with fucose, galactose, and/or sialic acid residues to form a diverse array of structures, which can be grouped into three categories: high mannose, hybrid, and complex glycans.<sup>9a</sup> High mannose glycans are enriched in mannose residues, complex glycans contain mannose residues but also terminal N-acetylglucosamine, fucose, and/or sialic acid residues, and hybrid glycans contain a mixture of the two. It is estimated that over 50% of all human proteins are glycosylated.<sup>10</sup> The location, extent, and type of glycosylation affect protein functions, folding, interactions with other proteins, and degradation, and they lend physicochemical properties (i.e., charge) to the protein.<sup>9b</sup> Specifically, to how many and to which sites the glycans are attached, and the composition and structure of the glycans—whether they are sialylated, fucosylated, or both—can all affect protein function.<sup>9a</sup> For example, the extent of sialylation on ApoE in cerebrospinal fluid affects its binding to beta-amyloid, which affects the development of Alzheimer's disease.<sup>11</sup>

Not much is known about the specificity of glycosylation in HDL-associated proteins, yet there is tantalizing evidence that it is likely to be important in determining both the function and metabolism of HDL. Sialylated ApoA-II associates only with HDL<sub>3</sub>, whereas nonsialylated ApoA-II associates with HDL of all sizes.<sup>12</sup> ApoE Leiden, an aberrantly glycosylated variant of ApoE, shows defective binding to the LDL receptor.<sup>13</sup> Loss of sialic acid  $\alpha$ 2,6-Neu5Ac containing structures in ApoC-III was found in lung cancer patients.<sup>14</sup> These data suggest important as yet not fully characterized mechanistic links between apoprotein glycosylation and function. Our group recently developed a method for the comprehensive profiling of the HDL glycome, and showed that HDLs are highly sialylated particles, suggesting that glycosylation may be important both functionally and diagnostically.<sup>15</sup> Another recent paper showed changes in both the amount of HDL-associated proteins, such as ApoC-III and SAA, and their glycosylation in the HDL fraction from patients with MetS compared to healthy controls.<sup>16</sup> However, the utility of glycomic profiling of HDL and other lipoproteins for the development of biomarkers has not yet been demonstrated.

We previously reported a method to comprehensively assess the HDL glycome.<sup>15</sup> In the present pilot study, we combined this approach with proteomics to compare the combined glycomic and proteomic profiles of HDL from subjects with and without CAD. Our objective was to test whether the combined proteomic and glycomic profiling of isolated HDL can be used as a novel analytical approach for developing biomarkers of disease. We hypothesized that the use of combined proteomic and glycomic profiling of HDL particles can be used to distinguish CAD patients from non-CAD subjects. This study is a proof of concept for the long-term objective of developing novel biomarkers of risk associated with abnormal lipid metabolism.

## METHODS

### Sample Collection

The samples used for this case-control study were a subset from a previously conducted study, which included 648 patients (401 men, 247 women) scheduled for diagnostic coronary arteriography at either Harlem Hospital Center in New York City or the Bassett Hospital in Cooperstown, NY.<sup>17</sup> The 20 subjects for this study were randomly selected from the parent study, with the following criteria: 10 subjects with CAD and 10 subjects without CAD, with equal number of males and females in each group, HDL-C levels below normal (i.e., <40 mg/dL) but no difference in HDL-C between the CAD and non-CAD group. If the subjects that were randomly selected resulted in a significant difference in HDL-C between groups the subjects with the outlier values were replaced by another set of subjects that had values that more closely matched the group average. The investigators performing the HDL analyses were blinded to the CAD assignment and all other subject clinical data until all of the HDL separations, and proteomic and glycomic analyses were completed. CAD was defined as the presence of >50% stenosis in any 1 of 15 coronary artery segments, and characterized using a composite cardiovascular score (0–75) based on determination of presence of stenosis on a scale of 0–5 of the 15 predetermined coronary artery segments. The subject characteristics are shown in Table 1. The average values for several of the traditional biomarkers of CAD in both groups of subjects included in this study were

outside of the clinically recommended range (i.e., total cholesterol, LDL-C, fasting glucose, C reactive protein (CRP), and HDL-C). Fasting blood samples were drawn 2–4 h before the catheterization procedure, and plasma samples were stored at  $-80^{\circ}\text{C}$ . The study was approved by the institutional review boards at Harlem Hospital, the Mary Imogene Bassett Hospital, and University of California Davis, and informed consent was obtained from all subjects.

### HDL Isolation

Preparation of HDL was performed as described previously using a fast micromethod.<sup>15,18</sup> Briefly, plasma samples were overlaid with KBr solution ( $d = 1.084\text{ g/mL}$ ) and ultracentrifugation was performed using a Sorvall RC M120 GX microultracentrifuge equipped with a S120-AT2 fixed-angle rotor (Thermo Scientific, Waltham, MA, USA) for 2.5 h,  $8^{\circ}\text{C}$ , 435,680g. The combined VLDL and LDL fractions with a density lower than  $1.063\text{ g/mL}$  were removed from the top of the tubes (0.9 mL). The remaining infranate (1.0 mL) was transferred before being overlaid with 0.9 mL of KBr solution ( $d = 1.34\text{ g/mL}$ ), and subjected to ultracentrifugation for 3 h at  $8^{\circ}\text{C}$  and 435,680g. Density solutions of KBr, at  $d = 1.084\text{ g/mL}$  and  $1.34\text{ g/mL}$  were made weekly and verified using the Densito30PX portable densitometer (Mettler Toledo, Columbus, OH, USA). The HDL fraction ( $1.21\text{--}1.063\text{ g/mL}$ ) was collected from the top of the tube (0.6 mL) and subjected to diafiltration using Amicon ultra-3K centrifugal filter devices. During this process the HDL fractions were desalted by washing out the KBr salts with water (Optima LC/MS) by two consecutive steps of centrifugation for 25 min at  $8^{\circ}\text{C}$  and 14,000 xg. Then, the concentrated HDL fraction was recovered in Optima Water for further analysis.

### HDL Proteomic Analysis

Shotgun proteomics was used to investigate the protein composition of the separated HDL fractions. Briefly, fractions were desiccated and reconstituted in  $100\ \mu\text{L}$  ammonium bicarbonate solution (50 mM). Cysteine residues were reduced with  $2\ \mu\text{L}$  of 550 mM Dithiothreitol (DTT) for 50 min at  $60^{\circ}\text{C}$  and incubated for 30 min with  $4\ \mu\text{L}$  of 450 mM iodoacetamide (IAA) at room temperature in the dark. Samples were digested using  $1\ \mu\text{L}$  trypsin ( $1\ \mu\text{g}/\mu\text{L}$ ) for 18 h at  $37^{\circ}\text{C}$ . The digests were purified on a reverse-phase C18 pipet zip-tip according to the manufacturer suggestions. HDL hydrolysates were dried down and reconstituted in  $20\ \mu\text{L}$  nanopure water prior to analysis by mass spectrometry.

Samples were analyzed by liquid chromatography mass spectrometry (LC-MS) on an Agilent 6520 nano-HPLC-Chip Q-TOF instrument in the positive mode. C18 was used as a stationary phase. Mobile phases consisted of 0.1% formic acid (FA) in 3% acetonitrile (ACN) (solvent A) and 0.1% FA in 90% ACN (solvent B). LC separation was performed with the following gradient: 1% to 8% B (0.00–5.00 min); 8% to 26.5% B (5.00 min–48.00 min); 26.5% to 73% B (48.00 min–75 min); 73% to 99% (75.00 min–77.00 min); 99%B (77.00 min–87.00 min). Mass range in the MS mode was  $m/z$  500–3,000 and  $m/z$  50–3,000 for MS/MS. Acquisition rates were 0.63 spectrum/s for both MS and MS/MS. All mass spectra were internally calibrated. For MS/MS mode, the collision energies for each compound were calculated as follows:

$$V_{\text{collision}} = 3.6V\left(\frac{m/z}{100\text{ Da}}\right) - 4.2V$$

MS/MS data was analyzed using X!Tandem ([www.thegpm.org](http://www.thegpm.org)) against the Swissprot human complete proteome. Precursor

ion and fragments were allowed 20 and 100 ppm error, respectively. Cysteine carbamidomethylation was specified as a fixed modification; asparagine and glutamine deamination and methionine and tryptophan oxidation were specified as variable modifications. Only proteins found in at least two samples (one CAD and one non-CAD subject) and with  $\log(e)$  values equal to or less than  $-2$  (corresponding to a false discovery rate of 99%) were considered. Some studies suggest that normalized LC-MS peak areas for detected proteins can be used to compare relative abundances between similar samples.<sup>19</sup> Hence,  $\log(I)$ , the base  $-10$  log of the sum of the fragment ion intensities in the tandem mass spectra was used to quantify proteins in HDL particles. Values were adjusted for initial sample volume as shown in eq 1 and eq 2.

$$a = 10^{(-\log(I))} \quad (\text{eq 1})$$

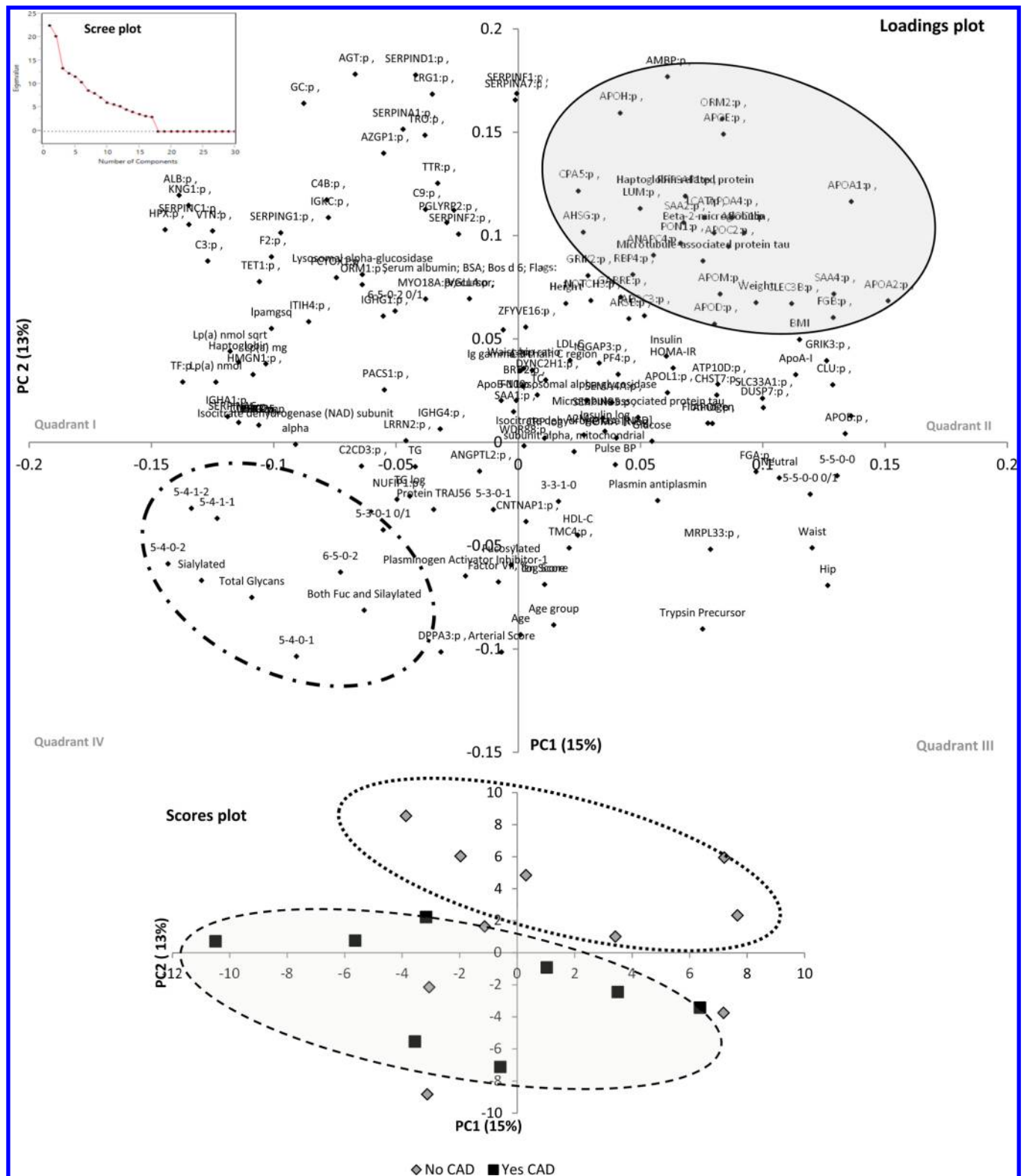
$$\text{Normalized } \log(I) = -\log\left(\frac{a}{\text{initial volume}}\right) \quad (\text{eq 2})$$

### N- and O-Glycan Analysis

In our previously published study<sup>20</sup> we analyzed both the composition of the released glycans and protein-specific glycosylation in HDL, however, in this study sample volumes were too low to allow for protein-specific glycosylation analysis. Thus, analysis of only the released glycans from all HDL-associated proteins was performed on the separated HDL fractions as described previously.<sup>15,21</sup> Briefly, N-glycans were released using the enzyme PNGase F and enriched using standard procedures. N-glycans were reduced in the presence of  $\text{NaBH}_4$  and desalted by solid phase extraction (SPE) using porous graphitized carbon (PGC) cartridges to obtain the correspondent alditols and avoid the presence of anomeric isomers prior to nano-LC-Chip-QTOF analysis. O-glycans were chemically released by  $\beta$ -elimination reaction. Briefly, O-glycans were released in the alditol form by treatment with 1.0 M  $\text{NaBH}_4$ , 0.1 M NaOH for 16–24 h at  $42^{\circ}\text{C}$ . Then, samples were transferred to an ice bath and 1.0 M HCl was added to stop the reaction and to neutralize excess  $\text{NaBH}_4$ . The resulting mixture of oligosaccharides was purified and fractionated on a PGC cartridge. Released and purified N- and O-glycans were analyzed by LC-MS on an Agilent 6210 nano-HPLC-Chip TOF in the positive mode. PGC was used as a stationary phase. The mobile phase consisted of the same solvents (A and B) previously described. The glycan compositions were identified by accurate mass, matched to a theoretical glycan mass library<sup>21c</sup> using a mass error tolerance of 20 ppm. Data analyses were performed with the MassHunter Qualitative Analysis software ver. B.03.01 (Agilent Technologies, Inc., Santa Clara, CA).

Each glycan peak was assigned a glycan composition based on the specific number of each type of monosaccharide contained within it. Each glycan contains a certain number of hexose residues, which include glucose, mannose, and galactose, N-acetylglucosamine residues, fucose residues, and N-acetylneuraminic acid or sialic acid residues. Glycan compositions are named according to their content of these four different saccharide categories such that the number of hexose residues is the first number, followed by the number of N-acetylglucosamine residues, then the number of fucose residues, and finally the number of sialic acid residues. For example, a glycan containing 5 hexose, 4 N-acetylglucosamine, 1 fucose and 2 sialic acid residues is designated as 5–4–1–2. On the other hand, a glycan with the composition 5–4–0–1 contains 5 hexose, 4 N-



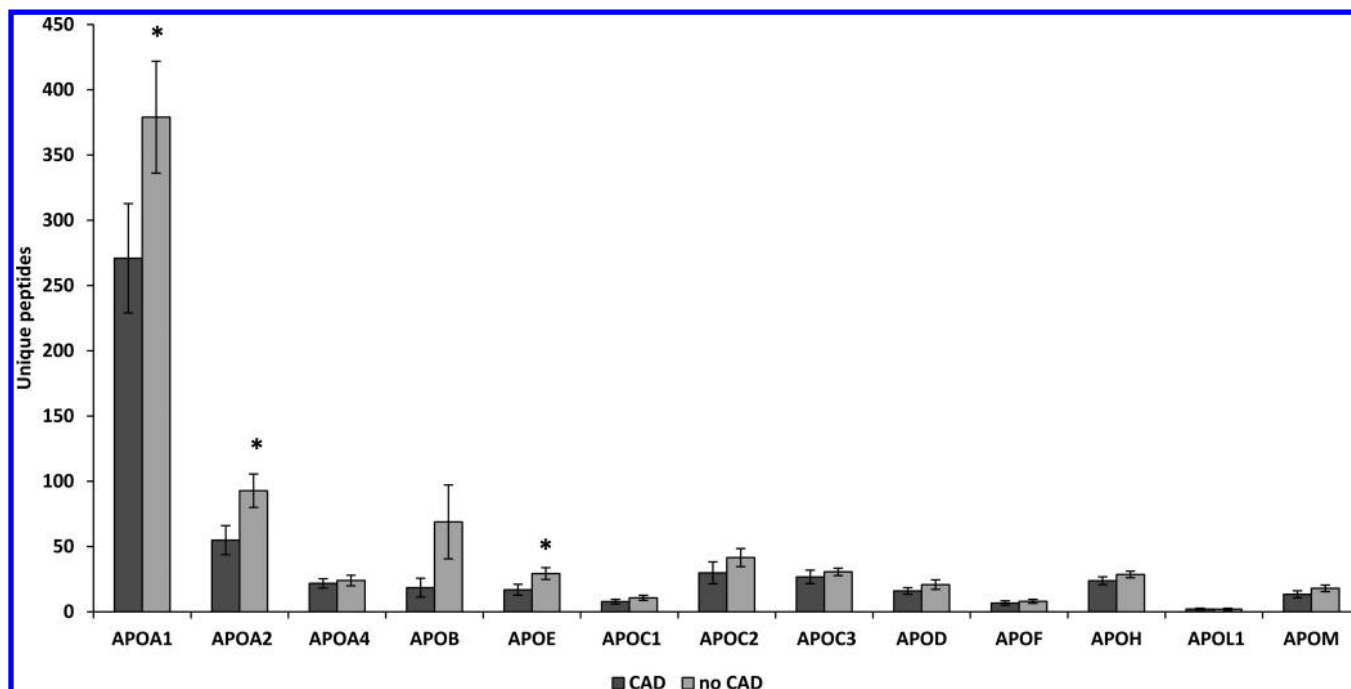


**Figure 1.** Scree, Scores, and Loadings plot of PCA using a standardized data array with metabolic, proteomic, and glycan data, performed on 18 subjects and excluding 2 subjects who were outliers for the PCA analysis. The glycans and apolipoproteins (especially ApoA-II, ApoE, and ApoM) are separated by PC2 in the Loadings plot. The CAD subjects are also clustered together in the lower half of PC2, while non-CAD subjects appear to be predominantly in the upper half of PC2, but not as clearly clustered together as the CAD subjects.

acetylglucosamine, 0 fucose and 1 sialic acid residue. The first glycan, 5-4-1-2, is both a sialylated and a fucosylated glycan. Specifically, it is monofucosylated and disialylated. The second glycan, 5-4-0-1, is nonfucosylated and monosialylated.

### Anionic Glycolipid (ganglioside) Analysis

Ganglioside analysis was performed on the separated HDL fractions as previously described.<sup>15</sup> Briefly, isolated HDL was mixed with water, methanol and chloroform. After phase



**Figure 2.** Apolipoproteins in CAD vs non-CAD subjects in the separated HDL fractions. ApoA-I, ApoA-II, and ApoE were significantly higher in non-CAD subjects compared to CAD subjects ( $p < 0.05$  “\*”).

separation, gangliosides in the aqueous upper layer were recovered and further enriched by a C8 SPE cartridge (Supelco, Bellefonte, PA, USA). A reverse-phase nano-HPLC Chip (G4240–62001, Agilent Technologies, Inc., Santa Clara, CA) with a 40 nL enrichment column and  $43 \times 0.075$  mm ID ZORBAX C18 analytical column was used for analysis. The mobile phases used for gangliosides were water (solvent C) and 15% isopropanol in methanol (v/v) (solvent D), with both containing 20 mM ammonium acetate and 0.1% acetic acid. The Agilent 6520 Q-TOF MS and MS/MS were operated in the negative mode. Data analyses were performed with the MassHunter Qualitative Analysis software ver. B.03.01 (Agilent Technologies, Inc., Santa Clara, CA). Molecular Fraction Extraction (MFE) was performed to generate a peak list ( $m/z$ , retention time and peak area) taking all ions into account exceeding 1,000 counts. Focused postprocessing precursor ion scan analysis was performed in order to select ganglioside peaks, through a mode of the “Find by Auto MS/MS”. The NeuAc ions ( $m/z$  290.095) were the fragment ions used to determine the precursor ion masses representing gangliosides. All metabolite identifications were done at level 2 of the MSI standards.<sup>22</sup>

### Statistical Analysis

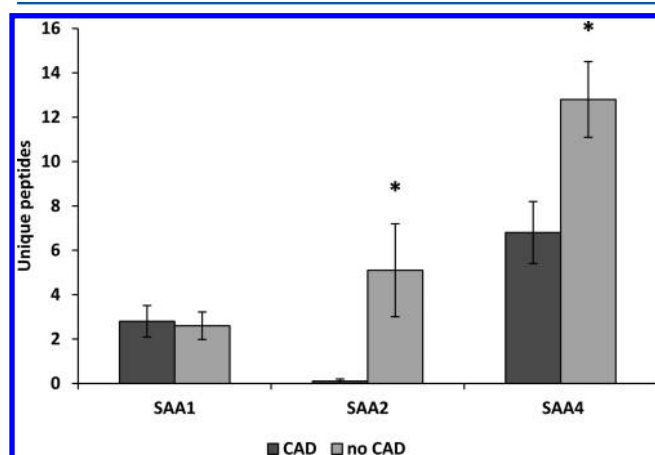
A preliminary mean-centered (since different variables had dramatically different ranges of magnitude), unsupervised principal components analysis (PCA) revealed two multivariate outliers (both from the CAD group—one female and one male), which we removed for the multivariate analysis. Hence, 18 from the total 20 subjects were used for the PCA analysis, however all 20 subjects were used for the rest of the analyses. PCA was used to explore the variations in a total of 164 variables including proteomic (128 proteins—listed in Supplemental Table 1), glycomic (7 individual glycans: 5–4–0–2, 5–4–1–2, 5–4–0–1, 6–5–0–2, 5–5–0–0, 3–3–1–0, 5–3–0–1; 3 individual glycans converted to a dichotomous variable showing either presence or absence of the glycan in each sample: 6–5–0–2 0/1, 5–3–0–1 0/1, 5–5–0–0 0/1; and 5 glycan categories: Total

Glycans, Sialylated, Fucosylated, Both Fucosylated and Sialylated, Neutral (i.e., nonfucosylated, nonsialylated)), and clinical (27 clinical and anthropometric parameters: Systolic BP, Diastolic BP, Age, BMI, TC, LDL-C, HDL-C, TG, Age group, Pulse BP, Height, Weight, Hip, Waist, Waist-hip ratio, TG log, Glucose, Insulin, HOMA-IR, CRP, Fibrinogen, ApoA-I, ApoB-100, Lp(a) nmol, Factor VII, %, Plasmin antiplasmin, Plasminogen Activator Inhibitor-1) data. The Scree plot (included in Figure 1) was used to determine which PCs to use for interpreting results. Following the PCA, Student’s *t* tests were used to identify differences between subjects with CAD compared to those without CAD. All final data analyses were performed using the R statistical software (packages—lme, multcomp, Rcmdr)<sup>23</sup> and JMP (SAS Institute, Cary NC).

## RESULTS

The anthropometric and fasting clinical characteristics of the subjects are summarized in Table 1. The CAD subjects were older (57.2y vs 50.3y), had a lower BMI ( $26.3 \text{ kg/m}^2$  vs  $30.6 \text{ kg/m}^2$ ), higher fasting triglycerides ( $195.3 \text{ mg/dL}$  vs  $146.2 \text{ mg/dL}$ ), and higher comprehensive cardiovascular score (25.2 vs 4.5) compared to the non-CAD subjects. Both CAD and non-CAD subjects had high total cholesterol ( $>200 \text{ mg/dL}$ ), high LDL-C ( $>130 \text{ mg/dL}$ ), high fasting glucose ( $>100 \text{ mg/dL}$ ), high CRP ( $>3 \text{ mg/L}$ ), and low HDL-C ( $<40 \text{ mg/dL}$ ). Both CAD and non-CAD subjects had HOMA-IR values  $>3$ , indicative of insulin resistance.<sup>24</sup> Thus, while both groups of subjects were at risk for CAD based on traditional biomarkers, only half had developed extensive atherosclerosis indicative of CAD based on coronary arteriography. The non-CAD group was comprised of 30% diabetic, 70% hypertensive, and 30% smoker volunteers, while the CAD group had 40% diabetic, 90% hypertensive and 30% smokers as volunteers. A chi square test for difference in these distributions in CAD vs non-CAD groups did not indicate a statistically significant difference ( $p = 0.96$ ,  $\chi^2 = 0.083$ ).

Figure 1 is a compilation of the Scree, scores, and loadings plot from the PCA conducted with all unique proteins, glycans, anthropometric, and fasting clinical parameters. The first 10 components explained ~100% of the variance, with the explained variance dropping dramatically following the first two components. PC1 and PC2, explaining ~30% of the total variance in this population, were used to represent results from the PCA. While CAD subjects appear to cluster together in the lower half of PC2, the non-CAD subjects display more variance across both PCs. The loadings plot, along similar lines, indicates a partitioning between apolipoproteins and glycans across PC2 (as well as PC1, but to a lesser degree). Apolipoproteins including ApoA-I, ApoA-II, ApoM, ApoD, ApoA-IV, ApoH, and ApoE appear above the intersection of PC1 and PC2 in the loadings plot, while the fucosylated and sialylated glycans cluster below the intersection, indicating a separation across PC2. Also of interest, some proteins (C3, C4b and C9) that belong to the complement activation system clustered in the fourth quadrant across PC1, while apolipoproteins clustered together on the other end of PC1. Univariate statistical analyses confirmed these observations since ApoA-I ( $p = 0.044$ ), ApoA-II ( $p = 0.019$ ), and ApoE ( $p = 0.030$ ) were all significantly higher in non-CAD subjects compared to the CAD subjects (Figure 2); however, no statistically significant differences in the complement activation system proteins were identified (data not shown). Serum amyloid A 2 (SAA2) ( $p = 0.020$ ) and SAA4 ( $p = 0.007$ ) were also significantly higher in the non-CAD subjects compared to the CAD subjects (Figure 3).



**Figure 3.** Serum Amyloid A (SAA) proteins in CAD vs non-CAD subjects in the separated HDL fractions. SAA2 and SAA4 were significantly different between CAD and non-CAD subjects ( $p < 0.05$ ), but there was no difference in SAA1.

Figure 4 depicts the N-glycan profile from a representative HDL sample. Most of the identified glycan compositions included two or more peaks corresponding to either structural and/or linkage isomers. Because the majority of the glycans observed in HDL were complex or hybrid type glycans, groupings were based on fucosylation and/or sialylation. Again, univariate analyses confirmed the PCA observations that total sialylated glycans were higher in CAD subjects compared to non-CAD subjects ( $p = 0.049$ ), and that the individual glycan 5-4-0-2 (a disialylated pentasaccharide) was significantly higher in CAD compared to non-CAD subjects ( $p = 0.039$ ) (Figure 5). There were no statistically significant differences in gangliosides between CAD and non-CAD patients.

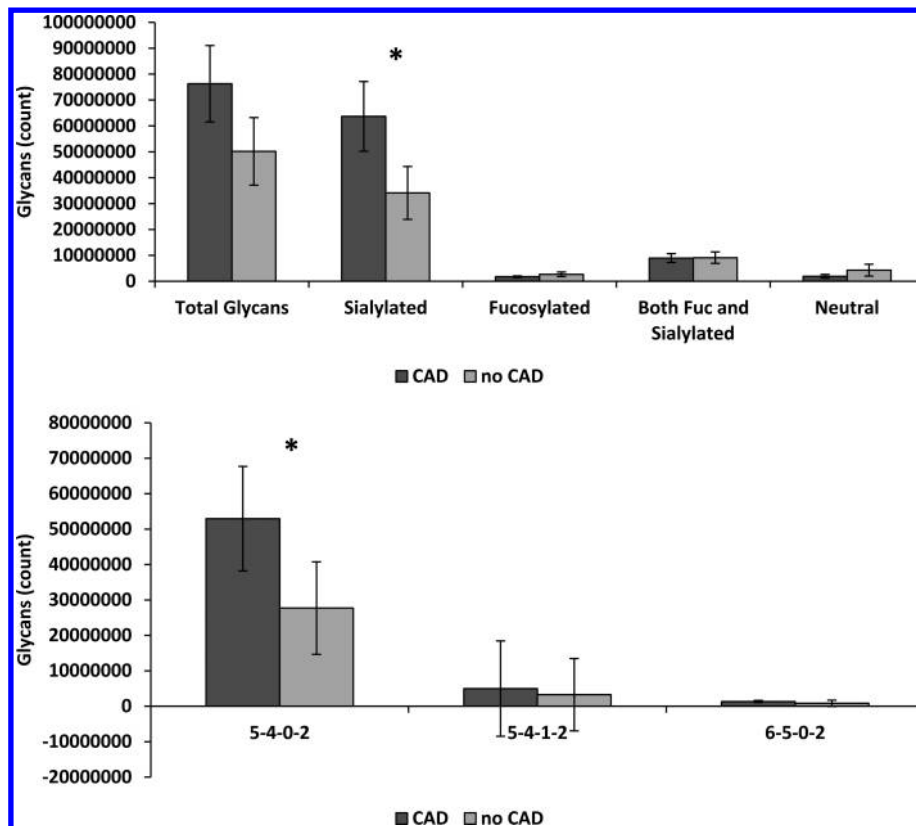
## DISCUSSION

In this pilot study, combined proteomic and glycomic profiles associated with HDL were characterized in CAD vs non-CAD subjects and found to be different in several aspects. CAD subjects had lower apolipoprotein content, including ApoA-I, ApoA-II, and ApoE, than non-CAD subjects. In contrast, total sialylated glycans were significantly higher in CAD subjects. Thus, our results indicate that HDL particles with lower apolipoprotein concentrations but a higher degree of sialylation were characteristic of CAD subjects. The CAD subjects were also significantly older, and had a lower BMI and higher TG than the non-CAD subjects. Thus, the differences in the proteomic and glycomic profiles between CAD and non-CAD patients in this study may be due to the presence of CAD itself, or to these other factors such as age, adiposity, and plasma TG levels, or a combination of all of these. Future studies using a larger cohort are needed to distinguish the influence of age vs metabolic phenotype on the combined glycomic and proteomic profiles, as well as disease outcomes.

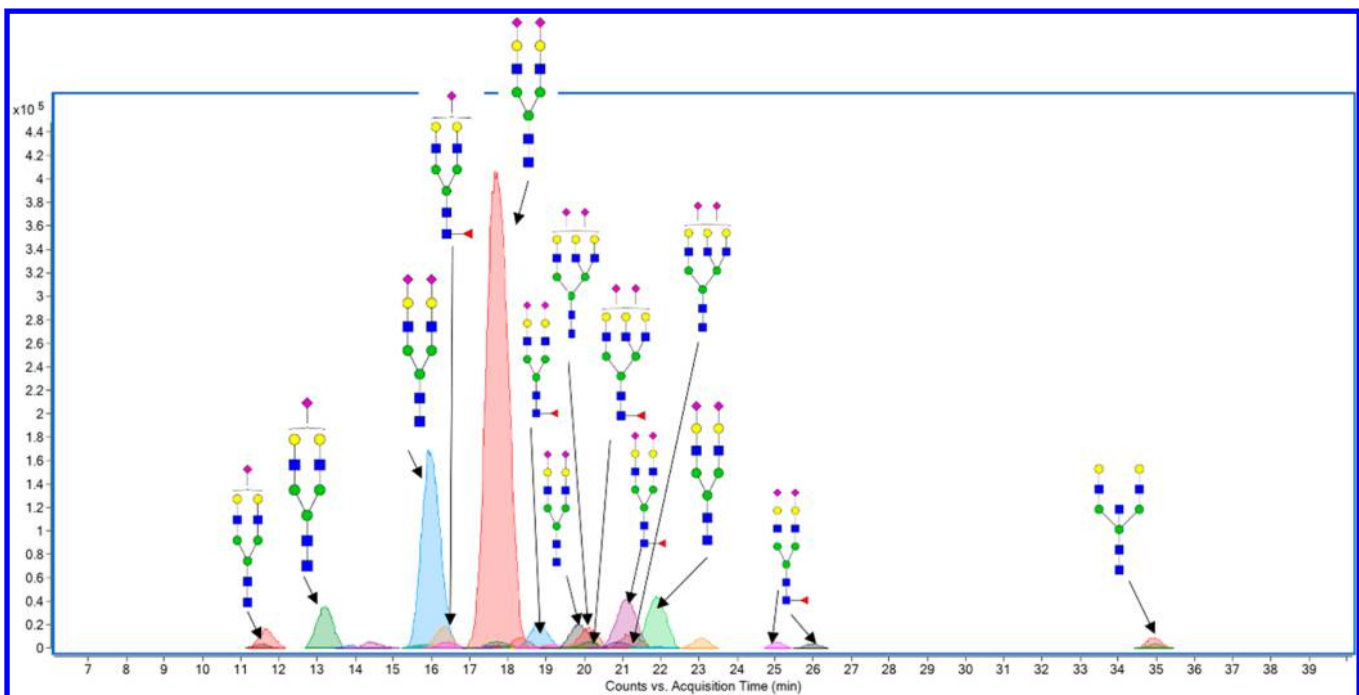
We found the HDL-associated apolipoprotein ApoA-I level to be higher in non-CAD subjects compared to CAD subjects. ApoA-I has long been associated with the cardioprotective, antiatherogenic effects of HDL, and its abundance in HDL particles is positively associated with protection from CAD.<sup>25</sup> We also found higher levels of ApoA-II and ApoE in HDL in non-CAD subjects compared to CAD subjects, but we did not detect any differences with regard to ApoC-III. Utilizing a similar shotgun proteomics approach to compare CAD vs healthy control individuals, Vaisar et al. detected higher HDL levels of ApoE, ApoA-IV, ApoC-IV, and complement factor C3 in CAD patients compared to healthy controls.<sup>5</sup> Heinecke et al. also found that levels of ApoE in the HDL proteome may be a crucial marker for cardiovascular disease risk.<sup>26</sup> In the current study, instead of comparing healthy individuals to individuals with disease, we compared a cohort of subjects who were all equally at risk for CAD based on traditional clinical biomarkers, but who were revealed to either have extensive stenosis or not following coronary arteriography.

In this study we detected ApoB in our HDL fractions. Other HDL proteomics studies, in fact most (10 out of 14), have also reported detection of ApoB in the HDL fraction (reviewed in ref 27). This is likely due to the high sensitivity of MS instruments, which can detect proteins present at low abundances.<sup>28</sup> Our proteomic data indicate that the levels of ApoA-I were about 10-fold higher on average than the levels of ApoB in these samples, which demonstrates that although a small amount of contamination of ApoB-containing lipoproteins (i.e., LDL) may have remained in these fractions, we did indeed successfully separate HDL with this method. Furthermore, comparing our proteomic results with those previously published<sup>27</sup> indicates that the separated HDL fractions contained predominantly HDL, as all of the proteins are known to be HDL-associated proteins.

Previous studies have reported that HDLs from patients with heart disease are enriched in SAA, a family of acute phase proteins associated with HDLs.<sup>29</sup> The SAA present in HDLs represents a multigene from four different loci: SAA1, SAA2, SAA3, and SAA4.<sup>30</sup> Of these, SAA1 and SAA2 are the dominant acute phase response proteins, whereas SAA4 is considered a "constitutive" form, which does not change acutely. In the current study, we found higher levels of SAA2 and SAA4 in non-CAD subjects and no difference across CAD status for SAA1.



**Figure 4.** Differences between CAD and non-CAD subjects in glycan peptides measured in the separated HDL fractions. Total sialylated peptide counts were significantly higher in the CAD subjects vs non-CAD subjects ( $p = 0.049$ , indicated by “\*” – top panel). The bottom panel depicts individual glycans –5–4–0–2, 5–4–1–2, and 6–5–0–2. While 5–4–0–2 was significantly higher in the CAD subjects ( $p = 0.039$ ), the other two were not significantly different between the groups.



**Figure 5.** Glycan structures identified in the separated HDL fractions. Green circles, mannose; yellow circles, galactose; blue squares, N-acetylglucosamine; red triangles, fucose; purple diamonds, N-acetylneuraminic acid (or sialic acid).

Several functions of the SAA proteins have been identified including endotoxin detoxification,<sup>31</sup> stimulation of cytokine production, and effectively rendering a pro-inflammatory

milieu.<sup>32</sup> Furthermore, SAA interferes with HDL's ability to perform cholesterol efflux,<sup>33</sup> decreasing the potential for cholesterol removal.<sup>34</sup> Some studies have associated higher



SAA protein levels with cardiovascular and coronary artery disease outcomes.<sup>35</sup> However, in a joint statement of the American Heart Association and the CDC, a need for further studies to substantiate this claim was voiced.<sup>36</sup> The SAA3 isoform is not expressed in humans,<sup>37</sup> and SAA 1, 2, and 4 contribute to the overall plasma concentration.<sup>38</sup> Whereas most previous studies have reported total SAA levels, the current study examined the individual isoforms of SAA (SAA 1, 2, and 4) separately. Combining the SAA1, 2, and 4 peptides measured in this study indicated higher (not statistically significant) total SAA levels in non-CAD subjects compared to CAD subjects ( $20.5 \pm 13.9$  vs  $9.7 \pm 6.8$  number of peptides respectively,  $p = 0.19$ ). As SAA1 and SAA2 are highly homologous genes,<sup>39</sup> further studies are needed to better understand any physiological significance due to variation in their levels. Both SAA1 and 2 have been shown to impact the cholesterol esterase enzyme,<sup>40</sup> and macrophage cholesterol efflux,<sup>41</sup> but there is limited knowledge regarding SAA4. In addition, SAA protein levels have been associated with adiposity with a 20% increase in SAA in obese individuals compared to lean counterparts.<sup>42</sup> Thus, it is possible that the difference in SAA levels between the CAD and non-CAD subjects in this study was entirely due to the difference in adiposity rather than the extent of stenosis, since the non-CAD subjects had a higher BMI.

In this pilot study we found a higher degree of sialylation in CAD compared to non-CAD subjects. Total plasma sialic acid has been associated with higher CVD risk,<sup>43</sup> especially in large epidemiological studies.<sup>44</sup> Total serum sialic acid levels increase prior to and during an atherosclerotic event.<sup>45</sup> On the other hand, a recent study found reduced glycosylation across lipoproteins in MetS patients compared to healthy controls, and specifically lower glycosylation in ApoC-III and SAA.<sup>16</sup> Deglycosylation was found to render LDL particles more pro-atherogenic by increasing their aggregation and ability to stimulate intracellular cholesterol uptake.<sup>46</sup> Desialylated LDL particles were also found to be internalized faster than native LDL particles by arterial smooth muscle cells.<sup>47</sup> In patients with atherosclerosis and diabetes, LDL was found to be desialylated, more electronegative, and with a higher capacity to stimulate cellular cholesterol uptake compared to LDL from normolipidemic subjects.<sup>48</sup> On the other hand, others have not found any difference in total glycan composition among LDL subclasses (small-dense vs large LDL), either among normolipidemic or hypercholesterolemic subjects.<sup>49</sup> These conflicting results emphasize the need for robust and sensitive methodologies, as older methods were less likely to differentiate between glycans that were part of glycoproteins vs glycolipids, such as gangliosides, and in fact could not distinguish between free sialic acid and sialic acid attached to proteins and lipids. In this study, due to low sample volumes, we analyzed the composition of all glycans that were cleaved from glycoproteins rather than examining the specific glycan composition of individual proteins. More studies are needed to examine whether protein-specific glycosylation changes affect disease outcomes and HDL function.

In this study we did not find any differences in the levels of gangliosides between CAD and non-CAD patients. It is possible that due to our small sample size we did not have the power to detect the differences. Previous studies have found differences in the GM3/GD3 ratio in atherosclerotic plaques,<sup>50</sup> and higher GD3 and lower GM3 content in atherosclerotic plaques compared with unaffected intima sections.<sup>51</sup> However, there is a general lack of knowledge of any effect of changes in HDL

ganglioside composition on disease outcomes or HDL function.<sup>44</sup>

Our study has several limitations. The sample size was limited and our findings therefore need to be verified in larger groups so that additional known modifiers of CAD risk can be included in the analysis (e.g., high vs low TG, BMI, insulin, and other clinical parameters, menopausal status, use of estrogens, kidney function, etc.). Also, while the shotgun proteomic approach is high throughput and useful, it is not quantitative. Rather than as a confirmatory or conclusive analysis, this study was undertaken as a proof of concept to explore whether the combined proteomic and glycomic profiling of HDL would be useful to assess markers of HDL function (and dysfunction).

## CONCLUSIONS

This proof of concept study demonstrated that the application of a comprehensive glycomic profiling method combined with proteomics may be a promising approach for the development of novel biomarkers of diseases related to lipoprotein metabolism. Future studies using larger sample sizes are needed to further examine the combined glycomic and proteomic differences between different disease groups, and the functional and mechanistic implications of those differences in HDL composition.

## ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jproteome.5b00730.

Supplemental Table 1: List of all proteins identified and used in statistical analysis in this report (PDF)

## AUTHOR INFORMATION

### Corresponding Author

\*Telephone: (530) 554-2534. Fax: (530) 752-8966. Email: amzivkovic@ucdavis.edu.

### Notes

The authors declare no competing financial interest.

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