Longitudinal Analysis of Maternal Plasma Apolipoproteins in Pregnancy: A Targeted Proteomics Approach*

Shannon K. Flood-Nichols‡§, Deborah Tinnemore‡§, Mark A. Wingerd‡, Ali I. Abu-Alya‡, Peter G. Napolitano‡, Jonathan D. Stallings‡, and Danielle L. Ippolito‡¶

Minimally invasive diagnostic tests are needed in obstetrics to identify women at risk for complications during delivery. The apolipoproteins fluctuate in complexity and abundance in maternal plasma during pregnancy and could be incorporated into a blood test to evaluate this risk. The objective of this study was to examine the relative plasma concentrations of apolipoproteins and their biochemical modifications (i.e., post-translationally processed, sialylated, cysteinylated, dimerized) over gestational time using a targeted mass spectrometry approach. Relative abundance of modified and unmodified apolipoproteins A-I, A-II, C-I, C-II, and C-III was determined by surface-enhanced laser desorption/ionization-time of flight-mass spectrometry in plasma prospectively collected from 11 gravidas with uncomplicated pregnancies at 4–5 gestational time points per patient. Apolipoproteins were readily identifiable by spectral pattern. Apo C-III2 and Apo C-III, (doubly and singly sialylated Apo C-III subtypes) increased with gestational age (r^2 > 0.8). Unmodified Apo A-II, Apo C-I, and Apo C-III, showed no correlation (r^2 = 0.01–0.1). Pro-Apo C-II did not increase significantly until third trimester (140 ± 13% of first trimester), but post-translationally cleaved, mature Apo C-II increased in late pregnancy (702 ± 130% of first trimester). Mature Apo C-II represented 6.7 ± 0.9% of total Apo C-II in early gestation and increased to 33 ± 4.5% in third trimester. A label-free, semiquantitative targeted proteomics approach was developed using LTQ-Orbitrap mass spectrometry to confirm the relative quantitative differences observed by surface-enhanced laser desorption/ionization-time of flight-mass spectrometry in Apo C-III and Apo C-II isoforms between first and third trimesters. Targeted apolipoprotein screening was applied to a cohort of term and preterm patients. Modified Apo A-II isoforms were significantly elevated in plasma from mothers who delivered prematurely relative to term controls (p = 0.02). These results support a role for targeted proteomics profiling approaches in monitoring healthy pregnancies and assessing risk of adverse obstetric outcomes. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.018192, 55–64, 2013.

The maternal physiology during pregnancy is characterized by inflammation and hyperlipidemia. Plasma protein composition fluctuates dynamically throughout gestation to reflect these physiological changes. Apolipoproteins, a diverse subset of triglyceride transport proteins, contribute to the hyperlipidemia of pregnancy by modulating lipid homeostasis in maternal plasma (1–3). Exaggerated hyperlipidemia and peripheral apolipoprotein burden are associated with inflammatory insult and signal obstetric complications (4–5). Numerous post-translationally modified apolipoprotein isoforms are reported in plasma, but it is unclear how these modifications affect apolipoprotein function and plasma distribution. For example, changes in the glycosylation status of apolipoprotein variants predate the onset of clinical symptoms in patients with preeclampsia, a hypertensive disorder of pregnancy with clinical features in common with cardiovascular disease (6–8). The identification and functional characterization of plasma apolipoprotein isoforms and their post-translationally modified subtypes may reveal important diagnostic and/or therapeutic targets for hypertensive disorders of pregnancy (6).

Mass spectrometry and targeted proteomics analyses afford unprecedented sensitivity and specificity for detecting apolipoproteins and their numerous isoforms and subtypes (9–12). Mass spectrometry approaches overcome limitations inherent in biochemical approaches (e.g., ELISA [enzyme-linked immunosorbant assays] and Western blot analysis), especially the lack of specificity of antibodies for post-translationally modified variants of plasma proteins. The objective of this study was to longitudinally evaluate maternal plasma apolipoprotein profile over gestational time by SELDI-TOF-MS (surface-enhanced laser desorption/ionization-time of flight-mass spectrometry) analysis of intact proteins and a

---

1 The abbreviations used are: MS/MS, tandem mass spectrometry; Apo C-I’, Apo C-I without N-terminal threonine-proline; Apo C-III2, di-sialylated Apo C-III; Apo C-III1, mono-sialylated Apo C-III; Apo C-III0, unsialylated Apo C-III.

From the ‡Madigan Healthcare System, Joint Base Lewis-McChord, 9040 Fitzsimmons Drive, Tacoma, Washington 98431
Received February 21, 2012, and in revised form, September 25, 2012
Published, MCP Papers in Press, October 10, 2012, DOI 10.1074/mcp.M112.018192

© 2013 by The American Society for Biochemistry and Molecular Biology, Inc.
This paper is available online at http://www.mcponline.org

Molecular & Cellular Proteomics 12.1
complementary targeted LTQ-Orbitrap XL MS approach. We evaluate changes in 13 post-translationally modified subtypes of the plasma apolipoproteins A-II, C-I, C-II, and C-III over gestational time.

**EXPERIMENTAL PROCEDURES**

**Patients**—The study was approved by the Madigan Healthcare System Institutional Review Board (Joint Base Lewis-McChord, WA). Patients were invited to participate during their initial OB-GYN appointment if they presented under 12 weeks of gestation, were 18 years of age or older, and were nulliparous. Patients were excluded if they had a history of chronic disease (including hypertension) or infertility treatment. All study participants provided informed consent. Antepartum records were reviewed to determine whether patients experienced an uncomplicated course of pregnancy according to the guidelines of the American College of Obstetrics and Gynecologists (ACOG). Prematurity was defined as delivery at less than 37 weeks of age as per ACOG guidelines (13). Nonpregnant patients were healthy women in the same age range as the pregnant cohort. Plasma was collected during the menstrual, luteinizing hormone (LH) surge, and luteal phases of the menstrual cycle in each patient. Plasma Collection—The target gestational age ranges for specimen collection were 4–12, 16–22, and 24–28, and 35–38 weeks. In some cases, patients provided two specimens in the 4–12 week range (see Supplemental Table SI). Blood was collected in EDTA Vacutainer tubes (BD Biosciences) and spun at 1500 × g for 15 min at 4 °C. Plasma supernatant was placed on ice and mixed with protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany) and stored in single-use aliquots at −150 °C. Time from phlebotomy to storage was recorded. Specimens with freeze times exceeding 90 min were not analyzed. Typical processing time was ∼30–45 min.

**Enzyme-Linked Immunosorbent Assays (ELISA)**—Antibodies directed against the analytes Apo C-II, Apo C-III, Apo A-I, and Apo A-II were used in ELISA assays to probe patient plasma using a colorimetric assay in which cholesterol oxidase reacts with free cholesterol to produce a color change in the presence of 3,5,3’,5’-tetramethylbenzidine (TMB) (Sigma Chemical, St. Louis, MO) and peroxidase and/or neuraminidase (Roche Diagnostics). Resultant peaks were assigned mass values of 9421.3 Da (neuraminidase) or 8764.7 Da (neuraminidase and O-glycosidase) corresponding to Apo C-III, and Apo C-II, respectively, as described (8). Samples were analyzed by SELDI-TOF-MS using matrix attenuation of 1 kDa, mass range of 20 kDa, focus mass of 10 kDa, and laser energy determined empirically (range: 700–2000 nJ). Parameters for a second, mid-mass range analysis were applied for protein candidates greater than 15 kDa: matrix attenuation of 1 kDa, mass range of 10–100 kDa, focus mass of 25 kDa, and empirically determined laser energy (1250–2000 nJ).

**RP HPLC MS/MS Analysis by NanoAcquity LC and Orbitrap XL MS**—High abundance plasma proteins were extracted by acetonitrile precipitation as described (16). An AQUA peptide standard (10pmol, Sigma Chemical, St. Louis, MO) was added to the specimens. Samples were digested with trypsin as described (17) and desalted using UltraMicroSpin silica C18 column chromatography according to the manufacturer’s instructions (The Nest Group, Inc., Southborough, MA, SUM SS18V). Nanoscale liquid chromatography-tandem mass spectrometry was performed using a NanoAcquity system for chromatographic separation (Waters Corporation, Milford, MA) and an LTQ-Orbitrap XL mass spectrometer (ThermoFisher Scientific, West Palm Beach, FL). Peptides were concentrated and desalted on a reversed phase C18 trapping column (0.34 × 5 mm; Waters Corporation, Milford, MA) for 5 min at 25 μl/min (2.5 μl injection volume). Peptides were eluted from the reversed phase C18 analytical column (75 μm × 250 mm; Waters Corporation) at a flow rate of 300 nl/min using a gradient of 0–40% acetonitrile for 100 min and 40–90% acetonitrile for 12.5 min. A wash program was used to flush the column by 7 alternating saw-tooth gradients (85–2% water/0.1% formic acid and 2–85% acetonitrile, 5 min each gradient). The column was re-equilibrated for 51 min with 100% water/0.1% formic acid. Mass spectral survey scans were acquired by the Orbitrap operated in data independent MS/MS mode at 60,000 resolution from 350–1800 m/z. Four precursor ions per full scan were selected for MS/MS by the linear trap with a normalized collision-induced dissociation (CID) energy of 35%.

**Mass Assignment of Post-translationally Modified Isoforms of Tryptic Peptides**—The m/z values for N-terminal tryptic peptides for Apo C-I’ (apo C-I lacking the N-terminal Thr-Pro sequence) and all Apo A-II subtypes were derived from experimentally determined mass assignments in the literature (15, 18) and sequence analysis by in silico tryptic digest calculations (2). The m/z for doubly and triply charged ions corresponding to the predicted glycoconjugate frag-
ments of C-terminal Apo C-III1 and Apo C-III2 subtypes was calculated based on literature assignments (15, 19–21). Relative quantification was obtained by area under the extracted ion chromatogram (AUC) of the most abundant isotope normalized to an internal standard AQUA peptide FSEFWDLDPEVRPT(13C6,15N2)SAAA (commercially synthesized, Sigma Chemical). To confirm identifications of singly and doubly sialylated Apo C-III1 and Apo C-III2 tryptic peptides, another (MS3) scan was triggered in the neutral loss mode when one of the 4 most intense peaks from the MS2 spectrum corresponded to a neutral loss event of $m/z$ 147.01, 227.97, or 329.39 (empirically derived from pilot data).

Data Processing and Database Searching—Data were processed using the Xcalibur software package, v. 2.0.7 (ThermoFisher Scientific) with post-processing by Proteome Discoverer, v. 1.2 (ThermoFisher Scientific). Searches were conducted against custom-built FASTA files from NCBI sequences for a total of 13 target apolipoprotein subtype sequences (v.2010.07.26). Database searches were applied with precursor $m/z$ tolerances of 2.5 Da and fragment mass tolerances of 1.0 Da. Trypsin was the enzyme specified with an allowance of two missed cleavages. Cysteine residues were searched as carbamidomethylated (+ 57.021 Da). Oxidation of methionine residues was allowed (+ 15.995 Da) (22). False discovery rates (FDR) were determined by searching against a decoy database (target FDR of 0.01). Peptide assignments were filtered by peptide score based on false positive rate estimated by the target/decoy database approach described by Gygi and colleagues (23, 24).

Statistics—SELDI-TOF-MS data were evaluated by one-way analysis of variance by ranks (Kruskal-Wallis) with significance set at $p < 0.05$. Comparisons between groups were evaluated by analysis of variance by repeated measures. Pairwise comparisons were made with post hoc Bonferroni correction. Areas under the extracted ion chromatogram (AUC) for first and third trimester LTQ Orbitrap XL data were evaluated by nonparametric Wilcoxon signed rank test with significance set at $p < 0.05$.

RESULTS

SELDI-TOF-MS Identification of Target Apolipoproteins—Maternal plasma was longitudinally collected at 4 gestational ages from each of 11 nulliparous patients: 4–12 weeks, 16–22 weeks, 24–28 weeks, and 35–38 weeks gestation. Antepartum records review confirmed uncomplicated pregnancies in these patients. Patient characteristics are summarized in Supplemental Table SI. Plasma proteins were fractionated in triplicate on-spot using hydrophobic chemically derivatized surfaces (HS50 arrays) and proteomic pattern was analyzed by SELDI-TOF-MS. Signature patterns of Apo C-I, Apo C-II, Apo A-II, Apo C-III, and Apo A-I in maternal spectra were identified based on molecular weight and spectral pattern (Fig. 1; Table I) (15, 18, 25–27). The apolipoproteins are stable features of the human plasma proteome readily detectable by published matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) spectral patterns as previously described (Fig. 1; Table I) (10, 15, 19–21, 28). The patterns and relative abundances of Apo C-I, Apo C-II, Apo A-II, Apo A-I, Apo C-III, transthyretin tetramers, and Apo A-II dimers were compared in plasma from a single representative patient in trimester 1 (Fig. 1). All presumptive apolipoprotein peaks had signal-to-noise ratios (S/N) greater than 2.5. Coefficients of variance averaged 12.5 ± 2% among replicates.

Unmodified Apo C-I was the predominant form identified, with a lesser percentage of Apo C-I’ lacking the N-terminal proline and threonine residues (Fig. 1A). Most of the apolipoproteins A-II, C-II, and C-III identified from maternal plasma corresponded to post-translationally modified isoforms (Figs.
Apolipoproteins in Pregnancy

### Table I
Calculated and observed molecular masses for apolipoproteins in maternal plasma

| Peak identification | Accession #a | Peak code | Calculated (Da)b | Reference | SELDI measured (m/z)c | S.D.d | Delta M (m/z)c |
|---------------------|-------------|----------|------------------|-----------|----------------------|-------|---------------|
| Apo C-I without N-terminal Thr-Pro | GI178834 | CL’ | 6432.4 | 15 | 6434.1 | 0.2 | 1.7 |
| Apo C-I | GI178834 | CL | 6630.6 | 15, 18 | 6631.6 | Calib | Calib |
| Apo C-II, mature form | PO2655 | CII | 8204.1 | 15, 27 | 8204.3 | 0.8 | 0.2 |
| Apo A-II, cysteinylated, minus C-terminal Gin | PO2652 | All’ | 8682.9 | 18 | 8689.9 | 0.9 | 7.0 |
| Apo C-III0 | PO2656 | CIII0 | 8764.7 | 15, 27 | 8765.7 | Calib | Calib |
| Apo A-II, cysteinylated | PO2652 | All | 8810.9 | 18 | 8815.0 | 0.4 | –0.4 |
| pro Apo C-II | PO2655 | proCII | 8914.9 | 15, 26 | 8915.6 | 0.4 | 0.7 |
| Apo C-III1 | PO2656 | CIII1 | 9421.3 | 15, 26 | 9422.3 | Calib | Calib |
| Apo C-III2 | PO2656 | CIII2 | 9712.6 | 15, 26 | 9713.4 | 0.4 | 0.8 |
| Apo A-II dimer minus two C-terminal Gin | PO2652 | Alla’ | 17123.4 | 18 | 17130.2 | 3.7 | 6.8 |
| Apo A-II dimer minus one C-terminal Gin | PO2652 | Alla’ | 17251.5 | 18 | 17249.6 | 1.2 | –1.9 |
| Apo A-II dimer | PO2652 | Alla’ | 17379.5 | 18 | 17378.1 | 1.2 | –1.4 |
| Apo A-I, mature form | GI296635 | Al | 28078.6 | 18 | 28079.8 | Calib | 1.2 |

a Accession numbers from NCBI database; 13 amino acid sequences were added to a custom-built FASTA file representing pertinent subtypes.
b Calculated molecular mass.
c Experimental molecular mass, this study.
d S.D., standard deviation.
e Calib, peak used for internal calibration.
f Delta M, molecular mass, (observed - calculated).

1A and 1C. Thanshyretin tetramers were also present in the 10,000–15,000 Da mass range (Fig. 1B). Both pro-Apo-CII and Apo-CII (mature, proteolytically cleaved) were present in detectable abundance (Table I, Fig. 1A) (10, 28). Both singly and doubly sialylated Apo C-III were the predominant forms of Apo C-III in maternal plasma, although an appreciable peak was detected at the predicted molecular weight for Apo C-III0 (Fig. 1A; Table I) (2, 15). Mass assignment and spectral pattern for Apo C-III0 and the sialylated subtypes were confirmed by combination neuraminidase and O-glycosidase enzyme treatment as described (data not shown and (8)). Linear regression analysis for Apo A-II relative to the 3 Apor-A-II dimers showed a positive correlation, with slopes statistically different from zero ($r^2 = 0.652$, $r^2 = 0.600$, and $r^2 = 0.305$ for All0, All1’, and All0’). Apo A-II’ was not correlated with any of the dimerized subtypes ($r^2 = 0.02–0.08$).

Quantification of Apolipoproteins and Cholesterols in Plasma—HDL, VLDL/LDL cholesterol, Apo C-III, and Apo A-II were quantified by ELISA at 4 gestational age ranges in 5 pregnant individuals and three nonpregnant controls (Table II). Apo C-II was quantified in first and third trimesters only (Table II). In support of the current literature, apolipoproteins uniformly increased over the course of pregnancy (Table II and Supplemental Fig. S1).

**SELDI-TOF-MS Relative Quantification—SELDI-TOF-MS ion intensity for each protein peak was used to determine relative abundance of the median peak amplitude for each presumptive apolipoprotein peak in patient plasma across gestational time. Ion intensities were plotted as a function of gestational age and correlation coefficients were calculated.**

Pro-Apo-CII showed a modest, increasing correlation with gestational age (2B and Figs. 3A, 3B). The positive correlation between gestational age and ion intensities for mature Apo C-II (with N-terminal TQPKQQ residues proteolytically cleaved) was more striking, showing a statistically significant increase with greater interpatient variability as gestation progressed ($r^2 = 0.342$) (Fig. 2A). Mature Apo C-II composed an increasingly greater proportion of total Apo C-II (mature plus pro-Apo-CII) as gestation progressed ($r^2 = 0.469$) (Fig. 2C). Mature Apo C-II composed 6.7 ± 0.9% of total Apo C-II in early gestation and increased to 33 ± 4.5% in third trimester. By third trimester, pro-Apo-CII was 140 ± 13% of first trimester (4–12 weeks) abundance (Figs. 3A, 3B, $p < 0.05$). Ion intensity peak amplitudes for mature Apo C-II at 16–22, 26–28, and 36–38 weeks gestation were 252 ± 54, 312 ± 56, and 783 ± 152% higher than samplings at 4–12 weeks gestation (first trimester, $p < 0.05$) (Fig. 3A). Apo C-II peak amplitudes decreased relative to nonpregnant controls in the first trimester, then increased progressively over gestational time in the pregnant cohort (Fig. 3A). In contrast, pro-Apo-CII did not significantly differ from baseline first trimester until late pregnancy (140 ± 13% above first trimester at 35–38 weeks gestation) ($p < 0.05$) (Fig. 3B).

The peak amplitudes for the a-sialylated, mono-, and disialylated isoforms of Apo C-III (Apo C-III0, Apo C-III1, and Apo C-III2) were plotted as a function of gestational age (Figs. 2D–2F and Figs. 3C–3E). Relative abundance of Apo C-III0 did not correlate with gestational age ($r^2 = 0.066$; Fig. 2D), whereas mono-sialylated Apo C-III1 and di-sialylated Apo C-III2 correlated strongly ($r^2 = 0.805$ and 0.815, Fig. 2E and Fig. 2F, respectively). Ion intensities for Apo C-III0 and sialylated variants were normalized to Apo C-III0 and plotted as a function of gestational age ($r^2 = 0.725$). Kruskal Wallis analysis of variance by ranks indicated a statistically significant
increase in Apo C-III1 and Apo C-III2 over gestational time (Figs. 3D, 3E) with no significant difference in Apo C-III0 (Fig. 3C). To quantify the elevation in Apo C-III concentrations in third trimester maternal plasma in terms of μg/ml, we generated standard curves by spiking escalating amounts of Apo C-III protein standard into first trimester maternal plasma as described (29). Calibration curves for 0, 12.5, 25.0, 50.0, and 100 μg/ml protein standard showed a linear increase in the 9421.3 Da peak at 2500 nJ laser energy (Supplemental Fig. S2). Based on interpolation to the standard curve, third trimester plasma was estimated to have 65.6 μg/ml more Apo C-III than first trimester plasma.

**TABLE II**
Quantification of apolipoproteins and cholesterols in maternal plasma and nonpregnant controls

| Apolipoprotein A-II (mg/dL) | Apolipoprotein A-I (mg/dL) |
|----------------------------|---------------------------|
| Menstrual                  | 4–12 weeks                | 16–22 weeks | 24–28 weeks | 35–38 weeks |
| Averages                   | 25.3                      | 23.7        | 30.0        | 32.9        | 34.1        |
| S.E.                       | 2.5                       | 2.5         | 1.8         | 1.8         | 4.1         |
| p value*                   | **p = 0.009**             |             |             |             |             |

| Apolipoprotein C-III (mg/dL) | Apolipoprotein C-II (mg/dL) |
|----------------------------|-----------------------------|
| Menstrual                  | 4–12 weeks                  | 16–22 weeks | 24–28 weeks | 35–38 weeks |
| Averages                   | 10.5                        | 9.6         | 16.6        | 24.3        | 41.5        |
| S.E.                       | 1.1                        | 1.9         | 2.6         | 2.8         | 6.8         |
| p value*                   | **p < 0.0001**              |             |             |             |             |

| VLDL/LDL cholesterol (mg/dL) | HDL (mg/dL) |
|------------------------------|-------------|
| Menstrual                    | 4–12 weeks | 16–22 weeks | 24–28 weeks | 35–38 weeks |
| Averages                     | 139.9       | 142.0       | 223.0       | 250.1       | 274.2       | 42.1        | 35.7        | 48.2        | 52.8        | 46.0        |
| S.E.                         | 18.4        | 15.8        | 15.1        | 27.8        | 23.6        | 5.8         | 8.2         | 6.3         | 5.0         | 5.5         |
| p value*                     | **p = 0.003** |             |             |             |             |             |             |             |             |             |

* p values calculated by paired t-test (2 groups) or repeated measures ANOVA (4 groups) for pregnant cohorts.

**Fig. 2.** Correlation of maternal plasma Apo C-II and C-III isoforms with gestational age. SELDI-TOF-MS peak amplitude (ion intensity) was plotted as a function of gestational age. A, Mature Apo C-II lacking the N-terminal TQQPQQ sequence (m/z 8204.1) and B, pro-Apo C-II (m/z 8915.6) were plotted according to gestational age. C, SELDI-TOF-MS ion intensities for peaks corresponding to mature Apo C-II (m/z 8204.3) were plotted as percent total Apo C-II (mature + pro-Apo C-II) over gestational time. D, Apo C-III0 (m/z 8765.7), E, Apo C-III1 (m/z 9422.3), and F) Apo C-III2 (m/z 9713.4) were plotted as a function of gestational age and correlation coefficients were determined. n = 11 patients at 4–5 time points per patient.
Relative abundance of Apo A-II increased slightly over gestational time whereas the subtype ApoA-II’ showed no significant correlation with gestational age (Figs. 3F, 3G).

Comparison with Nonpregnant Controls—Apolipoprotein profiles were compared between a nonpregnant control group of \( n = 3 \) women at 3 successive stages in the menstrual cycle and pregnant cohorts (Fig. 3). Apo C-II (mature) was significantly elevated in all phases of the menstrual cycle relative to pregnant individuals at 4–12 weeks gestation (Fig. 3A). Pro-Apo C-II showed a slight but statistically insignificant increase (Fig. 3B). The sialylated variants of Apo C-III were differentially expressed in nonpregnant controls relative to first trimester (Fig. 3C–3E). Apo C-III0 was lower in abundance in luteal and LH (luteinizing hormone) phases of the menstrual cycle relative to 4–12 weeks gestation (Fig. 3C), whereas Apo C-III2 was elevated relative to 4–12 weeks gestation (Fig. 3E). No significant differences were observed for Apo A-II subtypes in non-pregnant and first trimester patients (Figs. 3F, 3G).

To determine whether apolipoprotein SELDI-TOF-MS pattern could be used to accurately predict gestational age range, a test set of plasma specimens derived from \( n = 11 \) patients at 4–5 time points per patient.

**FIG. 3.** Repeated measures comparison of apolipoprotein subtypes by gestational age range. SELDI-TOF-MS peak amplitude (ion intensity) was plotted as a function of gestational age and normalized to 4–12 week ion intensities for each patient. Nonpregnant patient values were normalized to the average intensities of all 4–12 week intensities. A, Mature Apo C-II lacking N-terminal TQQPQQ sequence (m/z 8204.1), B, pro-Apo C-II (m/z 8915.6), C, Apo C-III0 (m/z 8765.7), D, Apo C-III1 (m/z 9422.3), E, Apo C-III2 (m/z 9713.4), F, Apo A-II (cysteinylated) (m/z 8810.5), G, Apo A-II’ (cysteinylated without C-terminal Gln) (m/z 8689.9). *, \( p < 0.05 \) relative to first trimester (100%), \( n = 11 \) patients at 4–5 time points per patient.
Apo-III is necessary for the detection of sialylated Apo C-III and Apo C-II in maternal plasma. Identification of sialylated Apo C-III and Apo C-II required MS3 validation (Figs. 4A–4C and Supplemental Table S2). To distinguish among the apolipoprotein subtypes in the tryptic peptides from the Orbitrap analysis, we quantified spectral counts (Supplemental Table S3) and area under the extracted ion chromatogram for only the peptides that effectively differentiate among the apolipoprotein subtypes (Supplemental Table S4). The area under the extracted ion chromatogram (AUC) for each differentiable N- or C-terminal peptide was normalized to an internal standard AQUA peptide (C-terminal tryptic peptide of Apo C-III, FSEFWDLPFPRPTSAA) as described, and relative abundances of pro-Apo C-II, mature Apo C-II, and the Apo C-III glycovariants were determined in first and third trimester plasma (17) (Fig. 4D, 4E, Supplemental Table S4).

SELDI-TOF-MS Analysis of Apolipoproteins in Prematurity—A new cohort composed of n = 8 term and n = 3–4 preterm specimens in trimesters 1 and 2 was evaluated by SELDI-TOF-MS to test the ability of the apolipoprotein targeted mass spectrometry assay to differentiate among normal patients and patients with a significant obstetrics complication (i.e. prematurity) (Supplemental Table S5). Because all individuals...
in the preterm cohort delivered before 37 weeks, third trimester specimens were unavailable from this group of women. Apolipoprotein panels were evaluated in first and second trimester plasma specimens from both sets of patients. The unmodified Apo A-II cysteinylated dimer (AIId) (m/z 17344) was significantly elevated in preterm cohorts relative to their gestationally age-matched term counterparts in both first and third trimester (59.4 ± 7.8 μA in term and 102.3 ± 6.6 μA in preterm patients in trimester 1 and 74.7 ± 6.3 μA in term and 97.0 ± 3.8 μA in trimester 2; Table III; Supplemental Table S5).

### DISCUSSION

In this study, we use SELDI-TOF-MS and LTQ-Orbitrap targeted proteomics to isolate and semiquantitatively characterize post-translationally modified subtypes of plasma apolipoproteins throughout gestation. We demonstrate the novel findings that a mature, cleaved form of Apo C-II increases over gestational time, and provide evidence that a panel of apolipoprotein subtypes may be useful in predicting preterm birth.

To our knowledge, this study is the first to report a disproportionate increase in mature Apo C-II relative to pro-Apo C-II subtypes in pregnancy using the detection capabilities unique to mass spectrometry. Studies using SELDI-TOF-MS have reported the increase in abundance of an 8204 Da peak coincident with mature Apo C-II in clinical conditions associated with increased inflammation. Systemic IL-2 administration resulted in an increase in the 8204 Da Apo C-II peak, suggesting a correlation between mature Apo C-II and inflammatory insult (21). In normal physiology, only about 5% of Apo C-II exists in adult plasma in its cleaved, mature form (lacking the N-terminal TQQPQQ sequence) (25). Pro-Apo C-II represents the remaining 95%. Mass spectrometry-based studies confirm a proportionally smaller quantity of mature Apo C-II in plasma relative to pro-Apo-CII (28).

ELISA and immunoturbidimetric assays show that Apo A-I, C-I, and A-II increase proportionally between first and second trimesters (3, 30–33). As expected in pregnancy, we observe a significant decrease in ratios of Apo A-I/Apo A-II and Apo A-II_{stable} subtypes from trimester 1 to trimester 3, and an appreciable increase in VLDL/LDL cholesterol but not HDL cholesterol. Immunological methods lack sufficient sensitivity to account for variation in apolipoprotein subtypes in pregnancy. The discrepancy between the SELDI-TOF-MS and ELISA data could also be explained by competitive binding of proteins present in high molar ratios to the array surfaces. The changes in subtypes reported in this study warrant further investigation in larger cohorts of complicated pregnancies.

Caution must be exercised when comparing proteomic profiles in physiological states such as pregnancy which are characterized by dynamic changes in plasma protein complement. Because SELDI-TOF-MS arrays preferentially bind proteins of greater molar abundance, significant changes in protein complement can artificially affect intensities of other spectral features (20). This limitation of the SELDI-TOF-MS technique may be overcome by fractionating specimens to limit the competitive inhibition of high molar abundance species in maternal plasma (34). However, one of the limitations of most of the mass spectrometry techniques described in this study is the ability to detect only relative changes in abundance. The concentrations of Apo C-III calculated by interpolation to an internal standard are lower than predicted by ELISA (an average difference of 290 μg/ml, Supplemental Table S4), suggesting that the sialyl groups caused significant attenuation of ionization in the SELDI-TOF technique. During laser desorption in mass spectrometry, variable loss of sialic acid occurs in proportion to laser energy and other factors. From a biochemical standpoint, the ELISA antibodies detect all subtypes of Apo C-III, whereas our mass spectrometry techniques examine the subtypes individually. Therefore, both the biochemical and mass spectrometry approaches have inherent limitations making it difficult to obtain accurate quantitation of individual subtypes in a given sample.

Bondarenko and colleagues demonstrated the utility of electrospray mass spectrometry in characterizing apolipoprotein subtypes by analyzing intact proteins (15, 35). More recently, novel fucosylated Apo C-III subtypes were identified by mass spectrometry analysis of Schistosoma mansoni from urine of infected individuals (36). Serum isoforms of Apo C-I, Apo C-II, Apo C-III, Apo A-II, dimerized Apo A-II, and Apo A-I were predictive markers for Alzheimers disease in MALDI-TOF-MS studies (35).

Although we acknowledge that our findings of changes in concentration for dimerized Apo A-II in preterm patients must be validated in a larger cohort, our results warrant further investigation into the use of apolipoprotein subtype analysis in
obstetric populations. Shotgun proteomics strategies in obstetric cohorts have yielded some success in biomarker identification in blood for early diagnosis of pregnancy complications (6–7, 37–43), but most of these studies require prefractionation and depletion of the most abundant proteins in plasma. These preprocessing modifications risk concomitant depletion of lower abundance proteins. Acetonitrile precipitation of high abundance proteins may account for the discrepancies between the SELDI-TOF-MS and Orbitrap data in our study, particularly among the Apo A-II and Apo C-I subtypes. Further, we did not investigate differences of 2 atomic mass units among our discriminatory peptides to differentiate monomeric from dimeric Apo A-II. Our quantification methods using 10 pmol of internal standard AQUA peptides (Orbitrap) or protein standard (SELDI-TOF-MS) are also limited in that variable loss of sialic acid which could result from laser desorption (in the case of SELDI-TOF-MS), depending on factors such as the laser energy used (20). Thus, our quantification probably underestimates the true content of Apo C-III in plasma. Alternatively, the antibody used in the ELISA is not specific for multiple apolipoprotein subtypes. The elevated concentration reported in the ELISA relative to the SELDI-TOF-MS quantification could reflect greater specificity of the mass spectrometry approach in detecting unique subtypes.

In this study, we evaluate LTQ-Orbitrap XL mass spectrometry as a complementary experimental approach to estimate changes in apolipoprotein subtype abundance. Our approach relies on determining the area under the extracted ion chromatogram of differentiable peptides, thus allowing for discrimination between apolipoprotein subtypes. The accurate mass capability of the Orbitrap renders this level of specificity possible, allowing for improved differentiation among the subtypes in the Orbitrap platform.

In conclusion, this study demonstrates the utility of a SELDI-TOF-MS proteomic profiling strategy to (1) detect apolipoprotein subtypes in plasma with minimal sample manipulation and (2) observe changes in protein profile in an obstetrically relevant biological problem (prematurity). The LTQ-Orbitrap XL relative quantification and targeted identification of modified peptides provide a complementary experimental approach to SELDI-TOF-MS for the Apo C-III, Apo C-II, and Apo A-II subtypes investigated. This study reports novel evidence for the presence and fluctuation of modified isoforms of apolipoproteins in maternal plasma. In particular, we describe for the first time an increase in mature Apo C-II relative to pro-Apo C-II over gestational time. Because database searching algorithms frequently do not incorporate post translational modifications of apolipoprotein isoforms, this study provides important evidence that many isoforms of proteins might be overlooked in conventional shotgun proteomics approaches.

Acknowledgments—We thank all mothers who volunteered for this project. We thank Patrick McNutt, PhD, for contributing to the conception of the Pregnancy Proteome Project; the Madigan OB-GYN nursing staff for recruiting patients; and the Madigan Pathology Laboratory for phlebotomy. We thank Phil Gafken, PhD, for LTQ-Orbitrap XL consultation and experimentation. We gratefully acknowledge the contributions of Mariano Mesngon, PhD, in manuscript review and figure generation. We thank David McCune, MD, and Richard Burney, MS, MD, for scientific and clinical guidance. We thank Troy Patience and Raywin Huang, PhD, for computational and statistical support, and Cindy Kirker for library assistance. D.L.I., D.T., and S.K.F.-N. jointly conceived of the study; D.L.I., D.T., S.K.F.-N., M.A.W., and A.I.A.-A. designed and performed the experiments; S.K.F.-N. reviewed patient antepartum medical records; D.L.I., J.D.S., and D.T. analyzed the data and interpreted the results; D.L.I. and D.T. wrote the paper; D.L.I., P.G.N., and J.D.S. supervised the project.

* This work was supported by internal funding from Madigan Healthcare System Graduate Medical Education Program. We gratefully acknowledge a grant from the Madigan Foundation for the computing cluster running the proteomics database searching algorithms.

This article contains supplemental FS1 and Tables S1 to S5.

§ Both authors contributed equally to this work.

¶ To whom correspondence should be addressed: Madigan Healthcare System, Joint Base Lewis-McChord, Tacoma, WA, 98431; Tel.: 253–968-0127; Fax: 253–968-1044; E-mail: Danielle.Ippolito@us.army.mil.

Disclaimer—The views expressed in this presentation are those of the authors and do not reflect the official policy of the Department of the Army, the Department of Defense, or the U.S. Government.

REFERENCES

1. Aouizerat, B. E., Kulkarni, M., Heilbron, D., Drown, D., Raskin, S., Pullinger, C. R., Malloy, M. J., and Kane, J. P. (2003) Genetic analysis of a polymorphism in the human apoa-V gene: effect on plasma lipids. J. Lipid Res. 44, 1167–1173

2. Mahley, R. W., Innerarity, T. L., Rail, S. C., Jr., and Weisgraber, K. H. (1984) Plasma lipoproteins: apolipoprotein structure and function. J. Lipid Res. 25, 1277–1294

3. Montes, A., and Knopp, R. H. (1977) Lipid metabolism in pregnancy. IV. C Apolipoprotein changes in very low and intermediate density lipoproteins. J. Clin. Endocrinol. Metab. 45, 1060–1063

4. Nagy, B., Rigdi, J., Jr., Fintor, L., Romics, L., Papp, Z., and Karádi, I. (1999) Distribution of apolipoprotein(a) isoforms in normotensive and severe preeclamptic women. J. Matern. Fetal. Med. 8, 270–274

5. Redman, C., and Sargent, I. L. (2007) Immunological factors and placentation: implications for preeclampsia. Preeclampsia: Etiology and Clinical Practice, Cambridge University Press, Cambridge, United Kingdom, 103–120

6. Atkinson, K. R., Blumenstein, M., Black, M. A., Wu, S. H., Kasabov, N., Taylor, R. S., Cooper, G. J., and North, R. A. (2009) An altered pattern of circulating apolipoprotein E3 isoforms is implicated in preeclampsia. J. Lipid Res. 50, 71–80

7. Blumenstein, M., McMaster, M. T., Black, M. A., Wu, S., Prakash, R., Cooney, J., McCowan, L. M., Cooper, G. J., and North, R. A. (2009) A proteomic approach identifies early pregnancy biomarkers for preeclampsia: novel linkages between a predisposition to preeclampsia and cardiovascular disease. Proteomics 9, 2929–2945

8. Flood-Nichols, S., Stallings, J. D., Gotkin, J. L., Tinnemore, D. T., Napolitano, P. N, and Ippolito, D. L. (2011) Elevated Ratio of Maternal Plasma Apolipoprotein C-III Relative to C-II in Preeclampsia. Repro. Sci. 18(5), 493–502

9. Levels, J. H., Bleijlevens, B., Rezaee, F., Aerts, J. M., and Meijers, J. C. (2007) SELDI-TOF mass spectrometry of High-Density Lipoprotein. Proteome Sci. 5, 15

10. Nelsestuen, G. L., Zhang, Y., Martinez, M. B., Key, N. S., Jilma, B., Verneris, M., Sinaiko, A., and Kasabov, N. Sinaiko, A., and Kasthuri, R. S. (2005) Plasma protein profiling: unique and stable features of individuals. Proteomics 5, 4012–4024

11. Shankar, R., Cullinane, F., Brenneck, S. P., and Moses, E. K. (2004) Applications of proteomic methodologies to human pregnancy research: a growing gestation approaching delivery? Proteomics 4, 1909–1917
28. Timms, J. F., Arslan-Low, E., Gentry-Maharaj, A., Luo, Z., T’Jampens, D., Ito, Y., Breslow, J. L., and Chait, B. T. (1989) Apolipoprotein C-III lacks density fractions by mass spectrometry. J. Lipid Res. 40, 643–655

26. Hospattankar, A. V., Fairwell, T., Ronan, R., and Brewer, H. B., Jr. (1984) Amino acid sequence of human plasma apolipoprotein C-II from normal density lipoprotein subclasses, and postheparin lipases during gestation in women. J. Lipid Res. 37, 299–308

MazzuKierczak, J. C., Watts, G. F., Warburton, F. G., Slavin, B. M., Lowy, C., and Koukkou, E. (1994) Serum lipids, lipoproteins and apolipoproteins in pregnant non-diabetic patients. J. Clin. Pathol. 47, 728–731

Piechota, W., and Staszewski, A. (1992) Reference ranges of lipids and apolipoproteins in pregnancy. Eur. J. Obstet. Gynecol. Reprod. Biol. 45, 27–35

21. Rossi, L., Martin, B. M., Hortin, G. L., White, R. L., Foster, M., Moharram, R., Hortin, G. L., Kasthuri, R. S., Verneris, M. R., Lund, T. C., Ely, E. W., Bernard, G. R., Zeisler, H., Homoncik, M., Jilma, B., Swan, T., and Kellogg, T. A. (2009) O-glycoside biomarker of apolipoprotein C-III response to obesity, bariatric surgery, and therapy with metformin, to chronic or severe liver disease and to mortality in severe sepsis and graft vs host disease. J. Proteome Res. 8, 603–612

20. Hortin, G. L. (2006) The MALDI-TOF mass spectrometric view of the plasma proteome and peptidome. Clin. Chem. 52, 1223–1237

19. Harvey, S. B., Zhang, Y., Wilson-Grady, J., Monkkonen, T., Nelsestuen, G. L., Kasthuri, R. S., Vermeris, M. R., Lund, T. C., Ely, E. W., Bernard, G. R., Zeisler, H., Homoncik, M., Jilma, B., Swan, T., and Kellogg, T. A. (2009) O-glycoside biomarker of apolipoprotein C-III response to obesity, bariatric surgery, and therapy with metformin, to chronic or severe liver disease and to mortality in severe sepsis and graft vs host disease. J. Proteome Res. 8, 603–612

18. Jin, Y., and Manabe, T. (2005) Direct targeting of human plasma for matrix-assisted laser desorption/ionization and analysis of plasma proteins by time of flight-mass spectrometry. Electrophoresis 26, 2823–2834

17. Bondarenko, P. V., Cockrill, S. L., Watkins, L. K., Cruzado, I. D., and Macfarlane, R. D. (1999) Mass spectral study of polymorphism of the apolipoproteins of very low density lipoprotein. J. Lipid Res. 40, 643–655

16. Barton, C., Kay, R. G., Gentzer, W., Vitzthum, F., and Pleasance, S. (2010) Development of high-throughput chemical extraction techniques and quantitative HPLC-MS/MS (SRM) assays for clinically relevant plasma proteins. J. Proteome Res. 9, 333–340

15. Kierczak, J. C., Watts, G. F., Warburton, F. G., Slavin, B. M., Lowy, C., and Koukkou, E. (1994) Serum lipids, lipoproteins and apolipoproteins in pregnant non-diabetic patients. J. Clin. Pathol. 47, 728–731

14. Bons, J. A., de Boer, D., van Dieijen-Visser, M. P., and Wodzig, W. K. (2006) Interrelationships of serum paraxoxonase, serum lipids and apolipoproteins in normal pregnancy. A longitudinal study. J. Obstet. Gynaecol. Invest. 38, 10–13

13. American College of Obstetrics and Gynecology (2003) ACOG Practice Bulletin Management of Preterm Labor

12. Shankar, R., Gude, N., Cullinane, F., Brennecke, S., Purcell, A. W., and Moses, E. K. (2005) An emerging role for comprehensive proteome analysis in human pregnancy research. Reproduction 129, 685–696

11. Vorderwulbecke, S., Cleverley, S., Weinberger, S. R., and Wiesner, A. (2005) Protein quantification by SELDI-TOF-MS-based ProteinChip System. Nature Methods 2, 393–395

10. Alvarez, J. J., Montelongo, A., Iglesias, A., Lasunccion, M. A., and Herrera, E. (1996) Longitudinal study on lipoprotein profile, high density lipoprotein subclass, and postheparin lipases during gestation in women. J. Lipid Res. 37, 299–308