An altered pattern of circulating apolipoprotein E3 isoforms is implicated in preeclampsia

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Abstract Preeclampsia is a common pregnancy complication that is an important cause of preterm birth and fetal growth restriction. Because there is no diagnostic test yet available for preeclampsia, we used a proteomic approach to identify novel serum/plasma biomarkers for this condition. We conducted case control studies comparing nulliparous women who developed preeclampsia at 36–38 weeks of gestation with healthy nulliparous women matched by gestational age at sampling. Serum/plasma was depleted of six abundant proteins and analyzed by two-dimensional gel electrophoresis (n = 12 per group) and difference gel electrophoresis (n = 12 per group). Differences in abundance of protein spots were detected by univariate and multivariate statistical analyses. Proteins were identified by mass spectrometry and expression of selected proteins was validated by immunoblotting. Proteins whose concentrations were selectively associated with preeclampsia included apolipoprotein E (apoE), apoC-II, complement factor C3c, fibrinogen, transthyretin, and complement factor H-related protein 2. An increase in a deglycosylated isoform of apoE3 and concomitantly decreased amounts of one apoE3 glycoisoform were identified in preeclamptic plasma and confirmed by immunoblotting. Altered production of these preeclampsia-related apoE3 isoforms might impair reverse cholesterol transport, contributing to arterial damage. These findings point to a novel mechanistic link between preeclampsia and subsequent cardiovascular disease.—Atkinson, K. R., M. Blumenstein, M. A. Black, S. H. Wu, N. Kasabov, R. S. Taylor, G. J. S. Cooper, and R. A. North, on behalf of the SCOPE Consortium. **An altered pattern of circulating apolipoprotein E3 isoforms is implicated in preeclampsia. *J. Lipid Res.* 2009.** 50: **71–80.**

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Preeclampsia is a syndrome that complicates 5% of first pregnancies and is characterized by the presence of hypertension and proteinuria, and in severe cases, can include coagulopathy, renal and liver dysfunction, and eclamptic seizures. A third of the resulting babies are premature and a quarter are growth restricted, with associated increased morbidity and mortality. Women who develop preeclampsia are at increased risk of cardiovascular disease in later life (1).

The complex pathophysiology of preeclampsia involves aberrant placentation triggering a systemic maternal response that involves widespread endothelial dysfunction, activated coagulation, and inflammation, leading to multiorgan damage (2). Diagnosis of preeclampsia is currently based on a set of clinical symptoms and signs, along with routine laboratory tests assessing platelet count and liver and renal function. Recently, the ratio of soluble vascular endothelial growth factor receptor 1 (sFlt-1) to placental growth factor has been proposed as a late predictive or diagnostic test (3). Given the multiple pathogenic processes that culminate in preeclampsia, however, it is likely that a set of biomarkers will be required to achieve clinical utility in a diagnostic test.

It is increasingly recognized that the systematic application of proteomic approaches in pregnancy research will be needed to discover novel biomarkers for preeclampsia (4, 5). Recently, two-dimensional gel electrophoresis (2DE) proteomic techniques have been used to investigate changes in the plasma proteome in preeclampsia (6, 7). A 2DE study of HELLP syndrome, a severe form of preeclampsia,

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Abbreviations: apoE, apolipoprotein E; 2DE, two-dimensional gel electrophoresis; DIGE, difference gel electrophoresis; ECF, evolving connectionist functions; FDR, false discovery rate; LC, liquid chromatography; MS/MS, tandem mass spectrometry; NSC, nearest shrunken centroids; RFE, recursive feature elimination.

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identified one downregulated and five upregulated plasma proteins (7). In another study, serum clusterin was reportedly increased in preeclampsia with fetal growth restriction (6). Although these studies indicated the potential of proteomic approaches, only small numbers of women with severe disease were included. Severe-disease subsets are not representative of the majority of women with preeclampsia. Furthermore, steroids may have been given to promote fetal lung maturation, modifying the plasma proteome, and limited statistical approaches were employed. Combinations of univariate statistical methods and multivariate classification methodologies have recently been proposed for the analysis of complex proteomic datasets (8). This approach pinpoints protein combinations that may be able to distinguish between healthy and disease states (9).

Here, we employed two different two-dimensional (2-D) gel-based proteomic approaches, conventional 2DE and difference gel electrophoresis (DIGE), to identify preeclampsia-specific markers in serum/plasma of women presenting with disease at 36–38 weeks of gestation in comparison with healthy pregnant women. Our comprehensive bioinformatic analysis identified several serum/plasma candidates in preeclampsia. Specifically, altered glycosylation of circulating apolipoprotein E (apoE) isoforms was a novel finding, confirmed by Western blot analysis. Furthermore, multivariate classification methods revealed that apoE and fibrinogen β, in combination with other proteins, could serve as discriminators between preeclampsia and healthy pregnancy.

MATERIALS AND METHODS

Study groups and sample collection

For 2DE analysis, serum from nulliparous women presenting with preeclampsia at 36–38 weeks (n = 12) and gestational age-matched healthy control participants (n = 12) was used. Similarly, n = 12 cases and controls were used for the DIGE study, analyzing plasma from women who were recruited into the SCOPE study (SCreening fOr Pregnancy Endpoints, Australian and New Zealand Clinical Trials Registry ACTRN12607000551493), a prospective screening study of nulliparous women. Specimen collection at this gestational age avoided any steroid exposure for fetal lung maturation prior to sampling. Study protocols were approved by the Auckland Regional Ethics Committee (2000/157 and AKX/02/00/364), and written informed consent was obtained from each woman.

Preeclampsia was defined as systolic blood pressure (BP) ≥140 mm Hg and/or diastolic BP ≥90 mm Hg on two or more occasions after 20 weeks of gestation but prior to the onset of labor, or postpartum systolic BP ≥140 mm Hg and/or diastolic BP ≥90 mm Hg postpartum on at least two occasions 4 h apart, combined with either proteinuria (spot protein:creatinine ratio ≥30 mg/mmol, or 24 h urinary protein ≥0.3 g per 24 h, or dipstick proteinuria ≥2+) or any multi-organ complication (10). Severe preeclampsia was defined by the presence of one or more of the following additional findings: coagulopathy, hemolysis, liver impairment, acute renal insufficiency, imminent eclampsia, or eclampsia. Babies who were small for gestational age had a birth weight less than the 10th customized centile (adjusted for infant sex and maternal ethnicity, height, and weight) (11).

For serum samples, blood was collected by venipuncture into plain tubes, allowed to clot on ice, and centrifuged (2,400 *g*, 10 min, 4°C). The resulting serum was centrifuged again (3,200 *g*, 15 min, 4°C) and stored at −80°C. For plasma samples, blood was collected into EDTA tubes, centrifuged (2,400 *g*, 10 min, 4°C), and stored at −80°C. All samples were processed within 3 h of collection.

Sample preparation

To remove the six most abundant proteins (albumin, transferrin, IgG, IgA, haptoglobin, and α-1-antitrypsin), serum/plasma was immunodepleted using the Multiple Affinity Removal System (MARS; Agilent, Santa Clara, CA) according to the manufacturer's instructions. Before depletion, a protease inhibitor cocktail was added (Roche Applied Science, Auckland, New Zealand). Depleted samples were buffer exchanged using 5 kDa molecular mass cutoff centrifugal filters into the respective 2DE and DIGE rehydration solutions. Protein content was determined using the 2D Quant protein assay (GE Healthcare, Auckland, New Zealand).

2DE

Depleted serum (150 μg) was diluted in rehydration solution (9 M urea, 2% CHAPS, 65 mM DTT, 0.5% immobilized pH gradient (IPG) Buffer pH 4–7, 0.002% bromophenol blue) and passively rehydrated overnight using 18 cm pH 4–7 IPG strips (GE Healthcare). IPG strips were focused on a PROTEAN cell (Bio-Rad, Hercules, CA), followed by SDS-PAGE using ExcelGel XL 12–14% polyacrylamide gels (GE Healthcare). Gels were stained with SYPRO® Ruby (Invitrogen, Carlsbad, CA) and imaged on a FLA–2000 phosphorimager (Fujifilm, Tokyo, Japan). Digitized 2DE images were analyzed by ImageMaster™ 2D Platinum v5.0 (GE Healthcare). Spot volume data for each gel were exported from ImageMaster for bioinformatic analyses.

DIGE

Depleted and buffer-exchanged plasma samples were labeled with 200 pmol per 50 μg of protein each with CyDye minimal dyes (GE Healthcare) according to the manufacturer's protocol. For the pooled internal standard, equal amounts of representative specimens (n = 6 cases and n = 6 controls) were combined. Samples were labeled with their respective CyDyes: the internal standard with Cy2, and controls and cases with either Cy3 or Cy5, with each case and control group being dye-balanced. IPG strips (11 cm, pH 4–7) were rehydrated with a multiplexed plasma sample comprising 50 μg protein for each CyDye-labeled case, control, and the pooled internal standard in rehydration buffer (7 M urea, 2 M thiourea, 1% C7BzO detergent, 1% IPG buffer, pH 4–7, 65 mM DTT, and 0.002% bromophenol blue). First-dimension separation was performed on a Multiphor II flatbed (GE Healthcare) followed by SDS-PAGE using Criterion 8–16% Tris-HCl midigels run in a Dodeca cell (Bio-Rad) for 30 min at 15 mA per gel, and 90 min at 30 mA per gel. All twelve DIGE gels were run simultaneously. Typhoon 9410 (GE Healthcare) digitized images were imported into DeCyder 2-D Differential Analysis software v6.5 (GE Healthcare) for spot detection and matched to an automatically chosen master gel using an estimated number of spots of 10,000 and applying a spot volume filter of >26,000. Standardized abundance data were exported from DeCyder for bioinformatic analyses.

Multiplexed immunoassays

ApoE and apoC-II were measured in native (undepleted) plasma using a multiplexed immunoassay (LINCOplex; Millipore, Billerica, MA) according to the manufacturer's instructions. Assays were read on a Luminex 100 instrument (Luminex, Austin, TX).

2-D Western blot analysis of apoE isoforms

First-dimension separation of depleted serum (200 μg) was performed on 7 cm pH 4–7 IPG strips followed by SDS-PAGE using NuPAGE 4–12% Bis-Tris minigels (Invitrogen), which were blotted onto Immobilon-FL membranes (Millipore) according to the manufacturer's instructions. Membranes were blocked with 5% BSA in PBS, 0.1% Tween 20 overnight at 4°C. Detection of apoE (Abcam #ab7620, Cambridge, UK, at 1:5,000) was achieved with Qdot® 655-conjugated IgG (Invitrogen, 1:1,000). Blots were imaged on a Typhoon 9410 imager. Spots were quantified using Phoretix 2D Evolution v2004 software (Nonlinear Dynamics, Newcastle upon Tyne, UK).

Statistical analysis

Clinical, immunoassay, and 2-D Western blot data. Continuous clinical variables, immunoassay data, and 2-D Western blot data were compared using Student's *t*-test. Categorical clinical data were compared using Fisher's exact test. A *P* value of less than 0.05 was considered significant. Analyses were performed using GraphPad Prism version 4.03 for Windows (GraphPad Software, San Diego, CA).

Bioinformatic analysis of proteomic data

Data transformation. Standardized abundances exported from DeCyder 2D were transformed (log base 2). 2DE and DIGE data were normalized by centering each gel at zero using median subtraction. Box plots demonstrated no outlying gels. Logged data were then used for univariate and multivariate analyses.

Univariate analysis

2DE data were analyzed by Student's *t*-test and DIGE data by the modified *t*-test implemented in Limma (linear models for microarray data) to determine differential expression between healthy and preeclamptic samples. A false discovery rate (FDR) correction (12) was applied to correct for multiple comparisons. An FDR-corrected *P* value of less than 0.05 in either of the above tests was considered significant.

Multivariate classifier analysis

2DE data were analyzed by three different classifier generation methods: nearest shrunken centroids (NSC), recursive feature elimination (RFE), and evolving connectionist functions (ECF). Missing values were imputed using the minimum nonzero spot volume for the 2DE data set. DIGE data were analyzed by the NSC method alone after imputing the median spot volume for missing values.

The NSC approach uses a variant of discriminant analysis to classify samples with a modified *t*-statistic used to rank spots according to the degree of separation exhibited between data classes (preeclamptic and healthy pregnant) (13). The scores for each spot were shrunk toward zero using an approach designed to eliminate noninformative spots from the analysis and determine sets of spots that best discriminate disease classes. The RFE method used an approach based on support vector machine methodology coupled with an iterative method for variable selection (14). At each iteration, the predictive ability of the model was assessed with cross-validation. An ECF model (15) was used in conjunction with 24-fold leave-one-out cross-validation to generate a classifier able to distinguish sample groups. The number of spots used in the final model was chosen as the number of spots that achieved the best overall cross-validated classification rate.

Selection criteria for protein spots of interest

Protein spots of interest were selected with the following criteria: *1*) protein spots that were significantly upregulated or downregulated ($P < 0.05$ with FDR correction) by univariate methods; or *2*) protein spots that were selected by a classifier method. Spots of interest were manually inspected and any gel artifacts excluded. The resulting spots were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS).

Protein identification by LC-MS/MS

Spots were excised from gels and digested with trypsin according to published methods (16), and submitted for LC-MS/MS analysis on a QSTAR XL ESI-qTOF (Applied Biosystems, Foster City, CA) at the Maurice Wilkins Centre for Molecular Biodiscovery, The University of Auckland. Tryptic peptides of spots corresponding to apoE isoforms were analyzed by LC-MS/MS (LTQ FT-ICR; Thermo, San Jose, CA) at the Maurice Wilkins Centre. The SALSA algorithm was used to search for posttranslational modifications (17).

MS/MS data were extracted from raw spectra using Mascot Distiller (Matrix Science, London, UK). Data were searched against the Swiss-Prot database (version 52.2, date 14 April 2007) using the Mascot search engine v2.2.0 with the following parameters. Taxonomy: human, semitrypsin cleavage with up to 1 missed cleavage allowed; fixed modification: carbamidomethylation (serum) or propionamidation (plasma); variable modification: oxidation (M), mass tolerances ±0.1 Da, peptide charges 2+ and 3+. Positive identifications reported here had at least three unique peptides match the database entry.

RESULTS

Two separate case control studies were conducted using either serum (2DE) or plasma (DIGE) collected from women diagnosed with preeclampsia compared with healthy pregnant women matched by gestational age at sampling. The clinical characteristics of participants and the maternal and fetal outcomes are detailed in **Table 1**.

2DE serum analysis

Univariate analysis of our 2DE data revealed 23 differentially expressed spots that were up- or downregulated ($P < 0.05$) in the serum of preeclamptic women. After correcting for multiple comparisons, no differences remained significant. Using multivariate classification methods, six spots were identified as part of a set of proteins capable of separating preeclampsia from healthy pregnancy (**Table 2**). Spot members of these classification models were identified by LC-MS/MS as apoC-II (spot 28), retinol binding protein (spot 125), apoE basic isoform (spot 161), apoE acidic isoform (spot 168), complement factor C3c (spot 194), and inter-α-trypsin inhibitor H4 (spot 428) (**Fig. 1** and **Table 3**). Levels of the two apoE isoforms varied oppositely between preeclampsia and healthy pregnancy. Specifically, the basic isoform (spot 161, pI 5.7) was found to be upregulated in preeclampsia, whereas the more acidic isoform (spot 168, pI 5.3) was downregulated.

Because conventional 2DE has an inherently high technical variability (18), we also employed a DIGE approach in a separate case control study. In contrast to the 2DE study, in which 42 gels per group were necessary to achieve an 80% power to detect three-quarters of the proteins with a 2-fold difference between cases and controls, the DIGE

TABLE 1. Maternal and neonatal clinical characteristics for 2DE and DIGE samples

Study Characteristic	2DE			DIGE		
	Preeclamptic (n = 12)	Healthy Pregnant (n = 12)	*P*	Preeclamptic (n = 12)	Healthy Pregnant (n = 12)	*P*
Maternal						
Gestational age at sampling (weeks)	37.6 (0.8)	37.3 (1.0)	0.38	37.3 (1.0)	37.0 (1.1)	0.44
Systolic blood pressure at sampling (mm Hg)	138 (14)	116 (9)	0.0001	137 (9)	113 (12)	<0.0001
Diastolic blood pressure at sampling (mm Hg)	94 (11)	72 (7)	<0.0001	95 (8)	70 (8)	<0.0001
Age (years)	31.4 (4.2)	31.8 (3.4)	0.83	30.2 (5.0)	31.6 (3.6)	0.43
Ethnicity						
Caucasian	7	8		7	9	
Maori or Pacific Islander	3	0	0.20	4	0	0.10
Other	2	4		1	3	
Body mass index at booking (kg/m^2)	26.4 (4.1)	23.2 (4.4)	0.09	26.8 (4.0)	25.2 (5.1)	0.39
Gestational age at first visit (weeks)	12.2 (2.7)	11.9 (4.1)	0.88	13.8 (2.7)	13.6 (3.3)	0.91
Systolic blood pressure at <20 weeks (mm Hg)	114 (14)	116 (12)	0.73	111 (15)	112 (11)	0.84
Diastolic blood pressure at <20 weeks (mm Hg)	70 (11)	63 (7)	0.09	67 (10)	63 (7)	0.33
Maximum systolic blood pressure (mm Hg)	165 (19)	125 (7)	<0.0001	155 (13)	127 (8)	<0.0001
Maximum diastolic blood pressure (mm Hg)	106 (6)	77 (8)	<0.0001	103 (6)	77 (9)	<0.0001
Proteinuria (g per 24 h; median, range, n)	0.87 (0.39–6.98, n = 10)	0		1.05 (0.39–8.94, n = 9)	0	
Severe preeclampsia	2 (16.6%)[a]	0		3 (25%)[b]	0	
Neonatal						
Gestational age at delivery (weeks)	38.1 (0.6)	40.1 (1.5)	0.0003	37.8 (0.8)	40.2 (1.4)	<0.0001
Infant birth weight (g)	2,943 (340)	3,628 (329)	<0.0001	3,107 (333)	3,671 (399)	0.0001
Small for gestational age	4 (33.3%)	0		2 (17%)	0	

2DE, two-dimensional gel electrophoresis; DIGE, difference gel electrophoresis. Values are reported as mean (SD) or n (%) unless otherwise noted.

[a] Imminent eclampsia (n = 1), HELLP (n = 1).

[b] HELLP (n = 1), acute renal insufficiency (n = 2).

protocol proved to be more efficient, requiring a sample size of 12 cases and 12 controls.

DIGE plasma analysis

DIGE plasma analysis (n = 12 per group) revealed 63 spots that had significantly different ($P < 0.05$) expression ratios by univariate statistical methods. None of these spots remained significant after FDR correction for multiple comparisons. NSC analysis identified a set of four spots associated with preeclampsia (**Fig. 2**). Based on their high Mascot scores and the large number of unique peptides per spot, these proteins were identified by LC-MS-MS as fibrinogen β (spots 355, 356), apoE (spot 521), and transthyretin (spot 569) (Table 3). Three of these spots (spots 355, 356, and 569) contained several additional protein species with more than two unique peptides (Table 3), likely to be due to comigration of plasma proteins on 2-D gels (19). Taking into account their molecular weights, other possible protein components were hemopexin (spot 355), fibrinogen γ (spot 356), and complement H-related protein 2 (spot 569). The set of the above six proteins correctly classified between preeclampsia and healthy pregnancy with 80% accuracy. It is noteworthy that the upregulation of the basic isoform of apoE (spot 521) was significant upon univariate analysis prior to FDR correction, and had also been identified in our prior 2DE-based study, further supporting its association with preeclampsia.

TABLE 2. Results from multivariate classifier analysis of 2DE data

Spot Number	Method		
	Nearest Shrunken Centroids	Recursive Feature Elimination	Feature Selection Using Evolving Connectionist Functions
28	x	x	x
125		x	
161	x	x	x
168		x	x
194	x	x	
428	x	x	x

Differential glycosylation of apoE in preeclampsia

To further explore the different production of preeclampsia-specific apoE isoforms revealed by 2DE and DIGE, we utilized 2-D Western blot analysis of depleted serum. Western blot analysis (**Fig. 3A, B**) confirmed our 2DE data and showed the upregulation of a basic apoE isoform (corresponding to spot 161 in Fig. 1A) in serum from women with preeclampsia, whereas an acidic isoform of apoE (corresponding to spot 168 in Fig. 1A) was downregulated in disease. The basic apoE isoform (spot 161) was found in eleven of twelve women with preeclampsia,

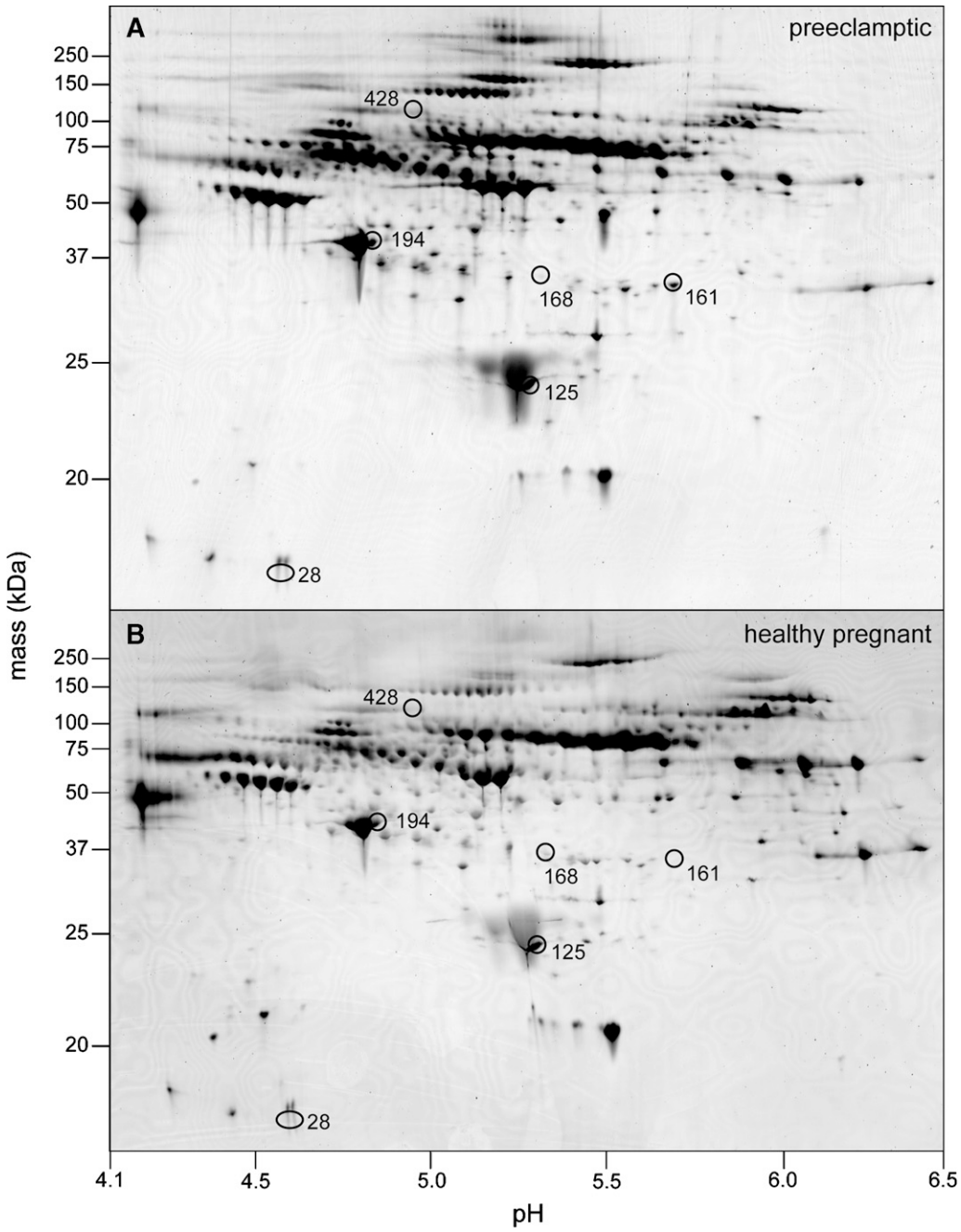

Fig. 1. Protein spots identified following two-dimensional (2-D) gel electrophoresis (2DE) analysis. Spots identified following classifier analysis of 2DE data are indicated on representative 2DE images. A: Gel from a preeclamptic sample at 36.9 weeks of gestation. B: Gel from a healthy pregnant sample at 36.1 weeks of gestation.

of whom two had severe disease and four had a baby that was small for gestational age, and in only three of twelve healthy pregnant women, indicating that disease severity is an unlikely explanation for this abnormality.

The apoE isoform spot volume changes observed on 2-D Western blots were significantly different between women with preeclampsia and healthy pregnant women ($P < 0.05$ for each of five isoforms; Fig. 3C). We explored the post-translational modifications of apoE by using the SALSA search algorithm to interrogate our peptide sequences for such modifications. By this method, the acidic isoform (spot 168) was found to be *O*-glycosylated with a linkage comprising Thr194-*N*-acetyl hexosamine-hexose-sialic acid (**Fig. 4** and **Tables 4 and 5**). Peptide sequences obtained from these LC-MS/MS data confirmed that spot 168 represented the E3 form of the apoE protein. Repeated analysis of peptides from spot 161 (basic apoE isoform) confirmed protein identity, but glycosylation was not detected.

Circulating apoE and apoC-II were further assessed by multiplexed immunoassay using native, undepleted plasma.

TABLE 3. Proteins identified from 2DE and DIGE classifier spots

Study	Spot Number	Fold Change (PE/Healthy)	Protein[a]	Accession	Sequence Coverage[b]	Peptides (Unique)	Calculated Molecular Mass	Gel Molecular Mass	Gel pI
					%		kDa[c]		
2DE	28	2.5	Apolipoprotein C-II	P02655	71	8 (6)	8.9	10	4.6
2DE	125	2.1	Plasma retinol binding protein	P02753	56	11 (8)	21.1	24	5.3
2DE	161	2.0	Apolipoprotein E (basic isoform)	P02649	40	11 (10)	34.2	34	5.7
2DE	168	−1.7	Apolipoprotein E (acidic isoform)	P02649	21	5 (5)	34.2	35	5.3
2DE	194	−2.5	Complement c3c, C-terminal fragment of α′ chain[c]	P01024	29[c]	8 (7)	39.5	40	4.8
2DE	428	−3.6	Inter-α-trypsin inhibitor, heavy chain H4	Q14624	17	15 (14)	70.6	90	4.9
DIGE	355	1.9	Fibrinogen, β chain	P02675	59	39 (22)	50.7	50	6.1
			β-2-glycoprotein 1	P02749	45	14 (10)	36.2		
			Transferrin	P02787	25	12 (12)	75.1		
			Hemopexin	P02790	30	10 (8)	49.3		
			Fibrinogen, α chain	P02671	16	12 (10)	91.3		
DIGE	356	1.9	Fibrinogen, β chain	P02675	64	36 (20)	50.7	50	6.3
			Fibrinogen, α chain	P02671	25	20 (15)	91.3		
			Fibrinogen, γ chain	P02679	19	5 (5)	48.5		
			Complement C3 (peptides map to the β chain)[d]	P01024	14 vs. β chain[d]	5 (5)	71.3[d]		
DIGE	521	4.7	Apolipoprotein E	P02649	47	14 (12)	34.2	34	5.6
DIGE	569	1.9	Transthyretin	P02766	64	10 (5)	13.7	28	5.4
			Mannose binding protein C	P11226	31	7 (5)	24.0		
			Serum amyloid P component	P02743	24	5 (5)	23.2		
			Complement H-related protein 2	P36980	34	7 (7)	28.7		
			Fibrinogen, γ chain	P02679	17	6 (5)	48.5		
			apolipoprotein E	P02649	24	5 (5)	34.2		

PE, preeclampsia.

[a] Proteins are listed within each gel spot number in descending order of Mascot match score.

[b] Sequence coverage was calculated as part of the Mascot search process using the entire chain of the protein's Swiss-Prot database entry.

[c] Molecular mass was calculated on Swiss-Prot entries from the main chain or appropriate chain only using the Compute MW/pI tool (http://www.expasy.org/tools/pi_tool.html).

[d] Complement C3 is a multi-chain protein derived from a single polypeptide precursor; sequence coverage, molecular mass, and pI were calculated from the C3 β chain only.

Plasma levels of total apoE from eighteen preeclamptic women (86.8 ± 17.9 μg/ml) were not significantly different from those in healthy controls (88.7 ± 21.0 μg/ml, n = 18). There were also no differences in plasma apoC-II levels in preeclampsia (103.2 ± 23.6 μg/ml, n = 18) compared with those in healthy pregnancy (89.5 ± 30.7 μg/ml, n = 18) (data not shown).

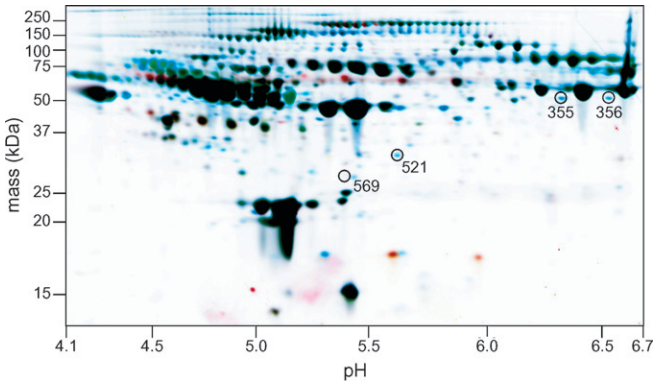

Fig. 2. Protein spots identified following difference gel electrophoresis (DIGE) analysis Spots resulting from classifier analysis of DIGE data are indicated on a representative overlaid image of a single DIGE gel imaged in three channels. The gel contains 50 μg of protein from a pooled internal standard (Cy2, yellow) and 50 μg of protein from depleted plasma samples from a preeclamptic woman at 35.7 weeks of gestation (Cy5, blue) and a healthy pregnant woman at 35.6 weeks of gestation (Cy3, red). Areas of color represent differentially expressed proteins, and black areas show spot coincidence.

DISCUSSION

Here we report for the first time that specific alterations in circulating apoE isoforms are associated with preeclampsia. Although we found no changes in total apoE plasma concentrations in preeclampsia, our proteomic analysis revealed a different pattern of glycosylation of apoE in women with preeclampsia compared with healthy pregnant controls. A basic apoE isoform that appeared to be deglycosylated was increased in preeclampsia, and an acidic apoE isoform containing *O*-linked oligosaccharide chains and sialic acid was decreased. These findings were confirmed by immunoblotting. Previous reports concerning total plasma apoE levels in preeclampsia are mutually inconsistent, with both unchanged and increased levels having been reported in the literature (20, 21).

ApoE is synthesized and secreted primarily by the liver, but also by the placenta and by tissue macrophages. ApoE has a complex isoform pattern due to genetically determined alleles and posttranslational modifications (glycosylation), both of which can influence its functional and structural properties. Three alleles, ε2, ε3, and ε4, encode the corresponding apoE variant proteins apoE2, apoE3, and apoE4, respectively; these are sequence variants that differ by sin-

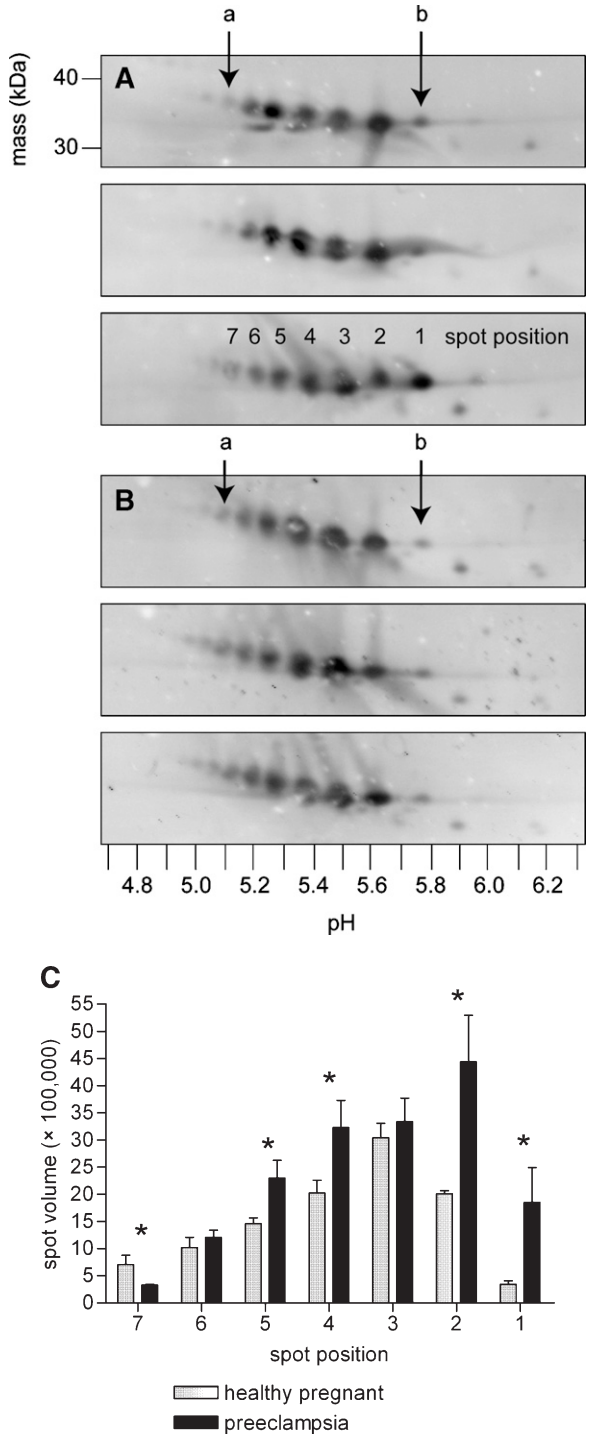

Fig. 3. Specific detection and quantitation of apolipoprotein E (apoE) isoforms in serum using 2-D Western blot analysis. 2-D Western blot analysis of 200 μg depleted serum from three women with preeclampsia (A) and three healthy pregnant women (B). Acidic (arrow a) and basic (arrow b) apoE isoforms, based on pI positions from serum 2DE, are indicated as shown. The volumes of individual spots 1–7 are shown as the mean ± SEM of values derived from all six blots (C). Asterisks indicate significant differences between groups (all $P < 0.05$).

gle amino acid substitutions leading to characteristic shifts in their isoelectric points (22). Glycosylated (sialylated) apoE isoforms arise from the attachment of a carbohydrate moiety, which is variably sialylated, at a single threonine residue, Thr194 (23). In the general population, the ε3 allele is most common (with a prevalence of over 60%), and E3 is the predominant circulating apoE variant. An association between the ε2 allele and preeclampsia was formerly suggested by Nagy et al. (24), but this hypothesis was not confirmed in several following studies (25–28). Moreover, Belo et al. (26) reported no differences in the prevalence of the ε3 allele in a large cohort of pregnant women with respect to their pregnancy outcome (healthy or complicated by preeclampsia). Our 2-D Western blot results are consistent with the apoE3 variant, which we additionally identified from LC-MS/MS data used to determine the glycosylation state of the acidic apoE isoform at pI 5.3 by SALSA analysis. If either apoE2 or apoE4 had been present in our samples, characteristic shifts in the isoelectric point from that of apoE3, the predominant isoform, would have been observed, consistent with previous reports (29, 30). Because these shifts were not observed, we conclude that differential glycosylation of apoE3, rather than protein sequence variants, is the probable explanation for our findings.

Our SALSA analysis showed that one singly sialylated *O*-glycan chain is present on the acidic apoE isoform. It was reported previously that some of the more acidic apoE isoforms are multiply sialylated (31). The possible discrepancy between that report and our current findings might be explained by the difficulties inherent in quantitating glycopeptides using MS alone, particularly if complex carbohydrate side-chains are present. Mancone et al. (32) reported apparently similar difficulties in identifying certain complex *O*-glycan structures present in acidic apoE glycoisoforms using MALDI-MS analysis. More often, orthogonal analysis methods involving the construction of mutants or selective digestion of sugar residues by specific enzymes followed by extensive MS analyses are necessary to elucidate glycoisoform structures. This is beyond the scope of our present study but would warrant further investigation.

Newly synthesized apoE is heavily sialylated, whereas approximately 80% of apoE present in serum/plasma is nonsialylated (31, 33). Decreased glycoslyation of other proteins has been reported in preeclampsia (34, 35). Recent work from Gu, Lewis, and Wang (36) also indicated differential glycosylation of soluble endoglin and sFlt-1 in term placental tissue from women with preeclampsia.

The presence of a maternal susceptibility locus for preeclampsia has been identified by linkage studies and mapped to chromosome 2q23 (37). This locus, termed PREG1 (preeclampsia, eclampsia gene 1) encompasses, among other genes, the polypeptide *N*-acetylgalactosaminyltransferases (GalNAc transferases). GalNAc transferases initiate mucin-type *O*-glycan biosynthesis. Taken together, the evidence of a preeclampsia susceptibility gene encompassing transferases responsible for *O*-linked glycosylation and our findings of differentially glycosylated apoE isoforms support the concept that wider dysregulation of protein glycosylation occurs in preeclampsia.

The biological significance of deglycosylation of apoE is uncertain. Sialylation of apoE does not appear to be obligatory for secretion or to affect its receptor binding (38, 39). Given the relationship between preeclampsia and later

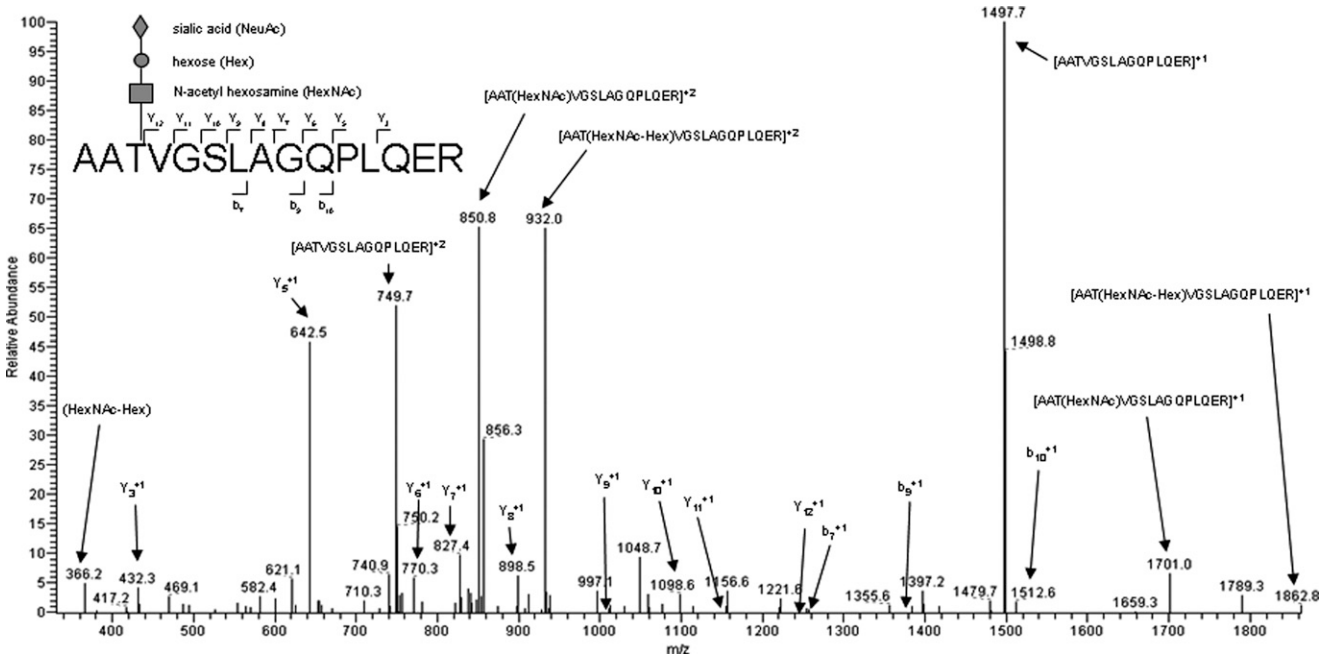

Fig. 4. Product ion spectrum of the *O*-glycosylated peptide from serum 2DE spot 168, which contains Thr194. The tandem mass spectrometry spectrum of *m/z* 1,077.9 (+2) is shown. This spectrum shows a characteristic carbohydrate-diagnostic oxonium ion at *m/z* 366 (HexNAc-Hex).

cardiovascular disease (1), desialylation of apoE may alter its anti-atherogenic actions and, in particular, its role in reverse cholesterol efflux from lipid-laden macrophages. To protect the arterial wall from the toxic effects of free cholesterol, macrophages scavenge cholesterol and form lipid-laden macrophages or "foam cells" in atherosclerotic plaques. Elevated plasma cholesterol has been reported prior to preeclampsia, and foam cells have long been recognized in this disorder in the lesions of "acute atherosis" of the spiral arteries supplying the intervillous space (40). Reverse cholesterol transport is largely mediated by apoA-I, but also apoE (41). ApoE, complexed to HDL, promotes cholesterol efflux from lipid-laden macrophages by promoting cholesteryl ester enrichment of HDL_2 (41). Marmillot and coworkers (42) found desialylation of apoE dramatically decreased its binding to HDL and reduced the uptake of esterified cholesterol from reconstituted HDL by HepG2 cells. Moreover, in cardiovascular disease, apoE levels have been reported to be decreased in HDL_2 and elevated in HDL_3 particles (43, 44). We speculate that desialylation of apoE in preeclampsia may impair its binding to HDL_2 and diminish reverse cholesterol transport. This may be a potential mechanism linking preeclampsia to an increased risk of later cardiovascular disease.

Moreover, we identified a preeclampsia-associated abnormality in serum/plasma proteins involved in pathways of lipid metabolism (apoE, apoC-II), coagulation (fibrinogen β and fibrinogen γ), complement activation (complement C3c, complement H-related protein 2, mannose-binding protein C, serum amyloid P), and acute phase responses

TABLE 4. Observed fragment ion series of the acidic apolipoprotein E *O*-glycosylated peptide shown in Fig. 4

Residue	Mass	b[a]	y
A	71.0	72.0	—
A	71.0	143.1	2,083.0
T#	757.2	900.3	2,011.9
V	99.1	999.4	1,254.7
G	57.0	1,056.4	1,155.6
S	87.0	1,143.5	1,098.6
L	113.1	1,256.5	1,011.6
A	71.0	1,327.6	898.5
G	57.0	1,384.6	827.4
Q	128.1	1,512.7	770.4
P	97.1	1,609.7	642.4
L	113.1	1,722.8	545.3
Q	128.1	1,850.8	432.2
E	129.0	1,979.9	304.2
R	156.1	—	175.1

[a] All *m/z* values shown are for singly charged ions. Fragment ions shown in Fig. 4 are underlined.

TABLE 5. Mass-to-charge ratios (*m/z*) of variant [peptide+oligosaccharide] ions from the acidic apolipoprotein E *O*-glycosylated peptide shown in Fig. 4

m/z	Assignment[a]
1,862.8	$[M\text{-}NeuAc]^{+1}$
1,701.0	$[M\text{-}(Hex\text{-}NeuAc)]^{+1}$
1,497.7	$[M\text{-}(HexNAc\text{-}Hex\text{-}NeuAc)]^{+1}$
932.0	$[M\text{-}NeuAc]^{+2}$
856.3	$[b_{10}\text{-}(HexNAc\text{-}Hex\text{-}NeuAc)]^{+1}$
850.8	$[M\text{-}(Hex\text{-}NeuAc)]^{+2}$
749.7	$[M\text{-}(HexNAc\text{-}Hex\text{-}NeuAc)]^{+2}$
671.2	$[b_8\text{-}(HexNAc\text{-}Hex\text{-}NeuAc)]^{+1}$
600.4	$[b_7\text{-}(HexNAc\text{-}Hex\text{-}NeuAc)]^{+1}$
487.3	$[b_6\text{-}(HexNAc\text{-}Hex\text{-}NeuAc)]^{+1}$
366.2	$[HexNAc+Hex]^{+1}$

[a] Assignment details show the modified peptides or peptide fragments (containing HexNAc-Hex-NeuAc) as M (peptide) or b ions respectively. Oligosaccharide losses from these peptides/fragments are indicated as M-(group lost).

(retinol binding protein, also known as retinol binding protein 4, and transthyretin). NSC analysis of our proteomic data revealed apoC-II as part of a classifier set of proteins that also included apoE, C3c, retinol binding protein, and inter-α-trypsin inhibitor heavy chain H4 (Table 3). Immunoassay of total plasma apoC-II did not confirm the 2.5-fold increase found by our proteomic approach, suggesting that different isoforms of apoC-II are responsible for the increased expression in preeclampsia. All of the above-mentioned pathways are reported to contribute to the pathogenesis of preeclampsia (45).

Circulating levels of total fibrinogen increase 2-fold during the course of a healthy pregnancy (46) and are significantly increased in preeclampsia (7, 47), providing a link with the hypercoagulable states of pregnancy and with preeclampsia in particular. Moreover, increased fibrinogen levels have been linked to an increased risk of coronary artery disease (48). Transthyretin, a homotetrameric protein that transports retinol binding protein and thyroxine, was identified as part of a second protein cluster implicated in preeclampsia. Given its apparent molecular weight by 2DE, the upregulated transthyretin in our study may well have been dimeric. Monomeric transthyretin has been reported at higher levels in amniotic fluid of women who subsequently developed preeclampsia, possibly as a result of oxidative stress that acts as a major destabilizing factor of the functional dimeric form (49). Once transthyretin dissociates into monomers, it can undergo tertiary structural changes to form amyloid fibrils (50). Other proteins identified in this study also have a demonstrated propensity to form amyloid fibrils, including apoE and apoC-II (51). Deposition of amyloid fibrils in the arterial wall has been implicated in the pathogenesis of atherosclerotic plaques (51), but this has not been investigated in preeclampsia.

In summary, the identification of several proteins involved in lipid metabolism, complement activation, and coagulation in our study supports the importance of these pathways in the pathogenesis of preeclampsia. Our finding of a preeclampsia-specific pattern of apoE glycoisoforms is novel. We postulate that increased deglycosylation of apoE may decrease its binding to HDL, impairing its role in reverse cholesterol transport from lipid-laden macrophages, and thereby contributing to vascular damage. Furthermore, it may point to a wider dysregulation of *O*-linked glycoslyation in preeclampsia and represent a mechanistic link between preeclampsia and subsequent cardiovascular disease.

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