RESEARCH ARTICLE

A proteomic approach identifies early pregnancy biomarkers for preeclampsia: Novel linkages between a predisposition to preeclampsia and cardiovascular disease

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Preeclampsia (PE) is a common, potentially life-threatening pregnancy syndrome triggered by placental factors released into the maternal circulation, resulting in maternal vascular dysfunction along with activated inflammation and coagulation. Currently there is no screening test for PE. We sought to identify differentially expressed plasma proteins in women who subsequently develop PE that may perform as predictive biomarkers. In seven DIGE experiments, we compared the plasma proteome at 20 wk gestation in women who later developed PE with an appropriate birth weight for gestational age baby ($n = 27$) or a small for gestational age baby ($n = 12$) to healthy controls with uncomplicated pregnancies ($n = 57$). Of the 49 differentially expressed spots associated with PE-appropriate for gestational age, PE-small for gestational age or both ($p < 0.05$, false discovery rate corrected), 39 were identified by LC-MS/MS. Two protein clusters that accurately (>90%) classified women at risk of developing PE were identified. Immunoblots confirmed the overexpression of fibrinogen γ chain and α-1-antichymotrypsin in plasma prior to PE. The proteins identified are involved in lipid metabolism, coagulation, complement regulation, extracellular matrix remodeling, protease inhibitor activity and acute-phase responses, indicating novel synergism between pathways involved in the pathogenesis of PE. Our findings are remarkably similar to recently identified proteins complexed to high-density lipoprotein and linked to cardiovascular disease.

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Abbreviations: A2M, α-2-macroglobulin; **AGA,** appropriate birth weight for gestational age; **ECM,** extracellular matrix; **FDR,** false discovery rate; **FWER,** family-wise error rate; **hDL,** high-density lipoprotein; **HRP,** haptoglobin-related protein; **LTQ,** linear trap quadrupole; **MARS,** multiple affinity removal system; **NSC,** nearest shrunken centroids; **PE,** preeclampsia; **PZP,** pregnancy zone protein; **SAP,** serum amyloid P; **SGA,** small for gestational age; **TTR,** transthyretin

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1 Introduction

Preeclampsia (PE) is a disorder specific to human pregnancy that complicates 4–7% of first pregnancies. This rapidly progressive syndrome is diagnosed when the mother develops hypertension and proteinuria, and in severe cases may cause seizures, coagulopathy, haemolysis, liver dysfunction and kidney failure [1]. A quarter of the babies born to women with PE are small for gestational age (SGA). The only cure for PE is delivery, resulting in a third of the babies being born prematurely. It is estimated that more than 8 million women are affected each year, resulting in over 70 000 maternal deaths worldwide. Moreover, women who develop PE have an increased risk of hypertension, cardiovascular disease and cardiovascular related death in later life [2].

An accurate and reliable test to predict PE in early pregnancy would be a major healthcare advance. Currently there is no biomarker-based screening test that accurately predicts PE in early pregnancy. As prediction is a prerequisite for prevention, there is a clinical need for such a screening test, especially for women in their first pregnancy [3]. This imperative has led to extensive research to identify potential biomarkers in early pregnancy plasma [3, 4]. To date, attempts have been largely based on the knowledge of biological processes involved in PE or microarray data from placental studies, but these have not provided a robust and reliable approach to the prediction of PE [4, 5].

The pathogenesis of PE is complex, with an interaction occurring between abnormal placentation and a maternal response to placenta-derived factors in the maternal circulation, ultimately culminating in the clinical manifestation of the disease [6]. PE is characterized by abnormal remodeling of the spiral arteries, resulting in ischemic reperfusion injury and oxidative stress damage to the placenta [6, 7]. Placenta-derived factors in the maternal circulation include syncytiotrophoblast debris and angiogenesis-associated factors such as vascular endothelial growth factor (VEGF) receptor 1 (also known as sFlt-1) [5, 8]. In response, widespread maternal endothelial cell dysfunction and vasoconstriction develop, along with activation of inflammatory and coagulation pathways [1, 6].

There is substantial evidence that women who develop PE have a predisposition to the vascular, inflammatory and coagulation changes characteristic of the condition including increased vascular responsiveness, as well as dyslipidemia. Alteration of relevant plasma proteins and lipids may be evident before pregnancy [9], in early pregnancy prior to the onset of disease [10–12] or on follow-up months to years after PE [13–15]. Several features of the metabolic syndrome including obesity, elevated blood pressure and reduced insulin sensitivity may be present before or after clinical onset of disease [9, 15, 16]. These susceptibility characteristics may modify a pregnant woman's response to placental triggers.

Disease-specific changes in protein expression at the placental level or in the maternal vasculature may be reflected as changes in plasma proteins in women who subsequently develop PE [12, 17, 18]. We hypothesized that changes in placenta-derived proteins and proteins released in the interaction between the endothelium, and inflammatory and coagulation pathways may be detected in early pregnancy blood using an unbiased proteomic approach. Our goal was to identify a set of biomarkers in plasma that may enable reliable early detection of women at risk of PE and, as a secondary objective, to improve understanding of disease mechanisms.

In the present investigation, we employed a DIGE-based approach to identify changes in the proteome in early pregnancy plasma. In a series of case–control studies, we investigated the plasma proteome at 20 wk gestation in women who subsequently developed PE with either a baby of appropriate birth weight for gestational age (PE-AGA) or a SGA baby (PE-SGA) compared with healthy pregnant controls.

Here we provide new evidence for a synergism between pathways involved in the pathogenesis of PE, which were previously believed to be unrelated. We report significant changes in the expression levels of proteins implicated in pathways of complement regulation, coagulation, lipid metabolism, acute-phase responses, heme scavenging, protease inhibitor activity and extracellular matrix (ECM) remodeling. Together, these may serve as early pregnancy biomarkers for PE.

2 Materials and methods

2.1 Study group and plasma collection

A series of seven case–control studies were conducted to identify changes in the proteome in early pregnancy plasma prior to the onset of PE. All women were participants in the Screening for Pregnancy Endpoints study (SCOPE study, Australian, New Zealand Clinical Trials Registry ACTRN12607000551493), a prospective screening study of nulliparous women. The study protocol was approved by the Auckland Human Ethics Committee and written informed consent was obtained from each participant.

EDTA-plasma from women at 20 ± 1 wk gestation who had uncomplicated pregnancies (controls, $n = 57$) or who subsequently developed PE-AGA ($n = 27$) or PE-SGA ($n = 12$) were obtained from the SCOPE biobank (Auckland, New Zealand). Different women were included in experimental groups except for the PE-SGA group due to limited specimen availability. Blood was collected by venipuncture into BD EDTA-Vacutainer®, placed on ice and centrifuged at $2400 \times g$ for 10 min at 4°C. Plasma was stored at −80°C within 4 h of collection.

PE was defined as systolic blood pressure ≥ 140 mm Hg and/or diastolic blood pressure ≥ 90 mm Hg on two or more occasions after 20 wk gestation but prior to the onset of labor

on at least two occasions 4 h apart, combined with either proteinuria (spot protein: creatinine ratio $\geq$30 mg/mmol or 24 h urinary protein $\geq$0.3 g/24 h or dipstick proteinuria $\geq$2+), or any multiorgan complication [19]. Severe PE was defined as any of the following: persistent severe hypertension (blood pressure $\geq$170/110 mmHg), coagulopathy (platelets $<100 \times 10^9$/L or disseminated intravascular coagulation) haemolysis; liver dysfunction, acute renal insufficiency or imminent eclampsia. SGA was defined as a customized birth weight less than the tenth centile for gestational age [20].

2.2 Materials

All reagents were of ultrapure, MB grade. The CyDye DIGE minimal dye kit, 11 cm Immobiline^{TM} pH 4–7 DryStrips and IPG pH 4–7 buffer were purchased from GE Healthcare (Auckland, New Zealand); detergent C7BzO, DMF and thiourea (ACS reagent) from Sigma-Aldrich (Auckland, New Zealand), CHAPS from AppliChem (Darmstadt, Germany); ultrapure DTT and Tris-Glycine-SDS gel-running buffer from Invitrogen (Auckland, New Zealand); Tris-HCl 8–16% Criterion gradient gels from Bio-Rad (Auckland, New Zealand).

2.3 Depletion of high-abundance plasma proteins by MARS

To remove the six most abundant proteins (albumin, transferrin, IgG, IgA, haptoglobin and α-1-antitrypsin) in experiments I and II, plasma was immunodepleted using the multiple affinity removal system (MARS; Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions. Plasma samples used in experiments III–VII were depleted of the seven most abundant proteins (top 6 plus fibrinogen) using a 4.6 × 100 mm Top7 MARS column. Before depletion, a protease inhibitor cocktail was added (Roche Applied Science, Auckland, New Zealand). Depleted samples were first desalted by washing three times with 0.1% TFA (centrifugation at 3700 × *g* at 18°C for 30 min) followed by a buffer exchange into 7 M urea, 2 M thiourea, 2% CHAPS using 5 kDa molecular weight cut-off centrifugal filters (Vivascience, Hannover, Germany). Protein content was determined using the 2-D Quant protein assay (GE Healthcare). Depleted plasma was stored at −80°C until DIGE analysis. Representative gel images for top6- and top7-depleted plasma samples are shown in Figs. 1A and B, respectively.

2.4 DIGE protocol

Depleted plasma samples were labeled with 200 pmol *per* 50 μg of protein with Cy dyes (GE Healthcare) according to the manufacturer's protocol. For the internal standards, equal amounts of all samples included in an experimental set (or in some cases representative specimens; $n = 4$–6 *per* group) were pooled and labeled with Cy2. Plasma from healthy and preeclamptic women was labeled with either Cy3 or Cy5, with each case and control group being dye-balanced. IPG strips were rehydrated with multiplexed plasma samples comprising 50 μg protein for each CyDye-labeled case, control and 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 electrophoresis system (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. Twelve DIGE gels were run simultaneously. Gels were scanned on a Typhoon 9410 (GE Healthcare) and imported into DeCyder 2-D Differential Analysis software v6.5 (GE Healthcare) for spot detection and matching. Comparisons of control *versus* PE-AGA and PE-SGA were performed as outlined in Table 1. The total number of detected spots ranged from 823 to 1094 *per* master gel after applying a spot volume filter (set at $>$17 000). Standardized abundance data were then exported from DeCyder for bioinformatics analyses.

2.5 Bioinformatics analysis of DIGE data

2.5.1 Clinical data

Continuous clinical variables were compared between groups using ANOVA with the *post hoc* Dunnett's test (SAS v9.1., Cary, USA). Categorical data were analyzed using the Chi-Squared or Fisher's Exact Test. A *p*-value of <0.05 was considered to be statistically significant.

2.5.2 Data transformation

Standardized abundances of down-regulated spots were transformed from negative fold-changes to values ranging from 0 to 1. Log-transformation (base 2) was then applied, and the data from each gel were normalized by zero-centering through median subtraction. Box plots were used to assess outlying gels and two gels in experiment III were removed from analysis. The logged, median-centered relative abundances were used for both univariate analyses to identify differentially expressed proteins, and for nearest shrunken centroids (NSC) analysis for constructing disease classifiers.

2.5.3 Univariate statistical tests for differential protein expression

Mann–Whitney tests and the moderated *t*-test implemented in Limma (Linear Models for Microarray Data [21]) were carried out independently using the log-transformed standardized

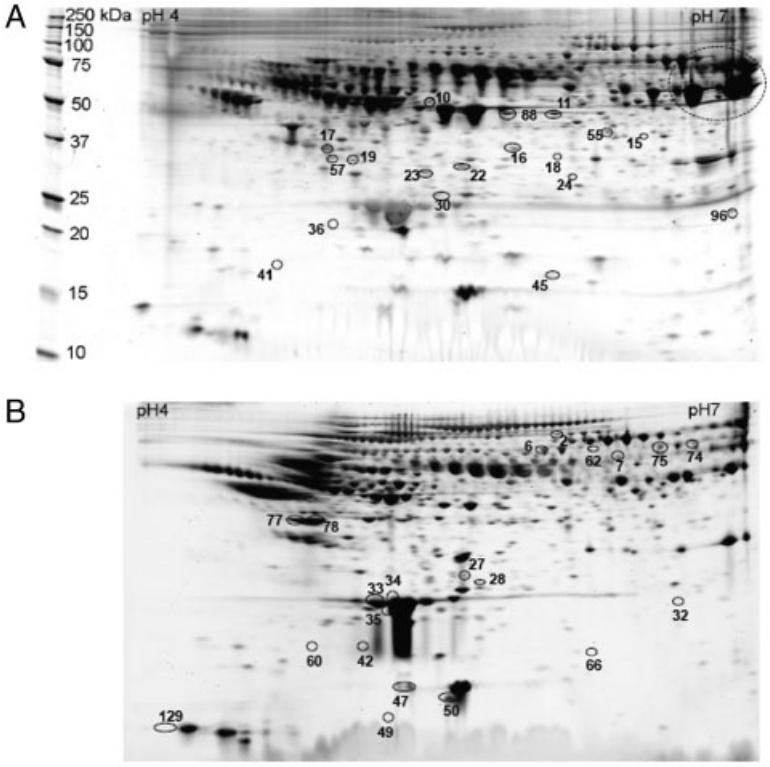

Figure 1. Protein spots identified following DIGE analysis of top6- and top7-depleted pregnancy plasma. Spots identified by LC-MS/MS (see Table 4) are indicated on a representative three-channel overlaid DIGE image containing 50 μg each of pooled internal standard (Cy2-labeled) and depleted plasma from a preeclamptic (Cy3/Cy5-labeled) and a healthy pregnant control (Cy5/Cy3-labeled) sample at 20 wk of gestation. (A) Representative gel of top6-depleted plasma, circled area to the right depicts fibrinogen, which is depleted in addition to top6 most abundant proteins using Agilent's top7 MARS column (B).

Table 1. DIGE experimental design

Experiment	Number of subjects			No. of gels	No. of spots (master)[a)]	Matching (%)[b)]
	Control	PE-AGA	PE-SGA			
I	8	8	6	11	889	81.2±2.8
II	8		8	8	1094	79.2±2.7
III	12	12		10	823	78.1±3.4
IV	12		12	12	851	78.9±2.9
V	12	12		12	1018	85.6±1.4
VI	12		12	12	880	80.2±3.0
VII	24	24		24	886	78.6±3.1

a) Total number of spots detected by DeCyder software after applying a 17 000 spot volume cut-off.
b) Matching is expressed for each gel as a ratio (mean percent±SD) of the total number of detected spots *per* respective master gel.

abundance of all gel spots. False discovery rate (FDR) correction was applied to correct for multiple testing, except for experiments III and IV where a large number of spots remained statistically significant using FDR correction [22]. Here the more stringent family-wise error rate (FWER) was used, effectively decreasing the likelihood of false-positive results. An adjusted *p*-value of <0.05 was considered statistically significant.

2.5.4 NSC for disease classification

DIGE data were analyzed by the NSC method for identification of disease classifiers (preeclamptic *versus* healthy) after imputing the spot volume for missing values from the median for all cases and controls in that experiment [23]. The scores for each spot were shrunk toward zero using an approach designed to eliminate non-informative spots from the analysis and determine sets of spots that best discriminate disease classes.

The generalizable performance of this classifier was then estimated by assessing the unbiased performance of this model selection procedure [24]. The parametric confidence interval around this estimate was calculated based on a binomial likelihood function with $n = 24$ and the number correctly classified equal to 24 multiplied by the bias-corrected misclassification rate.

2.6 Selection criteria for protein spots of interest

Spots were defined as ''proteins of interest'' based on the following criteria: (i) significantly ($p < 0.05$) ''up-regulated'' or ''down-regulated'' with FDR correction (all experiments except III and IV) or FWER correction (experiments III and IV) by Limma or Mann–Whitney tests and also significant in at least one other experiment (with or without correction) or (ii) identified as classifier by NSC analysis with a bias-corrected performance $\geq 90\%$.

2.7 Identification of proteins by LC-MS/MS

Protein spots of interest were excised from preparative 2-D gels, digested with trypsin according to an online published method (www.ucsf.edu/brc), and submitted for LC-MS/MS analysis on a QSTAR XL ESI-qTOF (Applied Biosystems) at the Maurice Wilkins Centre, University of Auckland or by nanospray LC-MS/MS using an linear trap quadrupole (LTQ) mass spectrometer at HortResearch (Ruakura, New Zealand). Separations using the QSTAR were carried out on an Ultimate capillary LC system (LC Packings, San Francisco, CA, USA) fitted with a Zorbax 300 SB C18 column (Agilent Technologies). MS was performed in positive ion mode with a mass range of 300–1600 m/z with the two most abundant peptides selected for fragmentation. Analysis of tryptic peptides by nanospray LTQ LC-MS/MS was performed as described previously [25].

2.8 Database searching and data interpretation

QSTAR MS/MS data were extracted from raw spectra using Mascot Search v1.6b13 (Matrix Science, London, UK) and searched against a mammal subset of MSDB v2005 (Imperial College, London, UK) or IPI-human database v3.27 (European Bioinformatics Institute) using Mascot search engine v2.0.05 (Matrix Science) with the following parameters: semi-trypsin cleavage with one missed cleavage allowed; fixed modification: propionamide; variable modifications: oxidation (M), deamidation (N and Q), pyroglutamic acid conversion (NH_2-terminal Q & E), acetylation; peptide tolerance: ± 0.1 Da; MS/MS tolerance ± 0.1 Da; peptide charge 2^+ and 3^+.

LTQ nanospray LC-MS/MS data were extracted using SEQUEST software. Spectra were searched against a human subdatabase from the latest available version of the public non-redundant protein database of the National Center for Biotechnology Information (Bethesda, MD, USA) with the following search parameters: mass type: monoisotopic precursor and fragments; enzyme and enzyme limits: trypsin (KR), fully enzymatic cleaving at both ends allowing up to two missed cleavages; peptide tolerance: 1.4000 atomic mass units; fragment ion tolerance: 1.0 atomic mass unit; number of results scored: 250; ion and ion series calculated: B and Y ions; static modifications: C-cysteine (propionamide); differential modifications: NH_2-terminal peptide (acetylation), methionine (oxidation).

For positive peptide identification the following filter criteria were set in Bioworks v3.3 (Thermo Electron): single, double and triple charge peptides with a correlation factor (XCorr) greater than 1.30, 1.80 and 2.30, respectively; delta cross-correlation factor (dCn) greater than 0.08 indicating a significant difference between the best match reported and the next best match; (Sp) preliminary score greater than 250; protein probability 1×10^{-2}; different (unique) peptides only. All matched peptides were confirmed by visual examination of the spectra.

2.9 Western blot analysis

Six differentially expressed proteins found by proteomic analysis were further evaluated by Western blot. Native plasma (20 μL) from week 20 of gestation was separated on 4–12% NuPAGE gradient gels (Invitrogen) according to the manufacturer's instructions. Proteins were transferred to Immobilon FL transfer membrane (Millipore) and blocked overnight at 4°C in 5% BSA. Detection of α-1-antichymotrypsin (DakoCytomation, Glostrup, Denmark, ♯A0022 at 1:5000), fibrinogen (Dako ♯A0080 at 1:10 000), complement 3c (Dako ♯A0062 at 1:10 000), gelsolin (Dako at 1:10 000), clusterin isoform 1 (Alexis Biochemicals, ♯ALX-210–451) and serum amyloid A4 (R&D Systems, ♯AF3856) was accomplished with the respective quantum dots Q655 Qdot conjugated IgGs (Invitrogen) at 1:1000 dilution. A FujiLAS-3000 CCD camera (FujiFilm) was used for signal detection (UV light excitation with a 655 nm WB20 filter). Densitometric analysis of total signal intensity of Western blot bands was performed with Quantitiy-One 1-D analysis software version 4.6.2 (Bio-Rad) using local background subtraction.

3 Results

3.1 Subject characteristics

In a series of seven DIGE experiments, we studied 27 pregnant women who developed PE-AGA, 12 who developed PE-SGA and 57 healthy controls (Table 1). The clinical characteristics of the women and maternal and neonatal outcomes are summarized in Table 2. PE was diagnosed according to strict criteria and more than half had severe disease. In the PE-SGA group, half the babies were born preterm, whereas 11% of the babies in the PE-AGA group were premature, $p < 0.0001$. There were no perinatal or maternal deaths.

3.2 Detection of plasma proteins differentially expressed in women who developed PE

As conventional 2-DE gel-based methods utilized for plasma are notorious for high inter-gel variability, resulting in low

Table 2. Maternal and fetal characteristics[a)]

	PE-AGA $n = 27$	PE-SGA $n = 12$	Controls $n = 57$	Significance p-value
Maternal characteristics				
Age (years)	30.0 (4.5)	30.3 (5.6)	29.7 (4.9)	0.92
Ethnicity				
Caucasian	20 (74.1%)	10 (83.4%)	46 (80.7%)	0.60
Maori/pacific islander	2 (7.4%)	0 (0%)	2 (3.5%)	
Chinese	0 (0%)	1 (8.3%)	1 (1.7%)	
Indian	1 (3.7%)	1 (8.3%)	3 (5.3%)	
Other	4 (14.8%)	0	5 (8.8%)	
BMI at 15 wk (w) (kg/m^2)	26.0 (3.5)	28.8 (6.6)	24.5 (3.2)	0.002[b)]
Smokers at 15 w	0	1 (8.3%)	3 (5.3%)	0.32
Gestation at sampling (w)	19.9 (0.7)	20.1 (0.7)	20.0 (0.8)	0.78
Blood pressure at 15 w				
Systolic (mm Hg)	113 (9)	118 (14)	106 (11)	0.001[c)]
Diastolic (mm Hg)	71 (7)	72 (12)	62 (9)	<0.0001[c)]
Blood pressure at 20 w				
Systolic (mm Hg)	113 (11)	116 (8)	109 (12)	0.09
Diastolic (mm Hg)	68 (8)	71 (9)	63 (7)	0.001[c)]
Maximum blood pressure at the end of pregnancy				
Systolic (mm Hg)	158 (14)	158 (24)	118 (10)	<0.0001[c)]
Diastolic (mm Hg)	105 (8)	104 (8)	75 (7)	<0.0001[c)]
Twenty-four-hour protein excretion (g/24 h)	0.71 (0.5–1.14) $n = 21$	0.92 (0.34–2.7) $n = 9$	—	
Protein/creatinine ratio (mg/mmol creatinine)	84 (46–126) $n = 23$	103 (50–219) $n = 10$	—	
Severe PE[d)]	16 (59%)	7 (58%)	—	
Placental abruption	2 (7.4%)	0	0	0.33
Fetal characteristics				
Gestational age at delivery (w)	38.1 (2.3)	35.4 (3.1)	40.2 (1.4)	<0.0001[c)]
Gestation at delivery				
<34 wk	2 (7.4%)	3 (25%)	0	<0.0001
34–36 wk	1 (3.7%)	3 (25%)	0	
≥ 37 wk	24 (88.9)	6 (50%)	57 (100%)	
Infant birth weight (g)	3207 (596)	1994 (533)	3620 (449)	<0.0001[c)]
Customized birth weight centile[e)]	52.4 (26.7)	3.1 (2.6)	55.3 (25.1)	<0.0001[b)]

a) Results are mean (SD), median (IQ range) or n (%). Customized birth weight centile
b) Comparisons using ANOVA. Significant PE-SGA *versus* controls $p < 0.05$ by Dunnett's t-test.
c) Comparisons using ANOVA. PE-AGA *versus* controls and PE-SGA *versus* controls $p < 0.05$ by Dunnett's t-test.
d) Severe PE includes severe hypertension (BP $>$ 170/110 mmHg), imminent eclampsia, liver dysfunction, acute renal insufficiency or coagulopathy.
e) Birth weight adjusted for gestational age, fetal sex, maternal parity, ethnicity, height and weight.

Table 3. Differentially expressed protein spots in week 20 plasma from women with subsequent PE with either an appropriate for gestational age baby (PE-AGA) or a small for gestational age baby (PE-SGA) compared with healthy controls

PE-AGA				PE-SGA				Overlapping PE-AGA and PE-SGA					
Spot ID[a)]	FC[b)]	*p*-value after FDR/FWER correction[c)]	No. of experiments[d)]	Spot ID[a)]	FC[b)]	*p*-value after FDR/FWER correction[c)]	No. of experiments[d)]	Spot ID[a)]	FC PE-AGA	*p*-value[c)]	FC PE-SGA	*p*-value[c)]	Number of experiments[d)]
43 (C)	7.8	<0.05	2	88	2.1, 1.6	<0.05	2	41 (C)	5.0, 2.5	<0.05	12.2, 2.4	<0.05	4
49 (C)	7.5	<0.00001		96	3	<0.01	2	11 (C)	6.4	<0.01	4.3, 1.9	<0.05	3
32 (C)	6.9	<0.01	2	27 (C)	1.5	<0.05	2	15 (C)	4.4	<0.0001	2.4, 1.7	<0.05	3
50	3.8	<0.05	2	10	1.3	<0.05	2	24	3.3	<0.05	4.6	<0.01	2
28 (C)	2.7	<0.01	2	34	−1.4	<0.05	2	23	3.0	<0.01	3.4	<0.05	2
42	2.5	<0.01	2	122	−1.4	<0.05		16	3.0	<0.01	3.6	<0.01	2
76	2.4	<0.05		33 (C)	−1.6	<0.05	2	45 (C)	2.9	<0.01	2.3, 1.5	<0.05	3
35	2.3	<0.01	2	117 (C)	−1.6	<0.01		19	2.8	<0.05	2.2	<0.05	2
75	2	<0.05		129 (C)	−1.9	<0.05		36 (C)	2.4	<0.001	5.0	<0.05	2
60 (C)	1.9	<0.01		123 (C)	−2	<0.05		18	2.2	<0.05	1.7	<0.05	2
72	1.8	<0.05		128 (C)	−2	<0.0001		22	2.1	<0.05	2.2	<0.05	2
74	1.7	<0.05		130	−2	<0.05		17 (C)	2.0	<0.0001	3.1	<0.05	2
78	1.6	<0.05		132	−2.6	<0.05		55 (C)	1.7	<0.01	5.5, 3.1	<0.01	3
7 (C)	1.5	<0.05	4					30 (C)	−1.6	<0.01	−2	<0.01	2
80	1.4	<0.05						2 (C)	−1.7	<0.05	−1.9	<0.05	2
77	1.4	<0.05	2					47 (C)	−2.2	<0.05	−1.8	<0.05	2
81	1.3	<0.05						6	−1.4	<0.05	−1.3	<0.05	2
79	1.3	<0.05											
71	−1.4	<0.05											

a) Master spot number after visual matching across all experiments; ''C'' depicts spots which were also found to be a member of a disease classifier for either PE-AGA or PE-SGA by NSC analysis with a bias-corrected performance above 90%.

b) FC, fold change between healthy *versus* PE-AGA or PE-SGA using median-standardized abundances. FC has been shown only for spots significant with FDR/FWER correction.

c) *p*-values after applying FDR or FWER correction.

d) Presence of spot in one or more DIGE experiments, statistically significant by univariate analysis ($p < 0.05$) with and without FDR/FWER correction.

power of detecting true disease-specific differences in protein expression profiles, we used DIGE. This proteomics approach greatly reduced inter-gel variability by simultaneously analyzing plasma from preeclamptic women and healthy controls in relation to a pooled Cy2-labeled internal standard [26]. We determined that at 80% power, three quarters of protein expression levels above a twofold difference between cases and controls would be detected in a sample size of 12 women *per* group using DIGE. In our hands, this was a considerable improvement over conventional 2-DE, whereby a sample size of 42 individuals *per* group would be required to detect significant differences at this level.

Comparison of plasma protein expression by univariate analyses identified 131 protein spots significantly up- or down-regulated in PE-AGA or PE-SGA ($p < 0.05$ after FDR or FWER correction). Differentially expressed spots were visually inspected in the gel images and technical artifacts excluded. Spots of interest were then selected for further analysis if they were significant in repeated experiments or were members of a classifier set of spots as determined by multivariate analyses. By these inclusion criteria, 49 spots were identified; 19 spots were differentially expressed in PE-AGA samples, 13 in PE-SGA samples and 17 spots were up- or down-regulated in both conditions (Table 3). Of these 49 differentially expressed protein spots, 22 spots were also included in one or more classification models generated by NSC analysis and with a bias-corrected performance above 90%. It was notable that 9 of the 13 protein spots associated with only PE-SGA were down-regulated (2.6- to 1.3-fold) compared with healthy controls. This contrasted with findings in the PE-AGA group where 18 of the 19 differentially expressed protein spots were up-regulated (1.5- to 7.8-fold) compared with healthy controls (Table 3).

3.3 Identification of proteins of interest by LC-MS/MS

The gel locations of 36 differentially expressed protein spots and a further 3 spots that were part of a disease classifier only identified by LC-MS/MS (two or more peptides *per* protein; Table 4) are shown in 2-DE-maps of top6- and top7-depleted pregnancy plasma in Fig. 1. We were unable to obtain the protein IDs for 10 spots due to being very low abundance on the 2-D gel.

For several spots, multiple proteins were identified by LC-MS/MS analysis from the gel plug. The peptide sequences and scores are listed in Supporting Information Table 1. While the phenomenon of spot overlapping in 2-D gels due to co-migration of proteins has widely been ignored, at present 2-DE is still considered one of the most powerful techniques for differential analysis of complex protein mixtures [27, 28]. Ultimately, other methods such as ELISA or multiple reaction monitoring are required to assess the contribution of each protein to the observed differential expression. The majority of proteins identified are involved in the biological processes of lipid metabolism, coagulation, complement regulation, ECM remodeling, protease inhibitor activity and acute-phase responses (Table 5).

3.4 Classification of PE-AGA and PE-SGA patients by protein clusters

We performed multivariate analysis in order to identify groups of markers that are co-regulated in plasma samples from women who developed either PE-AGA or PE-SGA compared with controls. Using the NSC approach three different models with a bias-corrected performance above 90% (classification accuracy) were identified for the PE-AGA group and six models for the PE-SGA group (Table 6). In PE-SGA, various combinations of three key proteins (fragments of apoA-I with estimated molecular weights of 17 and 21 kDa, pregnancy zone protein (PZP) or α-2-macroglobulin (A2M), fibrinogen) performed equally well as classifiers in experiment I with a classification accuracy of 99%. Classifier I-C4 also included clusterin isoform I, α-1-antichymotrypsin and complement factor I. In experiment IV, the same fragments of fibrinogen (spot 11) and apoA-1 (spot 41) were again part of the classifier for PE-SGA, along with serum amyloid P (SAP) precursor (spot 30) and transthyretin (TTR) with an approximate molecular weight of 12 kDa (spot 47).

3.5 Western blot analysis of candidate markers

Six of the potential PE markers were selected for immunodetection in undepleted plasma using specific antibodies to fibrinogen, α-1-antichymotrypsin, clusterin isoform I, complement 3c, human serum amyloid A4 (constitutive) and gelsolin. Western blot analysis confirmed differential plasma levels for fibrinogen and α-1-antichymotrypsin consistent with our DIGE findings (Fig. 2). Two fibrinogen fragments corresponding to 27 and 25 kDa appear to be highly up-regulated in two of the four PE-SGA patients. Densitometric analysis confirmed up-regulation of fibrinogen γ in PE-SGA (274 ± 75 SD, mean intensity units $\times 10^3$) *versus* controls (181 ± 19 SD), $p < 0.05$ (Student's *t*-test, one-tailed). Immunoblot analysis showed increased α-1-antichymotrypsin in the plasma of women who later developed PE-SGA with a mean band intensity of 309 ± 42 SD in the PE-SGA group *versus* controls (218 ± 21 SD), $p < 0.01$. No differences could be detected by immunoblot in the levels of plasma gelsolin, serum amyloid A4, complement 3c and clusterin isoform I (data not shown). This may be due to being unable to detect differential expression of isoforms in 1-D gels, a lack of sensitivity to detect differences between cases and controls by Western blot or possibly the difference was due to other proteins identified in a differentially expressed spot.

Table 4. LC-MS/MS identification of plasma proteins/peptides detected by DIGE as differentially expressed in pregnancy plasma at week 20 of gestation from women with subsequent PE-AGA and/or PE-SGA

Spot ID[a)]	PE-AGA FC[b)]	PE-SGA FC	Unique peptides[c)]	Accession number[d)]	Protein name[e)]	Classifier[f)]
Differentially expressed proteins by univariate analyses						
2	−1.7	−1.9	7	gi|4559406	Complement component 6 precursor	
			11	gi|4557225	α-2-Macroglobulin precursor	
			5	gi|4557385	Complement component 3 precursor	
			2	gi|4506355	PZP	
			4	gi|31542984	Inter-α (globulin) inhibitor H4	
			2	gi|45580688	Complement component 7 precursor	
6	−1.4		12	gi|38044288	Gelsolin isoform b	
			14	gi|9961357	Coagulation factor XIII B subunit precursor	
			4	gi|66932947	α-2-Macroglobulin precursor	
			3	gi|4506355	PZP	
7	1.5		4	gi|16933542	Fibronectin 1 isoform 3 preproprotein	√
			2	gi|38044288	Gelsolin isoform b	
			3	gi|4557321	Apolipoprotein A-I precursor	
			2	gi|4557385	Complement component 3 precursor	
			2	gi|4502503	Complement component 4-binding protein, α	
			4	gi|4502027	Albumin precursor	
			14	gi|4557871	Transferrin	
			6	gi|66932947	α-2-Macroglobulin precursor	
10		1.3	8	ANHU	Angiotensin precursor (angiotensinogen)	
			5	ITHU	α-1-Antitrypsin precursor	
			2	FAHUP	Gelsolin precursor, plasma	
			2	MAHU	α-2-Macroglobulin precursor	
11	6.4	4.3, 1.9	27	gi|11761631	Fibrinogen, β chain preproprotein	√
			2	MAHU	α-2-Macroglobulin precursor	
			2	ITHU	α-1-Antitrypsin precursor	
			2	gi|10645195	H2A histone family, member A	
			2	gi|11415030	H4 histone family, member E	
15	4.4	2.4, 1.7	5	gi|4557225	α-2-Macroglobulin precursor	
			3	gi|4557385	Complement component 3 precursor	
			3	gi|4506355	PZP	
			2	gi|4758502	Hyaluronan-binding protein 2	
16	3.0	3.6	7	gi|11761631	Fibrinogen, β chain preproprotein	
			2	gi|4557032	Lactate dehydrogenase B	
17	2.0	3.1	3	gi|42716297	Clusterin isoform 1	√
			3	gi|4504579	Complement factor I	
			4	gi|4501843	α-1-Antichymotrypsin	
			8	gi|11761633	Fibrinogen, γ chain isoform γ-B precursor	
18	2.2	1.7	2	gi|21361198	Serine (or cysteine) proteinase inhibitor, clade A	
19	2.8	2.2	6	gi|11761633	Fibrinogen, γ chain isoform γ-B precursor	
			2	gi|4501843	α-1-Antichymotrypsin, precursor	
22	2.1	2.2	16	gi|4557325	Apolipoprotein E precursor	
			3	gi|4507725	TTR	
			15	gi|11761633	Fibrinogen, γ chain isoform γ-B precursor	
			4	gi|4557225	α-2-Macroglobulin precursor	
23	3.0	3.4	5	gi|11761633	Fibrinogen, γ chain isoform γ-B precursor	
			2	gi|4557225	α-2-Macroglobulin precursor	
24	3.3	4.6	2	gi|4502945	α1-Type 1 collagen preproprotein	
			8	gi|4502027	Albumin precursor	
27		1.5	2	gi|4557225	α-2-Macroglobulin precursor	
			4	gi|4557321	Apolipoprotein A-I precursor	
			3	gi|4502133	SAP component precursor	√
			2	gi|4507725	TTR	
			2	gi|4557325	Apolipoprotein E precursor	
			2	gi|11761631	Fibrinogen, β chain preproprotein	
28	2.7		2	gi|66932947	α-2-Macroglobulin precursor	
			2	gi|67190748	Complement component 4A preproprotein	

Table 4. Continued

Spot ID[a)]	PE-AGA FC[b)]	PE-SGA FC	Unique peptides[c)]	Accession number[d)]	Protein name[e)]	Classifier[f)]
30	−1.6	−2.0	4	gi|4502133	SAP component precursor	
			2	gi|4557321	Apolipoprotein A-I precursor	√
32	6.9		4	gi|11321561	Hemopexin	√
33		−1.6	2	gi|4502067	α-1-Microglobulin/bikunin precursor	
			3	gi|22091452	Apolipoprotein M	
			9	gi|4557321	Apolipoprotein A-I precursor	
			2	gi|4501843	α-1-Antichymotrypsin, precursor	
34	−1.4		3	gi|4557321	Apolipoprotein A-I preproprotein	
35	2.3		2	gi|56786155	Complement component 1q subcompt. γ polypeptide	
			15	gi|4557321	Apolipoprotein A-I precursor	
36	2.4	5.0	9	gi|4557321	Apolipoprotein A-I precursor	
41	5.0, 2.5	12.2, 2.4	2	gi|4557321	Apolipoprotein A-I precursor	√
42	2.5		8	gi|4557321	Apolipoprotein A-I precursor	
45	2.9	2.3, 1.5	3	gi|4502005	α-2-HS-glycoprotein	
			4	gi|11761633	Fibrinogen, γ chain isoform γ-B precursor	
			2	gi|10835095	Serum amyloid A4, constitutive	
47	−2.2	−1.8	5	gi|4507725	TTR	√
49	7.5		2	gi|4507725	TTR	√
			2	gi|4502027	Albumin precursor	
50	3.8		2	gi|4557321	Apolipoprotein A-I precursor	
			2	gi|4507725	TTR	
55	1.7	5.5, 3.1	3	gi|4557225	α-2-Macroglobulin precursor	
60	1.9		6	gi|4557321	Apolipoprotein A-I precursor	√
74	1.7		2	gi|119763	Coagulation factor XII precursor (Hageman factor)	
75	2		9	gi|110590599	Chain A, Apo-Hu serum transferrin (glycosylated)	
			4	gi|87919	Ig μ chain precursor, membrane-bound (clone 201)	
77	1.4		8	gi|4557385	Complement component 3 precursor	
			4	gi|4502337	α-2-Glycoprotein 1, zinc	
			2	gi|4506115	Protein C (inactivator of coagulation factors Va, VIIIa)	
78	1.6		26	gi|78101271	Chain C, human complement component C3c	
			6	gi|38026	Zn-α-2-glycoprotein	
88		2.1, 1.6	8	gi|11761631	Fibrinogen, β chain preproprotein	
96		3	2	gi|11761629	Fibrinogen, α chain isoform α preproprotein	
129		−1.9	2	gi|4557323	Apolipoprotein C-III precursor	
Disease classifiers[g)]						
57		2.4	4	gi|42716297	Clusterin isoform 1	√
			5	gi|11761633	Fibrinogen γ chain	
62	1.6		10	gi|4557871	Transferrin	√
66	2.0		5	gi|4557321	Apolipoprotein A-I precursor	√
			2	gi|45580723	HRP	
			2	gi|4502209	ADP-ribosylation factor 5	

a) Master spot number after spot matching across all experiments.
b) FC, fold change of protein spots by median of standardized abundances in PE-AGA or PE-SGA *versus* controls.
c) Number of unique peptides *per* protein.
d) Protein accession numbers as *per* NCBInr 2006/2007 or Mascot 20051220 databases.
e) Protein name from human ref (FASTA) database.
f) Member of a set of spots included in a disease classifier.
g) Spots included in a classifier but standardized abundance not significantly different in cases compared with controls after adjustment for FDR

Table 5. Plasma proteins associated with later development of PE, classified by main biological function

Protein name by proposed function	Spot number	Fold change PE-AGA[a)] across all experiments	Fold change PE-SGA[a)] across all experiments
Lipid metabolism			
Apolipoprotein A-I	*7*, 27, 33, 34, 35, 36, 41, 42, 50, 60, 66[b)]	*1.5*, −1.4, 2.3, 2.4, 5.0, 2.5, 2.5, 3.8, 1.9, 2.0[b)]	1.5, −1.6, 5.0, 12.2, 2.4
Apolipoprotein C-III	129		−1.9
Apolipoprotein E	22, 27	2.1	2.2, 1.5
Apolipoprotein M	33		−1.6
Apolipoprotein J (Clusterin isoform 1)	17, 57[b)]	2.0	3.1, 2.4[b)]
Serum amyloid A4 (SAA4)	45	2.9	2.3, 1.5
Complement activation			
Complement factor I	17	2	3.1
Complement component 1q, C-chain	35	2.3	
Complement component 3 precursor/C3c	*2*, 7, *15*, 77, 78	*−1.7*, *1.5*, 4.4, 1.4, 1.6	*−1.9*, 2.4, 1.7
Complement component 4 BP α	7	1.5	
Complement component 4A	28	2.7	
Complement component 6/7	2	−1.7	−1.9
SAP	27, 30	−1.6	1.5, −2.0
Coagulation			
Fibrinogen α chain	96		3
Fibrinogen β chain	11, 16, 27, 88	6.4, 3.0	4.3, 3.6, 2.1, 1.9, 1.5, 1.6
Fibrinogen γ chain	16, 17, 19, 22, 23, 45, 57[b)]	3.0, 2.0, 2.8, 2.1, 3.0, 2.9	3.6, 3.1, 2.2, 2.2, 3.4, 2.3, 1.5, 2.4[b)]
Factor XII (Hageman factor)	74	1.7	
Factor XIII B subunit	6	−1.4	−1.3
ECM remodeling			
α1-Type 1 collagen	24	3.3	4.6
Fibronectin 1 isoform 3	7	1.5	
Protease inhibitors			
α-1-Antichymotrypsin (SERPINA3)	17, *19*	2.0, *2.8*	3.1, *2.2*
α-2-Macroglobulin/PZP	*2*, *6*, 7, 10, 15, 23, 27, 28, 55	*−1.7*,*−1.4*, 1.5, 4.4, 3.0, 1.7, 2.7	*−1.9*, 1.3, 2.4, 1.7, 3.4, 1.5, 5.5, 3.1
Angiotensinogen (SERPINA8)	10		1.3
α-1-Antitrypsin (SERPINA1)	10, *11*, 18	*6.4*, 2.2	1.3, *4.3*, *1.9*, 1.7
α-2-HS-glycoprotein (human fetuin-A)	45	2.9	2.3, 1.5
α-1-Microglobulin/bikunin	33		−1.6
Acute-phase response			
Inter-α-trypsin inhibitor H4	2	−1.7	−1.9
TTR	27, 47, 49, 50	−2.2, 7.5, 3.8	1.5, −1.8
Heme scavenging and iron transporter proteins			
Hemopexin	32	6.9	
HRP	66[b)]	2.0	
Transferrin	7, 62[b)], 75	1.5, 1.6[b)], 2.0	
Others			
Albumin precursor	7, 24, *47*	1.5, 3.3, *−2.2*	4.6, *−1.8*
Zinc-α-2-glycoprotein	77, 78	1.4, 1.6	
Gelsolin	7, 6, 10	1.5, −1.4	1.3
ADP-ribosylation factor 5	66[b)]	2.0	

a) Differential protein expression is shown as the FC of the median of standardized spot abundances in PE-AGA or PE-SGA *versus* controls.

b) Spots included in a classifier but standardized abundance not significantly different in cases compared with controls.

Italics depict proteins, which have been identified, but are unlikely to be the dominant protein in a spot based on spot location in experimental 2-D gel compared with published human plasma 2-D gel maps.

Table 6. Classification models for PE-AGA or with PE-SGA[a)]

Experiment	Classifier			
PE-AGA				
III	III-C1 Fibrinogen β (11)			
V	V-C1 Apo-AI (41, 60)	V-C2 Apo-AI (41,60,66) HRP (66) Transferrin (7, 62) A2M (7) FN (7)[b)]		
PE-SGA				
I	I-C1 Apo-AI (41)	I-C2 Apo-AI (41) PZP/A2M (55)	I-C3 Apo-AI (41) PZP/A2M (55) Fibrinogen β (11)	I-C4 Apo-AI (41,36) PZP/A2M (55) Fibrinogen β (11) Fibrinogen γ (15, 57) Clusterin I (57, 17) α1-Antichymotrypsin (17) Complement factor I (17)
IV	IV-C1 Apo-AI (41) SAP (30) TTR (47)	IV-C2 Apo-AI (41) SAP (30) TTR (47) Fibrinogen β (11)		

a) Classification (C) models were generated by NSC analysis. Proteins (spot master number) included in each classifier are listed. Classification accuracy was for PE-AGA: Experiment III-C1 100%; Experiment V-C1 and C2, 90.9%; and for PE-SGA: Experiment I-C1 to C4, 99%; Experiment IV-C1 and C2, 99.6%.

b) Fibronectin 1 isoform 3.

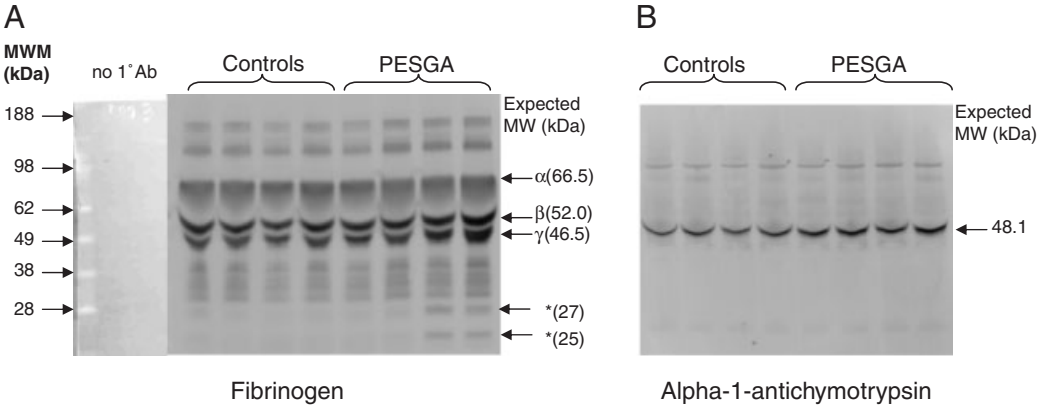

Figure 2. Specific detection of fibrinogen and α-1-antichymotrypsin by Western blot analysis. Western blots of 50 μg native plasma from four women at 20 wk gestation with subsequent PE-SGA compared with plasma from women with a healthy pregnancy outcome. Immunoblots confirmed our DIGE findings, indicating a significant overexpression of plasma fibrinogen γ chain in PE-SGA, $p<0.05$ (A) and α-1-antichymotrypsin, $p<0.01$ (B). Asterisks indicate low-molecular-weight fragments of fibrinogen overexpressed in PE-SGA requiring further validation.

4 Discussion

In a series of case–control studies, we employed a differential proteomic approach to screen for PE-specific biomarkers in plasma from women who subsequently developed PE. Our studies revealed 36 moderate to high-abundance plasma proteins that were differentially expressed at 20 wk gestation, preceding the onset of PE. The data provide evidence of novel synergism between pathways implicated in the etiology of PE. The proteins identified are involved in lipid metabolism, the complement cascade, coagulation, inflammation, ECM remodeling, protease

inhibition and heme scavenging, all of which have relevance to the pathogenesis of PE. During pregnancy there is constant traffic of fetal DNA, syncytiotrophoblast microparticles and placenta-derived proteins into maternal blood, and this is increased in women who later develop PE [8, 18, 29]. To remain healthy, pregnant women need to dampen down the inflammatory and coagulation responses to the placenta-derived substances in their blood and clear them from their circulation [6, 8]. We postulate that many of the proteins identified may be mediators or regulators of the maternal vascular, inflammatory and coagulation responses to placenta-derived triggers and may reflect a susceptibility to the condition.

Of note, 18 of the 36 proteins in our study overlapped with the unique protein cargo recently reported on high-density lipoprotein (hDL) cholesterol [30]. Vaisar and co-workers' proteomic analysis of proteins complexed to hDL particles in the context of cardiovascular disease revealed, among other proteins, apolipoproteins A-I, C-III, E, and M, serum amyloid A4, clusterin, complement C1q, C3, C4a, C4 binding protein, fibrinogen α, β and γ, angiotensinogen, hemopexin, haptoglobin-related protein (HRP), transferrin, α1-anti-trypsin, TTR, inter-α-trypsin inhibitor H4 and human fetuin-A. All these proteins were differentially expressed prior to PE in our study (Table 5). This suggests a similar convergence of abnormalities in lipid metabolism, innate immunity, inflammation and protease inhibition may be operating in PE [30, 31].

The overlap with the hDL proteome provides molecular evidence for the known association between PE and later cardiovascular disease [2]. Protective roles for hDL pertinent to both atherosclerosis and PE include reverse cholesterol transport from lipid laden vascular macrophages, reduced lipid oxidation, inhibition of complement activation and endothelial cell apoptosis, as well as increased phagocytic clearance of apoptotic cells, increased endothelial cell expression of adhesion molecules and inhibition of platelet activation [6, 31–34]. As the cargo proteins complexed to hDL may modify its ability to protect the vessel wall, our findings suggest a potential mechanism for the relationship between PE and later coronary artery disease [31, 35].

ApoA-I, the predominant lipoprotein in hDL, protects against lipid oxidation, facilitates cholesterol efflux from lipid-laden macrophages in atherosclerotic lesions and modifies endothelial expression of cell adhesion molecules [32, 35, 36]. We found the majority of ApoA-I fragments (MW 17, 21 and 30 kDa) were increased up to 12-fold prior to PE-AGA and PE-SGA. Only two ApoA-I fragments (MW 25 kDa) were down-regulated (1.5-fold). ApoA-I exhibits up to 34 distinct species in 2-D gels due to Kringle repeats, which account for the variation in different plasma ApoA-I species. 2-D-gel studies also reported increased serum ApoA-I when PE is manifest clinically [37, 38], but plasma levels measured by turbidimetry are conflicting [12, 39]. Increased ApoA-I is typically associated with a reduced risk of atherosclerosis. The elevated levels prior to PE may be a protective response to placental-derived stressors. Alternatively, Apo-A1 function may be modified through variants and post-translational modification [35, 40].

Displacement of ApoA-I by amyloid A on hDL reduces the anti-atherogenic activity of hDL and increased serum amyloid A has been implicated in atherosclerosis [35, 41]. We found elevated plasma amyloid A prior to the onset of PE, consistent with studies performed when PE was manifest clinically [37, 42]. While Heitner and co-workers confirmed their findings by ELISA, we were unable to confirm increased amyloid A in 20 wk plasma using less-sensitive Western blots.

To our knowledge, this is the first report of increased plasma ApoE levels before PE. Increased plasma levels of ApoE and altered glycosylation status of ApoE has been associated with PE [43, 44]. The anti-atherogenic actions of ApoE include facilitation of reverse cholesterol transport from cholesterol-loaded macrophages to hDL [45]. Of interest, lipid-laden macrophages are seen in spiral arteries from women with PE [46]. Whether the elevated ApoE reflects an intrinsic alteration in lipid metabolism, increased trophoblast secretion or a maternal response to protect the maternal vasculature, is unknown [47, 48].

We observed increased complement (C) components C1q, C3 precursor/C3c and C4 as well as C4BPα prior to PE, whereas C6 and C7 levels were decreased. In some spots more than one complement component was identified, which may reflect overlap of sequence or the presence of both the complement components. Elevated C3 precursor/C3c (a cleavage product of C3b) was the most common finding, suggesting predominant activation of the alternative pathway. C3d, also a cleavage product of C3b, has been reported to be increased in PE [49] and the complement fragment Bb from the alternative pathway was recently reported to be increased at 20 wk gestation prior to PE [50]. C3 and C1q deposits are increased in the placenta, decidual vessel walls and renal biopsies in PE and atherosclerotic lesions [34, 51, 52]. Complement may play an important role in the clearance of placenta-derived microparticles or apoptotic cells from the maternal circulation. In other settings, the binding of opsonins (such as C1q) in the complement cascade to microparticles and apoptotic cells facilitates their clearance by macrophages. Of interest, ficolins that activate the complement cascade *via* the lectin pathway are increased on apoptotic syncytiotrophoblast in the placenta [53]. Taken together, our observations are likely to reflect chronic low-grade complement activation, possibly in response to the fetal–maternal traffic of syncytiotrophoblast debris.

We identified aberrant expression of two other proteins related to the complement cascade, SAP and clusterin isoform 1 (also known as apolipoprotein J). SAP is a short pentraxin, like C-reactive protein, that binds to apoptotic cells and microparticles to facilitate their phagocytosis [54]. Clusterin is a glycoprotein that also induces cholesterol efflux from lipid laden macrophages and inhibits complement-mediated cell lysis [55, 56]. Increased serum

clusterin has been reported in PE [38], but this is the first report of clusterin as a predictive marker for PE. A single-nucleotide polymorphism (SNP) of clusterin has been associated with PE [57]. Clusterin was not increased on 1-D immunoblot analysis, suggesting there is differential up-regulation of specific clusterin isoforms. Further studies are required to confirm the increase in clusterin isoform 1 prior to PE and to elucidate its role in the disease pathogenesis.

Given the considerable cross talk between the complement cascade and coagulation system, it is intriguing that a number of differentially expressed proteins are involved in these pathways (Table 5). Activation of both cascades occurs in conditions associated with an increased risk of PE and in disseminated intravascular coagulation, a complication of PE [58]. We found an increase in plasma coagulation proteins, Factor XII (Hageman factor) and fibrinogen chains α, β and γ and reduced Factor XIIIb. The elevated fibrinogen γ prior to PE was confirmed by Western blot analysis. These findings are consistent with a propensity to clot formation, as seen in PE, with the reduced FXIIIb likely to be the result of consumption following activated coagulation [59].

Fibrinogen is a hexameric glycoprotein composed of two Aα chains, two Bβ chains and two γ chains. This is the first report of elevated levels of fibrinogen γ chain antedating the clinical signs of PE. Consistent with our data, a recent proteomics study reported fibrinogen γ was increased in severe PE [37]. Fibrinogen binds to platelets and leukocytes *via* the γ chain and to endothelium, and thus promoting adhesion of leukocytes and platelets, endothelial proliferation and angiogenesis [59–61]. We speculate that the increased fibrinogen γ contributes to the pathogenesis of PE by promoting platelet aggregation and leukocytes adhesion to the endothelium.

Fibrinogen is also part of the ECM in human placenta, interacting with fibronectin to play a major role in ECM remodeling [59]. Our data showed an increase in fibronectin type I isoform 3 and α1-type 1 collagen preceded PE. In early pregnancy, integrin signaling (including ligands for fibronectin) plays an important role in trophoblast invasion and vasculature remodeling. Fibronectin is believed to facilitate trophoblast differentiation [62] and is considered a promising biomarker to predict PE [63]. Alpha 1-type 1 collagen has not previously been considered a potential biomarker, but a recent study reported that among 775 SNPs investigated, the strongest SNP associations with PE were in the collagen type 1 α1 gene [64].

Our proteomics analysis indicates proteolytic activity in plasma is abnormal prior to the development of PE. Several serine protease inhibitors (SERPINS) were differentially expressed in plasma prior to PE, including a 2- to 3-fold increase in α-1-antichymotrypsin (SERPINA3). Our proteomics data were confirmed by immunoblot analysis. Elevated SERPINA3 has been reported in urine in PE and in amniotic fluid weeks before the onset of PE [65, 66]. A recent study of placental serpins revealed epigenetic changes in the promoter of trophoblast SERPINA3 in PE [67]. Overexpression of SERPINA3 in the preeclamptic placenta may result in deactivation of elastase [67], leading to perturbations of ECM remodeling necessary for placentation and could also result in increased maternal plasma levels. Alternatively, plasma SERPINA3 may be up-regulated in response to platelet activation that is present weeks before the onset of clinical PE. SERPINA3 inhibits two potent stimulators of platelet activation, platelet-activating factor [68, 69] and cathepsin G, a serine protease released from neutrophils [70, 71]. Binding of DNA to cathepsin G renders it almost completely resistant to SERPINA3 [70]. From early pregnancy, women who develop PE have increased free DNA in the plasma that may interfere with SERPINA3 binding to cathepsin G [29]. We postulate that the elevated plasma SERPINA3 may be a compensatory regulatory mechanism attempting to inhibit ongoing platelet activation.

The abundant endoprotease inhibitor, A2M or PZP, was also elevated in the plasma of women destined to develop PE. A2M inactivates a wide range of proteases and binds to angiogenic factors and cytokines, such as VEGF, PlGF and TNF-α [72]. PZP, another member of the A2M family, is up-regulated 10- to 100-fold in pregnancy. Our MS analysis of gel spots related to these proteins did not identify peptides unique to PZP due to the high homology (71%) to A2M. We are, therefore, unable to determine if A2M or PZP is contributing to the elevated levels observed. Elevated levels of A2M have previously been linked to PE and SGA babies [73, 74]. Others reported lower levels before the onset of PE, but their findings may have been confounded by modifiers of A2M levels, such as obesity [74, 75]. Of interest, infusion of human A2M into pregnant alymphoid mice or interferon γ gene ablated mice restored their ability to remodel decidual spiral arteries, indicating the importance of A2M in the development of normal utero-placental blood supply [76]. Further investigation of PZP and A2M as predictors of PE is warranted.

Given the complex biology of PE, it is likely a combination of biomarkers will be required for accurate prediction [3]. To reveal PE-specific clusters of plasma proteins, we performed a multivariate analysis using NSC. The analysis revealed two key proteins, fibrinogen and apoA-I, which overlapped both subgroups, PE-AGA and PE-SGA. ApoA-I in various combinations with PZP or A2M, fibrinogen, clusterin, α1-antichymotrypsin, TTR, or SAP accurately separated those women who later developed PE with an SGA baby and those who had uncomplicated pregnancies. The number of women included is small and these sets of proteins require further evaluation in larger screening studies. If primipara at high risk of PE could be identified, increased surveillance or therapeutic interventions utilizing low-dose aspirin, calcium or heparins may be used to improve their pregnancy outcome [77, 78]. Of interest, a number of the proteins identified here have heparin-binding motifs.

In conclusion, using a DIGE approach we have identified a set of proteins differentially expressed in plasma at 20 wk gestation in women who subsequently developed PE. There is remarkable overlap with a recently reported set of proteins complexed to hDL [30]. The proteins we identified may be placenta-derived or of a maternal response attempting to protect against endothelial damage, inflammation and activated coagulation in response to placental triggers. They indicate a convergence of abnormalities in lipid metabolism, innate immunity, coagulation, inflammation, protease inhibition and ECM remodeling that may reflect a susceptibility to the condition. Thirty years ago, Stark proposed PE may result from disequilibrium in proteolytic control [79]. Our data concurs with this concept and provides a new perspective on the protective and regulatory roles of major plasma proteins in the pathogenesis of PE.

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