

# Discovery of the serum biomarker proteins in severe preeclampsia by proteomic analysis

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Abbreviations: AHS<sub>2</sub>G,  $\alpha$ 2-HS-glycoprotein; AMBP,  $\alpha$ 1-microglobulin/bikunin; CID, collision-induced dissociation; ESI, electrospray ionization; FDR, false discovery rate; IGFBP-ALS, insulin like growth factor binding protein, acid labile subunit; MS, mass spectrometry; PE, preeclampsia; RBP4, retinol binding protein 4; SRM, selective reaction monitoring

## Abstract

**Preeclampsia (PE) is a severe disorder that occurs during pregnancy, leading to maternal and fetal morbidity and mortality. PE affects about 3-8% of all pregnancies. In this study, we conducted liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) to analyze serum samples depleted of the six most abundant proteins from normal and PE-affected pregnancies to profile serum proteins. A total of 237 proteins were confidently identified with < 1% false discovery rate from the two groups of duplicate analysis. The expression levels of those identified proteins were compared semiquantitatively by spectral counting. To**

**further validate the candidate proteins with a quantitative mass spectrometric method, selective reaction monitoring (SRM) and enzyme linked immune assay (ELISA) of serum samples collected from pregnant women with severe PE (n = 8) or normal pregnant women (n = 5) was conducted.  $\alpha$ 2-HS-glycoprotein (AHS<sub>2</sub>G), retinol binding protein 4 (RBP4) and  $\alpha$ -1-microglobulin/bikunin (AMBP) and Insulin like growth factor binding protein, acid labile subunit (IGFBP-ALS) were confirmed to be differentially expressed in PE using SRM ( $P < 0.05$ ). Among these proteins, AHS<sub>2</sub>G was verified by ELISA and showed a statistically significant increase in PE samples when compared to controls.**

**Keywords:**  $\alpha$ 2HS-glycoprotein; biomarkers; pre-eclampsia; spectrometry, mass, electrospray ionization; proteomics; serum

## Introduction

Preeclampsia (PE) is a disorder defined by new-onset hypertension and proteinuria after 20 weeks of gestation that can present as late as 4-6 weeks postpartum. PE affects 3-8% of all pregnancies and is a major cause of maternal morbidity and mortality (Noris *et al.*, 2005). However, the underlying pathogenesis of preeclampsia remains unknown, and the development of adequate biomarkers for its diagnosis and prognosis have not yet been developed (Jauniaux *et al.*, 2006). PE is generally characterized as follows: 1) during the early period of pregnancy, the placenta releases several growth factors into the maternal circulation, producing excessive systemic inflammation in response to ischemic hypoxia and oxidative stress (Borzychowski *et al.*, 2006). 2), the generalized endothelial dysfunction leads to maternal characteristic symptoms of preeclampsia such as hypertension and proteinuria during the late period of pregnancy (Taylor, 1997). Although PE has been studied for a long time, there are currently no reliable biomarkers to predict PE during early pregnancy.

Recently, many groups have been giving special attention to serum proteins related to obesity, low grade inflammation and hypertriglycerolemia, which are common symptoms in PE (Smets *et al.*, 2006). Various serum proteins are considered to

contribute to the symptoms of PE, such as placental growth factor (PIGF), vascular endothelial growth factor (VEGF), soluble vascular endothelial growth factor receptor-1 (sVEGFR-1), soluble fms-like tyrosine kinase-1 (sFlt-1), placental protein 13 (PP-13), pregnancy-associated plasma protein A (PAPP-A), human chorionic gonadotrophin (HCG), soluble endoglin (sEng), alpha fetoprotein (AFP), insulin like growth factor-1 and IGF-binding protein-1 (IGFBP-1) (Muller *et al.*, 1996; Bersinger and Odegard, 2004; Audibert *et al.*, 2005; Venkatesha *et al.*, 2006; Baumann *et al.*, 2007; Spencer *et al.*, 2008; Teixeira *et al.*, 2008).

Various studies have shown that the expression levels of these proteins increase or decrease according to the disorder and its degree in PE (Baumann *et al.*, 2007). However, it is not certain if these proteins would serve as new marker proteins for the diagnosis of PE in clinical practice. Because of these proteins relatively low specificities for the disease, further development is required for its application of clinical use.

To enable commercial use, it is necessary overcome challenges such as validation, reproducibility and specificity against novel marker proteins. Additionally, analysis of biological fluid that has high complexity and a dynamic range requires elaborate pre-preparation and more sensitive techniques (Hu *et al.*, 2006).

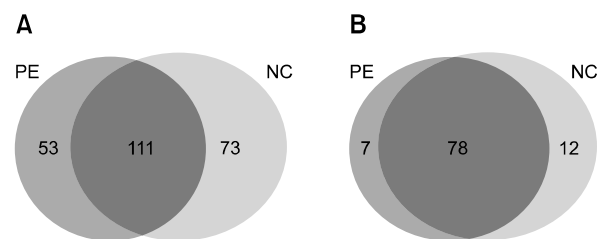
For these reasons, in this study, we systematically compared the maternal serum proteome in women with PE and normal pregnancy using LC-MS based proteomic technologies following depletion of highly abundant serum proteins, such as albumin, immunoglobulin, and transferrin, etc. A total of 237 serum proteins were confidently identified from serum samples of women with PE and normal from 1 % false discovery rate searches (FDR). Among them, 97 proteins having multiple unique peptides were retained for group comparison. The differential expression levels of the selected proteins were validated using a quantitative mass spectrometric method, selective reaction monitoring (SRM) (Ji *et al.*, 2003) and enzyme linked immune assay (ELISA).

## Results

Mining of novel biomarkers from human biological fluids poses many challenges that must be overcome through a proteomics approach, such as the wide dynamic range in abundance and complexity of protein components. Accordingly, various attempts have been made to discover new biomarker proteins with clinical values (Whiteaker *et al.*, 2007). Herein, we endeavored to identify new marker

**Table 1.** Patient's clinical characteristics

Clinical parameter	Pre-eclampsia (n = 8)	Normal outcome (n = 5)
Maternal age (yrs)	30.75 (25-33)	32.4 (28-38)
Gestational age (wks)	31.375 (27-39)	38.6 (37-41)
Birth weight (kg)	1.5025 (0.78-3.16)	3.184 (2.71-3.91)
Cesarean section (%)	8 (100%)	5 (100%)



**Figure 1.** Venn diagram of Identified proteins from both groups; (A) shows proteins identified with single peptide hit. Total of 111 proteins shared between the PE and NC group. (B) represents the proteins that have multiple unique peptides. Seventy eight proteins were shared in both group.

proteins for PE using data dependent LC-MS/MS for both identification and semi quantification. The expression level of the differentially expressed proteins was further validated by the mass spectrometric quantification method, SRM, and ELISA.

We analyzed individual serum samples from five patients per group in duplicate by LC-MS/MS to identify the serum proteins (Table 1). In this analysis, the collected serum samples were treated using a multiple affinity removal column (MARS) containing six antibodies against albumin, IgG, IgA, transferrin, haptoglobin, and alpha-1-antitrypsin (Ryu *et al.*, 2010). After high abundant protein depletion, the serum samples were digested using a sequence grade trypsin for LC-MS/MS analysis to compare the serum proteomes from women with PE and those from women undergoing normal pregnancy.

The resulting MS/MS spectra were searched with SEQUEST algorithms using a composite target decoy database strategy to evaluate the confidence of peptide (Ballif *et al.*, 2005). We acquired 5984 and 5744 MS/MS spectra from the PE group (n=5) and normal group (n=5), respectively. A total of 237 proteins with 1 % FDR were assigned from those spectra (Figure 1A). We limited the positive protein set to proteins identified by multiple peptides. Among them, 97 proteins were matched with  $2 \leq$  unique peptides (Figure 1B) (Table 2), and

**Table 2.** Total identified proteins; A total of 237 proteins were acquired from both groups with 1% FDR, with 97 of these proteins having multiple peptides. Each group had 85 and 90 serum proteins that were identified with multiple peptides

Group	Total number of IDed proteins (Multiple peptide Hit)	Total number of IDed proteins (Single peptide Hit)	Total number of spectra
PE	85	165	5984
Normal	90	185	5744

these were subjected to further annotation and validation. Consequently we identified 85 proteins and 90 proteins from serum samples of PE and serum samples of normal pregnancies, respectively. Among the 85 proteins identified in the PE groups, 49 proteins were identified in all five patients. Furthermore, 49 proteins were identified in all five patients among the 90 proteins identified in the normal group.

Among the 97 confidently identified proteins, 78 were identified in both groups. To screen potential

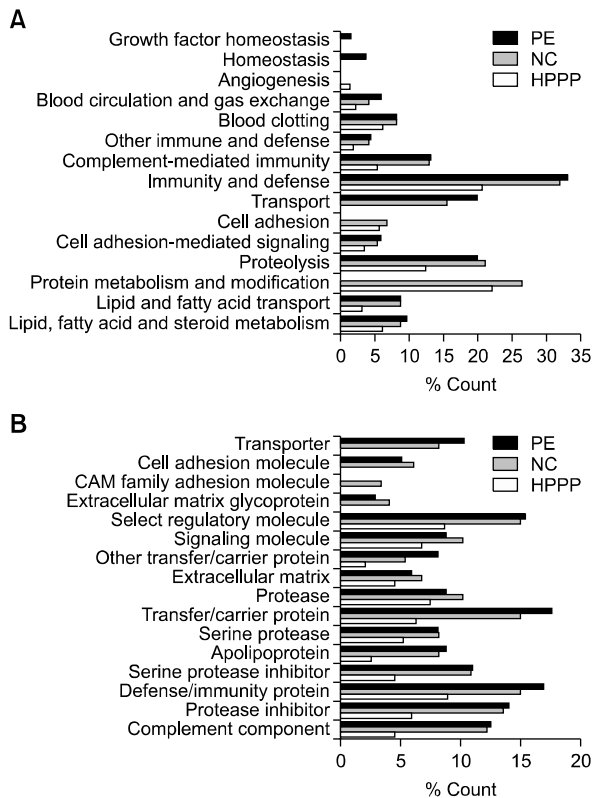
**Table 3.** The fold changes in protein levels by spectral counts, which is one of the relative quantitative methods used in proteomics; All proteins identified are listed based on their expression alteration and 62 proteins are differentially expressed with a 1.2 fold change cutoff. Among them, 27 proteins are up regulated, whereas 35 proteins are down regulated

Up-regulated Proteins in Preeclampsia patients		Unique peptide number		Spectral Counts		% Ratio	
Description	Accession <sup>a</sup>	NCgr <sup>b</sup>	PEgr <sup>c</sup>	NCgr	PEgr	NC/PE	PE/NC
Alpha 1 globin	4504347	0	4		42	0	only PE
Alpha-1-microglobulin/bikunin precursor	4502067	4	6	27	45	60	167
Alpha-2-HS-glycoprotein	156523970	12	14	194	240	81	124
Apolipoprotein A-II preproprotein	4502149	6	6	82	117	70	143
Apolipoprotein C-II precursor	32130518	2	2	14	17	82	121
Beta globin	4504349	0	7		45	0	only PE
Clusterin isoform 1	42716297	4	3	18	22	82	122
Complement component 1, q subcomponent, gamma polypeptide	56786155	2	2	4	7	57	175
Complement component 6 precursor	4559406	6	4	11	15	73	136
Complement component 8, gamma polypeptide	4557393	3	3	4	10	40	250
Complement factor H-related 3	54792787	2	3	5	7	71	140
Delta globin	4504351	0	3		20	0	only PE
Fibronectin 1 isoform 2 preproprotein	47132551	21	24	50	78	64	156
Gelsolin isoform a precursor	4504165	5	6	14	18	78	129
Insulin-like growth factor binding protein, acid labile subunit	4826772	2	4	4	6	67	150
Inter-alpha (globulin) inhibitor H3 preproprotein	133925809	3	2	3	4	75	133
Inter-alpha globulin inhibitor H2 polypeptide	70778918	16	12	69	101	68	146
Lumican precursor	4505047	5	3	8	12	67	150
Paraoxonase 1	19923106	4	6	18	22	82	122
Peptidoglycan recognition protein 2 precursor	156616294	5	3	5	10	50	200
Pregnancy specific beta-1-glycoprotein 9	156616284	2	3	4	6	67	150
Proteoglycan 2 preproprotein	46276889	0	2		3	0	only PE
Serine (or cysteine) proteinase inhibitor, Clade A (alpha-1 antiproteinase, Antitrypsin), member 1	50363221	0	7		12	0	only PE
Serpin peptidase inhibitor, clade A, member 3 precursor	50659080	5	5	20	35	57	175
Serum amyloid P component precursor	4502133	2	3	4	10	40	250
Transthyretin	4507725	8	10	23	60	38	261
Von Willebrand factor preproprotein	89191868	0	2	1	4	25	400

**Table 3.** Continued

Down-regulated Proteins in Preeclampsia patients		Unique peptide number		Spectral Counts		% Ratio	
Description	Accession <sup>a</sup>	NCgr <sup>b</sup>	PEgr <sup>c</sup>	NCgr	PEgr	NC/PE	PE/NC
Afamin precursor	4501987	11	14	62	51	122	82
Albumin precursor	4502027	38	15	120	27	444	23
Alpha-2-plasmin inhibitor	115583663	2	2	6	4	150	67
Apolipoprotein A-IV precursor	71773110	10	8	53	30	177	57
Apolipoprotein D precursor	4502163	4	2	7	5	140	71
Apolipoprotein M	22091452	2	0	2	1	200	50
Coagulation factor XII precursor	145275213	5	4	15	8	188	53
Complement component 4 binding protein, alpha chain precursor	4502503	4	0	9	1	900	11
Complement component 4A preproprotein	67190748	22	17	86	58	148	67
Complement component 4B preproprotein	158037400	21	11	79	34	232	43
Complement component 7 precursor	45580688	6	0	7	1	700	14
Complement component 8, alpha polypeptide precursor	4557389	3	2	9	4	225	44
Complement component 8, beta polypeptide preproprotein	4557391	2	0	2	1	200	50
Complement component 9	4502511	8	3	12	8	150	67
Complement factor H isoform b precursor	62739188	12	10	64	51	125	80
Complement factor H-related 1	118442839	2	0	4		only NC	0
Complement factor I	119392081	5	4	12	9	133	75
Corticosteroid binding globulin precursor	73858564	2	0	6	1	600	17
Fibulin 1 isoform A precursor	34734068	2	0	6		only NC	0
Fibulin 1 isoform C precursor	34734062	2	0	5		only NC	0
Fibulin 1 isoform D	34734066	2	0	2		only NC	0
Haptoglobin-related protein	45580723	2	0	2		only NC	0
Heparin cofactor II precursor	73858566	3	2	11	9	122	82
Plasma kallikrein B1 precursor plasminogen	78191798	2	2	6	3	200	50
	4505881	20	15	105	53	198	50
Pregnancy specific beta-1-glycoprotein 1	21361392	4	5	17	8	213	47
Pregnancy specific beta-1-glycoprotein 2	156616282	3	0	9		only NC	0
Pregnancy specific beta-1-glycoprotein 7 precursor	157805480	3	3	16	5	320	31
Pregnancy-associated plasma protein A preproprotein	38045915	3	5	9	6	150	67
Retinol-binding protein 4, plasma precursor	55743122	3	0	4	1	400	25
Serine (or cysteine) proteinase inhibitor, clade F (alpha-2 antiplasmin, pigment epithelium derived factor), member 1	39725934	4	2	7	4	175	57
Serum amyloid A4, constitutive	10835095	5	3	5	4	125	80
Sex hormone-binding globulin	7382460	9	7	39	27	144	69
Transferrin	4557871	21	2	56	4	1,400	7

<sup>a</sup>NCBI database accession number, <sup>b</sup>Negative control group, <sup>c</sup>Preeclampsia group.



**Figure 2.** Gene functional clustering as determined by the Panther functional annotation tool; We compared identified serum proteins from both groups with 889 high confidence HUPO plasma proteome project (HPPP) proteins by gene functional clustering at 95% confidence. Gene functional clustering showed that biological processes associated with transport, growth factor homeostasis, cell adhesion and protein metabolism/modification were altered significantly when compared to the HPPP proteins (A). In the case of molecular functions (B), cell adhesion related proteins were changed in Preeclampsia.

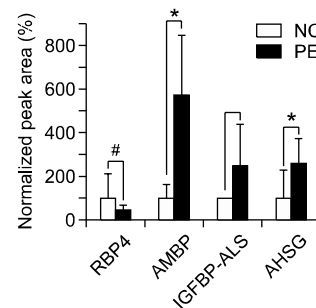
biomarkers specific for each group, the relative expression levels of the identified proteins were compared by a semi-quantitative analysis based on spectral counting (Choi *et al.*, 2008; Little *et al.*, 2010). Eventually, 62 proteins showed more than 1.2 fold differences in expression levels, 27 of which were up regulated and 35 of which were down regulated according to their spectral counts (Table 3).

Those proteins identified from the profiling procedure with 1 % FDR were further annotated based on their molecular function and biological process according to the gene ontology (GO) terms when compared with the control serum proteome acquired from the HUPO plasma proteome project (Adamski *et al.*, 2005; Omenn *et al.*, 2005) using the panther functional annotation tool (Thomas *et al.*, 2003).

PE specifically altered proteins were mainly enriched in gene ontology terms of biological pro-

**Table 4.** SRM transitions for target proteins to monitor the quantitative changes in the progression of preeclampsia

Protein name	Sequence	Precursor Ion (m/z)	Product Ion (m/z)
AHSG	TVVQPSVGAAGPVVPPCPGR	1009.2	683.7
IGFBP-ALS	LWLEGNPWDCGCPLK	923.6	1132.9
RBP4	YWGVASFLQK	600.6	849.9
AMBP	AFIQLWAFDAVK	750.3	836.9

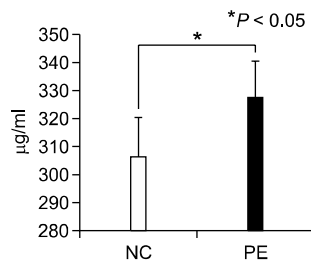


**Figure 3.** LC-SRM analysis of four candidate proteins, RBP4, AMBP, IGFBP-ALS and AHSG; We analyzed serum samples of patients with PE and normal pregnancy. After monitoring of the selected peptides, we calculated relative peak areas of transitions for the target proteins. The protein levels of AMBP, IGFBP-ALS and AHSG were increased in PE patients (\**P*-value < 0.05), while those of RBP4 were decreased in PE patients (<sup>#</sup>*P*-value = 0.08).

cesses of protein metabolism/modification and cell adhesion were mainly enriched in the normal pregnancy group (Figure 2). For the selection of SRM target proteins, we chose target proteins for SRM analysis according to the criteria as follows: i) the target proteins should be differentially altered the expression levels compared with normal group. ii) Their pathophysiological functions should be related to the pathology of PE based on previous reports. iii) Peptides of target proteins should have high SRM signal peaks in SRM scan mode, which guarantees a good analytical reproducibility with high probability.

According to these criteria, we selected four candidates as target proteins for further SRM ; alpha2-HS glycoprotein (AHSG), insulin like growth factor binding protein, acid labile subunit (IGFBP-ALS), retinol binding protein4 (RBP4) and alpha-1-microglobulin/bikunin (AMBP). These target proteins then were validated by SRM runs with well- established transitions (peptide sequences, precursor ions and product ions) (Table 4).

For protein quantification, the peak areas of the individual SRM transitions were compared. Consequently, we could determine the relative expression levels of target proteins between the PE group (n=8) and normal group (n=5). As shown in



**Figure 4.** The results of human AHSG ELISA; Serum AHSG levels of normal control (NC, hatched bar) and preeclampsia (PE, dotted bar) were determined by human AHSG ELISA as described in the method. AHSG levels were increased in the PE group when compared with NC group. Statistical significance was determined using a Student's *t*-test \* $P < 0.05$ .

Figure 3, the expression levels of IGFBP-ALS, AMBP and AHSG were higher in serum samples collected from the PE group. The expression level of RBP4 in the PE group was reduced when compared to the normal group.

In previous studies, AHSG, which is synthesized in the liver and secreted into the blood stream, has been shown to be related to angiogenesis and insulin resistance. It has also been shown that serum AHSG levels are increased in patients with obesity or diabetes (Danzer *et al.*, 1980; Thomas *et al.*, 2003). This is an important indicator that AHSG is involved in the insulin signaling pathway. Furthermore, it appears to boost the likelihood of developing gestational diabetes during pregnancy (Srinivas *et al.*, 1993; Kalabay *et al.*, 2002; Swallow *et al.*, 2004; Stefan *et al.*, 2006; Briana *et al.*, 2008). In fact, our results show that serum AHSG levels were increased in the PE group as determined by both the SRM assay (Figure 3) and ELISA assay (Figure 4).

A significant increase in AHSG was observed in serum samples with PE using a SRM assay (2.5 fold,  $P < 0.05$ ). In contrast, the serum level of RBP4 was reduced by 2.4 fold in the PE group. Accordingly, a serum AHSG- ELISA assay showed a 10 % increase of AHSG in the PE group ( $P < 0.05$ ).

## Discussion

Recent achievements in proteomics have opened a new chapter in protein biomarker discovery, making a contribution to diagnosis in clinical practice. However, novel marker proteins are still rarely used clinically due to their limited reproducibility and specificity.

In this study, we developed an analytic technique using mass spectrometer based SRM for the

quantification of biomarker proteins in the serum of pregnant women with severe PE. The results suggest that the mass spectrometry based quantification may be useful for the identification and quantification of differentially expressed proteins in clinical practice.

Although it has limitations, there is a need for a specific antibody against target proteins. However, it takes a long time to develop such assays; therefore, antibody-based immunoassays have been used as a powerful tool in the quantitative analysis of biological fluids. Furthermore, it is not possible to develop ELISA assays for all proteins in biological fluids in which the concentrations of proteins have an inherently large dynamic range and complexity. Therefore, mass spectrometry based assays have recently been introduced in this field (Aebersold and Mann, 2003).

In this study, we identified several proteins as potential diagnostic biomarkers for PE, IGFBP-ALS, AHSG and AMBP using proteomics technologies. In previous studies, some groups have reported that insulin like growth factors might be correlated with the pathophysiology of PE because of their abilities in fetal growth (Chard, 1994).

Ternary complex consisting of insulin like growth factor (IGF), insulin like growth factor binding protein (IGFBP) and IGFBP-ALS is believed to play a key regulatory role in the pathophysiology of PE (Lewitt *et al.*, 1995, 1998). One of these compounds, IGFBP-ALS, has been implicated in the stability of this complex and might regulate the supply of IGF in the blood stream and placenta. For these reasons, we speculated that IGFBP-ALS is important in the regulation of fetal and neonatal growth. Furthermore, our study showed that in PE pregnancy, serum levels of IGFBP-ALS were up-regulated by 50% when compared with normal pregnancies as determined by an SRM assay (Figure 4). Elevated levels of IGFBP-ALS might interact with IGF in the blood stream and interrupt the pathway to the placenta from blood. RBP4, one marker protein candidate for PE, is generally known to be a carrier protein for retinol in blood (Rask *et al.*, 1987). Moreover, in impaired insulin regulation, obesity or type 2 diabetes, RBP4 levels are up regulated in serum (Yang *et al.*, 2005), which is an important contribution to insulin resistance. These findings suggest that there are pathological differences between impaired insulin regulation caused by diabetes and PE. Temporarily increased insulin resistance in normal pregnancy is likely due to an imbalance between enhanced maternal adiposity and insulin desensitizing by the placenta. In addition, other studies have found lower RBP4 levels in gestational diabetes (Graham *et al.*, 2006).

Therefore, these candidates are correlated with fetal growth and pathophysiology of PE, even though they will not all be accepted as new marker proteins due to the limited numbers of patients and normal. Accordingly, further studies are needed to investigate the differential expression of candidate marker proteins through systematic analysis of large numbers of patients for their clinical application.

Additionally, the causes of this disease and how to cure PE without pre-mature delivery are still not certain. Accordingly, it will eventually be necessary to investigate the pathogenesis and diagnosis of this disease to prevent fetal and maternal death. From this aspect, the Mass Spectrometer based SRM described herein will be useful in the discovery of biomarker proteins.

## Methods

### Materials

A multiple affinity removal system (MARS) column was purchased from Agilent Technologies (Santa Clara, CA). Sequencing grade modified trypsin was obtained from Promega (Madison, WI). Acetonitrile (HPLC grade), water (HPLC grade) and formic acid (ACS reagent grade) were purchased from Aldrich (Milwaukee, WI). BCA protein assay reagent was acquired from PIERCE (Rockford, IL). A human Fetuin-A ELISA kit (BioVendor, Heidelberg, Germany) was used to determine the serum AHSG concentration.

### Study population and sample preparation

We recruited eight pregnant women with severe PE and five with uncomplicated pregnancies. Five pregnant women from each group were targeted for the profiling of global serum proteins and all of the women were used for the quantitative analysis, SRM and ELISA for validation. The phenotype of the study population is presented in Table 1. We selected women who underwent elective cesarean delivery to eliminate labor effects on gene expressions. Prior to delivery, 10  $\mu$ l of venous blood was sampled from the forearm and collected into a vacutainer tube. Severe preeclampsia was defined as a blood pressure (BP) of at least 160 mmHg (systolic) and/or 110 mmHg (diastolic), with proteinuria  $\geq$  2+ as determined via a dipstick test, measured on at least two occasions six hours apart after the 20<sup>th</sup> week of gestation. Women with pre-existing chronic hypertension, renal disease, lupus erythematosus, diabetes, and gestational hypertension without proteinuria were excluded. This study was approved by the Institutional Review Board at Gangnam CHA Medical Center, College of Medicine, CHA University, and written informed consent was obtained from each patient.

### Depletion of abundant serum proteins

To reduce the complexity of the serum protein, we removed the six most abundant proteins from the serum samples by MARS (Agilent Technologies, Santa Clara, CA) according to the manufacturer's instructions. This resulted in the depletion of albumin, transferrin, IgF, IgA, anti-trypsin, and haptoglobin from the human serum. The depleted serum samples were then desalted using a PepClean C18 spin column (thermo, Rockford, IL) to change the buffer (50 mM  $\text{NH}_4\text{HCO}_3$ ), after which they were subject to in-solution tryptic digestion as follows: 10  $\mu$ g of depleted serum samples and 10 mM of dithiothreitol were combined and incubated for 45 min at 60°C for protein reduction. Next, 5 mM iodoacetamide was added and the samples were incubated for 30 min in the dark at room temperature to induce alkylation. The prepared mixture was then subjected to digestion with 125 ng of trypsin for at least 4 h at 37°C, after which the digested samples were lyophilized using a Speed-Vac and then re-suspended in 10  $\mu$ l of 0.1% TFA in HPLC water followed by desalting with a ZipTip C-18 column (Millipore Co., Billerica, MA) to remove the detergent and salt from the peptide mixture.

### Identification of serum proteins by LC-MS/MS

The tryptic peptides were loaded onto a fused silica micro-capillary column (15  $\times$  75  $\mu$ m) packed with C18 (5  $\mu$ m, 200 Å) reversed phase resin and separated by LC using a linear gradient of 3-40% buffer B for 50 min followed by 40-85% buffer B over 10 min (buffer A: 0.1% formic acid in  $\text{H}_2\text{O}$ , buffer B: 0.1% formic acid in acetonitrile) at a flow rate of 200 nl/min. The column was connected directly to a LTQ ion-trap mass spectrometer (Finnigan, CA) equipped with a nano-electrospray ion source. The electrospray voltage was then set at 2.1 kV, and the threshold for switching from MS to MS/MS was 250. The normalized collision energy for MS/MS was 35% of the main RF amplitude and the duration of activation was 30 ms. All spectra were acquired in data-dependent mode. Each full MS scan was followed by five MS/MS scans corresponding to the most intense peak to the fifth most intense peak observed on the full MS scan. The repeat count of the peak for dynamic exclusion was 1, and its repeat duration was 30 s. The dynamic exclusion duration was set for 180 s and the exclusion mass width was  $\pm$  1.5 Da. The list size of the dynamic exclusion was 50 s. The acquired MS/MS data was searched using the SEQUEST database search algorithm (Thermo Fisher Scientific Inc., MA) against the human NCBI database (April 3, 2009 version) using a forward (target) and reverse (decoy) sequence. A decoy database was created with BioworksBrowser rev.3.3. Search parameters were as follows; specific to trypsin with two missed cleavage;  $\pm$  2.0 Da precursor-ion tolerance; and  $\pm$  1 Da fragment-ion tolerance; and fixed modifications, oxidation of Met (16 Da), alkylation of Cysteine (57 Da). For a more-reliable protein list, the identified peptides were filtered to 1% FDR with the target-decoy database strategy (Elias and Gygi, 2007).

### Selective reaction monitoring (SRM) assay

In this study, a quantitative SRM assay was conducted to determine the relative concentration of proteins that were selected from the differentially expressed proteins by comparing PE samples to normal samples. Serum samples for the SRM assay were not subjected to depletion with the MARS column to avoid systematic errors associated with the sample preparation. We also monitored a set of SRM transitions of representative peptides corresponding to the differentially expressed proteins that were selected by comparing two groups by spectral counts and GO analysis. SRM mode was conducted by using a series of several scan events, with SRM being executed in each scan event. For SRM analysis of the target protein, the *m/z* of a precursor ion, which corresponds to a peptide of protein interest, was selected and the selected precursor ion was then fragmented by collision induced dissociation (CID). Among the resulting MS/MS peaks from the precursor ion, a representative peak was selected for the identification and/or quantification of the corresponding protein. The SRM transitions selected for monitoring potential candidate proteins were as follows: (1009.2 → 683.7 for AHSG, TVVQPSVGAAGPVVPPC#PGR, C# indicates carbonyl-methylation of Cysteine), (923.6 → 1132.9 for IGFBP-ALS, LWLEGNPWDC#GC#PLK), (600.6 → 849.9 for RBP4, YWGVASFLQK), (750.3 → 836.9 for AMBP, AFIQLWAFDAVK) and (758.0 → 1160.0 for internal standard peptide for normalization, DHTGFLTEYVATR). The number of total scan events was six, and these consisted of one full MS scan event and five subsequent SRM scans. On the CID condition, the normalized collision energy was 35% of the main RF amplitude and the duration of activation was 30 ms. The peak width of the precursor and MS/MS daughter ion for SRM were  $\pm 1$  and  $\pm 2.5$  Da, respectively.

### ELISA assay

Equal amounts of sera were analyzed using a human AHSG ELISA kit (BioVendor, Heidelberg, Germany). Sandwich ELISA was conducted according to the manufacturer's instructions. Next, 100  $\mu$ l of diluted standards were added to each well of the quality control and samples and the plate was incubated on an orbital microplate shaker under room temperature for 1 h. After washing the well three times, 100  $\mu$ l of conjugate solution was added and the plate was then incubated for 1 h at room temperature while shaking at 300 rpm. The plate was washed then washed three times with wash solution, after which 100  $\mu$ l of substrate solution was added into each well and the plate was incubated for around 10 minutes to enable the reaction to develop. Finally, the absorbance at 450 nm was measured using an ELISA plate reader.

### Statistical analysis

The statistical significance of the measurements was compared using a student's *t*-test via MedCalc program (MedCalc software, Mariakerke, Belgium, version 11.3.3.0).

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