SUPPLEMENTARY APPENDIX

PROTEOMIC PROFILING OF URINE IDENTIFIES SPECIFIC FRAGMENTS OF SERPINA-1 AND ALBUMIN AS BIOMARKERS OF PREECLAMPSIA

by

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This appendix has been provided by the authors to give readers additional information about their work.

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I. SUPPLEMENTARY METHODS

a) Contributors to the "objective clinical score of preeclampsia severity (OCS-sPE)"

At the time of enrollment one of the investigators (CSB) abstracted the clinical information entered in the medical record into a single semi-quantitative variable which we named the "objective clinical score of preeclampsia severity (OCS-sPE)". Briefly, Boolean indicators (1=present and 0=absent) were assigned for each of the criteria listed above which are based on the ACOG clinical criteria of diagnosis and severity of preeclampsia (Table A1).[1] OCS-sPE was calculated by summing the point indicators for each case.

Table A1

Contribution to OCS-sPE	Criterion
1 point	systolic blood pressure ≥ 140 mmHg or diastolic blood pressure ≥ 90 mmHg and dipstick proteinuria ≥ +1
1 point	systolic blood pressure ≥ 160 mmHg
1 point	diastolic blood pressure ≥ 100 mmHg
1 point	dipstick proteinuria ≥ +3
1 point	neurological manifestations
1 point	elevated liver enzymes
1 point	trombocytopenia (platelets <100,000 cells/µl)
1 point	intrauterine growth restriction (IUGR)

AST: aspartate aminotransferase; ALT: alanine aminotransferase

b) Details of women who provided urine samples for the exploratory phase

Table A2

Variables	Asymptomatic pregnant women (n=21)	Severe preeclampsia (n=38)	P value
Demographic, clinical and laboratory characteristics at enrollment			
Age, *years* †	26 ± 6	26 ± 7	0.913
Parity ‡	0 [0-3]	0 [0-6]	0.697
Weight, *kg* †	87 ± 15	91± 27	0.499
Gestational age, *weeks* ‡	31 [22-42]	33 [24-41]	0.629
Systolic blood pressure, *mmHg* ‡	114 ± 11	168 ± 17	< 0.001
Diastolic blood pressure, *mmHg* ‡	67 ± 8	102 ± 9	< 0.001
Neurological symptoms ¶	0 [0 %]	17 [45 %]	< 0.001
Dipstick proteinuria ¶	0 [0-1]	3 [1-4]	< 0.001
24-h proteinuria, *grams/24h* ‡	NA	3.3 [0.2-13.1]	NA
Elevated liver enzymes ¶	NA	14 [37 %]	NA
Platelets <100,000 cells/mm^3 ¶	0 [0 %]	8 [21 %]	< 0.001
LDH, *U/L* ‡	NA	261 [206-1,300]	NA
Uric acid, *mg/dL* †	NA	6.7 ± 1.3	NA
OCS-sPE	0 [0-0]	4 [3-7]	< 0.001

Variables	Asymptomatic pregnant women (n=21)	Severe preeclampsia (n=38)	P value
Outcome characteristics			
Gestational age at delivery, *weeks* ‡	39 [37-42]	33 [28-41]	< 0.001
Indicated delivery ¶	0 [0 %]	38 [100 %]	< 0.001
Indicated delivery <34 wks ¶	0 [0 %]	21 [55 %]	< 0.001
Cesarean delivery ¶	8 [38 %]	26 [68 %]	0.029
Birth weight, *grams* ‡	3,360 [2,540-4,335]	1,775 [902-4,300]	< 0.001

† Data presented as mean ± standard deviation and analyzed by Student t test
‡ Data presented as median [range] and analyzed by Mann-Whitney test
¶ Data presented as n [%] and analyzed by Fisher's exact tests.

c) Details on Mass Restricted (MR) scoring as applied to extract the urinary proteomic profile characteristic of severe preeclampsia

The method of MR scoring is a stepwise strategy to extract relevant biomarkers based on filter principles applied sequentially. [2, 3, 4] Peaks were selected using the centroid tool built into the SELDI software and the mass and signal-to-noise ratio (S/N ratio) for all selected peaks exported to an Excel spreadsheet. A macro tool was constructed to further assign Boolean indicators of 1 to masses with S/N ratios above a pre-established cut-off value. Boolean indicators of 0 were assigned if the S/N ratio was below the cut-off. In this study the cut-off was chosen as average + 2SD of the S/N ratio at the same masses on the profiles obtained from the control samples. The principles (Table A3) are applied in sequence to either eliminate or retain peaks in the final profile. Only peaks of the final profile are designated as biomarkers.

Table A3. Criteria of Mass Restricted (MR) scoring method

Criterion	Principle
Criterion I	All biomarker peaks should be present in the "diseased" state (i.e. the search is for peaks that appear rather than disappear in preeclampsia)
Criterion II	The final profile should include peaks from at least two different experimental conditions (i.e. from both H4 and H5 arrays)
Criterion III	All peaks in the final profile should be significantly different at a level of <0.0001 between the "diseased" and "normal" state (i.e. for each remaining biomarker there should be a highly significant difference in S/N ratios between preeclamptic and control tracings)
Criterion IV (omitted)	Only parent peaks should be considered (singly ionized, least oxidized)
Criterion V	Peaks should not occur in areas where significant peaks appear in "normal" individuals as they could potentially interfere with informative peaks in "diseased" patients
Criterion VI (omitted)	The final diagnostic proteomic profile should be minimal (restricted)

d) Methodological details on biomarker identification

New arrays were prepared from urine samples in the *exploratory phase* cohort with highest UPSr scores. Tandem mass spectrometric (MS/MS) peptide sequencing was accomplished using a quadrupole time of flight instrument (Q-TOF™ II, Micromass) equipped with a PCI 1000 interface (Ciphergen Biosystems). [5] The interface allows for peptides to be sequenced directly from the arrays without any offline purification necessary. The instrument was calibrated externally using an acquired MS/MS spectrum of ACTH (18-39) peptide at 2465.2 m/z, where four fragment ions and the parent were used as calibration points. All mass spectra were acquired in electrospray positive-ion mode with a collisional gas pressure of approximately 100 mTorr. Matrix conditions were identical to SELDI-TOF analysis described previously and protein identification was achieved by database searching using Mascot software (Matrix Science, London, UK).

Following identification by MS/MS of some of the peaks of the UPS score, we conducted in vitro experiments by spiking urine samples devoid of biomarkers (n=3) with purified precursors (Sigma, St. Louis, MO) in the attempt to identify whether the biomarkers of the characteristic profile can be re-created by the interaction of the precursors with the urine milieu.

e) Methodological details on other biochemical, immunological and molecular estimates

Creatinine levels were measured using a colorimetric assay (Stanbio Laboratory, Boerne, TX) against standard curves derived from known concentrations.

Total protein was measured using a bicinchoninic acid/cupric sulphate reagent (BCA kit, Pierce, Rockford, Il).

ELISA assays for human unbound sFlt-1 and PlGF were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). Briefly, urine samples were assayed in duplicate in a 96-well plate pre-coated with a capture antibody directed against free sFlt-1 or PlGF. Incubation protocols were performed followed by washings in accordance with the procedure summary. Plates were read at 450nm with 570nm wavelength correction using a VERSAmax microplate reader with Softmax Pro 3.1.1 software (Molecular Devices, Sunnyvale, CA). The minimal detectable concentrations in the assays for sFlt-1 and PlGF were 5 and 7-pg/mL, respectively. The inter-assay and intra-assay coefficients of variation varied from 3 to 10%.

SERPINA-1 immunoassays microtiter plates (Immuno MaxiSorp, Nalge Nunc, Rochester, NY) were coated with capture antibody (10µg/mL sheep anti-human SERPINA-1 antibody, Affinity Biologicals, Ancaster, Canada). Urine and serum samples were assayed in duplicate at various dilutions (urine: 1:50-1:100,000, serum: 1:250,000) against a 7-point standard curve from 0.123 to 90 ng/mL.

Albumin immunoassays microtiter plates were coated with goat anti-human albumin antibody as capture antibody (10µg/mL; Bethyl Laboratories). The plates were washed, blocked and incubated with urine (1:1000 dilution) or serum (1:150,000) human albumin calibrants (Bethyl Laboratories) in a range from 6.25 to 400 ng/mL. Detection was accomplished using horseradish peroxidase conjugated secondary antibodies (sheep anti-human SERPINA-1, 1:5,000 dilution, Affinity Biologicals or anti-human albumin, 1:150,000 dilution, Bethyl Laboratories, respectively) and 3,3',5,5,'-tetramethylbenzidine (Vector Laboratories, Burlingame, CA) as substrate. The color reaction was stopped with 2M sulfuric acid and plates were read at 450 nm with 650 nm wavelength correction. The intra-assay coefficient of

variation was <5%. Fractional excretion calculations (amount of an analyte excreted in the urine relative to the amount filtered by the kidney) were based on the concentrations of SERPINA-1, albumin and total protein relative to creatinine in samples of blood and urine collected at the same time.[6,7]

Antielastase activity was monitored by the ability of serum or urine sample to inhibit cleavage of a p-nitroanilide substrate [8] by elastase in contolled kinetic conditions (α1-antiproteinase activity assay, Oxis Research, Foster City, CA). Results are reported as concentration equivalents of the elastase inactivated (μM).

Western blot: Gel electrophoresis was carried out on polyacrylamide separating gel and a 4% stacking gel using a BioRad Miniprotean II (Bio-Rad, Richmond, CA) gel apparatus under either denaturing (10% SDS-PAGE) or native (7.5% PAGE) conditions. Ten 10 μg total serum or urine protein, were diluted 1:2 v/v with electrophoresis sample buffer (BioRad) and reduced by boiling for 5 min. Two μg purified serum SERPINA-1 was loaded as positive control. After electrophoretic transfer to a PVDF membrane (Bio-Rad) at 100 V for 70 min. and blocking with 5% milk, the blots were incubated overnight at 4°C with anti-SERPINA-1 primary antibodies (1:1000) and subsequently subjected to Enhanced Chemiluminescence (ECL) using the western blotting detection system (Amersham, Arlington Heights, IL) with enzyme conjugate anti-rabbit IgG: horseradish peroxidase as secondary antibody. Autoradiography film was applied to the blot until satisfactory exposure was achieved.

Ex vivo preparation of SERPINA-1 oligomers was achieved by incubating purified plasma protein (Sigma) at 1 mg/mL in 0.015 mols/M Tris HCl containing 0.15 mol/L NaCl, pH7.4 overnight at 60C.[9] Aggregate formation was confirmed by 7.5% non-denaturing polyacrylamide gel electrophoresis (native PAGE) and a concurrent lost of antielastase activity.

Immunohistochemistry: Placental tissue sections (6 μm) were deparaffinized in xylene and rehydrated with graded ethanol to potassium-phosphate-buffered saline solution, pH 7.2. Following antigen retrieval with citrate buffer, the sections were pretreated with 1% hydrogen peroxide for 15 min. followed by overnight incubation (at 4°C) with rabbit anti-human SERPINA-1 (1:1000, LabVision Corp., Fremont, CA) or mouse monoclonal ATZ11 antibody (Alpco Diagnostics, Salem, NH) and then a 1-hour incubation at room temperature with biotinylated goat anti-rabbit or antimouse IgG (1:600, Jackson Immunoresearch, West Grove, PA), respectively. Detection was performed with avidin-biotin staining (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA) with 3,3´-diaminobenzidine/nickel sulfate as the chromogen solution. The tissue sections were dehydrated in graded ethanols, cleared, and mounted. Specific staining was evaluated semiquantitatively by examining 6 fields/slide and subjectively scoring on a scale from 0 (no staining) to 5 (intense blue-black staining) the intensity of the chromogen deposited in the trophoblast, villous stroma, villous endothelium and intravascular spaces. A median score was computed for each patient. Human liver carcinoma sections (Spring Bioscience, Fremont, CA) were used as SERPINA-1 positive control and sections incubated with rabbit and mouse IgG as negative controls. Some sections were counterstained with methyl green.

II. SUPPLEMENTARY RESULTS

a) Frequencies and experimental masses of individual biomarkers detected in the samples studied in the *exploratory phase*

Table A4

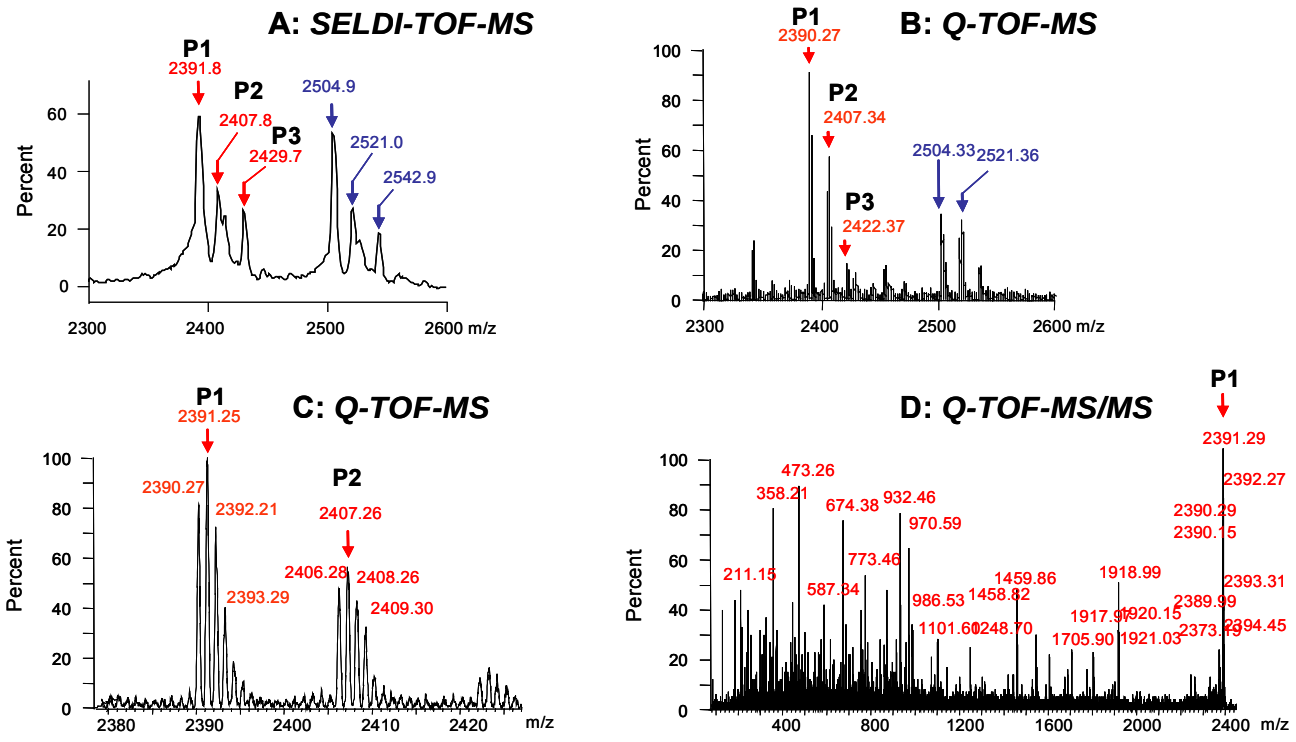
Peak	Observed SELDI mass average [95%CI] Da	Asymptomatic pregnant women (n=21)	Severe preeclampsia (n=38)	P value
H4 array				
P1	2393.1 [2392.9-2393.3]	0 [0 %]	15 [39 %]	< 0.001
P2	2408.6 [2408.4-2408.9]	0 [0 %]	16 [42 %]	< 0.001
P3	2426.0 [2425.6-2426.4]	1 [5 %]	12 [32 %]	< 0.001
P4	3486.9 [3486.6-3487.2]	0 [0 %]	14 [37 %]	< 0.001
H50 array				
P5	2755.3 [2755.1-2755.4]	10 [48 %]	38 [100 %]	< 0.001
P6	2939.4 [2939.2-2939.5]	0 [0 %]	35 [92 %]	< 0.001
P7	4010.1 [4009.9-4010.4]	0 [0 %]	33 [87 %]	< 0.001
P8	4302.3 [4302.1-4302.4]	3 [14]	38 [100 %]	< 0.001
P9	4414.7 [4414.3-4415.1]	2 [10]	36 [95 %]	< 0.001
P10	6399.3 [6398.5-6400.1]	7 [33]	38 [100 %]	< 0.001
P11	11101.7 [11098.9-11104.4]	3 [14]	38 [100 %]	< 0.001
P12	13319.2 [13316.4-13321.9]	5 [24]	38 [100 %]	< 0.001
P13	16652.7 [16648.2-16657.2]	3 [14]	38 [100 %]	< 0.001

Frequency data presented as n [%] and analyzed by Fisher's exact tests.

b) Correspondence of biomarker peaks between detection in the SELDI-TOF and the Q-TOF mass spectrometer

Appendix Figure A6 exemplifies the correspondence of peaks P1-P3 between detection in the PBSIIC SELDI-TOF mass spectrometer (Ciphergen, Panel A) and the Q-TOF mass spectrometer (Micromass, Panel B). The dominant mono-isotopic forms of the biomarkers P1 and P2 before and after fragmentation (of P1, 2390.2 Da) in the MS/MS mode are illustrated in Panels C and D, respectively. Masses for peaks part of P1-P3 complex (21 C-terminal aa of SERPINA-1) are noted in red (in Daltons). In blue are the peaks not part of the UPS score that accompany the complex P1-P3 and represent the 22-aa C-terminal fragment of SERPINA-1.

Figure A6



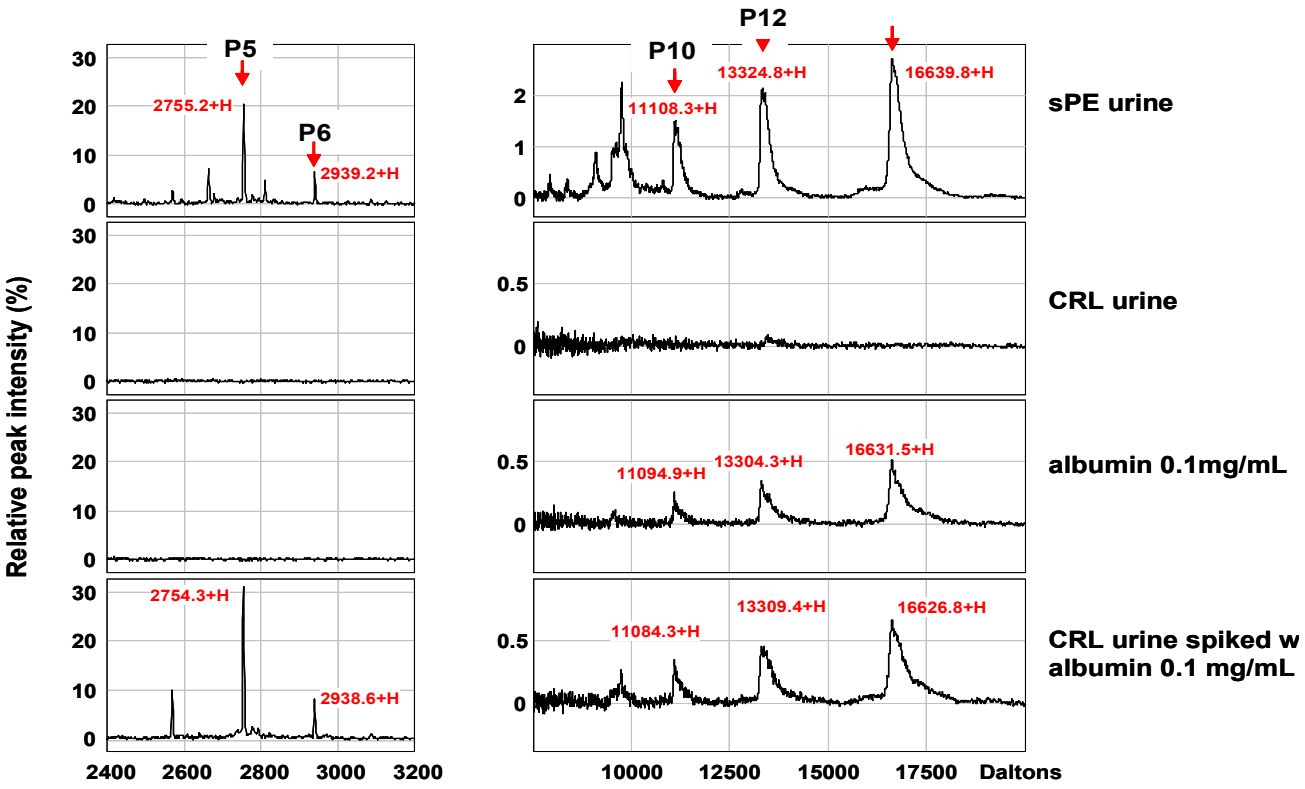
c) Strategy for identification of the origin of other biomarkers of the UPS profile

We reasoned that other peaks of the preeclampsia profile may correspond to fragments derived from the interaction of albumin and/or SERPINA-1 with the urinary milieu. To confirm, we spiked urine samples devoid of biomarkers (CRL urine with UPS scores of 0, n=3) with albumin or SERPINA-1 (Sigma, St. Louis, MO) purified from human serum in concentrations of 0.25 mg/mL or 0.1 mg/mL, respectively (chosen concentration reflects the median immunoreactivity of albumin and SERPINA-1 on urine of the sPE group) (Figure A7, experimental peak masses shown in red). We observed the appearance of P5 and P6 biomarkers in CRL urine samples spiked with albumin but not when either CRL urine or albumin alone were applied to the array. P5 has already been identified by MS/MS as the 24-aa N-terminus fragment of albumin.

Thus, we predict the peptide sequence of P6 is: DAHKSEVAHRFKDLGEENFKALVLIA [P02768 aa 25-50] with a computed mass of 2938.34 Da. We observed emergence of P8 and P10 in a similar pattern (not shown). This suggests that the origin of P5, P6, P8 and P10 in sPE urine is proteolytic in nature and perhaps secondary to albumin cleavage by urinary constituents. In contrast, P11, P12 and P13 appeared also when albumin alone was applied on the H50 array suggesting these peaks are either the consequence of fragmentation of albumin precursor (66 kDa) in the mass spectrometer or are multiple charged forms of the 66 KDa parent peak. However, regardless of their origin it is very likely that all these 3 fragments also originate from human albumin. When we spiked urine with purified SERPINA-1 we were unable to recreate the biomarkers identified as fragments of SERPINA-1. We thus concluded

that P1, P2, P3 (21-aa C-terminus cleavage fragment of SERPINA-1) and P7 (24-aa N-terminus cleavage fragment of SERPINA-1) originate only *in vivo* and to our knowledge only in the context of sPE.

Figure A7



III. LITERARURE CITED IN SUPPLEMENTARY APPENDIX

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