Regular Article

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Identification and Validation of Combination Plasma Biomarker of Afamin, Fibronectin and Sex Hormone-Binding Globulin to Predict Pre-eclampsia

Yasuo Uchida,[a,b,c,#] Tomoya Higuchi,[a,#] Matsuyuki Shirota,[c,d] Satoshi Kagami,[b] Daisuke Saigusa,[c,d] Seizo Koshiba,[c,d] Jun Yasuda,[c,e] Gen Tamiya,[c,d,f] Shinichi Kuriyama,[c,d] Kengo Kinoshita,[c,g] Nobuo Yaegashi,[c,d] Masayuki Yamamoto,[c,d] Tetsuya Terasaki,*[,a,b,c] and Junichi Sugawara*[,c,d]

[a] *Faculty of Pharmaceutical Sciences, Tohoku University; Sendai 980–8578, Japan:* [b] *Graduate School of Pharmaceutical Sciences, Tohoku University; Sendai 980–8578, Japan:* [c] *Tohoku Medical Megabank Organization (ToMMo), Tohoku University; Sendai 980–8574, Japan:* [d] *Graduate School of Medicine, Tohoku University; Sendai 980–8578, Japan:* [e] *Division of Molecular and Cellular Oncology, Miyagi Cancer Center Research Institute; Natori, Miyagi 981–1293, Japan:* [f] *Statistical Genetics Team, RIKEN Center for Advanced Intelligence Project; Tokyo 103–0027, Japan: and* [g] *Graduate School of Information Sciences, Tohoku University; Sendai 980–8578, Japan.*
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The purpose of the present study was to identify a plasma protein biomarker able to predict pre-eclampsia (PE). Comprehensive quantitative proteomics using mass spectrometry with sequential window acquisition of all theoretical fragment ion spectra (SWATH-MS) was applied to plasma samples of 7 PE and 14 healthy pregnant women (for PE subjects, plasma samples were taken before onset of PE), and 11 proteins were selected as candidates potentially able to differentiate the two groups. Plasmas collected at gestational weeks 14–24 from 36 PE and 120 healthy pregnant women (for PE subjects, plasma samples were taken before onset of PE) were used to conduct selected reaction monitoring quantification analysis, optimize protein combinations and conduct internal validation, which consisted of 30 iterations of 10-fold cross-validation using multivariate logistic regression and receiver operating characteristic (ROC) analysis. The combination of afamin, fibronectin, and sex-hormone-binding globulin was selected as the best candidate. The 3-protein combination predictive model (predictive equation and cut-off value) generated using the internal validation subjects was successfully validated in another group of validation subjects (36 PE and 54 healthy (for PE subjects, plasma samples were taken before onset of PE)) and showed good predictive performance, with the area under the curve (*AUC*) 0.835 and odds ratio 13.43. In conclusion, we newly identified a 3-protein combination biomarker and established a predictive equation and cut-off value that can predict the onset of PE based on analysis of plasma samples collected during gestational weeks 14–24.

Key words afamin; fibronectin; sex hormone-binding globulin; pre-eclampsia; biomarker; proteomics

INTRODUCTION

Pre-eclampsia (PE) affects about 2–3% of pregnancies and remains a major cause of maternal and perinatal mortality. It is a disease of the placenta, and results in widespread, multisystem, maternal vascular endothelial dysfunction and microangiopathy. It is clinically characterized by hypertension, proteinuria, and a decreased glomerular filtration rate, typically presenting after 20 weeks of gestation. Progression of PE may lead to other systemic complications such as the HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets), neurologic involvement (eclampsia), and even cardiac and renal failure. Fetal complications include oligohydramnios, intrauterine growth restriction, preterm delivery, and stillbirth. Although PE can be treated by delivery of the placenta, this is fatal for a small fetus. The timing of delivery requires balancing disease severity with potential fetal viability and well-being. Early identification of women at increased risk of developing PE is highly desirable, as it would enable close monitoring and early intervention. A prospective, multicenter observational study of about 500 women has demonstrated that a soluble fms-like tyrosine kinase-*1* (sFlt-1):placental growth factor (PlGF) ratio cut-off of 38 has clinical utility.[1] Specifically, in women in whom pre-eclampsia is suspected clinically, an sFlt-1:PlGF ratio of less than 38 can be used to rule out short-term development of PE. However, the detection of women at increased risk of developing PE based on this ratio is only possible if patient plasma samples are taken after about gestational week 20,[2] so there is still a need for earlier diagnosis.

Recent developments in high-throughput technologies, in particular, proteomics, which can identify circulating proteins in an unbiased and comprehensive manner, have made it easier to find new biomarkers. Biomarker screening is most frequently achieved by means of data-dependent acquisition (DDA) mass spectrometry. However, this approach has a critical limitation, in that it lacks reproducibility in the detection and quantification of individual proteins.[3,4] Consequently, although proteomic studies have found a number of biomarker candidate proteins for PE, it has not been possible to put the candidates in order of priority for later validation studies, making validation difficult. Indeed, among 24 reports on predictive markers for first trimester risk of PE, almost none attempted to validate the proposed markers, as described in

[#] These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: tetsuya.terasaki.d5@tohoku.ac.jp; jsugawara@med.tohoku.ac.jp

a systematic review.[5] Validation is extremely important, as unvalidated biomarkers may underperform in clinical practice if the new patient population differs from the original study group.

Proteomics technology based on data-independent acquisition (DIA), designated as sequential window acquisition of all theoretical fragment ion spectra mass spectrometry (SWATH-MS), was developed in 2012 by Aebersold's group.[6] Unlike DDA-based proteomics, SWATH-MS is a highly precise and reproducible technique for the detection and quantification of individual proteins, and can accurately quantify even small disease-associated changes in protein abundance in blood. Furthermore, it overcomes the major problem of DDA-based blood proteomics: that highly abundant proteins such as albumin and globulin interfere with the quantification of low-abundance proteins. For this reason, SWATH-MS is considered preferable to screen for candidate biomarkers at the discovery stage. In the validation stage of biomarker candidates, enzyme-linked immunosorbent assay (ELISA) assay is often used, but it still has issues related to the specificity and quantitative performance of antibodies. In contrast, selected reaction monitoring (SRM)-based targeted proteomics

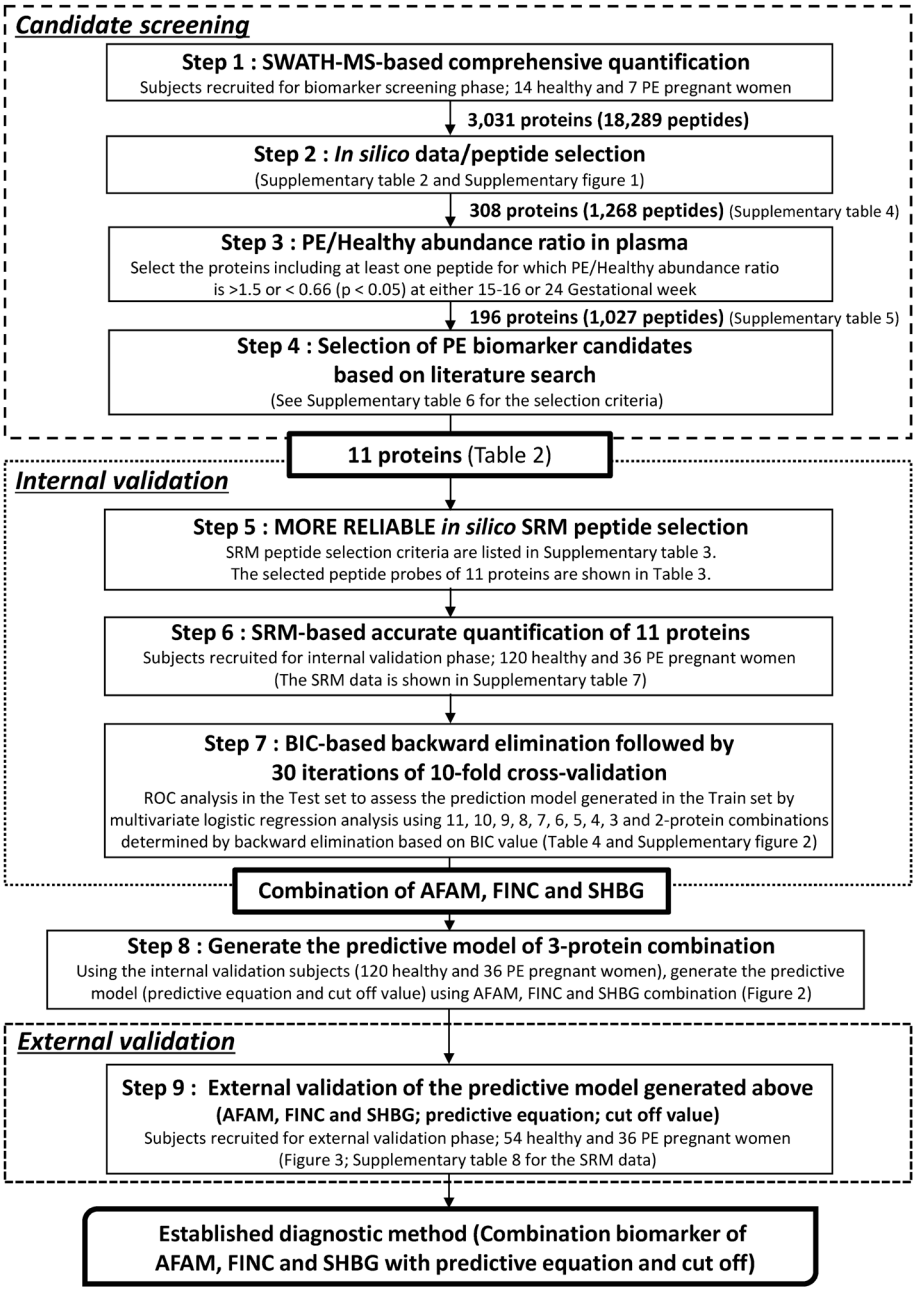


Fig. 1. Strategy of Discovery and Validation for PE Predictive Markers and Summary of Results in the Present Study

SWATH-MS enables highly reproducible and precise relative quantification of proteins compared to other comprehensive proteomic methods. The present study further increases the reliability of candidate screening by applying *in silico* data/peptide selection criteria to the raw SWATH-MS data. A new, larger number of subjects different from the subjects recruited in discovery phase is recruited for the internal validation of candidates by SRM analysis using stable-isotope-labeled peptides which were selected by *in silico* peptide selection criteria more reliable than those used in the data analysis of SWATH-MS. The biomarker combination is optimized by BIC-based backward elimination, and validated by multiple iterations of K-fold cross-validation which is a highly reliable approach among different internal validations. The predictive model (predictive equation using multiple protein combination and cut off value) generated using the subjects recruited in the internal validation phase is validated in the new subjects (external validation phase) in order to evaluate whether it is useful to predict the risk of PE.

using stable-isotope-labeled peptides makes it possible to specifically and accurately quantify proteins of interest with a wide dynamic range ($>1 \times 10^5$).[7,8] We have previously established *in silico* criteria to select suitable peptides for the precise and sensitive quantification of target proteins,[7,9] and we showed that the combination of these *in silico* criteria with SWATH/SRM analysis enabled successful identification of blood biomarkers for glioblastoma.[10]

Risk prediction markers should ideally be validated in a new cohort different from the original one used to identify them, in order to confirm their value in clinical practice. However, it is difficult to collect sufficient numbers of blood samples from pregnant women who subsequently develop PE. Strategies are available for internal validation in a cohort of limited size, including hold-out validation, leave-one-out cross validation and single or multiple-iteration K-fold cross validation. Among them, multiple-iteration K-fold cross-validation is a highly reliable approach.[11] We considered that biomarker candidates validated in this way by internal validation followed by external validation would be likely to work in clinical practice. A birth and three-generation cohort study was established by the Tohoku Medical Megabank Organization (ToMMo) (TMM BirThree Cohort Study) in order to elucidate the mechanisms of complicated multifactorial diseases in mothers and children in the wake of the Great East Japan Earthquake in 2011.[12,13] The epidemiological data of this large-scale cohort is well-organized on the basis of extensive questionnaire surveys and accurate clinical records including birth outcomes. Therefore, we considered that this large-scale cohort would be a useful resource for identifying risk prediction markers of PE.

Based on the above considerations, we chose to use the combination of SWATH-MS-based discovery and SRM-based internal and external validations using highly reliable peptides (Fig. 1) for the proteomics-based identification of candidate blood biomarkers to predict the risk of PE. The purpose of the present study was to apply this strategy according to the flowchart in Fig. 1, using plasma samples obtained at gestational weeks 14–24, in order to identify and validate risk predictors of PE.

MATERIALS AND METHODS

Ethics Statement Written informed consent was obtained from each subject. The research protocols for the present study were reviewed and approved by the Ethics Committee of ToMMo (2014-0002-2, 2019-4-006, and 2019-4-035). Additionally, the study's progress has been periodically monitored and reviewed by the committee specifically set up in ToMMo. This study was conducted in accordance with the Declaration of Helsinki.

Materials The stable isotope-labeled and unlabeled peptides listed in Supplementary Table 1 were synthesized at SCRUM, Inc. (Tokyo, Japan) or COSMO BIO Inc. (Tokyo and Sapporo, Japan). All other reagents were commercial products of analytical grade.

Clinical Samples Plasma samples (summarized in Table 1) were obtained from ToMMo biobank.[12,13] Control samples were randomly selected from uncomplicated healthy pregnancies. PE was diagnosed according to the guideline of the American College of Obstetricians and Gynecologists.[14] For PE subjects, plasma samples were taken before onset of PE. In Table 1, the samples used for candidate screening, internal validation and external validation were independent (no overlap). The plasma samples were anonymized, and there was no information about the institutes where the plasma samples had been collected.

Screening of Candidate Predictive Biomarkers for PE by Comprehensive SWATH-MS Analysis Plasma samples from the discovery phase, which consists of 14 healthy pregnant women and 7 subjects who subsequently developed PE, were randomly selected and screened for candidate PE biomarkers. They were prepared for LC-tandem-mass spectrometry (LC-MS/MS) analysis as described previously,[15] with minor modifications. Five-fold-diluted plasma samples (10 μL) were solubilized in 8 M urea in 100 mM Tris–HCl (pH 8.5), and *S*-carbamoylmethylated with dithiothreitol and iodoacetamide as described.[15] The *S*-carbamoylmethylated samples were diluted with 100 mM Tris–HCl (pH 8.5) so that the urea concentration was 1.2 M or less, and then treated with lysyl

Table 1. Clinical Characteristics of Study Samples

	Candidate screening phase		Internal validation phase		External validation phase	
	Control	PE	Control	PE	Control	PE
	($n = 14$)	($n = 7$)	($n = 120$)	($n = 36$)	($n = 54$)	($n = 36$)
Gestational week at blood collection	19 ± 4	19 ± 4	18 ± 3	19 ± 3	19 ± 2	19 ± 2
Gestational week at delivery	39.2 ± 1.2	29.4 ± 1.6*	39 ± 1.3	35.5 ± 4.3*	39.1 ± 1.0	38.2 ± 1.7*
Birth weight (BW) (g)	3044 ± 251	1084 ± 232*	3048 ± 329	2373 ± 906*	3031 ± 365	3016 ± 481
Percentile of BW	52.5 ± 24.5	14.1 ± 9.8*	56.4 ± 27.6	47.8 ± 36.8	50.9 ± 29.1	55.9 ± 30
Standard deviation of BW	0.168 ± 0.902	−1.28 ± 0.73*	0.226 ± 0.923	−0.245 ± 1.530	0.0856 ± 1.0808	0.326 ± 1.191
Maternal age	30 ± 5	36 ± 4	31 ± 5	31 ± 6	30 ± 4	29 ± 5
Maternal height (cm)	158 ± 5	157 ± 6	158 ± 6	158 ± 6	159 ± 4	158 ± 6
Maternal body weight (kg)	52.8 ± 5.9	56.8 ± 14.0	53 ± 7.3	59.9 ± 11.4*	53.3 ± 7.9	58.3 ± 12.8
Maternal BMI	21.2 ± 2.8	23 ± 4.5	21.2 ± 2.7	24.1 ± 4.5*	21.2 ± 3.0	23.4 ± 5.6
Gravidity	1.57 ± 1.16	1.71 ± 0.76	1.47 ± 1.61	1.43 ± 1.33	1.26 ± 1.49	1.36 ± 1.22
Parity	1.07 ± 1.00	1.29 ± 0.76	0.892 ± 1.027	0.657 ± 1.027	0.778 ± 0.984	0.833 ± 0.971
Period from blood collection to PE onset	—	10.4 ± 4.1	—	16.9 ± 5.7	—	19.5 ± 2.7

Clinical characteristics of study samples were summarized. Control samples were randomly selected from uncomplicated healthy pregnancies. PE was diagnosed according to the guideline of the American College of Obstetricians and Gynecologists.[14] For PE subjects, plasma samples were taken before onset of PE. The period from blood collection to PE onset was calculated by subtracting the gestational week at blood collection from that at delivery, because that at delivery approximates that of PE onset. The data represent the mean ± standard deviation (S.D.) *n* means the number of subjects. *$p < 0.01$, significantly different from the corresponding control group (Student's *t*-test).

endopeptidase (Wako Pure Chemical Corporation, Osaka, Japan) at an enzyme/substrate ratio of 1 : 100 at 30 °C for 3 h. Next, the samples were digested with sequence-grade modified trypsin (Promega, Madison, WI, U.S.A.) at an enzyme/substrate ratio of 1 : 100 at 37 °C for 16 h. The tryptic digests were cleaned up with a self-packed SDB-XD 200 μL tip (3 M, MN, U.S.A.). The samples were acidified with formic acid and subjected to SWATH-MS analysis.

SWATH-MS analysis (Fig. 1) was performed by coupling a nano-LC ultra 2D plus (Eksigent Technologies, Dublin, CA, U.S.A.) to an electrospray-ionization Triple TOF 5600 mass spectrometer (SCIEX, Framingham, MA, U.S.A.). SWATH-MS measurement (twice per subject) and peptide identification were performed as previously described.[10] Using a cHiPLC nanoflex system (Eksigent Technologies) with the nano-LC, injected peptides were loaded onto a trap column (200 μm × 6 mm, ReproSil-Pur 3 μm, C18-AQ 120 A˚) (Eksigent Technologies) and separated on an analytical column

Table 2. The PE/Healthy Abundance Ratios of Peptides That Showed >1.5-Fold Difference between the PE and Healthy Groups at Gestational Week 15–16 or 24 for the 11 Candidate Proteins Discovered by SWATH-MS Analysis

Uniprot accession No.	Protein	Peptide sequence	Precursor charge	PE/healthy abundance ratio			
				Gestational week			
				15–16	18	21	24
>1.5-Fold upregulated in PE group both at week 15–16 and week 24							
None	None	None	—	—	—	—	—
>1.5-Fold downregulated in PE group both at week 15–16 and week 24							
Q9UGM5	Fetuin-B (FETUB)	LVVLPFPK	2	0.347	1.008	0	0.448
P08185	Corticosteroid-binding globulin (CBG)	WSAGLTSSQVDLYIPK	3	0.427	0	3.238	0.332
>1.5-Fold upregulated at week 15–16 and >1.5-fold downregulated at week 24 in the PE group							
P04278	Sex hormone-binding globulin (SHBG)	TWDPEGVIFYGDTNPK	2	1.735	2.137	1.236	0.480
P04196	Histidine-rich glycoprotein (HRG)	DSPVLIDFFEDTER	3	1.503	1.047	0.268	0.599
>1.5-Fold downregulated at week 15–16 and >1.5-fold upregulated at week 24 in the PE group							
P20742	Pregnancy zone protein (PZP)	TLLVEAEGIEQEK	2	0.037	0.421	1.153	2.612
P20742	Pregnancy zone protein (PZP)	VFTVHPNLC[CAM] FHYSWVAEDHQGAQHTANR	6	0.074	0.468	1.451	2.301
P20742	Pregnancy zone protein (PZP)	AVGYLITGYQR	3	0.296	0.456	1.579	2.567
P01877	Ig alpha-2 chain C region (IGHA2)	HYTNPSQDVTVPC[CAM]PVPPPPPC[CAM] C[CAM]HPR	4	0.365	1.228	0.877	2.879
P01877	Ig alpha-2 chain C region (IGHA2)	HYTNPSQDVTVPC[CAM]PVPPPPPC[CAM] C[CAM]HPR	5	0.372	1.412	0.914	2.796
P20742	Pregnancy zone protein (PZP)	FEIENC[CAM]LANK	2	0.373	0.183	1.068	2.528
P20742	Pregnancy zone protein (PZP)	HQDGSYSTFGER	3	0.398	0.553	1.442	2.098
P20742	Pregnancy zone protein (PZP)	DLFHC[CAM]VSFTLPR	2	0.407	0	1.518	2.821
P20742	Pregnancy zone protein (PZP)	GSFALSFPVESDVAPIAR	2	0.410	1.070	1.458	2.223
P20742	Pregnancy zone protein (PZP)	AVGYLITGYQR	2	0.412	0.537	1.457	2.167
P20742	Pregnancy zone protein (PZP)	TLLVEAEGIEQEK	3	0.416	0	1.387	3.473
P20742	Pregnancy zone protein (PZP)	ALLAYAFSLLGK	2	0.454	0.613	1.664	2.465
P20742	Pregnancy zone protein (PZP)	ASPAFLASQNTK	2	0.460	0.440	1.436	2.003
P20742	Pregnancy zone protein (PZP)	DLFHC[CAM]VSFTLPR	3	0.470	0.479	1.456	2.340
P20742	Pregnancy zone protein (PZP)	IAQWQSLK	2	0.485	0.465	1.386	2.135
P20742	Pregnancy zone protein (PZP)	LEAGINQLSFPLSSEPIQGSYR	3	0.492	0.479	1.326	2.615
P20742	Pregnancy zone protein (PZP)	SLFTDLVAEK	2	0.497	0.475	1.385	2.328
P02751	Fibronectin (FINC)	ITYGETGGNSPVQEFTVPGSK	3	0.513	0.991	0	2.535
P20742	Pregnancy zone protein (PZP)	IQHPFTVEEFVLPK	3	0.534	0.451	1.393	2.137
P20742	Pregnancy zone protein (PZP)	ISEITNIVSK	2	0.546	0.461	1.645	2.205
P01877	Ig alpha-2 chain C region (IGHA2)	DLC[CAM]GC[CAM]YSVSSVLPGC[CAM] AQPWNHGETFTC[CAM]TAAHPELK	4	0.551	1.154	1.153	1.848
P20742	Pregnancy zone protein (PZP)	VVVQTESGGR	2	0.580	0.574	1.332	2.129
>1.5-Fold upregulated at week 15–16 in the PE group (<1.5-fold changed at week 24)							
P01860	Ig gamma-3 chain C region (IGHG3)	TPEVTC[CAM]VVVDVSHEDPEVQFK	4	2.298	0.946	1.774	0.707
P01860	Ig gamma-3 chain C region (IGHG3)	TPEVTC[CAM]VVVDVSHEDPEVQFK	3	2.044	0.993	1.277	0.697
P01860	Ig gamma-3 chain C region (IGHG3)	TPLGDTTHTC[CAM]PR	2	1.992	0.876	1.320	0.755
P01860	Ig gamma-3 chain C region (IGHG3)	TPLGDTTHTC[CAM]PR	3	1.948	0.932	1.122	0.682
P01860	Ig gamma-3 chain C region (IGHG3)	SC[CAM]DTPPPC[CAM]PR	2	1.924	1.125	1.308	0.752
>1.5-Fold downregulated at week 15–16 in the PE group (<1.5-fold changed at week 24)							
P01877	Ig alpha-2 chain C region (IGHA2)	DASGATFTWTPSSGK	2	0.438	0.973	0.894	1.272
P02751	Fibronectin (FINC)	NLQPASEYTVSLVAIK	2	0.477	0.933	8.515	0.937

Table 2. Continued

Uniprot accession No.	Protein	Peptide sequence	Precursor charge	PE/healthy abundance ratio			
				Gestational week			
				15–16	18	21	24
>1.5-Fold upregulated at week 24 in the PE group (<1.5-fold changed at week 15–16)							
P02679-2	Fibrinogen gamma chain (FIBG)	LTYAYFAGGDAGDAFDGFDFGDDPSDK	3	1.091	1.141	0.596	4.108
P02751	Fibronectin (FINC)	TGLDSPTGIDFSDITANSFTVHWIAPR	3	1.351	0.328	1.190	2.229
P02751	Fibronectin (FINC)	IGDQWDK	2	1.033	0	N.D.	2.176
P02751	Fibronectin (FINC)	VPGTSTSATLTGLTR	3	1.041	1.371	0	2.072
P02751	Fibronectin (FINC)	EESPLLIGQQSTVSDVPR	3	1.143	0.987	0.728	2.046
P02751	Fibronectin (FINC)	SYTITGLQPGTDYK	2	1.184	0.961	1.486	1.972
P02751	Fibronectin (FINC)	NTFAEVTGLSPGVTYYFK	2	1.350	0.981	3.338	1.883
P02679-2	Fibrinogen gamma chain (FIBG)	VAQLEAQC[CAM]QEPC[CAM]K	2	1.256	1.002	1.108	1.759
P02751	Fibronectin (FINC)	SSPVVIDASTAIDAPSNLR	2	1.325	0.856	1.890	1.741
P02751	Fibronectin (FINC)	GEWTC[CAM]IAYSQLR	3	1.141	0.791	1.186	1.705
P43652	Afamin (AFAM)	AIPVTQYLK	2	0.846	1.571	1.483	1.638
P02751	Fibronectin (FINC)	VVTPLSPPTNLHLEANPDTGVLTVSWER	4	1.216	0.895	1.636	1.514
>1.5-Fold downregulated at week 24 in the PE group (<1.5-fold changed at week 15–16)							
P08185	Corticosteroid-binding globulin (CBG)	SETEIHQGFQHLHQLFAK	5	1.338	5.151	2.045	0.208
P02751	Fibronectin (FINC)	EATIPGHLNSYTIK	3	1.183	2.042	0.428	0.259
P04196	Histidine-rich glycoprotein (HRG)	DSPVLIDFFEDTER	2	1.043	1.047	0.534	0.308
P04278	Sex hormone-binding globulin (SHBG)	VVLSSGSGPGLDLPLVLGLPLQLK	3	1.010	1.119	0.876	0.471
P08185	Corticosteroid-binding globulin (CBG)	SETEIHQGFQHLHQLFAK	3	0.848	3.528	2.827	0.487
P08185	Corticosteroid-binding globulin (CBG)	WSAGLTSSQVDLYIPK	2	0.882	2.037	1.740	0.551
P08185	Corticosteroid-binding globulin (CBG)	EENFYVDETTVVK	2	1.140	3.856	0	0.564
P04278	Sex hormone-binding globulin (SHBG)	IALGGLLFPASNLR	3	0.710	0.906	0.821	0.627
P08185	Corticosteroid-binding globulin (CBG)	IVDLFSGLDSPAILVLVNYIFFK	3	0.915	2.046	1.874	0.631
P06727	Apolipoprotein A-IV (APOA4)	LAPLAEDVR	2	0.762	0.712	0.739	0.637
P04196	Histidine-rich glycoprotein (HRG)	GGEGTGYFVDFSVR	2	1.039	1.107	0.458	0.643

The tryptic digests of plasma samples collected at gestational weeks 15–24 from 14 healthy pregnant women and 7 who became PE later (candidate screening phase) were measured by LC-MS/MS in the SWATH-MS mode with two replicates (each peptide was quantified with 3 to 6 transitions). According to the workflow shown in Fig. 1, 11 proteins were discovered as biomarker candidates to predict the onset of PE. For the 11 proteins, the PE/Healthy abundance ratios of peptides which showed >1.5-fold difference between the PE and healthy groups at gestational weeks 15-16 or 24 were calculated at each gestational week (15–16, 18, 21, and 24). The number of PE subjects (the plasma samples were taken before onset of PE) was 3, 1, 1 and 2 at gestational weeks 15–16, 18, 21, and 24, respectively. The numbers of healthy subjects were 6, 2, 2 and 4 at gestational weeks 15–16, 18, 21, and 24, respectively. C[CAM] represents a carbamoyl-methylated cysteine residue. N.D. indicates that the peptide was not detected in the healthy group. Zero indicates that the peptide was not detected in the PE group.

(75 μm × 15 cm, ReproSil-Pur 3 μm, C18-AQ 120 A°) (Eksigent Technologies). The flow rates were 2 μL/min (six minutes runtime) for loading on the trap column and 300 nL/min for separation on the analytical column. The injected peptides were eluted in (A = 0.1% formic acid in Milli-Q water, B = 0.1% formic acid in 100% acetonitrile) 0–20% B (0–60 min), changed to 20–40% B (60–75 min), increased to 40–100% B (75–77 min), maintained at 100% B (77–82 min), reduced to 0% B (82–84 min), and then maintained at 0% B (84–115 min). SWATH-MS data acquisition was conducted as previously described.[10] Targeted data extraction of DIA samples was performed by using the SWATH-MS Processing Micro App in Peakview (Version 2.0, SCIEX) with an extraction window of 8 min and FDR set at <10%. The FDR was set at <10% in order to discover as many candidates as possible in the screening phase, since promising candidates can be missed with an FDR 1% cut-off in the Peakview software. Among identified peptides, unreliable ones were removed based on data selection (see Supplementary Fig. 1) and amino acid sequence-based peptide selection (Supplementary Table 2) for SWATH-MS (Fig. 1). The peak areas of peptides were compared between PE and healthy pregnant women. DIA raw files were loaded in the Peptide Atlas database with the identifier PASS01451.

Peptide Selection for the Validation Study of 11 Candidate Proteins by Means of SRM Analysis Peptide probes for the reliable quantification of the 11 candidate proteins by SRM analysis were selected as follows (Fig. 1). The peptides of the 11 candidate proteins listed in Table 2 satisfied the peptide selection criteria for SWATH-MS analysis (Supplementary Table 2), but we adopted more stringent peptide selection criteria for SRM analysis (Supplementary Table 3) to find the most accurate, reproducible and sensitive peptide probes among the peptides listed in Table 2. For each candidate protein, if only one peptide (among the peptides in Table 2) satisfied the SRM peptide selection criteria, it was selected for the SRM analysis. When two or more peptides (among the peptides in Table 2) satisfied the SRM peptide selection criteria for a candidate protein, we selected the peptide that showed the largest difference in the abundance level between PE and healthy subjects at gestational week 15–16 or 24. If no peptide listed in Table 2 satisfied the SRM peptide selection criteria, a peptide meeting the SRM peptide selection criteria was chosen from among all tryptic peptides of the target protein. The peptide probes thus selected for 11 candidate proteins were chemically synthesized with stable isotope labeling at C-terminal arginine or lysine (Supplementary Table 1), and used as internal standards in the following SRM analysis.

LC-MS/MS-Based Accurate Protein Quantification of 11 Candidate Proteins in the SRM Mode Plasma samples from the internal validation phase, which consists of 120 healthy pregnant women and 36 subjects who subsequently developed PE (156 subjects in total), were randomly selected and used for the internal validation of candidate proteins. Plasma samples from the external validation phase, which consists of 54 healthy pregnant women and 36 subjects who subsequently developed PE (90 subjects in total), were randomly selected and used for the external validation of candidate proteins. They were prepared for LC-MS/MS analysis as described in the previous section "Screening of candidate predictive biomarkers for PE by comprehensive SWATH-MS analysis." After trypsin digestion, the digests were mixed with stable-isotope-labeled peptide mixture as internal standard peptides and then cleaned up with SDB-Tip and GC-Tip (GL Science, Tokyo, Japan). The samples were acidified with formic acid and subjected to LC-MS/MS analysis.

Simultaneous quantification of the 11 (internal validation) or 3 (external validation) candidate proteins was performed with the microLC-QTRAP 5500 system (Fig. 1). The micro-LC system (eksigent ekspert microLC 200; SCIEX) was equipped with a C18 column (HALO C18, 0.5 mm i.d. $\times$ 100 mm, 2.7 μm particles; SCIEX) and coupled to an electrospray ionization (ESI)-triple quadrupole mass spectrometer (QTRAP 5500; SCIEX). The digested peptides were separated and eluted (55 min run time at a flow rate of 10 μL/min) as follows: maintained at 1% B (0–2 min), increased from 2 to 30% B (2–32 min), increased from 30 to 100% B (32–35 min), maintained at 100% B (35–37 min), reduced from 100 to 1% B (37–39 min), and then maintained at 1% B (39–55 min). Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. As listed in Supplementary Table 1, four transitions for each target peptide (selected as described in the previous section) were monitored in the positive electrospray SRM mode in the QTRAP 5500. The corresponding four transitions were monitored for the stable-isotope-labeled internal standard peptide. The SRM quantification data was extracted with Skyline software.[16] A peak with an area of over 1000 counts, detected at the same retention time as the stable-isotope-labeled peptide, was defined as a positive peak. The quantitative value was calculated as the peak area count ratio (unlabeled peptide/labeled peptide) in each of the 4 transitions, and the peptide abundance level was calculated as the mean of the quantitative values determined with the 4 transitions. The quantitative values of peptides that were not detected were taken as 0.

Statistical Analysis for Internal Validation To determine the candidate proteins that would be used in each combination of 11, 10, 9, 8, 7, 6, 5, 4, 3, and 2 proteins, Bayesian information criterion (BIC)-based backward elimination was conducted (Fig. 1). Multivariate logistic regression analysis was conducted using the data of all 156 subjects (internal validation phase) to maximize the predictive performance of the combination of multiple candidate proteins for the onset of PE. In the case of the 10-protein combination (1 protein removed), the BIC values were calculated for all 11 combinations of 10 out of 11 candidate proteins, and then the 10-protein set with the minimum BIC value was selected. The 9-protein set was similarly determined from the 10 proteins selected above. The 8-, 7-, 6-, 5-, 4-, 3-, and 2-protein sets were also similarly determined.

For the 11, 10, 9, 8, 7, 6, 5, 4, 3, and 2 protein sets, 30 iterations of 10-fold cross-validation[17] were performed as follows (Fig. 1; the flow diagram is shown in Supplementary Fig. 2). [First step] The internal validation subjects (36 PE and 120 healthy pregnant women (for PE subjects, plasma samples were taken before onset of PE)) were randomly divided into 10 subgroups. The numbers of healthy and PE subjects (for PE subjects, plasma samples were taken before onset of PE) were the same among different subgroups. One subgroup was used as the Test set, and the remaining 9 subgroups were used for the Train set. Multivariate logistic regression analysis was performed with the Train set to build a predictive model for PE. Receiver operating characteristics (ROC) analysis was performed with the Test set to assess the predictive model created with the Train set. ROC curves were created by plotting the sensitivity (y-axis) and 1-specificity (x-axis) at various thresholds, and the values of the area under the curve (*AUC*) were calculated. This process was also conducted in the other 9 combinations of Train and Test sets. The mean of the 10 *AUC*s was calculated. [Second step] We repeated this processing 30 times; in other words, we divided the same 156 pregnant women (36 PE and 120 healthy (for PE subjects, plasma samples were taken before onset of PE)) into 10 subgroups at random followed by 10-fold cross validation as described above, 30 times. [Third step] The mean of the "mean 10-fold *AUC*" of the 30 repeats was calculated. [Additional step] In addition to the *AUC* calculation using the Test set, the mean of the "mean 10-fold *AUC*" of 30 trials for the Train set was also calculated using the *AUC* values obtained when ROC analysis to assess the predictive model created with the Train set was conducted using the Train set itself, for reference. R language (version 4.0.4) was used for the above analysis.

Statistical Analysis for External Validation of the Predictive Model Generated Using Internal Validation Subjects Multivariate logistic regression analysis was conducted using the protein abundance levels from all 156 subjects (internal validation phase) to generate a predictive model for PE with the combination biomarker of afamin (AFAM), fibronectin (FINC) and sex hormone-binding globulin (SHBG) (Fig. 1). The generated predictive model (predictive equation and cut-off value) was applied to the external validation phase (Fig. 1). The ROC analysis was performed using the probability calculated by the generated predictive equation, and then the *AUC* was calculated. The cut-off value determined in the internal validation phase was applied to external validation phase to calculate the sensitivity, specificity and odds ratio. R language (version 4.0.4) was used for the above analysis.

RESULTS

Clinical Characteristics of Study Samples Maternal and infant characteristics are summarized in Table 1. Women who developed PE did not significantly differ from women with healthy pregnancies as regards gestational week at blood collection, maternal age, height, body weight, body mass index (BMI), gravidity and parity in each study phase. The PE subjects (the plasma samples were taken before onset of PE) in the candidate screening phase had remarkably shorter gestational week at delivery and remarkably smaller birth weight than healthy pregnancies. By contrast, the PE subjects

(the plasma samples were taken before onset of PE) in the internal and external validation phases had relatively similar gestational week at delivery and birth weight to the healthy pregnancies.

SWATH-MS-Based Biomarker Candidate Screening for PE (Fig. 1, Steps 1–4) Figure 1 summarizes the strategy for discovery and validation of PE predictive markers, as well as the results. To identify biomarker candidate proteins for predicting PE, SWATH-MS analysis was performed using plasma samples collected at gestational weeks 15–24 from 14 healthy pregnant women and 7 who were later diagnosed as PE (candidate screening phase). Eighteen thousand two hundred eighty-nine peptides (3031 proteins) were identified at FDR <10% (Fig. 1, Step 1). These SWATH-MS data include unreliable data and peptides, and the reliable data and peptides were extracted from them (Fig. 1, Step 2) using the data-selection criteria shown in Supplementary Fig. 1 and the amino acid sequence-based peptide selection criteria shown in Supplementary Table 2. In this way, 308 proteins (1268 peptides) were identified as reliable data (Supplementary Table 4). To identify up- or down-regulated proteins, we calculated the PE/Healthy abundance ratios of individual peptides in each gestational week (15–16, 18, 21, and 24), as shown in Supplementary Fig. 1. Among the 308 proteins, 196 proteins (1027 peptides) (Supplementary Table 5) were selected as up- or down-regulated proteins having at least one peptide for which the PE/Healthy abundance ratio was >1.5 or <0.66 ($p < 0.05$) at either gestational week 15–16 or 24 (Fig. 1, Step 3). To select potential PE biomarker candidates among them, we searched the literature to find proteins likely to be involved in PE biology or whose expression levels change in PE, including late onset PE, and related diseases (Fig. 1, Step 4). Based on the rationales summarized in Supplementary Table 6, we selected 11 proteins.

For these 11 candidate proteins, the peptides whose PE/Healthy abundance ratio is >1.5 or <0.66 ($p < 0.05$) at either gestational week 15–16 or 24 are listed in Table 2. (1) The peptide of fetuin-B (FETUB) showed PE/Healthy abundance ratios of less than 1 at gestational weeks 15–16, 21, and 24. (2) Corticosteroid-binding globulin (CBG) had 4 peptides whose abundance levels were downregulated in the PE group, especially at gestational week 24. (3) For SHBG, all 3 peptides in Table 2 were reduced in the PE group at gestational week 24. (4) The 2 peptides of histidine-rich glycoprotein (HRG) were downregulated in the PE group at gestational weeks 21 and 24. (5) For pregnancy zone protein (PZP), 15 peptides listed in Table 2 showed PE/Healthy abundance ratios of less than 1 at gestational weeks 15–16 and 18, but greater than 1 at gestational weeks 21 and 24. (6) Immunoglobulin (Ig) alpha-2 chain C region (IGHA2) had 3 peptides with PE/Healthy abundance ratios of less than 1 at gestational week 15–16, but greater than 1 at gestational week 24. (7) The 3 peptides of Ig gamma-3 chain C region (IGHG3) were upregulated in the PE group at gestational weeks 15–16. (8) The 12 peptides listed in Table 2 for FINC were upregulated in the PE group at gestational week 24. (9) Fibrinogen gamma chain (FIBG) had 2 peptides, and the levels were upregulated in the PE group at gestational week 24. (10) One peptide is listed in Table 2 for AFAM, and it was continuously upregulated in the PE group at gestational weeks 18, 21, and 24. (11) Apolipoprotein A-IV (APOA4) also had 1 peptide, which showed a PE/healthy abundance ratio of around 0.7 at all gestational weeks tested.

More Reliable Peptide Selection and Quantification of the 11 Candidate Proteins by LC-MS/MS with SRM Analysis (Fig. 1, Steps 5–6) As we cannot exclude false-positive detection and the possibility of inaccurate peak areas due to sample-dependent ion-suppression in SWATH-MS analysis, we conducted SRM analysis of the 11 candidate proteins using stable-isotope-labeled peptides as internal standards for the internal validation phase (plasma samples collected at gestational weeks 14–24 from 120 healthy pregnant women and 36 women who were subsequently diagnosed as PE), which is different from the candidate screening phase, in order to evaluate the reliability and validity of the 11 candidate proteins as predictive markers of PE.

The criteria shown in Supplementary Table 3 were applied to the peptides shown in Table 2 to select suitable peptides for SRM quantification (Fig. 1, Step 5), as described in Materials and Methods. The peptides selected for SRM analysis are listed in Table 3. The target peptides of AFAM, FETUB, HRG, IGHA2, IGHG3, PZP, and SHBG were selected from Table 2,

Table 3. The Peptide Sequences of 11 Candidate Proteins Selected by More Reliable *in Silico* Peptide Selection Criteria for SRM Analysis

Uniprot accession No.	Protein name (abbreviation)	Sequence of target peptide probe	Sequence of Internal standard (IS) peptide probe	Peptide included in Table 2
P43652	Afamin (AFAM)	AIPVTQYLK	AIPVTQYL**K***	✓
P06727	Apolipoprotein A-IV (APOA4)	LTPYADEFK	LTPYADEF**K***	
P08185	Corticosteroid-binding globulin (CBG)	GTWTQPFDLASTR	GTWTQPFDLAST**R***	
Q9UGM5	Fetuin-B (FETUB)	LVVLPFPK	LVVLPFP**K***	✓
P02679-2	Fibrinogen gamma chain (FIBG)	AIQLTYNPDESSK	AIQLTYNPDESS**K***	
P02751	Fibronectin (FINC)	STTPDITGYR	STTPDITGY**R***	
P04196	Histidine-rich glycoprotein (HRG)	GGEGTGYFVDFSVR	GGEGTGYFVDFSV**R***	✓
P01877	Ig alpha-2 chain C region (IGHA2)	DASGATFTWTPSSGK	DASGATFTWTPSSG**K***	✓
P01860	Ig gamma-3 chain C region (IGHG3)	TPLGDTTHTC[CAM]PR	TPLGDTTHTC[CAM]P**R***	✓
P20742	Pregnancy zone protein (PZP)	ASPAFLASQNTK	ASPAFLASQNT**K***	✓
P04278	Sex hormone-binding globulin (SHBG)	TWDPEGVIFYGDTNPK	TWDPEGVIFYGDTNP**K***	✓

The peptides satisfying the more reliable SRM peptide selection criteria (Supplementary Table 3) were selected from those listed in Table 2. For each candidate protein, if only one peptide (among the peptides in Table 2) satisfied the SRM peptide selection criteria, it was selected as the peptide probe of the candidate protein for SRM analysis. When two or more peptides (among the peptides in Table 2) satisfied the SRM peptide selection criteria for each candidate protein, the peptide that showed the largest difference in the abundance level between PE and healthy subjects at gestational week 15–16 or 24 was selected. If no peptide listed in Table 2 satisfied the SRM peptide selection criteria, the peptide that satisfies the SRM peptide selection criteria among all tryptic peptides of target protein was selected as the peptide probe for SRM analysis. Bold letters with asterisks represent amino acids labeled with stable isotope (^{13}C and ^{15}N). C[CAM] represents a carbamoyl-methylated cysteine residue.

Table 4. Bayesian Information Criterion-Based Backward Elimination Followed by 30 Iterations of 10-Fold Cross-Validation of 11- to 2-Protein Combinations as the Biomarker to Predict the Onset of PE

Number of proteins	Combination of proteins	BIC	Mean *AUC* of test set	± S.D.	Mean *AUC* of train set	± S.D.
11	AFAM + APOA4 + FETUB + FIBG + IGHA2 + IGHG3 + PZP + CBG + FINC + HRG + SHBG	193.96	0.730	0.0240	0.811	0.0008
10	AFAM + APOA4 + FETUB + FIBG + IGHA2 + PZP + CBG + FINC + HRG + SHBG	189.00	0.741	0.0245	0.810	0.0011
9	AFAM + APOA4 + FETUB + IGHA2 + PZP + CBG + FINC + HRG + SHBG	184.46	0.744	0.0194	0.812	0.0007
8	AFAM + APOA4 + FETUB + PZP + CBG + FINC + HRG + SHBG	180.35	0.752	0.0242	0.804	0.0007
7	AFAM + FETUB + PZP + CBG + FINC + HRG + SHBG	176.59	0.741	0.0206	0.792	0.0009
6	AFAM + FETUB + CBG + FINC + HRG + SHBG	172.90	0.749	0.0256	0.783	0.0008
5	AFAM + CBG + FINC + HRG + SHBG	169.18	0.752	0.0267	0.771	0.0007
4	AFAM + CBG + FINC + SHBG	165.77	0.754	0.0266	0.769	0.0006
3	AFAM + FINC + SHBG	163.09	0.739	0.0196	0.746	0.0005
2	FINC + SHBG	164.80	0.716	0.0255	0.701	0.0006

The sets of 11, 10, 9, 8, 7, 6, 5, 4, 3, and 2-proteins were determined by bayesian information criterion (BIC)-based backward elimination with multivariate logistic regression analysis using the data of protein abundance levels in 120 healthy and 36 PE subjects (internal validation phase) (for PE subjects, plasma samples were taken before onset of PE) as described in the materials and methods. In the backward elimination, the protein combination which gave the minimum BIC value was selected in each set of 11, 10, 9, 8, 7, 6, 5, 4, 3 and 2 proteins. For the 11- to 2-protein sets determined, 30 iterations of 10-fold cross-validation were performed according to the flow diagram shown in Supplementary Fig. 2. The mean of "mean 10-fold *AUC*" of 30 trials is described as "mean *AUC* of test set" in this table. In addition to the *AUC* calculation using the test set, the mean of "mean 10-fold *AUC*" of 30 trials for the train set was also calculated using the *AUC* values obtained when the ROC analysis to assess the predictive model created with the train set was conducted using the train set itself; this is described as "mean *AUC* of train set" in this table. *AUC*, area under the curve of ROC curve; S.D., standard deviation. AFAM, afamin; APOA4, apolipoprotein A-IV; CBG, corticosteroid-binding globulin; FETUB, fetuin-B; FIBG, fibrinogen gamma chain; FINC, fibronectin; HRG, histidine-rich glycoprotein; IGHA2, Ig alpha-2 chain C region; IGHG3, Ig gamma-3 chain C region; PZP, pregnancy zone protein; SHBG, sex hormone-binding globulin.

whereas those of APOA4, CBG, FIBG, and FINC were newly designed. The chemically synthesized stable-isotope-labeled peptides (Table 3) were spiked in the tryptic digests of 156 plasma samples in the internal validation phase, and then the 11 peptides (11 proteins) were quantified using the SRM mode of the microLC-QTRAP5500 system (Fig. 1, Step 6). As shown in Supplementary Table 7, all 11 peptides were successfully quantified.

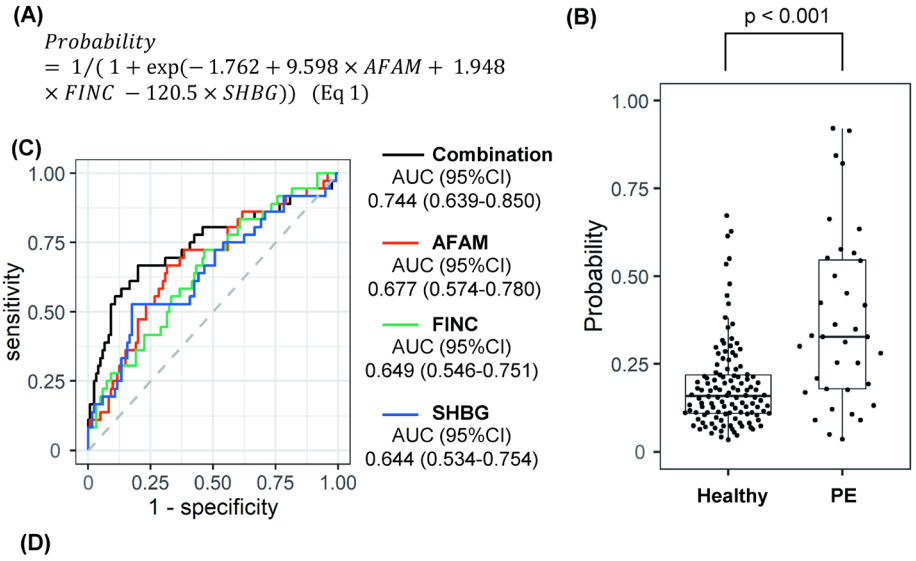
Selection of Optimum Protein Combination and Internal Validation by Means of 30 Iterations of 10-Fold Cross Validation (Fig. 1, Step 7) To select the optimum protein combination among the 11 candidate proteins (Table 4), we carried out multivariate logistic regression analysis with successive BIC-based backward elimination using the SRM data of all 156 subjects (internal validation phase) as described in the materials and methods. The BIC value gradually decreased as the number of proteins was reduced from 11, and was minimum in the 3-protein set (BIC 163.09), which contained AFAM, FINC and SHBG (Table 4).

In order to evaluate the predictive performance of the sets of 11, 10, 9, 8, 7, 6, 5, 4, 3, and 2 proteins determined above, we carried out 30 iterations of 10-fold cross validation as described in the materials and methods. The mean *AUC* values of 30 trials (test set) are shown in Table 4. The mean *AUC* values were within a range from 0.730 to 0.754 for the 11- to 3-protein combinations. Because the number of proteins in a combination biomarker should be small so that it can be a useful predictor of PE in different cohorts as well, the 3-protein combination (AFAM + FINC + SHBG; $AUC = 0.739$) was considered to be a promising biomarker. For reference, the mean *AUC* of the Train set was also calculated (Table 4) as described in the materials and methods.

Generation of Predictive Model (Predictive Equation and Cut-Off Value) with the AFAM, FINC and SHBG Combination Using the Internal Validation Subjects and Comparison with Each Single Protein (Fig. 1, Step 8) To generate the predictive equation for the AFAM, FINC and SHBG combination, we conducted multivariate logistic regression analysis using the data of 156 subjects (internal validation phase). Using the generated predictive equation (Fig. 2A), the probabilities of PE were calculated for the 156 subjects on the basis of the protein abundance levels (Supplementary Table 7) of AFAM, FINC and SHBG. We found a significant difference between the healthy and PE groups ($p < 0.001$, Fig. 2B), and values were higher in the PE subjects (the plasma samples were taken before onset of PE) than in the healthy subjects over all the gestational weeks examined (Supplementary Fig. 3B).

We performed ROC analysis using the probabilities for the combination of the 3 proteins. For the single proteins, the ROC analysis was conducted using the protein abundance level of each protein in the 156 subjects (Supplementary Fig. 3A, Supplementary Table 7). The *AUC* of the 3-protein combination was 0.744, which was higher than the *AUC* of each of the single proteins (Fig. 2C). The optimum cut-off values to distinguish the PE and healthy subjects were determined to be 0.250, 0.245, 0.271 and 0.0181 for the 3-protein combination, AFAM alone, FINC alone and SHBG alone, respectively (Fig. 2D). When these cut-off values were used, the odds ratio for the 3-protein combination was 8.00, which was 1.52-fold (8.00/5.27) greater than that of the best single protein (Fig. 2D).

External Validation of the Predictive Model Generated Using the Internal Validation Subjects (Fig. 1, Step 9) The predictive equation (Eq. 1 in Fig. 2A) and the cut-off values (Fig. 2D) established using the internal validation subjects were applied to the external validation phase (36 PE and 54 healthy pregnant women (for PE subjects, plasma samples were taken before onset of PE)) to calculate the probability, *AUC*, sensitivity, specificity and odds ratio. The probabilities were significantly different between the healthy and PE groups ($p < 0.001$, Fig. 3A), and were higher in the PE subjects (the



(D)

Name	Cut off	Sensitivity	Specificity	Odds ratio
Combination of 3 proteins (Eq 1)	0.250	0.667	0.800	8.00
AFAM alone	0.245	0.667	0.683	4.32
FINC alone	0.271	0.722	0.533	2.97
SHBG alone	0.0181	0.528	0.825	5.27

Fig. 2. Predictive Model Generated by the Multivariate Logistic Regression Using the Internal Validation Subjects (36 PE and 120 Healthy Women) for AFAM, FINC, and SHBG Combination, and the Comparison with Single Protein

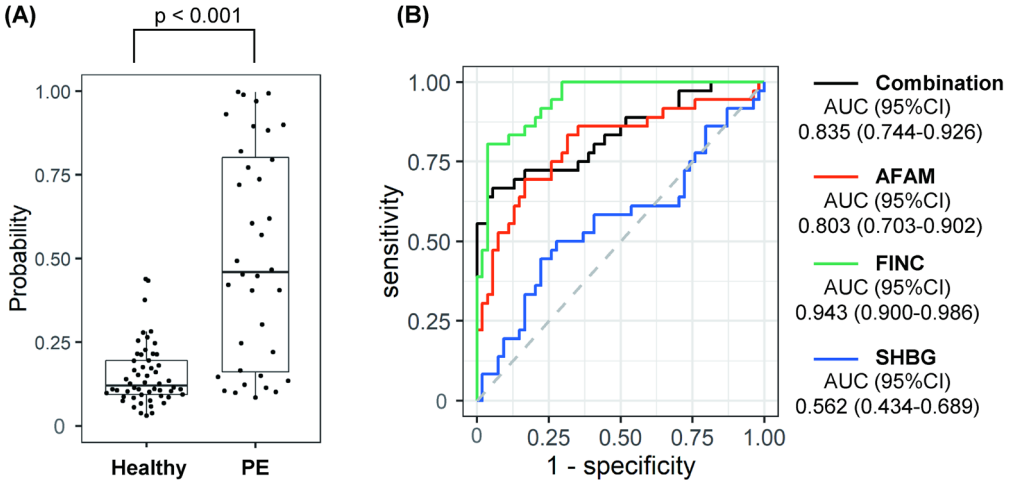
(A) Eq. 1 shows the multivariate logistic regression model (predictive equation) for 3-protein combination (AFAM + FINC + SHBG), which was generated by using the data of protein abundance levels of 3 proteins in all subjects used for internal validation phase (36 PE and 120 healthy pregnant women (for PE subjects, plasma samples were taken before onset of PE)). **(B)** Each dot represents the probability of PE calculated from Eq 1 in an individual subject. $p < 0.001$, significantly different between two groups (Student's *t*-test). **(C)** ROC analysis was performed using the probability (Eq. 1) and the protein abundance level of each protein obtained by SRM analysis (AFAM, FINC, or SHBG). *AUC* is the area under the ROC curve, and the 95% CI is shown. **(D)** The performance to predict the onset of PE for the internal validation subjects (156 subjects) was compared between each protein alone and 3-protein combination model (Eq. 1). The cut off value was determined by the ROC analysis above. Odds ratio was calculated as sensitivity × specificity/(1 − sensitivity) × (1 − specificity). AFAM, afamin; FINC, fibronectin; SHBG, sex hormone-binding protein. (Color figure can be accessed in the online version.)

plasma samples were taken before onset of PE) than in the healthy subjects over all the gestational weeks examined (Supplementary Fig. 4B). The *AUC* of the 3-protein combination was 0.835 (Fig. 3B). For the single proteins, the *AUC* was calculated using the protein abundance level of each protein (Supplementary Table 8, Supplementary Fig. 4A), and the values were 0.803, 0.943, and 0.562 for AFAM alone, FINC alone and SHBG alone, respectively (Fig. 3B). When the cut-off values established using the internal validation subjects were applied to the external validation phase, the odds ratio was 13.43 for the 3-protein combination (Fig. 3C). Interestingly, the odds ratio was infinite for FINC alone.

DISCUSSION

Numerous studies have confirmed that the sFlt1/PlGF ratio is a good predictor of the signs and symptoms of PE.[18–20] However, its usefulness is limited to diagnosis after 20 weeks of gestation.[2,18] Early prediction would be highly desirable to guide therapeutic planning, including low-dose aspirin. In an expert review, Hahn *et al.*[21] pointed out that among many proteomics studies for PE biomarker discovery, only a few have focused on maternal blood samples obtained prior to the onset of symptoms. To address this issue, in the present study we used plasma samples collected at gestational weeks 14–24 weeks from subjects who were subsequently diagnosed as PE. By employing SWATH-based proteomics for comprehensive discovery and SRM-based target quantification using *in silico* peptide selection criteria for validation (Fig. 1), we were able to identify a 3-protein combination biomarker (AFAM, FINC, and SHBG) that can predict effectively during gestational weeks 14–24 whether pregnant women would subsequently develop PE. As shown in Table 1, the periods from blood collection to PE onset were 16.9 and 19.5 weeks for the internal and external validation cohorts, respectively. This would be sufficiently early to guide therapeutic planning, including low-dose aspirin. Because ELISA kits are commercially available for all of these proteins, AFAM, FINC, and SHBG, it should be possible to introduce the 3-protein combination biomarker smoothly into clinical practice in the future.

A major concern with the detection of new proteomic biomarkers is the validation of their usefulness.[21,22] DDA-based proteomics lacks sufficient accuracy and reproducibility of quantification, and so has a high risk of failing to identify promising candidates or mistakenly identifying as candidates proteins whose expression levels actually do not change. Because of this problem, Hahn *et al.*[21] proposed that it would be the best to use methods developed for systems biology analyses, such as SWATH-MS and SRM.[23,24] Another concern is that most articles on PE biomarker identification have not attempted to validate the proposed biomarker candidates.[5] Therefore, in the present work, we used SWATH-MS and SRM,



(C)

Name	Cut off	Sensitivity	Specificity	Odds ratio
Combination of 3 proteins (Eq 1 in Figure 2A)	0.250	0.667	0.870	13.43
AFAM alone	0.245	0.694	0.741	6.49
FINC alone	0.271	1.000	0.685	∞
SHBG alone	0.0181	0.861	0.148	1.08

Fig. 3. External Validation of Predictive Model Generated Using the Internal Validation Subjects

The predictive equation (Eq. 1 in Fig. 2A) and the cut off value (Fig. 2D) which were established using the internal validation subjects were applied to the external validation subjects (36 PE and 54 healthy pregnant women (for PE subjects, plasma samples were taken before onset of PE)), and validated. **(A)** Each dot represents the probability of PE calculated from Eq. 1 (Fig. 2A) in an individual subject. $p < 0.001$, significantly different between two groups (Student's *t*-test). **(B)** ROC analysis was performed using the probability (Fig. 3A) and the protein abundance level of each protein obtained by SRM analysis in the external validation subjects. *AUC* is the area under the ROC curve, and the 95% CI is shown. **(C)** The performance to predict the onset of PE in the external validation subjects was compared between each protein alone and 3-protein combination model. The cut off values determined in Fig. 2D were applied to the external validation subjects to calculate the sensitivity and specificity. Odds ratio was calculated as sensitivity × specificity/(1 − sensitivity) × (1 − specificity). AFAM, afamin; FINC, fibronectin; SHBG, sex hormone-binding protein. (Color figure can be accessed in the online version.)

and conducted the validations in two different subject groups (Fig. 1). For the above reasons, we expect that the established diagnostic method using the AFAM, FINC, and SHBG combination biomarker with the predictive equation Probability = $1/(1 + \exp(-1.762 + 9.598 \times \text{AFAM} + 1.948 \times \text{FINC} - 120.5 \times \text{SHBG}))$, *i.e.*, Eq. 1 in Fig. 2A, and a cut-off value of 0.250 as probability would be useful in clinical practice (*AUC* = 0.835 and Odds ratio = 13.43 in external validation, Fig. 3). Because the accuracy of the candidate screening phase affects the diagnostic performance of candidates at the subsequent validation phase, we believe our use of SWATH-MS in the screening phase of this work was especially advantageous.

In the external validation phase, FINC alone showed a higher predictive performance than the 3-protein combination (Fig. 3). But, in contrast, the *AUC* of FINC alone was small in the internal validation phase (Fig. 2), thereby indicating that its ability to distinguish PE and healthy subjects is low. Based on the clinical characteristics, we found no significant difference between external and internal validation subjects (Table 1). Thus, it is unclear why the result for FINC was significantly different between these two groups, although this result does indicate that these two groups have different feature in terms of the quantitative molecular profile. Nevertheless, the 3-protein combination showed high predictive performance in both validation phases (Table 4, Figs. 2, 3). This suggests that the predictive model using the 3-protein combination can stably predict in different cohorts whether subjects would develop PE later. These results support the value of validating biomarker candidates in two different cohorts to establish robust performance.

In PE, angiogenic imbalance in the placenta causes impaired remodeling of the maternal spiral arteries and endothelial damage, which leads to the release of FINC from the endothelial cells into the maternal blood circulation.[25] The plasma level of FINC is reported to be upregulated at gestational weeks 14–24 or 19–25 in PE.[26,27] However, the predictive performance of FINC for PE has not been validated in a different cohort. We found in the present study that the inter-individual difference in plasma levels of FINC is large, and the range of plasma levels in the PE subjects (the plasma samples were taken before onset of PE) overlapped that in the healthy subjects, especially in the internal validation phase (Supplementary Fig. 3). These large inter-individual differences might explain why FINC alone has not been successfully validated as a biomarker so far. Nevertheless, endothelial damage occurs from the first trimester, so FINC can reasonably be included in a combination biomarker at the early stage before the onset of PE.

AFAM is a vitamin E-binding glycoprotein from human plasma which is also found in extravascular fluids.[28] A significant association between AFAM and vitamin E levels has been found in extravascular fluids, such as follicular fluid.[29] Vitamin E is an antioxidant that blocks the oxidation of lipids.[30] It has been reported that serum AFAM levels are increased in response to various kinds of oxidative stress, for example in the peritoneal fluid of women with endometrio-

sis.[31] Serum AFAM levels are also elevated among patients developing PE compared to controls, but substantial differences are observed among patients with late onset PE.[32] Elevated oxidative stress is a feature of PE, and therefore, the increase of AFAM level could be a compensatory mechanism to transport more antioxidant vitamin E to stressed locations where the vitamin E is rapidly consumed. Thus, AFAM could be a useful component of a combination biomarker.

SHBG is secreted from liver into the blood circulation, and its secretion is inhibited by insulin.[33] In PE, the insulin level is elevated compared to that in healthy pregnant women.[34] Therefore, the decreased level of SHBG in the plasma of PE patients (Supplementary Fig. 3) could be explained by the inhibition of SHBG secretion due to elevated insulin. A validation study of SHBG as a predictive biomarker for PE has been conducted using a test set cohort different from the original one, and *AUC* 0.61 was obtained at gestational weeks 9–13; however, the difference between the PE and healthy groups did not reach statistical significance.[35] The present study gave a similar *AUC* for SHBG alone (*AUC* 0.562, Fig. 3B), but notably, the combination of SHBG with the other 2 proteins in the 3-protein combination biomarker resulted in an *AUC* of 0.835 in the external validation (Fig. 3B), and a statistically significant difference between PE and healthy subjects was obtained in the probability of PE (Fig. 3A) calculated using the predictive equation (Eq.1) in the external validation phase. Therefore, the present study is the first to statistically demonstrate the usefulness of SHBG in PE prediction.

In the present study, the predictive model was established using the plasma samples from all gestational weeks between 14 and 24 weeks without consideration of difference in gestational weeks. This predictive model was validated in the external validation phase, and it clearly distinguished the PE and healthy subjects from gestational week 14 until 24 (Supplementary Fig. 4). The probability values for difference between the PE and healthy subjects were similar at each week between 14 and 24, and since the number of subjects in each week is small, we analyzed all subjects between 14 and 24 weeks together without distinguishing the gestational weeks. It is considered that changes in the blood concentrations of AFAM, FINC, and SHBG are associated with abnormality in the period of placental formation (until 14 weeks). Thus, application of the present predictive model for diagnosis immediately after placental formation (around 14 weeks) would be biologically rational for early prediction of PE risk. The previous biomarker, *i.e.*, sFlt1/PlGF ratio, is used for the prediction of PE onset after 20 weeks. Thus, the predictive model using AFAM, FINC, and SHBG should be able to predict the PE risk earlier than the use of sFlt1/PlGF ratio.

In the cross validation in the present study, the samples were divided into 10 subgroups, even though the number of PE subjects per subgroup then becomes quite small. The reason for this is that taking average values of *AUC*s from 10 iterations repeated 30 times increases the reliability of cross validation. If the number of subgroups is less than 10, the results become less reliable.

The present study has several limitations. First, analyzed samples in screening, internal and external validation phases were randomly selected, however, the number of each sample was relatively small and various biases should be considered. Second, external validation phase was conducted in the same cohort study. Therefore, further validation was needed to be preferred in other datasets from large scale cohort study.

CONCLUSION

In the present study, we employed SWATH-MS-based screening of blood proteins and subsequent SRM-based validation using highly reliable *in silico* peptide selection criteria for proteomics-based identification of blood biomarkers of PE. From among the identified biomarkers, AFAM, FINC and SHBG were selected as the best candidates for a combination biomarker. The 3-protein combination predictive model (predictive equation and cut-off value) generated using the internal validation subjects was successfully validated in a different group of validation subjects and showed good predictive performance, with *AUC* 0.835 and odds ratio 13.43. This combination biomarker is expected to be clinically useful to predict the onset of PE based on analysis of plasma samples obtained at gestational weeks 14–24.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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