Hypertension
Journal of the American Heart Association

Integrated Proteomics Pipeline Yields Novel Biomarkers for Predicting Preeclampsia

Jenny E. Myers, Robin Tuytten, Grégoire Thomas, Wouter Laroy, Koen Kas, Griet Vanpoucke, Claire T. Roberts, Louise C. Kenny, Nigel A.B. Simpson, Philip N. Baker and Robyn A. North

Hypertension. published online April 1, 2013;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563

Integrated Proteomics Pipeline Yields Novel Biomarkers for Predicting Preeclampsia

Jenny E. Myers, Robin Tuytten, Grégoire Thomas, Wouter Laroy, Koen Kas, Griet Vanpoucke, Claire T. Roberts, Louise C. Kenny, Nigel A.B. Simpson, Philip N. Baker, Robyn A. North

Abstract—Preeclampsia, a hypertensive pregnancy complication, is largely unpredictable in healthy nulliparous pregnant women. Accurate preeclampsia prediction in this population would transform antenatal care. To identify novel protein markers relevant to the prediction of preeclampsia, a 3-step mass spectrometric work flow was applied. On selection of candidate biomarkers, mostly from an unbiased discovery experiment (19 women), targeted quantitation was used to verify and validate candidate biomarkers in 2 independent cohorts from the SCOPE (SCreening fOr Pregnancy Endpoints) study. Candidate proteins were measured in plasma specimens collected at 19 to 21 weeks' gestation from 100 women who later developed preeclampsia and 200 women without preeclampsia recruited from Australia and New Zealand. Protein levels (n=25), age, and blood pressure were then analyzed using logistic regression to identify multimarker models (maximum 6 markers) that met predefined criteria: sensitivity ≥50% at 20% positive predictive value. These 44 algorithms were then tested in an independent European cohort (n=300) yielding 8 validated models. These 8 models detected 50% to 56% of preeclampsia cases in the training and validation sets; the detection rate for preterm preeclampsia cases was 80%. Validated models combine insulin-like growth factor acid labile subunit and soluble endoglin, supplemented with maximally 4 markers of placental growth factor, serine peptidase inhibitor Kunitz type 1, melanoma cell adhesion molecule, selenoprotein P, and blood pressure. Predictive performances were maintained when exchanging mass spectrometry measurements with ELISA measurements for insulin-like growth factor acid labile subunit. In conclusion, we demonstrated that biomarker combinations centered on insulin-like growth factor acid labile subunit have the potential to predict preeclampsia in healthy nulliparous women. **(*Hypertension*. 2013;61:00-00.)** • **Online Data Supplement**

Key Words: mass spectrometry ■ preeclampsia ■ screening ■ selective reaction monitoring ■ sensitivity ■ specificity

Preeclampsia continues to be a major cause of maternal mortality, resulting in >50 000 maternal deaths worldwide each year, and is the leading cause of iatrogenic preterm birth.[1] To prevent preeclampsia, women at high risk of the condition need to be identified early in pregnancy. Although there is significant interest in the prediction of preeclampsia using combinations of clinical risk factors, biophysical measurements, and biochemical tests, to date no screening test has achieved the requisite sensitivity and specificity to be useful and cost-effective in a clinical setting.[2–5]

Prediction of preeclampsia in healthy nulliparous women is particularly challenging, despite the greatest proportion of cases occurring in this population. The best known combination of markers tested in a low-risk nulliparous population had a sensitivity of 46% for a specificity of 80%, equating to a PPV of around 15.5%.[4] Other reports of better prediction have studied general obstetric populations that include high-risk women[3] or have used a nested case–control design with controls comprising uncomplicated pregnancies with the consequent overestimation of predictive performance.[6]

A screening test is likely to require multiple biomarkers that reflect different aspects of the complex pathological processes that culminate in preeclampsia.[7] Several proteins indicative of abnormal placentation, such as placental growth factor (PlGF) and pregnancy-associated plasma protein A, have been demonstrated to be predictive of preeclampsia, especially preterm disease.[8] Novel plasma biomarkers, representative of placentation or the maternal vascular and inflammatory response in preeclampsia, may be discovered using an unbiased proteomic approach. Unfortunately, to date, most proteomic research, which has aimed to discover biomarkers, has failed to incorporate adequate biomarker validation studies in independent sample sets. These are necessary steps in the translation of potential biomarkers into

Received February 6, 2013; first decision March 1, 2013; revision accepted March 1, 2013.

From the Maternal & Fetal Heath Research Centre, Manchester Academic Health Science Centre, University of Manchester, Manchester, United Kingdom (J.E.M., P.N.B.); Pronota, Zwijnaarde, Belgium (R.T., G.T., W.L., K.K., G.V.); Discipline of Obstetrics and Gynaecology, University of Adelaide, Adelaide, Australia (C.T.R.); The Anu Research Centre, Department of Obstetrics and Gynaecology, University College Cork, Ireland (L.C.K.); Department of Obstetrics and Gynaecology, University of Leeds, Leeds, United Kingdom (N.A.B.S.); and Division of Women's Health, King's College London, London, United Kingdom (R.A.N.).

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.113.01168/-/DC1.

Correspondence to Jenny Myers, Maternal & Fetal Health Research Centre, 5th Floor (Research), St Mary's Hospital, Oxford Rd, Manchester, M13 9WL, UK. E-mail Jenny.myers@manchester.ac.uk

clinical practice. Recent development of sophisticated mass spectrometry–based quantitation of multiple proteins has enabled the validation of large sets of candidate biomarkers in plasma.[9]

The objective of this study was to identify, verify, and validate panels of biomarkers, which are predictive of preeclampsia. Given that in current practice women with an estimated ≥20% risk of developing preeclampsia are referred for specialist prenatal care,[10] we aimed to develop a test with ≥50% sensitivity for a positive predictive value (PPV) of 20%. Selective reaction monitoring (SRM) was used to verify and validate panels of biomarkers in 2 independent sample sets from a prospective, international cohort of nulliparous women (www.scopestudy.net). In the preeclampsia prediction panels developed, insulin-like growth factor acid labile subunit (IGFALS), a novel preeclampsia biomarker, carries the most predictive weight.

Methods

Participants and Specimens

Local ethical committee approval was granted, and written informed consent was obtained from all participants.

Biomarker Discovery

Healthy, normotensive, nulliparous, and multiparous women (n=222) were recruited at Ninewells Hospital, Dundee, UK, after assessment of uterine artery Doppler waveform at a routine clinical visit, and an EDTA plasma was obtained at 22 and 26 weeks of gestation.[11] Pregnancy outcome data were available in all women, of whom 26 women (12%) developed preeclampsia defined using standard criteria.[12] Ten women with preeclampsia were matched for parity, ethnicity, and gestation at sampling to women with uncomplicated pregnancies (n=9).

Biomarker Verification and Validation

Women who were recruited into the SCOPE (SCreening fOr Pregnancy Endpoints) study, a prospective screening study of low-risk nulliparous women recruited in Australia, New Zealand, United Kingdom, and Ireland between November 2004 and July 2011 (ACTRN12607000551493), participated in this study.[2] A research midwife interviewed participants at 14 to 16 weeks' and 19 to 21 weeks' gestation, and pregnancy outcomes were prospectively tracked. At the time of interview, data were entered on the Internet-accessed central database (MedSciNet). Two consecutive manual blood pressure measurements were recorded. Blood samples were collected on EDTA at 14 to 16 and 19 to 21 weeks, and plasma was stored at −80°C within 4 hours of collection.

Training Set

One hundred women who developed preeclampsia and 200 controls were randomly selected from the 3182 women recruited in Australia and New Zealand. Controls were selected 2:1 from those who did not have preeclampsia at the same center and included women with uncomplicated pregnancies and those with complications, such as small for gestational age, preterm birth, gestational hypertension, and gestational diabetes mellitus. Preeclampsia was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg, or both, on ≥2 occasions 4 hours apart after 20 weeks' gestation but before the onset of labor, or postpartum, with either proteinuria (24-hour urinary protein ≥300 mg or spot urine protein:creatinine ratio ≥30 mg/mmol creatinine or urine dipstick protein ≥++) or any multisystem complication of preeclampsia.[2]

Validation Set

Fifty cases of preeclampsia and 5:1 controls (no preeclampsia), stratified by center, were randomly selected from women recruited to the European centers (London, Manchester, Leeds, UK, and Cork, Ireland; n=2423).

Mass Spectrometry Methods

N-Terminomics Discovery Platform

An N-terminomics platform, described in Mebazaa et al,[9] was used to identify candidate biomarkers in the 22- and 26-week discovery samples. In brief, N-terminomics COFRADIC (COmbined FRActional DIagonal Chromatography)[13,14] for complexity reduction and spotting on MALDI (matrix-assisted laser desorption/ionization) targets was used. Experimental details are provided in the online-only Data Supplement.

Quantification of Candidate Biomarkers

The candidate proteins were quantified in the training sample set with targeted mass spectrometry assays based on an SRM peptide quantification method,[15] using custom-built assays. In brief, plasma samples were depleted of albumin and IgG, denatured and spiked with a mixture of isotopically labeled peptides serving as internal reference. After tryptic digestion, and peptide separations, quantitative data were obtained with a triple quadrupole mass spectrometry (MS) instrument. The readout of an assay in each sample was the ratio of the analyte signal area (endogenous peptide) over the common internal standard signal area. Comparison of ratios between different samples represents the relative quantification of the protein. Detailed MS methods are described in the online-only Data Supplement.

The sample order was randomized before every analytic step, and laboratory personnel were blinded to the pregnancy outcome related to each sample. Technical variation was estimated by preparing and measuring in duplicate 10% of the samples in a randomized order.

PlGF was measured in all samples using DELFIA time resolve fluorescence technology (PerkinElmer, Turku, Finland). Interassay coefficients of variation were 3% at 16.8 pg/mL and 8% at 852 pg/mL. In the validation samples, only IGFALS was also measured, in duplicate, by ELISA (Mediagnost, Reutlingen, Germany); samples were randomized and blinded to the Mediagnost laboratory. Coefficients of variation (CVs) were <8% for all 5 reference samples.

Biomarker Panel Development

The size of the training set was chosen to achieve an accuracy for the sensitivity of ±10% with a confidence level of 95% for the minimum accepted performance (50% detection rate [sensitivity] at a PPV of 20%). The minimum total number of samples required to achieve this performance was 288.

R and bioconductor were used to perform all statistical analyses.[16] The characteristics of the preeclampsia group and controls were compared using Student *t* test, Wilcoxon rank sum test, and χ^2 test. Logistic regression was used to develop multivariable models. The clinical parameters (maternal age and mean arterial pressure [MAP]; no missing values) obtained at 20 weeks', protein assays (log transformed) with <20% missing values, and a ≤25% CV were used for the multivariable analysis.

The modeling aimed to discover all marker combinations predictive of preeclampsia using a maximum of 6 covariates to limit the risk of overfitting the data. For each combination, a logistic regression model was fitted on the participants with complete data; observations with outlying values were discarded. A conservative stepwise approach was used to select the models. First, the statistical significance of all coefficients was estimated using the Wald test. A model was ignored when the Wald test for one of the coefficients associated with a covariate was $P>0.05$. For the retained models, the discriminatory power was then estimated using the area under receiver-operator curve (AUC). Models with an AUC below 0.70 were ignored (this AUC corresponds to the AUC of the best univariate predictor; IGFALS). Finally, the sensitivity at 20% PPV was computed for the remaining models, and those with a sensitivity of ≥50%, the preset threshold, were retained for external validation.

Biomarker Panel Validation

The selected models were evaluated in the European samples (validation set). The performance was computed in the validation set using the models developed in the training set without any refitting.

Models were considered externally validated if the sensitivity was ≥50% at 20% PPV (Figure S3B in the online-only Data Supplement). Given the validation set comprised 50 cases and 250 controls, the accuracy of the sensitivity observed is expected to be ±14% for the target performance of 50% sensitivity at 20% PPV. The possibility of a validated model occurring by chance was also assessed (see the online-only Data Supplement).

Results

An overview of the steps taken to develop, verify, and validate the prediction models is outlined in Figure 1. The participants from the SCOPE study included in the training and validation data sets for biomarker verification and validation, respectively, are shown in Figure S1.

Biomarker Discovery

An N-terminomics platform was used to compare the plasma proteomes of women destined to develop preeclampsia (n=10) with women who had uncomplicated pregnancies (n=9) at 22- and 26-week gestation (Table S1). From this discovery experiment, 64 proteins were selected for verification (Table S2). Previously reported markers for preeclampsia, such as soluble endoglin (sEng), disintegrin, and metalloproteinase domain–containing protein 12 (ADAM12) were identified. In addition to these 64 proteins, 9 proteins previously identified in a cardiovascular biomarker study[9] with biology relevant to preeclampsia (Table S2) and 3 proteins (PlGF, soluble fms-like tyrosine kinase-1, and placental protein 13) with a recognized association with preeclampsia were also taken forward to the verification experiments.

Biomarker Verification and Model Development

The characteristics of participants in the training and validation sets are shown in Table 1. SRM assays were successfully developed for 51 proteins from the list of candidate biomarkers. SRM data with a CV ≤25% and ≤20% missing values were obtained for 24 different proteins (Table S2).

Univariate analysis revealed that IGFALS was significantly elevated in 19 to 21-week plasma from women who later developed preeclampsia compared with controls (Table S3; Figure S2). Furthermore, IGFALS was increased before both preterm (<37 weeks; n=30) and term preeclampsia (n=70). IGFALS had the highest performance as a single marker with 48% (95% confidence interval [CI], 37% to 59%) sensitivity at 80% specificity (Table S4). PlGF, sEng, ADAM12, and 20-week MAP also significantly discriminated women destined to develop preterm preeclampsia from control pregnancies (P<0.001; Figure S2).

Development of Models in Training Set

Forty-four models had a prediction performance higher than the predefined cutoff (sensitivity ≥50% at 20% PPV; Figure S3A). There was significant overlap of protein biomarkers in these prediction models, with a small number of biomarkers (PlGF, IGFALS, melanoma cell adhesion molecule [MCAM], sEng, ADAM12, serine peptidase inhibitor Kunitz type 1 [SPINT1]) appearing in the majority of algorithms.

Validation of Prediction Models

Of the 44 models, 8 reached the target performance of 50% sensitivity at 20% PPV for a 5% prevalence in the validation set (Figure S3B). These validated models included combinations of the proteins IGFALS, sEng, ADAM12, SPINT1, MCAM, selenoprotein P, multimerin-2, extracellular matrix protein 1, microtubule-associated protein RP/EB family member 1 or 3, fructose-bisphosphate aldolase A, PlGF, and blood pressure (MAP), Table 2. The likelihood of validating 1 model by chance was computed to be <1%.

The 8 validated models all showed very similar performance for overall preeclampsia prediction (Tables S5 and S6). With the exception of 1 model, these models combine IGFALS and sEng and a selection of 3 or 4 markers out of SPINT1, PlGF, MCAM, selenoprotein P, and MAP. The model that combines the 6 most frequently occurring covariates was selected as an

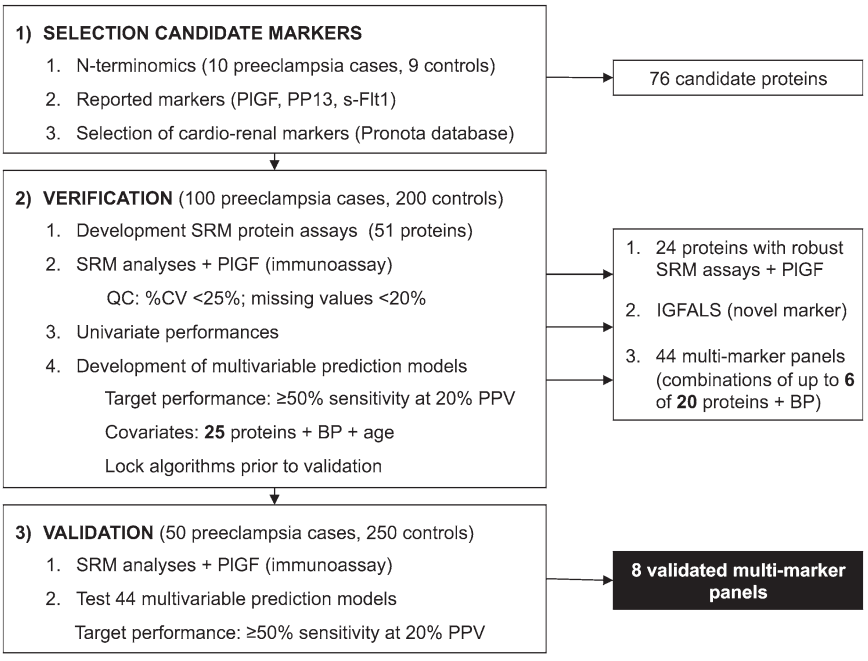


Figure 1. Flow chart describing the experimental steps taken during the identification and verification of novel markers, and the development and testing of predictive models. BP indicates blood pressure; CV, coefficient of variation; IGFALS, insulin-like growth factor acid labile subunit; PlGF, placental growth factor; PPV, positive predictive value; and SRM, selective reaction monitoring.

Table 1. Baseline Characteristics and Pregnancy Outcome of Participants

	Training Set		Validation Set	
Characteristics	Preeclampsia (n=100)	No Preeclampsia (n=200)	Preeclampsia (n=50)	No Preeclampsia (n=250)
Maternal age, y	26.6 (6.0)	26.8 (6.4)	29.7 (5.5)	28.9 (5.3)
White ethnicity	84 (84)	179 (90)	45 (90)	224 (90)
Smoker at 15 wk	8 (8%)	34 (17)†	4 (8)	27 (11)
Body mass index at 15 wk, kg/m^2	28.3 (23.1–31.3)	25.8‡ (21.8–28.2)	27.2 (23.2–29.5)	24.9‡ (21.6–27.4)
Gestation at sampling, wk	20.2 (0.6)	20.1 (0.8)	20.9 (0.6)	20.6 (0.8)
Blood pressure at 20 wk, mm Hg				
Mean arterial pressure, 1st	(84) (8)	80 (7)‡	84 (10)	79 (7)‡
Mean arterial pressure, 2nd	84 (8)	80 (7)‡	85 (10)	80 (7)‡
Pregnancy outcome				
Gestation age at delivery, wk	37.7 (2.6)	39.8 (1.9)	38.2 (3.1)	39.9 (1.9)
Preterm birth (<37 wk)	30 (30)	9 (4)	12 (24)	14 (6)
Maximum blood pressure				
Systolic, mm Hg	163 (18)	122 (13)	167 (21)	125 (13)
Diastolic, mm Hg	103 (10)	74 (10)	107 (15)	79 (11)
Proteinuria*	95 (95)	2 (1)	47 (94)	
Multiorgan complications	34 (34)	…	15 (30)	…
Other pregnancy complications				
Small for gestational age	22 (22)	16 (8)	17 (34)	33 (13)
Gestational hypertension	…	13 (7)	…	…
Gestational diabetes mellitus	7 (7)	6 (3)	1 (2)	2 (1)

Results are expressed as mean (SD), median (interquartile range), or n (%).
*Urine dipstick ≥2+ or 24-h urine protein excretion ≥300 mg or spot urine protein:creatinine ratio ≥30 mg/mmol.
†P<0.05; ‡P<0.001 cases vs controls.

example (Figure 2). Using the model, a risk index (relative risk to develop preeclampsia) was computed for each patient. A risk index cutoff corresponding to 20% PPV was computed on the training set. The cutoff corresponds to a detection rate (sensitivity) of 54% (95% CI, 37% to 66%) in the training set and 50% (95% CI, 36% to 68%) in the validation set. Preterm preeclampsia occurred in 30 women in the training and 12 women in the validation sets. Using the model for all preeclampsia and the same risk index cutoff, the detection of preterm preeclampsia was 72% (95% CI, 48% to 88%) in the training set and 80% (95% CI, 50% to 100%) in the validation set (Figure 2; Tables S5 and S6). Application of this model to a theoretical population of 1000 women would classify 125 women as being high risk of developing preeclampsia. Twenty percent of this high-risk group would later develop preeclampsia, and 10 of the 13 women who would develop preterm preeclampsia would be detected. In the test negative group, 2.9% would develop preeclampsia with 0.29% having preterm disease compared with an unstratified nulliparous population where 1.2% of pregnancies would develop preterm preeclampsia.

The incremental value of the novel biomarkers over the known markers was investigated by calculating the performances of any combination of sENG, PlGF, ADAM12, and MAP: within the training data set. The best model (combination of PLGF, ADAM12, and MAP) had a sensitivity of 30% at 20% PPV; data not shown). A comparison with the performance of the National Institute for Health and Clinical Excellence (NICE) risk factor model[17] and the best combination of markers in a comparable population[4] are presented in Table 3.

Substitution of SRM Data With ELISA Data for IGFALS

There was good correlation between the SRM and ELISA measurements of IGFALS (r=0.63; P<0.001; Spearman rank correlation; Figure S4). Substitution of IGFALS SRM data with ELISA measurements in the example model did not change its performance. The risk index of the model using the SRM readouts also correlated well with the risk index using the ELISA measurements (r=0.89; P<0.001; Spearman rank correlation; Figure S4), resulting in a detection rate of 59% (95% CI, 41% to 73%) at 20% PPV.

Discussion

In this study, we identified a number of novel biomarkers associated with the later development of preeclampsia in low-risk nulliparous women. These biomarkers, together with known biomarkers, were then used to develop predictive models that met à priori criteria (detection of ≥50% of preeclampsia cases with a PPV of 20%, given a disease prevalence of 5%). During the development of predictive models, 4 of these novel biomarkers, IGFALS, MCAM, selenoprotein P, and SPINT1, were highly recurrent. In combination with known biomarkers (PlGF and sEng) and MAP, these markers achieved predictive performances with the potential to identify a subgroup of healthy nulliparous women who could receive specialist prenatal care. Overall preeclampsia detection rates ranged from 50% to 56% in the training set and 50% to 54% on

Table 2. Performance in the Training and Validation Sets of the Eight Models That Have a Performance Equal or Higher Than the Target in Both Sample Groups

				Sensitivity at 20% PPV*		Sensitivity at 80% Specificity	
		Total (n)	Missing Data (n)	Sens	95% CI	Sens	95% CI
Training							
Model No.							
1	MAP, sEng, SPINT1, IGFALS, MCAM, PIGF	279	19	54	36%–66%	67	54%–80%
2	MAP, ADAM12, ECM1, MCAM, PIGF	280	18	53	37%–63%	62	51%–73%
3	MAP, MMRN2, sEng, MAPRE1/3, IGFALS, ALDOA	290	8	51	34%–61%	59	47%–72%
4	MAP, sEng, SEPP1, IGFALS, MCAM, PIGF	279	19	50	37%–64%	64	53%–74%
5	MAP, MMRN2, sEng, SPINT1, SEPP1, IGFALS	293	5	56	39%–67%	64	52%–74%
6	MAP, sEng, SPINT1, SEPP1, IGFALS, PIGF	286	12	53	35%–65%	64	54%–76%
7	sEng, SPINT1, SEPP1, IGFALS, MCAM, PIGF	279	19	54	39%–67%	64	52%–75%
8	sEng, SPINT1, IGFALS, MCAM, PIGF	279	19	53	38%–65%	64	52%–76%
Validation							
Model No.							
1	MAP, sEng, SPINT1, IGFALS, MCAM, PIGF	273	27	50	36%–68%	59	45%–73%
2	MAP, ADAM12, ECM1, MCAM, PIGF	284	16	51	32%–64%	57	43%–74%
3	MAP, MMRN2, sEng, MAPRE1/3, IGFALS, ALDOA	266	34	53	35%–68%	60	43%–75%
4	MAP, sEng, SEPP1, IGFALS, MCAM, PIGF	281	19	54	35%–67%	56	42%–71%
5	MAP, MMRN2, sEng, SPINT1, SEPP1, IGFALS	287	13	53	38%–69%	53	40%–71%
6	MAP, sEng, SPINT1, SEPP1, IGFALS, PIGF	286	14	53	38%–67%	58	44%–73%
7	sEng, SPINT1, SEPP1, IGFALS, MCAM, PIGF	273	27	50	32%–64%	59	43%–73%
8	sEng, SPINT1, IGFALS, MCAM, PIGF	273	27	50	32%–64%	59	41%–73%

ADAM12 indicates disintegrin and metalloproteinase domain–containing protein 12; ALDOA, fructose-bisphosphate aldolase A; CI, confidence interval; ECM1, extracellular matrix protein 1; IGFALS, insulin-like growth factor acid labile subunit; MAP, mean arterial pressure 20 wk; MARE1/3, microtubule-associated protein RP/EB family member 1 or -3; MCAM, melanoma cell adhesion molecule; MMRN2, multimerin-2; PIGF, placental growth factor; PPV, positive predictive value; sEng, soluble endoglin; SEPP1, selenoprotein; and SPINT1, serine peptidase inhibitor Kunitz type 1.

*Calculated for 5% prevalence.

external validation, with ≈3 quarters of preterm preeclampsia cases detected.

Fundamental to the identification of novel algorithms to predict preeclampsia was the application of the discovery–verification-validation proteomics pipeline. Our discovery proteomic approach capitalized on sensitivity gains achievable by using N-terminomics[13] to identify novel biomarkers associated with later preeclampsia. The samples used for the biomarker discovery experiments were taken from a cohort of samples completely independent to the SCOPE cohort,[11] at different gestational ages and with different risk factors for the development of preeclampsia. Although it is probable that a modified list of proteins would have been identified had the discovery experiment been performed in a subset of the SCOPE cohort, independent verification of several biomarkers across these 2 independent populations adds further credibility to the findings. Our quantitative MS assays enabled simultaneous determination of the concentration of 20+ lower abundant plasma proteins (many without established immunoassays) in 25 µL of plasma. Our study highlights the capability of LC-SRM assays to bridge the gap between discovery experiments (many candidates, large number of false positives) and clinical validation studies where fewer markers are studied in 100s of women. The importance of verification and validation of biomarkers in clinical proteomic studies cannot be overstated. Even with the use of highly sensitive unbiased MS techniques, there is a high attrition of biomarkers when measured in a larger independent sample set. Furthermore, in a heterogeneous clinical condition, the univariate performance of individual proteins is superseded by the performance of a combination of proteins within predictive models. Predictive models will always have the highest accuracy in the population in which they were created, even where steps have been taken to minimize overfitting. External validation in an independent sample set provides a much more robust estimate of the predictive performance if translated into clinical settings.

The algorithms devised in this study were selected to enrich, within a population of low-risk nulliparous women where risk factor screening is not adequate,[2] a subgroup with a disease prevalence equivalent to current high-risk obstetric clinics,[10] that is, a PPV of 20%. Such a screening test would allow stratification of prenatal care, with nulliparous women who screen positive receiving increased antenatal surveillance along with intervention to prevent preeclampsia.[18] In comparison with reported multimarker combinations,[4] and the NICE risk factor assessment tool, the models devised in this study perform favorably (Table 3) with better detection rates and lower numbers of false positives in healthy nulliparous women. Comparison with other algorithms is problematic as general populations, comprising high-risk and low-risk women, have been studied.[3] There are several additional clinical variables, including body mass index,[2] which could improve the performance of the biomarkers measured in this study. In future validation studies, which would require the measurement of far

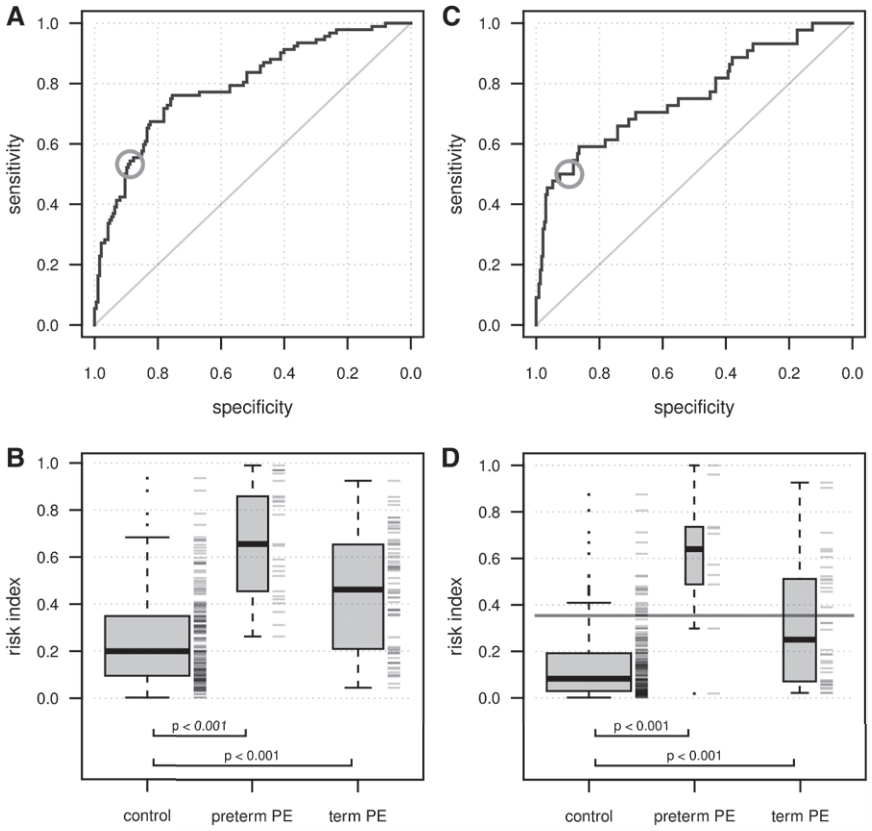


Figure 2. Performance of the example model in the training and validation sets. **A**, Performance of the example model in the training set for the discrimination of controls from women destined to develop preeclampsia. **B**, Distribution of the risk index for the example model in the training set. **C**, Performance of the example model in the test set for the discrimination of controls from women destined to develop preeclampsia; the circle indicates the sensitivity at 20% positive predictive value (PPV). **D**, Distribution of the risk index for the example model in the test set; the horizontal line corresponds to the maximum sensitivity for a PPV of 20%. *P* values: Mann–Whitney test. PE indicates preeclampsia.

fewer biomarkers, it may be appropriate to include body mass index and other clinical variables within the model.

IGFALS, which was increased in the plasma of women who developed preeclampsia, is part of the ternary insulin-like growth factor complex. It is known to complex with IGFBP3 and IGFBP5 (insulin-like growth factor binding protein), proteins that control the bioavailability of IGFs, which are crucial for placental development and growth. The acid labile subunit prevents the transport of the insulin-like growth factor complex across the endothelium into tissues confining them to the circulation. High levels of placental IGFALS mRNA have previously been reported in small for gestational age babies[19] and increased serum levels in women with established severe preeclampsia (n=8).[20] The other novel biomarkers, MCAM and SPINT1, decreased in women destined to develop preeclampsia consistently improved performance within the multivariable models. MCAM is an endothelial adhesion molecule, important in maintenance of the endothelial monolayer. It is highly expressed by placental trophoblasts with decreased expression in placentas from women with preeclampsia.[21] SPINT1 is a cell surface–binding protein of hepatocyte growth factor activator, which regulates hepatocyte growth factor activity. Hepatocyte growth factor contributes to the repair of injured tissues, is abundantly expressed by villous cytotrophoblasts,[22] and thought to be essential for placental development.[23]

Although this study benefits from rigorous protocols related to sample and data collection across 6 centers in 4 countries, it has some limitations. The study size is modest and certain ethnic groups are under-represented. Samples taken at 20-week gestation were used because of their temporal proximity to samples used in the discovery experiment. Although this time point has the advantage of coinciding with a standard antenatal care milestone (fetal anomaly scan), this must be balanced against the greater potential benefit of prophylactic aspirin, if

Table 3. Screening Performance in a Theoretical Population of Low-Risk Nulliparous Women (n=1000) Based on the Algorithm Shown in Figure 2, in Comparison With Application of the Existing National Institute for Health and Clinical Excellence (NICE) Criteria[17] and the Best Combination of Published Markers[4]

Theoretical Nulliparous Population Screened (n=1000)	Example Model (Figure 2)	Existing NICE[17] Risk Factor Model*	Current Best Marker Combination[4]†
Number who develop preeclampsia (preterm preeclampsia; n)	50 (13)	50 (13)	70
Number with positive screening test	125	120	218
Number of cases of preeclampsia detected	25	12	32
Number of cases of preterm preeclampsia detected	10	4	
Number of false-positive screening tests	100	108	186
Ratio false positives to true positives	4:1	9:1	7:1

*As applied to the SCOPE cohort.[3]

†Numbers extracted from published data as applied to a low-risk nulliparous population.

commenced before 20 weeks.[18] The benefit of aspirin may well be retained in high-risk nulliparous women if commenced at 20 weeks, but this would need to be assessed in a prospective trial.

Multivariable panels will always be difficult to establish in the context of a low-prevalence disease; the study design used here attempts to limit the chance of over fitting and selection of false positives variables by limiting the number of variables available to the models and using 2 independent sample sets. Validation of only 8 of the 44 models in the test set indicates that over fitting is likely in the training data. The validated models are expected to be generalizable. Given the consistency of the proteins retained in all validated models, it is likely that the combination of proteins identified in this study is robust.

The proteins in this study have been quantified using mass spectrometric methods, which are not currently used in the clinical setting. Commercial ELISA kits are available for several of the markers (PlGF, ADAM12, sEng), and importantly, measurement of IGFALS using ELISA measurements produced equivalent predictive performance to the SRM quantification. Before any clinical application of the biomarker combinations identified in this study, further prospective studies will need to be undertaken with the biomarkers measured on a platform used in clinical laboratories (eg, ELISA) and the predictive algorithms evaluated in an adequately sized cohort of nulliparous women.

Perspectives

By definition, low-risk nulliparous women do not have a history of significant medical disease or previous hypertensive disease in pregnancy, and therefore conventional clinical risk factor models do not perform well in this group. In current high-risk clinic settings (eg, previous preeclampsia, chronic medical disease), the rate of preeclampsia is ≈20%, and therefore, we set out to develop a model that had a PPV of ≥20%. This level of risk would justify referral of women with a positive test for specialist care with a manageable number of false positives. Novel biomarkers relevant to the prediction of preeclampsia were confirmed in 2 independent sample sets in this study, and IGFALS has emerged as a novel marker, predictive of term and preterm preeclampsia. In the future, it is likely that biochemical markers will be combined with a modest number of easily recordable clinical risk markers to improve the prediction of preeclampsia in this low-risk population.

Ethical Approval

Ethical approval for the longitudinal cohort recruited in Dundee was given by Tayside Medical Research Ethics committee. Ethical approval for the SCOPE study was obtained from local ethics committees [New Zealand AKX/02/00/364; Australia REC 1712/5/2008; London, Leeds, and Manchester 06/MRE01/98; and Cork ECM5 (10) 05/02/08].

Acknowledgments

We thank Dr Mires, M. Macleod, and all the women who participated in the study at Ninewells Hospital, Dundee. We also thank all the women who participated in the SCOPE Study, the SCOPE PIs, Professor L. Poston (King's College London), Professor L.M.E. McCowan (University of Auckland), Professor J.J. Walker (University of Leeds), and Professor G.A. Dekker (University of Adelaide); the SCOPE international coordinator, R. Taylor; the SCOPE Country Coordinators, D. Healy (University of Adelaide), A. Briley (Kings College London), and N. Murphy and E. Snapes (University College Cork); and the database and statistical support provided by E.H.Y. Chan, University of Auckland.

Sources of Funding

The SCOPE study was sponsored by the New Enterprise Research Fund, Foundation for Research Science and Technology, New Zealand; Health Research Council 04/198; Evelyn Bond Fund, Auckland District Health Board Charitable Trust; Australia: Premier's Science and Research Fund, South Australian Government; London: Guy's and St Thomas' Charity, United Kingdom, Tommys the Baby Charity; Manchester: UK Biotechnology and Biological Sciences Research Council GT084, UK National Health Services NEAT Grant FSD025, University of Manchester Proof of Concept Funding, Tommy's the Baby Charity, NIHR; Leeds: Cerebra, UK; and Cork, Ireland: Health Research Board, Ireland CSA/2007/2. The study at Ninewells hospital Dundee was funded by the Chief Scientist Office. J. Myers is also supported by Action Medical Research Endowment Fund and NIHR Manchester Biomedical Research Center. These study sponsors had no role in study design, data analysis, or writing this report. All mass spectrometry data acquisition was funded by Pronota Zwijnaarde, Belgium.

Disclosures

R. Tuytten, G. Thomas, W. Laroy, K. Kas, and G. Vanpoucke are employed by Pronota, which has a commercial interest in the development of predictive tests for preeclampsia. J. Myers, P.N. Baker, and R.A. North have received consultancy fees (paid to their institution) from Pronota. The other authors have no conflicts to report.

References

1. World Health Organisation. The world health report 2005—make every mother and child count. 2005. http://www.who.int/whr/2005/whr2005_en.pdf. Accessed March 20, 2013.
2. North RA, McCowan LM, Dekker GA, Poston L, Chan EH, Stewart AW, Black MA, Taylor RS, Walker JJ, Baker PN, Kenny LC. Clinical risk prediction for pre-eclampsia in nulliparous women: development of model in international prospective cohort. *BMJ*. 2011;342:d1875.
3. Akolekar R, Syngelaki A, Sarquis R, Zvanca M, Nicolaides KH. Prediction of early, intermediate and late pre-eclampsia from maternal factors, biophysical and biochemical markers at 11-13 weeks. *Prenat Diagn*. 2011;31:66–74.
4. Myatt L, Clifton RG, Roberts JM, et al; Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD) Maternal-Fetal Medicine Units (MFMU) Network. First-trimester prediction of preeclampsia in nulliparous women at low risk. *Obstet Gynecol*. 2012;119:1234–1242.
5. Kenny LC, Broadhurst DI, Dunn W, Brown M, North RA, McCowan L, Roberts C, Cooper GJ, Kell DB, Baker PN; Screening for Pregnancy Endpoints Consortium. Robust early pregnancy prediction of later preeclampsia using metabolomic biomarkers. *Hypertension*. 2010;56:741–749.
6. Lim JH, Kim SY, Park SY, Yang JH, Kim MY, Ryu HM. Effective prediction of preeclampsia by a combined ratio of angiogenesis-related factors. *Obstet Gynecol*. 2008;111:1403–1409.
7. Roberts JM, Lain KY. Recent Insights into the pathogenesis of preeclampsia. *Placenta*. 2002;23:359–372.
8. Kuc S, Wortelboer EJ, van Rijn BB, Franx A, Visser GH, Schielen PC. Evaluation of 7 serum biomarkers and uterine artery Doppler ultrasound for first-trimester prediction of preeclampsia: a systematic review. *Obstet Gynecol Surv*. 2011;66:225–239.
9. Mebazaa A, Vanpoucke G, Thomas G, et al. Unbiased plasma proteomics for novel diagnostic biomarkers in cardiovascular disease: identification of quiescin Q6 as a candidate biomarker of acutely decompensated heart failure. *Eur Heart J*. 2012;33:2317–2324.
10. Caritis S, Sibai B, Hauth J, Lindheimer MD, Klebanoff M, Thom E, VanDorsten P, Landon M, Paul R, Miodovnik M, Meis P, Thurnau G. Low-dose aspirin to prevent preeclampsia in women at high risk. National Institute of Child Health and Human Development Network of Maternal-Fetal Medicine Units. *N Engl J Med*. 1998;338:701–705.
11. Myers J, Mires G, Macleod M, Baker P. In preeclampsia, the circulating factors capable of altering *in vitro* endothelial function precede clinical disease. *Hypertension*. 2005;45:258–263.

12. Davey DA, MacGillivray I. The classification and definition of the hypertensive disorders of pregnancy. *Am J Obstet Gynecol*. 1988;158:892–898.
13. Gevaert K, Goethals M, Martens L, Van Damme J, Staes A, Thomas GR, Vandekerckhove J. Exploring proteomes and analyzing protein processing by mass spectrometric identification of sorted N-terminal peptides. *Nat Biotechnol*. 2003;21:566–569.
14. Staes A, Impens F, Van Damme P, Ruttens B, Goethals M, Demol H, Timmerman E, Vandekerckhove J, Gevaert K. Selecting protein N-terminal peptides by combined fractional diagonal chromatography. *Nat Protoc*. 2011;6:1130–1141.
15. Lange V, Picotti P, Domon B, Aebersold R. Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol Syst Biol*. 2008;4:222.
16. Gentleman R, Hornik K, Leisch F. R 1.5 and the Bioconductor 1.0 releases. *Comput Stat Data Anal*. 2002;39:557–558.
17. NICE. Hypertension in Pregnancy. 2010. http://guidance.nice.org.uk/CG107.
18. Duley L, Henderson-Smart DJ, Meher S, King JF. Antiplatelet agents for preventing pre-eclampsia and its complications. *Cochrane Database Syst Rev*. 2007;CD004659. Accessed March 20, 2013.
19. Iñiguez G, Argandoña F, Medina P, González C, San Martín S, Kakarieka E, Johnson MC, Cassorla F. Acid-labile subunit (ALS) gene expression and protein content in human placentas: differences according to birth weight. *J Clin Endocrinol Metab*. 2011;96:187–191.
20. Park J, Cha DH, Lee SJ, Kim YN, Kim YH, Kim KP. Discovery of the serum biomarker proteins in severe preeclampsia by proteomic analysis. *Exp Mol Med*. 2011;43:427–435.
21. Liu Q, Yan X, Li Y, Zhang Y, Zhao X, Shen Y. Pre-eclampsia is associated with the failure of melanoma cell adhesion molecule (MCAM/CD146) expression by intermediate trophoblast. *Lab Invest*. 2004;84:221–228.
22. Kataoka H, Meng JY, Itoh H, Hamasuna R, Shimomura T, Suganuma T, Koono M. Localization of hepatocyte growth factor activator inhibitor type 1 in Langhans' cells of human placenta. *Histochem Cell Biol*. 2000;114:469–475.
23. Tanaka H, Nagaike K, Takeda N, Itoh H, Kohama K, Fukushima T, Miyata S, Uchiyama S, Uchinokura S, Shimomura T, Miyazawa K, Kitamura N, Yamada G, Kataoka H. Hepatocyte growth factor activator inhibitor type 1 (HAI-1) is required for branching morphogenesis in the chorioallantoic placenta. *Mol Cell Biol*. 2005;25:5687–5698.

Novelty and Significance

What Is New?

- This study identified insulin-like growth factor acid labile subunit, selenoprotein, serine peptidase inhibitor Kunitz type 1, and melanoma cell adhesion molecule as novel biomarkers for preeclampsia.
- The combination of insulin-like growth factor acid labile subunit with blood pressure, along with other biomarkers, has the potential to be part of a clinically relevant predictive test for preeclampsia.

What Is Relevant?

- Application of this test could improve our ability to identify a subgroup of women at significant risk of preeclampsia among nulliparous women. Women with a positive test would have a 1 in 5 chance of developing preeclampsia, which equates to the risk in current high-risk obstetric clinics.
- The algorithm developed in this study could detect up to 50% of all preeclampsia and 80% of preterm preeclampsia cases arising in a low-risk nulliparous population. Detection of the majority preterm preeclampsia cases would allow intervention strategies, such as low-dose aspirin.
- A negative test does not adequately risk stratify women at very low risk of preeclampsia to modify management.

Summary

This study has identified insulin-like growth factor acid labile subunit as a novel candidate biomarker for preeclampsia; predictive models containing this marker have been validated in 2 independent sample sets.

Supplementary information

Integrated proteomics pipeline yields novel biomarkers for predicting preeclampsia

Jenny E Myers[1], Robin Tuytten[2], Grégoire Thomas[2], Wouter Laroy[2], Koen Kas[2], Griet Vanpoucke[2], Claire T Roberts[3], Louise C Kenny[4], Nigel AB Simpson[5], Philip N Baker[1], Robyn A North[6]

[1]Maternal & Fetal Heath Research Centre, Manchester Academic Health Science Centre, University of Manchester, Manchester, United Kingdom;
[2]Pronota, Zwijnaarde, Belgium; [3]Discipline of Obstetrics and Gynaecology, University of Adelaide, Adelaide, Australia; [4]The Anu Research Centre, Department of Obstetrics and Gynaecology, University College Cork, Ireland; [5]Department of Obstetrics and Gynaecology, University of Leeds, Leeds, United Kingdom; [6]Division of Women's Health, King's College London, London, United Kingdom

MASS SPECTROMETRY METHODS

N-terminomics Discovery Platform

All individual samples were measured against a reference sample, i.e., a pool of all samples under investigation. Individual samples were ^{18}O-labeled whereas the reference sample was ^{16}O-labeled (see below). This so-called "reference design" enabled comparison of the proteomic profiles of each individual sample within a population as well as across populations.

Sample preparation:

Plasma samples were depleted from their 14 most abundant proteins using the Multiple Affinity Removal System level II (MARS) (Agilent Technologies, Waldbronn, Germany) as described by the manufacturer. Proteins were denatured by adding guanidine hydrochloride to a final concentration of 3M (GdnHCl) (Merck), reduced with a 25 molar excess over protein of Tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) (Pierce, Rockford, IL, USA) and alkylated with a 50 molar excess over protein of iodoacetamide (IAA) (Fluka). Following a buffer exchange to 1.4 M GndHCl in 50mM sodium phosphate pH 8 ($(Na_2HPO_4.7H_2O)$ (Merck) using a PD-10 desalting column (GE Healthcare), all primary amines were acetylated by incubating with 75 times molar excess over protein of sulfo-N-hydroxysuccinimide acetate (sulfoNHS acetate) (Pierce) at 30°C for 90 min. Next, 3.5 molar excess over sulfoNHS acetate of hydroxylamine (Merck) was added to undo any acetylation of serine and threonine residues (30°C for 30 min).

Protein solutions in 10mM ammonium bicarbonate pH8.0 (Fluka), following buffer exchange (PD-10), were heated to 99°C for 5 minutes, and then digested using sequencing grade modified trypsin (Promega) in a 50:1 (w/w) substrate:trypsin ratio for 16h digestion at 37°C in a thermomixer (Eppendorf). Proteolytic activity was stopped by acidification of the samples to pH 5-6 (KH_2PO_4, pH 2) (Merck). After drying of the samples under vacuum, peptides were reconstituted in $H_2{}^{16}O$ (reference pool) or $H_2{}^{18}O$ (sample) (Cambridge Isotope Laboratories); the peptide mixtures were kept at pH 6. Incorporation of the ^{18}O-label by trypsin involved a minimum incubation of 26h at 37°C (thermomixer); ≥90% incorporation was confirmed using MALDI-MS. 6M GdnHCl mixed with 1000 molar excess over trypsin of TCEP and IAA, dissolved in $H_2{}^{16}O$ or $H_2{}^{18}O$ was then added to the samples to inactivate trypsin.

Peptide signature selection (N-terminomics):

In line with the reference design, individual samples were prepared by mixing 175µg of each ^{18}O-labeled sample with 175µg of ^{16}O-labeled reference pool. Back-exchange of the ^{18}O-labeled peptides was prevented by adjusting the samples pH to pH 4 (10% acetic acid solution). Prior injection on the LC system, samples volumes were corrected to 520 µL with 3M GndHCl/10mM sodium acetate in 96:4 (v/v) H_2O:ACN (HPLC-grade) (Biosolve). All COFRADIC separations were carried out on Zorbax SB300-C18 columns (2.1mm x 150mm; 5µm particle size, 300Å pore size) (Agilent Technologies, Waldbronn, Germany) using 1100 LC systems (Agilent Technologies, Waldbronn, Germany). The mobile phases were 10mM ammonium acetate 96:4 (v/v) H_2O:ACN (solvent A) and 10mM ammonium acetate in 30:70 (v/v) H_2O:ACN (solvent B). 500 µL sample injections were made and peptides separated by application of a

linear gradient of 1%B/min at a constant flow rate of 80µL/min. For the primary separation step, twelve 4-minute LC fractions were collected. The fractions were repeatedly (3x) dried under vacuum and dissolved in 50mM boric acid (Merck) buffer pH 9.5; the final residue was reconstituted in 50 µL 50mM boric acid pH 9.5. Then, N-terminal labeling was achieved by adding 4 times 10 µL of 10mM 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Fluka) in 50mM borate buffer pH 9.5 to each of the 12 fractions with 30 min intervals. Prior re-injection on the LC-system the 12 fractions were acidified to pH 4 (10% HOAc) and supplemented with 10mM sodium acetate in 96:4 (v/v) H_2O:ACN to 510 µL. All 12 reacted fractions were then separated under identical chromatographic conditions as used during the primary separation step. For each of the 12 secondary chromatography's the original 4 minute collection window was further distributed in sixteen 15s fractions, with the collection recipients being the injection vials for the below nano-LC separations. All uneven secondary separations (1,3,5,7,9,11) were collected in one set of sixteen vials and all even secondary separations (2,4,6,8,10,12) in another set of sixteen vials totaling in thirty-two different pooled collections. The 32 peptide pools (COFRADIC fractions) were then dried under vacuum and stored at -20°C till further processing by nano-LC.

Peptide separation:

Each of the 32 COFRADIC fractions was reconstituted in 44 µL of 0.1% formic acid (FA, ULC-MS grade; H_2O; ULC-MS grade) (Biosolve) and further separated using an Ultimate-3000 nano-LC system (Dionex, Amsterdam, The Netherlands). The LC unit was directly coupled to a Probot system (Dionex) equipped for direct spotting on Opti-TOF LC/MALDI inserts (Applied Biosystems). The mobile phases were 0.1% FA (ULC-MS grade) (solvent A) and 0.1% formic acid in 20:80 (v/v) H_2O:ACN (ULC-MS grade) (solvent B). 20µL sample injections were made on a column-switching set up combining a 300 µm x 5 mm C18 PepMap pre-column and a 75µm x 15cm C18 PepMap nano-column (Acclaim PepMap100; 3µm particle size, 100Å pore size) (Dionex). Peptides trapped on the pre-column were back-flushed to the analytical column and separated using a 57 minute gradient from 4% to 55% solvent B at a flow rate of 300nL/min. The column efflux was mixed inline (T-piece) in 1:4 ratio with a matrix solution, consisting of 4mg/mL alpha-cyano-4-hydroxy-cinnamic acid (LaserBio Labs, Cedex, France) in 0.1% trifluoroacetic acid in 30:70 (v/v) H_2O:ACN (ULC-MS grade). 65 minutes of the chromatography was spotted with a spotting interval of 15 seconds, i.e., 260 spots per nano-LC run. Per original plasma sample this culminated in 32 x 260 spots or 8320 spots. To the matrix solution also an internal standard of 5 peptides (PepMix 4, LaserBio Labs) was added for later mass calibration in the mass spectrometer.

Feature selection using MALDI:

MS spectra were acquired with MALDI-TOF/TOF instrumentation (4800 series, Applied Biosystems, Darmstadt, Germany) operated in the MS reflector positive ion mode, using the 4000 Series Explorer™ software. For each MS spectrum 1,000 laser shots were fired (25 sub-spectra; 40 shots per sub-spectrum). Focus mass was set at 1,800Da and peptides in a mass range from 500 to 4,000 were detected. Internal calibration was based on the five standard peptides (pepMix4).

The resulting sets of LC x LC-MS signals were deconvoluted to obtain for each detectable peptide a relative quantitation across all samples: an in-house developed software suite performs peak

deisotoping, accurate $^{16}O/^{18}O$ ratio determination and clustering of all mass spectrometric peaks belonging to the same peptide together (Pronota, Zwijnaarde, Belgium). The resulting features take into account the three dimensions of the pipeline: COFRADIC fraction (from 1 to 32), retention time in the Nano-LC run and mass-to-charge ratio (m/z). The so-obtained unambiguously definition of peptides permitted their identification at a later stage by targeted tandem MS analysis (see below).

Statistical analysis:

Within the experimental set-up, both the 22 weeks and the 26 weeks plasma samples of the 10 preeclampsia destined women were analysed (10+10; paired samples), whereas the 9 controls were split over the 2 gestation time points (5+5; all but 1 unpaired samples). The final data analysis was performed on 28 samples following unaccounted for contaminations in 2 samples (1: preeclampsia 22 weeks; 2: Control 26 weeks) preventing their proper alignment with the other samples.

Upon establishment of the relative concentration using the ratio unlabeled vs. labeled signal of all features (peptides) over all samples, a feature expression set was compiled for further analysis. To identify and rank differentially expressed features Significance analysis of Microarrays (SAM) [1] adapted for proteomics as well as the more generic 1R classifier[2] were applied. To accommodate the dynamics of pregnancy as well as any transient effects, multiple pairwise-comparisons were considered:

- generic / gestational age independent: all cases (19) vs. all controls (9);
- transient effects: 22 weeks cases (9) vs. all controls (9),
  22 weeks controls (5) vs. all others (23);
- late pregnancy effects: 26 weeks cases (10) vs. all controls (9)
- pregnancy effects: 22 weeks cases (9) vs. 26 weeks cases (9), i.e., paired analyses
  22 weeks Controls (5) vs. 26 weeks controls (4)

All features showing differential expression in any of the above comparisons constituted a targeted inclusion list for MALDI-MS/MS (see below). Features that showed discriminative behaviour were manually validated in order to discard any merged features (grouping of MS signals of unrelated peptides) or split features (distribution of a single peptide signal) purged. Based on the final list of 1147 features with discriminatory behaviour, a targeted inclusion list was compiled taking into account retention times (MALDI plate identifiers and spot numbers) and then submitted to tandem MS analysis to obtain a peptide/protein identification.

In addition comprehensive inclusion lists were generated from 2 individual samples (a 22 wks and a 26 wks case sample; different donors) to enable mapping of non-differential features on the data matrix.

Biomarker candidate identification:

For the acquisition of MS/MS spectra the MALDI-TOF/TOF instrument was operated in the MS reflector positive ion mode. For each MS/MS spectrum 3,600 (60 shots for each of the 60 sub-spectra) laser shots were fired. Timed ion selector window was set at 450 full width half maximum. No internal calibration was done. Precursor masses were selected according to the above inclusion list. Generation of mgf files was done without any filtering based on S/N or number of signals. Masses below 60Da and above the precursor mass minus 20Da were excluded. Protein identification was performed using an analysis pipeline compliant with the COFRADIC principle. [3] For linking peptides to proteins a peptide representative approach was followed whereby a protein is reported when at least one valid, peptide identification was available. Spectra that were not identified with Mascot or were identified with low scores were evaluated with other search engines such as ProteinPilotTM 2.0 (Applied Biosystems, Darmstadt, Germany) and PEAKS Studio 4.5 (Bioinformatics Solutions Inc., Waterloo, Canada). In some cases, spectra of interest were evaluated manually using SPIDER (part of PEAKS Studio 4.5).

When multiple peptide identifications accounted for a single protein, the protein was discarded when the expression profiles of the different peptides diverged. From the resulting list of proteins with eventual biomarker potential, abundant plasma proteins like complement and coagulation factors were further eliminated. The 64 retained candidate biomarker proteins are listed in Table S2.

Quantification of Candidate Biomarkers

Assay development:

For each of the proteins in the list of biomarker candidates as derived from the N-terminomics effort, proteotypic tryptic peptides were selected. Criteria for selection include size, mass, hydrophobicity, absence of amino acids and amino acid combinations (C, M, W, RP, KP), presence of amino acids (P), uniqueness in the human proteome, absence of potential sites for posttranslational modification, etc. as described in Lange et al.[4]

Isotopically labeled variants of the selected peptides (crude FasTrack peptides) (Thermo Biopolymers, Thermo Fisher Scientific, Ulm, Germany) were used to determine optimal experimental conditions (fractionation, nano-LC, MS and MS/MS).

SRM analyses:

Plasma samples (25µL) were depleted using the ProteoPrep immunaffinity albumin and IgG depletion kit (Sigma) as described by the manufacturer, but with 20mM ammonium bicarbonate buffer pH 8.0 as binding buffer. The non-bound proteins were denatured by heat at 99°C for 15 min followed by immediate cooling on ice. At this stage, a spike mixture of isotopically labeled peptides (heavy AQUA peptide, Thermo Scientific), 2000 fmol each, was added as internal reference. Next, proteins were digestion with sequencing grade modified Trypsin (Promega) for 16h at 37°C ; digestion was halted by immediate storing of the samples at -80°C (till further processing). Peptides were separated based on different physicochemical parameters before quantification using a triple quadrupole MS instrument (Vantage TSQ; Thermo Scientific) equipped with a NanoSpray ion source (Thermo Scientific). The mass spectrometer was on-line coupled to an Ultimate-3000 nano-LC system (Dionex). The mobile phases were 0.1% FA (ULC-MS grade), solvent A; and 0.1% formic acid in 20:80 (v/v) H_2O:ACN (ULC-MS grade), solvent B. Peptides were separated on a C18 nano-column (Acclaim PepMap100 – 75µm x 25cm; 3um particle size, 100Å pore size) (Dionex) using a 30 minute gradient from 4% to 55% solvent B at of 200nl/min. The mass spectrometer was operated in ESI+ mode, with application of a spray voltage of 1600V and a capillary temperature of 150°C. Both Q1 and Q3 resolution were set at 0.7 Da (FWHM). Different peptides/transitions were monitored within one run using multiplexed scheduled SRM: transitions were measured for a time period starting 3 minutes before and ending 3 minutes after the determined peptide retention times, each peak needed at least 10 datapoints.

The SRM data were analysed using LCQUAN (Thermofisher Scientific). The readout of the assay in each sample was the ratio of the analyte signal area (endogenous peptide) and the common internal standard signal area (AQUA peptide). Comparison of ratios between different samples represents the relative abundance of each protein.

Technical variation was estimated by preparing and measuring, in duplicate, 10% of the samples in a randomized order throughout the analytical process. Only assays with interassay coefficients of variation (CV) ≤ 25% and not more than 20% missing data in the 300 samples of the “Training Set” were

retained for biomarker panel development. From the 300 plasma samples from the Training Set 2 samples were lost during preparation, *i.e*, 1 case and 1 control pregnancy.

For 51/64 proteins satisfactory SRM assays were available (Table S2); from these 51 proteins, 24 protein assays met the predefined QC criterion of ≤25% CV and ≤20% missing data. For these 24 protein assays %CVs were ≤10% for 3 proteins, ≤15% for 12 proteins and 4 protein assays showed a CV ≥20%; all protein assays but one had less than 5% missing values (Table S1).

Computation of type 1 error

The training and validation procedure was repeated 1500 times on randomised datasets to empirically determine the significance level of the observations. At each iteration, the outcome labels together with the known pre-eclampsia markers (PlGF, sENG, ADAM12) and risk factors (age, Bp) were randomly permutated. The relationship between these parameters was kept in order to preserve the diagnostic performance of the known markers and risk factors. In only four of the 1500 iterations could we find models that complied with the success criteria. In three of these iterations, one valid model was found and in a fourth three valid models were found. This indicates that the likelihood to observe at least one valid model by chance is lower than 1% (type I error).

References

1. Tusher VG, Tibshirani R, Chu G. Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci U S A.* 2001;98:5116-5121.
2. Holte R. Very Simple Classification Rules Perform Well on Most Commonly Used Datasets. *Machine Learning.* 1993;11:63-91.
3. Martens L, Vandekerckhove J, Gevaert K. DBToolkit: processing protein databases for peptide-centric proteomics. *Bioinformatics.* 2005;21:3584-3585.
4. Lange V, Picotti P, Domon B, Aebersold R. Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol Syst Biol.* 2008;4:222.

SUPPLEMENTARY TABLES

Table S1 Demographic details for the women in the discovery sample set (median, range)

	PE (n=10)	Controls (n=9)
Body mass index	29 (21-35)	23 (20-30)
Parity	0 (0-1)	0 (0-1)
Max BP (systolic)	160 (130-180)	120 (96-142)
Max BP (diastolic)	105 (90-130)	78 (60-86)
Gestation age (delivery)	38.1 (30.6-40.1)	41+3 (39.6-42.0)
Gestation age (diagnosis)	36.7 (29.6-39.4)	NA
Birthweight	2820 (1430-4040)	3470 (2980-4060)
Birthweight centile	31 (1-90)	44 (12-84)

Table S2: Summary of the biomarker candidates retained at each level of the workflow; including protein candidate selection, successful SRM assay development and quality control following verification in the training set.

Swiss prot entry	HNGC	Protein description	Protein origin	SRM assay	Robust SRM assay*
ABRAL_HUMAN	ABRACL	Costars family protein ABRACL	Discovery		
ACE_HUMAN	ACE	Angiotensin-converting enzyme	Discovery	✓	
ADA12_HUMAN	ADAM12	Disintegrin and metalloproteinase domain-containing protein 12	Discovery	✓	✓
AHSP_HUMAN	AHSP	Alpha-hemoglobin-stabilizing protein	Discovery		
ALDOA_HUMAN	ALDOA	Fructose-bisphosphate aldolase A	cardio-renal	✓	✓
ALS_HUMAN	IGFALS	Insulin-like growth factor-binding protein complex acid labile subunit	Discovery	✓	✓
ANFB_HUMAN	NPPB	Natriuretic peptides B	cardio-renal	✓	
ANGI_HUMAN	ANGI	Angiogenin	Discovery		
ANGL6_HUMAN	ANGPTL6	Angiopoietin-related protein 6	Discovery	✓	✓
ANXA3_HUMAN	ANXA3	Annexin A3	Discovery	✓	
ATS4_HUMAN	ADAMTS4	A disintegrin and metalloproteinase with thrombospondin motifs 4	Discovery	✓	
CAN1_HUMAN	CAPN1	Calpain-1 catalytic subunit	Discovery	✓	
CGHB_HUMAN	CGB	Choriogonadotropin subunit beta	Discovery	✓	
CO6A3_HUMAN	COL6A3	Collagen alpha-3(VI) chain	Discovery	✓	✓
CRP_HUMAN	CRP	C-reactive protein	Discovery	✓	✓
CSF1R_HUMAN	CSF1R	Macrophage colony-stimulating factor 1 receptor	Discovery		
CSH_HUMAN	CSH1	Chorionic somatomammotropin hormone	Discovery		
CYTC_HUMAN	CST3	Cystatin-C	cardio-renal	✓	✓
DAG1_HUMAN	DAG1	Dystroglycan	Discovery		
DKK3_HUMAN	DKK3	Dickkopf-related protein 3	Discovery	✓	
DPEP2_HUMAN	DPEP2	Dipeptidase 2	Discovery		
DPP4_HUMAN	DPP4	Dipeptidyl peptidase 4	Discovery	✓	
DSG2_HUMAN	DSG2	Desmoglein-2	Discovery	✓	
ECM1_HUMAN	ECM1	Extracellular matrix protein 1	Discovery	✓	✓
EGLN_HUMAN	ENG	Endoglin	Discovery	✓	✓
ENPP2_HUMAN	ENPP2	Ectonucleotide pyrophosphatase/phosphodiesterase family member 2	Discovery	✓	✓
ENSA_HUMAN	ENSA	Alpha-endosulfine	Discovery	✓	
EZRI_HUMAN	EZR	Ezrin	Discovery	✓	
FBLN1_HUMAN	FBLN1	Fibulin-1	Discovery	✓	
FBN2_HUMAN	FBN2	Fibrillin-2	Discovery		
GFRA3_HUMAN	GFRA3	GDNF family receptor alpha-3	Discovery		
GOLM1_HUMAN	GOLM1	Golgi membrane protein 1	cardio-renal	✓	
GPR126	GPR126	G-protein coupled receptor 126	Discovery		
HGFL_HUMAN	MST1	Hepatocyte growth factor-like protein	Discovery		

HTRA_HUMAN	HTRA1	Serine protease HTRA1	Discovery		
ICAM3_HUMAN	ICAM3	Intercellular adhesion molecule 3	Discovery		
IL6RB_HUMAN	IL6ST	Interleukin-6 receptor subunit beta	Discovery	✓	✓
KISS1_HUMAN	KISS1	Metastasis-suppressor KiSS-1	Discovery		
LCAP_HUMAN	LNPEP	Leucyl-cystinyl aminopeptidase (pregnancy serum form)	Discovery	✓	✓
LCAT_HUMAN	LCAT	Phosphatidylcholine-sterol acyltransferase	Discovery	✓	✓
LTBP2_HUMAN	LTBP2	Latent-transforming growth factor beta-binding protein 2	cardio-renal	✓	
MARE1_HUMAN; MARE3_HUMAN	MAPRE1/3	Microtubule-associated protein RP/EB family member 1 or -3	Discovery	✓	✓
MFAP5_HUMAN	MFAP5	Microfibrillar-associated protein 5	Discovery		
MMRN2_HUMAN	MMRN2	Multimerin-2	Discovery	✓	✓
MUC18_HUMAN	MCAM	Melanoma cell adhesion molecule	cardio-renal	✓	✓
NET3_HUMAN	NTN3	Netrin-3	Discovery		
PCD12_HUMAN	PCDH12	Protocadherin-12	Discovery	✓	✓
PCP_HUMAN	PRCP	Lysosomal Pro-X carboxypeptidase	Discovery	✓	✓
PCYOX_HUMAN	PCYOX1	Prenylcysteine oxidase 1	Discovery	✓	
PGBM_HUMAN	HSPG2	Basement membrane-specific heparan sulfate proteoglycan core protein	Discovery	✓	
PGRP2_HUMAN	PGLYRP2	N-acetylmuramoyl-L-alanine amidase	Discovery		
PHLD_HUMAN	GPLD1	Phosphatidylinositol-glycan-specific phospholipase D	Discovery	✓	
PLGF_HUMAN	PGF	Placenta growth factor	Literature		
PP13_HUMAN	LGALS13	Galactoside-binding soluble lectin 13	Literature		
PRDX1_HUMAN	PRDX1	Peroxiredoxin-1	Discovery	✓	
PRDX2_HUMAN	PRDX2	Peroxiredoxin-2	Discovery	✓	✓
PROC_HUMAN	PROC	Vitamin K-dependent protein C	Discovery	✓	✓
PSG3_HUMAN	PSG3	Pregnancy-specific beta-1-glycoprotein 3	Discovery	✓	
PTPRS_HUMAN	PTPRS	Receptor-type tyrosine-protein phosphatase S	Discovery	✓	
PTX3_HUMAN	PTX3	Pentraxin-related protein PTX3	cardio-renal		
PXDC2_HUMAN	PLXDC2	Plexin domain-containing protein 2	Discovery		
QSOX1_HUMAN	QSOX1	Sulfhydryl oxidase 1	cardio-renal	✓	✓
RFX5_HUMAN	RFX5	DNA-binding protein RFX5	Discovery	✓	
ROBO4_HUMAN	ROBO4	Roundabout homolog 4	Discovery	✓	✓
S10A9_HUMAN	S100A9	Protein S100-A9	Discovery	✓	
S1PR3_HUMAN	S1PR3	Sphingosine 1-phosphate receptor 3	Discovery		
SAA4_HUMAN	SAA4	Serum amyloid A-4 protein	Discovery		
SEPP1_HUMAN	SEPP1	Selenoprotein P	Discovery	✓	✓
SPIT1_HUMAN	SPINT1	Serine Peptidase inhibitor Kunitz type 1	Discovery	✓	✓
SPON2_HUMAN	SPON2	Spondin-2	Discovery		
TENX_HUMAN	TNXB	Tenascin-X	Discovery	✓	
TFF3_HUMAN	TFF3	Trefoil factor 3	Discovery		
VASH1_HUMAN	VASH1	Vasohibin-1	Discovery	✓	
VGFR1_HUMAN	FLT1	Vascular endothelial growth factor receptor 1	Literature	✓	
VGFR3_HUMAN	FLT4	Vascular endothelial growth factor receptor 3	Discovery	✓	
XPP2_HUMAN	XPNPEP2	Xaa-Pro aminopeptidase 2	Discovery	✓	

*SRM assay which met the technical specification of CV ≤ 25% and ≤20% missing data

Table S3. Proteotypic peptides quantified for proteins available to the prediction modeling; for each peptide the univariate C-statistic: (AUC; 95%CI) observed in the training sample set is given.

swiss prot entry	HNGC	Proteotypic peptides applied in SRM	CV*	Univariate c-statistic	
				AUC	95% CI
ADA12_HUMAN	ADAM12	ELIINLER	12%	0.63	0.56-0.70
ALDOA_HUMAN	ALDOA	GILAADESTGSIAK	13%	0.56	0.49-0.63
ALS_HUMAN	**IGFALS**	**LAELPADALGPLQR**	**10%**	**0.71**	**0.64-0.77**
ANGL6_HUMAN	ANGPTL6	LAAADGAVAGEVR	20%	0.55	0.48-0.62
CO6A3_HUMAN	COL6A3	SLDEISQPAQELK	11%	0.55	0.48-0.62
CRP_HUMAN	CRP	ESDTSYVSLK	12%	0.46	0.39-0.53
CYTC_HUMAN	CST3	ALDFAVGEYNK	16%	0.61	0.54-0.67
ECM1_HUMAN	ECM1	EVGPPLPQEAVPLQK	13%	0.56	0.49-0.63
EGLN_HUMAN	ENG	LPDTPQGLLGEAR	11%	0.58	0.51-0.66
ENPP2_HUMAN	ENPP2	DIEHLTSLDFFR	21%	0.62	0.55-0.68
IL6RB_HUMAN	IL6ST	ILDYEVTLTR	13%	0.53	0.47-0.60
LCAP_HUMAN	LNPEP	YISIGSEAEK	14%	0.58	0.51-0.65
LCAT_HUMAN	LCAT	LEPGQQEEYYR	9%	0.63	0.56-0.70
MARE1_HUMAN; MARE3 _HUMAN	MAPRE1/3	FFDANYDGK	23%	0.53	0.46-0.60
MMRN2_HUMAN	MMRN2	EAEPLVDIR	10%	0.53	0.46-0.60
MUC18_HUMAN	MCAM	GATLALTQVTPQDER	22%	0.59	0.52-0.66
PCD12_HUMAN	PCDH12	NPAYEVDVQAR	20%	0.55	0.48-0.62
PCP_HUMAN	PR**C**P	YYGESLPFGDNSFK	19%	0.58	0.51-0.65
PRDX2_HUMAN	PRDX2	EGGLGPLNIPLLADVTR	24%	0.57	0.49-0.65
PROC_HUMAN	PROC	GDSPWQVVLLDSK	16%	0.63	0.55-0.71
QSOX1_HUMAN	QSOX1	LAGAPSEDPQFPK	19%	0.50	0.44-0.57
ROBO4_HUMAN	ROBO4	EDFQIQPR	20%	0.56	0.49-0.63
SEPP1_HUMAN	SEPP1	LPTDSELAPR	17%	0.51	0.45-0.58
SPIT1_HUMAN	SPINT1	YTSGFDELQR	13%	0.56	0.49-0.63

*CV: coefficient of variation

Table S4. Discriminative performance of IGFALS, PlGF, sEng, ADAM12 and MAP in the training sample set

Training set	Control Median (IQR)	PE Median (IQR)	p-value*	Sensitivity at 80% Specificity		Univariate c-statistic	
				Sens (%)	95% CI	AUC	95% CI
IGFALS	1.7 (1.5 - 1.9)	1.9 (1.7 - 2.2)	<0.001	48	37–59%	0.71	0.64-0.77
PlGF (pg/mL)	135 (97 - 180)	108 (81 - 144)	<0.001	32	23-45%	0.63	0.56-0.70
sEng	3.9 (3.5 - 4.4)	4.1 (3.5 - 5.2)	0.021	37	26-47%	0.58	0.51-0.66
ADAM12	0.16 (0.13 - 0.20)	0.19 (0.15 - 0.24)	<0.001	34	23-45%	0.63	0.56-0.70
20 wks MAP (mm Hg)	80 (75 - 84)	83 (78 - 90)	<0.001	40	31-53%	0.64	0.58-0.71

Data are expressed as a ratio of peak area LC-SRM signal endogenous proteotypic peptide / peak area LC-SRM signal isotopically labeled proteotypic peptide. *Mann Whitney test.

Table S5. Eight models that have a performance equal or higher than the target both in the training and test sets –formulas

model	covariates	Covariates (n)	formula
1	**MAP; sEng SPINT1; IGFALS; MCAM; PlGF**	**6**	**0.04469 * MAP_1st - 1.278 * LOG[SPINT1] - 1.253 * LOG[MCAM] + 2.406 * LOG[sEng] - 0.8219 * LOG[PlGF] + 2.942 * LOG[IGFALS] - 3.416**
2	MAP;ADAM12;ECM1;MCAM;PlGF	5	1.68 * LOG[ECM1] - 1.595 * LOG[MCAM] + 0.08714 * MAP_1st + 1.529 * LOG[ADAM12] - 1.042 * LOG[PlGF] - 2.131
3	MAP;MMRN2;sEng;MARE1/3;IGFALS;ALDOA	6	-0.7088 * LOG[MAPRE1/3] + 1.248 * LOG[sEng] + 1.219 * LOG[ALDOA] - 1.996 * LOG[MMRN2] + 0.05915 * MAP_1st + 3.178 * LOG[IGFALS] - 7.7
4	MAP;sEng;SEPP1;IGFALS;MCAM;PlGF	6	-1.13 * LOG[MCAM] - 1.81 * LOG[SEPP1] + 1.626 * LOG[sEng] + 0.06725 * MAP_1st - 0.8142 * LOG[PlGF] + 3.298 * LOG[IGFALS] - 1.245
5	MAP;MMRN2;sEng;SPINT1;SEPP1;IGFALS	6	-1.72 * LOG[MMRN2] + 0.05188 * MAP_1st - 1.758 * LOG[SEPP1] - 1.453 * LOG[SPINT1] + 2.54 * LOG[sEng] + 3.854 * LOG[IGFALS] - 11.2
6	MAP;sEng;SPINT1;SEPP1;IGFALS;PlGF	6	0.0527 * MAP_1st - 1.238 * LOG[SPINT1] - 1.739 * LOG[SEPP1] - 0.5985 * LOG[PlGF] + 2.057 * LOG[sEng] + 3.436 * LOG[IGFALS] - 2.848
7	sEng;SPINT1;SEPP1;IGFALS;MCAM;PlGF	6	-1.394 * LOG[SEPP1] - 1.181 * LOG[MCAM] - 1.5 * LOG[SPINT1] - 0.782 * LOG[PlGF] + 2.507 * LOG[sEng] + 3.907 * LOG[IGFALS] - 2.044
8	sEng;SPINT1;IGFALS;MCAM;PlGF	5	-1.329 * LOG[MCAM] - 1.476 * LOG[SPINT1] + 2.394 * LOG[sEng] - 0.8283 * LOG[PlGF] + 3.369 * LOG[IGFALS] - 1.695

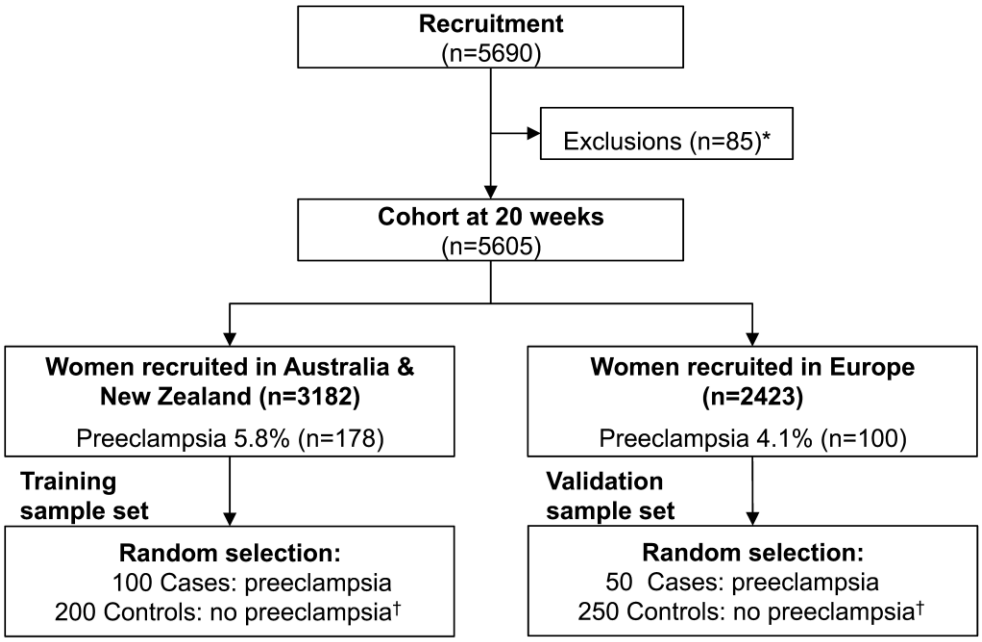
The protein levels are expressed as the normalized ratio peak area LC-SRM signal endogenous proteotypic peptide /_peak area LC-SRM signal isotopically labeled proteotypic peptide; PlGF pg/ml. Of the 27 features available to the models, 22 had <1% missing values, 25 <5% missing values and 2 >10% missing values.

MAP Mean arterial pressure 20 weeks; IGFALS Insulin-like growth factor acid labile subunit; sEng soluble Endoglin, PlGF placental growth factor; SPINT1 Serine peptidase inhibitor Kunitz type 1; MCAM Melanoma cell adhesion molecule; SEPP1 Selenoprotein ; MARE1/3 Microtubule-associated protein RP/EB family member 1 or -3; ALDOA Fructose-bisphosphate aldolase A; MMRN2 Multimerin-2

Table S6. Eight models that have a performance equal or higher than the target both in the training and test sets - additional metrics.

Model		Total (n)	missing data (n)	Max. p value	AUC	95% CI	% Preterm detected at 20% PPV cut-off %	95% CI
Training								
1	**MAP, sEng;SPINT1;IGFALS;MCAM;PlGF**	**279**	**19**	**0.04**	**0.79**	**0.74 - 0.85**	72	**48 - 88%**
2	MAP;ADAM12;ECM1;MCAM;PlGF	280	18	0.01	0.77	0.71 - 0.83	76	56 - 92%
3	MAP;MMRN2;sEng;MAPRE1/3;IGFALS;ALDOA	290	8	0.03	0.78	0.72 - 0.84	64	39 - 79%
4	MAP;sEng;SEPP1;IGFALS;MCAM;PlGF	279	19	0.03	0.80	0.74 - 0.85	64	44 - 84%
5	MAP;MMRN2;sEng;SPINT1;SEPP1;IGFALS	293	5	0.02	0.79	0.73 - 0.84	86	62 - 97%
6	MAP;sEng;SPINT1;SEPP1;IGFALS;PlGF	286	12	0.02	0.78	0.72 - 0.84	74	44 - 89%
7	sEng;SPINT1;SEPP1;IGFALS;MCAM;PlGF	279	19	0.05	0.79	0.73 - 0.84	72	52 - 88%
8	sEng;SPINT1;IGFALS;MCAM;PlGF	279	19	0.01	0.78	0.72 - 0.84	76	48 - 92%
Validation								
1	**MAP;sEng;SPINT1;IGFALS;MCAM;PlGF**	**273**	**27**		**0.76**	**0.67 - 0.85**	80	**50 - 100%**
2	MAP;ADAM12;ECM1;MCAM;PlGF	284	16		0.76	0.67 - 0.84	75	42 - 100%
3	MAP;MMRN2;sEng;MAPRE1/3;IGFALS;ALDOA	266	34		0.75	0.65 - 0.84	56	22 - 89%
4	MAP;sEng;SEPP1;IGFALS;MCAM;PlGF	281	19		0.75	0.66 - 0.83	75	50 - 100%
5	MAP;MMRN2;sEng;SPINT1;SEPP1;IGFALS	287	13		0.77	0.68 - 0.85	80	50 - 100%
6	MAP;sEng;SPINT1;SEPP1;IGFALS;PlGF	286	14		0.77	0.69 - 0.85	80	50 - 100%
7	sEng;SPINT1;SEPP1;IGFALS;MCAM;PlGF	273	27		0.75	0.67 - 0.84	90	60 - 100%
8	sEng;SPINT1;IGFALS;MCAM;PlGF	273	27		0.74	0.66 - 0.83	80	50 - 100%

MAP Mean arterial pressure 20 weeks; IGFALS Insulin-like growth factor acid labile subunit; sEng soluble Endoglin, PlGF placental growth factor; SPINT1 Serine peptidase inhibitor Kunitz type 1; MCAM Melanoma cell adhesion molecule; SEPP1 Selenoprotein ; MARE1/3 Microtubule-associated protein RP/EB family member 1 or -3; ALDOA Fructose-bisphosphate aldolase A; MMRN2 Multimerin-2



* Protocol violations (n=14); no preeclampsia outcome available (n=53); fetal loss prior to 20 weeks (n=18)
† Controls included other pregnancy complications

Figure S1. Flowchart describing the selection of subjects in the training and validation sample sets.

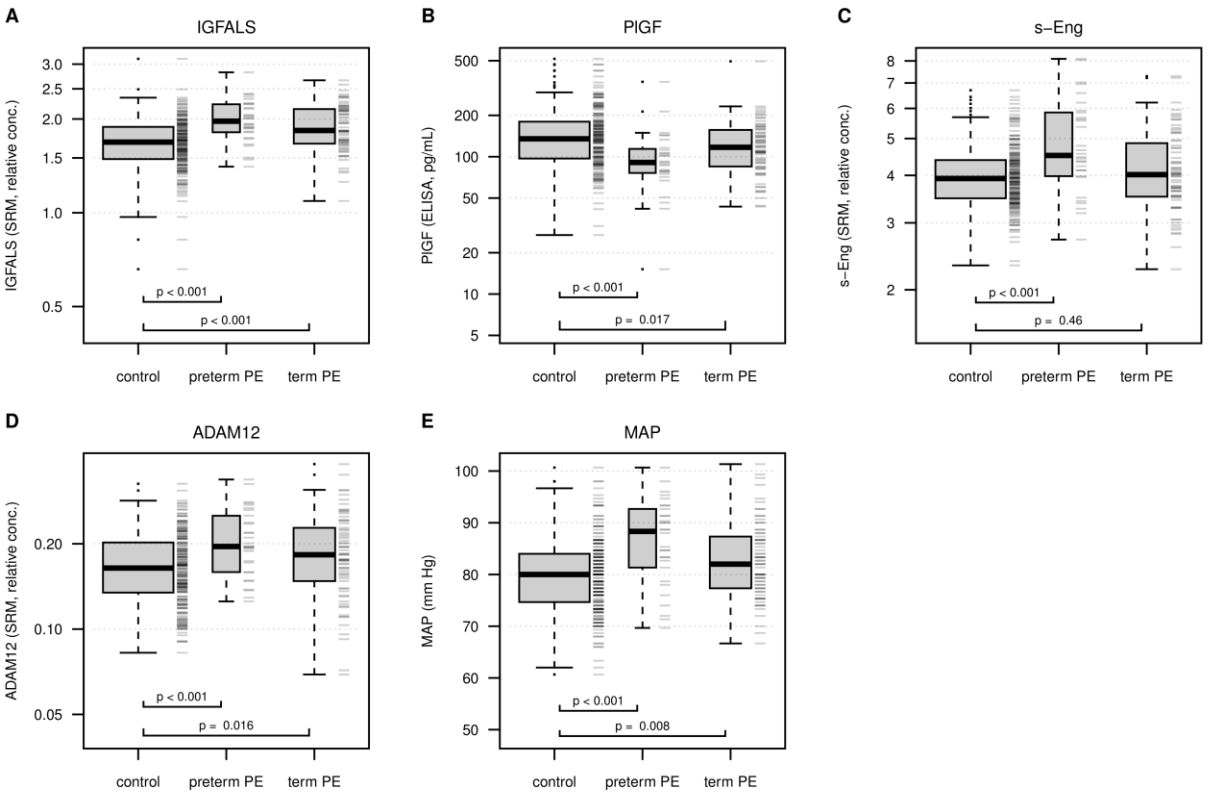


Figure S2. Univariate analyses of biomarkers in the training set.
Box and whisker plots displaying the levels of IGFALS, sEng, ADAM12 (measured by SRM), PlGF (immunoassay), and MAP (20 weeks) are shown for controls, preterm preeclampsia (<37 weeks, n=30) and term preeclampsia (≥ 37 weeks, n=70). P values: Mann-Whitney test.

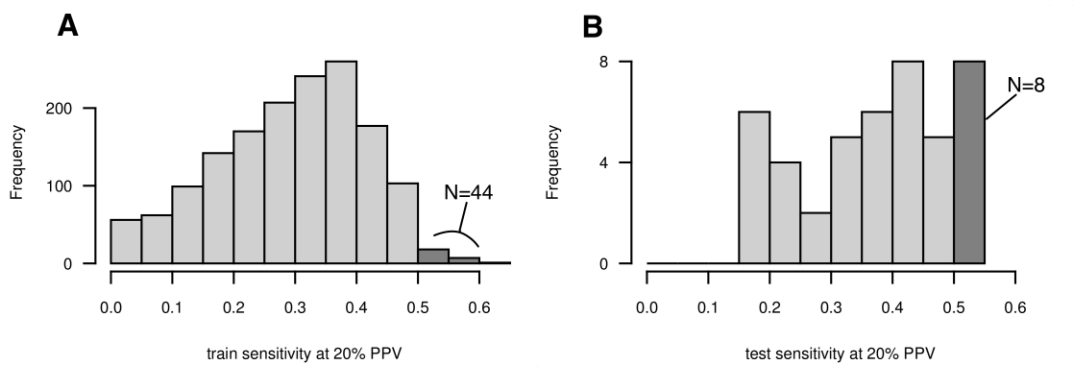


Figure S3 A: Distribution of sensitivities for the predictive models at 20% PPV with p-values for the Wald test < 0.05 and AUC ≥ 0.70 in the training set; the models with sensitivity ≥ 0.50 are highlighted. **B**: Distribution of sensitivities for the predictive models at 20% PPV of all 44 models which met the success criteria in the validation set; the models with sensitivity ≥ 0.50 are highlighted.

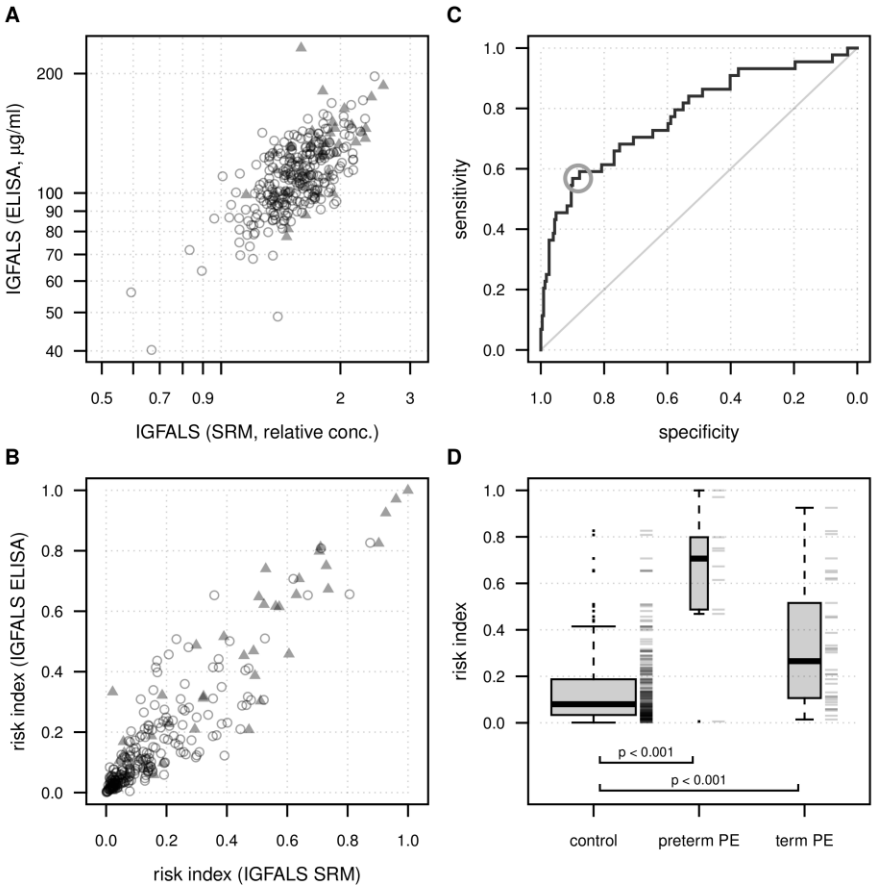


Figure S4. Correlation between SRM readouts and ELISA measurements for IGFALS.

A: Correlation between IGFALS SRM measurements and IGFALS ELISA measurements, cases: triangles; controls: circles (r = 0.63; Spearman's rank correlation). **B** Correlation of risk indexes for the example model using either SRM or ELISA read outs for IGFALS (r = 0.89; Spearman's rank correlation). **C** Receiver operating characteristic curve for the example model using ELISA measurements for IGFALS; the circle indicates the sensitivity at 20% PPV. **D** Risk index for the example model using ELISA read outs for IGFALS. P values: Mann-Whitney test.