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Abstract—Preeclampsia is a pregnancy-specific syndrome that causes substantial maternal and fetal morbidity and mortality. The etiology is incompletely understood, and there is no clinically useful screening test. Current metabolomic technologies have allowed the establishment of metabolic signatures of preeclampsia in early pregnancy. Here, a 2-phase discovery/validation metabolic profiling study was performed. In the discovery phase, a nested case-control study was designed, using samples obtained at 15±1 weeks' gestation from 60 women who subsequently developed preeclampsia and 60 controls taking part in the prospective Screening for Pregnancy Endpoints cohort study. Controls were proportionally population matched for age, ethnicity, and body mass index at booking. Plasma samples were analyzed using ultra performance liquid chromatography-mass spectrometry. A multivariate predictive model combining 14 metabolites gave an odds ratio for developing preeclampsia of 36 (95% CI: 12 to 108), with an area under the receiver operator characteristic curve of 0.94. These findings were then validated using an independent case-control study on plasma obtained at 15±1 weeks from 39 women who subsequently developed preeclampsia and 40 similarly matched controls from a participating center in a different country. The same 14 metabolites produced an odds ratio of 23 (95% CI: 7 to 73) with an area under receiver operator characteristic curve of 0.92. The finding of a consistent discriminatory metabolite signature in early pregnancy plasma preceding the onset of preeclampsia offers insight into disease pathogenesis and offers the tantalizing promise of a robust presymptomatic screening test. **(*Hypertension*. 2010;56:741-749.)**

Key Words: preeclampsia ■ metabolomics ■ biomarkers ■ screening ■ hypertension

Preeclampsia (PE) affects 5% of nulliparous pregnancies and globally afflicts ≈4 million women annually. It remains a leading cause of maternal death throughout the world and is responsible for significant baby morbidity and mortality.[1] Furthermore, PE has healthcare implications for the women later in life with an increased risk of hypertension, coronary artery disease, stroke, and type 2 diabetes mellitus.[2]

Although the precise etiology of the disease is unclear, accumulating evidence suggests that the disease results from complex interaction between a poorly perfused placenta, because of defective remodeling of the uteroplacental arteries in early pregnancy, and a maternal response to placental-derived triggers, which results in widespread vascular endothelial cell dysfunction.[1,3,4]

Widespread plasma alterations precede the clinical onset of PE, and there is intense interest in the identification of predictive biomarkers.[5] Numerous candidate biomarkers have been proposed for prediction of disease, including placental hormones, angiogenic factors, and lipids.[3,6–8] To date, none (nor any combination) has emerged with the requisite specificity and sensitivity to be of clinical use.[5] Consequently, clinicians are unable to offer either targeted surveillance or potential preventative therapies to those nulliparous women at greatest risk.

Metabolic profiling is a powerful strategy for investigating the low molecular weight (bio)chemicals (metabolites) present in the metabolome of a cell, tissue, or organism.[9] Its position as the final downstream product of gene expression enables the provision of a high-resolution multifactorial phenotypic signature of disease etiology, manifestation, or pathophysiology.[10–12]

We previously reported results of an anonymous metabolomic screen of plasma from women with established PE.[13,14] Subsequently, we identified highly discriminatory metabo-

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lites that effectively distinguished cases with PE from matched controls. We, therefore, sought to take a similar metabolomics approach for the detection and development of predictive early pregnancy biomarkers for PE.

A significant issue limiting the discovery of biomarkers in general is the availability of adequate numbers of quality samples from patients with well-characterized phenotypes, where disease prevalence is low (≈5% in PE). This is particularly the case when searching for predictive biomarkers early in pregnancy at a time remote from disease presentation. In the present study, the women were participants in the multinational Screening for Pregnancy Endpoints (SCOPE) Study (www.scopestudy.net), a prospective cohort study of healthy nulliparous women. These samples are extremely well curated, accompanied by comprehensive metadata, and are well matched to avoid potential sources of bias.[15] We performed 2 independent nested case-control studies within the SCOPE cohort, using samples for the discovery and validation phases from 2 different study centers. First, in a biomarker discovery study, plasma samples obtained at 15±1 weeks from 60 women who subsequently developed PE and 60 proportionally matched controls were analyzed using ultra performance liquid chromatography-mass spectrometry (UPLC-MS). The resulting metabolic profiles were investigated using a combination of both univariate and multivariate statistics. A univariate screen was performed to reduce the many thousand metabolite features detected by UPLC-MS down to several hundred that showed any biological variance, thus reducing the multivariate biomarker search space. Multivariate statistics were then used to investigate the underlying correlation between the remaining metabolites and to discover a multifactorial metabolite signature for PE. This signature was then validated using an independent nested case-control study on plasma obtained at 15±1 weeks from 39 different women within the SCOPE cohort who subsequently developed PE and 40 proportionally matched controls.

Methods

Participants and Specimens

The SCOPE Study is a prospective cohort study with the main aim of developing accurate screening methods for later pregnancy complications, including PE (ACTRN12607000551493). Full ethical approval has been obtained, and all of the patients gave written informed consent. Healthy nulliparous women with a singleton pregnancy were recruited between 14 and 16 weeks and tracked throughout pregnancy. For further details of the study population, please see the online Data Supplement at http://hyper.ahajournals.org.

In the discovery phase of our investigation, we performed a nested case-control study within the initial 1628 recruits in Auckland, New Zealand, of whom pregnancy outcome was known in 1608 (98.8%). Sixty-seven women (4.2%) developed PE, and 1021 (63.5%) had uncomplicated pregnancies. The remainder had other pregnancy complications. Sixty women who developed PE were proportionally population matched for age, ethnicity, and body mass index to 60 controls who had uncomplicated pregnancies. The study was limited to 120 samples to guarantee optimal measurement reproducibility from the UPLC-MS systems.[16]

In the validation-phase of our investigation we performed a nested case-control study within the initial 596 recruits in Adelaide, Australia, of whom pregnancy outcome was known in 595 (99.8%). Forty-six women (7.7%) developed PE, and 267 (44.9%) had uncomplicated pregnancies. The remainder had other pregnancy complications. Thirty-nine women who developed PE were proportionally population matched for age, ethnicity, and body mass index to 40 controls who had uncomplicated pregnancies.

PE was defined as a blood pressure ≥140/90 mm Hg after 20 weeks' gestation (but before the onset of labor) or in the postnatal period, with either proteinuria (24-hour urinary protein ≥300 mg, spot urine protein:creatinine ratio ≥30 mg/mmol, or urine dipstick ≥++) and/or evidence of multiorgan complications.[17]

Venipuncture was performed at 15±1 weeks' gestation in nonfasting patients, and plasma samples were collected into BD EDTA-Vacutainer tubes, placed on ice and centrifuged at 2400*g* at 4°C according to a standardized protocol. Plasma was stored in aliquots at −80°C. The collection and storage conditions were identical for cases and controls, with the time between collection and storage being 2.07 (SD 0.90) and 2.02 (SD 0.96) hours, respectively (P=0.78).

Reagents, Sample Preparation, and Mass Spectral Analysis

All of the chemicals and reagents used were of Analytic Reagent or high-performance liquid chromatography grade and purchased from Sigma-Aldrich or ThermoFisher Scientific. Plasma samples were allowed to thaw on ice for 3 hours, vortex mixed to provide a homogeneous sample, and deproteinized. A total of 450 μL of methanol (high-performance liquid chromatography grade) was added to 150 μL of plasma followed by vortex mixing (15 seconds, full speed) and centrifugation (15 minutes, 11 337 *g*). Three 170-μL aliquots of the supernatant were transferred to separate 2 mL tubes and lyophilized (HETO VR MAXI vacuum centrifuge attached to a Thermo Svart RVT 4104 refrigerated vapor trap, Thermo Life Sciences). Quality control (QC) samples were obtained by pooling 50-μL aliquots from each plasma sample prepared. This was defined as the pooled QC sample, and 150-μL aliquots were deproteinized as described above.

Deproteinized samples were prepared for UPLC-MS analysis by reconstitution in 70 μL of high-performance liquid chromatography grade water followed by vortex mixing (15 seconds), centrifugation (11 337 *g*, 15 minutes), and transfer to vials. Samples were analyzed by an Acquity UPLC (Waters Corp) coupled to a hybrid LTQ-Orbitrap mass spectrometry system (Thermo Fisher Scientific) operating in electrospray ionization mode. Samples were analyzed in batches of 120 samples, with an instrument maintenance step at the end of each batch involving mass spectrometer ion source and liquid chromatography column cleaning. For each analytic batch a number of pooled QC samples were included to provide quality assurance. The first 10 injections were pooled QC samples (to equilibrate the analytic system), and then every fifth injection was a pooled QC sample. For each of the analytic experiments (discovery/validation), sample preparation order was randomized from sample picking and rerandomized before sample analysis to ensure no systematic biases (eg, analysis order correlates with sample preparation order). The samples were also blinded to the analytic scientists to avoid any subjective bias. The discovery and validation analyses were performed 3 months apart, such that the 2 studies can be considered independent both in terms of sample population and chemical analysis.

Raw profile data were deconvolved into a peak table using XCMS software.[18] Data were then subjected to strict quality assurance procedures so that statistical analysis was only performed on reproducible data. For full details of all of the methods pertaining to sample preparation, UPLC-MS analysis, and quality assurance, please see the online Data Supplement at http://hyper.ahajournals.org.

Statistical Analysis

Comparisons of clinical data between cases and controls were performed using the Student t test, Mann–Whitney test, χ^2 test or Fisher exact test, as appropriate (SAS system 9.1).

Table 1. Characteristics and Pregnancy Outcome of Women Who Later Developed PE and Controls

	Auckland			Adelaide		
Variables	Preeclampsia (n=60)	Controls (n=60)	*P*	Preeclampsia (n=39)	Controls (n=40)	*P*
Maternal characteristics						
Age, y	30.2 (4.9)	30.4 (4.7)	0.79	22.0 (4.8)	23.2 (5.3)	0.30
Ethnicity						
White	46 (77%)	52 (87%)	0.16	39 (100%)	39 (97.5)	1.0
Other	14 (23%)	8 (13%)		0 (0%)	1 (2.5%)	
At 15 weeks' gestation						
Body mass index, kg/m^2	27.3 (4.9)	26.0 (3.9)	0.12	27.5 (6.2)	26.7 (4.6)	0.48
Systolic blood pressure, mm Hg	115 (11)	107 (12)	0.0003	113 (11)	108 (10)	0.05
Diastolic blood pressure, mm Hg	72 (9)	63 (9)	<0.0001	67 (7)	65 (7)	0.17
Current smoker	1 (1.7%)	4 (6.7%)	0.36	11 (28.2%)	12 (30%)	0.86
Gestation at blood sampling, wk	15.0 (0.9)	15.0 (0.8)	0.59	15.2 (0.7)	15.0 (0.7)	0.19
Pregnancy outcome						
Systolic blood pressure (highest recorded), mm Hg	156 (15)	119 (9)	<0.0001	158 (10)	124 (8)	<0.0001
Diastolic blood pressure (highest recorded), mm Hg	103 (8)	74 (9)	<0.0001	99 (10)	74 (7)	<0.0001
Proteinuria*	54 (90%)	…	…	32 (82%)	…	…
Protein:creatinine ratio, mg/mmol	70 (42, 117)	…	…	52 (26, 172)	…	…
n	53			38		
24-h proteinuria, g	0.6 (0.4, 1.2)	…	…	0.7 (0.2, 2.2)	…	…
n	42			14		
Severe preeclampsia						
Severe hypertension	20 (33.3%)	…	…	6 (15.4%)	…	…
Thrombocytopenia	7 (11.7%)	…	…	2 (5%)	…	…
Liver involvement	12 (20.0%)	…	…	11 (28%)	…	…
Renal involvement	7 (11.7%)	…	…	2 (5%)	…	…
Imminent eclampsia	4 (6.7%)	…	…	2 (5%)	…	…
Gestation at delivery, wk	37.5 (2.8)	40.1 (1.1)	<0.0001	38.1 (2.3)	40.0 (1.3)	<0.0001
Preterm delivery, <37 wk	21 (35%)	…	…	8 (21%)	…	…
Birth weight, g	2925 (753)	3628 (415)	<0.0001	3057 (784)	3583 (391)	0.0004
Customized birth weight centile	40 (11, 70)	50 (35, 75)	0.02	40 (9, 76)	47 (36, 67)	0.24
Small for gestational age	15 (25%)	…	…	10 (25.6%)	…	…

Values are mean (SD), median (interquartile range), or n (%).
*Data are defined as dipstick ≥2+, Protein:creatinine ratio ≥30 mg/mmol, or 24-hour urinary protein ≥0.3 g/24 hours.

Discovery Phase

For each metabolite peak reproducibly detected in the discovery phase study, the null hypothesis that the means of the case and control sample populations were equal was tested using either the Mann–Whitney test or Student *t* test, depending on data normality. The critical *P* value for significance was set to 0.05. No correction for multiple comparisons was performed at this point, because the aim was to reduce the many thousands of detected features down to a subset of potentially "information-rich" peaks while keeping the number of probable false negatives (type II error) to a minimum. False-positive candidate biomarkers are removed during the cross-validation of multivariate analysis and subsequent modeling of the validation data set.

To uncover multivariate latent structure in the data, which, in turn, helps assess the combinatorial predictive ability of the candidate biomarkers, the significant peaks were combined into a single multivariate discriminant model using partial least-squares discriminant analysis (PLS-DA).[19–21] The optimal number of latent factors used in the PLS-DA model was selected using stratified 5-fold cross-validation and model quality assessed using the standard R^2 and Q^2 measures,[19] where R^2, the squared correlation coefficient between the dependant variable and the PLS-DA prediction, measures "goodness of fit" (a value between 0 and 1, where 1 is a perfect correlation) using all of the available data to build a given PLS-DA model. Q^2 provides a measure of "goodness of prediction" and is the averaged correlation coefficient between the dependent variable and the PLS-DA predictions for the 5 holdout data sets generated during the cross-validation process.

Further validation was performed to check the robustness of the final PLS-DA model by comparing the R^2 value to a reference distribution of all of the possible models using permutation testing (N=1000) following the standard protocol for metabolomic studies.[22] Here a reference R^2 distribution is obtained by calculating all of the possible PLS-DA models under random reassignment of the case/control labels for each measured metabolic profile. If the correctly labeled model's R^2 value is close to the center of the reference

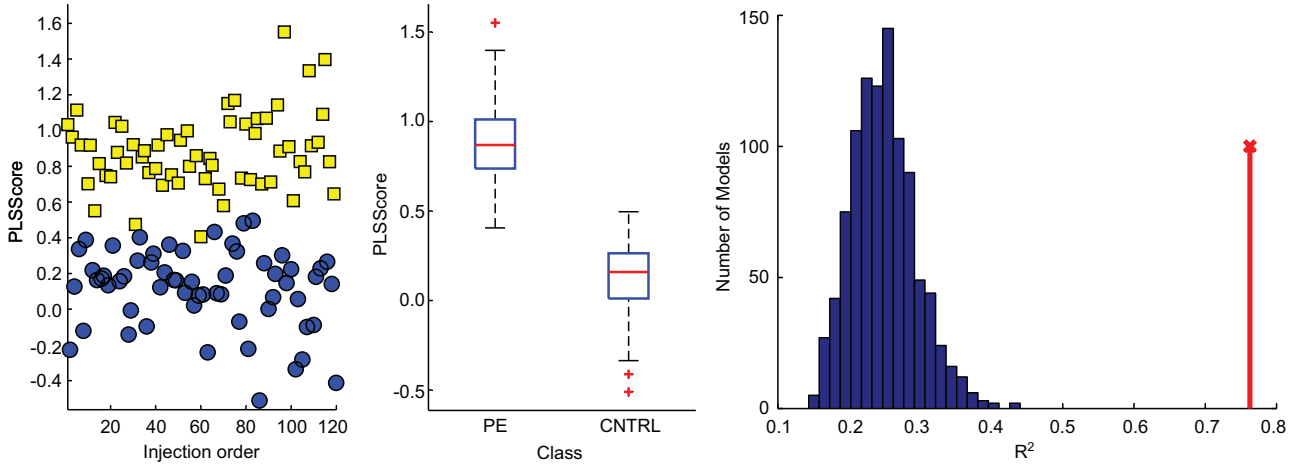

Figure 1. The scores plot for a PLD-DA model using the optimal number of latent vectors (n=1) for data taken from the "discovery" nested case-control study (yellow indicates preeclampsia; blue, controls). Model construction was performed using 5-fold cross-validation resulting in an R^2 of 0.76 and Q^2 of 0.68. The R^2 distribution plot shows that the chosen model's R^2 value is significantly distant from the H_0 randomly classified permutation distribution (n=1000); thus, the probability of the presented model randomly occurring is <0.001. Partial least-squares (PLS) score can be considered as the weighted linear combination of the "information-rich" peaks, which best discriminate between the preeclampsia and control samples. AUC curve was 0.99.

distribution, then the model performs no better than a randomly assigned model and is, therefore, invalid. For all of the PLS-DA models described here, the associated reference distribution plots are provided, from which an estimate of the probability of the candidate model randomly occurring can be estimated. In addition, for each PLS-DA model, a receiver operator characteristic (ROC) curve was determined so that an accurate assessment of discriminatory ability could be made.

As a preprocessing step to remove any structured noise in the data set, direct orthogonal signal correction[23] was performed using a single correction factor and a tolerance setting of 1×10^{-3}. All of the peak data were scaled to unit variance before multivariate analysis.[19,24]

For identification of UPLC-MS–related peaks, the accurate mass for each peak was searched against the Manchester Metabolomics Database[25] constructed with information from both the Human Metabolome Database (http://www.hmdb.ca/) and Lipidmaps (http://www.lipidmaps.org/). A metabolite name(s) was reported when a match with a mass difference between observed and theoretical mass was <5 ppm. Using UPLC-MS, metabolites are often detected multiple times because of chemical adduction, dimerization, multiple charging, isotope peaks, and fragmentation. After removal of duplicate identifications, a list of unique metabolites was compiled. Definitive identifications were reported only for metabolites with retention time errors <10 seconds and an accurate mass match <5 ppm. Once identified, the metabolites were grouped into metabolite classes using the Human Metabolome Database "Class" hierarchy.

For each named metabolite, an ROC curve was determined to assess each metabolite's effectiveness as a univariate discriminatory biomarker. In addition, for each metabolite, the optimal unbiased discriminatory decision boundary was estimated using the optimal Youden index method, and then the associated discriminatory odds ratios with 95% CIs were calculated.[26,27]

Validation Phase

The identified metabolites found to be significant in the discovery phase study were matched to the metabolite peaks detected in the validation study. If a match was found, then the metabolite was univariately assessed as a potential biomarker using the same protocol as for the discovery stage. A PLS-DA model was constructed to assess the multivariate discriminatory ability of the validation peaks.

Finally, we searched for an optimal multivariate discriminatory model drawn from the named metabolites observed in both the discovery and validation studies. A genetic algorithm-based search program was used to obtain the subset of metabolites that produced an effective predictive rule for the onset of PE. This search method has been shown to be very successful in previous studies.[9,28–32] In this algorithm, a set of candidate solutions evolves over time toward an optimal state. The evolution is pushed by computational techniques inspired by evolutionary biology. In our algorithm, each candidate solution (subset of metabolites) is assessed by building 2 independent linear discriminant analysis models, one modeling the discovery data and the other modeling the validation data. A candidate's fitness is proportional to the sum of the root mean square error of prediction of these 2 models. Once the optimal subset of metabolites was found, its predictive ability was assessed using PLS-DA and the Hotelling T^2 test.[33] Assessment was performed independently for the discovery and validation data.

All of the statistical analyses were carried out using the Matlab scripting language (http://www.mathworks.com/). All of the univariate algorithms were implemented such that any missing values are ignored. All of the multivariate algorithms were implemented such that missing values were imputed using the nearest-neighbor method.[34] The Genetic Algorithm search program was written in house.[28] Scripts are available on request.

Results

Discovery Phase

Maternal characteristics and pregnancy outcome in the women with PE and controls are shown in Table 1. After quality assurance, preprocessing, and univariate screening (see Methods section), the UPLC-MS analysis revealed 457 information-rich metabolite peaks. PLS-DA was performed. The resulting model had an R^2 of 0.76, Q^2 of 0.68, and area under the ROC curve (AUC) of 0.99. Model selection was performed using 5-fold cross-validation, and the final model was further validated using permutation testing (see Methods section). The final model used a single latent factor and the probability of this model randomly occurring was <0.001. Figure 1 shows the PLS-DA scores plot and the permutation testing.

Of the 457 candidate biomarker metabolite peaks detected by the UPLC-MS, 70 were successfully identified chemically as known metabolites, of which 45 were "unique" in the sense of being defined molecular entities (Table 2). When grouped

Table 2. Metabolites Identified in Discovery and Validation Phases

Metabolite		Auckland				Adelaide				
Identified as	Metabolite Class	*P*	AUC	Odd Ratio (95% CI)	Up/down in PE?	*P*	AUC	Odd Ratio (95% CI)	Up/down in PE?	Final Rule?
Isobutyrylglycine and/or *N*-butyrylglycine	Acyl glycines	0.05	0.64	2.0 (0.9 to 4.1)	Up					
Taurine	Amino acids	0.01	0.65	3.4 (1.4 to 7.8)	Up					
5-Hydroxytryptophan	Amino acids	0.01	0.67	23.8 (3.0 to 187.3)	Down	0.833	0.61	2.4 (0.8 to 7.1)	Down	√
Urea	Amino ketones	0.01	0.66	2.9 (1.3 to 6.3)	Down	0.949	0.59	1.8 (0.8 to 4.5)	Down	
12-Ketodeoxycholic acid*	Bile acids	0.02	0.67	2.6 (1.3 to 5.6)	Up	0.715	0.58	3.6 (0.9 to 14.4)	Up	
Monosaccharide(s)	Carbohydrates	0.01	0.71	6.1 (2.5 to 15.0)	Up	0.097	0.65	2.8 (1.1 to 7.4)	Up	√
Sedoheptulose	Carbohydrates	0.02	0.67	3.6 (1.5 to 8.4)	Down					
Palmitoylcarnitine	Carnitines	0.001	0.71	3.8 (1.7 to 8.2)	Up	0.244	0.63	3.4 (1.1 to 10.6)	Up	
Stearoylcarnitine	Carnitines	0.006	0.69	3.3 (1.5 to 7.4)	Up	0.610	0.61	2.7 (1.0 to 7.5)	Up	
Decanoylcarnitine	Carnitines	0.007	0.68	3.1 (1.4 to 6.9)	Up	0.624	0.59	1.6 (0.4 to 6.1)	Up	√
Octanoylcarnitine	Carnitines	0.01	0.7	3.0 (1.4 to 6.5)	Up	0.494	0.61	1.9 (0.7 to 5.3)	Up	
Acetylcarnitine	Carnitines	0.02	0.66	2.3 (1.1 to 5.0)	Up	0.207	0.65	4.7 (1.2 to 18.3)	Up	
Dodecanoylcarnitine	Carnitines	0.05	0.69	3.2 (1.2 to 8.8)	Up	0.349	0.63	4.6 (0.9 to 23.5)	Up	
Methylglutaric acid and/or adipic acid*	Dicarboxylic acid	0.01	0.64	2.6 (1.2 to 5.9)	Down	0.010	0.72	3.8 (1.4 to 10.0)	Down	√
8,11,14-Eicosatrienoic acid	Eicosanoids	0.003	0.64	8.7 (2.5 to 29.9)	Up	0.144	0.64	2.1 (0.8 to 5.3)	Up	
20-Carboxyleukotriene B4	Eicosanoids	0.005	0.69	3.1 (1.5 to 6.6)	Up	0.268	0.64	2.1 (0.8 to 5.0)	Up	
Eicosapentaenoic acid and/or retinoic acid	Eicosanoids and/or retinoids	0.03	0.61	3.2 (1.3 to 7.7)	Up					
Isovaleric acid and/or Valeric acid	Fatty acids	0.007	0.68	3.8 (1.7 to 8.6)	Up					
Oleic acid	Fatty acids	0.007	0.68	3.1 (1.4 to 6.7)	Up	0.276	0.63	2.0 (0.8 to 4.8)	Up	√
Linoleic acid	Fatty acids	0.01	0.66	3.5 (1.6 to 7.9)	Up	0.441	0.60	2.3 (0.8 to 6.5)	Up	
Docosahexaenoic acid and/or docosatriynoic acid	Fatty acids	0.01	0.66	5.6 (1.9 to 16.3)	Up	0.204	0.65	2.8 (1.0 to 8.0)	Up	√
Hydroxy-octadecanoic acid and/or oxo-octadecanoic acid	Fatty acids	0.01	0.66	3.5 (1.4 to 8.4)	Up	0.498	0.58	2.0 (0.6 to 6.6)	Up	
Hexadecanoic acid	Fatty acids	0.02	0.67	7.5 (2.1 to 27.3)	Up	0.317	0.62	2.0 (0.8 to 5.2)	Up	
Eicosatetraenoic acid	Fatty acids	0.02	0.67	3.1 (1.4 to 7.1)	Up	0.244	0.63	4.1 (1.0 to 16.3)	Up	
Octadecanoic acid	Fatty acids	0.02	0.67	3.0 (1.4 to 6.5)	Up	0.133	0.64	2.1 (0.8 to 5.3)	Up	
Docosahexaenoic acid	Fatty acids	0.02	0.67	2.6 (1.2 to 5.9)	Up					
γ-Butyrolactone and/or oxolan-3-one	Fatty acids and/or ketones	0.0004	0.72	4.3 (1.8 to 10.0)	Up	0.513	0.60	1.6 (0.6 to 4.1)	Up	√
2-Oxovaleric acid and/or oxo-methylbutanoic acid	Fatty acids or keto acids	0.03	0.66	2.6 (1.2 to 5.4)	Up	0.010	0.72	4.7 (1.8 to 12.3)	Up	√
3-hydroxybutanoic acid and/or 2-hydroxybutanoic acid	Keto or hydroxy FA	0.002	0.71	5.1 (1.9 to 13.8)	Up	0.459	0.61	1.8 (0.7 to 4.7)	Up	
Oxo-tetradecanoic acid and/or hydroxytetradecenoic acid*	Keto or hydroxy FA	0.006	0.72	3.6 (1.5 to 8.8)	Up					
Acetoacetic acid	Keto or hydroxy FA	0.01	0.67	2.9 (1.3 to 6.4)	Up	0.069	0.70	4.2 (1.6 to 11.1)	Up	√
Oxoheptanoic acid	Keto or hydroxy FA	0.02	0.66	2.4 (1.1 to 5.3)	Up					
Di-(heptadecadienoyl)-eicosanoyl-sn-glycerol*	Lipids	0.002	0.66	3.5 (1.5 to 8.0)	Down	0.170	0.65	2.78 (1.2 to 6.9)	Down	

(*Continued*)

Table 2. Continued

Metabolite		Auckland				Adelaide				
Identified as	Metabolite Class	*P*	AUC	Odd Ratio (95% CI)	Up/down in PE?	*P*	AUC	Odd Ratio (95% CI)	Up/down in PE?	Final Rule?
Hexadecenoyl-eicosatetraenoyl-sn-glycerol*	Lipids	0.01	0.69	3.0 (1.4 to 6.9)	Up	0.035	0.69	2.8 (1.1 to 6.9)	Up	√
Di-(octadecadienoyl)-sn-glycerol*	Lipids	0.05	0.65	2.2 (1.0 to 4.5)	Up	0.007	0.73	5.6 (2.1 to 14.6)	Up	√
Octadecenoyl-hexadecanoyl-sn-glycero-3-phosphoserine*	Phosphatidylserines	0.01	0.64	3.6 (1.4 to 9.0)	Down	0.883	0.58	1.7 (0.7 to 4.1)	Down	
Octadecenoyl-sn-glycero-3-phosphoserine*	Phosphatidylserines	0.02	0.65	2.8 (1.2 to 6.1)	Up	0.494	0.61	1.9 (0.8 to 4.6)	Up	
Dioctanoyl-sn-glycero-3-phosphocholine*	Phospholipids	0.01	0.67	3.0 (1.4 to 6.3)	Up	0.605	0.60	2.5 (0.9 to 7.2)	Up	
Sphingosine 1-phosphate	Phospholipids	0.01	0.68	3.3 (1.5 to 7.2)	Up	0.037	0.69	4.2 (1.6 to 11.1)	Up	√
Sphinganine 1-phosphate	Phospholipids	0.03	0.66	2.6 (1.3 to 5.6)	Up	0.939	0.59	1.8 (0.7 to 4.5)	Up	√
Bilirubin	Porphyrins	0.006	0.68	3.2 (1.5 to 6.9)	Up					
Biliverdin	Porphyrins	0.01	0.67	3.1 (1.4 to 6.8)	Up					
Heme	Porphyrins	0.02	0.63	2.9 (1.3 to 6.8)	Up					
Vitamin D3 derivatives	Steroids or steroid derivatives	0.002	0.69	6.2 (2.3 to 16.4)	Up	0.153	0.63	2.8 (1.0 to 7.4)	Up	√
Steroid and/or etiocholan-3-α-o17-one 3-glucuronide*	Steroids or steroid derivatives	0.01	0.68	2.5 (1.2 to 5.2)	Up	0.979	0.58	1.4 (0.6 to 3.5)	Up	

*Metabolite identification included other similar metabolites of the same class.

into metabolite classes (based on the Human Metabolome Database), 11 clear classes emerged. These were amino acids, carbohydrates, carnitines, Eicosanoids, fatty acids, keto or hydroxy acids, lipids, phospholipids, porphyrins, phosphatidylserine, and steroids.

A PLS-DA was performed using only the 45 named metabolites (1 latent factor). This produced a predictive model with R^2 of 0.58, Q^2 of 0.57, and AUC of 0.96 (Figure S1, available in the online Data Supplement at http://hyper.ahajournals.org). This proved to be only a slight reduction of diagnostic performance when compared with the full 457-peak model.

Validation Phase

The maternal characteristics and pregnancy outcome in the women with PE and controls are shown in Table 1. Of the 45 significant metabolites named in the discovery study, 34 were also detected in the validation study. All of these metabolites showed similar changes in peak response (29 were raised in patients who went on to develop PE; 5 were lowered). A PLS-DA model using the 34 metabolites (1 latent factor) proved to be predictive, with R^2 of 0.57, Q^2 of 0.53, and AUC of 0.95 (Figure S2).

Metabolite Signature of PE

Finally, data from both studies were mined using a genetic algorithm-based search program to find the subset of named metabolites that produced the most robust predictive general model. The Genetic Algorithm chose 14 metabolites (Table 2). Figure 2 shows the PLS-DA model predictions using these metabolites for both the discovery study and the validation study. For the discovery data, the 14-metabolite model had an R^2 of 0.54, Q^2 of 0.52, an AUC of 0.94, and an optimal odds ratio of 36 (95% CI: 12 to 108). For the validation data, the 14-metabolite model had an R^2 of 0.43, Q^2 of 0.39, an AUC of 0.92, and an optimal odds ratio of 23 (95% CI: 7 to 73). Permutation testing showed that the probability of both of these models randomly occurring was <0.001 (Figure S3). The combined effect of the 14 metabolites was also tested using the Hotelling T^2 statistic. For the discovery study data, this produced a P value of 2×10^{-6}, and for the validation study data, a P value of 0.006. The P values were obviously affected by the differing sample sizes (discovery n=120; validation n=79).

Discussion

PE is a complex syndrome with multiple biological pathways contributing to its etiology. We have, therefore, taken a holistic and data-driven systems biology approach to identify a metabolic signature in plasma that is predictive of subsequent PE.[35]

We identified 40 organic molecules to be significantly elevated and 5 that were reduced in plasma at 14 to 16 weeks' gestation from healthy nulliparous women who later developed PE, as compared with matched controls composed of women who had uneventful pregnancies. During the discovery phase, we showed that there is clear multifactorial disruption of plasma because of onset of PE (Figure 1). The 45 identified molecules, whose molecular weights ranged

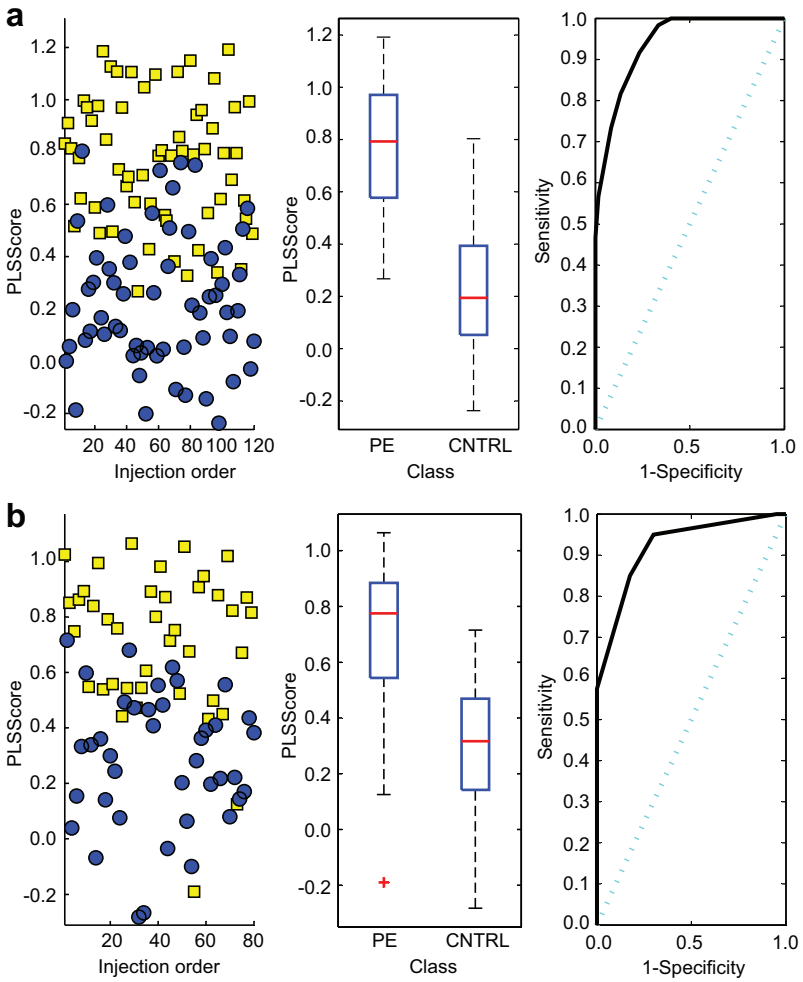

Figure 2. The PLS-DA model predictions for the final 14-metabolite signature found by the genetic algorithm search program (C indicates controls, blue circles; PE, preeclampsia, yellow squares). a, Model predictions for the discovery phase data; R^2=0.54, Q^2=0.52, an AUC of 0.94, an optimal odds ratio of 36 (95% CI: 12 to 108), and Hotelling T^2 $P=2\times10^{-6}$. b, Model predictions for the validation data; R^2=0.43, Q^2=0.39, an AUC of 0.92, an optimal odds ratio of 23 (95% CI: 7 to 73), and Hotelling T^2 $P=2\times10^{-3}$.

between 60.06 and 883.42, were sufficiently well characterized to enable their allocation into 5 broad functional categories, as detailed in Table 2. A thorough discussion of the biological significance of this metabolic fingerprint is outside the scope of this article. However, we note that there appears to be a significant overlap of scope of markers with what is already well known about the pathogenesis of this disease.

Using robust data mining and modeling techniques, and using an independent validation cohort, we have shown that a combination of 14 metabolites representing the latent systems-wide interaction in the metabolome is sufficient to produce a robust predictive model with AUC of >0.9 (Figure 2). For both the discovery and validation studies, each individual metabolite in this panel is not highly significant; however, when these metabolites are combined into a single multifactorial model, the power of such data-driven technology proves its worth.

From the 14 metabolite ROC curves (Figure 2) we can also determine potential screening performance. At a 10% false-positive rate, the estimated respective detection rates of subsequent PE for the discovery data and validation data are 77% and 73%. Conversely, for a detection rate of 90%, it is estimated that the false-positive rate would be 21% and 24%. The predictive power of the 14-metabolite rule compares highly favorably with that of other proposed first trimester screening tests, including those based on first trimester levels of placental hormones, such as placental protein 13 and pregnancy-associated plasma protein A. In a longitudinal study by Akolekar et al,[36] the comparative AUCs for placental protein 13 and pregnancy-associated plasma protein A alone are 0.818 and 0.872, respectively. For both placental protein 13 and pregnancy-associated plasma protein A the AUC is 0.878. The comparative values for our 14-metabolite rule in the discovery and validation sets are 0.94 and 0.92, respectively. Similarly, our 14-metabolite rule compares favorably with the predictive power of early pregnancy maternal levels of angiogenic factors. In a longitudinal study by Kusanovic et al,[37] the AUCs for placental growth factor alone and for the ratio of placental growth factor:soluble endoglin are 0.647 and 0.662, respectively. Poon et al[38] have generated first-trimester predictive models combining pregnancy-associated plasma protein A and placental growth factor together with a combination of maternal characteristics. For early onset PE, their model shows excellent (if not yet validated) predictive power that, given a 5% false-positive rate, produces a detection rate of 93%. However, for late-onset PE, the equivalent detection rate is only 36%. Based on the same false-positive rate assumptions, our metabolite model (early and late PE combined) produces detection rates of 71% (discovery) and 68% (validation). It is expected that

the detection rates of our model will increase significantly when combined with maternal characteristics. One potential limitation of this study is the lack of ethnic variation in the validation cohort. However, ongoing work in a larger cohort containing women from different ethnic groups will further validate the model presented here.

Perspectives

The present study is one of the most detailed metabolic screens performed in any human disease to date. The finding of discriminatory metabolites in early pregnancy plasma preceding PE offers insight into disease pathogenesis and the potential for early prediction. Most importantly, ongoing metabolomics work with a larger prospective cohort of healthy nulliparous women offers the prospect of combining demographic details and clinical data with metabolite measurements. These additional data will potentially improve the sensitivity and specificity of the final algorithm for the prediction of PE as early as 15 weeks' gestation and also provide further validation of the work presented here. A predictive rule at 15 weeks' gestation will have a significant impact on clinical care, allowing scarce resources to be concentrated on those at greatest risk. As an early indicator of PE, such a test will also present a platform for developing therapeutic interventions that could minimize the likelihood of serious complications later in pregnancy, significantly reducing morbidity and mortality rates.

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Disclosures

None.

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Title: On line supplement for: ROBUST EARLY PREGNANCY PREDICTION OF LATER PREECLAMPSIA USING METABOLOMIC BIOMARKERS

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Short title: Early pregnancy biomarkers of preeclampsia

Supplementary Methodology

Study Population

Healthy nulliparous women with singleton pregnancies were recruited to the SCOPE (Screening for Pregnancy Endpoints) study between November 2004 and July 2007 in Auckland, New Zealand and Adelaide, Australia. SCOPE is a prospective, multi-centre cohort study with the aim of developing screening tests to predict preeclampsia, small for gestational age (SGA) infants and spontaneous preterm birth. Ethical approval was obtained from local ethics committees (New Zealand AKX/02/00/364 and Australia REC 1712/5/2008) and all women provided written informed consent.

Women attending hospital antenatal clinics, obstetricians, general practitioners or community midwives prior to 15 weeks' gestation were invited to participate in the SCOPE study. Exclusion criteria included 1) considered at high risk of preeclampsia, SGA or spontaneous preterm birth due to underlying medical conditions (chronic hypertension, diabetes, renal disease, systemic lupus erythematosus, anti-phospholipid syndrome, sickle cell disease, human immunodeficiency virus), gynaecological history, ≥3 previous terminations or ≥3 miscarriages; 2) had a known major fetal anomaly or abnormal karyotype or 3) received intervention that may modify pregnancy outcome (e.g. aspirin therapy). Participants were interviewed and examined by a research midwife at 15+1 and 20+1 weeks of gestation and underwent an ultrasound scan at 20+1 weeks. At the time of interview, data were entered into an internet accessed, central database with a complete audit trail.

Data collected at 15 weeks included detailed demographic, obstetric and medical and family information. Current pregnancy data included an early pregnancy scan to accurately calculate the estimated date of delivery. If the woman had a certain last menstrual period (LMP) date, the estimated date of delivery was only adjusted if either 1) a scan performed at <16 weeks' gestation found a difference of ≥7 days between the scan gestation and that calculated by the LMP or 2) on 20-week scan a difference of ≥10 days was found between the scan gestation and that calculated from the LMP. If her LMP date was uncertain, then scan dates were used to calculate the estimated date of delivery. Information was collected on current pregnancy complications such as vaginal bleeding and dietary information pre-conception and during pregnancy was obtained using food frequency questions. Use of folate and multivitamin, cigarettes, alcohol and recreational drugs was recorded for preconception, 1st trimester and at 15 weeks. A lifestyle questionnaire was completed by participants asking about work, exercise and sedentary activities, snoring, domestic violence and social supports. Validated psychological scales measuring perceived stress (Perceived Stress Scale ref), depression (Edinburgh Postnatal Depression Scale ref), anxiety (Short Form of the State Trait Anxiety Index measuring anxiety ref) and behavioural responses to pregnancy (adapted from the Behavioural Responses to Illness Questionnaire) were completed. Maternal physical measurements included two blood pressure recordings with mercury or aneroid sphygmomanometers, height, weight and the circumference of her waist, hip, arm and head. Proteinuria in a midstream urine specimen was measured by dipstick or a protein creatinine ratio. At 20 ± 1 weeks' gestation, the information collected included any pregnancy complications since the 15 week interview, maternal physical measurements and the participant completed the lifestyle questionnaire. Ultrasound examination at 20 ± 1 weeks included measurements of the fetus (biparietal diameter, head circumference, abdominal circumference and femur length) and Doppler studies of the umbilical and uterine arteries.

Participants were followed prospectively, with pregnancy outcome data and baby measurements collected by research midwives. Data monitoring included 1) individually checking all data for each participant, including for any data entry errors of the lifestyle questionnaire, and 2) using customised software to detect any systematic data entry errors.

Primary Outcome Measure

The primary outcome was preeclampsia, defined as gestational hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90mmHg on at least two occasions four hours apart after 20 weeks of gestation, but before the onset of labour, or postpartum systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90mmHg on at least two occasions four hours apart) with proteinuria (24 hour urinary protein ≥ 300 mg or spot urine protein:creatinine ratio ≥ 30 mg/mmol creatinine or urine dipstick protein ≥ 2+) or any multi -system complication of preeclampsia. ref Multi-system complications included any of the following 1) acute renal insufficiency defined as a new increase in serum creatinine ≥100 umol/L antepartum or >130 umol/L postpartum; 2) liver involvement defined as raised aspartate transaminase and/or alanine transaminase >45 IU/L and/or severe right upper quadrant or epigastric pain or liver rupture; 3) neurological included eclampsia, imminent eclampsia (severe headache with hyperreflexia and persistent visual disturbance) or cerebral haemorrhage and 4) haematological included thrombocytopenia (platelets <100 x 109/L), disseminated intravascular coagulation or haemolysis.

An uncomplicated pregnancy was defined as a pregnancy not complicated by preeclampsia, SGA, spontaneous pre-term birth or any other pregnancy complication such as gestational hypertension.

UPLC-MS analysis

Samples were prepared by reconstitution in 70µl HPLC grade water followed by vortex mixing (15 seconds), centrifugation (11 337*g*, 15 minutes) and transfer to vials. Samples were analysed by an Acquity UPLC (Waters Corp. Milford, USA) coupled to a LTQ-Orbitrap mass spectrometry system (Thermo Fisher Scientific, Bremen, Germany) operating in electrospray ionisation mode. Samples were analysed consecutively in positive ion mode followed and then consecutively in negative ion mode. Chromatographic separations were performed employing an ACQUITY UPLC BEH 1.7µm-C_{18} column (2.1 x 100mm, Waters Corp. Milford, USA). Solvent A and solvent B were 0.1% formic acid in water and 0.1% formic acid in methanol, respectively. In positive ion mode a flow rate of 0.40ml.min^{-1} was applied with a gradient elution profile (100% A for 1 minute and subsequently ramped to 100% B (curve 5) over 15 minutes, followed by a 4 minute hold at 100% B before a rapid return to 100% A and a hold for 2 minutes). In negative ion mode a flow rate of 0.36ml.min^{-1} was applied with a gradient elution program (100% A for 2 minutes and subsequently ramped to 100% B (curve 4) over 15 minutes, followed by a 5 minute hold at 100% B before a rapid return to 100% A and a hold for 2 minutes). The column and samples were maintained at temperatures of 50°C and 4°C, respectively. A 10µl sample volume was introduced onto the column and 50% of the column effluent was transferred to the mass spectrometer. Centroid MS scans were acquired in the mass range of 50-1000Th using the Orbitrap mass analyser operating with a target mass resolution of 30 000 (FWHM as defined at m/z 400) and a scan time of 0.4s. Mass calibration was performed before each analytical batch using an instrument

manufacturer defined calibration mixture (ThermoFisher Scientific, Bremen, Germany).

Data processing of UPLC-MS data

All data was converted to netCDF format using the FileConverter program in the XCalibur software package (ThermoFisher Scientific, Bremen, Germany). Raw data processing. All raw data (in.raw file format) were converted to netCDF file format with the FileConverter program available in XCalibur (ThermoFisher Scientific, Bremen, Germany).

XCMS deconvolution

XCMS is an open-source deconvolution program available for LC-MS data.(1) Deconvolution using the XCMS program was performed using identical settings to those reported previously(2) with the exception of s/n threshold = 3, step = 0.02, m/z diff = 0.05 and for grouping bandwidth = 10 and mzwidth = 0.05. The esi program (http://msbi.ipb-halle.de/msbi/esi/) available with the XCMS software package was used to write peak output files to an annotated version (as a .csv file) which is more appropriate for these studies. XCMS and esi were run using R version 2.6.0.

Quality Assurance

The performance of analytical instrumentation has to be assessed robustly to ensure that data are of comparable high quality within an analytical run. An approach based on the periodic analysis of a standard biological Quality Control sample (QC sample) together with the patient samples is now accepted as a quality assurance strategy in metabolic profiling.(3), (4) A similar Quality Assurance protocol has been followed in this metabolomic study to assess the repeatability for thousands of endogenous metabolites. A set of pooled QC samples were prepared by mixing equal aliquots from all the samples in a single study. A QC sample is then injected after every fourth patient sample in each analytical run (a *lead-in* of 10 consecutive QC injections was performed at the start of every analytical run to equilibrise the IPLC column response). At the end of the experimental run, and after XCMS deconvolution each detected peak is normalised to the QC sample using robust Loess signal correction (R-LSC). Here Locally Weighted Scatterplot Smoothing (LOESS) is performed on the QC data with respect to the order of injection. A cubic spline correction curve for the whole analytical run is then interpolated, to which the total data set for that peak is normalized. Using this procedure any attenuation of peak response over an analytical run (i.e. confounding factor due to injection order) is minimised.(4, 5) After R-LSC each peak is required to pass strict Quality Assurance criteria. While there are no generally accepted criteria for the assessment of repeatability in metabolomic data sets, the UK Food and Drug Administration (FDA) suggests a range of criteria that should be applied. In the guidance for bioanalytical method validation in industry (6) the FDA recommends for single analyte tests that tolerance limits are set such that the measured response detected in two-thirds of QC samples is within 15% of the QC mean, except for compounds with concentrations at or near the limit of quantification (LOQ), in these cases a tolerance of 20% is acceptable. In our case, the methods are not specific for one analyte of interest, but instead we aim to detect thousands of analytes, therefore an acceptance tolerance of 20% would seem to be appropriate. Any peak that did not pass the QA criteria was removed from the dataset and thus ignored in any subsequent data analysis.

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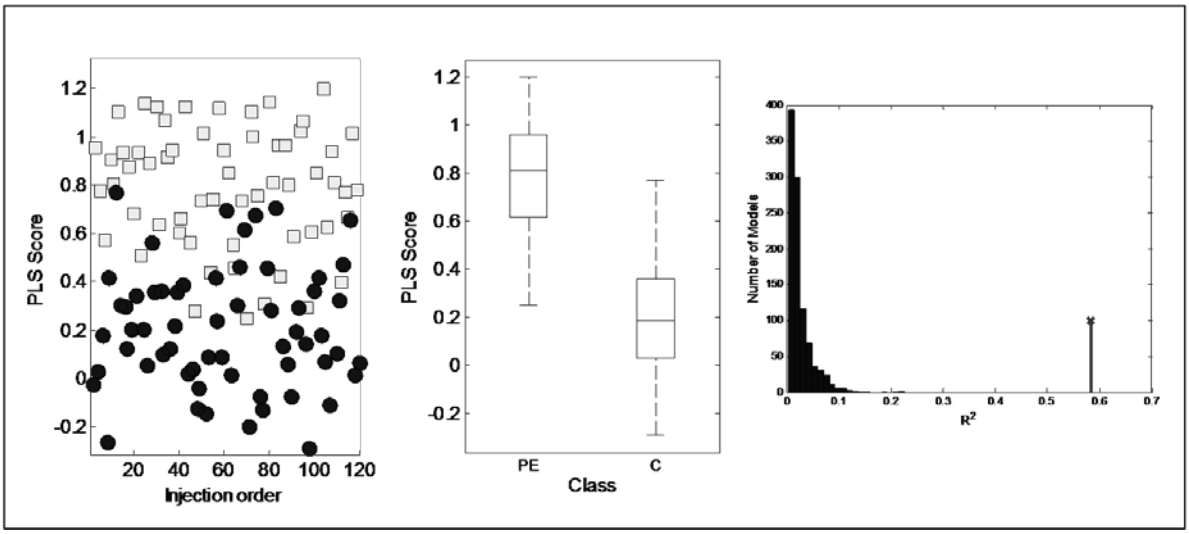
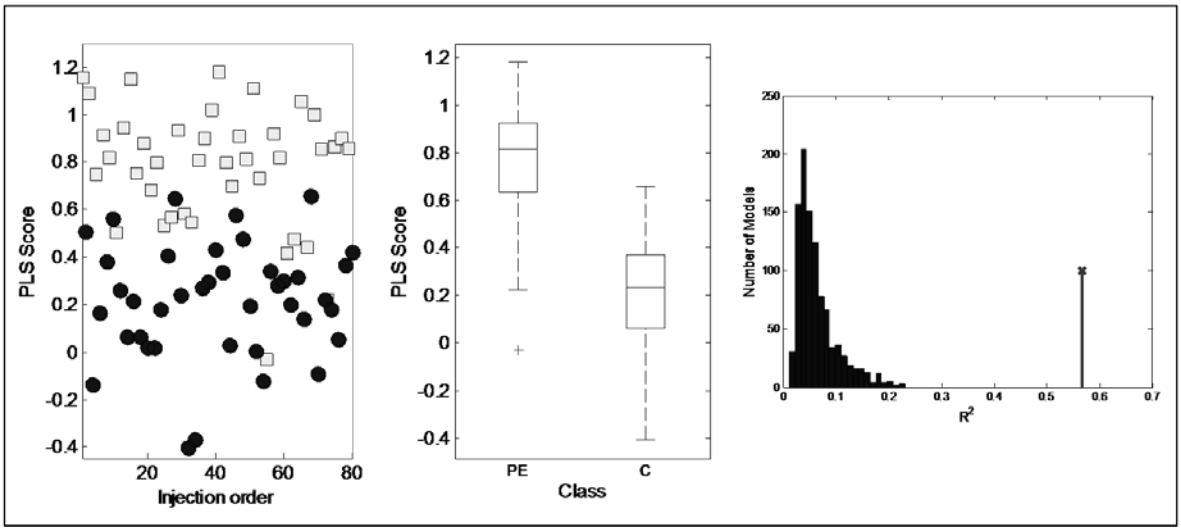
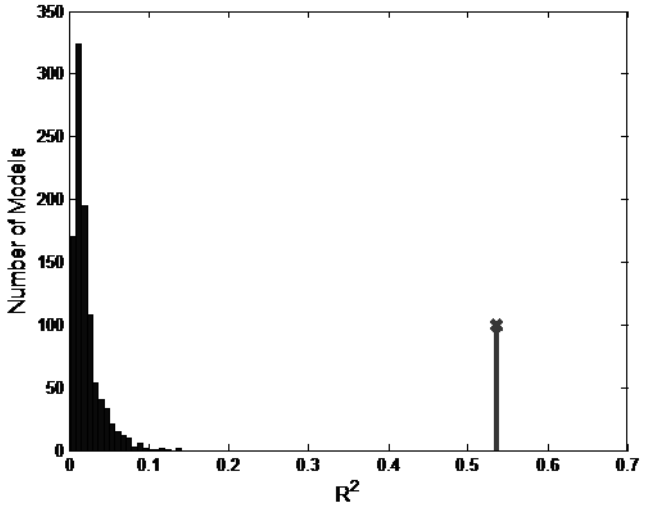


S1. The scores plot for a PLD-DA model using the optimal number of Latent Vectors (n=1) for the 45 named metabolites taken from the 'discovery' nested case-control study. Grey =preeclampsia; Black=controls. Model construction was performed using 5-fold cross validation resulting in an R^2 = 0.58 and Q^2 = 0.57 . The R^2 distribution plot shows that the chosen model's R^2 value is significantly distant from the H_0 randomly classified permutation distribution (n=1000); thus the probability of the presented model randomly occurring is < 0.001. Area under ROC curve was 0.96.

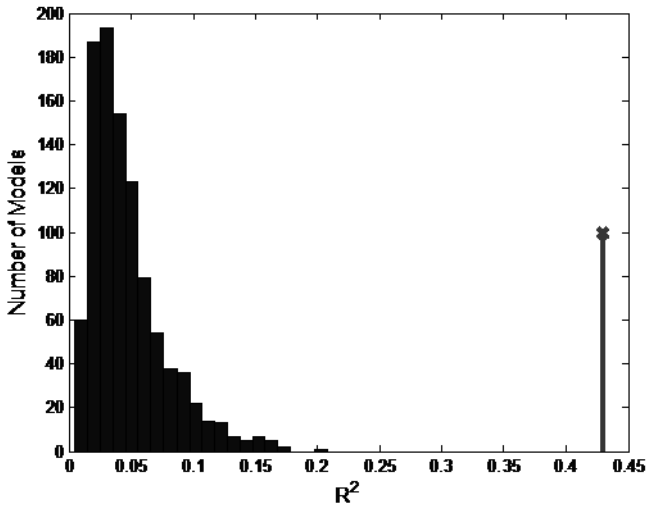


S2. The scores plot for a PLD-DA model using the optimal number of Latent Vectors (n=1) for 38 named metabolites taken from the 'validation' nested case-control study. The 38 metabolites were those of the 45 metabolites named in the discovery study that were detected in the validation study. Grey =preeclampsia; Black=controls. Model construction was performed using 5-fold cross validation resulting in an $R^2 = 0.57$ and $Q^2 = 0.53$. The R^2 distribution plot shows that the chosen model's R^2 value is significantly distant from the H_0 randomly classified permutation distribution (n=1000); thus the probability of the presented model randomly occurring is < 0.001. Area under ROC curve was 0.95.

S3(a)



S3(b)



S3. The R^2 distribution plots for (a) the 14 metabolite discovery model and (b) the 14 metabolite validation model. Both show that the chosen models' R^2 values are significantly distant from the H_0 randomly classified permutation distribution (n=1000); thus the probability of the presented model randomly occurring is < 0.001.