

# Metabolomics and first-trimester prediction of early-onset preeclampsia

Ray O. Bahado-Singh<sup>1</sup>, Ranjit Akolekar<sup>2</sup>, Rupasri Mandal<sup>3</sup>, Edison Dong<sup>3</sup>, Jianguo Xia<sup>4</sup>, Michael Kruger<sup>1</sup>, David S. Wishart<sup>3,4</sup> & Kypros Nicolaidis<sup>2</sup>

<sup>1</sup>Department of Obstetrics and Gynecology, Wayne State University, Detroit, MI, USA, <sup>2</sup>Department of Obstetrics and Gynecology, King's College Hospital, London, UK, <sup>3</sup>Departments of Biological Sciences, University of Alberta, Edmonton, Alberta, Canada, and <sup>4</sup>Department of Computing Sciences, University of Alberta, Edmonton, Alberta, Canada

**Objective:** To evaluate the use of metabolomics for the first-trimester detection of maternal metabolic dysfunction and prediction of subsequent development of early-onset preeclampsia (PE). **Study design:** This was a case-control study of maternal plasma samples collected at 11–13 weeks' gestation from 30 women who had subsequently developed PE requiring delivery before 34 weeks and 60 unaffected controls. Nuclear magnetic resonance (NMR) spectroscopy was used to identify and quantify metabolomic changes in cases versus controls. Both genetic computing and standard statistical analyses were performed to predict the development of PE from the metabolite concentrations alone as well as the combination of metabolite concentrations with maternal characteristics and first-trimester uterine artery Doppler pulsatility index (PI). **Results:** Significant differences between cases and controls were found for 20 metabolites. A combination of four of these metabolites (citrate, glycerol, hydroxyisovalerate, and methionine) appeared highly predictive of PE with an estimated detection rate of 75.9%, at a false-positive rate (FPR) of 4.9%. The predictive performance was improved by the addition of uterine artery Doppler PI and fetal crown-rump length (CRL) and with an estimated detection rate of 82.6%, at a FPR of 1.6%. **Conclusion:** A profound change in the first-trimester metabolite profile was noted in women who had subsequently developed early-onset PE. Preliminary algorithms appeared highly sensitive for first trimester prediction of early onset PE.

**Keywords:** First-trimester screening, metabolomics, preeclampsia, uterine artery Doppler

## Introduction

Maternal death remains a significant health problem and preeclampsia (PE), which affects about 2% of pregnancies, is a major cause of maternal and perinatal mortality and both short- and long-term morbidity [1–3]. Recent evidence suggests that PE can be subdivided into early-onset disease or early-PE requiring delivery before 34 weeks gestation and late-PE with the former being associated with a higher incidence of fetal growth restriction and fetal morbidities and both short-term and

long-term maternal mortality and morbidities [4–7]. The underlying mechanism for the development of early-PE is thought to be impaired trophoblastic invasion of the maternal spiral arteries and placental ischemia [8]. In contrast, late-onset PE is often due to preexisting maternal vascular disorders or metabolic abnormalities with associated mild or nonexistent failed trophoblast invasion [9].

Metabolomics is a rapidly developing field of omics technology that employs high-throughput analytical chemistry to characterize the metabolome. The metabolome is defined as the complete collection of metabolites (<1500 Da) or small molecules found in an organism or in its cells, tissues, and biofluids [10]. These metabolites include, but are not limited to amino acids, sugars, lipids, vitamins, bases, and other organic molecules. There is now the realization, after years of research, that even if we had a complete understanding of the genes, transcripts, and proteins in a living system this would not come close to describing the phenotype of that organism [11]. Metabolomics, in cataloguing the large number of metabolites that connect all the different pathways in the living cell, appears to come the closest to describing the cell phenotype in both normal and disease states. Because of its potential for systematic and unbiased cataloguing of such a large number of metabolites [12], metabolomics is increasingly being used to elucidate disease mechanisms and to detect complex disorders. Examples of some of the diseases being investigated by metabolomics include coronary artery disease, cancer, and diabetes [13].

The objective of the current study is to evaluate the use of metabolomics for the first-trimester detection of maternal metabolic dysfunction and prediction of subsequent development of early-PE.

## Methods

### Study population

The data for this case-control study were derived from prospective screening for adverse obstetric outcomes in women attending their routine first-hospital-visit during pregnancy. In this visit, which is held at 11<sup>+0</sup>–13<sup>+6</sup> week's gestation, we record maternal characteristics and medical history, perform an ultrasound scan

to determine gestational age from the fetal crown-rump length (CRL) [14] and to diagnose major fetal abnormalities [15]. We also carry out Doppler ultrasound to measure the uterine artery pulsatility index (PI) bilaterally and record the average of the two PI values [16]. Serum samples were also obtained and stored at  $-80^{\circ}\text{C}$  for subsequent laboratory analysis. The women were screened between March 2006 and September 2009, and they all gave written consent to participate in the study, which was approved by the King's College Hospital research ethics committee.

Metabolomic studies were carried out in 30 singleton pregnancies that subsequently developed early-PE requiring delivery before 34 weeks and 60 unaffected controls. The cases were drawn from the screening study population. The controls were also from the same population from pregnancies with no complications and normal outcome matched to the cases for storage time. The definition of PE used was that proposed by the International Society for the Study of Hypertension in Pregnancy [17]. To summarize, diagnostic features of PE were systolic pressures  $\geq 140$  or diastolic  $\geq 90$  mm Hg on two or more occasions 4 h apart after 20 weeks of gestation in women who were previously normotensive. In addition, the patient must have had proteinuria of 300 mg in a 24-h urine collection or two readings of at least  $2^{+}$  proteinuria on a midstream or catheterized urine specimen in the absence of a 24-h urine collection.

#### Nuclear magnetic resonance spectrometry

Nuclear magnetic Resonance (NMR) spectrometry uses radio frequency (RF) radiation to obtain information about the constituent atomic nuclei of chemical compounds being investigated. Exposure of a sample (under a high magnetic field) to RF radiation results in a change in energy state of the nuclei. When the RF energy pulse is discontinued the nuclei return to the lower energy state and reemit the stored energy in the form of atom-specific or molecule-specific RF waves. The emitted RF energy provides a "fingerprint" for identifying the atomic nuclei from which the energy is emitted. Based on this, the constituent atoms can be identified, and thus the chemical composition of the substance(s) being investigated was determined.

#### Sample preparation and NMR spectroscopy

Plasma and serum samples contain a significant concentration of large molecular weight proteins and lipoproteins, which affect the identification of the small molecular weight metabolites by NMR spectroscopy. A deproteinization step, involving ultrafiltration as previously described [18], was therefore introduced in the protocol to remove plasma proteins. Prior to filtration, 3 KDa cutoff centrifugal filter units (Amicon Microcon YM-3), were rinsed three times each with 0.5 mL of  $\text{H}_2\text{O}$  and centrifuged (10,000 rpm for 30 min) to remove residual glycerol bound to the filter membranes. Aliquots (350  $\mu\text{L}$ ) of each plasma sample were then transferred into the centrifuge filter devices and spun (10,000 rpm for 20 min) to remove macromolecules (primarily protein and lipoproteins) from the sample. If the total volume of the sample was under 300  $\mu\text{L}$ , an appropriate amount from a 50 mM  $\text{NaH}_2\text{PO}_4$  buffer (pH 7) was added until the total volume of the sample was 300  $\mu\text{L}$ . Any sample that had to have buffer added to bring the solution volume to 300  $\mu\text{L}$ , was annotated with the dilution factor, and metabolite concentrations were corrected in the subsequent analysis. Subsequently, 35  $\mu\text{L}$  of  $\text{D}_2\text{O}$  and 15  $\mu\text{L}$  of a standard buffer solution (11.667 mM DSS [disodium-2, 2-dimethyl-2-silcepentane-5-sulphonate], 730 mM imidazole, and 0.47%  $\text{Na}_3\text{N}$  in  $\text{H}_2\text{O}$ ) was added to the sample.

The plasma sample (350  $\mu\text{L}$ ) was then transferred to a standard Shigemi microcell NMR tube for subsequent spectral analysis. All  $^1\text{H}$ -NMR spectra were collected on a 500-MHz Inova (Agilent Inc., Palo Alto, CA) spectrometer equipped with a 5-mm ITCN Z-gradient PFG cold-probe.  $^1\text{H}$ -NMR spectra were acquired at  $25^{\circ}\text{C}$  using the first transient of the NOESY-presaturation pulse sequence, chosen for its high degree of quantitative accuracy [19]. All free induction decays (FIDs) were zero-filled to 64 K data points and subjected to line broadening of 0.5 Hz. The singlet produced by the DSS methyl groups was used as an internal standard for chemical shift referencing (set to 0 ppm) and for quantification all  $^1\text{H}$ -NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional Software package version 7.0 (Chenomx Inc, Edmonton, Alberta, Canada). The Chenomx NMR Suite software allows for qualitative and quantitative analysis of an NMR spectrum by manually fitting spectral signatures from an internal database to the spectrum. Specifically, the spectral fitting for metabolite was done using the standard Chenomx 500 MHz metabolite library. Typically, 90% of visible peaks were assigned to a compound and more than 90% of the spectral area could be routinely fit using the Chenomx spectral analysis software. Most of the visible peaks are annotated with a compound name. It has been previously shown that this fitting procedure provides absolute concentration accuracy of 90% or better. Each spectrum was processed and analyzed by at least two NMR spectroscopists to minimize compound misidentification and misquantification. We used sample spiking to confirm the identities of assigned compound. Sample spiking involves the addition of 20–200  $\mu\text{M}$  of the suspected compound and examination of the resulting spectra to determine whether the relative NMR signal intensity changed as expected.

#### Statistical analysis

Data normalization is critical to creating a normal or Gaussian distribution of metabolite values. This allows conventional statistical tests to be performed, and it simplifies data interpretation. There are many different normalization options available – here we used log scaling normalization. Principle component analysis (PCA) is an unsupervised classification technique for transforming a complex collection of data points such that the important properties of the sample can be more simply displayed along the X- and Y- axes. PCA involves calculating correlation coefficients between sets of data and then determining eigenvalues and eigenvectors through linear algebraic transformations. The result is a set of "vectors" of different metabolites which can be used to plot out the metabolite data on an X–Y cluster plot. The first and most significant vector is called the first-principal component (X axis), and the second most significant vector is called the second-principal component (Y axis). If the data are separable, it should be possible to see two sets of clusters. Two clusters indicate that there are some significant metabolic or metabolite differences between the two sets of samples, that is, disease versus normals [20].

Partial least squares discriminant analysis (PLS-DA) is a supervised classification technique that is widely utilized in metabolomic analysis. PLS-DA is used to enhance the separation between the groups by rotating the PCA components for maximum separation. This procedure allows a better identification and understanding of the variables most responsible for separating the two groups and is the next step used if no PCA separation is observable. In this technique, the samples are labeled (early-PE and normal). The data are then processed in a manner similar to PCA, but with the requirement that the method must try to separate

the two samples as best it can. However, false determination of separation between the two groups can occur. As a test to see if this separation is not just due to chance, permutation testing was used. This involves randomly re-labeling the metabolomic data and then running the PLS-DA again. This is performed repeatedly (2000 times), with different random labelings. After this is done, one calculates the *p* value or the probability that the first separation was a random event due to chance. The MetaboAnalyst program was used to perform all univariate and multivariate (PCA and PLS-DA) statistical analyses of the metabolomic data [21]. The important metabolites were ranked by Variable Importance in Projection or VIP score, which is a weighted sum of squares of the PLS loadings. This is a plot of the significant metabolites with their relative importance expressed as a numerical value on the X-axis. The higher the VIP value, the greater is the metabolites relative contribution in distinguishing the early-PE from control groups. Widely recommended statistical procedures were followed for metabolomic analysis [22].

A predictive model for PE: Mean (*SD*) metabolite concentrations in early-onset PE versus controls were compared. Stepwise logistic regression analyses were performed with early-onset PE as the dependent variable and metabolites as the independent or determinant variables. All metabolites with  $p \leq 0.10$  on univariate analysis were initially entered into the regression. Other variables including fetal CRL and maternal demographic and medical status (racial origin, weight, height, smoking, method of conception, previous pregnancy with or without PE, diabetes mellitus, chronic hypertension) were combined with metabolite concentrations and run in the regression analyses. Finally, regression analyses including first-trimester uterine artery PI and the preceding metabolomic and other maternal markers were performed for the prediction of early-PE. Based on these analyses, several regression equations for predicting the individual risk of early-PE were developed. Individual risk or probability of early-PE was calculated for each patient in the study. Different probability thresholds (e.g. individual probability of early-PE from  $1 > 1/2$ ,  $>1/10$ ,  $>1/20$ ,  $>1/30$ , up to  $>1/100$ ) were each used serially to define an increased risk of early-PE. Using each cutoff value as a screening test, paired sensitivity (defined as the percentage of early-PE cases with probability value above this threshold) and specificity (percentage of normal cases with calculated probability of having early-PE below this threshold), were calculated. FPR, defined as  $1 - \text{specificity}$ , can then be easily determined from the specificity value. Using multiple different probability threshold values, a series of paired sensitivity and FPR values were generated. Thereafter, a receiver-operator characteristic curve is plotted with sensitivity values on the Y-axis and the corresponding FPR on the X-axis. The area (AUC [23]) under the ROC curve indicates the accuracy of a test for correctly identifying a disorder, that is, early-PE cases from controls, with an AUC = 1 indicating a perfect test. The 95% CI and *p* values for the AUC curves were also calculated.

Genetic computing: More recent approaches to the analysis of the torrent of data generated by “omics” analyses have been developed [24,25]. Genetic computing or programming is a branch of evolutionary computing which has been around since the 1950s. Genetic computing is thought to be superior to conventional statistical analysis in explaining the differences between healthy and diseased individuals and in finding the most significant and interesting differences between groups. It generates rules by which an optimal number of variables can be selected from a large number of exploratory variables, for example, metabolite concentrations, and also optimally selects the interactions between these variables for the prediction of the outcomes of interest such as

the presence or absence of PE. A highly accurate classification of individuals into disease and nondisease groups is generated. TheGmax (from Genetics Consultant Ltd) software program is an evolutionary modeling tool that optimizes nonparametric data-mining objectives. Version 11.09.23 (www.thegmax.com) was used for evolutionary computing analysis.

## Results

Pertinent maternal and fetal characteristics in the early-PE and controls are compared in Table I. The only significant difference was in uterine artery PI which was higher in the PE group. A total of 42 metabolites were identified and quantified in the maternal plasma samples, and significant differences between the early-PE cases and controls were found for 20 of the metabolites when appropriate nonparametric analysis is made for metabolites that are nonnormally distributed. Five metabolites were at borderline significance using conventional statistical analysis (Table II). It is possible that significance may be found in the latter group, if a larger number of cases are analyzed. A number of the control patients in our study had exceptionally high levels (40× normal) of choline, whereas most of the early-PE patients had normal or below normal levels ( $<20 \mu\text{M}$ ) of choline. This suggests that these high choline controls could possibly be due to the use of choline supplements (400–500 mg/day). The separation between the cases of early-PE and controls from the PCA analysis of the NMR data is shown in Figure 1. The PLS-DA analysis resulted in a clear separation between the groups (Figure 2). Permutation testing demonstrated that the observed separation was not by chance ( $p < 0.005$ ). A VIP plot in which the metabolites were ranked by their contribution to distinguishing the cases of early-PE from controls is shown in Figure 3. The greater the distance from the Y-axis, the greater is the contribution of a particular metabolite in distinguishing cases from controls. This plot also indicates whether the metabolite concentration is increased or decreased in cases relative to controls.

Two models were developed, using logistic regression analysis, for the prediction of early-PE; one evaluated the following independent variables: four metabolites (citrate, glycerol, hydroxyisovalerate, methionine) in combination with maternal characteristics (weight and the presence/absence of medical disorders) and another evaluated three metabolites (citrate, glycerol, hydroxyisovalerate) in combination with maternal characteristics (parity and the presence or absence of medical disorders) and uterine artery PI and fetal CRL. The estimated

Table I. Comparison of the early preeclampsia and control groups.

Parameter	Early preeclampsia	Control	<i>p</i> value
Number of cases	30	60	–
Maternal age in years, mean ( <i>SD</i> )	30.6 (6.8)	31.8 (5.8)	0.41
Racial origin, <i>n</i> (%)			0.13
White	32 (52.5)	10 (34.5)	
Black	22 (36.1)	16 (55.2)	
Asian	7 (11.5)	2 (6.9)	
Other	0 (0)	1 (3.4)	
Nullipara (%)	47.5	41.4	0.65
Weight in kg, mean ( <i>SD</i> )	72.4 (17.0)	66.8 (14.9)	0.10
Crown-rump length in mm, mean ( <i>SD</i> )	63.3 (8.6)	65.4 (8.5)	0.28
Uterine pulsatility index (MoM), mean ( <i>SD</i> )	1.5 (0.5)	0.95 (0.3)	$<0.001$

MoM, multiples of median for gestational age.

Table II. Metabolite concentrations in early preeclampsia and controls.

Metabolite	Early-PE (concentration: μM/L)	Controls (concentration: μM/L)	p value
	Mean (SD)	Mean (SD)	
Number of cases	30	60	-
Hydroxybutyrate <sub>2</sub>	21.4 (5.6)	19.5 (7.4)	0.25
Hydroxybutyrate <sub>3</sub>	31.6 (19.1)	27.0 (16.2)	0.44
Hydroxyisovalerate <sub>3</sub> <sup>a</sup>	9.2 (1.7)	7.6 (3.1)	0.01
Acetamide	9.6 (5.4)	10.0 (6.3)	0.96
Acetate <sup>a</sup>	17.9 (6.0)	48.4 (50.4)	<0.001
Acetoacetate	14.5 (5.1)	18.8 (9.7)	0.59
Acetone	14.5 (3.6)	17.2 (20.3)	0.57
Alanine <sup>a</sup>	263.8 (51.5)	332.1 (184.2)	0.02
Arginine	113.4 (16.5)	124.2 (28.8)	0.056
Asparagine	36.5 (17.2)	34.5 (14.5)	0.48
Betaine	29.2 (6.9)	25.8 (8.8)	0.31
Carnitine	30.2 (6.3)	31.8 (14.1)	0.70
Choline <sup>a</sup>	10.4 (2.6)	143.9 (277.4)	0.001
Citrate	85.9 (19.1)	79.3 (19.3)	0.06
Creatine	34.7 (10.6)	37.4 (13.0)	0.06
Creatinine	56.8 (11.8)	56.7 (14.5)	0.74
Dimethylamine	3.5 (1.4)	4.0 (2.7)	0.219
Ethanol	33.1 (22.3)	49.3 (30.2)	0.08
Formate <sup>a</sup>	12.4 (4.5)	19.3 (14.8)	0.032
Glucose <sup>a</sup>	4194.1 (1229.6)	3702.2 (713.5)	(0.019)
Glutamine <sup>a</sup>	331.0 (57.2)	288.2 (82.0)	0.007
Glycerol <sup>a</sup>	166.5 (42.5)	484.2 (341.4)	<0.001
Glycine <sup>a</sup>	188.0 (42.8)	239.6 (106.1)	(0.035)
Isobutyrate	6.3 (2.1)	6.3 (2.1)	1.0
Isoleucine <sup>a</sup>	37.6 (9.6)	46.2 (19.3)	0.023
Isopropanol <sup>a</sup>	5.9 (4.1)	38.4 (98.8)	(0.034)
Lactate	1006.2 (396.8)	1035.6 (505.9)	0.78
Leucine <sup>a</sup>	67.5 (15.5)	96.9 (74.9)	0.003
Malonate	16.8 (6.7)	18.9 (9.8)	0.34
Methionine <sup>a</sup>	20.6 (4.2)	23.5 (7.1)	(0.024)
Ornithine	36.1 (11.9)	38.2 (15.7)	0.18
Phenylalanine <sup>a</sup>	62.9 (12.1)	79.6 (42.7)	0.002
Proline	138.6 (52.0)	142.6 (58.5)	0.067
Propylene_glycol <sup>a</sup>	9.9 (4.9)	8.0 (3.4)	(0.04)
Pyruvate <sup>a</sup>	89.3 (27.0)	71.3 (26.7)	(0.006)
Serine <sup>a</sup>	115.5 (23.8)	150.3 (84.3)	0.004
Succinate <sup>a</sup>	4.3 (1.6)	12.4 (13.9)	0.007
Threonine <sup>a</sup>	127.1 (29.0)	145.9 (60.3)	0.025
Trimethylamine <sup>a</sup>	13.3 (2.0)	11.2 (3.1)	(0.008)
Tyrosine	54.1 (15.3)	58.0 (22.3)	0.21
Valine	119.1 (29.5)	129.8 (51.1)	0.22
Methylhistidine	50.7 (12.9)	50.0 (14.6)	0.75

The p values in brackets are based on Mann-Whitney U test for biomarkers with non-normal distributions.

<sup>a</sup>Metabolites with statistically significantly different concentrations in the early-PE cases compared with controls. PE, preeclampsia.

performance of screening for early-PE by these models at an approximate risk threshold of >1 in 2 for early-PE is summarized in Table III which is based on standard statistical analysis with the corresponding ROC curves without and with inclusion of Doppler measurements demonstrated in Figures 4 and 5, respectively. In the first model (without Ut-PI) Table III, the estimated detection

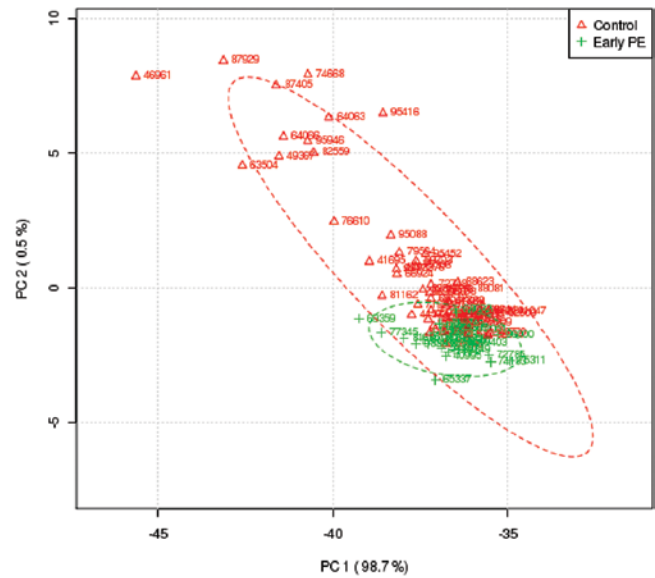


Figure 1. Principle component analysis plot showing the separation between early preeclampsia (in green) and control (in red) for nuclear magnetic resonance spectrometry.

rate of early-PE was 75.9%, at a FPR of 4.9%, and the respective values for the second model which included uterine Doppler PI were 82.6% and 1.6%. Ut PI by itself had a 40% detection rate at 8.2% FPR.

The results of genomic computing analysis using a minimum (parsimonious model) number of predictors are shown in Table IV. Two models were developed, one using metabolites (glutamine, pyruvate, propylene glycol, trimethylamine, hydroxybutyrate) in combination with maternal characteristics (weight and medical disorder) and another using metabolites (glutamine, pyruvate, propylene glycol, trimethylamine, hydroxybutyrate, carnitine, hydroxyisovalerate) in combination with uterine artery PI. The diagnostic sensitivities at low FPRs are shown in Table IV. The model that excluded uterine Doppler measurements also had a 70% detection rate at 15% FPR, whereas the model that included uterine Doppler measurements showed an increase in detection rate to 90% while simultaneously lowering the FPR to 11%. Complex models using a larger number of predictors achieved even greater areas under the ROC curves and thus diagnostic accuracy (not shown).

## Discussion

The findings of this case-control study demonstrate the potential value of metabolomics in the first-trimester prediction of early-PE. Algorithms combining metabolites in maternal plasma with uterine artery PI at 11–13 weeks' gestation could identify more than 80% of pregnancies that subsequently developed early-PE at a FPR of less than 2%.

Metabolomics is often regarded as the newest member of the "omics" family and is now being extensively applied for the development of disease biomarkers and to understand the pathogenesis of complex and formerly poorly understood disorders. As a consequence, there has been a meteoric increase in the number of publications on this topic, from 1 publication in 1997 to 1503 in 2009 [26]. We applied NMR-based metabolomics to determine the diagnostic accuracy of using small molecule metabolites from maternal plasma to predict the development of early-onset PE. Using standardized metabolomic analytic

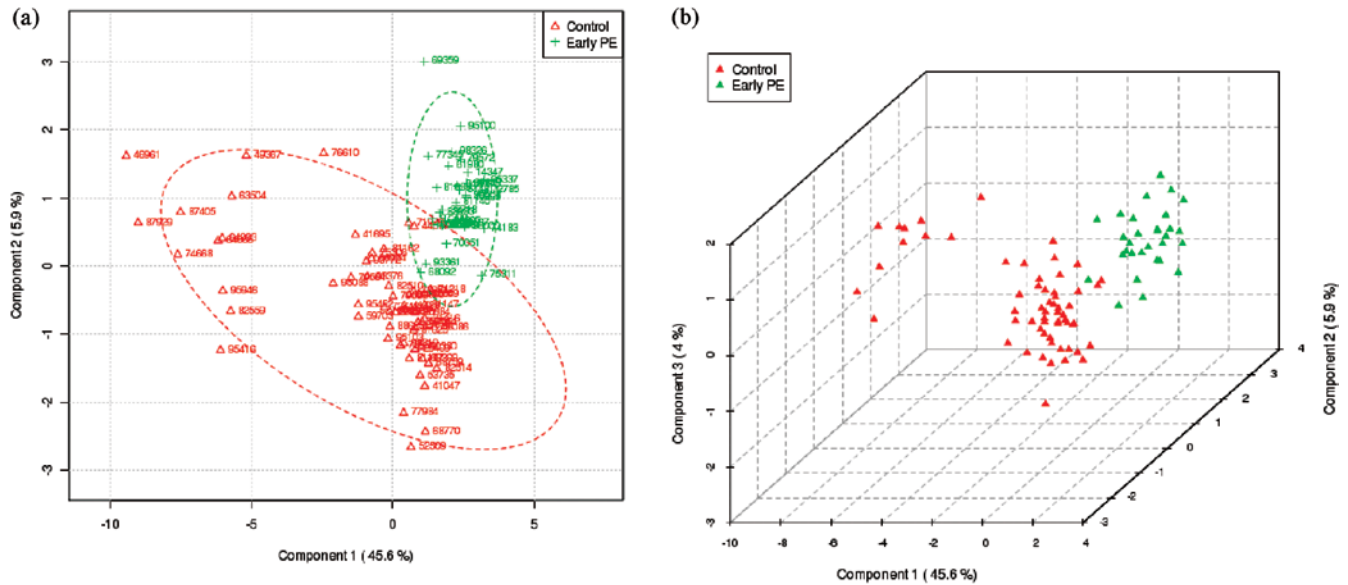


Figure 2. Separation between the cases of early preeclampsia (in green) and controls (in red) in the partial least squares discriminant analysis two dimensional score plot (a) and three dimensional score plot (b).

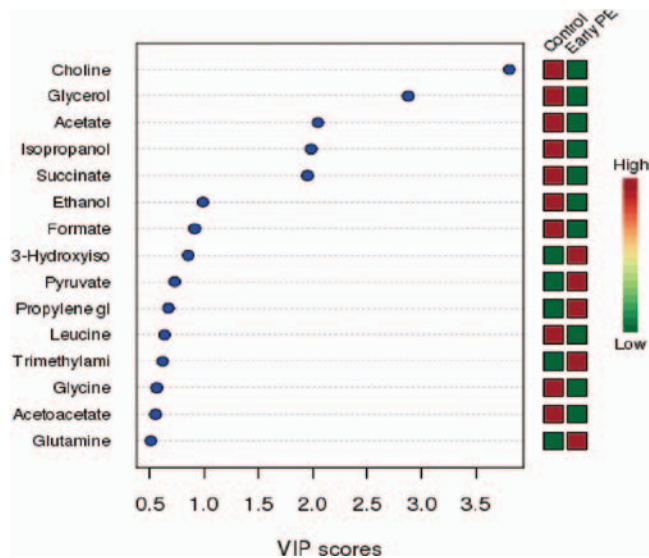


Figure 3. Variable Importance in Projection (VIP) plot indicating the most discriminating metabolite in descending order of importance.

Table III. Estimated performance of screening for early preeclampsia.

Regression model	AUROC (95% CI)	Sensitivity (%)	FPR (%)
Metabolites <sup>a</sup>	0.904 (0.828, 0.98)	75.9	4.9
Metabolites <sup>b</sup> , uterine artery PI, and fetal CRL	0.98 (0.95, 1.00)	82.6	1.6

<sup>a</sup>Citrate, glycerol, hydroxyisovalerate, methionine.

<sup>b</sup>Acetate, glycerol, hydroxyisovalerate.

AUROC, area under the receiver-operating characteristic curve; CI, confidence interval; CRL, crown-rump length; FPR, false-positive rate; PI, pulsatility index.

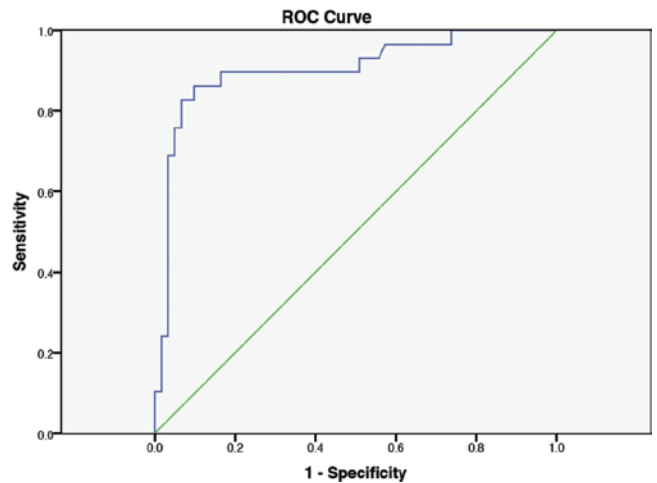


Figure 4. Receiver-operator characteristic (ROC) curve for prediction of early-onset preeclampsia: Metabolite-only Algorithm. Area under ROC curve = 0.904 ( $p < 0.001$ ).

Table IV. Estimated performance of screening for early preeclampsia using genetic computing analysis.

Regression model	AUROC	Sensitivity (%)	FPR (%)
Metabolites <sup>a</sup> , maternal weight, and medical disorders	0.84	50	5.0
Metabolites <sup>b</sup> , uterine artery PI	0.94	60	3.0

<sup>a</sup>Glutamine, pyruvate, propylene glycol, trimethylamine, hydroxy butyrate (parsimonious model).

<sup>b</sup>Pyruvate, propylene glycol, trimethylamine, hydroxy isovalerate-3 (parsimonious model).

AUROC, area under the receiver-operating characteristic curve; FPR, false-positive rate; PI, pulsatility index.

techniques and commonly recommended statistical approaches [27], we found marked differences in the metabolomic profile in the first trimester of women that several months later developed early-PE compared with normal controls. The results are all the more striking because we did not control for the many other potential sources of variability in the metabolite profile, including

time of day, diet, fasting versus nonfasting state and stress. Normalizing for these many potential variables could possibly have further improved the separation achieved between early-PE and normal cases.

The strength of metabolomics is that it is not hypothesis driven and can provide new understanding into the pathogenesis of

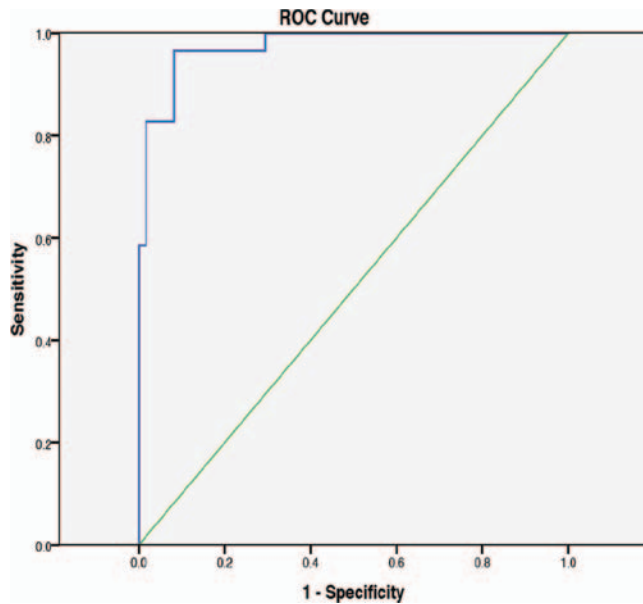


Figure 5. Receiver-operator characteristic (ROC) curve for prediction of early-onset preeclampsia: Metabolite Plus Uterine Artery Doppler Algorithm. Area under ROC curve = 0.975 ( $p < 0.001$ ).

complex disorders. In our study, the majority of discriminating metabolites were decreased in cases that subsequently developed early-PE. The biological role of the top three metabolites (glycerol, choline, and acetate) was reviewed in the Human Metabolome Database [10], and potential explanations for alterations of their concentrations are advanced. The decreased levels glycerol in PE patients appear to be a consequence of the increased conversion of glycerol to triglycerides into the blood stream. Significant dyslipidemia, particularly hyper-triglyceridemia has been previously described as a feature of PE [28]. In addition to glycerol, another significantly altered metabolite was choline. A number of the control patients in our study had exceptionally high levels ( $40\times$  normal) of choline, whereas most of the early-PE patients had normal or below normal levels ( $<20\ \mu\text{M}$ ) of choline. This suggests that these high choline controls could possibly be due to the use of choline supplements ( $400\text{--}500\ \text{mg/day}$ ). Although a dietary history was not obtained from these study participants, choline supplementation has previously been recommended for pregnant women, as low choline levels are known to be associated with elevated homocysteine levels [29], which increases the risk for PE, premature birth, and very low birth weight. Our results seem to suggest that choline supplementation may indeed protect women against early-PE as none of the individuals with these unusually high choline levels developed PE. Acetate when bound to coenzyme q plays a central role in the metabolism of carbohydrates and fats and point further to the potential role of dysfunction of lipid and carbohydrate (insulin resistance) metabolism in PE.

A second objective of our study was to identify potential biomarkers for the early prediction of subsequent PE. We evaluated metabolites by themselves and in various combinations with maternal characteristics and uterine artery PI. Metabolomic markers strongly predicted the future development of early-PE even without the usual pregnancy and demographic information such as gestational length, maternal weight, and race. The current sample size is limited, and thus future studies using larger number of cases will need to be performed to determine the variability introduced by standard demographic factors.

Due to its unfamiliarity to many readers a more detailed explanation of evolutionary computation is warranted. Evolutionary computation has been around since the 1950s. These computational methods are tools for predicting outcomes from a complex mass of data. Evolutionary computation included a number of approaches such as genetic algorithms. The latter is most widely utilized for problem solving based on the use of the three principles of natural evolution: selection, mutation, and recombination [30,31]. Applications extend from chemistry, economics, engineering, pharmaceuticals to metabolomics. Goodcare [32] outlined the acute challenge of analyzing the vast volume of data generated from new analytic platforms such as metabolomics. He used as an example the analysis of 250 metabolites, a plausible number in metabolomics, to discriminate plants resistant to drought from normal control plants. A complete search to determine whether or not a particular metabolite would be included in the model would require  $2^{250}$  or  $1.8 \times 10^{75}$  computations. An ultrafast computer would require more than an estimated  $3 \times 10^{62}$  years to perform the required computations [32]. Evolutionary computation is an automated method for providing a good solution or predicting the outcome of interest from a large mass of data in a much shorter time.

Evolutionary computation selects “chromosomes” (which is a “string” or a combination of different metabolites and their concentrations) that are optimally suited to “survive” meaning predict the outcome of interest. Each predictor variable (e.g. metabolite) represents a “gene” on this “chromosome” string. The “fitness” to survive of each chromosome is a numerical value from 0 to 1, assigned by the computer program. Fitness indicates how well these combination of parameters ensures “evolutionary survival”, or otherwise stated provides the best answer to the problem [32].

The combination of the “chromosome” and the “fitness” represents an “individual” [33]. A population of such “individuals” represents the “first generation” of the organisms. The “individuals” are ranked according to their fitness. This begins the evolutionary process. The selection operator creates the next generation by choosing the fittest individuals from the first generation which have the best chance of “survival,” that is, predicting the outcome of interest. In addition new “individuals” for the second generation are created by crossover with random rearrangement of segments of the “chromosome,” that is, a change in a “chromosome” segment with its string of constituent predictors (metabolites) which form the sequence of “genes.” Finally, “mutation” is produced where changes in an individual is introduced. The mutation could mean either changes in constituent predictors or input variables (metabolites) with or without any change in their numerical values (concentrations). The evolutionary parallel between genes and an input variable, in this case metabolites should be recognized by the reader.

Thus, genetic algorithms take high performing “individuals” and select “mutates” and “recombine” them with other high fitness or high performing “individuals” to eventually achieve the optimal combination of “genes” or input predictors on the “chromosome” that will predict the outcome of interest. The similarities to the well-recognized principles of evolution are obvious. Evolutionary computing including genetic algorithms produces progressively better solutions to the problem through continuous reevaluation and adjustment [30]. The process identifies key components and patterns form a large data set to achieve the highest predictive accuracy. The process is rapid, automated, and does not required any statistical or other assumption about the input variables or outcomes of interest. It is unaffected by missing data, impervious

to background noise, and does not require parametric distribution. Overall, it is said to be superior to regression analyses and neural networks and equally handles both small and extremely large data sets.

Uterine artery Doppler has emerged as the single most accurate predictor of PE [16,34–36]. By combining uterine artery PI with metabolite concentrations highly accurate algorithms were developed for the prediction of early-PE. Both conventional statistical analysis and genomic computing methods confirmed the accuracy of uterine artery PI and metabolomics-based methods for the prediction of early-PE. A recent study on the use of metabolomics-based markers found high predictive accuracy for the later development of PE [33]. The 45 significantly discriminating metabolites reported in that study were largely different from those found in the current study. There are several potential explanations for the differences in the metabolite lists for the two studies. First, different metabolomic platforms were used. We used an absolutely quantitative NMR-based platform, whereas the study of Kenny et al. [37] used a semiquantitative liquid chromatography–mass spectrometry (LC–MS)–based platform. Sharp differences in the types of metabolites identified (polar for NMR, nonpolar for LC–MS) and sensitivity (micromolar for NMR, nanomolar for LC–MS) are known to exist between different analytical platforms [18,20]. Second, the metabolites identified by the LC–MS study of Kenny et al. [37], were obtained via mass-matching only, whereas the metabolites identified/quantified by our NMR study were identified by authentic standards and comprehensive spectral matching. Third, we focused on early-PE, rather than PE overall as in the prior publication [37] because there are considerable differences both in the pathophysiology and consequences between early and late disease. Finally, there were differences in the racial origin of patients (about half were Black in our study, whereas all White in the study of Kinney et al. [37]) and in the gestational age at sampling (12 weeks in our study, whereas 15 weeks in the study of Kinney et al. [37]).

A recent publication of Odibo et al. [38] used LC–MS metabolomic methods of first-trimester specimens to identify individuals at risk for PE and found three amino acids, phenylalanine, glutamate, and alanine to be significantly elevated in women who subsequently developed PE. Interestingly, in our study, we found no significant difference in amino acid levels within our cohort. We are uncertain as to why these discrepancies exist, although the Odibo study did not measure choline, glycerol, acetate, or other metabolites that we reported. This may partly be explained by the use of different metabolomic platforms and the fact that the latter study grouped early- and late-onset PE cases in some of its analyses.

In conclusion, this study has identified novel first-trimester biomarkers that are associated with early-PE. Predictive models consisting of metabolites alone or in combination with uterine artery PI and maternal characteristics can potentially provide an effective early screening for the disease.

## Acknowledgments

All the metabolomics analyses were done at the Metabolomics Innovation Centre (TMIC), Edmonton, Alberta, Canada, funded by Genome Canada.

**Declaration of Interest:** This study was partly supported by a grant from the Fetal Medicine Foundation (Charity No: 1037116).

## References

- Villar J, Say L, Gulmezoghe AM, Merialdi M, Lindheimer M, Beltran AP, et al. Eclampsia and preeclampsia: a worldwide health problem for 2000 years. In: Critchley H, MacLean A, Poston L, Walker J, editors. Preeclampsia. London, UK: RCOG; 2003.
- Maternal Mortality in 2005. Estimates developed by WHO, UNICEF, UNFPA and the World Bank. Geneva, Switzerland: World Health Organization 2007.
- Bellamy L, Casas JP, Hingorani AD, Williams DJ. Pre-eclampsia and risk of cardiovascular disease and cancer in later life: systematic review and meta-analysis. *BMJ* 2007;335:974.
- Yu CK, Khouri O, Onwudiwe N, Spiliopoulos Y, Nicolaidis KH; Fetal Medicine Foundation Second-Trimester Screening Group. Prediction of pre-eclampsia by uterine artery Doppler imaging: relationship to gestational age at delivery and small-for-gestational age. *Ultrasound Obstet Gynecol* 2008;31:310–313.
- Witlin AG, Saade GR, Mattar F, Sibai BM. Predictors of neonatal outcome in women with severe preeclampsia or eclampsia between 24 and 33 weeks' gestation. *Am J Obstet Gynecol* 2000;182:607–611.
- Irgens HU, Reisaeter L, Irgens LM, Lie RT. Long term mortality of mothers and fathers after pre-eclampsia: population based cohort study. *BMJ* 2001;323:1213–1217.
- von Dadelszen P, Magee LA, Roberts JM. Subclassification of preeclampsia. *Hypertens Pregnancy* 2003;22:143–148.
- Brosens I, Pijnenborg R, Vercruyse L, Romero R. The “Great Obstetrical Syndromes” are associated with disorders of deep placentation. *Am J Obstet Gynecol* 2011;204:193–201.
- Wikström AK, Larsson A, Eriksson UJ, Nash P, Nordén-Lindeberg S, Olovsson M. Placental growth factor and soluble FMS-like tyrosine kinase-1 in early-onset and late-onset preeclampsia. *Obstet Gynecol* 2007;109:1368–1374.
- Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, et al. HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res* 2009;37:D603–D610.
- Villas-Bôas SG, Mas S, Akesson M, Smedsgaard J, Nielsen J. Mass spectrometry in metabolome analysis. *Mass Spectrom Rev* 2005;24: 613–646.
- Wishart DS. Advances in metabolite identification. *Bioanalysis* 2011;3: 1769–1782.
- Gowda GA, Zhang S, Gu H, Asiago V, Shanaiah N, Raftery D. Metabolomics-based methods for early disease diagnostics. *Expert Rev Mol Diagn* 2008;8:617–633.
- Robinson HP, Fleming JE. A critical evaluation of sonar “crown-rump length” measurements. *Br J Obstet Gynaecol* 1975;82:702–710.
- Syngelaki A, Chelemen T, Dagklis T, Allan L, Nicolaidis KH. Challenges in the diagnosis of fetal non-chromosomal abnormalities at 11–13 weeks. *Prenat Diagn* 2011;31:90–102.
- Plasencia W, Maiz N, Bonino S, Kaihura C, Nicolaidis KH. Uterine artery Doppler at 11 + 0 to 13 + 6 weeks in the prediction of pre-eclampsia. *Ultrasound Obstet Gynecol* 2007;30:742–749.
- Brown MA, Lindheimer MD, de Swiet M, Van Assche A, Moutquin JM. The classification and diagnosis of the hypertensive disorders of pregnancy: statement from the International Society for the Study of Hypertension in Pregnancy (ISSHP). *Hypertens Pregnancy* 2001;20: IX–XIV.
- Psychogios N, Hau DD, Peng J, Guo AC, Mandal R, Bouatra S, Sinelnikov I, et al. The human serum metabolome. *PLoS ONE* 2011;6:e16957.
- Saude EJ, Slupsky CM, Sykes BD. Optimization of NMR analysis of biological fluids for quantitative accuracy. *Metabolom ICD* 2006;2: 113–123.
- Wishart DS. Computational approaches to metabolomics. *Methods Mol Biol* 2010;593:283–313.
- Xia J, Psychogios N, Young N, Wishart DS. MetaboAnalyst: a web server for metabolomic data analysis and interpretation. *Nucleic Acids Res* 2009;37:W652–W660.
- Bijlsma S, Bobeldijk I, Verheij ER, Ramaker R, Kochhar S, Macdonald IA, van Ommen B, Smilde AK. Large-scale human metabolomics studies: a strategy for data (pre-) processing and validation. *Anal Chem* 2006;78:567–574.
- Hanley JA, McNeil BJ. The meaning and use of the area under a receiver operating characteristic (ROC) curve. *Radiology* 1982;143:29–36.
- Kell D. Genetic computing: defence against the flood: a solution to the data mining and predictive modeling challenges of today. *Bioinforma World* 2002;1:16–18.

25. Kell DB. Metabolomics and machine learning: explanatory analysis of complex metabolome data using genetic programming to produce simple, robust rules. *Mol Biol Rep* 2002;29:237–241.
26. Dunn WB, Broadhurst DI, Atherton HJ, Goodacre R, Griffin JL. Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy. *Chem Soc Rev* 2011;40:387–426.
27. Bijlsma S, Bodelijk I, Verheij E, Ramaker R, Kochhar S, MacDonald IA, et al. Large-scale human metabolomics studies: a strategy for data (pre-) processing and validation. *Anal Chem* 2006;78:567–574.
28. Demircio O, Tugriel AS, Dolgun N, Sozen H, Eren S. Serum lipid levels assessed in early pregnancy and the risk of pre-eclampsia. *J Obstet Gynecol Res* 2011 doi: 10.1111/j.1447-0756.2011.01562
29. Acilmis YG, Dikensoy E, Kutlar AI, Balat O, Cebesoy FB, Ozturk E, Cicek H, Pence S. Homocysteine, folic acid and vitamin B12 levels in maternal and umbilical cord plasma and homocysteine levels in placenta in pregnant women with pre-eclampsia. *J Obstet Gynaecol Res* 2011;37:45–50.
30. Peña-Reyes CA, Sipper M. Evolutionary computation in medicine: an overview. *Artif Intell Med* 2000;19:1–23.
31. Whitley D. An overview of evolutionary algorithms: practical issues and common pitfalls. *Info Software Tech* 2001;43:87–31.
32. Goodacre R. Making sense of the metabolome using evolutionary computing: seeing the wood with the trees. *J Exp Bot* 2005;56:245–254.
33. Miranda V, Srinivasan D, Proenca LM. Evolutionary computation in power systems. *Elec Power Energy Sys* 1998;20:89–98.
34. 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.
35. Poon LC, Kametas NA, Maiz N, Akolekar R, Nicolaides KH. First-trimester prediction of hypertensive disorders in pregnancy. *Hypertension* 2009;53:812–818.
36. Bahado-Singh RO, Jodicke C. Uterine artery Doppler in first-trimester pregnancy screening. *Clin Obstet Gynecol* 2010;53:879–887.
37. Kenny LC, Broadhurst DI, Dunn W, Brown M, North RA, McCowan L, Roberts C, et al.; Screening for Pregnancy Endpoints Consortium. Robust early pregnancy prediction of later preeclampsia using metabolomic biomarkers. *Hypertension* 2010;56:741–749.
38. Odibo AO, Goetzinger KR, Odibo L, Cahill AG, Macones GA, Nelson DM, Dietzen DJ. First-trimester prediction of preeclampsia using metabolomic biomarkers: a discovery phase study. *Prenat Diagn* 2011; 31:990–994.