VI Using first-trimester urinary metabolomics profiling to identify markers of preeclampsia

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Abstract

INTRODUCTION

Preeclampsia is a severe pregnancy complication with high maternal and fetal morbidity and mortality rates. The pathophysiology of preeclampsia is poorly understood, and no viable screening test exists. We therefore evaluated whether urinary metabolomics can be used to identify predictive biomarkers of preeclampsia in the first trimester.

METHODS

Urine samples were obtained from pregnant women between 11 weeks 0 days and 13 weeks 6 days of gestation. We performed a nested case-control study that included 73 women who later developed preeclampsia and 138 control subjects who were matched for Body Mass Index and gestational period at the time of sample collection. Urine samples were analyzed using Proton Nuclear Magnetic Resonance (^{1}H-NMR), and the concentrations of 36 metabolites were compared between the two groups. Because ethnicity appeared to be a strong confounding factor, a stratified analysis was performed for the two largest ethnic subgroups, Caucasians and Blacks.

RESULTS

In the first trimester, the Caucasian patients who later developed preeclampsia had significantly different concentrations of 18 metabolites compared to the control Caucasian subjects. In contrast, the Black patients who later developed preeclampsia had significantly different concentrations of three metabolites compared to the control Black subjects.

DISCUSSION

This is the first study to use urinary metabolomics in an attempt to identify markers for predicting preeclampsia early in pregnancy. Our finding of differential metabolite concentrations in urine samples obtained before the onset of preeclampsia may facilitate early disease prediction and provide insight into the pathogenesis of preeclampsia.

Introduction

Preeclampsia is a severe pregnancy-related condition characterized by hypertension and proteinuria after gestational week 20.[1] Preeclampsia is associated with high maternal morbidity and mortality rates,[2, 3] and fetal outcome is often compromised by a combination of intra-uterine growth restriction and preterm delivery.[1] Although the pathophysiology of preeclampsia is poorly understood, evidence suggests that the disease results from a complex interaction between poor placental perfusion—as a consequence of ineffective remodeling of the spiral arteries in early pregnancy—and a maternal response to placenta-derived components causing systemic endothelial dysfunction.[1, 4]

Currently available models for predicting preeclampsia depend upon a combination of maternal factors (including demographics, medical history, and obstetric history) and clinical tests (including booking blood pressure and uterine artery Doppler velocimetry).[5, 6] However, the development of high-end analytical technologies in the past two decades has opened new possibilities for using exploratory, hypothesis-free approaches to screen for new markers of preeclampsia. Metabolomics—and in particular metabolomics of bodily fluids—is one such promising approach.[7] Metabolomics is a discipline that arose from the explosive development of analytical sciences and a re-discovery of the holistic nature of biology.[7] The most promising aspect with respect to the medical sciences is the ability to provide a methodological framework for exploratory

studies, thereby generating new ideas and facilitating the discovery of novel associations between disease pathogenesis and the metabolic composition of bio-fluids.[7]

In the setting of preeclampsia, metabolomics analysis has been performed using plasma samples.[8-11] Recently, a pilot study measured urinary metabolomics in patients with preeclampsia at gestational week 36.[12] Changes in the plasma metabolome are reflected in the urine; importantly, urine samples are relatively easy to obtain and are usually free of proteins and lipids.[13] Moreover, urinary metabolomics has been used successfully to study other diseases.[14, 15] Here, we present the first study that uses urinary metabolomics to identify first-trimester biomarkers for predicting preeclampsia.

Methods

PATIENTS AND URINE COLLECTION

This nested case-control study is part of a larger prospective study currently being conducted by the Fetal Medicine Foundation in, London, UK to predict important fetal and obstetric disorders in the first trimester. The long-term objective of the project is to develop and evaluate existing and newly identified biomarkers that can be used to predict preeclampsia. The details of the larger study have been described previously.[8, 9] Pregnant women residing in the London area were prospectively screened from March 2006 through September 2009. Each participant provided written informed consent, and the study was approved by the King's College Hospital Research Ethics Committee. In brief, pregnant women at 11 weeks 0 days through 13 weeks 6 days gestation were recruited. Maternal characteristics and medical history were documented, and first-trimester ultrasound was performed, including crown-rump length (CRL) and uterine artery Doppler pulsatility index, was performed. Maternal urine samples were obtained and stored at -80°C for subsequent laboratory analysis. The study cohort consisted of 80 single-fetus pregnancies that subsequently developed preeclampsia and a matched group of 160 control subjects who did not develop preeclampsia. Each preeclamptic patient was matched for both Body Mass Index (BMI) and maternal age with two control subjects who delivered a healthy, full-term neonate with appropriate birth weight; in addition, the control subjects did not develop a hypertensive disorder of pregnancy, and they each provided a urine sample within three days of assessment of their matched preeclampsia case. Preeclampsia was defined based on the International Society for the Study of Hypertension in Pregnancy.[16] No HELLP syndrome or gestational hypertension cases were included.

NUCLEAR MAGNETIC RESONANCE (NMR) SAMPLE PREPARATION

Samples were thawed, centrifuged at 3,000´*g* for 5 min at 4°C to remove any precipitates, then transferred into deep 96-well plates. For sample preparation, 540 µl urine was mixed with 60 µl of pH 7.4 buffer (1.5 M K_2HPO_4 in D_2O) containing 4 mM sodium 3-trimethylsilyl-tetra-deuteriopropionate (TSP) and 2 mM NaN_3. The samples were mixed and transferred to NMR tubes using two Gilson 215 liquid handlers controlled by a Bruker Sample Track LIMS system (Bruker BioSpin, Karlsruhe, Germany).

NMR SPECTROSCOPY

^{1}H NMR data were collected at 27°C using a Bruker 600 MHz AVANCE II spectrometer equipped with a 5 mm TCI cryogenic probe head and a z-gradient system. After automatic tuning and matching of the probe head, 90° pulses were automatically calibrated for each individual sample using a homonuclear-gated nutation experiment[17] on the locked and shimmed samples. One-dimensional (1D) ^{1}H-NMR spectra were recorded using the first increment of a NOESY pulse sequence with presaturation (γB_1 = 50 Hz) during a relaxation delay of 4 seconds, with a mixing time of 10 msec to ensure efficient

water suppression.[18] Sixteen scans of 65,536 points covering 12,335 Hz were recorded and zero-filled to 65,536 complex points. Prior to Fourier Transformation (FT), an exponential window function was applied with a line-broadening factor of 1.0 Hz. The spectra were automatically phased, baseline corrected, and referenced to the internal standard (TSP ; δ 0.0). 2D *J* resolved (*JRES*)[19] spectra with presaturation (γB_1 = 50 Hz) were acquired with 12,288 data points over a spectral width of 10,000 Hz (F2 dimension), performing two scans for each of the 40 increments. Pre-FT data were weighted in both dimensions using a sine-bell function, and the spectra were tilted by 45° to provide orthogonal chemical shift (F2) and coupling constant (F1) axes; the spectra were subsequently symmetrized about the F1 axis. The chemical shift scale was referenced to an internal standard (TSP; δ 0.0). 2D-NMR spectra (^{1}H-^{1}H COSY, ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C HSQC-DEPT135) were also recorded for selected samples and were used for spectra annotation.

DATA ANALYSIS

The metabolites were annotated using reference spectra from the Bruker Bioref database and an in-house reference data set. The identities of the metabolites were confirmed using Statistical Total Correlation SpectroscopY (STOCSY) method.[20] The identified metabolites were quantified using deconvolution and subsequent integration of their resonance via in-house automation routine. Absolute concentrations were calculated using TSP as an internal reference. Metabolite concentrations were normalized to creatinine and log-transformed. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were performed to identify trends in the data using SIMCA software. The Mann-Whitney U test was used to compare the metabolite concentrations between the study groups. Differences with a *p*-value <0.05 were considered statistically significant.

Results

CLINICAL CHARACTERISTICS

The clinical characteristics of the preeclamptic patients and control subjects are summarized in Table 1. After excluding some samples because of insufficient sample volume or analytical failure, ^{1}H-NMR spectra of the urine samples from 73 patients with preeclampsia and 138 control subjects were included in the final analysis. The two study groups differed significantly with respect to ethnicity and birth weight.

MULTIVARIATE ANALYSIS

To investigate the primary sources of variance within our data, we subjected the entire data set to principal component analysis (PCA). The score plot of the PCA model (29 components explaining 66% of the variability; see Figure 1A) shows that the differences between the preeclampsia patients and control subjects are not reflected by the first two principal components (which account for approximately 18% of the total variance). Moreover, an attempt to build a regression model with diagnosis as class identity—preeclampsia versus non-preeclampsia—resulted in a poor, unstable model (data not shown). One possible explanation for this observation is that a strong confounding factor may have influenced the data. Because ethnic background differed significantly between the two groups, ethnicity may be one such confounding factor. To test this possibility, we re-plotted the data in Figure 1A according to each subject's ethnic background (Figure 1B). The two largest ethnic subgroups in our cohort—Caucasians (i.e., people of European descent) and Blacks (i.e., people of African descent)—influenced the data significantly. Because the remaining ethnic subgroups—South Asian, East Asian, and Mixed ethnicity—comprised only 10% of the entire cohort, we performed the subsequent analyses using the Caucasian (*n*=119) and Black (*n*=69)

subgroups only. A PCA model based on this subset of the data (25 components accounting for 66% of the variance) shows that the differences between these two major ethnic groups are represented in the first two principal components (Figure 2A). Thereafter, using a supervised analysis, we built a two class PLS-DA regression model using ethnicity as class identity (Figure 2B). This approach yielded a robust model with a cumulative explained variance (RY2) of 0.828 and predictive fraction (Q2Y) of 0.498.

Given the abovementioned results, ethnicity is likely the confounding factor that obscured the differences in urinary metabolic profiles between our preeclampsia patients and control subjects. Therefore, we examined the differences between the Caucasian preeclampsia patients and Caucasian control subjects, and between the Black preeclampsia patients and Black control subjects. However, multivariate modeling of these data did not yield a statistically sound model (data not shown).

Next, we explored whether ethnicity was an equally strong confounder among the control subjects and/or among the patients with preeclampsia. In the control group, a two class PLS-DA regression model with ethnicity as a class identity yielded a strong, statistically valid model. However, we did not obtain a valid model when we applied the same approach to the preeclampsia group (Supplemental Figure S1).

UNIVARIATE ANALYSIS

Because our supervised multivariate modeling was inconclusive, we next performed a targeted analysis. We selected a set of 36 physiologically relevant metabolites with consistent resonances in each preeclampsia patient and control subject. The identified metabolites were then quantified using spectral deconvolution and analyzed using the nonparametric Mann-Whitney U test with one-tailed exact *p*-value. Because ethnicity appeared to be a strong confounder, we compared the metabolite concentrations between the patients and control subjects within the Caucasian and Black subgroups. Table 2 compares the concentrations of 36 individual metabolite concentrations between the Caucasian preeclamptic patients and control subjects, and between the Black preeclamptic patients and control subjects. Remarkably, within the Caucasian subgroup, 18 of the 36 metabolites differed significantly between the preeclamptic patients and the control subjects. Within the Black subgroup, three metabolites differed significantly between the preeclamptic patients and control subjects. Two of these metabolites—2-hydroxyisobutyric acid and trigonelline—differed significantly in the Caucasian subgroup as well; the third significant metabolite—formate—did not differ significantly between the Caucasian preeclamptic patients and controls.

Discussion

Preeclampsia is a multifactorial syndrome, and understanding its pathophysiology requires a broad, bias-free view provided by modern "omics" technologies such as metabolomics. Here, we performed an exploratory metabolomics analysis in an attempt to identify a first-trimester urinary metabolomics signature among women who develop preeclampsia later in their pregnancy. We found that ethnic background was a confounding factor, prompting us to analyze the two primary ethnic groups separately. Within the Caucasian subgroup, 18 of the 36 metabolites differed significantly between the women who later developed preeclampsia and the control subjects. Within the Black subgroup, three metabolites differed significantly between the women who later developed preeclampsia and the control subjects.

Measuring the urinary metabolomics profile can be useful for assessing specific conditions; however, the eventual predictive signatures of those profiles are closely correlated with various factors, including the patient's lifestyle, diet, and ethnicity.[21, 22] Indeed, in our cohort, ethnicity appeared to be a strong confounder, as shown by the two-class PLS-DA model in which ethnic origin

was used as a class identity (Figure 2B). A straightforward multivariate case-control based model provided poor results, and it is unknown why we were unable to identify strong metabolic correlates when we used a case-control modeling strategy on each ethnic subgroup separately; however other confounding factors should certainly be considered. Moreover, given the complex physiological and metabolic changes that occur during pregnancy, in combination with the abovementioned confounding factors, a prospective follow-up study throughout pregnancy may yield more insight into pregnancy-related physiology.

Here, using a more targeted analysis and simple nonparametric univariate tests, we found that the metabolites that are associated with preeclampsia differed between the two major ethnic subgroups. Because this is the first study to investigate preeclampsia-related urinary metabolomics in the first trimester of pregnancy, we can only compare our results with studies that investigated metabolomics differences in plasma and with a recent pilot study of the urinary metabolome at gestational week 36.[12] Because the studies that measured plasma included either ethnically diverse groups[8, 9, 11] or one ethnic subgroup[10] of patients with preeclampsia and controls subjects, it is certainly conceivable that ethnicity might have influenced the outcome of these studies.

In our study, we observed a striking number of metabolites that differed significantly between the ethnic subgroups. However, these results should be interpreted with caution, as the disproportionate number of preeclamptic patients and control subjects in the Caucasian subgroup may have influenced the results. In contrast, the number of patients and control subjects in the Black subgroup was more balanced, and only a relatively small number of metabolites differed significantly between the preeclampsia patients and control subjects. Two of the metabolites that differed in the Black subgroup—2-hydroxyisobutyric acid and trigonelline—overlapped with the Caucasian subgroup. Thus, the pathophysiology of preeclampsia might differ—at least partially—among different ethnic groups.

A thorough discussion of the functional significance of the metabolites that differed significantly in our cohort is beyond the scope of this article. However, in both ethnic subgroups, the concentrations of two metabolites—2-hydroxyisobutyric acid and trigonelline—were significantly lower in the patients with preeclampsia than in their respective control subjects. The first metabolite, 2-hydroxyisobutyric acid, is a relatively well-studied compound, and increasing concentrations of this metabolite are associated with conditions that involve energy metabolism deficiency.[23] Remarkably, in our study we observed significantly lower concentrations of 2-hydroxyisobutyric acid in the patients with preeclampsia. In a recent pilot study, Austdal *et al.* reported that samples collected at gestational week 35-36 contained similar concentrations of 2-hydroxyisobutyric acid between preeclamptic patients and control subjects.[12] The second metabolite, trigonelline, also differed significantly between preeclamptic patients and control subjects in both ethnic subgroups. Although trigonelline excreted through the urine is usually dietary in origin,[24] trigonelline can be produced endogenously via methylation of nicotinic acid and involving S-adenosyl-methionine. To the best of our knowledge, experimental evidence for endogenous trigonelline synthesis exists for rodents only, and the endogenous synthesis of this metabolite is largely unexplored in humans.[25]

Low concentrations of trigonelline are associated with impaired lung function,[26] helminth infection,[27] and diabetes mellitus.[28] In particular, studies regarding diabetes mellitus lend credence to the notion that trigonelline is functionally significant in hypertension.[29] Administering trigonelline to rats with type 2 diabetes normalized several hypertension-related enzymes, including angiotensin converting enzyme (ACE).[29] Austdal *et al.* also reported that trigonelline levels are lower in patients with preeclampsia at 35-36 weeks of gestation.[12] This finding is consistent with our observation that trigonelline levels were significantly lower in the women who later developed preeclampsia than in the control subjects, regardless

of their ethnicity. Whether low levels of trigonelline are associated with the development of hypertension, or whether preeclampsia (and its associated hypertension) induces low levels of trigonelline—or both—remains to be investigated.

In conclusion, this study is the first urinary metabolomics screen in pregnant women who later developed preeclampsia. Our finding that the levels of specific metabolites differ in first-trimester urine samples between patients who develop preeclampsia and control subjects may provide a viable method for predicting preeclampsia early, even before symptoms develop, and may provide key insight into the disease's pathogenesis. Future studies using a larger cohort of patients are needed to expand these findings—particularly with respect to the role of ethnicity. Ideally, these studies should combine ^{1}H-NMR with other metabolomics techniques such as mass spectrometry. A comprehensive metabolomics analysis will likely increase our understanding of the pathophysiology of preeclampsia, which in turn will facilitate the development of preventive and therapeutic strategies.

Table 1

Characteristics	PE (n=73)	Controls (n=138)
Maternal age, years (SD)	32.3 (5.9)	32.5 (5.7)
Nulliparity, *n* (%)	40 (54.8)	61 (44.2)
Smoking, *n* (%)	2 (2.7)	7 (5.1)
Ethnicity, *n* (%) *		
Caucasian	24 (32.9)	95 (68.8)
Black	35 (47.9)	34 (24.6)
South Asian	7 (9.6)	2 (1.4)
East Asian	1 (1.4)	1 (0.7)
Mixed	6 (8.2)	6 (4.3)
BMI (SD)	28.6 (5.2)	28.0 (5.1)
PE early, *n* (%)	15 (20.5)	NA
Live birth, *n* (%)	71 (97.3)	138 (100)
Birth weight, g (%)	2575.8 (945.9)	3541.9 (363.5)*

Patient characteristics

*BMI: Body Mass Index; PE: preeclampsia. PE early: preeclampsia requiring delivery before gestational week 34. *p<0.001.*

Metabolite	Caucasian subjects (n=119)			Black subjects (n=69)		
	PE, n=24	Control, n=95	p-value	PE, n=35	Controls, n=34	p-value
1-Methylnicotinamide	9.6 (13.4)	17.8 (13.8)	0.160	18.0 (36.9)	17.4 (13.9)	0.877
2-Hydroxyisobutyric acid	23.1 (37.9)	53.7 (23.8)	0.002	41.3 (35.4)	51.2 (39.3)	0.037
Acetate	169.5 (118.5)	198.4 (131.4)	0.022	217.9 (140.8)	238.1 (130.9)	0.366
Acetoacetate	36.7 (93.8)	70.3 (96.2)	0.071	60.0 (128.3)	57.3 (83.7)	0.658
Acetone	6.6 (5.9)	11.5 (7.7)	0.017	12.0 (12.1)	11.6 (67.5)	0.995
Alanine	190.1 (375.3)	381.9 (311.4)	0.025	403.9 (435.0)	467.5 (275.4)	0.204
Carnitine	617.6 (1593.7)	1047.8 (1250.5)	0.119	651.7 (1094.6)	901.0 (886.4)	0.333
Citrate	1778.5 (2134.5)	3832.4 (2742.0)	0.003	3520.3 (3803.8)	3846.2 (2334.0)	0.734
Creatine	572.6 (827.8)	567.1 (836.1)	0.496	309.1 (865.3)	676.1 (632.1)	0.173
Creatinine	4714.0 (5518.2)	8165.4 (5360.8)	0.019	8364.9 (7433.7)	8237.3 (5662.4)	0.503
Dimethylamine	128.1 (162.7)	212.6 (126.3)	0.032	211.3 (380.2)	268.4 (180.3)	0.241
Dimethylglycine	41.0 (42.2)	63.6 (53.6)	0.014	60.1 (53.1)	59.21 (51.56)	0.269
Formate	132.4 (136.7)	171.1 (123.7)	0.174	139.1 (135.6)	212.8 (99.6)	0.030
Fumarate	0.8 (1.1)	4.1 (4.5)	0.001	4.2 (6.9)	3.4 (6.0)	0.807
Glucose	86.75 (207.4)	239.7 (192.2)	0.015	261.4 (228.5)	215.3 (208.5)	0.368
Glycine	1604.8 (2034.8)	2269.9 (1562.0)	0.105	2266.1 (3147.1)	2370.1 (1987.8)	0.571
Guanidinoacetate	515.7 (683.6)	856.5 (466.9)	0.141	770.6 (733.2)	823.7 (575.7)	0.734
Hippurate	690.4 (867.4)	1102.6 (829.9)	0.094	1133.6 (801.2)	1300.9 (950.5)	0.139
3-Hydroxybutyrate	28.5 (37.4)	31.1 (53.0)	0.952	33.5 (40.0)	22.6 (37.8)	0.584
3-Hydroxyisovaleriate	135.7 (214.6)	210.3 (174.1)	0.100	189.6 (197.7)	239.0 (229.8)	0.102
Isobutyrate	14.6 (22.7)	35.9 (31.4)	0.002	38.0 (35.9)	44.4 (43.6)	0.338
Lactate	262.7 (465.2)	602.6 (456.8)	0.006	578.8 (409.9)	533.8 (314.4)	0.620
Methylguanidine	213.2 (205.9)	321.0 (186.7)	0.031	324.9 (286.4)	329.8 (283.0)	0.676
Phenylacetylglycine	266.7 (391.3)	511.6 (330.4)	0.034	412.7 (446.4)	555.1 (519.1)	0.159
Propylene glycol	31.9 (50.2)	43.0 (48.4)	0.194	41.3 (38.7)	28.1 (36.3)	0.813
Pyruvate	29.9 (48.4)	51.9 (44.3)	0.135	37.8 (47.8)	47.1 (47.4)	0.146
Scyllo-inositol	36.4 (54.4)	80.7 (52.0)	<0.001	85.2 (60.8)	75.1 (59.3)	0.461
Succinate	47.5 (54.1)	102.5 (59.1)	<0.001	77.2 (106.6)	91.8 (77.8)	0.536
Sucrose	17.5 (31.9)	24.0 (37.4)	0.153	8.7 (32.8)	10.3 (35.9)	0.898
Trigonelline	7.8 (18.8)	26.7 (41.6)	<0.001	30.1 (46.4)	80.5 (71.4)	0.009
TMAO	253.9 (382.3)	391.5 (280.6)	0.130	316.5 (353.5)	402.6 (255.3)	0.610
Tyrosine	63.6 (69.6)	64.6 (50.7)	0.595	71.7 (62.6)	77.9 (67.6)	0.516
Valine	18.3 (18.9)	28.2 (15.5)	0.037	27.0 (20.9)	31.6 (29.1)	0.284
Pseudouridine	158.8 (190.1)	256.7 (155.6)	0.062	282.8 (262.0)	285.6 (210.3)	0.541
Glyoxylate	1.5 (1.1)	1.2 (1.3)	0.723	0.9 (2.5)	1.4 (2.5)	0.842
Hypoxanthine	9.4 (16.9)	20.9 (20.8)	0.055	13.2 (38.4)	15.1 (18.9)	0.945

Table 2 (previous page): Concentrations of metabolites in the urine of the indicated groups measured using nuclear magnetic resonance

Values represent the concentrations of metabolites in micromolar. Data are shown as median (standard deviation). PE: preeclampsia.

Figure 1

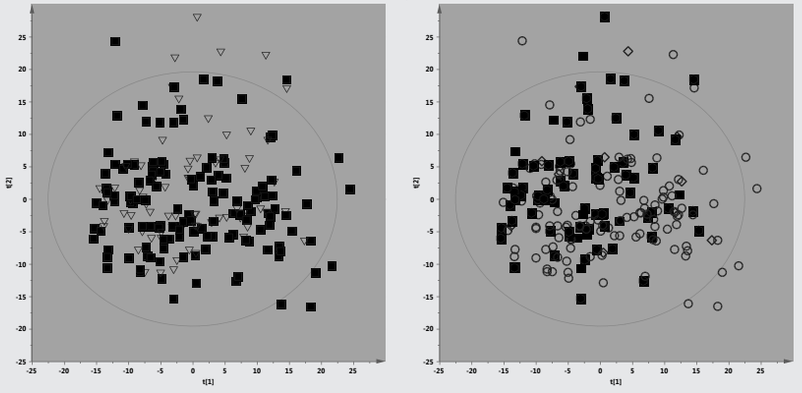

Figure 1A *Figure 1B*

Principal components analysis plot

Figure 1A shows a principal components analysis plot of the patients with preeclampsia (in open triangles) and control subjects (in solid squares). The x-axis represents the first principal component (the most significant vector), and the y-axis represents the second principal component (the second most significant vector). No separation was observed between the patients with preeclampsia and the control subjects based on 29 components that explained approximately 70% of the variance. Figure 1B shows the same principal components analysis plot, with the subjects coded according to their ethnic background. The open circles represent Caucasians, the solid hexagons represent Blacks, the open diamonds represent Asians (both South and East Asian), and the grey triangles represent mixed ethnicity.

Figure 2

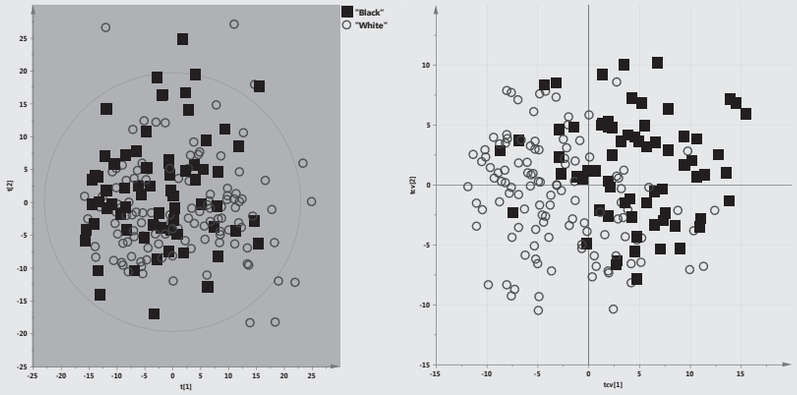

Figure 2A *Figure 2B*

Principal component analysis plot based on ethnicity

Principal component analysis plot based on the two largest ethnic groups in the cohort, Caucasians (shown in open circles) and Blacks (shown in solid hexagons). 25 components explain approximately 66% of the variance (Figure 2A). Figure 2B shows a partial least squares discriminant analysis (PLS-DA) model (cross-validated score plot). R2X = 0.202, R2Y = 0.828, Q2 = 0.498; p=4.59058e-24.

Figure S1

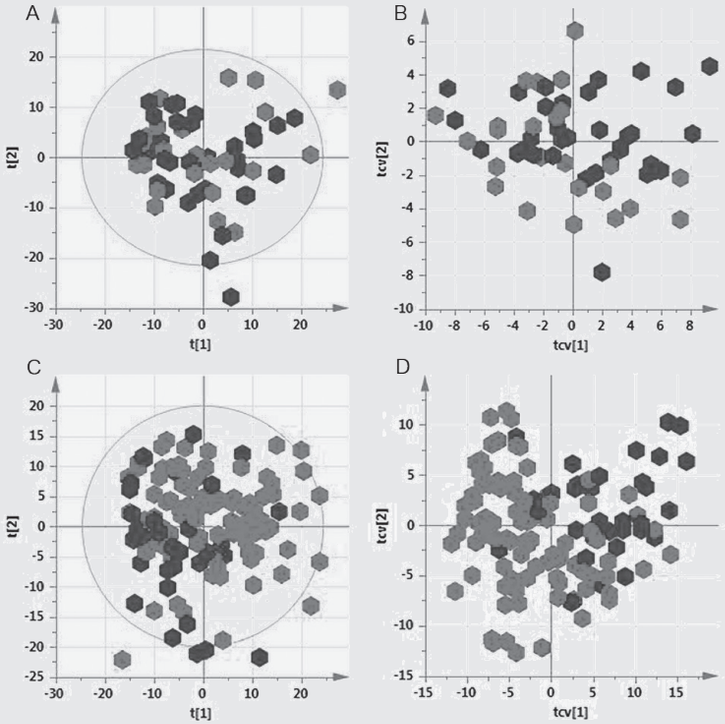

Figure S1: Influence of ethnic background

Principal Component Analysis (PCA; panels A and C) and PLS-DA (panels B and D) models with ethnic origin as class identity. The dark grey symbols represent Blacks, and the light grey symbols represent Caucasians. For the patients with preeclampsia, the PCA (A) resulted in a non-significant PLS-DA model (B; R2X = 0.102, R2Y = 807, Q2 = 0.009, p=0.683). For the control subjects, the PCA (C) resulted in a significant PLS-DA model (D; R2X = 0.202, R2Y = 0.771, Q2 0.404, p=2.99147e-12).

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