Metabolic profiles of placenta in preeclampsia using HR-MAS MRS metabolomics

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Abstract

Introduction: Preeclampsia is a heterogeneous gestational disease characterized by maternal hypertension and proteinuria, affecting 2-7% of pregnancies. The disorder is initiated by insufficient placental development, but studies characterizing the placental disease components are lacking. **Methods:** Our aim was to phenotype the preeclamptic placenta using high-resolution magic angle spinning nuclear magnetic resonance spectroscopy (HR-MAS MRS). Placental samples collected after delivery from women with preeclampsia (n=19) and normotensive pregnancies (n=15) were analyzed for metabolic biomarkers including amino acids, osmolytes, and components of the energy and phospholipid metabolism. The metabolic biomarkers were correlated to clinical characteristics and inflammatory biomarkers in the maternal sera. **Results:** Principal component analysis showed inherent differences in placental metabolic profiles between preeclamptic and normotensive pregnancies. Significant differences in metabolic profiles were found between placentas from severe and non-severe preeclampsia, but not between preeclamptic pregnancies with fetal growth restricted versus normal weight neonates. The placental metabolites correlated with the placental stress marker sFlt-1 and triglycerides in maternal serum, suggesting variation in placental stress signaling between different placental phenotypes. **Discussion:** HR-MAS MRS is a sensitive method for defining the placental disease component of preeclampsia, identifying several altered metabolic pathways. Placental HR-MAS MRS analysis may improve insight into processes affected in the preeclamptic placenta, and represents a novel long-required tool for a sensitive placental phenotyping of this heterogeneous disease.

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Introduction

Preeclampsia is a gestational disease that originates in the placenta and affects 2-7% of pregnancies [1]. Preeclampsia may reflect an excessive maternal inflammatory response to insufficient placentation or to pregnancy itself [2, 3]. The current hypothesis in regard to its development states that the uterine spiral arteries develop insufficiently during placentation, causing placental ischaemia and abnormal inflammation as the pregnancy develops [1, 4]. The oxidatively stressed placenta releases increasing amounts of inflammatory and angiogenic factors to the maternal circulation, eventually causing the manifestations of preeclampsia; endothelial dysfunction, intravascular inflammation and activation of the hemostatic systems clinically evidenced by proteinuria and hypertension [1, 4].

Preeclampsia is a heterogeneous disease, and markers in the placenta identifying disease subgroups are scarce [5]. Common subtypes of preeclampsia are today defined by clinical characteristics such as severity of maternal features, time of diagnosis, and presence of fetal growth restriction (FGR) [6, 7]. These subgroups are based on end stage maternal or fetal factors, and placental histology findings and maternal serum markers overlap between groups [2]. Similar end stage presentations of preeclampsia may stem from different pathologic placental processes [8]. Studying the altered metabolism of the placenta in preeclampsia, and how it is affected with disease severity and when combined with FGR, may give insight into which processes are shared and specific for these disorders. Placental phenotyping by metabolic expression may aid in identifying potential targets for treatment in the future.

Metabolite expression is the final level of regulation over gene and protein expression, and can be measured directly in tissue samples using high resolution magic angle spinning magnetic resonance spectroscopy (HR-MAS MRS) [9]. HR-MAS MRS has been used successfully in investigating molecular subtypes of breast cancer for improved treatment and outcome stratification [10]. HR-MAS MRS of intact placental tissue from normal or preeclamptic pregnancies has not been performed previously.

Our aim is to identify differences in placental metabolic expression between normotensive and preeclamptic pregnancies using HR-MAS MRS metabolomics. We further aim to investigate whether the placental metabolites correlate to maternal serum measurements of placental stress and inflammatory markers. We hypothesize that placental metabolic profiling provides a sensitive method for detailed identification of the placental component of preeclampsia.

Materials & Methods

Study participants, serum samples and placental tissue biopsies

The study was approved by the Norwegian Regional Committee for Medical and Health Research Ethics (REC 2012/1040) and informed consent was obtained from all participants. Women with singleton pregnancies delivering by cesarean section (CS) were recruited at Haukeland University Hospital (Bergen, Norway) from 2009 to 2012. Women with pregnancies complicated by preeclampsia were included as cases. Healthy pregnant women with no previous history of pregnancies with preeclampsia or FGR were included as normotensive controls. Preeclampsia was defined as persistent hypertension (systolic/diastolic blood pressure 140/90 mmHg) plus proteinuria (≥0.3g/24h or ≥1+ by dipstick) developing after 20 weeks of gestation [6]. Superimposed preeclampsia was defined as pre-existing hypertension where the women developed proteinuria after 20 weeks of gestation [6] and was included in the preeclampsia group. Preeclampsia was sub-classified as severe if diagnosed with one or more of the severe features: hypertension ≥160/110mmHg, proteinuria ≥3 g/24 h, pulmonary symptoms, seizures/eclampsia, oliguria of <500mL/24 h, or central nervous system symptoms [7]. Preeclamptic women not meeting the criteria for severe preeclampsia were designated as non-severe. FGR was diagnosed by serial ultrasound measurements showing reduced intrauterine growth defined as an estimated fetal weight gain between ultrasound examinations below the 10^{th} percentile [11, 12]. In absence of serial ultrasound measurements, neonates were defined with FGR if their birth weights were below the 5^{th} percentile for gestational age (GA) according to Norwegian fetal weight reference curves [13]. In the FGR subgroup in the study cohort, one pregnancy was defined as FGR based on SGA criteria alone, while two pregnancies had FGR diagnoses but with neonatal birth weights above the 5^{th} percentile.

A complete overview of clinical characteristics of the pregnancies studied are available in the Supplementary Tab. S.1 and S.2 [14].

Women with gestational hypertension, HELLP syndrome, or pregnancies with fetal chromosomal or congenital abnormalities were not included in the study. The primary indication for CS for the cases was preeclampsia, while indications for the normotensive women included breech presentation, previous CS or maternal request. None of the participants experienced signs or symptoms of labor. Clinical information was collected from the medical journals and through interviews.

Maternal venous blood was collected prior to CS, left to clot for ≤30 minutes, centrifuged at 1800G for 10 minutes, and serum aliquots (1mL) were stored at -80^{o}C until analysis. A tangential section (100 mg) from the maternal central side of the placenta was collected after delivery, placed in a cryotube and frozen either in liquid nitrogen or directly at -80^{o}C within 101 ± 49 minutes (mean ± SD) after delivery. The tissue was collected at a maximum depth of 0.5 cm and did not include the chorionic plate, but consisted of tissue from the basal plate and intervillous space, including placental parenchyma. Visible blood clots were carefully removed. Sample processing times were not significantly different between the preeclamptic and normotensive groups.

Maternal serum analyses

Soluble fms-like tyrosine kinase receptor 1 (sFlt-1) was measured in duplicate using a quantitative sandwich ELISA according to the manufacturer's instructions (#DVR100B, R&D Systems, Abingdon, UK). High sensitivity C-reactive protein (hs-CRP) (turbidimetric assay, Modular P analyzer, Roche, Burgess Hill, UK), total cholesterol, high-density lipoprotein (HDL), triglyceride and creatinine (enzymatic colorimetric assays, Modular P analyzer) were

measured by accredited methods at the Department of Clinical Chemistry, St. Olavs Hospital, Trondheim, Norway.

HR-MAS MRS placental analyses

The placental biopsies were analyzed in random order, blinded to pregnancy outcome. Samples were prepared on a metal plate cooled in liquid nitrogen in order to minimize the effect of tissue degradation, as described in [15, 16]. Biopsies (7.5 ± 1.4 mg) were cut to fit 30µL disposable inserts (Bruker Biospin Corp, USA) filled with 3µL D_2O containing 25mM formate for shimming. Spin-echo spectra were acquired on a Bruker Avance DRX600 spectrometer with a $^{1}H/^{13}C$ MAS probe with gradient (Bruker Biospin GmbH, Germany) using the following parameters: 5KHz spin rate, 5°C probe temperature, cpmgpr1d pulse sequence (Bruker Biospin) with 78ms total echo time, spectral width of 20 ppm and 256 scans. Two samples were additionally analyzed by ^{13}C-^{1}H spectroscopy (HSQC, HMBC) for aid in metabolite identification.

Data analysis

Spectra were Fourier transformed into 65.5k points following 0.3 Hz line broadening, and automatically phased and baseline corrected (Topspin 3.1, Bruker Biospin). The spectra were restricted to the region 0.5 to 4.7 ppm, peak aligned using iCoshift to correct for slight variations in peak positions [17], and divided by the total area under the spectrum curve to account for variation in sample weight. Metabolites were identified by comparing chemical shift values to spectral databases [18] and correlation of metabolite peaks using Statistical Total Correlation Spectroscopy [19]. Semi-quantitative metabolite levels were measured by integrating the spectral regions of identified metabolites (Matlab r2013b, The Mathworks Inc., Natick, MA, USA). The concentrations of the metabolites are given in arbitrary units, but nevertheless correspond to the normalized concentration of the metabolite in the tissue

sample. The normalized metabolite data are available as a supplementary file (Supplementary Table S.7).

Multivariate analysis was performed in PLS Toolbox 7.3.1 (Eigenvector Research Inc., WA, USA), and with MetaboAnalyst [20]. Quantified metabolite levels were autoscaled before multivariate modeling. The metabolic profiles were evaluated by principal component analysis (PCA) for initial data exploration [21]. Partial least squares discriminant analysis (PLS-DA) discriminated metabolic features between subgroups of preeclampsia (presence of severe maternal features or FGR), and between normotensive and preeclamptic pregnancies. Eight preeclamptic women had both FGR neonates and severe preeclampsia features, and these were included both in the FGR group and the severe preeclampsia group in subgroup analyses. The performance of the PLS-DA models was evaluated by five-fold cross-validation which was repeated twenty times. The number of latent variables was chosen as the number giving the highest accuracy of classification. Sensitivity, specificity and classification accuracy were reported as averages from the validation set for each model. To evaluate the validity of the regression and classification results, 1000 permutation tests were performed with models considered valid at $p \leq 0.05$. In permutation testing, classes or dependent values are shuffled and models built on the random data [22]. The classification results from the true model should then be outside the 95% confidence limit for the permuted models to be considered valid.

Univariate statistical analyses were performed in SPSS v. 20 (SPSS, Chicago, IL). Clinical characteristics were compared between disease subgroups using the one-way ANOVA with Tukey post-hoc test for groupwise normally distributed data (GA, birth weight) or the Kruskal Wallis test with post-hoc pairwise Mann-Whitney U tests for nonparametric data (maternal age, blood pressure, body mass index (BMI), placental weight, parity), or Fishers exact test

for categorical variables. Metabolite levels and serum measurements were compared using the Mann-Whitney U test. False discovery rate correction for multiple testing (Benjamini-Hochberg) was applied [23]. To adjust for confounding effects of GA, linear regression models with log-transformed metabolites as dependent variable and preeclampsia and GA as independent variables were generated. Interaction terms between preeclampsia and covariates were included in the models if significant, otherwise excluded. Standardized residuals were assessed with the Kolmogorov Smirnov test. Quantitative metabolite set enrichment analysis (MSEA) was performed for inferring the metabolic pathways associated with disease [24].

Results

Clinical characteristics

The clinical characteristics of the study participants are described in Tab. 1. A total of 34 pregnant women were included in the study (preeclampsia; n=19 and normotensive; n=15). Birth weight and GA were lower in women with preeclampsia than in normotensive pregnancies, and preeclamptic women were more likely to be primiparous. Maternal serum levels of creatinine, uric acid and sFlt-1 were significantly higher before delivery in women with preeclampsia compared to normotensive women (Tab. 2).

HR-MAS analyses

In total 25 metabolites were identified in the HR-MAS MRS analysis of placental biopsies (Supplementary Tab. S.3). The metabolite levels are shown in Tab. 2, grouped by metabolic pathway [25]. Median spectra from placental biopsies from normotensive and preeclamptic pregnancies are shown in Fig. 1.

Placental metabolic profiles in preeclampsia

154 The results from PCA are shown in Fig. 2. The score plots show the between-samples
155 variation and are colored by ellipses of 95% confidence intervals for preeclampsia subgroups
156 defined by presence or absence of preeclampsia (Fig. 2A), severe or non-severe preeclampsia
157 (Fig. 2B) or preeclampsia with or without FGR (Fig. 2C). Superimposed preeclampsia
158 appeared in the overlapping region in the score plot between preeclampsia and normal
159 pregnancy (Fig. 2A). Normotensive pregnancies were clearly different from preeclamptic
160 pregnancies in placental metabolic expression profile. The preeclamptic pregnancies showed a
161 more heterogeneous metabolic expression than the more unified group of normotensive
162 pregnancies (Fig. 2A). Placentas from preeclamptic pregnancies showed enrichment of
163 phospholipid biosynthesis and depletions in bile acid biosynthesis, taurine metabolism,
164 ammonia and urea cycles and protein biosynthesis, compared to placentas from normotensive
165 pregnancies (Supplementary Tab. S.4). No effect of fetal sex, maternal age or body mass
166 index on the placental metabolic profiles was found using PLS-DA.

167 The preeclampsia subgroups defined by severe preeclampsia or FGR had overlapping
168 metabolic expressions (Fig. 2B and C). The severe preeclampsia placental profiles were more
169 similar to the normotensive profiles than preeclampsia without severe features (Fig. 2B). In
170 contrast, placental metabolic profiles of preeclampsia with FGR were more separated from
171 normotensive profiles than preeclampsia without FGR (Fig. 2C). The loading plot (Fig. 2D)
172 identified the metabolites contributing to the sample distributions. The first principal
173 component (PC1) showed an increase in aspartate, phosphocholine and
174 glycerophosphocholine and decrease in glutamate, taurine, ascorbate and glutamine
175 corresponding to a preeclamptic phenotype. The second PC (PC2) showed variation not
176 related to group separation: relative to samples with low scores on PC2, samples with high
177 scores on PC2 had increased phosphocholine, glycerophosphocholine, myo-inositol and
178 threonine, and lower lactate, glycine and glycerol.

PLS-DA defined metabolic profiles for preeclampsia and its subgroups (Tab. 3). A significant difference in placental metabolic profiles was found between preeclamptic and normotensive pregnancies, and between placentas from severe and non-severe preeclampsia. There was no significant difference in the metabolic profiles between preeclampsia with or without FGR. Receiver-operator characteristic curves for the predictions, and results from the permutation tests, are shown in Supplementary Figure S.1. PLS regression revealed correlations between the placental metabolic profiles and maternal serum markers; triglycerides, sFlt-1, uric acid, and creatinine (Tab. 4). Serum sFlt-1 showed the highest correlation to placental metabolites (R^2=0.49, $p<0.001$). Of special interest, maternal serum sFlt-1 was clearly correlated with increased placental glycerophosphocholine levels, and decreased glutamate, taurine, glutamine, valine and ethanolamine.

Twelve out of 25 metabolites were significantly different between the placentas from preeclamptic and normotensive pregnancies after correction for multiple testing (Tab. 2). Placental levels of choline and lysine were increased in severe preeclampsia (Supplementary Tab. S.5). Linear regression modeling of the placental metabolites differing between preeclamptic and normotensive pregnancies revealed that GA significantly affected only the levels of ethanolamine and glycine (increasing with GA). Glutamine, glutamate, taurine, valine, 3-hydroxybutyrate and ascorbate remained significantly different between preeclamptic and normotensive groups after correction for GA (Supplementary information, Tab. S.6).

Discussion

We have for the first time uncovered metabolic profiles of placental tissue using HR-MAS MRS technology, reporting a novel method for defining the placental disease component in preeclampsia. The metabolic placenta profiles showed a highly significant altered metabolic

state in preeclampsia compared to normotensive pregnancies. Our study demonstrated metabolic differences between severe and non-severe preeclampsia, and showed that the presence of FGR was not reflected in the placental metabolites. The metabolic placenta profile correlated with maternal serum markers for angiogenic imbalance, uric acid and lipid levels. This is the largest metabolic profiling of placental tissue from preeclamptic pregnancies published to date.

Our study uses a novel technique in placental analyses to expand upon previous metabolomic studies on placental explants and cell culture from preeclamptic pregnancies [26-28]. Metabolic profiling of whole placental tissue by HR-MAS MRS is a nondestructive analysis allowing further proteomic or genomic analysis of the same sample. Studies in cancer have found metabolic profiles to correlate to prognostic factors and survival [29]. We have shown placental metabolites correlating with the disease severity and with maternal serum factors reflecting the placental disease. The scattering of the metabolic placental profiles in preeclampsia compared to normotensive placentas clearly reflected that preeclampsia is a heterogeneous disease and indicates that several different pathologic processes in the placenta may underlie similar clinical signs in the mother. This holistic approach may provide a sensitive classification of the placental component of preeclampsia. This method provides a novel tool for understanding the underlying placental disease mechanisms, and thereby phenotyping the disease based on placental involvement, not only end stage maternal and fetal features. The findings warrant further investigation in a larger cohort.

Several metabolic pathways were affected in preeclampsia; notably the taurine, glutamate and phospholipid metabolism. Taurine is an essential nutrient in fetal metabolism, as the fetus and placenta lack the enzyme for taurine synthesis [30]. Reduced activity of a placental taurine transporter has been found in preeclampsia and FGR [31, 32]. Reduced taurine in the placenta

may impair syncytiotrophoblast cell renewal and lead to decreased nutrient transfer to the fetus [32]. In our study, taurine levels were similar in placentas from preeclampsia with or without FGR, suggesting that taurine depletion is not specific for FGR. Glutamine and glutamate are crucial to the fetal carbon and nitrogen metabolism as precursors to protein, purine and pyrimidine synthesis [33]. Glutamate is also a precursor to glutathione, an important antioxidant, and has been shown to be lower in medium of placental explants from hypoxic normal tissue and preeclamptic tissue [26]. Consistent with this finding, placental ascorbate was significantly lower in preeclamptic placentas. Intracellular ascorbate protects endothelial cells from apoptosis induced by hypoxia followed by reoxygenation, and may be depleted as a response to hypoxia [34].

Two possible reasons for increased glycerophosphocholine in preeclampsia are suggested. First, the increase may be due to excessive cell death in preeclamptic placentas [35, 36]. This conforms to our findings of increased glycerophosphocholine especially in those pregnancies with increased maternal serum sFlt-1. Phosphatidylcholine catabolism releases glycerophosphocholine and arachidonic acid by the phospholipase PLA_2, possibly playing a role in increased inflammation, a central process in the preeclamptic placenta. PLA_2 activity is increased in preeclamptic placental tissue [37]. Second, the increase may stem from placental cell membrane catabolism for regeneration of choline methyl groups due to folate deficiency [38]. In our study, glycerophosphocholine was no longer significantly different between groups after adjustment for gestational age, as the metabolite decreases towards term in the preeclampsia group. The metabolite may still play a role in early onset disease. An MR imaging study of *in utero* placentas found increased placental phosphodiester components including glycerophosphocholine in early onset preeclampsia compared to gestational age matched controls [39], supporting that the increase is disease-related and not only dependent on gestational age. However, our data cannot separate these two effects. Choline levels were

252 in this study similar in preeclamptic and normotensive placentas, indicating a compensatory
253 mechanism. Another component of phospholipid biosynthesis, ethanolamine, was decreased
254 in the preeclamptic placenta. Ethanolamine kinase deficient mice have low birth weight
255 offspring and increased placental thrombosis and apoptosis, indicating an important role of
256 ethanolamine in placental and fetal development [40]. An earlier metabolomics study using
257 mass spectrometry of placental extracts found increased choline, succinate and
258 glycerophosphocholine species in placentas from preeclamptic pregnancies [28], the two
259 former of which we found no difference. However, many metabolites measured with MS are
260 not observed in MRS spectra.

261 The correlation of placental metabolic profiles to maternal serum sFlt-1 and triglycerides
262 suggests that different placental phenotypes may be recognized by targeted measurements of
263 maternal markers. Placental sampling must be done after delivery, but importantly, increased
264 understanding of underlying placental disease components will enable a more targeted search
265 for disease markers that more accurately reflect the diversity of the placental disease.
266 Identification of a placental phenotype correlating to maternal serum markers is an important
267 step in this direction. Novel markers reflecting the placental disease more directly will still
268 need to be identified.

269 Limitations of our study include the variation in GA between preeclamptic and normotensive
270 pregnancies. Differences were accounted for using linear regression, but variation due to GA
271 cannot easily be overcome in placenta research due to the nature of the preeclampsia
272 diagnosis. Additionally, only one sample per placenta was analyzed, thus, intra-individual
273 variability was not assessed. However, previous metabolomic analyses of the placenta found
274 no or few spatial differences in metabolites [28, 41]. Finally, the time passed between CS and
275 tissue freezing was longer than recommended for metabolomics studies, and therefore the

metabolic profiles may not directly reflect the in vivo situation [41, 42]. Strengths of our study are the whole tissue profiling without need for extraction and derivatization, and the sensitivity as reflected by metabolite correlation to the placental derived stress factor sFlt-1 in maternal serum. All deliveries were by CS precluding any labor-induced variation. Sensitive placental profiling as shown here is missing from preeclampsia research.

Metabolomics represents the closest measure to the phenotype, and this is reflected in the highly significant differences between placentas from normotensive and preeclamptic pregnancies. In this study we investigated pathways affected by preeclampsia, and found metabolic profiles specific for severe preeclampsia and correlating to increased maternal serum markers. An interesting direction of further research will be to metabolically classify the placental component of preeclampsia in larger cohorts, and identify unique factors that may benefit from separate treatments as has been done previously using gene expression analysis [43]. We present the HR-MAS MRS method as an excellent novel tool for placental disease phenotyping in pregnancy research possibly leading to future improved screening, prediction and follow-up of pregnant women at risk for preeclampsia.

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Table 1: Clinical characteristics of study participants

Variable	Total PE (n=19)	PE+FGR (n=11)	PE severe (n=14)	Normotensiv (n=1
Maternal age in years, md (IQR)	28 (9)	25 (6)*,***	28 (10)	34 (
Gravidity, md (IQR)	1 (2)	1 (2)	1 (2)	2 (
Primipara, n (%)	9 (47.4)*	6 (54.5)*	7 (50.0)	2 (13.
Systolic BP in mmHg, md (IQR)[a]	160 (29)**	160 (30)**	170 (20)**	124 (2(
Diastolic BP in mmHg, md (IQR)[a]	100 (15)**	104 (11)**	105 (36)**	75 (1
BMI in kg/m^2, md (IQR)[b]	24.2 (13.0)	24.2 (13.4)	23.8 (11.1)	23.6 (4.
CVD diagnosis[c], n (%)	3 (15.8)	1 (9.1)	3 (21.4)	0 ((
GA at delivery in weeks, md [range]	32.3 [25.6 -39.1]	31.7 [27.4-33.7]	32.3 [25.6-37.0]	39.3 [38.3 - 40.(
Birth weight in g, mn (SD)	1707 (1074)**	1212 (342)**	1621 (785)**	3501 (30
Placental weight in g, md (IQR)	300 (290)**	275 (125)**	295 (277)**	620 (15(
FGR, n (%)[d]	11 (57.9)**	11 (100)	8 (57.1)	0 ((
Severe PE, n (%)	14 (73.7)	8 (72.7)	14 (100)	0 ((
Superimposed PE, n (%)	2 (10.5)	1 (9.1)	2 (14.3)	
Early onset PE (<34 weeks) n (%)	17 (89.5)	11 (100)	13 (92.9)	0 ((
Gestational diabetes, n (%)	2 (10.5)	0 (0)	2 (14.2)	0 ((

P values are from a one-way ANOVA with Tukey post-hoc test or the Kruskal Wallis test with post-hoc pairwise Mann-Whitney U tests. Categorical values were compared using Fishers exact test.

[a] Blood pressure measured at the last regular prenatal visit, 0-2 weeks before cesarean section.

[b] Maternal weight for BMI calculation was measured at the first prenatal care visit, before week 12 of pregnancy. Weight information was missing for one woman with severe PE and FGR, and one normotensive woman.

[c] Pregestational CVD diagnoses included: pregestational hypertension, cardiomyopathies, congenital cardiac defects.

[d] Birth weight <5th percentile according to fetal weight reference curves [11] was used as a proxy for one of the FGR diagnoses.

* $p<0.05$ compared to normotensive.

** $p<0.001$ compared to normotensive.

*** $p<0.05$ compared to preeclampsia.

Abbreviations: BMI; body mass index; BP; blood pressure; CVD, cardiovascular disease; GA; gestational age; FGR, fetal growth restriction; IQR; interquartile range; NA, not applicable; md; median; mn, mean; n, number; PE, preeclampsia; SD, standard deviation

Table 2: Metabolite levels in placentas and maternal serum concentrations

Metabolite, median (IQR)	PE (n=19)	Normotensive (n=15)	P-value	P (adj)[a]	P (GA)[b]
Phospholipid biosynthesis					
Ethanolamine	6.6 (1.3)	9.80 (3.1)	<0.001	0.005	0.075
Choline	75.5 (11.1)	74.7 (20.3)	0.864	0.891	
Glycerophosphocholine	22.5 (12.1)	13.2 (14.8)	0.007	0.018	0.344
Phosphocholine	11.3 (5.6)	9.0 (4.5)	0.056	0.097	
Dihydroxyacetone	1.04 (1.25)	2.3 (1.9)	0.003	0.010	0.142
Glycerol	25.9 (9.7)	25.9 (4.0)	0.945	0.945	
Myoinositol	16.8 (3.8)	16.5 (3.6)	0.811	0.863	
Ammonia recycling, Urea cycle, Bile acid biosynthesis					
Glutamine	5.4 (2.3)	7.6 (2.4)	0.004	0.013	0.029
Aspartate	8.3 (4.4)	6.1 (2.4)	0.047	0.097	
Glutamate	16.4 (4.0)	21.7 (1.3	<0.001	0.005	<0.001
Acetate	2.8 (1.1)	3.0 (0.8)	0.372	0.512	
Glycine	8.5 (2.5)	11.1 (2.0)	0.001	0.005	0.070
Alanine	9.1 (3.4)	9.4 (3.0)	0.179	0.281	
Taurine	20.9 (7.5)	29.5 (4.6)	<0.001	0.005	0.031
Protein biosynthesis					
Leucine	11.23 (3.65)	11.77 (1.9)	0.286	0.410	
Isoleucine	1.8 (0.4)	2.0 (0.3)	0.056	0.097	
Valine	3.2 (0.8)	3.6 (0.9)	0.006	0.017	0.019
Threonine	3.6 (1.1)	4.3 (0.7)	0.021	0.046	0.054
Lysine	8.3 (3.5)	10.0 (0.9)	0.006	0.017	0.398
Glycolysis, ketone body metabolism					
Lactate	42.2 (10.7)	41.9 (7.2)	0.391	0.515	
Glucose	2.5 (2.0)	3.3 (3.1)	0.111	0.183	
Succinate	3.0 (1.3)	3.7 (1.3)	0.056	0.097	
3-Hydroxybutyrate	2.7 (0.9)	4.4 (3.0)	0.019	0.045	0.023
Catecholamine biosynthesis					
Ascorbate	2.2 (0.6)	2.9 (0.4)	0.001	0.005	0.045
Glycine and serine metabolism					
Creatine	5.5 (1.9)	6.0 (1.9)	0.256	0.384	
Serum markers, median (IQR)[c]					
Cholesterol [mM]	6.8 (1.9)	6.1 (2.2)	0.580	0.660	
Creatinine [μM]	66.0 (20.0)	58.0 (9.0)	0.001	0.005	
Uric acid [μM]	399 (157)	279 (105)	0.002	0.008	
HDL [mM]	1.6 (0.4)	1.7 (0.5)	0.656	0.722	
Triglycerides [mM]	3.7 (2.4)	2.7 (1.1)	0.421	0.515	
Calcium [mM]	2.3 (0.3)	2.4 (0.2)	0.421	0.515	
hsCRP [μg mL^{-1}]	4.8 (15.6)	3.3 (5.4)	0.486	0.573	
sFlt-1 [ng mL^{-1}]	960 (1350)	239 (162)	<0.001	0.005	

Metabolite levels are in arbitrary units relative to total spectral intensity. Metabolites and serum values were compared between preeclamptic and normotensive groups using the Mann-Whitney U test. Metabolites grouped by metabolic pathways described in the small molecule pathway database [21]. The metabolites may be involved in several pathways. Abbreviations: GA, gestational age; HDL, high density lipoprotein; hsCRP, high sensitivity C-reactive protein; IQR, interquartile range; sFlt-1, soluble Fms-like tyrosine kinase receptor 1; PE,

preeclampsia.
[a] Corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate.
[b] Corrected for gestational age. Linear regression models were made with log transformed metabolite levels as dependent variable and gestational age as independent variable. The metabolite is then evaluated at the average values GA=245 days.
[c] One placenta-serum pair was excluded due to missing blood sample.

Table 3: PLS-DA results

PLS-DA Model	LVs	Sens.	Spec.	Accuracy	P-value[a]	Metabolites (Rel to 1st mentioned)
Total PE (n=19) vs. normotensive (n=15)	1	0.870 (0.863-0.877)	0.984 (0.973-0.996)	0.927 (0.912-0.934)	<0.001	Increase: GPC, PCho, Asp Decrease: EtAm, Tau, Glu, Asc, Gly
Severe PE (n=14) vs. non-severe PE (n=5)	1	0.770 (0.707-0.832)	0.879 (0.859-0.897)	0.832 (0.802-0.862)	0.003	Increase: Cho, Lys, Ala, Glucose, Myo, Tau, Asp, Gln Decrease: 3-HB
PE (n=8) vs. PE+FGR (n=11)	1	0.631 (0.579-0.683)	0.723 (0.688-0.758)	0.677 (0.649-0.705)	0.163	Not significant

Placental metabolic profiles were compared between groups using partial least squares discriminant analysis (PLS-DA), and the discriminatory ability assessed with 5-fold cross validation.

[a] p value from 1000 permutation tests.

Abbreviations: 3-HB, 3-hydroxybutyrate; Ala, alanine; Asc, ascorbate; Asp, aspartate; Cho, choline; EtAm, ethanolamine; FGR, fetal growth restriction; Gln, glutamine; Glu, glutamate; GPC, glycerophosphocholine; Gly, glycine; Lys, lysine; LVs, latent variables; Myo, myo-inositol; Tau, taurine; PCho, phosphocholine; PE, preeclampsia; PLS-DA, partial least squares discriminant analysis; rel, relative; sens, sensitivity; sFlt-1, soluble fms-like tyrosine kinase receptor 1; spec, specificity; Suc, succinate; Val, valine.

Table 4: PLS results

PLS regression Metabolites vs Y [a]	LVs	R^{2} [b]	Y explained	P-value [c]	Metabolites
Serum sFlt-1	1	0.482 (0.463-0.500)	0.614	<0.001	Increase: GPC Decrease: Glu, Tau, Gln, Val, EtAm, Suc
Serum creatinine	1	0.102 (0.085-0.119)	0.343	0.032	Increase: GPC, Asp, PCho, Cre Decrease: Gly, EtAm
Serum uric acid	1	0.128 (0.110-0.147)	0.348	0.009	Increase: Asp, GPC Decrease: Glu, Tau, Gln, Val, EtAm, Suc
Serum triglycerides	2	0.164 (0.130-0.198)	0.166	<0.001	Increase: Cho, Gln, Gly Decrease: 3-HB, Cre

Results from partial least squares regression for correlation between metabolic placenta profiles and maternal serum measurements. The maternal serum values are for 33 placenta/serum pairs (34 placentas - 1 excluded from serum measurements due to missing blood sample). Results were assessed with 5-fold cross validation.

[a] Y denotes the dependent variable, e.g, the serum measurement

[b] R^{2} values give the correlation of the cross-validated predicted Y values to the real Y values.

[c] p value from 1000 permutation tests.

Abbreviations: 3-HB, 3-hydroxybutyrate; Asp, aspartate; Cho, choline; Cre, creatine EtAm, ethanolamine; Gln, glutamine; Glu, glutamate; GPC, glycerophosphocholine; Gly, glycine; LVs, latent variables; Tau, taurine; PCho, phosphocholine; PLS; partial least squares regression; sFlt-1, soluble Fms-like tyrosine kinase receptor 1; Suc, succinate; Val, valine.

Supplementary information

(See Supplementary tables 1 and 2 in separate documents).

Table S.3

Identified metabolites in ^{1}H and ^{1}H-^{13}C nuclear magnetic resonance (NMR) spectra of placental biopsies (n=34).

Metabolite name	**NMR shifts, ^{1}H (^{13}C)**
3-Hydroxybutyrate	1.197d
Acetate	1.927s (26.9)
Alanine	1.478d (19.0), 3.779q (53.4)
Ascorbic acid	4.515s
Aspartic acid	2.818dd (39.5)
Choline	3.207s (56.7), 3.527t (70.4), 4.051b (58.5)
Creatine	3.029s (41.8), 3.937s
Dihydroxyacetone	4.417s
Ethanolamine	3.135t/dd(44.3), 3.82d (61.1)
Glucose	3.269t (77.14), 3.394m (72.4), 3.468m (78.8), 3.521m (74.3), 3.898m (63.5), 4.652d (97.8)
Glutamic acid	2.055m (27.4), 2.338m (36.4), 3.759t (57.0)
Glutamine	2.138m (27.4), 2.444m (35.9)
Glycerol	3.650dd (65.4), 3.780m (74.9)
Glycerophosphocholine	3.234s (56.7), 4.33m (62.0)
Glycine	3.650s (53.6)
Isoleucine	0.955t, 1.003d
Lactic acid	1.318d (23.0), 4.123q (71.3)
Leucine	0.967d
Lysine	1.718quin 1.902m, 3.021t (42.2)
Myoinositol	3.27t (77.1), 3.556dd (74.2), 3.620t (75.1)
Phosphocholine	3.222s (56.7)
Succinic acid	2.410s (36.8)
Taurine	3.263t (50.9), 3.423t (38.2)
Threonine	1.318d (23.0), 3.586d (65), 4.255m (69.4)
Valine	0.988d, 1.042d (20.42)

Multiplicity of peaks are given as follows: s, singlet; d, doublet; t, triplet; q, quartet, quin, quintet; dd, doublet of doublet; m, multiplet.

Table S.4

Metabolite Set Enrichment Analysis of quantitative metabolite data from placenta from women with preeclampsia (n=19) and normotensive pregnancies (n=15).

Pathway	Total Cmpd[a]	Hits[b]	Q Statistic[c]	Expected Q[d]	Raw p[e]	FDR[f]
Bile acid biosynthesis	49	2	39.30	3.03	1.90×10^{-7}	6.07E-06
Taurine and hypotaurine metabolism	7	1	51.60	3.03	1.72×10^{-6}	2.76×10^{-5}
Phospholipid biosynthesis	19	2	29.17	3.03	6.07×10^{-6}	6.47×10^{-5}
Ammonia recycling	18	3	21.32	3.03	2.82×10^{-5}	2.25×10^{-4}
Protein biosynthesies	19	6	15.75	3.03	1.44×10^{-4}	9.24×10^{-4}
Urea cycle	20	2	18.48	3.03	9.32×10^{-4}	0.005
Glutathione metabolism	10	1	26.99	3.03	0.002	0.007
Porphyrin metabolism	22	1	26.99	3.03	0.002	0.007
Pyrimidine metabolism	36	1	25.47	3.03	0.002	0.007
Purine metabolism	45	1	25.471	3.03	0.002	0.007
Glycine, serine and threonine metabolism	26	3	13.69	3.03	0.003	0.007
Propanoate metabolism	18	1	24.97	3.03	0.003	0.007
Glutamate metabolism	18	2	17.69	3.03	0.009	0.022
Methionine metabolism	24	2	13.50	3.03	0.010	0.024
Valine, leucine and isoleucine degradation	36	2	14.90	3.03	0.016	0.033
Lysine degradation	13	1	13.93	3.03	0.030	0.056
Biotin metabolism	4	1	13.93	3.03	0.030	0.056
Beta-alanine metabolism	13	1	11.49	3.03	0.050	0.080
Aspartate metabolism	12	1	11.49	3.03	0.050	0.080
Malate-aspartate shuttle	8	1	11.49	3.03	0.050	0.080
Citric acid cycle	23	1	9.90	3.03	0.070	0.102
Mitochondrial electron transport chain	15	1	9.90	3.03	0.070	0.102

Metabolite set enrichment analysis results (Performed on Metaboanalyst online, www.metaboanalyst.ca [1]).

[a] Total number of compounds (metabolites) in the pathway

[b] Number of measured metabolites found in pathway

[c] Q-statistic describing the correlation between compound concentration profiles and phenotype labels

[d] Expected Q statistic given no correlation between compounds and phenotype labels

[e] P value for the probability of obtaining the Q statistic

[f] P values corrected for multiple correction using false discovery rates

Table S.7

Placental metabolite levels in severe vs non-severe preeclampsia.

Metabolite, median (IQR)	Non-severe PE (n=5)	Severe PE (n=14)	P-value	P (FDR)[a]
Phospholipid biosynthesis				
Ethanolamine	6.6 (3.8)	6.7 (1.2)	0.964	1.000
Choline	67.7 (18.2)	78.1 (7.4)	0.003	0.038*
Glycerophosphocholine	21.3 (20.8)	23.1 (16.02)	1.000	1.000
Phosphocholine	8.0 (8.3)	11.4 (4.2)	0.931	1.000
Dihydroxyacetone	1.0 (0.7)	1.1 (1.4)	0.559	0.736
Glycerol	20.9 (16.9)	25.9 (7.1)	0.500	0.694
Myoinositol	13.3 (6.4)	17.0 (2.0)	0.107	0.243
Ammonia recycling, urea cycle, bile acid biosynthesis				
Glutamine	4.8 (2.0)	5.6 (2.5)	0.070	0.242
Aspartate	6.8 (12.7)	8.3 (4.6)	0.687	0.818
Glutamate	13.8 (4.3)	17.0 (3.4)	0.044	0.242
Acetate	2.6 (1.7)	2.8 (1.0)	0.444	0.653
Glycine	7.3 (2.5)	8.8 (2.1)	0.219	0.391
Alanine	6.3 (3.4)	9.7 (2.0)	0.056	0.242
Taurine	18.0 (7.3)	21.9 (6.1)	0.130	0.271
Protein biosynthesis				
Leucine	9.0 (2.5)	11.6 (3.1)	0.087	0.242
Isoleucine	1.6 (0.4)	1.8 (0.5)	0.257	0.402
Valine	3.0 (0.3)	3.5 (0.7)	0.107	0.243
Threonine	2.9 (1.0)	3.8 (0.7)	0.070	0.242
Lysine	5.9 (1.5)	9.1 (3.8)	0.003	0.038*
Glycolysis, ketone body metabolism				
Succinate	2.7 (1.9)	3.1 (1.2)	0.257	0.402
Lactate	42.2 (7.7)	41.3 (11.1)	0.823	0.935
Glucose	1.6 (1.0)	2.8 (1.6)	0.034	0.242
3-Hydroxybutyrate	3.2 (2.0)	2.7 (0.8)	0.186	0.358
Catecholamine biosynthesis				
Ascorbate	1.9 (0.9)	2.3 (0.6)	0.087	0.242
Glycine and serine metabolism				
Creatine	5.7 (5.1)	5.3 (2.2)	0.687	0.818

Metabolites were compared between groups using the Mann-Whitney U test. Metabolites grouped by metabolic pathways described in the small molecule pathway database [2]. The metabolites may be involved in several pathways. Metabolite levels are in arbitrary units relative to total spectral intensity.

Abbreviations: FDR, false discovery rate; IQR, interquartile range; PE, preeclampsia.

[a] Corrected for multiple comparisons using the Benjamini-Hochberg false discovery rate.

* Significantly different between severe and non-severe preeclampsia after correction for multiple testing.

Table S.6

Comparison between metabolite levels in placentas from women with preeclamptic and normotensive pregnancies, with adjustments for gestational age.

Metabolite	P [a] (PE vs Normotensive)	P (adjusted for GA)
Ethanolamine	0.005	0.075
Glycerophosphocholine	0.018	0.344
Dihydroxyacetone	0.011	0.142
Glutamine	0.013	0.029*
Glutamate	0.005	<0.001*
Glycine	0.005	0.070
Taurine	0.005	0.031*
Valine	0.017	0.019*
Lysine	0.017	0.398
Threonine	0.046	0.054
3-HB	0.045	0.023*
Ascorbate	0.005	0.045*

For preeclamptic vs normotensive groups, the Mann-Whitney U test (adjusted for multiple comparisons) is given. For the gestational age adjusted p values, linear regression models were made with log transformed metabolite levels as dependent variable and gestational age as independent variable. The metabolite is then evaluated at the average values GA=245 days.
Abbreviations: 3HB, three-hydroxybutyrate; GA, gestational age; PE, preeclampsia.
[a] Adjusted for multiple comparisons using the Benjamini-Hochberg false discovery rate for all tested metabolites.
* Significantly different between normotensive and preeclamptic placentas after correction for difference in gestational age.

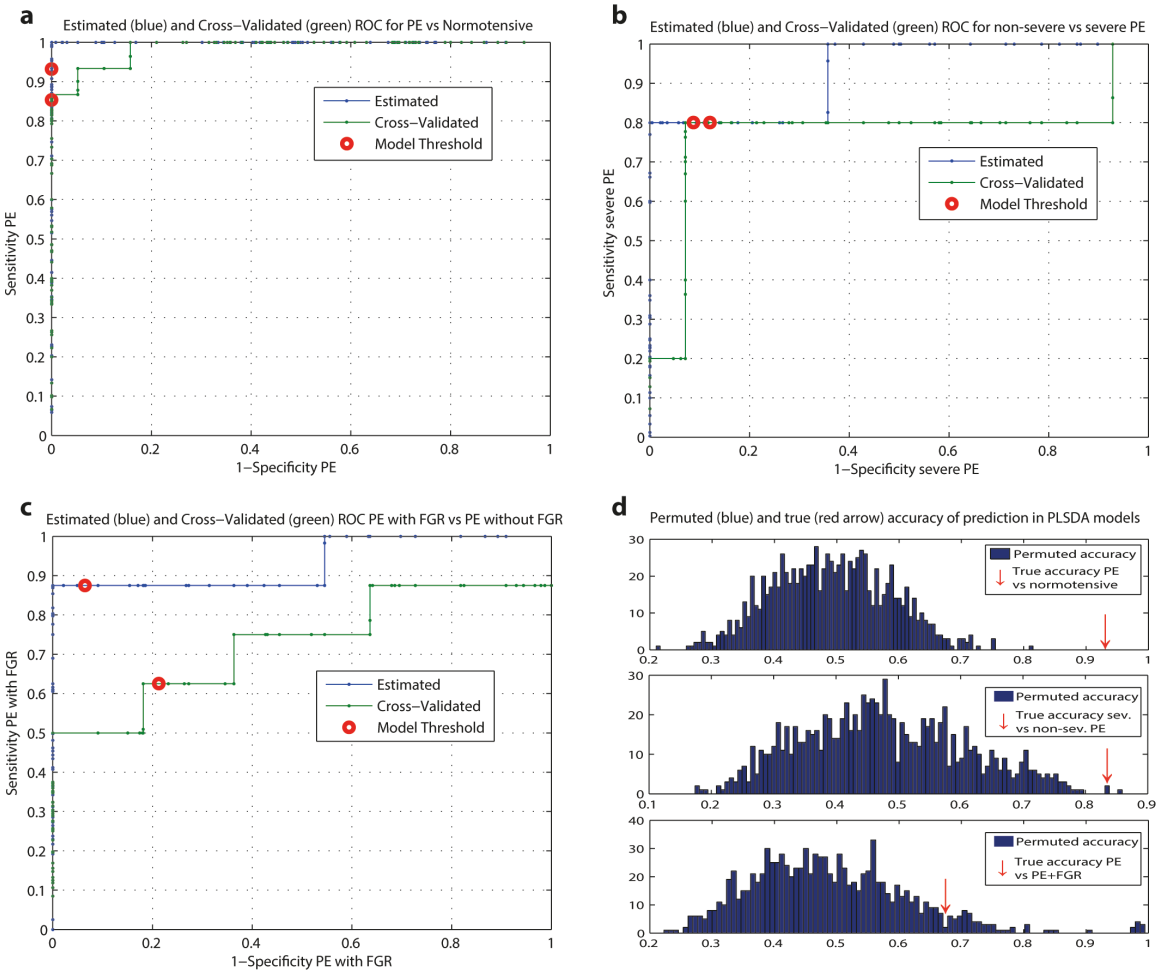

Figure S.1. Receiver-operator characteristic curves for prediction of class from metabolic profile of the placenta. a) Discrimination of preeclampsia from normotensive. b) Discrimination of severe preeclampsia from non-severe preeclampsia. c) Discrimination of preeclampsia with fetal growth restriction from preeclampsia without fetal growth restriction. d) Results from permutation testing showing true vs. permuted classification accuracy for the three classification models.
Abbreviations: FGR, fetal growth restriction; non-sev., non-severe; PE, preeclampsia, ROC, receiver-operator characteristic.

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