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ORIGINAL ARTICLE

Metabolomic determination of pathogenesis of late-onset preeclampsia

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Abstract

Objective: Our primary objective was to apply metabolomic pathway analysis of first trimester maternal serum to provide an insight into the pathogenesis of late-onset preeclampsia (late-PE) and thereby identify plausible therapeutic targets for PE.
Methods: NMR-based metabolomics analysis was performed on 29 cases of late-PE and 55 unaffected controls. In order to achieve sufficient statistical power to perform the pathway analysis, these cases were combined with a group of previously analyzed specimens, 30 late-PE cases and 60 unaffected controls. Specimens from both groups of cases and controls were collected in the same clinical centers during the same time period. In addition, NMR analyses were performed in the same lab and using the same techniques.
Results: We identified abnormalities in branch chain amino acids (valine, leucine and isoleucine) and propanoate, glycolysis, gluconeogenesis and ketone body metabolic pathways. The results suggest insulin resistance and metabolic syndrome, mitochondrial dysfunction and disturbance of energy metabolism, oxidative stress and lipid dysfunction in the pathogenesis of late PE and suggest a potential role for agents that reduce insulin resistance in PE.
Conclusions: Branched chain amino acids are known markers of insulin resistance and strongly predict future diabetes development. The analysis provides independent evidence linking insulin resistance and late-PE and suggests a potentially important therapeutic role for pharmacologic agents that reduce insulin resistance for late-PE.

Keywords

Insulin resistance, metabolomics, preeclampsia

History

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Introduction

The heterogeneous nature of preeclampsia (PE) continues to challenge efforts to develop a unifying theory of its pathogenesis. An emerging view is that PE is actually two disorders namely early- and late-onset PE (late-PE) [1,2]. Early-onset PE has thus been defined as PE manifesting by, and resulting in, delivery before 34 weeks gestation whereas late-PE has been defined as the sub-group that manifests clinically at or after 34 weeks gestation [3]. An alternative, but not irreconcilable view, is that rather than being two distinct disorders, PE is a single disorder with a wide spectrum of clinical manifestations [4]. Despite its milder presentation late- PE is not a benign disorder. In a large population study, it was associated with increased perinatal death and morbidities, higher rates of small-for-gestational-age (SGA) infants and is significantly more common than the early onset variety [5].

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Metabolites are the small molecules that are transformed during metabolism and provide a readout of cellular biochemistry. Metabolites are downstream to genes and proteins thus metabolomics is thought to provide a more precise description of cell phenotype compared to genomics and proteomics [6]. Metabolomics is a very effective technique for new biomarker development and its use has been previously reported for first-trimester prediction of PE. First trimester metabolite markers have been used for the prediction of early-onset-PE [7–13]. Beyond mere biomarker development, however, metabolomics can generate significant insights into the etiology and pathogenesis of complex and heterogeneous disorders [14–16]. This potential has not been explored as it relates to PE.

Late-PE is considered a maternal disorder while early-PE is considered a fetal/placental disorder [2]. Our primary objective in this study was to use metabolomics to identify dysregulated metabolic and biochemical pathways and thereby to generate insights into the basic pathogenesis of late-PE. In addition to the decades-long interest in understanding the pathogenesis, there is now increasing scientific [3] and commercial [17] interest in the early prediction of PE.

A secondary objective was to use the pathway analysis data on pathogenesis to develop insights into novel potential therapies for late-PE. Finally, we also wanted to evaluate the accuracy of new metabolomics algorithms for the first trimester prediction of late-PE.

Methods

The details of patient recruitment, evaluation, specimen collection and handling have been previously published [7,10]. This is part of an on-going prospective study being conducted by the Fetal Medicine Foundation (UK Charity No: 1037116), London, England, for the first trimester prediction of pregnancy complications. Institutional Review Board Project #02–03-033 approval was obtained on 14 March 2003. An average-risk population of British women were prospectively screened between March 2003 and September 2009 [10,18] and additional patients from cases recruited between 2009 and 2012. All patients gave written consent to participate. The study was approved by the King's College Hospital research ethics committee. Pregnant women were recruited at $11^{+0–13+6}$ week's gestation. Maternal demographics and medical history were documented. First trimester ultrasound assessment including fetal crown-rump length (CRL) and uterine artery Doppler pulsatility index (PI) measurements were performed. The lower of the left and right uterine artery Doppler PI value was used for PE prediction. Maternal serum from singleton pregnancies was obtained and transferred to the lab within 5 min of collection. Specimens were left to stand for 10–15 min at room temperature to allow the blood to clot. The specimens were centrifuged at 3000 rpm for 10 min in order to separate serum from clotted material. The serum was aliquoted in 0.5 mL quantities and placed into screw cap tubes. The samples were temporarily stored in a $-20\,^{\circ}C$ freezer and then transferred to a $-80\,^{\circ}C$ freezer within 24 h. The long-term objective of the project is to develop and evaluate new markers and existing biomarkers of PE.

In conducting this study, we combined the previously published 29 late-PE cases, requiring delivery at or after 34 weeks, and 57 unaffected controls [10] with a new group consisting of 30 late- PE cases and 58 controls that had not been previously used in our metabolomic analyses to achieve sufficient statistical power. In the prior study [10], we evaluated new biomarker combinations for late-PE prediction. No pathway analysis was performed in that study. The late-PE cases were selected at random from our database of available stored samples. As with our prior dataset, the controls were from non-hypertensive pregnancies that delivered a phenotypically normal term neonate with appropriate birth weight for gestational age. Each control had blood collected within 3 d of assessment of a late-PE case. PE was defined as proposed by the International Society for the Study of Hypertension in Pregnancy [19] with systolic blood pressure (BP) $\geq$ 140 or diastolic BP $\geq$90 mm Hg on two or more occasions 4 h apart after 20 weeks of gestation, in previously normotensive women. Proteinuria was defined as a total of 300 mg in a 24 h urine collection or two readings of at least 2^{+} proteinuria on a mid-stream or catheterized urine specimen in the absence of a 24 h urine collection, which must also have been present in addition to the hypertension. Cases diagnosed with HELLP syndrome or gestational hypertension were excluded.

NMR-based metabolomic analysis

Our previously reported study of late-PE [10] was limited to nuclear magnetic resonance (NMR) analysis. The metabolomic methods used to analyze the samples followed published protocols [7,18]. We briefly summarize the previously published description in the Supplemental Metabolomics Method section. A single metabolomics lab performed the NMR analyses and there were no changes in the protocols or analytical methods employed for the analysis of the two groups of specimens used in this study.

Statistics

The statistical methods used were as previously described by us and others and are detailed in the Supplemental Metabolomic and Statistical Method section. The MetaboAnalyst (version 3.0) computer program was used to perform all PCA and PLS-DA analyses [20–22]. Custom programs written using the R statistical software package (http://www.r-project.org) and STATA version 12.0 (StataCorp, College Station, TX; http://www.stata.com) were used to perform all other statistical analyses. Data were log-transformed as indicated, and metabolites with a p values <0.3 (using univariate analysis) were selected for model generation. A k-fold cross-validation (CV) technique was used to ensure that the logistic regression models were robust [23]. To develop and test the predictive models, the combined dataset (previously published plus new patients) was randomly split into a discovery set (''training set''; 60% of the cohort) and a validation set (''test- set''; 40% of the cohort).

For the selection of predictor variables in the regression models, Least Absolute Shrinkage and Selection Operator (LASSO) [24] and stepwise variable selection were utilized for optimizing all the model components [25] via 10-foldCV. The threshold used for inclusion of a metabolite or other clinical/phenotypic variables required that the particular variable be selected >8 times of the 10 CVs. Three different logistic regression models based on metabolite subsets were developed with 10-foldCV. Areas under the Receiver Operating Characteristic curve (AUROC or AUC) were calculated [23].

Pathway topology analysis

Metabolites that were found to be significantly different ($p < 0.05$) between controls and late-PE patients were applied to the pathway topology search tool in MetaboAnalyst (version 3.0) [20–22]. The pathway library chosen was that for *Homo sapiens* and all compounds in the selected pathways were used when referencing the specific metabolome. Fisher's exact test was applied for over-representation analysis and relative ''betweenness centrality'' was chosen for pathway topology testing. Pathways that had a Holm corrected p values and a false discovery rate less than 0.05 were considered to be altered due to the disease.

Results

A total of 59 late-PE cases and 115 controls were used in this analysis. Table 1 compares the demographic and clinical

Table 1. Comparison of demographic and clinical characteristics: combined late-onset preeclampsia*.

	Combined Group		
Parameter	Late-PE	Control	*p* Values
No. of cases	59	115	–
Maternal age, years, mean (SD)	30.5 (6.0)	31.3 (5.7)	0.396
Racial origin, *n* (%)			0.001
White	23 (39.0)	75 (65.2)	
Black	32 (54.2)	32 (27.8)	
Asian	2 (3.4)	7 (6.1)	
Mixed	2 (3.4)	1 (0.9)	
Nullipara, *n* (%)	34 (57.6)	51 (44.4)	0.097
Weight, kg, mean (SD)	74.6 (14.8)	68.6 (13.0)	0.007
Crown-rump length, mm, mean (SD)	61.6 (8.3)	62.8 (7.2)	0.344
Uterine pulsatility index, MoM*, mean (SD)	1.51 (0.61)	1.29 (0.49)	0.019

*Multiples of median for gestational age.

Table 2. Over-represented metabolic pathways: late onset preeclampsia.

Annotations	Pathways	Total	Expected	Hits	Holm adjusted *p* values	FDR
2	Pyruvate metabolism	32	0.27919	2	1	0.20485
3	Citrate cycle (TCA cycle)	20	0.17449	2	0.92749	0.14324
4	Glycolysis or gluconeogenesis	31	0.27046	3	0.1694	0.043999
5	Butanoate metabolism	40	0.34898	2	1	0.26484
6	Valine, leucine and isoleucine biosynthesis	27	0.23556	4	0.0052191	0.0026426
7	Propanoate metabolism	35	0.30536	5	0.00066666	0.00066666
8	Synthesis and degradation of ketone bodies	6	0.052347	2	0.08308	0.028403
9	Valine, leucine and isoleucine degradation	40	0.34898	3	0.34937	0.073551
10	Glycine, serine and threonine metabolism	48	0.41878	3	0.57704	0.10258
11	Vitamin B6 metabolism	32	0.27919	2	1	0.20485
12	Arginine and proline metabolism	77	0.67179	2	1	0.63832
–	Pantothenate and CoA biosynthesis	27	0.23556	2	1	0.20485
–	Aminoacyl-tRNA biosynthesis	75	0.65434	3	1	0.20485
–	Pentose phosphate pathway	32	0.27919	2	1	0.20485

characteristics of the cases and controls. There were significant differences in racial composition, maternal body weight and uterine artery PI mean values. Regression analyses therefore considered all these potential confounders. The uterine artery PI was found to be significantly elevated in the late-PE compared to controls. Significant differences in concentrations of multiple metabolites between late-PE and controls were also observed, Supplemental Table 1. A total of 15 metabolites displayed significant elevation (FDR *p* values <0.05) in maternal first-trimester serum among late-PE patients compared to normal cases. Therefore, significant metabolite disturbances in the first-trimester appeared to be a major feature of late-PE. Pathway analysis, Table 2, revealed an over-representation of the branched-chain amino acid (valine, leucine and isoleucine) and propanoate metabolic pathways which was statistically significant. The details of the significantly affected pathways are shown in Figures 1 and 2. L-Valine, L-leucine, L-isoleucine and pyruvic acid, which are all part of the branched chain amino acid pathway, were significantly elevated ($p<0.05$) in late-PE. For the propanoate pathway, L-valine, 2-hydroxybutyric acid, lactic acid, acetone and propan-2-ol were found to be significantly elevated ($p<0.05$) in late-PE. Abnormalities in the glycolysis and gluconeogenesis pathways as well as in the synthesis and degradation of ketone bodies were also observed in late-PE patients. These showed a trend but did not achieve statistical significance by the strict criteria that we used. In view of the relatively small number of cases analyzed, the lack of statistical significance is likely due to insufficient study power. The potential significance of the observed metabolic aberrations is discussed later.

A second objective of this study was to assess the performance of algorithms for the prediction of late-PE. Demographic and clinical characteristics were compared between late-PE and control groups in both the discovery and validation subgroups (Supplemental Table 2). Predictive models based on (1) uterine artery PI alone, (2) metabolites alone, (3) metabolites + uterine artery PI, as well as (4) metabolites + uterine artery PI and maternal weights were generated in the discovery/training set (Supplemental Table 3). The VIP plot (Supplemental Figure 1, ranks the metabolites based on their ability to identify late-PE. Higher VIP values (on the *x*-axis) correlate with higher discriminating ability. The performance of the algorithms in the validation group is shown in Table 3. Uterine artery PI by itself was not a significant predictor of late-PE (data not presented). Metabolite based algorithms had statistically significant screening accuracies, as evidenced by the confidence intervals of the AUC. However, the diagnostic performance for late-PE detection was only modest. The persistence of metabolites in the regression analyses while maternal weight did not (Supplemental Table 3), indicates that the metabolite differences between the groups could not be explained by weight differences. The same applies to the other demographic and clinical factors, e.g. age, parity and ethnicity.

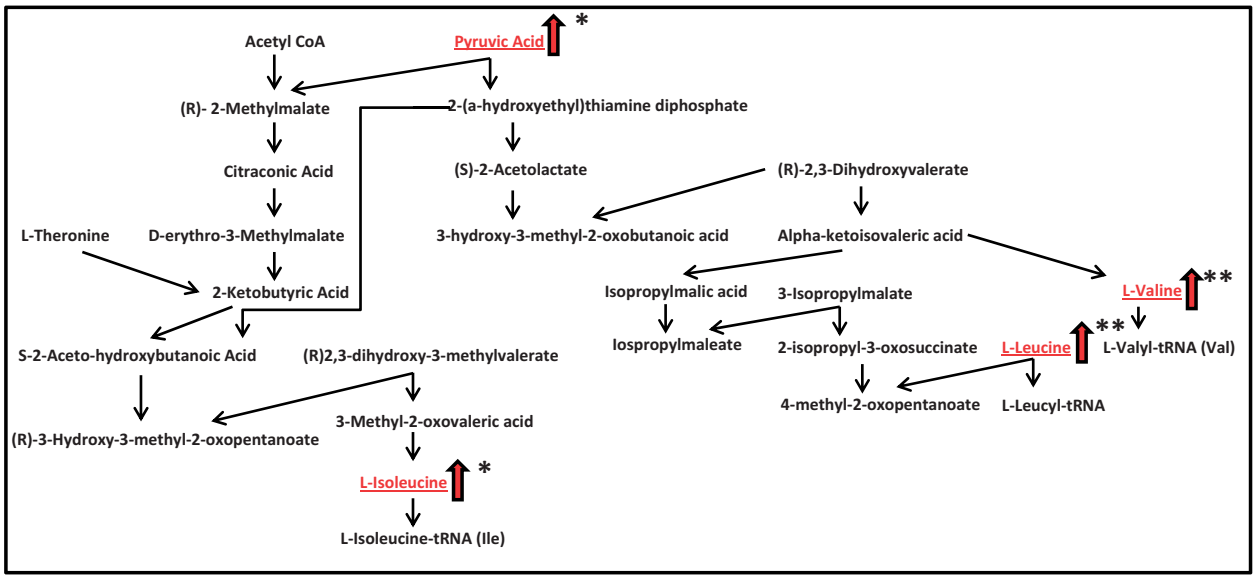

Figure 1. Valine, leucine and isoleucine metabolism. The metabolites that are underlined are those which are significantly up-regulated in late preeclampsia samples. *$p < 0.05$; **$p < 0.01$.

Discussion

In this study, we found significant alterations in the concentrations of multiple metabolites in the first trimester serum of women destined to develop late-PE. This is consistent with our smaller initial pilot study [7]. We found alterations in a number of metabolic pathways involved in lipid, oxidative stress and energy metabolism. There was strong evidence for insulin resistance as discussed below. Metabolite biomarkers performed better than clinical factors such as maternal BMI or race in predicting late-PE. Further, uterine artery Doppler by itself was not a significant predictor of late-PE, which contrasts with what is generally found in first trimester prediction of early-PE consistent with prior evidence that late-PE is a maternal disease, while early-PE is considered a fetal or placental disorder [2]. An independent validation analysis confirmed that first-trimester metabolites had modest sensitivity for prediction of late-PE.

VIP analysis indicated that carnitine was the most significantly affected metabolite in late-PE. Increased carnitine levels were observed in the first-trimester maternal serum of late-PE cases compared to controls. Review of the Human Metabolome Database (HMDB) [26–28] reveals that carnitine is a non-essential amino acid that is partly synthesized in the body. Carnitine is critical to fatty acid metabolism as it binds to fatty acids to form acyl-carnitines and shuttles the fatty acids across the mitochondrial membranes to undergo mitochondrial oxidative metabolism. This process of shuttling fatty acids occurs particularly in skeletal and cardiac muscles. Glycerol is a three-carbon alcohol (i.e. contains –OH) group that constitutes the backbone to which fatty acids and lipids are bound to form triglycerides and phospholipids. The synthesis occurs in the liver and adipose tissue. The metabolism of stored lipids leads to the release of glycerol and fatty acids into the bloodstream. Glycerol itself is metabolized to glucose, which thereafter plays its accustomed role in energy metabolism. Our VIP analysis found increased glucose and glycerol levels in late-PE cases consistent with perturbations of lipid metabolism. The changes in these two metabolites implicate abnormalities in lipid metabolism as playing an important role in late-PE pathogenesis. Late-PE cases are known to have significantly higher BMI than unaffected controls in a population study [1]. The changes observed in this study cannot merely be attributed to maternal obesity however since BMI did not persist as a significant independent predictor of late-PE in any of the regression analyses. The essential amino acids, L-valine, L-leucine and L-isoleucine, were significantly up-regulated in the first-trimester serum samples of late-PE patients. Leucine, isoleucine and valine account for a third of the nine essential amino acids in humans. According to data in the HMDB [26–28], branched chain amino acids play a critical role in stress and energy metabolism. Valine is metabolized and directed to carbohydrate synthesis while leucine goes to fat synthesis and isoleucine contributes to both. L-Leucine is known to play an important role in fetal nutrition [29,30]. The transport of this amino acid across the placenta is reportedly impaired in fetal growth restriction [31], which is a recognized complication of PE [5].

Leucine is also known to stimulate insulin secretion by being both a metabolic fuel and by activating glutamate dehydrogenase to increase glutaminolysis. Leucine also regulates gene transcription and protein synthesis in pancreatic beta cells through both mTOR-dependent and -independent pathways at physiological concentrations [32]. Branched chain amino acids were found to be powerful predictors of future development of diabetes in normoglycemic patients followed over 12 years [33]. Thus the abnormalities in branched chain amino acid concentrations found in this study support a link between potential insulin resistance and PE development that had been previously reported [34–38]. Metabolomic evidence of insulin resistance does indeed support and validate the role of high BMI and race (which

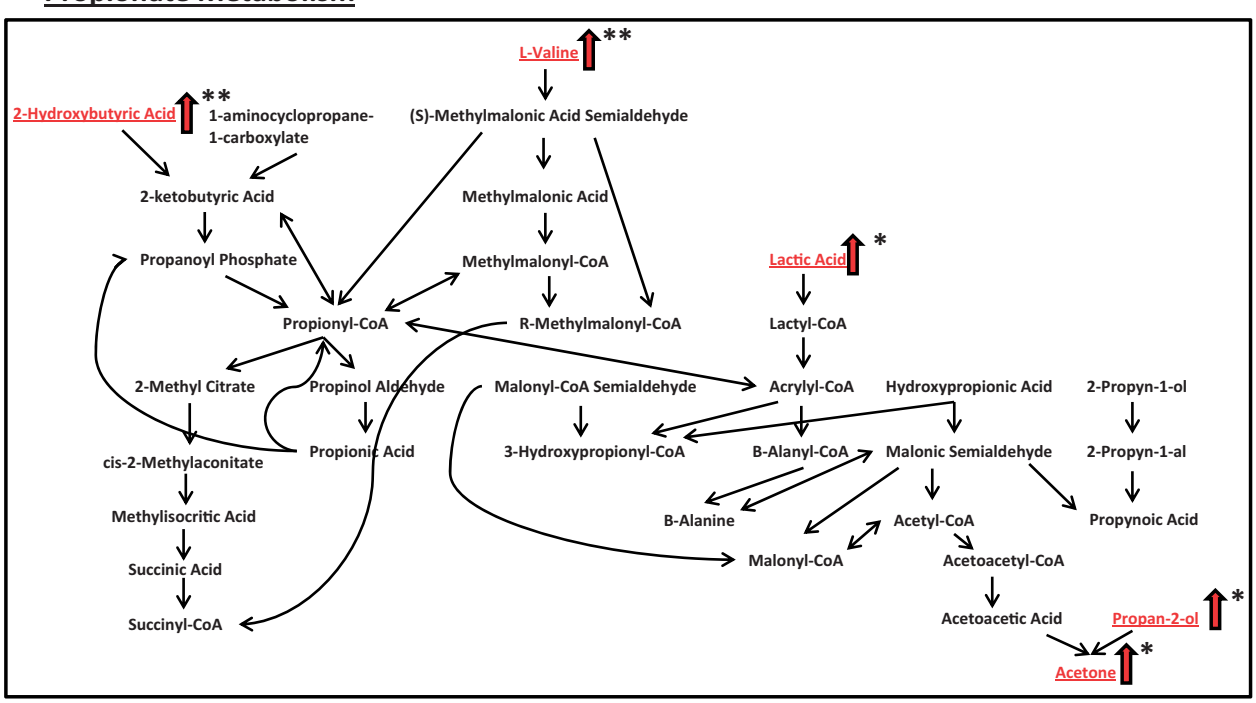

Figure 2. Propionate metabolism. The metabolites that are underlined are those which are significantly upregulated in late preeclampsia samples. $*p < 0.05$; $**p < 0.01$.

Table 3. Metabolomics based models for the prediction of late-onset preeclampsia: performance in an independent validation group.

	Validation group		
Model	AUC (95% CI)	Sensitivity	Specificity
Metabolites only	0.629 (0.490–0.767)	0.304	0.804
Metabolites + UtPI	0.722 (0.595–0.849)	0.391	0.804
Metabolites + UtPI + weight	0.734 (0.612–0.857)	0.348	0.826

Metabolites only = carnitine, pyruvate and acetone.
Metabolites + UtPI = UtPI, pyruvate, carnitine + glycerol.
Metabolites + UtPI + weight = weight + UtPI + pyruvate + carnitine.
UtPI (Uterine artery pulsatility index) – This was not a significant predictor by itself.

influences BMI frequency) in the development of late-PE. Therefore, our results do not in any way negate the importance of these two demographic predictors of late-PE.

Metabolomics is also a tool for identifying potential therapeutic opportunities. Our study found elevated branch chain amino acids, which are markers of insulin resistance and predictors of future development of diabetes as discussed. This raises the prospect of sulphonylurea prophylaxis against late-PE. Metformin for example is widely utilized for treatment of type 2 diabetes; however, there is excitement about its possible role in treatment of insulin resistance (pre-diabetes) and prevention of the metabolic syndrome [39]. A meta-analysis of polycystic ovary syndrome (PCOS) patients, a disorder characterized by insulin resistance, revealed that metformin treatment reduced the rate of PE by close to 50% [40]. A recent randomized study published subsequent to the initial submission of our manuscript found that that first-trimester prophylaxis with metformin in non-diabetic pregnancies resulted in a greater than 75% reduction in the frequency of PE [41]. This validates the use of metabolomics pathway analysis for identifying novel therapeutic options in obstetric disorders in general and in particular the findings in this study regarding the pathogenesis and potential treatment of late-PE. Oxidative stress with lipid dysfunction and oxidation is at the heart of the PE phenotype. The known antioxidant effects of statins and in particular their ability to prevent lipid oxidation gives strong support to their plausibility as therapeutic agents against PE [42,43].

We also report an elevation in the concentration of pyruvic acid in the first-trimester serum of cases destined to develop late-PE cases. The pathway analysis supported a disturbance of both glycolysis (the conversion of glucose to pyruvate) and gluconeogenesis (the conversion of pyruvate to glucose). Pyruvate is an intermediate product in the anaerobic metabolism of glucose, i.e. the ‘‘glycolytic’’ pathway. Further, metabolism of pyruvate leads under conditions of oxygen depletion, to its conversion to lactate. Hypoxia and oxidative stress are features of PE and we found elevated lactate levels in late-PE cases.

In the propanoate pathway, 2-hydroxybutyric acid levels were found to be significantly increased in the late-PE cases. This metabolite is a byproduct of the transulfuration pathway for homocystine metabolism leading to the synthesis of glutathione, an important antioxidant. 2-Hydroxybutyrate is released as a byproduct when cystathione is cleaved cysteine with incorporation of the latter into glutathione. Two hydroxybutyrate is said to be an early marker for both insulin resistance a condition known to be associated with PE [5] and impaired glucose regulation [42,43].

Using an updated data set we demonstrated in an independent subgroup that metabolite based algorithms

were statistically significant first-trimester predictors of late-PE; however, the diagnostic accuracy was modest. In our prior publication using fewer cases and based only on NMR analysis, glycerol alone had 40% sensitivity at 94.1% specificity for late-PE prediction [10].

One limitation of our study was the relatively small number of cases used despite the fact that we actually combined two cohorts to achieve higher case numbers. Having even greater numbers of cases and controls may have rendered other pathways statistically significant, thereby providing greater insights into the mechanisms of late-PE. Having BMI rather than maternal weight data might have provided a greater insight into how obesity affects risk of development of PE.

We utilized previously analyzed specimens and a new group of cases to perform this analysis. Both groups were similar in terms of timing of sample collection and duration of specimen storage. The only potential source of bias from combining these two case groups was the possibility that the metabolomics assays may have performed differently over time in the previously published versus the new patient group. Since both previously published and those undergoing more recent metabolomics analysis were randomly assigned to the current discovery and validation groups, this final potential source of bias was eliminated. Further, we developed novel algorithms based on the new discovery group and tested them in the new validation group. The cases in the validation group were independent of the cases used to generate the algorithm. This meets the statistical criteria required for an independent validation group.

Evaluating PE patients at the time of clinical presentation makes it difficult to determine whether observed changes are a part of the causal pathway or merely an expression of pathophysiological alterations [44]. This study was performed in the first-trimester, approximately 5 months before clinical manifestation. We believe this kind of early sample collection provides the best opportunity to identify disease etiology and to map its evolving pathogenesis.

Finally, the performance of these metabolomics markers by themselves for late-PE detection was modest. This is consistent with the overall literature reporting that predictive algorithms tend to have higher accuracy in early- compared to late-PE. Current standard of care requires the prediction of PE based on historical and clinical risk factors. This approach has a worse diagnostic performance with a sensitivity at 93.0% and false positives rate of 64.1% i.e. positive likelihood ratio 1.45 [1] compared to metabolites alone; sensitivity 30.4% and FPR of 19.6% and positive likelihood ratio of 1.55, for late-PE prediction. Additional metabolite markers will be needed to improve diagnostic accuracy.

Conclusion

To summarize, a complex mix of potential pathways for disease development was identified in this study. An analysis in a larger patient population would completely capture the diversity of pathogeneses and clinical heterogeneity and potential of late-PE. Given the promising results we have obtained for late-PE, we believe that such a large-scale metabolomics study into its pathogenesis is both justified and warranted.

Declaration of interest

The authors report no conflicts of interest.

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