Urinary metabolic profiles at first trimester are associated with preterm birth and foetal growth restriction in the RHEA mother-child cohort study

Léa Maitre\textsuperscript{1,4,5}, Eleni Fthenou\textsuperscript{2,3}, Toby Athersuch\textsuperscript{1,4}, Muireann Coen\textsuperscript{1,4}, Mireille B. Toledano\textsuperscript{4,5}, Elaine Holmes\textsuperscript{1}, Manolis Kogevinas\textsuperscript{3,6,7,8}, Leda Chatzi\textsuperscript{2}, Hector C Keun\textsuperscript{1}\textsuperscript{*}

\textsuperscript{1} Computational & Systems Medicine, Department of Surgery and Cancer, Faculty of Medicine, Imperial College London, London, SW7 2AZ, United Kingdom
\textsuperscript{2} Department of Social Medicine, Faculty of Medicine, University of Crete, PO Box 2208, 71003, Heraklion, Greece
\textsuperscript{3} Centre for Research in Environmental Epidemiology (CREAL), Barcelona, Spain
\textsuperscript{4} MRC-HPA Centre for Environment and Health, Imperial College London, W2 1PG, UK
\textsuperscript{5} Department of Epidemiology and Biostatistics, School of Public Health, Faculty of Medicine, Imperial College London, London, W2 1PG, UK
\textsuperscript{6} National School of Public Health, Alexandras Avenue 196, 115 21, Athens
\textsuperscript{7} IMIM (Hospital del Mar Research Institute), Barcelona, Spain
\textsuperscript{8} CIBER Epidemiologia y Salud Pública (CIBERESP), Barcelona, Spain

\textsuperscript{*}To whom correspondence should be addressed
Abstract

**Background.** Preterm birth (PB) and foetal growth restriction (FGR) convey the highest risk of perinatal mortality and morbidity, as well as increasing the chance of developing chronic disease in later life. Identifying unfavourable maternal conditions early in pregnancy that predict poor birth outcomes could help their prevention and management.

**Methods.** Here we used an exploratory metabolic profiling approach to investigate the association between birth outcomes and metabolites in maternal urine (n=438) collected early in pregnancy (first trimester) as part of the prospective mother-child cohort “Rhea” study. In addition to PB we used FGR in weight as a study endpoint. Major metabolites (34) in urine samples were measured using $^1$H-NMR spectroscopy.

**Results.** Our key observations included a link between FGR and decreased urinary acetate, formate, tyrosine and trimethylamine adjusting for maternal education, maternal age, parity and smoking during pregnancy. These metabolites were inversely correlated to blood insulin. Women with clinically induced PB had a significant increase in the $N$-acyetyl resonances visible in their NMR profile, attributed to glycoproteins and linked to increased BMI. Spontaneous PB cases presented elevated urinary lysine in addition to lower formate.

**Conclusions.** Urinary metabolic markers measured in the first trimester could indicate increased risk of adverse pregnancies and provide novel information regarding the possible mechanisms of negative birth outcomes in the Rhea cohort where metabolic abnormalities syndrome in early pregnancy have been identified as a key risk factor. This study underlines the potential of metabolic profiling in pregnancy research and in defining the human exposome, specifically the impact of the *in utero* environment on health.

**Key words**: Foetal growth restriction (FGR), Intrauterine growth restriction (IUGR), Small for gestational age (SGA), Preterm birth (PB), NMR, metabonomics, metabolomics, *in utero* environment, exposome
Introduction

Foetal growth restriction (FGR) and preterm birth (PB) are the main predictors of perinatal morbidity and mortality [1, 2]. These birth outcomes are associated with growth failure and accelerated weight gain during childhood. As a consequence, adverse child health and predisposition to metabolic and cardiovascular disorders can appear later in life [3, 4]. Over the past 10 years, PB has increased by 19.4% in developed regions with the USA accounting for 42% of all preterm births in 2010 [5]. PB can either be medically induced on the basis of maternal or foetal indications, or spontaneously induced. Spontaneous PB (SPB) occurs at varying prevalence in different ethnic groups and is believed to be associated with intrauterine infection (25-40% of cases), uterine overdistension due to multiple gestations, PB history, or shortened cervix [6]. Medically induced PBs (IPB), which depend upon the clinician decision, often reflect underlying conditions involved with obesity such as pregnancy-induced hypertension or pre-gestational diabetes. FGR, which represents pathological inhibition of foetal growth and failure of the foetus to attain its growth potential, can be due to foetal genetic abnormalities or congenital infections (e.g. toxoplasmosis, malaria, rubella). However, the vast majority of FGR cases are the result of extrinsic factors comprising maternal and placental conditions such as placental ischemia and uteroplacental deficiency [7]. In the developed world, FGR is prevalent among women with hypertensive disorders, toxic exposures (in particular cigarette smoke) and poor nutritional status [8-10].

A recent report evoked the sharp increase in late preterm birth in Greece for the past 20 years, in a similar fashion to what has been noted in other middle or high income countries, potentially associated with increased maternal age and a change in obstetric interventions [11]. Other factors were reported in several studies with associations between pre-pregnancy metabolic disease, such as obesity [12-14], chronic hypertension [15, 16], dyslipidemia and inflammation in early pregnancy [17] and high risk of preterm birth. To better understand the underlying mechanisms that give rise to PB and FGR the present study used data from the Rhea cohort, a large population based mother-child cohort initiated in Crete in 2007 [18]. Among this cohort, women with metabolic syndrome early in pregnancy were at high risk for PB (Relative Risk (RR) = 2.93, 95% CI: 1.53, 5.58), with the highest risk observed for IPB (RR=5.13, 95% CI: 1.97, 13.38). Because routine prenatal care fails to identify a large proportion of women at risk, a better understanding of birth outcomes is crucial to improve their prediction and prevention. The application of metabolic profiling (metabolomics/metabonomics) to pregnancy research has mainly emerged as a non-targeted approach, to explore potential biomarkers of reproductive outcomes and identify underlying biological mechanisms [19-
Since novel biomarkers fail to predict PB and FGR accurately in clinical settings, it was suggested that the use of biomarkers in combination with biophysical parameters and maternal characteristics may be more useful [22, 23]. Further large, prospective cohort studies with rich maternal lifestyle and medical records are therefore needed. To the best of our knowledge our study represents the largest human study (n=438) to date in which urinary metabolomics has been used to assess PB and FGR risk factors early in pregnancy (at first trimester).

Material and methods

Ethics Statement

The study was conducted according to the guidelines laid down in the Declaration of Helsinki and all procedures involving human subjects were approved by the ethical committee of the University Hospital in Heraklion, Crete, Greece. Written informed consent was obtained from all women participating in the study.

The mother-child cohort in Crete, Rhea study

The “Rhea” project is a mother-child study which examines prospectively a population-based cohort of pregnant women and their children at the prefecture of Heraklion, Crete, Greece [18]. Female residents (Greek and immigrants) who had become pregnant during the twelve-month period starting in February 2007 were contacted at the 4 maternity clinics (2 public and 2 private) in Heraklion and asked to participate in the study. Study enrolment and urinary collection were made at the time of the first major ultrasound examination (Mean: 11.96 weeks, SD 1.49). Questionnaires regarding health behaviours, pregnancy history, lifestyle characteristics and dietary habits during pregnancy were administered by trained interviewers at enrolment, during the third trimester, and at delivery.

During this study period 1,317 women were followed up until delivery. Women with incomplete diagnostic information, multiple pregnancies, diagnosed preeclampsia (a condition associated with PB), spontaneous or induced abortion, or who gave birth to stillborn infants were not included in the study [18]. Our metabolomics study was designed as a case-control study nested within the Rhea cohort. Mothers giving birth preterm and for which biological samples (first trimester urines) where available, were matched to controls (in a ~1:3 ratio) based on age (±2 years), origin and parity (n=464). From these urine specimens ¹H NMR spectra were acquired of which 26 spectra were excluded (due to high dilution or high excretion of drug metabolites), leaving 438 spectra available for modelling the metabolite profile with respect to birth outcome.
Definition of the outcomes

PB, the primary outcome of interest, is defined as premature delivery at less than 37 weeks of gestation [24]. The gestational age was estimated as the period between the last menstruation and the delivery. When the date did not match the ultrasound measurement estimation by seven days or more, the gestational age was corrected using its relationship to the crown-rump length [18]. Among the preterm births, some were classified as spontaneous deliveries (SPB, n=88) when the birth was vaginal or when the labour was not documented as having been induced. The PBs requiring either an induction of labour or prelabour caesarean, or both, were defined as medically induced deliveries (IPB, n=26) [25]. In addition, neonates were classified as FGR in weight (FGR) if their birth weight fell below the 10th percentile of their predicted birth weight distribution adjusted for genetic growth potential. This customised estimation of growth impairment allows for the better detection of neonates which fail to reach their genetic growth potential or their constitutional potential because of maternal, foetal, placental, or external factors and excludes constitutionally small babies [26]. A multivariable fractional polynomial linear regression model was used to predict birth weight, allowing polynomial terms for continuous variables in the linear regression models. The final model included as covariates: gestational age, infant gender, maternal and paternal height, pre-pregnancy maternal weight as well as the interaction of gestational age with maternal weight. Gestational age and type of PB were known for 438 women, whereas FGR data were available for 401 women due to missing values necessary to define the outcome.

Metabolic syndrome variables

Data on plasma triglycerides, total cholesterol, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol of 227 fasting pregnant women at the first prenatal visit were available [18]. The insulin concentrations were measured for 369 women and the diastolic and systolic blood pressures were available for 338 participants. The body mass index (BMI) calculated on reported weight before pregnancy and height, measured at the first prenatal visit, was used to classify women as underweight, BMI <18.5 kgm⁻², normal weight BMI >18.5 to <25 kgm⁻², overweight BMI 25 to 30 kgm⁻² or obese BMI >30 kgm⁻², according to standard international classification.

¹H NMR spectroscopic analysis of urine

Samples handling and preparation

Urine samples were stored at -80°C until analysis. An aliquot of 400 µL of urine was added to 200 µL phosphate buffer solution (0.2 M Na₂HPO₄/NaH₂PO₄, pH 7.4) to minimise variations in chemical shift values.
in the acquired $^1$H NMR spectra due to minor pH differences. This buffer contained sodium 3-trimethylsilyl-(2,3,3,4,4,4)-1-propionate (TSP, 1 mM) in 20% D$_2$O and the bacteriostatic agent sodium azide (NaN$_3$, 3 mM). TSP is a chemical shift reference ($\delta$ 0.00) and D$_2$O provided a field-frequency lock. The buffered urine sample was then centrifuged at 16000 g for 5 minutes to remove any debris. 550 µL of the resulting supernatant was pipetted into standard 5 mm NMR tubes [27].

$^1$H NMR experiments and data processing

$^1$H NMR spectra of the urine samples were acquired using a Bruker Avance 600 spectrometer (Bruker Biospin, Rheinstetten, Germany) operating at 600.13 MHz. The $^1$H NMR spectra of the urine samples were acquired using a standard one-dimensional pulse sequence with water pre-saturation (recycle delay-90°-$t_1$-90°-$t_m$-90°-acquisition; XWIN-NMR 3.5) during both the recycle delay (2 s) and mixing time ($t_m$, 100 ms). The 90° pulse length was adjusted to approximately 10 µs and $t_1$ was set to 3 µs. For each sample, 128 free induction decays (FIDs) were collected into 32K data points using a spectral width of 12,000 Hz. The FIDs were multiplied by an exponential weighting function corresponding to a line broadening of 0.3 Hz prior to Fourier Transformation [27].

All NMR spectra (spectral region $\delta$ 10 – 0.5) were imported into MATLAB 7.3.1 (MathWorks) and were referenced and corrected for phase and baseline distortion using in-house script developed by Dr. Rachel Cavill, Dr. Hector Keun and Dr. Tim Ebbels at the Imperial College London, UK. The spectral region $\delta$ 4.0-5.4 containing residual water and urea resonances were removed prior to median fold change normalisation [28]. Integrals of well resolved peaks were calculated. Certain metabolites were quantified using the Profiler and Library Manager modules in Chenomx NMRSuite 5.11 (Chenomx Inc, Edmonton, Canada) where overlapping signals were present in the integration window or for those with a low signal-to-noise ratio (specifically creatine, creatinine, tyrosine, dimethylamine and 1-methylnicotinamide). The advantage in using Chenomx for these metabolites was to account for quantification error by fitting experimental spectra of pure compounds on all the resonant peaks for the metabolite [29]. The statistical analysis presented later was applied on the peak integrals for all the metabolites, except for the metabolites cited above where Chenomx values were used.

$^1$H NMR spectroscopic signals were assigned to metabolites with reference to the literature [30, 31], online databases (HMDB) [32] and/or confirmed by 2D NMR experiments on a selected sample including homonuclear $^1$H-$^1$H correlation spectroscopy (COSY) and $^1$H-$^1$H total correlation spectroscopy (TOCSY).

Statistical analysis
All statistical analyses were performed using the R project software (http://www.R-project.org). Continuous distributed variables were displayed as median with interquartile range and were tested by using Mann-Whitney non parametric statistical tests. Categorical variables were tested using the chi-square test. The threshold statistical significance was set at a p-value <0.05 and conducted with a 2-side alternative hypothesis.

Statistical analyses were conducted on 34 metabolites to assess their variation in relation to birth outcomes e.g. PB, IPB, SPB and FGR and to maternal parameters (biochemical measures and dietary intake). A five-step analysis was conducted in order to select metabolites significantly associated with birth outcomes and associated with metabolic syndrome. In order to identify metabolites associated with birth outcomes, a non-parametric test, i.e. the Mann-Whitney test, was used, given the non-normal distribution of the metabolite relative concentrations. The impact of multiple testing was considered by calculating the false discovery rate (FDR) i.e. the expected proportion of the tests misclassified as significant for any given p-value cut-off [33]. To test for a dose response association between metabolite levels and birth outcomes, a trend test, i.e. chi-squared test for trend in proportions, was used to assess the frequency distribution of women with pregnancy outcomes according to the quartiles of the metabolites [34]. For the metabolites identified as ‘of interest’ by the above analyses, their association with birth outcomes was tested after adjusting for confounding factors using multivariate logistic regression models. Inter-quartile-range odds ratios (IORs) with 95% confidence intervals (95% CI) were calculated for PB, IPB, SPB and FGR by using interquantile range for standardization. We used the change from the outer quartiles as a measure, because metabolite integrals/predictors are not always normally distributed. Using the difference in the outer quartiles as a measure (0.25 and 0.75 quantiles), the odds ratio is called the interquartile range or half-sample odds ratio. Potential confounders with an established or potential association with PB or FGR were included in the logistic regression models.

In order to assess whether the metabolite panel associated with birth outcomes is also associated with known metabolic syndrome traits (BMI, blood pressure, blood glucose, insulin, lipids), Spearman’s correlation coefficients were calculated. Metabolites with significant association with birth outcomes in logistic regression models and significant correlation coefficients with metabolic syndrome traits were selected for the final analysis. A stratified analysis by maternal BMI before pregnancy and insulin levels, was performed using multivariate logistic regression models on log-transformed metabolite levels, correcting for potential confounders (same as above).
Results

Descriptive statistics of the study population

Our metabolomics study was designed as a case-control study nested within the Rhea cohort. Table 1 shows the demographic characteristics within each case group and control group and their comparison. Mothers of cases and controls tended to be of similar age (median 30 and 31 years respectively), and (with the exception of SGA) possessed no significant differences in parity or in proportion of smokers. However, less educated women were more likely to develop pregnancy outcomes such as preterm birth (32.7%) and FGR (27.8%) compared to controls (13.5%). The observations with respect to BMI and maternal education were consistent with associations reported in the wider cohort [18]. Extreme maternal BMI before pregnancy (either underweight or obese) occurred more in PB cases, especially more obese women had IPB (24% versus 11% in controls). Maternal BMI was not associated with FGR since parental height and weight were accounted for in the assessment of FGR.

Metabolomic analysis

In total 34 urinary metabolites were identified by $^1$H NMR spectroscopic analysis (a representative assigned $^1$H NMR spectrum from a healthy pregnant woman is displayed in Figure 1). Urinary NMR spectra contained prominent resonances from organic acids such as acetate, citrate and hippurate, and further metabolites including aliphatic amines such as creatinine, dimethylamine (DMA), trimethylamine (TMA) and trimethylamine-$N$-oxide (TMAO). Other metabolites such as p-cresol sulfate, niacin metabolites ($N$-methyl-2-pyridone-5-carboxamide or 2-Py) and amino acids (i.e. alanine, leucine and tyrosine) were also assigned in the urine spectra.

A systematic comparison of these metabolites was performed to detect associations between birth outcomes (PB, IPB, SPB, SGA and FGR) and metabolite abundance. As a single molecular species may give rise to multiple resonances (peaks) in an NMR spectrum, we chose to select a single representative peak for each metabolite (based on sufficient intensity and absence of overlap with other signals) to provide the measurement, with most metabolites exhibiting well-resolved peaks analysed by spectral integration. Our strategy was to use two different univariate approaches for initial candidate selection and look for agreement between these to define a consensus set of metabolites. These candidate metabolites were then subject to multivariate regression analysis to control for major confounding in our study. The first selection approach tested for significant median differences in metabolite abundance between cases and controls for
each outcome. The integral regions used for all metabolites are presented in supporting information S1. Of an initial 34 metabolites, eight metabolites displayed significant median differences between FGR and controls (see Table 2). Five metabolites were significant for SGA in common with FGR and two metabolites were specifically associated with SGA, namely leucine and N-acetyl neuraminic acid.

The analysis of preterm birth outcomes was conducted on all the clinical subtypes combined (PB) and separately (SPB and IPB). Formate and a singlet at 0.63 ppm, likely to derive from a steroid moiety, displayed significant median differences between PB and control cases (Mann-Whitney test, Table 2). Formate, N-methyl-2-pyridone-5-carboxamide (2-Py), glycine, trimethylamine oxide (TMAO), lysine and the singlet at 0.63 ppm significantly varied between SPB and control groups. The IPB group exhibited specifically higher levels of N-acetyl glycoproteins and lower levels of phenylacetylglutamine compared to controls.

Among the metabolites with significant differences in pairwise tests, we next examined the trend in the proportion of women with pregnancy outcomes across metabolite levels (dataset split in quartiles). Out of the eight candidate metabolites for FGR and two metabolites both for PB and IPB, all metabolites showed a trend in frequency of birth outcomes across quartiles, therefore showing a dose-response relationship between candidate metabolites and the outcome incidence. However, only three candidate metabolites for SPB out of six showed a significant trend, namely formate, lysine and the singlet at 0.63 ppm (Table 3).

Finally, risk estimates of pregnancy outcomes were computed using candidate metabolites as predictors in a logistic regression model adjusted for confounding factors such as maternal education, maternal age, parity and smoking habits (Table 4). The interquartile odd ratios (IORs) between the outer quartiles (0.25 and 0.75 quantiles) of the candidate metabolite level was used to determine a significant association. Models for FGR indicated that high levels of tyrosine, acetate, trimethylamine and formate were significantly associated with a decreased incidence of FGR (IORs between 0.27 and 0.14). High levels of N-acetyl glycoproteins were associated with a dramatic increased risk of IPB (IOR=5.84, 95% confidence interval 1.44 and 39.5). High lysine and low formate levels were significantly associated with a higher risk of SPB. IORs between all quartiles for metabolites significantly discriminating pregnancy outcomes are presented in Figure 2. Some metabolites levels are associated with a linear increase in outcome incidence such as 2-Py in SPB cases, other metabolites are associated with a steep increase in outcome incidence only at a high level such as N-acetyl glycoproteins in IPB cases.
Urinary metabolites characterising pregnancy outcomes and adverse metabolic status

The presence of metabolic syndrome in early pregnancy is related to increased risk of PB and FGR within the Rhea cohort participants according to a previous analysis [18]. Metabolic syndrome is a cluster of metabolic abnormalities related to increased risk of cardiovascular diseases and diabetes [35]. We hypothesised that the candidate metabolites associated to pregnancy outcomes might reflect aspects of metabolic syndrome and that clinical parameters associated with metabolic syndrome would correlate to levels of the urinary metabolites (Figure 3). Insulin was the parameter with the most significant correlations with urinary metabolites showing significant negative correlations to acetate, formate and tyrosine levels (Spearman $\rho=-0.22, \rho=-0.21, \rho=-0.15$ respectively, $p<0.05$). Increased BMI was associated with elevated levels of N-acetyl glycoprotein fragments in the urine ($\rho=0.14$). Blood pressure was poorly correlated to urinary metabolites. These findings suggest that some of the variation in the urinary metabolites associated with the birth outcomes could be related to underlying maternal metabolic disease such as obesity and insulin resistance. Stratified analysis by maternal BMI as two categories underweight and normal ($<25$) vs. overweight and obese ($>25$), confirmed that N-acetyl glycoprotein and IPB are significantly associated in overweight and obese women only ($p$-value=0.008 in overweight and obese group vs. $p$-value=0.40 in underweight and normal group). Figure 3 illustrates that N-acetyl glycoprotein levels are particularly high in IPB women with high BMI before pregnancy. A stratified analysis was performed also according to insulin levels (low levels $\leq 6$ mU/mL vs. high levels $>6$ mU/mL). Tyrosine, acetate and formate associations with FGR were not significant in the high insulin group.
Discussion

Although more than 90% of the foetal growth occurs in the second half of gestation, maternal metabolism in the first trimester undergoes deep changes in lipid storage, nitrogen species excretion and other metabolic pathways in order to facilitate foetal development [36]. Thus early maternal metabolic abnormalities could indicate, or even cause, abnormal implantation, foetal growth impairment or other adverse birth outcomes, before clinical symptoms appear. Using a $^1$H NMR-based metabolic profiling approach we found early (first trimester) differences in urinary metabolic phenotypes in pregnant women from the Rhea cohort study in whom PB and FGR subsequently occurred. These potentially predictive metabolic signatures of birth outcomes were correlated with aspects of metabolic syndrome. Furthermore we observed a distinction between the metabolic signature of "medically-indicated"/induced and "non-indicated"/spontaneous PB suggesting a range etiological metabolic factors contributing to PB.

Despite the relative small number of induced preterm pregnancies (N=28), a significant increase of N-acetyl glycoprotein fragments was observed in these women. The N-acetyl proton resonances arising at δ 2.04 ppm are frequently associated to the inflammation-induced acute phase proteins such as alpha-1 glycoprotein when reported in serum [37], but the urinary source is less certain. One candidate is uromodulin, also called the Tamm-Horsfall glycoprotein, the most abundant protein found in urine [38]. The N-acetyl glycoprotein resonance was positively correlated in this study with BMI. In the Rhea cohort, pregnant women with metabolic syndrome (and in particular the obesity component) had a high risk of encountering induced preterm birth (relative risk 5.13, 95% CI: 1.97, 13.38). The mechanisms relating N-acetyl glycoproteins to obesity and IPB, remain unclear, however it is widely reported that high levels of adipose tissue can lead to systemic inflammation through release of cytokines such as IL-6, which could lead to an acute phase response [39]. Higher N-acetyl glycoprotein in NMR spectra of women with PB was also found in a study profiling maternal serum and cord blood at birth [40]. Spontaneous PB was specifically associated to higher urinary lysine, an essential amino acid that is limiting for growth and is elevated in the plasma of premature infants [41]. The steroid-conjugate at 0.63ppm, possibly arising from a soluble metabolite of pregnanediol, was also increased in SPB cases by 19%. This signal was identified in previous studies in urine of second trimester pregnant women with subsequent foetal malformation and trisomy 21 [20, 42]. In our study, this steroid was also positively correlated to cholesterol and LDL, known sources for progesterone synthesis by the placenta.
With the exception of formate, a different metabolic profile (decreased urinary acetate, citrate, formate, glycine, tyrosine and trimethylamine) was associated specifically with poorer foetal growth. FGR remains difficult to assign owing to healthy biological variability in human size, hence in this study we used a definition based on customized birth-weight percentiles designed to differentiate better between infants who are small because of restricted in utero growth from infants who are small but have reached their likely individual growth potential (see methods and [18, 26]. A similar pattern of associations was observed to this parameter as for the more conventional classification of small for gestational age, however FGR resulted in more statistically significant associations because of larger sample size. The FGR metabolite profile was broadly inversely associated with plasma insulin and positively correlated to HDL levels, among these metabolites, formate, tyrosine and trimethylamine levels were all found to be significantly positively correlated to each other, suggesting a common source of variation ($\rho_{\text{formate-tyrosine}}=0.38$, $\rho_{\text{formate-trimethylamine}}=0.21$ and $\rho_{\text{tyrosine-trimethylamine}}=0.26$). Elevations of several of these metabolites in blood have been previously associated to risk of insulin resistance [43, 44], however the biological significance of low urinary levels of these molecules is less clear. Low urinary formate has been previously associated with increased hypertension a large multinational study [45] and interestingly, hypertension in the first trimester of pregnancy was the most significant risk factor for PB and FGR in the Rhea cohort [18]. However the association between formate and blood pressure observed was not statistically significant in our study cohort. Several of the metabolites in the FGR signature (acetate, formate, tyrosine, trimethylamine) are known to be consumed or produced in significant quantities by gut microbes [46-49], hence the association might reflect a specific gut microbial distribution or a dietary pattern selective of this. A recent study reports dramatic change of gut microbial composition throughout pregnancy, shifting to a disease-associated dysbiosis characterised by increased insulin resistance and greater adiposity [50]. This indicates that the composition of gut microbiota in pregnant women could influence their metabolic homeostasis and their pregnancy outcomes. Daily intake of 5-mg supplemental folic acid in the whole Rhea population (n=1,279) was associated with a 66 % decrease in the risk of delivering a small for gestational age (SGA) neonate (relative risk, 0.34; 95 % CI, 0.16, 0.73) [51]. However, formate levels were not correlated to supplementary folate intake in our study population ($\rho=-0.05$ and p-value= 0.23). Decreased levels of urinary trimethylamine, tyrosine and, although not significant in the last test, glycine, are consistent with results from a previous study in women with subsequent FGR [42].

Our results are not directly comparable to previous metabolomics studies investigating pregnancy outcomes in part due to differences in the analytical platform used and biofluids studied (often cord blood serum or
amniotic fluid); also our samples are taken in the first trimester whereas most metabolomics studies are examining late pregnancy samples. It is also possible that our observations reflect etiologic factors specific to the Rhea cohort, which experience an abnormally high rate of PB, and not generalizable to the broader European population. Specimens from an independent cohort would be needed to validate our findings. We are aware, however, of several efforts to complete comparable studies in large birth cohorts (e.g. HELIX) so we hope that such comparisons between populations will be possible in the near future [52]. Our study has a number of important other limitations. Firstly, our study was not specifically designed to examine FGR and only a limited number of these cases were available within our dataset. Secondly, although our study is unique in defining associations between metabolism during the first trimester and birth outcomes, it is not possible at this stage to distinguish between pregnancy-induced effects and underlying metabolic risk factors. However this does not negate the potential value of these signatures as biomarkers of risk of negative birth outcomes and our exploratory study has generated several hypotheses for future investigation.

In conclusion, we believe this report to be a clear indication of the potential of metabolomics to reveal novel links between metabolite exposure and pregnancy outcomes, and evidence in support of the inclusion of such approaches in studies that attempt to link the exposome [53] to neonatal health.

**Abbreviations**


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The authors declare that they have no competing interests.
<table>
<thead>
<tr>
<th>Categorical variables</th>
<th>SPB (n=88)</th>
<th>IPB (n=26)</th>
<th>FGR (n=36)</th>
<th>SGA (n=19)</th>
<th>Control (n=275)</th>
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<tbody>
<tr>
<td></td>
<td>Maternal education</td>
<td>Low</td>
<td>Medium</td>
<td>High</td>
<td></td>
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<tr>
<td>Low</td>
<td>31 (35.6%)</td>
<td>6 (23.1%)</td>
<td>10 (27.8%)</td>
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<td>4 (21.1%)</td>
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<td>Medium</td>
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<td>11 (42.3%)</td>
<td>10 (27.8%)</td>
<td>7 (36.8%)</td>
<td>139 (50.5%)</td>
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<td>High</td>
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<td>9 (34.6%)</td>
<td>16 (44.4%)</td>
<td>8 (42.1%)</td>
<td>99 (36.0%)</td>
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<td>Greek origin</td>
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<td>35 (97.2%)</td>
<td>19 (100.0%)</td>
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<td>Parity (Multiparous)</td>
<td>58 (65.9%)</td>
<td>19 (73.1%)</td>
<td>20 (55.6%)</td>
<td>12 (63.2%)</td>
<td>187 (68.0%)</td>
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<td>Smoking during pregnancy</td>
<td>19 (22.9%)</td>
<td>6 (24.0%)</td>
<td>10 (27.8%)</td>
<td>8 (42.1%)</td>
<td>*</td>
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<td>Pre-pregnancy BMI</td>
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<td>1 (4.0%)</td>
<td>1 (2.8%)</td>
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<td>Normal</td>
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<td>181 (66.8%)</td>
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<td>4 (11.1%)</td>
<td>2 (10.5%)</td>
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<td>6 (24.0%)</td>
<td>4 (11.1%)</td>
<td>1 (5.3%)</td>
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<td>3 (4.0%)</td>
<td>3 (14.3%)</td>
<td>36 (100.0%)</td>
<td>***</td>
<td>17 (94.4%)</td>
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<td>Maternal age, years</td>
<td>29.0 (26.0-33.0)</td>
<td>31.0 (27.2-36.0)</td>
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<td>31.0 (27.0-34.5)</td>
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<tr>
<td>Gestational age, weeks</td>
<td>35.5 (35.0-36.0)</td>
<td>***</td>
<td>36.0 (35.5-36.0)</td>
<td>***</td>
<td>39.0 (37.5-40.0)</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>2715 (2430-2980)</td>
<td>***</td>
<td>2800 (2570-2890)</td>
<td>***</td>
<td>2610 (2482-2802)</td>
</tr>
<tr>
<td>Cholesterol (n=227)</td>
<td>215.0 (189.5-237.0)</td>
<td>222.0 (212.8-233.0)</td>
<td>*</td>
<td>205.0 (163.0-225.0)</td>
<td>225.0 (198.0-234.0)</td>
</tr>
<tr>
<td>Triglycerides (n=227)</td>
<td>112.0 (86.5-134.5)</td>
<td>149.0 (104.0-159.2)</td>
<td>99.0 (89.0-119.0)</td>
<td>95.0 (89.0-131.0)</td>
<td>111.0 (85.5-138.0)</td>
</tr>
<tr>
<td>Insulin (n=369)</td>
<td>6.3 (2.3-14.8)</td>
<td>10.6 (5.0-17.5)</td>
<td>*</td>
<td>8.3 (3.2-26.6)</td>
<td>5.1 (2.7-37.9)</td>
</tr>
<tr>
<td>LDL (n=227)</td>
<td>128.0 (101.5-138.5)</td>
<td>130.0 (121.2-135.8)</td>
<td>116.0 (90.0-142.0)</td>
<td>142.0 (112.2-149.0)</td>
<td>114.0 (98.5-142.0)</td>
</tr>
<tr>
<td>HDL (n=227)</td>
<td>61.0 (52.0-71.0)</td>
<td>70.5 (59.8-79.2)</td>
<td>59.0 (49.0-69.0)</td>
<td>63.0 (49.0-67.0)</td>
<td>60.0 (49.0-68.5)</td>
</tr>
<tr>
<td>Systolic BP (n=338)</td>
<td>107.7 (101.0-115.7)</td>
<td>110.7 (105.7-117.3)</td>
<td>105.0 (96.0-112.1)</td>
<td>99.7 (94.2-110.2)</td>
<td>106.3 (100.3-112.0)</td>
</tr>
<tr>
<td>Diastolic BP (n=338)</td>
<td>69.7 (64.3-76.0)</td>
<td>74.5 (69.6-79.3)</td>
<td>69.3 (61.3-77.0)</td>
<td>65.2 (58.2-77.3)</td>
<td>69.7 (63.7-76.0)</td>
</tr>
</tbody>
</table>

Values are mean (SD), median (interquartile range), or n (%). P values were calculated using the Chi-squared test (categorical variables) or Mann-Whitney test (continuous) between cases and controls, significant tests are labelled as following: * P < 0.05, **P < 0.01 and ***P < 0.001. Gestational age and type of PB were known for 438 women, whereas FGR data were available for 401 women.
### Table 2. Urinary metabolites significantly associated with birth outcomes.

<table>
<thead>
<tr>
<th>Selected peak</th>
<th>All PB types (n=114)</th>
<th>SPB (n=88)</th>
<th>IPB (n=26)</th>
<th>FGR (n=36)</th>
<th>SGA (n=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>δ (ppm)</td>
<td>p-value</td>
<td>q-value</td>
<td>Fold change %</td>
<td>p-value</td>
<td>q-value</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.87 (d), 7.18 (d)*</td>
<td>0.055</td>
<td>0.264</td>
<td>-15%</td>
<td>0.161</td>
</tr>
<tr>
<td>Steroid-conjugate – 0.63 (s)</td>
<td>0.63 (s)</td>
<td>0.039</td>
<td>0.252</td>
<td>17%</td>
<td>0.045</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.96 (t)</td>
<td>0.364</td>
<td>0.578</td>
<td>2%</td>
<td>0.227</td>
</tr>
<tr>
<td>Lactate</td>
<td>1.33 (d)</td>
<td>0.342</td>
<td>0.562</td>
<td>-5%</td>
<td>0.259</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.48 (d)</td>
<td>0.627</td>
<td>0.702</td>
<td>-1%</td>
<td>0.426</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.73 (m)</td>
<td>0.056</td>
<td>0.264</td>
<td>1%</td>
<td>0.016</td>
</tr>
<tr>
<td>Acetate</td>
<td>1.92 (s)</td>
<td>0.423</td>
<td>0.613</td>
<td>-2%</td>
<td>0.885</td>
</tr>
<tr>
<td>N-acetyl glycoprotein fragments</td>
<td>2.04 (s)</td>
<td>0.390</td>
<td>0.594</td>
<td>3%</td>
<td>0.783</td>
</tr>
<tr>
<td>N-acetyl neuraminic acid</td>
<td>2.06 (s)</td>
<td>0.806</td>
<td>0.752</td>
<td>0%</td>
<td>0.701</td>
</tr>
<tr>
<td>Citrate</td>
<td>2.55 (d)</td>
<td>0.818</td>
<td>0.754</td>
<td>-2%</td>
<td>0.882</td>
</tr>
<tr>
<td>Trimethylamine</td>
<td>2.87 (s)</td>
<td>0.094</td>
<td>0.277</td>
<td>-2%</td>
<td>0.218</td>
</tr>
<tr>
<td>TMAO</td>
<td>3.27 (s)</td>
<td>0.067</td>
<td>0.270</td>
<td>-3%</td>
<td>0.032</td>
</tr>
<tr>
<td>Glycine</td>
<td>3.57 (d)</td>
<td>0.103</td>
<td>0.279</td>
<td>-5%</td>
<td>0.049</td>
</tr>
<tr>
<td>Phenylacetlyglutamine</td>
<td>7.37 (d)</td>
<td>0.071</td>
<td>0.271</td>
<td>-9%</td>
<td>0.356</td>
</tr>
<tr>
<td>N-methyl-2-pyridone-5-carboxamide</td>
<td>8.33 (s)</td>
<td>0.065</td>
<td>0.269</td>
<td>8%</td>
<td>0.049</td>
</tr>
<tr>
<td>Formate</td>
<td>8.46 (s)</td>
<td>0.004</td>
<td>0.105</td>
<td>-11%</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Alterations in the median metabolite content are expressed as fold change relative to controls. Metabolite differences with P < 0.05 are in bold; q-values indicate estimated false discovery rate.

* Metabolite measured in chenomx using multiple signals.
Table 3. Dose-response relationships between candidate metabolites and birth outcomes.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Metabolite</th>
<th>Q1</th>
<th>Q2</th>
<th>Q3</th>
<th>Q4</th>
<th>P-value for trend</th>
</tr>
</thead>
<tbody>
<tr>
<td>All PB types (n=114)</td>
<td>Steroid-conjugate – 0.63 (s)</td>
<td>21%</td>
<td>22%</td>
<td>25%</td>
<td>32%</td>
<td>0.01 *</td>
</tr>
<tr>
<td></td>
<td>Formate</td>
<td>32%</td>
<td>25%</td>
<td>23%</td>
<td>20%</td>
<td>0.02 *</td>
</tr>
<tr>
<td>SPB (n=88)</td>
<td>Steroid-conjugate – 0.63 (s)</td>
<td>20%</td>
<td>22%</td>
<td>24%</td>
<td>34%</td>
<td>0.01 *</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>14%</td>
<td>30%</td>
<td>26%</td>
<td>31%</td>
<td>0.03 *</td>
</tr>
<tr>
<td></td>
<td>TMAO</td>
<td>26%</td>
<td>28%</td>
<td>28%</td>
<td>17%</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>26%</td>
<td>34%</td>
<td>20%</td>
<td>19%</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>N-methyl-2-pyridone-5-carboxamide</td>
<td>19%</td>
<td>19%</td>
<td>30%</td>
<td>32%</td>
<td>0.03 *</td>
</tr>
<tr>
<td></td>
<td>Formate</td>
<td>32%</td>
<td>25%</td>
<td>24%</td>
<td>19%</td>
<td>0.02</td>
</tr>
<tr>
<td>IPB (n=26)</td>
<td>N-acetyl glycoprotein fragments</td>
<td>8%</td>
<td>23%</td>
<td>19%</td>
<td>50%</td>
<td>0.01 **</td>
</tr>
<tr>
<td></td>
<td>Phenylacetylglutamine</td>
<td>42%</td>
<td>27%</td>
<td>12%</td>
<td>19%</td>
<td>0.03 *</td>
</tr>
<tr>
<td>FGR (n=36)</td>
<td>Tyrosine</td>
<td>33%</td>
<td>36%</td>
<td>17%</td>
<td>14%</td>
<td>0.03 *</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>33%</td>
<td>33%</td>
<td>17%</td>
<td>17%</td>
<td>0.05 *</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>39%</td>
<td>31%</td>
<td>11%</td>
<td>19%</td>
<td>0.03 *</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>39%</td>
<td>31%</td>
<td>22%</td>
<td>8%</td>
<td>0.004 **</td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td>33%</td>
<td>39%</td>
<td>14%</td>
<td>14%</td>
<td>0.02 *</td>
</tr>
<tr>
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<td>Trimethylamine</td>
<td>50%</td>
<td>19%</td>
<td>17%</td>
<td>14%</td>
<td>0.002 **</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>33%</td>
<td>39%</td>
<td>11%</td>
<td>17%</td>
<td>0.03 *</td>
</tr>
<tr>
<td></td>
<td>Formate</td>
<td>39%</td>
<td>28%</td>
<td>19%</td>
<td>14%</td>
<td>0.02 *</td>
</tr>
<tr>
<td>SGA (n=19)</td>
<td>Leucine</td>
<td>16%</td>
<td>16%</td>
<td>26%</td>
<td>42%</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>37%</td>
<td>37%</td>
<td>16%</td>
<td>11%</td>
<td>0.05 *</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>42%</td>
<td>32%</td>
<td>16%</td>
<td>11%</td>
<td>0.03 *</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>42%</td>
<td>21%</td>
<td>32%</td>
<td>5%</td>
<td>0.05 *</td>
</tr>
<tr>
<td></td>
<td>N-acetyl neuraminic acid</td>
<td>21%</td>
<td>11%</td>
<td>16%</td>
<td>53%</td>
<td>0.04 *</td>
</tr>
<tr>
<td></td>
<td>Trimethylamine</td>
<td>47%</td>
<td>16%</td>
<td>21%</td>
<td>16%</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>42%</td>
<td>37%</td>
<td>11%</td>
<td>11%</td>
<td>0.02 *</td>
</tr>
</tbody>
</table>

Percentage of pregnant women with birth outcomes across four groups of candidate metabolite levels (quartiles: Q1, Q2, Q3, Q4). Significant p-values are labelled as following: * P < 0.05 and **P < 0.01.
Table 4. Logistic regression models predicting pregnancy outcomes with regards to candidate metabolite levels.

<table>
<thead>
<tr>
<th>Outcomes</th>
<th>Metabolite</th>
<th>IQR</th>
<th>CI 95%</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All PB types (n=114)</td>
<td>Steroid-conjugate – 0.63 (s)</td>
<td>1.90</td>
<td>0.99</td>
<td>3.69</td>
</tr>
<tr>
<td></td>
<td>Formate</td>
<td>0.51</td>
<td>0.26</td>
<td>0.99</td>
</tr>
<tr>
<td>SPB (n=88)</td>
<td>Steroid-conjugate – 0.63 (s)</td>
<td>1.99</td>
<td>0.94</td>
<td>4.32</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>2.79</td>
<td>1.20</td>
<td>6.98</td>
</tr>
<tr>
<td></td>
<td>N-methyl-2-pyridone-5-carboxamide</td>
<td>2.05</td>
<td>0.96</td>
<td>4.51</td>
</tr>
<tr>
<td></td>
<td>Formate</td>
<td>0.42</td>
<td>0.19</td>
<td>0.94</td>
</tr>
<tr>
<td>IPB (n=26)</td>
<td>N-acetyl glycoprotein fragments</td>
<td>5.84</td>
<td>1.44</td>
<td>39.50</td>
</tr>
<tr>
<td></td>
<td>Phenylacetylglutamine</td>
<td>0.37</td>
<td>0.09</td>
<td>1.28</td>
</tr>
<tr>
<td>FGR (n=36)</td>
<td>Tyrosine</td>
<td>0.27</td>
<td>0.08</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>0.37</td>
<td>0.12</td>
<td>1.04</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>0.38</td>
<td>0.13</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>0.18</td>
<td>0.04</td>
<td>0.60</td>
</tr>
<tr>
<td></td>
<td>Citrate</td>
<td>0.33</td>
<td>0.09</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>Trimethylamine</td>
<td>0.14</td>
<td>0.04</td>
<td>0.40</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>0.36</td>
<td>0.11</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>Formate</td>
<td>0.24</td>
<td>0.07</td>
<td>0.71</td>
</tr>
<tr>
<td>SGA (n=19)</td>
<td>Lactate</td>
<td>0.20</td>
<td>0.03</td>
<td>0.89</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>0.19</td>
<td>0.03</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Acetate</td>
<td>0.12</td>
<td>0.01</td>
<td>0.70</td>
</tr>
<tr>
<td></td>
<td>N-acetyl neuraminic acid</td>
<td>2.23</td>
<td>0.64</td>
<td>9.10</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>0.19</td>
<td>0.03</td>
<td>0.88</td>
</tr>
</tbody>
</table>

Interquartile odds ratios (1st vs 4th) with 95 % confidence intervals are presented for the incident risk for pregnancy outcomes according to candidate metabolite relative concentrations. Statistical analysis (z-score) of the beta values indicates if the metabolites significantly contributed to the model. Models were adjusted for maternal education, maternal age, parity and smoking.
Figure legends:

Figure 1. Representative $^1$H NMR spectrum (600 MHz) recorded for first trimester urine of a healthy pregnant woman. 1, Steroid-conjugate; 2, Leucine; 3, Valine; 4, Lactate; 5, Alanine; 6, Acetate; 7, N-acetyl of glycoprotein fragments; 8, N-acetyl neuraminic acid; 9, Phenylacetylglutamine; 10, p-cresol sulphate; 11, Citrate; 12, Dimethylamine; 13, Creatine; 14, Creatinine; 15, Proline Betaine; 16, Choline-containing moieties; 17, Trimethylamine-N-oxide (TMAO); 18, Glycine; 19, Hippurate; 20, Tyrosine; 21, N-methyl-2-pyridone-5-carboxamide (2Py); 22, Formate; 23, N-methyl nicotinic acid (trigonelline); 24, 1-methylnicotinamide.

Figure 2. Interquartile Odds ratios (IOR) for pregnancy outcomes according to candidate metabolite relative concentrations. Models were adjusted for maternal education, maternal age, parity and smoking.

Figure 3. Spearman’s correlation heatmap between metabolic syndrome components and discriminatory metabolites for pregnancy outcomes. Black squares indicate p-values<0.05.

Figure 4. N-acetyl of glycoproteins in IPB cases and maternal BMI. Box plots represent median and range of metabolite concentration with numbers in white corresponding to individual counts per categories.


<table>
<thead>
<tr>
<th></th>
<th>Formate</th>
<th>Acetate</th>
<th>Citrate</th>
<th>Trimethylamine</th>
<th>Tyrosine</th>
<th>Lysine</th>
<th>N-acetyl glycoprotein fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cholesterol (n=226)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Triglycerides (n=226)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Insulin (n=369)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Fasting glucose (n=218)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BMI (n=427)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>LDL (n=226)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HDL (n=226)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Systolic BP (n=396)</strong></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Diastolic BP (n=396)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
Figure 4
Additional files provided with this submission:

Additional file 1: revised paper RHEAmetabonomics BMC Med submission LM.docx, 121K
http://www.biomedcentral.com/imedia/9810406351285550/supp1.docx