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^{1}H NMR-based metabolomics study on the physiological variations during the rat pregnancy process

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ABSTRACT

In this study, NMR-based metabolomics in combination with multivariate pattern recognition technologies were employed to evaluate the physiological variations in the Wistar rats' plasma that are induced by pregnancy on the gestational days (GDs) 11, 14, 17 and 20. Untargeted metabolomics analysis revealed some possible mechanism of physiological effects for healthy pregnancies and showed a metabolic trajectory during pregnancy process. The levels of 24 metabolites were found to change significantly throughout pregnancy in maternal plasma. These metabolite changes involved in varied kinds of metabolic pathways including synthesis of biological substances, microbial metabolism in diverse environments, protein digestion and absorption, carbohydrate metabolism, digestion and absorption, mineral absorption, and ATP (Adenosine Triphosphate)-binding cassette transporters (ABC transporters). The substantial cores of all the metabolic pathways are promoting fetal growth and development and regulating maternal physiological state. This work showed relevant metabolic pathways perturbation in the maternal plasma due to normal pregnancy and provided the physical basis of time-dependent metabolic trajectory against which disease-related maternal physiological responses may be better understood in future studies.

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1. Introduction

Pregnancy is an extraordinarily dynamic process, in which the maternal body must change its physiological and homeostatic mechanisms including metabolic, hematologic, renal, and respiratory patterns in order to ensure the continuous availability of conditions and substrates for fetal development (Bo et al., 2015; Redman and Sargent, 2005; York et al., 2014). Substantially, these physiological variations come from the metabolic changes during the pregnant period (King, 2000; Luan et al., 2014), which are complex and widespread, resulting in the maternal metabolism working from a different baseline compared with the non-pregnant state. Among the reported changes, those in amino acid metabolism, energy and gut microflora metabolisms were observed in connection to pregnancy (Pinto et al., 2015). These metabolic changes of pregnancy directly influence the health conditions of the mother and the development of the fetus (Laughon et al., 2014). However, the reasons for these changes in metabolic process relating to the metabolism of pregnant mother are still not well understood. Conventional clinical pregnancy monitoring mainly rely on routine physical and histological examinations (Diaz et al., 2011; Halscott et al., 2014; Hourrier et al., 2010; Legardeur et al., 2011). These examinations such as ultrasound testing are often carried out in combination with chorionic villus sampling, amniocentesis, and cordocentesis, as well as the measurement of specific maternal serum markers for chromosomal disorders. Despite their importance in prenatal diagnostics, some of these procedures still carry some risks of miscarriage, infection, or premature birth and could not get the sound physiological or metabolic information

Abbreviations: NMR, nuclear magnetic resonance; GD, gestational day; PCA, principal components analysis; PLS-DA, partial least squares-discriminant analysis; OPLS-DA, orthogonal partial least squares discriminant analysis; PC, principal component; ABC, ATP-binding cassette; ATP, adenosine-triphosphate; LC-MS, liquid chromatograph-mass spectrometer; BH_2, dihydrobiopterin; LPC, lysophosphatidylcholine; PHCs, phosphatidylcholines; LPEs, lysophosphatidylethanolamines; LPSs, lysophosphatidylserines; SPF, specific pathogen free; HILIC-MS, hydrophilic interaction chromatography-mass spectrometry; GC–MS, gas chromatography–mass spectrometry.

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(Halscott et al., 2014; Legardeur et al., 2011). Therefore, it is necessary to exploit maternal or fetal metabolic characteristics to define new biomarkers and develop new methods for noninvasive pregnancy monitoring, aiming at early disease detection and prediction.

Metabolomics is a feasible and promising approach that applies advanced separation and detection methods to investigate the global metabolite change and obtain the related biochemical pathways to elucidate specific sites of perturbations (Nicholson and Lindon, 2008). In recent years, the analysis of biological fluids or tissues has been used successfully in identification of typical compounds of various physiologic or pathologic conditions by providing the underlying metabolic changes during the pregnancy period, and a steady inflow of research development has been reported in this context by using maternal blood and urine, amniotic fluid, and umbilical cord blood to characterized the relevant prenatal diseases such as preeclampsia (Austdal et al., 2014; Luque et al., 2014; Redman and Sargent, 2005), fetal malformations (Diaz et al., 2011), gestational diabetes mellitus (Bo et al., 2015; Hernandez et al., 2014), poor pregnancy outcome (Halscott et al., 2014; Hourrier et al., 2010), preterm delivery (Alexandre-Gouabau et al., 2013) and small for the gestational age (Cohn et al., 2010). Moreover, protein and nitrogen metabolism were found to play extremely important roles in pregnancy and the development of the fetus (Altobelli et al., 2013). The conservation and accretion of nitrogen in mother and fetal bodies, for example, fat accumulation and lipogenesis, were started in early pregnancy (Altobelli et al., 2013; Laughon et al., 2014). In addition, carbohydrate metabolism received particular concerns due to the well-recognized clinical disorder known as diabetes mellitus (Hernandez et al., 2014). Recently, an untargeted metabolomics analysis with LC-MS and shotgun lipidomics of maternal plasma showed that the levels of dihydrobiopterin (BH_2), carnitine, acetylalanine, leucyl-phenylalanine, lysophosphatidylcholine (LPC) (18:1), and LPC (20:4), were found to change significantly throughout pregnancy, as well as the changes in the total concentrations of LPCs, phosphatidylcholines (PHCs), lysophosphatidylethanolamines (LPEs) and lysophosphatidylserines (LPSs) (Luan et al., 2014). Meanwhile, the dynamic metabolic adaptations of maternal urine from healthy pregnancies has been evaluated by NMR-based metabolomics techniques, in which the levels of 21 metabolites including choline, creatinine, 4-deoxyerythronic acid, 4-deoxythreonic acid, furoylglycine, guanidoacetate, 3-hydroxybutyrate, and lactate were found to change significantly throughout pregnancy (Diaz et al., 2013). In addition, some targeted studies of amniotic fluid have shown that the levels of alanine, glutamine, valine, creatinine, glucose, and succinic acid reflect gestational age (Athanasiadis et al., 2011; Cohn et al., 2010; Ottolenghi et al., 2010). The advances in these previous studies consistently demonstrated the potential of metabolomics to reveal prenatal disorders and corresponding individual responses, thus setting the reference for the detection of deviant trajectories in biofluids in the context of the metabolic follow-up of pregnancy. However, the normal metabolic trajectory from the metabolomes closer related to the maternal metabolism, such as plasma metabolome, will be more helpful to understand the maternal and fetal physiological conditions during healthy pregnancies. Suitable validated metabolic biomarkers, especially those relating to the metabolic trajectory and their influence on the physiological variations during the pregnancy process may trigger important steps toward an improvement in prenatal health management (Pinto et al., 2015).

In the present study, a global ^{1}H NMR-based metabolomics approach was carried out to map the metabolic trajectory in the maternal plasma that are collected from the healthy pregnant rat models fed a normal diet throughout pregnancy. The objective of our work is to describe critical maternal metabolic processes in rat plasma and analyze the physiological variations during the middle and late of pregnant period, and elucidate the physiological role and functions of biomolecules binding in the biological systems during pregnancy. Our work would provide insight into an overall metabolic trajectory and underlying metabolic mechanism during pregnancy process and help to understand physiological variations of pregnancy and fetal development.

2. Materials and methods

2.1. Animal handling procedure and sampling

All experimental procedures and protocols were approved by the Xiamen University Institutional Animal Care and Use Committee. Specific Pathogen Free (SPF) female (weighing 200 ± 20 g) and male (weighing 280 ± 20 g) Wistar rats were provided by Xiamen University Laboratory Animal Center (XMULAC, Xiamen, China) and housed under a controlled condition of 12 h light/12 h dark cycle at 23 ± 2 °C and 50 ± 10% humidity. The rats were allowed free access to standard laboratory chow diet and water. Following a two-week accommodation period, the female rats were mated with male rats in 2:1 overnight. The next day was taken as gestational day (GD) 0 if spermatozoa were found in the vaginal smear or vaginal plug (Feng et al., 2014).

Ten pregnant rats were randomly selected for the blood collections. Four hundred microliters of pregnant rat blood were withdrawn into heparinized tube via lateral tail vein on the GD11, GD14, GD17 and GD20 under isoflurane anesthesia, respectively. The blood was kept on ice one by one in the collection order and stored for another 5 min after the collection, then centrifuged at 16,000 g for 10 min at 4 °C. The plasma was then carefully removed out and frozen in liquid nitrogen immediately and stored at −80 °C for NMR experiments, and the cell and buffy coat were discarded.

2.2. Sample preparation and ^{1}H NMR spectroscopy

Rat plasma samples were prepared by mixing 200 μL of plasma with 400 μL of 90 mM phosphate buffer (pH 7.4) in 0.9% saline solution (50% D_2O/H_2O, v/v). The plasma-buffer mixture was placed at room temperature for 5 min, and then centrifuged at 16,000 g and 4 °C for 10 min to remove suspended debris. The supernatant (500 μL) was then pipetted into a 5-mm NMR tube. After the sample preparation, the samples were stored at 4 °C before analysis by a NMR spectrometer. The analysis time for one sample is about 6 min and the maximum interval analysis time between the first and last sample is about 400 min.

All of the samples were analyzed randomly at 298 K by using a Bruker AMX-600 NMR spectrometer with TXI CryoProbe (Bruker Biospin, Germany) at 600.13 MHz ^{1}H NMR spectra were acquired for plasma samples using the water-suppressed Carr-Purcell-Meiboom-Gill (CPMG) spin-echo pulse sequence. The 90° pulse length was adjusted to approximately 10 μs, and 64 transients were collected into 32 K data points for each spectrum with a spectral width of 20 ppm. And spin-echo loop time was set to 70 ms and relaxation delay was 2.0 s. The assignments of endogenous metabolites in the plasma ^{1}H NMR spectra were made with reference to published data (Bollard et al., 2005; Feng et al., 2014; Nicholson et al., 1995) and confirmed further by public HMDB database (Wishart et al., 2013).

2.3. Preprocessing of NMR spectra and pattern recognition

All free induction decays were multiplied by an exponential

function equivalent to a 1 Hz line-broadening factor prior to Fourier transformation. The collected NMR spectra were phased and baseline-corrected manually using the software MestReNova (V7.1.0-9185, Mestrelab Research S.L.). The chemical shifts in plasma spectra were referenced to the internal lactate CH_3 resonance at δ1.33.

The CPMG spectrum over the range of 9.0–0.5 ppm for each pregnant rat plasma sample was reduced to 4250 regions, each 0.002 ppm wide, and the signal intensity in each region was integrated. The regions of residual water resonance (δ5.22–4.30) and urea resonance (δ5.90–5.65) were excluded prior to pattern recognition analysis. To minimize statistical bias resulted from the concentration variations of different metabolites, each spectrum was normalized to its total integrated area.

The NMR data were imported into SIMCA-P software (version 11.0, Umetrics AB, Umeå, Sweden) for analysis and visualization by multivariate statistical methods including PCA, PLS-DA and OPLS-DA. PCA was performed by using a mean-centered approach in order to identify intrinsic trends and obvious outliers within the data set. Metabolic trajectory with different gestation periods was constructed using the PCA scores for each sample point. A more sophisticated discriminant technique, PLS-DA, was further applied to achieve global profile separation between the different gestation periods through maximizing systematic variance. The quality of model was described by the cross-validation parameter Q^2, indicating the predictability of the model, and R^2, indicating the total explained variation for the NMR data. And an additional validation method, permutation test (permutation number = 200) was also conducted using 10-fold cross validation. Finally, the OPLS-DA method was used to inspect the significance of metabolites variations between different gestation periods under a unit-variance scaling pattern.

The model coefficients were then back-calculated from the coefficients incorporating the weight of the variance in order to enhance interpretability of the model: in the coefficient plot, the intensity corresponds to the mean-centered model (variance) and the color-scale derives from the unit variance scaled mode (correlation). Thus, biochemical components responsible for the differences between samples detected in the score plot were extracted from the corresponding loadings with the weight of the variable contributing to the discrimination. The coefficient plots were generated with MATLAB scripts (downloaded from http://www.mathworks.com) with some in-house modifications and were color-coded with absolute value of coefficients. In our study, the correlation coefficient of $|r| > 0.602$, which was determined according to the test for the significance of the Pearson's product-moment correlation coefficient, was used as the cutoff value for the statistical significance based on the discrimination significance at the level of $p < 0.05$ and degree of freedom = 9. In addition, univariate statistical analyses of identified metabolites were carried out to evaluate the metabolite changes between different gestation periods. The fold changes of metabolite were calculated and the color coded according to the $\log_2$(fold) was used to show up or down regulation of the important metabolites by red the increased and blue the decreased in each groups. Furthermore, *p*-value of metabolite between different gestation periods was also calculated by *t*-test.

3. Results

3.1. Metabolic profiles of maternal plasma from different gestational periods

For ^1H NMR spectra of plasma, CPMG pulse sequence was used to suppress the resonances from macromolecules such as proteins and lipoproteins which partially obscure the sharp peaks from small molecules, thus revealing subtle biochemical information of samples. Typical 600 MHz ^1H NMR spectra of pregnant rat plasma obtained at gestational days 11, 14, 17, and 20 were shown in Fig. 1, in which most of detectable resonances have been assigned and marked according to the literature (Bollard et al., 2005; Feng et al., 2014; Nicholson et al., 1995) and public NMR database (Wishart et al., 2013). According to Fig. 1, maternal rat plasma from different gestational periods shared similar spectral profiles and also gave a few variations by a direct visual comparison.

3.2. Metabolic trajectory of maternal plasma during middle and late gestational days

A global PCA was conducted on the NMR data of maternal plasma at the different gestation days in order to display the overall metabolic trajectory during the middle and late gestation periods and find the possible outliers (Fig. 2). The first two principal components (PCs) explained 89.7% of the total variance in the data. Spectra of maternal plasma from different gestation periods formed distinct clusters and further displayed the metabolic trajectory along the first PC from gestation day (GD) 11 to GD20. We noticed a certain overlap between the clusters from GD11 and GD17, but they were well separated from those of GD20. It indicated that the most influential factor in describing the metabolic difference was the gestation periods, which explained 69.6% of the total variance in the first PC, followed by the difference of intra-groups in the second PC (20.1% of total variance was explained by individual difference). This PCA trajectory plot implied that significantly and unevenly maternal physiological changes were induced during the pregnancy.

3.3. Comparison of metabolic variations between different gestation periods

PLS-DA was further applied to achieve global profile separation between the two different gestation periods through maximizing inter-group variance. The quality of model was described by the cross-validated parameter Q^2, indicating the predictability of the model, R^2X, indicating the total explained variance for the NMR data, and R^2Y, indicating the total explained by the class membership.

Scores plots and validate model plots by permutation test of PLS-DA derived for ^1H NMR spectra of pregnant rat plasma at different pair-wise gestation periods were shown in Fig. 3. As shown in the scores plots (left panels in Fig. 3), the different gestation periods were well separated with a reasonable Q^2 value (greater than 0.400), indicating the strong predictability of the model and the reliable subsequent analysis of the metabolites. In addition, the parameters of Q^2 and R^2 are also the indicators of statistical significance of metabolic differences between different gestation periods. Further analysis found that the R^2Y and Q^2 values of the adjacent gestation period, such as GD11-GD14 ($R^2Y = 0.819$, $Q^2 = 0.443$), GD14-GD17 ($R^2Y = 0.849$, $Q^2 = 0.497$), GD17-GD20 ($R^2Y = 0.848$, $Q^2 = 0.642$), are smaller than the other different pair-wise period groups such as GD11-GD17 ($R^2Y = 0.933$, $Q^2 = 0.776$), GD11-GD20 ($R^2Y = 0.971$, $Q^2 = 0.926$), and GD14-GD20 ($R^2Y = 0.942$, $Q^2 = 0.814$), while the R^2 and Q^2 values increased gradually with the increase of gestation days, for example from GD11-GD14 and GD11-GD17 to GD11-GD20. The validation plots from permutation test also give similar trends (right panels in Fig. 3), where the steeper the regression line, the better the NMR data fits the model for R^2Y and the more significant the metabolic differences, and big difference between the R^2 and Q^2 may be an indication of an overfit model and also indicate an indistinct

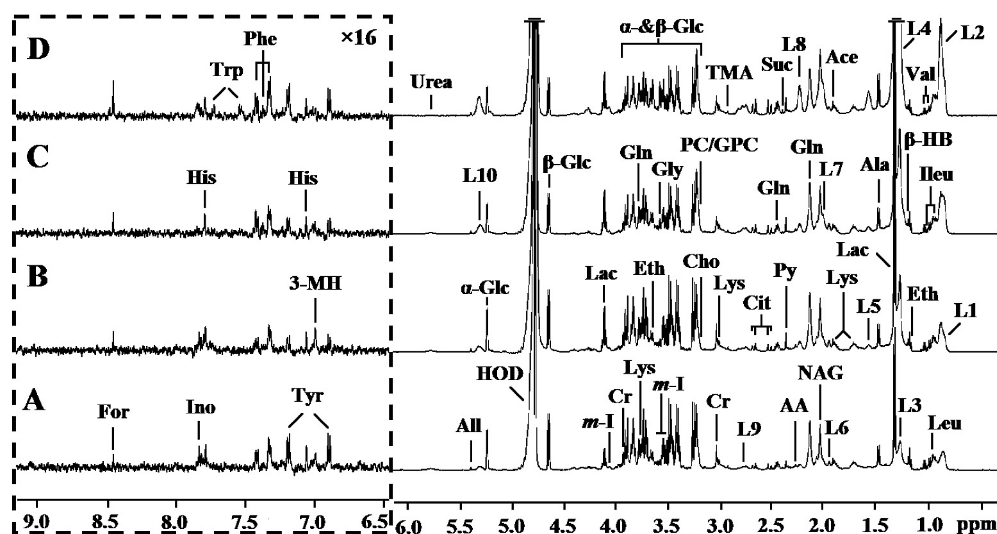


Fig. 1. Representative 600 MHz water-suppressed ^{1}H CPMG NMR spectra (δ0.4–6.2 and δ6.4–9.2) of pregnant rat plasma at (A) gestational day 11, (B) gestational day 14, (C) gestational day 17, and (D) gestational day 20. The spectra in the region δ6.4–9.2 (in the dashed box) were magnified 16 times and scaled in a different chemical shift expansion compared with the region δ0.4–6.2 for the purpose of clarity. Keys: 3-MH, 3-Methylhistidine; AA, Acetoacetate; Ace, Acetate; Ala, Alanine; All, Allanotin; Cho, Choline; Cit, Citrate; Cr, Creatine; Eth, Ethanol; For, Formate; Gln, Glutamine; Gly, Glycine; GPC, Glycerolphosphocholine; His, Histidine; Ileu, Isoleucine; Ino, Inosine; L1, LDL, $CH_3-(CH_2)_n-$; L2, VLDL, $CH_3-(CH_2)_n-$; L3, LDL, $CH_3-(CH_2)_n-$; L4, VLDL, $CH_3-(CH_2)_n-$; L5, VLDL, $-CH_2-CH_2-C{=}O$; L6, Lipid, $-CH_2-CH{=}CH-$; L7, Lipid, $-CH_2-CH{=}CH-$; L8, Lipid, $-CH_2-C{=}O$; L9, Lipid, $=CH-CH_2-CH=$; L10, Lipid, $-CH{=}CH-$; Lac, Lactate; Leu, Leucine; Lys, Lysine; *m*-I, *myo*-Inositol; NAG, N-acetyl glycoprotein signals; PC, Phosphocholine; Phe, Phenylalanine; Py, Pyruvate; Suc, Succinate; TMA, Trimethylamine; Trp, Tryptophan; Tyr, Tyrosine; Val, Valine; α-Glc, α-Glucose; β-Glc, β-Glucose; β-HB, β-Hydroxybutyrate.

difference between gestation periods. Accordingly, metabolic changes of maternal body are a gradual process of accumulation, that is, a relatively short pregnancy period did not induce obvious physiological changes, but the physiological metabolic changes would become more significant as the time increases.

3.4. Physiological and metabolic responses in the middle and late period of pregnancy

To understand the detailed metabolic information following pregnancy, OPLS-DA was conducted on the spectral data of maternal rats from different gestation periods. The plasma metabolome changes could be derived from NMR data of the different pair-wise gestation periods by OPLS-DA. Similar to PLS-DA, clear separation was also present between the two different gestation days in the OPLS-DA score plots (left panels in Fig. 4). The corresponding coefficient loading plots (middle and right panels in Fig. 4) showed the metabolites contributing to the class discrimination, and the correlation coefficients (with color-coded scale) for NMR signals indicated the significance of the metabolites' contribution, where a hot-colored signal (red) indicates more significant contribution to the class separation than a cold-colored one, and peaks in the positive direction indicate the metabolites that are more abundant in the longer gestation period group, vice versa.

Metabolite changes in rat plasma from different gestation periods were assessed using correlation coefficients, fold change values, color codes according to the $\log_2$(fold) and associated *p*-values obtained by the independent *t*-test (summarized in Table 1). In Table 1, the results of the multivariate statistical analysis and univariate analysis were most consistent with each other. According to the results of these discriminant analyses, the levels of 24 metabolites were found to change significantly during the gestation periods, which involved in the metabolic processes of glucose, lipid, amino acid, etc. These changes in maternal plasma are summarized as follows: (1) a gradual decrease of glucose level (α- and β-glucose) during the gestation periods; (2) a relatively decrease of pyruvate, lactate and acetate contents at the late gestation days (according to the results of GD11-GD20 and GD14-GD20); (3) a general increase of LDL and VLDL with the pregnancy periods except the period from GD14 to GD17; (4) a decrease of GPC/PC, choline, *myo*-inositol and ethanol with the progress of the pregnancy. Such changes are very obvious in the pair-wise groups of GD11-GD20, GD14-GD20 and GD17-GD20, however, they are unconspicuous in other pair-wise groups including GD11-GD14, GD11-GD17, and GD14-GD17, which implied a more prominent response in the late gestation period; (5) decreased contents of some amino acids including essential amino acid (i.e. lysine, phenylalanine, tyrosine, valine, isoleucine, leucine) except tryptophan and non-essential amino acid (glutamine, histidine and 3-methylhistdine) and some protein catabolic products (i.e. creatine, N-acetyl glycoprotein) in the late gestation period.

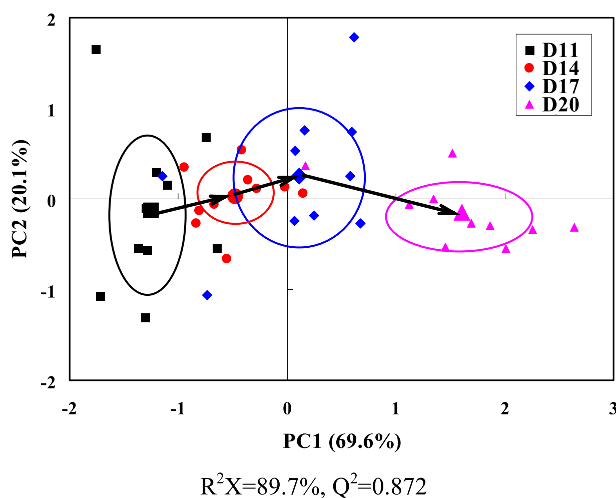


Fig. 2. PCA scores plot based on CPMG ^{1}H NMR spectra of pregnant rat plasma at different gestation periods. The big marks in the center of each ellipse indicate the mean, and the margin indicates one standard deviation at each gestation period.

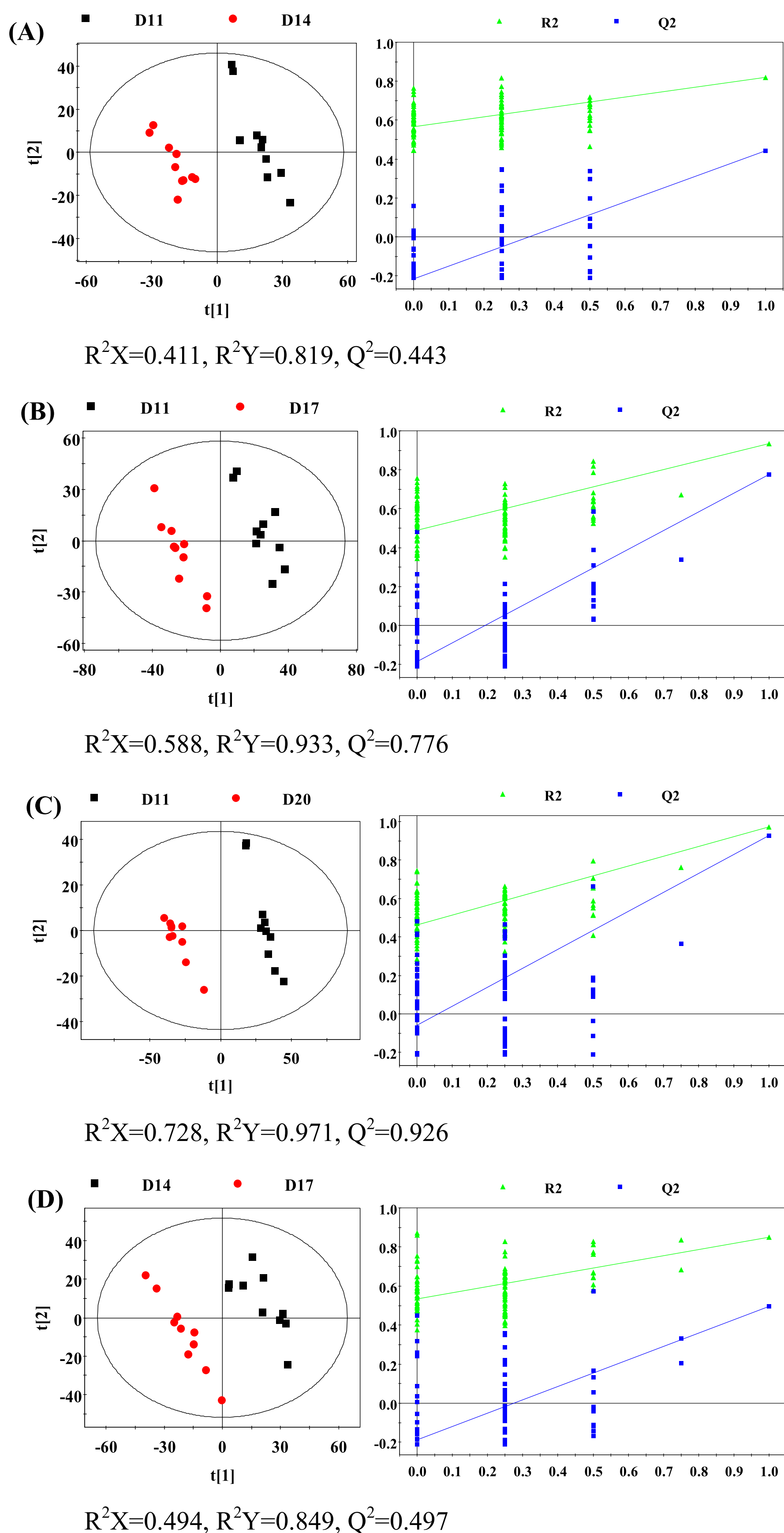


Fig. 3. Score plots (left) and validate model plots (right) by permutation tests (n = 200) of PLS-DA for ^{1}H NMR spectra of pregnant rat plasma at different gestation periods. (A) GD11-14; (B) GD11-17; (C) GD11-20; (D) GD14-17; (E) GD14-20; (F) GD17-20.

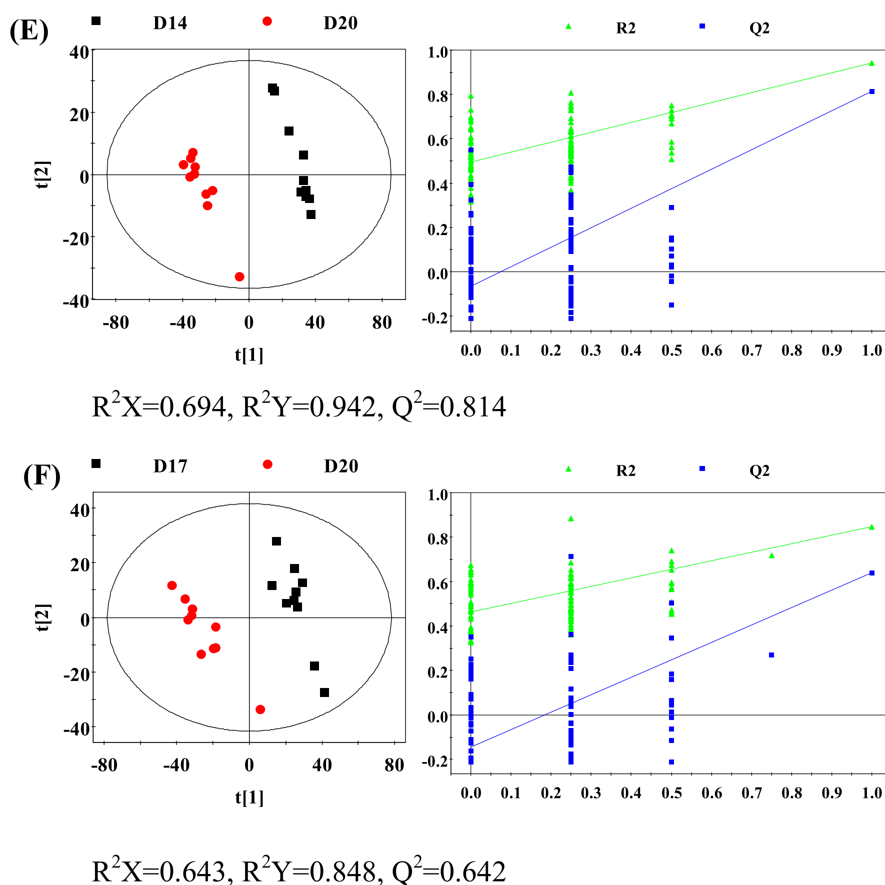


Fig. 3. (*continued*).

Besides, gradually lower concentrations of inosine were also detected with the development of the pregnancy. (6) Other metabolites including trimethylamine and allantoin were overall decreased in the late gestation periods (GD14, GD17 and GD20).

3.5. Metabolic pathways involved in the middle and late pregnancy periods

It is obvious that the maternal physiological and metabolic processes are affected markedly during the pregnancy. With the help of KEGG database (www.kegg.jp), the metabolic pathways corresponding to the differential metabolites in the maternal plasma metabolome could be rationally derived. According to the KEGG pathway analysis, these metabolites basically involved in the varied kinds of metabolic pathways including biosynthesis of biological substances, microbial metabolism in diverse environments, protein digestion and absorption, carbohydrate metabolism, digestion and absorption, mineral absorption, and ABC transporters, and glycolysis/gluconeogenesis were also obviously involved between a longer interval such as GD11-GD17, GD11-GD20, and GD14-GD20 (Table 2).

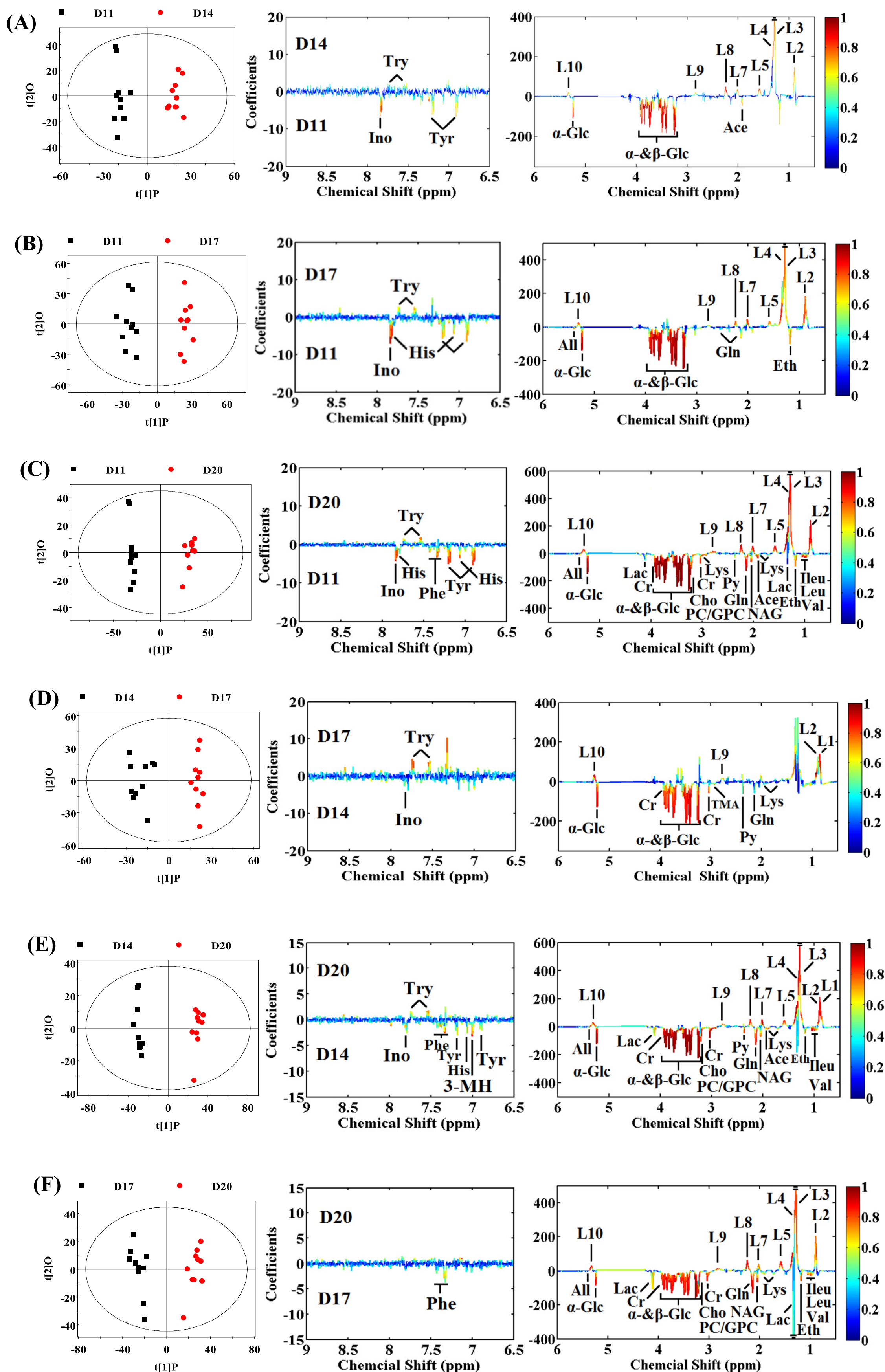
Previous study has shown that microbial metabolism in diverse environments was correlated with fetal development and growth (Ma et al., 2014), and it will be helpful to the stabilizing of internal microbial environment of fetus. The gradually increased biosynthesis of biological substances with the gestation period, including secondary metabolites, amino acid, aminoacyl-tRNA, glucosinolate and alkaloids, should be consistent with the fetal developmental process (Vining, 1992).

4. Discussion

All of the results show that the significant metabolic changes observed in maternal plasma throughout pregnancy relate mainly to amino acid metabolism, energy metabolism, lipid metabolism, protein metabolism and some of their derivatives.

4.1. Amino acid and energy metabolisms

Pregnancy is known to be accompanied by increased placental transfer of amino acids, favoring nitrogen conservation for fetal growth (King, 2000), and thus leading to decreased levels of many amino acids in plasma, a condition known as hypoaminoacidemia. The lower levels noted here for lysine, phenylalanine, tyrosine, valine, histidine, glutamine, and inosine identify these amino acids as particularly important serving the fetus, particularly in middle and later pregnancy, which are in broad agreement with the previous literature (Dasarathy et al., 2010). They are also correlated with ATP-binding cassette transporters (ABC transporters), protein digestion and absorption with the other metabolic variations of glucose, pyruvate, *myo*-inositol, acetate, and choline (Table 2), and utilized the energy of ATP to transport a wide variety of substance across extra- and intracellular metabolic products including lipids and sterols in order to maintain a continuous supply of nourishment for the growing fetus (Broehan et al., 2013). Previous reports also associated decreases in glucose and pyruvate due to the inability of the mother to sustain glucose production and gluconeogenesis in the presence of increased demands (Kalhan et al., 1997). Furthermore,


(A)
D11
D14
D11
Ino
Tyr
Try
L10
L9
L8
L7
L5
L4
L3
L2
α-Glc
α-&β-Glc
Ace
(B)
D11
D17
D17
D11
Ino
His
Try
Gln
Eth
All
(C)
D11
D20
Phe
Lac
Cr
Lys
Py
Cho
PC/GPC
NAG
Ileu
Leu
Val
(D)
D14
D17
TMA
L1
(E)
D14
D20
3-MH
(F)
D17
D20
Coefficients
Chemical Shift (ppm)
Chemcial Shift (ppm)
t[1]P
t[2]O

with the extension of the pregnancy process, the changes of these metabolites especially α- and β-glucose become more significant as shown in Table 1. The significantly decreased concentrations of saccharides in maternal plasma implicated either a reduced supply of these metabolites or an elevated consumption of them by fetal development process. In fact, glucose, pyruvate, acetate and ethanol are also involved in the pathways glycolysis and gluconeogenesis. The previous study have proven that pyruvate is an intermediate compound in the metabolism of carbohydrates, proteins, and fats (Wishart et al., 2013), and it can be served to generate glucose as the first designated substrate of gluconeogenic pathway. Moreover, all citric acid cycle intermediates, through conversion to oxaloacetate, amino acids, and glycerol, can also function as substrates for gluconeogenesis (Wishart et al., 2013). Thus, glucose, lipid, and amino acids can transform to each other via pyruvate in the tricarboxylic acid cycle. Glucose can be synthesized in the liver and kidneys from non-carbohydrate intermediates such as pyruvate and glycerol by gluconeogenesis process. However, the substantial decrease of pyruvate was accompanied by a simultaneous decrease of glucose levels during the late gestation period, which indicate that glucose may be the main energy source of fetal development and the consumption of maternal body. As energy compensation, lactate is transported back to the liver where it is converted into pyruvate by the Cori cycle using lactate dehydrogenase and further obtains energy from glycolysis. As a results of the huge demand in the energy in the late gestation period, lactate decreases more obviously between the GD11 and GD20, GD14 and GD20, GD17 and GD20.

It should be pointed out that tryptophan showed a significant increase between the middle and late gestation period, i.e. the GD11 and GD14, GD11 and GD17, GD11 and GD20, GD14 and GD17, GD14 and GD20, but it was not so obvious in the late gestation period, especially between GD17 and GD20. Tryptophan is an essential amino acid and cannot be synthesized by the organism, and therefore it must be part of daily diet including dietary proteins. Furthermore, tryptophan acts as building blocks in protein biosynthesis (Koopmans et al., 2006), and functions as a biochemical precursor for serotonin (a neurotransmitter) (Rosebrough, 1996). Higher concentrations of tryptophan in the maternal plasma mean the greater demand of dietary proteins intake by the pregnant mothers, and higher speed of protein digestion and absorption. Therefore, the significant increase of tryptophan might be a natural stress response in fetal development, and it indicated that the fetal development is faster in the late gestation period than in the middle period, but at a steadier pace in the late period (i.e. GD17 and GD20). In short, tryptophan is vital for fetal development, especially with respect to the brain, but it cannot be synthesized by the organism and must be obtained from the diet (Koopmans et al., 2006). That is why the high speed protein synthesis should not be associated with tryptophan depletion. Therefore, it is particular important for pregnant mothers to receive enough tryptophan from their diet during pregnancy. The amino acid variations shown here are agreement with the literature (King, 2000), but the specific mechanisms of the exact amino acid fluctuations accompanying healthy pregnancies need to be further investigated.

4.2. Protein metabolism

In addition, protein digestion and absorption in maternal hepatic and adipose tissues will be activated to subsidize and preserve energy needs by fetus. As a consequence, a decreased concentration of amino acids was observed in maternal blood. Changes in plasma concentration of amino acids may play an important role in determining appropriate fetal growth. Consistent with reported changes in several amino acids found in pregnant mother (Di Giulio et al., 2004), many kinds of amino acids also demonstrated different degrees of deletion in our study. The decreased concentrations of some metabolites such as N-acetyl glycoprotein, creatine and creatinine related to the decomposition of the protein (Delanghe et al., 1989; Pinto et al., 2015). Previous reports have also associated decreases in creatine and creatinine to the lower circulating levels and increased excretion, together with ammonia and uric acid late in pregnancy (Dasarathy et al., 2010; Pinto et al., 2015). The decreases of these intermediate and end-products of energy storage during the middle and late pregnancy indicate the strong demand of energy in the second and third trimesters. All of these results indicated that the characteristic alterations of maternal metabolome are particularly important in serving the fetus and probably associated with the rapid growth of the fetus. Maternal protein deficiency in late gestation seems to more impact on birth weight than does energy deficiency (Robinson, 1996). Severe or prolonged maternal protein deficiency not only can result in intrauterine growth retardation of the fetus, but also negatively impact viability through decreased thermogenic capacity and reduced production of quality colostrum. It is obvious that the content of almost all the lipid increased during pregnancy process, which might be related to maternal obesity during pregnancy in order to meet the maternal needs and provide the mother with sufficient energy stores and substrates required for labor and lactation.

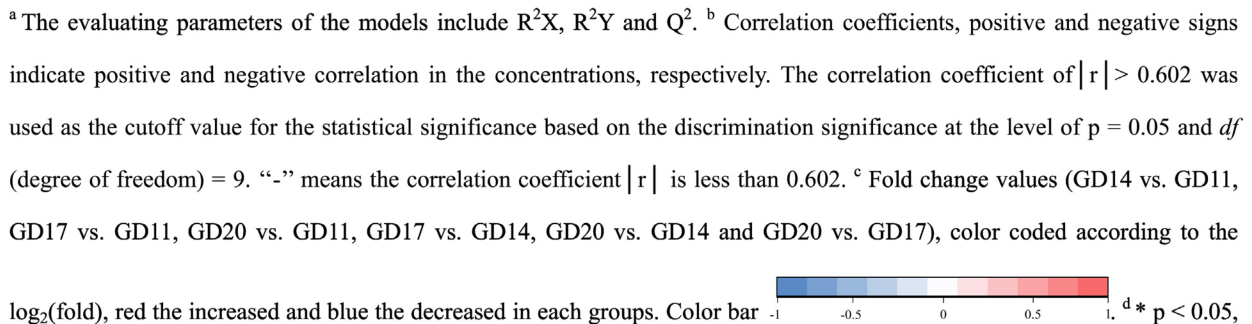
4.3. Lipid metabolism

An increase of LDL, VLDL and lipid in plasma indicates that lipid metabolism was accelerated in order to counter negative energy balance as a result of decreased plasma glucoses being decreasingly regulated. Our results further support the expected increases in lipoproteins described in several reports (Butte, 2000; Hadden and McLaughlin, 2009; Lippi et al., 2007). The obvious decrease of some metabolites related to lipid metabolism such as choline, phosphorylcholine, glycerolphosphocholin, inositol and ethanol may be due to some reasons. Firstly, the lipid concentrations are very sensitive to energy balance in contrast to blood glucose concentrations as blood glucose is homeostatically regulated. Secondly, it should be pointed out that the choline signals indicated as phosphocholine/glycerophosphocholine in Fig. 1 are actually mainly from glycerophosphocholines present in high concentration in lipoprotein particles (Sun et al., 2012). The decreases of the choline, phosphorylcholine, and glycerolphosphocholin show the slowdown of the lipid metabolism. Pregnant women normally sustain an elevated blood glucose level and undergo fat storage for fetal growth and development from their non-gravid state and early pregnancy towards middle and late pregnancy (Herrera and Ortega-Senovilla, 2014). When maternal blood glucose decreases,

Fig. 4. OPLS-DA scores plots (left panel) derived from ^{1}H NMR spectra of pregnant rat plasma and corresponding coefficient loading plots (right panel) obtained from different gestation periods. (A) GD11-14; (B) GD11-17; (C) GD11-20; (D) GD14-17; (E) GD14-20; (F) GD17-20. The color map shows the significance of metabolites variations between the two classes. Peaks in the positive direction indicate metabolites that are more abundant in the groups in the positive direction of first principal component. Consequently, metabolites that are more abundant in the groups in the negative direction of first primary component are presented as peaks in the negative direction. Keys of the assignments were shown in Fig. 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
OPLS-DA coefficients and significant change of the metabolites derived from the NMR data of rat plasma obtained at different gestation periods.

Metabolites	GD11-GD14		GD11-GD17		GD11-GD20		GD14-GD17		GD14-GD20		GD17-GD20	
	R^2X: 0.411[a]		0.588		0.728		0.494		0.694		0.643	
	R^2Y: 0.819		0.933		0.971		0.849		0.942		0.848	
	Q^2: 0.476		0.782		0.925		0.476		0.819		0.653	
	r[b]	Fold[c]	r	Fold	r	Fold	r	Fold	r	Fold	r	Fold
Glucose metabolism												
α-Glucose	-0.918	0.85 *[d]	-0.965	0.69 **	-0.995	0.52 *	-0.840	0.81 *	-0.966	0.61 *	-0.880	0.76 *
β-Glucose	-0.901	0.85 *	-0.967	0.76 **	-0.997	0.56 **	-0.837	0.89 *	-0.962	0.66 **	-0.951	0.74 **
Pyruvate	-	1.06	-	0.93	-0.706	0.86 *	-0.726	0.88 *	-0.881	0.81 *	-	0.93
Lactate	-	1.10	-	1.21	-0.673	1.19	-	1.11	-0.739	1.09	-0.749	0.98 *
Acetate	-0.709	0.87 *	-	0.84	-0.855	0.71 *	-	0.97	-0.833	0.82 *	-	0.85
Lipid metabolism												
LDL: $C\underline{H}_3$-$(CH_2)_n$-	-	0.92	-	1.24	-	1.22	0.778	1.35	0.757	1.32 *	-	0.98
LDL: CH_3-$(C\underline{H}_2)_n$-	0.703	1.42 *	0.748	1.62 *	0.921	2.40 *	-	1.14	0.893	1.69 *	0.766	1.48 *
VLDL: $C\underline{H}_3$-$(CH_2)_n$-	0.681	1.27	0.842	1.59 *	0.922	2.23 *	0.832	1.26 *	0.917	1.75 *	0.796	1.40 *
VLDL: CH_3-$(C\underline{H}_2)_n$-	0.725	1.47 *	0.819	1.88 *	0.940	2.69 **	-	1.28	0.907	2.05 *	0.867	1.60 *
VLDL, -$C\underline{H}_2$-CH_2-C=O	0.745	1.54 *	0.745	1.77	0.887	2.79 *	-	1.15	0.812	2.01 *	0.837	1.74 *
Lipid, $C\underline{H}_2$-C=O	0.844	1.36 *	0.775	1.39 *	0.921	2.13 *	-	1.02	0.860	1.56 *	0.874	1.53 *
Lipid, -$C\underline{H}_2$-CH=CH-	0.727	1.18	0.841	1.30 *	0.915	1.51 *	-	1.10	0.927	1.28 **	0.725	1.16 *
Lipid, =CH-$C\underline{H}_2$-CH=	-	1.12	0.685	1.26	0.875	1.66 *	0.688	1.13	0.823	1.49 *	0.914	1.32 **
Lipid, -CH=CH-	0.763	1.15 *	0.846	1.38 *	0.898	1.74 *	0.871	1.20 *	0.890	1.52 *	0.853	1.27 *
GPC/PC	-	0.92	-	0.97	-0.860	0.60 *	-	1.06	-0.849	0.66 *	-0.859	0.62 *
Choline	-	0.97	-	1.03	-0.804	0.63 *	-	1.06	-0.848	0.65 *	-0.886	0.61 *
myo-Inositol	-	1.01	-0.809	1.03	-0.951	1.08 **	-	1.03	-0.939	1.07 *	-0.901	1.04 *
Ethanol	-	0.76	-0.765	0.71	-0.904	0.56 *	-	0.94	-0.872	0.74 *	-0.909	0.79 *
Amino acid metabolism												
Lysine	-	1.03	-	1.04	-0.971	0.85 *	-0.674	1.02 *	-0.944	0.83 **	-0.906	0.82 *
Phenylalanine	-	0.86	-	0.85	-0.856	0.66 *	-	0.99	-0.865	0.77 *	-0.719	0.78 *
Tryptophan	0.685	1.96 *	0.715	2.89 *	0.806	2.54 *	0.724	2.12 *	0.775	2.09 *	-	0.99
Tyrosine	-	0.56	-0.744	0.43 *	-0.873	0.38 *	-	0.77	-0.699	0.69 *	-	0.90
Valine	-	0.98	-	0.94	-0.911	0.75 *	-	0.96	-0.887	0.76 *	-0.798	0.79 *
Isoleucine	-	1.18	-	1.10	-0.841	0.99 *	-	0.93	-0.834	0.84 *	-0.691	0.91 *
Leucine	-	1.01	-	1.02	-0.747	0.88 *	-	1.01	-	0.88	-0.694	0.87 *
Histidine	-	0.73	-0.669	0.51 *	-0.825	0.39 *	-	0.70	-0.807	0.54 *	-	0.77
3-Methylhistidine	-	1.19	-	0.81	-	0.54	-	0.69	-0.882	0.46 *	-	0.67
Glutamine	-	0.95	-0.913	0.96 **	-0.977	0.96 **	-0.684	1.01 *	-0.924	1.01 *	-0.884	0.99 *
N-acetyl glycoprotein	-	1.05	-	1.04	-0.831	1.01	-	0.99	-0.749	0.96 *	-0.810	0.97 *
Creatine	-	0.98	-	0.84	-0.858	0.56 *	-0.723	0.86 *	-0.935	0.57 **	-0.840	0.66 *
Inosine	-0.849	0.39 *	-0.911	0.21 *	-0.929	0.35 *	-0.869	0.54 *	-0.927	0.91 **	-	1.68
Other												
Trimethylamine	-	1.02	-	0.79	-0.814	0.68 *	-0.668	0.77 *	-0.913	0.67 *	-	0.86
Allantoin	-	0.85	-0.784	0.74 *	-0.800	0.67 *	-	0.87	-0.688	0.78	-0.682	0.90


[a] The evaluating parameters of the models include R^2X, R^2Y and Q^2. [b] Correlation coefficients, positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The correlation coefficient of $|r| > 0.602$ was used as the cutoff value for the statistical significance based on the discrimination significance at the level of $p = 0.05$ and *df* (degree of freedom) = 9. "-" means the correlation coefficient $|r|$ is less than 0.602. [c] Fold change values (GD14 vs. GD11, GD17 vs. GD11, GD20 vs. GD11, GD17 vs. GD14, GD20 vs. GD14 and GD20 vs. GD17), color coded according to the $\log_2$(fold), red the increased and blue the decreased in each groups. Color bar -1 -0.5 0 0.5 1. [d] * $p < 0.05$, ** $p < 0.01$, p-values were calculated by *t*-test.

maternal body will adjust the blood glucose level to meet the needs of the fetus through a variety of glucose regulation mechanism with increased lipolysis (Butte, 2000), and plasma levels of free fatty acids rise as well. All of these metabolites are not only associated with the lipid metabolism, but also the intermediates of the amino acid metabolism during pregnancy. Furthermore, the pregnancy

Table 2
The involved metabolic pathways corresponding to differential metabolites in maternal plasma between pair-wise gestation periods.

Gestation periods	Differential metabolites	Metabolic pathways
GD11 −GD14	α-&β-glucose, acetate, tryptophan	Biosynthesis of biological substance including secondary metabolites and indole alkaloid
	Acetate	Microbial metabolism in diverse environments
	Acetate, tryptophan	Protein digestion and absorption
	α-&β-glucose, tryptophan	Mineral absorption
GD11 −GD17	α-&β-glucose, tryptophan, histidine, *myo*-inositol, ethanol, glutamine	Biosynthesis of biological substance including secondary metabolites, amino acid and aminoacyl-tRNA
	Glutamine, *myo*-inositol, ethanol	Microbial metabolism in diverse environments
	α-&β-glucose, glutamine, histidine, *myo*-inositol	ABC transporters
	α-&β-glucose, *myo*-inositol	Mineral absorption
	α-&β-glucose, ethanol	Glycolysis/Gluconeogenesis
GD11 −GD20	Pyruvate, α-&β-glucose, acetate, lysine, tryptophan, phenylalanine, leucine, histidine, *myo*-inositol, valine, isoleucine, ethanol	Biosynthesis of biological substance including secondary metabolites, amino acid, aminoacyl-tRNA and glucosinolate
	Pyruvate, acetate, lysine, glutamine, *myo*-inositol, ethanol, trimethylamine, trimethylamine N-oxide	Microbial metabolism in diverse environments
	α-&β-glucose, lysine, glutamine, phenylalanine, choline, leucine, histidine, *myo*-inositol, valine, isoleucine	ABC transporters
	Acetate, lysine, glutamine, tryptophan, phenylalanine, leucine, histidine, valine, isoleucine	Protein digestion and absorption
	Pyruvate, lysine, tryptophan, phenylalanine, leucine, valine, isoleucine	Carbohydrate metabolism, digestion and absorption
	α-&β-glucose, glutamine, tryptophan, phenylalanine, leucine, valine, isoleucine	Mineral absorption
	Pyruvate, α-&β-glucose, acetate, ethanol	Glycolysis/Gluconeogenesis
GD14 −GD17	Pyruvate, α-&β-glucose, lysine, tryptophan, glutamine	Biosynthesis of biological substance including secondary metabolites, amino acid and aminoacyl-tRNA
	Pyruvate, lysine, glutamine, trimethylamine, trimethylamine N-oxide	Microbial metabolism in diverse environments
	α-&β-glucose, lysine, glutamine	ABC transporters
	Lysine, glutamine, tryptophan	Protein digestion and absorption
	Pyruvate, lysine, tryptophan	Carbohydrate metabolism, digestion and absorption
	α-&β-glucose, glutamine, tryptophan	Mineral absorption
	Pyruvate, α-&β-glucose	Glycolysis/Gluconeogenesis
GD14 −GD20	Pyruvate, α-&β-glucose, acetate, lysine, tryptophan, phenylalanine, leucine, histidine, *myo*-inositol, valine, isoleucine, ethanol	Biosynthesis of biological substance including secondary metabolites, amino acid, aminoacyl-tRNA, glucosinolate and alkaloids
	Pyruvate, acetate, lysine, glutamine, *myo*-inositol, ethanol, trimethylamine, trimethylamine N-oxide	Microbial metabolism in diverse environments
	α-&β-glucose, lysine, glutamine, phenylalanine, choline, leucine, histidine, *myo*-inositol, valine, isoleucine	ABC transporters
	Acetate, lysine, glutamine, tryptophan, phenylalanine, leucine, histidine, valine, isoleucine	Protein digestion and absorption
	Pyruvate, lysine, tryptophan, phenylalanine, leucine, valine, isoleucine	Carbohydrate metabolism, digestion and absorption
	α-&β-glucose, glutamine, tryptophan, phenylalanine, leucine, valine, isoleucine	Mineral absorption
	Pyruvate, α-&β-glucose, acetate, ethanol	Glycolysis/Gluconeogenesis
	Pyruvate, tryptophan, choline, creatine	Amino acid metabolism
GD17 −GD20	α-&β-glucose, lysine, phenylalanine, leucine, *myo*-inositol, valine, isoleucine, ethanol, glutamine	Biosynthesis of biological substance including secondary metabolites, amino acid, aminoacyl-tRNA and glucosinolate
	Lysine, glutamine, *myo*-inositol, ethanol	Microbial metabolism in diverse environments
	α-&β-glucose, lysine, glutamine, phenylalanine, choline, leucine, *myo*-inositol, valine, isoleucine	ABC transporters
	Lysine, glutamine, phenylalanine, leucine, valine, isoleucine	Protein digestion and absorption
	Phenylalanine, leucine, valine, isoleucine	Carbohydrate metabolism, digestion and absorption
	α-&β-glucose, glutamine, phenylalanine, leucine, valine, isoleucine	Mineral absorption

process exerted more significantly effects on amino acid metabolism than on lipid metabolism. All in all, the deficient energy supply derived from glucose metabolism during the gestation period would facilitate proteolysis, presenting that a majority of lipid increase and the amino acids decreased in maternal plasma (Butte, 2000). Prolonged or extreme lipid mobilization during pregnancy process can lead to elevated liver fat infiltration and increased periparturient disease problems (Cai et al., 2005).

5. Conclusion

This work employed an NMR-based metabolomics approach to study the metabolic variations in maternal plasma of healthy pregnant Wistar rats during pregnancy process from gestation day 11 to day 20, representing two pregnancy trimesters. Our result highlighted 24 differential metabolites and their changes throughout pregnancy were investigated. The gradual decreased concentrations of glucose, pyruvate, lysine, phenylalanine, leucine, valine and isoleucine were involved in the energy storage, protein digestion and absorption. In addition, the general increase of tryptophan was also suggested to be somehow involved in protein biosynthesis, and confirmed the more rapid growth of the fetus in the late gestational period than in the middle period. Furthermore, a number of amino acids also demonstrated different degrees of reduction in our study, with N-acetyl glycoprotein, creatine and creatinine being more significantly excreted than expected based on the literature, possibly in connection to lower circulating levels of ammonia and uric acid later in pregnancy. Meanwhile, the increase (LDL, VLDL and lipid) or decrease (such as choline, phosphorylcholine, glycerolphosphocholin, inositol and ethanol) concentration of some metabolites further confirmed that lipid metabolism was accelerated in order to counter energy balance during the middle and late pregnancy.

The variations in these metabolites were basically involved in

the varied kinds of metabolic pathways including synthesis of biological substances, microbial metabolism in diverse environments, protein digestion and absorption, carbohydrate metabolism, digestion and absorption, mineral absorption, and ABC transporters were found to be associated with normal pregnancy. The changes in maternal plasma reflect both maternal physiological state and fetal development and growth. During the middle and late gestation periods, gradual increase of energy demand and utilizing should be crucial for promoting fetal development and regulating maternal physiological state. The results reveal metabolite changes and related relationships in the process of pregnancy, which may provide an important reference for the prenatal care related diet, nutrition and health monitoring during pregnancy and offer guidance on the implementation of eugenics in metabolism. The comprehensive coverage of the entire metabolome might bring in new discoveries. In future study, the combination of NMR technology with additional analytical technologies such as hydrophilic interaction chromatography-mass spectrometry (HILIC-MS) and gas chromatography-mass spectrometry (GC–MS) may be employed to get more insight into the detailed metabolic phenotype during pregnancy process.

Conflicts of interest

All of the authors confirm that there is no conflict of interest in relation to this work.

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