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RESEARCH ARTICLE

Metabolic Profiling of Plasma in Gestational Diabetes Mellitus Using Liquid Chromatography and Q-TOF Mass Spectrometry

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SUMMARY

Background: **To delineate the metabolomic profiling and identify early diagnostic biomarkers in maternal plasma from the pregnant women who subsequently developed gestational diabetes mellitus (GDM) using liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (LC-Q-TOF MS).**
Methods: **Plasma samples were collected from GDM pregnant women (n = 30) and healthy controls (n = 30) at the 20th gestational week in Huzhou Central Hospital and Huzhou Maternal and Child Health Hospital. The principle component analysis (PCA), partial least-squares discriminant analysis (PLS-DA), and orthogonal PLS (OPLS) were sequentially applied to discover the differential metabolites for GDM diagnosis. Further, we analyzed the identified biomarkers in the MetPA database in order to reveal the key relevant metabolism in GDM.**
Results: **Twenty-four out of 975 aligned metabolites were distinguished among GDM plasma and healthy controls. In particular, the level of linolenic acid and arachidonic acid was significantly elevated in GDM.**
Conclusions: **The linolenic acid and arachidonic acid could be selected as new potent biomarkers for GDM diagnosis and prognosis in early pregnancy; however, they still need to be confirmed from large samples in future.**
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KEY WORDS

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INTRODUCTION

Gestational diabetes mellitus (GDM) is defined as a condition in which women without a diabetic history exhibit carbohydrate intolerance during pregnancy. Affecting 7 - 15% of all pregnancies worldwide [1,2], GDM patients have an increased risk of adverse perinatal outcomes, including macrosomia, neonatal jaundice as well as maternal type 2 diabetes mellitus [3-5]. It has been reported that early diagnosis and intervention of GDM is a key to improving long-term health and well-being of both mother and child [6]. However, routine blood screening and oral glucose tolerance test (OGTT), which was first proposed by O'Sullivan and Mahan in 1964 [7], are still in use today as the diagnostic criteria for GDM in the late second trimester [8,9]. Therefore, it is important to discover the potential biomarkers for GDM diagnosis, prognosis, and therapies early in gestation [10].

Metabolomics allows for the comprehensive assessment of the metabolic state of biological objects [11] and attempts to systematically identify the endogenous metabolites in biofluids [12]. During the past decade, there has been a rapidly growing interest in applying metabolomics into the prediction of GDM onset [13-19]. Although a multitude of studies have been devoted to revealing the potential pre-diagnostic biomarkers from urine, amniotic fluid or plasma, e.g., acetate, formate, creatinine, betaine, 3-hydroxyisovalerate, and 2-hydroxyisobutyrate, GDM prediction seems to require an altered set of multivariate profiles rather than univariate changes. In order to systematically find consistent biomarkers for GDM diagnosis, we pursued a metabolomics strategy in this study, based on recently developed liquid chromatography coupled with the quadrupole time-of-flight mass spectrometry (LC-Q-TOF MS) method. LC-Q-TOF MS, which provides highly accurate analytical information at the sub-ppm level, was employed to depict the global plasma profiles of GDM patients and healthy controls. Furthermore, multivariate analysis and database stimulation were used to identify the regulated metabolites along with their relevant metabolic pathways, which might be the clinical biomarkers for the pre-diagnosis of GDM.

MATERIALS AND METHODS

Subjects

The research was approved by the Ethics Committee of Huzhou Central Hospital and Huzhou Maternal and Child Health Hospital. Three hundred pregnancies were enrolled in this study after signing the written informed consent. The fasting venous plasma was drawn into sodium heparin-containing tubes, collecting from pregnancies at the 20th gestational week, and stored at -80°C until analysis. All women were requested to undertake a standardized OGTT between the 24th and 28th gestational week. After a 75 g anhydrous glucose load, fasting plasma glucose (FPG) and venous plasma glucose values at 1 hour (1-h PG) and 2 hours (2-h PG) were recorded and analyzed. According to the International Association of Diabetes and Pregnancy Study Groups (IADPSG) guidelines [8], diagnostic criteria of GDM were defined as FPG $\geq$ 5.1 mmol/L, 1-h PG $\geq$ 10.0 mmol/L or 2-h PG $\geq$ 8.5 mmol/L.

Finally, 30 GDM patients and 30 gestational-age matched healthy controls were chosen for the further study. The characteristics of GDM patients and healthy controls are provided in Table 1.

LC-Q-TOF MS analysis

Plasma samples for metabolomics testing were thawed at room temperature for 15 minutes and vortexed for 5 seconds. Subsequently, 300 µL methanol was added to 100 µL plasma. After shaking for 30 seconds, the mixture was left standing for 20 minutes at 4°C, followed by a centrifugation at 12000 rpm for 15 minutes at 4°C. The supernatant was then transferred into an autosampler vial. Quality control (QC) samples were prepared by pooling equal volumes of each sample and were injected every 6 sample injections and at the beginning or end of each analysis.

LC-Q-TOF MS measurements were performed on an Agilent 1290 Infinity LC system coupled with a 6530 UHD and Accurate-Mass Q-TOF MS (Agilent, Santa Clara, CA, USA). 4 µL plasma extract was injected into a C18 column (100 x 2.1 mm, 1.8 µm, Agilent). The LC gradient elution started from 95% mobile phase A (water containing 0.1% formic acid) to 5% mobile phase B (acetonitrile containing 0.1% formic acid) at a flow rate of 0.4 mL/min for 16 minutes and then held with 5% A and 95% B at 0.4 mL/min for 3 minutes.

The Q-TOF MS analysis was carried out with a capillary voltage of 4 kV and sampling cone voltage of 35 kV. The desolvation gas flow was kept at 600 L/h. And the desolvation and source temperature were set to 350°C and 100°C, respectively. The Q-TOF MS data were acquired over a mass range of 50 - 1000 m/z with a scan time of 0.03 seconds and interscan delay of 0.02 seconds. Leucine enkephalin was used as the lock mass (m/z 556.2771 in ESI positive mode).

Data processing and statistical analysis

All raw data obtained from the mass spectra were converted into mzData formats using Masshunter acquisition software (Agilent) and analyzed by XCMS for peak identification, alignment, and normalization. The resulting matrix was exported into SIMCA-P 13.0 (Umetrics AB, Umea, Sweden) for multivariate data analysis. Mean-centering and unit variance scaling (UV-scaling) were applied in order to adjust the importance of high and low abundance metabolites to an equal level, prior to principal component analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA). PCA was performed to explore the clustering behavior of metabolites, while PLS-DA was employed to maximize the variance between GDM and healthy controls. Orthogo-

nal PLS (OPLS) was also used for picking out the discriminating ions that contribute to the classification of samples and remove non-correlated variations contained within spectra. Subsequently, receiver operating characteristic (ROC) curve analysis was performed to investigate the diagnostic value of the different metabolites with SPSS V20.0 software (SPSS Inc., Armonk, NY, USA). The quality of the model was assessed by two parameters, the relevant R^2 (goodness of fit), and Q^2 (predictive ability).

Furthermore, MetPA (http://metpa.metabolomics.ca/MetPA/faces/Home.jsp), database was used to systematically analyse the selected diagnostic GDM biomarkers, and explore key metabolic pathways involved in GDM pathogenesis.

All the results were presented as mean ± SD. Shapiro-Wilk test was used to determine the normality of the distribution. Mann-Whitney *U* test or Student's *t*-test was conducted to determine the differential metabolites obtained from OPLS modeling. A p-value of < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics

The clinical characteristics of the enrolled patients and healthy controls are shown in Table 1. There were no significant differences in age, parity, week of gestation, BMI, and blood pressure (SBP and DBP) between the two groups ($p > 0.05$). The GDM patients exhibited higher hyperglycemia in FPG, 1-h PG, and 2-h PG.

Metabolic profiling of LC-Q-TOF MS

Nine hundred seventy-five peaks of positive ions were obtained from LC-Q-TOF MS in the ESI-positive mode (Figure 1). The spectrum of all the samples (30 GDM patients, 30 healthy controls) demonstrated the excellent stability and reproducibility of chromatographic separation and mass measurement throughout the entire process (Figure 1A). Figure 1B and C exhibited a representative total ion chromatogram (TIC) of a random sample from the control group and GDM, respectively. Differences in peak intensities were found between the two different groups. All the metabolites were identified as endogenous metabolites by METLIN database.

Multivariate statistical analyses

In this study, PCA and PLS-DA of plasma spectra were constructed to disclose the global metabolic differences between GDM and healthy controls. In the PCA model, the parameter of the modeling R2X was only 0.146. The clusters were not distinctly separated in the scores plots, which might be due to the complexity and variation of clinical samples (Figure 2A). Red spots represented healthy controls, and the green spots represented GDM patients. There was a certain overlap between the samples from the two groups, but most of the samples were at the 95% confidence interval. In contrast, the two-dimensional scores of PLS-DA plot showed a clearer separation between the healthy controls and the GDM patients and aided in identification of differential metabolites (Figure 2B). The PLS-DA model was validated by describing 99.3% of the variation in the response Y ($R^2Y = 0.993$) and predicting 78.5% of the variation in the response Y ($Q^2 = 0.785$). The PLS-DA model was valid based on $Q^2 > 0.4$.

Where after, OPLS class prediction model was further trained with a dataset, consisting of 20 GDM patients and 20 healthy pregnancies ($R^2Y = 0.997$, $Q^2 = 0.851$). Shown in Figure 3, 10 samples in each group were correctly tested as GDM (cross stars) and non-GDM (stars) individuals. It successfully demonstrated the establishment of the OPLS class prediction model.

Identification of potential biomarkers

To identify potential biomarkers, OPLS was finally chosen as an effective approach to filter the unrelated variations. Plasma metabolic profiles from GDM and healthy subjects were revealed in OPLS score plots with $R^2Y = 0.843$ and $Q^2 = 0.823$ (Figure 4). Potential differential metabolites were defined according to the variable importance in the projection (VIP) values. VIP value larger than 1 was considered as a threshold for identification of potent biomarkers. As a result, expressions of 24 metabolites were altered between GDM and controls (Table 2). The plasmatic levels of linolenic acid and arachidonic acid were significantly increased in the GDM patients compared to healthy controls (Figure 5). Moreover, ROC curve analysis with high area under the curve (AUC) indicated that linolenic acid and arachidonic acid could differentiate the GDM patients from healthy controls (Table 3 and Figure 6).

Metabolic pathways and function analysis

Ingenuity pathway analysis and networks were presented by MetPA, which is established based on the KEGG database. Based on the differentiated metabolites between the GDM patients and healthy controls, five top canonical pathways have been revealed, namely glycolysis/gluconeogenesis metabolism, fatty acid metabolism, urine cycle, citrate cycle, and tryptophan metabolism (Figure 7). These five pathways, which are related to the identified metabolites, were also previously proven in the development of GDM. Among the five pathways, the arachidonic acid metabolism and linolenic acid metabolism were the top altered pathways in GDM, which is shown in Figure 8 in detail.

DISCUSSION

In the present study, we focused on the differential metabolites in plasma between GDM patients and healthy controls using LC-Q-TOF MS methods along with multivariate data modeling and metabolic pathway analyses. Among the regulated 24 metabolites, linolenic acid and arachidonic acid, which are significantly upregulat-

Table 1. The characteristics of the pregnancies included in the study.

Characteristics	GDM group (n = 30)	Healthy controls Group (n = 30)	p-value
Age (years)	28.4 ± 0.3	28.1 ± 0.4	ns
Parity (number)	1.3 ± 0.4	1.2 ± 0.5	ns
Week of gestation	25.3 ± 1.6	26.3 ± 1.3	ns
BMI (kg/m^2)	24.8 ± 8.5	24.3 ± 3.2	ns
FPG (mmol/L)	5.3 ± 0.9	4.5 ± 0.3	< 0.0001
1-h PG (mmol/L)	11.1 ± 1.4	5.8 ± 0.7	< 0.0001
2-h PG (mmol/L)	9.4 ± 2.2	5.3 ± 0.8	< 0.0001
Systolic blood pressure (mm Hg)	115.2 ± 6.2	115.3 ± 9.2	ns
Diastolic blood pressure (mm Hg)	75.3 ± 7.8	74.3 ± 8.5	ns

Table 2. 24 metabolites exhibited different regulation between GDM and normal control pregnant women. The potential biomarkers were identified by variable importance in the projection (VIP) values (VIP > 1).

RT	Mass	Name	VIP	p-value	Fold (B/A) *
0.730733	226.0958	Porphobilinogen	1.736	0.001	0.57
0.823933	181.0741	L-Tyrosine	1.86314	0.003	0.77
1.137967	117.0793	Betaine	1.69489	0.012	0.90
1.1389	204.022	Oxaloglutarate	1.77264	0.003	0.55
1.141117	203.1163	Acetylcarnitine	1.4964	0.028	0.84
4.709083	204.0915	Tryptophan	2.11144	0.001	0.85
4.70925	187.064	Indoleacrylic acid	2.03866	0.002	0.86
6.661167	254.0798	L-Arginine phosphate	1.18673	0.014	0.86
7.566867	175.0636	3-Indoleacetic Acid	1.4204	0.000	0.54
8.488867	362.2103	Cortisol	2.13027	0.003	0.81
8.5553	360.1946	Cortisone	2.21625	0.013	0.73
9.519533	315.2421	Decanoylcarnitine	1.34181	0.049	0.71
10.6753	343.2734	Lauroylcarnitine	1.44647	0.033	0.72
11.22675	449.3152	Glycodeoxycholic acid	1.47688	0.000	0.52
11.52273	666.9481	Adenosine 5'-pentaphosphate	1.76055	0.000	0.408
12.7442	501.2872	Glycerophospho-N-Arachidonoyl Ethanolamine	1.67144	0.034	0.73
13.0424	314.2254	Progesterone	1.72043	0.005	0.74
13.14028	517.316	PC (18:3)	1.3434	0.049	0.88
13.51793	479.3024	Glycerophospho-N-Oleoyl Ethanolamine	1.74091	0.034	0.86
13.97665	521.3494	PC (18:1)	1.48515	0.014	0.59
14.89313	523.3678	PC (18:0)	1.48318	0.029	0.93
16.03007	278.2254	γ-Linolenic Acid	1.49843	0.000	2.27
16.31102	148.0377	2-Hydroxyglutarate	1.36447	0.002	0.69
16.68292	304.2409	Arachidonic Acid	1.51205	0.000	2.5

Table 3. ROC analysis parameters of selected plasma biomarker between the control and GDM status.

Marker	Sensitivity (%)	Specificity (%)	p-value	AUC[1]	95% CI[2]
Linolenic acid	86.7%	93.3%	< 0.001	0.930	0.867 - 0.993
Arachidonic acid	90.0%	90.0%	< 0.001	0.942	0.887 - 0.997

[1] AUC stands for area under the curve.
[2] 95% confidence interval of the difference.

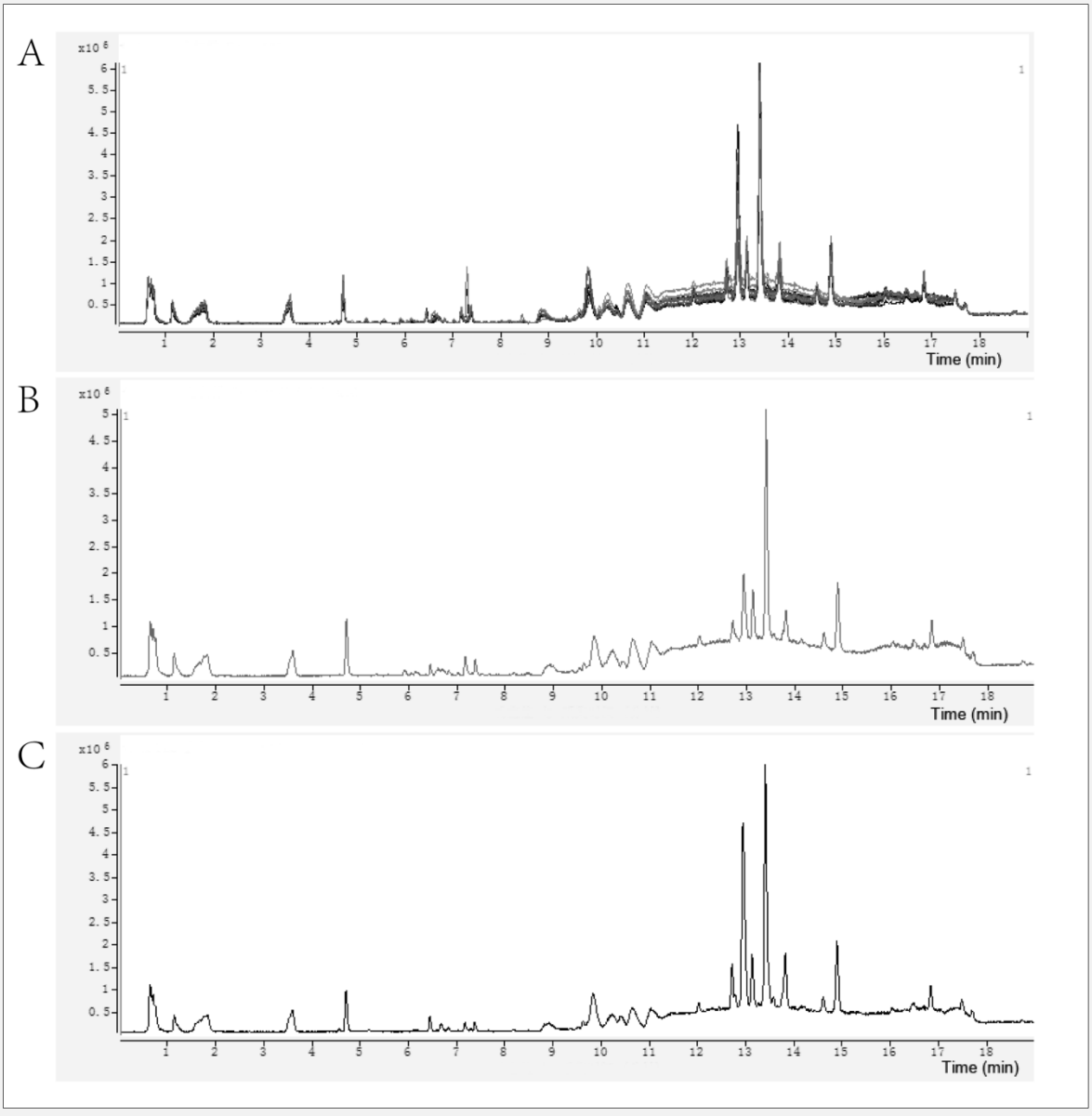

Figure 1. Total ion chromatogram (TIC) obtained from LC-Q-TOF MS at 50-1000 m/z in the ESI-positive mode.

(A) Unabridged TIC chromatograms at for 30 GDM and 30 healthy controls. (B and C) The representative TIC obtained from random control sample and GDM patient, respectively.

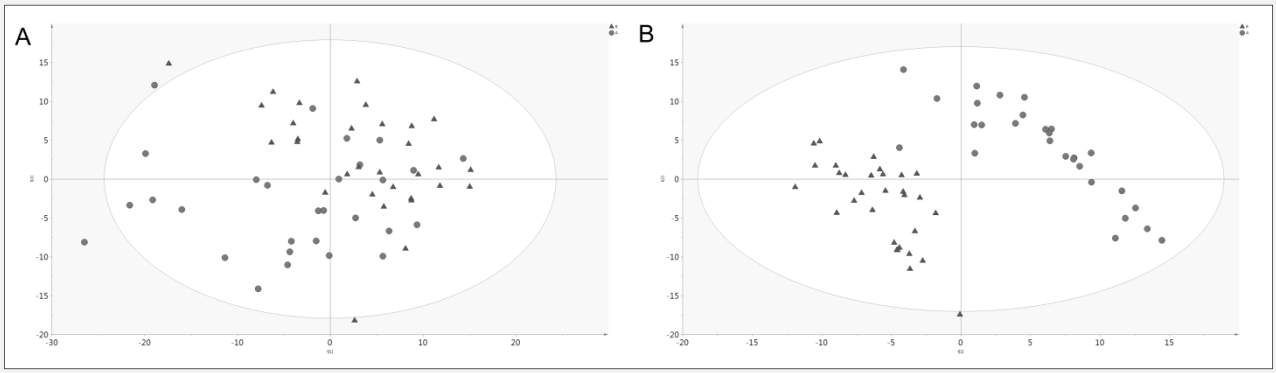

Figure 2. Principle component analysis (PCA) scores plot (A) and partial least-squares-discriminant analysis (PLS-DA) scores plot (B) of the healthy controls (circles) and GDM patients (triangles) in ESI-positive ion mode.

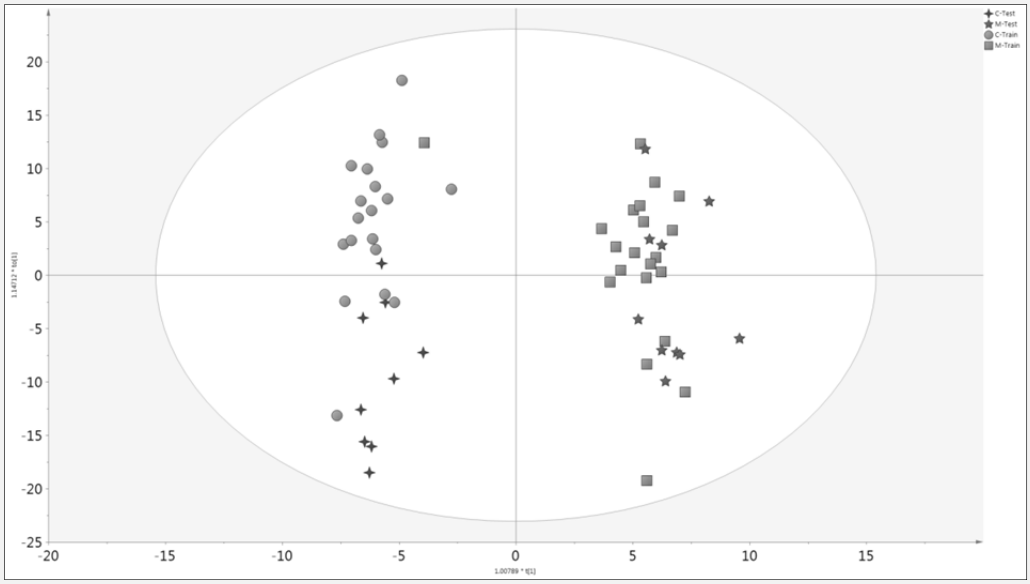

Figure 3. Orthogonal partial least-squares-discriminant analysis (OPLS) class prediction model was trained with 20 GDM subjects (m-train, squares) and 20 healthy pregnancies (c-train, circle) dataset (R2Y = 0.997, Q2 = 0.851), and tested with 10 GDM patients (m-test, stars) and 10 healthy persons (c-test, cross stars) as testing set.

ed in GDM pregnancy, may act as clinical biomarkers for GDM diagnosis during early pregnancy. Moreover, we have further analyzed the pathways of potential GDM biomarkers using the MetPA database and revealed that the metabolism of arachidonic acid and linolenic acid were the top altered pathways in GDM.

Linolenic acid and/or arachidonic acid also play an increasing important role in the diagnosis and prognosis as well as in the evaluation of therapeutic responses in other diseases, such as nonalcoholic steatohepatitis [20], lepromatous leprosy [21], colorectal cancer [22], hepatocellular carcinoma [23], atherosclerosis [24], renal

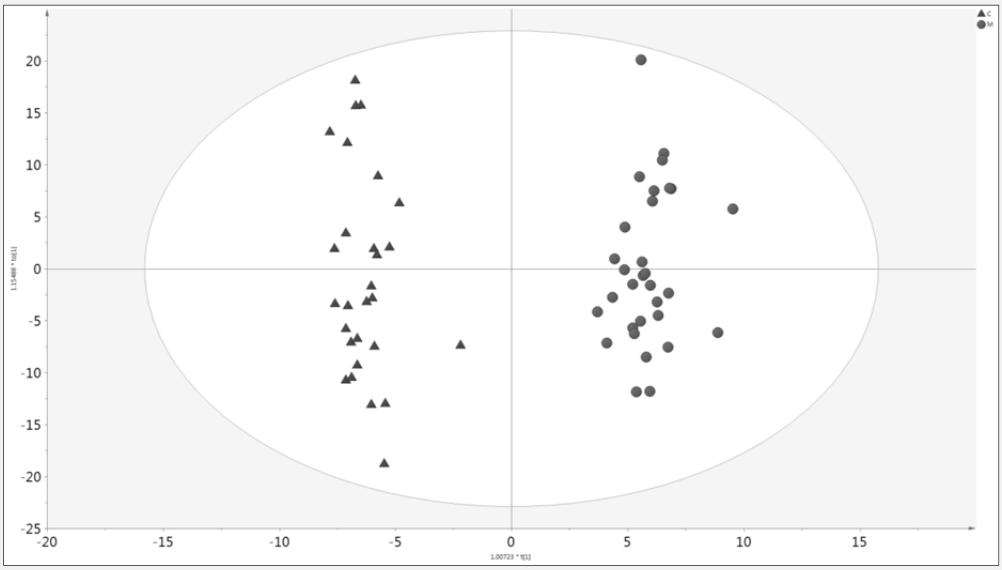

Figure 4. OPLS score plots of the plasma metabolic profiles obtained from GDM patients (triangles) and healthy control (circles) ($R^2Y = 0.843$, $Q^2 = 0.823$).

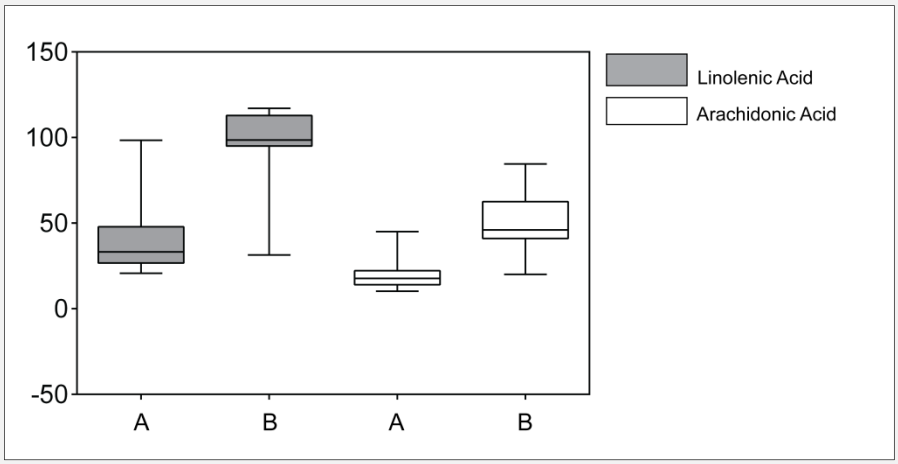

Figure 5. Linolenic acid and arachidonic acid were significantly elevated in GDM patients (B), as compared to healthy controls (A). P values from Manu-Whitney *U* test for linolenic acid and arachidonic acid were both less than 0.01.

cell carcinoma [25], and post-traumatic cognitive impairments [26].

In this work, we have revealed the high contents of polyunsaturated fatty acids in GDM plasma, which is consistent with the previous studies from De Seymour JV [18], Ghebremeskel [27], and Thomas [28]. Therefore, fatty acid metabolism is the top altered pathway in GDM, as shown in Figure 7. Linolenic acid is consid-

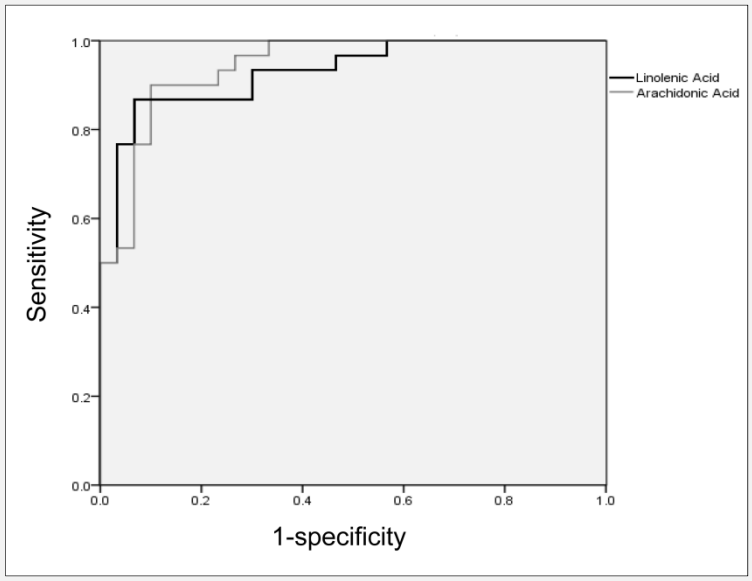

Figure 6. Receiver operating characteristic (ROC) curve analysis of linolenic acid and arachidonic acid in order to differentiate the GDM patients from controls.

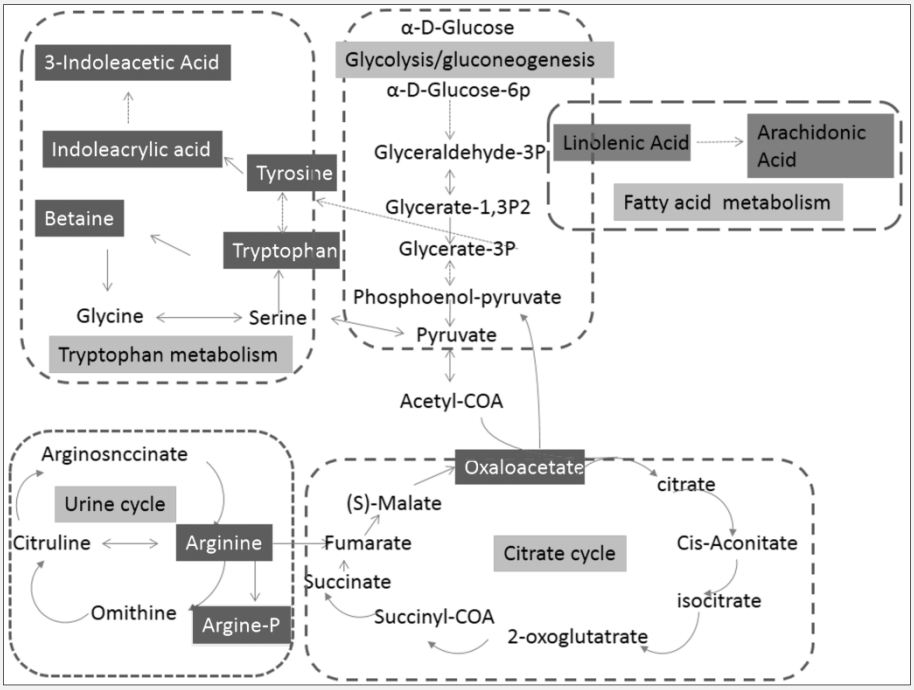

Figure 7. Linolenic acid and arachidonic acid have been involved in various metabolic pathways, including glycolysis/gluconeogenesis metabolism, Fatty acid metabolism, urine cycle, citrate cycle, tryptophan metabolism.

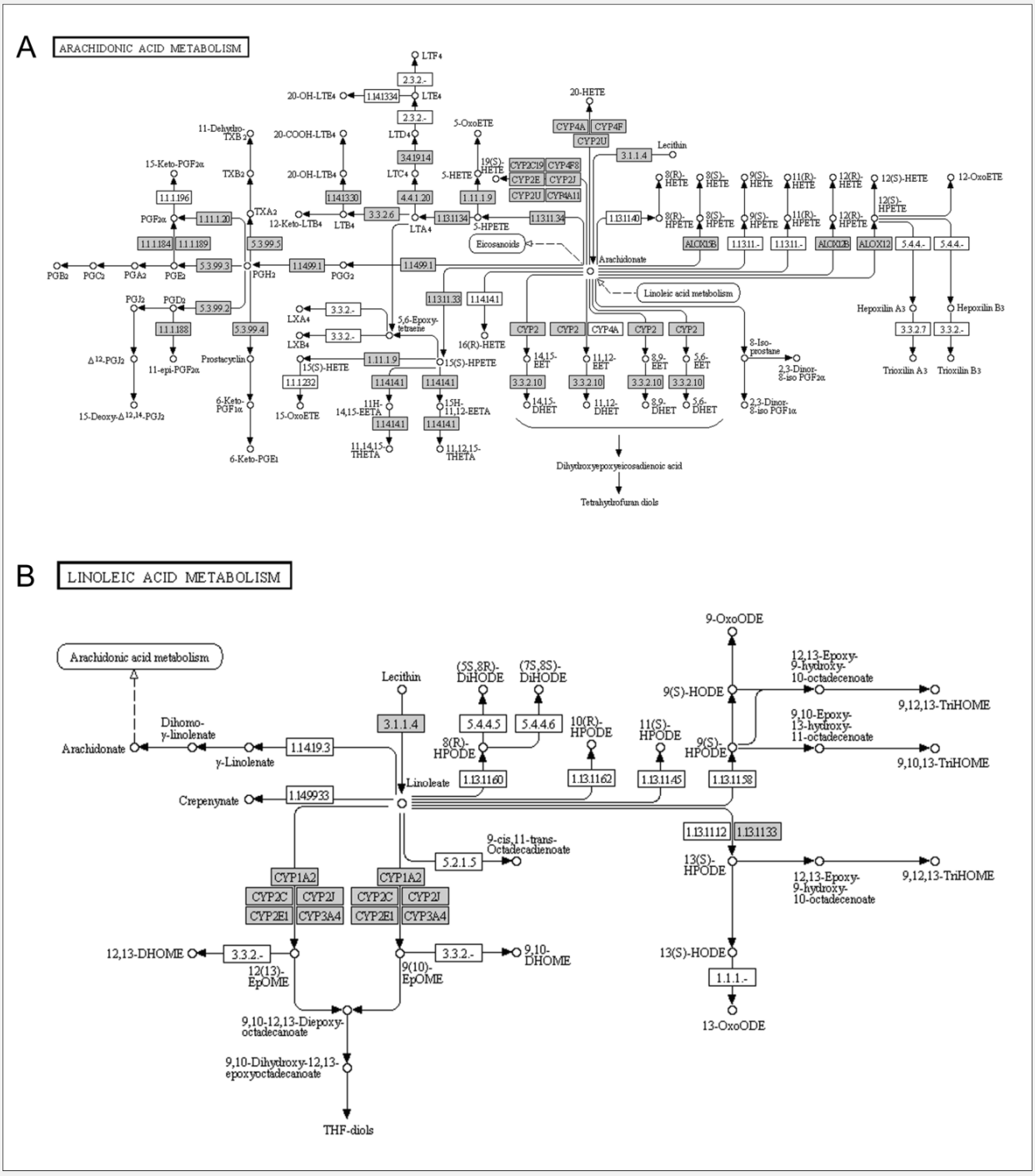

Figure 8. The construction of pathways in detail: including arachidonic acid metabolism (A) and linolenic acid metabolism (B).
The map was generated using the KEGG reference map.

ered an essential fatty acid and can be converted into arachidonic acid through desaturation and elongation reactions. The ability of the placenta and fetus to synthesize long-chain polyunsaturated fatty acids from essential fatty acids is relatively low [29]. Therefore, fetal linolenic acid and arachidonic acid are mainly supplied

by maternal circulation [30]. Increased fatty acid levels in the plasma of GDM patients might due to the high maternal esterification and limited placental-fetal transfer [31], which then impair the fetal and neonatal growth and development [32].

Long-chain polyunsaturated linolenic acid and arachidonic acid function not only act as the source of energy, but also as important signaling molecules in various cellular processes, such as insulin secretion and inflammation activation [33]. In GDM, hyperglycemia may mobilize the fatty acids in the blood, which were transported from liver and adipose tissue [33]. Sustained elevation of fatty acids then induces the apoptosis process in pancreatic beta cells. As a consequence, beta cells reduce the production and secretion of insulin, which are associated with a longer duration of diabetes and a greater prevalence of microvascular complication [34, 35]. On the other hand, long chain fatty acids are always believed to exert inflammatory action in cells, particularly in diabetes mellitus [36]. As the inflammation may act as the precursor during GDM development, monitoring the alteration of linolenic acid and arachidonic acid in early pregnancy could clinically help the prevention of GDM and improve the diagnosis of GDM.

CONCLUSION

This work reported the characterization of the maternal plasma using untargeted LC-Q-TOF MS in order to identify the metabolite markers for pre-diagnosis of GDM in the early pregnancy. The expression of linolenic acid and arachidonic acid were significantly increased in the GDM patients compared to healthy controls. The metabolic differences may provide a better understanding of the metabolic changes prior to GDM onset, which is important to allow early diagnosis and intervention of GDM.

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Declaration of Interest:
The authors have no conflicts of interest.

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