MEDICAL SCIENCE MONITOR

CLINICAL RESEARCH

e-ISSN 1643-3750
© Med Sci Monit, 2018; 24: 4038-4045
DOI: 10.12659/MSM.907653

Received: 2017.10.20
Accepted: 2018.01.23
Published: 2018.06.13

Metabolomic Profiling of Plasma Samples from Women with Recurrent Spontaneous Abortion

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Source of support: This study was funded by the Interdisciplinary Program of Shanghai Jiao Tong University (project number YG2014MS35)

Background: Gas chromatography coupled with mass spectrometry (GC-MS) and liquid chromatography coupled with mass spectrometry (LC-MS) metabolomics have been deployed to detect novel differential metabolites in cases with recurrent spontaneous abortion (RSA).

Material/Methods: Fifty patients who had recurrent spontaneous abortions (RSAs) and 51 control patients (age, gestational age, and body mass index (BMI) match) were enrolled in this study. Untargeted GC-MS and targeted LC-MS were combined to discover and validate the different metabolomic profiles between groups. Score plots of orthogonal partial least-squares discriminant analysis (OPLS-DA) clearly separated the RSA group from the control group. The variable importance in projection (VIP) generated in OPLS-DA processing represented the contribution to the discrimination of each metabolite ion between groups. Variables with a VIP >1 and $P<0.05$ were considered to be different variables. We also used MetaboAnalyst 3.0 to analyze the pathway impact of potential metabolite biomarkers.

Results: Fifty-four metabolites were significantly different between the two groups, as indicated by a VIP >1 and $P<0.05$. The metabolic pathways involving glycine, serine, threonine ($P=0.00529$, impact=0.26), beta-alanine ($P=0.0284$, impact=0.27), and phenylalanine metabolism ($P=0.0217$, impact=0.17), along with the tricarboxylic acid (TCA) cycle ($P=0.0113$, impact=0.19) and the glycolysis pathway ($P=0.037$, impact=0.1) are obviously related to RSA. Verification by LC-MS showed that the concentration of lactic acid in RSA was higher than that in the control group ($P<0.05$), while the concentration of 5-methoxytryptamine was significantly lower in the RSA group ($P<0.05$).

Conclusions: In our study, untargeted GC-MS was used to detect disturbance of metabolism occurs in RSA and targeted LC-MS further was used to show that plasma concentrations of two metabolites (lactic acid and 5-methoxytryptamine) were different in the RSA compared to the control group.

MeSH Keywords: Abortion, Habitual • Chromatography, Liquid • Gas Chromatography-Mass Spectrometry • Metabolomics

Full-text PDF: https://www.medscimonit.com/abstract/index/idArt/907653

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Background

Recurrent spontaneous abortion (RSA) is a prevalent complication of pregnancy that occurs in 1–2% of all pregnant women, and it is defined as the occurrence of 2 or more consecutive spontaneous abortions [1]. A number of factors are thought to play a role in recurrent abortions, including genetic mutations, chromosomal abnormalities in the fetus, infectious agents, and a variety of environmental factors such as alcohol, tobacco, and caffeine [2]. Nevertheless, nearly 50% of RSA cases remain unexplained, and it is likely that this condition is multifactorial in nature [3]. Therefore, there is a critical need to identify factors involved in RSA to obtain a clear understanding of its causes. However, RSA is often asymptomatic, and therefore identifying RSA is very difficult. RSA sometimes represents a serious clinical condition that can cause physical and psychological trauma to patients. It would be clinically valuable to identify highly selective biomarkers for RSA to inform patients and minimize trauma. Thus far, a range of factors have been isolated from the plasma of RSA patients and evaluated as predictors of RSA, particularly indoleamine 2,3-dioxygenase and human chorionic gonadotropin (hCG) [4]. However, current studies have failed to investigate the role of metabolites in plasma as potential RSA biomarkers.

Metabolomics, which evolved from genomics, transcriptomics, and proteomics, is the systematic analysis of the inventory of metabolites to highlight disease-specific metabolic signatures, which could be used as biomarkers [5]. Several techniques can then be used to identify and characterize these metabolites, including nuclear magnetic resonance (NMR), liquid chromatography (LC) coupled with mass spectrometry (MS), and gas chromatography MS (GC-MS) [6]. Metabolomics has been successfully applied in the diagnosis of a wide range of diseases, including cancer, cardiovascular diseases, neurodegenerative diseases, and rheumatic diseases [7]. Mass-spectrometry-based metabolic profiling is increasingly used to uncover new biomarkers for diagnosis [8], prognosis [9], pathogenesis clarification and potential therapeutic targets for clinical treatment [10].

Chee et al. reported that metabolomics was used to detect the potential of urine metabolites as a non-invasive screening tool for predicting threatened miscarriage [11]. And metabolomics was also applied for non-invasive embryo assessment *in vitro* fertilization (IVF) [12]. However, this valuable tool has not yet been used for the discovery of new biomarkers for RSA. Consequently, the use of metabolomics might create new options for researchers to identify novel mechanisms and pathways involved in the pathogenesis of RSA.

In the present study, we collected plasma samples from patients with RSA and from women with normal pregnancies which have matched in maternal age, gestational age and body mass index (BMI). We used a variety of GC-MS techniques to discover the different metabolic compositions of the plasma samples. Then, differentially expressed metabolites were verified using a targeted metabolomics method via UPLC-MS. Our overall aim was to identify differential metabolites that could be used as new biomarkers for predicting the RSA.

Material and Methods

Clinical samples

Fifty RSA patients and 51 control patients were enrolled at the International Peace Maternity and Child Health Hospital of China Welfare Institute (IPMCH) between January 2016 and May 2017. The RSA group included patients who had experienced more than 2 unexplained and consecutive spontaneous abortions at less than 10 weeks of gestation. Those who had genital abnormalities, chronic hypertension; diabetes; liver, kidney, cardiovascular, and thyroid diseases; autoimmune diseases; or infectious diseases were excluded. The control group was composed of women undergoing induced abortions of a normal pregnancy at a gestational age matched to that of the RSA group. None of the study participants had experienced any previous spontaneous abortions and had delivered at least one healthy baby at term. The patients in the control group were not treated with any drugs and had no pregnancy risk factors.

Gestational age was determined by last menstruation period. All patients provided informed written consent, and this investigation was approved by the Ethics Committee of IPMCH. Plasma samples were kept at –80°C until analysis.

Sample preparation and analysis by GC-MS

Peripheral venous blood was collected from all study participants using ethylene diamine tetra-acetic acid (EDTA) for anticoagulation. Blood samples were allowed to coagulate for approximately 10–20 minutes and then centrifuged at 2000–3000 rpm for 20 minutes. Then, the supernatant (plasma) was stored at –80°C until analyzed.

Plasma samples were thawed and analyzed by GC-MS using the method described by Peng et al. [13]. A 100 µL aliquot of plasma was centrifuged at 15 000 rpm for 10 minutes at 4°C. Then, 50 µL of plasma was spiked with 10 µL of an internal standard (2-chlorophenylalanine, 0.3 mg/mL) and vortexed for 10 seconds. This mixture was then extracted with 150 µL of methanol-acetonitrile (2: 1, v/v) and vortexed for 30 seconds. Samples were cooled for 10 minutes at -20°C and then centrifuged at 15 000 rpm for 10 minutes at 4°C. An aliquot of the 150 µL supernatant was placed in a glass sampling vial

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Table 1. Demographic and clinical details of study participants with RSA compared to the control group.

	RSA	Control	*P*
Sample numbers	50	51	
Maternal age (y)	30.9±3.77	30.35±4.59	NS
Gestational age (wk)	9.7±2.34	8.5±2.25	NS
BMI (kg/m^2)	22.3±3.42	21.5±2.51	NS
Number of spontaneous abortions	2.75±0.45	0	<0.001

RSA – recurrent spontaneous abortion; BMI – body mass index; Data are expressed as mean ±SD. Gestational age of subjects represents the time at which plasma samples were collected. NS – not significant.

and dried under vacuum at room temperature. The residue was first reconstituted in 80 µL of methoxyamine (15 mg/mL in pyridine), vortexed for 30 seconds, and kept at 37°C for 90 minutes. Finally, 80 µL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) (1% trimethylchlorosilane) and 20 µL of n-hexane were added, and the extract was kept at 70°C for 60 minutes.

A 1-µL aliquot of the sample extract was injected in splitless mode onto an Agilent GC-MS system (Agilent, USA). A non-polar DB-5 capillary column (30 m×250 µm ID, J&W Scientific, Folsom, CA, USA) was used for separation, with high purity helium as the carrier gas at a constant flow rate of 1.0 mL/minute. GC temperature programming began at 50°C. This was then followed by ramps of 15°C/minute to 125°C, 5°C/minute to 210°C, 10°C/minute to 270°C, and 20°C/minute to 305°C, with final 5-minute incubation at 305°C. The electron impact (EI) ion source was held at 230°C with a filament bias of –70 V. We used full scan mode (m/z 50–600) and an acquisition rate of 20 spectrum/second in the MS setting.

GC-MS data analysis

Data acquired from GC-MS were then characterized by Chroma TOF software (v 4.34, LECO, St Joseph, MI, USA). The resulting data sets were then imported separately into the SIMCA-P+ 14.0 software package (Umetrics, Umeå, Sweden). After mean centering and unit variance scaling, we used principle component analysis (PCA) and orthogonal partial least-squares-discriminant analysis (OPLS-DA) to identify differences between the RSA and control groups. The variable importance in projection (VIP) generated in OPLS-DA processing represents the contribution to the discrimination of each metabolite ion between groups. Variables with a VIP >1 and $P<0.05$ were considered to be different variables.

We used a variety of statistical techniques to investigate the significance of each of the metabolites identified, including multivariate analysis, the Student's *t*-test, and the Wilcoxon-Mann-Whitney test. Metabolites responsible for differentiating the RSA group from the control group were those in which multivariate and univariate analysis showed statistical significance (VIP >1.0 and $P<0.05$). We then compared the extract masses of all selected metabolites using the Human Metabolome Database [14] (*http://metlin.scripps.edu*) to identify these compounds. Lastly, we used MetaboAnalyst 3.0 (*http://www.metaboanalyst.ca/*) to characterize the potential impact of our selected metabolites [15]. The *P* value and impact value threshold, as calculated via the analysis of pathway topology, were set to 0.05 and 0.1.

Validation of deferential metabolites between the RSA and control groups

We selected 8 metabolites (lactic acid, 5-methoxytryptamine, pyruvic acid, phenylalanine, proline, alanine, threonine, and 3-hydroxybutyric acid) that were quantitatively measured by LC-MS analysis. LC-MS analysis was performed using a Waters UPLC I-class system equipped with a binary solvent pump and a sample manager, coupled with a Waters TQ-XS mass spectrometer equipped with an electrospray interface (Waters, USA). Chromatographic separation was accomplished on an ACQUITY UPLC BEH Amide column (100×2.1 mm ID, packed with 1.7 µm particles, Waters, USA). The mobile phases consisted of 20 mM ammonium formate and 0.1% formic acid (A) and 95% ACN with 20 mM ammonium formate and 0.1% formic acid (B). The mobile phase gradient proceeded from 5% A: 95% B to 60% A: 40% B at a flow rate of 0.4 mL/minute. The autosampler was maintained at 4°C. In order to test the repeatability and stability of the system, the QC sample was randomly inserted into the sample sequence.

Results

Study participants

The participants' detailed characters are shown in Table 1.

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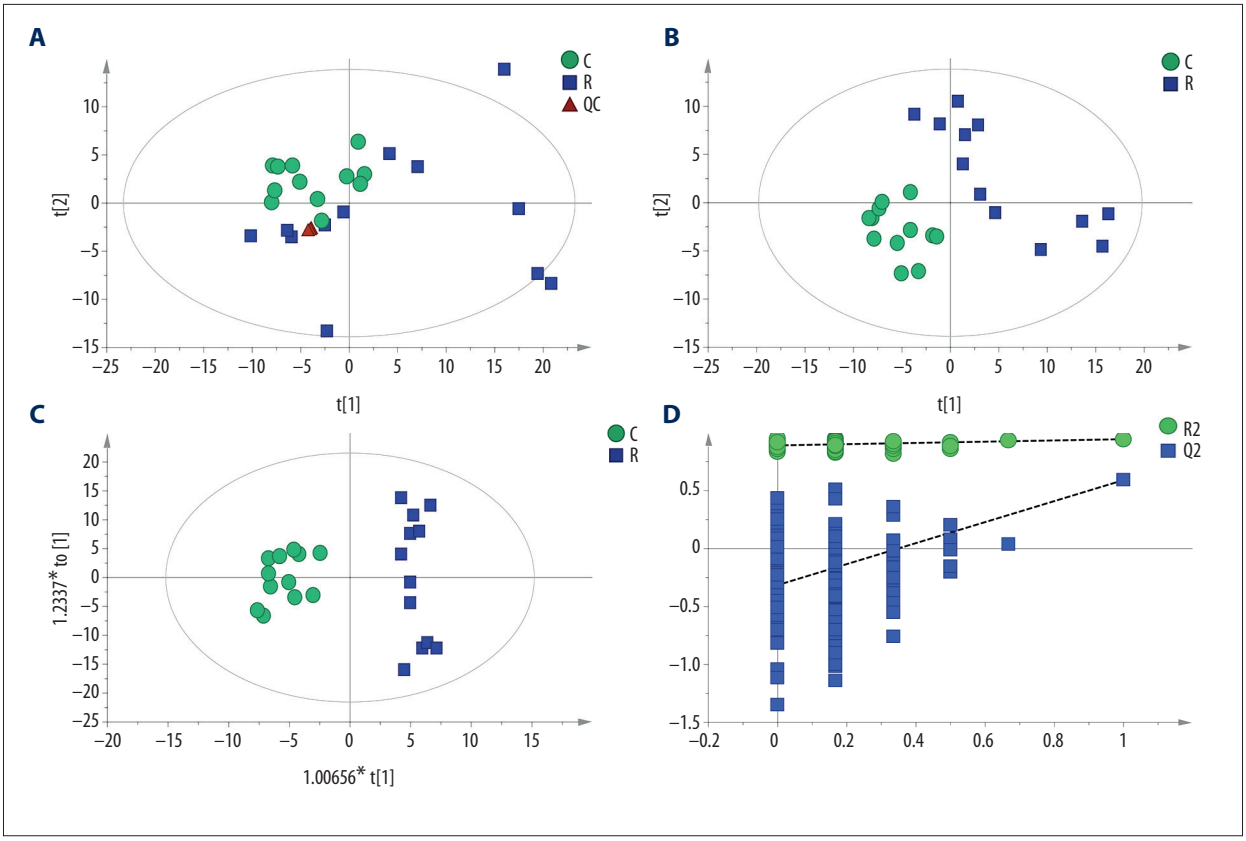

Figure 1. Score scatter plot of (**A**) PCA showing significant differences between the RSA and control groups, (**B**) PLS-DA showing improved discrimination, and (**C**) OPLS-DA showing optimized discrimination. (**D**) Comparison of the true model parameters in the validation test and those of permutated models. Y-axis intercepts: R2=(0.0, 0.891) and Q2=(0.0–0.314).

The average maternal age, gestational age, and the body mass index (BMI) of the patients in the RSA group were 30.9±3.77 years, 9.7±2.34 weeks, and 22.3±3.42 kg/m^2, respectively. The average age, gestational age, and the BMI of the patients in the control group were 30.35±4.59 years, 8.5±2.25 weeks, and 21.5±2.51 kg/m^2, respectively. No significant differences were shown in age, gestational age, or BMI between the 2 groups (P>0.05). Therefore, the 2 groups were suitable for the comparison studies. The mean number of spontaneous abortion in the RSA group was 2.78, compared to 0 in the control group (P<0.001).

Metabolomic profiling of plasma samples from the RSA and control groups

The PCA score plot, which used 5 components (R2Xcum=0.62, Q2cum=0.0996), showed separate trends when comparing maternal plasma from the RSA group and the control group, as shown in Figure 1A. Class separation improved following PLS-DA analysis (Figure 1B), which identified three key parameters: R2Xcum, R2Ycum, and Q2cum (0.376, 0.87, and 0.661, respectively). OPLS-DA identified an optimized class separation (R2Xcum=0.448, R2Ycum=0.945, Q2cum=0.592), which indicated that the model exhibited good fit and could predict RSA in an efficient manner (Figure 1C). Permutation testing (Figure 1D) was performed on the quality of the model and indicated that the model was not over-fitted.

Fifty-four metabolites (VIP >1and P<0.05) are shown in Table 2. To more clearly characterize the plasma profile of RSA, we used a novel software called Heatmap Illustrator, version 1.0 (HemI) to draw a heat map based on the intensity levels of 54 markers between the 2 groups, and the heat map is presented in Figure 2 [16].

Metabolic pathway and function analysis

Metabolic pathways involving glycine, serine and threonine metabolism (P=0.00529, impact=0.26) and beta-alanine (P=0.0284, impact=0.27) along with the TCA cycle (P=0.0113, impact=0.19), phenylalanine metabolism (P=0.0217, impact=0.17), and glycolysis (P=0.037, impact=0.1) were highlighted as targets for investigating the pathological mechanisms underlying RSA (showed in Figure 3).

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Table 2. Potential metabolites associated with RSA and their metabolic pathways.

Mass	R.T. (min)	Metabolites	Related pathway	VIP value	FC (R/C)	*p* Value
234	7.29	3-hydroxybutyric acid	Fatty acid Biosynthesis	1.23	0.41	0.02
180	19.40	Oleic acid	Not available	1.29	0.43	0.02
174	5.79	Pyruvic acid	TCA cycle	1.42	0.58	0.005
201	12.73	Alpha-ketoglutaric acid	TCA cycle	1.07	0.63	0.03
174	18.38	5-Methoxytryptamine	Not available	1.38	0.67	0.01
218	10.00	Serine	Glycine, serine, and threonine metabolism	1.39	1.33	0.009
152	5.70	2-hydroxypyridine	Not available	1.39	1.34	0.03
83	6.48	20alpha-Hydroxycholesterol	Steroidogenesis	1.37	1.34	0.04
117	14.89	2-Deoxyerythritol	Not available	1.38	1.34	0.03
185	22.99	Methyl Palmitoleate	Not available	1.40	1.34	0.04
218	10.34	Threonine	Glycine, serine, and threonine metabolism	1.17	1.35	0.02
218	13.38	Phenylalanine	Phenylalanine and tyrosine metabolism	1.10	1.35	0.02
149	21.68	Dioctyl phthalate	Not available	1.41	1.35	0.02
121	5.22	p-benzoquinone	Pyrimidine metabolism	1.40	1.36	0.04
285	9.55	Phenylacetic acid	Phenylacetate metabolism	1.37	1.37	0.03
215	10.07	Pelargonic acid	Not available	1.36	1.38	0.04
192	12.70	L-kynurenine	Tryptophan metabolism	1.06	1.41	0.04
218	8.06	Valine	Valine, leucine, and isoleucine	1.23	1.43	0.02
130	4.97	N-Ethylglycine	Not available	1.27	1.45	0.04
174	18.49	Noradrenaline	Not available	1.03	1.46	0.02
211	24.29	D-(glycerol 1-phosphate)	Glycerolipid metabolism	1.37	1.48	0.04
369	25.35	Cholesterol	Steroid biosynthesis	1.37	1.48	0.04
130	7.46	N-Methyl-DL-alanine	Not available	1.20	1.49	0.02
218	10.81	Aminomalonic acid	Not available	1.34	1.50	0.004
217	11.87	Threitol	Not available	1.38	1.51	0.007
245	9.91	Fumaric acid	TCA cycle	1.25	1.52	0.04
262	13.49	6-deoxy-D-glucose	Not available	1.37	1.52	0.006
180	18.30	Guanidinosuccinic acid	Not available	1.25	1.54	0.02
217	17.09	Galactinol	Galactose metabolism	1.43	1.54	0.009
158	9.13	Isoleucine	Valine, leucine, and isoleucine	1.22	1.55	0.008
164	6.31	4-hydroxyphenylpyruvate	Phenylalanine and tyrosine metabolism	1.27	1.55	0.04
216	13.05	3-hydroxy-L-proline	Not available	1.46	1.56	0.002
117	5.19	Lactic acid	Pyruvate metabolism	1.09	1.57	0.02
217	13.91	Threo-beta-hydroxyaspartate	Not available	1.37	1.58	0.006
202	21.49	Indolelactate	Not available	1.26	1.65	0.015
218	16.13	D-Arabitol	Not available	1.26	1.66	0.017
158	12.49	N-acetyl-L-aspartic acid	Aspartate metabolism	1.43	1.67	0.004

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Table 2 continued. Potential metabolites associated with RSA and their metabolic pathways.

Mass	R.T. (min)	Metabolites	Related pathway	VIP value	FC (R/C)	*p* Value
369	7.36	Gallic acid	Not available	1.16	1.67	0.03
248	5.30	Beta-Alanine	Beta-alanine metabolism	1.13	1.68	0.04
212	7.74	4-Androsten-19-ol-3,17-dione	Not available	1.27	1.70	0.03
179	19.75	dl-p-Hydroxyphenyllactic acid	Not available	1.22	1.72	0.01
212	7.67	Phosphomycin	Not available	1.39	1.73	0.02
156	9.66	Pipecolinic acid	Not available	1.17	1.74	0.02
248	14.72	Aconitic Acid	TCA cycle	1.72	1.75	<0.001
217	15.68	Xylitol	Not available	1.42	1.86	0.006
116	6.51	Alanine	Alanine metabolism	1.47	1.87	0.005
262	11.22	Erythrose	Not available	1.63	1.89	<0.001
221	10.51	Malonic acid	Aspartate metabolism	1.15	1.92	0.02
262	12.41	N-Methyl-L-glutamic acid	Not available	1.31	1.93	0.007
188	9.43	2,3-Dihydroxypyridine	Not available	1.55	2.00	<0.001
142	9.22	Proline	Proline metabolism	1.57	2.03	0.004
202	19.28	Trehalose-6-phosphate	Not available	1.76	2.56	<0.001
102	6.77	3-Aminoisobutyric acid	Pyrimidine metabolism	1.63	2.63	<0.001
295	15.23	Terephthalic acid	Not available	1.57	2.64	0.002

VIP – variable importance in projection; FC (R/C) – the ratio of relative amounts of RSA group to control group; TCA cycle – tricarboxylic acid cycle.

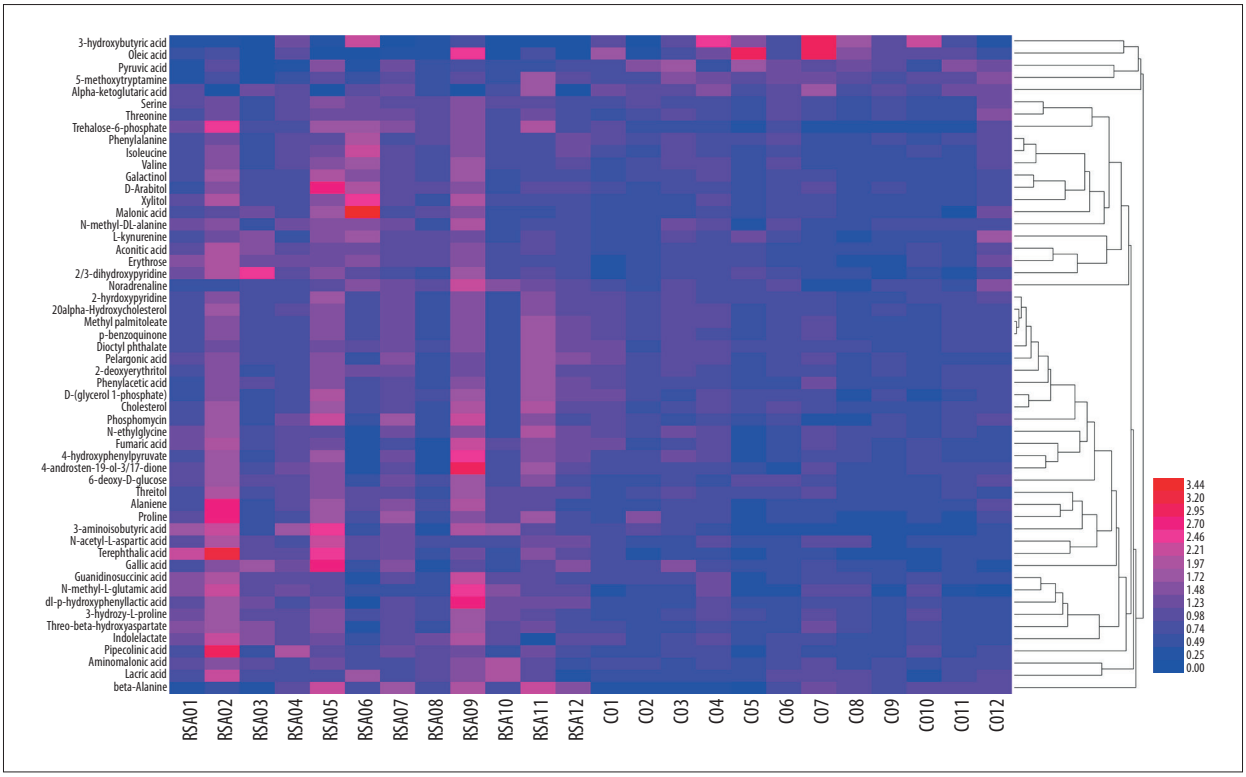

Figure 2. Heat map based on the normalized quantities of potential marker metabolites in the RSA and control groups.

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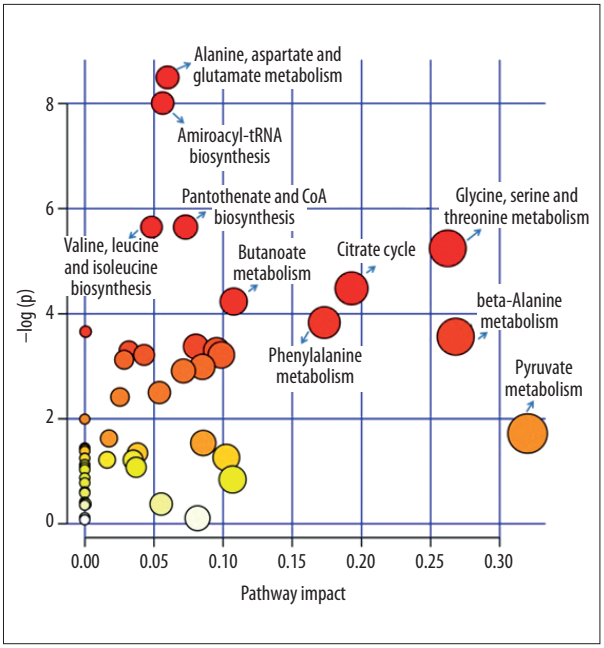

Figure 3. Summary of pathway analysis using MetaboAnalyst 3.0.

Validation of metabolites in the RSA and control groups

Eight amino acids and biogenic amines (lactic acid, 5-methoxytryptamine, pyruvic acid, phenylalanine, proline, alanine, threonine, and 3-hydroxybutyric acid) were quantitatively measured. The concentration of lactic acid in RSA was higher than that in the control group ($P<0.05$). Additionally, 5-methoxytryptamine concentration was significantly lower in the RSA group ($P<0.05$). There was no significant difference in pyruvic acid, phenylalanine, proline, alanine, threonine, and 3-hydroxybutyric acid between groups.

Discussion

Our study indicated that metabolic profiling in unexplained RSA were significantly different from the control group and 54 potential metabolites were identified by using GC-TOFMS. The OPLS-DA models derived from the metabonomic analysis showed clear separation between groups. Furthermore, targeted UPLC-MS/MS was performed to validate metabolites differentially expressed between the RSA and control groups in a more reliable and robust manner [17].

Feng et al. [18] discovered the metabolic profile in plasma in the threatened abortion of women with polycystic ovary syndrome (PCOS) was significantly altered. The metabolomic approach was also applied in our study, showing distinct profiles of metabolites between RSA and control group. Other research reported [19] that in missed abortion, metabolism was disturbed significantly and glyceric acid, indole and sphingosine were found satisfactory metabolites in diagnosis for missed abortion. While the metabolism in RSA is still not reported. In our present study, metabolic pathway impact analysis identified glycine, serine, threonine metabolism, beta-alanine metabolism, the TCA cycle, and phenylalanine metabolism as key biological pathways in the development of RSA. Furthermore, we found significant differences in 5-methoxytryptamine and lactic acid concentrations in RSA patients using LC-MS. Lactic acid arises as a byproduct of anaerobic metabolism. In cases involving sepsis and septic shock, there is a clear increase in the production and clearance of lactic acid leading to lactic acidosis. This condition can be caused by arterial hypotension, microcirculatory dysfunction, and the reduced extraction of oxygen from the peripheral tissues. Albright et al. found that elevated levels of lactic acid in pregnant women were associated with adverse maternal outcomes [20]. The process of implantation shares similarities with the pro-inflammatory response. Similar to the findings of Albright et al., we also found elevated lactic acid levels in our RSA group. Metabolic pathways showed that the impact of glycolysis was 0.1 ($P=0.037$), which implies that lactic acid may play a role in the pathological mechanisms underlying RSA. 5-methoxytryptamine is biosynthesized via the deacetylation of melatonin, which occurs naturally in the body at low levels [21]. It is a strong *in vitro* or *in vivo* antioxidant [22]. Lower 5-methoxytryptamine concentration may play a role in the pathogenesis of RSA. Further exploration is necessary to verify that.

There were some potential limitations of this study when interpreting our conclusions. First, the number of patients in this study was relatively small. Second, it is imperative that further validation experiments are performed in future prospective investigations that involve a larger number of people with RSA. Therefore, our findings can only provide indirect evidence for the pathogenesis of RSA.

To summarize, we investigated differences in metabolites in the plasma of RSA patients. This study suggests that plasma metabolic profiling has great potential in differentiating RSA patients from control patients, and the metabolism of lactic acid and 5-methoxytryptamine is significantly different between RSA and control groups, implying that the differential metabolites might be novel biomarkers of RSA.

Conclusions

Metabolic screening was conducted via GC-MS to identify clear metabolic differences between RSA patients and a control group. LC-MS quantitative analysis showed that lactic acid and 5-methoxytryptamine concentrations in RSA patients were significantly different from a control group.

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Conflict of interests

None.

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