Early potential metabolic biomarkers of primary postpartum haemorrhage based on serum metabolomics

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ABSTRACT

Objectives: Postpartum hemorrhage (PPH) is the leading cause of maternal death, accounting for 1/4 of maternal deaths worldwide. Determining sensitive biomarkers in the peripheral blood to identify postpartum haemorrhage (PPH) is essential for the early diagnosis and management of PPH. The purpose of this study is to identify predictive serum metabolic biomarkers of PPH. Thirty healthy pregnant women and 30 cases of postpartum hemorrhage were studied for our research.

Material and methods: The serum metabolites of all pregnant were detected by liquid chromatography-quadruple time-of-flight mass spectrometry (LC-QTOFMS) and the corresponding biomarkers were identified.

Results: 34 significantly altered metabolites in PPH-pre-group were identified. They were mainly involved in fatty acid, and glycerophospholipid metabolism.

Conclusions: The LysoPCs, PCs, PGs, PIs were effective biomarkers for identifying PPH. The disturbed signaling pathways, mTOR signaling, acute phase response signaling, AMPK signaling and eNOS signaling pathways might be related to the etiopathogenesis of PPH. Our study provided a valuable attempt to screen early diagnostic markers of PPH and to further understand its pathogenesis.

Key words: biomarker; metabolomics; primary postpartum haemorrhage

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INTRODUCTION

Postpartum haemorrhage is the leading cause of death during pregnancy and preterm birth, accounting for 1/4 of maternal deaths worldwide [1]. It is defined as blood loss of more than 500 mL after the birth of the genital tract (or > 1000 mL after a caesarean section). It is estimated that 140,000 women die of PPH each year [2]. Of postpartum deaths, 45% occur within the first 24 hours and 66% occur within the first week. The prevalence of PPH worldwide is between 6% and 10% [3]. The reason for deaths of PPH is that is not easy to notice immediately or earlier which is important to early diagnosis and intervention for PPH. Women who have prolonged childbirth, multiple pregnancies, amniotic fluid, and fetal macrosomia, obesity or fever during childbirth are at increased risk. Previous studies have identified risk factors associated with the PPH [4, 5], such as history of PPH, overdistended uterus, nullipara or low parity, multiple birth, high blood pressure, ante-partum hemorrhage and increased maternal BMI have been significantly associated with PPH [5]. While these associations are not the sensitive predictive biomarkers, women with these risk factors are not suffering from postpartum hemorrhage, while some pregnant women without these risk factors may be suffering from postpartum hemorrhage.

Metabolomics is a discipline that studies the changes of metabolites induced by physiological stimuli or genetic modifications in living systems [6]. Until now, metabolomics has been applied to identify early diagnostic markers of various diseases and study pathogenesis of them. Furthermore, metabolomics is a rapid method for qualitative and semiquantitative analysis of metabolites in cells, biological fluids and tissues. Due to its unique advantages and significant efficiency, metabolomics has been widely used in studying diseases of obstetrics and gynecology in various

aspects. For example, a series of experimental investigations had been conducted on gestational diabetes mellitus [7, 8], preeclampsia [9, 10] and cancers [11, 12], and to identify the potential markers in maternal serum, plasma, amniotic fluid (AF), cord blood, urine, feces, trophocyte, or tissues. While until now, there is no metabolomics study of PPH for the early diagnostic biomarker identification before childbirth.

In our study, we first employed a LC-MS-based metabolomics study to characterize the metabolic profiles of PPH-pre, and then used bioinformatics analysis software to identify corresponding pathogenesis of PPH. Our aim is to search for effective biomarkers and further understanding the pathogenesis of PPH.

MATERIAL AND METHODS

Patients

Pregnant women with no previous history of pregnancies were included in our study. The subjects were assessed for their health status. Their weight and height information was also recorded, and body mass index was calculated to be kg/m^2. These women were asked to fast for one hour prior to serum collection to prevent the effects of diet on metabolism. All subjects signed informed consent when they were admitted to this study. This study was approved by the research ethics committee of Tongren Hospital, School of Medicine, Shanghai Jiaotong University. Collection of pregnant women's serum within 24 hours prior to the production as the study subjects. After childbirth, calculation of the amount of bleeding in pregnant women with parturition. Women with total bleeding volume more than 500 mL following vaginal delivery were included as the PPH, and these serums collected within 24 hours prior to the production were included as PPH-pre-group. The serum collected within 24 hours before the production from women with total bleeding volume less than 500 mL was included as control group. Lastly, 30 cases of postpartum hemorrhage and 30 women in control group were collected. The patient's age, gestational age, total blood loss during PPH were recorded.

Serum Sample Collection

Venous blood was drawn into a non-heparin tube, placed for 20 minutes at 25°C, and then centrifuged for 10 minutes at 3000 rpm. One Hundred μL serum samples were added with 300 μL methanol with internal standard (2-chlorobenzene alanine, 0.2 mg/mL) and then the sample was vortexed and placed for 1 min then the supernatant was collected after a 12,000 rpm centrifuge for 10 mins at 4°C.

LC-Q-TOF/MS

A 3 μL aliquot of sample was injected into Agilent 1290 LC-Agilent 6545 QTOF/MS (Agilent Technologies,

Santa Clara, CA, USA). All samples were separated with a HSS T3 C18 column (100 mm × 2.1 mm, 1.7 μm, Waters, USA) and the column was heated at 45°C. The following gradient program was used water and acetonitrile. The elution procedure was 5% acetonitrile for 0–2 min; 2–95% acetonitrile 0.1% formic acid for 2–13 min; elution with 95% acetonitrile 0.1% formic acid for 13–15 min; post-time step for 3 min. The flow rate was 0.3 mL/min. Mass detection was operated with an electrospray source operating in either positive or negative ion mode. The mass spectrum parameters are set as follows: drying gas (N2) flow rate was set at 7 L/min; gas temperature was set at 330°C; pressure of nebulizer gas was 35 psig; Vcap was 4200V; fragmentor was 165V; skimmer was 60V; scan range (m/z) was 80–1000. The MS/MS detection was used the targeted MS2 mode with collision energy 10EV, 20EV and 40EV. Serum samples were run randomly, and the quality control (QC) sample was analyzed every 10 serum samples. The analysis should be stopped when the QC sample was abnormal. Then rinse the column and ion source and correct the mass axis until the QC test was normal.

Data Processing and Statistical Analysis

The Agilent Mass Hunter Qualitative Analysis Software 7.0 (Agilent Technologies, Palo Alto, CA, USA) was used for peak extraction, deconvolution and peak matching from the UPLC-QTOFMS ESI+ and ESI- raw data. After that a list of each peak detected was created including the retention time, m/z and ion intensity information of each ion. And then the SIMCA-P13.0 software (Umetrics AB, Umea, Sweden) was used for Principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). The default 7-fold cross-validation was applied for guard against PLS-DA model over-fitting. Those significantly changed ions were identified and those with the VIP (variable importance in the projection) value more than 1 obtained from the PLS-DA model, as well as P < 0.05 obtained from the Student's t test, were selected as potential biomarkers. Those ions were identified by comparison of exact molecular weight and fragment ion acquired in targeted MS/MS mode with the Human Metabolome Database (http://www.hmdb.ca/) and Metlin (https://metlin.scripps.edu/). Pathway analysis of the markers was used the Web-based software, MetaboAnalyst 3.0 (http://www.metaboanalyst.ca/).

IPA Analysis

To systematically understand the biomarkers of PPH, we uploaded the excel file with the deferentially expressed metabolites (with HMDB IDs and KEGG IDs) and the fold changes information onto an online software Ingenuity Pathway Analysis (IPA) server (IPA, Ingenuity® Systems, http://www.ingenuity.com). Then the bioinformatics analysis was per-

formed in accordance with operational guidelines, and the pathways analysis and networks analysis were carried out based on the knowledge sorted in the database of IPA.

RESULTS

Identification of the Differential Metabolites

After removing those peaks with missing value in the two groups, a total of 1070 peaks of ESI+ and 670 peaks of ESI- were obtained from the acquired data. The PCA score plot showed a separation tendency between the control samples and PPH-pre-individuals, and the QC samples were clustered together illustrating the stability of the method through the whole run (Fig. 1). The PLS-DA score also showed the same separation trend between the control samples and PPH-pre-individuals (Fig. 2A, B), The permutation test of the model also showed that there was no overfitting, which proved that the model was reliable as shown in Figure 2C, D. Thirty-four metabolites with VIP > 1 and p < 0.05 calculated from the PLS-DA model and the Student's t test (p < 0.05) were identified showed in Table 1. Some of metabolites showed increased concentration in PPH patients, such as

LysoPC [20:4 (5Z, 8Z, 11Z, 14Z)], while several lipids, such as PI (O-20:0/0:0), PG (18:0/0:0), octadecanedioic acid and PC (15:0/0:0) were observed in decreased levels in the PPH-pre group.

Metabolic Pathway Analysis with IPA

To study the relationship between metabolites, we used IPA software to conduct molecular pathways and networks analysis. The interaction Network Analysis of differential metabolites between the PPH-pre and control was shown in Figure 3. These metabolites were correlated with mTOR signaling, acute phase response signaling, AMPK signaling and eNOS signaling. IPA revealed that Myo-inositol Biosynthesis (1/9), Urate Biosynthesis (1/22), Guanosine Nucleotides Degradation (1/22), Maturity Onset Diabetes of signaling (1/25), and Adenosine Nucleotides Degradation (1/27) (as shown in Tab. 2) ,which were the top five significantly changed pathways in PPH-pre-compared to control. The metabolic functions analysis by IPA analysis were summarized in Table 2. The most significantly perturbed biological functions
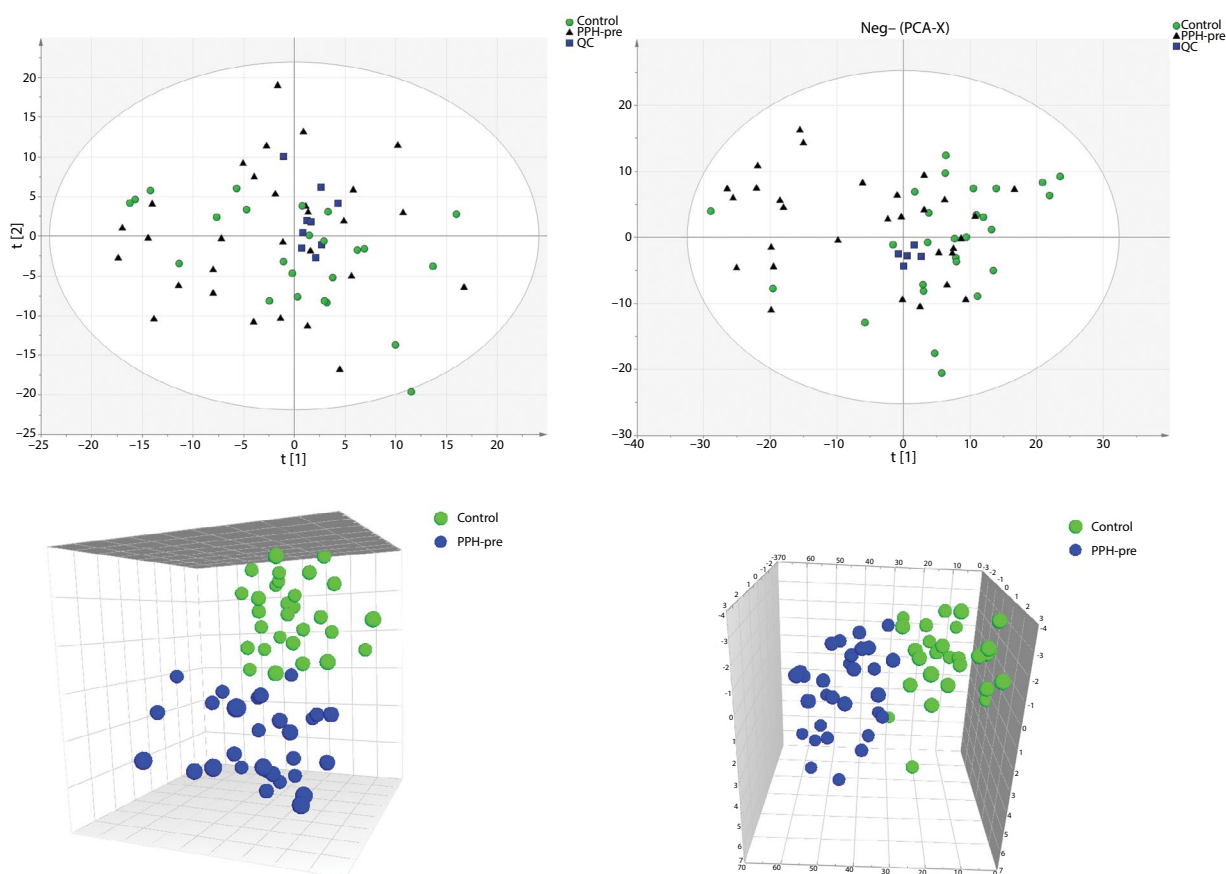
Figure 1. Principle component analysis (PCA) scores plot and 3D scores plot discriminating the metabolic profiles in serum of PPH-pre and those in healthy control. (A), POS (two components model: R2X = 0.34, Q2 = 0.2). (B), NEG (two components model: R2X = 0.41, Q2 = 0.2); ■ — QC group; ● — Healthy Control group; ▲ — PPH-pre group; PPH — Primary Postpartum haemorrhage; QC — Quality Control
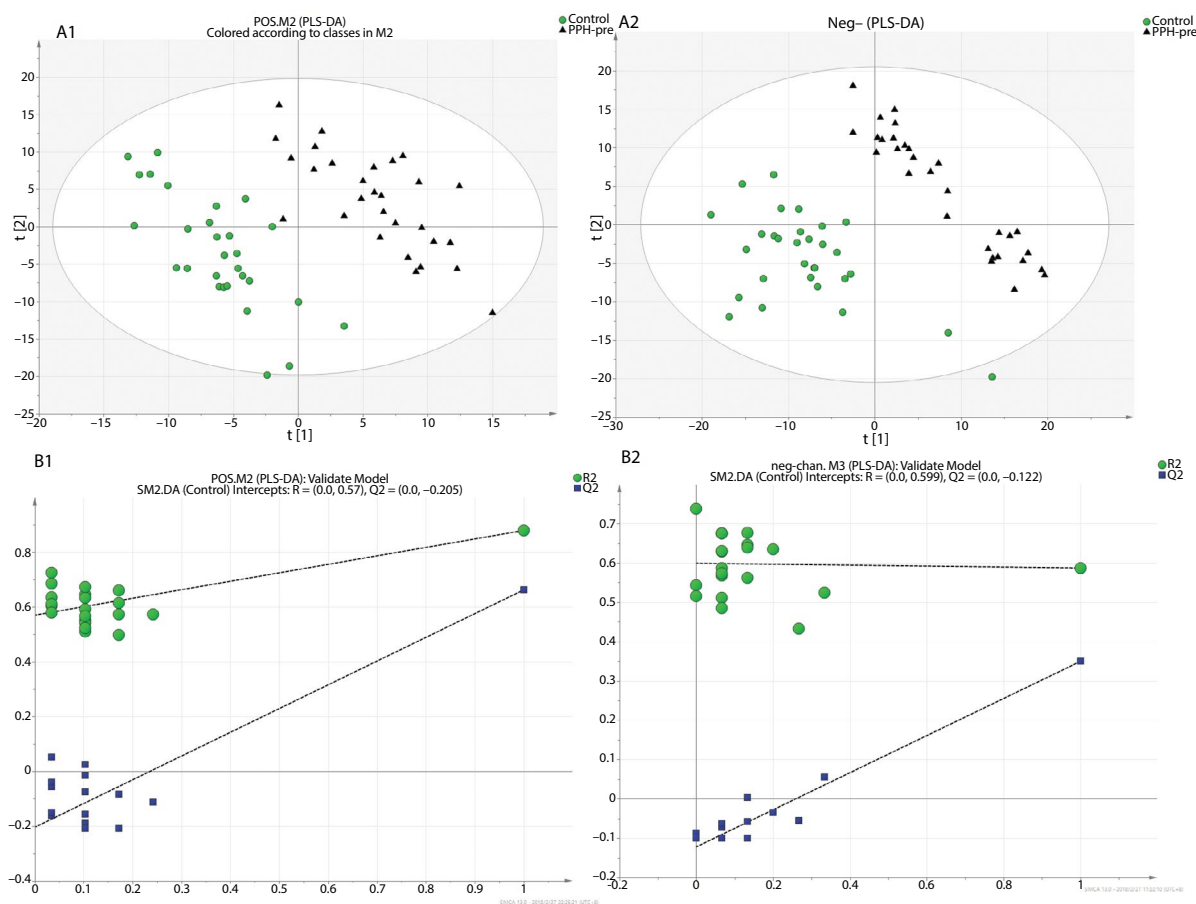
Figure 2. Partial least squares-discriminant analysis (PLS-DA) scores plot and permutation test for the model discriminating serum samples from PPH-pre group and healthy control; ● — Healthy Control group; ▲ — PPH-pre group; (A1) POS-PLS-DA scores plot. The model parameters were: R2Xcum = 0.34, R2Ycum = 0.87, Q2 = 0.89; (A2) NEG-PLS-DA scores plot. The model parameters were: R2Xcum = 0.44, R2Ycum = 0.89, Q2 = 0.87; (B1) A 999-times permutation test for the corresponding model. The Y-axis intercepts were: R2 (0, 0.55), Q2 (0, –0.13). (B2) A 999-times permutation test for the corresponding model. The Y-axis intercepts were: R2 (0, 0.52), Q2 (0, –0.12); PPH — Primary Postpartum haemorrhage

were connective Tissue Disorder (p = 6.02e-04-8.6e-05), Developmental Disorder (p = 8.60e-05-8.60e-05), Hematological Diseases (p = 3.78e-02-8.60e-05), Hereditary Disorder (p = 8.60e-05-8.60e-05) and Inflammatory Diseases (p = 1.21e-02-8.60e-05) (Tab. 2). The top 5 significant molecular and cellular functions were Free Radical Scavenging, Lipid metabolism, Small molecule biochemistry, Cellular Movement and Cell death and survival (Tab. 2). Then we performed pathway enrichment analysis of significantly changed metabolites in PPH group, As shown in Figure 3 and Table 3, Glycerophospholipid metabolism, D-Glutamine and D-glutamate metabolism, Purine metabolism, Linoleic acid metabolism, Citrate cycle (TCA cycle), Vitamin B6 metabolism.

DISCUSSION

PPH causes approximately 14 million deaths each year and the prevalence of PPH worldwide is between 6% and 10%. The major reason for deaths of PPH is that related medical emergencies are not easy to early diagnosis even intervene immediately. Until now the diagnosis of PPH has been dependent on the estimate the amount of bleeding with low predictive ability and accuracy. Metabolomics is a relatively new tool for biomarkers identification for diseases like genomics, transcriptomics, and proteomics. We firstly applied metabolomics study on PPH using LC/MS to screen the predictive biomarkers of PPH. In our study, vaginal delivery pregnant women without PPH and pregnant women with PPH were recruited, then we detected the metabolites of serum obtained prior to childbirth. In our study, 34 significantly altered metabolites in PPH-pre-group were identified. They were mainly involved in fatty acid, and glycerophospholipid metabolism.

Fatty acids, especially the unsaturated derivatives, affect many physiological functions and a wide range of mechanisms [13]. In addition to the source of energy, polyunsaturated fatty acids (PUFA) also endow cell mem-

Table 1. The potential biomarkers of PPH detected by UPLC-Q-TOF/MS and their variation tendency

RT	Mass	Name	HMDB	KEGG	vip	p	PPH/Control
0.58	801.7194	SM (d17:1/24:0)	HMDB0011695	–	1.72	0.01	0.47
0.93	173.0046	Quinaldic acid	HMDB0000842	C06325	2.00	0.00	0.24
13.63	750.5649	PG (16:0/18:0)	HMDB0010572	–	2.17	0.00	0.49
14.24	757.5686	PC [20:2(11Z, 14Z)/14:0]	HMDB0008328	C00157	1.83	0.02	1.60
0.56	145.989	oxoglutarate	HMDB0000208	C00026	1.86	0.02	1.13
12.43	281.2718	Oleamide	HMDB0002117	C19670	1.73	0.03	1.32
4.14	145.0527	N-Butyrylglycine	HMDB0000808	–	1.88	0.02	0.89
10.11	543.3333	LysoPC [20:4 (5Z, 8Z, 11Z, 14Z)]	HMDB0010395	C04230	1.57	0.04	1.58
11.28	547.3641	LysoPC [20:2 (11Z, 14Z)]	HMDB0010392	C04230	1.70	0.03	1.84
9.65	517.3172	LysoPC [18:3 (6Z, 9Z, 12Z)]	HMDB0010387	C04230	2.34	0.00	1.30
2.26	119.0739	L-Threonine	HMDB0000167	C00188	1.44	0.04	0.89
1.45	161.0509	Indole-3-carboxylic acid	HMDB0003320	C19837	1.54	0.04	1.39
8.32	248.1988	hexadecatetraenoic acid	–	–	1.77	0.00	0.24
12.20	413.3507	Heptadecanoyl carnitine	HMDB0006210	–	1.70	0.03	0.44
9.40	184.1462	hendecenoic acid	–	–	1.63	0.05	0.37
10.68	196.146	dodecadienoic acid	–	–	1.83	0.02	0.38
10.21	519.3365	1-Linoleoylglycerophosphocholine	HMDB0010386	C04230	2.14	0.01	0.80
1.24	168.0287	Uric acid	HMDB0000289	C00366	1.32	0.04	2.00
9.39	379.2497	Sphingosine-1-phosphate	–	–	1.75	0.00	0.83
12.72	464.351	SM (d18:1/0:0)	HMDB0006482	C03640	1.36	0.03	0.59
9.65	539.324	PS (19:0/0:0)	–	–	1.89	0.00	2.17
7.46	614.3683	PI (O-20:0/0:0)	–	–	1.94	0.00	0.76
11.91	586.3609	PI (O-18:0/0:0)	–	–	1.50	0.02	0.81
10.61	558.3299	PI (O-16:0/0:0)	–	–	1.41	0.03	0.79
8.08	510.2843	PG [18:1 (9Z)/0:0]	–	–	1.48	0.02	0.70
7.62	512.3004	PG (18:0/0:0)	–	–	1.30	0.04	0.81
6.70	482.2532	PG [16:1 (9Z)/0:0]	–	–	1.53	0.01	0.41
10.61	481.3185	PC (15:0/0:0)	–	–	1.80	0.00	0.73
10.20	555.3109	Pateamine	–	–	2.44	0.00	0.71
7.73	368.1668	PA (6:0/6:0)	–	–	1.84	0.00	3.96
10.24	450.263	PA [19:1 (9Z)/0:0]	–	–	1.32	0.04	0.77
11.50	314.2455	Octadecanedioic acid	HMDB0000782	–	1.24	0.05	0.55
0.66	60.0211	Urea	HMDB0003344	C00266	1.38	0.03	0.71
0.73	260.0293	D-Glucose 6-phosphate	HMDB0001401	C00092	1.27	0.04	2.08

branes with unique structural and functional properties, modulate cellular and intercellular communication and gene expression [14]. Certain fatty acids regulate transcription by peroxisome proliferator activated receptor family (PPARs). PPARs may play a role in placental metabolism, fetal development and preeclampsia [15, 16]. During pregnancy, fatty acids accumulated in developing tissues [17] and the supply of maternal PUFA is crucial for mothers and neonates [18, 19]. The γ-3 fatty acid can inhibit the thrombosis and reduce the incidence and mortality of cardiovascular diseases. Other research

suggested that γ-3 fatty acid also could reduce the blood pressure, triglyceride concentration, and risk of vascular endothelial dysfunction [20, 21]. In our study, the fatty acids were decreased in PPH-pre, this may be the warning signal for PPH. Most importantly is that we may also give the pregnant women ω-3 fatty acids supplemental diet for prevent PPH.

Phospholipids are hydrolyzed to produce lysophospholipids and free fatty acids. Lysophospholipids exists in biological fluids and plays an important role in cell proliferation, migration and survival [22, 23]. Lysophos-
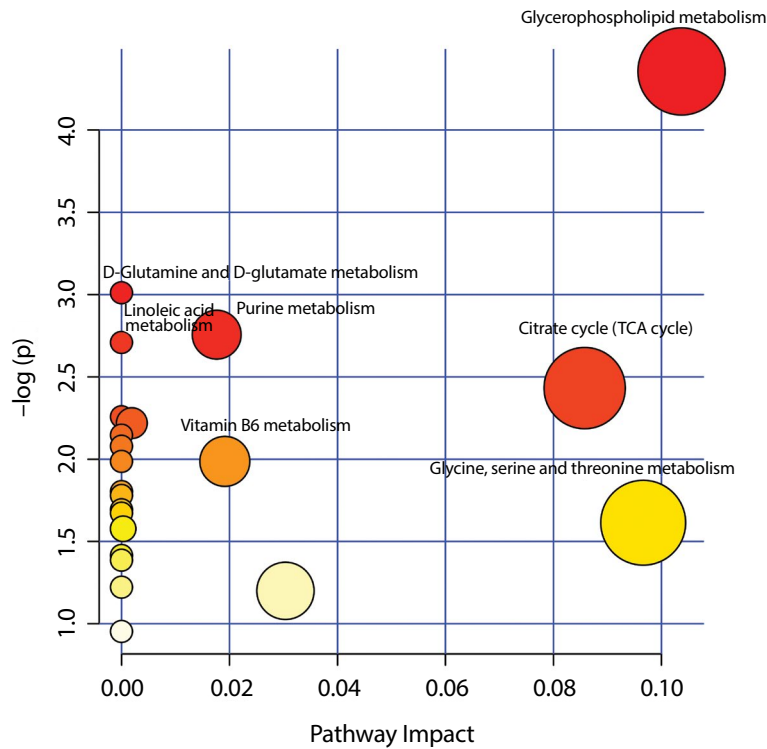
Figure 3. Summary of the pathway analysis of biomarkers of Primary Postpartum haemorrhage

Table 2. Summary of Biomarkers Function Analysis			
Top canonical pathways		**p-value**	**Overlap**
Myo-inositol Biosynthesis		7.74e-04	11.1%,1/9
Urate Biosynthesis		1.89e-03	4.5%, 1/22
Guanosine Nucleotides Degradation		1.89e-03	4.5%, 1/22
Maturity Onset Diabetes of signaling		2.15e-03	4%, 1/25
Adenosine Nucleotides Degradation		2.32e-03	3.7%, 1/27
Diseases and bio functions		**p-value range**	**Molecules**
Connective Tissue Disorder		6.02e-04-8.6e-05	1
Developmental Disorder		8.60e-05-8.60e-05	1
Hematological Diseases		3.78e-02-8.60e-05	1
Hereditary Disorder		8.60e-05-8.60e-05	1
Inflammatory Diseases		1.21e-02-8.60e-05	1
Molecular and cellular functions		**p-value range**	**Molecules**
Free Radical Scavenging		8.58e-03-6.57e-05	2
Lipid metabolism		1.22e-02-1.26e-04	2
Small molecule biochemistry		3.98e-02-1.26e-04	2
Cellular Movement		2.08e-02-1.72e-04	1
Cell death and survival		2.92e-02-2.58e-04	1

ID	Molecules in Network	Score	Focus	Top Diseases and Functions
1	APOA1, ASB7, cholesterol sulfate, COL4A1, Collagentype VII, CPB2, CPN1, F13A1, F13B, factor XIII, FGA, FGB, FGG, Fibrin, Fibrinogen, glycosylphosphatidylinositol, GPLD1, HDL, Hmgn3, HSPG2, ITIH4, LAMA3, LAMB3, LPA, miR-18a-5p (and other miRNAs w/seed AAGGUGC), NID1, P38 MAPK,PDGF-DD, Pzp, SERPINF2, Stat3-Stat3, Tgf beta, TGFB1, TLL1, TLL2	27	10	Organismal Injury and Abnormalities, Hematological System Development and Function, Developmental Disorder
2	ABCB4, ACOX1, AIRE, C1QA, choline, CKMT1A/CKMT1B, CXCR6, CYCS,D-glucose, FST, FTL, G6PC, Gm15807/Hmgn5, GPD1, Hbb-b2,Hmgn3, IFNG, IL16, LAMA4, LAMB2, LTB, MAFK, Mbl1, MLXIPL, Mup1(includes others), NR1I2, NRF1, PEPCK, PLAC8, RETNLB, RORA, RORC, SCD, SMARCB1, TP53	9	4	Inflammatory Response, Cancer, Organismal Injury and Abnormalities

Table 3. Pathway analysis of biomarkers of Primary Postpartum haemorrhage

Pathway Name	Match Status	p	-log (p)	Holm p	FDR	Impact
Glycerophospholipid metabolism	2/39	0.012832	4.3558	1.0	0.9181	0.1037
D-Glutamine and D-glutamate metabolism	1/11	0.049237	3.0111	1.0	0.9181	0.0
Purine metabolism	2/92	0.06351	2.7566	1.0	0.9181	0.01763
Linoleic acid metabolism	1/15	0.066588	2.7092	1.0	0.9181	0.0
Citrate cycle (TCA cycle)	1/20	0.087871	2.4319	1.0	0.9181	0.08577
Alanine, aspartate and glutamate metabolism	1/24	0.10458	2.2578	1.0	0.9181	0.0
Sphingolipid metabolism	1/25	0.10871	2.219	1.0	0.9181	0.00191
Valine, leucine and isoleucine biosynthesis	1/27	0.11693	2.1462	1.0	0.9181	0.0
alpha-Linolenic acid metabolism	1/29	0.12507	2.0789	1.0	0.9181	0.0
Lysine biosynthesis	1/32	0.13716	1.9866	1.0	0.9181	0.0
Vitamin B6 metabolism	1/32	0.13716	1.9866	1.0	0.9181	0.01914
Inositol phosphate metabolism	1/39	0.16479	1.8031	1.0	0.9181	0.0
Butanoate metabolism	1/40	0.16867	1.7798	1.0	0.9181	0.0
Histidine metabolism	1/44	0.18402	1.6927	1.0	0.9181	0.0
Ascorbate and aldarate metabolism	1/45	0.18782	1.6723	1.0	0.9181	0.0
Glycine, serine and threonine metabolism	1/48	0.19912	1.6139	1.0	0.9181	0.09661
Glyoxylate and dicarboxylate metabolism	1/50	0.20657	1.5771	1.0	0.9181	0.0
Starch and sucrose metabolism	1/50	0.20657	1.5771	1.0	0.9181	3.1E-4
Pyrimidine metabolism	1/60	0.2429	1.4151	1.0	0.99993	0.0
Arachidonic acid metabolism	1/62	0.24998	1.3864	1.0	0.99993	0.0
Aminoacyl-tRNA biosynthesis	1/75	0.29457	1.2223	1.0	1.0	0.0

phatidylcholine (LPC) is a bioactive lysophospholipids, mainly produced by the action of phospholipase A2 (PLA2) enzyme on the plasma membrane. After cell uptake, the free LPC reaction produces PC or deacylation to produce FA and choline. These endogenous lysophosphatidates regulate the PPAR gamma function required for vascular wall pathology and metabolic related diseases. The imbalance of PPAR gamma can induce cell function changes, including ROS production, NOS and cytokines expression. In our study, the level of PCs, PSs, PIs were decreased in the PPHand the lysoPCs were increased in the PPH, in which the latter may be cause the coagulation dysfunction in the PPH. According to our study, it may be useful to monitor the changes of lipids to assess the risk of PPH, especially for the lysoPCs.

Although the occurrence of PPH is associated with a variety of causes, in most cases, PPH is caused by bleeding from the site of the placenta, which is caused by uterine inertia [24, 25]. Due to high uterine artery blood flow at the end of pregnancy, uterine inertia can lead to severe bleeding. The PPH stepwise initiative management scheme can improve the prognosis. So far, there is no unified conclusion about the pathogenesis of PPH. In addition, we need to find markers for PPH risk prediction and find new

precise mechanisms. According to our pathway analysis with IPA, these potential makers found in our study were related to mTOR signaling, acute phase response signaling, AMPK signaling and eNOS signaling, as shown in Figure 4. These signaling pathways may provide guidance for further studies on the pathogenesis of postpartum hemorrhage.

CONCLUSIONS

In our study, we found the LysoPCs, PCs, PGs, PIs were effective biomarkers for predicating PPH. The disturbed signaling pathways, mTOR signaling, acute phase response signaling, AMPK signaling and eNOS signaling might be related to the etiopathogenesis of PPH. Our study provided a valuable attempt to screen early diagnostic markers of PPH and to further understand its pathogenesis.

Path Designer Networks 1,2 Merged 1
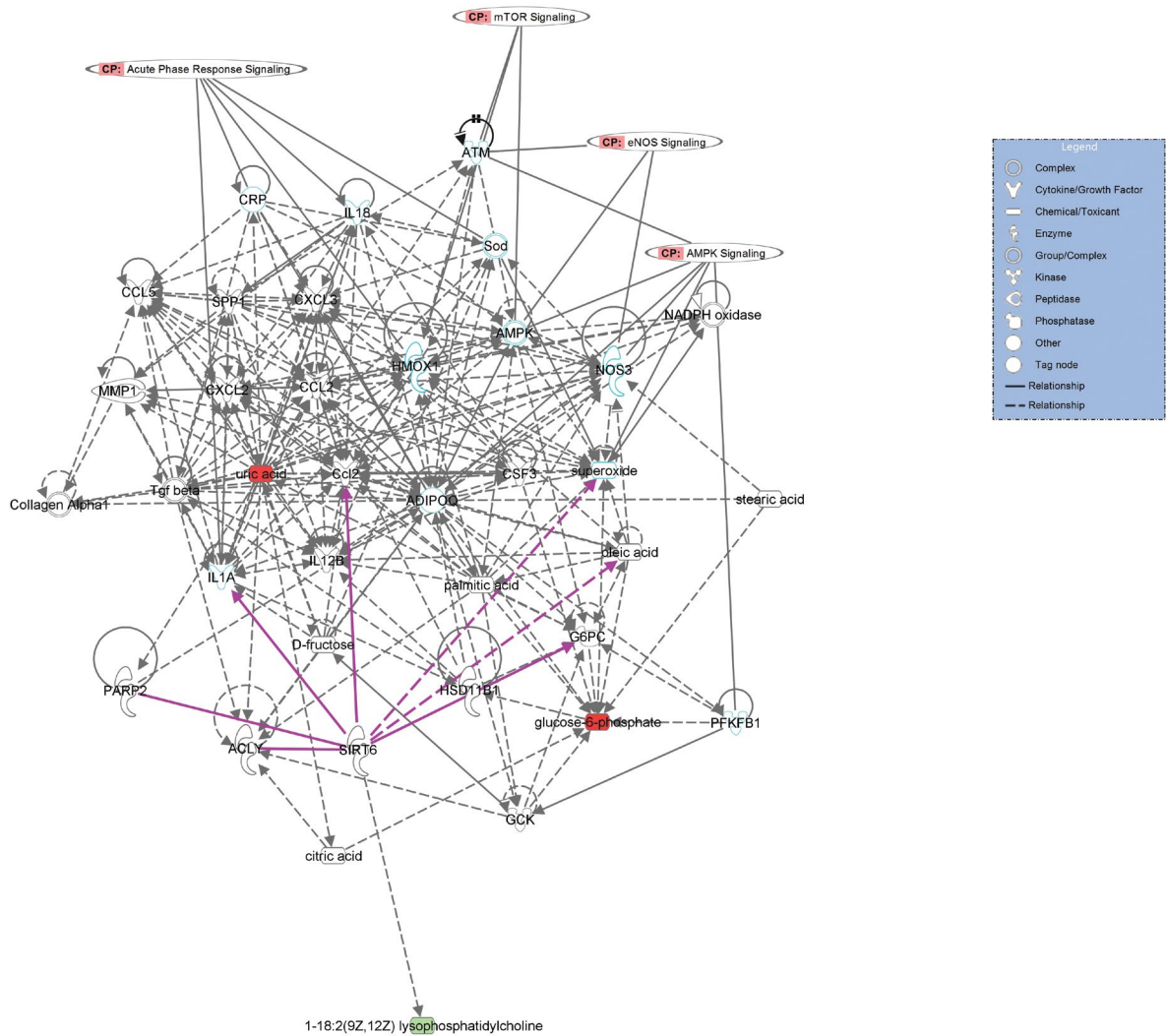
Figure 4. Biological network, canonical pathways and functions related to the identified metabolites. In the network, molecules are represented as nodes, and the biological relationship between two nodes is represented as a line. Red symbols represent up-regulated metabolites; blue symbols represent down-regulated metabolites; while the green symbols represent canonical pathways that are related to the identified specific metabolites. Solid lines between molecules show a direct physical relationship between molecules, while dotted lines show indirect functional relationships

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