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ORIGINAL RESEARCH

Serum Proteomic Signatures in Umbilical Cord Blood of Preterm Neonates Delivered by Women with Gestational Diabetes

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Background: Women who develop diabetes during pregnancy are at higher risk of preterm birth. Here, we identified differentially expressed proteins (DEPs) in the serum of umbilical cord blood samples obtained from preterm neonates delivered by women with gestational diabetes to provide therapeutic targets for clinical drug development.

Materials and Methods: Umbilical cord blood was collected after delivery of preterm neonates by women with gestational diabetes and after delivery of healthy neonates by women without diabetes. DEPs in the serum samples were identified using liquid chromatography–tandem mass spectrometry. Gene Ontology (GO), cluster analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) were used to determine the biological functions associated with these DEPs. Enzyme linked immunosorbent assay was used to confirm the key DEPs.

Results: We found that 21 proteins were significantly upregulated, and 51 proteins were significantly downregulated in 72 DEPs in serum samples. GO analyses showed that the DEPs were mainly associated with the GO terms cellular process, biological regulation, cellular anatomical entity, and binding. KEGG signaling pathway analysis indicated that most of the upregulated DEPs were associated with the complement and coagulation cascades, *Staphylococcus aureus* infection, pertussis, HIF-1 signaling pathway and PPAR signaling pathway and that most of the downregulated DEPs were associated with the complement and coagulation cascades, dilated cardiomyopathy, pathways in cancer, Chagas disease, and hypertrophic cardiomyopathy. The results of KEGG pathway annotation and enrichment analyses indicated that changes in the complement and coagulation cascades may be importantly associated with preterm delivery of neonates by women with gestational diabetes. The key DEPs were confirmed by enzyme linked immunosorbent assay.

Conclusion: Our proteomics and bioinformatics analyses identified several key proteins and the complement and coagulation cascades pathway that warrant further investigation as potential novel therapeutic targets in preterm delivery among women with gestational diabetes.

Keywords: label-free LC-MS/MS, proteomics, gestational diabetes, preterm birth, complement and coagulation cascades

Introduction

The number of people with diabetes increases every year, including among pregnant women.[1] An increasing number of women in China have received a diagnosis of type 1 or type 2 diabetes, often before they become pregnant.[2,3] A reported prevalence of gestational diabetes is 11.91% in China in 2018, but another study showed that the prevalence of gestational diabetes is various in different cities of China.[4,5] One potential reason for an increase in gestational diabetes is that women are waiting to become pregnant at older ages (>35 years).[6] Women who develop diabetes during pregnancy are at higher risk of developing complications related to the perinatal period and to diabetes.[1,7] Many pregnancies are unplanned and may occur when diabetes is not well controlled. This leads to higher risk of preterm

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birth, cesarean delivery, eclampsia, preeclampsia, or complications from advanced diabetes.[1,7–9] To discover novel therapeutic targets for clinical drug development and to better control the impact of gestational diabetes on the fetus and the occurrence of diabetes complications, two studies in China have used proteomics techniques to identify metabolic profiling of blood serum from gestational diabetes patients and animal model to find novel biomarkers for precision or personalized diagnosis for gestational diabetes.[10,11] In addition, another study using proteomics techniques to identify plasma protein changes of gestational diabetes mellitus at 16-18 weeks.[12] However, it is still necessary to further understand the mechanisms underlying the development of gestational diabetes and the effect of gestational diabetes on fetus.

Preterm birth, accounting for 11% of pregnancies worldwide, is the leading cause of perinatal death.[13] The causes undergirding preterm birth are various and complex and include gestational diabetes, periodontal health, preeclampsia, intrauterine infection, chorioamnionitis, and multiple births.[14,15] In China, a study reported that the prevalence of preterm birth of gestational diabetes is 13.41%.[16] Prevention of preterm birth is considered a public health priority because it has the potential to reduce morbidity and mortality in infants and children. Therefore, the discovery of new therapeutic targets and treatment strategies is important for preventing the preterm birth.

Proteomics is a robust method used to detect and quantify serum proteins that are expressed differentially in persons with a condition of interest. In the present study, we used label-free proteomics techniques to detect the differentially expressed proteins (DEPs) in neonatal cord blood from women with gestational diabetes who delivered preterm neonates and from women without diabetes who delivered healthy full-term neonates. Using bioinformatics analyses, we identified the functional roles of the various DEPs and their related signaling pathways to find those DEPs that may be key players in the occurrence and development of preterm birth associated with gestational diabetes. Those DEPs warrant further study as potential novel therapeutic targets for the treatment of the preterm birth.

Materials and Methods

Clinical Information and Sample Collection

All our experiments comply with the Declaration of Helsinki. Neonatal cord blood samples were collected from four women with gestational diabetes who delivered preterm neonates and from four women without diabetes who delivered healthy full-term neonates, all of whom had been admitted to the Anhui Province Maternity and Child Health Hospital. All pregnant women have complete clinical medical records and regular pregnancy examination without hereditary diseases. The delivery was successful. In healthy control group, inclusion criteria were: (1) ≥37 weeks gestation; (2) no disease history before or during pregnancy. Exclusion criteria were: (1) <37 weeks gestation; (2) disease history before or during pregnancy; (3) hereditary disease history. In diabetes group, inclusion criteria were: (1) <37 weeks gestation; (2) no history of diabetes before pregnancy; (3) pregnant with diabetes mellitus and using insulin to control blood glucose. Exclusion criteria were: (1) ≥37 weeks gestation; (2) diabetes history before pregnancy; (3) pregnant with diabetes mellitus and blood glucose was not controlled in the standard reference range; (4) hereditary disease history. Gestational diabetes mellitus diagnosis was conducted according to the World Health Organization (WHO) criteria.[17] All women with gestational diabetes were diagnosed at the middle of pregnancy and treated during pregnancy with sufficient insulin to maintain blood glucose levels within the standard reference range. This study was approved by the Medical Ethics Committee of Anhui Province Maternity and Child Health Hospital (approval number YYLL2020-2020xkj235-02-01), and all participants provided written informed consent. The study was carried out from 03/2021 to 12/2021. Inclusion criteria were: (1) all women conformed to the clinical diagnostic criteria; (2) regular pregnancy examination; (3) successful delivery; (4) no hereditary diseases of coagulation system; (5) complete clinical medical records; and (6) pregnant women and family members gave informed consent to participate in this study. Exclusion criteria were: (1) women were diagnosed with diabetes mellitus or impaired glucose regulation before pregnancy; (2) women with other pregnancy-related diseases; (3) heart, liver or kidney dysfunction; (4) hematological diseases; (5) other diseases that may affect blood glucose; and (6) mental illness or retardation.

After the neonate was delivered, the blood samples from the umbilical cord were collected and placed at room temperature for 1 h prior to being centrifuged at 3000 × *g* for 15 min. The obtained serum was stored at −80°C for use in the following experiments.

Protein Preparation and Gel Analysis

High-abundance proteins in the plasma were removed using a ProteoMiner Protein Enrichment Kit (Bio-Rad). The protein concentrations were determined using the Bradford method. From each serum sample, 35 μg of total protein was mixed with 5 × sample buffer, boiled, and loaded onto 10% gels for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were electrophoresed at a constant current of 14 mA for 90 min. Coomassie blue was used to stain the resultant protein bands.

Enzymatic Hydrolysis of Proteins and High-Performance Liquid Chromatography with Tandem Mass Spectrometry (LC-MS/MS) Analysis

A flowchart of the study analyses is provided in Supplementary Figure 1. Dithiothreitol (5 μL of 1 M) was added to samples containing 200 μg of each protein. Iodoacetamide (20 μL of 1 M) was added to the samples following incubation at 37°C for 1 h. After another incubation in the dark at room temperature for 1 h, the samples were centrifuged at 14,000 × *g* for 20 min at 4°C. The precipitate was collected, and 100 μL of UA buffer (8 M urea, 100 mM Tris-HCl, pH 8.0) was added. After ultracentrifugation, the resulting pellet was digested with trypsin according to the (mass) protein to enzyme ratio of 50:1 for 12–16 h at 37°C. Next, we used nanoliter flow rate, high-performance liquid chromatography with a liquid phase system to separate the samples. Before adding samples, the chromatographic column was balanced with 95% phase A solution (0.1% formic acid in water). The mobile phases included phase A and phase B (0.1% formic acid in acetonitrile). The phase B gradient parameters were 0–2 min for a linear increase from 6% to 9%; 2–10 min for an increase to 13%; 10–50 min for an increase to 26%; 50–70 min for an increase to 38%; and 70–71 min for an increase to 100%, which was maintained for 71–78 min. We used capillary LC-MS/MS with an Orbitrap Fusion Mass Spectrometer (Thermo Scientific) to separate and analyze all samples.

Database Retrieval and Bioinformatics Analyses

We used Proteome Discoverer, version 2.4 (Thermo Fisher Scientific) software to analyze the LC-MS/MS data. Briefly, human proteome databases containing reviewed UniProt sequences were used to identify peptides with the SEQUST search engine. We used P values < 0.05 and 1.5-fold changes (FC) to define DEPs between the two groups of samples. A FC ≥ 1.5 and $P < 0.05$ represented upregulated proteins, whereas a FC ≤ 0.667 and $P < 0.05$ represented down-regulated proteins. If the FC was between 0.667 and 1.5 or if $P > 0.05$, this was defined as no change detected in protein expression levels between the two groups. For bioinformatics analyses, we obtained gene ontology (GO) results of these DEPs using Metascape, which is a web-based resource (http://metascape.org). Through this analysis, DEPs were mapped to the terms in the database, and the number of proteins per term was determined. In addition, the hypergeometric test was used to identify GO entries that were significantly enriched.[18,19] The signaling pathways were analyzed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology-Based Annotation System (KOBAS) online analysis tool (http://kobas.cbi.pku.edu.cn/),[20] through which we identified those signaling pathways that were significantly enriched with DEPs.

Enzyme Linked Immunosorbent Assay (ELISA)

ELISA experiment was used to confirm the key proteins identified by LC-MS/MS. The kit was purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China). After we collected the blood samples, optical density (OD) values were measured at a wavelength of 450 nm using a microplate reader. The protein concentrations of the blood samples were calculated using a standard curve.

Statistical Analysis

We performed two-tailed Mann–Whitney and Fisher exact tests with SigmaPlot software. Unless otherwise stated, values are expressed as means ± SEM. A two-sided value of $P < 0.05$ was considered statistically significant.

Results

Participants in LC-MS/MS Test

No significant differences were found in mother age and weight gain during pregnancy between the four healthy women and the four women with diabetes (Table 1). However, significant differences were detected for the gestational age, birth weight of neonates and body mass index (BMI) before pregnancy (Table 1). The results showed that the gestational age of neonates delivered by women with gestational diabetes was significantly younger, and the birth weight of the preterm neonates delivered by women with gestational diabetes was significantly lower than neonates delivered by healthy women, and BMI before pregnancy of women with gestational diabetes was significantly higher than that in healthy women.

SDS-PAGE

Proteins in the serum of neonatal cord blood obtained from four women with gestational diabetes who delivered preterm neonates and from four women without diabetes who delivered full-term neonates were separated by SDS-PAGE. The molecular weights of the total proteins ranged from 25 to 180 kDa in all eight samples. The proteins were effectively separated in similar patterns without degradation, indicating that the samples were of sufficient quality to be used in the following experiments. Compared with the bands of the original serum (Lane E in Supplementary Figure 2), the high-abundance proteins were significantly decreased, whereas the bands of other molecular weight regions were significantly increased. The total protein content met the requirements for use in the present study (Supplementary Figure 2).

LC-MS/MS Spectrum Analysis to Identify DEPs

LC-MS/MS was used to detect the proteins. A total of 450 proteins were identified among all serum samples (Supplementary Tables 1 and 2). Among them, 72 serum proteins from neonate cord blood were differentially expressed between preterm and full-term neonates, with 21 upregulated proteins and 51 downregulated proteins (Figure 1A; Table 2). We used cluster analyses to find that the protein expression pattern of the DEPs differed significantly between the two groups (Figure 1B).

Table 1 Demographic Characteristics of Participants

Group	Identification No.	Age of Mother (Years)	Gestational Age (Days)	Neonate Birth Weight (g)
Healthy control	1	32	280	3090
	2	33	287	3550
	3	31	286	3100
	4	35	280	3360
Diabetes	1	35	238	2380
	2	31	257	2510
	3	32	229	1900
	4	31	258	2220

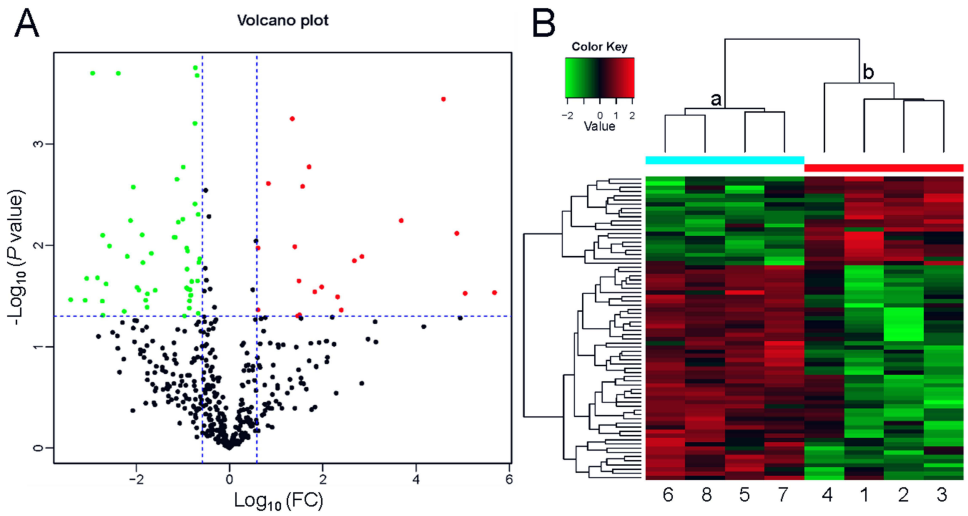

Figure 1 Profile of the differentially expressed proteins. (**A**) Volcano plot. Red dots represent proteins with a significant fold change (FC) > 1.5; green dots, proteins with a significant FC < 0.667; black dots, proteins with no significant change. (**B**) Heatmap showing the upregulation and downregulation of different proteins observed by cluster analysis. Each band in the image represents a protein, each column is a sample (1–4, full-term infant; 5–8, preterm infant), and colors represent various expression levels.

GO Functional Annotation and Enrichment Analysis

GO has three categories indicating how gene functions may be described: biological process, cellular component, and molecular function. GO analysis, a useful method and tool in bioinformatics analyses, has functional annotations

Table 2 Differentially Expressed Proteins in Serum from Umbilical Cord Blood of Full-Term Neonates Delivered by Healthy Women vs Preterm Neonates Delivered by Women with Gestational Diabetes

Change	Protein Accession Number	Protein Symbol	Protein Description	FC
Increased	A0A0G2JPR0	*C4A*	C4a anaphylatoxin	51.43
Increased	P0DJI8	*SAA1*	Serum amyloid A-1 protein	33.22
Increased	Q15848	*ADIPOQ*	Adiponectin	29.42
Increased	P13727	*PRG2*	Bone marrow proteoglycan	24.09
Increased	P55058	*PLTP*	Phospholipid transfer protein	12.87
Increased	P07988	*SFTPB*	Pulmonary surfactant-associated protein B	7.18
Increased	Q9BTY2	*FUCA2*	Plasma alpha-L-fucosidase	6.41
Increased	F8WDD7	*ARPC4*	Actin-related protein 2/3 complex subunit 4	5.28
Increased	C9JVG0	*TF*	Serotransferrin (Fragment)	4.99
Increased	A0A3B3IS80	*ALDOB*	Fructose-bisphosphate aldolase	3.94
Increased	B4DLR2	*FAP*	Prolyl endopeptidase FAP	3.56
Increased	P08185	*SERPINA6*	Corticosteroid-binding globulin	3.27
Increased	Q9UBQ6	*EXTL2*	Exostosin-like 2	2.97
Increased	P02771	*AFP*	Alpha-fetoprotein	2.84
Increased	P06681	*C2*	Complement C2	2.81

(*Continued*)

Table 2 (Continued).

Change	Protein Accession Number	Protein Symbol	Protein Description	FC
Increased	P13645	*KRT10*	Keratin, type I cytoskeletal 10	2.76
Increased	P02760	*AMBP*	Protein AMBP	2.65
Increased	P29622	*SERPINA4*	Kallistatin	2.55
Increased	P05546	*SERPIND1*	Heparin cofactor 2	1.79
Increased	P02679	*FGG*	Fibrinogen gamma chain	1.53
Increased	P01011	*SERPINA3*	Alpha-1-antichymotrypsin	1.53
Decreased	P14151	*SELL*	L-selectin	0.64
Decreased	P19823	*ITIH2*	Inter-alpha-trypsin inhibitor heavy chain H2	0.64
Decreased	Q96PD5	*PGLYRP2*	N-acetylmuramoyl-L-alanine amidase	0.63
Decreased	Q12805	*EFEMP1*	EGF-containing fibulin-like extracellular matrix protein 1	0.63
Decreased	P27169	*PON1*	Serum paraoxonase/arylesterase 1	0.62
Decreased	Q9NZP8	*C1RL*	Complement C1r subcomponent-like protein	0.62
Decreased	P43652	*AFM*	Afamin	0.60
Decreased	P19827	*ITIH1*	Inter-alpha-trypsin inhibitor heavy chain H1	0.60
Decreased	D6RAR4	*HGFAC*	Hepatocyte growth factor activator	0.60
Decreased	C9JB55	*TF*	Serotransferrin (Fragment)	0.57
Decreased	Q7Z7M0	*MEGF8*	Multiple epidermal growth factor-like domains protein 8	0.57
Decreased	H0YD13	*CD44*	CD44 antigen	0.56
Decreased	P55290	*CDH13*	Cadherin-13	0.56
Decreased	P23470	*PTPRG*	Receptor-type tyrosine-protein phosphatase gamma	0.55
Decreased	G3XAP6	*COMP*	Cartilage oligomeric matrix protein	0.55
Decreased	P02747	*C1QC*	Complement C1q subcomponent subunit C	0.54
Decreased	K7ER74	*APOC4-APOC2*	Apolipoprotein C-II	0.54
Decreased	X6RLJ0	*C1QA*	Complement C1q subcomponent subunit A (Fragment)	0.53
Decreased	P41222	*PTGDS*	Prostaglandin-H2 D-isomerase	0.53
Decreased	A0A0C4DGZ8	*GP1BA*	Glycoprotein Ib (Platelet), alpha polypeptide	0.53
Decreased	Q6UWP8	*SBSN*	Suprabasin	0.51
Decreased	P04217	*A1BG*	Alpha-1B-glycoprotein	0.50
Decreased	P00748	*F12*	Coagulation factor XII	0.50
Decreased	E7END6	*PROC*	Vitamin K-dependent protein C	0.47
Decreased	P02647	*APOA1*	Apolipoprotein A-I	0.46
Decreased	P01042	*KNG1*	Kininogen-1	0.45
Decreased	V9GYM3	*APOA2*	Apolipoprotein A-II	0.44

(*Continued*)

Table 2 (Continued).

Change	Protein Accession Number	Protein Symbol	Protein Description	FC
Decreased	P0DOY3	*IGLC3*	Immunoglobulin lambda constant 3	0.33
Decreased	P68871	*HBB*	Hemoglobin subunit beta	0.31
Decreased	P08637	*FCGR3A*	Low affinity immunoglobulin gamma Fc region receptor III-A	0.30
Decreased	A0A6Q8PHJ8	*CDH2*	Cadherin-2 (Fragment)	0.29
Decreased	Q06830	*PRDX1*	Peroxiredoxin-1	0.29
Decreased	P23284	*PPIB*	Peptidyl-prolyl cis-trans isomerase B	0.28
Decreased	P62942	*FKBP1A*	Peptidyl-prolyl cis-trans isomerase FKBP1A	0.27
Decreased	Q99497	*PARK7*	Parkinson disease protein 7	0.26
Decreased	H0Y984	*BST1*	ADP-ribosyl cyclase/cyclic ADP-ribose hydrolase (Fragment)	0.25
Decreased	P18065	*IGFBP2*	Insulin-like growth factor-binding protein 2	0.24
Decreased	P02790	*HPX*	Hemopexin	0.23
Decreased	A0A1B0GVK0	*FGL2*	Fibroleukin	0.22
Decreased	A0A2R8YGX3	*TPM4*	Tropomyosin alpha-4 chain	0.21
Decreased	O95467	*GNAS*	Neuroendocrine secretory protein 55	0.19
Decreased	A0A0G2JHD9	*COL11A2*	Collagen alpha-2(XI) chain (Fragment)	0.17
Decreased	P21333	*FLNA*	Filamin-A	0.16
Decreased	P02775	*PPBP*	Platelet basic protein	0.15
Decreased	A0A087X089	*CCL14*	C-C motif chemokine	0.15
Decreased	P08833	*IGFBP1*	Insulin-like growth factor-binding protein 1	0.15
Decreased	P35579	*MYH9*	Myosin-9	0.14
Decreased	P05019	*IGF1*	Insulin-like growth factor 1	0.13
Decreased	A0A087WWU8	*TPM3*	Tropomyosin alpha-3 chain	0.12
Decreased	P02042	*HBD*	Hemoglobin subunit delta	0.12
Decreased	O75144	*ICOSLG*	ICOS ligand	0.09

Abbreviation: FC, fold change (ratio of the protein level in the two groups).

indicating the number of DEPs in each category, and GO functional enrichment provides GO functional terms related to these DEPs. Thus, through GO analysis, the distribution of DEPs in these three categories and the involvement of the DEPs in cellular functions were identified. In addition, the most important biochemical metabolic pathways and signal transduction pathways were determined.

Our GO functional annotation analysis showed that the highest percentage of DEPs in the biological process classification were related to the term "cellular process" for the upregulated proteins (n = 17 proteins) (Figure 2A), with the top three proteins being SAA1, ADIPOQ and PRG2, and to the term "biological regulation" for the downregulated proteins (n = 37 proteins) (Figure 2B), with the top three proteins being ICOSLG, HBD and IGF1. For the cellular component classification, the highest percentage of DEPs were related to the term "cellular anatomical entity" for both the upregulated proteins (n = 20 proteins) (Figure 2A)—with the top three proteins being C4A, SAA1 and ADIPOQ—and for the downregulated proteins (n = 46 proteins) (Figure 2B)—with the top three proteins being ICOSLG, HBD and TPM3. For the molecular function classification, the highest

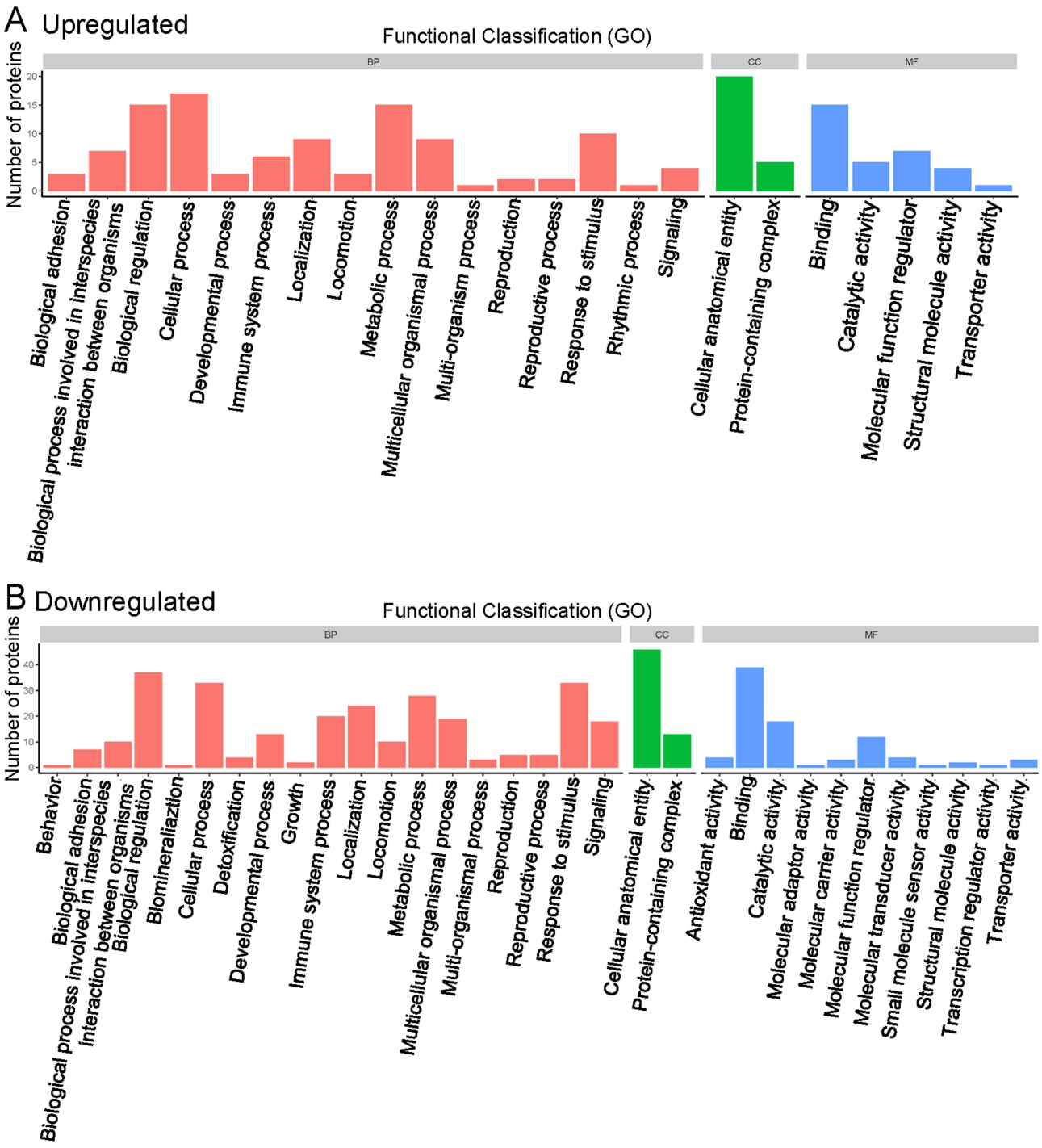

Figure 2 Functional classification of gene ontology analysis. (**A**) Upregulated proteins. (**B**) Downregulated proteins.
Abbreviations: BP, biological process; CC, cellular component; MF, molecular function.

percentage of DEPs were related to the term "binding" for both the upregulated proteins (n = 15 proteins) (Figure 2A)—with the top three proteins being SAA1, ADIPOQ and PRG2—and for the downregulated proteins (n = 39 proteins) (Figure 2B)—with the top three proteins being ICOSLG, HBD and TPM3.

In the GO functional enrichment analysis, the top five upregulated GO terms were serine-type endopeptidase inhibitor activity, endopeptidase inhibitor activity, endopeptidase regulator activity, peptidase inhibitor activity, and peptidase regulator activity (Figure 3A). The top five downregulated GO terms were protein stabilization, positive regulation of

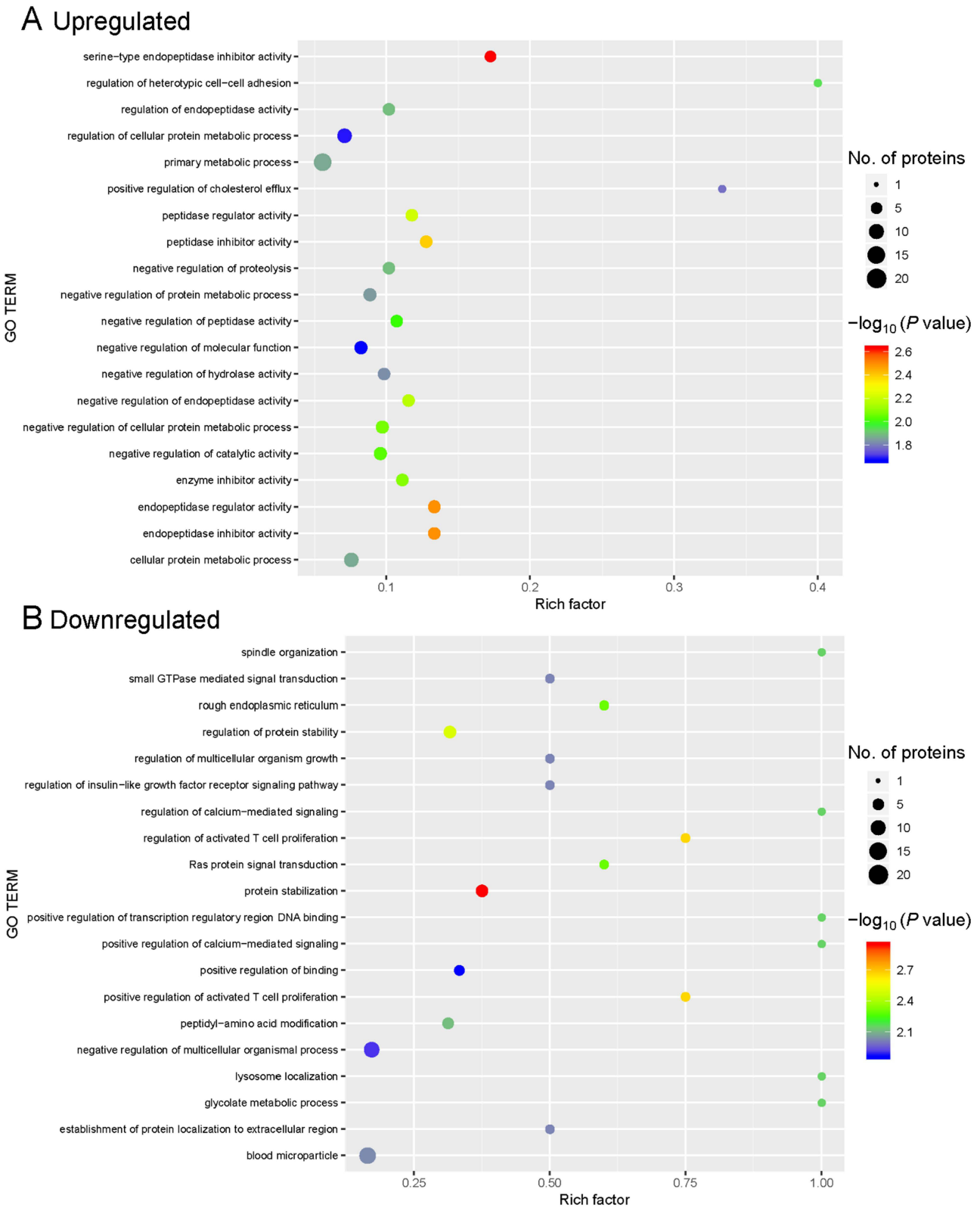

Figure 3 Gene ontology (GO) enrichment for functional terms. (**A**) Upregulated proteins. (**B**) Downregulated proteins. The abscissa represents the enrichment factor, showing the percentage of differentially expressed proteins in the GO classification relative to the identified proteins in that classification. The ordinate indicates the GO term description. The size of the bubble represents the number of proteins in the GO classification. The enrichment test P value was obtained using the Fisher exact test; −log10 (*P* value): the logarithmic conversion of the Fisher exact test *P* value.

activated T cell proliferation, regulation of activated T cell proliferation, regulation of protein stability, and Ras protein signal transduction (Figure 3B).

KEGG Pathway Annotation and Enrichment Analysis

We used KEGG signaling pathways analysis to identify the signaling pathways that were altered in cord blood from preterm neonates delivered by women with gestational diabetes. In the KEGG pathway annotation analysis, we found that the top five upregulated KEGG pathways were the complement and coagulation cascades, *Staphylococcus aureus* infection, pertussis, HIF-1 signaling pathway, and PPAR signaling pathway (Figure 4). The top five downregulated KEGG pathways were complement and coagulation cascades, dilated cardiomyopathy, pathways in cancer, Chagas disease, and hypertrophic cardiomyopathy (Figure 4).

The results of our KEGG pathway enrichment analysis indicated seven KEGG signaling pathways were enriched for the DEPs: inflammatory mediator regulation of TRP channels, Chagas disease, adrenergic signaling in cardiomyocytes, long-term depression, PPAR signaling pathway, ovarian steroidogenesis, and longevity regulating pathway (Figure 5). All but one pathway, PPAR signaling, were downregulated.

On the basis of our KEGG pathway annotation and enrichment analyses, we concluded that the complement and coagulation cascades may be importantly associated with preterm delivery of neonates by women with gestational diabetes. The DEPs in the serum obtained from the cord blood of these neonates involved in the complement and coagulation cascades included C4A, C2, SERPIND1, FGG, C1QC, C1QA, F12, PROC, and KNG1 (Figure 6).

Concentrations of Coagulation-Associated Proteins in DEPs

To confirm the DEPs identified by the LC-MS/MS, we examined the concentrations of coagulation-associated proteins in DEPs in neonatal cord blood samples using ELISA experiment and expended samples. Beside the samples used in LC-MS/MS experiment, we further collected 9 neonatal cord blood samples respectively from women with gestational diabetes who delivered preterm neonates and from women without diabetes who delivered full-term neonates. Our data

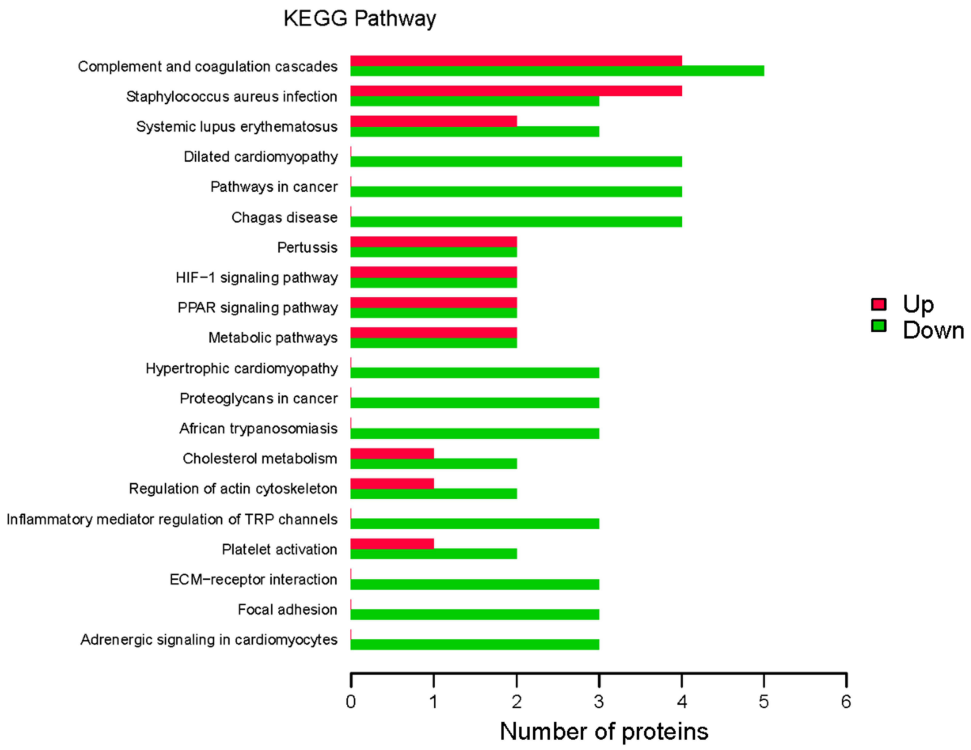

Figure 4 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway annotation. KEGG signaling pathways were identified in differentially expressed proteins (DEPs). Abscissa represents the number of DEPs; ordinate, KEGG pathways associated with the DEPs; green bars, upregulated pathways; and red bars, downregulated pathways.

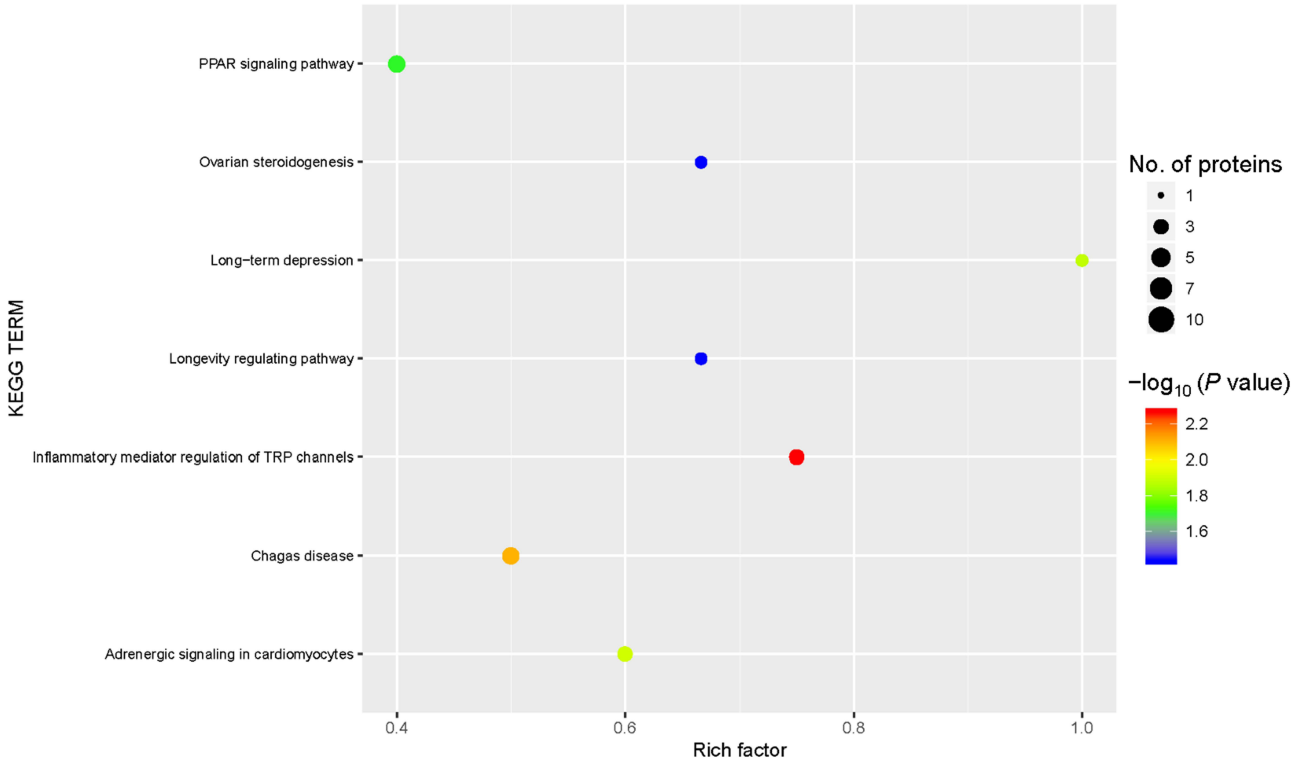

Figure 5 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment. KEGG signaling pathways were enriched for the differentially expressed proteins (DEPs). Abscissa: the enrichment factor showing the total proportion of DEPs in the KEGG signaling pathway as a proportion of the identified proteins in the classification. Ordinate: the KEGG term description. Bubble size indicates the number of DEPs in the KEGG pathway. The enrichment test *P* value was obtained using the Fisher exact test; −log10 (*P* value): the logarithmic conversion of the Fisher exact test *P* value.

showed that the expressions of SERPIND1 and FGG were significantly upregulated in the cord blood samples of preterm neonates compared to those of full-term neonates. On the contrary, the expressions of F12, KNG1and PROC were significantly downregulated in the cord blood samples of preterm neonates compared to those of full-term neonates (Figure 7). Therefore, these results are consistent with the data from our LC-MS/MS experiment.

Discussion

The incidence of diabetes in China continues to increase every year, including among pregnant women. Gestational diabetes is a serious threat to the health of pregnant women and their fetuses. Thus, understanding the pathogenesis and development of gestational diabetes is critical for finding new targets for prevention and treatment. In the present study, we used label-free LC-MS/MS analysis to identify key proteins that were differentially expressed between the serum obtained from neonatal cord blood of healthy women who delivered full-term neonates and of women with gestational diabetes who delivered preterm neonates. Overall, we identified 450 proteins, of which 21 DEPs were upregulated and 51 DEPs were downregulated. Our KEGG pathway annotation and enrichment analyses determined that many of these DEPs were involved in complement and coagulation cascades.

Hemorrhagic or thrombotic complications are important factors inducing neonate morbidity and mortality.[21–25] One study reported that the coagulation system is similar in preterm and full-term infants and does not show a rapid change between 30 and 40 weeks of life.[26] Even in extremely preterm neonates (24–31 weeks), the initial clotting tests, including platelets, fibrinogen, K-dependent factors, and factors VIII and V, are within standard references ranges.[27] However, gestational diabetes is a risk factor associated with coagulation abnormalities, with reduced factor V and prothrombin-proconvertin, a prolonged prothrombin time, elevated hematocrit, and reduced fibrinolytic activity in cord blood, which may cause preterm birth.[28,29] In the present study, we used label-free LC-MS/MS to find that the levels of coagulation-associated proteins, including SERPIND1, F12, FGG, KNG1, and PROC, were significantly changed in neonatal cord

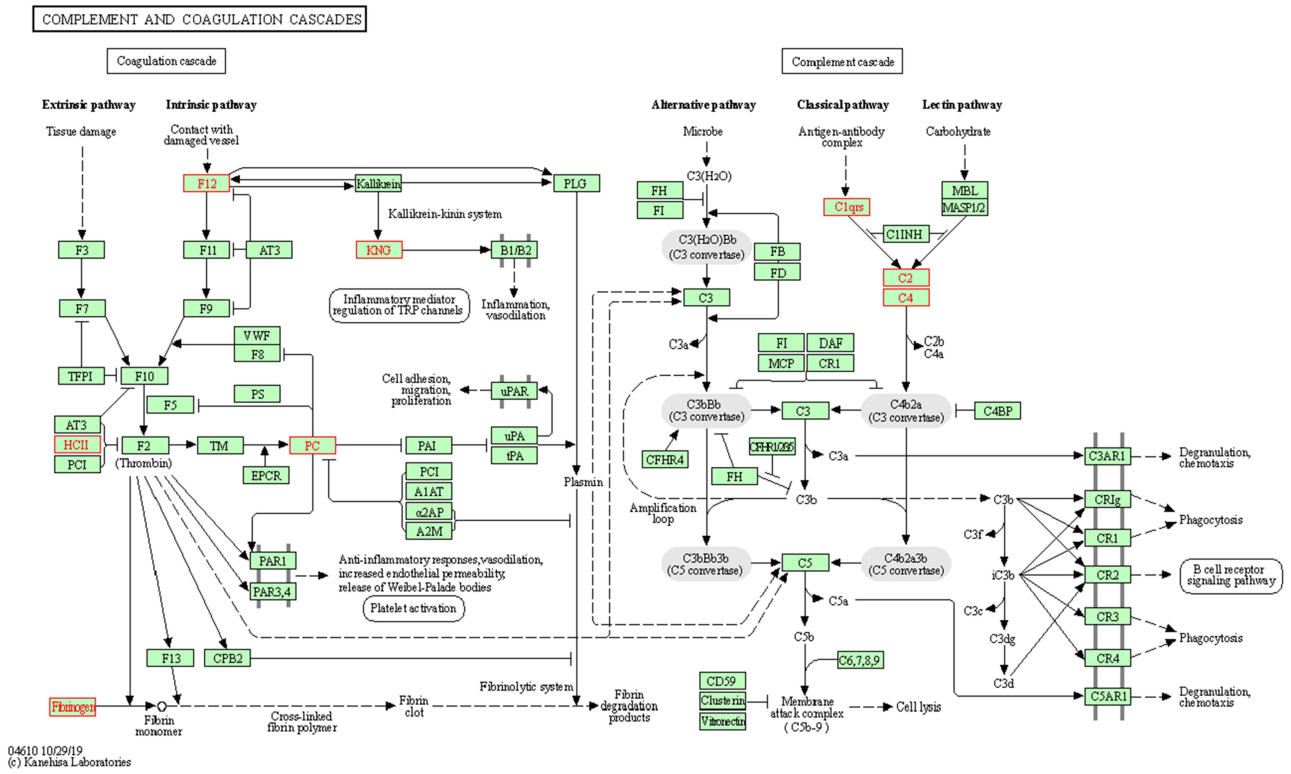

Figure 6 Complement and coagulation cascades. The differentially expressed proteins (DEPs), highlighted in a red frame, participating in the complement and coagulation cascades. The upregulated DEPS are C4A (C4, C4a anaphylatoxin), C2 (complement C2), SERPIND1 (HCII, heparin cofactor 2) and FGG (fibrinogen, fibrinogen gamma chain). The downregulated DEPs are C1QC (complement C1q subcomponent subunit C), C1QA (complement C1q subcomponent subunit A), F12 (coagulation factor XII), PROC (PC, vitamin K-dependent protein C) and KNG1 (KNG, kininogen-1).

blood from women with diabetes who delivered preterm neonates. Among these five coagulation-associated DEPs, SERPIND1 and FGG were increased, and F12, KNG1 and PROC were decreased. SERPIND1 (heparin cofactor 2) is a thrombin inhibitor activated by the glycosaminoglycans, heparin or dermatan sulfate.[30] FGG (fibrinogen gamma chain), a primary component of blood clots and involved in hemostasis, polymerizes with fibrinogen alpha and fibrinogen beta to form an insoluble fibrin matrix.[31] F12 (coagulation factor XII) is a glycoprotein involved in coagulation initiation, fibrinolysis, and the bradykinin and angiotensin production.[32] KNG1 (kininogen-1) is an inhibitor of thiol proteases and participates in the aggregation of thrombocytes and blood clotting.[33] PROC (vitamin K-dependent protein C) is a vitamin K-dependent serine protease that inactivates factors Va and VIIIa to regulate blood coagulation and to protect the endothelial cell barrier.[34,35] Our results suggest that these five DEPs are potential targets for future study of pregnancy-induced hemorrhagic or thrombotic complications in preterm infants delivered by women with gestational diabetes.

In general, the complement system is activated during pregnancy; for example, increased levels of C3A, C4A, and C5A are observed in plasma.[36] However, in women with gestational diabetes, plasma levels of C3A, C4A, and C5A are decreased, while C3 and C4 are increased in the second trimester of pregnancy.[37,38] In the present study, the levels of C2, C4A, C1QA, and C1QC were significantly altered in the neonatal cord blood of preterm neonates delivered by women with gestational diabetes, with C2 and C4A significantly increased, and C1QA and C1QC significantly decreased. C2 (component C2), part of the classic pathway of the complement system, is cleaved by activated factor C1 into two fragments: C2b and C2a. C2a, a serine protease, then combines with complement factor C4b to generate C3 or C5 convertase.[39,40] C4A (C4a anaphylatoxin) is an anaphylatoxin generated when complement is activated. However, because the receptor(s) for C4A remain uncertain, the functions of C4A have not been well identified.[41] Zhao et al reported that C4A inhibits C5a-induced neointima formation following arterial injury.[42] C1QA (complement C1q subcomponent subunit A) and C1QC (complement C1q subcomponent subunit C) are subcomponent subunits of C1q, which associates with proenzymes C1r and C1s

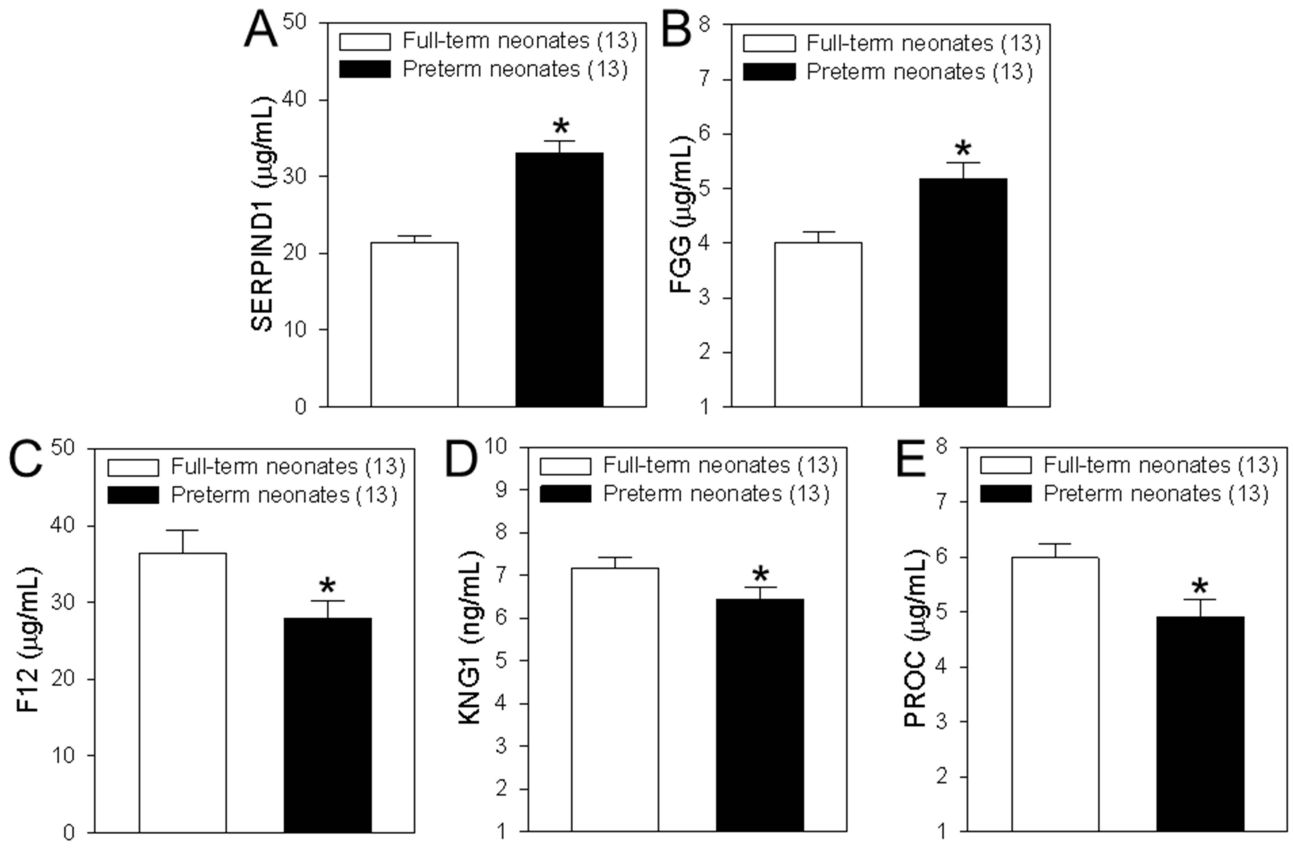

Figure 7 Concentrations of coagulation-associated proteins in differentially expressed proteins (DEPs). (**A**–**E**) Summary data showing the concentrations of heparin cofactor 2 (SERPIND1, **A**), fibrinogen gamma chain (FGG, **B**), coagulation factor XII (F12, **C**), kininogen-1 (KNG1, **D**) and vitamin K-dependent protein C (PROC, **E**) in serum samples from cord blood of healthy women who delivered full-term neonates and women with gestational diabetes who delivered preterm neonates. Among of them, SERPIND1and FGG are upregulated DEPs, but F12, KNG1and PROC are downregulated DEPs. Values are shown as the mean ± SEM (n = 13); *$P < 0.05$ for full-term neonates vs preterm neonates.

to yield C1.[43] C1q uses collagen-like regions to interact with the calcium-dependent C1r2C1s2 proenzyme complex.[44] C1q interacts with the Fc regions of IgG or IgM antibody present in immune complexes to activate C1r, C1s, and the classic complement pathway.[44] Thus, the results of our study may provide potential new therapeutic targets for disorders of the immune system in preterm infants delivered by women with gestational diabetes. These complement components may also warrant investigation as potential therapeutic targets.

Among the key signaling pathways of these DEP_S, our bioinformatics analysis identified the complement and coagulation cascades. Some of the proteins identified in the present study may warrant further study as new therapeutic targets for preterm birth among women with gestational diabetes. In this study, even we found these interesting DEPs, there are some limitations including that clinical samples used in LC-MS/MS experiments are small, and verification using larger samples from multiple hospitals is needed for the translational application in future.

In conclusion, we used label-free LC-MS/MS to identify and quantify key DEPs in serum derived from neonatal cord blood and the complement and coagulation cascades pathway and their potential roles in pathogenesis and the preterm birth of neonates delivered by mothers with gestational diabetes.

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Disclosure

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