

# Comparative proteomic analysis of maternal peripheral plasma and umbilical venous plasma from normal and gestational diabetes mellitus pregnancies

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## Abstract

Gestational diabetes mellitus (GDM) increases many health risks in offspring. The study aims to investigate the underlying mechanism in fetal risk of GDM.

We collected maternal peripheral plasma and umbilical venous plasma samples from 4 GDM and 4 control patients during their delivery at a university-based women's hospital. An isobaric tag for relative and absolute quantitation-labeled proteomics analysis was performed. The enzyme-linked immunosorbent assay was used to confirm the change of cholesteryl ester transfer protein (CETP). Bioinformatic analysis was performed with Ingenuity Pathway Analysis (IPA) software package.

We identified 19 up-regulated proteins and 15 down-regulated proteins in GDM peripheral plasma, 29 up-regulated proteins and 69 down-regulated proteins in GDM umbilical venous plasma. CETP concentration was significantly lower in both GDM peripheral plasma and umbilical venous plasma. Upstream regulator analysis predicted follicle-stimulating hormone (FSH) as the activated regulator of differentially expressed proteins.

The protein profiles in both GDM peripheral plasma and umbilical venous plasma between normal and GDM patients were significantly different. The results indicated that CETP and FSH might associates with health problem of GDM offspring.

**Abbreviations:** CETP = cholesteryl ester transfer protein, FSH = follicle-stimulating hormone, GDM = gestational diabetes mellitus, HDL = high-density lipoprotein, IPA = Ingenuity Pathway Analysis, iTRAQ = isobaric tags for relative and absolute quantitation labeling, LDL = low-density lipoprotein.

**Keywords:** cholesteryl ester transfer protein (CETP), follicle-stimulating hormone (FSH), gestational diabetes mellitus, proteomics, umbilical

## 1. Introduction

Gestational diabetes mellitus (GDM) is defined as any degree of glucose intolerance with onset or first recognition during pregnancy. GDM occurs in up to 14% of all pregnancies.<sup>[1]</sup>

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YL and G-FX are considered similar in author order.

This study was approved by the Ethics Committee of the Women's Hospital of Zhejiang University and conducted in accordance with the Declaration of Helsinki.

Written informed consents for publication of individual data were obtained from all patients.

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Maternal hyperglycemia causes increased glucose delivery to the fetus, resulting in fetal hyperinsulinemia and increased fetal growth. Complications of excessive fetal growth include birth trauma, increased cesarean deliveries, and the long-term risk of glucose intolerance and obesity.<sup>[2,3]</sup> However, the mechanism of the offspring problems is unclear.

The umbilical vein carries oxygenated blood with nutrition and some factors from mothers to the growing fetus. Changes in the placental transportation of nutrients and proteins may affect fetal programming.<sup>[4]</sup> Therefore, the characterization of proteins in umbilical venous blood can help us to discover the underlying mechanism in the offspring risk of GDM.

The study aims to explore the differentially expressed proteins in maternal peripheral plasma and umbilical venous plasma from normal and GDM pregnancies with isobaric tags for relative and absolute quantitation labeling (iTRAQ). Further bioinformatics analysis on biological pathway, network and upstream regulator was performed with Ingenuity Pathway Analysis (IPA) software package.

## 2. Materials and methods

### 2.1. Ethics statement

This study was approved by the Ethics Committee of the Women's Hospital of Zhejiang University and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients.

## 2.2. Study subjects

All GDM women were diagnosed in the Women's Hospital of Zhejiang University from June 2012 to September 2013. The GDM was diagnosed according to the new IADPSG criteria.<sup>[5]</sup> Briefly, GDM was diagnosed if 1 or more plasma venous glucose values in the 75-g oral glucose tolerance test during weeks 24 to 28 of gestation met the following conditions:  $\geq 5.1$  mmol/L (0 h); 10.0 mmol/L (1 h); 8.5 mmol/L (2 h). All women diagnosed with GDM were put on a controlled diet to maintain a fasting plasma glucose level below 5.1 mmol/L and a 2-hour postprandial level below 6.7 mmol/L. Insulin treatment was administered only in cases when dietary control alone failed to maintain the above 2 levels.

Control women (normal pregnancy) were matched with GDM women for maternal body mass index (BMI), gestational age, and birth weight.

The exclusion criteria for both GDM and control group were: pre-gestational diabetes, pregnancy-induced hypertension, assisted reproductive treatment-related pregnancy, multiple pregnancies, hyperthyroidism, hypothyroidism, and major fetal malformation.

Clinical data were collected from inpatient database. The clinical information was provided in Tables 1 and 2.

## 2.3. Sample collection

For GDM and 4 control women were enrolled in this study. Additional 12 GDM and 12 control women were included for cholesteryl ester transfer protein (CETP) concentration validation. Maternal peripheral blood samples were collected at admission (about 1 day to 1 week before delivery). Umbilical venous blood was collected during labor or Cesarean section. Samples were placed in EDTA-treated tubes. Plasma was obtained by centrifugation at 3000 rpm for 15 minutes at 4°C. Protease inhibitors were then added (protease inhibitor: supernatant = 1:100) and samples were stored at -80°C for further use.

## 2.4. Protein preparation

To reduce the complexity of samples, the highly abundant proteins were depleted with ProteoMiner TM Kits (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol. Samples were eluted in Lysis buffer (7M Urea, 2M Thiourea, 4% CHAPS, 40 mM Tris-HCl, pH 8.5) and reduced with 10 mM Dithiothreitol (DTT) (final concentration) at 56°C for 1 hour, followed by alkylation with 55 mM iodine acetamide (final concentration) in the darkroom for 1 hour. The reduced and alkylated protein mixtures were precipitated by adding 4 volumes of chilled acetone at -20°C overnight. After centrifugation at 4°C and 30,000 g, the pellet was dissolved in 0.5 M tetraethylammonium bromide (TEAB) (Applied Biosystems, Milan, Italy) and sonicated in ice. Protein concentration was determined in the Bradford assay. The proteins in the supernatant were kept at -80°C for further analysis.

## 2.5. iTRAQ labeling and strong cation-exchange chromatography (SCX) fractionation

Total protein (100 µg) was taken out of each sample solution and then the protein was digested with Trypsin Gold (Promega, Madison, WI) with the ratio of protein: trypsin = 30:1 at 37°C for 16 hours. After trypsin digestion, peptides were dried by vacuum centrifugation. Peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's protocol for 8-plex iTRAQ reagent (Applied Biosystems). Briefly, 1 unit of iTRAQ reagent was thawed and reconstituted in 24 µL of isopropanol. Samples were labeled with the iTRAQ tags. The peptides were labeled with the isobaric tags and then incubated at room temperature for 2 hours. The labeled peptide mixtures were then pooled and dried by vacuum centrifugation.

SCX chromatography was performed with a LC-20AB high-performance liquid chromatography (HPLC) Pump system (Shimadzu, Kyoto, Japan). The iTRAQ-labeled peptide mixtures were reconstituted with 4 mL of Buffer A (25 mM NaH<sub>2</sub>PO<sub>4</sub> in 25% ACN, pH 2.7) and loaded onto a 4.6 × 250 mm Ultremex SCX column containing 5-µm particles (Phenomenex). The

**Table 1**

**Clinical characteristics of control and GDM groups.**

Characteristics	Control (N=4)	GDM (N=4)	P values
Maternal Age, yrs	30.3 (3.5)	32.0 (3.4)	.50
Gravidity	1.75 (0.95)	1.50 (0.57)	.67
Parity	1.00 (0)	1.25 (0.50)	.35
Maternal BMI before pregnancy, kg/m <sup>2</sup>	20.83 (2.44)	20.09 (1.69)	.63
Maternal BMI at delivery, kg/m <sup>2</sup>	27.20 (3.38)	25.58 (1.82)	.43
OGTT at 24 to 28 weeks, fast, mmol/L	4.67 (0.45)	5.06 (0.43)	.26
OGTT 1 hour at 24 to 28 weeks, mmol/L	7.60 (0.87)	11.02 (1.20)	<.01
OGTT 2 hours at 24 to 28 weeks, mmol/L	6.55 (0.93)	8.71 (1.33)	.03
HbA1c, %	4.92 (0.38)	5.45 (0.52)	.15
Neonatal fasting glucose concentration at delivery, mmol/L	5.59 (0.97)	5.57 (1.16)	.98
Triglyceride, mmol/L	3.72 (1.24)	4.67 (1.80)	.42
Total cholesterol, mmol/L	5.80 (1.19)	7.97 (1.34)	.05
High-density lipoprotein, mmol/L	1.42 (0.16)	2.39 (0.56)	.04
Low-density lipoprotein, mmol/L	2.50 (0.48)	4.56 (0.99)	.01
Delivery data			
Gestational age at delivery, week	39.2 (0.9)	38.8 (0.9)	.52
Birth weight, g	3412 (228)	3475 (239)	.71
Cesarean section, %	100	100	NA
Neonatal gender, Male%	25	75	.20

BMI = body mass index, GDM = gestational diabetes mellitus, NA = not applicable, OGTT = oral glucose tolerance test. Data are expressed as mean (standard deviation). P values were calculated by independent Student t-test, Chi-square test or Fisher's exact test.

**Table 2****Clinical characteristics of control and GDM groups tested for CETP validation.**

Characteristics	Control (N = 16)	GDM (N = 16)	P values
Maternal Age, yrs	32.7 (3.6)	33.6 (3.6)	.50
Gravidity	2.6 (1.1)	2.1 (0.8)	.16
Parity	1.7 (0.6)	1.6 (0.6)	.57
Maternal BMI before pregnancy, kg/m <sup>2</sup>	22.0 (3.2)	21.8 (3.2)	.85
Maternal BMI at delivery, kg/m <sup>2</sup>	27.5 (3.1)	26.7 (2.8)	.45
OGTT at 24 to 28 weeks, fast, mmol/L	4.51 (0.37)	5.25 (0.50)	<.01
OGTT 1 hour at 24 to 28 weeks, mmol/L	9.29 (1.51)	11.17 (1.14)	<.01
OGTT 2 hours at 24 to 28 weeks, mmol/L	7.38 (1.28)	8.71 (1.33)	.04
HbA1c, %	5.30 (0.44)	5.51 (0.70)	.31
Neonatal fasting glucose concentration at delivery, mmol/L	4.09 (1.26)	4.79 (0.79)	.07
Triglyceride, mmol/L	3.57 (2.43)	3.72 (1.88)	.84
Total cholesterol, mmol/L	5.78 (1.40)	7.35 (1.47)	.04
High density lipoprotein, mmol/L	1.58 (0.39)	2.16 (0.41)	.04
Low density lipoprotein, mmol/L	2.58 (1.07)	4.06 (0.90)	.01
Delivery data			
Gestational age at delivery, week	38.8 (1.0)	39.3 (0.9)	.52
Birth weight, g	3275 (329)	3337 (366)	.71
Neonatal gender, Male%	31	69	.03

BMI = body mass index, CETP = cholesteryl ester transfer protein, GDM = gestational diabetes mellitus, NA = not applicable, OGTT = oral glucose tolerance test. Data are expressed as mean (standard deviation). P values were calculated by independent Student *t*-test, Chi-square test or Fisher exact test.

peptides were eluted at a flow rate of 1 mL/min according to the procedure: Buffer A for 10 minutes, 5% to 60% Buffer B (25 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 M KCl in 25% ACN, pH 2.7) for 27 minutes, and 60% to 100% Buffer B for 1 minute. The system was then maintained in 100% Buffer B for 1 minute before equilibrating with Buffer A for 10 minutes before the next injection. Elution was monitored by measuring the absorbance at 214 nm, and fractions were collected every 1 minute. The eluted peptides were pooled into 20 fractions, desalted with a Strata X C18 column (Phenomenex) and vacuum-dried.

### 2.6. Liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) analysis

Each fraction was resuspended in Buffer A (5% ACN, 0.1% FA) and centrifuged at 20000g for 10 minutes. Then, 10 μL of supernatant was loaded on an LC-20AD nano HPLC (Shimadzu, Kyoto, Japan) by the auto-sampler onto a 2-cm C18 trap column. Then, the peptides were eluted onto a 10-cm analytical C18 column (inner diameter of 75 μm) packed in-house. The samples were loaded at 8 μL/min for 4 minutes. Then the 35-minute gradient was run at 300 nL/min starting from 2% to 35% B (95% ACN, 0.1% FA), followed by 5-minute linear gradient to 60% B, then, followed by 2-minute linear gradient to 80% B, maintained at 80% B for 4 minutes, and finally restored to 5% B within 1 minute.

Data acquisition was performed with a TripleTOF 5600 System (AB SCIEX, Concord, ON) fitted with a Nanospray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA).

### 2.7. Protein identification

Raw data files acquired from the Orbitrap were converted into MGF files using Proteome Discoverer 1.2 (PD 1.2, Thermo), [5600 ms converter] and the MGF files were searched. Protein identification was performed by using Mascot search engine (Matrix Science, London, UK; version 2.3.02). A mass tolerance of 0/05 Da (ppm) was permitted for intact peptide masses and 0.1

Da for fragmented ions with the allowance for 1 missed cleavage in the trypsin digests. For protein quantitation, it is required that a protein contains at least 2 unique spectra. The quantitative protein ratios were weighted and normalized by the median ratio in Mascot. Only proteins with *P*-values < .05, and fold changes of >1.2 were considered to be significant.

### 2.8. Human CETP enzyme-linked immunosorbent assay (ELISA)

A human CETP ELISA kit (Biovision) was used to measure CETP protein concentration in plasma. The assay was performed as recommended by the manufacturer.

### 2.9. IPA

IPA (Ingenuity Systems, Redwood City, CA) was performed to identify canonical pathways, diseases, and biological functions, networks of interacting partners and upstream analysis of the identified differently expressed proteins between GDM and normal samples. A detailed description is given in the online repository (<http://www.ingenuity.com>).

### 2.10. Statistical analysis

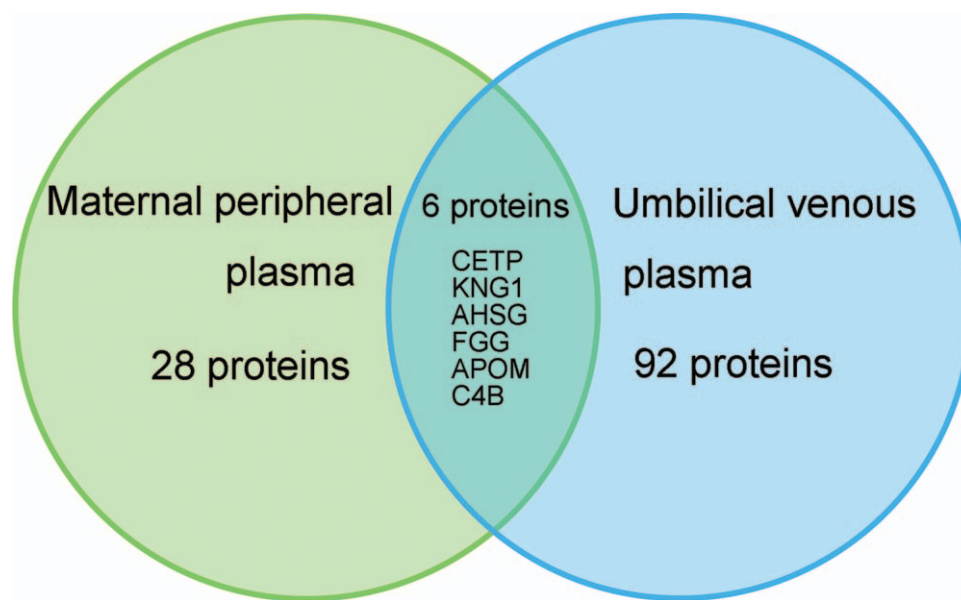
We compared continuous variables by independent Student *t*-test. Categorical variables were compared by using 2-tailed Chi-square tests or Fisher exact tests. All reported *P* values are 2-sided.

We also used multivariate linear model to test the difference of CETP concentration. CETP concentration was adjusted for age and neonatal gender. We used Pearson correlation to test correlations.

## 3. Results

### 3.1. Identification of differentially expressed proteins in maternal peripheral plasma and umbilical venous plasma between control and GDM pregnancies

In maternal peripheral plasma, we identified 523 proteins. Of these, 34 proteins were recognized as differentially expressed



**Figure 1.** Proteins identified as differentially expressed in GDM and control group. GDM=gestational diabetes mellitus.

(fold change  $>1.2$ ,  $P < .05$ ) between groups, including 19 up-regulated proteins and 15 down-regulated proteins in the GDM group (Supplemental Table 1, <http://links.lww.com/MD/C458>).

In umbilical venous plasma, we identified 780 proteins. Of these, 98 proteins were recognized as differentially expressed (fold change  $>1.2$ ,  $P < .05$ ) between groups, including 29 up-regulated proteins and 69 down-regulated proteins in the GDM group (Supplemental Table 1, <http://links.lww.com/MD/C458>).

Combined together, we identified 6 proteins with consistent change in both maternal peripheral and umbilical blood. Of these, 2 were up-regulated and 4 were down-regulated in GDM (Fig. 1, Table 3).

We did literature research and found CEPT and APOM were the most GDM-relevant proteins. And CEPT was the protein with highest fold change. So we confirmed the changes of CETP using ELISA. CETP was down-regulated in both maternal peripheral and umbilical venous plasma in GDM women using student *t* test (Fig. 2A, B). We also used multivariate linear model to test the difference of CETP concentration. CETP concentration in maternal peripheral and umbilical venous plasma was also decreased when adjusted for age and neonatal gender ( $P < 0.01$  and  $P = .01$ ).

### 3.2. CETP concentration correlate with maternal metabolic variables

We tested correlation of CETP concentration and maternal metabolic variables. We found that CETP concentration in maternal peripheral plasma correlates with high-density lipoprotein (HDL), low-density lipoproteins (LDL), and maternal fasting glucose concentration. We also found that CETP concentration in umbilical venous plasma correlates with LDL and maternal fasting glucose concentration (Table 4).

### 3.3. Bioinformatics analysis of differentially expressed proteins in umbilical venous plasma

All the 98 differentially expressed proteins in umbilical venous plasma identified in proteomics were analyzed by IPA.

Canonical pathway analysis showed top 10 altered canonical pathways in GDM umbilical blood. Integrin-linked kinase (ILK) signaling was the most inhibited pathway (z-score =  $-1.604$ ) (Fig. 3A).

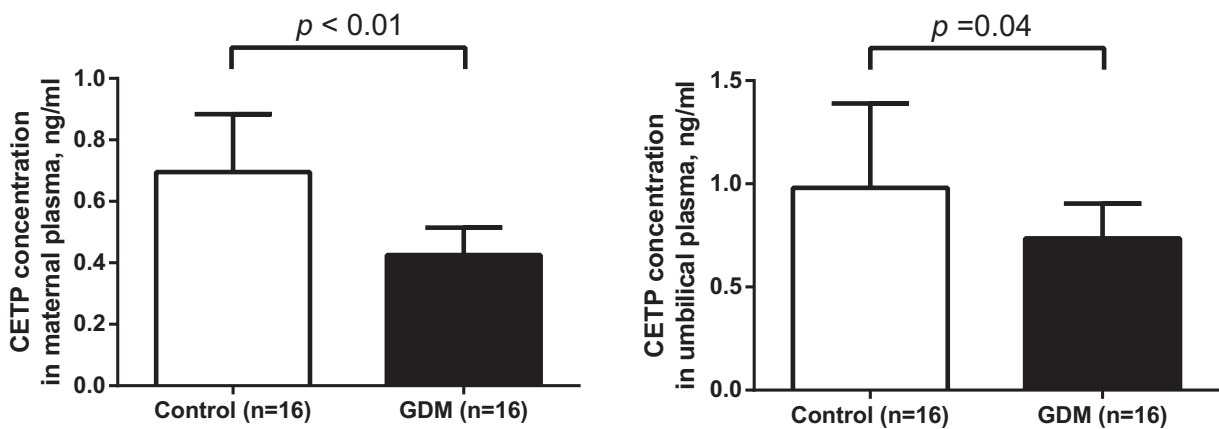
Diseases and functions analysis showed that the aggregation of blood platelets was the most inhibited altered function (z-score =  $0.106$ ) (Fig. 3B).

**Table 3**

**Consistently differentially expressed proteins in both maternal peripheral and umbilical venous plasma from control and GDM women.**

Description	Fold change in maternal peripheral plasma	Fold change in umbilical plasma
cDNA FLJ76868, highly similar to Homo sapiens cholesteryl ester transfer protein, plasma (CETP)	-2.92	-2.35
cDNA FLJ53075, highly similar to Kininogen-1 (KNG1)	-1.76	-2.15
cDNA FLJ55606, highly similar to Alpha-2-HS-glycoprotein (AHSG)	-1.49	-1.76
Fibrinogen gamma chain, isoform CRA_a (FGG)	-7.36	-9.85
Apolipoprotein M (APOM)	1.53	1.38
C4B (C4B)	2.26	1.58

Negative value in fold change means down-regulation.GDM

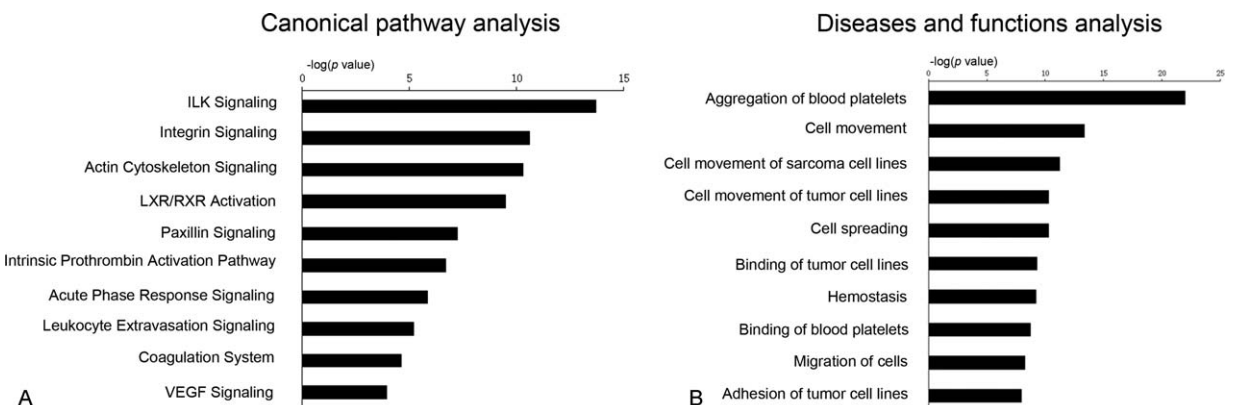


**Figure 2.** CETP concentration in maternal peripheral and umbilical venous plasma. CETP concentration was significantly lower in GDM maternal peripheral plasma. (B) CETP concentration was significantly lower in GDM umbilical venous plasma. CETP=cholesteryl ester transfer protein, GDM=gestational diabetes mellitus.

**Table 4**  
Correlation analysis of CETP concentration and maternal variables.

	HDL	LDL	BMI before pregnancy	BMI at delivery	Maternal fasting glucose
CETP maternal peripheral plasma					
r	-0.50	-0.62	-0.13	0.00	-0.41
p	<0.01	<0.01	0.44	0.98	0.01
CETP umbilical venous plasma					
r	-0.23	-0.51	0.05	0.09	-0.04
p	0.21	<0.01	0.79	0.61	0.82

BMI=body mass index, CETP=cholesteryl ester transfer protein, HDL=high density lipoprotein, LDL=low density lipoprotein. Value r was calculated with Pearson correlation.



**Figure 3.** Pathway and function analyses of GDM umbilical vein blood. (A) Top 10 altered pathways in canonical pathway analysis. (B) Top 10 altered functions in diseases and functions analysis. GDM=gestational diabetes mellitus.

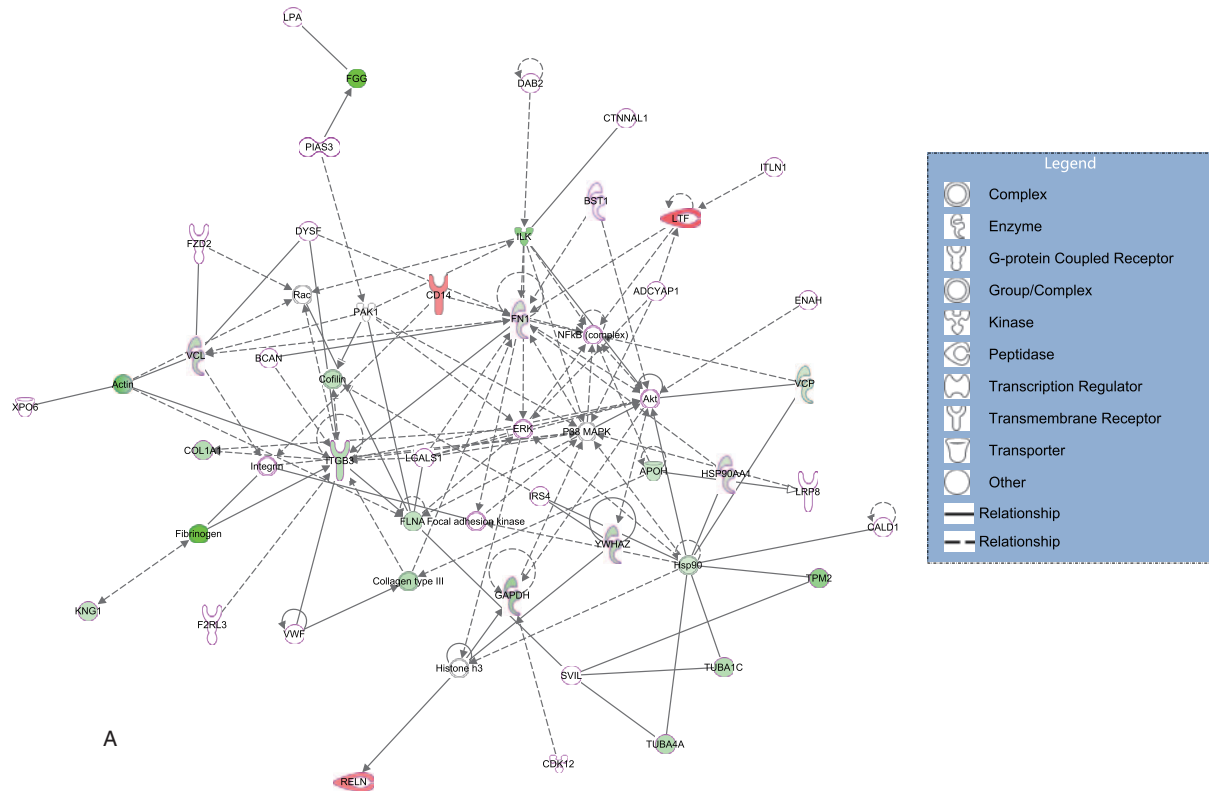
Network analysis showed that differentially expressed proteins were enriched in 2 subcategory networks: “Cellular Movement, Cell Morphology, Cellular Assembly, and Organization” and “Cell-To-Cell Signaling and Interaction, Hematological System Development, and Function, Cell Death and Survival” (Fig. 4).

Upstream regulator analysis predicted follicle-stimulating hormone (FSH) as the activated regulator of the differentially expressed proteins (Fig. 5).

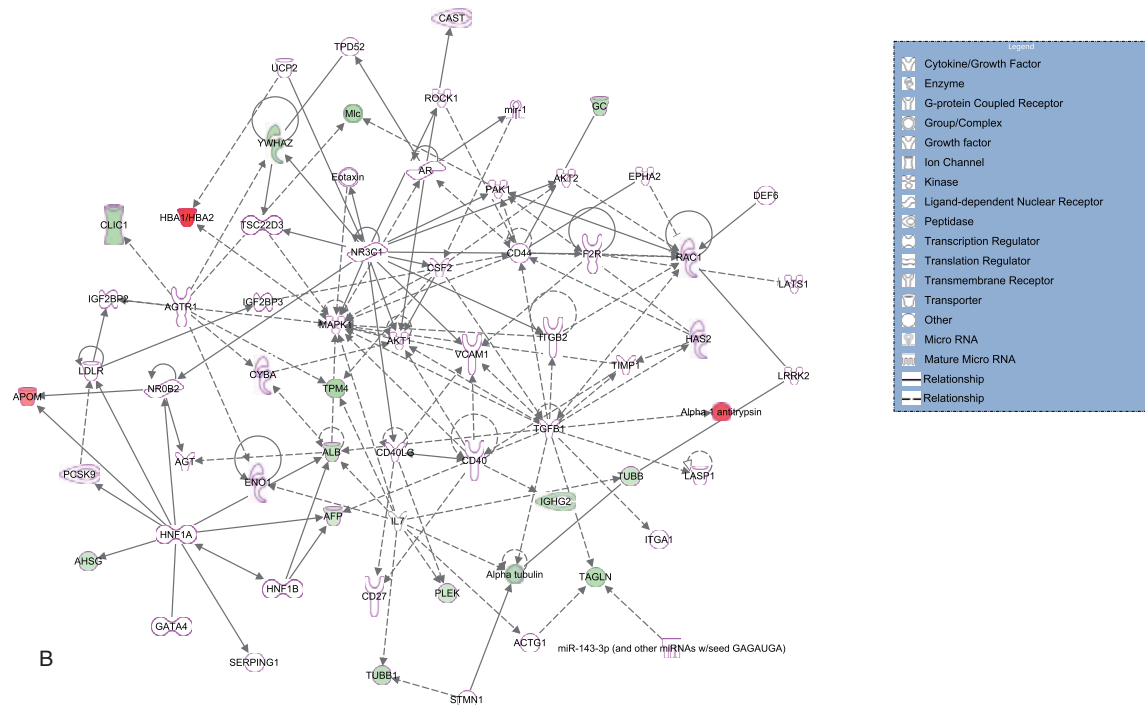
**4. Discussion**

GDM is independently associated with adverse maternal and neonatal outcomes.<sup>[6]</sup> The health problems in offspring of GDM mothers can be very serious, the effect of GDM on offspring may be huger than that on mothers themselves.<sup>[7,8]</sup> This may explain that more differentially expressed genes were found in umbilical blood than in maternal blood. Moreover, the molecular mechanisms underlying these impairments are poorly understood. The umbilical cord blood is the most important channel

Cellular Movement, Cell Morphology, Cellular Assembly and Organization



Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, Cell Death and Survival



**Figure 4.** Network of interacting proteins and modules involved in the main diseases and biological functions. Proteins in red are up-regulated and those in green are down-regulated. The solid lines mean direct relationship and the dotted lines mean indirect relationship.

through which the mother can affect the health of the fetus. Thus, in this study, we tried to analyze the proteins in maternal peripheral and umbilical venous plasma in order to reveal the possible mechanisms underlying GDM.

Six proteins displayed consistent change in both maternal peripheral and umbilical venous plasma. Of these, CETP is related to lipid metabolism and also GDM. CETP, also called plasma lipid transfer protein, is a plasma protein that facilitates

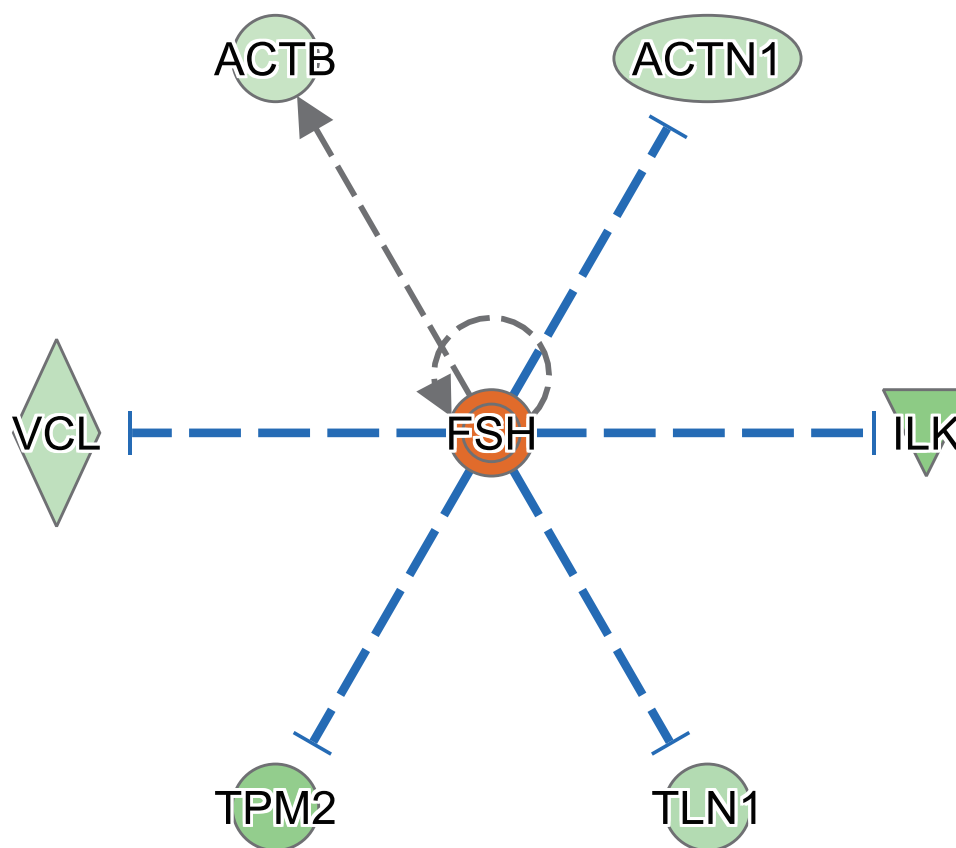


Figure 5. FSH identified as an upstream regulator of the differentially expressed proteins. FSH=follicle-stimulating hormone.

the transport of cholesteryl esters and triglycerides between the lipoproteins. It collects triglycerides from very-low-density (VLDL) or LDL and exchanges them for cholesteryl esters from HDL, and vice versa. CETP participates in the lipid dysregulation of GDM patients.<sup>[9]</sup> Furthermore, CETP also plays a role in health problems in GDM offspring. Analysis of HDL isolated from GDM and control neonates showed GDM neonatal HDL composition is altered, CETP mass and activity alterations may be related.<sup>[10]</sup> We also found a correlation between CETP and HDL/LDL. Combined with our results, we assume that GDM might contribute to the development of diabetes/obesity later in life through CETP-regulated lipid metabolism. Trans-generation studies are required to prove the effects of CETP on the health problem of GDM offspring.

FSH was predicted as the upstream regulator in the IPA analysis. In previous studies, low FSH was associated with diabetes,<sup>[11]</sup> but no evidence of direct association between GDM and FSH was reported. In fact, FSH is an important regulator in the metabolism process. A recent review indicated that FSH could regulate the onset of cardiovascular and metabolic disorders during androgen deprivation therapy.<sup>[12]</sup> It was reported that FSH could regulate fat accumulation in adipose tissue.<sup>[13,14]</sup> FSH also regulated glucose transporters in Sertoli cells.<sup>[15]</sup> Because adipose tissue and glucose transporter are both crucial factors in the pathophysiologic process in diabetes, we suggested that FSH might participate in the etiology of diabetes. Further exploration will be performed in the future.

There are several limitations and considerations with regard to the study. First, some maternal information, such as weight gain, social class and income, which might be influencing factors, was

not obtained. Second, we did not follow up the offspring further. Samples from offspring at 1 year old, or even older, will give more information. Third, the limited sample size weakened the validation of the study.

#### Author contributions

**Conceptualization:** Qiong Luo.

**Data curation:** Ying Jiang.

**Formal analysis:** Ying Jiang.

**Funding acquisition:** Qiong Luo.

**Investigation:** Li-Juan Sun.

**Methodology:** Li-Juan Sun, Rong Peng, Qiong Luo.

**Validation:** Hong Zhu.

**Writing – original draft:** Yun Liao, Gu-feng Xu.

**Writing – review & editing:** Yun Liao, Gu-feng Xu.

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