A proteomic analysis of human follicular fluid: proteomic profile associated with embryo quality

Jingjuan Ji (jiaoshou75@126.com)
The First Affiliated Hospital of USTC: Anhui Provincial Hospital
https://orcid.org/0000-0001-6161-7432

Xinyi Zhu
The First Affiliated Hospital of USTC: Anhui Provincial Hospital

Yan Zhang
The First Affiliated Hospital of USTC: Anhui Provincial Hospital

Lijun Shui
The First Affiliated Hospital of USTC: Anhui Provincial Hospital

Shun Bai
The First Affiliated Hospital of USTC: Anhui Provincial Hospital

Lingli Huang
The First Affiliated Hospital of USTC: Anhui Provincial Hospital

Haoyu Wang
The First Affiliated Hospital of USTC: Anhui Provincial Hospital

Lihua Luo
The First Affiliated Hospital of USTC: Anhui Provincial Hospital

Bo Xu
The First Affiliated Hospital of USTC: Anhui Provincial Hospital

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Abstract

Embryo selection is a key point of in vitro fertilization (IVF). The most commonly used method for embryo selection is morphological assessment. However, it is sometimes inaccurate. Follicular fluid (FF) contains a complex mixture of proteins that are essential for follicle development and oocyte maturation. Analyzing human FF proteomic profiles and identifying predictive biomarkers might be helpful for evaluating embryo quality. A total of 22 human FF samples were collected from 19 infertile women who underwent IVF/intracytoplasmic sperm injection (ICSI) treatment between October 2021 and November 2021. FFs were grouped into two categories on the basis of the day 3 embryo quality, grade  or  in the hqFF group and grade  in the nhqFF group. FF was analyzed by liquid chromatography-tandem mass spectrometry (LC/MS/MS). The key differentially expressed proteins (DEPs) were validated by parallel reaction monitoring (PRM). Differentially expressed proteins were further analyzed using DAVID software. A total of 558 proteins were identified, of which 50 proteins were differentially expressed in the hqFF vs. nhqFF group, including 32 upregulated proteins (>1.20-fold, P<0.05) and 18 downregulated proteins (<0.67-fold, P<0.05). Bioinformatics analyses showed that the upregulated DEPs were enriched in components of the coagulation and complement systems and negative regulation of peptidase activity, while the downregulated DEPs were enriched in molecular function of extracellular matrix structural and constituent collagen binding. Our results suggested that a number of protein biomarkers in FF were associated with embryo quality. It may help develop an effective and noninvasive method for embryo selection.

Introduction

Many breakthroughs have taken place in in vitro fertilization (IVF) since the first IVF child was born in 1978. The goal of IVF has changed from “pregnancy” to ‘birth of a healthy singleton baby at term, without compromising the health and safety of the woman and baby achieved at the lowest possible cost’ [1]. How can we achieve this goal? Single embryo transfer is an important method. To balance the pregnancy rate and single embryo transfer, selecting the best embryo is crucial. The most commonly used method for embryo selection is morphological assessment. However, this method is sometimes inaccurate [2]. It is necessary to develop an effective and noninvasive method for embryo selection.

Follicular fluid (FF) is produced by the selective transfer of blood plasma and the secretion of granulosa and thecal cells. It is the microenvironment of follicles, which means it may be an important determinant of oocyte development [3]. What is the protein composition of human FF? What role do these proteins play in follicle and oocyte development? Information about these questions has aroused wide attention. Ambekar, A. analyzed the proteome of FF and compared it with the plasma proteome. They reported sixty-four proteins that had not been reported in the plasma proteome database [4]. Jarkovska, K found that in women undergoing successful IVF, the proteome of FF is different from the proteome of plasma/serum. They concluded that these proteins together with their regulatory pathways may play a vital role in reproduction [5]. Bayasula et al. compared proteomic profiling of FFs containing oocytes that were fertilized and resulted in pregnancy with FFs containing oocytes that were not fertilized in the same patients. They found 53 differentially expressed proteins. Their results suggested that the differentially expressed proteins might be involved in folliculogenesis and might serve as biomarkers for oocyte maturation and the success of IVF [6]. Chen, F., et al. analyzed peptide profiles and found 7 peptides as potential biomarkers predicting fertilization outcomes. Bioinformatics analyses were not performed in the abovementioned two studies [7]. In the study of Severino, V., et al, an iTRAQ labeling strategy was performed, and 30 differentially expressed proteins were identified in FFs with successful compared to unsuccessful IVF outcomes. In that study, proteins from different samples were pooled based on IVF outcome, which means that the precise correlation of the protein profile in FFs and IVF outcome could not be established. In addition, no information could be obtained on which key point of IVF the differentially expressed proteins affected [8].

Although protein profile analysis of FF has been performed and protein changes in FFs associated with IVF outcome have been found, for embryos originating from normal fertilization, to date, no study has investigated the association of the proteomic profile in FF and embryo quality. Therefore, identifying potential biomarkers to predict embryo development...
competence may be useful in embryo selection. In addition, FF can be easily collected in IVF treatment without harming oocyte quality, which means it is a convenient material for research.

Proteomic analyses in a high-throughput mode have been widely used in the postgenomic era. In addition to discovering predictive biomarkers for IVF, FF proteomic analysis provides better insight into the physiological process of follicle development and oocyte maturation. The proteomic analysis of FF has become an active research area in recent years [6–7, 9–11].

In the present study, we performed a proteomic analysis of FF based on liquid chromatography-tandem mass spectrometry (LC/MS/MS) to identify biomarkers associated with embryo quality.

Materials And Methods

Patients

The whole workflow is described in Fig. 1. A total of 22 FF samples were collected from 19 females undergoing IVF/intracytoplasmic sperm injection (ICSI) treatment at The First Affiliated Hospital of USTC (Anhui Provincial Hospital) between October 2021 and November 2021. All patients ranged from 23 to 33 years old and were undergoing their first treatment cycle, with infertility caused by tubal factor or male factor infertility. Patients with chromosomal abnormalities, polycystic ovary syndrome, serum follicle-stimulating hormone (FSH) > 10 IU/L and/or anti-Müllerian hormone (AMH) ≤ 1.1 ng/mL, endometriosis or endocrine disease were not considered. The body mass indexes of the patients were between 18.5 and 23.9 kg/m². This study was approved by the Ethics Committee of The First Affiliated Hospital of USTC.

IVF Procedure

All patients were treated with exogenous gonadotropin and gonadotropin-releasing hormone (GnRH) analogs (agonists and antagonists). In brief, ovarian stimulation was started with a starting dose from 150 U.I. to 225 U.I. The doses were adjusted individually according to the ovarian response. When the leading follicle exceeded 18 mm in diameter, 250 µg of recombinant hCG (Merck Serono, Switzerland) was injected intramuscularly. Transvaginal ultrasound–guided oocyte retrieval was performed 36 h after triggering. A clear visible follicle between 16–18 mm was aspirated at the beginning of the ovum retrieval procedure with the use of a double lumen aspiration needle. Flushing was carried out when no oocytes were achieved after aspiration. We collected each oocyte and FF individually. Only yellow or light reddish FFs containing oocytes were included. The FF samples were individually placed into 10-mL conical tubes. Collected oocytes were inseminated either via conventional IVF or ICSI. Embryos were examined on day 3 after insemination to assess their quality according to the number and regularity of blastomeres and the degree of embryonic fragmentation. Embryos were graded based on the ESHRE Istanbul consensus on embryo assessment [12]. FFs were grouped into two categories on the basis of day 3 embryo quality. FFs containing an oocyte that resulted in a grade  or  embryo were distributed to the high-quality embryo FF (hqFF) group (n = 11), while FFs containing an oocyte that resulted in a grade  embryo were distributed to the nonhigh-quality embryo (nhqFF) group (n = 11). Thereafter, we compared each of the 11 hqFF to the 11 nhqFF.

Preparation Of Ff

The FF samples were centrifuged at 1500 * g for 10 min. The supernatant was then separated and stored at − 80°C for further use.

Lc–ms/ms Analysis (Tandem Mass Tags, Tmt) Of Proteins In Pct-based Protein Digestion
An 800 µl sample solution was dried in a SpeedVac and then resuspended in 30 µL 6 M urea/2 M thiourea/100 mM triethylammonium bicarbonate (TEAB). After transfer into the PCT-MicroPestle device, 10 µL 100 mM Tris (2-carboxyethyl) phosphine (TCEP) and 2.5 µL 800 mM iodoacetamide (IAA) were added for reduction and alkylation under 45000 psi, with 30 s high pressure and 10 ambient pressure per cycle, 30 °C for 90 cycles. Eighty-five microliters of LTEAB was added to the PCT tube to decrease the concentration of urea to lower than 1.5 M. Then, 10 µl trypsin (0.5 µg/µl) and 5 µl Lys-C (0.25 µg/µl) were added for protein digestion under 20000 psi, with 50 s high pressure and 10 ambient pressure per cycle, 30 °C for 120 cycles. Tryptic peptides were transferred into 1.5 mL tubes, and digestion was then terminated by 15 µL of 10% TFA.

Desalting And Tmt Labeling

Confirm that the pH of the samples was between 2 and 3. SOLAµ (Thermo Fisher Scientific™, San Jose, USA) was applied for desalting, and the TMT pro 16plex Isobaric Label Reagent Set was applied for TMT labeling according to their user guide.

High Ph Fractionation

Fractionation was performed with a Waters X Bridge Peptide BEH C18 column (300 Å, 5 µm × 4.6 mm × 250 mm) under a DIONEX UltiMate 3000 Liquid Chromatogram. Mobile phase A was 10 mM ammonium hydroxide (pH = 10), and mobile phase B was 98% ACN and 10 mM ammonium hydroxide (pH = 10). Peptides were collected every minute from 5% ACN to 35% ACN with a flow rate of 0.5 ml/min in 60 min and then combined into 30 fractions. After SpeedVac drying, the 30 fraction samples were resuspended in 2% ACN and 0.1% formic acid and then sent for LC‒MS analysis.

Dda Data Acquisition

LC‒MS/MS with the nanoflow DIONEX UltiMate 3000 RSLCnano System coupled to an Orbitrap Exploris 480 mass spectrometer (Thermo Scientific™, San Jose, USA), which was equipped with a FAIMS Pro™ (Thermo Scientific™, San Jose, USA), in data-dependent acquisition (DDA) mode. Buffer A. 2% ACN, 98% H2O containing 0.1% FA; Buffer B. 98% ACN in water containing 0.1% FA. All reagents were MS grade for each acquisition, and peptides were loaded onto a precolumn (3 µm, 100 Å, 20 mm*75 µm i.d.) at a flow rate of 6 µL/min for 4 min and then injected using a 30 min LC gradient (from 7–30% buffer B) at a flow rate of 300 nL/min (analytical column, 1.9 µm, 120 Å, 150 mm*75 µm i.d.) Buffer A was 2% ACN and 98% H2O containing 0.1% FA, and buffer B was 98% ACN in water containing 0.1% FA. All reagents were MS grade. The m/z range of MS1 was 375–1800 with a resolution of 60,000, a normalized AGC target of 300% with an intensity threshold of 2e4, and a maximum ion injection time (max IT) of 50 ms. MS/MS experiments were performed with a resolution of 30,000, a normalized AGC target of 200%, and a max IT of 86 ms. The isolation window was set to 0.7 m/z, and the first mass was set to 110 m/z. Differentially expressed proteins (DEPs) were identified as those with over 1.2- or < 1/1.2-fold change as the cutoff value. Proteins with a P < 0.05 calculated by Student’s t test were included in the DEP list.

Bioinformatics

Gene Ontology (GO) enrichment analysis of FF proteins was performed by using DAVID software v6.7 (Database for Annotation, Visualization and Integrated Discovery). Proteins were classified by GO annotations based on three categories: biological process, cellular component, and molecular function. Enrichment analysis of the functional annotation was conducted by a two-tailed Fisher's exact test. P < 0.05 was considered significant. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.genome.jp/kegg/) was used to perform the enrichment analysis of pathways. Protein–protein interaction (PPI) information was obtained from the STRING database (https://string-db.org/).

Validation By Parallel Reaction Monitoring (Prm)
The differential abundance of proteins was validated by PRM using the same FF samples as TMT. PRM validation was performed on 7 selected proteins. The proteins were chosen from differentially expressed proteins (DEPs) with adjusted P values less than 0.05. The selected proteins are involved in different biological processes. Twenty peptide precursors from these 7 proteins were successfully monitored through the PRM assay with the following limitations: unique peptide, no dynamic modification, no missed cleavage, peptide length ranging from 8 to 20 using SKYLINE (Version 21.1), and clear mass-fragment spectrum. PRM analysis was performed on a Thermo Q Exactive™ HF system with an UltiMateTM 3000 RSLCnano System (Thermo Fisher Scientific). Peptides from FF samples were separated at a flow rate of 300 nL/min along a 60 min 10–30% linear LC gradient of buffer B. The PRM acquisition method was the same as the previous publication of Sun et al. [13].

**Statistical analysis**

Statistical analyses were performed using SPSS software version 23.0 (IBM Software, New York, USA). The results are presented as the mean ± standard deviation. Comparative analysis of the quantitative data was performed using the two-tailed Student’s t test between the hqFF and nhqFF groups, and P < 0.05 was considered statistically significant.

**Results**

**Quantitative mass spectrometry analysis of FF by TMT labeling**

To compare the global proteome profiles of FFs in the hqFF and nhqFF groups based on embryo quality, a TMT labeling-based strategy was applied. A total of 558 proteins were identified. Among them, there were 50 differentially expressed proteins in the hqFF group compared with the nhqFF group, including 32 upregulated proteins (> 1.20-fold, P < 0.05) and 18 downregulated proteins (< 0.67-fold, P < 0.05) (Table 1, Fig. 2).
Table 1
Differentially expressed proteins and their expression levels quantified by TMT.

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<th>P_value</th>
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<td>ARSG</td>
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<td>Ribonuclease</td>
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<td>0.010174</td>
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Bioinformatics analysis of differentially expressed FF proteins associated with embryo quality.

To investigate the biological roles of the DEPs, functional enrichment analyses were performed. Gene Ontology (GO) annotation of these DEPs was performed using the DAVID Functional Annotation tools. These proteins were classified by GO annotations based on three categories: biological process, cellular component, and molecular function (Fig. 3). In the biological process category, upregulated DEPs were mainly enriched in negative regulation of peptidase activity, negative regulation of endopeptidase activity, acute inflammatory response, and protein activation cascade, while the downregulated DEPs were mainly enriched in regulation of cell adhesion, extracellular structure organization and external encapsulating structure. Regarding cellular component and molecular function categories, the upregulated DEPs were associated with terms including blood microparticles, high-density lipoprotein particles, endopeptidase inhibitor activity, serine-type endopeptidase inhibitor activity and peptidase inhibitor activity. The downregulated DEPs were associated with the molecular function of extracellular matrix structural and constituent collagen binding. They were also enriched in the processes of the protein activation cascade (Supplementary Table 1, Fig. 3). KEGG pathway-based enrichment analysis showed that the upregulated proteins were significantly enriched in complement and coagulation cascades, Staphylococcus aureus infection, systemic lupus erythematosus, and coronavirus disease, while the downregulated proteins were enriched in tyrosine metabolism (Supplementary Table 1). In addition, STRING analysis was used to construct an interaction network between these DEPs (Fig. 4).

PRM

To confirm our TMT results, we verified the FF levels of 7 proteins by PRM in hqFF and nhqFF samples. Among the 7 proteins detected by PRM, 6 peptide precursors from 4 proteins were upregulated, and 1 peptide precursor was downregulated (Supplementary Table 2). This result is consistent with the TMT analysis (Fig. 5).

Discussion

FF is the microenvironment of oocytes in vivo. Some components of FF may affect folliculogenesis. Hence, exploring these components may contribute to discovering predictive biomarkers for oocyte quality and reproductive outcomes as well as understanding reproductive processes.

In the present study, TMT-based proteomic analysis was applied to identify FF protein profiles associated with embryo quality. We identified 558 proteins, of which 50 were significantly differentially expressed between the hqFF and nhqFF groups. Among them, 32 were upregulated, and 18 were downregulated. The result of PRM validation is consistent with TMT analysis. Through GO and KEGG analyses, components of the coagulation and complement systems (C7, C1R, SERPINC1), negative regulation of peptidase activity (SERPINC1, SERPINF1, SERPINA6), ITIH1, ITIH2, and KRT1 (structural constituent of cytoskeleton) were upregulated in the hqFF group.

KEGG analyses showed that complement and coagulation cascades were the pathways in which the most upregulated genes were enriched (P = 3.06E-04). Not only does complement promote coagulation, but coagulation also activates complement. They cross talk with each other extensively [14]. The role of the complement system in follicle development and pregnancy is controversial. In a study of pigs, it was demonstrated that iCO3b, a derivative of CO3, positively influenced oocyte maturation [15]. Hashemitabar et al found that compared to younger women (20–32 years old), the level of the complement cascade (components C3, C4) decreased in older women (38–42 years old) [16]. This finding supported the positive effect of the complement cascade on oocyte maturation. Some previous studies also showed that complement component 3 is positively correlated with fertilization [7, 17]. However, in a study by Jarkovska et al., it was demonstrated that inappropriate complement activation causes a deficiency in free vascular endothelial growth factor (VEGF), the angiogenic factor required for oocyte maturation [5]. Kushnir et al indicated that inhibition of the complement system is needed for oocytes to maintain their viability; a lack of inhibition was associated with miscarriage [9]. In our study, several components of the complement system (e.g., C7 and C1R) were upregulated in hqFF versus nhqFF.
In our study, SERPINC1 was upregulated in hqFF versus nhqFF. The role antithrombin-III in reproduction is controversial. The study of Severino showed that antithrombin-III was upregulated in a positive IVF outcome FF sample versus a negative sample [8]. The study of Kim YS showed that antithrombin plays an important role in maintaining normal pregnancy [18]. However, the study of Estes showed that antithrombin is decreased in the FF of live birth patients versus nonpregnant patients. However, in that study, oocyte number was a dependent variable; hence, the predictive value of proteomic analysis for IVF outcome might be affected by the ovarian response [10]. Consistent with the studies of Severino and Kim YS, our study suggested that SERPINC1 is positively related to embryo quality. In IVF, the function of the complement and coagulation cascades may be very complicated. More work is still needed in both mechanistic research and clinical practice to investigate the role it plays in the reproductive process.

In this study, we found that ITIH1 and ITIH2 were upregulated in hqFF compared to nhqFF. ITIH1 and ITIH2 are two subunits (heavy chain) of intera-trypsin inhibitor [19]. They are designated the only serum-derived hyaluronan-associated proteins (SHAP) that have been shown to bind covalently to hyaluronan (HA) [20, 21]. The cumulus layer of cumulus-oocyte complexes (COCs) is a unique structure that contains numerous cumulus cells plus an extracellular matrix that binds these cells together [22]. Under ovulatory stimulus, ITIH1 and ITIH2 enter the follicle from serum and crosslink with HA, which is synthesized and secreted by cumulus cells [23, 19]. The SHAP-HA (ITIH1/ITIH2-HA) complex is essential for stabilization of the COC extracellular matrix [19]. At the same time, COC undergoes expansion. This process is essential for ovulation and fertilization [23, 24]. Therefore, the upregulation of ITIH1 and ITIH2 may benefit ovulation and fertilization.

Corticosteroid binding globulin (CBG) protein, the protein encoded by the SERPINA6 gene, is a member of the serine protease inhibitor (SERPIN) family. CBG is the major cortisol-binding protein modulating circulating plasma corticosteroids and their bioavailability [25, 26]. Glucocorticoids (GCs) are essential steroid hormones for the life of mammals [27]. Many studies have focused on the direct effect of GCs on oocytes, but the results are controversial. Andersen et al. showed that both free and total cortisol in FF were not associated with the developmental capacity of oocytes [28]. However, in the study of Jimena, the level of cortisol in FF was found to be negatively associated with fertilization outcome [29]. Prasad et al. found that cortisol damages oocyte quality by decreasing estradiol production [30]. However, in the study of Simerman, it was shown that FF cortisol levels positively correlated with oocyte quality during IVF [31]. The study of YDING showed that in IVF treatment, the cortisol-binding protein concentration in FF was positively associated with IVF outcome and can be used as a predictor of IVF outcome [32]. Through GO analysis, we found that the SERPINA6 protein was upregulated. Our result was consistent with the study of YDING.

In previous studies, FF from multiple mature follicles was pooled. Hence, it was difficult to ascertain the point-to-point correlation between protein profiles and oocyte quality. In the present study, we collected FF at the beginning of OPU from one 16–18 mm follicle and confirmed that there was one oocyte in this follicle. The embryo development outcome was accurately recorded. The predictive value of FF protein profiles on embryo development competence can be assumed.

**Conclusions**

In conclusion, using a proteomic analysis, we successfully identified 50 differentially expressed proteins in the FF that were associated with embryo quality. Our study was helpful to find potential biomarkers for predicting embryo development competence. It also provides valuable data for understanding reproductive processes.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>AMH</td>
<td>anti-Müllerian hormone</td>
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<tr>
<td>CBG</td>
<td>corticosteroid binding globulin</td>
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complement C1r subcomponent
C7
complement component C7
COCs
cumulus-oocyte complexes
DDA
data-dependent acquisition
DEP
differentially expressed protein
FF
follicular fluid
GCs
glucocorticoids
GnRH
gonadotropin releasing hormone
GO
Gene Ontology
ICSI
intracytoplasmic sperm injection
ITIH1
inter-alpha-trypsin inhibitor heavy chain H1
ITIH2
inter-alpha-trypsin inhibitor heavy chain H2
IVF
in vitro fertilization
KEGG
Kyoto Encyclopedia of Genes and Genomes
LC/MS/MS
liquid chromatography-tandem mass spectrometry
PPI
protein–protein interaction
PRM
parallel reaction monitoring
SERPIN
serine protease inhibitor
SERPINA6
serine protease inhibitor A6
SERPINC1
serpin peptidase inhibitor, clade C
SHAP
serum-derived hyaluronan-associated proteins
TMT
tandem mass tags
VEGF
vascular endothelial growth factor

Declarations
Acknowledgments

We thank all the patients for participating in this research.

Author Declarations

Competing interests

The authors declare that they have no competing interests.

Ethics approval and consent to participate

The Ethics Committee of The First Affiliated Hospital of USTC approved this study protocol (2022-KY-354), and informed consent was obtained from each individual.

Consent for publication

Consent for publication All authors consent to the publication of the manuscript.

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Data Availability

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Code Availability

Not applicable.

Authors’ contributions

BX and JJ conceived of the study, SB BX LH and JJ participated in its design and coordination and reviewed the final manuscript for submission. JJ, XZ, LS, SB and HW carried out the studies and drafted the manuscript. BX, SB carries out the bioinformatics analysis. LL participated in the study design and performed the HFF collection. All authors read and approved the final manuscript.

References


Figures
Figure 1

The workflow for ovarian follicular fluid biomarkers of maturation and quality of oocytes.
Figure 2

(A) Volcano plot highlights differentially expressed proteins with the threshold of $| \log_2(\text{fold change}) | \geq 2.5$ and $P$ value $< 0.05$. Red points indicate upregulated proteins, blue points indicate downregulated proteins, and black and gray points denote proteins that are not differentially expressed. (B) Heatmap of 50 differentially expressed proteins.
Figure 3

Gene Ontology enrichment of follicular fluid from hqFF and nhqFF.
Figure 4

STRING analyses.
Figure 5

PRM-MS analysis for seven selected proteins. (A) Representative peak group chromatography of a peptide precursor. (B) The box plot shows the protein abundance in hqFF and nhqFF. Statistical significance was calculated by two-tailed Student’s t test. (C) Average protein relative expression folds of the three proteins (CO7, CBG, ITIH2, VWF) by TMT and PRM.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarydata.docx