Original Article

Urinary proteomic analysis during pregnancy and its potential application in early prediction of gestational diabetes mellitus and spontaneous abortion

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Background: The maternal physiological changes which occur during gestation are complex and affect diverse systems in the body. Elucidating the various changes that occur during pregnancy may assist with understanding maternal health and the factors affecting pregnancy outcomes.

Methods: A longitudinal cohort of 84 pregnant women was established. The urinary proteomes of women in different trimesters of pregnancy (6–8, 22–24, and 32–34 weeks) were characterized using data-independent acquisition tandem mass spectrometry. Gestational diabetes mellitus (GDM) was diagnosed at 24 to 28 weeks. Functional analysis of serial changed proteins was performed.

Results: Fifteen women had GDM, 50 were healthy, and 19 experienced spontaneous abortion (SA). Functional analysis showed that the urinary proteome reflected physiological and pathological changes during pregnancy. Compared to those of women with a normal pregnancy, the urinary proteomes of women with GDM and SA showed significant disease-related changes in insulin secretion and estrogen receptor activity, respectively, during the first trimester. Urinary protein during the first trimester of pregnancy achieved an area under the curve of 0.91 and 0.81 for GDM and SA, respectively.

Conclusions: The urinary proteome has the potential to reflect serial changes of pregnancy progression; therefore, its use might facilitate early diagnosis of pregnancy complications.

Keywords: Gestational diabetes mellitus (GDM); proteomic analysis; spontaneous abortion

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Introduction

Maternal physiological adaptation during gestation is a complex process characterized by metabolic, anatomical, and immune changes (1), which have a significant impact on diverse systems in the body. The hematological, cardiovascular, respiratory, renal, and endocrine systems undergo changes to meet the demands of fetal growth and development, and prepare the mother for parturition (2). Pregnancy has been reported to affect protein expression in maternal serum and urine (3), and thus, a full description of protein expression may aid in understanding the physiological changes during pregnancy and the factors affecting maternal health and pregnancy outcomes. With the rapid development of mass spectrometry (MS) technology in recent years, proteomics has become an important tool for assessing protein expression profiles and can provide an understanding of physiological changes and disease pathogenesis.

Biological specimens, including maternal plasma (1,4), umbilical cord plasma (5), amniotic fluid (6), and urine (3), have been used to characterize protein and peptide changes in normal pregnancy. Unlike plasma and other biofluids *in vivo*, urine is not responsible for maintaining the homeostatic microenvironment, which makes it superior as a source of biomarkers (7). Urine has also been shown to have great potential in reflecting systemic physiological characteristics, which are influenced by factors such as sex, age, diet, hormone status, lifestyle, and extreme environments (8). To date, only one urinary proteomic study on the process of normal pregnancy has been published (3). In urine samples collected before and after vaginal delivery, 16 differential urinary phosphoproteins were found (3). However, whether the urinary proteome reflects the physiological changes of normal pregnancy remains largely unknown.

Urinary proteome analysis has so far been employed to understand and characterize prenatal complications such as hypertension (9), preeclampsia (10-12), and gestational diabetes mellitus (GDM) (13,14). GDM is considered the most common metabolic disorder of pregnancy, and it has severe implications (15). Clinical diagnostic guidelines for GDM recommend that a 75-g oral glucose tolerance test (OGTT) be performed at 24 to 28 weeks (16). However, as the diagnosis is established during the second trimester of pregnancy, drug therapy and dietary intervention are started relatively late. Consequently, inevitable and sometimes irreversible adverse effects on the mother and fetus can occur during the first trimester; early diagnosis and intervention are therefore extremely important. Previous urinary proteome research studied differential proteins during the second or third trimester (13,14), and none of them focused on early biomarkers of GDM.

Spontaneous abortion (SA)—defined as pregnancy loss without intervention before 20 gestational weeks—is a complication that affects approximately 20% of recognized pregnancies (17). Around half of SAs are attributable to genetic abnormalities, such as chromosomal abnormalities (18). There are also multiple other risk factors, including maternal infections, medications, toxins, uterine abnormalities, and chronic maternal diseases (17). It is vital that predictive biomarkers for women who are at increased risk of SA are developed. To the best of our knowledge, no urinary proteomic study has been published on the early diagnosis of SA.

This prospective cohort study aimed to collect urine samples from pregnant women during the first, second, and third trimesters (6–8, 22–24, and 32–34 weeks, respectively). The aim of the study was to perform serial urinary proteome analyses using a data-independent acquisition (DIA) approach to compare physiological and pathophysiological changes during gestation in normal pregnancy, pregnancy affected by GDM, and SA. The differential proteins in the GDM group and the SA group were then verified using a parallel reaction monitoring (PRM) approach (*Figure 1*). This study showed that the urinary proteome has the potential to reflect the changes of pregnancy progression and might facilitate early diagnosis of pregnancy complications. We present the following article in accordance with the STROBE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-21-3497/rc).

Methods

Ethical statement

The research protocol of this study was approved by the Ethics Committee of Peking Union Medical College Hospital (No. ZS-976). The study was conducted under the guidance of the Major New Drugs Innovation and Development Program (Clinical Trial No. NCT03246295). Between October 2015 and May 2016, all pregnant women treated at the Peking Union Medical College Hospital were recruited. All study participants were given a verbal explanation of the study before enrollment, and each participant signed an informed consent form. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

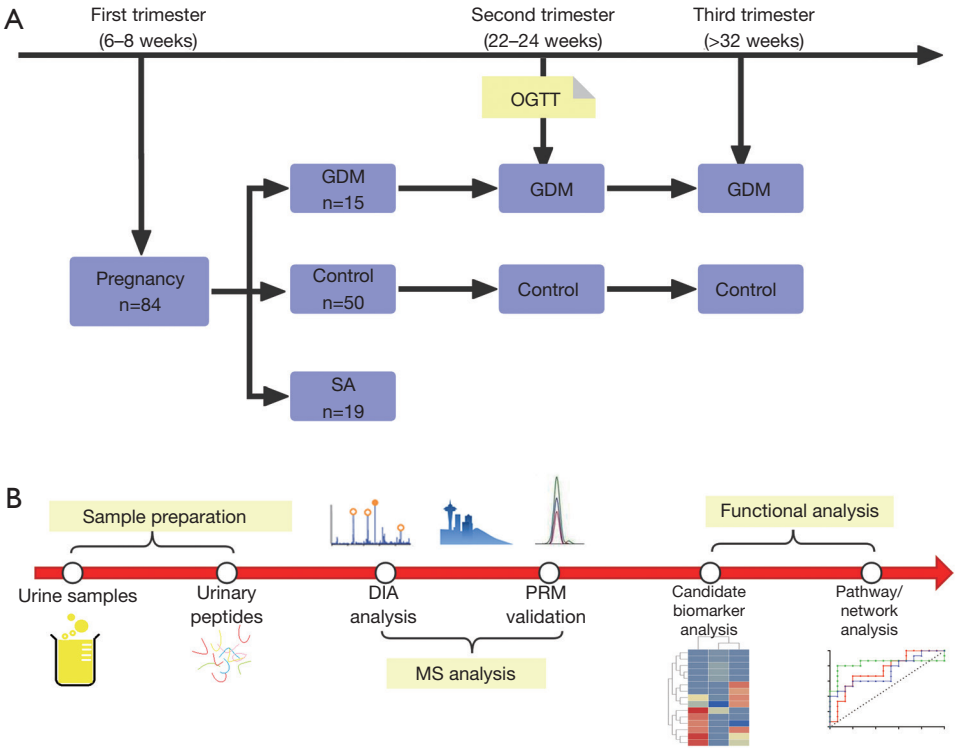

Figure 1 Workflow of the urinary proteome analysis of women with normal pregnancy, GDM, and SA. (A) Participants were divided into four groups. (I) The GDM group: urine samples were collected from 15 GDM patients in each trimester during pregnancy; (II) the Control-1 group: urine samples were collected during each trimester from 15 healthy pregnant women, who were matched to GDM group based on clinical characteristics; (III) the SA group: urine samples were collected from the 19 women with SAs; and (IV) the Control-2 group: urine samples were collected from the 19 women who were matched to SA group based on clinical characteristics. (B) The experimental design of sample preparation, and mass spectrometry and data analysis. OGTT, oral glucose tolerance test; GDM, gestational diabetes mellitus; SA, spontaneous abortion; DIA, data-independent acquisition; PRM, parallel reaction monitoring; MS, mass spectrometry.

Patient groups

A total of 84 pregnant women were recruited, and urine samples were collected at 6 to 8 weeks of gestation. GDM was diagnosed at 24 to 28 gestational weeks using an OGTT test. For a diagnosis to be made, at least one of the following criteria needed to be present: fasting blood glucose ≥5.1 mmol/L, 1-hour blood glucose ≥10.0 mmol/L, or 2-h blood glucose ≥8.5 mmol/L. Fifteen pregnant women developed GDM at 24 to 28 weeks, and 19 women were diagnosed as having had an SA before 12 weeks. Women diagnosed with GDM implemented lifestyle (diet and exercise) interventions to control blood glucose.

The participants were divided into four groups. (I) The GDM group: urine samples were collected at 6–8 weeks, 22–24 weeks, and >32 weeks from the 15 women who developed GDM; (II) the Control-1 group: urine samples were collected during each trimester from 15 healthy pregnant women, who were matched to GDM group based on clinical characteristics; (III) the SA group: urine samples were collected at 6–8 weeks from the 19 women who were diagnosed as having had an SA before 12 weeks; and (IV) the Control-2 group: urine samples were collected during the first trimester from who were matched to SA group based on clinical characteristics. The clinical characteristics of patients in the GDM, SA, and control groups are shown in *Tables 1,2*. Second-morning midstream urine samples were collected from all the participants, centrifuged, and stored at −80 ℃ for analysis.

Sample preparation

Urinary proteins were extracted from 10 mL of urine from each sample via acetone precipitation, and then digested using a filter-aided sample preparation method (19). To

Table 1 The clinical characteristics of patients with normal pregnancies and gestational diabetes mellitus

Characteristics	GDM group	Control-1 group	P value
Sample size (N)	15	15	
Maternal age (years)	33.8±3.61	32±5.01	0.31
Prepregnancy BMI (kg/m^2)	24.2±2.88	23.8±2.33	0.34
24–28 weeks fasting glucose (mmol/L)	4.98±0.42	4.4±0.21	0.01
24–28 weeks OGTT 1-h glucose (mmol/L)	10.28±1.45	7.2±2.08	0.02
24–28 weeks OGTT 2-h glucose (mmol/L)	8.49±1.06	6.4±1.06	0.02
Gravity	1.7±0.9	2±1.31	0.21
Deliveries	1.3±0.49	1.2±0.41	0.21

GDM, gestational diabetes mellitus; OGTT, oral glucose tolerance test.

Table 2 The clinical characteristics of patients with normal pregnancies and spontaneous abortions

Characteristics	SA group	Control-2 group	P value
Sample size (N)	19	19	
Maternal age (years)	34.71±4.57	34.11±3.95	0.34
Gravity	2.6±1.45	2.21±1.2	0.21
Deliveries	0.6±0.49	1.32±0.46	0.00
Systolic blood pressure (mmHg)	107.19±10.27	106.17±7.57	0.38
Diastolic blood pressure (mmHg)	68.44±9.08	69±7.8	0.43
Gestation age at delivery		275.17±8.44	

SA, spontaneous abortion.

generate a protein library and for quality control, a pooled sample with equal amounts of protein from all participants was digested simultaneously. Briefly, proteins were denatured through incubation with 50 mM dithiothreitol at 56 ℃ for 1 h and alkylated in the dark for 45 min in 55 mM iodoacetamide at room temperature. The proteins were then treated with trypsin (1:50) and incubated at 37 ℃ for 8 h. The tryptic peptides were desalted using Oasis HLB cartridges (Waters, Milford, MA, USA).

High-pH reversed-phase liquid chromatography separation

To generate a library for DIA analysis, urinary peptides from the pooled sample were loaded on offline high-pH reversed-phase liquid chromatography (RPLC) columns (4.6 mm × 250 mm, C18, 3 μm; Waters, Milford, MA, USA) in buffer A1 (H_2O, pH 10). The peptides were eluted for 60 minutes with 5–30% buffer B1 (90% acetonitrile, pH 10; flow rate, 0.7 mL/min) and collected at one fraction per minute. In total, 30 fractions were lyophilized, resuspended, and concatenated into 10 fractions by combining fractions 1, 11, 21 and so on.

Liquid chromatography–MS analysis

The individual samples were analyzed using DIA mode. According to the previously described DIA-MS workflow (20,21), firstly, a pooled sample was obtained by mixing the same amount of digested peptide from each individual in the GDM, SA, and Control-1 and Control-2 groups. Then, an extensive data-dependent acquired (DDA) spectral library of the pooled samples was generated using two-dimensional liquid chromatography with tandem MS (LC-MS/MS) in DDA mode. Finally, the individual samples were analyzed using the DIA mode.

The Orbitrap Fusion Lumos MS (Thermo Scientific, Dreieich, Germany) coupled with an EASY-nLC 1000 liquid chromatography system was used for MS analysis. The digested peptides were dissolved in 0.1% formic acid and separated on an RP C18 self-packing capillary LC column (75 μm × 100 mm, 3 μm). The eluted gradient was 5–30% buffer B2 (0.1% formic acid, 99.9% ACN; flow rate, 0.3 μL/min) for 60 min.

To generate the spectral library, the peptide fractions separated by RPLC were analyzed in DDA mode. The parameters were set as follows: the full scan was acquired at a resolution of 60,000, and tandem MS scans were performed at a resolution of 15,000 with an isolation window of 1.6 Da and higher-energy collisional dissociation (HCD) energy of 32%.

The DIA data were acquired with the maximum injection time set to 50 ms for full and DIA scans, and the cycle time set to 1.55 s. The full scan was set at a resolution of 120,000 over an m/z range of 400 to 900, and was followed by DIA scans with a resolution of 30,000 and HCD collision energy of 32%.

Data analysis

To generate the spectral library, the DDA data acquired were processed using the Proteome Discoverer (Thermo Scientific, Dreieich, Germany) and searched against the human UniProtfasta database (Homo sapiens, 71,592 sequences, 2017_09 version) appended with the iRT fusion protein sequence. The search allowed two missed cleavage sites in the trypsin digestion; cysteine carbamidomethylation was set as a fixed modification, parent ion mass tolerances were set to 10 ppm, and fragment ion mass tolerances were set to 0.02 Da. The applied false discovery rate (FDR) cutoff was 0.01 at the protein level. The spectral library was then generated by importing the results to the Spectronaut™ Pulsar software (Biognosys, Schlieren, Switzerland).

The DIA-MS raw files were analyzed using Spectronaut Pulsar 14.10 (Biognosys, Schlieren, Switzerland). Briefly, the DIA raw data were loaded to Spectronaut™ to calculate peptide retention time based on iRT data. The Spectronaut™ software identified and quantitated proteins by matching the retention time and m/z to the peptide library. The proteins were filtered with at least two unique peptides, at an FDR threshold of 1% at the protein level. Then, those proteins that were identified in less than 70% of the samples were removed for further analysis in the GDM and SA groups. Missing values were imputed with the median value of each group.

Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were conducted using SIMCA 13.0.2 (Umetrics, Umeå, Sweden) statistical software.

The area under the receiver operating characteristic (ROC) curve (AUC) was calculated using MedCalc 15.8 (www.medcalc.org). A GDM diagnostic panel was constructed using a logistic regression algorithm available from MedCalc.

Bioinformatics analysis

For ingenuity pathway analysis (IPA), the UniProt accession numbers were uploaded to IPA software (QIAGEN, Germanland, MD, USA). The proteins were mapped to disease and function categories and canonical pathways available in Ingenuity and other databases, and were ranked according to the P value. For each function and network, the enrichment significance was calculated using a one-sided Fisher's exact test.

Targeted proteomic analysis

Data derived from the spectral library using HCD collision were imported into Skyline (v.3.6) (22). The most intense peptide transitions were selected. Up to five transitions per peptide were traced on a TripleTOF 5600 mass spectrometer (AB Sciex, Framingham, MA, USA). Between one and three peptides from each protein were selected for quantification. For ionization, a spray voltage of 2.20 kV and a capillary temperature of 150 ℃ were used. The peptides were monitored by using the PRM acquisition mode to perform tandem MS scans of the precursor ions for the allpeptide markers along the complete chromatographic run. The normalized collision energy was fixed to 35%, and the accumulated time was 100 ms. The resulting tandem MS data were processed using Skyline for further visualization, transition detection, and quantity calculation.

Statistical analysis

PCA and orthogonal partial least squares discriminant analysis (OPLS-DA) were conducted using SIMCA 13.0.2 (Umetrics, Umeå, Sweden) statistical software. One hundred permutation tests was performed to estimate there was no over-fitting in these models.

A comparison of the proteins between multiple trimesters were analyzed using one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered to be serially changed proteins. A comparison of the differential proteins between disease and control groups was analyzed using unpaired student *t*-test in DIA and PRM analysis. Proteins with fold change >1.5 and P<0.05 was considered to be significant differential proteins.

The area under the receiver operating characteristic (ROC) curve (AUC) was calculated using MedCalc 15.8 (www.medcalc.org). A GDM diagnostic panel was constructed using a logistic regression algorithm available from MedCalc.

Results

Demographic characteristics of the study participants

A cohort of 84 pregnant women was initially recruited for this prospective study. Among the initial cohort, 19 women were diagnosed as having had an SA before 12 weeks of gestation, and 15 women were diagnosed with GDM through an OGTT test performed at 24 to 28 weeks of gestation. Fifteen healthy pregnant women were also included for the purpose of studying the longitudinal

dynamic urinary proteomic changes in different trimesters during pregnancy. These healthy women (Control-1 group) were matched to the 15 patients with GDM by age, body mass index, and clinical laboratory data. Furthermore, another 19 healthy pregnant women (Control-2 group) were matched to the 19 women with SAs based on above clinical characteristics. *Tables 1,2* shows the general clinical characteristics of women who had a normal pregnancy and those diagnosed with GDM or who had an SA.

Library generation, and protein identification and quantification

To generate the urinary proteome spectral library, the pooled urinary peptides from all samples were separated into 30 fractions using offline high-pH RPLC and analyzed using DDA LC/MS/MS analysis. The raw data were searched and filtered with Proteome Discoverer software. Finally, a spectral library containing 21,190 precursors, 10,173 peptides, and 2,015 proteins was generated.

Urinary peptides from each sample were analyzed using a DIA approach, and the data were loaded to the Spectronaut Pulsar software for qualitative and quantitative analysis. A total of 1,783 proteins which had at least two unique peptides and met an FDR threshold of 1% at the protein level were identified. For each sample, the identification protein number ranged from 1,112 to 1,620 (Figure S1A and available online: https://cdn.amegroups.cn/static/public/atm-21-3497-1.xlsx). A total of 1,328 proteins remained for further analysis after missing values had been filtered.

The urinary peptides from all pooled samples were also assessed as a quality control measure, whereby every eighth sample was assessed through a standard DIA analysis. Fifteen replicates were then generated to evaluate the technical performance. For the 15 technical replicates, the average number of proteins identified was 1,465, and 1,277 proteins were identified in all 15 runs. The median and 90% quantile Coefficient of variation (CV) of the quantity of all the identified proteins were 0.14 and 0.34, respectively. The Spearman's correlation coefficients between replicates were calculated, and the median R-squared value was 0.97 for all 15 replicates (Figure S1B). The above results evidenced that the integrated analysis system was stable and the technical reproducibility was acceptable.

Longitudinal urinary proteomic study of normal pregnancy

The urinary proteomes of patients with normal pregnancies were characterized during each trimester (at 6–8, 22–24, and 32–36 weeks) using high-throughput LC-MS/MS methods. The DIA data were subjected to multivariate statistical analysis using SIMCA 13.0.2 software. The PCA score plot showed that there is a considerable intergroup distinction of the proteins altered in 1st, 2nd and 3rd trimester of gestation of healthy controls (*Figure 2A*). The first trimester differed significantly from the second and third trimesters. The OPLS-DA score plots showed greater significant differences between the three trimesters (*Figure 2B*). One hundred permutation tests showed that there was no overfitting (Figure S2A). The above data indicated that the urinary proteome exhibited differences across the trimesters of normal pregnancies.

To illustrate the variability in the urinary proteome with the progression of pregnancy, the differential proteins across the three trimesters were analyzed using one-way analysis of variance (ANOVA). In total, 249 urinary proteins (available online: https://cdn.amegroups.cn/static/public/atm-21-3497-2.xlsx) were identified as serially changed proteins ($P<0.05$). *Figure 2C* depicts the functional annotation analysis (IPA) of the differentially expressed proteins during the three trimesters. The proteins that were highly expressed during the first trimester were mostly involved in the cell movement/migration and leukocyte migration. Most of the differential proteins during the second trimester were associated with vasculature branching, anion quantity, and homeostasis. For the third trimester, cellular protein metabolism, body cavity, and morphology were enriched. The functional and pathway analyses of the three trimesters demonstrated that the urinary proteome differed in terms of protein composition and function during gestation, suggesting that it may reflect the physiological changes of pregnancy.

A cross-sectional study of predictive markers in GDM pregnancy

As with normal pregnancies, it was possible to separate the first, second, and third trimesters of pregnancies affected by GDM using PCA and F-DA analysis (*Figure 3A*, Figure S2). In the GDM group, 216 urinary proteins were identified as serially changed proteins (ANOVA) (available online: https://cdn.amegroups.cn/static/public/atm-21-3497-2.xlsx). The functional annotation analysis (Figure S2) of urinary proteins in the GDM group showed that during the first trimester, proteins were enriched in cell movement and migration, which was similar to what was observed in normal pregnancies. However, during the second trimester,

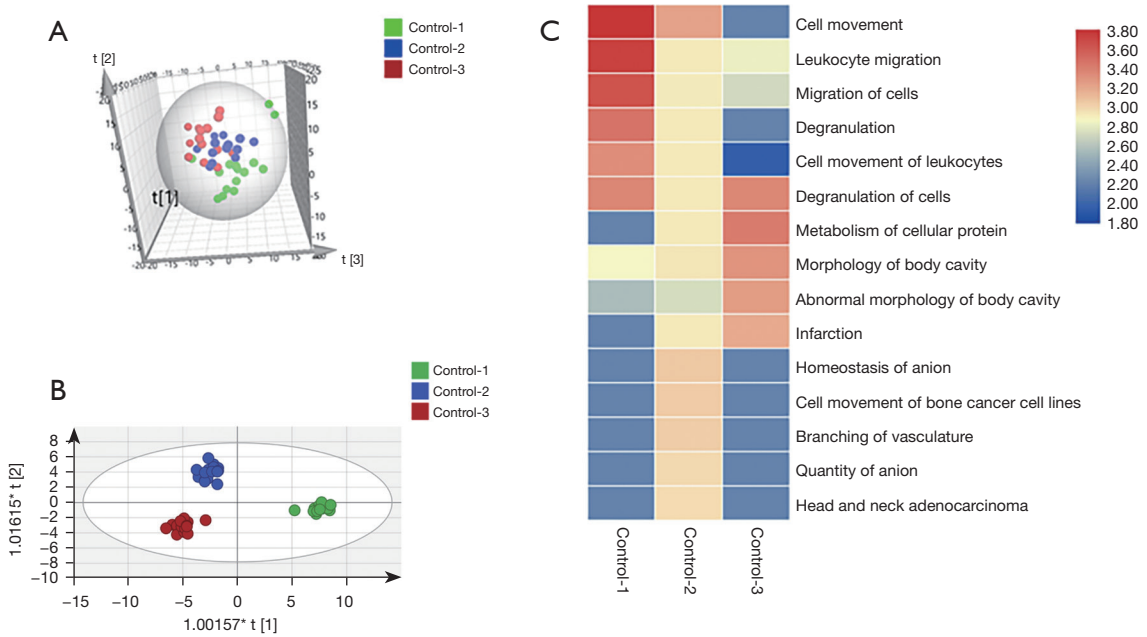

Figure 2 The urinary proteome exhibited differences in the three trimesters of normal pregnancy. (A) The gestational diabetes mellitus score plot shows that there was an intergroup distinction of the proteins altered during the first, second, and third trimester of gestation. (B) The orthogonal partial least squares discriminant analysis score plots show more significant differences between the three trimesters. (C) Functional analysis of longitudinal proteomics study reveals that the urinary proteome differed between the different trimesters. Functional annotations were analyzed using Ingenuity Pathway Analysis software. The significance of enrichment for each function and network were calculated using a one-sided Fisher's exact test.

proteins were functionally annotated in receptor-mediated endocytosis and the classical complement pathway. During the third trimester, proteins participated in cellular invasion and organismal injury and abnormalities. The longitudinal urinary proteomic results of pregnancies affected by GDM differed from those of normal pregnancies, especially during the second and third trimesters.

Comparisons of normal pregnancies and pregnancies affected by GDM across the different trimesters through multivariate analysis using OPLS-DA showed a marked difference between the two groups in all three trimesters (*Figure 3A*, Figure S3). In particular, women with GDM and the normal controls exhibited different urinary proteome patterns, even during the first trimester. To probe the underlying difference, a cross-sectional differential proteome analysis was performed. A total of 48, 90, and 82 urinary proteins were found to be differentially expressed between the two groups during the first, second, and third trimesters, respectively (available online: https://cdn.amegroups.cn/static/public/atm-21-3497-3.xlsx). The functional annotations of the differential proteins during the three trimesters are shown in *Figure 3B*. In the first trimester, the differential proteins were associated with intracellular activities, such as cell movement, migration, and viability. The differential proteins during the second trimester were functionally related to necrosis, immune response, and reactive oxygen species (ROS). The third-trimester differential proteins participated in fatty acid metabolism, apoptosis, and endocytosis. The cross-sectional urine proteome analysis of GDM indicated that the differential expressed proteins in the three trimesters are functionally distinct.

As the early diagnosis of GDM is highly important but has not been implemented in clinical practice, the 21 altered urinary proteins during the first trimester were validated using targeted LC-PRM-MS. To evaluate the stability of the platform, a pooled urine sample was randomly analyzed for quality control. The median CV of all replicates was 0.19, which indicated the stability of the MS signal throughout the analysis. Significant abundance changes ($P<0.05$ using a two-sided *t*-test) were observed for the 28 peptides corresponding to 21 proteins (available online: https://cdn.amegroups.cn/static/public/atm-21-3497-4.

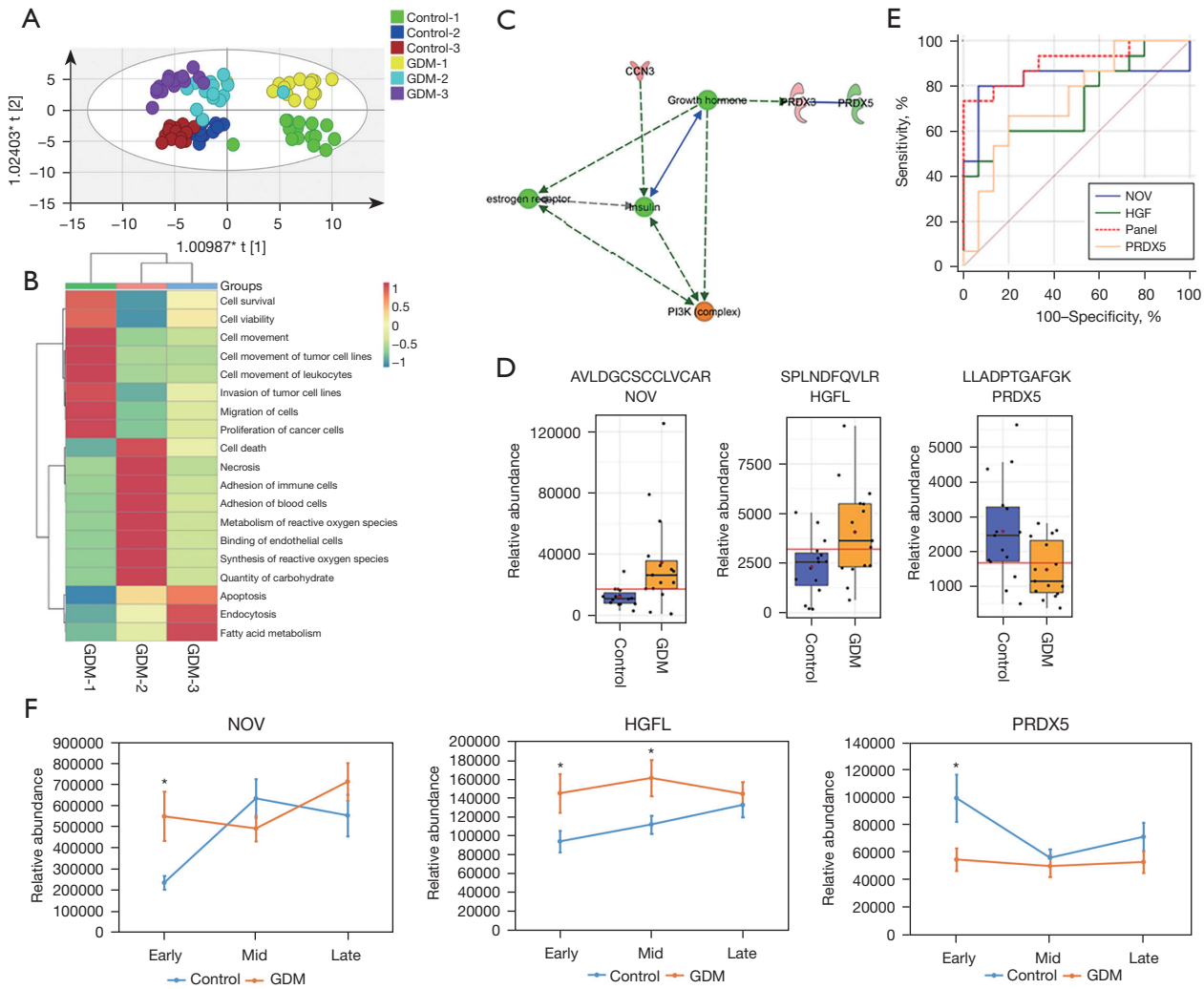

Figure 3 Urinary proteome provides valuable clues for early diagnosis of GDM. (A) The orthogonal partial least squares discriminant analysis score plot of controls and the GDM group makes it possible to separate the urinary proteome during the three trimesters. (B) Functional analysis of the GDM group and controls in different stages of pregnancy. Functional annotations were analyzed using the Ingenuity Pathway Analysis software. (C) The network of early differentially expressed proteins in the GDM group correlated to insulin secretion during the first trimester. (D) The protein quantity distribution of NOV, PRDX5, and HGFL in the normal and GDM groups during the first trimester. (E) The AUC values of the three proteins calculated for discriminating patients with GDM and healthy controls during the first trimester ranged from 0.72 to 0.83. The panel consisting of NOV, PRDX5, and HGFL had an AUC value of 0.91 (95% confidence interval: 0.76–1), with sensitivity and specificity of 73.3% and 100%, respectively. (F) The line plot for the relative abundance of NOV, PRDX5, and HGFL during the first, second, and third trimesters of pregnancy in the control and GDM groups. The average relative abundance and standard error of each group is shown. *P<0.05. GDM, gestational diabetes mellitus; AUC, area under the curve.

xlsx); the tendency of the 21 proteins was consistent with the DIA results.

The differentially expressed proteins were then imported into IPA to generate the molecular network. As shown in *Figure 3C*, insulin served as the core component of the network, and was directly and positively connected to growth hormone secretion. Insulin secretion is modulated by upstream negative elements like CCN3 and PI3K, while the elevation of growth hormone has a negative effect on another molecule, PRDX3.

Marked differences were observed in three urinary proteins, NOV, PRDX5, and HGFL (HGFL/MSP-

Table 3 Seven function-related proteins that were validated in the parallel reaction monitoring analysis

Peptides	ID	Protein	PRM			DIA			Related diseases
			ROC	Disease/CON	P value	ROC	Disease/CON	P value	
AVLDGCSCCLVCAR	P48745	NOV_HUMAN	0.83	2.84	0.02	0.78	2.14	0.02	GDM
LLADPTGAFGK	P30044	PRDX5_HUMAN	0.74	0.57	0.02	0.76	0.55	0.03	GDM
SPLNDFQVLR	P26927	HGFL_HUMAN	0.72	1.77	0.02	0.71	1.47	0.04	GDM
VADPAYLPTQQDVLR	P50148	GNAQ_HUMAN	0.78	0.44	0.01	0.83	0.55	0.01	SA
LFVSGACDASAK	P62873	GBB1_HUMAN	0.80	0.55	0.00	0.74	0.65	0.03	SA
TDTGVSLQTYDDLLAK	Q99988	GDF15_HUMAN	0.81	0.41	0.02	0.85	0.28	0.00	SA
MFDVGGQR	P08754	GNAI3_HUMAN	0.75	0.63	0.03	0.81	0.61	0.01	SA

PRM, parallel reaction monitoring; DIA, data-dependent acquisition; ROC, receiver operating characteristic curve; CON, control; GDM, gestational diabetes mellitus.

induced motility in cells is known to be mediated by PI3K pathways (23), that also play central parts in the network of insulin effect. The predictive performance of these three proteins was further evaluated using the ROC curve (*Tables 2*,*3*). The AUC values of the three proteins for discriminating GDM and healthy pregnancies ranged from 0.72 to 0.83 (*Figure 3D*). According to the above functional annotation and ROC analyses, a panel of NOV, PRDX5, and HGFL could achieve an AUC value of 0.91 (95% confidence interval: 0.76–1), with sensitivity and specificity of 73.3% and 100%, respectively (*Figure 3E*). These three proteins are functionally associated with the insulin secretion network and might serve as valuable predictive biomarkers for the early diagnosis of GDM.

To understand the changing trends of these three proteins during pregnancy, their relative abundance during the first, second, and third trimesters was analyzed in the control and GDM groups. Using normal pregnancies for comparison, NOV was upregulated during the first trimester in women with GDM pregnancies, but recovered to a normal level during the second and third trimesters. HGF was upregulated during the first and second trimesters in the GDM group, but decreased to a normal level during the third trimester. PRDX5 was downregulated during the first trimester in the GDM group, before recovering to a normal level during the second and third trimesters (*Figure 3F*).

A cross-sectional study of predictive markers in SA

In this study, 19 urine samples from women who experienced SAs and 19 from Control-2 group, collected at 6 to 8 gestational weeks, were used to study potential predictive biomarkers of SA. The PCA and OPLS-DA of the urinary proteome showed a significant difference between the two groups (*Figure 4A*,*4B*). The differential proteins were defined by P<0.05 and fold change >1.5. A total of 86 differentially expressed proteins (56 upregulated and 30 downregulated) were found. IPA functional analysis of these proteins revealed a series of pathways and functions to be involved in the pathogenesis of SA. The top canonical pathways (*Figure 4C*) included the synaptogenesis signaling pathway, nNOS signaling, and superoxide radical degradation. An upstream analysis of differential proteins indicated that estrogen receptors play important roles in their regulation. Nine proteins (RAB2A, EVPL, ECM1, SPRR1A, GNA/B, EFNA, AHNAK, GNAQ, and GDF) were found to be modulated by the estrogen hormone upstream pathway (*Figure 4D*).

Next, 42 proteins (available online: https://cdn.amegroups.cn/static/public/atm-21-3497-4.xlsx) were validated using the PRM method. The protein change tendency of these 42 proteins in PRM validation was consistent with the DIA results. The predictive performance of these proteins was further evaluated using ROC curve analysis. Four proteins (GDF-15, GNAQ, GBB1, and GNAI3) included in the upstream estrogen metabolism pathway had higher AUC values, ranging from 0.75 to 0.81, than the other proteins (*Figure 4E*,*4F*, *Tables 3*,*4*). The above data showed that the cause of SA was multifactorial and that the profile of the urinary proteome might provide some clues for early diagnosis.

Discussion

In this study, comprehensive urinary proteomic profiling

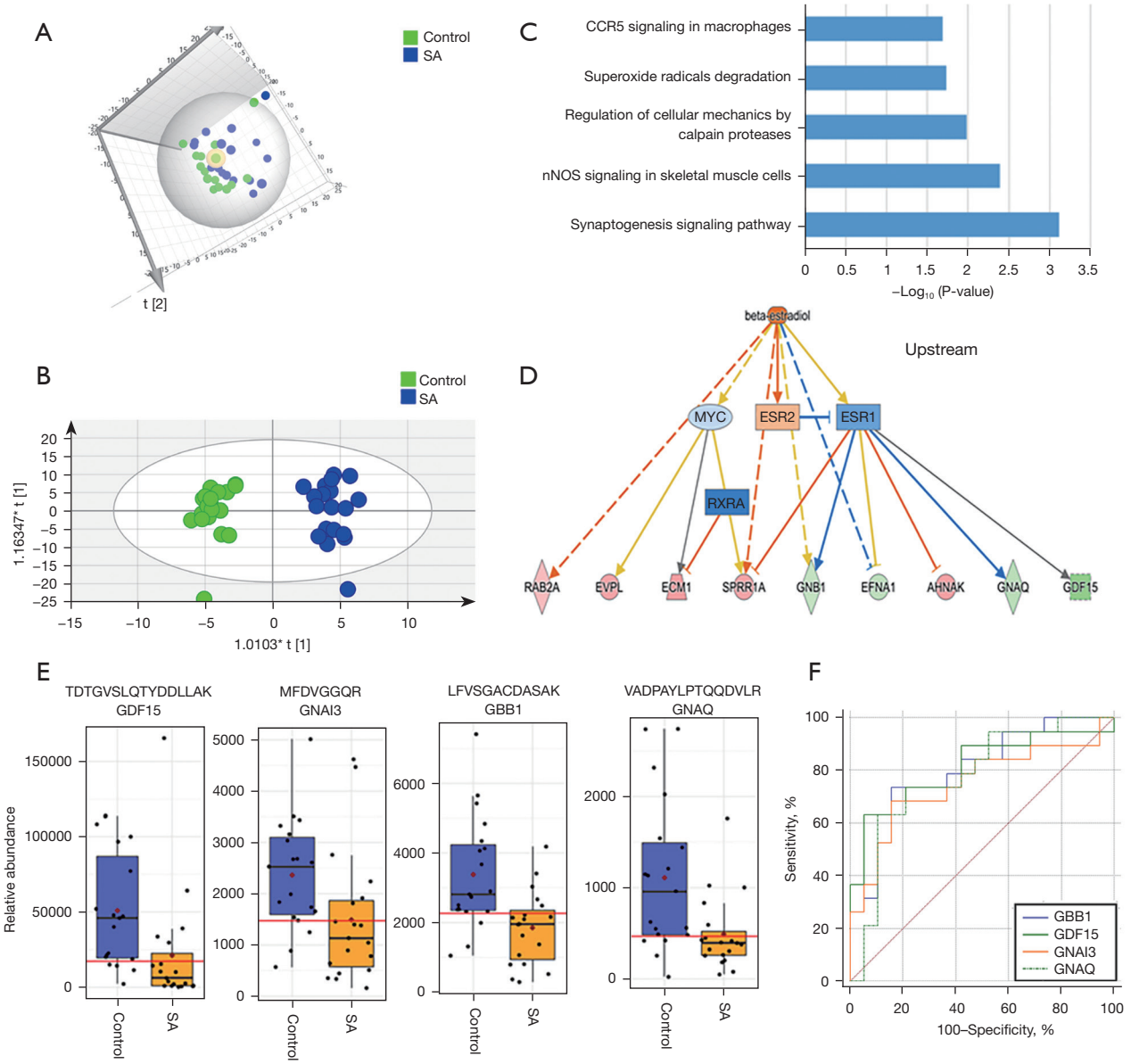

Figure 4 The urinary proteome provides valuable clues for early diagnosis of SA. (A) The principal component score plot shows differences between the SA and the control groups. (B) The orthogonal partial least squares discriminant analysis score plot of SA and control groups make the difference more evident. (C) The canonical pathways annotated using the ingenuity pathway analysis software. The top canonical pathways included the synaptogenesis signaling, nNOS signaling, and superoxide radical degradation pathways. (D) Upstream analysis of the differential proteins. Nine proteins (RAB2A, EVPL, ECM1, SPRR1A, GNA/B, EFNA, AHNAK, GNAQ, and GDF) were modulated by the estrogen hormone upstream pathway. (E) The protein abundance distribution of the GDF-15, GNAQ, GBB1, and GNAI3 proteins in the control and SA groups. (F) The area under the curve of the four proteins. SA, spontaneous abortion; nNOS, neuronal nitric oxide synthase.

of pregnant women during each trimester was undertaken using the DIA LC-MS/MS approach. During the three trimesters, the urinary proteome showed different patterns and suggested various important biofunctions, which indicated that the longitudinal urinary proteome may reflect the physiological changes during normal pregnancy. Urinary proteomic analysis applied to women with GDM pregnancy and SA further revealed proteomic differences

Table 4 AUC, sensitivity, specificity, accuracy, and MCC of biomarkers for GDM and spontaneous abortion diagnosis

Protein name	Disease	AUC	Sensitivity	Specificity	Accuracy	MCC
NOV	GDM	0.742	0.667	0.8	0.733	0.471
HGF	GDM	0.827	0.8	0.933	0.867	0.74
PRDX5	GDM	0.724	0.6	0.8	0.7	0.408
combine	GDM	0.902	0.733	1	0.867	0.761
GDF15	SA	0.812	0.632	0.947	0.79	0.61
GNAQ	SA	0.778	0.632	0.895	0.763	0.546
GBB1	SA	0.801	0.737	0.842	0.789	0.582
GNAI3	SA	0.751	0.684	0.842	0.763	0.533

AUC, area under the curve; MCC, Matthews correlation coefficient; GDM, gestational diabetes mellitus; SA, spontaneous abortion.

in both protein composition, and canonical pathways and networks, indicating that the urinary proteome may reflect changes related to pregnancy disorders. In summary, the urinary proteome might be used to monitor pregnancies and study pregnancy complications.

The urinary proteome could reflect the changes in normal pregnancy

The urinary proteomic analysis showed that cell movement and leukocyte/cell migration were significantly elevated during the first trimester of normal pregnancy. During this time, cell movement and migration increase to meet the needs of placental development, implantation, and pregnancy. A previous study reported that endometrial epithelial cell-derived interleukin-8 stimulated the migration and survival of first-trimester villous cytotrophoblast cells (24). In terms of the immune responses during the early stage of pregnancy, the elevated leukocyte activity may be attributable to possible changes in progesterone levels, which affects innate immune cells, pathways, and humoral immunity (25).

In the second trimester, the functions of anion homeostasis and branching of vasculature, which might serve in maintaining acid or base balance and optimal solute transport rates, were more significant. Fetal growth accelerates toward the end of the second trimester. In order to meet these alterations in the fetal growth rate, the capacity of the placenta to exchange solute and water also has to increase over the gestation period (26), which may be the cause of the anion homeostasis elevation.

During the third trimester, cellular aggregation, cellular protein metabolism, and the humoral immune response were significantly changed. Cellular aggregation, especially platelet aggregation, may be related to complications in pregnancy or childbirth. Platelet reactivity has been reported to drop during the first trimester of pregnancy but is significantly elevated during the third trimester (27). In the current study, the increased cellular aggregation changes in the late phase might provide some useful clues about platelet reactivity changes throughout pregnancy. Regarding protein metabolism, metabolites such as fatty acid and glucose are well known to be elevated during late pregnancy; however, protein metabolism is also an important component of fetal growth. Some proteins that play a role in metabolic processes, like apolipoprotein C-II, are reported to increase significantly in plasma during the third trimester (1). Based on the above, urinary proteomes in different stages of pregnancy are closely related to known physiological processes and may also provide some new findings.

The urinary proteome might be used for early diagnosis of GDM

When compared with healthy pregnancies, significant differences in physiology were observed for pregnancies affected by GDM depending on the pregnancy stage. In the first trimester, compared to the controls, the GDM group showed upregulation of intracellular activities such as cell survival, viability, movement, and migration. As mentioned previously, in a normal pregnancy, cell movement and migration are significantly elevated during the first trimester to support morphogenesis and body shape establishment. In GDM pregnancies, these changes in basic physiological activities are even more obvious. Previous reports show that in the early stage, the damage to insulin sensitivity

is unknown (28). In the current study, PRM was used to validate differential urinary proteins during this early phase of the insulin-related network, which indicated that possible islet damage may occur during the early trimester, even when blood glucose is normal.

During the second trimester, it is evident that increased blood glucose, which results in elevated ROS production (29), may eventually cause pancreatic β-cell damage and cell apoptosis (30). Our results are comparable with the previously reported finding that β-cell levels are significantly lower in women with GDM during the second and third trimesters (31). Functional analysis of the urinary proteome in the GDM group showed more differences in cell death or necrosis and immune response (immune cell adhesion, metabolism, and ROS synthesis), which is consistent with the above reports.

Our urinary proteomic analysis indicated that the fatty acid metabolism pathway varies significantly during the third trimester. As reported previously (1), fatty acid metabolism is closely related to insulin resistance and progress in late pregnancy. A previous study also reported that fatty acid metabolism in women with GDM was different to that of controls (32). Also, cell apoptosis in the third trimester was as significant as that during the second trimester, indicating that the body is recovering during this time.

The above data showed that the urinary proteome can reflect the disease progression of GDM. During the first trimester, the network of differential proteins targeted at insulin-related functions and the glucose level remains normal, which may be the result of tissue changes in the compensatory stage. With the increase of stress stimulation in the second trimester, insulin secretion is impaired, ROS production and blood glucose are increased, and cell apoptosis occurs; this may reflect a decompensation stage. During the third trimester, with lifestyle intervention, blood glucose returns to normal and cell damage is reduced, but fatty acid metabolism remains abnormal, indicating that the body is recovering.

We also attempted to identify urinary biomarkers for the early diagnosis of GDM. Through functional analysis, three proteins related to the insulin network were defined: NOV, PRDX5, and HGFL. The circulating NOV level is reported to be positively correlated with HbA1c, body mass index (BMI) and fat mass (33). NOV, which is highly expressed in smooth muscle cells of the arterial vessel wall (34), inhibits β-cell proliferation via different mechanisms to impair β-cell insulin secretion (35). Therefore, the upregulation of NOV in the early trimester might be related to β cells. However, NOV has also been reported to be a proangiogenically secreted molecule involved in placental development, and is increasingly expressed in the placenta and serum during normal pregnancy. NOV recovers to normal levels during normal pregnancy (36). PRDX5, a member of the family of antioxidant enzyme, participates in eliminating hydrogen peroxide and neutralizing other ROS (37). PRDX5 has been found to significantly prevent high glucose-induced apoptotic cell death, and it has been reported as an early sign of retinal pathology in diabetic patients (38). In the first trimester of pregnancy, when maternal–fetal blood circulation has only just been established, the oxygen concentration increases rapidly in the placenta, leading to an increase in oxidative stress and the subsequent activation of the antioxidant enzymes to protect against oxidative stress. The generation of ROS causes a decrease in endogenous antioxidants. Therefore, the early downregulation of PRDX5in patients with GDM might be related to its overconsumption in the process of protecting against oxidative stress. During the second and third trimesters of GDM pregnancy, the consumption of PRDX5 of GDM and normal pregnancy was similar and showed the similar expression level (39-41). HGF, a vital component of insulin resistance pathophysiology, plays roles in β-cell homeostasis, inflammatory response mediation, and the glucose metabolic flux in diverse insulin-sensitive cell types (42). Increased levels of HGF are associated with the occurrence of insulin resistance, and HGF has been regarded as a serum biomarker of macroangiopathy in DM (43). The decrease of HGF during the third trimester of GDM pregnancy might reflect a clinical treatment effect. The above three urinary proteins have not been reported in previous urinary proteomic studies of GDM, but all of them have been reported to play roles in the pathogenesis of insulin resistance or the inflammatory response. Therefore, changes in the protein panel might be used for the early diagnosis of GDM.

The urinary proteome could potentially be used for early diagnosis of SA

According to previous reports, the causes of SA are multifactorial. In our study, analysis of differential urinary proteins showed that the synaptogenesis signaling, nNOS signaling, and superoxide radical degradation pathways were the top canonical pathways involved (*Figure 4C*). According to IPA annotations, synaptogenesis signaling and nNOS signaling play important roles in the brain and skeletal

muscle, while superoxide radical degradation signaling is related to cell death and survival. Estrogen receptor alpha (*ESR1*) and estrogen receptor beta (*ESR2*), two major isoforms of estrogen receptors, act directly or indirectly on these differentially expressed molecules. Estrogen modulates multiple reproductive functions, including progesterone production and uteroplacental blood flow (44), and is thus important for fertilization and gestation. Some polymorphisms in the *ESR1* gene, such as *PvuII* and *XbaI*, have been linked to increased abortion risk (45). However, the relationships between gene polymorphisms in *ESR2* and abortion still need further research.

Four proteins (GDF-15, GNAQ, GBB1, and GNAI3) that were validated by PRM in the current study with high confidence are modulated by *ESR1* and *ESR2* (*Figure 4B*). GDF-15, which modulates food intake, energy consumption, and body weight when metabolic and toxin-induced stress increases (46), was elevated in all of the SA samples this study. This suggests that the metabolic and stress pathway may play important roles in SA. Oxidative stress-induced placental dysfunction has been put forward as a common cause of multifactorial and polygenic etiologies of abortion (47). GNAQ, GNAI3, and GBB1 are subunits of guanine nucleotide-binding protein G (G proteins) which are involved as modulating or transduction proteins in various transmembrane signaling cascades (48). Also, G-protein coupled receptors, in response to hormones, neurotransmitters, and environmental stimulants, may serve as potential therapeutic targets for a broad spectrum of patients (49). The changes of growth/differentiation factor and G proteins in urine of SA patients may provide some clues for the causes such as metabolic/toxin-induced stresses, GTPase activity and G protein-effector interaction. However, these proteins need to be validated in future studies, and the causes of the SAs in participants in this study remain unknown.

Conclusions

In this study, a longitudinal and cross-sectional cohort urinary proteomic study of 84 pregnant women was conducted. Analysis of the urinary proteome in each trimester of normal pregnancy reflected the progression of pregnancy and can be utilized to understand the physiological changes that take place during pregnancy. The application of urinary proteome analysis in GDM and SA provide candidate biomarkers for the early diagnosis of GDM during the first trimester and clues as to the pathogenesis of SA. Therefore, the urinary proteome has the potential to reflect the changes with the progression of normal pregnancies, and our candidate biomarker panel may facilitate early diagnosis of pregnancy complications. With the advancement of proteomics technology, in-depth and high-throughput analysis will be provided more comprehensive data in the future. Multiomics studies with large sample sizes and multicenter clinical trials may provide more reliable results in the future.

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Footnote

Reporting Checklist: The authors have completed the STROBE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-21-3497/rc

Peer Review File: Available at https://atm.amegroups.com/article/view/10.21037/atm-21-3497/prf

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-21-3497/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-21-3497/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the Ethics Committee of Peking Union Medical College Hospital

(No. ZS-976), and informed consent was obtained from all participants.

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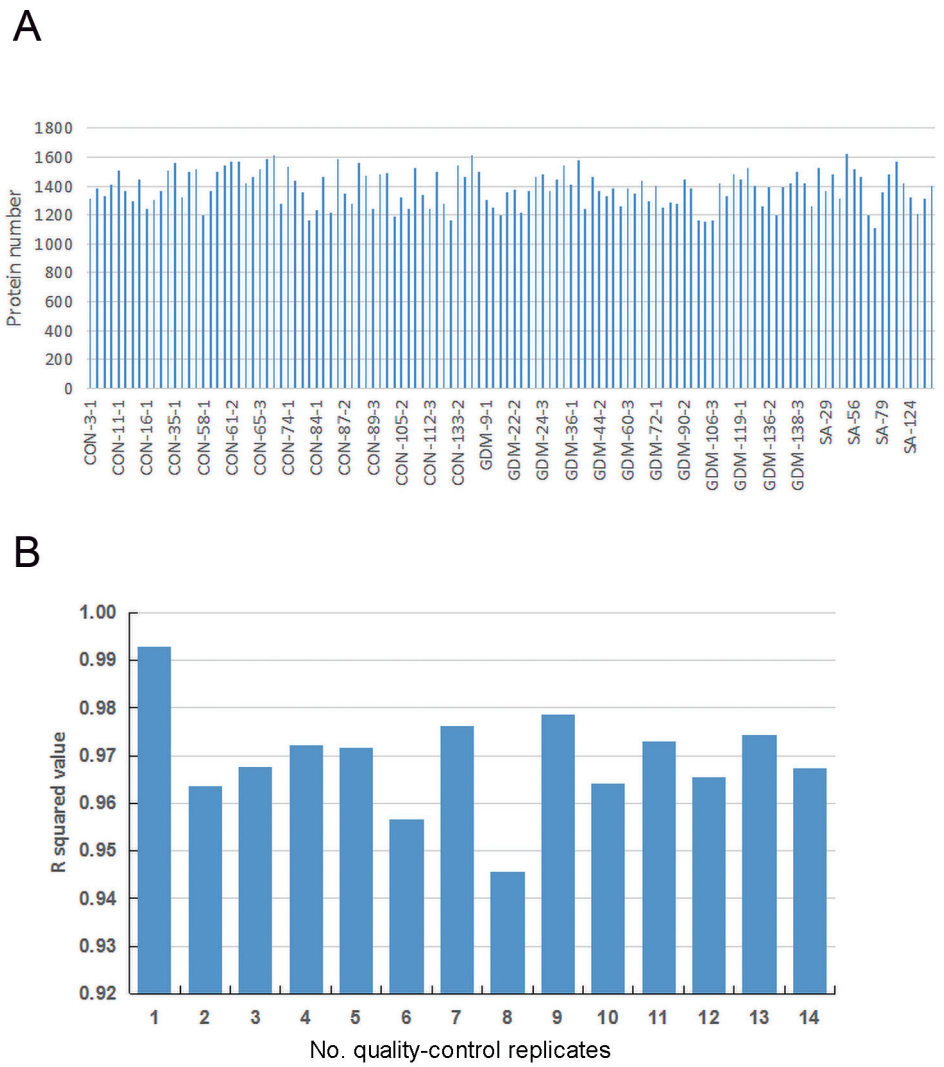

Figure S1 Quality control of the DIA proteomic data. (A) The identification protein numbers across patients with normal pregnancies, gestational diabetes mellitus, and spontaneous abortion; (B) the R-squared value distribution of Spearman's correlation coefficient analysis of all the quality-control replicates. DIA, data-independent acquisition.

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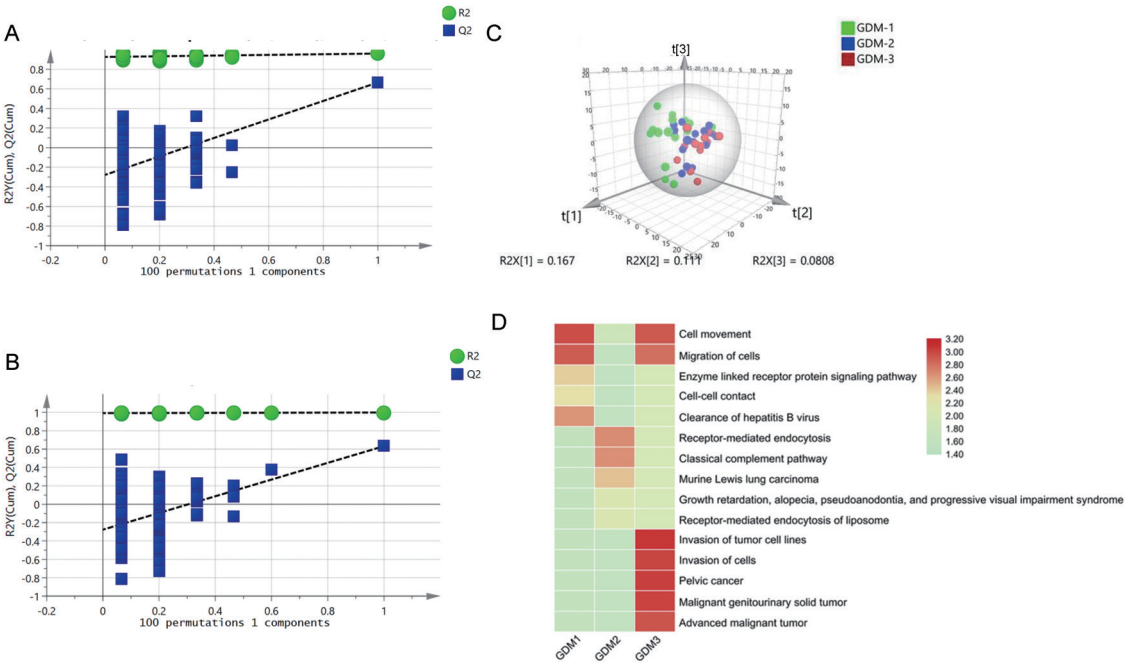

Figure S2 The urinary proteome exhibited differences in the three trimesters of normal pregnancy and GDM. (A) The permutations tests showed that there was no overfitting in the OPLS-DA for normal pregnancy. (B) The principal component analysis made it possible to separate the three trimesters of pregnancy affected by GDM. (C) The permutations tests showed that there was no overfitting in the OPLS-DA analysis for GDM. (D) The function of urinary proteins of pregnant women with GDM annotated using the Ingenuity Pathway Analysis software. GDM, gestational diabetes mellitus; OPLS-DA, orthogonal partial least squares discriminant analysis.

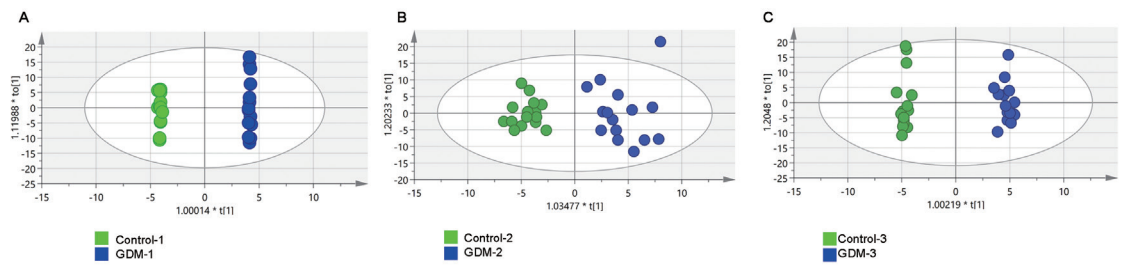

Figure S3 Multivariate analysis using orthogonal partial least squares discriminant analysis showed a marked difference between the GDM and control groups in all three trimesters. (A) 1st trimester; (B) 2nd trimester; (C) 3rd trimester.

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