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RESEARCH ARTICLE

Is there any difference between metabolomic profiles of mothers who progress to gestational diabetes versus healthy women during pregnancy?

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Abstract

Background Gestational diabetes (GD) is associated with a variety of numerous metabolic changes. Discovering related biomarkers by the metabolomic studies can provide a better understanding of the pathological processes involved in the development and progression of GD.

Methods Blood samples were taken from 400 naturally conceived healthy women aged 25–40 years old in the first trimester of pregnancy. Participants were followed up again at 28 weeks of gestation and reevaluated for GD based on American Diabetes Association (ADA) criteria. After identifying 32 women with GD as the case group, 32 healthy matched women selected as the control group. Plasma samples in the first and third trimester, were sent for nuclear magnetic resonance (NMR) testing. Altered biochemical pathways were identified in MetaboAnalyst 4.0 using Human Metabolism Database (HMDB). The comparison of altered metabolomes in two groups was assessed using multivariate logistic regression analysis in SPSS 23 software.

Results In the first trimester, the amount of increase in steroid hormones level was greater in women who progressed to GD (Impact = 0.344). In the third trimester, although we had lower levels of steroid hormones, prostaglandins and bile acids in the diabetic group vs healthy subjects, however the level of glycine conjugated bile acid was higher in affected women by GD ($P = 0.016$).

Conclusions For the first time, we reported new disrupted pathways such as steroid hormone pathways and their related altered metabolites in a group of Iranian population with GD. This may provide a better and faster way to predict, diagnose and prevent GDM in the future. Surely, further studies are required for the validation of the results.

Keywords Gestational diabetes · Metabolomic profile · Nuclear magnetic resonance (NMR)

Introduction

Gestational diabetes mellitus (GDM) is defined as impaired glucose tolerance, first diagnosed in pregnancy, and is one of the most important problems affecting approximately 10% of pregnancies in the United States [1, 2]. Its prevalence in Iran is about 8% [3] and can lead to important complications such as maternal and fetal morbidity and mortality [4]. Maternal complications include gestational hypertension, increased cesarean delivery and lactation disorders [1, 5]. It also increases the future risk of type 2 diabetes, cardiovascular disease and metabolic syndrome in these mothers [1, 6, 7]. Fetal complications include an increased risk of macrosomia [8], congenital hypoglycemia, jaundice, polycythemia, hypocalcemia, and preterm delivery [9].

The exact underlying cause of GDM is not yet known. Although some hypotheses have been put forward, but some researchers have suggested that GDM is caused by a dysregulation of sex hormones. The main physiological disorders in GDM are insulin resistance and decreased insulin secretion, which are most often diagnosed in the third trimester of pregnancy using fasting glucose tolerance test (GTT) and insulin level measurement. Increased levels of hormones such as placental lactogen, placental-derived growth

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hormone, progesterone, cortisol, and prolactin may play a role in reducing insulin sensitivity in peripheral tissues by interfering with insulin receptor signals [10]. Most women with GDM are obese and obesity-related insulin resistance or biomolecular mechanisms involving the secretion of adipokines and cytokines from white adipose tissue may lead to the development of GDM [11].

Despite numerous recommendations for the diagnosis of gestational diabetes, there is still no consensus on the early detection of GDM. Therefore, the identification of metabolites or metabolic pathways that can predict the development of GDM is considered very valuable. Metabolomic studies allow us to determine a fingerprint of specific metabolic pattern that guides us to decipher complex biological processes and may predict a disease onset even before it begins. The powerful science of metabolomics can identify new biomarkers in different diseases within different breeds [12]. This method has already been used as a biomarker detection tool in GDM epidemiological studies [13–15].

Changes in metabolome composition may directly reflect the amount of gene expression, physiological status of diseases, and the effects of environmental stimuli. These important metabolites, once further confirmed, can be used to diagnose and predict susceptibility to a disease and even evaluate response to treatments especially in pregnant women with gestational diabetes and their fetuses [16].

This study was conducted to identify the metabolites of pregnant women who will later develop gestational diabetes compared to healthy mothers in the first trimester of pregnancy and to discover altered metabolites in the third trimester of pregnancy. The main goal of this study was to discover biomarkers for early detection of pregnant mothers who will progress to gestational diabetes.

Materials and methods

Study design and participants

Considering the average prevalence of 8% of GD in Iran, initially a total of 400 blood samples were collected from naturally conceived women, who were in the first trimester of pregnancy. Samples were in the age range of 25–40 years and had no previous history of overt or gestational diabetes. The samples were centrifuged and their plasma was stored separately at −80 °C until use. A questionnaire including information's such as age, height, weight, BMI, previous history of stillbirth, abortion, number of deliveries and family history of diabetes was prepared. Pregnant women with a history of overt diabetes, hypertension, thyroid disorders, PCO, known kidney or liver disease, those taking medications such as corticosteroids and ovulation induction drugs and those who could not be properly followed; were excluded from the study. Participants were followed up again at 26 weeks of gestation and were evaluated for occurrence of gestational diabetes based on the 75 g -Oral GTT test and its interpretation based on ADA guidelines. BS and insulin levels were measured and the HOMA-IR index [(Fasting Glucose mg/dl * Fasting Insulin µIU/ml)/405] was calculated before insulin injection. HOMA-IR values above 2.1 were considered abnormal [17]. After identifying 32 women with gestational diabetes as the case group, 32 healthy matched women in terms of number of live births, pre-pregnancy weight and body mass index were selected as the control group.

Sample collection and preparation

Fasting whole blood samples were collected in vacutainer tubes. The samples were remained in the refrigerator temperature for 1 h and then centrifuged at 3000 g for 10 min at 4 °C. The supernatant serum was aliquoted and stored at −80 °C until required. Written consent was obtained from the patients. Subjects included 32 patients with GD, and 32 healthy control all from the same age.

NMR spectroscopy and data processing

All spectroscopy experiments were performed using a Germany Bruker-Avance 400 MHz NMR, equipped with a 5 mm probe at 298 K. 400 µl of samples were mixed with an equal amount of buffer (including 70% D2O, 4% KH2PO4, 0.01% NaN3 and 2.5% of TSP as internal standard).

All primary NMR spectra based on Carr-Purcell-Meibooms-Gill (CPMG) protocol were entered in Free Induction Decay (FID) format in Mest ReNova software (version 6.0.2–5475). By converting the spectrum to a frequency domain, the FID data provided appropriate and meaningful data that could be used for multivariate analysis methods of chemometrics. After correcting the baseline data; the NMR spectra were imported to MATLAB (R2098a; Math Works, Natick, MA) as an American Standard Code for Information Interchange (ASCII) file for further analysis. The spectra were normalized with the thermal shift assay (TSA) method, aligned to a reference spectrum and log transformed. The 4.5–5.5 ppm region of spectra was removed to eliminate water suppression variation efficiency and then the spectra were normalized.

Statistical and bioinformatics analysis

The multivariate statistical analysis was used to identify significant metabolites between studied groups by MATLAB software. The unsupervised principal component analysis (PCA) was performed to detect similarities in different samples. Since the separation of groups based on the results

of PCA analysis was not suitable, therefore, the supervised method, Partial least squares-discriminant analysis (PLS-DA) analysis was done for predictive and descriptive modelling as well as for discriminative variable selection. In the PLS-DA analysis using the variable importance in projection (VIP) test, each chemical shift was assigned a rank in order to identify the shift of the altered metabolites among the studied groups. Finally, the chemical shifts with rank >1 were identified as the shifts responsible for the important alteration of metabolites.

Metabolites identification

After maximal class segregation, the Human Metabolism Database (HMDB) was used to identify the significant metabolites based on the chemical displacements of the class separation. Two-sided student's t test was used to evaluate *P* values for each metabolite. The VIP was used to find the significant discriminating metabolites in every comparison. Metabolites with VIP > 1, *P* value<0.05, and fold changes of more than 1.5 were marked as significant variables. In addition, Metaboanalyst 4.0 web-based platform was used for pathway enrichment analysis of altered metabolites. The relationship between patients' demographic data and some specific findings of metabolomes as well as comparing altered metabolomes in two groups was performed using SPSS 23 software and multivariate logistic regression analysis.

Results

Statistical analysis results

In the 26th week of pregnancy, 32 pregnant women with GD were identified which were classified as the case group. 32 healthy pregnant women matched in terms of number of live births and deliveries, pre-pregnancy weight and body mass index were included as the control group. The mean levels of HOMA-IR, BMI and fasting blood sugar did not show any significant difference between two groups. However, the mean blood glucose levels after performing 75 g- oral glucose tolerance test were significantly different (Table 1). Multivariate analysis was performed to find metabolites that mostly discriminated the study groups. PLS-DA analysis can reveal trends in the data and groups of observations. The PLS-DA results including loading and score plots are shown in Fig. 1. The PLS-DA analysis of the NMR metabolic profiles of subjects could successfully separate clusters for comparison of first trimester and third trimester, which is visualized in loading plots in Fig. 1A, C, respectively. In addition, the multivariate analysis indicate that there is a high correlation between subjects in each of the case and control groups, while difference in the expression of metabolites in the two studied groups, the groups were separated (Fig. 1B, D).

Table 1 Comparison of BMI, fasting blood sugar and HOMA-IR Index between the case and the control groups

Variable	Groups		
	Case ($n=32$) Mean ± SD	Control ($n=32$) Mean ± SD	*p* Value
BMI (Kg/m^2)	28.23 ± 3.12	28.06 ± 2.66	>0.05
FBS (mg/dl)	79.4 ± 8.87	80.73 ± 11.52	>0.05
HOMA-IR Index	2.07 ± 0.69	1.79 ± 0.74	>0.05

Identification of differential metabolites

In the present study, the differentially expressed metabolites were determined between control and GD subjects in the first and third trimester of pregnancy, separately. The spectral bins of the highest importance based on VIP values were detected. The results showed a significant difference between the metabolites of two comparison (first & third trimester) according to VIP values more than 1 and the *p* value<0.05.

In the first trimester, comparison of involved metabolites in case and control groups, showed an increase in the concentration of Aldosterone synthesis mediators, 17α-Hydroxy pregnenolone, Deoxycorticosterone, Corticosterone, Testosterone, Etiocholanolone, Estradiol, Hydroxyestradiol, Hydroxyprogesterone, Cholesterol sulfate, Hydroxyestrone, 17α-Estradiol, prostaglandin D2, prostaglandin E2, Taurocholic acid, L-Isoleucine, alpha Tocopherol and decrease in concentrations of Androstenedione, Methoxyestrone and 17-alpha,20-alpha Dihydroxypregn 4-en-3-one (Table 2). Analysis of altered serum metabolites in the third trimester of pregnancy revealed an increase in the level of Androstanedione and primary bile acid metabolite named Glycocholic acid and a decrease in the level of other metabolites that emphasized in the first trimester of pregnancy (Table 3). Although common altered metabolites have been identified in two comparisons, however, the regulation of these common metabolites in each of the comparison was reverse, predominantly.

Pathway analysis results

The metabolic pathway analysis base on altered metabolites were performed using the MetaboAnalyst 4.0 database. In this analysis method, the metabolites list (significant differentially expressed metabolites between studied groups in every comparison) are measured relative to the predefined metabolic pathways in the database. The

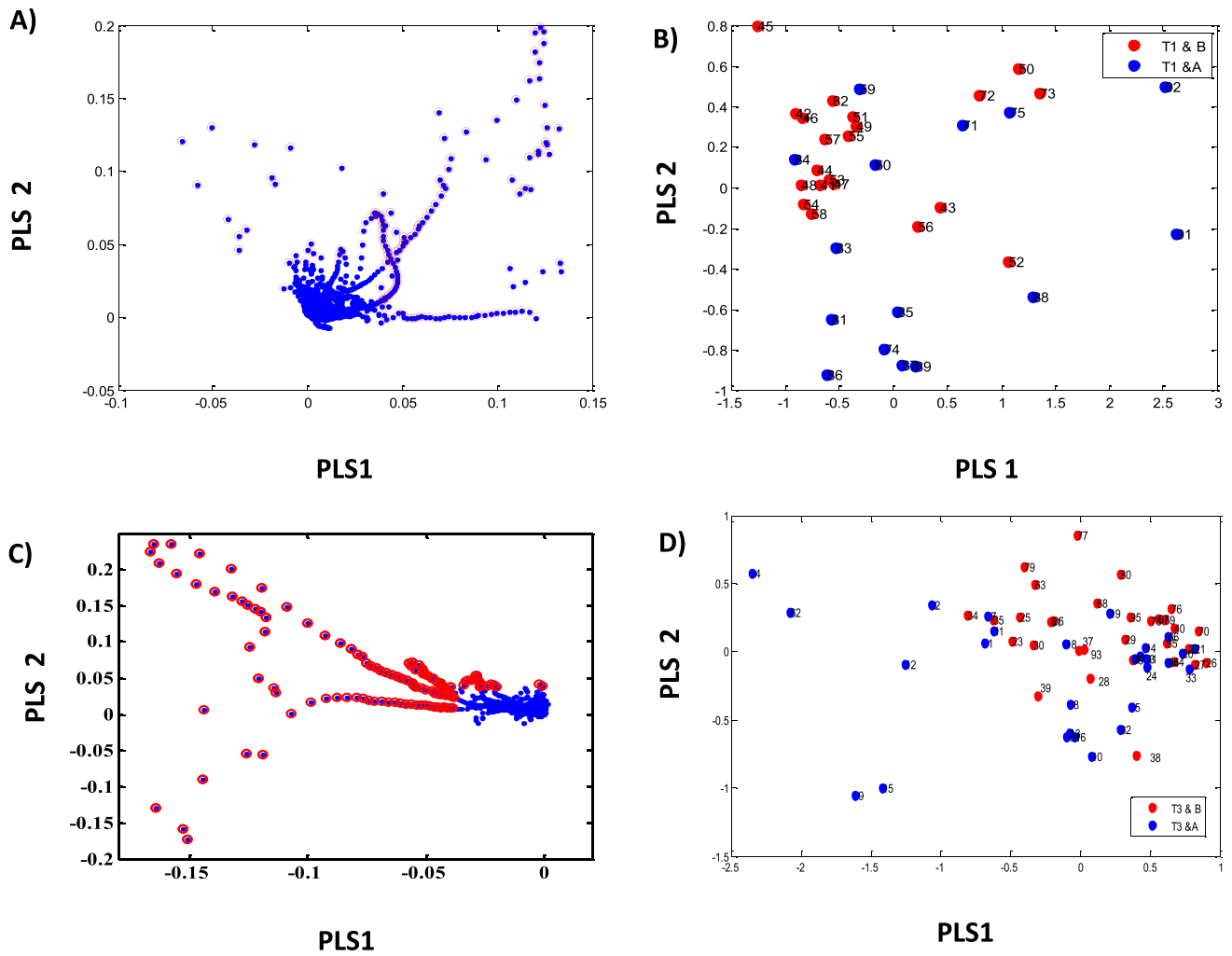

Fig. 1 PLS-DA analysis for the discrimination between **A**) Loading plot from case and control groups in the first trimester of pregnancy, **B** Score plot from case and control groups in the first trimester of pregnancy, **C** Loading plot from case and control groups in the third trimester of pregnancy, and **D**) Score plot from case and control groups in the third trimester of pregnancy. **T3&A**: Metabolites from case group; **T3&B**: Metabolites from control group

topographic diagram obtained from MetaboAnalyst 4.0 software showed the three most important altered metabolic pathways as follows: i) Steroid hormone biosynthesis, ii) Biotin metabolism, and iii) Arachidonic acid metabolism in comparison of case and control group in the first trimester of pregnancy (Fig. 2A). Topographic diagram obtained from MetaboAnalyst 4.0 software showed an extra metabolic pathway-primary bile acid biosynthesis pathway- potentiated in the third trimester of pregnancy, in addition to significant changes of steroid hormone biosynthesis pathway (Fig. 2B). In this diagram, each circle revealed a different pathway. The size and color of each circle was the result of the pathway impact and the value of p index. Red circle was the most important pathway.

Pregnant mothers who progressed to gestational diabetes showed significant reductions in the levels of steroid hormones, Taurocholic acid, prostaglandin E2 and D2, and alpha-Tocopherol, compared to mothers who remained healthy.

The significant altered biochemical pathways in A) the first trimester and B) third trimester of pregnancy in the case group (GD patients) compared control group are presented in Table 4. According to results, the significant metabolic pathways with p value<0.05 include steroid hormone biosynthesis in two comparison, and primary bile acid biosynthesis in third trimester comparison (Table 4). According to above explanations, the steroid hormones biosynthesis pathway showed the most significant changes in both groups. This important and common pathway is demonstrated in Fig. 3.

Table 2 The significantly altered metabolites between control and GD group in first trimester. (Metabolites which had VIP values>1, *p* value<0.05 have been considered)

Metabolite Name	HMDB ID	KEGG ID	VIP	Regulation
Aldosterone	HMDB0000037	C01780	1.34975	Up
17a-Hydroxypregnenolone	HMDB0000363	C05138	1.3803	Up
Deoxycorticosterone	HMDB0000016	C03205	1.36502	Up
Corticosterone	HMDB0001547	C02140	1.38946	Up
Testosterone	HMDB0000234	C00535	1.39252	Up
2-Androstenedione	HMDB0000053	C00280	1.34364	Down
Etiocholanolone	HMDB0000490	C04373	1.36197	Up
Estradiol	HMDB0000151	C00951	1.40474	Up
Methoxyestrone	HMDB0000010	C05299	1.34669	Down
Hydroxyestradiol	HMDB0000338	C05301	1.9974	Up
Cholesterol sulfate	HMDB0000653	C18043	3.17661	Up
11a-Hydroxyprogesterone	HMDB0000920	C03747	1.40779	Up
16a-Hydroxyestrone	HMDB0000335	C05300	1.37724	Up
17a-Estradiol	HMDB0000429	C02537	2.000452	Up
17-alpha,20-alpha Dihydroxy-pregn 4-en-3-one	HMDB0011653	C04518	1.35891	Down
Prostaglandin D2	HMDB0001403	C00696	1.39557	Up
Prostaglandin E2	HMDB0001220	C00584	1.423068	Up
Taurocholic acid	HMDB0000036	C05122	1.334472	Up
L-Isoleucine	HMDB0000172	C00407	1.38641	Up
Alpha-Tocopherol	HMDB0001893	C02477	3.70206	Up

VIP Variable Importance In Projection, *Up* Up-regulated (Increased), *Down* Down-regulated (Decreased)

Table 3 The significantly altered metabolites between control and GD group in third trimester. (Metabolites which had VIP values>1, *p* value<0.05 have been considered)

Metabolite Name	HMDB ID	KEGG ID	VIP	Regulation
17a-Hydroxypregnenolone	HMDB0000363	C05138	1.334472	Down
Androstenedione	HMDB0000899	C00280	1.316142	Up
Estradiol	HMDB0000151	C00951	1.38641	Down
Hydroxyestradiol	HMDB0000338	C05301	1.994342	Down
Hydroxyestrone	HMDB0000335	C05300	1.3192	Down
Cholesterol sulfate	HMDB0000653	C18043	1.39252	Down
17a-Estradiol	HMDB0000429	C02537	1.38335	Down
Prostaglandin D2	HMDB0001403	C00696	1.3803	Down
Prostaglandin E2	HMDB0001220	C00584	1.37724	Down
Taurocholic acid	HMDB0000036	C05122	1.310032	Down
Chenodeoxycholic acid	HMDB0000172	C00407	1.31309	Down
Hydroxyprogesterone	HMDB0000920	C03747	1.35586	Down
Eticholanolone	HMDB0000490	C04373	1.316142	Down
Alpha-Tocopherol	HMDB0001893	C02477	3.445448	Down
Glycocholic acid	HMDB0000138	C01921	1.33142	Up

VIP Variable Importance In Projection, *Up* Up-regulated (Increased), *Down* Down-regulated (Decreased)

Discussion

The metabolic profile study is considered a valuable approach for the identification of predictive, diagnostic, and prognostic biomarkers. In addition, metabolomic is a tool for full understanding of the molecular mechanism of disease pathology. Meanwhile the metabolomic study of body fluids that are collected non-invasively methods such as blood is more acceptable. Since, blood serum contains metabolites from all body organs, it seems hard to detect a specific metabolic pattern for diseases, and however, metabolomics studies along with multivariate statistical

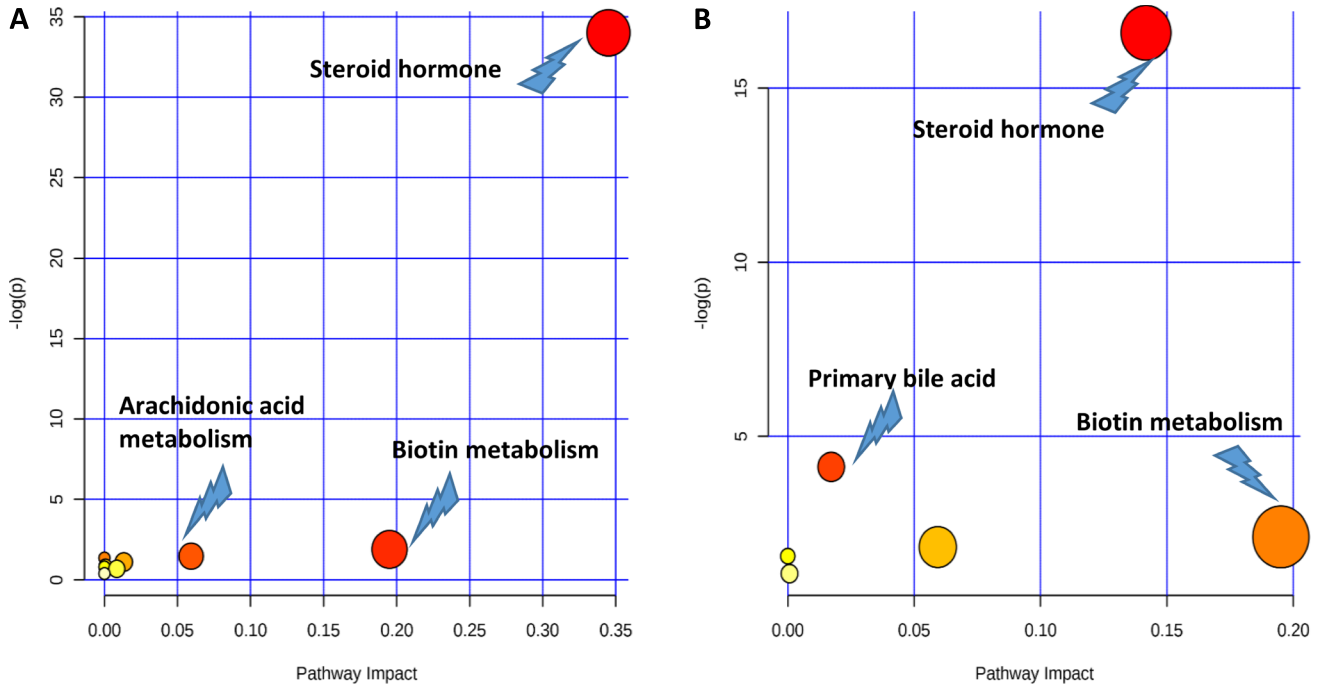

Fig. 2 Topological analysis of the most important altered metabolic pathways in the **A**) first trimester of pregnancy, **B** third trimester of pregnancy in the case group compared to the control group

Table 4 The significant altered biochemical pathways in **A**) the first trimester and **B**) third trimester of pregnancy in the case (GD patients) compared control group

Pathway Name	metabolites	Total	Hits	*p* value	FDR	Impact
A) First Trimester						
Steroid hormone biosynthesis	Aldosterone,17a-Hydroxypregnenolone, Deoxycorticosterone, Corticosterone Testosterone, 2-Androstenedione Etiocholanolone, Estrone Estradiol, Methoxyestrone Hydroxyestradiol, Cholesterol sulfate 11a-Hydroxyprogesterone,16a-Hydroxyestrone, 17a-Estradiol 17-alpha,20-alpha Dihydroxypregn 4-en-3-one	99	17	1.7028E-15	1.3622E-13	0.34
Biotin metabolism	Dethiobiotin	11	1	0.1491	1.0	0.19
Arachidonic acid metabolism	Prostaglandin D2, Prostaglandin E2	62	2	0.22724	1.0	0.05
B) Third Trimester						
Steroid hormone biosynthesis	17a-Hydroxypregnenolone, Etiocholanolone, Androstanedione, Estrone, Estradiol, Hydroxyestradiol, Cholesterol Sulfate, Hydroxyprogestrone, Hydroxyestrone, 17a-Estradiol	99	10	6.2389E-8	4.9911E-6	0.14177
Primary bile acid biosynthesis	Glycocholic acid, Taurocholic acid, Chenodeoxycholic acid	47	3	0.01624	0.64961	0.01719
Biotin metabolism	Dethiobiotin	11	1	0.12101	1.0	0.19512

Total The Total Number of Compounds in The Pathway, *Hits* The Metabolite Matches, *FDR* False Discovery Rate

analysis make it possible. Several studies were conducted in this region and various potential biomarkers have been offered based on omics studies including genomics, proteomics, and metabolomics [18]. This study compared the most important altered metabolic pathways involved in the first and third trimesters of pregnancy between diabetic

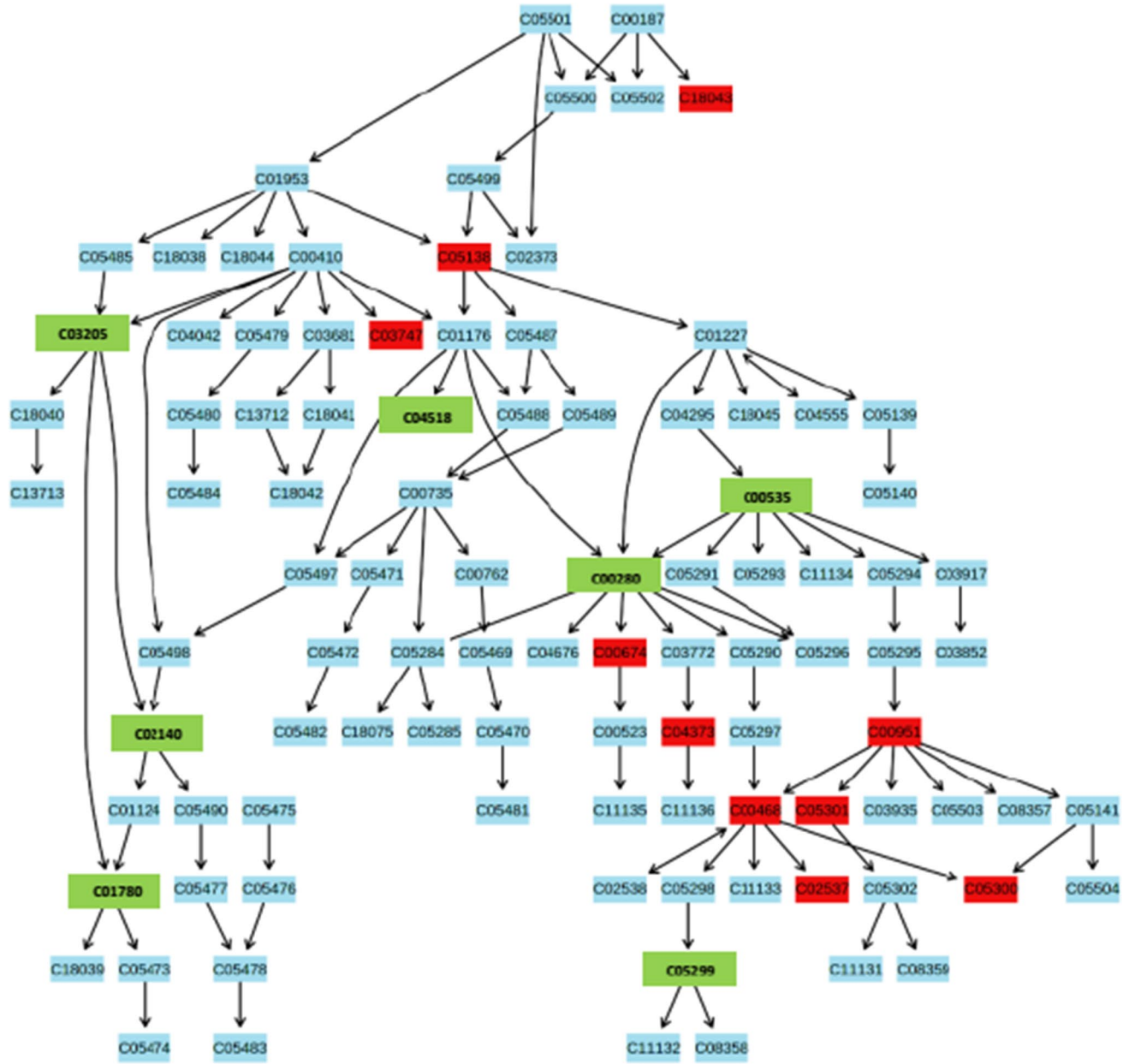

Fig. 3 The most significantly enriched pathway involved in the pathogenesis of gestational diabetes, including steroid hormone biosynthesis. The metabolites in the red boxes were significantly altered in both of first and third trimester of pregnancy in case compared to control group, and the metabolites in the green boxes were significantly altered only in first trimester of pregnancy. **C18043**: Cholesterol sulfate; **C5138**: 17a-Hydroxypregnenolone; **C03747**: 11a-Hydroxypregestrone; **C03205**: Deoxycorticosterone; **C04518**: 17a-alpha-20-alpha-Dihydroxypregn-4-en-3-one; **C00535**: Testosterone; **C00280**: Androstenedione; **C04373**: Eticholanolone; **C00951**: Estradiol; **C02140**: Corticosterone; **C01780**: Aldosterone; **C00468**: Estrone; **C05301**: 2-Hydroxyestradiol; **C02537**: 17a-Estradiol; **C05300**: 16a-Hydroxyestrone; **C05299**: 2-Methoxyestrone

and non-diabetic women. Three pathways including steroid hormones biosynthesis, biotin metabolism and arachidonic acid biosynthesis were the most important altered pathways in the first trimester which of these, the only significant altered pathway was the steroid hormones biosynthesis. In the third trimester, a metabolite of the primary bile acid pathway called Glycocholic acid, in addition to other pathway metabolites involved in the first trimester, showed significant changes between the gestational diabetic women and healthy individuals.

Our study found that the steroid hormone biosynthesis pathway was different in women with gestational diabetes compared to healthy women. In addition, a primary bile acid named Taurocholic acid, was the only detected bile acid

metabolite which had a higher level in the first trimester of women with progression to GDM. Vejrazkova et al. pointed to changes in steroid hormones biosynthesis as an important factor in causing changes from normal pregnancy to glucose intolerance and finally GDM [19]. In this study elevated levels of a number of steroid hormones during pregnancy, such as progesterone and corticosteroids, lead to decreased insulin sensitivity, hyperinsulinemia, and increased androgen production, which exacerbated peripheral insulin resistance. In a study of postmenopausal women, Ding et al. noted the role of steroid hormones in the development of diabetes. In these women, high levels of Estradiol and Testosterone led to progression to overt diabetes, emphasizing the role of these hormones as an independent risk factor for diabetes occurrence [20]. A study by Lopez et al. examining urinary metabolites in the third trimester of pregnancy in women with gestational diabetes, showed an increased metabolite of steroid hormones, suggesting that these hormones affect beta cell function and insulin sensitivity at the end of pregnancy [21]. The results of a study by Jawerbaum et al., pointed to the role of prostaglandins and arachidonic acid pathway in gestational diabetes, showed that lower levels of some prostaglandins were associated with more severe complications of diabetes [22]. Changes in the metabolism of arachidonic acid leads to a change in the production of prostaglandins and eventually a change in the amount of nitric oxide (NO). Our study also showed a decrease in prostaglandin D2 and E2 levels (Arachidonic acid metabolism) with the development of gestational diabetes in the third trimester. Additionally, the amino acid pathway, especially the production of the branched-chain amino acid isoleucine, showed an increased activity in the first trimester of women who progressed to gestational diabetes. This finding, consistent with the findings of previous studies, highlights the role of branched-chain amino acids in insulin resistance, obesity, and diabetes [14]. In the Ubiquinone biosynthesis pathway, the amount of alpha-Tocopherol decreased in the third trimester of the case group compared to the control group. In a study by Ihara et al., chronic hyperglycemia has been shown to act as a damaging oxidant on pancreatic beta cells, exacerbating the complications of diabetes. Conversely, treatment with antioxidants improves the course of diabetes. In the present study, the production of alpha-Tocopherol antioxidant was reduced in women with gestational diabetes; which supports the hypothesis of treatment with antioxidant agents for the prevention of gestational diabetes [23]. Recent studies have shown that many bile acids, some of which are unknown, are involved in the pathophysiology of GDM. They believe that serum bile acids increase with the progression of pregnancy. The study by Jao et al., showed an increase in the level of 8 types of sulfated bile acids in GDM subjects compared to the control group, suggesting their protective role against cytotoxicity of bile acids [24]. Another studies have also shown a link between bile acid metabolites and the development of large for gestational age or the development of GDM and type 2 diabetes in the future [25]. In our study, the Glycocholic acid level of diabetic women increased in the third trimester, while the level of Cenodeoxycholic acid (CDCA) and Taurocholic acid decreased compared to increased level of these metabolites in the first trimester.

Conclusion

This study has reported new metabolites for the first time focusing on the metabolic profile of patients with GDM in an Iranian population. Pregnant women who progressed to gestational diabetes had higher levels of steroid hormones, prostaglandins, the amino acid isoleucine, and bile acid Taurocholic acid in the first trimester, and higher level of conjugated bile acid- Glycocholic acid - in the third trimester of pregnancy. According to the increasing prevalence of GMD, the development of molecular markers could aid in better diagnosis of this disease. Validation of the results in larger populations is also of utmost importance for application of the finding to the clinic. These findings may provide a better and faster way to diagnose and prevent GDM in the future.

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Declarations

Conflict of interest The authors declare no conflict of interest.

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