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A metabolomic signature of FIGO stage I and II endometrial cancer

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Endometrial cancer (EC) is a malignant tumor of the female reproductive tract. Due to its rapid growth and invasiveness, EC is currently the only gynecological neoplasm with rising incidence and mortality rates. It is of great significance to explore the metabolomics signature of stage I and II EC for the diagnosis and treatment. A mass spectrometry-based untargeted metabolomics approach was used to explore preoperative serum metabolites in the normal and stage I and II EC patients. The metabolites were mapped to the Ingenuity pathway analysis (IPA) database to determine the potential biomarkers and metabolic pathways that differ between EC patients and healthy controls. The top analysis-ready molecules of upregulated D-glucose thiamine and downregulated cholesterol, arachidonic acid, palmitic acid, oleic acid, stearic acid, linoleic acid may be the most related metabolites. These potential biomarkers have essential functions in regulating vital metabolic pathways associated with stage I and II EC. Additionally, our pathway analysis revealed five significantly related pathways according to the metabolite differentials. Finally, the disease and function prediction of the initial pathway analysis suggested that small molecule biochemistry, lipid metabolism, and organismal injury and abnormalities were associated with EC cases. Over 25 metabolites were differentially expressed in stage I and II EC. In addition, the six most significant metabolites were related to stage I and stage II EC cases. Ingenuity pathway analysis revealed potential biomarkers and metabolic pathways revolved to EC. In this paper, candidate endogenous biomarkers were defined as the basis for disease diagnosis and individualized treatment monitoring and revealed the mechanism of EC occurrence and development.

Key words: endometrial cancer, metabolomics, stage I and II, potential biomarkers, mass spectrometry

Endometrial cancer (EC) is a malignant gynecological cancer originating from female endometrial epithelial tissues, primarily in Western countries and some economically developed cities in China [1–3]. Due to its rapid growth and invasiveness, EC is one of the female reproductive system's primary malignant tumors [4, 5]. It is currently the only gynecological neoplasm with rising incidence and mortality rates [6–8]. EC accounts for approximately 7% of all malignant tumors in females (excluding skin cancers) and up to 25% of female reproductive system cancers. The vast majority of endometrial cancers are related to estrogen and occur in women of all ages. However, most cases occur in peri- and postmenopausal women, since the peak of incidence is between 55 and 65 years old. The risk factors for EC include polycystic ovary syndrome, glucose metabolism disorders, long-term hormone therapy, endocrine diseases, and being overweight [9, 10].

Most cases of EC are diagnosed at advanced stages of the disease [11–13]. Stage I EC is the early-stage EC and can often be cured through normal surgery. The cure rate can reach more than 90% in patients with early-stage EC. In stage I of EC, the lesion is confined to the muscularis, while the lesion penetrates the muscularis but does not metastasize in stage II. The disease-related death rates in Federation International of Gynecology and Obstetrics (FIGO) stages 2B-4 are high (20–80% and higher). In the more frequently diagnosed early-stage FIGO 1-2A, the death rates are still significant, ranging from 5–15% [14]. Therefore, it is meaningful to compare stage I and stage II EC cases to search for biomarkers and metabolic pathways to explore the pathological mechanisms of EC [15].

Metabolomics is a field of study that comprehensively analyzes the metabolites of living organisms. The relatively new field plays a decisive role in explaining the pathogenesis, diagnosis, and treatment of many diseases. This holds especially true in exploring tumor pathogenesis-related biomarkers. For instance, findings related to oncometabolite-related discovering have shown unsuspected cellular pathways, with potential diagnostic or prognostic biomarkers, may be excellent therapeutic targets for treatment [16]. However, only a few studies have assessed the utilization of metabolomics for EC. More than 30 global and targeted mass spectrometry (MS)-based metabolomic studies have been published on ovarian cancer. These studies have demonstrated the strong connection between metabolic pathway dysregulation and tumorigenesis [17]. This study compares the preoperative serum metabolite profiles between healthy controls and women with stage I and II EC. In addition, we employed global metabolomics profiling to identify specific biomarkers of EC in postmenopausal women.

Patients and methods

Study populations and specimen collection. The recruitment of healthy postmenopausal women and the blood sample collections were conducted at the Department of Laboratory Medicine, Shanghai General Hospital. Women were of postmenopausal status and were all undergoing hysterectomy, and had not received hormone replacement therapy (HRT) for at least three weeks prior to the blood collection. Women who had any other types of cancer were not eligible for inclusion in the study. All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Shanghai General Hospital (2020KY219). On the morning of surgery, blood samples were collected and kept on ice before being transported by the medical courier and processed within 2–3 h.

The tubes were centrifuged to collect the serum, which was stored at –80 °C until analysis. The histopathological characteristics of the hysterectomy specimens were assessed by a trained pathologist. The systematic compilation and review of medical records and clinical databases were performed by one of the treating gynecologic oncologists. This allowed for the grouping of patients by preoperative and postoperative histology and grade using the FIGO classification system. Using these criteria, samples at FIGO stage I and II were selected. The controls had no history or current diagnosis of any type of cancer, and no history of radiation or chemotherapy for any reason. There were 30 cases of early-stage EC (FIGO stage I), classified as group A, with tumors that did not extend beyond the uterus. In addition, there were 30 cases of FIGO stage II, classified as group B, with tumors that spread the cervical. Finally, 30 control subjects were classified as group C. All patients were between 40 and 85 years of age.

Specimen processing. The metabolomics platform was used to assess the metabolites in the serum samples (Shanghai Sensichip Biotechnology Co., Ltd., Shanghai, China). The serum samples were thawed at room temperature, vortex-mixed for 15 s, and then 100 µl of the sera were mixed with 1 ml of pre-cooled anhydrous methanol. A total of 10 µl of DL-o-chlorophenylalanine (2.9 mg/ml) was used for the internal standards and was added to each sample. After mixing the samples for 30 s, they were subsequently centrifuged at 12,000×g for 15 min at 4 °C. Next, 200 µl of supernatant was collected into the glass derivatization bottle and evaporated to complete dryness under a nitrogen gas stream. The dried samples were subsequently oximized by adding 30 µl of 20 mg/ml methoxamine pyridine hydrochloride solution into the glass derivatization vial, with vigorous shaking (30 s) at 37 °C for 90 min. The samples were derivatized with 30 µl of bis(trimethylsilyl)-trifluoroacet-amide (BSTFA) and 1% trimethylchlorosilane at 70 °C for 1 h. Samples were incubated at room temperature for 30 min and prepared for GC/MS metabolomics analysis [18].

GC-MS analysis. The GC-MS analysis was conducted using a 7890A/5975C GC-MS system (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5MS fused silica capillary column (30 m × 250 µm, 0.25 µm) (Agilent Technologies) at Shanghai Sensichip Infotech Co. Ltd. (Shanghai, China). Helium (99.99%) was used as carrier gas with a constant flow rate of 1.0 ml min^{-1} through the column. A small amount of the same (1 µl) was injected with a splitless mode. The injection temperature of 280 °C was maintained. The column temperature was maintained at 80 °C for 2 min initially, followed by an increase to 320 °C at a rate of 10 °C/min. The temperature was maintained at 320 °C for 6 min. A quadrupole mass spectrometer was used as the detector with a temperature of quadrupole and ion source of 150 °C and 230 °C, respectively. Full scan mode was used to acquire the data from m/z 50 to 550. Continuous sample analysis was carried out in a random order to avoid the influence of signal fluctuations from the instrument.

Data processing and pattern recognition. After the GC-MS analysis, each sample was represented by a GC/MS TIC. The peak area of each compound was first integrated, and then the peak area ratio was calculated based on the corresponding internal standard. The two-sample t-test was used to compare the differences in metabolite levels between the EC groups and the normal group. Any p-values ≤ 0.05 were considered statistically significant. The GC-MS data were preprocessed in the R-software platform using the XCMS software package. Impurity peaks were removed to account for losses from the column and sample preparation. The results were organized in a two-dimensional matrix that incorporated the variable (retention time/mass-to-charge, rt/m/z), observation value (samples), and peak strength.

A total of 950 features were obtained, and the data were normalized to the total signal integral. The normalized data were imported into SIMCA-P (Version 11.0, Umetri

AB, Umeå, Sweden) for analysis with the principal component analysis (PCA) and orthogonal-projection to latent structure-discriminant analysis (OPLS-DA) models, with the first principal component of variable importance in the projection (VIP) values greater than 1. These findings were combined with a t-test to determine which metabolites were differentially expressed ($p<0.05$). The NIST (http://www.nist.gov/index.html) and KEGG (http://www.genome.jp/kegg/) databases were used to search for the metabolites.

Ingenuity pathway analysis (IPA). IPA is an all-in-one integrated online biological pathway analysis software based on life sciences data collected by the Ingenuity Knowledge Base. IPA helps researchers understand the properties of biomarkers and their relation to genetic, proteins, chemical pathways, along with the network of interactions among them. In addition, these experimental data gathered from the metabolic, genome, and protein groups can be analyzed to predict biological functions and diseases. The network of differentiated serum metabolites between groups A, B, and C was constructed using IPA. Their attributes and corresponding variables were added to our analysis to obtain the most relevant and probable candidate biomarkers and canonical pathways (CPs).

Results

Multivariate analysis of GC-MS results from the serum samples: Differences between the normal, stage I, and stage II groups. The GC-MS results from the serum samples were used for the multivariate data analysis to measure changes in the serum metabolic profiles of stage I and II groups, as compared to the normal group. This allowed us to explore the biomarker candidates and disturbances in the metabolic patterns of EC. Unsupervised principal component analysis (PCA) analysis was used to examine the clustering of samples for differences in metabolic patterns among the three groups, which revealed an obvious separation among the groups (Figure 1). PCA accurately grouped the samples according to their classification labels, and the distribution showed an apparent separation.

The orthogonal partial least squares-discriminant analysis (OPLS-DA) can filter out orthogonal variables in the metabolites unrelated to the categorical variables. This makes it possible to analyze the non-orthogonal and orthogonal variables separately and more intuitively distinguish the degree of difference between different groups. Hence, it is possible to determine which metabolite variables cause differences between groups and obtain the contribution rate of the different substances for each group. Therefore, it is simpler to find the main variables that lead to differences in the spatial distribution of the principal components of the samples. The OPLS-DA scores are presented in Figure 2. All samples were included to fit OPLS-DA models (Figures 2A–2C). EC samples could be clearly distinguished from normal samples, and stage I and II EC samples could be clearly distinguished.

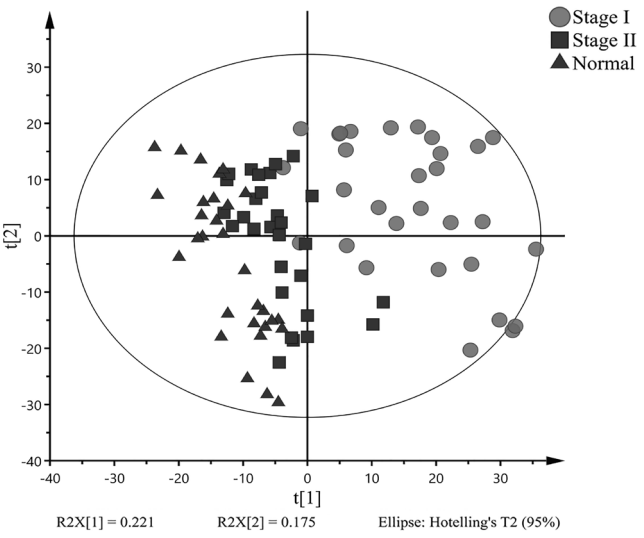

Figure 1. PCA score plot of the stage I EC, stage II EC, and normal groups.

Table 1. Differential analysis of the metabolites in stage I EC and normal groups.

Name	VIP	m/z [1]	rt [2]	p-value	Fold [3] (stage I/normal)
Urea	1.732	79	9.27	7.56E-12	−2.193
Arachidonic acid	1.675	80	20.93	5.32E-13	−2.236
Mannose	1.652	221	17.04	1.71E-12	1.815
Phosphoric acid	1.638	181	9.64	3.33E-12	−1.659
Threose	1.621	75	17.06	7.56E-12	1.296
GABA	1.567	155	11.92	7.69E-11	−1.359
1-Monopalmitoylglycerol	1.557	370	22.69	1.13E-10	−1.711
Ethylamine	1.543	155	11.58	2.00E-10	−0.777
Cholesterol	1.526	57	27.67	3.86E-10	−1.154
Glycine	1.432	248	9.97	1.01E-08	−2.431
Oxalic acid	1.414	133	7.73	5.42E-06	−1.415
D-Galactose	1.38	321	16.98	4.91E-08	1.813
3-Oxaoct-4-en-11-imine	1.37	258	7.43	6.52E-08	−2.002
Pyroglutamic acid	1.361	157	12.70	8.37E-08	−2.101
Aminomalonic acid	1.341	248	12.07	1.47E-07	−1.939
β-D-Allopyranose	1.34	206	17.70	1.48E-07	2.316
Serine	1.324	188	10.66	2.28E-07	−1.783
D-Glucose	1.265	73	17.06	1.04E-06	0.414
Glucopyranose	1.191	205	16.89	5.74E-06	2.039
L-Isoleucine	1.18	299	9.77	7.17E-06	−1.898
diphosphate	1.176	451	14.43	7.89E-06	−4.861
N-α-Acetyl-L-Lysine	1.172	84	16.32	8.52E-06	−1.446
Allose	1.168	217	17.22	9.37E-06	−0.737
Hydroxylcyclohexene	1.131	170	6.52	1.99E-05	−1.559
Carbamate	1.102	77	6.22	3.47E-05	−1.585
Inositol	1.099	432	18.81	3.69E-05	−1.907
Oleic acid	1.031	137	19.63	1.26E-04	−2.031

Notes: [1]mass-to-charge ratio; [2]retention time; [3]the fold change of related metabolites

As shown in Figures 2D–2F, the models possessed satisfactory fit, which indicated the discrimination of the serum metabolomics signature between groups.

Differential metabolites of EC cases relative to the normal group. The preoperative serum metabolites from each group were also examined. Variance importance projec-

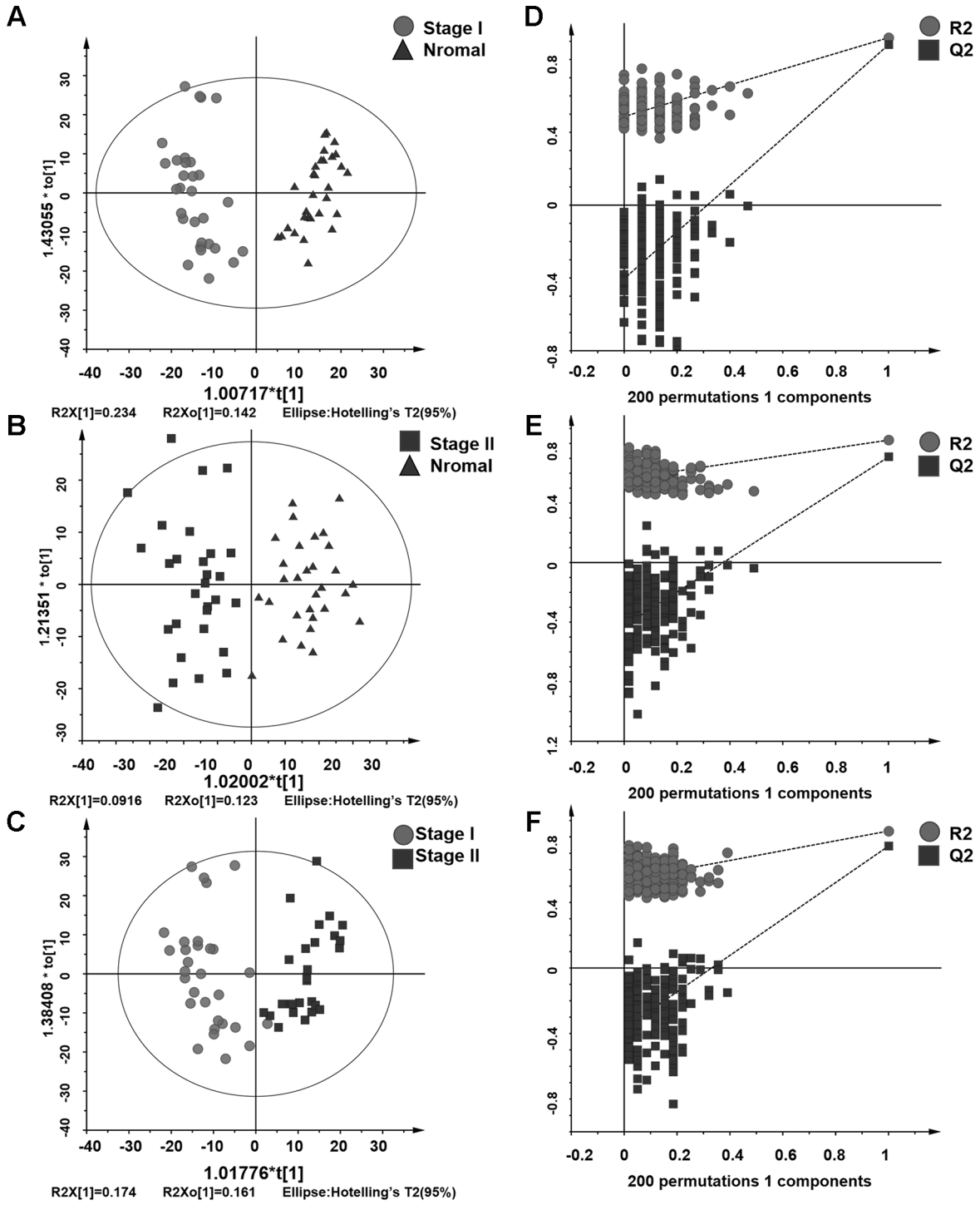

Figure 2. The OPLS-DA scatter plots were based on the serum metabolic profiles of EC patients and normal women. OPLS-DA scatter plots: A) between stage I EC and normal groups; B) between stage II EC and normal groups; C) between stage I and stage II EC groups. The 200 permutation plots of two OPLS-DA models (D–F).

tion (VIP) scores demonstrated the interrelated significance of each metabolite for distinguishing between the groups. VIP scores provide the means to understand the importance of metabolites better, as higher VIP scores indicate a greater discrimination contribution. VIP scores greater than 1 for the first principal component of the OPLS-DA model, combined with the p-value (threshold value of 0.05) of the Student's t-test (t-test), can be used to determine the differential metabolites. As shown in Figure 3 and Supplementary Figures S1A, S1B, volcano plots were produced to determine the most considerable metabolites by both fold change (1.5; x-axis) and p-value ($p<0.05$; y-axis).

When stage I EC cases were compared to the normal group, 27 metabolites were significantly altered (7 upregulated and 20 downregulated, $p<0.05$; Table 1). The top 9 most altered features in the EC cases were urea, arachidonic acid, mannose, phosphoric acid, threose, γ-aminobutyric acid (GABA), 1-monopalmitoylglycerol, ethylamine, and cholesterol (VIP >1.5) as the most changed metabolites. Moreover, there were 28 different metabolites in the serum samples of stage II EC cases and normal group, including diphosphate, 3-oxaoct-4-en-11-imine, β-D-allopyranose, serine, L-isoleucine, glycine, arachidonic acid, 1-monopalmitoylglycerol, GABA, aminomalonic acid, oxalic acid, and urea (VIP >1.5) (Supplementary Table S1). Among the 28 different metabolites, 22 metabolites in the stage II EC group were downregulated compared to normal females, while six metabolites were upregulated. There were 21 differential metabolites that were the same between stage I and stage II EC compared to the normal group, as shown in Table 1 and Supplementary Table S1, respectively.

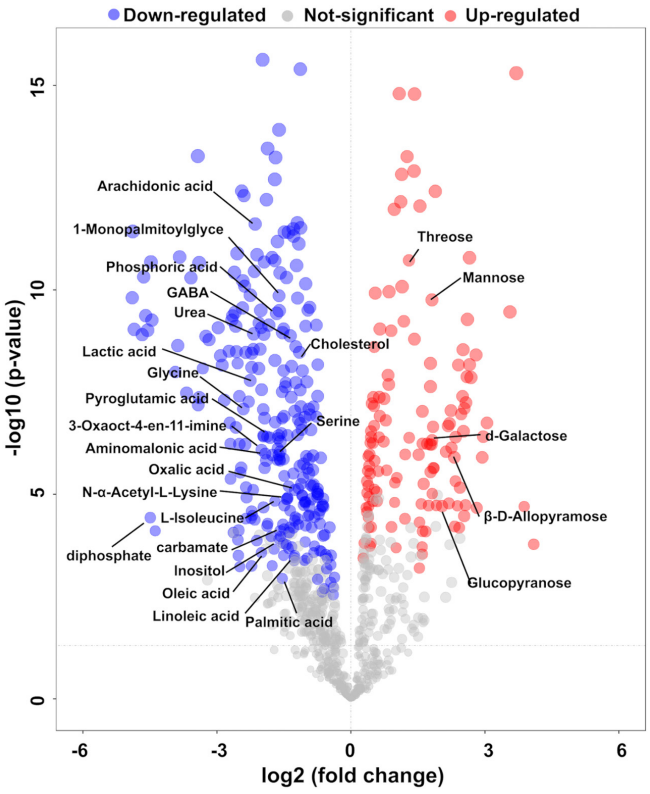

Figure 3. The volcano plot between stage I EC and normal groups were generated according to VIP values; fold changes and p-values are log-transformed. Significant metabolites were represented as red dots and the farther the red dot is away from the (0,0), the more significant the feature is.

Differential metabolites of EC cases relative to the normal group. A total of 25 metabolites were significantly distinguishable between stage I EC and stage II EC groups (Supplementary Table S2), including D-galactose, phosphoric acid, threose, urea, 5-hydroxycaproic acid, cholesterol, mannose, GABA, and β-D-allopyranose (VIP >1.5). Among the 25 different metabolites, 18 metabolites in group A were downregulated in comparison with group B, while seven metabolites were upregulated. Particular comparisons of several representative metabolites are presented in Figure 4. As demonstrated, urea, cholesterol, arachidonic acid, stearic acid, D-glucose, and linoleic acid displayed clear differences between the stage I EC and stage II EC cases.

Network of identified biomarkers and their functions in the IPA. The networks of differentiated serum metabolites between groups A–B and A–C were constructed by the IPA to identify the potential candidate biomarkers and CPs for cases of stage I and stage II EC. In this designer network (Figure 5), the upregulated D-glucose (red mark) and downregulated arachidonic acid (green mark) were observed. Seven top CPs, which were associated with stage I or stage II EC were identified, including tRNA Charging; Bile Acid Biosynthesis, Neutral Pathway; Stearate Biosynthesis I (Animals); Urea Cycle; Glycine Biosynthesis I; γ-linolenate Biosynthesis II (Animals); Cysteine Biosynthesis III (Mammalia). As shown in the interactive diagram of the Biomarker (BM), D-glucose is a biomarker of EC. Moreover, small molecule biochemistry, lipid metabolism, and organismal injury and abnormalities were presumed to be associated with stage I or stage II EC from the IPA predictive analysis of disease and function. In general, multiple targets and multiple pathways were associated with EC, and the representative metabolites might be the targeting regulatory molecular biomarkers (e.g., D-glucose and arachidonic acid). In addition, tRNA Charging; Bile Acid Biosynthesis, Neutral Pathway; Stearate Biosynthesis I (Animals), and the Urea Cycle might be significant interfering signaling pathways.

Discussion

EC is the most frequent gynecological cancer diagnosed each year. Metabolomics can provide substantial evidence for predicting the diagnosis of various diseases, including cancer. To the best of our knowledge, this is the first study reporting on the metabolite profiles found in stage I and stage II EC. The findings of this important pilot study showed

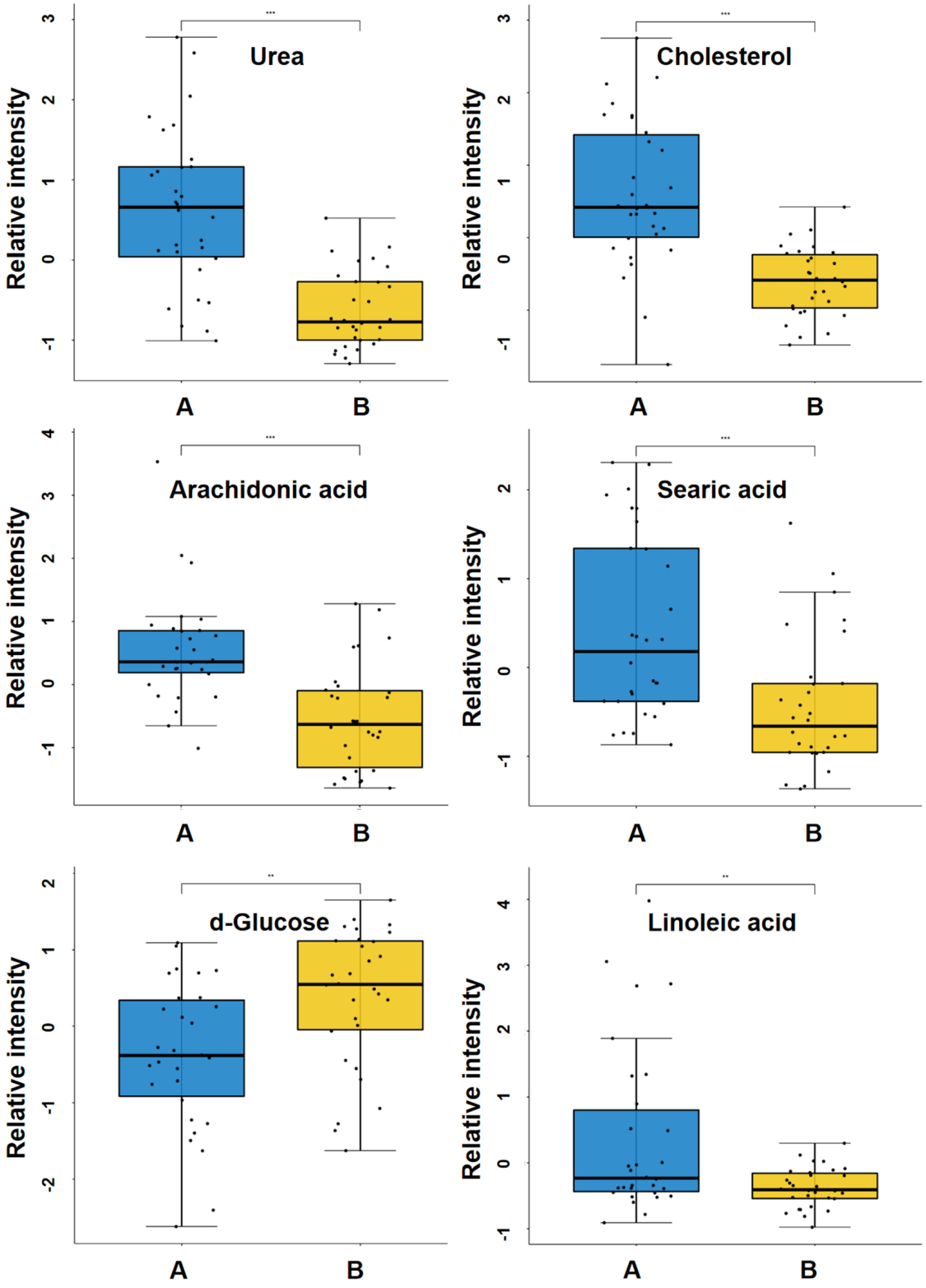

Figure 4. Boxplots showing normalized values of several representative metabolites significantly regulated in A (stage I EC) vs. B (stage II EC) group. The horizontal line located within the box respected the grand mean. The y-axis illustrates normalized, log-transformed, and scaled peak area. The samples were represented as dots, a horizontal line within the box represents the group average. Asterisks indicate statistical significance based on unpaired two-sided Welch's t test. p-value: *<0.05; **<0.01; *<0.001**

several putative serum biomarkers that may be useful for detecting stage I and stage II EC. In this work, we aim to characterize the pathways dysregulated in EC tumorigenesis and the changes experienced by metabolites along with the progression of the disease. A variety of different metabolites were screened through our analysis of serum metabolomics, and several key metabolic pathways were tracked, including tRNA Charging; Bile Acid Biosynthesis, Neutral Pathway; Stearate Biosynthesis I (Animals); Urea Cycle; Glycine Biosynthesis I; γ-linolenate Biosynthesis II (Animals); and Cysteine Biosynthesis III (Mammalia).

D-glucose (upregulation) has been used as a biomarker for measuring the efficacy of metformin in the treatment of EC [19]. D-glucose increases the release of arachidonic acid (downregulation) [20]. Arachidonic acid (5,8,11,14-eicosatetraenoic acid) is a member of the omega-6 polyunsaturated fatty acid family and is a common precursor of many inflammatory factors. Arachidonic acid is related to the production of biologically active prostaglandins and 5-lipoxygenase (5-LOX) leukotrienes. In recent years, leukotrienes and prostaglandins have been shown to promote tumorigenesis. For example, Bellamkonda et al. found that leukotriene D4 and prostaglandin E2 can encourage the growth of colon cancer cell lines by altering the tumor microenvironment and upregulating specific downstream inflammatory factors [21]. Leukotriene B4 is also closely related to epidermal tumors. As another possible player, oleic acid (downregulated in the comparison of A–C and B–C) mediates the production of arachidonic acid [22], while linoleic acid (downregulated in the comparison of A–B and B–C) increases the production of arachidonic acid [23]. We found that the metabolic pathway of arachidonic acid was always downregulated, which may be associated with the occurrence and development of EC.

Lipid metabolism disorders are closely related to the occurrence and development of EC. Fatty acid biosynthesis in patients with estrogen and progesterone receptor-positive EC has become a research target for many targeted therapies [24]. In the follicular membrane cells, cholesterol (downregulation) produces androstenedione and testosterone under the action of cyclic adenosine monophosphate (cAMP), and androstenedione then enters the granular cells, where it is combined with estradiol under the catalysis of aromatase [25]. Women with EC also present with co-morbidities, such as a high BMI index, obesity, hyperlipidemia, hypertension, or diabetes [26, 27]. Patients with high BMIs often have high levels of valine, palmitic acid, oleic acid, stearic acid, and linolenic acid in their serum [28]. Among them, palmitic acid is the most common saturated fatty acid, accounting for about 20–30% of the human body's fatty acid content. Its physiological role is primarily to ensure the integrity of the cell membrane structure and physiological functions. It can be obtained from food or synthesized through endogenous fatty acid synthesis [29]. Special pathophysiological conditions can strongly induce the endogenous *de novo* synthesis of palmitic acid, thereby breaking the dynamic balance of palmitic acid content. In addition, palmitic acid is less desaturated than stearic acid [30], and it is associated with insulin resistance and hypercholesterolemia [31, 32]. We have shown that the concentration of multiple intermediates related to lipid metabolism in the serum samples of EC cases was significantly lower than that of the normal group,

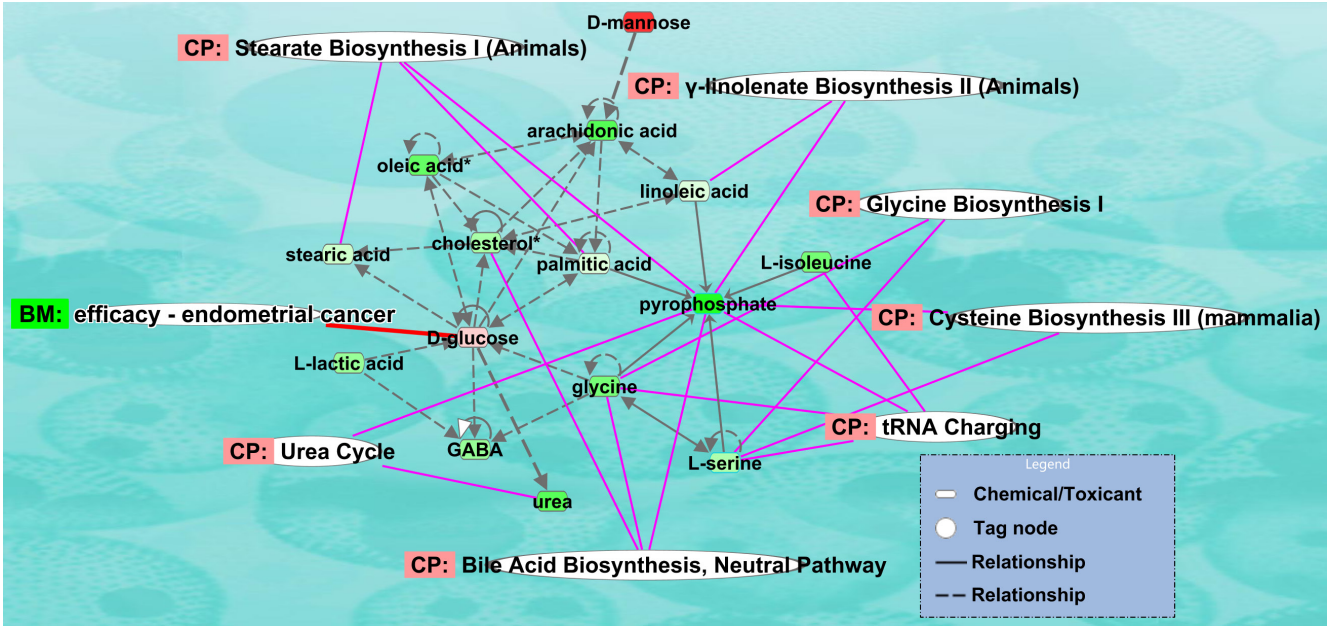

Figure 5. The network of pathways involved in EC.

including palmitic acid (comparison of B–C), oleic acid, stearic acid, linoleic acid (comparison of A–B and B–C). In another study, Altadill et al. found that in the metabolomics study of postmenopausal EC cases, lipid metabolism disorders were more common than in normal endometrial cases, the content of various lipid metabolites in these women had changed [33].

The inconsistent changes in serum lipid metabolite levels in EC of the offspring may be related to the choice of research subjects. In order to eliminate the disturbance of metabolic diseases on the results from EC serum samples, patients with lipid metabolism disorders, such as hypertension, diabetes, and coronary heart disease, were excluded. The subjects of this study only had EC. Therefore, the admission criteria of the subjects in the study ensured the accuracy of the non-target serum metabolomics research results of EC. In addition, the downregulated lipid metabolism intermediate products, such as palmitic acid, oleic acid, stearic acid, and linoleic acid, may also be related to the high-level energy metabolism processes in patients with EC. These intermediate products may be associated with energy metabolism through different biochemical reaction pathways. Hence, the relationship between EC and lipid metabolism disorders warrants further exploration.

In summary, our work suggested that serum metabolomics, as a robust platform to explore the pathogenesis of EC, exhibits considerable potential in elucidating the response mechanisms of stage I or stage II EC cases. A total of 27 different metabolites were identified in stage I EC and the normal group (7 upregulated and 20 downregulated), 28 different metabolites were identified in stage II EC and the normal group (6 upregulated and 22 downregulated), and 25 different metabolites were identified in stage I and stage II EC cases (7 upregulated and 18 downregulated). The path designer network analysis by the IPA of the EC groups compared to the normal group revealed that tRNA Charging; Bile Acid Biosynthesis, Neutral Pathway; Stearate Biosynthesis I (Animals), and the Urea Cycle played important roles in the occurrence and development of EC. In addition, the six most significant metabolites (D-glucose, cholesterol, arachidonic acid, palmitic acid, oleic acid, stearic acid, and linoleic acid) were related to stage I and stage II EC cases. Furthermore, small molecule biochemistry, lipid metabolism, and organismal injury and abnormalities were also presumed to be associated with stage I and stage II EC cases from the IPA predictive analysis of disease and function.

Supplementary information is available in the online version of the paper.

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A metabolomic signature of FIGO stage I and II endometrial cancer

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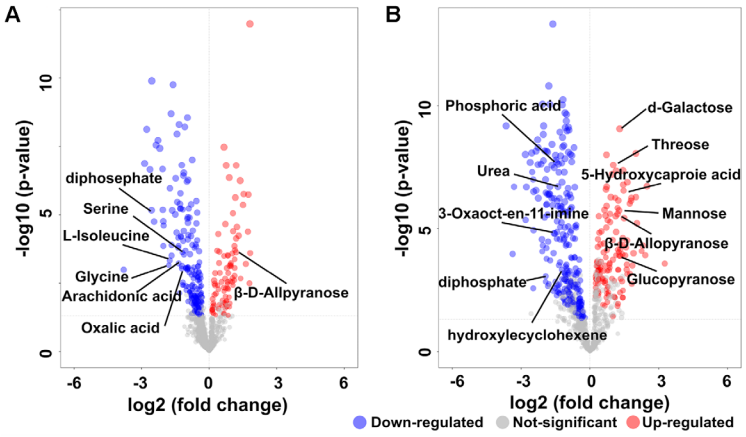
Supplementary Information



Supplementary Figure S1. The volcano plot: A) between stage II EC and normal groups; B) between stage I and stage II EC groups.

Supplementary Table S1. Differential analysis of the metabolites in stage II EC and normal groups.

Name	VIP	m/z	rt	p-value	Fold (stage I/normal)
Diphosphate	2.215	451	14.43	6.31E-07	–2.959
3-Oxaoct-4-en-11-imine	1.944	258	7.43	2.12E-05	–0.572
β-D-Allopyranose	1.724	206	17.70	2.14E-04	1.167
Serine	1.689	188	10.66	2.99E-04	–1.050
L-isoleucine	1.662	299	9.77	3.83E-04	–1.829
Glycine	1.602	248	9.97	6.54E-04	–1.801
Arachidonic acid	1.587	80	20.93	7.42E-04	–1.278
1-Monopalmitoylglycerol	1.571	370	22.69	8.50E-04	–1.027
GABA	1.581	155	11.92	7.81E-04	–0.515
Aminomalonic acid	1.877	248	12.07	1.06E-03	–0.869
Oxalic acid	1.607	60	7.73	6.24E-04	–1.060
Urea	1.547	79	9.27	1.04E-03	–0.813
Mannose	1.359	221	17.04	4.38E-03	0.522
N-α-Acetyl-L-Lysine	1.219	84	16.32	1.12E-02	–0.822
Lactic acid	1.331	75	6.63	5.34E-03	–1.353
Phosphoric acid	1.293	181	9.64	6.91E-03	–0.392
Oleic acid	1.232	137	19.63	1.03E-02	–0.820
Cholesterol	1.263	57	27.67	8.44E-03	–0.339
Glucopyranose	1.18	205	16.89	1.42E-02	0.781
Ethylamine	1.188	155	11.58	1.36E-02	–0.325
d-Galactose	1.133	321	16.98	1.89E-02	0.506
Linoleic acid	1.18	79	19.59	1.43E-02	–0.377
5-Hydroxycaproic acid	1.159	132	9.22	1.62E-02	–0.515
Stearic acid	1.083	342	19.83	2.52E-02	–0.663
d-Glucose	1.008	73	17.06	3.76E-02	0.141
Threose	1.136	75	17.06	1.86E-02	0.297
Carbamate	1.022	77	6.22	3.50E-02	–0.619
Palmitic acid	1.004	97	18.08	3.85E-02	–0.460

Supplementary Table S2. Differential analysis of the metabolites in stage I EC and stage II EC groups.

Name	VIP	m/z	rt	p-value	Fold (stage I/normal)
d-Galactose	1.904	321	16.98	2.42E-10	1.307
Phosphoric acid	1.838	181	9.64	1.75E-09	–1.267
Threose	1.713	75	17.06	4.51E-08	0.999
Urea	1.675	79	9.27	1.08E-07	–1.381
5-Hydroxycaproic acid	1.661	132	9.22	1.49E-07	1.500
Cholesterol	1.624	57	27.67	3.28E-07	–0.815
Mannose	1.637	221	17.04	2.53E-07	1.293
GABA	1.561	155	11.92	1.18E-06	–0.844
β-D-Allopyranose	1.574	206	17.70	9.12E-07	1.150
3-Oxaoct-4-en-11-imine	1.489	258	7.43	4.53E-06	–1.430
Arachidonic acid	1.448	80	20.93	9.26E-06	–0.958
Glucopyranose	1.307	205	16.89	8.49E-05	1.258
1-Monopalmitoylglycerol	1.273	370	22.69	1.36E-04	–0.684
Ethylamine	1.221	155	11.58	2.76E-04	–0.452
Stearic acid	1.167	341	19.82	5.42E-04	–0.617
Citric acid	1.214	273	16.06	2.99E-04	–1.039
Hydroxylcyclohexene	1.177	170	6.52	4.78E-04	–1.118
Diphosphate	1.148	451	14.43	6.81E-04	–1.902
Aminomalonic acid	1.247	248	12.07	1.94E-04	–1.070
Pyroglutamic acid	1.107	157	12.70	1.10E-03	–1.082
Oxalic acid	1.06	133	7.73	1.86E-03	–0.867
Phosphoric acid propyl ester	1.059	299	15.47	1.89E-03	–1.703
d-Glucose	1.108	73	17.06	1.09E-03	0.273
Linoleic acid	1	79	19.59	3.49E-03	–0.895
Allose	1	217	17.22	3.50E-03	–0.402