



## Searching for the primary metabolic alterations of polycystic ovary syndrome by application of the untargeted metabolomics approach

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### ABSTRACT

Despite a large number of studies, the pathogenesis of polycystic ovary syndrome (PCOS) still remains unexplained. In light of ambiguous observations reported in metabolomics, there is a need to carry out studies focusing on confirming the discriminating power of the proposed metabolomics biomarkers. Our research aimed to perform a validation study of metabolites detected in our previous study from serum samples, on the new set of samples obtained from PCOS women and healthy controls to confirm previously selected compounds. Additionally, the second biological matrix – urine – was used to get a more comprehensive insight into metabolic alterations. We applied two analytical techniques – gas chromatography and liquid chromatography coupled with mass spectrometry to analyze both serum and urine samples obtained from 35 PCOS patients and 35 healthy women. Thank to our approach, we identified and described a comprehensive set of metabolites altered in PCOS patients. Results of our study indicate increased steroid hormone synthesis, alteration in sphingo- and phospholipids metabolism, and disturbed fatty acids metabolism. Moreover, the citric acid cycle,  $\gamma$ -glutamyl cycle, vitamin B metabolism, and a few primary amino acids like tryptophan, phenylalanine, histidine, and alanine are altered.

### 1. Introduction

Polycystic Ovary Syndrome (PCOS) is recognized as one of the main causes of infertility in women of reproductive age. It is a heterogeneous endocrine disorder whose diagnosis is currently based on ovulatory dysfunction, androgen excess, and the presence of polycystic ovarian morphology (PCOM). Depending on which clinical signs are more pronounced, the appropriate phenotype of PCOS is recognized as per Rotterdam criteria. Even though the mechanism of PCOS is not fully understood, insulin resistance (IR) seems to play a critical role in its pathogenesis [1]. Many studies support alterations in insulin activity or the insulin signaling pathways to be a central disorder in PCOS development [2]. Hyperinsulinemia is being recognized in more than 80% of

PCOS women and impacts ovarian androgen overproduction. Additionally, it contributes to the development of dyslipidemia and diabetes in PCOS patients [3]. Apart from that, additional metabolic abnormalities such as cardiovascular diseases are described to be connected with this endocrinopathy [4].

The number of studies concerning the search for biomarkers of this endocrinopathy is increasing. However, only a small group of identified metabolites are confirmed and validated. The promising approach, which is more often applied for this purpose, is metabolomics. Metabolomic analysis delivers much information about biochemical processes occurring in living organisms and enables monitoring the changes in metabolomes arising during the disease.

This study aimed to validate the results obtained with our previous

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study focusing on the determination of metabolites with the most discriminating power. For this purpose, a new set of serum samples obtained from women suffering from PCOS and healthy controls, was used. Analysis was carried out following the methodology applied in our first study [5]. Additionally, another biological matrix - urine - was analyzed to complete the full picture of metabolite alteration characteristics for PCOS. Two complementary techniques such as gas chromatography, and liquid chromatography coupled with mass spectrometry were applied to analyze serum and urine samples. In untargeted metabolomic analysis, the essential aspect is the determination of the largest possible panel of compounds. Currently, existing analytical platforms lack the capability to comprehensively assess the entire human metabolome, mostly due to the diversity of the physicochemical properties of the metabolites. However, the majority of metabolomics studies predominantly relied on a single analytical technique. While the application of the GC-MS technique is widely used for analyzing volatile and thermostable organic compounds, LC-MS is suitable for the analysis of more polar, non-volatile, and thermolabile molecules. Hence, the different metabolites can be determined by distinct techniques. Consequently, a combination of GC-MS and LC-MS analytical techniques is necessary to examine the metabolomic fingerprint fully. Moreover, both techniques show high sensitivity and resolution, which is highly desirable while analyzing of thousand metabolites in biological samples.

Our integrated strategy enabled us to select a comprehensive set of metabolites with a wide range of physicochemical properties. Thank to that, we identified compounds which showed the most significant differences between PCOS women and controls and determined the specific metabolic pathways deranged in PCOS.

## 2. Experimental

### 2.1. Material and methods

#### 2.1.1. Study design and sample collection

Seventy women, divided into two groups - PCOS patients ( $n = 35$ ) and healthy controls ( $n = 35$ ), were enrolled in the study. From each woman, serum and urine were sampled. Therefore, in total, 70 serum samples and 70 urine samples were obtained. Each of the women enrolled in the reported study was examined by a specialist in endocrinology at the Department of Clinical and Experimental Endocrinology of the Medical University of Gdańsk. The study group consisted of 35 patients with diagnosed PCOS. The control group comprised 35 women with normal serum androgen concentration and regular menses. PCOS diagnosis was based on AES&PCOS criteria. One of the crucial parameters required the presence of clinical or biochemical signs of androgen excess. Moreover, transvaginal ultrasonography (TV USG) was performed by a gynaecologist/endocrinologist. This examination confirmed PCOS in the case of the study group, and the absence of ovary disorders in patients qualified to the control group. Additionally, groups were matched by age and BMI. Clinical characteristics of the studied subjects are presented in Table 1. Diabetes, hormonal therapy, and pregnancy excluded the patient from participating in the study. To minimize the influence of the fluctuation of hormones during the period, the samples were collected in the morning between the 6th and 10th day of the menstrual cycle. Obtained serum and urine sample were placed at  $-80\text{ }^{\circ}\text{C}$  and stored frozen up to the day of analysis. The collection of serum and urine samples was approved by an Independent Bioethics Committee for Scientific Research at Medical University of Gdańsk (NKBBN/27/2018) and informed consent was obtained from all participants.

#### 2.2. Sample preparation

Serum and urine samples have been prepared according to the optimized procedures. Detailed preparation procedure of serum samples for both LC-MS and GC-MS analyses has been described in our previous

**Table 1**

Clinical characteristics of the studied subjects.

Variable	PCOS (n = 35)	Control group (n = 35)	p-value
Age (yrs)	26.9 (17; 40)	26.7 (18, 41)	0.883
BMI	24.5 (16.3; 42.7)	24.5 (16.1; 39.5)	0.962
Waist circumference (cm)	88.3 (63; 129)	85.7 (68; 136)	0.415
Serum LH (U/L)	10.3 (3.6; 29.2)	8.4 (3.4; 66.8)	0.311
Serum FSH (U/L)	8.8 (3.7; 59.1)	6.8 (3.8; 16)	0.185
LH/FSH ratio	1.4 (0.4; 3.4)	1.3 (0.5; 10.3)	0.872
Serum E2 (pmol/l)	260.3 (28.2; 841.9)	314.2 (45.1; 1340)	0.290
Serum PRL ( $\mu\text{IU/ml}$ )	420 (85.2; 935.3)	396 (143; 748.3)	0.558
Serum total TST (nmol/l)	1.8 (0.7; 3.0)	1.1 (0.5; 1.7)	< 0.001
Serum SHBG (nmol/l) <sup>1</sup>	64.5 (24.4; 170.1)	75.7 (15.6; 159.8)	0.136
FAI	3.3 (0.6; 8.5)	1.9 (0.6; 9.2)	< 0.001
Serum DHEA-S ( $\mu\text{g/dl}$ )	300.9 (116.9; 612.2)	228.9 (95.1; 343.2)	< 0.001
Serum TCh (mg/dl)	188.5 (93; 289)	185.1 (129, 252)	0.702
Serum HDL-Ch (mg/dl)	67.8 (34; 117)	69.4 (26; 99)	0.664
Serum LDL-Ch (mg/dl)	106.9 (53; 193)	99.7 (26; 165)	0.337
Serum TG (mg/dl)	87.2 (34; 221)	79.9 (43; 182)	0.412
Fasting plasma glucose (mmol/l)	4.8 (4.1; 5.4)	4.8 (4.0; 5.6)	0.847
Serum fasting insulin (mUI/l)	10.6 (3.8; 33.4)	8.66 (1.2; 17.2)	0.106
Serum CRP (mg/l)	0.7 (0; 3.4)	1.0 (0; 7.1)	0.182
HOMA-IR	2.3 (0.8; 6.6)	1.8 (0; 3.69)	0.072

BMI: body mass index, LH: luteinizing hormone, FSH: follicle-stimulating hormone, E2:  $17\beta$ -estradiol, PRL: prolactin, TST: testosterone, SHBG: sex hormone binding globulin, FAI: free androgen index, DHEA-S: dehydroepiandrosterone sulphate, TCh: total-cholesterol, HDL-Ch: high-density lipoprotein cholesterol, LDL-Ch: low-density lipoprotein cholesterol, TG: triglycerides, CRP: C-reactive protein, HOMA-IR: Homeostasis Model of Assessment-Insulin Resistance

study [5].

In order to prepare urine samples for LC-MS analysis following steps were performed. After thawing to room temperature, samples were vortexed (1 min) and centrifuged (15 min, 13 000 rpm,  $4\text{ }^{\circ}\text{C}$ ). Then, 500  $\mu\text{l}$  of urine was placed in glass tube and diluted with 500  $\mu\text{l}$  of deionized water. The mixture was vortexed and centrifuged again (15 min, 13 000 rpm,  $4\text{ }^{\circ}\text{C}$ ). Finally, samples were filtered through nylon syringe filters (0.2  $\mu\text{m}$ ) to LC-MS dedicated vials.

Preparation procedure of urine sample for GC-MS analysis was more complex. After thawing to room temperature, samples were vortexed (1 min) and centrifuged (15 min, 13 000 rpm,  $4\text{ }^{\circ}\text{C}$ ). 200  $\mu\text{l}$  of urine was transferred to glass tube and 50  $\mu\text{l}$  of a previously prepared urease solution (0,0085 g/ml) was added. The mixture was incubated for 30 min at  $37\text{ }^{\circ}\text{C}$ . Next, to each sample, 800  $\mu\text{l}$  of cold methanol ( $-80\text{ }^{\circ}\text{C}$ ) was added. Additionally, 10  $\mu\text{l}$  of n-pentadecanoic acid (methanol solution, 1 mg/ml), as internal standard, was added. The mixture was vortexed and centrifuged again (15 min, 13 000 rpm,  $4\text{ }^{\circ}\text{C}$ ). Then, 200  $\mu\text{l}$  of supernatant was transferred into GC-MS dedicated vials. Samples were placed in a Quatro-Vac vacuum and evaporated to dryness (2 h,  $36\text{ }^{\circ}\text{C}$ ). In the case of GC-MS analysis, additional step of sample preparation, derivatization process, is required. At first, dry residues were reconstituted with 30  $\mu\text{l}$  of methoxyamine (pyridine solution, 15 mg/ml) and vortexed (10 min). Then, samples were incubated in a dark place (16 h, at room temperature). Afterwards, 30  $\mu\text{l}$  of 1% TMCS in BSTFA was added and samples were vortexed (5 min) and incubated for 1 h at  $70\text{ }^{\circ}\text{C}$ . The last step consisted of addition of 70  $\mu\text{l}$  of heptane and vortexing (10 min).

To prepare quality control samples (QC), the same volume of each of the urine samples were pooled and mixed in a glass vial. Depending on the analysis, required amount of pooled urine was distributed to a few tubes and prepared. 500  $\mu\text{l}$  of the pooled urine (for LC-MS analysis) and 200  $\mu\text{l}$  of pooled urine (for GC-MS analysis) was prepared according to the procedures described above. QC samples injection has been scheduled every 8 urine samples within the sequence run.

### 2.3. Untargeted LC-TOF/MS and GC-QqQ/MS metabolomic analysis

Two complementary analytical techniques such as high-performance liquid chromatography and gas chromatography coupled with mass spectrometry have been applied to analyze serum and urine samples.

HPLC-TOF/MS analyses were conducted using HPLC 1260 system coupled with 6224 TOF mass spectrometer equipped with an electrospray ionization (ESI) source (Agilent Technologies, Germany). Analysis of urine samples was carried out with the use of mobile phase consisted of 0.1% formic acid (phase A) and 0.1% formic acid in acetonitrile (phase B) with a flow rate of 0.35 ml/min. The gradient program, increasing from 2% to 98% of phase B within the 14 min was applied. Then, the composition of mobile phase including 98% of phase B was kept within 4 min 2  $\mu$ l of urine was injected and chromatographic separation was carried with the use Zorbax SB-C18 reversed-phase (RP) column (2.1 mm  $\times$  50 mm, 1.8  $\mu$ m; Agilent, USA) within 18 min. The equilibration time was 10 min. Analysis was carried out in scan mode and spectra were registered in the mass range from 50  $m/z$  to 1100  $m/z$ , in both positive and negative modes. Following MS parameters were applied: nebulizer gas flow rate (11 L/min), nebulizer gas pressure (50 psig), nebulizer gas temperature (350  $^{\circ}$ C), capillary voltage (3250 V).

GC-QqQ/MS analyses were conducted using GCMS-TQ8030 system (Shimadzu, Japan) equipped with electron ionization (EI) source. 1  $\mu$ l of urine was injected using spitless injection mode. The separation was performed with the use Zebtron ZB-5MS column (30 m  $\times$  0.25 mm, 0.25  $\mu$ m; Phenomenex, USA) and temperature gradient increasing from 60  $^{\circ}$ C to 320  $^{\circ}$ C. Helium was used as a carrier gas and following parameters were applied: injector temperature (250  $^{\circ}$ C), interface temperature (300  $^{\circ}$ C), and ion source temperature (200  $^{\circ}$ C). Data was collected at mass range from 50  $m/z$  to 600  $m/z$ .

In the case of serum sample, LC-MS and GC-MS analyses have been performed with the use the same equipment. Detailed parameters applied have been described in our previous work [5].

### 2.4. Data treatment and statistical analysis

Both serum and urine raw data sets obtained by LC-MS were deconvoluted with the selection of H<sup>+</sup>, Na<sup>+</sup>, adducts in positive ionization as well as -H, +HCOO adducts in negative ionization. Neutral loss of water was also considered. Next, data was extracted using MassHunter Qualitative Analysis software to separate searched compounds peaks from background noise, and aligned with Mass Profiler Professional software in order to minimize shift of retention times (RT). Then, data filtration was performed with the application of quality assurance criteria (QA), and presence in the studied groups. Variables which were presented in at least 50% of QC samples with coefficient of variation (CV) < 20%, as well as variables which were presented in at least 80% of serum/urine samples, in at least one of the studied groups, were selected for further analysis. Normalization of serum data was conducted with the use of internal standard (IS), namely 1-(4-fluorobenzyl)-5-oxoproline (Sigma Aldrich), and proceeded for statistical comparisons. Regarding urine data, probabilistic quotient normalization (PQN) was applied.

In the case of serum and urine data obtained by GC-MS, deconvolution, retention index (RI) calculation and RT alignment was performed with the use of Automated Mass Spectral Deconvolution and Identification Software (AMDIS). Then, data were filtered with the application of similar criteria as described above. Variables which were presented in at least 50% of QC samples with coefficient of variation (CV) < 50%, as well as variables which were presented in at least 80% of serum/urine samples, in at least one of the studied groups, were selected for further analysis. Normalization of data was performed using the IS (n-pentadecanoic acid, Sigma Aldrich).

Selection of statistically significant variables was performed by application of univariate and multivariate statistical analysis. The normality of the data distribution was checked using the Shapiro-Wilk

test and then parametric Welch's *t*-test or nonparametric Mann-Whitney *U*-test was applied for normally distributed or not normally distributed variables respectively. As an unsupervised multivariate method, principal component analysis (PCA) was applied using Simca P + 13.0.3 software (Umetrics, Umea, Sweden). Additionally, in order to select the most discriminant variables a supervised partial least squares-discriminant analysis (PLS-DA) and orthogonal partial last squares discriminant analysis (OPLS-DA) were conducted. Based on the obtained models, variable importance into projection (VIP) and selectivity ratio (SR) values were calculated using Matlab 2016b software (Mathworks, Natick, Ma, USA). The variables with *p*-values < 0.05, VIP > 1.0, and SR > 1.0 were assigned as statistically significant and proceeded for metabolite identification.

The statistically significant variables obtained by LC-MS were putatively identified based on monoisotopic mass, isotopic distribution, formula, and hits found in publicly available databases such as HMDB (www.hmdb.ca), METLIN (www.metlin.scripps.edu), KEGG (www.genome.jp/kegg), LIPIDMAPS (www.lipidmaps.org/), and CEU Mass-Mediator (http://ceumass.eps.uspceu.es/mediator). In the case of urine data, identification has been confirmed using an Ultimate 3000 nano-LC system (Thermo Fisher Scientific, USA) connected in-line to a TripleTOF 6600 + apparatus (Sciex, Framingham, MA) and MS/MS NIST or All-in-one Sciex spectra library.

Identification of compounds obtained by GC-MS was performed based on RI, RT, and mass spectra and comparison with data included in the NIST 11 and in-house spectral libraries.

## 3. Results

Metabolic fingerprints of serum and urine samples obtained with the application of LC-MS and GC-MS techniques are provided in [Supplementary Material](#).

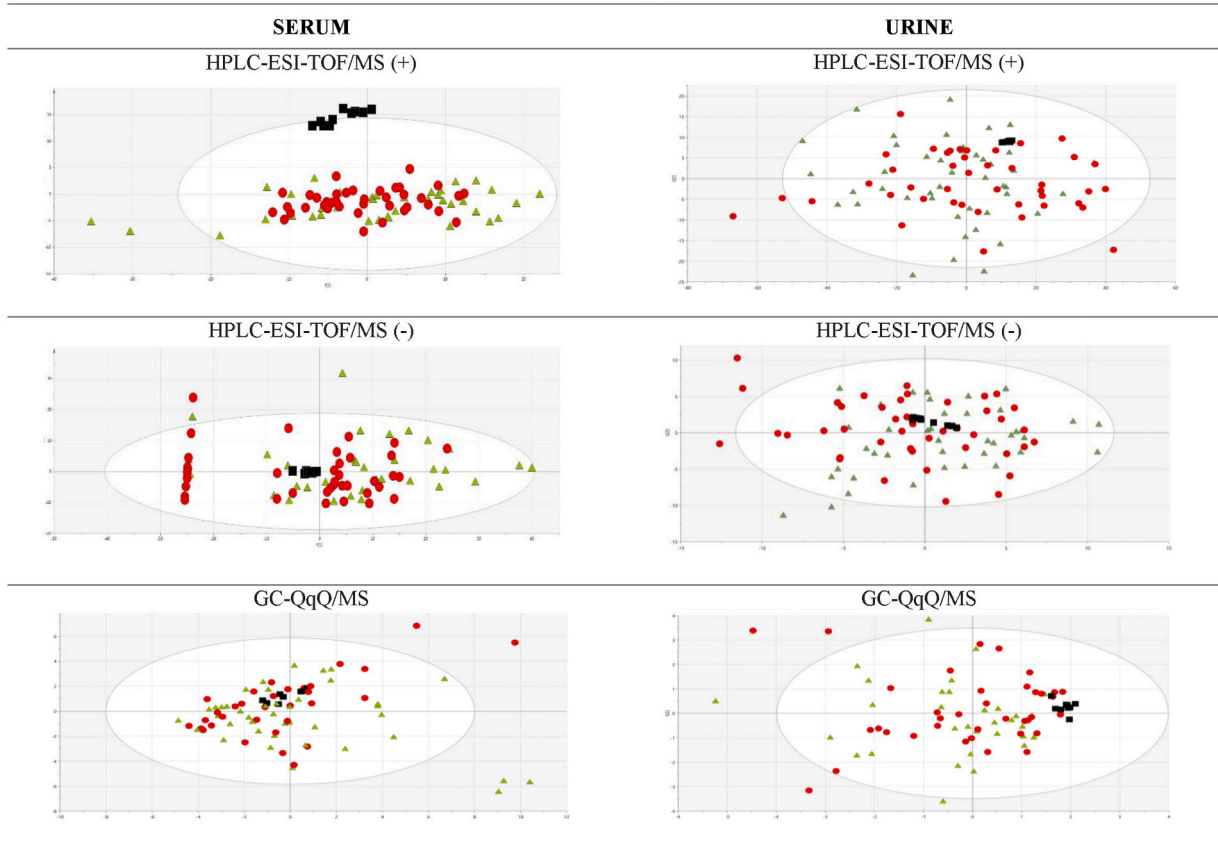
### 3.1. Validation of the analytical methodology

Validation of the analytical methodology in our study was performed with the use of pooled QC samples and blank samples. The blank samples were injected at the beginning and at the end of each analytical run to verify the system stability, method and instrument. Lack of peaks in blanks proves the purity of the system and excludes sample contamination, which translates into the quality of the obtained data and demonstrates, that the differences in the data are due to the samples. Additionally, QC samples were analyzed in regular intervals throughout the analytical run in order to monitor the quality of the data and reproducibility of the method applied. The application of QC samples also enabled to control the analytical variability. The PCA model modes were built to verify the analytical process's validity. The obtained PCA models are presented in [Fig. 1](#). Good clustering of QC samples confirms the stability of the system and reproducibility of the methods applied during both serum and urine analysis. It also confirms, that statistically significant changes observed between compared groups reflect true biological differences, not changes occurring due to analytical variability. Additionally, data were checked with the application of the Hotelling-T2 test to evaluate potential outlier results.

In the case of serum data, two samples were not included in further statistical analyses in the positive ionization mode, three samples were found as outliers in the negative ionization mode of the LC-MS technique, and five samples were rejected concerning GC-MS.

Regarding urine data, five samples were not included in further statistical analyses in the positive ionization mode, five samples were found as outliers in the negative ionization mode of the LC-MS technique and six samples were rejected concerning GC-MS.

PCA models



PLS-DA models

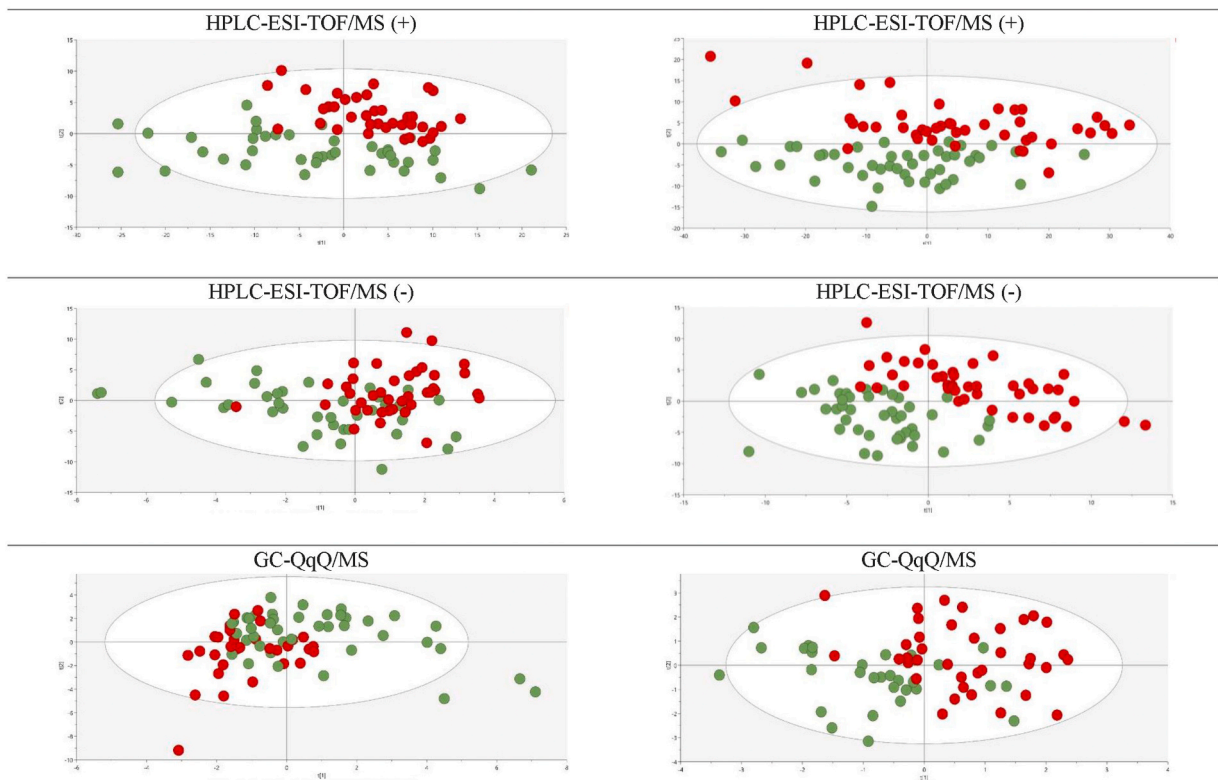


Fig. 1. PCA and PLS-DA models built on data obtained with serum and urine LC-MS analysis in positive & negative ionization and GC-MS analysis. Green triangles, red dots, and black squares correspond to healthy controls, PCOS patients, and QC samples, respectively.



3.2. Statistics

3.2.1. Univariate statistics

In the case of data obtained from serum analysis, univariate methods, based on the U'Mann-Whitney test, applied to LC-MS results, let to select 6 statistically significant masses ( $p < 0.05$ ) in positive ionization mode

and 3 statistically significant masses ( $p < 0.05$ ) in negative ionization mode. Regarding GC-MS results, 3 metabolites demonstrated statistically significant differences between serum samples of PCOS women and healthy control.

In the matter of data obtained from urine analysis, the U'Mann-Whitney test applied to LC-MS results, enabled to select 5 and 3

VIP method results

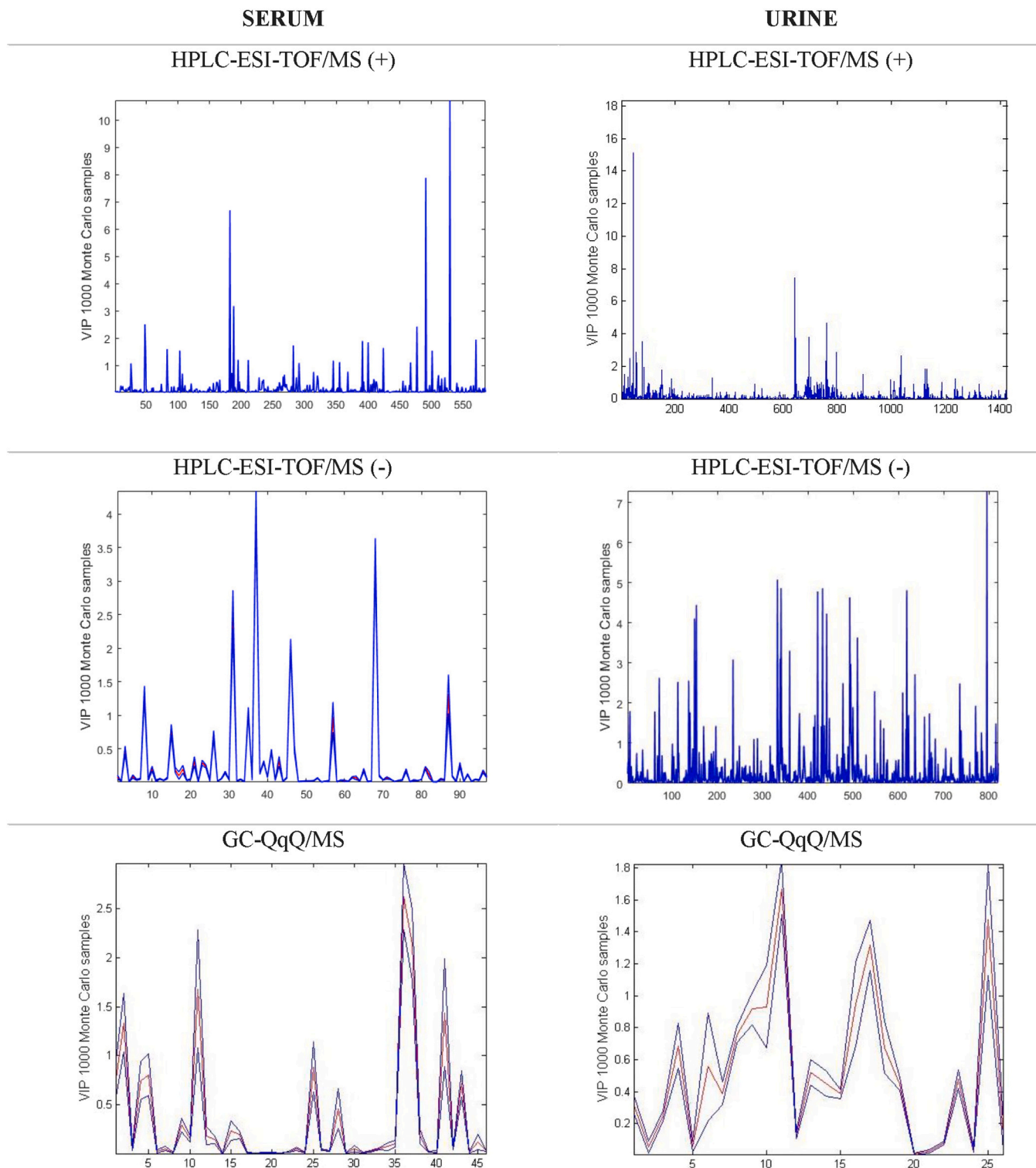


Fig. 2. Multivariate statistics with the use VIP method.



statistically significant masses ( $p < 0.05$ ), in positive and negative ionization modes, respectively. In the case of GC-MS results, 3 compounds demonstrated statistically significant differences between urine samples of two tested groups.

### 3.2.2. Multivariate statistics

Based on the obtained data, PLS-DA (Fig. 1) and OPLS-DA models were prepared. The OPLS-DA model plots built for data obtained for urine and serum for all techniques are present in [Supplementary Material](#).

The application of the VIP method enabled to select the most discriminant variables. Compounds with a VIP value higher than 1 were selected as the most significant. The higher the VIP value, the most discriminating power of the selected metabolite. Multivariate statistics with the use VIP method are presented in Fig. 2.

In the data obtained from serum analysis, the VIP method led to select 14 variables in positive ionization mode and 3 variables in the negative ionization mode of HPLC-ESI-TOF/MS. In the case of GC-QqQ/MS results, 4 compounds were selected. Each peak corresponds to one metabolite.

Regarding data obtained from urine analysis, 5 and 9 variables with VIP value  $> 1$  have been selected, in positive and negative ionization modes of HPLC-ESI-TOF/MS, respectively. In the case of data GC-QqQ/MS results, 3 variables were selected with VIP value  $> 1$ . Additionally, applying the SR method to urine data enabled the selection of 4 variables with SR value  $> 1$ , obtained with the use of HPLC-MS, only in positive ionization mode, and 6 compounds with SR value  $> 1$ , obtained with the use of GC-MS.

### 3.3. Serum metabolite identification

The metabolite identification results obtained by HPLC-ESI-TOF/MS technique are shown in Table 2. The information about measured mass, retention time (RT), potential metabolite identified, method used for statistical selection, p-value and/or VIP value, and regulation of detected metabolites between both groups are included. Identified serum

**Table 2**

Metabolites significantly changed in serum samples of PCOS patients compared to healthy control identified by the HPLC-ESI-TOF/MS technique.

Measured mass [Da]	RT [min]	Metabolite	Statistical analysis	p value/ VIP value	Ionization mode + / -	Regulation PCOS/Control
273.2669 *	18.7	C16 Sphinganine	Univariate & Multivariate	$p = 6.0 \times 10^{-3}$ VIP = 1.73	+	↓
317.2928	18.8	Phytosphingosine	Univariate	$p = 1.1 \times 10^{-2}$	+	↓
465.3086	18.3	Glycocholic acid	Univariate	$p = 1.4 \times 10^{-2}$	+	↑
511.3269	18.8	PS(O-18:0)	Univariate	$p = 1.7 \times 10^{-2}$	+	↓
162.1257	15.6	1,3,7-Octanetriol	Univariate & Multivariate	$p = 3.6 \times 10^{-2}$ VIP = 1.19	+	↓
481.3172	23.9	LysoPE(18:0)	Univariate	$p = 3.8 \times 10^{-2}$	+	↓
567.3568 *	27.4	LysoPS (21:0)	Univariate & Multivariate	$p = 2.3 \times 10^{-3}$ VIP = 1.31	-	↓
368.1686 *	21.2	Dehydroepiandrosterone sulfate (DHEA-Sulfate)	Univariate & Multivariate	$p = 2.8 \times 10^{-2}$ VIP = 4.13	-	↑
213.0107	13.7	Indoxyl sulfate	Univariate	$p = 4.4 \times 10^{-2}$	-	↓
521.3487 *	27.6	LysoPC(18:1)	Multivariate	VIP = 10.73	+	↓
495.3330 *	26.6	LysoPC(16:0)	Multivariate	VIP = 7.88	+	↓
678.5056	14.1	DG(17:0/PGE2/0:0)	Multivariate	VIP = 6.69	+	↓
165.0788 *	9.9	Phenylalanine	Multivariate	VIP = 2.50	+	↓
545.3486	26.2	LysoPC(20:3)	Multivariate	VIP = 2.42	+	↓
519.3338 *	24.4	LysoPC(18:2)	Multivariate	VIP = 1.84	+	↓
543.3332	24.8	LysoPC(20:4)	Multivariate	VIP = 1.64	+	↓
204.0901 *	11.3	Tryptophan	Multivariate	VIP = 1.59	+	↓
467.3014	22.9	LPC(14:0)	Multivariate	VIP = 1.17	+	↓
541.3174 *	23.1	LysoPC(20:5)	Multivariate	VIP = 1.11	+	↓
495.3340 *	25.9	PE(19:0)	Multivariate	VIP = 1.10	+	↓
131.0947	8.1	Leucine	Multivariate	VIP = 1.07	+	↓
370.1836	22.7	Dihydrotestosterone sulfate (DHT -Sulfate)	Multivariate	VIP = 2.05	-	↑

\*Masses detected and selected as statistically significant during two independent analyses.

metabolites detected by GC-QqQ/MS are presented in Table 3. Finally, 5 up-regulated and 21 down-regulated metabolites have been identified in the serum of PCOS women as compared to healthy control.

Based on the above results, there are four compounds which were selected as statistically significant both by univariate and multivariate tests: C16 sphinganine (monoisotopic mass 273.2669 Da) and 1,3,7-octanetriol (monoisotopic mass 162.1257 Da) detected in positive ionization mode as well as dehydroepiandrosterone sulfate (monoisotopic mass 368.1686 Da) and LysoPS (21:0) (monoisotopic mass 567.3568 Da) detected in negative ionization mode. Additionally, phenylalanine (monoisotopic mass 165.0788 Da) was selected as a significant compound detected by both LC-MS and GC-MS techniques.

Referring to one of the aims of this study, obtained results enabled us to confirm most of the metabolites, which were detected in serum samples and selected as statistically significant in our previous study [5]. It proves the significance of these metabolites in PCOS pathomechanism independently of the set of samples taken for testing. The validated metabolites obtained by LC-MS and GC-MS techniques are marked in the above tables.

**Table 3**

Metabolites significantly changed in PCOS patients' serum samples compared to healthy control identified by GC-QqQ/MS technique.

Metabolite	Fragment ions	Statistical analysis	p value/ VIP value	Regulation PCOS vs. Control
Aspartic acid	74.0; 70.0	Univariate	$p = 1.4 \times 10^{-2}$	↑
Phenylalanine*	146.0; 120.0; 80.0	Univariate	$p = 2.1 \times 10^{-2}$	↑
Threonine acid	292.0; 220.0; 205.0	Univariate	$p = 3.1 \times 10^{-2}$	↓
Cholesterol*	129.0; 368.0; 329.0	Multivariate	VIP = 1.43	↓

\*Metabolites detected and selected as statistically significant during two independent analyses.

### 3.4. Urine metabolite identification

Identified compounds detected in urine samples are presented in Table 4. The information about measured mass, retention time (RT), potential metabolite identified, method used for statistical selection, p value and/or VIP or SR value, and regulation change observed. Identified urine metabolites detected by GC-QqQ/MS are presented in Table 5. In summary, compared to healthy control, 13 up-regulated and 16 down-regulated metabolites have been identified in the urine of PCOS women.

According to the presented results, there are three compounds detected by the LC-MS technique in the positive ionization: phenylacetylglutamine, pyroglutamic acid, and 4-pyridoxic acid, which passed both VIP and SR methods and have the most discriminating power. VIP and SR values were the highest for phenylacetylglutamine and were 17.84 and 5.47, respectively. Additionally, two compounds: androsterone 3-glucuronide and 3-hydroxyglutaric acid detected in negative ionization mode, were selected as statistically significant both by univariate and multivariate tests.

Regarding GC-MS urine results, Threonic acid was selected both by VIP and SR method. In turn 1,5-anhydrosorbitol, fructose and alanine were selected as statistically significant both by univariate and multivariate tests.

## 4. Discussion

In this study, we received a comprehensive set of metabolites characteristic for Polycystic Ovary Syndrome. Untargeted metabolomic analysis of two biological matrices and utilization of two complementary techniques enabled us to select a wide range of metabolites in urine and serum samples. Application of GC-MS allowed to detect amino acids (like aromatic phenylalanine, aspartic acid, and alanine) as well as several carbohydrates (1,5-anhydrosorbitol, N-acetyl-D-glucosamine, fructose, threonic acid, glycerol). In addition, cholesterol from the steroids group was identified. In the case of metabolites detected by LC-MS, similarly steroid compounds (DHEA-S, DHT-S and glucuronide derivatives of testosterone and androsterone) were identified, as well as several

amino acids (glutamine, tryptophan, and leucine). Apart from them, other chemical groups like glycerophospholipids (LysoPC), keto acids (pyruvic acid, oxoglutaric acid), carboxylic acid (citric acid, glutaconic acid) and fatty acids (2-octenoylcarnitine) were detected by LC-MS. The obtained results confirm the legitimacy of using two platforms. Thanks to this, it was possible to select metabolites with such different physicochemical properties. Received data indicate the complexity of the disease. The use of two techniques gives us a panel of compounds that may be crucial for the pathogenesis of PCOS, and after appropriate verification by quantitative analysis, their diagnostic significance can be confirmed.

Majority of identified metabolites came from the same biochemical pathways, what confirms that alterations occurring in these paths are mostly disturbed during PCOS development. The most significant metabolic pathways disturbed in PCOS women, have been presented in Table 6.

The important metabolite validated in serum samples and also detected in urine is sphinganine, which indicates the alteration in sphingolipid metabolism. Down-regulation of sphinganine was observed in our study. A similar observation of sphinganine levels in PCOS women was reported by C. Jia et al. [6]. Sphingolipids are a relatively minor class of lipids in human cells, however, they have gained more attention, because of their responsibility in cell-signaling and their role in the pathogenesis of some metabolic disorders. Sphingolipids may directly impact on insulin signaling. Due to the blocking translocation of the insulin-sensitive glucose transporter GLUT4 to the plasma membrane, they may lead to a decrease in glucose uptake in cells [7]. Apart from sphinganine, decreased level of phytosphingosine (PHS) has been observed in our study. A similar observation has been reported by Dong et al. [8]. According to the literature, PHS has an impact on the stimulation of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), which plays a crucial role in glucose and lipid metabolism [9].

In the range of lipids, phospholipids were detected in serum samples as metabolites significantly changed in PCOS women, what was observed in our previous study and confirmed in the validation approach. Phospholipids are the main component of the cell membrane, and their altered profile may profoundly impact cell function [10]. They

**Table 4**

Metabolites significantly changed in urine samples of PCOS patients in comparison to healthy control identified by HPLC-ESI-TOF/MS technique.

Measured mass [Da]	RT [min]	Fragment ions	Metabolite	Statistical analysis	p value / VIP or SR value	Ionization mode + / -	Regulation PCOS/Control
316.2388	12.6	159.1134, 281.2279, 299.2419	Pregnenolone	Univariate	p = 2.3 × 10 <sup>-3</sup>	+	↑
138.0419	2.1	121.0455, 93.0499, 66.0312	Urocanic acid	Univariate	p = 5.0 × 10 <sup>-3</sup>	+	↓
219.1109	3.5	88.0431, 146.0891, 71.0578	Pantothenic acid	Univariate	p = 8.6 × 10 <sup>-3</sup>	+	↓
301.2973	13.3	284.2919, 254.2812, 60.4576	C18 Sphinganine	Univariate	p = 2.1 × 10 <sup>-2</sup>	+	↓
187.0633	5.9	142.0781, 116.0678, 140.0518	Indoleacrylic acid	Univariate	p = 4.7 × 10 <sup>-2</sup>	+	↑
466.2564	12.6	75.0235, 85.0281, 287.2141	Androsterone 3-glucuronide	Univariate & Multivariate	p = 1.3 × 10 <sup>-3</sup> VIP = 4.72	-	↑
464.2403	12.3	97.0678, 109.0678, 289.2213	Testosterone glucuronide	Univariate	p = 2.8 × 10 <sup>-3</sup>	-	↑
148.0368	0.8	85.0413, 87.0293, 59.0231	3-Hydroxyglutaric acid	Univariate & Multivariate	p = 2.5 × 10 <sup>-2</sup> VIP = 1.11	-	↓
264.1102	6.9	130.0491, 84.0543, 147.0819	Phenylacetylglutamine	Multivariate	VIP = 17.84 SR = 5.47	+	↓
179.0579	6.5	105.0251, 77.0398, 51.0391	Hippuric acid	Multivariate	VIP = 7.18	+	↓
285.1935	10.2	73.0318, 55.0295, 57.0451	2-Octenoylcarnitine	Multivariate	VIP = 3.01	+	↓
183.0532	1.7	166.0561, 148.0358, 65.0328	4-Pyridoxic acid	Multivariate	VIP = 2.28 SR = 2.46	-	↓
376.1379	7.7	243.0891, 172.1039, 198.0691	Riboflavin	Multivariate	VIP = 1.61	+	↓
129.0419	6.9	84.0421, 56.0193	Pyroglutamic acid	Multivariate	VIP = 1.28 SR = 1.40	+	↓
146.0683	1.4	84.0219, 130.0491, 56.0614	Glutamine	Multivariate	SR = 2.56	+	↓
192.0285	0.9	111.0241, 87.0291, 173.0192	Citric acid	Multivariate	VIP = 4.09	-	↓
168.0283	0.9	92.0612, 126.0312, 70.0315	Uric acid	Multivariate	VIP = 3.88	-	↓
130.0265	0.8	85.1252, 107.0981, 97.1099	Glutaconic acid	Multivariate	VIP = 3.24	-	↑
182.0445	1.7	69.0219, 112.0591, 140.0512	Methyluric acid	Multivariate	VIP = 2.94	-	↓
88.0173	1.0	88.0412, 69.9517, 63.1295	Pyruvic acid	Multivariate	VIP = 2.19	-	↑
146.0219	0.8	101.0319, 73.0419, 57.0298	$\alpha$ -Ketoglutaric acid (Oxoglutaric acid)	Multivariate	VIP = 1.45	-	↓

**Table 5**  
Metabolites significantly changed in urine samples of PCOS patients in comparison to healthy control identified by GC-QqQ/MS technique.

Metabolite	Fragment ions	Statistical analysis	p value/ VIP or SR value	Regulation PCOS vs. Control
1,5-Anhydrosorbitol	117.0; 191.0; 133.0	Univariate & Multivariate	$p = 2.1 \times 10^{-2}$ VIP = 2.22	↑
Fructose	217.0; 205.0; 103.0	Univariate & Multivariate	$p = 4.1 \times 10^{-2}$ SR = 2.21	↓
Alanine	116.0; 190.0; 218.0	Univariate & Multivariate	$p = 4.7 \times 10^{-2}$ SR = 1.20	↑
Creatinine	115.0; 100.0; 171.0	Multivariate	VIP = 2.29	↑
Threonic acid	292.0; 220.0; 205.0	Multivariate	VIP = 1.94 SR = 2.62	↑
N-Acetyl-D-glucosamine	217.0; 103.0; 205.0	Multivariate	SR = 1.45	↑
Propanoic acid	131.0; 205.0; 233.0	Multivariate	SR = 1.35	↑
Glycerol	177.0; 205.0; 133.0	Multivariate	SR = 1.20	↑

constitute the barrier to the passage of specific molecules into and out of the cell. It was observed that phospholipids might impact insulin action and its secretion in cells in different ways and play a key role in insulin sensitivity [11].

Referring to disturbed fatty acids metabolism, 2-octenoylcarnitine has been detected as a significantly changed metabolite in urine samples. 2-octenoylcarnitine belongs to acylcarnitines, which play an important role in the transport of fatty acids into the mitochondrial matrix, where they are oxidized and turned into energy. After acylcarnitines are transported into the mitochondria, they are converted back to carnitines and acyl-CoA. Then, Acyl-CoA groups can participate in  $\beta$ -oxidation and subsequently in the citric acid cycle (Fig. 3).

According to the literature, alteration in this process, connected with incomplete fatty acid  $\beta$ -oxidation and acylcarnitines accumulation, leads to increased oxidative stress, what may be linked with insulin resistance development [12]. Additionally, it was noticed that decreased carnitine level in PCOS women correlates with insulin resistance and hyperandrogenism [13]. Low levels of carnitine could limit the fatty acids availability to produce ATP, so the energy needed by cells is insufficient [14]. Moreover, higher abundance of CPT I has been detected in ovarian tissue of PCOS women indicating increased fatty acid  $\beta$ -oxidation [15]. A. M. Ismail et al. noticed that treating insulin resistant PCOS women with carnitine positively impacted ovulation improvement and increased pregnancy rate [16].

Steroids hormones are one of the most important groups of metabolites disturbed in women with PCOS. Alterations in steroid metabolism are very visible and often described in metabolomic studies [6]. Some of them were detected in our study both in serum and urine samples. Dehydroepiandrosterone sulphate (DHEA-S) has been identified primarily in serum samples and was confirmed during validation study. Apart from that, dihydrotestosterone sulfate (DHT -S) was detected in serum, while glucuronide conjugates of androsterone and testosterone have been detected in urine samples. Moreover, pregnenolone has been identified in urine. All of identified steroids are up-regulated in PCOS group. The first research describing the association between alteration in carbohydrate metabolism and hyperandrogenism appeared in the nineteen twenties [3]. It was assumed that there exists a mechanism connecting abnormal glucose metabolism and steroid hormone levels in PCOS women. Moreover, hyperandrogenemia, considered as the main element of PCOS, is closely associated with hyperinsulinemia [17]. Insulin can lower hepatic production of sex hormone-binding globulin (SHBG) which could explain the frequently occurring contrary correlation between SHBG and peripheral insulin levels [2]. Some observations suggest, that hyperinsulinemia can stimulate ovarian androgen and estrogens production in PCOS women, whereas in healthy women, under physiological conditions, insulin does not show any impact on ovarian function. It was also noticed that the application of insulin-sensitizing compounds resulted in a decrease of DHEA-S levels in PCOS patients. It could be also connected with the direct impact of insulin on increasing sensitivity of adrenocorticotrophic hormone (ACTH) [3].

Tryptophan has been confirmed in our study as the most significant compound in the case of PCOS pathophysiology. Tryptophan metabolites, including kynurenine, have been reported as strongly associated with insulin resistance, pancreatic cells functions, and glucose homeostasis in the research conducted by T. Chen et al. [18]. Additionally, in the study performed by E. Hou et al., tryptophan was detected in follicular fluid of PCOS women as significantly altered. Authors claimed that tryptophan metabolism may be considered as a key role in PCOS development [19]. In our study, apart from tryptophan itself, indoleacrylic acid (IA) has been detected. Indoleacrylic acid is one of the tryptophan metabolites, which is produced by *Lactobacillus* bacterial via indole pyruvate pathway (Fig. 4).

According to the newest literature data, changes in gut microbiota composition are common feature of PCOS [20]. M. Mathew reported, that indoleacrylic acid is an activator of the anti-oxidant erythroid 2-related factor 2 (NRF2), which regulates the iron absorption in the intestine and promotes antioxidant and anti-inflammatory immune response [21]. Author also noticed, that some proteins connected with iron are dysregulated in PCOS patients due iron overload, which could be connected with microbial dysbiosis regarding *Lactobacilli spp.* causing increased absorption of iron. It has been also reported, that disturbed gut microbiota is associated with hyperandrogenism in PCOS women [22]. G. Yourtdas proposed potential mechanisms of PCOS development connected with gut microbiota dysbiosis. One of them is connected with activation of the immune system by altered gut microbiota. An activated immune system causes interference of insulin receptors leading to hyperinsulinemia. Hyperinsulinemia, in turn, stimulates ovarian androgens production and inhibits the normal growth of the follicles [20].

Among urinary metabolites selected in our study, we observed, that three of them: citric acid, pyruvic acid, and  $\alpha$ -ketoglutaric acid (oxoglutaric acid) are the main components of the citric acid cycle. Alteration in these metabolites proves, that this is one of the main metabolic pathways affected in PCOS patients (Fig. 5). Impairment in the TCA cycle during PCOS development has been confirmed in the literature [23]. Furthermore, changes in the level of pyruvate indicate on impairment of glucose metabolism pathway. Elevated level of pyruvate can be also linked with increased level of alanine observed in PCOS women. Oxoglutaric acid is reported as a key metabolite during the PCOS development. J. Chen et al. selected  $\alpha$ -ketoglutaric acid as potential biomarker of PCOS [24].

Women with PCOS are often deficient in vitamins, especially in B group vitamins [25]. In our study, we identified pantothenic acid (vitamin B<sub>5</sub>), riboflavin (vitamin B<sub>2</sub>) and 4-pyridoxic acid - metabolite of vitamin B<sub>6</sub>, at a significantly decreased level in urine of PCOS women. Riboflavin is a strong antioxidant and its deficiency results in increased oxidative stress, which is a contributing factor to presence of numerous symptoms of the polycystic ovary syndrome, such as improper ovary function, insulin resistance and disturbed lipid metabolism [26]. Moreover, correlation between riboflavin and androgens has been



**Table 6**  
The most significant metabolic pathways changed in PCOS patients.

Metabolic pathway	Metabolite	Analytical technique	Fold change ratio PCOS vs Control
Androgen metabolism	Dehydroepiandrosterone sulfate (DHEA-Sulfate)	LC-MS (-)	1.3 ↑
	Dihydrotestosterone sulfate (DHT -Sulfate)	LC-MS (-)	1.7 ↑
	Testosterone glucuronide	LC-MS (-)	1.3 ↑
	Androsterone 3-glucuronide	LC-MS (-)	1.6 ↑
	Pregnenolone	LC-MS (+)	1.5 ↑
Phospholipid metabolism	Cholesterol	GC-MS	0.9 ↓
	PS(O-18:0), LysoPE(18:0), LPC (14:0)	LC-MS (+)	0.8 ↓
	LysoPS (21:0)	LC-MS (-)	0.7 ↓
	LysoPC(18:1), LysoPC(16:0), LysoPC(20:3), LysoPC(18:2)	LC-MS (+)	0.9 ↓
	LysoPC(20:4), LysoPC(20:5), PE(19:0)	LC-MS (+)	0.9 ↓
Sphingolipids metabolism	LPC(14:0)	LC-MS (+)	0.8 ↓
	Sphinganine	LC-MS (+)	0.7 ↓
TCA cycle	Phytosphingosine	LC-MS (+)	0.6 ↓
	Oxoglutaric acid	LC-MS (-)	0.9 ↓
Glutathione metabolism	Pyruvic acid	LC-MS (-)	1.3 ↑
	Citric acid	LC-MS (-)	0.9 ↓
Vitamin B metabolism	Pyroglutamic acid	LC-MS (+)	0.9 ↓
	Pantothenic acid	LC-MS (+)	0.6 ↓
Tryptophan metabolism & Indole pyruvate pathway	4-Pyridoxic acid	LC-MS (-)	0.7 ↓
	Riboflavin	LC-MS (+)	0.4 ↓
Phenylalanine metabolism	Indoxyl sulfate	LC-MS (-)	0.7 ↓
	Tryptophan	LC-MS (+)	0.9 ↓
Histidine metabolism	Indoleacrylic acid	LC-MS (+)	1.4 ↑
	Phenylalanine	GC-MS/LC-MS (+)	1.31/0.9 ↓
Branched-chain amino acids	Phenylacetylglutamine	LC-MS (+)	0.8 ↓
	Glutamine	LC-MS (+)	0.8 ↓
Alanine metabolism	Hippuric acid	LC-MS (+)	0.8 ↓
	Urocanic acid	LC-MS (+)	0.9 ↓
Fatty acid metabolism	Aspartic acid	GC-MS	1.7 ↑
	Leucine	LC-MS (+)	0.9 ↓
Carbohydrate metabolism	Alanine	GC-MS	1.3 ↑
	1,3,7-Octanetriol	LC-MS (+)	0.9 ↓
Bile acid metabolism	DG(17:0/PGE2/0:0)	LC-MS (+)	0.8 ↓
	Glutaconic acid	LC-MS (-)	1.4 ↑
Glycerolipid metabolism	3-Hydroxyglutaric acid	LC-MS (-)	0.8 ↓
	2-Octenoylcarnitine	LC-MS (+)	0.9 ↓
Glycerolipid metabolism	Propanoic acid	GC-MS	1.3 ↑
	1,5-Anhydrosorbitol	GC-MS	2.2 ↑
Glycerolipid metabolism	N-Acetyl-D-glucosamine	GC-MS	1.3 ↑
	Fructose	GC-MS	1.5 ↑
Glycerolipid metabolism	Threonic acid	GC-MS	1.5 ↑
	Glycocholic acid	LC-MS (+)	1.2 ↑
Glycerolipid metabolism	Glycerol	GC-MS	1.5 ↑

reported in the literature [27]. Riboflavin is a natural, strong inhibitor of testosterone 5 $\alpha$ -reductase, which catalyzes the conversion of testosterone to dihydrotestosterone [28].

Pyroglutamic acid was the next metabolite detected in urine samples and selected as the most significant in our study. This compound was also detected in urine by Zou et al. and in follicular fluid by E. Hou et al. It has been observed a positive association between pyroglutamic acid and serum levels of androstenedione [19]. Pyroglutamic acid is a cyclic amino acid that takes part in the  $\gamma$ -glutamyl cycle. Therefore, an altered level of pyroglutamic acid has a direct impact on disturbances of glutathione or glutamic acid metabolism. According to data presented by Chen et al., glutamic acid is an essential compound for the growing oocytes [29].

The metabolite which has been selected as the most discriminative

between PCOS women and healthy control was phenylacetylglutamine (PAG). The conjugation of phenylacetyl-CoA forms Phenylacetylglutamine with glutamine during phenylalanine metabolism (Fig. 6). According to the latest data, phenylacetylglutamine has been assessed as one of the biomarkers of type 2 diabetes [30]. Apart from PAG, both phenylalanine, and glutamine have been selected in our study as the significantly decreased in PCOS women. A similar observation regarding the down-regulation of glutamine in PCOS women has been reported by Sun et al. [31]. G. Wu et al. observed that supplementation of glutamine has a positive impact on inflammation and oxidative stress occurring in PCOS [32].

Taking into account described abnormalities, it can be observed, that most of them have a common denominator – a connection with insulin action. Insulin has numerous target points, such as stimulation of glucose uptake, transport of glucose through GLUT4 translocation, protein and lipid synthesis, cell growth, or gene regulation. The insulin mechanism of action is mediated by a ligand-activated tyrosine kinase receptor which triggers a variety of intracellular signaling pathways through insulin receptor substrate proteins (IRS) and phosphoinositide 3-kinase (PI3-K) cascades, leading to the pleiotropic actions [33] (Fig. 7). It has been reported, that in muscle cells biopsied from PCOS patients, the impaired insulin-stimulated connection of PI3-K with IRS-1 was observed to be accompanied by in vivo decreased glucose transport. Therefore, alterations in early steps in the insulin signaling cascade may be responsible for defective metabolic action of insulin in women with PCOS [34].

## 5. Conclusions

The results of our study allowed us to indicate the most specific metabolic paths disturbed in women with PCOS. The identified metabolites showed increased steroid hormone synthesis, alteration in sphingo- and phospholipids metabolism, and disturbed fatty acids metabolism. Moreover, altered citric acid cycle,  $\gamma$ -glutamyl cycle, vitamin B metabolism, and the alteration in a few main amino acids like tryptophan, phenylalanine, histidine and alanine have been observed. Most metabolic abnormalities in women with PCOS, identified in our study, are linked to perturbations in insulin action or inappropriate glucose utilization. Taking into consideration the pleiotropic actions of insulin and the fact, that insulin resistance is highly prevalent in PCOS women it is advisable to emphasize further assessment of the influence of insulin action on the pathophysiology of PCOS in future research endeavours.

We to point out, that in the field of PCOS untargeted metabolomic studies this is the first study conducted with the use of two biological matrices (serum and urine) obtained from the same study cohort and analyzed by two complementary analytical techniques. In most of the other studies, analytes are mainly identified based on the only one matrix and a single analytical technique within one study. Based on proposed approach, we identified and described a comprehensive set of metabolites altered in PCOS patients. Our findings determine the direction of future metabolomic studies focusing on searching for the specific biomarkers of PCOS and indicate, that more profound insight into the alteration of the selected pathways may deliver valuable information regarding the pathomechanism of this complex disorder. According to the obtained data, compounds were selected for further quantitative analysis to evaluate their significance in the pathogenesis of PCOS. Nevertheless, an untargeted metabolomics approach applied in our study, focus on semiquantitative evaluation, therefore, there is a need to confirm selected metabolites on a larger number of samples with the use of a targeted approach and determine exact quantities of these compounds to find a specific direction of biochemical reactions.

## Ethics statement

The study was approved by the Bioethics Committee of the Medical

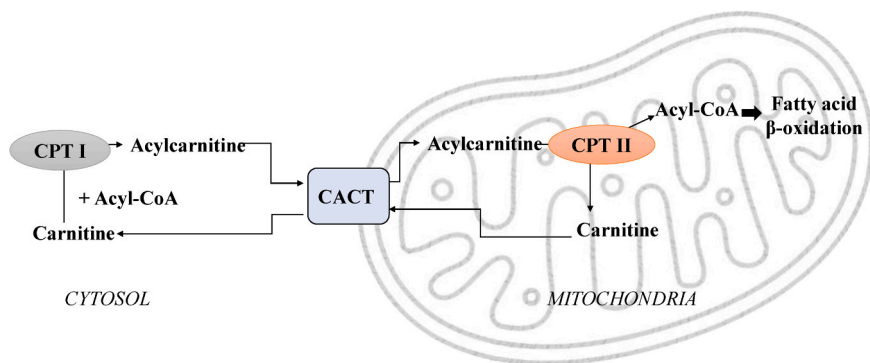


Fig. 3. Interconversion of carnitine and acylcarnitine. Carnitine-acylcarnitine translocase (CACT), Carnitine palmitoyltransferase I (CPT I), Carnitine palmitoyltransferase II (CPT II).

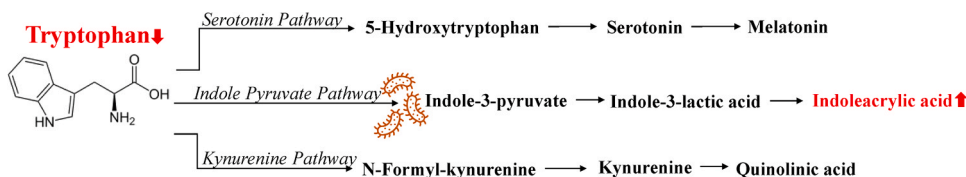


Fig. 4. Tryptophan metabolic pathways.

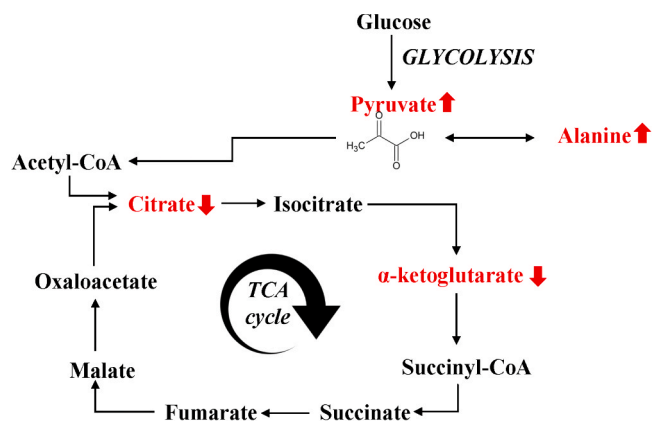


Fig. 5. Citric acid cycle metabolites altered in POCS women.

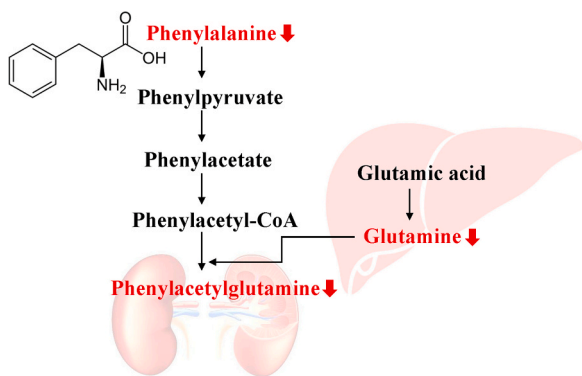


Fig. 6. Phenylalanine metabolism alteration observed during PCOS development.

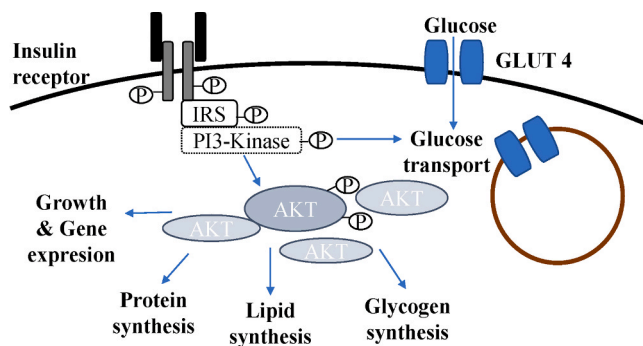


Fig. 7. Mechanism of insulin action.

University of Gdańsk (permission number NKBBN/27/2018) and all participants provided a written consent to participate in this study.

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CRediT authorship contribution statement

The authors confirm contribution to the paper as follows: **Conceptualization:** Anna Rajska, Magdalena Buszewska-Forajta. **Methodology:** Anna Rajska, Magdalena Buszewska-Forajta, Szymon Macioszek, Renata Wawrzyniak. **Validation:** Anna Rajska, Magdalena Buszewska-Forajta, Renata Wawrzyniak, Paweł Wityk. **Formal analysis:** Anna Rajska, Magdalena Buszewska-Forajta, Szymon Macioszek. **Investigation:** Anna Rajska, Magdalena Buszewska-Forajta, Renata Wawrzyniak. **Resources:** Agnieszka Kowalewska, Dominik Rachoń, Andrzej Berg. **Data Curation:** Anna Rajska, Magdalena Buszewska-Forajta, Szymon Macioszek. **Writing - Original Draft:** Anna Rajska, Magdalena

Buszewska-Forajta. **Writing - Review & Editing:** Dominik Rachoń, Michał J. Markuszewski. **Visualization:** Anna Rajksa. **Supervision:** Dominik Rachoń, Michał J. Markuszewski, Magdalena Buszewska-Forajta. **Project administration:** Anna Rajksa. **Funding acquisition:** Anna Rajksa.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

### Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jpba.2023.115602](https://doi.org/10.1016/j.jpba.2023.115602).

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