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Metabolome analysis as a potential source of endometriosis biomarkers with the use of multiomics approach in its diagnosis

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Endometriosis poses diagnostic challenges. This study aimed to analyze the metabolomic profiles of plasma and peritoneal fluid samples obtained from women with endometriosis compared to controls. Our multicenter study involved sample collection from women undergoing laparoscopic surgery. The metabolomic profiles of plasma samples obtained from 73 women with endometriosis and 35 controls, as well as peritoneal fluid samples from 53 women with endometriosis and 34 controls, were analysed using mass spectrometry techniques. Differences in lipid profiles were observed between the groups. Chemometric analyses identified a set of 20 metabolites present in peritoneal fluid and 26 compounds in plasma, which serve as potential diagnostic tools for endometriosis. Then, we used a simple approach to build a classification model based on the sets of metabolites in combination with autoantibodies selected using protein microarrays from our previous study. The classification performance obtained on the joined metabolomic and proteomic feature sets exceeds that achievable for separate assays (sensitivity/specificity for plasma and peritoneal fluid were respectively 0.98/0.86 and 0.92/0.82). Identified metabolites present promising candidates for biomarkers. Utilizing these metabolites in a diagnostic panel may enhance endometriosis detection. Moreover, we observed the potential benefits of a multi-omics approach based on integrated metabolomic and proteomic analysis to endometriosis research.

Keywords Endometriosis, Metabolomics, Multiomics, Lipids, Biomarkers

Endometriosis is a common gynecological condition in which tissue histologically similar to the endometrium is located outside the uterus[1]. Currently, the diagnosis of endometriosis is primarily possible through laparoscopic surgery, during which the lesions can be visualized and their presence confirmed through histopathological analysis. Recent technological advancements have enabled disease diagnosis using imaging techniques such as transvaginal ultrasound and magnetic resonance imaging[2]. Although these methods allow for the diagnosis of some cases of deep endometriosis and ovarian endometrioma, they still present diagnostic challenges in particular for peritoneal lesions. Hence, there is a need to develop a non-invasive diagnostic test for the detection of endometriosis. Despite extensive research, no single biomarker for endometriosis with high accuracy has been identified. Therefore, the idea of integrating multiple biomarkers, even those with low diagnostic sensitivity and specificity, to create an algorithm seems to be a good solution that could increase the efficiency of the diagnostic process. Large-scale "omics" technologies could play a key role in developing non-invasive diagnostic method for endometriosis.

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The use of metabolomics to search for new biomarkers in various clinical areas is founded on the premise that diseases alter biochemical pathways, resulting in the formation of a metabolic "signature" characteristic of the location and nature of the disease[3]. The metabolome reflects the actual biological state of the organism, making its analysis particularly useful in assessing diseases such as endometriosis, which can progress to a more advanced stage[4,5]. Metabolomics can provide information not only about the presence of a disease itself but also about its stage and progression. Moreover, it can offer insights into the biological changes underlying the disease, thereby facilitating the identification of molecular biomarkers that arise from disease-induced alterations, as well as factors contributing to its development[5]. Although metabolomic analysis appears to be a promising method for identifying novel biomarkers of endometriosis, the number of identified and analyzable metabolites remains limited[6,7]. For this reason, combining this method with the results of complementary omic approaches offers a greater chance of identifying biomarkers for a disease with a limited understanding of its pathophysiology. Other techniques that, along with metabolomics, can be used to create diagnostic tools for endometriosis and have already provided promising results in this field include proteomics and glycomics[8,9].

This study aimed to analyze the metabolomic profiles of plasma and peritoneal fluid samples from women with endometriosis compared to controls, employing mass spectrometry (MS). Then, we conducted a multi-omic analysis to build a classification model based on the sets of metabolites in combination with autoantibodies selected using protein microarrays from our previous study[8]. The study analyzed autoantibody profiles in endometriosis patients and controls, finding no significant differences in overall autoantibody levels between groups. However, stage-specific elevations (e.g., ANAPC15 and GABPB1 in Stage II peritoneal fluid) and menstrual cycle-dependent variations (e.g., NEIL1, MAGEB4 and TNIP2 in luteal-phase samples) were observed. Thirty proteins were prioritized based on overlapping uncorrected $p < 0.01$ signals in both plasma and peritoneal fluid, suggesting potential biological relevance despite lacking statistical significance after false discovery rate correction.

Methods

Sample collection

A multicenter, cross-sectional study was conducted between 2018 and 2019 in eight centers in Poland. Biological material (plasma and peritoneal fluid) was obtained from women undergoing laparoscopic surgery for the following reasons: ovarian cyst, pelvic pain and/or infertility. The exclusion criteria were as follows: age under 18 and over 45 years old, irregular menstruation (< 25 or > 35 days), any form of hormonal therapy during the last three months before surgery, pelvic inflammatory disease, uterine fibroids, polycystic ovary syndrome, any autoimmune diseases and malignant or suspected malignant. The details of the study are described elsewhere[8]. The cycle phase was calculated from the last menstrual period and average length of the menstrual cycle. Women from the endometriosis group were diagnosed through laparoscopic findings, and each case was histopathologically confirmed. As controls, we recruited patients without visible endometriosis during laparoscopy. In this study we analysed plasma samples obtained from 73 women with endometriosis and 35 controls and peritoneal fluid samples obtained from 53 women with endometriosis and 34 controls. Women from whom peritoneal fluid was collected represent the subgroup of patients from whom plasma was collected. All women completed a World Endometriosis Research Foundation clinical questionnaire and signed an informed consent form to participate in the study.

Diagnostic laparoscopy was performed in all patients by trained gynaecologists. After surgery, each woman diagnosed with endometriosis was assigned an appropriate stage of disease advancement according to the revised American Fertility Society (rAFS) classification[10]. Peritoneal fluid was collected through aspiration using a Veress needle under direct visualisation immediately upon introduction of the laparoscope to avoid contamination with blood. The procedure was meticulously performed in line with the standard operating procedures of the Endometriosis Phenome and Biobanking Harmonisation Project[11]. The collected peritoneal fluid was centrifuged at 1,000 × g for 10 min at 4 °C. The supernatant was transferred to a fresh 10 mL tube (Sarstedt) and divided into 500 µL tubes. Blood samples were collected before laparoscopy (before anaesthesia) in ethylenediaminetetraacetic acid (EDTA) 10 mL tubes (Sarstedt). The time lapse between sample collection (both plasma and peritoneal fluid) and processing was < 45 min. Blood samples were centrifuged at 2,500 × g for 10 min at 4 °C. Then, the plasma samples were split into 500 µL aliquots. Both materials were stored at −80 °C until further use.

Experimental protocols were approved by The Bioethics Committee operating at the Medical University of Warsaw (opinion No. KB 223/2017, issued on December 12, 2017) and by The Bioethics Committee operating at Calisia University (opinion 04/2021, issued on December 20, 2021). All methods were performed in accordance with the relevant guidelines and regulations.

Sample preparation

Before analysis, all plasma and peritoneal fluid samples were thawed on ice, centrifuged at 2 750 g, 4 °C for 5 min, and then centrifuged for 15 min at 1200 RPM as advised by AbsoluteIDQ p180 kit instruction. AbsoluteIDQ p180 kit procedure started with the preparation of derivatization mixture, extraction, and FIA solvents. 10 µl of internal standard (IS) was put onto each 96-well plate. Afterward, 10 µl of the respective sample was pipetted into the previously assigned well. The plate was dried under a nitrogen stream using a Positive Pressure-96 Processor (Waters) for 30 min. At the end of the drying process, 50 µl of derivatization mix was added to each well and left to derivatize for 25 min at room temperature. The plate was further dried using a positive pressure manifold for 60 min. 300 µl of extraction solvent was added to each well, left to vortex at 450 RPM for 30 min, and then centrifuged at 500 g for 2 min to elute the extracted metabolites. 150 µl of eluted sample was transferred to a 96-well LC plate, diluted with 150 µl of pure water, and 10 µl of eluted sample was transferred to a 96-well FIA plate

and diluted with 490 µl of FIA solvent. Before injection, plates were centrifuged at 600 RPM for 5 and 10 min, respectively.

Samples analysis

Liquid chromatography with tandem mass spectrometry (LC–MS/MS) and flow injection analysis using tandem mass spectrometry (FIA-MS/MS) analysis were performed using Waters Acquity Ultra Performance Liquid Chromatography coupled with Waters TQ-S triple-quadrupole mass spectrometer using AbsoluteIDQ® p180 kit (Biocrates inc.). The kit enables the analysis of 188 different metabolites in every biological sample, belonging to the following analyte classes: amino acids, biogenic amines, monosaccharides, acylcarnitines, glycerophospholipids and sphingomyelins. Quantification of metabolites in LC–MS/MS mode was done using a 7-point calibration curve. Identification of metabolites was possible thanks to the presence of their isotopically labeled standards. Calibration ranges and isotopically labeled internal standards used for quantitative analysis of metabolites by LC–MS/MS are presented in Supplementary Table S1 (see online). Mass spectrometer parameters were set as given by the Biocrates instruction (UM-p180 Waters v8-2021, Biocrates). MS spectra were obtained using multiple-reaction-monitoring mode. Amino acids and biogenic amines were analyzed using LC–MS in positive mode. Analytes separation was performed using a Waters BEH C18 column (1.7 µm, 2.1 mm × 50 mm) and Waters BEH C18 guard column (1.7 µm, 2.1 mm × 5 mm). The FIA extract was analyzed in positive mode to capture acylcarnitines, glycerophospholipids and sphingolipids, while hexoses were monitored in a subsequent run in negative mode. Data acquisition was made with the use of MassLynx 4.1 (Waters), TargetLynx XS 4.1 (Waters), and MetIDQ Oxygen-DB110-3005 (Biocrates). A list of metabolites covered by the AbsoluteIDQ® p180 kit and analytical methods for their analysis are presented in this Biocrates file[12].

Data analysis

Univariate tests were carried out using STATISTICA software version 13.1 (Tibco Software/Statsoft). Values below the limit of quantification were replaced with the 0.5*LOQ value for each variable. Normality of distribution and homogeneity of variation were verified with the Shapiro–Wilk and Levene's tests, respectively. A parametric Student's t-test was used for variables with normal distribution, although other variables were tested using a nonparametric Mann–Whitney test. The false discovery rate (FDR) based on the Benjamin–Hochberg procedure was applied for the tested variables. All univariate statistical tests were calculated at a significance level of $\alpha = 0.05$. Dispersion of variables was represented by the coefficient of variation (CV) for normally distributed data or by the coefficient of quartile variation (QCV). Assessment of classifier performance was characterized by receiver operating characteristic (ROC) curve.

Chemometric analysis was performed in PLS-Toolbox version 7.5 (Eigenvector Research, Inc.: Manson, IA, USA, 2020) and MATLAB software (v R2022b, Mathworks Inc. Natick, MA, USA, 2022). Discriminant analysis was performed using the partial least squares method (PLS-DA) or the orthogonal variant (OPLS-DA). Data were preprocessed by the log10 function and standardization. Cross-validation was made using the Venetian blinds method. The prediction effectiveness of the PLS-DA model is presented by a ROC. Variable selection was made with the procedure based on VIP (Variable Importance in Projection) or Selectivity Ratio values. The quality of the PLS-DA and OPLS-DA models was evaluated by the Root Mean Square Error of Calibration (RMSEC) or cross-validation (RMSECV) and a permutation test (100 permutations). The self-predicted and cross-validated residuals of each permutation were compared to the original residuals using the Wilcoxon test, Sign test, and Randomized t-test.

For two PLS-DA models based on samples collected in the luteal phase of the menstrual cycle (referred to as model 3 and model 7 in the Results section), we also attempted to determine the possibility of integrating knowledge from the metabolome and proteome levels. We used a simple approach to build a classification model based on the sets of metabolites identified by the chemometric analysis in combination with autoantibodies selected using protein microarrays in a previously conducted study[8]. First, we restricted the metabolomic and proteomic datasets to luteal phase samples common for both studies. Next, we trained separate classifiers for the two types of biological samples: one based on metabolites indicated by the chemometric analysis (20 for peritoneal fluid and 26 for plasma, see Supplementary Table S2 online) and one taking into account 30 autoantibodies presented in the microarray-based study (see Supplementary Table S3 online). Finally, multi-omics models were built on datasets created after merging the lists of metabolites and antibodies (in total, 50 features for peritoneal fluid and 56 for plasma). All the resulting PLS-DA classifiers were evaluated by repeated stratified threefold cross-validation and compared in terms of accuracy (ACC), sensitivity (SE), specificity (SP), F1-score and area under the ROC curve (AUROC). Additionally, an ensemble classifier (PLS-DA and logistic regression combined by soft-voting) with automatic feature selection was created to verify the potential to improve classification results further by a more advanced machine-learning approach. Data processing for the integrative metabolomic and proteomic analysis was performed using MStat (available at https://proteom.ibb.waw.pl/mstat) running in the MATLAB environment. Classifier training and evaluation procedures were implemented in Python with the scikit-learn package.

Results

Clinical characteristics of women are presented in Table 1. We did not observe the differences in terms of age, BMI, phase of the menstrual cycle, diagnosed infertility, and pelvic pain syndrome, between the groups of women diagnosed with endometriosis and the control group.

Statistically significant metabolites are presented together with descriptive statistics and area under ROC curve (AUROC) values in Table 2 and Fig. 1. Univariate analysis showed significant differences in phosphatidylcholine (PC ae C30:2) level in plasma (with FDR adjusted p-value of 1.19E-4) and 5 metabolites in peritoneal fluid: propenoylcarnitine (C3:1); lysophosphatidylcholine (lysoPC a C24:0); phosphatidylcholines

Variable	Peritoneal fluid			Plasma		
	Endometriosis (n = 53)	Control (n = 34)	*p*-value	Endometriosis (n = 73)	Control (n = 35)	*p*-value
Age (years)	31.4 ± 5.3	31.1 ± 6.1	0.811	31.7 ± 5.1	31.2 ± 6.1	0.647
BMI (kg/m^2)	22.1 ± 3.3	22.9 ± 3.5	0.423	22.1 ± 3.4	23.2 ± 3.9	0.191
Proliferative phase of the menstrual cycle, n (%)	33 (62.3%)	24 (70.6%)	0.425	50 (68.5%)	25 (71.4%)	0.756
Luteal phase of the menstrual cycle, n (%)	20 (37.7%)	10 (29.4%)		23 (31.5%)	10 (28.6%)	
rAFS Stage I, n (%)	14 (26.4%)	-	-	22 (30.1%)	-	-
rAFS Stage II, n (%)	8 (15.1%)	-	-	10 (13.7%)	-	-
rAFS Stage III, n (%)	25 (47.2%)	-	-	31 (42.5%)	-	-
rAFS Stage IV, n (%)	6 (11.3%)	-	-	10 (13.7%)	-	-
Infertility, n (%)	31 (58.5%)	19 (55.9%)	0.730	40 (54.8%)	20 (57.1%)	1.000
Primary infertility, n (%)	25 (80.6%)	9 (47.4%)	0.014	33 (82.5%)	10 (50.0%)	0.009
Secondary infertility, n (%)	6 (19.4%)	10 (52.6%)		7 (17.5%)	10 (50.0%)	
Endometrial cyst, n (%)	30 (56.6%)	-	-	38 (52.1%)	-	-
Non-endometrial cyst, n (%)	3 (5.7%)	12 (35.3%)	0.0004	4 (5.5%)	12 (34.3%)	0.0001
Pelvic pain syndrome, n (%)	4 (7.5%)	7 (20.6%)	0.074	6 (8.2%)	7 (20.0%)	0.082

Table 1. Clinical characteristics of women. Data of age and Body Mass Index (BMI) are mean ± Standard Deviation (SD). rAFS—revised American Fertility Society classification.

Material	Metabolite	Fold change (E/C)	CV or QCV (%)		FDR adjusted p-value	AUROC
			E	C		
Peritoneal fluid	Propenoylcarnitine (C3:1)	1.11[a]	13.30	12.28	1.95E-2	0.714
	lysoPC a C24:0	1.09[b]	7.47	9.47	1.95E-2	0.723
	PC ae C30: 2	1.08[b]	8.53	9.34	1.95E-2	0.725
	PC ae C42:5	1.08[a]	8.67	9.73	1.95E-2	0.710
	Nonaylcarnitine (C9)	1.07[b]	7.09	8.47	1.95E-2	0.699
Plasma	PC ae C30:2	1.14[b]	8.39	12.73	1.19E-4	0.768

Table 2. Statistically significant metabolites based on univariate analysis. Fold change calculated based on [a]mean, [b]median.; CV – coefficient of variation; QCV – quartile coefficient of variation; AUROC – area under ROC curve (Receiver Operating Characteristic); E – endometriosis; C – controls; PC – phosphatidylcholine; lysoPC—lysophosphatidylcholine.

(PC ae C30: 2 and PC ae C42:5) and nonaylcarnitine (C9) with FDR adjusted p-value of 1.95E-2. For those metabolites, fold change values and area under the curve were calculated. In plasma, phosphatidylcholine (PC ae C30:2) concentration was elevated in the endometriosis group. Whereas in peritoneal fluid, an increase in concentrations of propenoylcarnitine (C3:1), lysophosphatidylcholine (lysoPC a C24:0), phosphatidylcholines (PC ae C30:2 and PC ae C42:5) and nonaylcarnitine (C9) were identified. The highest change was observed for phosphatidylcholine (PC ae C30: 2) in plasma samples with fold change 1.14 and for propenoylcarnitine (C3:1) in peritoneal fluid with fold change 1.11. All these metabolites, except nonaylcarnitine (C9) in peritoneal fluid, showed AUROC values above 0.700.

Table 3 presents the results of chemometric analysis employed to evaluate the differentiation between the selected groups of samples. The primary metrics for assessing the discriminatory potential of PLS-DA models include the classification error. This metric is calculated as the average of the false positive rate and false negative rate for a class, using the formula: 1—(sensitivity + specificity)/2. For peritoneal fluid, PLS-DA models 2 and 3 exhibit the lowest classification errors for calibration and cross-validation, whereas for plasma, models 6 and 7 demonstrate the lowest values. Models 2 and 6 differentiate women with endometriosis in stages III and IV according to the rAFS classification[10] from the control group. In turn, models 3 and 7 distinguish women with endometriosis (all stages) and the control group among samples collected during the luteal phase of the menstrual cycle. In both analyzed biological materials (peritoneal fluid and plasma), a comparison of the groups of samples obtained in the luteal phase appears to have better discriminatory potential based on classification error. The variables that influenced the positions of observations in the OPLS-DA model 3 (peritoneal fluid samples) are presented in Fig. 2 A and for model 7 (plasma samples) in Fig. 2 B. The list of metabolites comprising the above models is presented in Supplementary Table S2 (available online).

The results presented in Supplementary Table S4 (available online) indicate that the classification performance obtained on the joined metabolomic and proteomic feature sets exceeds that achievable for separate proteomics and metabolomics assays (in terms of ACC, SE, SP, AUROC and F1-score), especially for plasma. The obtained sensitivity and specificity parameters were 0.98 and 0.86 for plasma and 0.92 and 0.82 for peritoneal fluid,

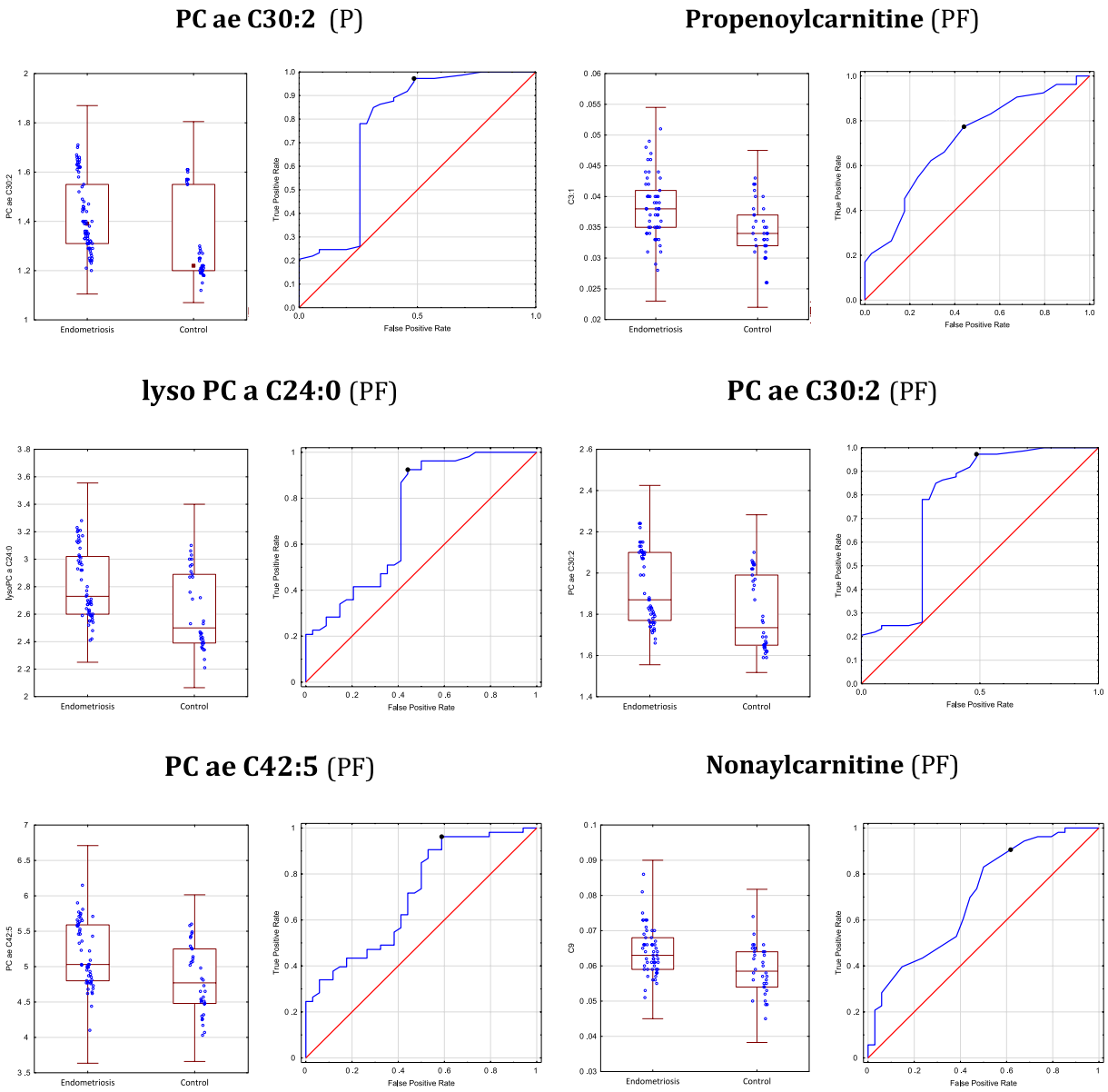

Fig. 1. Boxplots and receiver operating characteristic curves (ROC) for statistically important metabolites.

respectively. Moreover, further improvement of the results is possible by applying a more advanced machine-learning classification model (see Supplementary Table S5 online).

Discussion

We performed a targeted metabolomic study to identify specific metabolites in plasma and peritoneal fluid samples in women with endometriosis. The analyses conducted also allowed us to quantitatively assess the observed compounds and determine differences between women suffering from endometriosis and the control group. Our study based on LC–MS/MS and FIA-MS/MS analysis, showed significant differences in phosphatidylcholine (PC ae C30:2) observed in plasma and peritoneal fluid, as well as propenoylcarnitine (C3:1), lysophosphatidylcholine (lysoPC a C24:0), phosphatidylcholines (PC ae C30: 2 and PC ae C42:5) and nonaylcarnitine (C9) observed in peritoneal fluid, between endometriosis group and control group. All of the above compounds are lipids (glycerophospholipids and acylcarnitines). Phosphatidylcholine (PC ae C30:2) was the only metabolite increased in both plasma and peritoneal fluid samples. Simultaneously, the highest AUROC values were observed for this compound in both biological materials.

An altered lipid profile in endometriosis has been previously observed[13–17]. These observations were based on the analysis of various biological materials, such as eutopic endometrium, peritoneal fluid, serum and follicular

Model	Material	Binary classification	LV	RMSEC/RMSECV	Class. Error Cal/CV	R^2 Cal/CV	Permutation test*
1	Peritoneal fluid	E vs C	7	0.69	0.07/0.14	0.742/0.506	passed
2		E(III-IV) vs C	7	0.54	0.00/0.09	0.857/0.548	passed
3		EL vs CL	5	0.52	0.00/0.03	0.899/0.634	passed
4		EP vs CP	4	0.64	0.04/0.19	0.754/0.426	failed
5	Plasma	E vs C	4	0.73	0.06/0.20	0.624/0.332	passed
6		E(III-IV) vs C	9	0.46	0.01/0.09	0.893/0.552	passed
7		EL vs CL	4	0.59	0.00/0.00	0.914/0.754	passed
8		EP vs CP	4	0.68	0.05/0.20	0.652/0.320	passed

Table 3. Validation metrics of PLS-DA or OPLS-DA models. Quality measures were separately estimated for the training (calibration) set (Cal) and by cross-validation (CV). LV – number of latent variables; RMSEC/RMSECV – Root Mean Squared Error of Calibration and Cross-validation; E – endometriosis; E(III-IV) – endometriosis stage III and IV according to rAFS; C – controls; EL – endometriosis samples obtained in the luteal phase of the menstrual cycle; CL — controls samples obtained in the luteal phase of the menstrual cycle; EP — endometriosis samples obtained in the proliferative phase of the menstrual cycle; CP — controls samples obtained in the proliferative phase of the menstrual cycle. *passed means that for all three tests (Wilcoxon, Sign-test, Rand t-test) $p < 0.05$ at the 95% confidence level, whereas failed means that for at least one test $p > 0.05$; at the permutation test, 100 permutations were used.

fluid[18]. Vouk et al. identified elevated levels of five glycerophospholipids (PC ae C32:2; PC aeC34:2; PC ae C36:1; PC ae C34:0 and PC ae C30:0) and three sphingomyelins (SMOH C16:1; SMOH C22:2 and SM C16:1) in serum samples obtained from women with ovarian endometriosis[17]. In another study, Vouk et al. observed lower levels of four acylcarnitines (D,L-carnitine (C0); octenoyl-L-carnitine (C8:1); decanoyl-L-carnitine (C10:1) and fumaryl-L-carnitine/hexanoyl-L-carnitine (C4:1-DC or C6), four glycerophospholipids (PC aa C38:3; PC aa C38:4; PC aa C40:4 and PC aa C40:5) and two sphingomyelins (SM C16:1 and SM C18:1) in peritoneal fluid samples collected from women with endometriomas[15]. Our observations only partially confirm the above results. We observed an increased level of phosphatidylcholine (PC ae C30:2) in the plasma of women with endometriosis, which is also in line with the Dutta et al. study, where authors reported an increased total level of phosphatidylcholines in serum samples from endometriosis women[19]. However, we observed elevated levels of glycerophospholipids (lysoPC a C24:0; PC ae C30: 2; PC ae C42:5), acylcarnitine (propenoylcarnitine C3:1), and nonaylcarnitine (C9) in peritoneal fluid samples, not decreased like Vouk et al[15]. In fact, there is heterogeneity in the results obtained regarding the direction of changes in glycerophospholipids in different biological materials and studies[18].

Endometriosis is associated with developing an inflammatory process and oxidative stress and exhibits certain malignancy-like traits, including cell migration, proliferation, and invasion[20,21]. Phosphatidylcholine acts as a source of polyunsaturated fatty acids, which serve as precursors to eicosanoids like prostaglandins[14,22]. Hence, increased phosphatidylcholine levels may be connected to heightened prostaglandin production, acting as mediators of inflammation and promoting angiogenesis, increased cell proliferation, and invasion in endometriosis while inhibiting their programmed cell death[18]. Furthermore, phosphatidylcholine remains a marker of proliferation observed in gynecological cancer, which may be a common mechanism with endometriosis[23]. Acylcarnitines are linked to the incomplete oxidation of fatty acids and are also associated with oxidative stress[15,24,25]. Glycerophospholipids serve as lysophosphatidic acid precursors, a vital signaling compound. Its activity influences the proliferation and migration of endometrial cancer cells[26,27]. Therefore, endometriosis may have a similar mechanism[16,18,28].

Other metabolites have also been proposed as potential biomarkers for endometriosis. Jana et al. described a group of serum metabolites as discriminatory compounds between endometriosis and controls[29]. This group included biogenic amines (arginine, asparagine, isoleucine, leucine, lysine, and alanine), monosaccharides (glucose), succinic acid, creatine, pyruvate, adipic acid, citric acid, and 2-hydroxybutyrate. Murgia et al., in their study, indicated a potential role of hydroxybutyric acid, glutamine and tryptophan as compounds responsible for the differentiation between endometriosis and controls in serum samples[30]. Biogenic amines are often suggested as potential biomarkers for endometriosis, just as glycerophospholipids, acylcarnitines and sphingomyelins, described earlier. Metabolomic analyses of eutopic endometrial samples indicate the potential diagnostic role of arginine, tyrosine, leucine, asparagine, and lysine[31]. In peritoneal fluid samples, glycerophospholipids, sphingolipids and ceramides were significantly altered in the endometriosis group[32–34].

Our chemometric analyses using PLS-DA method identified a set of 20 metabolites present in peritoneal fluid and 26 compounds in plasma, which serve as potential diagnostic tools for endometriosis. These compounds belong to the group of glycerophospholipids, biogenic amines, acylcarnitines and sphingomyelins, which confirms observations of other authors, that these analyte classes can play a key role in noninvasive diagnostic test for endometriosis. Among the identified sets of metabolites, there are also those previously selected using univariate analysis (Model 7 for plasma includes PC ae C30: 2 and Model 3 for peritoneal fluid include C3:1, lysoPC a C24:0, PC ae C30: 2, PC ae C42:5 and C9). This observation demonstrates the consistency between both methods for selecting metabolites that differentiate the studied groups. Another important observation is that six metabolites (glycine, alpha-aminoadipic acid, PC ae C30:1, PC ae C30:2, PC ae C42:5 and lysoPC a C24:0)

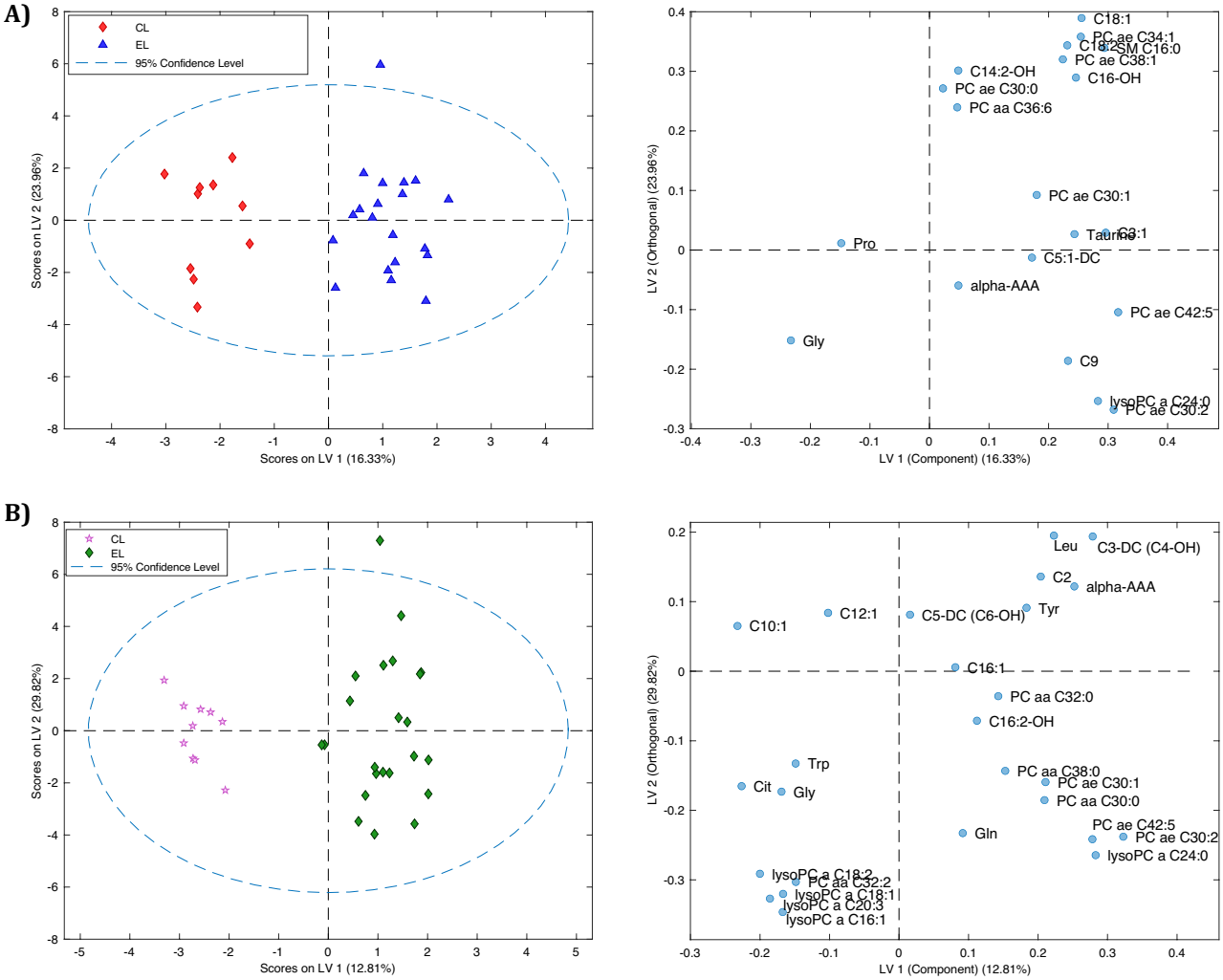

Fig. 2. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot (left) and corresponding loading plot (right) with quantified variables for (**A**) Model 3 (peritoneal fluid) and (**B**) Model 7 (plasma). CL — controls in luteal phase of the menstrual cycle; EL — endometriosis group in luteal phase of the menstrual cycle.

are included in both analyzed models, which may suggest similar changes occurring in both peritoneal fluid and plasma in the pathophysiology of endometriosis.

Metabolomic changes occur dynamically and can be influenced by various factors, including genetic, environmental, coexisting medical conditions and hormonal fluctuations during the menstrual cycle[35]. This presents an additional challenge in the search for endometriosis biomarkers. Hormonal changes that occur during the menstrual cycle can be a reason for the differences not only in the types of metabolites observed among studies by different authors but also in the fluctuating levels of these metabolites[35]. Therefore, basing a diagnostic tool on a specific phase of the menstrual cycle can enhance its discriminatory potential. This was observed in our results, where the most attractive diagnostic models (Model 3 and Model 7) were characterized by metabolites present in biological samples collected during the luteal phase of the menstrual cycle.

Combining metabolomic and proteomic analyses allowed us to obtain a better classification model than if it were based on separate metabolomic and proteomic analyses. Although based on a small sample size (a limited number of patients), this observation demonstrates the potential benefits of a multi-omics approach to endometriosis research.

The main limitation of this study is small sample size. To enhance the reliability of our findings, it is necessary to validate them in an independent and larger cohort of women. Nonetheless, the use of strict inclusion criteria ensured that the selected patients accurately represented the population under investigation.

Conclusion

In this study, we conducted a targeted metabolomic investigation to identify specific metabolites in plasma and peritoneal fluid samples from women with endometriosis. Our findings revealed significant alterations in lipid profiles, particularly glycerophospholipids and acylcarnitines, in individuals with endometriosis. Furthermore,

our analysis identified a set of metabolites that could be incorporated into a novel diagnostic tool for detecting endometriosis in both plasma and peritoneal fluid samples. We also recognized the potential advantages of using a multi-omics approach based on integrated metabolomic and proteomic analysis for advancing endometriosis research.

Data availability

This study is available at the NIH Common Fund's National Metabolomics Data Repository (NMDR) website, the Metabolomics Workbench, https://www.metabolomicsworkbench.org where it has been assigned Study ID ST003984. The data can be accessed directly via its Project https://doi.org/10.21228/M8D54V.

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Declarations

Competing interests
The authors declare no competing interests.

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