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Wide spectrum targeted metabolomics identifies potential ovarian cancer biomarkers

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ABSTRACT

Aims: Despite of almost a hundred years of research on cancer metabolism, the biological background of cancerogenesis and cancer-related reprogramming of metabolism remains not fully understood. In order to comprehensively and effectively diagnose and treat the deadliest diseases, the mechanisms underlying these diseases have to be discovered urgently. Among the gynecological malignancies, ovarian cancer is the most common cause of death. The aim of the study was to search for potential cancer-related differences in concentrations of metabolites and interactions between them in serum of women with ovarian cancer and benign ovarian tumor in comparison with healthy controls using targeted metabolomics. These metabolites might serve as biomarkers in the future.

Main methods: We used wide spectrum targeted metabolomics to evaluate serum concentrations of metabolites related to ovarian cancer and compared them against benign ovarian tumors and healthy controls. The measurements were performed using high performance liquid chromatography coupled with triple quadrupole tandem mass spectrometry technique in highly-selective multiple reaction monitoring mode.

Key findings: In this study we confirmed our previous findings about the role of histidine and citrulline in ovarian cancer as well as we indicated new lipid compounds (lysoPC a C16:1, PC aa C32:2, PC aa C34:4 and PC aa C 36:6) potentially involved in cancer metabolism.

Significances: We indicated interesting interactions between metabolites for further in-depth research which could potentially serve as clinically useful biomarkers in future. Moreover, the presented work attempts to visualize a possible 3D-network of relationships between the molecules found to be related to ovarian malignancy.

1. Introduction

Despite extensive research on ovarian cancer (OC) metabolism our knowledge is still very limited and the diagnostic methods are far from ideal. OC is the most frequent cause of death among gynecological cancers in women in Europe [1,2]. The main reason for the disease to have a generally poor prognosis is late diagnosis and this is mainly due to lack of early symptoms and lack of efficient early diagnostic methods. In consequence, > 70% of patients are diagnosed in advanced stages of the disease. The 5-year survival rates for stages III and IV of OC (according to the staging by the International Federation of Gynaecology and Obstetrics – FIGO) are approximately 30%, whereas for FIGO stage I it reaches 90% [3]. Therefore there is an urgent need for specific diagnostic methods in order to improve the treatment outcomes. Currently, there are no screening methods for OC. CA125 and HE4 are the most frequently used serum biomarkers, however their clinical application is rather in differential diagnosis of ovarian tumors and malignancy risk-assessment (e.g. as part of the ROMA test – Risk of Ovarian Malignancy Assessment) than in screening a healthy population. In addition, their sensitivity drops significantly in early-stage disease and in premenopausal women [4,5]. Imaging methods like transvaginal ultrasound or magnetic resonance have insufficient sensitivity and are too expensive to be used in screening.

The process of cancerogenesis and cancer-related metabolism remains not fully understood. Explaining biological mechanisms underlying OC is likely to give grounds for new diagnostic and/or treatment methods. Following our previous studies on OC biomarkers and its pathophysiology [5–9] we adopted yet another approach of searching

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for OC biomarkers. In the presented study we applied a technique of wide-spectrum targeted metabolomics to compare the differences in serum concentrations of 188 metabolites in patients with OC, benign ovarian tumors (BOT) and healthy controls (HC). The study suggests a few compounds which are promising targets for further evaluation as OC biomarkers. In addition, the study attempts to visualize a possible 3D-network of relationship between the molecules found to be related to OC.

2. Materials and methods

2.1. Study subjects

The protocol of the study was approved by the Bioethical Commission of the Poznan University of Medical Sciences, Poland (Consent No 165/16 and 80/17). The research was conducted in accordance with the Declaration of Helsinki. All study participants gave written informed consent prior to the sample collection. This study was performed using serum from 26 women with histologically confirmed diagnosis of ovarian cancer (OC), 25 women with benign ovarian tumor (BOT) and 25 age-matched control samples from patients who had no pathology of the ovaries (HC) (Table 1). The exclusion criteria were: presence of OC other than epithelial, presence of any other cancer currently or in anamnesis and any cancer treatment history (surgery, radiotherapy, chemotherapy, hormonal therapy) prior to the sample collection.

2.2. Blood sampling

The venous blood samples were collected into 7.5 mL S-Monovette (Sarstedt AG & Co., Nümbrecht, Germany) tubes with a clotting activator. Immediately after blood collection, samples were allowed to clot for 30 min in room temperature and centrifuged (15 min at 4000 rpm). After centrifugation the obtained serum was aliquoted, frozen and stored at −80 °C until analysis. Before loading on the plate the serum samples were thawed and centrifuged at 2800 ×*g* for 5 min.

2.3. Methodology

The targeted metabolomics assay was performed using AbsoluteIDQ p180 kit (Biocrates Life Sciences AG, Innsbruck, Austria). This methodology enables simultaneous quantification of 188 metabolites in 10 μL of serum, including acylcarnitines, amino acids, biogenic amines, hexoses, glycerophospholipids and sphingolipids. Sample preparation and measurements were done according to the manufacturer's specifications. In brief, 10 μL aliquots of serum were put onto the filter of the 96-well kit plate which already contained stable isotope labeled internal standards and dried under nitrogen flow. Then 50 μL of 5% phenylisothiocyanate as a derivatization agent was added and the samples were dried under nitrogen flow again. In the next step, the metabolite extraction with 300 μL of 5 mM ammonium acetate in methanol was performed. After 30 min of room-temperature shaking at 450 rpm, the extract was transferred via a filter plate to the capture plate using positive pressure nitrogen manifold. 200 μL of the final extract was transferred to a second 96-deepwell plate and after dilution with appropriate solvent both plates were covered by silicon mats preventing evaporation and placed into autosampler until LC-MS/MS analysis or FIA-MS/MS analysis.

The electrospray ionization liquid chromatography–triple quadrupole tandem mass spectrometry (LC-ESI-MS/MS) system comprising 1260 Infinity HPLC (Agilent Technologies, Santa Clara, CA, USA) coupled with 4000 QTRAP mass spectrometer (SCIEX, Framingham, MA, USA) was applied for metabolite separation and detection. Amino acids and biogenic amines were separated on ZORBAX Eclipse XDB-C18 (3.0 × 100 mm, 3.5 μm) column (Agilent Technologies, Santa Clara, CA, USA), with a pre-column (C18, 4.0 × 3.0 mm) SecurityGuard (Phenomenex, Torrance, CA, USA) followed by MS/MS quantification. The analyses of lipids, acylcarnitines and hexoses were performed using flow injection analysis (FIA-MS/MS method).

Metabolomic data was acquired using the Analyst software version 1.5.2 (Sciex, Framingham, MA, USA) and analyzed with Biocrates MetIDQ software version Boron (Biocrates Life Sciences AG, Innsbruck, Austria). Internal standards and quality control samples provided by Biocrates in p180 Kit were used to evaluate the overall quality of the assay and the calculation of the metabolites' concentrations.

Table 1
Study group characteristics.

Variables	Ovarian cancer	Benign ovarian tumors	Healthy controls
No. of subjects	26	25	25
%	34.2%	32.9%	32.9%
Age [years]			
Median	58.5	50.0	59.0
Range	32–72	26–72	28–69
Weight [kg]			
Median	67.6	69.6	67.0
Range	52.0–98.0	55.5–118.0	56.0–97.5
Body mass index [kg/m^2]			
Median	25.63	25.26	25.63
Range	20.69–36.89	21.16–39.89	20.94–40.06
Menopausal status			
Post-			
No	20	12	18
%	76.9%	48.0%	72.0%
Pre-			
No	6	13	7
%	23.1%	52.0%	28.0%
FIGO stage		n/a	n/a
I	6		
II	1		
III	18		
IV	1		
CA125			n/a
Median	496.4	14.58	
Range	46.7–5000.0	3.75–97.75	
HE4			
Median	321.05	50.59	
Range	55.35–1500.0	32.81–120.8	
ROMA			n/a
Median	91.0	8.5	
Range	11.3–99.7	2.8–44.1	
Histological type		n/a	n/a
Serous adenocarcinoma	15		
Mucinous adenocarcinoma	1		
Endometrioid adenocarcinoma	3		
Undifferentiated	7		

n/a - not applicable.
FIGO - staging according to classification by International Federation of Gynaecology and Obstetrics.
CA125 - cancer antigen 125.
HE4 - human epididymis protein 4.
ROMA - Risk of Ovarian Malignancy Algorithm.

2.4. Data analysis

After collection, the data was verified for completeness and the missing data imputation procedure was applied. The variables with missing data exceeding 20% were excluded from further statistical testing. For the remaining analytes (missing data below 20%), the missing values were replaced by half of the limit of detection (LOD).

In order to identify the most relevant metabolites involved in ovarian cancerogenesis, the following statistical analyses were performed. The normality of distribution was evaluated based on Shapiro-Wilk test. One-way analysis of variance (ANOVA) was applied for

normally distributed data with equal variances confirmed by Levene's test. The Kruskal–Wallis test was performed for non-parametric variables. For both ANOVA and Kruskal-Wallis tests, NIR post-hoc tests were performed to reveal the significance of differences between groups (Table 1). To control the false-discovery rate, Benjamini and Hochberg procedure was applied (with the Benjamini-Hochberg FDR of 0.05) [10]. Finally, the univariate receiver operating characteristic (ROC) curves were plotted for the selected metabolites. Data auto-scaling was used before drawing a heatmap to make the individual features more comparable. All statistical tests were carried out using the STATISTICA software version 13 (StatSoft, Tulsa, OK, USA) and the Metaboanalyst 4.0 [11].

2.5. Pathway analysis and network of interactions

The most affected metabolic pathways were identified and drawn based on a comprehensive analysis of different databases and web platforms such as: LIPID MAPS Lipidomics Gateway [12], Human Metabolome Database [13], Metaboanalyst 4.0 [11], network visual analytics system OmicsNet [14]. All possible isomeric/isobaric lipids according to 'Annotation of potential isobaric and isomeric lipid species measured with the AbsoluteIDQ p180 Kit' [15] with available HMDB IDs were selected as metabolites entering the pathway analysis. The interaction networks were drawn using the network visual analytics system OmicsNet [14].

3. Results

A total of 76 serum samples were analyzed using wide spectrum targeted metabolomics approach. 41 out of 188 metabolites measured had missing data exceeding 20% and were excluded from further analyses. After missing value imputation procedure with 0.5× LOD for variables with 20% or less of the missing data, 147 metabolites from 5 different chemical groups: acylcarnitines, amino acids and biogenic amines, glycerophospholipids, sphingolipids and hexoses were further analyzed.

The performed univariate ANOVA and Kruskal-Wallis tests of all serum samples ($n = 76$), after Benjamini-Hochberg correction, showed that the level of 21 metabolites differed significantly between the compared groups of patients. Age, BMI and FIGO stage had no impact on the concentrations of the evaluated metabolites (data not shown). To reveal the between-group differentiation, the post-hoc NIR tests were performed. Table 2 shows no separation between BOT and HC. On the contrary, in the case of HC vs OC, as well as BOT vs OC comparisons, there was an evident between-group separation. Diagnostic utility of the selected metabolites was further evaluated by ROC curve analysis. The highest area under the ROC curve values were found between OC patients and HC group (4 metabolites had the AUC value above 0.8). Metabolites with AUC exceeding 0.7, should be considered adequate for further biomarker research [16]. These results strongly suggest that the wide spectrum metabolomics approach is helpful in distinguishing OC from both, BOT and HC and therefore could be potentially useful for OC screening. 17 out of the 21 differentiating metabolites were lipid compounds, the remaining 4 were small molecules: histidine, citrulline, kynurenine, and spermine (Table 2). It is worth emphasizing that the highest AUC values were obtained by the following lipid compounds: lysoPC a C16:1, PC aa C32:2, PC aa C34:4 and PC aa C36:6.

The multivariate analyses were performed to confirm the findings revealed by the univariate tests. Fig. 1 presents a heatmap showing the differentiation of OC patients from HC using the top 25 metabolites ranked automatically by Metaboanalyst based on the *t*-tests. The signatures of each group stand out clearly. What is interesting, these results confirm the important role of lipid compounds in samples grouping (21 out of 25 metabolites were lipids). What is important, histidine and citrulline were found by us earlier as potentially related to ovarian cancerogenesis [6]. The results of the current study are in line with our previous findings concerning the potential involvement of these two amino acids in OC growth and/or development.

The pathway analysis revealed 11 metabolic pathways potentially related to OC (Table 3). Four of them are directly involved in lipid metabolism (linoleic acid metabolism, alpha-linolenic acid metabolism, glycerophospholipid metabolism and arachidonic acid metabolism). The other four (beta-alanine metabolism, arginine and proline metabolism, histidine metabolism, tryptophan metabolism) are closely associated with amino acids metabolism. The graphical representation of the results of the pathway analysis is shown in Fig. 2, and the summary of these results is provided in Table 3. In order to provide a comprehensive visualization of the network of molecular interactions, a three-dimensional scheme showing the relationships between the selected metabolites and genes/proteins was constructed (Fig. 3). Fig. 4 illustrates an analogous network of connections with molecules possibly related to pathways engaged in ovarian cancer highlighted in blue (10 out of 11 pathways were matched and highlighted).

4. Discussion

Our recent paper concerning the role of amino acids in OC identified histidine and citrulline as potential new OC biomarkers [6]. To make a step forward in better understanding the biological background of OC, we analyzed a wide spectrum of metabolites using the targeted metabolomics approach. It is worth pointing out that we decided to quantitate a panel of compounds, in which some metabolites overlapped the analytes tested previously (e.g. histidine, citrulline). The study aimed at confirming our previous hypotheses about the role of histidine and citrulline in metabolic pathways related to OC development and at indicating new compounds that could be potentially valuable in ovarian cancer diagnosis with special emphasize on lipids. In the presented study we confirmed the findings from our previous paper [6], namely that the levels of citrulline and histidine are significantly decreased in the serum of OC patients as compared to BOT and HC. It might be due to the linkage of histidine metabolism via alanine, aspartate and glutamate metabolism and TCA cycle with de novo nucleotide synthesis [17] and maintaining the upregulated mitochondrial biosynthesis, crucial for cancer cells [18]. The work of Lyssiotis et al. [19] is in line with our findings regarding the role of citrulline via arginine and proline pathways, by enhancing nitric oxide (NO) synthesis and intensifying glycolysis process in the malignant-transformed cells. Similar results of significantly altered citrulline level in OC were presented by Zeleznik et al. [20].

Kynurenine, a metabolite of the amino acid L-tryptophan, was another metabolite that was significantly altered in OC patients. Similar results were presented in a study on plasma biomarkers for epithelial ovarian cancer [21]. The researchers found a correlation between increased kynurenine level and shorter overall survival of epithelial OC patients. Moreover, the same team previously reported a disturbed tryptophan and kynurenine metabolism in epithelial OC patients [22]. In the prospective study by Zhang et al. [23] on 38 plasma samples from patients with epithelial OC, kynurenine was identified as one of the five potential biomarkers that predicted recurrence (the other four being tryptophan, bilirubin, lysoPC (14:0) and lysoPE (18:2)). The phenomenon of decreased tryptophan and increased kynurenine serum levels might be explained by disturbances in tryptophan metabolism (Figs. 2 and 4). The upregulated metabolism of tryptophan and the increase in kynurenine levels, through indoleamine 2, 3-dioxygenases (IDO1 and IDO2) and tryptophan 2, 3-dioxygenase (TDO) activity entails the immunosuppression of the function of the effector T and NK-cells through activation of T regulatory cell and myeloid-derived suppressor cells, as well as the promotion of neovascularization of solid tumors [24]. Thus, reversing immunosuppression by targeting IDO1 is one of the emerging strategies to enhance the immune response to OC and currently a 'hot topic' in anti-cancer therapy research. In our study we provided more evidence on the importance of the disturbances in tryptophan

Table 2
Univariate analyses of the metabolite concentrations.

Metabolites	Serum levels [mean ± SD]			Post hoc test *p*-values			AUC (CI)		
	HC	BOT	OC	HC vs BOT	HC vs OC	BOT vs OC	HC vs BOT	HC vs OC	BOT vs OC
Citrulline[a]	25.017 ± 7.401	25.544 ± 5.796	18.988 ± 6.082	NS	0,013438	0,003092	0.5448 (0.392–0.706)	0.74 (0,589-0,858)	0.75846 (0,612–0884)
Kynurenine[a]	1.699 ± 0.495	1.582 ± 0.47	2.601 ± 1.414	NS	0.041077	0.006356	0.5616 (0.410–0.726)	0.718 (0.572–0.847)	0.743 (0.583–0.875)
Spermine[a]	0.102 ± 0.008	0.101 ± 0.006	0.112 ± 0.015	NS	0.015810	0.011908	0.5088 (0.333–0.649)	0.733 (0.592–0.852)	0.735 (0.607–0.867)
lysoPC a C16:1[a]	3.016 ± 0.662	2.73 ± 1.129	2.034 ± 0.884	NS	0.000047	0.034963	0.672 (0.513–0.817)	**0.828 (0.708–0.922)**	0.728 (0.595–0.862)
PC aa C32:2[a]	2.864 ± 0.972	2.512 ± 0.907	1.691 ± 0.803	NS	0.000077	0.002842	0.5536 (0.290–0.616)	**0.866 (0.748–0.952)**	0.747 (0.6–0.882)
PC aa C34:3[a]	13.623 ± 3.996	12.16 ± 3.591	9.529 ± 3.883	NS	0.000955	0.044154	0.6016 (0.44–0.746)	0.789 (0.649–0.91)	0.708 (0.548–0.828)
PC aa C34:4[a]	1.615 ± 0.519	1.414 ± 0.496	0.932 ± 0.404	NS	0.000021	0.001268	0.5824 (0.432–0.736)	**0.866 (0.741–0.945)**	0.795 (0.648–0.905)
PC aa C36:0[a]	4.914 ± 0.914	4.881 ± 1.262	4.068 ± 0.888	NS	0.010451	0.013782	0.516 (0.343–0664)	0.762 (0.604–0.875)	0.715 (0.555–0.848)
PC aa C36:2[a]	166.12 ± 35.129	164.64 ± 34.251	134.265 ± 32.199	NS	0.012948	0.005622	0.5248 (0.352–0.681)	0.737 (0.596–0.856)	0.757 (0.601–0.869)
PC aa C36:6[a]	0.97 ± 0.357	0.927 ± 0.413	0.609 ± 0.232	NS	0.000776	0.003632	0.5152 (0.32–0.656)	**0.816 (0.696–0.918)**	0.745 (0.592–0.879)
PC aa C38:1[a]	0.577 ± 0.257	0.787 ± 0.467	0.41 ± 0.287	NS	NS	0.002040	0.6072 (0.432–0.769)	0.706 (0.541–0.838)	0.766 (0.622–0.887)
PC ae C34:2[a]	9.148 ± 2.438	9.267 ± 2.573	6.974 ± 2.009	NS	0.009592	0.003557	0.532 (0.378–0.696)	0.757 (0.601–0.865)	0.761 (0.626–0.886)
PC ae C36:3[a]	7.558 ± 1.755	7.514 ± 1.938	5.6687 ± 1.539	NS	0.001764	0.001785	0.5152 (0.348–0.670)	0.795 (0.668–0.897)	0.768 (0.636–0.872)
PC ae C38:0[a]	2.022 ± 0.497	2.02 ± 0.652	1.495 ± 0.449	NS	0.002146	0.005277	0.5056 (0.332–0.659)	0.798 (0.656–0.898)	0.738 (0.601–0.8740)
PC ae C38:2[a]	2.044 ± 0.489	1.961 ± 0.501	1.614 ± 0.411	NS	0.004276	0.028413	0.5432 (0.379–0.691)	0.765 (0.619–0.893)	0.706 (0.572–0.843)
PC ae C40:1[a]	1.049 ± 0.239	1.04 ± 0.248	0.813 ± 0.241	NS	0.001818	0.002618	0.506 (0.347–0.674)	0.789 (0.66–0.904)	0.768 (0.611–0.878)
PC ae C42:1[a]	0.276 ± 0.052	0.275 ± 0.062	0.231 ± 0.05	NS	0.005114	0.010476	0.5136 (0.374–0.694)	0.765 (0.617–0.875)	0.752 (0.591–0.877)
lysoPC a C18:0[a]	26.07 ± 7.80	26.23 ± 12.73	19.175 ± 10.07	NS	0.008773	NS	0.5552 (0.411–0.713)	0.74 (0.599–0.873)	0.696 (0.535–0.844)
PC aa C42:6[a]	0.434 ± 0.116	0.445 ± 0.149	0.360 ± 0.093	NS	0.021591	0.023323	0.5032 (0.35–0.667)	0.728 (0.567–0.855)	0.713 (0.571–0.848)
His[b]	84.82 ± 16,714	82.352 ± 12.7	69.396 ± 19,113	NS	0.001277	0.006266	0.5136 (0.326–0.651)	0.72462 (0.589–0.842)	0.721 (0.572–0.843)
PC ae C36:4[b]	15.052 ± 3.429	15.133 ± 3.843	12.035 ± 3.088	NS	0.002670	0.002076	0.524 (0.344–0.699)	0.751 (0.611–0.874)	0.747 (0.6–0.881)

NS - not significant, SD - standard deviation, CI - confidence interval, AUC > 0.8 in bold.
[a] Kruskal Wallis.
[b] ANOVA.

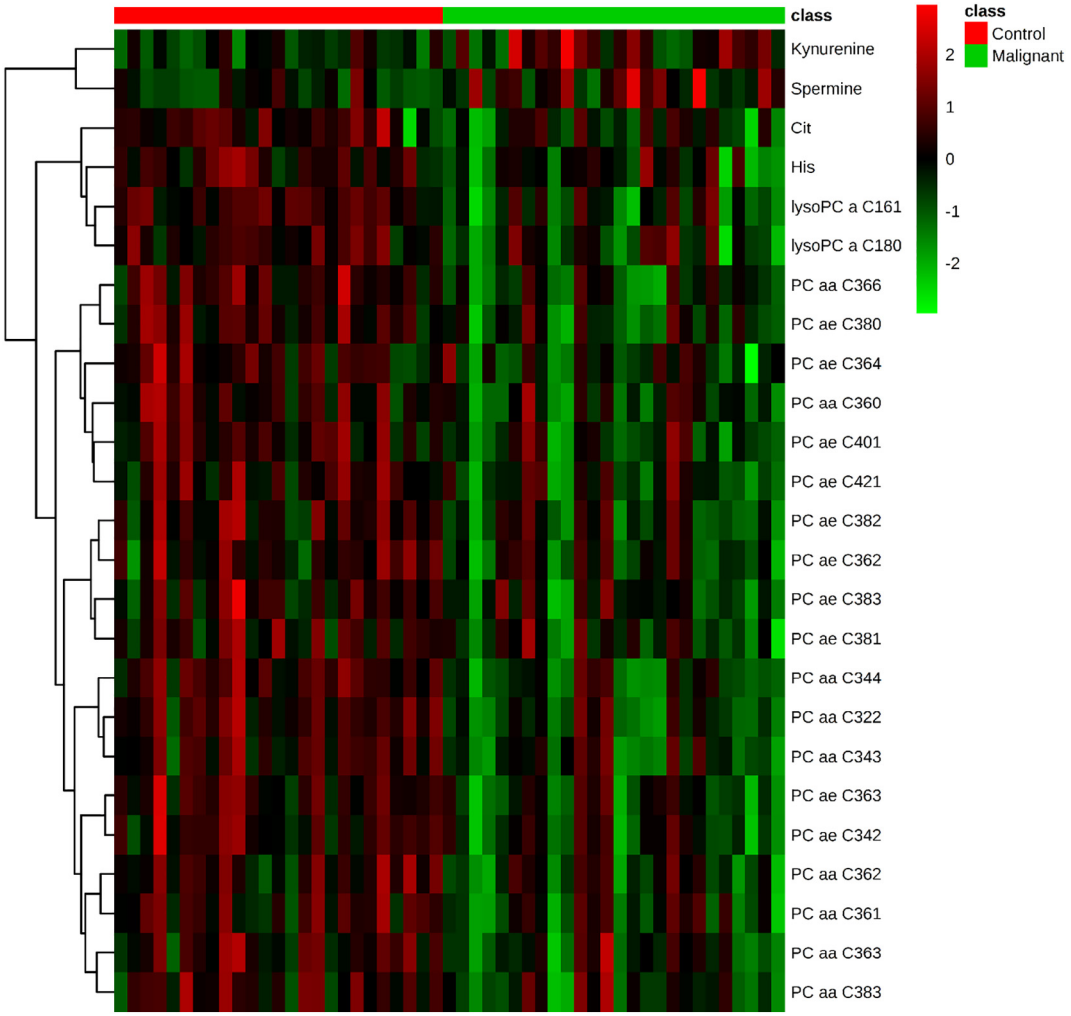

Fig. 1. Heat map combined with hierarchical clustering (Euclidean distance and Ward linkage) of 25 metabolites showing separation between OC and HC. Higher serum concentrations of metabolites were marked in red. Decreased metabolites levels were presented in green. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Metabolic pathways potentially related to ovarian cancer.

No	Pathway name	Match status	*p*	-LOG(*p*)	Holm *p*	FDR	Impact
1	beta-Alanine metabolism	2/28	0.0019023	6.2647	0.15219	0.14735	0.0
2	Glycerophospholipid metabolism	2/39	0.0036837	5.6038	0.29101	0.14735	0.1037
3	Arginine and proline metabolism	2/77	0.01394	4.273	1.0	0.37173	0.01997
4	Linoleic acid metabolism	1/15	0.036851	3.3009	1.0	0.73702	0.0
5	alpha-Linolenic acid metabolism	1/29	0.070217	2.6562	1.0	1.0	0.0
6	Glutathione metabolism	1/38	0.091154	2.3952	1.0	1.0	0.03456
7	Nitrogen metabolism	1/39	0.093456	2.3703	1.0	1.0	0.0
8	Histidine metabolism	1/44	0.10489	2.2548	1.0	1.0	0.13988
9	Arachidonic acid metabolism	1/62	0.14507	1.9305	1.0	1.0	0.0
10	Aminoacyl-tRNA biosynthesis	1/75	0.17315	1.7536	1.0	1.0	0.0
11	Tryptophan metabolism	1/79	0.18163	1.7058	1.0	1.0	0.03496

metabolism in cancer development and thus reinforced the foundations for research on IDO1 inhibitors and other immune-oncology drugs.

As we presented before [6] circulating citrulline might be involved, among other metabolites, in arginine biosynthesis through the urea cycle. Arginine, in turn, is the main substrate for polyamine biosynthesis. Spermine alongside spermidine and putrescine represent essential cellular polyamines. They are crucial for DNA and RNA metabolism, for maintaining the cellular membrane potential and for controlling gene expression and thus they contribute to growth and differentiation of cells [25]. The polyamines are thought to contribute to the creation of a permissive microenvironment via the influence on immunosurveillance processes [26]. We have presented spermine as a metabolite that significantly differed between OC and HC as well as between OC and BOT. The increased serum level of this polyamine in OC patients could be a consequence of accumulation of spermine in the fast-growing tumor tissue and/or in its microenvironment. On the molecular level the polyamines could be also involved in enhancing cellular invasion/metastasis. Under hypoxic conditions cancer cells demonstrate enhanced polyamines uptake from surrounding tissues. This phenomenon is accompanied by a deregulation of the homeostasis of the adhesion

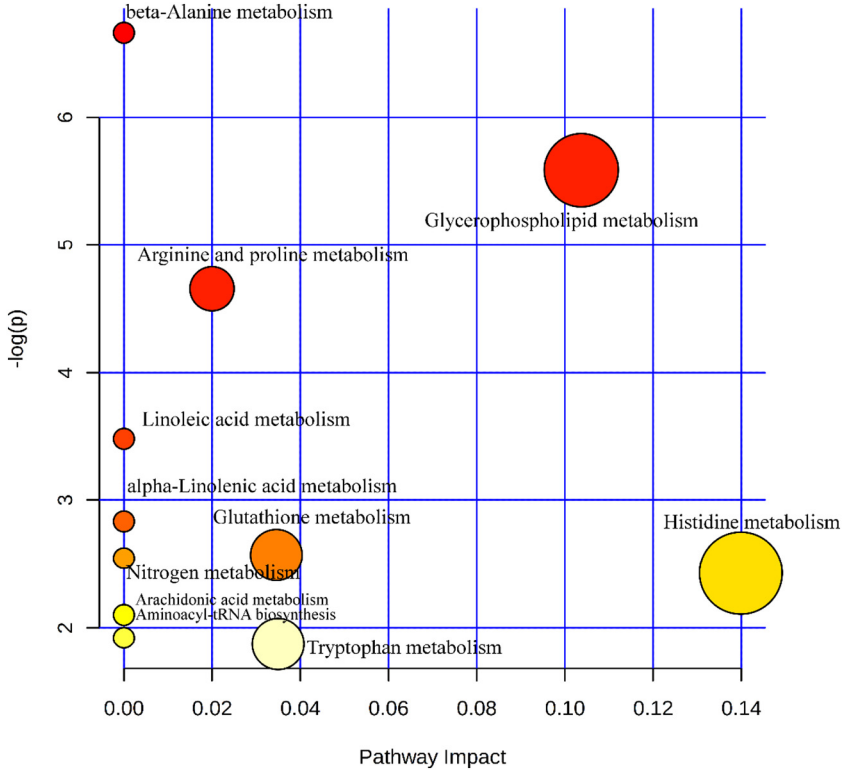

Fig. 2. Graphical representation of results of pathway analysis based on metabolites significantly altered between the groups (Kruskal-Wallis and ANOVA tests).

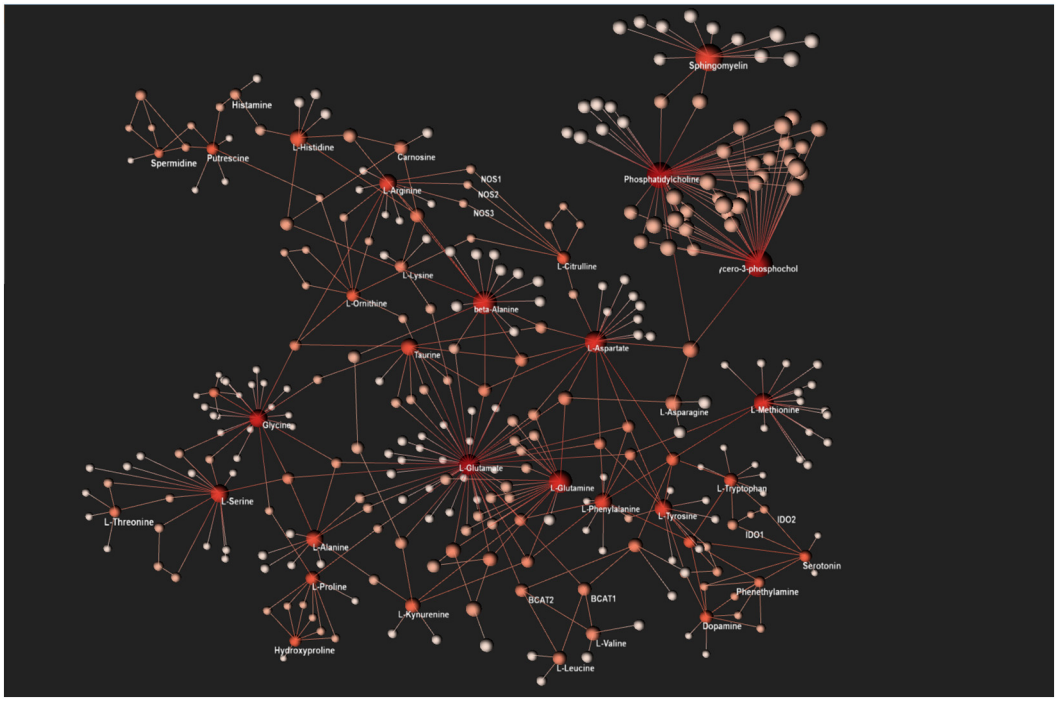

Fig. 3. The visualization of the network of molecular interactions created based on list of metabolites quantified using the applied methodology.

molecules (CD44), thus promoting cancer metastasis [27]. A vast majority of the significantly altered compounds identified in our research belongs to glycerophospholipids (Table 2), or more specifically – to phosphatidylcholines (PC). The aberrant lipid metabolism of this subgroup of glycerophospholipids seems to be directly engaged in cell apoptosis/necrosis, cell proliferation as well as cellular signaling. The proposal of integration of the metabolic pathways potentially related to the role of glycerophospholipids metabolism in cancerogenesis is presented on Fig. 5. The PC and phosphatidylethanolamine (PE) are synthesized de novo from choline and ethanolamine, respectively. These multistep reactions constitute the two branches of the Kennedy Pathway [28,29]. Furthermore, PC can be transformed into lysophosphatidylcholines (LPC) by phospholipases A1 and A2 (plA1 and plA2) [30]. Then LPC can be converted into lysophosphatidic acid (LPA) by

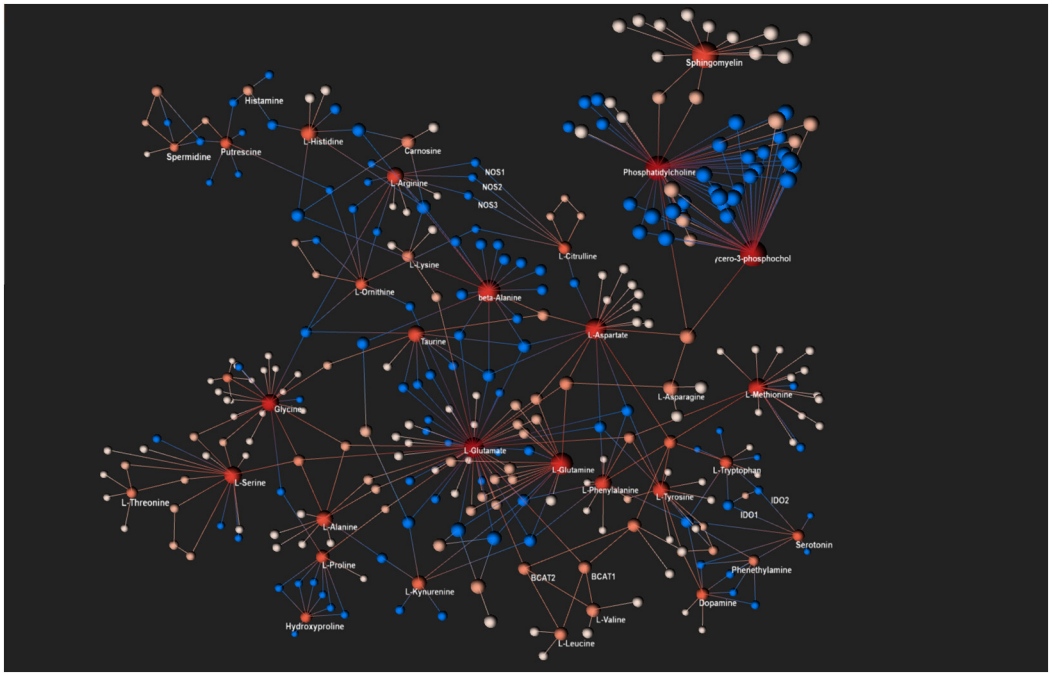

Fig. 4. The visualization of the network of interactions with highlighted molecules possibly related to pathways engaged in ovarian cancer. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

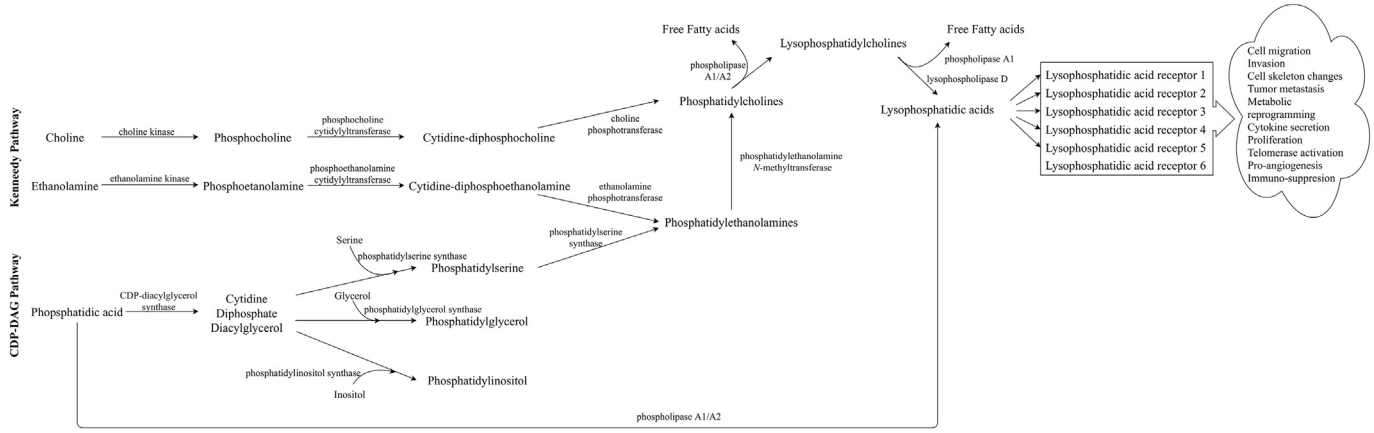

Fig. 5. The metabolic pathways presenting the potential role of glycerophospholipids metabolism on cancerogenesis.

lysophospholipase D enzyme (lpD) [31]. LPA in body fluids can consist of a mixture of various fatty acids (saturated and unsaturated), and thus it might play various roles in the body by activating different LPA receptors (LPAR) [32,33]. In our study, we observed decreased serum levels of the group of PC (Table 2). Fig. 1 shows group clustering of these metabolites showing correlation between OC and decreased concentration of PC. We speculate that it may be the result of an increased PC catabolism. We suppose that PC are converted into LPC followed by extensive transformation into LPA. The decreased levels of some LPC might be the result of overexpression of lpD, which, as mentioned before, is the enzyme responsible for conversion of LPC to LPA. In turn, LPA has been recently shown to be a pro-tumorigenic factor, involved in cell proliferation, differentiation, adhesion migration and invasion as well as promoting tumor cell survival and proliferation [33,34].

It should be noted that due to the specificity of the lipid particle composition, it is possible that over 1000 lipid species exist in a single eukaryotic cell. Moreover, they are geographically restricted within the cell [35]. Therefore, to uncover the molecular background of the lipids involvement into e.g. cancerogenesis, further lipidomic research with special emphasis on cell, tissue and biofluid compartmentation of lipids are needed.

Considering ROC curve analysis results (AUC $>$ 0.8) we propose 4 new lipid compounds for further investigation: lysoPC a C16:1, PC aa C32:2, PC aa C34:4 and PC aa C36:6 (Fig. 6). The same metabolites were previously found to correlate with the overall survival of high grade serous OC patients [36]. Moreover, Wang et al. [37] analyzed the effects of the knockout of branch chain amino-acid transaminase 1 (BCAT1) gene, which was previously found to be significantly hypomethylated in OC tissue [38]. The study revealed that silencing BCAT1 gene was related to inhibition of cell proliferation, migration and invasion and has indicated glycerophospholipids alterations in BCAT1 knockdown clones, which partially correspond with our results. For example, decreased levels of PC aa C32:2; PC aa 34:4 and PC aa 36:6 – metabolites selected by us, as the ones which most significantly differentiated OC patients from HC in ROC curve analysis, were observed. Taking into account such convergence with the cited studies, we believe that those lipid compounds are strongly correlated with OC-induced metabolic alterations and potentially involved in OC growth and progression. This finding could be a step forward to better understand the

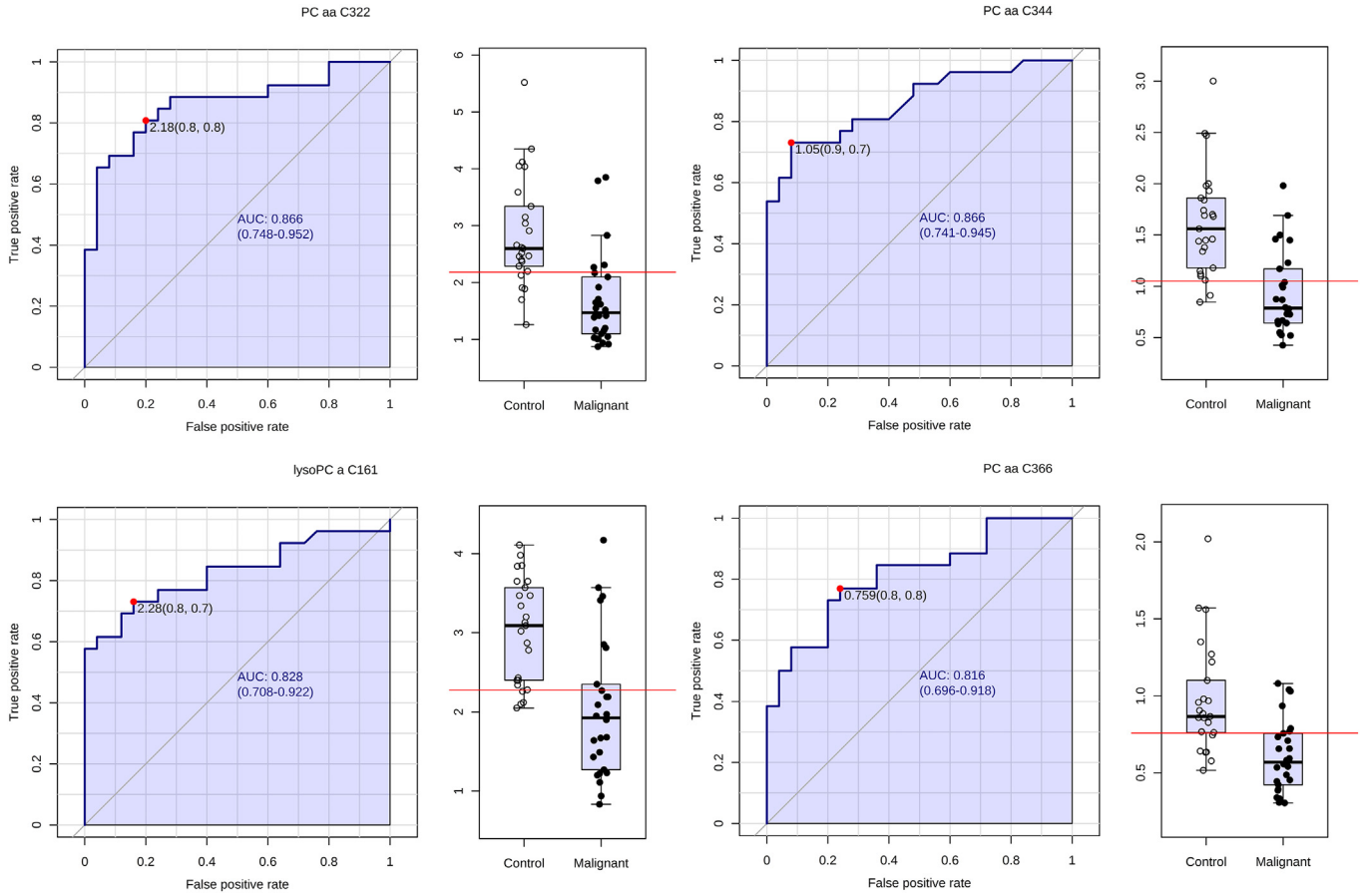

Fig. 6. Receiver operating characteristic curves with corresponding box and whiskers plots for compounds which yielded AUC above 0.8 for ovarian cancer vs healthy controls comparison.

molecular background of OC initiation and progression.

So far, acylcarnitines have been extensively investigated as potential indicators of metabolic diseases or different cancers: lung [39], prostate [40], or kidney [41]. One of the important findings of this research is that among the 40 quantified acylcarnitines none were significantly differentiating the analyzed groups (after Benjamini-Hochberg correction) and thus this set of acylcarnitines could practically be disqualified from further research as OC biomarkers. The aforementioned study on BCAT1-silenced ovarian cell lines [37] shows quite similar results – among the wide spectrum of evaluated acylcarnitines only 1 was substantially decreased in OC. Thus, we suggest that the development of ovarian cancer does not have a significant impact on the oxidative catabolism of fatty acids which is reflected in alterations of serum levels of acylcarnitines.

Bearing in mind the limitations of different kinds of multiple comparison corrections, we decided to apply the Benjamini-Hochberg procedure. Assuming that the quantified metabolites might be partially dependent on one another and to avoid type II errors (false negatives), a more sensitive and less strict correction was applied compared to the Bonferroni's one [42]. Nevertheless, we suspected that some metabolites with a potential role in OC growth/development could have been lost by applying the multiple comparison correction. To reveal these possibly hidden metabolites, we created the network of molecular interactions presented on Figs. 3 and 4. Based on that, it could be pointed out that glutamine-glutamate axis has the crucial role in OC growth/development. This phenomenon might be explained by the increased glutamine turnover during cancerogenesis and was described by us earlier [6]. This seems to be confirmed by another study where cancer cells exhibited increased glutamine dependency during growth and proliferation. This dependency is based on the conversion of glutamine to glutamate which is included in TCA cycle to produce energy for cancer cells [43]. Presented by us (Fig. 4) central role of glutamine-glutamate axis in cancer could be also explained by its connections with serine and proline, which were previously linked with regulation of cancer cell growth [44,45].

To the best of our knowledge, this is the first study that demonstrates such a comprehensive network of molecular interactions in OC on the basis of wide spectrum targeted metabolomics. The identified metabolic pathways might indicate new directions for further studies. We made an effort to explain the role of the main intermediates in cancer growth and development. Special attention to early stages of OC should be given in order to evaluate the possibilities of earlier OC detection The presented work should provide the basis for further in-depth, multi-omic research leading to novel translational opportunities in biomarkers searching, targeted anticancer therapy development and other medical interventions focused on cancer treatment.

5. Conclusions

In summary, we confirmed that citrulline and histidine play a role in ovarian cancerogenesis and should be considered as OC biomarkers. We found other metabolites that are most probably linked with some signaling and metabolic pathways of cancer growth and development. The serum levels of several metabolites (kynurenine, spermine) were altered in OC patients. We confirmed a dominant role of lipid alterations in OC. The following lipids: lysoPC a C16:1, PC aa C32:2, PC aa C34:4 and PC aa C36:6 were characterized by a satisfactory AUC > 0.8, setting new paths for further lipidomic studies, with high potential for clinical application. We suggest that the metabolism of acylcarnitines has little significance in OC development. In addition, the identified metabolic

pathways, with a presented for the first time - comprehensive network of molecular interactions in OC, might indicate new directions for further studies on bigger populations, which will be able to fully interpret these findings.

Ethical statement

The protocol of the study was approved by the Bioethical Commission of the Poznan University of Medical Sciences, Poland (Consent No 165/16 and 80/17). The research is in accordance with the Declaration of Helsinki. All study participants gave written informed consent prior the sample collection.

Author contributions

Szymon Plewa, Jan Matysiak and Zenon J. Kokot designed research; Agnieszka Horała and Ewa Nowak-Markwitz collected samples; Szymon Plewa and Paweł Dereziński performed research; Szymon Plewa and Paweł Dereziński collected data; Szymon Plewa, Paweł Dereziński and Jan Matysiak analyzed data; Szymon Plewa, Agnieszka Horała, Paweł Dereziński performed data interpretation; Szymon Plewa, Agnieszka Horała, Paweł Dereziński, Jan Matysiak, Ewa Nowak-Markwitz and Zenon J. Kokot wrote paper; All authors have read and approved the submitted manuscript.

Conflict of interest statement

All authors declare that there are no conflicts of interest.

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References

[1] I. San-Millán, G.A. Brooks, Reexamining cancer metabolism: lactate production for carcinogenesis could be the purpose and explanation of the Warburg Effect, Carcinogenesis 38 (2016), https://doi.org/10.1093/carcin/bgw127 bgw127.

[2] F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA Cancer J. Clin. 68 (2018) 394–424, https://doi.org/10.3322/caac.21492.

[3] E.S. Paik, Y.-Y. Lee, E.-J. Lee, C.H. Choi, T.-J. Kim, J.-W. Lee, D.-S. Bae, B.-G. Kim, Survival analysis of revised 2013 FIGO staging classification of epithelial ovarian cancer and comparison with previous FIGO staging classification, Obstet. Gynecol. Sci. 58 (2015) 124, https://doi.org/10.5468/ogs.2015.58.2.124.

[4] M.A. Karlsen, N. Sandhu, C. Høgdall, I.J. Christensen, L. Nedergaard, L. Lundvall, S.A. Engelholm, A.T. Pedersen, D. Hartwell, M. Lydolph, I.A. Laursen, E.V.S. Høgdall, Evaluation of HE4, CA125, risk of ovarian malignancy algorithm (ROMA) and risk of malignancy index (RMI) as diagnostic tools of epithelial ovarian cancer in patients with a pelvic mass, Gynecol. Oncol. 127 (2012) 379–383, https://doi.org/10.1016/j.ygyno.2012.07.106.

[5] A. Horała, A. Swiatly, J. Lorek, Z.J. Kokot, J. Matysiak, E. Nowak-Markwitz, Assessment of diagnostic utility of multivariate diagnostic models in differential diagnosis of ovarian tumors, Ginekol. Pol. 89 (2018) 568–572, https://doi.org/10.5603/GP.a2018.0097.

[6] S. Plewa, A. Horała, P. Dereziński, A. Klupczynska, E. Nowak-Markwitz, J. Matysiak, Z.J. Kokot, Usefulness of amino acid profiling in ovarian cancer screening with special emphasis on their role in cancerogenesis, Int. J. Mol. Sci. 18 (2017), https://doi.org/10.3390/ijms18122727.

[7] A. Swiatly, A. Horala, J. Hajduk, J. Matysiak, E. Nowak-Markwitz, Z.J. Kokot, MALDI-TOF-MS analysis in discovery and identification of serum proteomic patterns of ovarian cancer, BMC Cancer 17 (2017), https://doi.org/10.1186/s12885-017-3467-2.

[8] A. Horala, A. Swiatly, J. Matysiak, P. Banach, E. Nowak-Markwitz, Z. Kokot, Diagnostic value of serum angiogenesis markers in ovarian cancer using multiplex immunoassay, Int. J. Mol. Sci. 18 (2017) 123, https://doi.org/10.3390/ijms18010123.

[9] A. Swiatly, A. Horala, J. Matysiak, J. Hajduk, E. Nowak-Markwitz, Z. Kokot, Understanding ovarian cancer: iTRAQ-based proteomics for biomarker discovery, Int. J. Mol. Sci. 19 (2018) 2240, https://doi.org/10.3390/ijms19082240.

[10] M. J.H., Handbook of Biological Statistics, 3rd ed., (2014).

[11] J. Chong, O. Soufan, C. Li, I. Caraus, S. Li, G. Bourque, D.S. Wishart, J. Xia, MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis, Nucleic Acids Res. 46 (2018) W486–W494, https://doi.org/10.1093/nar/gky310.

[12] M. Sud, E. Fahy, D. Cotter, A. Brown, E.A. Dennis, C.K. Glass, A.H. Merrill, R.C. Murphy, C.R.H. Raetz, D.W. Russell, S. Subramaniam, LMSD: LIPID MAPS structure database, Nucleic Acids Res. 35 (2007) D527–D532, https://doi.org/10.1093/nar/gkl838.

[13] D.S. Wishart, Y.D. Feunang, A. Marcu, A.C. Guo, K. Liang, R. Vázquez-Fresno, T. Sajed, D. Johnson, C. Li, N. Karu, Z. Sayeeda, E. Lo, N. Assempour, M. Berjanskii, S. Singhal, D. Arndt, Y. Liang, H. Badran, J. Grant, A. Serra-Cayuela, Y. Liu, R. Mandal, V. Neveu, A. Pon, C. Knox, M. Wilson, C. Manach, A. Scalbert, HMDB 4.0: the human metabolome database for 2018, Nucleic Acids Res. 46 (2018) D608–D617, https://doi.org/10.1093/nar/gkx1089.

[14] G. Zhou, J. Xia, OmicsNet: a web-based tool for creation and visual analysis of biological networks in 3D space, Nucleic Acids Res. 46 (2018) W514–W522, https://doi.org/10.1093/nar/gky510.

[15] List of lipids the deep phenotyping company annotation of potential isobaric and isomeric lipid species measured with the AbsoluteIDQ® p180 Kit (and p150 Kit), (n.d.). https://www.biocrates.com/images/List-of-Isobaric-and-Isomeric-Lipid-Species_v1_2018.pdf (accessed November 21, 2018).

[16] J.V. Carter, J. Pan, S.N. Rai, S. Galandiuk, Education ROC-ing Along: Evaluation and Interpretation of Receiver Operating Characteristic Curves, (2016), https://doi.org/10.1016/j.surg.2015.12.029.

[17] K.N. Gonzalez Herrera, E. Zaganjor, Y. Ishikawa, J.B. Spinelli, H. Yoon, J.-R. Lin, F.K. Satterstrom, A. Ringel, S. Mulei, A. Souza, J.M. Gorham, C.C. Benson, J.G. Seidman, P.K. Sorger, C.B. Clish, M.C. Haigis, Small-molecule screen identifies de novo nucleotide synthesis as a vulnerability of cells lacking SIRT3, Cell Rep. 22 (2018) 1945–1955, https://doi.org/10.1016/j.celrep.2018.01.076.

[18] F. Jing, X. Hu, Y. Cao, M. Xu, Y. Wang, Y. Jing, X. Hu, Y. Gao, Z. Zhu, Discriminating gastric cancer and gastric ulcer using human plasma amino acid metabolic profile, IUBMB Life 70 (2018) 553–562, https://doi.org/10.1002/iub.1748.

[19] C.A. Lyssiotis, A.C. Kimmelman, Metabolic interactions in the tumor microenvironment, Trends Cell Biol. 27 (2017) 863–875, https://doi.org/10.1016/J.TCB.2017.06.003.

[20] Oana A. Zeleznik, Elizabeth M. Poole, Clary Clish, Heather A. Eliassen, Peter Kraft, Shelley S. Tworoger, Metabolomic analysis of ovarian cancer risk in the Nurses' Health Studies: Metabolite associations are more pronounced in non-serous tumors. [abstract], Proceedings of the AACR Conference: Addressing Critical Questions in Ovarian Cancer Research and Treatment; Oct 1-4, 2017, Clin. Cancer Res. 24(15_Suppl) AACR, Pittsburgh, PA. Philadelphia (PA), 2018Abstract nr A18.

[21] H. Xie, Y. Hou, J. Cheng, M.S. Openkova, B. Xia, W. Wang, A. Li, K. Yang, J. Li, H. Xu, C. Yang, L. Ma, Z. Li, X. Fan, K. Li, G. Lou, Metabolic profiling and novel plasma biomarkers for predicting survival in epithelial ovarian cancer, Oncotarget 8 (2017) 32134–32146, https://doi.org/10.18632/oncotarget.16739.

[22] C. Ke, Y. Hou, H. Zhang, L. Fan, T. Ge, B. Guo, F. Zhang, K. Yang, J. Wang, G. Lou, K. Li, Large-scale profiling of metabolic dysregulation in ovarian cancer, Int. J. Cancer 136 (2014), https://doi.org/10.1002/ijc.29010 n/a-n/a.

[23] H. Zhang, T. Ge, X. Cui, Y. Hou, C. Ke, M. Yang, K. Yang, J. Wang, B. Guo, F. Zhang, G. Lou, K. Li, Prediction of advanced ovarian cancer recurrence by plasma metabolic profiling, Mol. BioSyst. 11 (2015) 516–521, https://doi.org/10.1039/c4mb00407h.

[24] M. Liu, X. Wang, L. Wang, X. Ma, Z. Gong, S. Zhang, Y. Li, Targeting the IDO1 pathway in cancer: from bench to bedside, J. Hematol. Oncol. 11 (2018) 100, , https://doi.org/10.1186/s13045-018-0644-y.

[25] G. Miolo, E. Muraro, D. Caruso, D. Crivellari, A. Ash, S. Scalone, D. Lombardi, F. Rizzolio, A. Giordano, G. Corona, Pharmacometabolomics study identifies circulating spermidine and tryptophan as potential biomarkers associated with the complete pathological response to trastuzumab-paclitaxel neoadjuvant therapy in HER-2 positive breast cancer, Oncotarget 7 (2016) 39809–39822, https://doi.org/10.18632/oncotarget.9489.

[26] N.F. Evageliou, M. Haber, A. Vu, T.W. Laetsch, J. Murray, L.D. Gamble, N.C. Cheng, K. Liu, M. Reese, K.A. Corrigan, D.S. Ziegler, H. Webber, C.S. Hayes, B. Pawel, G.M. Marshall, H. Zhao, S.K. Gilmour, M.D. Norris, M.D. Hogarty, Polyamine antagonist therapies inhibit neuroblastoma initiation and progression, Clin. Cancer Res. 22 (2016) 4391–4404, https://doi.org/10.1158/1078-0432.CCR-15-2539.

[27] K. Soda, The mechanisms by which polyamines accelerate tumor spread, J. Exp. Clin. Cancer Res. 30 (2011) 95, , https://doi.org/10.1186/1756-9966-30-95.

[28] F. Gibellini, T.K. Smith, The Kennedy pathway—de novo synthesis of phosphatidylethanolamine and phosphatidylcholine, IUBMB Life 62 (2010), https://doi.org/10.1002/iub.337 n/a-n/a.

[29] C. Huang, C. Freter, Lipid metabolism, apoptosis and cancer therapy, Int. J. Mol. Sci. 16 (2015) 924–949, https://doi.org/10.3390/ijms16010924.

[30] E. Iorio, A. Ricci, M. Bagnoli, M.E. Pisanu, G. Castellano, M. Di Vito, E. Venturini, K. Glunde, Z.M. Bhujwalla, D. Mezzanzanica, S. Canevari, F. Podo, Activation of phosphatidylcholine cycle enzymes in human epithelial ovarian cancer cells, Cancer Res. 70 (2010) 2126–2135, https://doi.org/10.1158/0008-5472.CAN-09-3833.

[31] J. Xu, Y. Chen, R. Zhang, Y. Song, J. Cao, N. Bi, J. Wang, J. He, J. Bai, L. Dong, L. Wang, Q. Zhan, Z. Abliz, Global and targeted metabolomics of esophageal squamous cell carcinoma discovers potential diagnostic and therapeutic biomarkers, Mol. Cell. Proteomics 12 (2013) 1306–1318, https://doi.org/10.1074/mcp.M112.022830.

[32] J. Aoki, A. Inoue, S. Okudaira, Two pathways for lysophosphatidic acid production, Biochim. Biophys. Acta Mol. Cell Biol. Lipids 1781 (2008) 513–518, https://doi.

org/10.1016/J.BBALIP.2008.06.005.
[33] Y. Xu, Yan Xu, Lysophospholipid signaling in the epithelial ovarian cancer tumor microenvironment, Cancers (Basel) 10 (2018) 227, https://doi.org/10.3390/cancers10070227.
[34] L.C. Rogers, R.R. Davis, N. Said, T. Hollis, L.W. Daniel, Blocking LPA-dependent signaling increases ovarian cancer cell death in response to chemotherapy, Redox Biol. 15 (2018) 380–386, https://doi.org/10.1016/J.REDOX.2018.01.002.
[35] G. van Meer, D.R. Voelker, G.W. Feigenson, Membrane lipids: where they are and how they behave, Nat. Rev. Mol. Cell Biol. 9 (2008) 112–124, https://doi.org/10.1038/nrm2330.
[36] A. Bachmayr-Heyda, S. Aust, K. Auer, S.M. Meier, K.G. Schmetterer, S. Dekan, C. Gerner, D. Pils, Integrative systemic and local metabolomics with impact on survival in high grade serous ovarian cancer, Clin. Cancer Res. (2016), https://doi.org/10.1158/1078-0432.CCR-16-1647.
[37] Z.-Q. Wang, A. Faddaoui, M. Bachvarova, M. Plante, J. Gregoire, M.-C. Renaud, A. Sebastianelli, C. Guillemette, S. Gobeil, E. Macdonald, B. Vanderhyden, D. Bachvarov, BCAT1 expression associates with ovarian cancer progression: possible implications in altered disease metabolism, Oncotarget 6 (2015) 31522–31543, https://doi.org/10.18632/oncotarget.5159.
[38] W. Ju, B.C. Yoo, I.-J. Kim, J.W. Kim, S.C. Kim, H.P. Lee, Identification of genes with differential expression in chemoresistant epithelial ovarian cancer using high-density oligonucleotide microarrays, Oncol. Res. 18 (2009) 47–56 http://www.ncbi.nlm.nih.gov/pubmed/20066894 (accessed November 14, 2018).
[39] J. Ni, L. Xu, W. Li, L. Wu, Simultaneous determination of thirteen kinds of amino acid and eight kinds of acylcarnitine in human serum by LC-MS/MS and its application to measure the serum concentration of lung cancer patients, Biomed. Chromatogr. 30 (2016) 1796–1806, https://doi.org/10.1002/bmc.3755.
[40] G.F. Giskeødegård, A.F. Hansen, H. Bertilsson, S.V. Gonzalez, K.A. Kristiansen, P. Bruheim, S.A. Mjøs, A. Angelsen, T.F. Bathen, M.-B. Tessem, Metabolic markers in blood can separate prostate cancer from benign prostatic hyperplasia, Br. J. Cancer 113 (2015) 1712–1719, https://doi.org/10.1038/bjc.2015.411.
[41] H.I. Wettersten, A.A. Hakimi, D. Morin, C. Bianchi, M.E. Johnstone, D.R. Donohoe, J.F. Trott, O.A. Aboud, S. Stirdivant, B. Neri, R. Wolfert, B. Stewart, R. Perego, J.J. Hsieh, R.H. Weiss, Grade-dependent metabolic reprogramming in kidney cancer revealed by combined proteomics and metabolomics analysis, Cancer Res. 75 (2015) 2541–2552, https://doi.org/10.1158/0008-5472.CAN-14-1703.
[42] B. Wang, Z. Shi, G.F. Weber, M.A. Kennedy, Introduction of a new critical p value correction method for statistical significance analysis of metabonomics data, Anal. Bioanal. Chem. 405 (2013) 8419–8429, https://doi.org/10.1007/s00216-013-7284-4.
[43] S.K. Bharti, F. Wildes, C.-F. Hung, T.C. Wu, Z.M. Bhujwalla, M.-F. Penet, Metabolomic characterization of experimental ovarian cancer ascitic fluid, Metabolomics 13 (2017) 113, , https://doi.org/10.1007/s11306-017-1254-3.
[44] I. Amelio, F. Cutruzzolá, A. Antonov, M. Agostini, G. Melino, Serine and glycine metabolism in cancer, Trends Biochem. Sci. 39 (2014) 191–198, https://doi.org/10.1016/j.tibs.2014.02.004.
[45] J.M. Phang, W. Liu, C.N. Hancock, J.W. Fischer, Proline metabolism and cancer: emerging links to glutamine and collagen, Curr. Opin. Clin. Nutr. Metab. Care 18 (2015) 71–77, https://doi.org/10.1097/MCO.0000000000000121.