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Proteomic pattern of cervico-vaginal fluid (CVF) in an ovarian cancer diagnosis — pilot study

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ABSTRACT

Objectives: High grade serous ovarian cancer (HGSC) is the most common type of ovarian cancer and is responsible for about 90% of ovarian cancer deaths. The diagnostic tests currently used do not increase the detection rates for ovarian cancer. There is a great necessity to develop new and non-invasive diagnostic tests for ovarian cancer (OC). Cervico-vaginal fluid (CVF) seems to be a potential and valuable source of biomarkers for genital tract diseases including ovarian cancer. The aim of our pilot study was to undertake a preliminary proteomic analysis of CVF derived from ovarian cancer patients and to compare these with results from a control group.

Material and methods: We analysed and compared samples from a group of ovarian cancer patients and a control group of healthy patients. The study used MALDI-TOF coupled with nanoLC and ClinProTools software for MS, MS/MS spectra collection and proteomic analysis.

Results: We identified 404 different proteins in the OC group and 417 proteins in the control group. 239 of the proteins were found to be common to both study groups, 165 proteins were unique to the OC subjects, and 178 proteins were unique to the control subjects. We selected three proteins as the OC markers with the greatest potential: cysteine-rich secretory protein 3, fibronectin and Ly6/PLAUR domain-containing protein 3.

Conclusions: The proteins we selected seem to possess great potential as markers for the screening and early detection of OC, especially in non-invasive and low-cost diagnostic tests. However, our findings require more advanced and validated proteomic analysis to confirm the suitability of the selected proteins in everyday medical diagnoses.

Key words: ovarian cancer; proteomic pattern; tumor markers; cervico-vaginal fluid

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INTRODUCTION

High grade serous ovarian cancer (HGSC) is the most common type of ovarian cancer and is responsible for about 90% of ovarian cancer deaths [1]. Early detection of cancers, especially of serous ovarian cancer, is necessary to reduce mortality rates. Until now, various diagnostic tests and tumor markers have been used, but they have not increased the detection of the disease. A randomized study of the applicability and efficacy of these tests in postmenopausal women for ovarian cancer screening showed no reduction in mortality [2, 3]. It is therefore necessary to seek new tumor markers from body secretions other than blood serum. Cervico-vaginal fluid (CVF) is a potential source of biomarkers for genital tract diseases. It is easily available, and so it is possible to use it in repeatable, inexpensive and non-invasive tests. CVF is a complex body secretion consisting of a mixture of plasma proteins, inflammatory cells, enzymes and genital tract epithelial cells. The body's physiological and disease states are reflected in the proteomic profiles characteristic of CVFs [4]. Determining the proteomic profile for ovarian cancer may allow the identification of new markers of the early stages of this disease.

The aim of our pilot study was to undertake a proteomic analysis of cervico-vaginal fluid in patients with ovarian cancer and to compare the results with those derived from a healthy control group. The study attempted to compare the protein patterns of the ovarian cancer subjects with those of the control group, and in addition, to identify the

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proteins that differentiated the ovarian cancer patients from the healthy subjects.

MATERIALS AND METHODS

Study group

The total study group comprised eleven patients with a median age of 57 (range 35–76) admitted to the Gynecologic Oncology Department of Poznan University of Medical Sciences. Inclusion criteria in the cancer group: a patient with suspected ovarian cancer, prior to surgical treatment, with an open cervical os, with confirmed ovarian cancer diagnosis after surgery.The cancer group comprised seven patients with advanced ovarian cancer (5 GRADE 3 serous OC, 2 GRADE 2 mucinous OC) and one patient with a borderline tumor of the ovary. Inclusion criteria in the control group: healthy patients without any oncological disease, no changes in ultrasound in the uterus and ovaries.The control group comprised three healthy patients who had been admitted because of urine incontinence. Informed written consent was obtained from each patient. The study was approved by the Poznan Univeristy of Medical Sciences Bioethical Commission.

Sample processing

Cervico-vaginal fluid samples were obtained from each patient by placing a sterile swab into the cervical canal for 10 seconds until saturation. The swab was removed and placed directly into a sterile tube containing 2 mL of 0.9% NaCl. The sample was then centrifuged at 3000 g for 10 minutes, and the supernatant was collected and stored at -80°C. The Bradford method was used for the determination of total protein concentration in each sample that we analyzed from the cancer group and the control group. The sample processing for MS/MS analysis was strictly correlated with the data received from the Bradford method analyses, to obtain a constant concentration of proteins (20 μg/mL) for all the vaginal fluid samples we analyzed. Thereafter 10mL of each vaginal fluid sample was transferred to a 0.5mL Eppendorf tube, and 15 μL of ammonium bicarbonate solution (50 mM) plus 1.5 μL of DDT (100 mM) were added. The mixture was incubated for 5 minutes at 95°C. After cooling to room temperature, we added 3 μL of iodoacetamide (100 mM) to the reaction mixture and incubated in the dark for 20 minutes at room temperature. After this incubation, we added 3 μL of trypsin enzyme solution (0.1 mg/mL) to the protein mixture and incubated for 16 hours at 37°C. The digestion process was arrested by adding 1μL of 10% trifluoracetic acid (TFA) to the digestion mixture. Afterwards, samples were frozen and stored at -81°C until the MS and MS/MS analysis.

Protein identification by MALDI-TOF/TOF- mass spectrometry

Our study used the AnchorChip Standard (800 mm, Bruker, Germany) target plate. As a mass standard in these experiments, the Peptide Calibration Standard II (Bruker, Germany) was used. The defined masses of calibrates allowed for the proper calibration of the apparatus and covered the mass range of 700–3500 Da. Each of the vaginal fluid samples from both the cancer patients and the control subjects was separated and fractionated using the reversed phase nano-liquid chromatography technique (Easy nanoLC, Bruker, Germany). The nanoLC apparatus was equipped with: pre-column (C18, 5 μm, 120Å, L = 20 mm, NS-MP 10 BioSphere) and bioanalytical nano chromatographic column (C18, 75 mm × 15 cm, 3 μm, 100Å) (Acclaim PepMap, Thermo Scientific). The following mobile phases were used: A) 0.05% TFA in water and B) 0.05% TFA in 90% acetonitrile. Analytes were eluted from the analytical column at 300 nL/min flow during a 96-minute linear gradient from 2% to 50% of the mobile phase B. The eluent was mixed with HCCA (α-cyano-4-hydroxycinnamic acid) matrix and spotted onto AnchorChip standard plates (800 mm, Bruker, Germany). The 384 fractions with a 15-second deposition were automatically collected on the target plates using the PROTEINEER apparatus (Bruker, Germany).

The mass spectrometry experiments were performed with the MALDI-TOF/TOF apparatus (UltrafleXtreme, Bruker), equipped with FlexControl and FlexAnalysis modules, that allowed for data acquisition and data/spectra analysis. The MS spectra were acquired in the mass range of 700–3500 Da and analyzed using the MALDI-TOF/TOF mass spectrometry instrument (Bruker, UltrafeXtreme, Germany) using a fixed laser intensity and 2500 shots per spectrum.

We used the Peptide Calibration Standard II (Bruker, Germany) as the mass standard in these experiments. Prior to each MS-analysis, the apparatus was calibrated according to the reference masses that included: bradykinin 1–7 (*m/z* 757.3992 Da), angiotensin I and II (1046.5418 and 1296.6848 Da, respectively), substance P (1347.7354 Da), bombesin (1619.8223 Da), renin substrate (1758.9326 Da), ACTH clip1-17 and ACTH clip 18–39 (2093.0862 and 2465.1983 Da, respectively), and somatostatin 28 (3147.4710 Da).

Based on the list of masses obtained during the MS experiments, the MS/MS mode was applied. Protein identification was performed with the ProteinScape and Mascot platform using the SwissProt database, and results with less than 1% FDR (false discovery rate) were taken into consideration. The following protein modifications were taken into the consideration: carbamidomethyl, oxidation, acetyl (N)-term and Glu- > pyro-Glu (N-term E). Protein identification search parameters were set as follows: peptide tolerance 50 ppm and peptide charge +1; and up to 1 missed cleavage was permitted.

RESULTS

Our study was designed to focus on protein profile identification in cervico-vaginal fluid taken from ovarian cancer

patients in comparison with samples from healthy control group subjects.

Using MALDI-TOF/TOF mass spectrometry (MS/MS) experiments we identified 404 different proteins in the cancer CVF samples and a further 417 different proteins in the CVF samples from the control group. At the very beginning of the study, all the proteins that we identified were divided into three separate classes: those common to both the cancer and control groups, those unique to the cancer group, and those unique to the control group.

Detailed comparison of the proteins identified in both the cancer and control group samples enabled the selection of 239 different proteins that were common to both the cancer and the control samples; 178 different proteins that were unique to the control group, and 165 different proteins that were unique to the cervico-vaginal fluid samples derived from the cancer patients. Moreover, we used the Panther Classification System program to analyze the proteins groups according to their molecular functions, biological processes, cellular components, protein class and pathways [5]. Those proteins that were characteristic of cancer were identified (Tab. 1) and those found in at least 4 (50%) of the samples were identified (Tab. 2) and all these were analyzed according to their molecular functions, biological processes, cellular components, protein class and biological pathways. (Fig. 1). However, we particularly focused our attention on three different proteins: cysteine-rich secretory protein 3, fibronectin, and Ly6/PLAUR domain-containing protein 3, due to their presence in 5 of the 8 ovarian cancer samples we analyzed and because of their particular biological properties.

DISCUSSION

Human cervico-vaginal fluid (CVF) is a mixture of fluids originating from the vagina, cervix, endometrium and oviduct [6]. Its composition is influenced by many factors, both hormonal changes during the menstrual cycle, as well as pathogens present in the reproductive tract [4]. Cervico-vaginal fluid can be considered as a potential source of genital tract biomarkers. The secretion is readily available [7]. For several years, interest in vaginal and cervical secretions, as potential sources of ovarian cancer markers, has been increasing. In 1978, there were cases presented that showed that the presence of cancer cells in cytology cervical smears was caused by advanced ovarian and fallopian cancer [8]. Cytological changes caused by ovarian cancer have even occurred in 19.3% of patients in the absence of changes in the cervix and endometrium. The percentage of positive smears of lavage fluid from the uterine cavity was even higher [9,10]. Positive cytology results were usually found in the serous type of advanced ovarian cancer (HGSC) coexisting with ascites [11]. Microscopic assessment of CVF is

Table 1. Classification of proteins identified as unique to the cancer group due their molecular functions, biological processes, cellular components, protein class and biological pathways

Molecular Function	
Binding (GO:0005488)	44
Receptor activity (GO:0004872)	2
Structural molecule activity (GO:0005198)	5
Signal transducer activity (GO:0004871)	3
Catalytic activity (GO:0003824)	31
Transporter activity (GO:0005215)	5
Biological process	
Cellular component organization or biogenesis (GO:0071840)	17
Cellular process (GO:0009987)	62
Localization (GO:0051179)	16
Biological regulation (GO:0065007)	18
Response to stimulus (GO:0050896)	18
Developmental process (GO:0032502)	16
Multicellular organismal process (GO:0032501)	14
Biological adhesion (GO:0022610)	5
Locomotion (GO:0040011)	1
Metabolic process (GO:0008152)	51
Immune system process (GO:0002376)	7
Cellular component	
Synapse (GO:0045202)	1
Cell junction (GO:0030054)	3
Membrane (GO:0016020)	18
Macromolecular complex (GO:0032991)	7
Extracellular matrix (GO:0031012)	1
Cell part (GO:0044464)	41
Organelle (GO:0043226)	29
Extracellular region (GO:0005576)	5
Protein class	
Transporter (PC00227)	3
Transmembrane receptor regulatory/adaptor protein (PC00226)	1
Membrane traffic protein (PC00150)	1
Hydrolase (PC00121)	13
Oxidoreductase (PC00176)	4
Cell adhesion molecule (PC00069)	4
Cell junction protein (PC00070)	3
Enzyme modulator (PC00095)	13
Transfer/carrier protein (PC00219)	4
Transferase (PC00220)	2
Transcription factor (PC00218)	3
Nucleic acid binding (PC00171)	9
Defense/immunity protein (PC00090)	4
Calcium-binding protein (PC00060)	4
Cytoskeletal protein (PC00085)	7
Signaling molecule (PC00207)	10

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Table 1. cont. Classification of proteins identified as unique to the cancer group due their molecular functions, biological processes, cellular components, protein class and biological pathways

Pathway	
Axon guidance mediated by netrin (P00009)	2
Axon guidance mediated by Slit/Robo (P00008)	1
Axon guidance mediated by semaphorins (P00007)	1
Apoptosis signaling pathway (P00006)	2
Angiogenesis (P00005)	2
Alzheimer disease-presenilin pathway (P00004)	4
Integrin signalling pathway (P00034)	6
Inflammation mediated by chemokine and cytokine signaling pathway (P00031)	4
Hypoxia response via HIF activation (P00030)	1
Nicotine pharmacodynamics pathway (P06587)	1
Huntington disease (P00029)	1
p53 pathway (P00059)	1
Heterotrimeric G-protein signaling pathway-Gq alpha and Go alpha mediated pathway (P00027)	3
Heterotrimeric G-protein signaling pathway-Gi alpha and Gs alpha mediated pathway (P00026)	1
Wnt signaling pathway (P00057)	2
VEGF signaling pathway (P00056)	1
Ras Pathway (P04393)	1
T cell activation (P00053)	1
FGF signaling pathway (P00021)	1
ATP synthesis (P02721)	1
Plasminogen activating cascade (P00050)	1
Endothelin signaling pathway (P00019)	1
EGF receptor signaling pathway (P00018)	1
DNA replication (P00017)	1
Parkinson disease (P00049)	1
Cytoskeletal regulation by Rho GTPase (P00016)	2
PDGF signaling pathway (P00047)	2
Oxidative stress response (P00046)	1
Histamine H1 receptor mediated signaling pathway (P04385)	1
Cadherin signaling pathway (P00012)	1
Blood coagulation (P00011)	1
Dopamine receptor mediated signaling pathway (P05912)	1
Salvage pyrimidine ribonucleotides (P02775)	1
B cell activation (P00010)	2
Muscarinic acetylcholine receptor 1 and 3 signaling pathway (P00042)	1
Angiotensin II-stimulated signaling through G proteins and beta-arrestin (P05911)	2
Salvage pyrimidine deoxyribonucleotides (P02774)	1
CCKR signaling map (P06959)	3
Pyrimidine Metabolism (P02771)	1
Gonadotropin-releasing hormone receptor pathway (P06664)	2

Table 2. List of proteins that differentiate the ovarian cancer group (serous ovarian cancer, mucinous ovarian cancer and borderline tumor) and control group

Protein \ Diagnosis	SOC (n = 5)	MOC (n = 2)	BT (n = 1)
Alpha-2-macroglobulin-like protein 1	3		
Serpin B-13	4		
Acyl-CoA-binding protein	4		
Calmodulin-like protein 5	3		
Cytidine deaminase	2	1	
Cysteine-rich secretory protein 3	4	1	
Fibronectin	2	2	1
Interleukin-1 receptor antagonist protein	4		
Ly6/PLAUR domain-containing protein 3	5		

SOC — serous ovarian cancer; MOC — mucinous ovarian cancer; BT — borderline tumor, n — number of subjects in analyzed group

an imprecise method in comparison with analyses of protein composition [12]. Only a limited number of proteomic studies using mass-spectrometry have been performed on cervico-vaginal fluid [7]. Analysis of CVF proteins was used in the identification of biomarkers for premature delivery, premature rupture of membranes, bacterial vaginosis and even cervical cancer [4, 13]. Our study is the first proteomic analysis of cervico-vaginal fluid in ovarian cancer patients known to the authors, which has allowed the detection of new potential markers, that may be useful for the early diagnosis and progress monitoring of ovarian cancer.

For our preliminary proteomic study of ovarian cancer, we used MALDI-TOF/TOF MS/MS mass spectrometry coupled with the nanoLC technique for sample analysis. MALDI-TOF/TOF MS/MS is very fast and sensitive for proteomics analysis and it allows for a high throughput of protein identification [14]. However, it must be emphasized, that the presence of proteins identified by MALDI-TOF/TOF needs to be confirmed by other bioanalytical methods (e.g. immuno-assay methods).

The results of our study showed three proteins that can potentially differentiate patients with ovarian cancer from healthy patients: fibronectin, cysteine-rich secretory protein 3 and Ly6/PLAUR domain-containing protein 3. In ovarian cancer (OC), fibronectin has a well-established role in cancer metastasis. It has antiapoptotic functions, and promotes angiogenesis and the adhesion of OC cells to the peritoneal surface [15].

Cysteine-rich secretory protein3 (CRISP3) is involved in defense and immunity processes [16]. The expression of CRISP3 has been shown to be highly up-regulated in prostate cancer. It is associated with poor prognoses due to its role in cell invasion. Cysteine-rich secretory proteins are

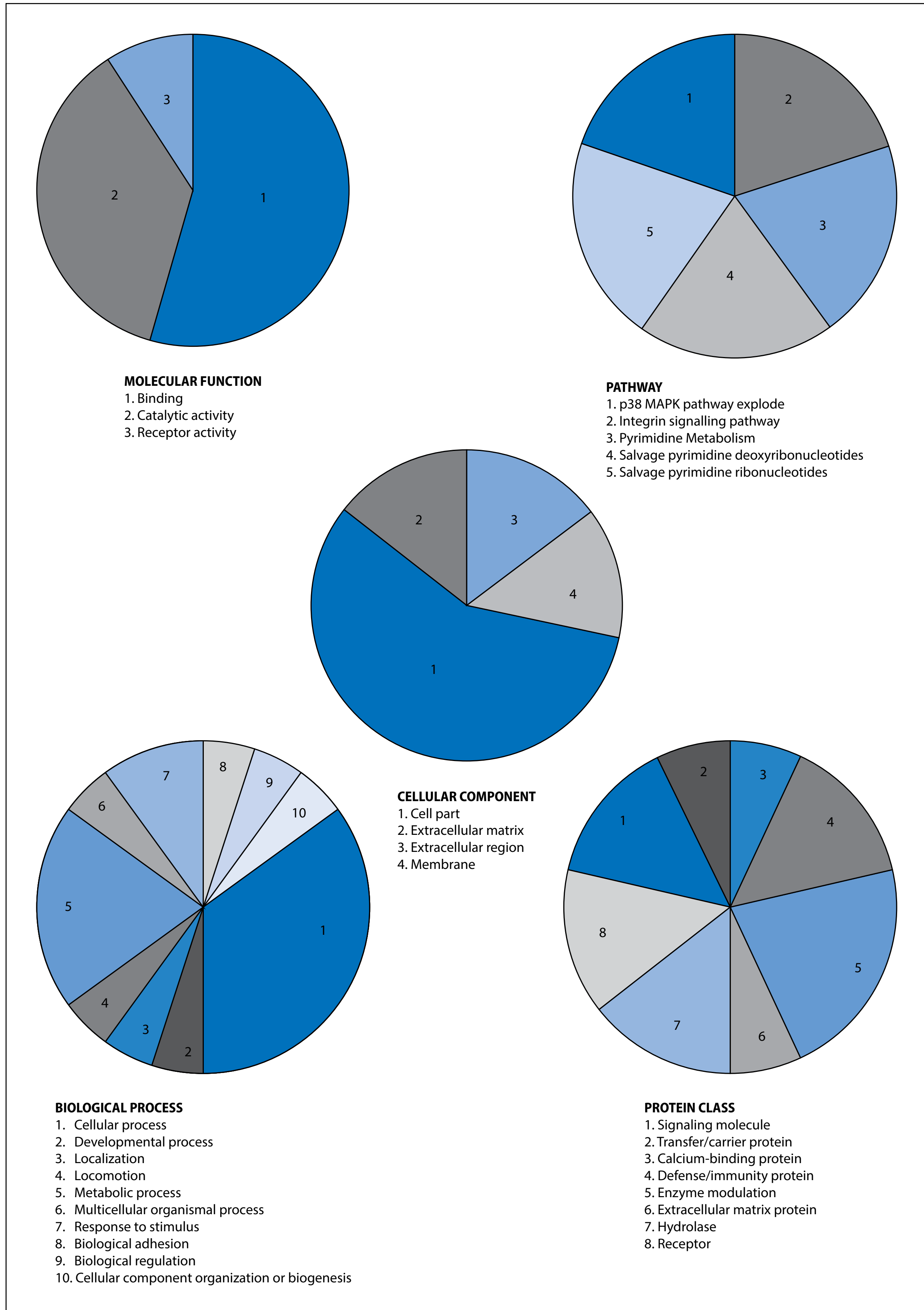

Figure 1. Graphic presentation of the functional classification (molecular functions, biological processes, cellular components, protein class and biological pathways) of all the proteins selected as characteristic of ovarian cancer patients gathered and presented at Table 1

strongly expressed in lung adenocarcinoma tissues where they promote the migration and invasion of carcinoma cells [17]. Also high expression of CRISP3 is found in ovarian cancer tissue. However, its role and importance in this disease is unknown [18, 19].

Ly6/PLAUR domain-containing protein3 (LYPD3) has been identified in non-small cell lung cancer, colorectal and breast cancer. It seems to be involved in cell migration, invasion and tumor progression [20–22]. LYPD3 protein deserves special attention because it occurred in all the serous OC samples in our study. It can be a marker of the most lethal serous type of ovarian cancer and enables recognition of this disease at all stages.

The proteins selected in our pilot study seem to be of great interest and to be potentially valuable markers for early ovarian cancer diagnosis. They may help in screening patients with BRCA mutation and differentiate healthy from ovarian cancer patients. However, further investigations will be necessary to prove their suitability and value as non-invasive biomarkers. It is strongly recommended that these possibilities are tested in large population studies so as to determine the levels of these proteins in vaginal fluid samples derived from ovarian cancer patients at various tumor progression stages when compared with control samples.

CONCLUSIONS

There is a characteristic proteomic pattern in the cervico-vaginal fluid of patients with ovarian carcinoma, which may allow the differentiation of cancer patients from healthy patients. Our findings indicated 3 proteins in the cervico-vaginal fluid of ovarian cancer patients: fibronectin, cysteine-rich secretory protein3 and Ly6/PLAUR domain-containing protein3. These proteins show a strong potential to be used in multi-marker tests for the screening and detection of ovarian cancer. Our findings provide the basis for further research aimed at the early diagnosis of ovarian cancer and the reduction of mortality due to this disease.

Conflicts of interest

The authors declare no conflicts of interest.

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Author contributions

We confirm that all co-authors have been included and have contributed to the final manuscript and have approved it. EG designed the study, collected samples, analyzed the data, wrote the manuscript and prepared figures for this manuscript. JL collected samples. BU performed the MALDI-TOF-TOF analysis, prepared figures and reviewed the manuscript. ENM provided the biological material for analysis and critically reviewed the manuscript. JM provided critical review of the manuscript.

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