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Microvesicles released from ectopic endometrial foci as a potential biomarker of endometriosis

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ABSTRACT

Objectives: Angiogenesis is engaged in endometriosis. It is regulated by regulatory factors and cytokines, transported in microvesicles. The purpose was to investigate the presence of MVs with vascular endothelial growth factor (VEGF) and metalloproteinase-9 (MMP-9) in peripheral blood and peritoneal fluid of women operated on for endometrioma or teratoma.

Material and methods: Microvesicles (MVs) were determined in blood samples and peritoneal fluid samples collected from women aged 20–60 years operated on for endometriosis (test group) and teratoma (control group). The final investigations were performed on 47 patients, who qualified for the study based on the meticulous inclusion criteria. MVs were analyzed by flow cytometry (FACS) using annexin V, antibodies for molecules characteristic of cells from endometriosis foci (keratin 18 (K18), CD105, CD146), and antibodies for intraepithelial vascular growth factor VEGF and metalloproteinase-9 (MMP-9). The sample was double "reading" using flow cytometry (FACSCantoII).

Results: Cytometry analysis confirmed MVs' presence in plasma and peritoneal fluid collected from patients with both endometriosis and teratomas. A statistically significant higher level of AnnexinV (+) MVs were observed in plasma samples of endometriosis patients. In the control group, there was a higher percentage of double-positive VEGF (+)/MMP-9 (+) and single MMP-9 (+) positive MVs in the serum. In the peritoneal fluid higher frequency of double-positive VEGF (+)/ /MMP-9 (+) MVs were found in the control group. However, the amount of VEGF (+) / MMP-9 (+) MVs object did not enable to differentiate between the test and control groups. The study was the first, in which MVs were confirmed in plasma and peritoneal fluid in benign adnexa tumors.

Conclusions: Microvesicles are present in peripheral blood and peritoneal fluid samples collected from patients with endometriosis and teratomas. Microvesicles with proangiogenic factors (VEGF and MMP-9) are more abundant in blood and peritoneal fluid samples from patients with teratomas.

Keywords: endometriosis; microvesicles; angiogenesis; VEGF MMP-9

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INTRODUCTION

Endometriosis is a common gynecological disorder defined by the proliferation of endometrial glands and stroma outside the normal uterine cavity [1]. It affects especially women of reproductive age, but girls before menarche and postmenopausal women are occasionally diagnosed as well [2]. Between 20% to 50% infertile women suffer from endometriosis [1, 2]. The physical, mental and social well-being is reduced significantly.

Clinical manifestation of endometriosis is not characteristic. The disease can be asymptomatic; however, permanent or temporary pain is common. Dysmenorrhea, dyspareunia, and infertility are the typical and common triad of endometriosis symptoms [3]. The symptoms also depend on the localization of ectopic endometrial lesions. Endometrial implants in the peritoneum of recto-uterine and vesicouterine excavation, Sacro-uterine ligaments, and rectovaginal septum cause pain and bleeding during urination and defecation, pain

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in the sacral spine, and pain radiating to the thighs. However, ovaries, fallopian tubes, and pelvic and abdominal peritoneum are more common localization for endometrial implants. Chocolate cysts filled with hemolyzed blood are a typical sign of ovarian endometriosis. Endometrial glands and stroma can grow into the myometrium layer and lead to uterine muscle tissue changes called adenomyosis. Extra pelvic endometriosis is rare and occurs in well-vascularized organs, for example, the intestine, lung, or brain [4–6].

The pathogenesis of endometriosis remains unclear. Many theories explain how endometrial implants form, but neither describes this process clearly. Retrograde menstruation is accepted as the most plausible sequence of events leading to lesions establishment [1, 7–9] According to the new hypothesis, endometriosis could also originate from endometrial stem cells or mesenchymal stem cells from the bone marrow homing peritoneal cavity and differentiating into endometrial cells. The viable endometrial cells spread and attach to the peritoneal surface. The success of the ectopic implants depends on the inflammatory response, neoangiogenesis, fibrosis, adhesion formation, avoidance of apoptosis, immune dysfunction of the host, and neuronal infiltration [4, 10–14]. The exact pathological mechanisms are observed both in endometriosis and neoplastic disease. Angiogenesis is one of the critical steps engaged in those pathologies.

Role of angiogenesis in endometriosis pathogenesis

Angiogenesis is a multistep process of forming new blood vessels and occurs both in physiological and pathological conditions. The new blood vessels form by budding endothelial cells into the extracellular matrix, which is strictly regulated. Endothelial cells and other cells (*e.g.*, macrophages, neoplastic cells) release growth factors characterize for angiogenesis (VEGF, bFGF) to degrade the existing vessel's wall. vascular endothelial growth factor (VEGF) is the main proangiogenic factor. It influences endothelial cells' proliferation, migration, and apoptosis. Activated endothelial cells release proteolytic enzymes to degrade the base membrane allowing more effortless movement of cells. Metalloproteinases (enzymes modeling matrix) cause changes in the composition of the base membrane. Metalloproteinases modify the extracellular matrix to promote the migration of epithelial cells and the process of neovascularization [15, 16]. Cells combine to form capillary buds. A synthesis of base membrane components and the development of the other layers of the vessel produces further stabilization of the vessel.

The web of blood vessels surrounding endometriosis foci is characteristic of this disease and confirmed in microscopic studies [1]. Cells of ectopic endometrium, immune cells, and mesothelium cells release the growth factors and proteolytic enzymes into the peritoneal cavity. Macrophages and mast cells from peritoneal fluid and endometriosis foci are the primary sources of VEGF [17–20]. Increased concentration of the soluble form of VEGF was observed in peritoneal fluid collected from patients with advanced endometriosis [1, 2, 18, 21–23]. Some studies also demonstrated the highest concentration of VEGF in endometrial cysts and red implants [4, 24, 25]. The expression of VEGF, VEGF-2, and the number of activated macrophages correlates to the expression of metalloproteinase 9 in both peritoneal fluid and endometrial implants [15, 16]. Moreover, increased amount of metalloproteinases MMP-2, MMP-3, MMP-7, and MMP-9 is observed in patients with endometriosis [26–28]. This fact confirms enhanced proteolysis in ectopic endometrium [26].

Role of peritoneal fluid in endometriosis pathogenesis

Peritoneal fluid is the most critical factor controlling the peritoneal cavity microenvironment and is observed in physiological and pathological conditions. Physiologically, peritoneal fluid ranges from 5 to 20 ml in size, and the amount depends on the menstrual cycle phase. Its production increases in endometriosis and infertility [29–32]. The fluid arises through plasma filtration, the ovaries secretion, tubal mucus production, retrograde menstruation, and macrophages activity to produce and secrete proinflammatory cytokines/chemokines. It contains cells elements (macrophages, natural killers cells, lymphocytes, eosinophils, mast cells, and mesothelial cells) and substances they produce (cytokines, prostaglandins, hydrolytic enzymes, complement components, and oxygen free radicals) [32].

Changes in the composition of peritoneal fluid in endometriosis result from immune responses and their direct contact with the endometrial implants. Chronic inflammation in the pelvic cavity is a consequence of humoral and cell-mediated responses. The concentration of T cells increases, followed by the increase of the T-helper/T-suppressor ratio [33]. The number of NK cells responsible for removing ectopic endometrial cells increases. However, they are dysfunctional and fail to kill ectopic implants [33–37]. A significant rise in macrophages is observed, and the cells demonstrate higher activin than macrophages from healthy women [32]. Macrophages and cells of endometrial implants produce the following cytokines: IL-8, IL-10, ICAM-1, MCP-1, RANTES, VEGF, IGF, EGF, M-CSF, HGF, which are found in higher concentrations in the peritoneal fluid [33]. This fact confirms the existence of subclinical inflammation inside the peritoneal cavity. Immunological and angiogenic factors present in the pelvic environment participate in the pathogenesis of endometriosis [33].

Microvesicles

Extracellular vesicles were described in 1967 by Wolf [38] as waste products of the human body. Further studies showed that cells could release more different vesicles and apoptotic bodies, exosomes, and microvesicles (MVs). Microvesicles have different shapes ranging from 100 to 1,000 nm in size [39–41]. The shape and size depend on MVs' origin and function. Regulated release from outward budding or blebbing on the plasma membrane [1] causes the formation of the MVs. Health cells shed MVs from selected areas, whereas tumor cells from the entire surface [1]. Specific markers for MVs have not been identified yet. Previous research has used selectins, integrins, flotillin-2, CD-40, and metalloproteinase [39, 42, 43]. The release of MVs was confirmed in normal cells types, including red blood cells, platelets, endothelial cells, and pathological mostly cancer cells [39, 44–46]. Microvesicles can transport enzymes, regulatory and growth factors, cytokines, lipids, and nucleic acid (mRNA, miRNA, ncRNA, genomic DNA) [9, 39, 47–51]. They are responsible for homeostasis in human organisms and the induction of pathological processes. Proteolytic enzymes and proangiogenic factors play a role in creating new vessels. They are the MVs' cargo, as confirmed in cancer, and could in similar promote and regulate the creation of new endometrial implants. Moreover, MVs with regulatory factors could theoretically induce the tolerance against foci of ectopic endometrium and mediate angiogenesis. That was the assumption leading to the start of the study.

Objectives

Time from first symptoms to the final diagnosis of endometriosis is essential. Nowadays, it takes from 7 to 11 years [52–55]. Diagnosis is based on clinical signs; however, surgery and histological examination are the strongest confirmation Clinicians are recommended to use imaging (US or MRI) in the diagnostic work-up for endometriosis, but they need to be aware that a negative finding does not exclude endometriosis, particularly superficial peritoneal disease [56]. It explains why new diagnostic tools are needed.

Blood markers have not been found yet. Our study was conducted to find microvesicles in peripheral blood and peritoneal fluid collected from women with benign ovarian lesions like ovarian endometrioma or teratoma. Teratoma is a germ cells tumor that differentiates toward somatic-type cell populations [57].

If the presence of MVs is confirmed, we decided to investigate if they were filled with essential angiogenesis mediators, like vascular endothelial growth factor (VEGF) and metalloproteinase-9 (MMP-9). Previous research revealed a higher VEGF in peritoneal fluid and foci of ectopic endometrium [19, 58]. The same observation concerned metalloproteinases 9 and 2 [26]. In addition, the correlation between MMP-9 and VEGF in endometriosis foci was found [59]. However, the transport of VEGF and MMP-9 in MVs has never been studied both in endometriosis and teratoma.

MATERIAL AND METHODS

The study was conducted in Dept. of Gynecology and Gynecologic Oncology PMHCRI from 2014 to 2017 based on two grants (NCN UMO-2014/13/N/NZ5/00446 — "Microvesicles released from ectopic endometrial foci as a potential biomarker of endometriosis"; Young Researcher PMHCRI Grant – "Microvesicles as a potential biomarker of endometriosis"). The isolation technique was tested and finally established during the preliminary phase of the study provided by Young Researcher Grant. The Ethical Commission of the Polish Mother Memorial Hospital Research Institute approved all studies (decision number 40/2013).

Patients

The patients operated on for benign lesions of ovaries in PMHCRI were qualified for the study. All patients were given information about endometriosis and signed informed consent. Forty-seven women aged 20–60 took part in the study. Women operated on for endometriosis were the test group, whereas women with teratoma formed the control group (Tab. 1). Moreover, patients with chronic diseases such as cardiac problems (blood hypertension, ischemic heart disease), thyroid diseases, and autoimmune diseases (Hashimoto's disease, psoriasis, atopic dermatitis, lupus, Crohn's disease, celiac disease) were excluded from the research due to the observations that MVs could be present and play a role in the pathogenesis of these diseases.

Clinical presentation and ultrasound examination determined patients' qualifications for to test or control group. The ovarian lesions were firstly examined using ultrasound performed by a doctor having great experience in ultrasonography. The doctor performed gynecological ultrasounds in PMHCRI for many years. During this time, he saw different cases of endometriosis and other gynecological diseases. That gives him the qualification to diagnose even small endometrial lesions, difficult to see for others. However, the diagnosis was confirmed during operation and finally in histopathological examination.

Samples

Microvesicles (MVs) were determined in samples of 5 mL blood and samples of 5 mL peritoneal fluid. The blood samples were collected upon admission while taking the blood sample for preoperative test. The fluid was collected from the peritoneal cavity during the g operation. Blood and peritoneal fluid samples were dispensed into tubes containing trisodium citrate solution serving as

Table 1. Distribution of patients in test and control groups

All patients		PFP	Peritoneal fluid	PFP and peritoneal fluid
Test group	35	23	19	7
Control group	12	7	8	3
Both group	47	30	27	10

PFP — platelet free plasma

an anticoagulant. Blood samples were collected through a 16-gauge needle (S-Monovette®-Needle) into a syringe (S-Monovette® 5ml, Citrate 3.2% (0.106 mol/L). Peritoneal fluid samples were collected at the beginning of operations. Fluid from the Douglas pouch was collected through a laparoscopic needle into a sterilized syringe. It was done after abdominal cavity insufflation when a camera and trocars were put through the abdominal wall. Fluid from the sterilized syringe was put into a blood collecting tube with 3.2% NaCitrate (5 mL volume). Samples of peritoneal fluid were not collected from all patients undergoing operations because either the fluid was not found, or its amount was too small to collect.

Thirty samples of plasma and 27 samples of peritoneal fluid were collected. Plasma samples were collected from 23 patients with endometriosis (test group) and seven patients with teratoma (control group); twenty-seven peritoneal fluid samples were collected: 19 in the test group and 8 in the control group. Both peritoneal fluid and plasma were collected from 10 patients (7 in the test group and 3 in the control group). Table 1 presents the distribution of patients the in test and control group.

MVs isolation and samples storage

Samples of blood and peritoneal fluid have undergone the process of getting platelet-free plasma (PFP)/ /platelet-free peritoneal fluid. The method was developed in the Central Research Laboratory (CoreLab) of Medical University in Lodz and is based on previously published studies [60, 61]. The first stage was performed in PMHCRI. The samples underwent centrifugation to remove platelets, erythrocytes, and other large fragments, which could interfere with subsequent analysis. The platelet free plasma (PFP) was prepared from a solution of anticoagulant and blood through singular centrifugation at 3000xg for 15 minutes in angle-headed rotor. The centrifugation to isolate MVs was performed in less than thirty minutes after blood and peritoneal fluid collection. PFP was collected from sediment and stored in special tubes for freezing. Samples of peritoneal fluid with anticoagulants have undergone the same process of centrifugation. Supernatant formed during centrifugation was collected as platelet-free peritoneal fluid. It was stored in tubes fit to freeze at minus 40° Celsius. All samples (PFP/platelet free peritoneal fluid) were frozen at minus 40° Celsius after centrifugation and stored for subsequent analysis.

The second stage of MV's isolation was performed in the Central Research Laboratory of Medical University in Lodz. Frozen samples of PFP and platelet-free peritoneal fluid were transported in an icebox to protect them from thaw and put at the same temperature (–40°C). Frozen aliquots (2 mL) were thawed at ambient temperature for 20–30 minutes and mixed well but delicately to limit the possible extent of *in vitro* MVs generation. Different times and speeds were tested to choose the optimal conditions. Data was not shown, due to the large number of them. Moreover, their presentation will not improve the value of our publication. Optimal conditions did not cause substantial loss of MVs but let pellet bigger debris. From each sample, portions of 2 × 650 µL were taken and centrifuged at 1000 × g for five minutes in a commonly used tabletop centrifuge (Eppendorf). To prepare the sample for further processing, 500 µL of supernatant was taken from each portion, then combined and mixed gently in one Eppendorf tube. As the next step, 100 µL aliquots of isolated MVs were used.

Antibodies and incubation

The study used six types of antibodies. Annexin V was chosen as a substance commonly used to stain MVs in previous research. One of the study assumptions was finding MVs revealed from ectopic endometrium cells (endometriosis focus). Antibodies for CD105, CD146, and cytokeratin (CK 18) were used to confirm this thesis. Those structures were found on the endometrial cell surface. Antibodies for VEGF and MMP-9 were the last group. Their presence inside microvesicles could confirm MVs' role in angiogenesis — one of the main processes observed in endometriosis development.

Aliquots of microvesicles (100 µl) were incubated at room temperature with proper antibodies for 25–30 minutes, protected from light. Three sets of antibodies mixtures were prepared:

- Set 1: Annexin V (5 µL + 13 µL buffer), CK 18 (10 µL), MMP — 9 (2 µL) and VEGF (10 µL);
- Set 2: Annexin V (5 µL + 13 µL buffer), CD 105 (10 µL), MMP — 9 (2 µL) and VEGF (10 µL);
- Set 3: Annexin V (5 µL + 13 µL buffer), CD 146 (10 µL), MMP — 9 (2 µL) and VEGF (10 µL).

Stained samples were analyzed after setting the correct conditions.

Three sets of four monoclonal antibodies were created to reduce possible interference. The sets (set 1, set 2, set 3) differed in the molecule found on the surface of endometrial cells (CK18, CD105, CD146, respectively) (Tab. 2). Objects with a different arrangement of monoclonal antibodies were

Table 2. Sets of four monoclonal antibodies	
Set 1	CK 18 + AnnexinV + VEGF + MMP-9
Set 2	CD 105 + AnnexinV + VEGF + MMP-9
Set 3	CD 146 + AnnexinV + VEGF + MMP-9

CK — cytokeratin; VEGF — vascular endothelial growth factor; MMP-9 — metalloproteinase-9

Table 3. Different confirmation of monoclonal antibodies in set 1, set 2, set 3

Set 1	Set 2	Set 3
VEGF+/MMP-9+	VEGF+/MMP-9+	VEGF+/MMP-9+
All+(AnnexinV+/CK18+)	All+1(AnnexinV+/CD105+)	All+-1(AnnexinV+/CD146+)
AnnexinV+/MMP-9+	AnnexinV+/MMP-9+	AnnexinV+/MMP-9+
Allq+(VEGF+/CK18+)	Allq+-1(VEGF+/CD105+)	Allq+-1(VEGF+/CD146+)
AnnexinV+/CK18+	AnnexinV+/CD105+	AnnexinV+/CD146+
VEGF+/CK18+	VEGF+/CD105	VEGF+/CD146
CK18+/MMP-9+	CD105+/MMP-9+	CD146+/MMP-9+

CK — cytokeratin; VEGF — vascular endothelial growth factor; MMP-9 — metalloproteinase-9

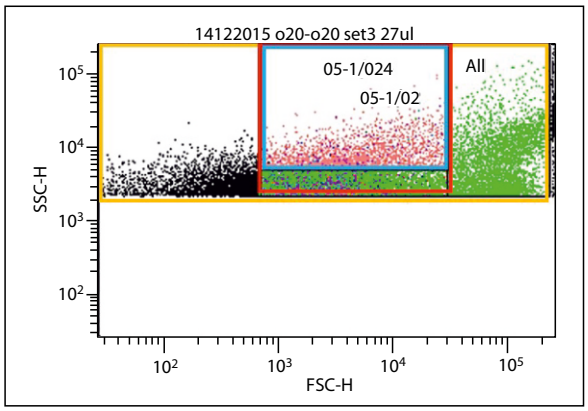

Figure 1. Two-dimensional scatter plot; SSC-H — side scatter height; FSC-H — forward scatter height

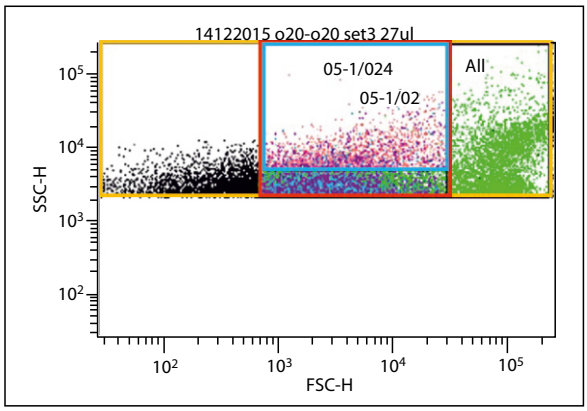

Figure 2. Two-dimensional scatter plot; SSC-H — side scatter height; FSC-H — forward scatter height

counted in each set (Tab. 3). Moreover, objects positively for single monoclonal antibodies were counted.

Flow cytometry

FACSCanto II Biosciences cytometer was used to analyze MVs in peripheral blood and peritoneal fluid collected from women with benign ovarian lesions (endometriosis and teratomas). Proper gating was set up for structures as small as MVs. Specialized beads were used to achieve it (Megamix — SSC Biocytex). They had a similar size to microvesicles. Their size was ranging from 0.1 µm to 1 µm. MVs stained by monoclonal antibodies were analyzed after setting the correct gate. The obtained data were quantified and plotted using the BD FACSDiva software (Becton Dickinson). Data on events ranging in size from 0.22 µm to 0.24 µm (220–240 nm) were analyzed statistically. The obtained data were quantified and plotted. An example of a two-dimensional scatter plot is presented below (Fig. 1 and 2), and the explanation is in Table 4.

Table 4. Explanation of two-dimensional scatters plot	
All	Rectangle with yellow frame shows all events detected by flow cytometry
05-1/024	Rectangle with blue frame shows events in size bigger than 0.24 µm (240 nm)
05-1/02	Rectangle with red frame shows events in size from 0.20 µm (200 nm)

Statistical analysis

STATISTICA was used to analyze obtained results. Median, 1st, and 3rd quartiles were counted for each type of mark in individual sets (set 1, set 2, set 3) and in each subgroup of patients. Median gave the average value in patients groups, whereas 1st and 3rd quartiles measured the scatter of

results. Mann–Whitney's test was used to compare both groups of patients. The null hypothesis was that analyzed data were from the same population or population with identical medians. p Values lower than 0.05 allowed to reject the null hypothesis with a significance level of 0.05 and accept the alternative theory that the medians in the groups are different.

Moreover, the correlation of MVs amount in plasma and peritoneal fluid was checked. The clustering of patients within groups (plasma and peritoneal fluid) was performed to show the cytometry results between groups. Logistic regression was used to predict from MVs profile whether the patient was in the test or control group.

RESULTS

Cytometry analysis confirmed MVs' presence in plasma and peritoneal fluid collected from patients with endometriosis and teratomas. A statistically significant higher level of AnnexinV (+) MVs were observed in plasma samples of endometriosis patients (Fig. 3). In the teratoma group, there was a significant percentage of double-positive VEGF (+)/ /MMP-9 (+) (Fig. 4) and single MMP-9 (+) positive MVs in the serum (Fig. 5). The error bars, in Figures 3 to 5, shoved standard deviation. Moreover, the above differences are summarized in Table 5.

In the peritoneal fluid higher frequency of double-positive VEGF (+)/MMP-9 (+) MVs were found in the control group. However, the amount of VEGF (+)/MMP-9 (+) MVs object did not differentiate between the test and control groups (Fig. 6). The above differences are summarized in Table 6.

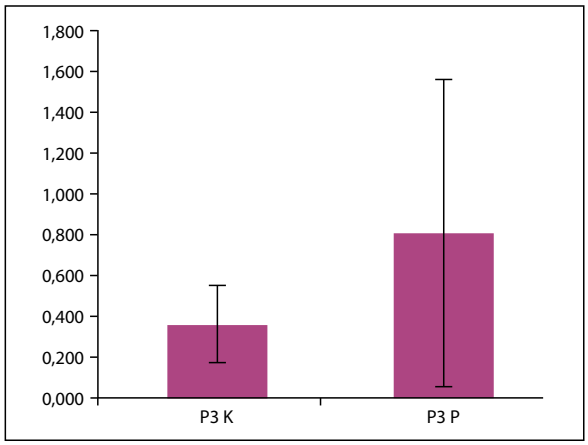

Figure 3. Objects Annexin V+ in plasma in test and control group for p = 0.03. The P3 K objects Annexin V positive detected in plasma in the control group, P3 P objects Annexin V positive detected in plasma in the test group

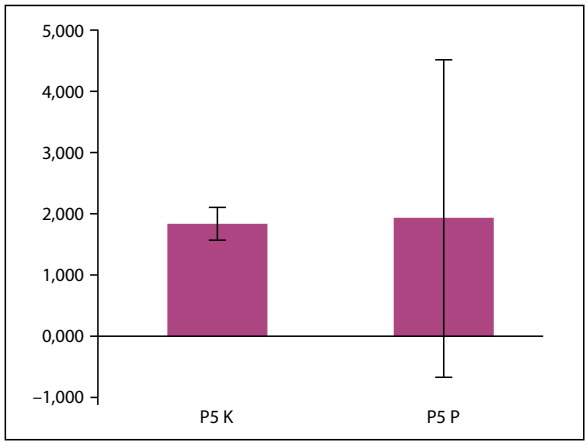

Figure 5. Objects metalloproteinase-9 (MMP-9)+ in plasma in test and control group p = 0.02. P5 K objects MMP-9 positive detected in plasma in the control group, P5 P objects MMP-9 positive detected in plasma in the test group

DISCUSSION

The study was the first in which MVs were confirmed in plasma and peritoneal fluid in benign conditions like endometriosis and teratoma. Different MVs are found in both physiological conditions and healthy bodies and diseases [62–65]. Studies in which MVs have been analyzed in gynecological disorders are not common. Researchers focused mostly on MVs present in ascites that accompany ovarian cancer, not on the MVs population in the benign lesions.

It is worth mentioning that the study group was the biggest group of patients with gynecological diseases, in which MVs were studied. Despite the fact, that the strict exclusion criteria influenced negatively the study participants numbers. Ten patients were excluded because of abnormal levels of thyroid gland hormones. Further tests confirmed hypothyroidism or Hashimoto's disease. MVs increase is observed in autoimmunological diseases

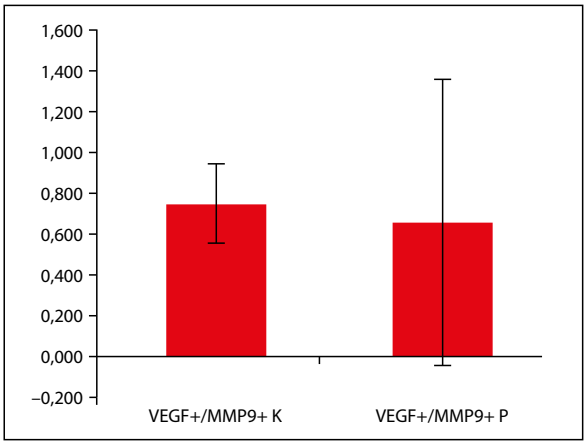

Figure 4. Objects vascular endothelial growth factor (VEGF)+/ /metalloproteinase-9 (MMP-9)+ in plasma in test and control group p = 0.03. VEGF+/MMP-9+ K objects VEGF and MMP-9 positive detected in plasma in the control group, VEGF+/MMP-9+ P objects VEGF and MMP-9 positive in plasma in the test group

Table 5. All data of counted Median, 1st, and 3rd quartiles and results. Mann–Whitney's test (plasma)

	Control group (N = 7)	Test group (N = 23)	P (Whitney's Mann test)
	Median (1st oraz 3rd quartile)		
All — events	144497.33 (108630.89–254163.11)	225603.44 (144497.33–398578.11)	0.2594
05-1/022 — events	100807.67 (67197.00–228570.89)	142806.44 (101802.78–353579.89)	0.3774
All — parent%	79.21 (74.81–83.17)	82.60 (76.73–87.59)	0.5238
05-1/022 — parent%	78.72 (71.41–86.81)	73.17 (59.51–85.30)	0.5238
set 1 VEGF+/MMP9+ — parent%	0.73 (0.40–0.97)	0.37 (0.30–0.63)	0.0528
set 1 AnnexinV+/MMP9+ — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.7499
set 1 AnnexinV+/CK18+ — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.6239
set 1 VEGF+/CK18+ — parent%	0.23 (0.10–0.27)	0.17 (0.13–0.33)	0.9024
set 1 CK18+/MMP-9+ parent%	0.00 (0.00–0.03)	0.00 (0.00–0.03)	0.3268
set 1 VEGF+/CD105? — parent%	0.10 (0.00–0.10)	0.00 (0.00–0.07)	0.1056
set 1 CD105?+/MMP-9+ — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.9804
set 2 VEGF+/MMP9+ — parent%	0.70 (0.40–0.87)	0.45 (0.33–0.60)	0.1938
set 2 AnnexinV+/MMP9+ — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.8831
set 2 AnnexinV+/CD105+ — parent%	0.00 (0.00–0.10)	0.00 (0.00–0.00)	0.2203
set 2 VEGF+/CD105 — parent%	0.10 (0.07–0.17)	0.10 (0.00–0.10)	0.2112
set 2 CD105+/MMP-9+ — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.9804
set 3 VEGF+/MMP9+ parent%	0.73 (0.60–0.90)	0.47 (0.37–0.65)	0.0350
set 3 AnnexinV+/MMP9+ parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.8831
set 3 AnnexinV+/CD146+ parent%	0.00 (0.00–0.13)	0.00 (0.00–0.00)	0.4620
set 3 VEGF+/CD146 parent%	0.27 (0.20–0.37)	0.20 (0.13–0.30)	0.1698
set 3 CD146+/MMP-9+ parent%	0.00 (0.00–0.10)	0.07 (0.00–0.10)	0.3515
set 1-3 AnnexinV+/VEGF+ — parent%	0.11 (0.10–0.17)	0.16 (0.11–0.31)	0.1938
P1 — CK18 — parent%	0.93 (0.80–1.23)	0.97 (0.53–1.50)	0.9609
P2 — VEGF — parent%	38.57 (22.71–41.37)	25.63 (23.44–34.19)	0.0861
P3 — AnnexinV — parent%	0.36 (0.20–0.50)	0.49 (0.41–0.87)	0.0310
P4 — CD105 — parent%	0.23 (0.20–0.27)	0.13 (0.10–0.27)	0.1005
P4 — CD146 — parent%	0.77 (0.70–1.00)	0.73 (0.63–1.07)	0.8254
P5 — MMP9 — parent%	1.83 (1.58–1.97)	1.31 (1.03–1.70)	0.0273

CK — cytokeratin; VEGF — vascular endothelial growth factor; MMP-9 — metalloproteinase-9

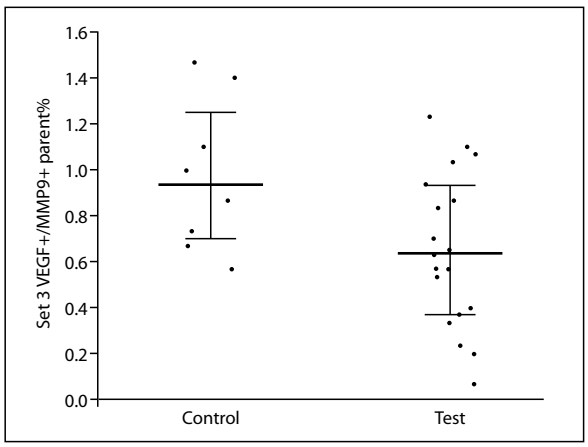

Figure 6. Double-positive vascular endothelial growth factor (VEGF)+/ /metalloproteinase-9 (MMP-9)+ objects in test and control groups

[66–68], kidney disorders [69], and vascular diseases [70]. MVs take part in immunological response through antigens transport and presentation [71–72]. Microvesicles are also described as proinflammation mediators [71]. Based on those facts, patients with endocrine disorders and infections were not qualified for the study. Microvesicles are also observed in cardiological problems, such as hypertension or atherosclerosis disease. MVs with procoagulant features are released from monocytes and can induce a formation of an atherosclerotic plaque [73–75]. MVs have influenced deterioration of the renal function in patients with hypertension [75–77].

We hoped to collect peritoneal fluid from every operated patient, but the peritoneal fluid was not found in every case. This fact made it impossible to check correlations between MVs numbers in peritoneal fluid and peripheral

Table 6. All data of counted Median, 1st, and 3rd quartiles and results. Mann–Whitney's test (peritoneal fluid)

	Grupa: kontrola (N = 8)	Grupa: badana (N = 19)	P (Whitney's Mann test)
	Median (1st and 3rd quartile)		
All — events	248201.06 (155898.61–400354.56)	325321.78 (201410.44–358509.56)	0.6139
05-1/022 — events	236642.56 (146211.89–358450.83)	314261.78 (183076.22–354250.44)	0.4735
All — parent%	95.93 (93.41–96.46)	94.14 (92.49–95.70)	0.2536
05-1/022 — parent%	93.69 (88.50–95.39)	96.60 (91.16–97.54)	0.1594
set 1 VEGF+/MMP9+ — parent%	0.72 (0.64–0.93)	0.67 (0.47–0.90)	0.3810
set 1 AnnexinV+/MMP9+ — parent%	0.00 (0.00–0.02)	0.00 (0.00–0.00)	0.5592
set 1 AnnexinV+/CK18+ — parent%	0.10 (0.07–0.18)	0.00 (0.00–0.10)	0.0594
set 1 VEGF+/CK18+ — parent%	4.87 (2.70–7.73)	3.47 (0.93–7.53)	0.5592
set 1 CK18+/MMP-9+ parent%	0.23 (0.15–0.35)	0.10 (0.00–0.27)	0.1371
set 1 VEGF+/CD105? — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.9788
set 1 CD105?+/MMP-9+ — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.9788
set 2 VEGF+/MMP9+ — parent%	0.85 (0.65–1.00)	0.57 (0.37–0.90)	0.0670
set 2 AnnexinV+/MMP9+ — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.9788
set 2 AnnexinV+/CD105+ — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.9788
set 2 VEGF+/CD105 — parent%	0.08 (0.03–0.10)	0.03 (0.00–0.10)	0.2648
set 2 CD105+/MMP-9+ — parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.9788
set 3 VEGF+/MMP9+ parent%	0.93 (0.70–1.25)	0.63 (0.37–0.93)	0.0384
set 3 AnnexinV+/MMP9+ parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.9788
set 3 AnnexinV+/CD146+ parent%	0.00 (0.00–0.00)	0.00 (0.00–0.00)	0.9788
set 3 VEGF+/CD146 parent%	0.50 (0.38–0.68)	0.30 (0.20–0.47)	0.1112
set 3 CD146+/MMP-9+ parent%	0.10 (0.10–0.12)	0.10 (0.00–0.10)	0.0526
set 1-3 AnnexinV+/VEGF+ — parent%	0.34 (0.25–0.56)	0.17 (0.14–0.42)	0.1172
P1 — CK18 — parent%	10.15 (7.23–15.99)	6.80 (2.83–12.43)	0.1933
P2 — VEGF — parent%	52.76 (42.99–54.24)	46.08 (34.78–57.98)	0.3810
P3 — AnnexinV — parent%	0.65 (0.45–0.87)	0.40 (0.28–0.61)	0.1053
P4 — CD105 — parent%	0.12 (0.10–0.17)	0.10 (0.10–0.13)	0.4105
P4 — CD146 — parent%	1.28 (0.92–1.45)	0.77 (0.60–1.37)	0.1757
P5 — MMP9 — parent%	1.54 (1.27–1.92)	1.17 (0.86–1.91)	0.1302

CK — cytokeratin; VEGF — vascular endothelial growth factor; MMP-9 — metalloproteinase-9

blood. Moreover, peritoneal fluid contaminated by blood or chocolate cysts could not be used.

First centrifugation of blood and peritoneal fluid samples was performed within 30 minutes from samples collection. Time range was vital because we wanted to analyze MVs presented in blood and peritoneal fluid at the moment of collection. Prior studies showed that MVs are released from activated cells *in vitro* [78]. Moreover, the correlation between MVs amount and time from collection of blood samples was observed [60]. The same assumption was made for the collection of peritoneal fluid samples. Microvesicles were not analyzed in peritoneal fluid in non-oncological diseases previously. Studies were performed on specimens collected from patients with ascites. The composition of ascites fluid differs from the peritoneal fluid. The differences are shown in cells, proteins, macro, and microelements [79,80]. Moreover, the peritoneal fluid composition is different from blood. It contains cells not observed in the blood, such as endometrial cells, adipocytes, and mesothelial cells.

The centrifugated samples were frozen to minus 40°C, which allowed safe storage and transport. Witwer et al. [60] froze FPF at minus 80°C, but Jayachandran et al. [8] revealed that freezing in higher temperatures did not influence MVs composition and amount. Moreover, the negative effect of dehydration was avoided [61].

Choice of isolation method was not easy because methods of MVs isolation are not united and standardized [81, 82]. Many researchers tried different times and speeds of centrifugation to get the optimal amount of microvesicles. Ultracentrifuges were also used; however, they led to a new MVs generation. We wanted to avoid that. We followed the methodology described by the papers in which MVs released from platelets, leukocytes, and epithelial cells were analyzed [83–89].

Based on them, we decided to reduce the number of centrifugations after samples defrosting. More than one centrifugation did not give a statistically significant difference in MVs amount [90]. Moreover, the speed was reduced, resulting in more MVs [83]. Shah MD et al. also showed more MVs originated from epithelial cells when they used a shorter time of centrifugation [89].

In prior studies, optical and non-optical methods were used to analyze small subjects like microvesicles. More research was conducted with flow cytometry than with TEM (transmission electron microscope) and AFM (atomic force microscope). Despite TEM and AFM having higher resolution, the process of MVs isolation is more complicated and requires a restrictive condition of samples preparation [82]. In flow cytometry, it is possible to measure the intensity of scattered light and fluorescence of a singular subject in a hydrodynamically focused fluid stream [84]. In addition, the results of flow cytometry analysis are repeatable and plausible [91].

The release of MVs from endothelium and epithelium cells was confirmed previously [62–65]. Based on those facts, we assumed that endometrium had adequate abilities. MVs were stained by monoclonal antibodies for cytokeratin 18, CD 105, and CD 146, typical markers for endometrial cells. Bokor et al. analyzed the cells composition of peritoneal fluid collected from women with endometriosis. They revealed cells with cytokeratins 18, 19, and vimentin in PF [92] which explained retrograded menstruation as a cause of endometriosis. Linden et al. [33] also analyzed peritoneal fluid composition in endometriosis. They found endometrial cells positive for CK 18 in both groups of patients, one with endometriosis and the other without [93]. In this research, we observed more subjects with CK18 positive in PF in both groups of patients.

Antibodies characteristic of endometrium were chosen to mark MVs in peritoneal fluid and blood samples. The choice was made based on previous studies. Zhang et al. [94] observed the increase of CD105 on endothelial cells in small vessels in the endometrium. Moreover, the expression of CD105 and CD146 was observed in the endometrium and its stroma [94, 95]. Annexin V was widely used as a marker for microvesicles released from most types of cells in the human body [96–98]. This research suggests the presence of MVs annexin V positive but not in all sets. The absence of MVs annexin V positive is surprising and it might suggest that not all MVs contain phosphatidylserine [99–102].

Endometriosis development depends on a web of new vessels. Endothelial cells are stimulated by growth factors to proliferation, whereas enzymes change the extrauterine matrix to make more space for new vessels. Endometrium epithelium cells can release vesicles, which transfer moleculesto the endometrium, also the ectopic one too [103]. Harper et. al isolated microvesicles in samples of endometrium collected from healthy individuals and patients with endometriosis [104]. VEGF and MMP-9 as angiogenesis markers should be observed in MVs analyzed in FPF and PF collected in the test group. However, MVs stained antibodies for VEGF and MMP-9 were found in set 3 in both media collected in the control group. Moreover, more MVs with single antibodies for MMP-9 were found in the same group of patients. It could indicate intensive angiogenesis in benign ovarian lesions such as teratomas. Tao et al. [105] had similar results in their study of the VEGF/VEGFR2 trail of angiogenesis. Several studies focused on miRNAs enabledto regulate angiogenesis. The regulation is based on the influenceon VEGF-A production [106]. Higher expressionof miR16–5p, miR-138, miR-29c-3p, and miR-424–5p was shown in endometriosis [106]

The strength of the study is the strict exclusion criteria and the methodology based both on the literature and personal testing of different conditions to optimize the MVs harvest. The weakness is the fact that based on the results it was not possible to bring patients under the category of endometriosis or teratoma. Even though the groups have been the greatest studied so far, it occurs that they could be too small to show properly the differences between the groups based on the peritoneal fluid samples.

CONCLUSIONS

Microvesicles are present in peripheral blood and peritoneal fluid samples collected from patients with endometriosis and teratoma.

Microvesicles with proangiogenic factors (VEGF and MMP-9) are more commonly observed in blood and peritoneal fluid samples from patients with teratoma.

Article informations and declarations

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Conflict of interest

All authors declare no conflict of interest.

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