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МИКРОВЕЗИКУЛЫ ЛЕЙКОЦИТАРНОГО ПРОИСХОЖДЕНИЯ В ПЕРИФЕРИЧЕСКОЙ КРОВИ ПАЦИЕНТОК С НАРУЖНЫМ ГЕНИТАЛЬНЫМ ЭНДОМЕТРИОЗОМ

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Резюме. Эндометриоз – хроническое гинекологическое заболевание, которое является серьезной проблемой с точки зрения диагностики и лечения. Несмотря на десятилетия исследований, нет ни специфических признаков и симптомов, ни анализов крови для клинического подтверждения диагноза, что затрудняет своевременную диагностику и лечение. Поэтому по-прежнему остается актуальным поиск новых маркеров для ранней неинвазивной диагностики заболевания. Перспективными биологическими маркерами наружного генитального эндометриоза можно считать различные субклеточные структуры, участвующие в межклеточных коммуникациях, в частности, микровезикулы. В связи с этим, целью исследования явилась оценка состава микровезикул лейкоцитарного происхождения в периферической крови пациенток с наружным генитальным эндометриозом I-II степени и возможность их использования в качестве маркеров неинвазивной диагностики перитонеальных форм эндометриоза. В исследование вошли 97 женщин с наружным генитальным эндометриозом I-II степени в возрасте от 26 до 40 лет, диагноз у которых был установлен интраоперационно и подтвержден гистологически. У всех пациенток основной группы отмечен болевой синдром, а также у 73,2% больных выявлено бесплодие. Контрольную группу составили 20 пациенток, средний возраст которых составил 25,5±1,1 лет, обследовавшихся в связи с мужским фактором бесплодия перед проведением процедуры экстракорпорального оплодотворения, у которых на основании проведенного интраоперационного обследования не было найдено гинекологических заболеваний, а также отсутствовал болевой синдром. Перед проведением оперативного вмешательства у всех пациенток осуществлялся забор периферической крови для определения содержания микровезикул лейкоцитарного происхождения. Для выделения микровезикул использовали ранее описанный Gelderman M. и Semak J. метод. Было установлено, что для пациенток с наружным генитальным эндометриозом I-II степени характерно увеличение в периферической крови количества микровезикул с фенотипом $CD14^+$, $CD16^+$ и $CD54^+CD14^+$ в 1,1, 1,38 и 1,55 раза соответственно, а также снижение содержания

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микровезикул с фенотипом $CD45^+CD4^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ в 1,2, 4 и 1,5 раза соответственно, по сравнению с женщинами из группы контроля. Таким образом, у пациенток с наружным генитальным эндометриозом I-II степени повышение в периферической крови относительного количества микровезикул с фенотипом $CD54^+CD14^+$ выше 5,22% может служить маркером для ранней неинвазивной диагностики заболевания с чувствительностью 80,5% и специфичностью 71%.

Ключевые слова: эндометриоз, неинвазивный маркер, клетки иммунной системы, микровезикулы, моноциты, NK-клетки

MICROVESICLES DERIVED FROM LEUKOCYTES IN THE PERIPHERAL BLOOD OF PATIENTS WITH EXTERNAL GENITAL ENDOMETRIOSIS

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Abstract. Endometriosis is a chronic gynecological disease, which poses a serious problem in terms of diagnosis and treatment. Despite decades of research, there are no specific signs and symptoms and no blood tests to clinically confirm the diagnosis, which makes timely diagnosis and treatment difficult. Therefore, the search for new markers for early non-invasive diagnosis of the disease remains relevant. Various subcellular structures involved in intercellular communication, in particular, microvesicles, can be considered promising biological markers for external genital endometriosis. The aim of this work was to assess the composition of microvesicles derived from leukocytes in the peripheral blood of patients with stage I-II of external genital endometriosis and the possibility of their use as markers of non-invasive diagnosis of peritoneal forms of endometriosis. The study involved 97 women aged 26-40 with stage I-II of external genital endometriosis, whose diagnosis was established intraoperatively and confirmed histologically. Pain syndrome was noted in all patients of the main group, with infertility also detected in 73.2% of the patients. The control group consisted of 20 patients, whose average age was 25.5±1.1 years, who were examined in connection with male infertility factor before the in vitro fertilization, and in whom, on the basis of intraoperative examination, presented no gynecological diseases, and no pain syndrome. Before the surgical intervention, peripheral blood was taken from all patients to determine the content of microvesicles derived from leukocytes. To isolate microvesicles, we used the previously described by M.P. Gelderman and J. Simak method. It was found that patients with stage I-II of external genital endometriosis experience an increase in the number of $CD14^+$, $CD16^+$ and $CD54^+CD14^+$ microvesicles in the peripheral blood by 1.1, 1.38 and 1.55 times, respectively, as well as a decrease in the number of $CD45^+CD4^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ microvesicles by 1.2, 4 and 1.5 times, respectively, compared with patients from the control group. Therefore, in patients with stage I-II of external genital endometriosis, an increase in the relative number of $CD54^+CD14^+$ microvesicles in the peripheral blood above 5.22% can serve as a marker for early non-invasive diagnosis of the disease with sensitivity of 80.5% and specificity of 71%.

Keywords: endometriosis, non-invasive marker, immune system cells, microvesicles, monocytes, NK cells

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Introduction

Endometriosis is a chronic inflammatory disease characterized by the migration of endometrial cells outside the uterine cavity. The main clinical manifestations include chronic pelvic pain, dysmenorrhea, dyspareunia and infertility. The latter is detected in 30-50% of cases, while in 20-25% of patients the disease is asymptomatic [50].

Patients with diverse manifestations of pain syndrome inherent in endometriosis are often treated by general physicians without proper therapy, since the symptoms of this disease may also be characteristic of other pathologies associated with chronic pelvic pain. Therefore, the gold standard for a definitive

diagnosis consists of surgical intervention followed by histological examination [2]. Under these conditions, a significant delay in diagnosis leads to 8-12 years of delayed appropriate treatment [10, 31]. At the moment, there are no reliable laboratory biomarkers for this gynecological pathology. The increased prevalence of endometriosis in women requires the development of new non-invasive diagnostic biomarkers for faster diagnosis, appropriate treatment and selection of potential patients for surgery [3, 20]. Therefore, a biomarker or a panel of biomarkers found in the biological fluids of affected patients may be an appropriate tool for diagnosing endometriosis, as well as an objective assessment of the effectiveness of treatment.

One of the new biomedicine directions is the study of the phenomenon of microvesicle formation by eukaryotic cells and their role in intercellular interactions. Almost all types of cells are capable of releasing microvesicles into the intercellular space after their activation or death due to apoptosis. Depending on their formation, microvesicles may differ in biochemical composition and biological properties [19]. They function as transporters of biologically active molecules between cells, and participate in the regulation of various processes, in particular, inflammation, hemocoagulation, vascular reactions, apoptosis and cell proliferation [24]. Endometriosis is accompanied by a chronic inflammatory reaction, which is characterized by a decrease in the activity of cytotoxic T cells and NK cells, a change in the secretion of cytokines by T helper [35] cells, and possibly the formation of microvesicles by leukocytes. Currently, there is a limited number of studies on the composition of microvesicles in the peripheral blood of patients with endometriosis. Thus, in one of the studies, higher levels of circulating microvesicles were found in patients with deep infiltrative endometriosis compared with patients with endometriomas and without this disease, which can be explained by a more intense inflammatory reaction and angiogenesis in this aggressive form of endometriosis. These results show that the levels of circulating microvesicles may play a role in the pathophysiological mechanisms of deep infiltrative endometriosis [29]. The same group of authors conducted a pilot study in 2019 in patients with endometriomas. The authors showed, that the number of microvesicles present in the peripheral blood increased after removal of endometriomas by the excision method, but not when using laser ablation. The increase in the level of circulating microvesicles is temporary and it returns to the basal level three months after the removal procedure. These results show that the excision method causes a more pronounced short-term inflammatory response when compared with that resulting from laser vaporization [30]. In this regard, the study of the composition of microvesicles derived from leukocytes present in the peripheral blood of patients with endometriosis seems relevant for understanding the pathogenesis of the disease and developing new approaches to both the diagnosis of the disease itself and for monitoring the effectiveness of treatment and predicting the risk of possible relapses.

In connection with the above, **the purpose of this study** was to assess the composition of microvesicles derived from leukocytes in the peripheral blood of patients with stage I-II of external genital endometriosis and the possibility of their use as markers of non-invasive diagnosis of peritoneal forms of endometriosis.

Materials and methods

A study was conducted among 97 women aged 26-40 years (average age 29.3±1.4 years) with stage I-II of external genital endometriosis. Inclusion criteria: age 18-40 years, first-time intraoperatively verified diagnosis of stage I-II of external genital endometriosis confirmed by histological examination, the presence of pain syndrome (pain in the pelvic region, pain during menstruation, pain during sexual activity), signing of voluntary informed consent to participate in the study. Exclusion criteria: decompensation of chronic somatic diseases, acute infectious diseases or exacerbation of their chronic forms, uterine fibroids, polycystic ovarian syndrome, taking immunomodulatory and hormonal medications three months before surgery for endometriosis, acute-stage pelvic inflammatory diseases, autoimmune diseases.

All patients of the main group were diagnosed intraoperatively with external genital endometriosis, which was confirmed by histological examination. When using the r-ASRM classification in this group of patients, it was revealed that the stage I of external genital endometriosis was present in 43.3% (42) of patients, stage II of external genital endometriosis – in 56.7% (55) of patients. Pain syndrome (algodismenorrhea, pelvic pain and dyspareunia) was noted in all patients and was the most common reason women consulted a gynecologist for examination. At the same time, before the start of the therapy, complaints of chronic pelvic pain were noted in 90.7% (88) of patients with endometriosis, while algodismenorrhea occurred in 93.8% (91) of patients with endometriosis, and dyspareunia was observed in 70.1% (68) of patients with endometriosis. A combination of three types of pain occurred in 72.2% (70) of patients. A visual analog pain scale was used to assess the pain syndrome. The severity of pelvic pain in patients with stage III of external genital endometriosis was 6.8±0.3 points, algodismenorrhea – 8.2±0.4 points, and dyspareunia – 5.3±0.6 points.

The second most important clinical symptom of endometriosis was infertility, which occurred in

73.2% (71) of patients, while primary infertility was observed in 69% (49) of patients, the proportion of secondary infertility was 31% (22) of patients. The duration of primary infertility in patients with external genital endometriosis was 5.3±2.2 years, secondary – 4.5±1.8 years.

The control group consisted of 20 patients, whose average age was 25.5±1.1 years (minimum – 21.2 years, maximum – 33 years), who were examined in connection with male infertility factor before the in vitro fertilization, and in whom, on the basis of intraoperative examination, presented no gynecological diseases, and no pain syndrome.

Before the surgical intervention, peripheral blood was taken from all patients to determine the content of microvesicles derived from leukocytes. To isolate microvesicles, the method described by M.P. Gelderman and J. Simak was used [13]. All solutions for working with microvesicles were filtered in advance through an ultrafilter with a pore diameter of 0.2 microns (Corning, USA).

For long-term storage of microvesicles obtained from peripheral blood, we used the plasma cryopreservation method developed by us (patent RU 2746950). The plasma samples were stored in liquid nitrogen in a Dewar flask at -196 °C and thawed immediately. The procedure was performed in a water bath at 37 °C. Further, the resulting plasma was centrifuged for 20 minutes at 19800 g +10 °C in order to precipitate microvesicles. Further, the obtained microvesicles were washed with a cold Hanks' solution without $CaCl_2$ (Sigma, USA) containing sodium heparin at a concentration of 30 IU/mL of solution by centrifugation for 20 minutes at 19800 g + 10°C. The resulting microvesicle precipitate was resuspended in Hanks` solution (BioloT, Russia) containing 0.35% of serum albumin (Sigma, USA) and heparin sodium at a concentration of 30 IU per 1 mL of solution, and treated with antibodies to CD4, CD3, CD8, CD45, CD41a, CD14, CD54, CD56 and CD16 (R&D Systems, USA) in accordance with the manufacturer's recommendations. The expression of these markers was analyzed, both individually and in various combinations. The expression of these markers was assessed using the BD FACS Canto II flow cytofluorimeter (BD, USA). In terms of light scattering, microvesicle gate was isolated using polystyrene beads calibrated in sizes of 1.0 microns and 0.2 microns (Invitrogen, USA). Isotypic controls (R&D, USA) were used for gating microvesicles according to fluorescence indicators. Staining with CD41a antibodies was used to separate platelets and their microvesicles, the assessment of which was not part of the objectives of this study.

Statistical processing of data was carried out using nonparametric analysis methods. Accumulation, correction, systematization of the initial information and visualization of the results were carried out in Microsoft Office Excel 2007 spreadsheets. Statistical analysis was carried out using the STATISTICA v. 10.0 software (Statsoft Inc., Tulsa, USA). The critical confidence level of the null hypothesis was assumed to be equal to a probability of at least 95% ($p < 0.05$). Quantitative indicators were evaluated for compliance with the normal distribution. For this purpose, the Shapiro–Wilk's test was used (with the number of patients under study less than 50). The distribution of quantitative indicators differed from normal, and therefore the values of the median (Me), lower and upper quartiles ($Q_{0.25}$-$Q0_{.75}$) were used. The Mann–Whitney U test was used to compare independent aggregates.

Results

In the peripheral blood of patients with endometriosis, an increase in the intensity of $CD16^+$ molecules expression on the surface of microvesicles by 1.38 times was shown, as well as an increase in the relative content of microvesicles with $CD14^+$ receptor on their surface by 1.1 times compared with these indicators in patients without the disease (Figures 1, 2).

Patients with stage I and stage II of external genital endometriosis are also characterized by a decrease in the relative content of microvesicles with $CD45^+CD4^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ antigens on their surface by 1.2, 4 and 1.5 times, respectively, as well as an increase in the relative content of microvesicles with $CD54^+CD14^+$ receptors by 1.55 times compared with these indicators in patients from the control group (Figure 3).

To assess the diagnostic effectiveness of the prognostic model of the relationship between the content of $CD14^+$, $CD16^+$ and $CD54^+CD14^+$ microvesicles in the peripheral blood and external genital endometriosis, we used the method of constructing a curve of mutual dependence of the probabilities of false positive and true positive results (ROC curve). The area under the ROC curve of the corresponding relationship between the prognosis of external genital endometriosis and the content of $CD54^+CD14^+$ microvesicles in blood plasma was 0.7±0.056 with 95% CI: 0.59-0.81 (Figure 4). The resulting model corresponds to good predictive quality and is statistically significant ($p < 0.01$). The threshold value of the content of $CD54^+CD14^+$ microvesicles at the classification threshold point is 5.22%. The relative content of $CD54^+CD14^+$ microvesicles in blood plasma equal to 5.22% or exceeding this value corresponds to the prognosis of the presence of external genital endometriosis. The sensitivity and specificity of the method were 80.5% and 71%, respectively. The areas under the ROC curve corresponding to the relationship between the prognosis of external genital

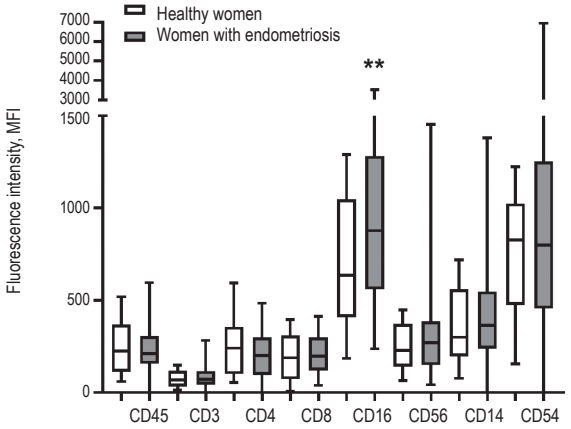

Figure 1. Intensity of surface markers expression on peripheral blood microvesicles in patients with external genital endometriosis compared with the control group

Note. **, p < 0.01, the group of patients with external genital endometriosis differs from the control group.

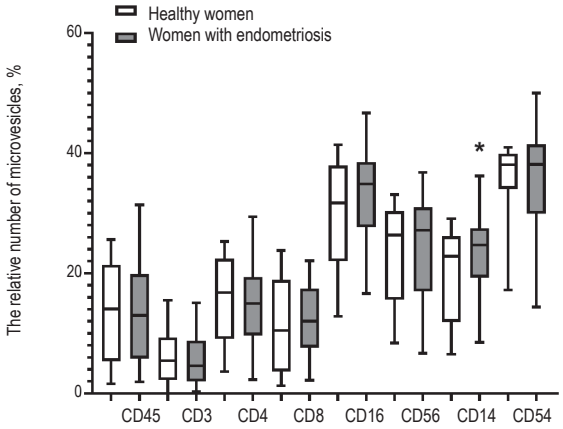

Figure 2. Relative content of microvesicles in the peripheral blood of patients with external genital endometriosis compared with patients from the control group

Note. *, p < 0.05, the group of patients with external genital endometriosis differs from the group of healthy patients.

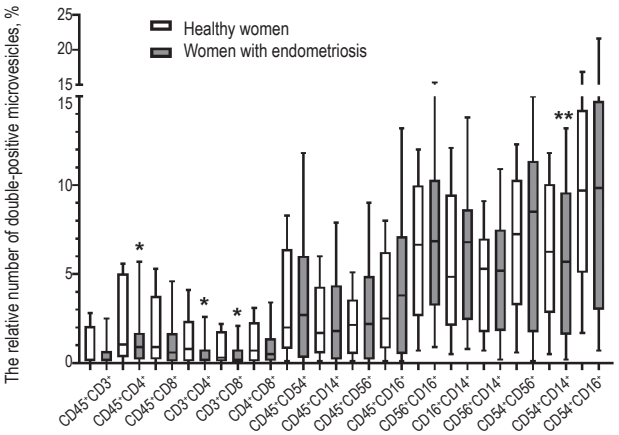

Figure 3. Relative content of microvesicles in the peripheral blood of patients with external genital endometriosis expressing various combinations of leukocyte markers compared with patients from the control group

Note. *, p < 0.05; **, p < 0.01, the group of patients with external genital endometriosis differs from the control group.

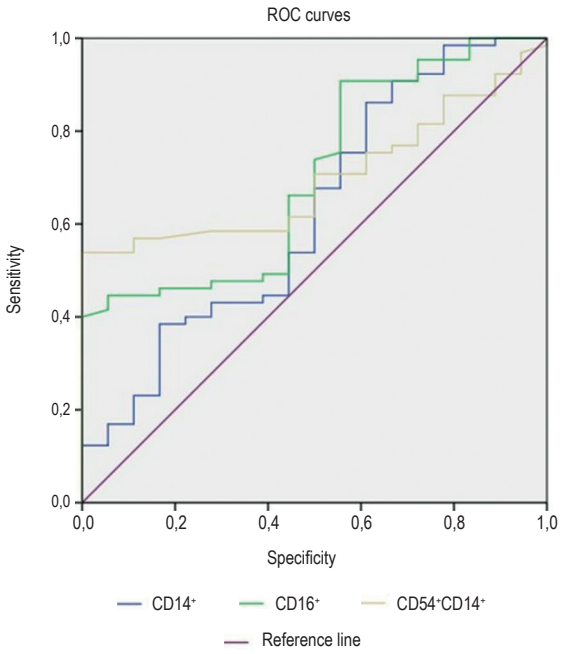

Figure 4. ROC-curves of the relationship between the content of $CD14^+$, $CD16^+$ and $CD54^+CD14^+$ microvesicles in the peripheral blood and external genital endometriosis

endometriosis and the content of $CD14^+$ and $CD16^+$ microvesicles in blood plasma were: 0.6±0.078 with 95% CI: 0.47-0.78 and 0.7±0.066 with 95% CI: 0.58-0.84, respectively (Figure 4). The obtained models also have good predictive quality, however, they are statistically insignificant.

Discussion

Microvesicles are extracellular structures surrounded by a bilipid membrane, specific transport systems capable of transferring biologically active substances and genetic material between cells [1]. At the moment, they are considered as the most important regulators of intercellular interactions [27] and participants in physiological and pathophysiological processes [5]. It is assumed that microvesicles can act as diagnostic markers indicating for various diseases [7].

The leukocyte surface markers identified in this study are not narrowly specific. Their expression is

TABLE 1. ANTIGENS ON THE SURFACE OF THE MAIN POPULATIONS OF LEUKOCYTES IN THE PERIPHERAL BLOOD

Antigens on the surface of leukocytes	Main populations of peripheral blood leukocytes, the surface of which carries these antigens
CD45	All leukocytes [34]
CD3	T lymphocytes [15], NKT cells [21]
CD4	T helper lymphocytes, regulatory T lymphocytes [6]
CD8	Cytotoxic T lymphocytes [42]
CD16	NK cells [42], monocytes [36], neutrophils [46]
CD56	NK cells, NKT cells [44], monocytes [11]
CD14	Neutrophils [16], monocytes [47]
CD54	T lymphocytes [43], monocytes [33], NK cells [45]
$CD45^+CD3^+$	T lymphocytes [38]
$CD45^+CD4^+$	T helper lymphocytes, regulatory T lymphocytes [17]
$CD45^+CD8^+$	Cytotoxic T lymphocytes [33]
$CD3^+CD4^+$	T helper lymphocytes [18]
$CD3^+CD8^+$	Cytotoxic T lymphocytes [49]
$CD4^+CD8^+$	Immature T lymphocytes
$CD45^+CD54^+$	T lymphocytes, monocytes, NK cells
$CD45^+CD14^+$	Neutrophils, monocytes
$CD45^+CD56^+$	NK cells, NKT cells [44], monocytes [11]
$CD45^+CD16^+$	Neutrophils [9], monocytes [17], NK cells [48]
$CD56^+CD16^+$	NK cells [26]
$CD16^+CD14^+$	Monocytes [23]
$CD56^+CD14^+$	Monocytes [25]
$CD54^+CD56^+$	NK cells [39]
$CD54^+CD14^+$	Monocytes [33]

characteristic of several types of cells present in the peripheral blood (Table 1).

We found that external genital endometriosis is accompanied by an increase in the number of microvesicles in the peripheral blood that express surface markers such as $CD14^+$ and $CD16^+$, as well as a combination of $CD54^+CD14^+$ antigens on their surface. Monocytes and neutrophils are the source of $CD14^+$, $CD16^+$ and $CD54^+CD14^+$ microvesicles. In addition, $CD16^+$ microvesicles can also be secreted by NK cells (Table 1).

Monocytes, neutrophils and NK cells are known to be involved in the pathogenesis of endometriosis. It was found, that the pancreas of patients with external genital endometriosis is characterized by increased neutrophil infiltration compared with patients without this disease [40]. This is probably the result of an increased concentration of a potent neutrophil chemoattractant, such as IL-8, which is present in the plasma and in the peritoneal fluid of patients with endometriosis [28]. Therefore, an increase in the number of microvesicles with surface

markers inherent in neutrophils probably indicates the activation of these cells and the severity of the inflammatory process in endometriosis.

Activated macrophages, whose precursors are monocytes, participate in the pathogenesis of endometriosis due to the secretion of soluble mediators, such as cytokines, prostaglandins, complement components and enzymes. Through the production of these immunomediators, macrophages can cause inflammation, tissue repair, neovascularization, and also promote the attraction of fibroblasts and endothelial cells [35]. Thus, the pancreatic macrophages in patients with endometriosis have an increased ability to produce MCP-1, which plays a role in attracting peripheral blood monocytes to areas of damage and inflammation [4].

The prognostic significance of the model of the relationship between the content of $CD54^+CD14^+$ microvesicles in the peripheral blood and external genital endometriosis based on the construction of the ROC curve, makes it possible to use microvesicles only with the above phenotype as a non-invasive diagnostic marker for the diagnosis of external genital endometriosis. The content of $CD54^+CD14^+$ microvesicles in blood plasma equal to or exceeding 5.22% (classification threshold point) corresponds to the prediction of the presence of external genital endometriosis with 80.5% sensitivity and 71% specificity.

NK cells are lymphocytes of innate immunity that have a cytotoxic effect against a variety of target cells and secrete cytokines, that are also involved in the pathogenesis of genital endometriosis [12]. NK cells are able to distinguish between damaged cells by expressing inhibitory and activating receptors. One of these activating receptors is CD16, which is able to bind with cells coated with immunoglobulin G (IgG) and initiate antibody-dependent cell-mediated cytotoxicity of NK cells [37]. The increased number of microvesicles with $CD16^+$ receptor in the peripheral blood of patients with external genital endometriosis indicates an increase in the content of activated NK cells. However, this does not coincide with the literature data on a decrease in the cytotoxic activity of NK cells in patients diagnosed with external genital endometriosis. It is possible, that the number of $CD16^+$ microvesicles in the peripheral blood of patients with endometriosis increases due to the activation of $CD16^+$, which belong to uterine NK cells, as part of uterine NK cells migrates from the peripheral blood. It has been shown, that the number of cytotoxic $CD16^+$ uterine NK cells in the eutopic endometrium is increased in patients with external genital endometriosis compared with fertile patients from the control group [14]. This indicates, that altered uterine NK cells in patients with endometriosis can cause an excessive inflammatory environment during implantation or decidualization, which in turn increases the risk of infertility and miscarriage [14]. This is also confirmed by our results, according to which 73.3% of patients with external genital endometriosis had primary infertility.

In the peripheral blood of patients with endometriosis, a decrease in the content of microvesicles with the $CD45^+CD4^+$ (T helpers, regulatory T lymphocytes), $CD3^+CD4^+$ (T helpers), $CD3^+CD8^+$ (cytotoxic lymphocytes) phenotypes was also detected (Table 1). T lymphocytes are a subpopulation of adaptive immunity that play an important role in the survival and proliferation of endometrial cells [32]. Studies, in which T lymphocytes were assessed in patients with endometriosis, showed a higher CD4/CD8 ratio and an increased concentration of each subgroup in the peritoneal fluid of these patients [41]. A higher concentration of T lymphocytes was detected in endometrioid heterotopias compared with eutopic endometrium, but with a similar CD4/CD8 ratio. An increase in the total number of T lymphocytes was noted in the peripheral blood of patients with external genital endometriosis, but no differences in the CD4/CD8 ratio were detected [35]. Another important subgroup of T lymphocytes are regulatory T cells (Tregs). They are considered as powerful suppressors of the immune response and are responsible for maintaining the antigen-specific tolerance and immune homeostasis [22]. The published systematic review assessed the role of Tregs in endometriosis. The authors concluded, that a higher concentration of Tregs and/or expression of their markers can be observed in the pancreas and endometrioid heterotopias of patients with external genital endometriosis compared with patients from the control group. However, there is no consensus on the concentration of Tregs in the eutopic endometrium and peripheral blood of patients with external genital endometriosis [8]. Thus, the reduced number of microvesicles secreted by T helpers, regulatory T lymphocytes and cytotoxic lymphocytes may indicate a decrease in their activation and a decrease in cytotoxic action against cells of endometrioid heterotopias.

Conclusion

The study of the phenomenon of the formation of microvesicles by eukaryotic cells in the peripheral blood of patients with endometriosis is a promising direction for understanding the pathogenesis of the disease, developing markers for its diagnosis and monitoring the effectiveness of treatment. Our results on the increase in the number of $CD14^+$ and $CD16^+$ microvesicles in the peripheral blood of patients with stage I and stage II external genital endometriosis and the decrease in the number of $CD45^+CD4^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ microvesicles inherent in

neutrophils, NK cells, T helpers, regulatory T lymphocytes, and cytotoxic lymphocytes, respectively, indicate a possible involvement of these microvesicles in the development and progression of endometriosis and require further study. Since it is precisely these cells of the immune system that play a key role in the pathogenesis of the disease by participating in the implementation of the inflammatory process. The increase in the relative number of $CD54^{+}CD14^{+}$ microvesicles in the peripheral blood of patients with stage I-II of external genital endometriosis can serve as a marker for early non-invasive diagnosis of the disease.

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