Original article

Determination of cytokines for assessment of inflammatory status at genital endometriosis

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Abstract:

Objective: The Aim of the research was to study the profile of serum cytokines in patients with genital endometriosis, as well as to evaluate the sensitivity and specificity of any single cytokine or combination of cytokines in predicting the inflammatory status in endometriosis. ***Material and methods:*** The study included 48 patients. They were divided into two groups: I group (experimental) – 28 women with genital endometriosis, II group (control) - 20 healthy non-pregnant women. For the determination of serum biomarkers (IL-6, IL-8, IL-18, TNF-α, MIF) the multiplex immunofluorescence method with XMap technology was used. ***Results:*** There were no statistical differences in IL-18 and MIF levels between the studied groups ($p = 0.630$ and $p = 0.421$). However, serum levels of IL-6, IL-8, TNF-α were significantly different (respectively, $p = 0.037$, $p < 0.001$, $p < 0.001$). In women with endometriosis the levels of IL-8 and TNF-α were increased 5 times, and level of IL-6 was increased 3 times. The diagnostic significance of IL-6, IL-8 and TNF-α levels for predicting genital endometriosis was 75.0% and 60.0%; 100.0% and 90.0%; 92.9% and 85.0%, respectively. ***Conclusions:*** The levels of IL-6, IL-8, and TNF-α were significantly higher in patients with genital endometriosis. The diagnostic significance of IL-6, IL-8 and TNF-α levels for predicting genital endometriosis was also established with the determination of the sensitivity and specificity of the model.

Keywords: endometriosis; IL-6; IL-8; IL-18; TNF; cytokines.

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Introduction:

Endometriosis is the third most common and as we can see its frequency is increasing every year. This pathology is caused by the abnormal development of endometrial-like tissues outside the inner layer of the uterus. It affects the quality of life and is considered one of the most complex diseases in gynecology. Endometrial-like tissues are most often found in the pelvis and in the peritoneal cavity. They can influence the closest organs, leading to chronic pelvic pain syndrome, infertility, and painful periods. Currently, the diagnosis of this disease is based on laparoscopic surgery with tissue sampling. Practical healthcare currently lacks specific, sensitive, non-invasive

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diagnostic tools for diagnosing this disease, taking into account the fact that not everyone is indicated for surgical intervention[1].

There are several etiological theories for the occurrence of endometriosis. The most common theory of endometriosis was presented by J. A. Sampson's hypothesis about the implantation of viable endometrial-like tissues in the pelvic area. As a result of retrograde menstruation, sloughing endometrial-like tissues enter the peritoneal cavity through the uterine tubes, attach to the peritoneum, penetrate into its epithelium, and proliferate[2]. However, this process is considered physiological, it is diagnosed in 70-90% of healthy women, and the disease develops only in 10% of cases. That is, processes occur in the body that should contribute to the overgrowth of endometrial-like tissues and the preservation of the inner layer of the uterus lesions. Therefore, much attention is currently paid to the changes in the immune system and inflammatory response in the pathogenesis of endometriosis. There is a study reporting that the peritoneal fluid of women with endometriosis contains an increased number of immune cells. They appear to increase the survival and proliferation of ectopic endometrial-like tissues by secreting various local products such as growth factors and cytokines[3]. In addition, there is evidence interleukin-1 beta (IL-1β), IL-6, IL-8, IL-10, IL-17A, and TNF-α, were increased in peritoneal fluid and blood in women with endometriosis[4,5,6]. Some authors noted that increased production of these markers in the peritoneal fluid was associated with increased levels of similar markers in the blood of patients with endometriosis[7].

It is not clear whether the observed increase of inflammatory markers is a consequence of inflammatory reactions during the disease process or one of the causes of the disease.

The objective of the research was to study the profile of serum cytokines in patients with genital endometriosis, as well as to evaluate the sensitivity and specificity of any single cytokine or combination of cytokines in predicting the inflammatory status in endometriosis.

Materials and methods:

All procedures performed in the study was conducted in accordance with the guidelines outlined in the Helsinki Declaration and its amendments. This study was approved by the Bioethics Committee of NJSC «Karaganda Medical University» (protocol №10 dated March 16, 2020). Informed consent was obtained from all participants included in the study.

The case-control study was conducted from September 2021 to January 2022 at the clinic of NJSC «Karaganda Medical University» (Karaganda, Kazakhstan). The study included 48 patients, who were divided into two groups: group I (experimental) – 28 women with genital endometriosis, operated on for specified diagnosi; group II (control) - 20 healthy non-pregnant women without clinical signs of endometriosis. Signs of endometriosis were confirmed by histological analysis. The stages of endometriosis were determined according to the r-AFS classification (American Society for Reproductive Medicine Revised American Society for Reproductive Medicine Classification of endometriosis, 1997)[8].

Criteria for inclusion into the experimental group: patients with genital endometriosis older than 18 years. Exclusion criteria for both groups: age under 18, pregnant and breastfeeding, patients with autoimmune and oncological diseases, patients with HIV-infection, patients taking drugs that affect the immune system or hormonal drugs, and patients with infectious process that occurred at least 4 weeks prior to the study.

The following data were collected for each patient: life and disease history, gynecological history, anthropometric data (weight, height, BMI), and data on age and symptom onset.

For the determination of serum biomarkers 5 ml of venous blood was taken from each patient. The samples were centrifuged at 1000g for 20 minutes with the resulting serum stored afterwards was stored at -70°C.

Assessment of cytokines. For the determination of serum biomarkers in blood serum by the immunofluorescence method using XMap technology, the Human Circulation Biomarker panel of the Milliplex Map series (manufactured by Millipor) was used. The kit included simultaneous immunofluorescence detection on magnetic spheres of the following analytes: IL-6, IL-8, IL-18, macrophage inhibitory factor (MIF), TNF-α. The sensitivity of the test was indicated according to the manufacturer's instruction.

Statistical analysis was carried out using the StatTech v. 2.6.5 (Stattech LLC, Russia). Quantitative indicators were assessed for compliance with the normal distribution using the Shapiro-Wilk test

(when the number of subjects was less than 50). Quantitative indicators with normal distribution were described using means (M), standard deviations (SD) and 95% confidence interval (95% CI). In the absence of a normal distribution, quantitative data were characterized using the median (Me) and the lower and upper quartiles (Q1 – Q3). Qualitative indicators were described with absolute values and percentages. Comparison of two groups in terms of a quantitative indicator, the distribution of which differed from the normal one, was performed using the Mann-Whitney U-test. Comparison of percentages in the analysis of four-field contingency tables was conducted using Fisher's exact test (with values of the expected phenomenon less than 10). Comparison of percentages in the analysis of multifield contingency tables was realized using Pearson's chi-square test (χ^2).

The ROC-curve analysis method was used to assess the diagnostic significance of quantitative indicators of studied biomarkers in predicting a certain outcome. The separating value of the quantitative trait at the cut-off point was determined by the highest value of the Youden's index.

Ethical clearance: All procedures performed in the study was conducted in accordance with the guidelines outlined in the Helsinki Declaration and its amendments. This study was approved by the Bioethics Committee of NJSC «Karaganda Medical University» (protocol №10 dated March 16, 2020). Informed consent was obtained from all participants included in the study.

Results:

Information about patients in the control and experimental groups is presented in Tables 1 and 2. In the control group mean age was 27 years [24; 40] in the experimental– 33 years [26; 42].

Table 1: Descriptive statistics of quantitative group variables

Index	Group	Me	$Q_1 – Q_3$	min	max	p
Age	Control group, (years)	27,0	24,0 – 40,0	21,0	43,0	0,062
	Experienced group, (years)	33,5	26,8 – 42,2	22,0	53,0	
Weight	Control group, (kg)	62,5	55,8 – 76,2	20	47,0	0,476
	Experimental group, (kg)	64,5	60,0 – 74,0	28	50,0	
Height	Control group, (cm)	1,6	1,6 – 1,7	1,5	1,8	1,000
	Experienced group, (cm)	1,6	1,6 – 1,7	1,5	1,8	
BMI	Control group	22,9	21,2 – 25,9	20	17,7	0,464
	Experienced group	24,4	21,8 – 26,5	28	19,3	
Menarche	Control group, (years)	12,0	12,0 – 13,0	11,0	14,0	0,077
	Experienced group, (years)	12,0	11,0 – 13,0	9,0	16,0	
Age of onset of sexual activity	Control group, (years)	19,0	11,2 – 22,0	-	26,0	
	Experienced group, (years)	19,5	17,8 – 22,2	-	29,0	
Age when the first signs of endometriosis appeared (years)	Control group, (years)	-	-	-	-	
	Experienced group, (years)	26,0	24,5 – 35,5	28	19,0	

There were no significant differences in age, height, weight, BMI, and menarche in the experimental and control groups. It was also not possible to compare the age of onset of sexual activity, since patients who were not yet sexually active were in both groups.

There were studies describing the role of early menarche in the development of endometriosis. Since, according to the theory of «retrograde menstruation», the young age of the onset of menarche due to earlier exposure to retrograde menstrual flow may be the cause of an increase in the volume of the endometrium in the pelvic cavity. Therefore, it may contribute to an increased risk of endometriosis. Early menarche (occurring before the age of 11-12 years) has been reported to be associated with an increased risk of endometriosis[9], although no such association was found in our study (p=0.077). This fact may be due to the small sample size.

Table 2: Descriptive statistics of categorical group variables

Index	Group	Categories	Абс.	%	p
Residence	Control group	City	19	5,0	
		Small town or community	1	95,0	
	Experienced group	City	24	85,7	0,385
		Small town or community	4	14,3	
Education	Control group	Secondary vocational	10	52,6	
		Incomplete higher	0	0	
		Higher	5	26,3	
		Post-graduate	4	21,1	
	Experienced group	Secondary vocational	8	28,6	
		Incomplete higher	4	14,3	
		Higher	13	46,4	0,092
		Post-graduate	3	10,7	
Marital status	Control group	Single	7	35,0	
		Married	11	55,0	
		Divorced	1	5,0	
		Widow	0	0,0	
		Civil marriage	0	0,0	
		Live separately	1	5,0	
	Experienced group	Single	6	21,4	
		Married	17	60,7	
		Divorced	2	7,1	0,482
		Widow	1	3,6	
		Civil marriage	2	7,1	
		Live separately	0	0	
Social status	Control group	Worker	7	35,0	
		Employee (official, soldier)	0	0,0	
		Intellectual or creative worker	12	60,0	
		Student (pupil, student)	1	5,0	
		Unemployed (housewife, pensioner, temporarily unemployed)	0	0,0	
	Experienced group	Worker	9	32,1	
		Employee (official, soldier)	3	10,7	0,031*
		Intellectual or creative worker	9	32,1	p = 0,047
		Student (pupil, student)	0	0	
		Unemployed (housewife, pensioner, temporarily unemployed)	7	25	
Place of work	Control group	State enterprise	16	80,0	
		Private enterprise or firm	3	15,0	
		Individual entrepreneurship	0	0,0	
		Other	1	5,0	
		Not working	0	0,0	
	Experienced group	State enterprise	13	46,4	
		Private enterprise or firm	8	28,6	
		Individual entrepreneurship	1	3,6	
		Other	0	0	0,049*
		Not working	6	21,4	
Infertility	Control group	Lack of infertility	20	100	
		Presence of infertility	0	0	
	Experienced group	Lack of infertility	24	85,7	
		Presence of infertility	4	14,3	0,130
Abdominal operations	Control group	Absence of abdominal operations	14	70	
		Presence of abdominal surgery	6	30	
	Experienced group	Absence of abdominal operations	19	67,9	
		Presence of abdominal surgery	9	32,1	1,000
Menstrual cycle nature	Control group	Regular	18	90	
		Not regular	2	10	
	Experienced group	Regular	22	78,6	
		Not regular	6	21,4	0,440
	Control group	Painless	11	55	
		Painful	9	45	
	Experienced group	Painless	8	28,6	
		Painful	20	71,4	0,065
	Control group	Meager	6	30	
		Moderate	13	65	
		Abundant	1	5	
	Experienced group	Meager	2	7,1	
		Moderate	21	75,0	0,068
		Abundant	5	17,9	
Endometriosis according to ICD 10	Control group	Adenomyosis	0	nan	
		Ovarian endometriosis	0	nan	
	Experienced group	Adenomyosis	12	42,9	
		Ovarian endometriosis	16	57,1	-
Stages of endometriosis	Control group	Stage I	0	nan	
		Stage II	0	nan	
		Stage III	0	nan	
	Experienced group	Stage I	5	17,9	
		Stage II	18	64,3	-
		Stage III	5	17,9	

* – differences in indicators are statistically significant (p<0.05)

It is assumed that the genetic profile, inflammation, hormonal background, menstrual cycle, prostaglandin metabolism, and immunological factors play a role in the onset and development of endometriosis[10]. From this perspective, several risk factors have been studied to suggest or support various etiological hypotheses. There were no significant differences in the indicators such as: «residence», «education», «marital status», «infertility», «abdominal operations», «menstrual cycle nature in terms of regularity, pain, quantity». Only for indicators "social status" and "place of work" statistically significant differences were found (p=0.031 and p=0.049, respectively).

Employees of intellectual or creative work (60%) dominated in the control group according to the «social status» indicator. In the experimental group, 32.1% of cases were employees of intellectual/creative labor and ordinary workers, 25% of cases were unemployed. Given the predominance of the working class in the experimental group, environmental factors such as exposure to PCBs and dioxins can be suggested as risk factors. All these factors support the potential role of hormonal levels and inflammation in the pathogenesis of endometriosis. According to «place of work» indicator, employees of state institutions (46%) and private organizations (28.6%) dominated in the experimental group, although they were 80% and 15% in the control group, respectively.

According to ICD-10, in the experimental group, 12 (42.9%) of 28 women were diagnosed with adenomyosis, 16 (57.1%) had ovarian endometriosis (external endometriosis). Almost all cases from the experimental group belonged to stage II (18-64.3%) and evenly 5 cases each (5-17.9%) belonged to stages I and III of endometriosis in accordance with the rASRM staging criteria.

Table 3 presents data on inflammatory markers considered in this study.

Table 3: Descriptive statistics of studied pro-inflammatory parameters

Index	Group	Me	$Q_1 – Q_3$	min	max	p
IL-6 (pg/mL)	Control group	0,43	0,41 – 2,98	0,38	10,23	0,037*
	Experienced group	1,43	1,18 – 6,33	0,42	15,83	
IL-18 (pg/mL)	Control group	184,89	140,69 – 225,37	111,14	386,11	0,630
	Experienced group	203,11	151,66 – 226,89	111,12	355,76	
IL-8 (pg/mL)	Control group	3,45	2,71-5,45	0,81	12,44	< 0,001*
	Experienced group	18,80	13,90 – 34,02	9,60	233,50	
MIF (pg/mL)	Control group	72,27	45,62 – 144,47	17,18	272,47	0,421
	Experienced group	74,12	35,35 – 100,06	16,76	630,07	
TNF (pg/mL)	Control group	6,54	2,23 – 16,87	1,01	28,08	< 0,001*
	Experienced group	31,75	26,48 – 43,87	15,55	51,01	

* – differences in indicators are statistically significant (p<0.05)

There were no statistical differences in IL-18 and MIF levels between the studied groups (p = 0.630 and p = 0.421). However, serum levels of IL-6, IL-8, TNF-α were significantly different (respectively, p = 0.037, p < 0.001, p < 0.001).). In women with endometriosis the levels of IL-8 and TNF-α were increased 5 times, and the level of IL-6 was increased 3 times.

Based on the results ROC-analysis was carried out to analyze biomarkers (IL-6, IL-8, TNF) diagnostic significance in predicting genital endometriosis.

The ROC-curve of the IL-6 levels to predict genital endometriosis is shown at Figure 1. The area under the ROC-curve was 0.678±0.077 (95% CI: 0.526 – 0.829). The resulting model was statistically significant (p=0.037). The threshold value of IL-6 at the cut-off point, which corresponded to the highest value of the Youden index, was 1.380 pg/mL. Genital endometriosis was predicted with IL-6 value higher than this value or equal to it. The sensitivity and specificity of this model were 75.0% and 60.0%, respectively.

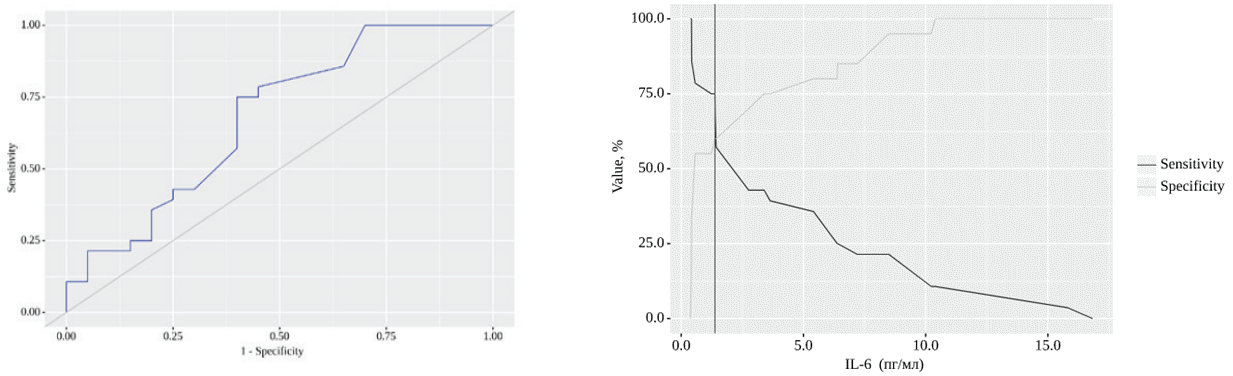

Figure 1. ROC-curve of the IL-6 level to predict genital endometriosis and sensitivity /specificity analysis of the model depending on the threshold values of IL-6

The ROC-curve of the IL-8 levels to predict genital endometriosis is shown at Figure 2. The area under the ROC-curve was 0,989±0,014 (95% CI: 0,961 – 1,000). The resulting model was statistically significant (p<0,001). The threshold value of IL-8 at the cut-off point, which corresponded to the highest value of the Youden index, was 9.600 pg/mL. Genital endometriosis was predicted with IL-8 value higher than this value or equal to it. The sensitivity and specificity of this model were 90.0% and 80.0%, respectively.

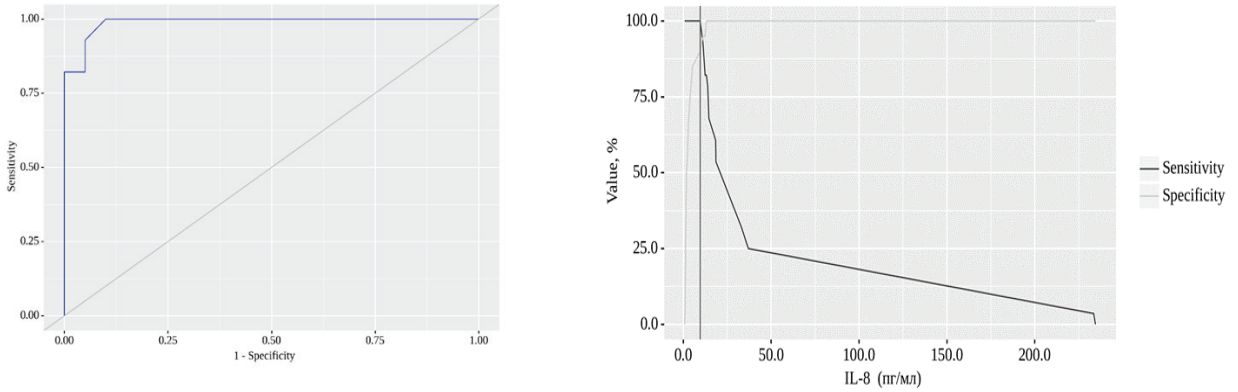

Figure 2. ROC-curve of the IL-8 level to predict genital endometriosis and sensitivity /specificity analysis of the model depending on the threshold values of IL-8

The ROC-curve of the TNF levels to predict genital endometriosis is shown at Figure 3. The area under the ROC-curve was 0,951±0,031 (95% CI: 0,890 – 1,000). The resulting model was statistically significant (p<0,001). The threshold value of TNF at the cut-off point, which corresponded to the highest value of the Youden index, was 21.190 pg/mL. Genital endometriosis was predicted with TNF value higher than this value or equal to it. The sensitivity and specificity of the model were 92.9% and 85.0%, respectively.

Discussion. Nowadays, interleukins are very widely studied in different types of diseases, especially in immunological aspect[11,12]. At the moment, many studies show that when endometriosis occurs, the following markers, such as IL-2, IL-4, IL-6, IL-8, IL-10, interferon γ (IFN-γ) and TNF-α have pathophysiological significance[13,14].

IL-6 is known to reduces endometrial cell growth, although the effect does not affect to work in endometriotic tissues. Cytokines, and in particular interleukin-6, have been studied in relation to the development of endometriosis, as well as a predictor of the disease. The next study found that serum levels of IL-1β, IL-6, and TNF-α were significantly higher in women with the genital endometriosis compared to women is the control group, and that there was no difference in serum IL-1 levels. More interestingly, a study showed that IL-1β and IL-6

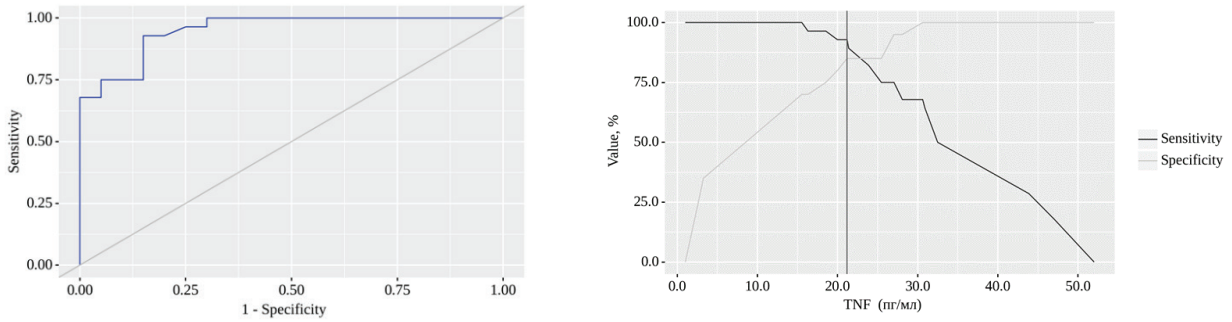

Figure 3. ROC-curve of the TNF level to predict genital endometriosis and sensitivity /specificity analysis of the model depending on the threshold values of TNF

can be used as a non-surgical diagnostic test for endometriosis[15]. Interleukin 6 is a T-cell derived cytokine produced by macrophages, lymphocytes, fibroblasts and endothelial cells. It is noted that the level of interleukin 6 is increased by macrophages in the case of endometriosis.

In the present study, we found that women with endometriosis had significantly higher serum levels of IL-6, IL-8, and TNF compared to controls. We also found that IL-18 and MIF did not significantly differ between women with and without endometriosis.

In our study the serum level of IL-6 is clearly increased in endometriosis ($p<0.037$ *) and can be considered as a new potential diagnostic marker, since the sensitivity and specificity of this model were 75.0% and 60.0%, respectively. At a cut-off point of 1.380 pg/mL, genital endometriosis was predicted.

IL-8 is a chemokine with potent neutrophil and T-cell chemotactic activity. IL-8 produced by the following cells: monocytes, macrophages, eutopic and ectopic endometrial stromal cells, as well as inflammatory cytokines such as IL-1, TNF-α, which can influence the release of this interleukin. It is known that IL-8 affects the penetration and proliferation of endometrial cells, along with increased expression of proteins that are involved in migration and invasion. This process leads to resistance to progesterone hormone of the corpus luteum. IL-8 has a feedback with apoptotic genes and proteins, thereby accelerating the growth of the lesion[16]. Our data showed the significance of IL-8 level in genital endometriosis: at a cut-off point of 9.600 pg/mL genital endometriosis was predicted. The resulting model was statistically significant ($p<0.001$). The sensitivity and specificity of the model were 100.0% and 90.0%, respectively, which may cast doubt on the performance of this model. Perhaps the results of the study turned out to be so because of the small sample size.

One of the most important factors in the development of endometriosis are mutations in the genes of cytokines. Tumor necrosis factor-alpha (TNF-α) is an important cytokine in the acute inflammatory response to infectious factors and is genetically variable. According to numerous studies, TNF-α is considered a molecular indicator of gynecological diseases. It is suggested that the inflammatory response in endometriosis is enhanced by cytokines such as TNF-α[17,18]. Galo S., Zúbor P. et al. in a prospective clinical study found statistical significance between TNF-α levels and in a group of women with endometriosis depending on the stage of the disease. At a TNF-α threshold of 30 pg/mL, sensitivity was 63.33%, specificity – 77.42%, positive predictive value – 73.07%, and negative predictive value – 68.57% [19,20]. In our study, the threshold value of TNF-α at the cut-off point was 21.190 pg/ml. Also, the sensitivity and specificity of this model were 92.9% and 85.0%, respectively.

The presented study has some limitations, since the statistical power of the study is limited (28 patients in the experimental group). However, this study assessed the diagnostic significance of serum biomarkers (IL-6, IL-8, IL-18, TNF-α, and MIF) in predicting genital endometriosis.

Conclusions:

Better understanding of the processes of inflammation and the involvement of cytokines in it helps to reveal the unclear pathophysiological and clinical aspects of genital endometriosis. This study evaluated the diagnostic value of markers in genital endometriosis. The results of the study showed that the levels of IL-6, IL-8, and TNF-α were significantly significantly higher in patients with genital endometriosis. The diagnostic significance of IL-6, IL-8 and TNF-α

levels for predicting genital endometriosis was also established with the determination of the sensitivity and specificity of the model. We believe that further study of inflammatory cytokines is necessary to optimize the diagnosis and prognosis of genital endometriosis.

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Conflict of interests. Authors declare that they have no conflict of interests.

Data Availability. Data will be available on request.

Authors's contribution.

Data gathering and idea owner of this study: LA, LA, IK,YT and ZhA

Study design: LA, LA, IK and YaT

Data gathering: LA, LA, IK,YaT and ZhA.

Writing and submitting manuscript: LA, LA and YT

diting and approval of final draft: LA, LA, IK,YT and ZhA

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