

Proliferative and Inflammatory Changes in Dogs with Pyometra Caused by *Escherichia coli*Aslıhan BAYKAL UĞUR^{1,a,✉}, Zeynep GÜNAY UÇMAK^{1,b}, Merve YILMAZ^{1,c}, Mert SARILAR^{1,d}, İbrahim KURBAN^{2,e}, Sevede İSLAMOĞLU^{3,f},
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Received

28.01.2026

Accepted

12.03.2026

Published

30.06.2026

DOI

10.47027/duvetfd.1872578

How to cite: Baykal Uğur A, Günay Uçmak Z, Yılmaz M et al. (2026). Proliferative and inflammatory changes in dogs with pyometra caused by *Escherichia coli*. *Dicle Univ Vet Fak Derg.*, 19(1):29-37.This journal is licensed under a
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Abstract

The purpose of the current study is to evaluate the proliferative changes by measuring Ki-67 levels in both the uterus and blood serum of bitches with pyometra caused by *Escherichia coli* (Group PYO; n=14) and healthy diestrous controls (Group C; n=18). The study also aims to examine the relationship between proliferative changes and hematological parameters (C-reactive protein, complete blood count, albumin, serum progesterone, monocyte-to-lymphocyte ratio, neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio). Uterine tissue Ki-67 level in Group PYO was significantly higher than in Group C (P<0.01). In Group PYO, albumin, red blood cell, hemoglobin, hematocrit, platelet, and platelet-lymphocyte ratio values were significantly lower compared to those in Group C, while an increase in C-reactive protein, white blood cell, neutrophil, monocyte-lymphocyte ratio, and neutrophil-lymphocyte ratio values was observed in Group PYO than in Group C. In conclusion, the present study showed that Ki-67 is measurable in serum samples from dogs with pyometra and healthy controls using the Enzyme-Linked Immunosorbent Assay method, and that uterine tissue Ki-67 levels can serve as an effective marker reflecting proliferative changes in canine pyometra cases. Also, decreased albumin, increased C-reactive protein and white blood cell counts, slight anemia, and changes in hematological ratios are considered predictive markers for evaluating inflammatory changes in pyometra cases in dogs.

Key Words: Canine, Ki-67, Pyometra*Escherichia coli* Kaynaklı Pyometralı Köpeklerde Proliferatif ve İnflamatuvar Değişiklikler

Öz

Bu çalışmada, *Escherichia coli* kaynaklı pyometra (Grup PYO; n=14) olan dişi köpeklerin ve sağlıklı diöstrus kontrol grubunun (Grup C; n=18) uterus dokusu ve kan serumundaki Ki-67 düzeylerini ölçerek proliferatif değişikliklerin değerlendirilmesi amaçlandı. Çalışma ayrıca proliferatif değişiklikler ile hematolojik parametreler (C-reaktif protein, tam kan sayımı, albumin ve serum progesteron ile monosit-lenfosit oranı, nötrofil-lenfosit oranı, trombosit-lenfosit oranı) arasındaki ilişkiyi incelemek amacıyla yapıldı. PYO grubunun uterus dokusunda belirlenen Ki-67 seviyesi, kontrol grubuna kıyasla daha yüksek bulundu (P<0.01). PYO grubunda, albümin, kırmızı kan hücresi, hemoglobin, hematokrit, trombosit ve trombosit-lenfosit oranı değerleri ise kontrol grubuna kıyasla anlamlı olarak daha düşük olarak belirlendi, ancak C-reaktif protein, beyaz kan hücresi, nötrofil, monosit-lenfosit oranı ve nötrofil-lenfosit oranı değerlerinde PYO grubunda C grubuna kıyasla artış gözlemlendi. Sonuç olarak, sunulan çalışma, Ki-67'nin pyometra olan köpeklerin ve sağlıklı kontrol grubunun serum örneklerinde Enzim Bağlantılı İmmünosorbent Analiz yöntemi ile ölçülebilir olduğunu ve uterus dokusu Ki-67 düzeylerinin köpek pyometra vakalarında proliferatif değişiklikleri yansıtan etkili bir belirteç olarak kullanılabileceğini göstermiştir. Ayrıca, albümin azalmasının yanında C-reaktif protein ve beyaz kan hücrelerinin artışı, hafif anemi ve hematolojik oranlardaki değişiklikler, köpeklerde pyometra vakalarında inflamatuvar değişikliklerin değerlendirilmesinde öngörücü belirteçler olarak kabul edilmektedir.

Anahtar Kelimeler: Ki-67, Köpek, Pyometra

INTRODUCTION

Cystic endometrial hyperplasia (CEH)-pyometra complex is a major reproductive pathology seen in unsprayed female dogs of middle age and older, in diestrus (1,2). Cystic endometrial hyperplasia is a subclinical disease characterized by the formation of fluid-filled cysts resulting from endometrial glandular proliferation and accumulation of their contents in the uterine lumen. Pyometra, on the other hand, has a clinical course characterized by purulent content that causes infertility and exacerbates the symptoms of the disease due to bacterial infection (3). In addition, pyometra is divided into two groups associated with cervical patency. In open cervix pyometra, clinical symptoms such as vaginal purulent discharge, depression, weakness, anorexia, vomiting, and polydipsia are observed. In closed cervix pyometra, symptoms are more severe because the discharge is not drained, and it is characterized by abdominal enlargement, dehydration, and depression (4). Diagnosis is based on anamnesis and gynecological examination, hematology and blood biochemistry analyses, abdominal ultrasonography, and/or radiographical findings (5). In veterinary medicine, hematological and biochemical abnormalities are commonly used to diagnose and monitor various diseases. Moreover, blood indices derived from hematological parameters, such as the monocyte-to-lymphocyte ratio (MLR), neutrophil-to-lymphocyte ratio (NLR), and platelet-to-lymphocyte ratio (PLR), are widely utilized to assess systemic inflammation (6,7). Although the etiology remains unknown, many factors contribute to the development of pyometra. These factors include the joint action of estrogen and progesterone (P4) during the sexual cycle, bacterial toxins produced by bacteria such as *Escherichia coli* (*E. coli*), and endometrial degeneration. As a result, this interaction leads to endometrial changes characterized by inflammatory reactions (8). Therefore, pyometra is an inflammatory disorder and involves a series of endometrial proliferative changes (9). In addition, several studies have demonstrated that proliferation and apoptosis play a role in the cyclic changes in the endometrium (10-12). Ki-67 is one of the most frequently used biomarkers for evaluating endometrial proliferation in dogs (9,11,12). Cell proliferation in the endometrium is affected by hormonal changes, and it is greater on postovulatory Day 40 than on Day 60 (13). The proliferative activity in the canine endometrium at different stages of the estrous cycle was evaluated by immunohistochemical expression of the Ki-67. As a result of this, proliferation in the canine endometrium was observed both during proestrus under estrogenic influence in the surface epithelium, stroma, blood vessels, crypts, and during estrus in the basal glands regulated by progestins (12). Immunohistochemical expression of Ki-67 in the late luteal phase was higher in dogs with pyometra than in CEH (9). In a study evaluating the tissue response to CEH-pyometra, an infectious agent identified by immunohistochemical analysis, uterine Ki-67 expression was found to increase (2).

In the present study, proliferative activity was assessed by measuring Ki-67 levels in uterine tissue and serum of bitches diagnosed with pyometra caused by *E. coli* and in healthy diestrous controls. Ki-67 levels were compared between groups, and their associations with P4, C-reactive protein (CRP), complete blood count (CBC) parameters, and hematological ratios (MLR, NLR, PLR) were analyzed. By simultaneously evaluating Ki-67 in both tissue and serum, this

study aimed to determine whether circulating Ki-67 levels reflect uterine proliferative changes and to assess the relationship between Ki-67 and systemic inflammatory and hematological alterations, thereby providing new insights into the pathogenic mechanisms of canine pyometra.

MATERIAL AND METHODS

Animals and Study Design

32 intact female dogs were included in the study. The dogs ranged in age from 2 to 13 years and weighed 5 to 22 kg. The groups were designed regardless of breed variation. Study groups were formed based on clinical examinations. Group C comprised 18 intact female dogs brought to the clinic for elective ovariohysterectomy. They were gynecologically healthy and in diestrus. The stage of estrus of all bitches was determined as reported by Maksimović et al. (14). The age and weight of the dogs in Group C ranged between 2-8 years and 5-20 kg, respectively. Group PYO consisted of 14 intact female dogs with pyometra, in which *E. coli* was isolated from the pus. They had no other gynecological pathologies on clinical examination, such as mammary tumor, ovarian tumor, or vaginal tumor, etc. The age and weight of the dogs in Group PYO ranged between 3-13 years and 5-22 kg, respectively. Ovariohysterectomy was performed for all dogs, as described by Günay et al. (15). Following ovariohysterectomy, a standard postoperative care protocol was implemented, and a postoperative medical treatment regimen, including antibiotic therapy, was prescribed (15).

Clinical Examination and Sample Collection

Diagnosis of the pyometra was based on signalement, anamnesis, vaginal inspection, vaginal cytology, and transabdominal ultrasonography. Vaginal cytology was performed for all dogs as defined by Kurban et al. (16). Staining of cytology smears was performed using the Diff-Quick stain set. Slides were examined under a light microscope at x 400 magnification (BAB-LAB, BAB, Türkiye). Transabdominal ultrasonography was performed without sedation by the same operator in both groups, using B-mode US with a micro-convex (C11-3s) transducer (Resona i9, Mindray, China). Uterine diameter, content of the uterine lumen, and ovaries were examined. CBC, red blood cell (RBC) count, haemoglobin (HGB), haematocrit (HCT), white blood cell (WBC) count, platelet (PLT) count, mean platelet volume (MPV), lymphocyte (LYM), monocyte (MONO), neutrophil (NEU) (ProCyte Dx, IDEXX Laboratories, USA), and CRP, albumin (ALB), and P4 levels (Catalyst One, IDEXX, USA) were evaluated in both groups. MLR, NLR, and PLR were calculated prior to surgery for all dogs. Additionally, 10 milliliters of peripheral blood were collected into plain tubes to obtain serum samples (Centrifuged at +4°C, 3000 rpm, 10 min). They were stored at -40°C until the Ki-67 measurements were performed.

Identification of Bacterial Agent

Samples from the luminal content of the uterus were collected with medium containing cotton swabs for isolation of the bacteriological agent. Samples for microbiological analysis were collected using sterile swabs with Cary-Blair carrier medium (Cat. 08602001, Isolab, Laborgeräte GmbH, Türkiye).

The samples were maintained in a cold chain and transported to the laboratory. The isolation was performed immediately. Swab samples delivered to the laboratory were inoculated on both nutrient agar supplemented with 5% sheep blood and MacConkey agar and incubated at 37°C for 24 hours under microaerobic conditions (17). At the end of the incubation period, Gram staining was performed on cultures that showed growth, and the Gram-specificity and microscopic morphology of the bacteria were determined. The morphologically determined colonies were purified again, and the pure Gram-negative isolates were identified using the NMIC/ID-433 test kit using a BD Phoenix M050 device (6).

ELISA Analysis

Following ovariohysterectomy, uterine tissue samples were sectioned (0.5 cm, from the middle of each uterine horn) and transferred into sterile Eppendorf tubes, and they were stored at -40°C until the ELISA analysis was performed. Before homogenization, tissue sections were weighed, and each tissue sample was cut into small pieces (100 mg). The sectioned tissues were homogenized in PBS (100 µl per 10 mg of tissue). The homogenate was centrifuged at 3000 rpm for 20 minutes. The supernatant was carefully collected and transferred to a separate Eppendorf tube. Both serum and uterine Ki-67 levels were measured by ELISA according to the manufacturer's instructions (Canine Ki-67 Protein ELISA Kit, Cat. No: MBS089640, Mybiosource, USA). The detection range of Ki-67 was 0.625 ng/ml to 20 ng/ml. The intra- and inter-assay coefficient of variations were 4.93% (n=3) and 8.45% (n=3), respectively.

Histopathological Evaluation

After tissue section samples were taken for ELISA analysis, the ovaries and uterus were examined histopathologically. Tissue samples were prepared for histopathological examination according to Enginler et al. (3). Uterine tissue samples exhibiting hyperplasia, cystic changes in glandular structures, and moderate-to-high cellular infiltration between glands and lumens, as well as pus within the uterine lumen, were classified as pyometra. The uterine tissue samples showing a thickened endometrium and oedematous stroma, with secretions in the glands, were classified as from healthy bitches at the diestrus stage (3). The histopathological evaluation was performed in a blinded manner by two pathologists.

Statistical Analysis

G*Power analysis was performed to determine the minimum sample size (G*Power 3.1.9.7 software, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany). Calculations revealed that a total sample size of n=24 would be sufficient to provide 85% effect size of 0.5, with a power of 0.05 and an α -error probability. SPSS 23.0 (IBM, USA) was utilized for statistical analysis. The Shapiro-Wilk test and Levene's test were used to assess the normality of the data and the homogeneity of variances, respectively. The comparisons of the hematological parameters and Ki-67 levels between the groups were performed by Student's T-test and Mann-Whitney U test. The Pearson correlation test was used to evaluate the relationships among the parameters. Values were given

as mean $\pm$ standard error of the mean (SEM). A P-value <0.05 was considered statistically significant.

RESULTS

The mean age of the dogs in Group PYO (8.39 ± 0.82 years) was significantly higher compared to the dogs in Group C (4.86 ± 0.41 years) ($P < 0.05$). However, the mean weight of the dogs in Group PYO (9.85 ± 1.42 kg) was not significantly different from the dogs in Group C (9.35 ± 1.09 kg) ($P > 0.05$). Group C consisted of gynecologically healthy female dogs, all of whom had proestrus bleeding approximately 45-60 days before presentation to the clinic. Bacterial growth was not observed in any samples collected from the uterus of the bitches in Group C. Only 14 intact female dogs with pyometra, in which *E. coli* was isolated from the pus, were included in the Group PYO. Hematometra, mucometra, and hydrometra cases were excluded from the study. However, loss of appetite, fever, abdominal enlargement (4 of 14 dogs), pussy vaginal discharge (10 of 14 dogs), polyuria, polydipsia, and vomiting were observed in Group PYO.

Clinical Examination Findings (Vaginal cytology, transabdominal ultrasonography)

During vaginal examination, purulent vaginal discharge indicative of open cervix pyometra was observed in 10 dogs of the Group PYO, whereas the remaining four dogs exhibited the closed cervix form of pyometra. All dogs had clustered intermediate cells on vaginal cytology, whereas neutrophils were dense in Group PYO. Also, the surface of cytology smears was not clean due to the presence of pus in dogs with open cervix pyometra. In Group C, the clustered intermediate cells less neutrophils existed while no evidence of pus was observed in smear samples, and animals confirmed to be in the diestrus phase based on recorded serum progesterone concentrations were included in the control group, as defined by Concannon et al. (18). Transabdominal ultrasonography revealed that enlargement of uterine diameter and increased thickness of the uterine wall were observed in Group PYO while they were within normal limits in diestrus female dogs in Group C. The widest cross-sectional uterine diameter in Group PYO (2.69 ± 0.53) was considerably higher compared to the dogs in Group C (0.72 ± 0.04) ($P < 0.001$). Group PYO consisted of dogs from which *E. coli* was isolated from the uterine contents, while no bacteria grew from the uterine contents of dogs in Group C.

Histopathological Evaluation Findings

In dogs diagnosed with pyometra, the uterine lumen was extensively occupied by neutrophils and abundant purulent exudate, largely originating from degenerated epithelial cells. Within the endometrium, there was a prominent inflammatory infiltrate composed mainly of neutrophils and lymphoplasmacytic cells. The endometrial glands exhibited moderate to severe cystic dilatation, frequently containing intraluminal neutrophilic exudate, together with hyperplastic and degenerative alterations of the glandular epithelium (Figure 1(a)-(b)-(d)). Stromal lymphoplasmacytic infiltration was consistently evident. Moreover, the myometrium demonstrated diffuse neutrophilic and lymphoplasmacytic in-

filtration (Figure 1(c)), indicating that the inflammatory response extends beyond the endometrium and highlighting the severity of the lesion. In contrast, the uterus of healthy diestrus bitches showed a thickened endometrium with well-developed glands lined by simple columnar epithelium.

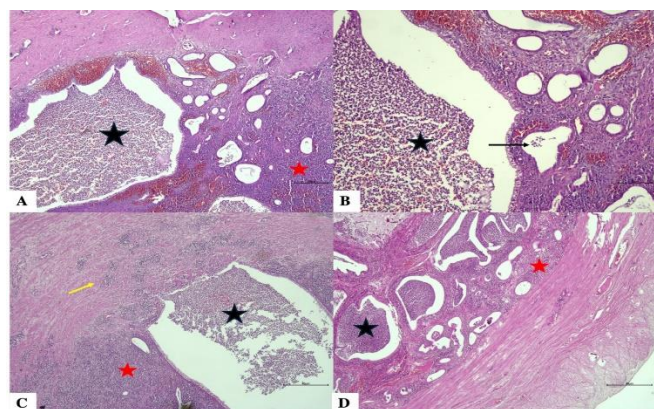


Figure 1. Pyometra in a dog. A-B: Diffuse neutrophilic and lymphoplasmacytic infiltration in the endometrium (red star), marked luminal neutrophil accumulation (black star), moderate glandular dilatation (black arrow) (H&E, bar: 30 μ m) C: Neutrophilic and lymphoplasmacytic infiltration in the myometrium (yellow arrow), lymphoplasmacytic infiltration in the endometrium (red star) and luminal neutrophil accumulation (black star) (H&E, bar: 40 μ m)

Evaluation of Hematological Parameters and Ki-67 Levels

The comparison of the groups with respect to hematological parameters, serum, and Ki-67 levels in uterine tissue is presented in Table 1. Significantly lower ALB ($P<0.001$), RBC ($P<0.001$), HGB ($P<0.001$), HCT ($P<0.001$), PLT ($P<0.01$), and PLR ($P<0.01$) levels were determined in Group PYO compared to Group C. However, considerably higher CRP ($P<0.001$), WBC ($P<0.001$), MON ($P<0.001$), NEU ($P<0.001$), MLR ($P<0.001$), P₄ ($P<0.01$), and uterine Ki-67 ($P<0.01$) levels were detected in Group PYO relative to Group C. Although serum Ki-67 values could be measured in both groups, the

In the late diestrus stage, stromal edema and secretory activity within the glands (Figure 2(a)-(b)) were clearly observed, findings consistent with the physiological influence of P₄ on the uterine tissue.

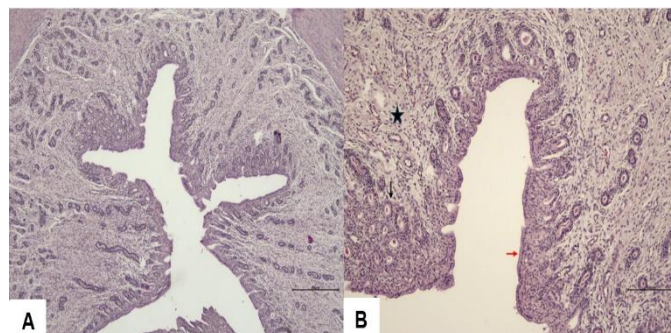


Figure 2. Diestrus stage in the canine uterus. A-B: Prominent endometrial glands (black arrow), stromal edema in the late phase (black star) and simple columnar epithelium lining the lumen (red arrow) are observed (H&E, bar: 40 μ m).

difference was not statistically significant ($P>0.05$). The relationships between the evaluated parameters in Group C and Group PYO were presented separately in Tables 2 and 3. In Group C, serum Ki-67 levels were moderately correlated with PLR ($r=0.657$, $P<0.01$), MPV ($r=-0.488$, $P<0.05$), and CRP ($r=-0.757$, $P<0.001$). Also, uterine Ki-67 levels in Group C were inversely associated with CRP ($r=-0.607$, $P<0.05$). In Group PYO, serum Ki-67 levels were moderately correlated with NLR ($r=0.683$, $P<0.01$), CRP ($r=0.554$, $P<0.05$), and uterine diameter ($r=0.586$, $P<0.05$). However, uterine tissue Ki-67 levels in Group PYO were not related to any of the evaluated parameters.

Table 1. The comparison of the groups related to the hematological parameters, serum and tissue Ki-67 levels.

Parameters	Group PYO (n=14)	Group C (n=18)	P
ALB (g/dL)	2.83 $\pm$ 0.09 ^a	3.29 $\pm$ 0.07 ^{b***}	<0.001
CRP (mg/dL)	7.55 $\pm$ 1.00 ^a	0.74 $\pm$ 0.09 ^{b***}	<0.001
WBC (K/ μ L)	53.1 $\pm$ 8.15 ^a	10 $\pm$ 0.41 ^{b***}	<0.001
RBC (M/ μ L)	5.59 $\pm$ 0.28 ^a	6.91 $\pm$ 0.11 ^{b***}	<0.001
HGB (g/dL)	13.3 $\pm$ 0.7 ^a	17.3 $\pm$ 0.33 ^{b***}	<0.001
HCT (%)	35.2 $\pm$ 1.72 ^a	47.1 $\pm$ 0.8 ^{b***}	<0.001
PLT (K/ μ L)	147 $\pm$ 31.7 ^a	264.9 $\pm$ 20.9 ^{b*}	0.003
MPV (fL)	10.3 $\pm$ 1.00 ^a	9.97 $\pm$ 0.38 ^a	0.458
LYM (10 ⁹ /L)	2.58 $\pm$ 0.79 ^a	3.09 $\pm$ 0.33 ^a	0.071
MONO (10 ⁹ /L)	4.91 $\pm$ 0.72 ^a	1.21 $\pm$ 0.36 ^{b***}	<0.001
NEU (10 ⁹ /L)	49.8 $\pm$ 7.83 ^a	6.19 $\pm$ 0.4 ^{b***}	<0.001
MLR	2.58 $\pm$ 0.46 ^a	0.36 $\pm$ 0.09 ^{b***}	<0.001
PLR	69.8 $\pm$ 46.3 ^a	106.5 $\pm$ 16.2 ^{b*}	0.002
NLR	51.4 $\pm$ 20.3 ^a	2.68 $\pm$ 0.42 ^{b***}	<0.001
P ₄	37.7 $\pm$ 4.6 ^a	23.8 $\pm$ 0.9 ^b	0.005
Uterine Diameter	2.69 $\pm$ 0.53 ^a	0.72 $\pm$ 0.04 ^{b***}	<0.001
Ki-67 (Tissue)	6.16 $\pm$ 0.23 ^a	5.37 $\pm$ 0.12 ^{b*}	0.004
Ki-67 (Serum)	3.30 $\pm$ 0.56 ^a	2.77 $\pm$ 0.59 ^a	0.180

Table 2. Association between the evaluated parameters in Group C

Association between the evaluated parameters in Group C.	ALB	CRP	WBC	RBC	HGB	HCT	MPV	LYM	MONO	NEU	MLR	PLR	NLR	P ₄	Uterine Diameter	Ki-67 (Tissue)	Ki-67 (Serum)
ALB	-	-0.141 NS	-0.281 NS	0.485 *	0.289 NS	0.312 NS	0.116 NS	-0.169 NS	-0.202 NS	-0.102 NS	-0.120 NS	-0.236 NS	0.006 NS	0.198 NS	-0.252 NS	-0.092 NS	-0.074 NS
CRP		-	0.372 NS	-0.538 *	-0.443 NS	-0.404 NS	0.439 NS	-0.128 NS	-0.072 NS	0.472 *	-0.084 NS	-0.086 NS	0.240 NS	-0.177 NS	-0.148 NS	-0.607 **	-0.757 ***
WBC			-	-0.662 **	-0.729 ***	-0.755 ***	0.366 NS	0.368 NS	0.266 NS	0.639 **	0.186 NS	-0.192 NS	0.001 NS	-0.597 **	0.086 NS	-0.187 NS	-0.180 NS
RBC				-	0.763 ***	0.815 ***	-0.291 NS	-0.035 NS	-0.254 NS	-0.618 **	-0.230 NS	-0.024 NS	-0.185 NS	0.515 *	-0.067 NS	0.280 NS	0.215 NS
HGB					-	0.953 ***	-0.464 NS	0.004 NS	-0.264 NS	-0.647 **	-0.260 NS	0.005 NS	-0.176 NS	0.360 NS	-0.142 NS	0.185 NS	0.215 NS
HCT						-	-0.453 NS	0.020 NS	-0.234 NS	-0.695 **	-0.228 NS	-0.027 NS	-0.255 NS	0.413 NS	-0.068 NS	0.135 NS	0.198 NS
MPV							-	-0.028 NS	-0.187 NS	0.310 NS	-0.214 NS	-0.338 NS	0.048 NS	-0.052 NS	-0.026 NS	0.009 NS	-0.488 *
LYM								-	0.340 NS	-0.418 NS	0.152 NS	-0.744 ***	-0.951 ***	-0.073 NS	0.248 NS	0.045 NS	-0.276 NS
MONO									-	-0.137 NS	0.923 ***	-0.258 NS	-0.388 NS	-0.208 NS	0.628 **	0.015 NS	-0.189 NS
NEU										-	-0.068 NS	0.389 NS	0.732 ***	-0.618 **	-0.202 NS	-0.230 NS	0.062 NS
MLR											-	-0.131 NS	-0.241 NS	-0.262 NS	0.529 *	-0.036 NS	-0.139 NS
PLR												-	0.750 ***	-0.283 NS	-0.178 NS	0.172 NS	0.657 **
NLR													-	-0.222 NS	-0.242 NS	-0.045 NS	0.316 NS
P ₄														-	-0.201 NS	-0.016 NS	-0.054 NS
Uterine Diameter															-	0.375 NS	-0.100 NS
Ki-67 (Tissue)																-	0.192 NS
Ki-67 (Serum)																	-

The difference between the means with different letters in the same row is significant ($P < 0.05$). ^{NS} Not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. PYO: Pyometra, C: Control, ALB: Albumin, CRP: C-reactive Protein, WBC: White blood cell, RBC: Red blood cell, HGB: Hemoglobin, HCT: Hematocrit, PLT: Platelet, MPV: Mean platelet volume, LYM: Lymphocyte, MONO: Monocyte, NEU: Neutrophil, MLR: Monocyte/Lymphocyte ratio, PLR: Platelet/Lymphocyte ratio, NLR: Neutrophil/Lymphocyte ratio, P₄: Progesterone.

Table 3. Association between the evaluated parameters in Group PYO

Association between the evaluated parameters in Group PYO.	ALB	CRP	WBC	RBC	HGB	HCT	MPV	LYM	MONO	NEU	MLR	PLR	NLR	P ₄	Uterine Diameter	Ki-67 (Tissue)	Ki-67 (Serum)
ALB	-	-0.552 *	-0.271 NS	0.275 NS	0.159 NS	0.109 NS	0.241 NS	-0.159 NS	-0.455 NS	-0.143 NS	0.128 NS	0.084 NS	-0.526 NS	0.449 NS	0.147 NS	0.047 NS	-0.114 NS
CRP		-	-0.067 NS	0.286 NS	0.316 NS	0.341 NS	-0.747 **	-0.389 NS	-0.222 NS	-0.037 NS	-0.295 NS	-0.076 NS	0.916 NS	-0.049 NS	0.100 NS	0.308 NS	0.554 *
WBC			-	0.135 NS	0.155 NS	0.157 NS	-0.135 NS	-0.061 NS	0.723 **	0.806 ***	0.333 NS	-0.423 NS	-0.177 NS	-0.360 NS	-0.156 NS	-0.229 NS	-0.279 NS
RBC				-	0.957 ***	0.965 ***	-0.157 NS	-0.572 *	-0.408 NS	0.137 NS	-0.029 NS	0.358 NS	0.073 NS	0.314 NS	-0.056 NS	-0.026 NS	0.033 NS
HGB					-	0.964 ***	-0.248 NS	-0.567 *	-0.353 NS	0.118 NS	-0.048 NS	0.127 NS	0.083 NS	0.183 NS	-0.123 NS	-0.043 NS	0.007 NS
HCT						-	-0.255 NS	-0.567 *	-0.361 NS	0.175 NS	-0.047 NS	0.103 NS	0.125 NS	0.145 NS	-0.106 NS	-0.103 NS	0.074 NS
MPV							-	0.608 *	-0.090 NS	-0.216 NS	-0.173 NS	-0.034 NS	-0.113 NS	0.301 NS	0.036 NS	0.034 NS	-0.351 NS
LYM								-	0.251 NS	-0.292 NS	-0.416 NS	-0.688 **	-0.215 NS	-0.003 NS	-0.329 NS	0.041 NS	-0.484 NS
MONO									-	0.560 *	0.488 NS	-0.418 NS	-0.230 NS	-0.430 NS	-0.089 NS	-0.285 NS	-0.229 NS
NEU										-	0.560 *	-0.313 NS	0.007 NS	-0.342 NS	0.295 NS	-0.318 NS	0.178 NS
MLR											-	-0.060 NS	-0.270 NS	-0.064 NS	0.454 NS	-0.276 NS	0.190 NS
PLR												-	-0.101 NS	-0.254 NS	0.004 NS	-0.362 NS	0.190 NS
NLR													-	-0.034 NS	0.555 *	0.301 NS	0.683 **
P ₄														-	0.121 NS	0.478 NS	-0.163 NS
Uterine Diameter															-	-0.092 NS	0.586 *
Ki-67 (Tissue)																-	0.038 NS
Ki-67 (Serum)																	-

The difference between the means with different letters in the same row is significant ($P < 0.05$). ^{NS} Not significant ($p > 0.05$); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. PYO: Pyometra, C: Control, ALB: Albumin, CRP: C-reactive Protein, WBC: White blood cell, RBC: Red blood cell, HGB: Hemoglobin, HCT: Hematocrit, PLT: Platelet, MPV: Mean platelet volume, LYM: Lymphocyte, MONO: Monocyte, NEU: Neutrophil, MLR: Monocyte/Lymphocyte ratio, PLR: Platelet/Lymphocyte ratio, NLR: Neutrophil/Lymphocyte ratio, P₄: Progesterone.

DISCUSSION AND CONCLUSION

The accumulation of purulent exudate in the uterine lumen is defined as pyometra. Therefore, the diameter of the uterus increases as a result of an increase in intrauterine fluids (19). Similarly, the mean uterine diameter was significantly higher in the pyometra group compared to healthy dogs in this study. The findings of our study, conducted on 32 female dogs, indicate that the incidence of pyometra increased with age. It was supported by Niskanen and Thrusfield (20), who reported that repeated exposure to endogenous P4 in nulliparous bitches increases the risk of pyometra. Paudel et al. (21) observed that the occurrence of closed pyometra decreased with age. There was a relationship between cervical patency and the age at which pyometra occurs in dogs, attributable to weakness and relaxation of the cervix, which may be caused by multiple pregnancies and estrus cycles (21). In this study, senior dogs (7–11 years) constituted Group PYO, and the occurrence of closed pyometra cases (n=4 dogs) was lower than that of open-cervix pyometra cases (n=10 dogs).

Pyometra can be a life-threatening illness, which may cause local and systemic inflammatory response with multiple organ dysfunction (22). Gram-negative bacteria, especially *E. coli*, play a significant role in the occurrence of the disease. *E. coli* has a higher affinity for the endometrium and myometrium layers. This situation constitutes a resistance to the uterus's defense mechanisms (23). The most isolated pathogen from pyometra is *E. coli* (24,25). The bacterial pathogen causes purulent inflammation in the uterus (26). In addition to diagnostic imaging (such as radiography and ultrasonography), haematology and blood chemistry analyses are performed to confirm the diagnosis (27). In accordance with previous reports, the current study examined pyometra cases caused by *E. coli* using ultrasonography, haematology, and blood chemistry analyses to evaluate the local and systemic effects of the disease. Chronic uterine inflammatory disease can cause normocytic and normochromic anemia by depressed erythropoiesis in the bone marrow (28). Similar to the literature (22,28,29), reduced levels of RBC, HGB, and HCT were observed in Group PYO. Anemia can usually be related to inflammatory exudates in cases of pyometra. Additionally, bacterial endotoxins suppress bone marrow function, and anemia can be severe, primarily due to *E. coli* (30). Sepsis, chronic inflammation, and endotoxemia cause protein loss (31). Hypoalbuminemia may occur in pyometra cases due to decreased ALB synthesis (16). As reported in canine pyometra (16,21,22), lower ALB concentrations were observed in Group PYO than in Group C. Leukocytes, also known as WBCs, are the primary biological components of inflammatory and immunological responses (32). Besides, CRP is a major predictor of inflammation and/or infection, and it can bind to bacteria, allowing phagocytes to pick up bacteria (33). Köse et al. (34) reported that CRP in dogs may be more useful when a marked increase in WBC above the cut-off value is detected in the CBC routinely used to assess inflammatory or infectious status. In the present study, CRP and WBC levels in Group PYO were significantly higher than in Group C, consistent with previous reports indicating the role of these parameters in inflammation and infection. Demeli and Meyer (22) reviewed 15 studies that were about the blood parameters associated with pyometra in dogs.

They reported that LYM levels increased in some studies (n=6) and decreased in others (n=9), whereas the MONO concentration was significantly increased in pyometra compared with control in all studies. Percentage band NEU and MONO counts were positively associated with keratinocyte-derived chemokine, which is a specific cytokine to inflammation of infectious origin (35). WBC, MONO, and NEU counts in the leucogram differ significantly in the Group PYO. These findings are compatible with the results of previous studies (36). Platelets play a significant role in the inflammatory response (36). MPV reflects the average platelet size, and the size of circulating PLTs may vary with the intensity of inflammation (37). There are various studies on PLT and MPV values in dogs with sepsis or inflammatory disease. Jasmin et al. (38) reported that PLT and MPV values in dogs with systemic inflammatory response signs with thrombocytopenia were found to be higher than those of healthy dogs. However, PLT values did not show significance with respect to cervical patency in dogs with pyometra (30). Also, changes in PLT and MPV in dogs and cats with pyometra were not significantly different from those in healthy animals (39). Although PLT and MPV levels were within reference ranges in both groups in the present study, PLT levels decreased significantly in Group PYO. The possible mechanism may be associated with cytokine release (40). Thrombocytopenia may occur due to severe sepsis as a consequence of decreased tissue perfusion or body dysfunction (28,41).

Inflammatory ratios have been investigated as novel inflammatory markers that can be used to investigate systemic inflammation in various diseases (41,42). Pierini et al. (7) indicated that NLR may be a useful marker of inflammation, whereas PLR and MLR may serve as prognostic markers in septic dogs. MLR is a simple biomarker of the immune system. Research has linked a high MONO count and the generation of monocyte/macrophage-related cytokines (43). Güneş et al. (6) indicated that MLR could be a diagnostic tool for canine pyometra. Similarly, MLR was found to be higher in Group PYO than in Group C in the present study. NLR reflects the balance between NEU and LYM numbers, and it is directly related to the inflammatory status in septic illness (7,40). Similarly, NLR was higher in dogs that suffered from pyometra compared to those in Group C in this study. This result supports that NLR is one of the inflammatory markers that can be used to investigate systemic inflammation, and it can be a useful tool for the diagnosis of pyometra in bitches, as previously reported (6). Cytokine levels during inflammatory disease increase megakaryocytes. The degree of PLR is influenced by the elevation in a variety of proinflammatory and prothrombotic states (36). Thrombocytopenia or thrombocytosis may occur in canine sepsis, but neither was associated with mortality (44). İncir et al. (40) evaluated the PLR levels in patients with a CRP value of <3 mg/L (non-inflammatory group), and CRP level between 3 to 9 mg/L (low-grade inflammatory group), but they didn't detect any significant changes. They indicated that the predictive ability of PLR may be lower at the beginning of the inflammation (40). In this study, PLR in Group PYO was significantly lower compared to Group C. This could be due to the presence of decreased PLT in Group PYO. The possible mechanism was explained previously as in both thrombocytopenia and thrombocytosis may occur in canine sepsis (44). The decre-

ase in neutrophil phagocytic activity is associated with increased P4 levels (45). Progesterone has immunosuppressive properties in dogs. These hormonal effects are accompanied by alterations in inflammation and coagulation consistent with a spontaneous sepsis model (46). Blood P4 concentration in dogs with pyometra was significantly higher than in healthy dogs (6). Similar results were obtained in this study. The probable mechanism is due to the destructive effect of P4 on the immune system.

Ki-67 is the most reliable biomarker of tumor proliferation and apoptosis in many tumor types (47). Cyclic changes are influenced by proliferation and apoptosis in the healthy canine endometrium (9). Proliferative changes in the canine endometrium were examined at different estrous stages using Ki-67 immunolabeling. A mild positive correlation was observed between the Ki-67 index and serum P4 concentrations (12). Ki-67-positive cells in stroma during the late luteal phase were lower in cystic endometrial hyperplasia than in canine pyometra (47). However, Anusha et al. (48) didn't achieve consistent results for the nuclear staining of Ki-67 in the glandular, stromal, and luminal epithelium; it was only they who observed cytoplasmic staining of stromal cells in a few cases of Type II canine pyometra (CEH with diffuse infiltration of plasma cells in the stroma). Although the analysis technique (ELISA) in our study is different from the previous reports (immunohistochemistry-IHC), similarly, the uterine tissue Ki-67 level in Group PYO was significantly higher than in healthy dogs. The result obtained reflects the proliferative activity of Ki-67 in canine endometrium, as previously reported.

In the study by Neumann et al. (49), serum Ki-67 concentrations were evaluated as biomarkers of malignant change using a canine-specific commercial ELISA assay. Their results demonstrated that Ki-67 serum concentrations were undetectable in healthy dogs and that malignancies were associated with elevated Ki-67 serum concentrations in dogs (49). Serum Ki-67 levels were evaluated both in canine pyometra cases and healthy controls, but not in neoplastic alterations in this study, Ki-67 serum concentrations were detectable in healthy dogs in contrast with Neumann et al. (49). Ki-67 index by IHC is already widely used to determine tumor growth and progression in dogs (47,50). Similarly, IHC is widely used to assess Ki-67 expression in many studies of canine endometrium (9,12,48). To the best of our knowledge, this is the first study to determine Ki-67 levels in serum samples from both dogs with pyometra and healthy controls. Although the mean serum Ki-67 level in Group PYO was higher than that of healthy controls, it did not reach significance. Therefore, serum Ki-67 levels were not able to distinguish canine pyometra from healthy dogs. However, in the present study, serum Ki-67 concentrations in Group PYO showed a moderate association with NLR, CRP, and uterine diameter. In light of these results, serum Ki-67 levels should be investigated in canine pyometra cases (CEH, acute inflammatory reaction with CEH, endometrial atrophy), with a larger number of cases in future studies.

One limitation of this study is that Ki-67 expression was evaluated only by ELISA, and IHC analysis at the tissue level could not be performed. While ELISA provides quantitative information on protein levels, it does not allow the determination of tissue localization or cellular origin. Therefore, this

limitation should be considered when interpreting the results. Another limitation of the study is that the higher mean age of animals in the pyometra group compared to the control group may be relevant when interpreting the results, given the potential influence of age on proliferative activity and hematological parameters. Finally, the lack of separate evaluation of open- and closed-cervix pyometra cases in terms of hematological parameters and Ki-67 levels may be considered a limitation of the study and should be supported by further investigations with larger sample sizes.

The present study showed that Ki-67 is measurable in serum samples from dogs with pyometra and healthy controls, and that uterine Ki-67 levels can serve as an effective marker of proliferative changes in canine pyometra. Also, decreased ALB, increased CRP and WBC, slight anemia, and changes in MLR and NLR are considered as predictive markers in the evaluation of inflammatory changes in pyometra cases and in the diagnosis of pyometra in dogs.

ACKNOWLEDGEMENT

The authors would like to thank Prof. Dr. Bülent Ekiz for his valuable support and contribution to the statistical analysis of this study.

FINANCIAL SUPPORT

No support was received from any organization in the conduct of this research.

CONFLICT OF INTEREST

There is no conflict of interest to be declared by the authors.

AUTHOR CONTRIBUTIONS

ABU contributed to data curation, methodology, and investigation. ZGU was responsible for writing the original draft, conceptualization, and supervision of the study. MY contributed to visualization and resources. MS contributed to software and resources. İK contributed to data curation and software. Sİ, BGG, SYG, and BH contributed to methodology and formal analysis. All authors have read and agreed to the published version of the manuscript.

ETHICAL STATEMENT

This study was approved by the Istanbul University-Cerrahpaşa Animal Experiments Local Ethics Committee (Approval number: 2025/05).

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