FULL PAPER *Internal Medicine*

Canine pyometra: a model for the analysis of serum CXCL8 in inflammation

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ABSTRACT. Interleukin-8 (IL-8 or CXCL8) is a highly selective pro-inflammatory chemokine, that is elevated in sera of humans and animals with various inflammatory diseases. CXCL8 is possibly involved in uncontrolled inflammation and the development of a systemic inflammatory response syndrome (SIRS) and sepsis. Nevertheless, its behavior and precise properties in the course of inflammation are not fully understood. Thus, we used naturally occurring canine pyometra as a model of inflammation, in order to examine the behavior of serum CXCL8 in relation to the disease intensity and commonly analyzed inflammatory mediators. Using a commercially available canine ELISA kit, a significant increase of CXCL8 was determined in the serum of 23 dogs with pyometra compared with 35 healthy dogs. Interestingly, serum CXCL8 did not increase in severely diseased patients and behaved contrary to white blood cells (WBC), neutrophils and C-reactive protein (CRP). The measurement of serum CXCL8 may provide valuable information about the extent of ongoing lesions and could be a useful complement for existing laboratory tests.
KEY WORDS: canine, CXCL8, inflammation, pyometra, serum

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Inflammation is a complex biological response of the innate immune system to protect the body from diverse harmful stimuli. However, a massive stimulation or an unbalanced immune reaction can lead to SIRS with fatal consequences due to generalized tissue destruction and organ dysfunction. The intricate mechanisms of this usually well orchestrated process, and the cause for an inflammatory overturn is not fully understood. Since uncontrolled inflammation leads to a devastating condition responsible for most intensive care unit deaths in human and veterinary medicine [15], all possibly involved mechanisms are considered important research subjects.

The chemokine CXCL8 plays a fundamental role in inflammation, as it promotes neutrophil migration and activation, inducing the full pattern of responses of these cells [3, 6, 31]. A local and systemic elevation of CXCL8 has been found in various inflammatory diseases [2, 13, 17, 22, 23, 25] as well as in SIRS [14] and sepsis [7, 10, 18], but its precise role in the pathomechanism of disease is unclear. Thus, knowledge about its behavior during acute and chronic inflammation is of great interest in human and veterinary medicine.

In order to assess this topic, serum CXCL8 was measured in canine pyometra. This is a common inflammatory disease of the uterus in intact, sexually mature bitches, caused by a secondary bacterial infection [11, 14, 28]. The presence of bacteria within the uterus induces the up-regulation of several cytokines and consequently the attraction of inflammatory cells. Pyometra is characterized by severe endometrial inflammation and accumulation of pus in the uterine lumen, frequently leading to endotoxemia and SIRS or sepsis, representing a life threatening condition [12, 24, 28]. Therefore, an examination of immunological components in dogs with pyometra may provide important insights into general mechanisms operating during diverse inflammatory reactions.

MATERIALS AND METHODS

Animals: Twenty-three client-owned female dogs admitted between 2011–2014 to the Small Animal Clinic of the University of Goettingen, for diagnosis and subsequent surgical treatment (ovariohysterectomy, OHE) of pyometra were included in this prospective study. Inclusion criteria were the presence of pyometra without previous treatment and the absence of further diseases. Enrolled patients underwent a detailed anamnesis, physical examination, complete blood cell count (CBC) and serum chemistry. Ultrasonography was performed to determine abnormalities of the uterus and their extent. Post-surgical gross evaluation of the uterus was carried out to establish the degree of uterine distension, as well as the presence and amount of pus in the uterine lumen.

The control group consisted of 12 healthy client-owned dogs admitted for routine health examinations or OHE and 23 healthy dogs from the animal shelter "ETN Tierschutzhof Wiesenfeld" in Bad Karlshafen (State of Lower Saxony). The dogs were confirmed to be healthy based on their medical history, detailed physical examination, CBC and serum chemistry. All samples were acquired with the owner's consent, and the procedure was in accordance with the German "Protection of Animals Act" and carried out under the

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Table 1. Clinical characteristics and grouping of dogs with pyometra

Characteristic	Clinical features	Groups	n=23
Symptomatology	Apathy	Mild	15
	Lethargy	Severe	8
	Polyuria		
	Polydipsia		
	Inappetance		
	Emesis		
	Diarrhea		
	Abdominal pain		
	Abdominal distension		
	Vaginal discharge		
Uterine distension	Intrauterine pus amount	Moderate filled uterus	10
		Massively filled uterus	13
Time for recovery	Days of hospitalization	<2 Days	14
		>2 Days	9
WBC count	6,000–12,000/μl	Normal	6
	12,000–25,000/μl	Moderate	9
	>25,000/μl	Severe	8
Neutrophil count	3,000–9,000/μl	Normal	7
	9,000–17,000/μl	Moderate	5
	>17,000/μl	Severe	11
Serum CRP	<15 mg/l	Normal	8
	>15 mg/l	Elevated	15

supervision of the animal welfare officer of the Faculty of Agriculture, University of Goettingen.

Dogs with pyometra had a median age of 8.5 years (range 3–14.6 years), being most of them older than 7 years (n=19). Fifteen different breeds were represented in this group. To analyze the behavior of serum CXCL8 in relation to pyometra, the following clinical characteristics were taken into consideration: symptomatology, uterine distension/filling, time of recovery, WBC, neutrophil count and serum CRP values. Dogs were grouped according to the extent of these characteristics, as listed in Table 1. The grouping of dogs according to the intensity of their symptoms was done using the ASA physical status classification system [27]. Dogs without substantive functional limitations were considered to have a mild symptomatology, whereas patients with severe symptoms showed substantive functional limitations due to systemic disease. Yet, it has to be considered that there is no absolute measure of severity, as this is a matter of clinical judgment. The classification of the degree of uterine distension/filling was based on the diameter of the uterus horns in relation to the dogs body weight. For dogs below 15 kg body weight, a uterus horn diameter of 3 cm was set as limit between the two groups. For dogs above 15 kg body weight, the limit was set at 4 cm.

Healthy dogs had a median age of 4.5 years (range 1.5–11.5 years), being 10 of them older than 7 years old. Seventeen dogs were female (8 neutered) and 18 male (13 neutered). Seven breeds were represented, but most dogs were of mixed breed (n=25).

Sampling and analysis procedures

Blood samples: Blood samples were taken from the cephalic vein previous to treatment. Blood samples for CBC were collected in polypropylene tubes with 1.6 mg EDTA/m*l* blood (Sarstedt AG & Co, Nuembrecht, Germany) and immediately analyzed with a CellDyn 3500 Analyzer (Abbott GmbH & Co KG, Wiesbaden, Germany). Serum samples required for serum chemistry (including CRP) were collected in standard serum tubes (Sarstedt AG & Co) and centrifuged in an Eppendorf centrifuge 5424 (Eppendorf AG, Hamburg, Germany) at 3,000 × *g* for 5 min. Serum was extracted from the tube and analyzed according to standardized procedures using a clinical chemistry analyzer (Konelab 20i; Thermo Fischer Scientific Inc., Dreieich, Germany) and commercial kits.

CXCL8 was measured in serum, since preliminary results showed a higher reliability than plasma values. Serum samples used to determine the CXCL8 concentrations were collected at the same time as the blood for CBC and serum chemistry and were then processed following the commercial ELISA kit protocol (R&D Systems, Minneapolis, MN, U.S.A.). Samples were kept at room temperature for two hr to allow clotting and were then centrifuged for 20 min at 1,000 × *g*. Serum was immediately aliquoted with a Pipetman P100 (Gilson, Villers Le Bel, France) and stored at −20°C until required for analysis. A storage time of more than three months and repeated freeze-thaw cycles were avoided.

Assessment of CXCL8: Serum CXCL8 levels were measured in triplicate using a commercially available canine CXCL8 quantitative ELISA kit (Quantikine, R&D Systems)

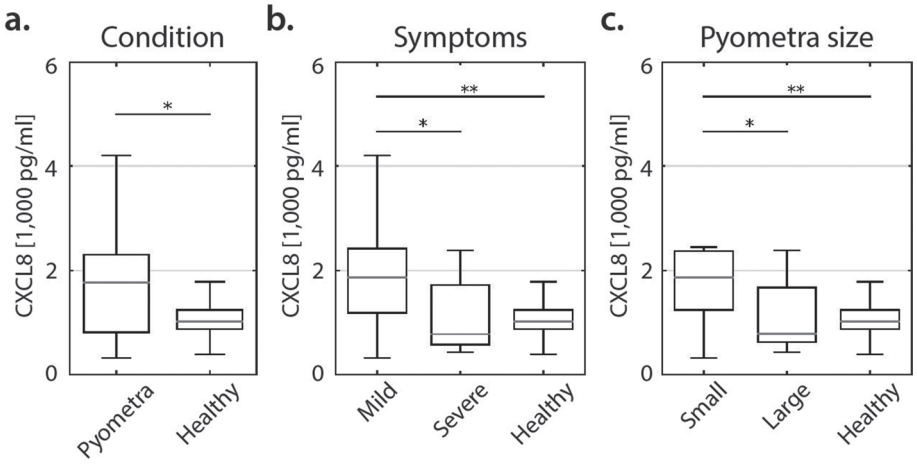

Fig. 1. Dogs with pyometra have higher serum CXCL8 values than healthy patients and patients with severe disease. a) Comparison of the serum CXCL8 levels among the healthy control group and dogs with pyometra. b) Serum CXCL8 concentrations of healthy dogs and dogs with mild and severe symptoms due to pyometra. c) Serum CXCL8 concentrations of healty dogs and dogs with a moderate and severe uterine distension. *P value <0.05. **P value <0.01.

following the manufacturer's instructions. The optical density of each well was determined within 30 min using a TECAN microplate reader at an optical density of 450 nm and a wavelength correction of 570 nm. Calibration was performed using a standard series of dilutions of recombinant canine CXCL8 provided in the kit. For matters of test accuracy, a canine CXCL8 control of known concentration was also provided in the kit. The CXCL8 concentration (*p*g/m*l*) of each sample was calculated through a data analysis software (Magellan, TECAN Austria GmbH, Groedig, Austria). According to the manufacturer's instructions, the assay measures the natural and recombinant CXCL8 isoforms of 74 and 79 amino acids, and the minimum detectable dose of CXCL8 is 1.26 *p*g/m*l*. The assay shows no cross-reactivity with a series of cytokines and growth factors. The manufacturer reported mean intra- and inter-assay coefficients of variation of 5.6 and 5.3%, respectively.

Statistical analysis: Statistical analysis was performed using a commercial software (MATLAB and Statistics Toolbox Release 2015b, The Math Works, Inc., Natick, MA, U.S.A.). CXCL8 concentrations were grouped according to goal criteria, and the null-hypothesis was tested using a non parametric Mann-Whitney-U-Test. Since clinical characteristics are given in ordinal categorical classes, the relation between the serum CXCL8 concentration and increase in category was analyzed using generalized linear models (GLM). For this, CXCL8 values were analyzed log transformed, and both process and observation error were assumed to have Gaussian distributions. A P value below 0.05 value was considered statistically significant.

RESULTS

No influence of the dogs age, gender and reproductive status on serum CXCL8 concentrations was found, validating the use of a mixed age and gender control group (data not shown).

The serum CXCL8 levels of dogs with pyometra ranged from 313.43 *p*g/m*l* to 5987.80 *p*g/m*l*, with a median of 1767.4 *p*g/m*l* (interquartile range: 813.35–2299.68 *p*g/m*l*). The average serum CXCL8 level in these patients was significantly higher (P=0.026) than in healthy controls (Range: 383.79–2506.50, median: 1019.50 *p*g/m*l* and interquartile range: 868.54–1239.0 *p*g/m*l*) (Fig. 1a). Nevertheless, when analyzing serum CXCL8 in relation to the severity of the symptoms, only dogs with mild symptoms had significantly higher values than healthy dogs (P=0.002). Patients with severe symptoms had much lower CXCL8 levels than those with mild symptoms (P=0.035) and were statistically indistinguishable from the healthy control group (Fig. 1b). A relationship between the severity of the symptoms and serum CXCL8 was further sustained by the use of GLM (P=0.04).

Similar results were found for the levels of serum CXCL8 in association to the uterine distension due to pus accumulation in the uterine lumen. Dogs with a less filled and distended uterus had significantly higher serum CXCL8 values than dogs with a massively filled and distended uterus (P=0.03). Instead, dogs with a massively filled uterus had similar values to healthy dogs (Fig. 1c). Using GLM, a significant association between the symptomatology and the uterus distension was found (P<0.001), explaining the similarity of the previous results. Moreover, patients with a faster and uncomplicated recovery after surgery had higher initial serum CXCL8 values than patients with a delayed recovery, but the difference was not significant (Data not shown).

The behavior of WBC and neutrophils was equivalent, since an increase in the WBC count during pyometra is mainly caused by a left shift neutrophilia. Given that the function of CXCL8 is almost restricted to neutrophils, only statistical results regarding the neutrophil count will be

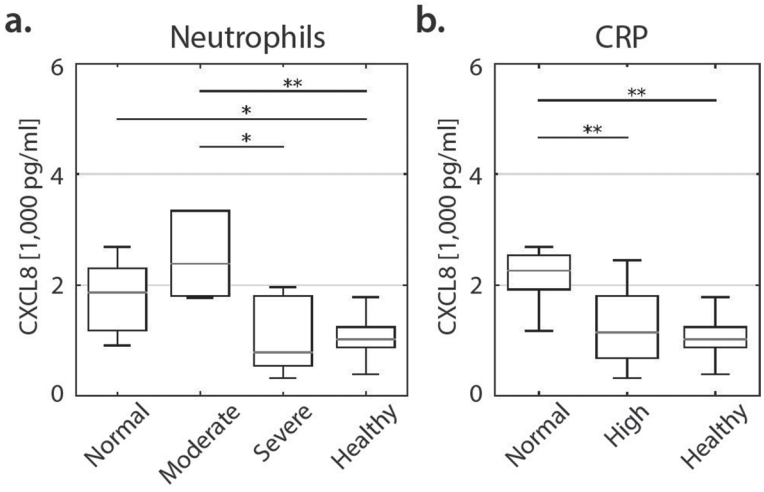

Fig. 2. Serum CXCL8 values behave oppositely to the neutrophil and CRP values. a) Serum CXCL8 concentrations in healthy dogs and dogs with different neutrophil counts. b) Comparison of serum CXCL8 among healthy dogs and dogs with normal and increased CRP values. *P value <0.05. **P value <0.01.

mentioned. An opposed behavior was observed when serum CXCL8 values and the neutrophil count were compared. Dogs with pyometra and a normal neutrophil count had significantly higher serum CXCL8 values compared to healthy dogs (P=0.01). In the presence of a moderate neutrophilia, serum CXCL8 was even higher (P=0.001), but the values were significantly lower when neutrophilia was severe. The serum CXCL8 values of dogs with severe neutrophilia were similar to the values of healthy dogs (Fig. 2a). Although, the neutrophil count tended to be higher in the presence of a more pronounced symptomatology, a marked uterine distension and a slow recovery, these associations were statistically not significant (Data not shown).

Similar results were found for CRP. Patients with normal CRP values had significantly higher serum CXCL8 concentrations than healthy dogs (P<0.001) and patients with increased CRP levels (P<0.01), while the last two groups were statistically indistinguishable (Fig. 2b). Although the neutrophil and CRP levels behave in a comparable way, a relationship between both could not be proven. As with the neutrophil count, CRP tended to raise with a pronounced symptomatology, a marked uterine distension and a slow recovery, but the results were statistically not significant (Data not shown).

A graphical representation of the CXCL8, neutrophil count and CRP values, and their association to the symptomatology and the uterine distension remarked the opposed behavior of these inflammatory mediators (Fig. 3).

DISCUSSION

Inflammatory reactions are part of the pathomechanism of multiple diseases, and their exacerbation is associated with a high mortality in human and veterinary medicine [18]. The inflammatory response is an intricate cross-talk between several cytokines, acute phase proteins (APPs) and cells, but many of these ongoing interactions remain unclear. The neutrophil chemoattractant CXCL8 is shown to be elevated in various inflammatory diseases, but its precise role in the pathophysiology of imbalanced inflammatory processes needs to be further clarified [2, 13, 17, 22, 23, 25]. As a common and well described naturally occurring inflammatory disease, canine pyometra represents a good model to study the behavior of serum CXCL8 and its relation to other inflammatory mediators during inflammation. Since pyometra is a disease that affects exclusively females, we first discarded the influence of gender on serum CXCL8 by comparing healthy male and female dogs. A difference between genders, as well as between neutered and unneutered dogs, was statistically discarded. Thus, a mixed gender control group was employed in order to increase the groups size and minimize a possible hormonal influence on our results.

We found that dogs with pyometra have higher serum CXCL8 values than healthy dogs. Since CXCL8 is fundamental for the migration and activation of neutrophils, it is expected to be elevated in diseased animals [3, 6, 31]. Serum CXCL8 levels in dogs with pyometra have seldom been analyzed, but it has been shown that various cell types within the uterus produce CXCL8 in response to diverse stimuli, including pyometra [5, 11, 16, 30]. The source of CXCL8 measured in sera is unknown and so, a local as well as a systemic secretion has to be taken into consideration. Therefore, local and systemic CXCL8 should be compared in future studies.

When analyzing serum CXCL8 in relation to the severity of the symptoms, only dogs with mild symptoms had significantly higher values than healthy dogs. On the other hand, the values of healthy dogs and dogs with severe symptoms were alike. Similar results were found by Karlsson *et al.* in a study about serum CXCL8 canine pyometra and by DeClue

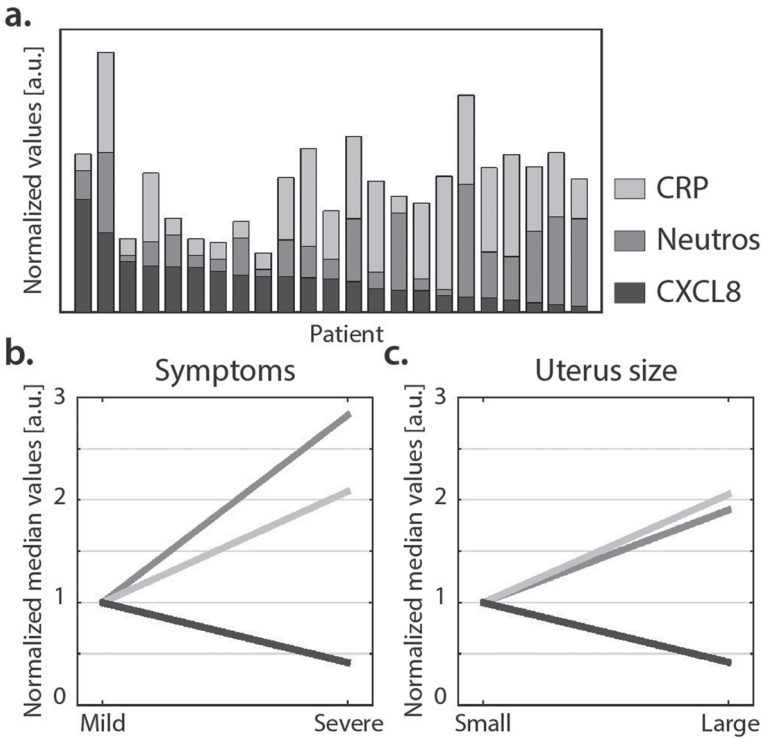

Fig. 3. Serum CXCL8 has an opposed behavior to that of neutrophils and CRP. a) Normalized values of serum CXCL8, neutrophils and CRP in each patient. b) Normalized values of serum CXCL8, neutrophils and CRP in relationship to the symptoms intensity. c) Normalized values of serum CXCL8, neutrophils and CRP in relationship and CRP in the relationship to the uterine distension.

et al. who analyzed plasma CXCL8 in septic dogs [7, 14]. Both, patients with SIRS or sepsis, had similar values to healthy dogs [7, 14]. Nonetheless, dogs without SIRS had lower values than healthy and SIRS positive patients [14], being contrary to our results. This can be due to grouping criteria, since the clinical signs used to diagnose SIRS are different from the symptoms included in our study. Thus, dogs with marked symptoms do not necessarily have SIRS.

There are various possible reasons for the lacking increase of CXCL8 in the group with marked symptoms. First, the raise of serum CXCL8 is transient and can change drastically very fast during the course of disease [1, 8, 15]. As the exact moment of this dynamic process at sampling time is not known, a physiological decrease cannot be discarded. This theory may be supported by the raise of neutrophils and CRP that accompanied the CXCL8 decrease, as this may be part of a physiologically orchestrated response. Second, low serum CXCL8 values can be due to a poor or depleted immune response and thus be related to severe symptoms. Third, progesterone is an inhibitor of CXCL8 in uteri and makes this organ more susceptible to infection [5, 16, 28, 29, 31]. If the CXCL8 in sera stems from the uterus, its decrease may be the result of high progesterone concentrations. Whether progesterone has the same effect on systemically produced CXCL8 has not been reported and should be considered for further research. Yet, our analysis regarding gender and neutering was statistically irrelevant, making an influence of progesterone on circulating CXCL8 unlikely.

Consistent with the previous results, dogs with a less filled uterus had higher serum CXCL8 values than the healthy group and dogs with a massively filled uterus, while the last two groups were akin. The similarity to the results regarding the symptomatology is not surprising, as the degree of uterus distension and pus accumulation within the uterine lumen influences the symptoms intensity. Although, it is not known if the lacking serum CXCL8 increase is the cause or the result of a more severe disease, it is reasonable that low CXCL8 levels may lead to an impaired neutrophil response within the uterus and consequently a worsening of the disease. Furthermore, CXCL8 is strongly involved in cervix ripening [5, 16, 30], allowing the expulsion of uterus content. Thus, decreased CXCL8 can lead to an impairment of this mechanism favoring the accumulation of pus in the uterine lumen [28]. On the other hand, if the CXCL8 measured in sera is produced in uteri, an increasingly damaged uterus tissue may lead to a decreased CXCL8 production.

In this study, serum CXCL8 was not increased in dogs with severe leucocytosis and neutrophilia when compared to dogs with normal and moderately elevated values. Although CXCL8 has a strong effect on neutrophils, this is a dynamic

process and their concentrations do not change simultaneously and proportionally [1, 8, 9, 21]. The raise of CXCL8 is transient, whereas WBCs and neutrophils may stay elevated for a longer period of time. In the presence of pyometra, apoptotic polymorphonuclear (PMN) rates are much lower, prolonging PMN survival. Interestingly, this is partly enhanced by CXCL8 [24]. Furthermore, a feedback mechanism is likely, so that when high levels of neutrophils are reached, a CXCL8 decrease is induced. Moreover, CXCL8 stimulates neutrophil migration into the tissue, causing a decrease in the amount of circulating cells. Thus, an opposed behavior of the CXCL8 and neutrophil concentrations in blood is reasonable.

When comparing CRP and serum CXCL8, contrary concentrations were found. CRP has been found to be elevated along with CXCL8 in severe or complicated cases of various diseases [4, 8, 20]. However, although the production of CRP and CXCL8 is partly regulated by the same cytokines, namely tumor necrosis factor (TNF) and interleukin-1 (IL-1), the stimulation and response of each protein vary depending on the stimulus, the activation pathway and if it is an acute or chronic inflammation. Distinct to CXCL8, CRP is mainly induced by IL-6, and this may determine how CRP behaves in relation to CXCL8 [8, 19, 26]. Contrary to CXCL8, CRP leads to an inhibition of chemotaxis and modulation of neutrophil function and may therefore be stimulated at a different point of the disease. Furthermore, some of the common APPs have recently been shown to share the ability to down-regulate the pro-inflammatory cytokine production and activity in monocytic cells [19]. Since monocytes represent an important source of CXCL8, low values may be caused by a CRP peak as part of a regulatory mechanism. The opposite behavior of these two parameters may also be explained by the location of their production. While CXCL8 may be produced in uteri and be therefore synthesized very early in the course of the disease, CRP is mainly produced in the liver and may require stronger or systemic changes to be stimulated.

In conclusion, serum CXCL8 is elevated in dogs with moderate pyometra compared to dogs in a severe state of the disease. Low concentrations of serum CXCL8 may be part of a well-orchestrated inflammatory response or the consequence of a depleted immune system. As it is elevated in dogs with moderate pyometra compared to dogs in a severe state of the disease, it is possible that CXCL8 plays a protective role in this disease. However, establishing through blood tests the exact role of this chemokine in the physiopathology of pyometra and inflammation is difficult. Its presence in serum can be due to multiple stimuli, and its source and exact functions are unclear. Furthermore, the concentrations of serum CXCL8 can change rapidly, and the timing of the assessment is unknown. Still, the analysis of serum CXCL8 canine pyometra gives an insight into its behavior in inflammation and its interaction with other components of the immune system. CXCL8 showed to be a good predictor for the clinical characteristics of pyometra and could be a useful complement for existing laboratory tests, such as the WBC, neutrophil and CRP levels. Due to its relatively short half life in serum and a high response in diseased animals, serum CXCL8 constitutes a possible indicator of a systemic reaction to a stimulus. Nonetheless, CXCL8 is only a small part of the complex cross-talk between several cell types and cytokines during inflammation. Its analysis is not suitable for establishing a specific diagnosis, but can provide valuable information about the physiopathology and extent of ongoing lesions in individual animals.

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