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Increased concentrations of C-reactive protein but not high-mobility group box 1 in dogs with naturally occurring sepsis.

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Abstract

Sepsis is difficult to diagnose and remains a common mortality cause worldwide in both humans and animals. The uterine infection pyometra causes sepsis in more than half of affected dogs and therefore allows the natural physiological development of sepsis to be studied. To find a sepsis-specific biochemical marker that could be combined with conventional clinical criteria for a more robust and quick diagnosis of sepsis, we measured systemic concentrations of high-mobility group box 1 (HMGB1) in 23 healthy control dogs and in 27 dogs with pyometra, 74% of which had sepsis. We also measured concentrations of the major acute phase protein C-reactive protein (CRP) and an indicator for endotoxaemia, prostaglandin F_{2α} metabolite (PGM) to assess the relative contribution of HMGB1 to the detection of systemic inflammation and endotoxaemia. We found that HMGB1 concentrations, in line with concentrations of CRP and PGM, were significantly increased in dogs with pyometra, and that concentrations of CRP, but not HMGB1, were significantly higher in dogs with sepsis compared to dogs without sepsis. Although serum HMGB1 did not differ between dogs with or without sepsis and was not correlated with either CRP or PGM concentrations, HMGB1 was correlated with the total white blood cell counts, suggesting an independent regulation and involvement in inflammation.

Keywords: sepsis, SIRS, biomarkers, diagnostics, cytokines, canine/dog, pyometra, bacterial uterine infection, inflammation, HMGB1, CRP, PGM.

Introduction

Sepsis, defined as systemic inflammatory response syndrome (SIRS) caused by an infection (Levy et al., 2003) is associated with high mortality rates worldwide in both humans and animals (Bone et al., 1992, Brun-Buisson et al., 1996, Angus et al., 2001, King et al., 2001, Harrison et al., 2006, Blanco et al., 2008, Boller and Otto, 2009 and Esper and Martin, 2009). There is currently no rapid and precise test to diagnose sepsis, which is why certain clinical criteria are used to identify patients more likely to suffer from sepsis. Clinical diagnostic criteria for sepsis are similar in humans and dogs, comprising elevated heart and respiratory rates, abnormal total white blood cell count (WBC) and abnormal body temperature (Hauptman et al., 1997). Such criteria are largely unspecific and allow for false and delayed sepsis diagnosis, increased mortality and erroneous choice of treatment. The need for novel criteria or markers that will allow for an accurate diagnosis of sepsis is therefore urgent (Lever and Mackenzie, 2007, Marshall and Reinhart, 2009 and Khair et al., 2010).

Dogs are currently recognized as a more suitable animal species than rodents for studying the development of sepsis (Otto, 2007), and natural sepsis models are highly physiologically relevant. The canine disease pyometra, caused by an opportunistic bacterial infection of the uterus (Hagman et al., 2006b), leads to sepsis in nearly 60% of the cases (Fransson et al., 2007) and is therefore a relevant natural model of sepsis (Conti-Patara et al., 2012). The findings obtained using this model may be important for both human and veterinary medicine.

Several biomarkers have previously been evaluated in canine SIRS or sepsis including procalcitonin, tumor necrosis factor α (TNF- α), interleukin 6 (IL-6),

interleukin 10 (IL-10), interleukin 8 (IL-8), endotoxin indicator prostaglandin F_{2α} metabolite (PGM) and a well-known inflammatory marker C-reactive protein (CRP) (Hagman et al., 2006a, Fransson et al., 2007, Nakamura et al., 2008, Yilmaz et al., 2008, DeClue et al., 2012 and Karlsson et al., 2012). However, none of the biomarkers studied in humans has yet been shown to differentiate non-infectious SIRS from sepsis in a clinical setting (Pierrakos and Vincent, 2010 and Hall et al., 2011).

It was previously shown that high-mobility group box 1 (HMGB1) serum/plasma concentrations were increased in sepsis and were correlated with sepsis severity in humans (Wang et al., 1999a and Karlsson et al., 2008). HMGB1 is a chromatin-binding nuclear factor that has been shown to act as a pro-inflammatory cytokine when secreted by activated monocytes and macrophages or passively released by damaged or killed cells (Wang et al., 1999a, Wang et al., 1999b and Scaffidi et al., 2002). Recent findings showed that HMGB1 concentrations were increased in a canine model of endotoxaemia (Yu and Park, 2011) and also in dogs with naturally occurring SIRS (Yu et al., 2010 and Ishida et al., 2011). The clinical applicability of HMGB1 in sepsis diagnosis in dogs has not yet been investigated.

The aims of this study were to 1) measure serum concentrations of HMGB1 in healthy intact female dogs, 2) determine whether HMGB1 concentrations were associated with severity of the disease, i.e. with the presence of SIRS in pyometra (sepsis), 3) determine whether HMGB1 concentrations in dogs with pyometra were associated with a well-known inflammatory marker CRP, and 4) assess whether

HMGB1 concentrations were associated with concentrations of the endotoxin indicator PGM.

Methods

Animals

Fifty female dogs of 21 different breeds with an age of 6.2 ± 3.1 years (mean \pm SD) were included in this study upon the owners' written agreement. The study was approved by Uppsala local ethical committee, permission number C242/7. A complete physical examination including assessment of body weight, heart and respiratory rates, and measurement of standard haematological and biochemical parameters were performed in all dogs in the study by trained personnel in the Clinical Pathology Laboratory, University Animal Hospital, Swedish University of Agricultural Sciences, Uppsala, Sweden. Measurement of the following parameters were analysed in haematological and biochemical tests: the total number of white blood cells (WBC) including the differential count, the concentrations of alanine aminotransferase, creatinine and bile acids. Clinical profiles of the subjects in each group are summarised in Table 1. SIRS-status determination was performed in all dogs and defined as positive if two or more of the following criteria were met: abnormal body temperature (<38.1 or $>39.2^{\circ}\text{C}$), elevated respiratory rate (>20 counts/min), elevated heart rate (>120 beats/min), and abnormal number of WBC (<6 or >16 cells $\times 10^9/\text{L}$) or $>3\%$ band neutrophils (Hauptman et al., 1997).

In total, 27 female dogs of 17 different breeds were preliminary diagnosed with pyometra using criteria described previously (Hagman et al., 2006b). The diagnosis of pyometra was verified postoperatively by histopathological examination of the uterus and ovaries and/or positive bacteriological culture from the uterine content. Dogs with pyometra included in the study had no apparent concurrent diseases and had received no anti-inflammatory medication within two months prior to the study.

Twenty dogs with pyometra fulfilled two or more of the criteria for SIRS (Hauptman et al., 1997), and SIRS-positive dogs with pyometra (P⁺SIRS⁺) were classified as having sepsis according to the definition by Levy and others (2003). Among other variables included in the complete physical examination was determination of the general condition (general attitude) as normal, slightly depressed, moderately depressed or severely depressed. Postoperative hospitalization at the University Animal Hospital for pyometra treatment is generally 1-2 days, and is only prolonged for dogs with slightly to severely depressed general condition or complications that require veterinary care. Postoperative hospitalization duration was used as a measurement for outcome (Hagman et al., 2006b) and was considered prolonged when more than two days. Eight SIRS-positive and one SIRS-negative dog with pyometra had a prolonged hospitalization (3-5 days). None of the dogs had lethal outcome due to pyometra or sepsis.

In total, 23 staff-owned sexually intact female dogs of 12 different breeds were used as controls. All control dogs were clinically healthy as determined by physical examination.

Sampling

All dogs with pyometra were sampled upon admission and before surgical treatment (ovariohysterectomy); nine of these dogs were also similarly sampled the day after the surgical treatment. Control dogs were sampled in metoestrus or anoestrus using an identical procedure; the same veterinary nurse performed procedures on dogs with pyometra and controls. Blood samples were first drawn for the blood cultures and then collected into non-additive EDTA- or heparin-coated plastic tubes (Becton-

Dickinson, Stockholm, Sweden) by venipuncture of the distal cephalic vein, and transported on ice directly to the Clinical Chemistry Laboratory, University Animal Hospital, Swedish University of Agricultural Sciences, Uppsala, Sweden. Sera and plasma were freeze-stored in aliquots at -80°C until assayed.

Bacteriological culturing

The presence of bacteria in the uterus of dogs with pyometra was verified by positive bacterial cultures from samples obtained from the uterine content using sterile cotton swabs (Culturette; Becton-Dickinson, Stockholm, Sweden) inserted into the uterine lumen directly after surgery. Bacteriological culturing was performed within 8 h according to the routine methods at the accredited laboratory at the Section of Bacteriology, National Veterinary Institute (SVA), Uppsala, Sweden. All uterine samples from dogs with pyometra included in this study were positive for bacterial growth. Blood samples for bacterial cultures were aseptically collected into a sterile syringe, and 3 mL blood was added to each of biphasic aerobic and anaerobic medium blood culture bottles (BOF, Substratlab, SVA, Uppsala, Sweden). The BOFs were transported to the accredited laboratory at Section of Bacteriology, SVA, Uppsala, Sweden and cultured in 37°C for 7 days or until growth. Bacteraemia, i.e. positive bacterial blood culture was detected in one dog with sepsis, i.e. SIRS-positive dog with pyometra (P⁺SIRS⁻), and two dogs with pyometra without SIRS (P⁺SIRS⁻).

HMGB1 measurement

Serum concentrations of canine HMGB1 were quantified using a commercially available human sandwich ELISA kit (ST51011; IBL-International, Hamburg,

Germany). The amino acid sequence of human HMGB1 is 100% homologous to that of canine HMGB1 (Murua et al., 2003) and the method has previously been validated for use in dogs (Ishida et al., 2011). Each assay was validated with assay control consisting of recombinant canine HMGB1 included in the kit and performed according to the manufacturer's instructions. All samples were analysed in duplicate. The intra-assay and inter-assay variation coefficients were calculated as described (Reed et al., 2002) and were below 9.6% and 16.7%, respectively. The lower detection limit of the assay was 0.97 ng/mL. The values above the concentration of the highest standard (20 ng/mL) that were not possible to re-evaluate due to the lack of additional sample volume (n = 2) were assigned values of 20 ng/mL.

CRP measurement

Canine serum CRP was analysed in 26 dogs with pyometra (19 P⁺SIRS⁺ and 7 P⁺SIRS⁻ dogs) and 17 healthy control dogs using an automated assay (High Linearity CRP, Randox Laboratories, Crumlin, United Kingdom) validated for dog serum (Kjelgaard-Hansen et al., 2003 and Klenner et al., 2010). The intra-assay and inter-assay variation coefficients were below 10% and 18%, respectively, and the lower detection limit of the assay was 5 µg/mL. All samples were assayed in duplicate and the assay was performed according to the manufacturer's instructions.

PGM measurement

The release of prostaglandin metabolite F_{2α} (PGF_{2α}) was monitored by measuring 15-keto-13,14-dihydro-PGF_{2α} concentrations, the main circulating PGM, in heparinized plasma from 16 dogs with pyometra (12 P⁺SIRS⁺ and 4 P⁺SIRS⁻) and 13 healthy control dogs using a radioimmunoassay (RIA) as previously described (Granström

and Kindahl, 1982). The intra-assay and inter-assay variation coefficients were below 8% and 14%, respectively, and the lower detection limit of the assay was 0.3 nmol/L. All samples were assayed in duplicate.

Statistical analysis

Data were analysed for normality using the following tests: Kolmogorov-Smirnov, D'Agostino and Pearson omnibus, Shapiro-Wilk. Concentrations of HMGB1 were normally distributed between the groups according to the Kolmogorov-Smirnov normality test, and unpaired *t*-test was used for statistical analyses. Mann-Whitney's *u*-test was used to analyse CRP and PGM because these variables passed none of the normality tests above. Analyses of pre- and postoperative values were performed similarly but using paired tests. Correlations of variables were evaluated using Spearman rank correlation and linear regression analyses. The biomarker performance was evaluated using receiver operator characteristic (ROC) analyses with sensitivity and specificity calculated for selected cut-off values. All analyses were performed using Graph Pad Prism 4.0c. A *p*-value < 0.05 was considered significant for all analyses performed.

Results

Increased HMGB1 concentrations in dogs with pyometra

Concentrations of HMGB1 were detectable in 94% of the serum samples. Dogs with pyometra had significantly higher concentrations of HMGB1 compared to healthy control dogs ($p < 0.05$; Fig. 1A). A cut-off value of 4.5 ng/mL for HMGB1 had sensitivity and specificity above 60% for differentiating between dogs with pyometra and healthy control dogs (Fig. 1B). Concentrations of serum HMGB1 did not differ significantly between dogs with or without SIRS in pyometra (Fig. 1C). Similarly, HMGB1 concentrations did not differ significantly between dogs with confirmed bacteraemia and those with negative blood cultures (Fig. S1A).

HMGB1 concentrations correlate with total leukocyte number

HMGB1 concentrations were significantly correlated with WBC ($p = 0.0002$; Fig. 1D), but not with the remaining SIRS criteria: PBN, body temperature, heart rate and respiratory rate (data not shown).

Higher CRP concentrations in pyometra and in dogs with sepsis

Serum CRP concentrations were significantly higher in dogs with pyometra compared to healthy control dogs ($p < 0.0001$; Fig. 2A), and a cut-off value of 29 $\mu\text{g/mL}$ had 85% sensitivity and 100% specificity for differentiating between dogs with pyometra and healthy control dogs (Fig. 2B). Moreover, CRP concentrations were able to differentiate between the SIRS-positive and SIRS-negative dogs with pyometra ($p < 0.05$; Fig. 2C) with 89.5% sensitivity and 71.4% specificity for a cut-off value 107 $\mu\text{g/mL}$ (Fig 2D). The CRP concentrations did not differ in the dogs with positive blood cultures ($n = 3$) and those with negative blood cultures ($n = 24$;

Fig. S1B). The concentrations of CRP did not correlate significantly with HMGB1 in canine serum (Fig. 2E).

Increased concentrations of HMGB1 but not CRP after surgery

Of dogs with pyometra sampled both before and after surgery 78% (7/9) had increased HMGB1 concentrations in the serum one day after surgery ($p < 0.05$; Fig. 3A). In contrast, CRP concentrations did not differ significantly between the pre- and postoperative samples (Fig. 3B).

Higher PGM concentrations in dogs with pyometra correlated with CRP

Plasma concentrations of PGM were significantly higher in dogs with pyometra compared to control dogs ($p < 0.001$; Fig. 4A). PGM concentrations in P⁺SIRS⁺ and P⁺SIRS⁻ dogs did not differ significantly (Fig. 4B). (Fig. 4B). PGM and HMGB1 were not correlated (Fig. 4C) but PGM concentrations were clearly correlated with CRP concentrations ($p < 0.0001$; Fig. 4D) in dogs with pyometra.

Discussion

A systemic inflammatory condition such as sepsis involves a massive release of cytokines (Stearns-Kurosawa et al., 2011) which might be detectable in the blood and therefore have a diagnostic potential or become targets for novel treatments. Whereas neutrophils generally are the first cells to appear on site during inflammation, the primary cell type recruited between 24 and 48 h after the initiation of inflammation are monocytes (Pepys, 1995). Human monocytes and murine macrophages are known to actively release HMGB1 in response to endotoxin challenge or pro-inflammatory cytokine stimuli (Wang et al., 1999a), but passive release of HMGB1 by necrotic non-immune cells is thought to be the source of systemically detectable HMGB1 in toxic shock as demonstrated in rats (Degryse et al., 2001). The results of the present study showed that serum concentrations of HMGB1 were increased in dogs with bacterial uterine infection (pyometra). This is in agreement with the previous findings showing that HMGB1 is a marker in both primary and secondary infection in humans (van Zoelen et al., 2007 and Allonso et al., 2012) and other findings supporting the idea that HMGB1 is one of the main diffusible signals of uncontrolled cell death (Scaffidi et al., 2002). However, concentrations of HMGB1 did not differ significantly between SIRS-positive and SIRS-negative dogs with pyometra, i.e. dogs with or without sepsis. Thus the potential of this cytokine to distinguish between local and systemic inflammation in pyometra, i.e. to detect the disease severity in a clinical setting, appears limited.

It is well established that increased CRP concentrations in the blood are strongly associated with inflammation in humans (Fagan et al., 1982 and Pepys, 1995) and animal species including dogs (Yamamoto et al., 1992 and Hayashi et al., 2001).

However, CRP has been shown to have a limited ability to distinguish sepsis from other inflammatory conditions in humans (Clyne and Olshaker, 1999). The results of the present study showed that CRP concentrations were increased in dogs with pyometra. Moreover, dogs with sepsis (P⁺SIRS⁺) had considerably higher concentrations of CRP compared to dogs without sepsis (P⁺SIRS⁻). These results are in agreement with our previous findings (Karlsson et al., 2012) and with an earlier study of 53 dogs with pyometra (Fransson et al., 2007). A study of 307 dogs with SIRS of both infectious and non-infectious origin showed that CRP concentrations were generally increased in plasma from dogs with SIRS (Ishida et al., 2011). Possibly CRP is a sensitive marker for SIRS, but it is not necessarily specific for sepsis, i.e. CRP concentrations may also be increased in dogs with inflammation of various other etiologies (Clyne and Olshaker, 1999, and Mitaka, 2005). Although it has been shown that CRP was able to differentiate between bacterial and viral infection in sepsis and complicated influenza in humans (Lindbäck et al., 1989), the clinical value of CRP for this differentiation in canine and possibly also human sepsis must be further evaluated.

Prostaglandin F_{2α} metabolite concentrations have been reported to be a reliable and sensitive marker of endotoxin release in cattle, pigs and goats (Fredriksson, 1984, Fredriksson et al., 1985 and Holst et al., 1993). We did not quantify the systemic concentrations of endotoxin in this study but instead measured PGM as an indicator for the release of endotoxin into the bloodstream due to the massive infection in the uterus during pyometra. Previously, we showed that PGM concentrations were highly increased in bitches with pyometra (Hagman et al., 2006a) and with sepsis caused by pyometra (Hagman et al., 2006b). In the present study, although PGM concentrations

were increased, the concentrations did not differ in dogs with or without sepsis. Interestingly, the PGM concentrations correlated significantly with concentrations of CRP but not with HMGB1. This supports the finding that HMGB1 and PGM are involved in different inflammatory pathways, and that HMGB1 might provide important independent information about the inflammatory processes caused by infection.

In the present study, CRP and HMGB1 concentrations were not correlated in dogs with pyometra or in dogs with pyometra and sepsis, which is in line with the results of the study by Ishida and coworkers (2011), in which the concentrations of CRP and HMGB1 were not correlated in dogs with SIRS. Systemic CRP is produced by hepatocytes, and hepatic CRP production is potentiated mainly by IL-6 with no effect of TNF α or IL-1 (Castell et al., 1989 and Yamashita et al., 1994). In contrast, HMGB1 secretion by macrophages is stimulated by TNF α , IL-1 and IFN- γ (Wang, 1999b and Rendon-Mitchell et al., 2003). This indicates that HMGB1 and CRP are differently regulated during the inflammatory response, which may explain why HMGB1 and CRP concentrations were not correlated in dogs with pyometra and sepsis. In addition, we found that concentrations of HMGB1, but not CRP, were correlated with one of the SIRS criteria, WBC. Elevated WBC is a typical sign of inflammation commonly used in the clinic, which suggests that serum HMGB1 concentrations reflect similar inflammatory processes as detected by WBC and any added value of HMGB1 analysis in dogs with sepsis remains to be further explored.

Sepsis in pyometra is caused by bacterial infection of the uterus, which induces local and subsequent systemic inflammation. When the source of infection is removed, i.e.

the uterus with bacterial content is surgically removed, the amount of endotoxin released into the blood is expected to gradually decrease and concentrations of biological markers sensitive to endotoxin in the blood or to ongoing inflammation should also decrease. The inflammation is, however, still active one day after surgery since the surgery itself causes inflammation albeit not at the same level as endotoxin. Our results showed that most dogs with pyometra had higher concentrations of HMGB1 one day after surgical treatment, which was not the case for CRP. Although it was shown previously that CRP concentrations were increased 24 h after surgery in dogs with pyometra (Dabrowski et al., 2007), our results are well in agreement with another independent study on dogs with SIRS. This study showed that CRP concentrations increased three days after ovariohysterectomy, whereas HMGB1 concentrations were higher on day one after surgery and then decreased (Ishida et al., 2011). Earlier reports suggest that a preformed protein pool of HMGB1 can be released by damaged or immune cells and peak within 16 h after *in-vitro* LPS stimulation in human and rodent cells, activating in turn other cells and leading to prolonged inflammation (Wang et al., 1999a and Scaffidi et al., 2002), and that systemic concentrations of HMGB1 peak as early as at 16 h in serum of LPS-treated mice (Wang et al., 1999a). In contrast, canine CRP is gradually produced in the liver starting from the onset of pro-inflammatory stimuli with a peak of systemic concentration reached first after 24 h (Conner et al., 1988, Yamamoto et al., 1992). HMGB1 could therefore be considered an earlier inflammatory marker than CRP when the presence and not the extent of the inflammation is of diagnostic interest.

Bacteriological blood cultures are routinely used to diagnose systemic infection, but they are time consuming (>24 h), have varying sensitivity (10-67%) (Kuruville et al.,

1998 and Lee et al., 2007) and can be inconclusive for slow-growing pathogens or in patients that have received antimicrobial treatment. There were few positive blood cultures in our study, which limits the possibility to detect any differences between the groups. Further studies including more dogs and possibly also repeated samples cultured from each dog to increase the possibility of detecting irregular bacteraemia would be useful for this purpose.

Although increased serum concentrations of HMGB1 have been associated with multiple organ dysfunction syndrome (MODS) and fatal outcome in severe sepsis, the kinetics of HMGB1 release has been shown to differ substantially depending on the primary source of infection in human sepsis (Wang et al., 1999a, Karlsson et al., 2008, van Zoelen et al., 2007). Measuring serum HMGB1 in dogs with pyometra did not allow detection of animals that had developed sepsis. It may be due to few dogs with severe sepsis and MODS in the present study, no dogs with lethal outcome and the possibility of false-positive SIRS-diagnosed subjects (Hauptman et al., 1997). However, the finding that HMGB1 is higher in dogs with pyometra compared to healthy dogs in the present study indicates that HMGB1 might be an important marker in uterine infection, and should be further evaluated as a potential screening parameter of infection and inflammation. Since HMGB1 concentrations were not correlated with concentrations of CRP or PGM, HMGB1 is likely to be involved in different inflammatory mechanisms than CRP and PGM, and it may thus be a source of new information about the inflammatory response during infection.

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Table 1. Clinical and laboratory variables in dogs with pyometra^a

Parameters ^b	P ⁺ SIRS ⁺ (n = 20)	P ⁺ SIRS ⁻ (n = 7)	Controls (n = 23)
Body temp (°C)	39.3 (38.9 – 39.7)	38.5 (38.2 – 38.9)	38.2 (38.1 – 38.3)
HR (counts/min)	100 (86.5 – 127)	100 (85 – 100)	96 (84 – 102)
RR (counts/min)	29 (24.5 – 82.5)	16 (16 – 18)	16 (16 – 21)
WBC (cells ×10 ⁹ /L)	20.7 (18.2 – 25.6)	11.5 (10.4 – 14.2)	9.8 (8.2 – 12)
PBN (%)	26.4 (10.8 – 74.7)	16.8 (2.9 – 36.7)	0.77 (0.0 – 4.76)
Lymph (cells ×10 ⁹ /L)	1.8 (1.0 - 2.6)	1.45 (0.95 – 1.75)	2.4 (1.7 – 3.3)
Mono (cells ×10 ⁹ /L)	2.35 (1.65 – 3.8)	0.4 (0.25 – 1.6)	0.4 (0.3 – 0.7)
Eosin (cells ×10 ⁹ /L)	0.0 (0.0 – 0.65)	0.4 (0.05 – 0.6)	0.5 (0.2 – 1.4)
Baso (cells ×10 ⁹ /L)	0.0 (0.0 – 0.0)	0.0 (0.0 – 0.0)	0.0 (0.0 - 0.1)
ALAT (μkat/L)	0.3 (0.2 – 0.4)	0.4 (0.3 – 1.4)	0.5 (0.4 – 0.7)
Bile acids (g/L)	1.85 (0.65 – 3.95)	2.5 (0.8 – 2.6)	3.8 (1.8 – 5.7)
Crea (μmol/L)	62 (54.5 – 76)	53 (48 – 62)	76 (61 – 80)

^aP, Pyometra; SIRS, Systemic inflammatory response syndrome; Controls, healthy intact female dogs. Data presented as median (interquartile range).

^bHR, heart rate; RR, respiratory rate; WBC, total number of white blood cells; PBN, percentage band neutrophils; Lymph, lymphocytes; Mono, monocytes; Eosin, eosinophils; Baso, basophils; ALAT, alanine aminotransferase; Crea, creatinine.

Figure legends

Figure 1. Increased concentrations of serum high-mobility group box 1 (HMGB1) in dogs with pyometra. Horizontal line in each group of samples represents the mean value. A) HMGB1 concentrations were higher in dogs with pyometra (Pyometra, n = 27) compared to healthy controls (Control, n = 23). B) Receiver-operator curve for HMGB1 concentrations in all samples. Area under the curve = 0.62, $p = 0.12$. C) HMGB1 concentrations in dogs with pyometra and SIRS, i.e. dogs with sepsis (P^+SIRS^+ , n = 20), compared to dogs with pyometra without SIRS (P^+SIRS^- , n = 7). D) Correlation between HMGB1 and total white blood cell count (WBC) in dogs with pyometra (n = 27) using linear regression analysis. * $P < 0.05$.

Figure 2. Higher C-reactive protein (CRP) concentrations in dogs with sepsis caused by pyometra. The figure shows concentrations of CRP measured in serum (n = 43). Bars denote the minimal and maximal values; boxes indicate 1st quartile, median and 3rd quartile. A) CRP concentrations in dogs with pyometra (Pyometra, n = 26) compared to healthy dogs (Control, n = 17). B) Receiver-operator curve for CRP concentrations in all samples. Area under the curve = 0.93, $p < 0.0001$. C) Concentrations of CRP in dogs with pyometra and SIRS, i.e. dogs with sepsis (P^+SIRS^+ , n = 19), compared to dogs with pyometra without SIRS (P^+SIRS^- , n = 7). D) Receiver-operator curve for CRP concentrations in samples from SIRS-positive and SIRS-negative dogs with pyometra. Area under the curve = 0.82, $p = 0.013$. E) Correlation between concentrations of CRP and HMGB1 in dogs with pyometra (n = 26) using linear regression analyses. ** $P < 0.01$; *** $p < 0.001$.

Figure 3. Increased high-mobility group box 1 (HMGB1) concentrations one day after surgery. The figure illustrates serum concentrations of HMGB1 (A) and CRP (B) measured before (Preop) and one day after (Postop) ovariohysterectomy in dogs with pyometra (n = 9). Paired *t*-test (CRP) and Wilcoxon matched pairs test (HMGB1); * $p < 0.05$.

Figure 4. Higher prostaglandin F_{2α} metabolite (PGM) concentrations in pyometra. The figure illustrates PGM concentrations measured in canine plasma (n = 29). Bars denote the minimal and maximal values; boxes indicate 1st quartile, median and 3rd quartile. A) Concentrations of PGM in dogs with pyometra (Pyometra, n = 16) compared to healthy dogs (Control, n = 13). B) Concentrations of PGM in dogs with pyometra and SIRS, i.e. dogs with sepsis (P⁺SIRS⁺, n = 12, $p = 0.19$), compared to dogs with pyometra without SIRS (P⁺SIRS⁻, n = 4). C) Correlation between PGM and HMGB1 serum concentrations in dogs with pyometra (n = 16) using linear regression analyses. D) Correlation between PGM and CRP serum concentrations in dogs with pyometra (n = 15) using linear regression analyses. *** $P < 0.001$.

Figure S1. Serum high-mobility group box 1 (A) and C-reactive protein (B) concentrations in dogs with positive (Blood culture⁺, n = 3) or negative (Blood culture⁻) for bacterial growth in the blood (n = 24 for HMGB1 and n = 17 for CRP). Unpaired *t*-test. Horizontal line in each group of samples represents mean value.