Systemic Inflammatory Response in Canine Pyometra

The Response to Bacterial Uterine Infection

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Abstract

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Research efforts have focused mainly on the hormonal aspects of canine pyometra for more than 6 decades. However, this disease is often manifested as systemic illness in response to the bacterial uterine infection.
Studies I-II were undertaken to clarify bacteriological aspects of canine pyometra; *i.e.* the origin of the infecting bacteria, the infecting bacteria's impact on severity of the systemic illness and the presence of bacterial endotoxin in the systemic circulation. Study I, a bacterio-epidemiologic study, investigated the predominant bacteria, *Escherichia coli*, using biochemical fingerprinting. The homogeneity among *E. coli* populations, isolated from various sites in bitches with pyometra and from faeces of healthy dogs, was determined. Study II, a clinical study of bitches with pyometra, determined uterine bacterial species, haematology, blood biochemical parameters and plasma endotoxin levels. The impact of infecting bacteria on blood parameters and clinical status was studied. Study III investigated if bitches with pyometra display the Systemic Inflammatory Response syndrome (SIRS) and if SIRS relates to outcome. Systemic levels of interleukin-6 (IL-6), C - reactive protein (CRP) and tumor necrosis factor α (TNFα) were determined, investigating a possible correlation between these inflammatory markers and SIRS or outcome. Study IV used clinical parameters, haematology, blood biochemical parameters, CRP and TNFα to clinically differentiate pyometra from the often preceding uterine condition cystic endometrial hyperplasia (CEH).
Study I revealed that *E. coli* isolated from bitches with pyometra show high level of homogeneity indicating that *E. coli* associated with pyometra may have properties, yet undetermined, in common. Identical clones of *E. coli* were found in the faeces and uterus of bitches with pyometra, indicating an ascending infection route. Study II failed to show systemic endotoxemia in bitches with pyometra, but showed many other signs of systemic affection in blood parameters. Study III showed that 57 % of pyometra cases fulfil clinical criteria for SIRS and that SIRS criteria are correlated to increased length of hospitalization. Body temperature, heart rate and CRP correlated to SIRS and CRP correlated to outcome. Study IV revealed that clinical signs and levels of percent band neutrophils and CRP aid in the differentiation of CEH from pyometra.

Keywords: PhenePlate® system, lipopolysaccharide, limulus amebocyte lysate assay, ELISA, interleukin 6 bioassay, acute phase protein, inflammatory mediators.

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To Claude,
without who's bright ideas, help and support this thesis would not have been completed

ΩΩΩ

Tucked away in our subconscious is an idyllic vision. We see ourselves on a long trip that spans the continent. We are traveling by train. Out the windows we drink in the passing scene of cars on nearby highways, of children waving at a crossing, of cattle grazing on a distant hillside, of smoke pouring from a power plant, of row upon row of corn and wheat, of flatlands, and valleys, of mountains and rolling hillsides, of city skylines and village halls.

But uppermost in our minds is the final destination. Bands will be playing and flags waving. Once we get there our dreams will come true, the pieces of our lives will fit together like a jigsaw puzzle. How restlessly we pace the aisles, damning the minutes for loitering—waiting, waiting, waiting for the station.

"When we reach the station, that will be it!" we cry.
"When I'm 18."
"When I buy a new Mercedes Benz."
"When I get a promotion, I shall live happily ever after!"

Sooner or later, we must realize there is no station, no one place to arrive once and for all. The true joy of life is the trip. The station is only a dream. It constantly outdistances us.

So stop pacing the aisles and counting the miles. Instead, climb more mountains, eat more ice cream, go barefoot more often, swim more rivers, watch more sunsets, laugh more, cry less. Life must be lived as we go along. The station will come soon enough. You should enjoy the ride, not wait for the station.

ΩΩΩ

After Robert J. Hastings "The Station"

Contents

Appendices

Papers I-IV

This thesis is based on the following 4 papers, which will be referred to by their Roman numerals:

I. Wadas (Fransson) B., Kühn I., Lagerstedt A-S. & Jonsson P. 1996. Biochemical phenotypes of *Escherichia coli* in dogs: Comparison of isolates isolated from bitches suffering from pyometra and urinary tract infection with isolates from faeces of healthy dogs. *Veterinary microbiology* 52: 293-300

II. Fransson B., Lagerstedt A-S., Hellmen E. & Jonsson P. 1997. Bacteriological findings, blood chemistry profile and plasma endotoxin levels in bitches with pyometra or other uterine diseases. *Journal of veterinary medicine series A* 44: 417-426

III. Fransson B.A., Lagerstedt A.-S., Bergstrom A., Hagman R., Park J.S., Chew B.P., Evans M.A. & Ragle C.A. 2003. Systemic inflammatory response in canine pyometra. Submitted for publication.

IV. Fransson B.A., Karlstam E., Bergstrom A., Lagerstedt A.-S., Park J.S., Evans M.A. & Ragle C.A. 2003. C-reactive protein can aid in the differentiation of pyometra from cystic endometrial hyperplasia in dogs. Accepted for publication in *Journal of the American Animal Hospital Association.*

Abbreviations

ALAT	Alanine aminotransferase
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase (am.)
AP	Alkaline phosphatase (am.)
ASAT	Aspartate aminotransferase
BUN	Blood urea nitrogen
CEH	Cystic endometrial hyperplasia
CK	Creatin kinase
CRP	C-reactive protein
ET	Endotoxin
IL	Interleukin
LAL	Limulus amebocyte lysate
LD	Lactate dehydrogenase
LPS	Lipopolysaccharide
MTT	3,[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
PBN	Percent band neutrophils
PCA	Perchloric acid
PCV	Packed red blood cell volume
SIRS	Systemic inflammatory response syndrome
TNF	Tumor necrosis factor
UTI	Urinary tract infection
WBC	White blood cell count

Introduction

The Pathogenesis of Canine Pyometra, a Background

History and definition

Mankind has struggled with existential questions like "where did we come from, and what are we doing here?" since before the time when Aristotle (884-322 B.C.) approached these questions in a scientific manner. Similar questions could be applied to the pathogenesis of canine pyometra, a topic that has triggered the interest of veterinarians since the 1930's (Teunissen, 1938). However, more than six decades of studies have still not led to a complete understanding of the pathogenesis of pyometra.

Canine pyometra is a disease affecting the adult intact bitch, causing a variety of clinical signs of genital and systemic disease. The concept of cystic endometrial hyperplasia (CEH) - Pyometra, was introduced by Dow (1957) and stated that hormonal changes lead to CEH, which predisposes the uterus to secondary infection, leading to pyometra. Before Dow's investigations the condition had been described under a variety of names. Dow (1957, 1958, 1959a, 1959b) described the development of pyometra from a pathological-anatomical point of view. He concluded that the pus-distended uterus seen in pyometra is the end result of a series of uterine changes. These changes range from the uncomplicated CEH, over inflammatory changes in the endometrium in combination with CEH, to the end stage; chronic purulent endometritis, with pus accumulation in the uterus. The gradual transition makes it challenging to define pyometra morphologically and clinically. However, definitions of pyometra as an acute or chronic polysystemic disease in mature bitches (Hardy & Osborne, 1974) or an acute or chronic endometritis (Boerresen, 1975) occurring in metestrus, appears generally accepted. Lately, the classical CEH-pyometra complex has been challenged. It has been suggested that in some cases the development of pyometra is a separate entity from CEH, with a hormonal component in the pathogenesis but mainly triggered by bacterial infection (De Bosschere *et al.*, 2001).

Hormonal component

Traditional theories suggest that hormonal changes render the uterus susceptible for infection. This understanding was originally based on work by Teunissen (1952) and Dow (1957, 1958, 1959a, 1959b), who investigated the importance of estrogen and progesterone in the development of endometritis. They found that the cystic and inflammatory changes of the uterus associated with endometritis could be reproduced by injections of progesterone. Estrogen alone seemed to play a less important role, but appeared to enhance the endometrial response to progesterone, and exogenous estrogen administration, used to terminate pregnancy, has been reported to increase the risk for pyometra (Bowen *et al.*, 1985). Progesterone stimulates endometrial glandular secretion and suppresses contractions of the uterus, and thus creates an intrauterine environment predisposing to bacterial growth (Cox, 1970). Several authors investigated whether prolonged or excessive

progesterone production would be responsible for the development of pyometra, but failed to show such disturbances (Christie, 1972; Hadley, 1975; Chauffaux & Thibier, 1978; Austad *et al.*, 1979). More recently, the possibility of an exaggerated endometrial response to progesterone and/or estrogen has been investigated (Dhaliwal *et al.*, 1999; De Bosschere *et al.* 2002). Presently, there is no conclusive evidence for changes in these hormon receptors being responsible for the development of pyometra. Interestingly enough, De Bosschere and co-workers (2001) found that the expression of estrogen and progesterone receptors differed significantly between uteri of bitches with CEH and uteri of bitches with pyometra, and suggested that these differences indicate different pathogenesis of the two conditions. The expression of androgen receptors in the uterus was found to be significantly decreased in bitches with pyometra as compared to healthy bitches (Sauerwein *et al.*, 1998).

Bacteriological component

In the past, investigations of the bacteria associated with canine pyometra have been few. However, multiple authors have reported a predominance of *Escherichia coli* (Asheim, 1965; Renton *et al.*, 1971; Grindlay *et al.*, 1973; Sandholm *et al.*, 1975; Kivisto *et al.*, 1977; Vandesplassche, 1991; Dhaliwal *et al.*, 1998). Sandholm and co-workers (1975) found that *E. coli* adheres to receptors in the progesterone-stimulated endometrium, which might be one explanation for the observed predominance of this bacterium. Grindlay and co-workers (1973) found that certain serotypes of *E. coli*, *e.g.* 02, 04, 06, and 075, were more commonly associated with pyometra than others. Dhaliwal and co-workers (1998) concluded that *E. coli* serotype 032 and 04 were the most commonly prevalent in their study population. In addition, they found that Cytotoxin Necrotizing Factor (CNF) was expressed in 7/16 (44%) serotypes associated with pyometra. Certain serotypes appeared to affect the endometrium more severely than others, and the presence of CNF was associated with more severe endometrial changes.

The route of infection of the uterus was early on suggested to be hematogenic or lymphogenic as well as ascending (Dow, 1959b). The ascending route was not supported by the work of Meyers-Wallen and co-workers (1986), who observed that the type of bacteria isolated from the vagina did not necessarily represent the bacterial species isolated from the uterus in pyometra. Sandholm and coworkers (1975) showed that cystitis was commonly associated with canine pyometra, and that the *E. coli* bacteria isolated from the urinary bladder and the isolate from the uterus showed many similarities. It was suggested that the urinary tract may serve as a bacterial reservoir, and bacteria ascend into the uterus during a susceptible stage in the estrous cycle (Sandholm *et al.*, 1975).

Clinical signs and laboratory parameters in pyometra

Common clinical signs of pyometra are not limited to the genital tract, *e.g.* vaginal discharge, but include systemic signs such as vomiting and/or inappetence, polyuria /polydipsia and lethargy. (Dow, 1957; Borresen, 1979; Nelson & Feldman, 1986; Stone *et al.*, 1988). It has been suggested that the clinical signs are

more severe in cases where the cervical canal is occluded (Dow, 1958, Borresen, 1975). However, the cervix may spontaneously open or close during the disease, causing intermittent vaginal discharge or a sudden deterioration in the clinical status of the bitch (Studdert, 1971).

The systemic effects of pyometra are reflected by several laboratory parameters. The most characteristic alteration is an inflammatory leukogram with marked elevation of the total white blood cell count (WBC) and usually a regenerative left shift in the differentiated WBC count (Dow, 1957; Sandholm *et al*., 1975; De Shepper *et al.*, 1986; Stone *et al.*, 1988; Wheaton *et al.*, 1989; Sevelius, 1990). Other common blood work abnormalities include a normocytic, normochromic anemia. The anemia has been suggested to be caused by decreased erythropoesis, so called anemia of chronic disease, and by loss of erythrocytes into the uterine lumen (Shalm, 1973). Anemia of chronic disease can be caused by a variety of disorders including chronic inflammation, in which lactoferrin and other acute-phase reactants mediate an iron sequestration within the myeloid cells in the bone marrow, withdrawing iron from the normal erythropoiesis (Nelson & Couto, 1998). Hyperglobulinemia with a pronounced hypoalbuminemia, bilirubinemia, hypercholesterolemia and increases in alkaline phosphatase (AP), aspartate aminotransferase (ASAT) and lactate dehydrogenase (LD) have also been associated with pyometra. In contrast, serum alanine aminotransferase (ALAT) is usually significantly decreased (Borresen, 1980; De Schepper *et al.*, 1987; Capiau *et al.*, 1987). Asheim (1964) suggested that the cause of hyperglobulinemia, concurrent with hypoalbuminemia was due to renal loss of albumin, but later studies have demonstrated only a mild to moderate urinary protein loss (Borresen & Skrede, 1980; Sevelius *et al.*, 1990), and interpreted the changes in serum proteins as part of an acute phase reaction (Borresen & Skrede, 1980). The elevated levels of ALP, bilirubin and serum cholesterol have been considered to be due to intrahepatic cholestasis (Borresen, 1980). The enzymes ASAT and LD, which are normally present in the liver as well as in skeletal and heart muscle, are often elevated in pyometra cases (Borresen & Skrede, 1980). Borresen & Skrede (1980) suggested that the elevated levels of ASAT and LD more likely are derived from muscle breakdown than hepatocellular insult. This suggestion was based on the observation of increases in the muscular enzyme creatine kinase (CK) and decreased activity of ALAT (Borresen & Skrede, 1980; De Schepper *et al.*, 1987a), indicating that no hepatocellular damage has occurred. Subsequent examinations of liver biopsy specimens showed fatty infiltration and bile pigments, consistent with cholestasis, but no gross hepatocellular necrosis (Borresen, 1980; Sevelius *et al.*, 1990). De Schepper and co-workers (1987a) suggested that endotoxin-related liver dysfunction may be responsible for the observed increase in the ASAT and the decrease in ALAT concentration.

Azotemia in canine pyometra is seen in 15-31 % of cases (increased serum creatinine and/or elevated blood urea nitrogen, BUN)(Borresen, 1980; De Schepper *et al.*, 1987a; De Schepper *et al.*, 1987b). Asheim (1963-1965) studied the renal dysfunction. He concluded that disturbances in the release of antidiuretic hormone from the hypothalamus-pituitary gland were unlikely reasons for the polyuria in bitches with pyometra (Asheim 1963). Supporting this conclusion, further studies revealed pathological changes in the kidney, which shared

morphological features with glomerulinephritis in humans and were reversible after ovariohysterectomy (Asheim, 1965; Obel *et al.*, 1964). Asheim suggested that the presence of bacteria in the uterus was responsible for the decreased urine concentration ability. Furthermore, he reported that injection of *E. coli* toxin induced a reversible reduction in the renal concentrating ability. Later authors have suggested that this effect was caused by endotoxin (Borresen, 1975). This theory was contradicted by Stone and co-workers (1988) who found neither structural glomeruli changes nor the presence of bacteria in the uterus to correlate with the loss of urinary concentration ability. However, De Schepper and co-workers (1989) found indications of glomerular damage in 54 of 74 (73%) bitches with pyometra, as reflected by proteinuria, either alone or in combination with tubular lesions, the latter indicated by elevated urinary levels of gamma-glutamyl transferase (GGT). More recent investigations of renal dysfunction in canine pyometra showed that 13/55 dogs (24%) showed increase of GGT and/or N-acetyl-beta-glucosaminidase, which are enzyme markers for tubular damage. The increase in urinary enzymes was sometimes associated with decreased glomerular filtration rate (Heinene, Moe & Molmen, 2001).

Endotoxin in pyometra

Blood endotoxin concentrations have been shown to relate to severity of clinical signs and mortality in canine pyometra. Endotoxin concentrations in dogs that died from pyometra were significantly higher than in bitches with a good outcome (74.2±18.3 pg/ml versus 9.5 ±18.3 pg/ml) (Okano *et al.*, 1998). Borresen & Naess (1977) did not find endotoxemia whereas another study demonstrated endotoxemia with mean plasma endotoxin concentrations of 438 pg/ml in 15 bitches with pyometra prior to surgery (Wessels & Wells, 1989).

The lipopolysachharide endotoxin (ET) is a cell wall component of *E. coli* and other gram-negative bacteria, and is released either from bacterial cell death and disruption, or during vigorous growth of the bacteria (Crutchley *et al.*, 1967). Normally, small amounts of ET from the intestinal bacterial flora are absorbed into the portal blood and transported to the liver, where it is eliminated in two steps (Nolan, 1988; Fox *et al.*, 1990). The Kuppfer cells trap the ET and modify it, to facilitate uptake by the hepatocytes. The hepatocytes then partially detoxify and eliminate the modified ET. Clearance of ET from the portal blood is very rapid and occurs within minutes (Fox *et al.*, 1990). The partially degraded ET is slowly excreted from the body, mainly through the gut. Another route of ET excretion, under normal circumstances less important, is through the lungs, where macrophages carrying ET migrate to the lung and pass into the alveolar and bronchiolar space (Freudenberg & Galanos, 1988).

Systemic effects of ET are seen only when the ET clearance capacity of the liver is exceeded (spill-over effect) (Okano *et al.*, 1993). When ET has gained entrance into the circulation, a broad spectrum of biological effects may occur. Among the beneficial effects are generalized stimulation of the immune system and microbial killing. On the other hand, a massive release of ET often causes irreversible shock and death (Rietschel & Brade, 1992). Experimentally, sub-lethal doses of ET have

been shown to cause fever, lethargy and an increase in heart and respiration rate (Van Miert & Frens, 1968). Higher ET doses in dogs lead to hemorrhagic diarrhea and vomiting (Hardie & Kruse-Elliot, 1990). Initial hemodynamic changes lead to portal hypertension, hepatosplanchnic pooling of blood, and a decrease in central venous blood pressure, cardiac output and systemic blood pressure (Hardie & Kruse-Elliot, 1990). In endotoxic shock, these early changes are transitory and normal values are regained, but with inadequate treatment the condition progresses to refractory hypotension. Myocardial failure and death are usually the outcome of severe endotoxic shock. Histopathologically, changes including congestion of liver and kidneys, hepatocellular necrosis, mild pulmonary edema as well as subendocardial, adrenal and gastrointestinal tract hemorrhage have been noted (Hardie & Kruse-Elliot, 1990). The molecule ET is not directly cytotoxic but interacts with inflammatory cells, mainly macrophages, platelets and vascular endothelium resulting in the release of a cascade of inflammatory mediators, such as cytokines (Tumor Necrosis Factor, Interleukin-1, -6, -8), lipid mediators (thromboxane, prostaglandins, platelet-activating factor) and oxygen free radicals (Rietchel & Brade, 1992). In response to these primary mediators, multiple secondary mediators are released and together the mediators induce inflammatory changes and cell death (Hardie & Kruse-Elliot, 1990).

Severity of clinical signs has also been related with degree of immunosuppression in dogs with pyometra (Faldyna *et al.*, 2001). Immunosuppression was reflected by a decrease in circulating actively phagocytosing neutrophils and monocytes in the peripheral blood, and by inhibition in lymphocyte activity. The lymphocyte inhibition activity could be transferred with serum of bitches with low lymphocyte activity to cell cultures of normal lymphocytes. Serum from bitches with pyometra showed higher immunoglobulin content, and circulating immune-complexes and lysozyme. The clinically most affected dogs also showed the most pronounced changes in these tests, and clinical status was directly proportional to the level of leukocytosis and lymphopenia in these dogs (Faldyna *et al.*, 2001). The suppression of lymphocyte activity may be induced by endotoxemia. Bacterial products or components could be one reason for impaired immune response and human endotoxemia has been associated with decreased production of pro-inflammatory cytokines (Granowitz *et al.*, 1993).

Systemic inflammatory response syndrome

Systemic inflammatory response syndrome (SIRS) is the clinical manifestation of a response to an inciting stimulus, severe enough to cause systemic release of circulating inflammatory mediators (Purvis & Kirby, 1994; Brady & Otto, 2001). Any severe injury or infection can potentially lead to SIRS, such as pancreatitis, heat stroke, burns, multiple trauma or pan-systemic neoplasia (Purvis & Kirby, 1994). Sepsis is a classical disease manifested as SIRS and sepsis is indeed defined as SIRS resulting from infection (Purvis & Kirby, 1994; Hardie, 1995). Canine pyometra is another common disease suggested to result in SIRS and is like sepsis associated with bacterial infection (Hardie, 1995). However, the frequency with which dogs with pyometra are displaying SIRS has not been determined. Early

identification of patients affected by SIRS is critical due to the risk for fatal complications resulting from progression of this syndrome. The critically ill patient with SIRS is at risk for development of Multiple Organ Dysfunction Syndrome (MODS), which carries a high mortality rate despite recent years advances in critical care (Brady & Otto, 2001; Deitch & Goodman, 1999). Even the less severely affected patient with SIRS is at risk for MODS if a reactivation of the inflammatory response occurs, often caused by a perceivable minor event such as a catheter site infection, the so called "two hit" theory (Sasdua & Schein, 1999). An imbalanced immune response to pro-inflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha (TNFα) has been considered the reason for progression of SIRS into multiple organ dysfunction (Brady & Otto, 2001; Deitch & Goodman, 1999). In humans, clinical criteria have been established in order to recognize SIRS (Bone *et al.*, 1992) and these criteria have shown a significant correlation with mortality and/or morbidity as reflected by physiologic deterioration and increasing organ dysfunction (Muckart & Bhagwanjee, 1997; Pietrantoni *et al.*, 2003; Bochicchio *et al.*, 2002; Buter *et al.*, 2002; Afessa *et al.*, 2001; Sun & Aikawa, 1999; Bossink *et al.*, 1998; Shoenberg, Weiss & Radermacher, 1998). The limits for clinical criteria used in people, including fever or hypothermia, tachycardia, hypocapnea or tachypnea, increased or decreased white blood cell count and/or increased band neutrophils, have been adapted for use in dogs. The clinical criteria as presented by Purvis & Kirby (1994), Hardie (1995) and Hauptman, Walshaw & Olivier (1997) are presented in Table 1.

Table 1. *Clinical criteria of systemic inflammatory response syndrome (SIRS) and their limits for use in dogs in three studies. SIRS is suspected if two of four criteria are present in a canine patient*

Criteria	**Purvis & Kirby (1994)**	**Hardie (1995)**	**Hauptman, Walshaw & Olivier (1997)**
Temperature (°C)	<37.8; >39.7	<38.0; >40.0	<38.1; >39.2
Heart rate (beats/ min)	>160	>120	>120
Respiratory rate (breaths/min)	>20	>20	>20
WBC (x 10^3/μL); band neutrophils	<4, >12; >10 %	<5, >18; >5 %	<6, >16; >3 %

Hauptman, Walshaw & Olivier (1997) investigated the sensitivity and specificity of the different limits for detection of sepsis. They found that the previously suggested limits showed sensitivity in the detection of sepsis of only 77-83% (Hauptman, Walshaw & Olivier, 1997). With further modifications of the limits for these criteria, Hauptman reached a sensitivity of 97%, minimizing the risk for the serious consequences that can result from failure to identify sepsis, *i.e.* SIRS, before the onset of organ dysfunction. However, with the limits defined to increase sensitivity, the numbers of false positive results in the identification of sepsis increased (specificity 64%) (Hauptman, Walshaw & Olivier, 1997).

Multiple human studies have evaluated different inflammatory mediator's ability to more specifically act as markers for sepsis/SIRS and predictors of outcome (Reny *et al.* 2002; Marik, 2002; Harbarth *et al.*, 2001; Whicher *et al.* 2001; Bossink *et al.*, 1998). In human critical care, plasma TNFα has been shown to increase with SIRS and MODS (Spielmann *et al.*, 2001) and increases in IL-6 and the acute phase protein C-reactive protein (CRP) were detected in infected patients with SIRS (Gogos *et al.*, 2001).

Human CRP was first detected in 1930 and was named for its ability to bind the C polysaccharide of Streptococcus pneumoniae (Tillet & Francis, 1930). C-reactive protein is mainly synthesized by the liver as part of the acute phase response after hepatocyte stimulation with pro-inflammatory cytokines such as IL-6, IL-1 and transforming growth factor β (Young , Gleeson & Cripps, 1999). The function of CRP is not fully understood but this protein does show anti-inflammatory properties (Zouki *et al.,* 1997; Tilgh *et al.,*1993). Alone or together with procalcitonin, CRP has been shown to be a valuable marker for sepsis in critically ill people (Reny *et al.,* 2002; Suprin *et al.,* 2000). In addition, CRP has shown to be of great diagnostic importance in atherosclerotic cardiovascular disease where it has predictive value (Kereiakes, 2003). In dogs, several studies have shown a significant increase of CRP over the base-line value in experimentally induced inflammation (Hayashi *et al.,* 2001; Otabe *et al.* 2000; Yamashita *et al.* 1994). However, only a couple of clinical studies of CRP in dogs are available. A group of veterinarians in Poland reported increased levels of CRP in bitches with pyometra (Krzyzanowski *et al.,* 2000) and another report showed CRP elevations in dogs with various disorders and surgical traumas (Yamamoto *et al.,* 1993).

Aims of the study

The purpose of this investigation was to:

- Determine the most prevalent bacterial species in canine pyometra and the origin of this bacterium.
- Determine if the bacterial species of the uterine infection is related to severity of disease or to the presence of endotoxemia.
- Identify the frequency of the systemic inflammatory response syndrome in canine pyometra.
- Identify biochemical markers for severity of the systemic inflammatory response in pyometra.
- Identify biochemical markers able to predict outcome in canine pyometra.
- Identify biochemical or clinical markers for differentiation between pyometra and cystic endometrial hyperplasia.

Material and methods

Animals, data and sample collection (I-IV)

All bitches with pyometra included in studies I through IV were client-owned clinical cases seen at the Dept. of Small Animal Clinical Sciences, Swedish University of Agricultural Sciences, Uppsala, Sweden. The studies were approved by the Swedish Animal Ethical Committee prior to onset of the investigations. All dogs obtained the presumptive diagnosis of pyometra after history review, physical examination and/or diagnostic imaging and they underwent subsequent ovariohysterectomy. Final diagnosis was verified through histopathologic examination of uteri.

In study I-II the bitches included were clinical cases seen at the Dept. of Small Animal Clinical Sciences, from 1991 to 1994. As control animals served clinically healthy laboratory beagles. Blood samples for hematology, biochemical panels and plasma endotoxin determinations were obtained immediately pre-operatively in all dogs. In dogs with the presumptive diagnosis pyometra, an additional set of blood samples were obtained approximately 48 hours after surgery. These samples were transported without delay to the clinical laboratory, Department of Clinical Chemistry, Uppsala, Sweden. Blood samples for hematology and biochemical panels were handled according to the routines for the laboratory, and plasma from endotoxin-free heparinized vials were separated by centrifugation and frozen in –20 ˚C for later endotoxin determination.

Study III-IV included bitches seen at the Dept. of Small Animal Clinical Sciences from 2001 to 2002. Control animals included one group of clinically healthy age-matched intact female dogs boarding at the clinic. In addition, controls included one group of dogs that previously had been enrolled in the study (pyometra cases) and where the owner of the dog agreed to return the healthy animal to the clinic for repeat physical examination and blood work, a minimum of 8 weeks after surgery. In all dogs, blood samples were obtained pre-operatively or, in control animals, after hospital admission of the dog. The samples were immediately placed on ice and then transported without delay to the clinical pathology laboratory. Blood samples for hematology and biochemical panels were handled according to the routines for the laboratory, and plasma from EDTA vials were separated and frozen in –70 ˚C for subsequent IL-6, TNFα and CRP determinations.

Bacteriological examination (I-II)

A 1x2 cm biopsy of the uterine wall was aseptically collected immediately following ovariohysterectomy and placed in thioglycollate medium USP (one liter of medium contained 5 g yeast extract, 15 g tryptone, 5.5 g dextrose, 0.5 g Na-thioglycollate, 2.5 g NaCl, 0.5 g L-cystine, 0.001 g resazurin and 0.5 g agar no.1). The specimens were subjected to bacteriological examination within 12 hours of collection and were cultured by aerobic incubation on 5% (v/v) horse blood and bromocresol-purple agar plates (one liter containing 10 g balanced pepton [LabM

MC4), 10 g Lab M agar No. 2 [MC6], 10 g lactose and 1 ml 1.6% bromocresol-purple in ethanol). For bacteriological examination standard procedures were used (Carter & Cole, 1990). For anaerobic examination the uterine biopsies were cultured on Fastidious Anaerobe Agar plates (Lab M, Axel Johnson Lab System Inc., Solna, Sweden).

Rectal swab samples from healthy dogs were cultured on Mac Conkey agar plates (Difco, Detroit, MI, USA). After 18 hours incubation at 37 ˚C, one *E. coli* colony, representing the macroscopically morphologically dominant colony type, from each dog was subcultured on 5% (v/v) horse blood agar plates. Rectal swabs from dogs with pyometra were cultured on Mac Conkey agar plates (Difco, Detroit, MI, USA). To ensure representation of at least one colony of the dominant *E. coli* in the samples, after 18 hours incubation at 37 ˚C, six colonies from each sample were subcultured on 5% (v/v) horse blood agar plates.

Identification of *E. coli* was based on positive response to B-nitrophenyl-B-D-glucopyranosiduronic acid (PGUA) and indol production. Isolates negative for one or both of these tests were identified using the API 20E system (API System S.A., La Balme les Grottes, France). *Escherichia coli* isolates from dogs with UTI were kindly provided by Dr. A. Franklin (National Veterinary Institute, Uppsala, Sweden). Further strains of *E. coli* from UTI were isolated at the Section of Clinical Microbiology, Swedish University of Agricultural Sciences, Uppsala, Sweden, according to routine methods.

Biochemical fingerprinting of bacteria (I)

Biochemical fingerprinting was performed using the PhenePlate® (PhP) System (BioSys Inova, Stockholm, Sweden) which is based on quantitative and kinetic measurements of biochemical reactions of the bacteria. The system consists of twenty-four biochemical reagents, selected based on high discriminating ability between non-related *E. coli* (Kühn, 1985; Kühn *et al.*, 1990), and the system software for numerical data analysis. The method has been described in great detail previously (Kühn, 1985). Briefly, 10 µl of bacteria from a 24 h (37 ˚C) *E. coli* culture on 5% (v/v) horse blood agar plate was suspended in 10 ml substrate containing 0.1% peptone and 0.1% bromthymol blue in distilled water. The suspension was thereafter vigorously shaken before 150 µl was dispensed into each of 24 wells, representing 24 biochemical reagents, of the microtiter plate. The absorbance (620 nm) for each reaction was read after 7, 24 and 48 hours incubation at 37 ˚C in an optical microplate reader. After the final reading the mean absorbance value from each test was calculated yielding ‘biochemical fingerprints’, for each isolate and defined by a numerical value ranging from 0 (acidic reaction) to 25 (alkaline reaction) (Kühn, 1985).

The relationship between each isolate was determined by pair-wise comparisons and calculated as a correlation coefficient (r). An $r > 0.975$ indicated that the isolates were of the same clone, and considered to be of the same biochemical phenotype (PhP-type) (Kühn 1990). A somewhat lower correlation coefficient ($r > 0.960$) indicated isolates of high similarity. Isolates with $r < 0.950$ were regarded as not related.

The phenotypic diversity of an *E. coli* population was measured using Simpson's index of diversity (D_i) (Kühn *et al.*, 1990). D_i is high in populations consisting of many different phenotypes and low if certain PhP-types dominate in the population (range 0.0-1.0).

The biochemical homogeneity of a population was calculated as the mean of the correlation coefficients (r_{mean}), indicating whether the population is derived from a normal, random population of bacteria or contains groups of related bacteria.

Plasma endotoxin determination (II)

Samples for endotoxin determination was collected in endotoxin-free heparinized vacutainer tubes (Endotubes, Chromogenix, Goteborg, Sweden). Plasma was separated by centrifugation and thereafter collected in plastic vials (A/S Nunc, Roskilde, Denmark) in which they were stored at –70 ˚C prior to transport. They were transported, cooled, over less than 24 hours to the analyzing laboratory Scan Dia Laboratory Services I/S, Charlottenlund, Denmark. This commercial laboratory utilizes a kinetic turbidometric Limulus amebocyte lysate (LAL) assay for endotoxin determinations. This assay is approved for endotoxin determinations by the Food and Drug Administration, USA (1987).

Pathologic examination (II-IV)

Following ovariohysterectomy the whole uterus including ovaries was fixed in 10 % neutral buffered formalin. The uterus' gross appearance was documented and the presence of yellow bodies, corpora lutea, in the ovaries noted. Representative sections from each uterine horn were embedded in paraffin and stained with Hematoxylin &Eosine.

The histopathological diagnosis of pyometra was based on fulfilment of the criteria for chronic purulent metritis, purulent endometritis or purulent metritis, as proposed by Boerresen (1975). Chronic purulent metritis was characterized by a macroscopically distended uterus with irregular endometrial surface. Microscopically, varying numbers of neutrophils, plasmacells and lymphocytes were observed in the mucosa. These cell types were also present to a lesser extent in the myometrium. The endometrial epithelium was hyperplastic and cystic with columnar cells indicating progesterone influence. Cysts often contained large amounts of neutrophils. In some cases a varying degree of fibrosis and/or erosions in the mucosa was seen. In purulent endometritis, observed inflammatory cells were mostly neutrophils and cellular infiltration was limited to the endometrium, whereas in purulent metritis inflammatory changes were also present in deeper layers of the uterine wall. A diagnosis of CEH was based on observation of hyperplasia of the endometrium, in combination with cysts of different sizes, without inflammatory changes. A diagnosis of mucometra was based on gross or microscopical evidence of mucus or serous fluid in the uterine lumen in combination with a hyperplastic/cystic or atrophic endometrium.

C-reactive protein assay (III-IV)

C-reactive protein was determined with a commercially available canine ELISA (Tridelta Phase™ range canine CRP kit, TriDelta Diagnostics Inc., Plains, NJ, USA), which is a solid phase sandwich immunoassay. Plasma samples and CRP standard, serially diluted to range from 7.8 to 250 ng/ml, were applied to a microplate, with the wells coated with a monoclonal anti-canine CRP antibody, and incubated for 15 minutes at 37 ˚C before the plate was decanted and washed with diluted buffer. Anti-canine CRP conjugate was added to the wells and the plate incubated for 15 minutes at 37 ˚C before the plate again was decanted and washed with diluted buffer. Finally, TMB substrate was added and the plate incubated for 15 minutes at room temperature, stop solution added and the absorbance of the wells read in a microtiter plate reader at 450 nm using 630 nm as a reference. A standard curve was generated using Microsoft® Excel software (1997) and the concentration of the samples obtained by inserting mean optical density into the function created by the standard curve.

Tumor necrosis factor α assay (III-IV)

The TNFα concentration in plasma was determined with an ELISA, with reagents kindly provided by Dr. W.A. Buurman (University of Maastricht, Maastricht, the Netherlands). ELISA tests utilizing this antibody experimentally in dog plasma have showed a detection limit of 15 pg/ml, which is 1/60 of the levels achieved after sub-lethal doses of endotoxin (Moeniralam *et al.*, 1998). This ELISA test has shown a good correlation with the Walter and Eliza Hall Institute of Medical Research (WEHI) bioassay and is specific for biologically active TNFα (Engelberts *et al.*, 1991). High-affinity microtiter plates (MaxiSorp F96, Nunc, Denmark) were coated with the provided monoclonal antibody for human TNFα and incubated for 15 hours at 4 ˚C. The plate was washed by adding diluted PBS buffert (one liter containing 2 g of KCl, 2 g of KH2PO4, 80 g NaCl and 29 g Na2HPO412H20 in distilled water) with 0.1% BSA (Bovine Albumin, Sigma Aldrich Inc., Saint Louis, MO, USA) and incubated for 1 hr at room temperature before decanting. Samples and standard titration curve of provided rhTNFα (range 5 pg/ml – 10 ng/ml) were applied in duplicate, rabbit anti-TNFα, diluted in PBS 0.1% BSA, was added, and finally goat anti-rabbit peroxidase applied (Cat. No. 111-035-045, Jackson Immuno Research Laboratories, Inc., West Grove, PA, USA). Between and after the three latter steps the plates were incubated at room temperature for one hour. TMB peroxidase substrate was added, the reaction stopped with 1 M H2SO4 after 15 min, and the plates read in a microtiter plate reader at 450 nm. A standard curve was generated using Microsoft® Excel software (1997) and the concentration of the samples obtained by inserting mean optical density into the function created by the standard curve.

Interleukin 6 determination (III)

The bioactivity of IL-6 in plasma samples was analyzed with a bioassay utilizing an IL-6 dependent B-9 hybridoma cell, kindly provided by Dr. Boon Chew, Dept. of Animal Science, Washington State University, Pullman, USA. This assay has previously shown to be specific for IL-6 (Helle *et al.,* 1988) and has been used experimentally in dogs with success (Chew *et al*., 2000). The IL-6 assay was performed as previously described (Fernandez, Botran & Vaclav, 1995). Briefly, the B9 hybridoma cell was harvested and washed with washing buffer and with culture medium. The cells were centrifuged (200 x g for 10 minutes) and resuspended to a density of 20000 cells/ml in culture medium. The diluted test samples and rIL-6 standard were incubated for 72 hours with the indicator cell and for the final 4 hours with MTT. The samples were analyzed in triplicate including a negative control, containing medium only. The cells were harvested and the formazan dye formation resulting from the MTT reaction was determined by spectrophotometry. Absorbance of the MTT reaction was read at 560 nm (test wavelength) and 690 nm (reference wavelength). The detection limit of this assay is 0.1 pg/ml.

Results and discussion

Bacterio-epidemiologic study (I)

Escherichia coli were isolated from the uteri of 79 dogs with pyometra (79 isolates), from the feces of 10 dogs with pyometra (60 isolates) and from the urine of 16 bitches (16 isolates) that were suffering from both pyometra and concurrent urinary tract infection (UTI). In addition, two separate *E. coli* populations were collected; 91 fecal isolates from 91 healthy, unrelated dogs, and 88 isolates from the urinary tracts of unrelated dogs with UTI were kindly provided Dr. A. Franklin, the National Veterinary Institute, Uppsala, Sweden.

Biochemical fingerprinting was used for bacterial epidemiological studies of the *E. coli* isolates. This method has proved to be a highly discriminative method, well suitable for epidemiological investigations of *E. coli* (Kühn *et al.*, 1990, Mollby *et al.*, 1993). It is rapid, cost effective and suitable for investigations of high numbers of samples. The diversity and homogeneity of uterine, urine and fecal *E. coli* populations are presented in table 2.

Table 2. *The diversity and homogeneity of 3 different E. coli bacterial populations; isolated from uterus of bitches with pyometra, from urinary tract infection (UTI) and from faeces of healthy dogs*

Samples	Number of isolates	Diversity index D_i	Homogeneity r_{mean}
Pyometra	79	0.98	0.79
UTI	88	0.97	0.70
Fecal	91	0.96	0.69
All samples	258	0.99	0.72

The results revealed that the level of homogeneity of the pyometra *E. coli* isolates was higher (0.79) than the homogeneity among epidemiologically unrelated fecal *E. coli* isolates (0.69). We suggest that this finding could be the result of certain serotypes being more prevalent in our pyometra material, in accordance with previous studies (Grindlay *et al.*, 1973, Dhaliwal *et al.*, 1998).

Bacteriological examinations were also performed on fecal samples from 10 bitches with pyometra and showed that at least one of the 6 fecal isolates was identical or very similar to the uterine *E. coli* isolate. Thus, an ascending route of infection from the fecal flora into the uterus seems a logical explanation in most cases [Wadas (Fransson) *et al.*, 1996]. An ascending infection route is accepted for other urogenital disease, such as both canine and human urinary tract infection (UTI), where the infecting bacteria most commonly are descendents from the intestinal flora (Low *et al.*, 1988, Stamey *et al.*, 1971, Ling *et al.*, 1979). Sandholm *et al.* suggested (1975) that the urinary tract may serve as a bacterial reservoir, and infect the uterus during a susceptible stage in the estrus cycle. Prospective studies would be necessary to prove such a theory. It appears more likely that the urinary tract might get secondarily infected by the purulent vaginal discharge from a pyometra, considering the anatomically dependent position of the urethra opening in the female dog.

Of the studied 16 pyometra bitches that also had UTI, the urinary isolate was identical or very similar in 14/16 cases (88%). In the remaining two cases (12%) the isolates were different. This indicates that the same clone of *E. coli* found in the uterus of bitches with pyometra also caused UTI in most of the bitches. These results have subsequently been repeated using another method of bacterial subtyping, *i.e.* restriction enzyme digestion and pulsed-field gel electrophoresis, reported by other members of our research group (Hagman & Kühn, 2002).

Bacteriological findings (II)

Sixty bitches, representing 34 different breeds with the age ranging from 2- 13 years were included in this study. Of the 60, 48 bitches received the histopathologic diagnosis pyometra, whereas 8 were diagnosed with CEH, mucometra, hydrometra or endometrial hyperplasia (Table 3) and 4 dogs showed uteri with normal morphology. Eleven clinically healthy laboratory beagles were included as controls.

The results of bacteriologic examination of the uteri of 48 bitches with pyometra and of 8 bitches with non-inflammatory uterine disease are presented in table 4. The study showed that *E. coli* is by far the most common bacterial isolate in canine pyometra, which is in accordance with results from previous studies (Sandholm *et al.*, 1975, Kivisto *et al.*, 1977, Dhaliwal *et al.* 1998). The study also showed that bacterial growth in the uterus is present in 98% of cases with histopathologically verified diagnosis of pyometra. The 8 cases of non-inflammatory uterine disease and the 4 normal uteri showed bacterial growth in only 1/12 cases (8%). The only non-pyometra case that did show bacterial growth, a morphologically normal uterus, had a mixed culture of low numbers, suggesting that contamination of the sample, rather than uterine bacterial infection was present in this case. Therefore, it seems reasonable to assume that if pyometra is histopatologically verified, there is bacterial infection present in the uterus. The results confirm that non-inflammatory uterine disorders such as CEH and mucometra, rarely are associated with infection.

Table 3. *Diagnosis in 8 bitches with non-inflammatory uterine conditions. The diagnosis is based on the histopathologic findings and/or the gross findings of mucus or fluid in the uterus. CEH=Cystic endometrial hyperplasia*

Bitch number	Diagnosis
1	CEH and adenomyosis
2	Mucometra and endometrial atrophy
3	Mucometra
4	Endometrial hyperplasia
5	Hydrometra
6	CEH
7	CEH and adenomyosis
8	CEH and adenomyosis

Table 4. *Results of bacteriologic examination of uteri from 48 bitches with pyometra and from 8 bitches with non-inflammatory uterine condition*

Bacterial Isolates	Pyometra N (%)	Non-inflammatory uterine conditions N (%)
Escherichia coli	43 (90%)	0
Pasteurella multocida	1 (2%)	0
Streptococcus canis	1 (2%)	0
Mixed culture	2 (4%)	0
No bacterial growth	1 (2%)	8 (100%)
Total	48	8

Systemic effects of pyometra (II-III)

Hematology and blood biochemical parameters (II-III)

Study II investigated total and differential WBC count, including percent band neutrophils (PBN) and serum biochemical parameters including; BUN, creatinine, glucose, bilirubin, preprandial bile acids, ALAT, ALP and glutamate dehydrogenase (GLDH). Three categories female dogs were included, *i.e.* pyometra cases (n=48), bitches with non-inflammatory uterine disorders (n=8), and control bitches (n=11). In order to compare the influence of the infecting uterine bacteria, the pyometra dogs were divided into pyometra with gram-negative (group A) vs. grampositive (group B) uterine infection. Before surgery the control dogs were all within normal limits for all the blood values whereas the pyometra dogs in group A showed an inflammatory leukogram with a left shift, a decreased BUN, increased ALP but decreased ALAT and GLDH. Blood samples were available in only 3 dogs in group B, making conclusions of results from group B difficult to draw. However, group B showed a significantly ($p=0.05$) less severe left shift (PBN 4.0±2.0) and lower creatinine (75.0±0) compared to the dogs in group A. After surgery (applicable only to dogs with pyometra, group A, and dogs with non-inflammatory uterine disorders), the only differences between groups A and controls noted were in the WBC, the PBN and in the ALP. Mean values and standard deviations for hematology, blood chemistry parameters and average grades of general attitude before surgery are presented in table 5. Table 6 presents postoperative hematology and blood biochemical parameters, significantly different between pyometra group A, and cases with non-inflammatory uterine disorders. Parameters not included in table 5 were not significantly different between any of the groups.

Table 5. *Mean and standard deviation of hematology and blood biochemistry parameters, and mean value of general attitude before surgery. Pyometra dogs include dogs with histologically confirmed pyometra and gramnegative uterine infection. Dogs with other uterine disorders include bitches with non-inflammatory uterine disease and controls include healthy dogs with normal uteri * = p-value<0.05, ** = p-value<0.01 and *** = p-value<0.001 (two-tailed Student's T-test, unequal variance) when compared to pyometra group. Lack of * indicate no signifificant difference.*

a) The general attitude was assigned numerical levels with 0=Bright, alert, 1=Quiet, alert, 1.5=mildly depressed, 2=moderately depressed (none of the dogs reached 3=severely depressed

Parameter	**Pyometra**		**Dogs with other uterine disorders**		**Controls**	
	Mean(±SD)	count	Mean(±SD)	count	Mean(±SD)	count
ALAT (µkat/L)	0.4 (±0.2)	27	0.7 (±0.4)	6	0.7 (±0.1)***	11
ALP (µkat/L)	2.3 (±1.7)	24	0.6 (±0.4)***	6	1.0 (±0.5)**	11
Band neutrophils (%)	9.6 (±12.5)	43	0.5 (±1.4)***	8	0.2 (±0.4)***	11
Bile acids (µmol/L)	6.4 (±8.9)	25	5.0 (±10.8)	6	16.4 (±15.5)	7
Bilirubin (µmol/L)	2.8 (±5.7)	25	2.1 (±1.4)	6	2.2 (±2.2)	7
BUN (mmol/L)	3.4 (±1.3)	27	3.6 (±0.7)	8	10.2 (±3.3)**	7
Creatinine (µmol/L)	82.2(±17.4)	27	79.7 (±12.9)	6	85.7 (±20.2)	11
GLDH (µkat/L)	32.6 (±20.0)	26	74.5(±103.0)	6	56.0 (±19.0)**	9
Glucose (mmol/L)	4.8 (±0.9)	17	5.5 (±0.5)	5	Not available	
Lymphocytes (%)	12.1 (±9.7)	43	18.5 (±9.5)	8	22.3 (±7.0)***	11
WBC (x 10^9/L)	27.0 (±17.0)	44	9.4 (±2.9)***	8	11.5 (±4.0)***	11
General attitude [a)]	0.67(±0.6)	41	0.2 (±0.4)*	6	Not applicable	

Table 6. *Mean and standard deviation of selected hematology and blood biochemistry parameters, and mean value of general attitude, 2 days after surgery. Pyometra dogs include dogs with histologically confirmed pyometra and gramnegative uterine infection. Dogs with other uterine disorders include dog with the presumptive diagnose of pyometra, but where histopathology revealed non-inflammatory uterine disorders. *** indicate p-value <0.001 (calculated by two-tailed Student's T-test, unequal variance). No * indicates no significant difference.*

a) The general attitude was assigned numerical levels with 0=Bright, alert, 1=Quiet, alert, 1.5=mildly depressed, 2=moderately depressed (none of the dogs reached 3=severely depressed)

	Pyometra Mean (±SD)	**N**	**Dogs with other uterine disorders** Mean (±SD)	**N**
ALP (μkat/L)	2.7 (±1.2)	16	1.2 (±0.4)***	4
Band neutrophils (%)	3.9 (±4.5)	26	0.2 (±0.4)***	6
WBC (x 10^9/L)	45.3 (±23.9)	26	15.2 (±6.0)***	6
General attitude [a)]	0.3 (±0.5)	39	0.2 (±0.4)	6

Study III, which included 53 pyometra cases and 19 control dogs, expanded on the hematology and blood biochemistry investigations as compared to study II. The following hematology parameters were determined: Hct, hemoglobin, RBC, WBC, differential count of white blood cells including total count and percentage band neutrophils (PBN), nucleated red blood cells, erythrocyte morphology, erythrocyte mean volume, erythrocyte hemoglobin content and platelet count. The following serum biochemical parameters were determined: ALAT, albumin, ALP, BUN, cholesterol, glucose, total protein, sodium, potassium, chloride and calcium. Of these parameters, the average values of WBC, PBN, platelet count, albumin and ALP, in study III, are presented in Table 6. The average of parameters not included in Table 6 differed between pyometra cases and control dogs in PCV, hemoglobin and RBC (p-value <0.0001), in serum urea nitrogen (p-value 0.02) and in s-cholesterol (p-value 0.0004). The average of other parameters did not differ from healthy control dogs.

Table 7. *Mean and standard deviations for clinical parameters (body temperature, heart rate, respiratory rate), hematology (WBC, percent band neutrophils, platelet count), blood biochemistry (AP, albumin), and inflammatory markers (CRP, TNFα and IL-6) in dogs with pyometra and in healthy control dogs. P-values calculated with Two-sample unequal variance Student's T-test (two-tailed test) AP = Alkaline Phosphatase; CRP = C-reactive protein; IL-6 = Interleukin 6; TNFα = Tumor necrosis factor alpha; WBC =White blood cell count*

	Pyometra group Mean (±SD)	**N**	**Control Group Mean (±SD)**	**N**	**Ttest p-value**
Albumin (g/L)	26.38 (±4.09)	45	33.9 (±3.0)	10	0.03
AP (μkat/L)	6.15 (±6.06)	53	1.37 (±0.58)	10	<0.0001
Band neutrophils (%)	12.04 (±9.5)	53	0.06 (±0.25)	16	<0.0001
CRP (mg/L)	207.66 (±92.46)	43	19.84 (±8.19)	10	<0.0001
Heart rate (beats/min)	111.29 (±24.81)	48	102.00 (±52.66)	10	0.61
IL-6 (pg/mL)	172.62 (±249.25)	46	167.58 (±144.9)	19	0.98
Platelet count (10^9/L)	241.54(±121.4)	52	345.33(±111.5)	16	0.0395
Respiratory rate (Breaths/min)	24.87 (± 9.68)	39	21.50 (±7.21)	10	0.23
Temperature (˚C)	39.02 (±0.53)	52	38.97 (±0.27)	10	0.64
TNFα (pg/mL)	0.13 (±0.073)	39	0.01(±0.012)	10	<0.0001
WBC (10^9/L)	23.29 (±11.47)	53	9.03 (±2.7)	16	<0.0001

In both studies (II-III) an inflammatory leukogram was a common finding, reflected by a high average WBC and PBN. Of the 53 pyometra cases in study III, the WBC exceeded 16 000/μL in 36 cases (68%) and PBN exceeded 3 % in 44 (83%) cases. The inflammatory leukogram is not a surprising finding in a disease characterized by chronic inflammation such as pyometra. Other common findings included increases in ALP (II and III), decreased platelet count, below 200 000/μL in 19 (37 %) of the dogs (III), decreased albumin (III), less than 25 g/L in 15 cases (33%), a non-regenerative anemia (III), and increases in the electrolytes sodium and chloride (III). Increased ALP was documented, in consistence with previous studies (Boerresen 1980, Boerresen & Skrede 1980). ALP exceeded the reference range (0-5 μkat/L) in 18 cases (34%) in study III. Intrahepatic cholestasis has been documented in bitches with pyometra (Boerresen & Skrede, 1980) and presents a reasonable explanation for the increases seen in ALP. Hypercholesterolemia, which was noted in 32/43 cases in study III (74%) (reference range 3.25-7.8 mmol/L), likely is another feature of intrahepatic cholestasis (Borresen & Skrede, 1980). A normocytic normochromic anemia, with PCV below 38%, was documented in

27/45 cases (60%) with an average mean corpuscular volume (MCV) of 67 fL (reference range 64-74) and average mean corpuscular hemoglobin content (MCHC) of 355g/L (reference range 335-363 g/L). This common finding is most likely representing anemia of chronic inflammation (Nelson & Couto, 1998). Hypernatremia was manifested in 13 of 45 cases (29%) (reference range 146-156 mmol/L), and hyperchloremia in 15/45 cases (33%) (reference range 113-123 mmol/L). Hypernatremia and hyperchloremia was thus seen in approximately 1/3 of the pyometra cases (III) and was considered consistent with dehydration.

Azotemia was an unusual finding among dogs with pyometra. In study III, azotemia was noted in 3/50 cases (6 %) with serum creatinine exceeding the reference range (40-130µmol/L) but with only 2 of these cases (4%) showing increased BUN. In fact, in both studies (II-III) the pyometra dogs were more commonly showing a decrease in BUN (reference range 2.5-11.4 mmol/L), which was observed in 10 % (5/48 dogs). The decreased BUN is likely due to a combination of factors. Polyuria, seen in 71 % of the cases in study IV, leads to increased BUN excretion (Barsanti *et al.*, 1999) and insufficient protein intake from inappetance may contribute to the decreased BUN (Barsanti *et al.* 1999). Hepatic insufficiency is another cause of decreased BUN, which theoretically could be associated with the noted ALP increases. However, study II showed that the bile acid test was in general normal which does not support decreased hepatic function. Also, increases of ALAT were rare. In study III only 4/54 cases (7%) showed increased ALAT (reference range <1.2 µkat/L). In study II, the ALAT among pyometra dogs were actually significantly lower than in the other groups. The decreases seen in the hepatocellular enzymes (ALAT and GLDH) in study II, are usually considered insignificant findings (Willard & Twedt, 1999).

In study III, blood glucose abnormalities, and electrolyte abnormalities other than sodium and chloride, were rare findings. Hypoglycemia was affecting only 9% (4/47) of dogs and hyperglycemia was seen in 4 % (2/47) of pyometra dogs (reference range 3-6.5 mmol/L). Hypokalemia was seen in 2/45 cases (4%) (reference range 3.9-5.5 mmol/L), hyperkalemia in none, and hypochloremia in only 1 case (2%). Three of 47 cases (6 %) showed mild hypocalcemia, and none showed hypercalcemia.

Of the 19 control dogs, 10 had previously been enrolled into the study and treated for pyometra. In these dogs, all hematology parameters had returned to normal after a median of 64 days (range 57-111 days). The blood biochemical panel in one of the dogs showed mild increases in serum creatinine and ALAT and moderate hypernatremia and hyperchloremia. Hemoconcentration from dehydration was assumed, which showed no other abnormalities in blood work, history or physical examination. In addition to this dog, 3 dogs showed mild hyperchloremia and two of them also showed hypernatremia. Mild dehydration was assumed in these dogs. Three dogs showed mild hypercholesterolemia and a postprandial response could not be ruled out. Thus, with none of these dogs showing significant abnormalities in the blood work, paired with a normal clinical status, the organ dysfunction in pyometra appears to be of a reversible nature after ovariohysterectomy.

Endotoxin (II)

Studies II and III primarily dealt with the systemic effects of the uterine infection. In study II the hypothes was that endotoxemia would be associated with more severely affected clinical status. Endotoxin determinations were performed on 92 plasma samples. However, endotoxinemia was detected in only four bitches with pyometra (in three of them before surgery and in one after surgery). Endotoxin was also detected in one bitch with non-inflammatory uterine disease and in 2 healthy control dogs. The ET levels in these 7 cases ranged from 14.2 pg/L to 14.5 ng/L. Of the 4 bitches with pyometra and detectable plasma ET, 2 had uterine growth of *E. coli*, 1 showed no growth and 1 showed growth of *Streptococcus canis*. The fact that endotoxin was detected in healthy control dogs indicated that contamination of the samples, alternatively assay errors, might have led to the inconsistent findings. These results are in sharp contrast to other studies (Wessels & Wells, 1989 and Okano et al 1998). One explanation for the absence of endotoxinemia might have been that the clinical status of the dogs was not severe enough to be consistent with "spill-over" effects of ET. In general, the clinical status of the population of dogs in study II was normal or only mildly impaired, and none showed the expected severely affected general attitude associated with a massive release of ET (Rietschel & Brade, 1992). The majority of the dogs in study II did however show a severe inflammatory leukogram that could represent a response to low concentrations of circulating ET (Van Miert & Frens, 1968). Certainly, the study population in study III-IV, which likely is very similar to the population in study II, showed many of the clinical signs associated with experimentally induced low dose endotoxemia, i.e. fever (33%), lethargy (71%), tachycardia (23%) and tachypnea (32%) (Van Miert & Frens, 1968). It is possible that ET indeed is responsible for the systemic effects of pyometra, but that the release of ET from the uterus is intermittent and difficult to demonstrate with a one-point-in-time test such as in study II.

Systemic inflammatory response syndrome (III)

Study III included a total of 53 pyometra dogs, of which 30 (57%) were positive for SIRS according to criteria levels defined by Hauptman, Walshaw & Olivier (1997) and 23 (43%) were SIRS negative. Dogs positive for SIRS showed an average onset of 8 (±6.39) days, whereas SIRS negative dogs showed a slightly shorter duration of clinical signs (4.5±4.32 days). The difference in duration was notable, but did not quite reach statistical significance (p-value 0.055).

Any clinical scoring system appears relatively non-specific, including the SIRS criteria in dogs (specificity 64%; Hauptman *et al.*, 1997). This is logical, considering that there are many reasons for hyperthermia, tachycardia and tachypnea, other than systemic inflammation, such as anxiety and pain (Lucas *et al.*, 2001). Multiple human studies have evaluated different inflammatory mediator's ability to more specifically act as markers for sepsis/SIRS and predictors of outcome (Reny *et al.,* 2002; Marik, 2002; Harbarth *et al.*, 2001; Whicher et al 2001; Bossink *et al.*, 1998). The consensus seems to be that out of many studied markers (*i.e.* TNFα, IL-1, IL-6, IL-8, IL-1 receptor antagonist, CRP, procalcitonin, E-selectin, soluble intercellular adhesion molecule-1, protein C,

leukocyte elastase, granulocyte colony-stimulating factor, complement 3a, erythropoietin, serum amyloid protein, neopterina and plasma nitrite/nitrate concentrations) procalcitonin and CRP appears to have the most utility (Reny *et al.*, 2002; Marik, 2002). Procalcitonin determinations are at present available through one manufactured assay, for humans only as this protein shows structural species differences, making cross-reactivity unlikely (pers. comm. Christiane Dinter, Brahms Aktiengesellschaft, Hennigsdorf, Germany, 2003). Therefore, procalcitonin determinations were not part of our study.

When we searched for parameters associated with positive or negative SIRS status in pyometra dogs we chose to investigate not only the classical SIRS parameters (body temperature, heart rate, respiratory rate, WBC and PBN) but in addition platelet count, ALP, albumin, IL-6, TNFα and CRP. The additional parameters were selected based on that the platelet count has previously been shown to significantly differ between dogs with and without sepsis (Hauptman, Walshaw & Olivier, 1997). Albumin was included due to its propensity to act as a negative acute phase protein (Werner & Turnwald, 1999). Alkaline phosphatase was studied based on previous finding of this enzyme being significantly increased in pyometra cases as compared to normal (study II) and this enzyme appears to reflect the level of hepatic impairment with respect to cholestasis (Borreson & Skrede, 1980). The inflammatory mediators IL-6 and TNFα, and the acute phase protein CRP, have been shown to be increased in human patients with SIRS (Spielmann *et al.*, 2001; Gogos *et al.*, 2001). Serum glucose was not included as a SIRS criteria based on a previous study indicating no significant differences in glucose concentration in dogs with and without sepsis (Hauptman, Walshaw & Olivier, 1997) which is in agreement with the results in study III. The mean values for the selected clinical criteria, haematology and blood biochemical parameters, IL-6, TNFα and CRP, in the SIRS positive group and the SIRS negative group are presented in Table 8.

Table 8. *Mean and standard deviations for clinical parameters (body temperature, heart rate, respiratory rate), hematology (WBC, percent band neutrophils, platelet count), blood biochemistry (AP, Albumin), and inflammatory markers (CRP, TNFα and IL-6) in dogs with pyometra. The SIRS group consists of dogs with 2 or more positive SIRS criteria17 and the No SIRS group of dogs with less than 2 positive SIRS criteria. P-values were calculated using Two-sample unequal variance Student's T-test (two-tailed test).*
AP=Alkaline Phosphatase; CRP=C-reactive protein; IL-6=Interleukin 6; PBN= Percent band neutrophils; TNFα=Tumor necrosis factor alpha; WBC =White blood cell count

	SIRS Group		**No SIRS**		**Ttest**
	Mean (±SD)	**N**	Mean (±SD)	**N**	p-value
Albumin (g/L)	26.21 (±4.19)	24	26.57 (±4.07)	21	0.77
AP (μkat/L)	6.67 (±7.3)	30	5.65 (±4.14)	21	0.53
PBN (%)	11.90 (±9.59)	30	11.88 (±9.62)	23	0.99
CRP (mg/L)	235.49(±79.8)	22	178.51 (±97.55)	21	0.0398
Heart rate (beats/min)	121.00(±26.9)	27	98.10 (±13.32)	21	0.0004
IL-6 (pg/mL)	180.90(±265.5)	24	156.05 (±232.13)	22	0.74
Platelets (10^9/L)	239.76(±124.4)	29	236.22 (±123.28)	23	0.91
Respiratory rate (breaths/min)	23.48 (±6.94)	25	27.36 (±13.21)	14	0.32
Temperature (˚C)	39.23 (±0.56)	30	38.74 (±0.33)	22	0.0002
TNFα (pg/mL)	0.12 (±0.06)	20	0.14 (±0.09)	19	0.61
WBC (10^9/L)	23.98 (±11.55)	30	22.47 (±11.33)	23	0.63

To the author's knowledge, study III was the first to evaluate the use of the inflammatory mediators TNFα and IL-6 or the acute phase protein CRP as markers for SIRS in dogs. None of the hematology and blood biochemical parameters or the inflammatory mediators TNFα and IL-6, chosen for their possible ability to predict SIRS, was confirmed to have such abilities. In our population CRP was the only biochemical marker that was significantly (p-value 0.0372) associated with SIRS. Of the classical criteria for SIRS (heart rate, respiratory rate, body temperature, WBC and PBN) the heart rate (p-value 0.0003) and the body temperature (p-value=0.0005) were significantly different in SIRS positive versus negative dogs. In fact, multiple logistic regression showed that in the study population (III) a combination of CRP, heart rate and body temperature, was the most effective way (p-value <0.0001) to discriminate between SIRS positive and SIRS negative dogs. Figure 1 presents a contour plot of the 95% estimated probability of a dog to be SIRS positive given the values for CRP, heart rate and body temperature. If the levels for CRP and heart rate bisect each other to the right

of the curve representing the body temperature, the probability is at least 95% for that dog to be SIRS positive. If the bisecting values are located to the left of the curve the probability is less than 95% for the case to represent a SIRS positive dog.

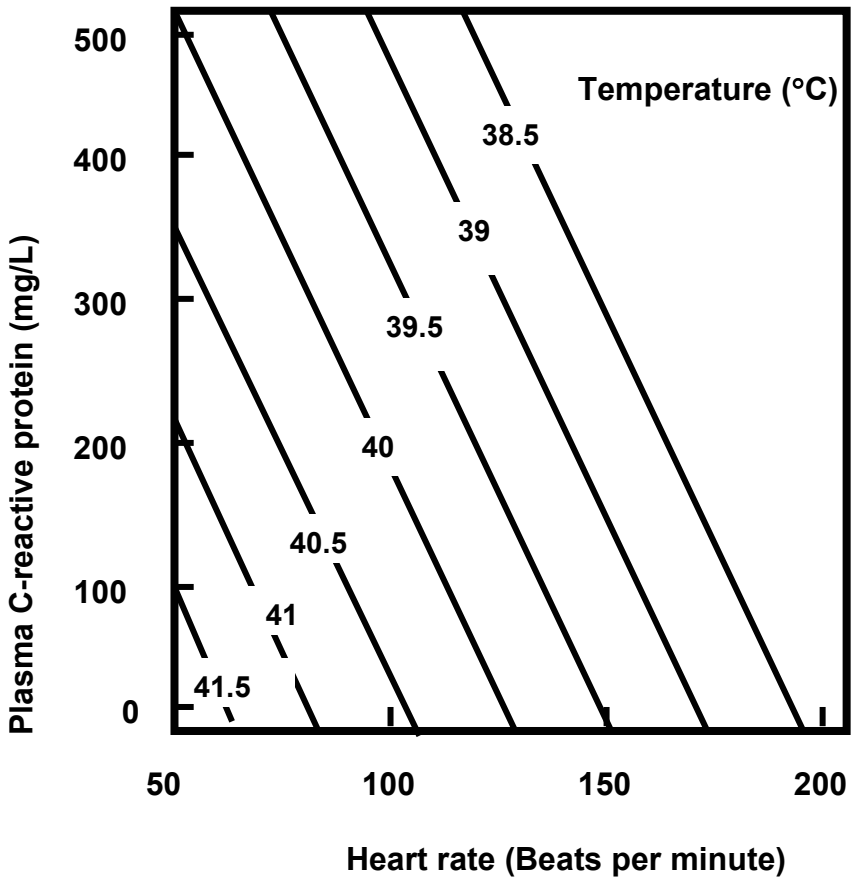

Figure 1. Estimated 0.95 probability of an animal being affected with systemic inflammatory response syndrome (SIRS) using values for body temperature, heart rate (HR) and plasma C-reactive protein (CRP). Bisection of the values for CRP and HR to the right of the curve corresponding with body temperature in an animal indicates a 95% probability of SIRS.

This graph (Figure 1) was generated through multiple logistic regression as a function of the formula: Estimated Probability of SIRS =

$$\frac{\exp(-198.5 + 4.6971*\text{temperature} + 0.0169*\text{CRP} + 0.1077*\text{heart rate})}{1 + \exp(-198.5 + 4.6971*\text{temperature} + 0.0169*\text{CRP} + 0.1077*\text{heart rate})}$$

Studies of human patients with positive SIRS criteria have clearly shown the association of SIRS with increased morbidity and/or inferior outcome (Bochicchio *et al.*, 2002; Buter *et al.*, 2002; Afessa *et al.*, 2001; Sun & Aikawa, 1999; Bossink, 1998 & Shoenberg *et al.*, 1998). Only two studies regarding outcome related to

SIRS in dogs have been presented prior to our study (Welzl *et al.*, 2001; Okano *et al.*, 2002). The former study did not find SIRS, nor the precense of MODS, able to predict outcome, manifested as death or euthanasia, in dogs with babesiosis and the authors requested more sensitive and specific definitions of SIRS in dogs. However, one may speculate if the systemic effects of babesiosis might reflect the effect of hemolysis and disseminated intravascular coagulation, as well as the systemic inflammatory response. In addition, the study appeared to define MODS with less stringent criteria compared to other studies. In contrast, Okano and co-workers (2002) showed a significant correlation between SIRS and increased mortality. Our study (III) did show a significant association between a positive SIRS status and outcome, estimated as number of hospitalization days. One can argue that the hospitalization length offers an extremely crude estimation of morbidity. None the less, this is a factor also used in human studies of SIRS (Afessa *et al.*, 2001).

Study III appears to be the first to evaluate the use of haematology and biochemical parameters and the inflammatory mediators TNFα and IL-6, or the acute phase protein CRP as predictors of outcome in dogs. In the population CRP was the only biochemical marker related to SIRS, and in addition, CRP was in itself a significant predictor of outcome. The regression analysis of plasma CRP versus length of hospitalization is illustrated in figure 2. The graph is based on the regression function: Days hospitalization =1.04553+0.0034538 CRP
This function reveals that a plasma CRP greater than 276.35 mg/L is associated with hospitalization exceeding 2 days. The results provide encouragement for further studies of CRP as a predictor of morbidity/mortality in dogs.

Cystic endometrial hyperplasia versus pyometra (II, IV)

The concept of CEH-pyometra introduced by Dow (1957) has lately been questioned (De Bosshere *et al.*, 2001). Regardless of the discussion of similarities or discrepancies in the underlying etiopathogenesis, the lack of bacterial infection in CEH, as reflected in study II, leads to many differences in the clinical presentation between this disorder and pyometra. Thus, the signalment, history and physical examination as well as haematology and blood biochemical findings have classically been considered of great importance when trying to predict a final diagnosis of pyometra vs. CEH/mucometra. Severe systemic affection as reflected by inflammatory leukogram or decreased general attitude has not been demonstrated in CEH. However, despite the differences in presentation and blood work, CEH and pyometra can be difficult to separate clinically. The diagnostic image of uteri with these disorders can be deceiving, since fluid can accumulate in the uterus in both diseases, in CEH mucus and in pyometra pus (Study II and IV, De Bosschere *et al.* 2002). For the clinician trying to decide whether to perform emergency surgery or to wait until more ideal circumstances are available, differentiation between the two diagnosis can be crucial in order to avoid the critical situation where delayed surgery of a pyometra dog leads to a ruptured uterus.

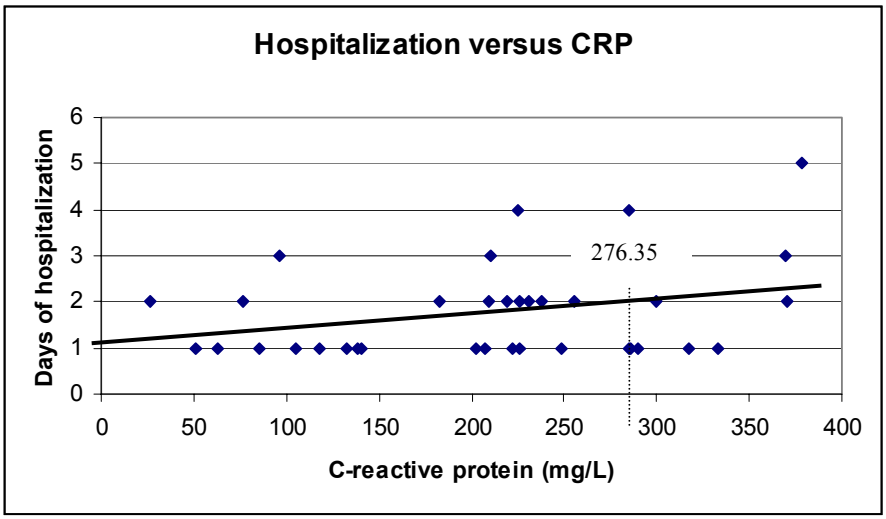

Figure 2. Length of hospitalisation versus plasma C-reactive protein (CRP) in 53 bitches with pyometra. A plasma CRP exceeding 276.35 mg/L predicts a hospitalisation length exceeding what is considered normal, *i.e.* 1-2 days.

Study IV investigated the benefit of signalment, history and physical exam findings, hematology parameters and biochemical parameters, including TNFα and CRP, in the differentiation between pyometra and CEH. The signalment (age and breed) did not show significant differences between pyometra dogs as compared to dogs with CEH. The average age of the CEH dogs was slightly lower (6.6 years) as compared to pyometra cases (8.4 years) but the respective ranges were wide and the difference in average age did not reach statistical significance. The frequency of clinical signs and the significance of the differences in clinical signs between bitches with pyometra and bitches with CEH/mucometra are presented in Table 9. The number of days of clinical signs prior to admission ranged from 1 to 28 days with a mean of 6.5 (±5.8) days. Of these four most commonly reported clinical signs, *i.e.* vaginal discharge, polyuria/polydipsia, lethargy and vomiting/inappetance, the latter three were significantly different between the two groups of dogs. In addition, a dog with only one of these clinical signs observed by the owner is significantly more likely to represent a dog with CEH, whereas a dog showing 3 or more of these signs is more likely to represent a pyometra case. In contrast to the multiple differences in clinical signs the general physical examination showed limited ability to differentiate between a dog with pyometra from a dog with CEH. The general attitude was the only discriminative finding, with a dog presenting bright and alert being significantly more likely to be diagnosed with CEH than pyometra. The frequencies of physical examination findings are presented in Table 10.

Table 9. *Differences in the presence of vaginal discharge, polyuria/polydipsia, lethargy and gastrointestinal signs, i.e. vomiting and/or inappetance, between dogs with pyometra and dogs with cystic endometrial hyperplasia (CEH)/Mucometra. P-values calculated with Fisher's exact test (two-tailed test)*

	Pyometra **N=48**	**CEH** **N=9**	**P-value**
Vaginal discharge	25 (52%)	6 (67%)	0.49
Polyuria/polydipsia	34 (71%)	2 (22%)	0.0090
Lethargy	34 (71%)	2 (22%)	0.0090
Vomiting/ inappetance	36 (75%)	1 (11%)	0.0005
Only 1 clinical sign	6 (12%)	6 (67%)	0.0015
≥3 clinical signs	29 (60%)	1 (11%)	0.0095

The results (IV) from hematology, blood biochemistry, plasma TNFα and plasma CRP determinations are presented in Table 11 and the significance of differences in these parameters are presented in Table 12. Multiple logistic regression showed that an elevated PBN in combination with high plasma CRP identified pyometra with few false negative results (sensitivity 97.7 %) as confirmed by histopathological examination. The specificity was fairly low (75 %) in this combination. The combination of elevated PBN and ALP showed a slightly higher specificity (77.8%) but with a lower sensitivity (96.1%). Any other combinations of two or more parameters did not increase the sensitivity or specificity in the prediction of pyometra as the diagnosis. Figure 3 presents a contour plot of the 95 % and 80 % estimated probability of a dog having pyometra, in contrast to CEH, given the values of PBN and CRP. This contour plot was generated from the regression analysis and illustrates the formula: Estimated Probability of Pyometra =

$$\frac{\exp(-2.3110 + 0.2643*\text{band percent} + 0.0202*\text{CRP})}{1 + \exp(-2.3110 + 0.2643*\text{band percent} + 0.0202*\text{CRP})}$$

For clinicians, a test with high sensitivity in the prediction of pyometra vs. CEH/mucometra is desirable. The sensitivity of the test is probably of higher importance than the specificity since it is reasonable to assume that emergency surgery on a dog that is not critically ill, *i.e.* CEH/ mucometra, would be less hazardous than the risk associated with a delay in surgery for a pyometra dog, which potentially could rupture an infected uterus. Thus, using the 80% probability plot in figure 3, associated with a high sensitivity but less specificity would be

safer. In contrast, the 95% probability plot provides a higher specificity in the diagnosis of pyometra versus CEH/mucometra.

Table 10. *Differences in the percentage of physical examination abnormalities between dogs with pyometra and dogs with cystic endometrial hyperplasia (CEH)/mucometra. P-values calculated with Fisher's exact test (two-tailed test)*

	Pyometra	**n=51**	**CEH**	**n=9**	**P-value**
Temperature > 39.2°C	17 (33%)	51	1 (11%)	9	0.26
Heart rate > 120 beats/minute	11 (23%)	47	0 (0%)	9	0.18
Respiratory Rate > 20 breaths/minute	13 (32%)	40	4 (50%)	8	0.43
Abdominal pain on palpation	37 (76%)	49	5 (56%)	9	0.24
Attitude Bright and Alert	7 (14%)	49	6 (67%)	9	0.0025
Attitude ≥ moderately depressed	15 (31%)	49	2 (22%)	9	1.00
Mucus membranes Pale or Hyperemic	12 (24%)	50	0 (0%)	9	0.18

C-reactive protein in canine uterine disorders (Study III and IV)

In study III the laboratory parameters that differed between diseased and healthy animals were albumin, AP, PBN, platelet count, TNFα, WBC and CRP. Of these, only the latter was able to distinguish between SIRS positive and SIRS negative dogs. Also, when evaluating possible correlation between all clinical and laboratory parameters previously found associated with SIRS (temperature, WBC, PBN, heart rate [HR], respiratory rate, platelet count, CRP, TNFα and IL-6), only the temperature, the HR and the CRP was statistically related to SIRS. CRP showed a less significant correlation with SIRS than temperature and HR (p-value=0.0003), but one must bear in mind that the latter two criteria are part of the definition of SIRS.

To the author's knowledge, CRP has not previously been used for prediction of severity in diseased animals or to predict outcome. A possible limitation of CRP as a predictor of outcome and severity of disease is the fact that CRP can show a high individual variability (Reny *et al.* 2002), which was also reflected by study III. This makes certain conclusions based on an individual value more difficult, and in human intensive care procalcitonin is emerging as a more novel and possibly more accurate tool in diagnosis of sepsis (Marik, 2002). Regardless, CRP and procalcitonin appears to be the most valuable predictors of sepsis available in human intensive care today and of these two, only CRP is available for use in dogs (Marik, 2002).

Table 11. *Mean and standard deviation for selected hematology parameters (hemoglobin, WBC, % band neutrophils, platelet count), blood biochemical parameters (AP, Albumin), CRP and TNFα in dogs with Pyometra or Cystic endometrial hyperplasia (CEH) or healthy dogs (Controls AP=Alkaline phosphatase; CRP=C-reactive protein; TNFα=Tumor necrosis factor alpha; WBC= White blood cell count*

	Pyometra **Mean (±S.D.)** **N=54**	**CEH** **Mean (±S.D.)** **N=10**	**Controls** **Mean (±S.D.)** **N=10**
Hemoglobin	139.23 (±24.52)	136.90 (±19.85)	168.80 (±16.83)
(g/L)	53	10	10
WBC	23.31 (±11.45)	10.19 (±5.44)	8.22 (±1.67)
(10^9/L)	53	10	10
Band neutrophils	12.58 (±9.77)	1.69 (±3.78)	0.00 (±0.00)
(%)	53	10	10
Platelet count	233.27 (±125.28)	251.67 (±110.50)	307.20 (±128.65)
(10^9/L)	52	9	10
AP	6.15 (±6.06)	2.26 (±2.30)	1.37 (±0.58)
(μkat/L)	54	9	10
Albumin	26.83 (±4.39)	30.57 (±3.64)	33.90 (±2.96)
(g/L)	48	7	10
CRP	200.28 (±93.51)	53.51 (±66.24)	19.84 (±8.19)
(mg/L)	47	8	10
TNFα	0.13 (±0.07)	0.12 (±0.05)	0.01 (±0.01)
(pg/mL)	43	7	10

The results from study IV showed that an elevated PBN in combination with high plasma CRP showed the highest sensitivity (97.7 %) of all possible combinations of the investigated parameters in the prediction of whether a dog with a fluid filled uterus will have the diagnosis pyometra confirmed by histopathological examination. For the clinician trying to decide whether to perform emergency surgery or to wait until more ideal circumstances are available, the sensitivity of the test is probably of higher importance than the specificity. It is reasonable to assume that emergency surgery on a dog that is not critically ill, *i.e.* CEH, would be less hazardous than the risk associated with a delay in surgery for a pyometra dog, that potentially could rupture an infected uterus. Therefore it seems logical to use the parameters with the highest sensitivity in the prediction of the diagnosis. However, clinically this requires that a reliable, rapid, and preferably cheap CRP assay is available at all times, as well as a differential WBC. In contrast to WBC, which is part of routine blood work at most small animal hospitals, this is not presently the case for CRP. The most popular commercially available canine assay today is a relatively expensive and time-consuming ELISA. This contrasts the automated CRP assays (nephelometric or turbidometric) available for humans. Structural differences in canine CRP as compared to human CRP have previously been considered to preclude the use of human diagnostics for dogs (Yamamoto *et*

al., 1993). However, a commercially available human turbidometric CRP assay was recently showed to reliably measure canine serum CRP levels as well (Kjelgard-Hansen, Jensen & Kristensen, 2003). The benefits of CRP determination in human medicine are extensive in several different fields, reflected by the development of this assay into a relatively inexpensive routine 24-hour blood work (Reny *et al.* 2002). Our studies are likely only the first in a multitude of future reports on benefits of CRP as a diagnostic aid in veterinary medicine.

Table 12. *Significance of differences in selected hematology parameters (hemoglobin, WBC, % band neutrophils, platelet count), blood biochemical parameters (AP, Albumin), CRP and TNFα in dogs with Pyometra or Cystic Endometrial Hyperplasia (CEH) or healthy dogs (Controls). Two-sample unequal variance Student's T-test (two-tailed test) was used to calculate p-values. A p-value of <0.05 is considered significant and these p-values are indicated with bold figures. AP=Alkaline phosphatase; CRP=C-reactive protein; TNFα=Tumor necrosis factor alpha; WBC=White blood cell count*

	Pyometra vs. Control p-value	**CEH vs. Control p-value**	**Pyometra vs. CEH p-value**
Albumin (g/L)	**<0.0001**	0.12	**0.0365**
AP (μkat/L)	**<0.0001**	**0.0437**	**0.0016**
Band neutrophils (%)	**<0.0001**	**0.004**	**<0.0001**
CRP (mg/L)	**<0.0001**	0.28	**0.0001**
Hemoglobin (g/L)	**<0.0001**	**0.0012**	0.93
Platelet count (10^9/L)	0.12	**0.0092**	0.66
TNFα (pg/mL)	**<0.0001**	**0.0012**	0.58
WBC (10^9/L)	**<0.0001**	**0.0023**	**<0.0001**

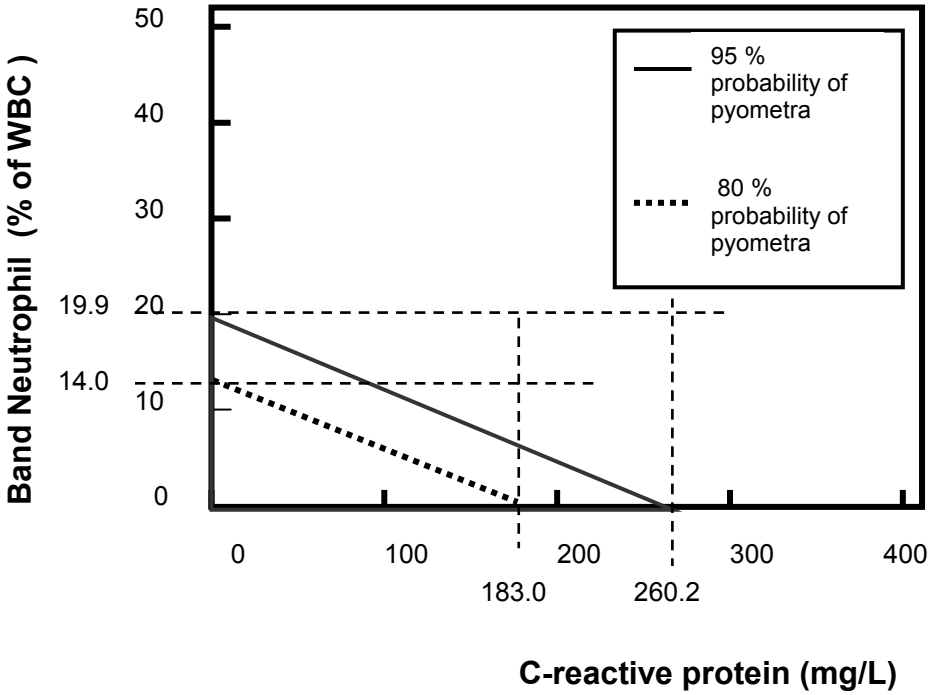

Figure 3. Contour plot of estimated 95% (solid graph) and 80 % (dotted graph) probability of the diagnose pyometra, based on plasma C-reactive (CRP) concentration and the percent band neutrophils (PBN) from the differential white blood cell count. If values for CRP and PBN are bisecting to the right of the graph there is a 95% or 80% , respectively, probability that the diagnosis is pyometra in contrast to Cystic Endometrial Hyperplasia (CEH).

General conclusions

The following conclusions can be drawn from this series of investigations:

• *Escherichia coli* isolated from the uterus of bitches with pyometra showed a higher degree of clone similarity than would have been expected from a random population. This indicates that *E. coli* causing pyometra are descendants of certain clones.

• Identical *E. coli* clones were found in the rectum and the uterus of bitches with pyometra, indicating that the infection ascends into the uterus from the intestinal bacterial flora.

• Identical *E. coli* clones were isolated from the uterus and the urinary tract in bitches with pyometra and concomitant urinary tract infection. The primary infection site is however not determined.

• Commmon hematology and blood biochemical abnormalities in bitches with pyometra versus non-inflammatory uterine disease or versus healthy controls include an inflammatory leukogram with a left shift, non-regenerative anemia of chronic inflammation, mildly elevated ALP, hypercholesterolemia, hypernatremia and hyperchloremia from hemoconcentration and decreased serum urea nitrogen. Uncommon abnormalities (<10 % of dogs) include azotemia, hypo- or hyperglycemia, hypocalcemia, hypokalemia, and elevations of ALAT. Results of preprandial bile acids, bilirubin or GLDH are in general within normal limits.

• Endotoxemia was not consistently demonstrated in bitches with pyometra.

• Two out of four criteria for systemic inflammatory response syndrome (SIRS) were fulfilled in 57 % of bitches with pyometra.

• Of hematology parameters, blood biochemical parameters, tumor necrosis factor α, interleukin 6 or C-reactive protein (CRP), CRP, despite notable individual variation, was the only parameter that was significantly related to SIRS, with the exception of the clinical criteria that defines this syndrome. A combination of body temperature, heart rate and CRP was most strongly related to SIRS as compared to all other parameters alone or grouped.

• In bitches with pyometra, a positive SIRS status, high plasma CRP concentration and high body temperature are significant predictors of increased morbidity as reflected by length of hospitalization.

• Of diagnostic laboratory work, the percent band neutrophils (PBN) in combination with CRP are highly sensitive in the prediction of the diagnosis pyometra versus cystic endometrial hyperplasia/mucometra (CEH). Alkaline phosphatase in combination with PBN showed a slightly increased specificity (77.8%) at the cost of decreased sensitivity in the prediction of pyometra. In

addition, the presenting clinical signs as well as the general attitude of the dog were shown to be helpful in the differentiation between pyometra and CEH.

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