Predictive Markers and Risk Factors in Canine Pyometra

Supranee Jitpean
Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences
Uppsala

Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2015

Acta Universitatis agriculturae Sueciae
2015:85

Cover: Drawing by Nuntarwat Wipoosak

ISSN 1652-6880
ISBN (print version) 978-91-576-8368-7
ISBN (electronic version) 978-91-576-8369-4

Print: SLU Service/Repro, Uppsala 2015

Predictive Markers and Risk Factors in Canine Pyometra

Abstract

Pyometra is a common and life-threatening disease in intact female dogs, which is generally treated by surgery. Early identification of dogs with high risk of complications or poor prognosis is valuable for optimising treatment and increase survival. The objectives of this thesis were to detect predictive markers for prognosis and outcome of pyometra by investigating clinical and pathophysiological responses and to explore the breed-dependent risk for pyometra and mammary tumours (MTs).

Leucopaenia was the most important predictive variable, associated with an 18-fold increased risk for peritonitis (present in 13% of the dogs) and an over 3.5-fold increased risk for prolonged postoperative hospitalisation. Fever or hypothermia was linked with an increased risk for peritonitis and dogs with moderate to severely depressed general condition or pale mucous membranes had an increased risk for prolonged postoperative hospitalisation. These results show that commonly explored clinical variables may be helpful for predicting prognosis.

Blood concentrations of the acute phase proteins, C-reactive protein and serum amyloid A (SAA) were found to be increased in dogs with pyometra, whereas concentrations of albumin, insulin-like growth factor-I, and iron were decreased. Importantly, SAA concentrations were higher in the dogs that also suffered from sepsis. Though unspecific, SAA could therefore be a potential marker for identifying more severely affected dogs. The neuroendocrine protein chromogranin A was measured by its breakdown products catestatin and vasostatin. Catestatin concentrations were decreased in pyometra whereas vasostatin concentrations did not differ compared to healthy dogs. None of these investigated inflammatory mediators or chromogranin A were useful for outcome prediction as measured by postoperative hospitalisation.

The incidence of pyometra in 110 different breeds was studied using insurance data. Before 10 years of age, 19% of all female dogs had suffered from the disease. Breed greatly affected the risk of both pyometra and MTs.

In summary, these findings show that clinical and laboratory data and analysis of inflammatory variables can be helpful for predicting prognosis and assessing severity in dogs with pyometra. Breed considerably affects the risk of pyometra and MTs, and the information presented in this thesis will be valuable for evaluating possible health benefits of spaying in individual dogs, based on the risk of developing these diseases.

Keywords: dog, biomarkers, C-reactive protein, serum amyloid A, insulin like growth factor-I, iron, albumin, SIRS, chromogranin A, catestatin, vasostatin.

Author's address: Supranee Jitpean, SLU, Department of Clinical Sciences,
P.O. Box 7054, SE-750 07 Uppsala, Sweden. *E-mail:* Supranee.Jitpean@ slu.se

Dedication

To my family, fellow teachers, and my dogs

Life is like riding a bicycle. 'You do not fall off unless you plan to stop pedalling'

"ชีวิตก็เหมือนการขี่จักรยาน ตราบใดที่คุณไม่คิดจะหยุดปั่นจักรยาน มันจะไม่มีวันล้ม"

Claude Pepper

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I **Jitpean S**, Holst BS, Emanuelson U, Höglund OV, Pettersson A, Alneryd-Bull C, Hagman R (2014). Outcome of pyometra in female dogs and predictors of peritonitis and prolonged postoperative hospitalization in surgically treated cases. *BMC Veterinary Research* 10(1):6.

II **Jitpean S**, Holst BS, Höglund OV, Pettersson A, Olsson U, Strage E, Södersten F, Hagman R (2014). Serum insulin-like growth factor-I, iron, C-reactive protein, and serum amyloid A for prediction of outcome in dogs with pyometra. *Theriogenology* 82(1):43-48.

III **Jitpean S**, Pettersson A, Höglund OV, Holst BS, Olsson U, Hagman R (2014). Increased concentrations of Serum amyloid A in dogs with sepsis caused by pyometra. *BMC Veterinary Research* 10:9.

IV **Jitpean S**, Stridsberg M, Pettersson A, Höglund OV, Holst BS, Hagman R (2015). Decreased plasma Chromogranin A361-372 (Catestatin) but not Chromogranin A17-38 (Vasostatin) in female dogs with bacterial uterine infection (pyometra). *BMC Veterinary Research* 11(1):14.

V **Jitpean S**, Hagman R, Holst BS, Höglund OV, Pettersson A, Egenvall A (2012). Breed variations in the incidence of pyometra and mammary tumours in Swedish dogs. *Reproduction in Domestic Animals* 47:347-350.

The contribution of Supranee Jitpean to the papers included in this thesis was as follows:

I Conception of study and study design, interpretation of results, drafting the article, and critical revision of article

II Conception of study, study design and data management, interpretation of results, drafting the article, and critical revision of article

III Conception of study, study design and data management, interpretation of results, drafting the article, and critical revision of article

IV Conception of study, study design and data management, interpretation of results, drafting the article, and critical revision of article

V Interpretation of results, drafting the article, and critical revision of article

Abbreviations

ALAT	Serum alanine aminotransferase
ALP	Alkaline phosphatase
BUN	Blood urea nitrogen
BW	Body weight
CEH	Cystic endometrial hyperplasia
CgA	Chromogranin A
CRP	C-reactive protein
CRT	Capillary refill time
Cst	Catestatin (Chromogranin A361-372)
DYAR	Dog-years at risk
ET	Endotoxin
Hb	Haemoglobin
Hct	Haematocrit
IGF-I	Insulin-like growth factor-I
IL	Interleukin
LPS	Lipopolysaccharide
MTs	Mammary tumours
OHE	Ovariohysterectomy
SAA	Serum amyloid A
SIRS	Systemic inflammatory response syndrome
TNF- α	Tumour necrosis factor-alpha
UDS	The University Animal Hospital (at SLU)
VS	Vasostatin (Chromogranin A17-38)
WBC	Total white blood cell count

1 Introduction

1.1 Canine pyometra - general background

Bacterial uterine infection (pyometra) is often described as the "major disease of female dogs" since it develops in more than 50% of all intact female dogs in certain breeds before 10 years of age (Egenvall *et al.*, 2000a). The disease is characterised as a hormone induced uterine bacterial infection and inflammation, leading to a pus-filled uterus and systemic illness.

Pyometra is potentially life-threatening and has deadly consequences if left untreated. Importantly, dogs with the disease often develop systemic inflammatory response syndrome (SIRS) (Fransson *et al.*, 2007; Hagman *et al.*, 2007). SIRS is defined as a generalised inflammatory response which can be initiated by infectious as well as non-infectious insults, and where inflammatory mediators stimulate the cascade-like release of other mediators, which in excess can be harmful for the individual (Muckart & Bhagwanjee, 1997). Sepsis *i.e.* "blood-poisoning" is defined as presence of SIRS caused by infection (Levy *et al.*, 2003). Despite commonly inducing sepsis, the mortality rate in pyometra is relatively low, about 3-4% (Egenvall *et al.*, 2001). In more severe cases, such as when shock has developed, the mortality rate increases to about 35% (Conti-Patara *et al.*, 2012), and can be as high as 50% if peritonitis is present (Oelzner & Munnich, 1997).

Early diagnosis and treatment of pyometra is essential for increased chance of survival. Clinically useable markers for identifying high-risk patients and predicting outcome are currently in high demand. Several studies in both human medicine and veterinary medicine have investigated a variety of markers for monitoring treatment response or to predict outcome in different diseases (Foushee *et al.*, 2012; Zhang *et al.*, 2008; Kosuge *et al.*, 2007; Rau *et al.*, 2007; Ceron *et al.*, 2005; Claeys *et al.*, 2002; Hogarth *et al.*, 1997). Among such markers, analysis of inflammatory mediators in dogs with various

diseases is gaining interest within veterinary research (Christensen *et al.*, 2014; Dabrowski *et al.*, 2013; Kjelgaard-Hansen & Jacobsen, 2011; Klenner *et al.*, 2010).

The safest and most effective treatment of pyometra is surgical removal of the ovaries and infected uterus, *i.e.* ovariohysterectomy (OHE). Ovariohysterectomy is the same procedure as is commonly performed for elective spaying of female dogs, after which pyometra is prevented. In Sweden, elective spaying was only performed in few dogs (7% in 1999, all ages) (Egenvall *et al.*, 1999) and though the proportion has been increasing lately to nearly 17% (Statistics Sweden, Sweden, 2012), most dogs remain intact (not neutered/spayed). In other countries, such as the USA, neutering/spaying is commonly performed to control the dog population and avoid a large number of unwanted stray dogs (Trevejo *et al.*, 2011; Salman *et al.*, 1998). The large proportion of intact Swedish dogs means that most female dogs are at risk of developing diseases associated with reproductive organs and hormones such as pyometra. Most Swedish dogs are insured, which is why data from insurance companies can be used to investigate the occurrence of diseases.

The main insurance reimbursement claims due to costly veterinary care in female dogs in Sweden are treatment of pyometra and MTs (Egenvall et al., 1999) with pyometra being the most frequent unique diagnosis in the female dog (Bonnett & Egenvall, 2010). Pyometra, and to some extent MTs, may be prevented by spaying, but OHE is also associated with some negative side-effects such as urinary incontinence, fur alterations, behavioural changes, overweight, and increased risk for malignant tumours such as osteosarcoma and haemangiosarcoma (Smith, 2014; Zink *et al.*, 2014; McGreevy *et al.*, 2005; Ru *et al.*, 1998; Thrusfield, 1985). There are breed- and age-related differences in the occurrence of both pyometra and MTs, but the combined risk of developing these diseases has not yet been assessed (Egenvall et al., 2005; Egenvall et al., 2001). Risk factors for pyometra include nulliparity and hormonal therapy (oestrogen and progesterone) (Whitehead, 2008; Niskanen & Thrusfield, 1998; Borresen, 1979) whereas overt pseudo-pregnancy has been proposed as protective (Fidler *et al.*, 1966). The effect of certain risk factors such as nulliparity may differ between dog breeds (Hagman *et al.*, 2011).

1.1.1 Pathogenesis of pyometra

Although the development of pyometra has been the focus of many research studies over the years, the pathogenesis is still unclear (De Bosschere *et al.*, 2002; Millerliebl *et al.*, 1994; Sandholm *et al.*, 1975; Hardy & Osborne, 1974; Dow, 1959; Lesbouyries & Berthelon, 1936). The development is complex and the aetiology includes a combination of effects of hormone interaction,

virulence of the causative bacterial strains and counteracting defence mechanisms (Sandholm *et al.*, 1975; Hardy & Osborne, 1974; Dow, 1959). Progesterone plays an important role by stimulating proliferation and secretion of the uterine glands, decreasing contractions of the myometrium and diminishing the function of the immune response (Dow, 1959; Teunissen, 1952). Endogenous or exogenous progesterone influences changes in the uterus which makes it suitable for foetal development, but such environment also facilitates intrauterine bacterial attachment and growth, creating optimal conditions for developing pyometra (Dow, 1959; Teunissen, 1952). Progesterone-induced changes of the endometrium facilitate adherence of *Escherichia coli* (*E. coli)* to specific receptors in the endometrium, and probably contribute to the development of the disease (Sandholm *et al.*, 1975). Administrating oestrogen hormone alone does not induce pyometra in dogs (Dow, 1959). Oestrogen, however, enhances the effects of progesterone in its action which in turn may lead to development of the disease (Lessey *et al.*, 1981; Teunissen, 1952). In most pyometra cases (70-90%), *E. coli* bacteria are isolated from pyometra uteri (Hagman & Greko, 2005; Fransson *et al.*, 1997). As a natural inhabitant of the vaginal flora, *E. coli* enter the uterus during cervical opening in proestrus and oestrus (Watts *et al.*, 1996). In healthy dogs, the defence mechanisms are able to eliminate the bacteria (Watts *et al.*, 1996).

Two different classifications are used in many research studies of pyometra (De Bosschere *et al.*, 2001; Dow, 1957). Based on histological changes of the uterus, Dow (1975) named the disease "the cystic endometrial hyperplasia (CEH)-pyometra complex" and classified it into four subtypes: (1) cystic hyperplasia (CEH), (2) cystic hyperplasia with acute endometritis, (3) cystic hyperplasia with plasma cell infiltration, and (4) chronic endometritis. The other classification was based on morphological and histological changes of the uterus together with reported clinical signs, dividing the disease into two entities, CEH-mucometra or endometritis-pyometra (De Bosschere *et al.*, 2001). Cystic endometrial hyperplasia is believed to predispose for development of pyometra, but CEH is generally not associated with clinical signs and may be present in middle-aged or older bitches without signs of disease (Dow, 1957). As a result, it is difficult to know how common CEH is in the dog population, and in how many of the cases with CEH, pyometra will follow, and how soon.

1.1.2 Clinical presentation

Pyometra is commonly diagnosed in metoestrus/dioestrus, in middle-aged to older dogs, with mean age at diagnosis of 6-9 years (range; 9 months - 18 years) (Niskanen & Thrusfield, 1998; Wheaton *et al.*, 1989; Dow, 1957).

Clinical signs depend on cervical patency, the severity of systemic inflammation and organ functions affected. Vaginal discharge, which is often odorous and sanguineous to muco-purulent, is considered a characteristic finding (Borresen, 1979). However vaginal discharge is only present in cases of open cervix pyometra, *i.e.* absent if the cervix is functionally closed, making diagnosis less clear in these cases. Other common signs of pyometra are more non-specific and include lethargy, depression, lack of appetite, polydipsia, polyuria and vomiting (Hagman *et al.*, 2009b). Lameness is often present, but generally disappears after treatment of the pyometra (Klainbart *et al.*, 2014).

1.1.3 Haematology and biochemistry variables

Common haematological changes in dogs with pyometra include leucocytosis, neutrophilia with a left shift and mild normocytic, normochromic, and non-regenerative anaemia, which also can be observed in many other diseases (Bartoskova *et al.*, 2007; Hagman *et al.*, 2006; Fransson *et al.*, 1997; De Schepper *et al.*, 1987a; Borresen, 1979). Leucopaenia is less commonly found (Borresen, 1980). Organ functions can be altered, including kidney and liver function, indicated by increased creatinine and blood urea nitrogen (BUN) concentrations, hypoalbuminemia and proteinuria (Maddens *et al.*, 2011; Fransson *et al.*, 1997; De Schepper *et al.*, 1987b; Borresen, 1979). The impaired renal function might be due to tubulointerstitial inflammation or immune-complex associated glomerulonephritis resulting in glomerular and tubular dysfunction (Maddens *et al.*, 2011; Heiene *et al.*, 2007; Asheim, 1964). Increased serum concentrations of alkaline phosphatase (ALP), bilirubin and cholesterol may indicate intrahepatic cholestasis which also has been suggested as a possible consequence of endotoxaemia (Sato *et al.*, 2002; Borresen, 1980; Borresen & Skrede, 1980).

1.1.4 Diagnosis

The diagnostic workup in a dog with suspected pyometra usually includes retrieving disease and general history data from the dog-owner, physical examination, laboratory blood tests of haematology and biochemistry variables and diagnostic imaging by radiography and/or ultrasonography to demonstrate uterine enlargement. Abdominal ultrasonographic examination allows for accumulating uterine fluid content to be detected, even minor amounts, and CEH and signs of ovarian disease may also be visible. Ultrasonography is considered a reliable diagnostic imaging investigation for diagnosis of uterine disease associated with fluid accumulation, but because it depends on the equipment and skills of the examiner, it is not always possible to detect minor endometrial cysts (Bigliardi *et al.*, 2004). Furthermore, accumulation of fluid

in the uterus is not always due to pyometra; it could also be caused by mucometra, hydrometra or haematometra, hence bacterial isolation and histopathological examination are necessary for accurate verification of the diagnosis.

1.1.5 Treatment

Early diagnosis of pyometra and assessment of its severity is a prerequisite for optimal treatment decisions *i.e.* whether to perform immediate surgery as soon as the patient's general condition is stabilized or if surgery can be delayed without risk for the patient.

Surgical treatment

For several decades, the most effective treatment of pyometra has been considered to be surgical removal of the ovaries and uterus (OHE) (Rootwelt-Andersen & Farstad, 2006), because it removes the site of infection and prevents disease recurrence. The OHE procedure is, however, not always life-saving and it results in permanent infertility. Stabilization prior to surgery e.g. by intravenous fluid administration, adjusting electrolyte and acid-base balance and antimicrobial therapy is generally performed in severe cases to increase survival and prevent complications associated with sepsis such as bacterial emboli or shock (Conti-Patara *et al.*, 2012). Antimicrobial drugs as sole treatment for pyometra are as a rule not considered completely curative.

Medical treatment

Purely medical treatment of pyometra is an option in selected cases such as for preserving fertility in a bitch with only mild disease condition or if surgery or anaesthesia is considered as associated with high risk for serious complications or death. Medical treatment protocols contain antimicrobial therapy administered in combination with drugs that induce cervical opening and emptying of the uterus and may include progesterone antagonists, dopamine agonists, prostaglandins or a combination of these drugs (Corrada *et al.*, 2006; Fieni, 2006; Gobello *et al.*, 2003). The reported recurrence rate is 19-48% but because the infected uterus is not removed, the risk of endotoxaemia and sepsis and associated complications remain during the treatment period (Ros *et al.*, 2014; Corrada *et al.*, 2006; Fieni, 2006). The prognosis after medical treatment is more favourable in pyometra without CEH and ovarian cysts (Trasch *et al.*, 2003; Arnold *et al.*, 1988) and in bitches of younger age (Jurka *et al.*, 2010).

Combinations of antimicrobial therapy and intrauterine administration of betadine-saline (De Cramer, 2010) or prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (Gabor *et al.*,

1999), or intrauterine catheters (Funkquist *et al.*, 1983) have been suggested as other non-surgical treatment alternatives for pyometra.

1.2 Biomarkers - general background

One definition of a "biomarker" is "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to therapeutic intervention" (Atkinson *et al.*, 2001). Studies on biomarkers have gained growing interest in both human and veterinary medicine for various diseases and conditions, with the goal to find suitable biomarkers for early detection and diagnosis. Not only rapid change in levels of the biomarker when the disease develops is warranted, but also rapid normalisation during recovery, to allow its usefulness when monitoring the effects of therapeutic intervention. Additionally, sampling should be easy, the biomarker relatively stable, analysis reliable, quick, cost-effective and practical to perform cage-side. Predictive biomarkers are biomarkers that are valuable as objective tools for prognostication *i.e.* in the prediction of outcome and/or treatment response, and such biomarkers have been identified in a variety of human and animal diseases (Foushee *et al.*, 2012; Eckersall & Bell, 2010; Gebhardt *et al.*, 2009; Kosuge *et al.*, 2007; Ceron *et al.*, 2005). Markers or variables useful for prognostication can be derived from case history or physical examination parameters, results from inflammatory response and various other laboratory analyses or even other biological factors.

1.2.1 Assessing the severity of pyometra

As mentioned earlier, pyometra leads to sepsis (SIRS) in more than 50% of dogs diagnosed (Hagman *et al.*, 2009b; Fransson *et al.*, 2007; Hagman *et al.*, 2007), and SIRS has been associated with prolonged hospitalisation (Fransson *et al.*, 2007). It is not only the bacteria *per se* that cause the severity of the disease but bacterial products such as endotoxin (ET), a potent inducer of inflammation, may contribute to a poorer prognosis. In one study of dogs with pyometra, higher blood concentrations of ET were associated with poor prognosis (death) (Okano *et al.*, 1998). Hyperlactataemia has been associated with increased risk of mortality in other severely ill dogs suffering from other diseases (Stevenson *et al.*, 2007; Nel *et al.*, 2004). However, in dogs with pyometra, hyperlactataemia is generally mild and present only in a few dogs with unaffected circulation (Conti-Patara *et al.*, 2012; Hagman *et al.*, 2009b). Disseminated intravascular coagulation (DIC) may develop in dogs with pyometra (Plavec *et al.*, 2006). Other variables that have value for

prognostication include BUN and creatinine with increased concentrations being associated with higher risk of mortality (Kuplulu *et al.*, 2009). The cause of death in dogs with the disese may also be due to cardiac malfunction. Cardiac troponin I (cTnI), a marker for myocardial damage, has been found to be mildly to moderately increased in nearly half of the dogs diagnosed with pyometra (Pelander *et al.*, 2008). Additionally, in one study highly increased cTnI postoperatively was found in a dog that died and that had myocarditis (Hagman *et al.*, 2007). In one study of dogs with sepsis or septic shock due to pyometra, higher central venous oxygen saturation ($ScvO_2$) and lower base deficit was associated with a more favourable prognosis (low risk of death) (Conti-Patara *et al.*, 2012).

1.2.2 Why biomarkers for sepsis in dogs with pyometra?

Sepsis, and especially severe sepsis which is defined as sepsis and dysfunction of one or more organs, remains an important and common cause of death in both human and veterinary intensive care units (Weiss *et al.*, 2015; Kenney *et al.*, 2010). Because the progression of pyometra to severe sepsis is potentially lethal, early recognition of sepsis and appropriate treatment is a prerequisite for treatment success and favourable outcome. Diagnosing sepsis is challenging because clinical signs and laboratory variables are generally unspecific, and reliable diagnostic markers are lacking (Borresen, 1979). Presence of bacterial infection and SIRS, established by certain clinical and laboratory criteria, is therefore currently used to identify patients with sepsis both in human and veterinary medicine (Kamisoglu *et al.*, 2015; Hagman *et al.*, 2009b; Fransson *et al.*, 2007; Otto, 2007; Afessa *et al.*, 2001; Hauptman *et al.*, 1997). SIRS can be initiated by several causes, *i.e.* viral infection, mechanical trauma, chemical agents, thermal injuries, radiation, immunological causes, ischaemia and necrotic tissue (Bone, 1992). Conventional diagnosis of sepsis through bacterial blood culturing can be time-consuming and antimicrobial sensitivity test results take additional time to obtain. In a study of septic human patients, it was found that antimicrobial treatment should be initiated within 6 hours, as the survival rate decreased by 7.6% for every hour that treatment was delayed (Kumar *et al.*, 2006). Other studies have also shown that hospital mortality rates increase if administration of antimicrobials is delayed in septic human patients (Bloos *et al.*, 2014; Dickinson & Kollef, 2011; Hounsom *et al.*, 2011; Blanco *et al.*, 2008). Biomarkers that could aid in the early detection of sepsis are thus valuable for optimising therapy and decreasing hospitalisation and mortality. In dogs, despite the benefits of antimicrobials with sepsis being currently debated (Keir & Dickinson, 2015), early detection and treatment initiation in sepsis is considered crucial for survival. Biomarkers that can be

helpful when selecting which patients should be treated with antimicrobials are urgently needed, because unnecessary or ineffective antimicrobial therapy will promote resistant bacteria and long-term increase of infections untreatable by commonly used drugs (Alanis, 2005).

In humans, concentrations of the acute phase proteins (APPs) C-reactive protein (CRP) and procalcitonin (PCT) are increased in sepsis and widely used as adjunctive tools to predict morbidity and mortality (Brunkhorst *et al.*, 2000; Yentis *et al.*, 1995). Other inflammatory mediators explored for this purpose include cytokines such as Interleukin-6 (IL-6) which is useful for diagnosis and prognostication in neonatal septic human patients (Kuster *et al.*, 1998). In comparison to cytokines, the APPs are more stable and long-lasting in the circulation, which is advantageous for use as a biomarker (Dandona *et al.*, 1994). CRP and serum amyloid A (SAA) are major acute phase proteins in dogs (Ceron *et al.*, 2005) and have been investigated for diagnostic and prognostic purposes in dogs with SIRS and sepsis (Chan *et al.*, 2009; Gebhardt *et al.*, 2009; Fransson *et al.*, 2007; Martinez-Subiela & Ceron, 2005). Blood concentrations of CRP increase in pyometra and to a higher extent if SIRS is present (Fransson *et al.*, 2007).

Other biomarkers, such as catecholamines have been studied in human patients with sepsis, and it was shown that sympathetic nerve activities are stimulated in sepsis and caused increased release of catecholamines (Marchuk *et al.*, 1977). Studies in veterinary medicine of catecholamines as markers for sepsis in dogs, however, are still lacking.

C-reactive protein

The acute phase protein, CRP, discovered in humans in the 1930's, is a major APP also in dogs (Yamashita *et al.*, 1994; Tillett & Francis, 1930). C-reactive protein is produced by the liver (Hurlimann *et al.*, 1966) when triggered by pro-inflammatory cytokines, foremost IL-6 (Yamashita *et al.*, 1994) and has been used as a marker for systemic inflammation in studies of both humans and dogs (Ceron *et al.*, 2005; Pepys & Hirschfield, 2003; Bigoszewski *et al.*, 2001; Yamamoto *et al.*, 1993). In humans, CRP is useful for prognostication, *i.e.* prediction of survival rate and duration of hospitalisation and to evaluate the response of treatment (Pierce *et al.*, 2009; Lobo *et al.*, 2003; Philip & Mills, 2000). In human patients, those with higher concentrations of CRP had worse outcome *i.e.* longer hospitalisation, higher risk of death, or non-responsiveness to treatment (Hogarth *et al.*, 1997; Yentis *et al.*, 1995). In veterinary medicine, increased serum CRP concentrations have been reported in various diseases (Christensen *et al.*, 2014; Yamamoto *et al.*, 1993) including dogs suffering from pyometra (Dabrowski *et al.*, 2013; Fransson *et al.*, 2007). However, CRP

concentrations increase in all diseases with systemic inflammation *i.e.* the increase is not specific for pyometra.

Serum amyloid A

Serum amyloid A is another major APP in dogs and its production is induced by pro-inflammatory mediators (Ceron *et al.*, 2005). Studies have investigated SAA in various diseases, in humans and animals, including dogs (Christensen *et al.*, 2014; Dabrowski *et al.*, 2013; Zhang *et al.*, 2012; Cho *et al.*, 2010) and blood concentrations can be 800-fold increased during inflammation (Yule *et al.*, 1997). In the uterus of dogs with pyometra, the gene for SAA is upregulated (Hagman *et al.*, 2009c). The usefulness of SAA as marker to diagnose sepsis in dogs with pyometra has not yet been explored.

Albumin

Hypoalbuminaemia is a common finding in dogs with pyometra (Borresen & Skrede, 1980). Albumin is considered a negative acute phase protein. The production decreases during inflammation as a possible result of increased hepatic production of positive APPs and other inflammatory components (Liao *et al.*, 1986; Schreiber *et al.*, 1982). Albumin concentrations have been shown to be a predictive marker in critically ill human patients, and also to be linked with malnutrition (Owen *et al.*, 1993). Hypoalbuminaemia may be an effect of decreased albumin production but also and/or reflect increased vascular permeability in response to inflammation or infection (Deysine & Stein, 1980) or increased loss via the kidneys (Levey *et al.*, 2003). Furthermore, in humans with renal disease and who required haemodialysis, hypoalbuminaemia was associated with increased risk of mortality (Wetmore *et al.*, 2008; Owen *et al.*, 1993). The value of albumin for prognostication in pyometra has not yet been explored.

Insulin- like growth factor-I

Insulin-like growth factor-I (IGF-I) is an anabolic peptide mediated by growth hormone which has many roles including control of cell proliferation, cell differentiation and anti-apoptosis (Heemskerk *et al.*, 1999; Rodriguez-Tarduchy *et al.*, 1992). Several studies in humans have reported decreased concentrations of plasma IGF-I in response to infection and inflammation (Heemskerk *et al.*, 1999; Timmins *et al.*, 1996). In endotoxaemia and chronic inflammation, IGF-I is downregulated, leading to decreased circulating IGF-I concentrations, which has been interpreted as IGF-I being a negative inflammatory marker in rats and dogs (Tvarijonaviciute *et al.*, 2011; Priego *et al.*, 2003; Lang *et al.*, 2000; Lopez-Calderon *et al.*, 1999). Recently, one study

showed that concentrations of IGF-I decreased in dogs with pyometra prior to surgery and increased after surgery, in contrast to CRP (Dabrowski *et al.*, 2015). The role of IGF-I in dogs suffering from pyometra has not been thoroughly investigated.

Iron

Plasma iron concentrations are regulated by the hormone hepcidin, originating from the liver. Production of hepcidin leads to decreased concentrations of iron in plasma (Nemeth *et al.*, 2004). The production of hepcidin can be induced by cytokines e.g. IL-6 (Nemeth *et al.*, 2003) and bacterial infection (Shike *et al.*, 2002). Studies evaluating iron concentrations in response to infection are rare in dogs (Konitzer *et al.*, 1956), and to the author's knowledge there has been no study focused on pyometra.

Chromogranin A (CgA)

Chromogranin A is a neuroendocrine secretory acid and water soluble protein that belongs to the granin family (Taupenot *et al.*, 2003). CgA is one of three "classic" granins which include also chromogranin B and secretogranin II (chromogranin C). Chromogranin A was first isolated from chromaffin granules in the adrenal gland (Helle, 1966; Banks & Helle, 1965) and it can also be found in neuroendocrine and various neuron cells (Wilson & Lloyd, 1984). CgA is co-released with catecholamines from chromaffin cells in the adrenal medulla by exocytosis (Oconnor & Bernstein, 1984) and is a precursor of several biologically active peptides. CgA has a various protease and peptidase cleavage sites and its breakdown products are named based on their different biological activities, e.g. catestatin (Cst; CgA361-372), vasostatin (VS; CgA17-38) and pancreastatin (Bartolomucci *et al.*, 2011). The autocrine inhibitory effect of Cst to nicotinic acetylcholine receptor in the adrenal gland impedes secretion of catecholamines (Mahata *et al.*, 2004). In addition, Cst induces proliferation, migration and anti-apoptosis in endothelial cells (Theurl *et al.*, 2010).

In humans, CgA measurement has been widely used as a marker for diagnostic and prognostic purposes in patients suffering from neuroendocrine tumours leading to increased CgA concentrations (Korse *et al.*, 2012; Portela-Gomes *et al.*, 2010; Hsiao *et al.*, 1990). Additionally, a high CgA concentration has been shown to be clinically useful for predicting severity and mortality in patients suffering from chronic heart disease (Angelone *et al.*, 2012; Ceconi *et al.*, 2002). In severely ill non-surgical patients at an intensive care unit, higher CgA concentrations were detected in nonsurvivors compared to survivors (Zhang *et al.*, 2008). Similarly, concentrations of CgA were shown

to be higher in human patients who died of sepsis compared to those who survived (Rosjo *et al.*, 2012).

There are a few published studies on CgA in dogs (Srithunyarat *et al.*, 2014; Akiyoshi *et al.*, 2005; Myers *et al.*, 1997). In one study of dogs with acute stress, the results indicated that CgA concentrations increased in response to stress induced by insulin injection and it was concluded that CgA could be a useful marker for stress (Akiyoshi *et al.*, 2005). Additionally, increased plasma concentrations of CgA might be an indicator of suspected neuroendocrine tumours such as insulinoma (Myers *et al.*, 1997). However, CgA has not yet been studied in dogs with sepsis or bacterial infection.

1.3 Breed risk for pyometra and mammary tumours

Earlier studies have been performed to evaluate the incidence of pyometra (Egenvall *et al.*, 2001) and MTs (Egenvall *et al.*, 2005) in Sweden. These studies have some limitations because data from only two years and 30 dog-breeds were included in the earlier pyometra study whereas, for MTs, data from an eight-year period and over 200 breeds were assessed. Information about the occurrence of pyometra is therefore missing for most dog breeds. Additionally, the combined risk for developing the two most important diseases of the reproductive organs (pyometra and MTs) has never been investigated. Because neutering may affect the occurrence of both these diseases, it is valuable to evaluate the risk of both diseases combined by breed when deciding whether or not spaying may have some health benefits for a female dog. Possible health benefits and the risk for unwanted side-effects in each specific breed should be considered when assisting the dog-owners in making the optimal choice for their pet regarding neutering/spaying.

2 Aims and hypotheses of the thesis

The objectives of this thesis were to investigate the clinical and pathophysiological responses to pyometra in dogs, to identify variables that are helpful for predicting outcome or severity of the disease, and to determine breed-dependent possible benefits of disease prevention by neutering. This new knowledge could increase the understanding of how findings from case history, clinical examination and laboratory testing and biomarkers can be helpful for assessing severity of the disease in each particular dog.

- In Paper I, the hypothesis that case history data, clinical signs, and findings on physical examinations or laboratory analyses may be useful for predicting peritonitis or outcome in pyometra bitches treated by surgery was tested.
- In Paper II, the hypothesis that concentrations of variables induced by the inflammatory response may be helpful in the diagnosis of pyometra or prediction of outcome was tested.
- In Paper III, the hypothesis that concentrations of inflammatory variables may be valuable for identifying sepsis in dogs with pyometra was tested.
- In Paper IV, the hypothesis that a neuroendocrine response is induced in pyometra and that associated variables may be valuable in the prediction of outcome was tested.
- In Paper V, the hypothesis that health benefits of spaying may differ between breeds as a result of the occurrence of two common diseases affecting the reproductive organs was tested.

3 Materials and methods

A general description of the materials and methods used in the studies is presented here. For additional details, see Paper I -V (appendices).

3.1 Study designs

In Paper I, a retrospective study using data from dogs diagnosed with pyometra and treated at the University Animal Hospital (UDS), Swedish University of Agricultural Sciences (SLU) was designed. For Paper II-IV, prospective clinical studies with dogs diagnosed with pyometra and treated at the UDS, SLU, were designed. In Paper V, an epidemiological study using data from the Agria Insurance Company database was constructed including data from more than 260 000 female dogs enrolled in both veterinary care and covered by life insurance (the two types of insurances offered) during the years 1995-2006.

3.2 Ethical permission

The studies were approved by the Uppsala Local Ethical Board (permission number C413/12), and a signed informed consent was obtained from each dog-owner before their dog participated, as described in Paper II-IV.

3.3 Animals

Study population/animals and data management

In Paper I, a retrospective study was carried out using data records from all bitches diagnosed with pyometra during the years 2006-2007 at UDS, SLU, Uppsala, Sweden. The bitches were identified by the diagnostic code for pyometra used in Sweden (Olson *et al.*, 1993). In total 356 bitches were included in the study of which 315 were surgically treated, 9 purely medically

treated and 32 euthanised instead of treated after the diagnosis. The UDS's patient records include data such as breed, weight, age, case history, findings on physical examination, results of radiographic and/or ultrasonographic examinations, laboratory analyses including haematology and serum biochemistry, treatments, date of leaving the UDS and follow-ups. The cases were generally admitted within two months of previous oestrus (in metoestrus/dioestrus). Exclusion criteria were parturition or pregnancy. All dogs had signs of systemic illness. The bitches were divided into three groups: 1) euthanised dogs 2) medically treated dogs and 3) surgically treated dogs. Euthanasia was performed at the request of the owner and in agreement with the veterinary surgeon in charge, in most cases due to concomitant diseases and not because of a poor prognosis. Only bitches with normal hydration status, unaffected or slightly depressed general condition and with no ovarian or endometrial cysts demonstrated on ultrasonographic examination were selected for medical treatment with aglepristone (Alizin vet®, Virbac, Montpellier, France) in combination with antimicrobials (enrofloxacin, amoxicillin, ampicillin, cephalosporin, sulfadiazine and trimethoprim or amoxicillin/clavulanic acid), performed according to the routines at UDS. The success of the medical treatment was evaluated by ultrasonography and laboratory tests including haematology, total white blood cell counts (WBC) and differential counts. Furthermore, data from the surgically treated bitches were analysed to explore possible indicators of pre-existing peritonitis or development of postoperative peritonitis or as predictors of dogs with subsequent prolonged postoperative hospitalisation. Intraocular pressure was measured in all bitches diagnosed with uveitis.

In Paper II-III, bitches admitted to UDS, SLU, Uppsala, Sweden, and diagnosed with pyometra were included in the studies. Thirty-one client-owned dogs with pyometra (Paper II and III) admitted to UDS, SLU, Uppsala, Sweden, during 2011, were included in the study prior to surgical treatment (OHE). Additionally, 17 healthy female staff-owned dogs of similar weight and in a comparable stage of the oestrus cycle were enrolled as the control group (Paper II).

The preliminary diagnosis pyometra was based on case history, and results of physical examination and diagnostic imaging by ultrasonography or radiography or both to demonstrate an enlarged, fluid-filled uterus. The presumptive diagnosis pyometra was further indicated by visual inspection during OHE and later confirmed by postoperative macroscopic and histopathological examination of the uterus and ovaries performed at the Department of Biomedical Sciences and Veterinary Public Health, SLU, Uppsala, Sweden and bacterial cultures and sensitivity tests were performed at

an accredited laboratory, Section of Bacteriology, National Veterinary Institute (SVA), Uppsala, Sweden. Bitches with solely cystic endometrial hyperplasia, mucometra, hydrometra, haematometra or endometritis were not included in the study.

In general, bitches subjected to OHE due to pyometra at UDS are hospitalised 1-2 days after surgery. Prolonged postoperative hospitalisation (defined as ≥ 3 days) is warranted if specific complications occur or the general condition of the bitch is moderately or severely depressed and additional veterinary care and monitoring required (considered an unspecific complication in the investigation).

In Paper IV, in total 114 bitches were included, including 50 with pyometra of 23 breeds, and 64 healthy bitches of 22 breeds. All bitches with pyometra were treated by OHE at the UDS, SLU, during the study period 2009-2013. A complete physical examination was performed by the veterinarian in charge, and the results recorded in a special form. The preliminary diagnosis of pyometra was based on case history data, findings on physical examination, laboratory test results, and diagnostic imaging by either abdominal ultrasonography or radiography or both.

In Paper V, data from more than 260 000 dogs insured during the years 1995-2006 was downloaded to a personal computer. All dogs included in the analyses were less than 10 years old. The variables used were: sex, breed, date of birth, date of death, postal code, dates when dogs entered or left the insurance programme and information on the type of insurance for which dogs were enrolled, insurance claims or reimbursement. Diagnostic codes, assigned by the attending veterinarian, were also downloaded. These codes were assigned based on a hierarchically constructed diagnostic registry with approximately 8 000 codes (Olson *et al.*, 1993). If dogs left the insurance during a year for reasons other than the evaluated diseases, they were regarded as censored (leaving the database during the year of analysis). For example, in the 12-month risk calculations (see below) censored dogs only contribute a "½-dog" to the denominator instead of a whole dog for the non-censored dogs.

Many dogs originally insured before 1995 had only the year of birth accurately recorded. These dogs were considered to have been born the 2nd of July the respective year. For analyses that required dogs to be assigned to age categories, these were assigned based on the age of the dog on the 1st of January 1995-2006 respectively (< 1, 1 < 2, ..., 9 < 10 years). Breeds were classified according to the Swedish Kennel Club breed classification system and some breeds were combined because they were considered to be the same. For example, "Dachshund miniature" included all miniature variants, "Dachshund normal-size" included all except long-haired Dachshunds,

"German Pointer" included both smooth-haired and wire-haired, and "Poodle" included Miniature and Toy Poodles. The breed varieties mentioned are considered to have a shared gene pool because there are no strict barriers for breeding between the varieties.

Veterinary care insurance has no age limit and reimburses the owner most of the fee if the dog receives costly veterinary care. Dogs can also be life-insured, but only up to 10 years of age. With life insurance, the owner generally will be reimbursed if the dog dies or is euthanised. Whether the dog died or was euthanised cannot be differentiated in the database. Most insured dogs have both types of insurance. The insurance process has earlier been described in detail (Egenvall *et al.*, 2000b).

3.4 Laboratory analyses

3.4.1 Haematology, biochemistry and hormonal analyses

Blood samples for haematology, serum biochemistry and hormonal analyses were collected from the distal cephalic vein and transferred into EDTA and non-additive collection tubes (Vacutainer®, Becton-Dickinson, Stockholm, Sweden), respectively. The non-additive tubes were placed on ice, centrifuged and serum separated before analysis.

Haematological (WBC including differential counts, haematocrit (EVF) and haemoglobin (Hb)) and biochemical (bile acids, alanine aminotransferase (ALT), glucose, blood urea nitrogen (BUN), and creatinine) analyses were performed using Advia 2120; Siemens Healthcare Diagnostics, Deerfield, IL, USA for haematology and Abbott Architect c4000, Abbott Park, IL, USA, for biochemistry (Paper I- IV).

Albumin was analysed with a colorimetric method (bromocresol green) using an automated analyser (Abbott Architect c4000, Abbott Park, IL, USA) with a commercial albumin reagent from Abbott Laboratories (Paper I-IV).

Progesterone analyses were performed using an enhanced chemoluminescence immunoassay (Immulite, Diagnostic Products Corporation, Los Angeles, CA, USA). The oestrous cycle stage was defined by vaginal cytology and progesterone analysis (Paper II).

Haematological and biochemical analyses were performed according to routine methods at the Clinical Pathology Laboratory, UDS, SLU, Uppsala, Sweden.

3.4.2 Acute phase proteins and other inflammatory markers

Analysis of CRP was performed with a human immunoturbidimetric test previously validated for dogs (Randox Laboratories Ltd, Crumlin, UK)

(Klenner *et al.*, 2010; Kjelgaard-Hansen *et al.*, 2003). The analyses were performed on Abbot Architect (Abbott Architect c4000, Abbott Park, IL, USA) and the method was calibrated with canine CRP (Life Diagnostics canine CRP, West Chester, USA). The lowest measurable concentration was 5 mg/L with mean intra- and interassay variations of 1.4% and 2.4%, respectively. Samples with high concentrations of CRP (above 217 and 225 mg/L for the two lots used) were autodiluted 1:3 with 0.9% NaCl and reanalysed to obtain exact values (Paper II, III and IV).

For SAA, the analyses were performed using a commercially available ELISA (Tridelta Phase^{TM} Range SAA Assay, Tridelta Development Limited, County Kildare, Ireland), with mean intra- and inter-assay coefficients of variation of 4.75% and 8.8%, respectively, and with the lowest measurable concentration of 10 mg/L. The absorbance was evaluated using Tecan Sunrise reader (Tecan Inc., Männedorf, Switzerland). The method has previously been validated for dogs (Martinez-Subiela & Ceron, 2005) (Paper II and III).

An IGFBP-blocked ELISA (Mediagnost, Reutlingen, Germany), validated for use in dogs, was used for evaluation of IGF-I concentrations, with intra- and inter-assay coefficients of variation of less than 10%, and the lowest measurable concentration of 22 ng/mL (Strage *et al.*, 2014; Strage *et al.*, 2011) (Paper II).

Measurement of iron concentrations was performed by direct colorimetric determination (Abbott Laboratories Inc., Illinois, USA) with a detection limit of 0.9 µmol/L (Paper II).

All laboratory tests for inflammatory variables were performed according to the manufacturer's instructions by trained laboratory staff at the Clinical Pathology Laboratory, UDS, Uppsala, Sweden (Paper I, II, and III).

The break-down products derived from CgA- Cst and VS- were measured in heparinised plasma by radioimmunoassays specific for Cst and VS (Stridsberg *et al.*, 2004; Stridsberg *et al.*, 2000). These assays have been described in detail in a previous study and are in-house assays performed at the Research Department of Clinical Chemistry, Uppsala University Hospital, Sweden (Stridsberg *et al.*, 2004). The method has been evaluated for use in dogs (Stridsberg M *et al.*, 2014).

3.4.3 Bacterial isolation

Samples were immediately collected from the uterine content of the removed uterus with sterile fibre cotton swabs (Culturette; Becton-Dickinson AG, Stockholm, Sweden). Bacterial cultures and sensitivity tests were performed at an accredited laboratory, Section of Bacteriology, SVA, Uppsala, Sweden as described earlier (Hagman *et al.*, 2009a) (Paper III).

3.5 Data analyses

3.5.1 Paper I

Univariable associations between potential risk factors within case history, physical examination and laboratory data and the outcomes prolonged hospitalisation and signs of peritonitis, respectively, were analysed by Chi-Square test and Fisher's exact test. Multivariable associations between potential risk factors and the outcomes were analysed by logistic regression models. All variables with a p-value ≤ 0.20 in the univariable analyses were considered as potential predictor variables. Categorical predictor variables were introduced in the models coded as dummy variables. Collinearity between potential predictor variables were assessed by Spearman rank correlation and considered present when $|r| > 0.6$, in which case the variable with a) least missing values or b) providing the best model fit was retained. Modelling was done manually by backward elimination of non-significant ($P > 0.05$) variables. At each step, previously eliminated variables were tested for re-entry. Confounding was assessed by comparing the change in estimated coefficients when variables were excluded from the model, and were considered present if a coefficient changed > 20%. The statistical models were developed separately with and without variables related to laboratory data because of many missing observations. All statistical analyses were performed using SAS (version 9.3, SAS Institute Inc., Cary, NC, USA).

3.5.2 Paper II

The program SAS 9.2 for Windows version 6.1.7601 (SAS Institute Inc. Cary, NC, USA) was used for statistical analyses. Student's t-test and Analysis of Variance (ANOVA) were used for normally distributed variables, and Van der Waerden Two-Sample test was used for variables when most healthy bitches had concentrations below the lowest measurable concentration (*i.e.* for CRP and SAA, levels were set to half the lowest measurable concentrations (5 mg/L and 10 mg/L, respectively, for the analyses). Pearson's product moment correlation coefficient was used for analyses the association of variables and duration of postoperative hospitalisation. Significance level was set to $P < 0.05$ for all tests in the study.

3.5.3 Paper III

All statistical analyses were performed using of SAS 9.3 package (SAS Institute Inc. Cary, NC, USA). ANOVA was used to evaluate the differences of SAA, CRP and albumin between septic and non-septic groups and to investigate a possible relation between inflammatory markers and morbidity as

measured by increased postoperative hospitalisation. Residual diagnostic plots were used to assess normality and homoscedasticity.

The diagnostic power of different markers was assessed using logistic regression (Olsson, 2002). The area under curve (AUC) was used as a measure of diagnostic ability. The cut-off value was selected as upper 5% limit in the non-septic group (Pape, 2003).

Descriptive data were described as mean ± SE. Bitches with concentrations below the lowest measurable concentration were assigned a value of half that value for the statistical analyses.

3.5.4 Paper IV

Statistical analyses were performed by using Minitab software programmes for Windows version 16 (Minitab Inc., State College, PA, USA). After checking that data were normally distributed, Student's t-test was used to test differences of haematology, biochemistry, Cst and VS between the control and pyometra group. The different concentrations of Cst and VS in pre- and postoperative samples were compared by using the paired t-test. In control dogs that had concentrations of CRP lower than 5 mg/L, the concentration was set to half the lowest measurable concentration (2.5 mg/L) for the statistical analyses. A Wilcoxon two-sample test was used for analysis when most control bitches had CRP concentrations below the lower measurable concentration. In the pyometra dogs, Pearson's correlation was used to evaluate associations between Cst and VS and other variables- including age, weight, haematology, biochemistry, concentration of CRP, and duration of hospitalisation. Pearson's product moment correlation coefficient was also used to investigate associations between Cst and VS in heparinised and EDTA plasma. The p-value was set at $P < 0.05$ for all tests used.

3.5.5 Paper V

Few bitches in Sweden are spayed electively. It was assumed, for the sake of this study, that none were electively spayed and accordingly that all insured bitches were at potential risk of developing pyometra, if not already having done so. If dogs had reimbursed claims for pyometra for veterinary care and/or life they were considered as pyometra cases. For life claims they were considered as cases irrespective of whether reimbursed or not. From the information in the database it could not be seen whether dogs claimed for life insurance died or were euthanised. Only reimbursed veterinary care events were included because the non-reimbursed group was not homogenous. Some people submitted all claims and others submitted only those that have a reasonable chance of being reimbursed. Almost all claims for pyometra and

MT however, were submitted and reimbursed. Accordingly, it was assumed that whether or not non-reimbursed claims are included should not make a quantitative difference.

Using data on all bitches < 10 years of age and covered by insurance both for life and veterinary care, the crude and breed-specific median ages were calculated and the crude and breed-specific median ages at development of pyometra and MTs were determined.

Dogs entering during a year (*i.e.* enrolling in insurance after January 1st) were not included in the analysis of that year. As few Swedish bitches are neutered/spayed and because neuter status was not included in the database, it was decided to adjust the number of dogs at risk in each age group (Egenvall *et al.*, 2001).

Age- and breed-specific adjusted 12-month risks were used to estimate the cumulative crude and breed-specific risk of pyometra and MTs in each age category, using a combined risk formula: total risk in n age category = 1- (1-risk $_{\text{age category <1}}$)*(1-risk $_{\text{age category 1<2}}$)*…*(1-risk $_{\text{age category n}}$)(Kleinbaum *et al.*, 1982). One minus the total risk was considered as the proportion that had, at that age, not yet developed pyometra (or MT, *i.e.* "the survival to pyometra or MT". The statistical software programme SAS (SAS Institute, Cary, NC) was used to analyse the data, and the procedure GENMOD used for the logistic regression.

4 Results and discussion

4.1 Pyometra - a common and life-threatening illness

On average, nearly one-fifth of all Swedish female dogs (19%) were diagnosed with pyometra before 10 years of age. In 10 of the studied breeds, a proportion of 50% or more of the bitches had been diagnosed with the disease by the age of 10 years (Paper V). These results show that pyometra remains a common diagnosis and that the disease is an important illness. The proportion of dogs overall affected by the disease was slightly lower than what has been reported earlier (23-24%), which probably depends on the fact that more breeds of low-risk were included in the present analyses (Egenvall *et al.*, 2001).

In many dogs with pyometra, the clinical signs are classic and the diagnosis straight-forward, but in some dogs the diagnosis can be more difficult because of diffuse clinical signs. In dogs with closed cervix pyometra, *i.e.* without vaginal discharge, the signs are less clear. The most common case history and clinical signs reported (in Paper I/III, respectively) were vaginal discharge (77%/65%) anorexia (69%/74%), depression (63%/100%), polydipsia (62%/45%) and polyuria (60%/42%). These findings illustrate the local and systemic effects of the disease (Hagman, 2012; Wheaton *et al.*, 1989; Borresen, 1979; Dow, 1957).

Ovariohysterectomy is considered the safest and most efficient treatment method for pyometra and most dogs are surgically treated, *i.e.* 315 of 356 dogs (Paper I). Only four of the surgically treated dogs died postoperatively leading to a mortality of 1% in these dogs. This is lower than the 5-27% mortality in previous reports (Kuplulu *et al.*, 2009; Hagman *et al.*, 2007; Wheaton *et al.*, 1989). The relatively low mortality could reflect that the disease is common and thereby recognised quickly in Sweden enabling appropriate treatment to be initiated early in the disease progression. Rapid initiation of treatment might

reduce the presence of severe complications result in shorter duration of hospitalisation and increase the survival rate in dogs with pyometra.

The different causes of mortality in the surgically treated dogs were unknown (2 dogs), splenic rupture (1 dog) and severe peritonitis (1 dog). As the disease is more common in bitches of middle-aged to older age, concurrent diseases may be present. Euthanasia instead of treatment was performed in 32 dogs (9%) (Paper I), not because of a poor prognosis of the pyometra treatment but by request from the owner and in agreement with the veterinary surgeon in charge due to concomitant diseases including severe hip dysplasia (n = 1), hepatic disease associated with ascites (n = 1), long-term polyuria/polydipsia (n = 1), several other diseases (n = 2), kidney malfunction (n = 1), MT (n = 1), multiple neoplasia in oesophagus (n = 1), or due to pyometra associated with old age of the bitch (n = 24). If the 32 euthanised dogs were included, the total mortality rate in dogs with pyometra was about 10% (36/356 bitches).

The severity of pyometra was also shown by the presence of complications (Paper I).

In total, specific complications were observed in 25% (n = 78) of the 315 surgically treated bitches. The most frequently observed complication was peritonitis (40 bitches), followed by urinary tract infection (19 bitches), wound infection (8 bitches), uveitis (6 bitches), and cardiac arrhythmia (5 bitches). Thus, although the mortality is relatively low, complications develop in one-fourth of surgically treated dogs. The most common complication was peritonitis which is serious and can be life-threatening, further indicating the severity of the disease and the urgency for early diagnosis and treatment.

Postoperative hospitalisation after surgical treatment of pyometra is generally 1-2 days at UDS, unless specific complications are present or the overall health status indicates that further monitoring and treatment is necessary. Prolonged postoperative hospitalisation (here estimated three or more days), considered as a non-specific complication, was observed in 19% of the cases (60/315 bitches) (Paper I) and 39% (12/31 bitches) (Paper II), respectively. In spite of pyometra being linked to a relatively low mortality, the increased postoperative hospitalisation observed in about 20-40% of the cases clearly shows the severity of the disease. If complications were possible to detect early or if it was possible to predict bitches at risk of becoming more severely affected than others, this would be highly valuable for providing early and optimal treatment that in turn would increase survival and potentially fewer dogs requiring prolonged postoperative hospitalisation.

4.2 Clinical parameters are helpful in the prediction of outcome in surgically treated dogs

Among the haematology variables analysed, neutrophilia (55.3%), leucocytosis (54.3%) and monocytosis (50.7%) were the most common findings in dogs with pyometra and present in more than 50% of the dogs (Paper I). These alterations are considered typical for the disease and the proportions are about the same magnitudes as reported previously (Fransson *et al.*, 1997; De Schepper *et al.*, 1987a). Concentrations of bile acids and albumin were lower in dogs with pyometra than in healthy dogs (Papers II and IV).

Hypoglycaemia was associated with both prolonged postoperative hospitalisation and peritonitis in surgically treated dogs (Paper I). The finding that hypoglycaemia has predictive value is in agreement with the results of a study of septic human patients, showing that low blood glucose was associated with a high risk of mortality in sepsis caused by *E. coli* (Alamgir *et al.*, 2006). However, as there were only few dogs with hypoglycaemia in the present study, these results should be interpreted with some caution and will need to be investigated further.

Decreased concentrations of iron were commonly found in pyometra (Paper II). It has been shown in mice that subcutaneously injected turpentine oil led to an inflammatory response and subsequently decreased circulating iron (Nicolas *et al.*, 2002). A similar study performed in dogs with injected turpentine or staphylococci, also led to decreased iron concentrations even before anaemia had developed (Gbeenberg *et al.*, 1947). Iron could perhaps be used as a negative marker for diagnosis of diseases associated with inflammation or infection, but further studies of its value as a possible biomarker are necessary.

In Paper I, medical treatment was chosen for nine dogs (2.5%). These bitches were treated with aglepristone in combination with antimicrobial therapy administered for a mean duration of 23 days. Regarding antimicrobial therapy, one fifth (n = 65) of all the dogs with pyometra (Paper I) had been treated prior to presentation at UDS. Of these, five dogs were not treated with antimicrobials because of pyometra but because of other disorders (dermatitis in one dog, cystic calculi in one dog, cystitis in two dogs, and arthritis in one dog). In 124 (35%) of the 315 surgically treated bitches, antimicrobials were administered postoperatively. During the past decades, the increasing threat of antimicrobial resistant microbes has become a major problem in both humans and animals. In Sweden, indications for antimicrobial therapy in pyometra are moderately to severely depressed general condition, high risk of or signs of sepsis or specific complications associated with disseminated infection, and antimicrobials are thus not administered to all bitches with the disease.

However, antimicrobials are more widely used in other countries (Adamovich-Rippe *et al.*, 2013; Dabrowski *et al.*, 2013; Kum *et al.*, 2013). Further studies of antimicrobial therapy and possible additional restrictions are warranted for optimising medical and surgical treatment routines at the same time as reducing the likelihood of antimicrobial resistant bacteria developing.

In Paper I, the risk of prolonged postoperative hospitalisation was investigated in relation to different clinical signs, physical examination findings and laboratory data. The results showed that vomiting, severe depression, pale mucous membranes, severe dehydration, abdominal pain on palpation, leucopaenia, neutropaenia, monocytopaenia, increased serum creatinine, hypoglycaemia and increased lactate concentrations were associated with prolonged hospitalisation. However, some parameters were analysed in only a few of the bitches for example lactate (n = 19); hence, missing data was a limitation for thorough evaluation of these variables.

Peritonitis was the most common complication in bitches with pyometra undergoing surgical treatment, present in about 13%, whereof one dog died postoperatively. Furthermore, 12 out of the 40 surgically treated dogs with peritonitis required prolonged postoperative hospitalisation. In pyometra, peritonitis may be caused by leakage of pus from the ovarian bursa (Rubio *et al.*, 2014) or uterine rupture. Septic peritonitis is associated with a high risk of death with reported mortality rates of 30-70% in dogs (Lanz *et al.*, 2001; Mueller *et al.*, 2001; Ludwig *et al.*, 1997). The mortality in dogs with peritonitis was lower in the present study (<1%) and also compared to a previous study in which 50% of dogs with pyometra diagnosed with peritonitis died (Oelzner & Munnich, 1997). These results indicate that if treated appropriately, the prognosis is more favourable in peritonitis caused by pyometra than earlier reported. The reason for the difference between studies of pyometra is unknown but could depend on the disease being well-known in Sweden, with a widespread knowledge of optimal treatment routines and possible complications detected at an early stage. There could also be a difference in severity of the peritonitis in the studied dogs.

Several potentially useful predictive markers among the routinely analysed laboratory and clinically registered parameters were found in the 356 bitches with pyometra in Paper I. Leucopaenia was demonstrated in seven of the surgically treated dogs and in six out of these (85%) the postoperative hospitalisation was prolonged which indicates a more severe disease. In bitches with leucopaenia there was an 18-fold increased risk of being diagnosed with peritonitis and 3.5-fold increased risk of needing prolonged postoperative hospitalisation. Although few dogs (only 8) had leucopaenia, these results clearly indicate that leucopaenia is a sign to consider when evaluating the

severity of pyometra. That leucopaenia can be associated with increased severity in pyometra, is in line with leucopaenia also being associated with increased risk of mortality in septic humans (Afessa *et al.*, 1995; Hook *et al.*, 1983).

4.3 Inflammatory variables may be helpful in the diagnosis and prediction of outcome

Despite concentrations of CRP being significantly increased in pyometra (Paper II and III), CRP was not valuable as a marker to identify sepsis as the concentrations did not differ between septic and non-septic dogs. Furthermore, CRP was not associated with prolonged hospitalisation (Paper II and III) in contrast to what has been reported previously (Fransson *et al.*, 2007). The differences between studies could reflect that the present study included fewer dogs because otherwise sampling methods and patient inclusion criteria were similar. Increased concentrations of CRP have been associated with increased mortality in dogs with SIRS and sepsis (Gebhardt *et al.*, 2009), but this association could not be investigated here as none of the dogs in which CRP was measured died. Serum amyloid A was another APP studied and increased concentrations of SAA were found in dogs with pyometra (Paper II and III). This finding is in agreement with increased SAA concentrations demonstrated also in two other studies (Christensen *et al.*, 2014; Dabrowski *et al.*, 2007).

Exclusively dogs with pyometra had SAA and CRP concentrations over the lowest measurable concentration (5 and 10 mg/L, respectively) (Paper II and III). It was therefore possible that CRP and SAA could be useful in differentiating dogs with pyometra from healthy dogs, when used in combination with other findings. However, it should be noted that one dog with pyometra had CRP and SAA concentrations below the lowest measurable concentration (Paper II). Considering that the APPs increase in all diseases associated with systemic inflammation and that pyometra did not always lead to increased concentrations, the diagnostic value of either APP for pyometra is therefore limited. In dogs with pyometra and sepsis, the number of band neutrophils, monocytes and basophils were significantly higher than in dogs without sepsis (Paper III). Because WBC and band neutrophils were used to classify dogs with SIRS in our study, these variables and those closely linked, were not independent and therefore not useful as markers to identify sepsis.

4.4 Novel variables for inflammation and stress – possible markers for prognostication of disease?

4.4.1 Insulin-like growth factor-I

In Paper II, it was shown for the first time that IGF-I concentrations are lower in bitches with pyometra than in healthy bitches. These results are confirmed in another more recent study of dogs with the disease (Dabrowski *et al.*, 2015). Mean IGF-I concentrations (± SE) in dogs with pyometra were 221.2 ± 22.5 ng/mL and in healthy dogs 366.7 ± 46.2 ng/mL. The findings are also in agreement with a previous study where systemic IGF-I concentrations decreased in response to chronic inflammation (De Benedetti *et al.*, 1997). However, other factors such as age, gender, nutrition status and diseases such as diabetes mellitus, could influence concentrations of IGF-I in the circulation (Chestnut & Quarmby, 2002; Heemskerk *et al.*, 1999; Juul *et al.*, 1994), which should be considered in further investigations of the use of this parameter to detect inflammation or infection. Furthermore, body size and age in dogs have been associated with the IGF-I levels *i.e.* a positive correlation with body size and a negative correlation with age was shown (Greer *et al.*, 2011; Eigenmann *et al.*, 1988). In the present study, the body weight was similar in the disease and control groups, and they could therefore be compared. However, the age difference cannot be excluded as possible confounder for the difference in IGF-I levels between the two groups. Moreover, IGF-I concentrations in dogs have been shown to decrease with restricted energy intake (Maxwell *et al.*, 1998). Most pyometra dogs had decreased appetite at admission, which is why low food intake also may have contributed to the lower serum IGF-I concentrations. Additionally there might be unknown breed differences to consider.

4.4.2 Chromogranin A

In Paper IV, two break-down products, Cst and VS, derived from CgA, were measured with methods recently evaluated for use in dogs (Stridsberg M *et al.*, 2014). Concentrations of Cst (mean ± SE) in the pyometra group were significantly lower (1.0 ± 0.05 nmol/L) compared to the healthy group (1.7 ± 0.03 nmol/L). Meanwhile, concentrations of VS did not differ between the two groups (mean ± SE, 0.40 ± 0.04 and 0.42 ± 0.30 nmol/L in pyometra dogs and healthy dogs, respectively). Concentrations of Cst and VS did not differ when comparing samples obtained before surgery (mean ± SE 0.89 ± 0.07 nmol/L for Cst and 0.36 ± 0.04 nmol/L for VS) and after surgery (Cst 0.86 ± 0.04 nmol/L and VS 0.36 ± 0.04 nmol/L). This study is the first report on evaluating CgA, known as a stress marker, in dogs suffering from infection. However, the

results were somewhat unexpected, because they were not similar to other studies in humans where increased circulating CgA was found in critically ill and intensive care patients with severe sepsis (Rosjo *et al.*, 2012; Zhang *et al.*, 2009). An explanation for this difference could be that most pyometra dogs in our study were not suffering from as severe sepsis, and had shown clinical signs within two weeks before admission at UDS as mentioned in Paper I (*i.e.* possibly less acute disease). Another explanation could be that a negative feedback control of Cst leads to inhibited secretion of catecholamines and CgA, and subsequently decreased CgA concentrations. In humans, the Cst is a potent and specific cholinergic antagonist in chromaffin cells, and increased Cst concentration could inhibit catecholamine secretion (Taupenot *et al.*, 2000; Mahata *et al.*, 1997). Such negative feedback would be more likely to occur in individuals with chronic (pyometra) as opposed to acute disease (severe illness or sepsis in the studied human patients). There may also be species differences or methodological differences that could be overlooked.

Why Cst concentrations but not VS concentrations were decreased remains to be determined. The relation between intact CgA and its fragments is complex and although this has not been shown, one possible explanation of the differences found between Cst and VS could be that Cst measurement comprises both intact CgA and Cst as well as other possible fragments of CgA that contain the Cst part. Because the assay is expressed in molar units it measures the number of molecules present in the sample *i.e.* the assay most probably measures the total CgA content. The same is true for VS measurements with the currently used assay, but when comparing results from these two assays (Cst and VS), diverging results were found. Other explanations for the different results of the two derived CgA in our study, would be because of a variety of post-transitional cleavages and the difference of its biologically active peptides (Helle *et al.*, 2007). However, more studies in dogs are needed to gain further information.

A method to measure the entire molecule of CgA in dogs is currently not commercially available. Methods used to detect CgA in humans vary and different peptides have been measured, which should be considered when comparing the results of different clinical studies (Biswas *et al.*, 2010; Mazza *et al.*, 2010; Stridsberg *et al.*, 2003). The results of Cst in our study are promising and indicate that Cst might be useful as a marker for CgA in a variety of diseases in dogs. Measurement of CgA could be valuable not only for dogs with infection but also with neuroendocrine tumours because CgA has been shown to be an excellent marker for neuroendocrine tumours in humans (Ferrari *et al.*, 2004; Oconnor & Bernstein, 1984). Whether CgA and its break-

down products Cst and VS are useful as biomarkers for diagnostic purposes or prognostication in dogs, remains to be further discovered.

4.5 Can sepsis be identified in pyometra?

Sepsis, *i.e.* SIRS-positive status, was found in nearly 75% of the studied dogs with pyometra (Paper III). The two major APPs and other variables were investigated for possible association with sepsis and SAA concentrations were significantly increased in pyometra dogs with sepsis. For SAA, the receiver operating characteristic curve (ROC) analysis displayed an area under the curve (AUC) of 0.74 in dogs with sepsis (P = 0.04). The specificity and sensitivity for detection of dogs with sepsis by SAA analysis was not very high (74% and 50% respectively) using a selected cut-off value at 109.07 mg/L. This means that one-fourth of dogs without sepsis would be falsely classified as septic dogs and, worse, truly septic dogs would be classified as non-septic, making SAA unsuitable as a sole marker for sepsis. Concentrations of CRP and albumin were not significantly different in dogs with or without sepsis according to the criteria selected. These results point to a possible higher clinical value for SAA in the detection of sepsis compared to CRP, and this potential deserves to be further investigated.

The finding that SAA possibly had a better clinical value compared to CRP is in line with other studies in which SAA has shown more promise as a diagnostic marker than CRP for the diseases linked with inflammation, even though there was some overlapping (Christensen *et al.*, 2014; Christensen *et al.*, 2013). In human medicine, SAA has been shown to be a better prognostic marker for severity of cancer associated with inflammation (Wang *et al.*, 2012; Zhang *et al.*, 2012; Cho *et al.*, 2010; Kosuge *et al.*, 2007) and for prediction of outcome in non-ST-segment elevation acute coronary syndromes (Kosuge *et al.*, 2007). Although both CRP and SAA are major APPs in dogs, the roles and abilities as biomarkers may differ, indicating that there may be conditions or diseases for which SAA could have a clinically higher diagnostic or prognostic value than the more frequently used CRP. Alternatively, that a panel of several APPs combined with other analyses with different abilities will be more optimal for diagnosis or prognostication.

Concentrations of CRP and albumin did not differ between septic and non-septic dogs although albumin concentrations were decreased and CRP increased in most bitches with pyometra compared to healthy dogs (Paper II, III). CRP was not associated with sepsis which is in contrast to a previous report (Fransson *et al.*, 2007). This difference between studies could be because of methodological differences, individual variation/severity of

systemic inflammation in the dogs included in the two studies that larger study is necessary to show this association. Concentrations of albumin were also not significantly different in bitches with or without sepsis. This finding was unforeseen because lower albumin concentrations have been reported in sepsis and circulating albumin decreases in experimentally induced endotoxaemia in dogs, cats and rats (Greiner *et al.*, 2008; Deysine & Stein, 1980; Powanda *et al.*, 1972). However, the clinical situation is different from experimental studies in that the studied dogs were admitted at various stages of disease progression and the effect of endotoxaemia may therefore vary. CRP and albumin might still have a diagnostic value in dogs with pyometra and more severe disease (septic peritonitis or septic shock), but this needs to be further studied.

4.6 To spay or not to spay? Considerations based on breed differences in disease occurrence

The development of pyometra depends on several factors, of which breed is one of the most important for predicting the risk for developing the disease. The risk for developing both pyometra and MTs varied considerably between breeds as shown in Paper V. The risk of developing both these diseases was high in several large or giant breed dogs and also increased with increasing age (Table 1). Although reproductive hormones may be involved to some extent in the development of both diseases, the high risk breeds differed, as illustrated by the 10 breeds with the highest risk of developing either pyometra or MTs or both pyometra and MTs up to 10 years of age (Table 1). This means that different factors are likely to be involved in the pathogenesis. The 10 breeds with highest risk to be diagnosed with either or both of the two diseases were Leonberger (73%), Irish Wolfhound (69%), Bernese Mountain Dog (69%), Great Dane (68%), Staffordshire Bull Terrier (66%), Rottweiler (65%), Bull Terrier (62%), Doberman (62%), Bouvier des Flandres (60%) and Airdale Terrier (60%) (Table 1).

Disease occurrence differed greatly among breeds, which is why breed is an important factor to consider when predicting the risk for pyometra in dogs on an individual basis. The breeds with highest risk of developing pyometra (proportion (%) by 10 years of age) included Bernese Mountain Dog (66%), Great Dane (62%), Leonberger (61%), Rottweiler (58%), Irish Wolfhound (58%), Staffordshire Bull Terrier (54%), Keeshond (52%), Bull Terrier (52%), Bouvier des Flandres (50%), and Newfoundland (50%) (Table 1).

Meanwhile, Finnish Spitz (3%), Norrbotten Spitz (4%), Coton de Tulear (5%), Maltese (8%), Gordon Setter (8%), Laika (8%), Saluki (10%), Tibetan

Terrier (10%), Lancashire Bull Terrier (10%) and Norwich Terrier (11%) were the breeds that had the lowest risk of developing pyometra (Table 1).

Indications for neutering/spaying healthy female dogs include pet overpopulation, owner convenience reasons, behaviour-related issues, contraception and disease prevention (Reichler, 2009; Rupprecht *et al.*, 2006; Blackshaw & Day, 1994). Possible health benefits of neutering female dogs are prevention of the common uterine diseases, such as cystic endometrial hyperplasia and pyometra, especially in high-risk breeds. Additionally, oestrus-cycle associated disorders such as severe overt pseudo-pregnancy and ovarian cysts are prevented by OHE (Akihara *et al.*, 2007; Patnaik & Greenlee, 1987; Bishop, 1972; Krook *et al.*, 1960). The risk of other diseases induced by reproductive hormone production such as neoplasia of the mammary glands (Schneider *et al.*, 1969) and genital tract, progestagen-induced diabetes type 2 and growth hormone excess is also reduced in neutered animals (Fall *et al.*, 2007; Eigenmann *et al.*, 1983). The age at which spaying is performed may influence the protective effect as shown by dogs neutered at an early age (before 1st oestrus) having a lower risk of developing malignant MTs (0.5%) which increased to 8% when neutering was performed at 2nd oestrus and 26% at any oestrus thereafter (Schneider *et al.*, 1969). Our results showed that the proportion of dogs developing pyometra and MTs increased by age (Table 1).

Spaying female dogs has been associated with some negative side-effects such as urinary incontinence, vaginitis, overweight, altered consistency of the coat and behavioural changes (Belsito *et al.*, 2009; Slauterbeck *et al.*, 2004; Cooley *et al.*, 2002; Hart, 2001; Sorenmo *et al.*, 2000). These side-effects can be serious and may lead to euthanasia if non-responsive to treatment (Arnold *et al.*, 1996). Urinary incontinence may be incurable and neutering was shown to lead to acquired urinary incontinence in nearly 10% of the studied bitches in one report (Stocklin-Gautschi *et al.*, 2001). The occurrence of urinary incontinence after neutering/spaying also varies greatly with breed, being more frequent in large breed dogs, *i.e.* individuals with a weight of over 20 kg (Stocklin-Gautschi *et al.*, 2001; Arnold *et al.*, 1989). Additionally, in one study the Boxer breed was shown to have high risk (65%) of developing urinary incontinence after spaying (Arnold *et al.*, 1989). Some large or giant breed dogs that have high risk of developing pyometra thus also have increased risk for acquiring urinary incontinence after spaying according to the results of other studies, as shown in Table 2. However, the occurrence of the unwanted side effects may also differ depending on the age at which age the spaying is performed (Stocklin-Gautschi *et al.*, 2001).

Neutered dogs are more likely to become overly overweight (Lund *et al.*, 2006), and obesity may in turn increase the risk for diseases such as diabetes

mellitus (DM) (German, 2006; Lund *et al.*, 2006). In contrast, one other study indicated that spaying was not associated with an increased risk of developing DM (Mattin *et al.*, 2014). Several factors influence the risk of DM including breed (Fall *et al.*, 2007), administered drugs or hormones (e.g. progesterone) (Krook *et al.*, 1960; Wilkinson, 1960). Neutering prevents the development of pyometra but the interrelationship between obesity, DM and the disease is complex and not yet clarified.

Neutered dogs have a higher risk of developing neoplastic conditions such as osteosarcoma and haemangiosarcoma (Smith, 2014). The risk of developing osteosarcoma was reported to be increased nearly two times in neutered dogs (Ru *et al.*, 1998). It has also been indicated that spayed dogs live longer than intact female dogs (Hoffman *et al.*, 2013; Michell, 1999). Because malignant diseases are more common in old dogs, the fact that neutered dogs live longer than intact female dogs might explain why some malignant diseases are more common in neutered dogs (Hoffman *et al.*, 2013). However, in another study, neutering at younger age (< 4 years of age) led to a shorter life span than when neutering was performed after four years of age (Waters *et al.*, 2009). Oestrogens enhance the immune response (Cutolo *et al.*, 2004) and have anti-oxidative stress properties (Borras *et al.*, 2003) which has been suggested to be one reason why women live longer than men (Austad, 2006). However, many factors might influence life expectancy and the role of oestrogen in dogs and its relationship with longevity is not yet clarified.

In conclusion, whether a bitch should be spayed or not is disputable, and there are many factors to consider. Health benefits and unwanted side-effects of spaying in the short- and long-term are known to differ by breed and may also depend on the age at which the procedure is performed. These factors and possible effects for each individual dog should be discussed with the owner to make the optimal choice for each bitch.

Table 1. *Proportion of bitches that had developed either mammary tumour (MT) or pyometra, or both diseases separately as illustrated in different breeds at < 10 years of age, < 8 years of age and < 6 years of age in insurance data from 1995-2006*

Variable	Proportion (%) at different age		
	< 10 years	< 8 years	< 6 years
Either MT or pyometra or both diseases			
Breed at high risk at < 10 years of age			
Leonberger	73	46	16
Irish Wolfhound	69	46	17
Bernese Mountain Dog	69	50	28
Great Dane	68	54	33
Staffordshire Bull Terrier	66	43	30
Rottweiler	65	43	20
Bull Terrier	62	39	24
Doberman Pinscher	62	37	21
Bouvier des Flandres	60	34	15
Airedale Terrier	60	26	6
Breed at low risk at < 10 years of age			
Finnish Spitz	9	3	1
Laika	10	9	4
Norwich Terrier	11	8	5
Lancashire Bull Terrier	12	7	5
Norrbotten Spitz	14	5	1
Finnish Lapphund	15	10	3
Basenji	16	13	10
Greyhound	16	10	5
Tibetan Spaniel	18	9	4
Siberian Husky	18	12	5

Table 1 cont'd.

Pyometra			
Breed at high risk at < 10 years of age			
Bernese Mountain Dog	66	50	28
Great Dane	62	51	31
Leonberger	61	41	15
Rottweiler	58	42	20
Irish Wolfhound	58	34	17
Staffordshire Bull Terrier	54	40	27
Keeshond	52	29	14
Bull Terrier	52	35	20
Bouvier des Flandres	50	33	15
Newfoundland	50	37	16
Breed at low risk at < 10 years of age			
Finnish Spitz	3	2	1
Norrbotten Spitz	4	2	1
Coton de Tulear	5	5	5
Maltese	8	2	0
Gordon Setter	8	5	5
Laika	8	7	4
Saluki	10	8	4
Tibetan Terrier	10	6	3
Lancashire Bull Terrier	10	7	5
Norwich Terrier	11	8	5

Table 1 cont'd.

MT			
Breed at high risk at < 10 years of age			
Leonberger	46	27	8
Doberman Pinscher	42	20	7
Irish Wolfhound	41	33	4
Welsh Terrier	37	12	0
English Springer Spaniel	36	17	4
American Cocker Spaniel	35	17	4
Boxer	35	16	6
Bedlington Terrier	33	11	4
Old English Sheepdog	29	13	2
Softcoated Wheaten Terrier	28	14	5
Breed at low risk at < 10 years of age			
Norwich Terrier	1	0	0
Collie	2	1	0
Lancashire Bull Terrier	2	0	0
Laika	2	2	0
Pug	3	3	1
Finnish Lapphund	3	1	0
Buhund	4	2	1
Pomeranian	4	2	1
Siberian Husky	5	3	0
Basenji	5	2	2

Table 2. *The top 10 breeds with highest risk of developing pyometra up to 10 years of age illustrated together with dogs breeds at increased risk of developing urinary incontinence after spaying according to other studies.*

Pyometra	Acquired urinary incontinence[¶]
Bernese Mountain Dog	Weimaraner[¶]
Great Dane	Doberman Pincher[¶]
Leonberger	Old English Sheepdog[¶]
Rottweiler	Rottweiler[¶]
Irish Wolfhound	Boxer*
Staffordshire Bull Terrier	Irish Setter[¶]
Keeshond	
Bull Terrier	
Bouvier des Flanders	
Newfoundland	

[¶]Holt and Thrusfield, 1993; [*]Arnold *et al*., 1989

5 Conclusions

- Several routinely explored clinical and laboratory findings such as leucopaenia, moderate to severe depression of the general condition, pale mucous membranes, fever or hypothermia were useful as markers to predict peritonitis and/or outcome as measured by prolonged postoperative hospitalisation after surgical treatment. Leucopaenia was the most important predictive variable, associated with both increased risk for peritonitis and prolonged postoperative hospitalisation.

- Concentrations of IGF-I and iron were decreased in pyometra, whereas concentrations of CRP and SAA were increased. Although unspecific, these variables might be helpful in the diagnosis of pyometra if used together with other clinical parameters.

- Concentrations of SAA were higher in dogs with pyometra that also were diagnosed with sepsis. This indicates that SAA might be clinically useful as a biomarker to aid in the differentiation of septic versus non-septic dogs.

- Concentrations of CRP and albumin did not differ between septic and non-septic dogs and were therefore not valuable as markers for sepsis.

- Catestatin concentrations were decreased in dogs with pyometra, whereas concentrations of VS were not significantly affected by the disease. These findings indicate a possible neuroendocrine response in pyometra. To evaluate the potential of CgA and its breakdown products as biomarkers, further studies are needed.

- None of the inflammatory variables investigated (CRP, SAA, albumin, iron, IGF-I) or CgA breakdown products (Cst and VS) were useful in the prediction of outcome as measured by postoperative hospitalisation.

- The occurrence of pyometra and MTs was investigated in female dogs of 110 different breeds, and was shown to vary greatly between different breeds. In 20 dog breeds, over 50% of the bitches had developed either pyometra or MTs or both diseases before 10 years of age. These findings show that breed is an important factor for predicting the risk of these diseases in individual dogs.

- Spaying is associated with possible health benefits as well as unwanted side-effects, and these differ by breed. The breed-specific data of pyometra and MTs presented in this thesis may be valuable when deciding whether or not to perform elective spaying in individual dogs of different breeds.

6 Future perspectives

Biomarkers are gaining increasing interest in both human and veterinary medicine *i.e.* for diagnostic purposes and to predict outcome by hospitalisation or survival. Early recognition of disease and appropriate treatment is crucial, especially for severely ill patients. Clinically useful biomarkers that can be helpful for diagnosis and prognostication will be most valuable, and this area needs to be studied further.

The mechanisms involved in the response to sepsis and the progression of severe bacterial infection is complicated. Because pyometra is caused by a bacterial infection, is treated by surgery and often leads to sepsis, the disease may serve as a model for studying surgical sepsis. The results of such studies are most likely applicable for sepsis induced also by other diseases or surgical procedures. Because dogs have similar inflammatory responses as humans, studying dogs that are surgically treated will provide a spontaneous disease model for surgical sepsis that is more similar to the real-life situation at veterinary and human hospitals than experimental research studies. Further studies on predictive markers for outcome in septic patients suffering from a variety of diseases will be most valuable clinically. Studies of dogs with pyometra could be helpful to identify early diagnostic and prognostic markers, and also for evaluating novel therapies or therapeutic strategies for sepsis.

So far there are no single or specific biomarkers available for diagnosing pyometra or for predicting the severity of the disease. Further studies in this area, and of panels of several biomarkers combined for the same purposes, are therefore needed. Other than establishing predictive or diagnostic markers, there is also a demand for increased knowledge about possible novel ways for preventing the development of pyometra.

Dogs with sepsis need to be recognised as early as possible, especially when requiring surgery which may be associated with high risk of life-threatening complications or death. There are no specific markers that can be

used for early diagnosis or prediction of sepsis in dogs. In human medicine, several biomarkers have been investigated, but many interesting variables still remain to be explored in dogs. Moreover, genetic factors are likely to predispose and/or to protect development of pyometra, as shown by the great breed differences in occurrence. It would be interesting to investigate possible genetic factors as well as up- and downregulated genes and products of the upregulated genes in the circulation, to identify novel diagnostic or prognostic markers and new targets for treatment of the disease.

References

Adamovich-Rippe, K.N., Mayhew, P.D., Runge, J.J., Culp, W.T.N., Steffey, M.A., Mayhew, K.N. & Hunt, G.B. (2013). Evaluation of Laparoscopic-Assisted Ovariohysterectomy for Treatment of Canine Pyometra. *Veterinary Surgery,* 42(5), pp. 572-578.

Afessa, B., Greaves, W.L. & Frederick, W.R. (1995). Pneumococcal bacteremia in adults- a 14-year experience in an inner-city university hospital. *Clinical Infectious Diseases,* 21(2), pp. 345-351.

Afessa, B., Green, B., Delke, I. & Koch, K. (2001). Systemic inflammatory response syndrome, organ failure, and outcome in critically ill obstetric patients treated in an ICU. *Chest,* 120(4), pp. 1271-1277.

Akihara, Y., Shimoyama, Y., Kawasako, K., Komine, M., Hirayama, K., Kagawa, Y., Omachi, T., Matsuda, K., Okamoto, M., Kadosawa, T. & Taniyama, H. (2007). Immunohistochemical evaluation of canine ovarian cysts. *The Journal of Veterinary Medical Science / the Japanese Society of Veterinary Science,* 69(10), pp. 1033-7.

Akiyoshi, H., Aoki, M., Shimada, T., Noda, K., Kumagai, D., Saleh, N., Sugii, S. & Ohashi, F. (2005). Measurement of plasma chromogranin A concentrations for assessment of stress responses in dogs with insulin-induced hypoglycemia. *American Journal of Veterinary Research,* 66(10), pp. 1830-1835.

Alamgir, S., Volkova, N.B. & Peterson, M.W. (2006). Prognostic value of low blood glucose at the presentation of *E. coli* bacteremia. *American Journal of Medicine,* 119(11), pp. 952-957.

Alanis, A.J. (2005). Resistance to antibiotics: are we in the post-antibiotic era? *Archives of Medical Research,* 36(6), pp. 697-705.

Angelone, T., Mazza, R. & Cerra, M.C. (2012). Chromogranin-A: a Multifaceted Cardiovascular Role in Health and Disease. *Current Medicinal Chemistry,* 19(24), pp. 4042-4050.

Arnold, S., Arnold, P., Hubler, M., Casal, M. & Rusch, P. (1989). Urinary-incontinence in spayed bitches-prevalence and breed disposition. *Schweizer Archiv Fur Tierheilkunde,* 131(5), pp. 259-263.

Arnold, S., Hubler, M., Casal, M., Fairburn, A., Baumann, D., Flueckiger, M. & Ruesch, P. (1988). Use of low-dose prostaglandin for the treatment of canine pyometra. *Journal of Small Animal Practice,* 29(5), pp. 303-308.

Arnold, S., Hubler, M., LottStolz, G. & Rusch, P. (1996). Treatment of urinary incontinence in bitches by endoscopic injection of glutaraldehyde cross-linked collagen. *Journal of Small Animal Practice,* 37(4), pp. 163-168.

Asheim, A. (1964). Renal function in dogs with pyometra- uterine infection and pathogenesis of renal dysfucntion. *Acta Pathologica Et Microbiologica Scandinavica,* 60(1), pp. 99-107.

Atkinson, A.J., Colburn, W.A., DeGruttola, V.G., DeMets, D.L., Downing, G.J., Hoth, D.F., Oates, J.A., Peck, C.C., Schooley, R.T., Spilker, B.A., Woodcock, J., Zeger, S.L. & Biomarkers Definitions Working, G. (2001). Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clinical Pharmacology & Therapeutics,* 69(3), pp. 89-95.

Austad, S.N. (2006). Why women live longer than men: sex differences in longevity. *Gender medicine,* 3(2), pp. 79-92.

Banks, P. & Helle, K. (1965). The release of protein from the stimulated adrenal medulla. *Biochemical Journal,* 97(3), pp. 40c-41c.

Bartolomucci, A., Possenti, R., Mahata, S.K., Fischer-Colbrie, R., Loh, Y.P. & Salton, S.R.J. (2011). The Extended Granin Family: structure, function, and biomedical implications. *Endocrine Reviews,* 32(6), pp. 755-797.

Bartoskova, A., Vitasek, R., Leva, L. & Faldyna, M. (2007). Hysterectomy leads to fast improvement of haematological and immunological parameters in bitches with pyometra. *Journal of Small Animal Practice,* 48(10), pp. 564-568.

Belsito, K.R., Vester, B.M., Keel, T., Graves, T.K. & Swanson, K.S. (2009). Impact of ovariohysterectomy and food intake on body composition, physical activity, and adipose gene expression in cats. *Journal of Animal Science,* 87(2), pp. 594-602.

Bigliardi, E., Parmigiani, E., Cavirani, S., Luppi, A., Bonati, L. & Corradi, A. (2004). Ultrasonography and cystic hyperplasia-pyometra complex in the bitch. *Reproduction in Domestic Animals,* 39(3), pp. 136-140.

Bigoszewski, M., Ryuchlik, A. & Depta, A. (2001). Acute phase proteins in domestic animals. *Medycyna Weterynaryjna,* 57(3), pp. 151-155.

Bishop, M.W. (1972). Genetically determined abnormalities of the reproductive system. *Journal of Reproduction and Fertility,* 15, pp. 51-78.

Biswas, N., Curello, E., O'Connor, D.T. & Mahata, S.K. (2010). Chromogranin/secretogranin proteins in murine heart: myocardial production of chromogranin A fragment catestatin (Chga(364-384)). *Cell and Tissue Research,* 342(3), pp. 353-361.

Blackshaw, J.K. & Day, C. (1994). Attitudes of dog owners to neutering pets: demographic data and effects of owner attitudes. *Australian Veterinary Journal,* 71(4), pp. 113-6.

Blanco, J., Muriel-Bombin, A., Sagredo, V., Taboada, F., Gandia, F., Tamayo, L., Collado, J., Garcia-Labattut, A., Carriedo, D., Valledor, M., De Frutos, M., Lopez, M.-J., Caballero, A., Guerra, J., Alvarez, B., Mayo, A., Villar, J., Grp, E. & Anal Cuidados, I. (2008). Incidence, organ dysfunction and

mortality in severe sepsis: a Spanish multicentre study. *Critical Care,* 12(6), p. 14.

Bloos, F., Thomas-Ruddel, D., Rueddel, H., Engel, C., Schwarzkopf, D., Marshall, J.C., Harbarth, S., Simon, P., Riessen, R., Keh, D., Dey, K., Weiss, M., Toussaint, S., Schaedler, D., Weyland, A., Ragaller, M., Schwarzkopf, K., Eiche, J., Kuhnle, G., Hoyer, H., Hartog, C., Kaisers, U., Reinhart, K. & Grp, M.S. (2014). Impact of compliance with infection management guidelines on outcome in patients with severe sepsis: a prospective observational multi-center study. *Critical Care,* 18(2), p. 10.

Bone, R.C. (1992). Toward an epidemiology and natural-history of SIRS (systemic inflammatory response syndrome). *Journal of the American Medical Association,* 268(24), pp. 3452-3455.

Bonnett, B.N. & Egenvall, A. (2010). Age patterns of disease and death in insured Swedish dogs, cats and horses. *Journal of Comparative Pathology,* 142 Suppl 1, pp. S33-8.

Borras, C., Sastre, J., Garcia-Sala, D., Lloret, A., Pallardo, F.V. & Vina, J. (2003). Mitochondria from females exhibit higher antioxidant gene expression and lower oxidative damage than males. *Free Radical Biology and Medicine,* 34(5), pp. 546-552.

Borresen, B. (1979). Pyometra in the dog- a pathophysiological investigation. II. Anamnestic, clinical and reproductive aspects. *Nordisk Veterinaer Medicin,* 31(6), pp. 251-7.

Borresen, B. (1980). Pyometra in the dog- a pathophysiological investigation. IV. Functional derangement of extra-genital organs. *Nordisk Veterinaer Medicin,* 32(6), pp. 255-268.

Borresen, B. & Skrede, S. (1980). Pyometra in the dog- a pathophysiological investigation V. The presence of intrahepatic cholestasis and an "acute phase reaction.". *Nordisk Veterinaer Medicin,* 32(9), pp. 378-86.

Brunkhorst, F.M., Wegscheider, K., Forycki, Z.F. & Brunkhorst, R. (2000). Procalcitonin for early diagnosis and differentiation of SIRS, sepsis, severe sepsis, and septic shock. *Intensive Care Medicine,* 26, pp. S148-S152.

Ceconi, C., Ferrari, R., Bachetti, T., Opasich, C., Volterrani, M., Colombo, B., Parrinello, G. & Corti, A. (2002). Chromogranin A in heart failure - a novel neurohumoral factor and a predictor for mortality. *European Heart Journal,* 23(12), pp. 967-974.

Ceron, J.J., Eckersall, P.D. & Martinez-Subiela, S. (2005). Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Veterinary Clinical Pathology,* 34(2), pp. 85-99.

Chan, D.L., Rozanski, E.A. & Freeman, L.M. (2009). Relationship among plasma amino acids, C-reactive protein, illness severity, and outcome in critically ill dogs. *Journal of Veterinary Internal Medicine,* 23(3), pp. 559-563.

Chestnut, R.E. & Quarmby, V. (2002). Evaluation of total IGF-I assay methods using samples from Type I and Type II diabetic patients. *Journal of Immunological Methods,* 259(1-2), pp. 11-24.

Cho, W.C.S., Yip, T.T., Cheng, W.W. & Au, J.S.K. (2010). Serum amyloid A is elevated in the serum of lung cancer patients with poor prognosis. *British Journal of Cancer,* 102(12), pp. 1731-1735.

Christensen, B.M., Langhorn, R., Goddard, A., Andreasen, B.E., Moldal, E., Tvarijonaviciute, A., Kirpensteijn, J., Jakobsen, S., Persson, F. & Kjelgaard-Hansen, M. (2013). Canine serum amyloid A (SAA) measured by automated latex agglutination turbidimetry is useful for routine sensitive and specific detection of systemic inflammation in a general clinical setting. *Journal of Veterinary Medical Science,* 75(4), pp. 459-66.

Christensen, B.M., Langhorn, R., Goddard, A., Andreasen, B.E., tvarijonaviciute, A., kirpensteijn, J., Jakobsen, S., Persson, F. & kjelgaard-Hansen, M. (2014). Comparison of serum amyloid A and C-reactive protein as diagnostic markers of systemic inflammation in dogs. *Canadian Veterinary Journal,* 55(2), pp. 161-8.

Claeys, R., Vinken, S., Spapen, H., Elst, K.V., Decochez, K., Huyghens, L. & Gorus, F.K. (2002). Plasma procalcitonin and C-reactive protein in acute septic shock: clinical and biological correlates. *Critical Care Medicine,* 30(4), pp. 757-762.

Conti-Patara, A., Caldeira, J.d.A., de Mattos-Junior, E., de Carvalho, H.d.S., Reinoldes, A., Pedron, B.G., Patara, M., Francisco Talib, M.S., Faustino, M., de Oliveira, C.M. & Gaido Cortopassi, S.R. (2012). Changes in tissue perfusion parameters in dogs with severe sepsis/septic shock in response to goal-directed hemodynamic optimization at admission to ICU and the relation to outcome. *Journal of Veterinary Emergency and Critical Care,* 22(4), pp. 409-418.

Cooley, D.M., Beranek, B.C., Schlittler, D.L., Glickman, N.W., Glickman, L.T. & Waters, D.J. (2002). Endogenous gonadal hormone exposure and bone sarcoma risk. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology,* 11(11), pp. 1434-40.

Corrada, Y., Arias, D., Rodriguez, R., Tortora, M. & Gobello, C. (2006). Combination dopamine agonist and prostaglandin agonist treatment of cystic endometrial hyperplasia-pyometra complex in the bitch. *Theriogenology,* 66(6-7), pp. 1557-1559.

Cutolo, M., Sulli, A., Capellino, S., Villaggio, B., Montagna, P., Seriolo, B. & Straub, R.H. (2004). Sex hormones influence on the immune system: basic and clinical aspects in autoimmunity. *Lupus,* 13(9), pp. 635-638.

Dabrowski, R., Kostro, K. & Szczubial, M. (2013). Concentrations of C-reactive protein, serum amyloid A, and haptoglobin in uterine arterial and peripheral blood in bitches with pyometra. *Theriogenology,* 80(5), pp. 494-497.

Dabrowski, R., Szczubial, M., Kostro, K., Wawron, W., Ceron, J.J. & Tvarijonaviciute, A. (2015). Serum insulin-like growth factor-1 and C-reactive protein concentrations before and after ovariohysterectomy in bitches with pyometra. *Theriogenology,* 83(4), pp. 474-477.

Dabrowski, R., Wawron, W. & Kostro, K. (2007). Changes in CRP, SAA and haptoglobin produced in response to ovariohysterectomy in healthy bitches and those with pyometra. *Theriogenology,* 67(2), pp. 321-327.

Dandona, P., Nix, D., Wilson, M.F., Aljada, A., Love, J., Assicot, M. & Bohuon, C. (1994). Procalcitonin increase after endotoxin injection in normal subjects. *Journal of Clinical Endocrinology & Metabolism,* 79(6), pp. 1605-1608.

De Benedetti, F., Alonzi, T., Moretta, A., Lazzaro, D., Costa, P., Poli, V., Martini, A., Ciliberto, G. & Fattori, E. (1997). Interleukin 6 causes growth impairment in transgenic mice through a decrease in insulin-like growth factor-I- a model for stunted growth in children with chronic inflammation. *Journal of Clinical Investigation,* 99(4), pp. 643-650.

De Bosschere, H., Ducatelle, R. & Tshamala, M. (2002). Is mechanically induced cystic endometrial hyperplasia (CEH) a suitable model for study of spontaneously occurring CEH in the uterus of the bitch? *Reproduction in Domestic Animals,* 37(3), pp. 152-157.

De Bosschere, H., Ducatelle, R., Vermeirsch, H., Van den Broeck, W. & Coryn, M. (2001). Cystic endometrial hyperplasia-pyometra complex in the bitch: should the two entities be disconnected. *Theriogenology,* 55(7), pp. 1509-1519.

De Cramer, K.G.M. (2010). Surgical uterine drainage and lavage as treatment for canine pyometra. *Journal of the South African Veterinary Association-Tydskrif Van Die Suid-Afrikaanse Veterinere Vereniging,* 81(3), pp. 172-177.

De Schepper, J., Vanderstock, J. & Capiau, E. (1987a). Anemia and Leukocytosis in 112 Dogs with Pyometra. *Journal of Small Animal Practice,* 28(2), pp. 137-145.

De Schepper, J., Vanderstock, J., Capiau, E. & Decock, I. (1987b). Renal Injury in Dogs with Pyometra. *Tijdschrift Voor Diergeneeskunde,* 112, pp. 124-126.

Deysine, M. & Stein, S. (1980). Albumin shifts across the extracellular-space secondary to experimental infections. *Surgery Gynecology & Obstetrics,* 151(5), pp. 617-620.

Dickinson, J.D. & Kollef, M.H. (2011). Early and adequate antibiotic therapy in the treatment of severe sepsis and septic shock. *Current Infectious Disease Reports,* 13(5), pp. 399-405.

Dow, C. (1957). The cystic hyperplasia-pyometra complex in the bitch. *Veterinary Record,* 69, pp. 1409-1415.

Dow, C. (1959). Experimental reproduction of the cystic hyperplasia-pyometra complex in the bitch. *Journal of Pathology and Bacteriology,* 78(1), pp. 267-78.

Eckersall, P.D. & Bell, R. (2010). Acute phase proteins: biomarkers of infection and inflammation in veterinary medicine. *Veterinary Journal,* 185(1), pp. 23-27.

Egenvall, A., Bonnett, B.N., Ohagen, P., Olson, P., Hedhammar, A. & von Euler, H. (2005). Incidence of and survival after mammary tumors in a

population of over 80,000 insured female dogs in Sweden from 1995 to 2002. *Preventive Veterinary Medicine,* 69(1-2), pp. 109-127.

Egenvall, A., Bonnett, B.N., Olson, P. & Hedhammar, A. (2000a). Gender, age and breed pattern of diagnoses for veterinary care in insured dogs in Sweden during 1996. *Veterinary Record,* 146(19), pp. 551-7.

Egenvall, A., Bonnett, B.N., Shoukri, M., Olson, P., Hedhammar, A. & Dohoo, I. (2000b). Age pattern of mortality in eight breeds of insured dogs in Sweden. *Preventive Veterinary Medicine,* 46(1), pp. 1-14.

Egenvall, A., Hagman, R., Bonnett, B.N., Hedhammar, A., Olson, P. & Lagerstedt, A.S. (2001). Breed risk of pyometra in insured dogs in Sweden. *Journal of Veterinary Internal Medicine,* 15(6), pp. 530-8.

Egenvall, A., Hedhammar, A., Bonnett, B.N. & Olson, P. (1999). Survey of the Swedish dog population: age, gender, breed, location and enrolment in animal insurance. *Acta Veterinaria Scandinavica,* 40(3), pp. 231-240.

Eigenmann, J.E., Amador, A. & Patterson, D.F. (1988). Insulin-like growth factor-I levels in proportionate dogs, chondrodystrophic dogs and in giant dogs. *Acta Endocrinologica,* 118(1), pp. 105-108.

Eigenmann, J.E., Eigenmann, R.Y., Rijnberk, A., Vandergaag, I., Zapf, J. & Froesch, E.R. (1983). Progesterone-controlled growth-hormone overproduction and naturally-occurring canine diabetes and acromegaly. *Acta Endocrinologica,* 104(2), pp. 167-176.

Fall, T., Hamlin, H.H., Hedhammar, A., Kampe, O. & Egenvall, A. (2007). Diabetes mellitus in a population of 180,000 insured dogs: incidence, survival, and breed distribution. *Journal of Veterinary Internal Medicine,* 21(6), pp. 1209-1216.

Ferrari, L., Seregni, E., Lucignani, G., Bajetta, E., Martinetti, A., Aliberti, G., Pallotti, F., Procopio, G., Della Torre, S., Luksch, R. & Bombardieri, E. (2004). Accuracy and clinical correlates of two different methods for chromogranin A assay in neuroendocrine tumors. *International Journal of Biological Markers,* 19(4), pp. 295-304.

Fidler, I.J., Brodey, R.S., Howson, A.E. & Cohen, D. (1966). Relationship of estrous irregularity pseudopregnancy and pregnancy to canine pyometra. *Journal of the American Veterinary Medical Association,* 149(8), pp. 1043-6.

Fieni, F. (2006). Clinical evaluation of the use of aglepristone, with or without cloprostenol, to treat cystic endometrial hyperplasia-pyometra complex in bitches. *Theriogenology,* 66(6-7), pp. 1550-1556.

Foushee, J.A., Hope, N.H. & Grace, E.E. (2012). Applying biomarkers to clinical practice: a guide for utilizing procalcitonin assays. *Journal of Antimicrobial Chemotherapy,* 67(11), pp. 2560-2569.

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-Physiology Pathology Clinical Medicine,* 44(7), pp. 417-426.

Fransson, B.A., Lagerstedt, A.S., Bergstrom, A., Hagman, R., Park, J.S., Chew, B.P., Evans, M.A. & Ragle, C.A. (2007). C-reactive protein, tumor

necrosis factor alpha, and interleukin-6 in dogs with pyometra and SIRS. *Journal of Veterinary Emergency and Critical Care,* 17(4), pp. 373-381.

Funkquist, B., Lagerstedt, A.S., Linde, C. & Obel, N. (1983). Intrauterine drainage for treatment of pyometra in the bitch. *Zentralblatt Fur Veterinarmedizin Reihe a-Journal of Veterinary Medicine Series a-Animal Physiology Pathology and Clinical Veterinary Medicine,* 30(1), pp. 72-80.

Gabor, G., Siver, L. & Szenci, O. (1999). Intravaginal prostaglandin F2 alpha for the treatment of metritis and pyometra in the bitch. *Acta Veterinaria Hungarica,* 47(1), pp. 103-8.

Gbeenberg, G.R., Ashenbrucker, H., Lauritsen, M., Worth, W., Humphreys, S.R. & Wintrobe, M.M. (1947). The anemia of infection. V. Fate of injected radioactive iron in the presence of inflammation. *Journal of Clinical Investigation,* 26, pp. 121-125.

Gebhardt, C., Hirschberger, J., Rau, S., Arndt, G., Krainer, K., Schweigert, F.J., Brunnberg, L., Kaspers, B. & Kohn, B. (2009). Use of C-reactive protein to predict outcome in dogs with systemic inflammatory response syndrome or sepsis. *Journal of Veterinary Emergency and Critical Care,* 19(5), pp. 450-458.

German, A.J. (2006). The growing problem of obesity in dogs and cats. *Journal of Nutrition,* 136(7), pp. 1940S-1946S.

Gobello, C., Castex, G., Klima, L., Rodriguez, R. & Corrada, Y. (2003). A study of two protocols combining aglepristone and cloprostenol to treat open cervix pyometra in the bitch. *Theriogenology,* 60(5), pp. 901-908.

Greer, K.A., Hughes, L.M. & Masternak, M.M. (2011). Connecting serum IGF-1, body size, and age in the domestic dog. *Age,* 33(3), pp. 475-483.

Greiner, M., Wolf, G. & Hartmann, K. (2008). A retrospective study of the clinical presentation of 140 dogs and 39 cats with bacteraemia. *Journal of Small Animal Practice,* 49(8), pp. 378-383.

Hagman, R. (2012). Clinical and Molecular Characteristics of Pyometra in Female Dogs. *Reproduction in Domestic Animals,* 47, pp. 323-325.

Hagman, R. & Greko, C. (2005). Antimicrobial resistance in Escherichia coli isolated from bitches with pyometra and from urine samples from other dogs. *Veterinary Record,* 157(7), pp. 193-6.

Hagman, R., Karlstam, E., Persson, S. & Kindahl, H. (2009a). Plasma PGF(2 alpha) metabolite levels in cats with uterine disease. *Theriogenology,* 72(9), pp. 1180-1187.

Hagman, R., Kindahl, H., Fransson, B.A., Bergstrom, A., Holst, B.S. & Lagerstedt, A.S. (2006). Differentiation between pyometra and cystic endometrial hyperplasia/mucometra in bitches by prostaglandin F-2 alpha metabolite analysis. *Theriogenology,* 66(2), pp. 198-206.

Hagman, R., Lagerstedt, A.-S., Hedhammar, A. & Egenvall, A. (2011). A breed-matched case-control study of potential risk-factors for canine pyometra. *Theriogenology,* 75(7), pp. 1251-1257.

Hagman, R., Lagerstedt, A.S., Fransson, B.A., Bergstrom, A. & Haggstrom, J. (2007). Cardiac troponin I levels in canine pyometra. *Acta Veterinaria Scandinavica,* 49, p. 6.

Hagman, R., Reezigt, B.J., Ledin, H.B. & Karlstam, E. (2009b). Blood lactate levels in 31 female dogs with pyometra. *Acta Veterinaria Scandinavica,* 51(2), p. 9.

Hagman, R., Ronnberg, E. & Pejler, G. (2009c). Canine Uterine Bacterial Infection Induces Upregulation of Proteolysis-Related Genes and Downregulation of Homeobox and Zinc Finger Factors. *Plos One,* 4(11), p. 14.

Hardy, R.M. & Osborne, C.A. (1974). Canine pyometra: pathophysiology, diagnosis and treatment of uterine and extra-uterine lesions. *Journal of the American Animal Hospital Association,* 10(3), pp. 245-268.

Hart, B.L. (2001). Effect of gonadectomy on subsequent development of age-related cognitive impairment in dogs. *Journal of the American Veterinary Medical Association,* 219(1), pp. 51-6.

Hauptman, J.G., Walshaw, R. & Olivier, N.B. (1997). Evaluation of the sensitivity and specificity of diagnostic criteria for sepsis in dogs. *Veterinary Surgery,* 26(5), pp. 393-397.

Heemskerk, V.H., Daemen, M. & Buurman, W.A. (1999). Insulin-like growth factor-1 (IGF-1) and growth hormone (GH) in immunity and inflammation. *Cytokine & Growth Factor Reviews,* 10(1), pp. 5-14.

Heiene, R., Kristiansen, V., Teige, J. & Jansen, J.H. (2007). Renal histomorphology in dogs with pyometra and control dogs, and long term clinical outcome with respect to signs of kidney disease. *Acta Veterinaria Scandinavica,* 49(1), p. 9.

Helle, K.B. (1966). Some chemical and physical properties of the soluble protein fraction of bovine adrenal chromaffin granules. *Molecular Pharmacology,* 2(4), pp. 298-310.

Helle, K.B., Corti, A., Metz-Boutigue, M.H. & Tota, B. (2007). The endocrine role for chromogranin A: a prohormone for peptides with regulatory properties. *Cellular and Molecular Life Sciences,* 64(22), pp. 2863-2886.

Hoffman, J.M., Creevy, K.E. & Promislow, D.E.L. (2013). Reproductive Capability Is Associated with Lifespan and Cause of Death in Companion Dogs. *Plos One,* 8(4), p. 7.

Hogarth, M.B., Gallimore, J.R., Savage, P., Palmer, A.J., Starr, J.M., Bulpitt, C.J. & Pepys, M.B. (1997). Acute phase proteins, C-reactive protein and serum amyloid A protein, as prognostic markers in the elderly inpatient. *Age and Ageing,* 26(2), pp. 153-158.

Hook, E.W., 3rd, Horton, C.A. & Schaberg, D.R. (1983). Failure of intensive care unit support to influence mortality from pneumococcal bacteremia. *The Journal of the American Medical Association,* 249(8), pp. 1055-7.

Hounsom, L., Grayson, K. & Melzer, M. (2011). Mortality and associated risk factors in consecutive patients admitted to a UK NHS trust with community acquired bacteraemia. *Postgraduate Medical Journal,* 87(1033), pp. 757-762.

Hsiao, R.J., Neumann, H.P.H., Parmer, R.J., Barbosa, J.A. & Oconnor, D.T. (1990). Chromogranin-A in familial pheochromocytoma- diagnostic screening value, prediction of tumour mass, and postresection kinetics indicating 2 compartment distribution. *American Journal of Medicine,* 88(6), pp. 607-613.

Hurlimann, J., Thorbecke, G.J. & Hochwald, G.M. (1966). The liver as the site of C-reactive protein formation. *The Journal of Experimental Medicine,* 123(2), pp. 365-78.

Jurka, P., Max, A., Hawrynska, K. & Snochowski, M. (2010). Age-Related Pregnancy Results and Further Examination of Bitches after Aglepristone Treatment of Pyometra. *Reproduction in Domestic Animals,* 45(3), pp. 525-529.

Juul, A., Bang, P., Hertel, N.T., Main, K., Dalgaard, P., Jorgensen, K., Muller, J., Hall, K. & Skakkebaek, N.E. (1994). Serum insulin-like growth factor-I in 1030 healthy-chidren, adolescents, and adults-relation to age, sex, stage of puberty, testicular size, and body-mass index. *Journal of Clinical Endocrinology & Metabolism,* 78(3), pp. 744-752.

Kamisoglu, K., Haimovich, B., Calvano, S.E., Coyle, S.M., Corbett, S.A., Langley, R.J., Kingsmore, S.F. & Androulakis, I.P. (2015). Human metabolic response to systemic inflammation: assessment of the concordance between experimental endotoxemia and clinical cases of sepsis/SIRS. *Critical Care,* 19(1), pp. 783-783.

Keir, I. & Dickinson, A.E. (2015). The role of antimicrobials in the treatment of sepsis and critical illness-related bacterial infections: examination of the evidence. *Journal of Veterinary Emergency and Critical Care,* 25(1), pp. 55-62.

Kenney, E.M., Rozanski, E.A., Rush, J.E., deLaforcade-Buress, A.M., Berg, J.R., Silverstein, D.C., Montealegre, C.D., Jutkowitz, L.A., Adamantos, S., Ovbey, D.H., Boysen, S.R. & Shaw, S.P. (2010). Association between outcome and organ system dysfunction in dogs with sepsis: 114 cases (2003-2007). *Journal of the American Veterinary Medical Association,* 236(1), pp. 83-87.

Kjelgaard-Hansen, M. & Jacobsen, S. (2011). Assay validation and diagnostic applications of major acute-phase protein testing in companion animals. *Clinics in Laboratory Medicine,* 31(1), pp. 51-70.

Kjelgaard-Hansen, M., Jensen, A.L. & Kristensen, A.T. (2003). Evaluation of a commercially available human C-reactive protein (CRP) turbidometric immunoassay for determination of canine serum CRP concentration. *Veterinary Clinical Pathology,* 32(2), pp. 81-87.

Klainbart, S., Ranen, E., Glikman, G., Kelmer, E., Bdolah-Abram, T. & Aroch, I. (2014). Hindlimb lameness and gait abnormalities in bitches with pyometra. *Veterinary Record,* 175(2), pp. 46-U52.

Kleinbaum, D., Kupper, L. & Morgenstern, H. (1982). Principles and Quantative Methods. In: *Epidemiologic Research*. 1st. ed. Newyork: Van Nostrand reinhold, pp. 108-109.

Klenner, S., Bauer, N. & Moritz, A. (2010). Evaluation of three automated human immunoturbidimetric assays for the detection of C-reactive protein in dogs. *Journal of Veterinary Diagnostic Investigation,* 22(4), pp. 544-552.

Konitzer, K., Endell, E. & Baetgen, G. (1956). Studies on iron metabolism. 1. Course of the decline in iron and transferrin in the serum in relation to the disturbance in blood proteins during infection. *Zeitschrift fur die Gesamte Innere Medizin und ihre Grenzgebiete,* 11, pp. 834-840.

Korse, C.M., Taal, B.G., Vincent, A., van Velthuysen, M.-L.F., Baas, P., Buning-Kager, J.C.G.M., Linders, T.C. & Bonfrer, J.M.G. (2012). Choice of tumour markers in patients with neuroendocrine tumours is dependent on the histological grade. A marker study of Chromogranin A, neuron specific enolase, progastrin-releasing peptide and cytokeratin fragments. *European Journal of Cancer,* 48(5), pp. 662-671.

Kosuge, M., Ebina, T., Ishikawa, T., Hibi, K., Tsukahara, K., Okuda, J., Iwahashi, N., Ozaki, H., Yano, H., Kusama, K., Nakati, T., Umemura, S. & Kimura, K. (2007). Serum amyloid A is a better predictor of clinical outcomes than C-reactive protein in non-ST-segment elevation acute coronary syndromes. *Circulation Journal,* 71(2), pp. 186-190.

Krook, L., Larsson, S. & Rooney, J.R. (1960). The Interrelationship of Diabetes Mellitus, Obesity, and Pyometra in the Dog. *American Journal of Veterinary Research,* 21(80), pp. 120-124.

Kum, C., Voyvoda, H., Sekkin, S., Karademir, U. & Tarimcilar, T. (2013). Effects of carprofen and meloxicam on C-reactive protein, ceruloplasmin, and fibrinogen concentrations in dogs undergoing ovariohysterectomy. *American Journal of Veterinary Research,* 74(10), pp. 1267-1273.

Kumar, A., Roberts, D., Wood, K.E., Light, B., Parrillo, J.E., Sharma, S., Suppes, R., Feinstein, D., Zanotti, S., Taiberg, L., Gurka, D. & Cheang, M. (2006). Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Critical Care Medicine,* 34(6), pp. 1589-1596.

Kuplulu, S., Vural, M.R., Demirel, A., Polat, M. & Akcay, A. (2009). The comparative evaluation of serum biochemical, haematological, bacteriological and clinical findings of dead and recovered bitches with pyometra in the postoperative process. *Acta Veterinaria-Beograd,* 59(2-3), pp. 193-204.

Kuster, H., Weiss, M., Willeitner, A.E., Detlefsen, S., Jeremias, I., Zbojan, J., Geiger, R., Lipowsky, G. & Simbruner, G. (1998). Interleukin-1 receptor antagonist and interleukin-6 for early diagnosis of neonatal sepsis 2 days before clinical manifestation. *Lancet,* 352(9136), pp. 1271-1277.

Lang, C.H., Frost, R.A., Jefferson, L.S., Kimball, S.R. & Vary, T.C. (2000). Endotoxin-induced decrease in muscle protein synthesis is associated with changes in eIF2B, eIF4E, and IGF-I. *American Journal of Physiology-Endocrinology and Metabolism,* 278(6), pp. 1133-1143.

Lanz, O.I., Ellison, G.W., Bellah, J.R., Weichman, G. & VanGilder, J. (2001). Surgical treatment of septic peritonitis without abdominal drainage in 28 dogs. *Journal of the American Animal Hospital Association,* 37(1), pp. 87-92.

Lesbouyries & Berthelon (1936). Pathogenesis and Treatment of Chronic Endometritis in the Female Dog and Cat. *Bulletin de l'Academie Veterinaire de France,* 9, pp. 346-349.

Lessey, B.A., Wahawisan, R. & Gorell, T.A. (1981). Hormonal/regulation of cytoplasmic estrogen and progesterone receptors in th Beagle uterus and oviduct. *Molecular and Cellular Endocrinology,* 21(2), pp. 171-180.

Levey, A.S., Coresh, J., Balk, E., Kausz, A.T., Levin, A., Steffes, M.W., Hogg, R.J., Perrone, R.D., Lau, J. & Eknoyan, G. (2003). National kidney foundation practice guidelines for chronic kidney disease: evaluation, classification, and stratification. *Annals of Internal Medicine,* 139(2), pp. 137-147.

Levy, M.M., Fink, M.P., Marshall, J.C., Abraham, E., Angus, D., Cook, D., Cohen, J., Opal, S.M., Vincent, J.L., Ramsay, G. & Int Sepsis Definitions, C. (2003). 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions Conference. *Critical Care Medicine,* 31(4), pp. 1250-1256.

Liao, W.S.L., Jefferson, L.S. & Taylor, J.M. (1986). Changes in plasma-albumin concentration, synthesis rate, and messenger- RNA level during acute inflammation. *American Journal of Physiology,* 251(6), pp. C928-C934.

Lobo, S.M.A., Lobo, F.R.M., Bota, D.P., Lopes-Ferreira, F., Soliman, H.M., Melot, C. & Vincent, J.L. (2003). C-reactive protein levels correlate with mortality and organ failure in critically ill patients. *Chest,* 123(6), pp. 2043-2049.

Lopez-Calderon, A., Soto, L. & Martin, A. (1999). Chronic inflammation inhibits GH secretion and alters the serum insulin-like growth factor system in rats. *Life Sciences,* 65(20), pp. 2049-2060.

Ludwig, L.L., McLoughlin, M.A., Graves, T.K. & Crisp, M.S. (1997). Surgical treatment of bile peritonitis in 24 dogs and 2 cats: a retrospective study (1987-1994). *Veterinary Surgery,* 26(2), pp. 90-98.

Lund, E.M., Armstrong, P.J., Kirk, C.A. & Klausner, J.S. (2006). Prevalence and risk factors for obesity in adult dogs from private US veterinary practices. *International Journal of Applied Research in Veterinary Medicine,* 4(2), pp. 177-186.

Maddens, B., Heiene, R., Smets, P., Svensson, M., Aresu, L., van der Lugt, J., Daminet, S. & Meyer, E. (2011). Evaluation of kidney injury in dogs with pyometra based on proteinuria, renal histomorphology, and urinary biomarkers. *Journal of Veterinary Internal Medicine,* 25(5), pp. 1075-1083.

Mahata, S.K., Mahata, M., Wen, G., Wong, W.B., Mahapatra, N.R., Hamilton, B.A. & O'Connor, D.T. (2004). The catecholamine release-inhibitory "catestatin" fragment of chromogranin A: naturally occurring human variants with different potencies for multiple chromaffin cell nicotinic cholinergic responses. *Molecular Pharmacology,* 66(5), pp. 1180-1191.

Mahata, S.K., Oconnor, D.T., Mahata, M., Yoo, S.H., Taupenot, L., Wu, H.J., Gill, B.M. & Parmer, R.J. (1997). Novel autocrine feedback control of catecholamine release- a discrete chromogranin A fragment is a noncompetitive nicotinic cholinergic antagonist. *Journal of Clinical Investigation,* 100(6), pp. 1623-1633.

Marchuk, J.B., Finley, R.J., Groves, A.C., Wolfe, L.I., Holliday, R.L. & Duff, J.H. (1977). Catabolic hormones and substrate patterns in septic patients. *Journal of Surgical Research,* 23(3), pp. 177-182.

Martinez-Subiela, S. & Ceron, J.J. (2005). Analytical validation of commercial assays for the determination of haptoglobin, C-reactive protein and serum amyloid A in dogs. *Archivos De Medicina Veterinaria,* 37(1), pp. 61-66.

Mattin, M., O'Neill, D., Church, D., McGreevy, P.D., Thomson, P.C. & Brodbelt, D. (2014). An epidemiological study of diabetes mellitus in dogs attending first opinion practice in the UK. *Veterinary Record,* 174(14), pp. 349-357.

Maxwell, A., Butterwick, R., Yateman, M., Batt, R.M., Cotterill, A. & Camacho-Hubner, C. (1998). Nutritional modulation of canine insulin-like growth factors and their binding proteins. *Journal of Endocrinology,* 158(1), pp. 77-85.

Mazza, R., Imbrogno, S. & Tota, B. (2010). The interplay between chromogranin A-derived peptides and cardiac natriuretic peptides in cardioprotection against catecholamine-evoked stress. *Regulatory Peptides,* 165(1), pp. 86-94.

McGreevy, P.D., Thomson, P.C., Pride, C., Fawcett, A., Grassi, I. & Jones, B. (2005). Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. *Veterinary Record,* 156(22), pp. 695-702.

Michell, A.R. (1999). Longevity of British breeds of dog and its relationships with sex, size, cardiovascular variables and disease. *Veterinary Record,* 145(22), pp. 625-629.

Millerliebl, D., Fayrerhosken, R., Caudle, A. & Downs, M. (1994). Reproductive-tract diseases that cause infertility in the bitch. *Veterinary Medicine,* 89(11), pp. 1047-1054.

Muckart, D.J.J. & Bhagwanjee, S. (1997). American College of Chest Physicians Society of Critical Care Medicine Consensus Conference definitions of the systemic inflammatory response syndrome and allied disorders in relation to critically injured patients. *Critical Care Medicine,* 25(11), pp. 1789-1795.

Mueller, M.G., Ludwig, L.L. & Barton, L.J. (2001). Use of closed-suction drains to treat generalized peritonitis in dogs and cats: 40 cases (1997-1999). *Journal of the American Veterinary Medical Association,* 219(6), pp. 789-794.

Myers, N.C., Andrews, G.A. & ChardBergstrom, C. (1997). Chromogranin A plasma concentration and expression in pancreatic islet cell tumors of dogs and cats. *American Journal of Veterinary Research,* 58(6), pp. 615-620.

Nel, M., Lobetti, R.G., Keller, N. & Thompson, P.N. (2004). Prognostic value of blood lactate, blood glucose, and hematocrit in canine babesiosis. *Journal of Veterinary Internal Medicine,* 18(4), pp. 471-476.

Nemeth, E., Tuttle, M.S., Powelson, J., Vaughn, M.B., Donovan, A., Ward, D.M., Ganz, T. & Kaplan, J. (2004). Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science,* 306(5704), pp. 2090-2093.

Nemeth, E., Valore, E.V., Territo, M., Schiller, G., Lichtenstein, A. & Ganz, T. (2003). Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. *Blood,* 101(7), pp. 2461-2463.

Nicolas, G., Chauvet, C., Viatte, L., Danan, J.L., Bigard, X., Devaux, I., Beaumont, C., Kahn, A. & Vaulont, S. (2002). The gene encoding the iron regulatory

peptide hepcidin is regulated by anemia, hypoxia, and inflammation. *Journal of Clinical Investigation,* 110(7), pp. 1037-1044.

Niskanen, M. & Thrusfield, M.V. (1998). Associations between age, parity, hormonal therapy and breed, and pyometra in Finnish dogs. *Veterinary Record,* 143(18), pp. 493-498.

Oconnor, D.T. & Bernstein, K.N. (1984). Radioimmunoassay of chromogranin-A in plasma as a measure of exocytosis sympathoadrenal activity in normal subjects and patients with pheochromocytoma. *New England Journal of Medicine,* 311(12), pp. 764-770.

Oelzner, J. & Munnich, A. (1997). Diagnosis and treatment of the endometritis-pyometra-complex in the bitch. *Tierarztliche Praxis,* 25(3), pp. 249-253.

Okano, S., Tagawa, M. & Takase, K. (1998). Relationship of the blood endotoxin concentration and prognosis in dogs with pyometra. *Journal of Veterinary Medical Science,* 60(11), pp. 1265-1267.

Olson, P., Kängström, L.E. & Svenska djursjukhusföreningen. (1993). Svenska djursjukhusföreningens diagnosregister för häst, hund och katt (Diagnostic Registry for the Horse, the Dog and the Cat). Taberg: Svenska djursjukhusfören.

Olsson, U. (2002). *Generalized linear models – an applied approach*. Lund: Lund: STudentlitteratur.

Otto, C.M. (2007). Clinical trials in spontaneous disease in dogs: a new paradigm for investigations of sepsis. *Journal of Veterinary Emergency and Critical Care,* 17(4), pp. 359-367.

Owen, W.F., Lew, N.L., Liu, Y., Lowrie, E.G. & Lazarus, J.M. (1993). The urea reduction ratio and serum-albumin concentration as predictors of mortality in patients undergoing hemodialysis. *New England Journal of Medicine,* 329(14), pp. 1001-1006.

Pape, M.S. (2003). *The Statistical Evaluation of Medicine tests for Classification and Prediction*. Oxford: Oxford University Press.

Patnaik, A.K. & Greenlee, P.G. (1987). Canine ovarian neoplasms: a clinicopathologic study of 71 cases, including histology of 12 granulosa cell tumors. *Veterinary Pathology,* 24(6), pp. 509-14.

Pelander, L., Hagman, R. & Haggstrom, J. (2008). Concentrations of cardiac Troponin I before and after ovariohysterectomy in 46 female dogs with pyometra. *Acta Veterinaria Scandinavica,* 50, p. 8.

Pepys, M.B. & Hirschfield, G.M. (2003). C-reactive protein: a critical update. *Journal of Clinical Investigation,* 111(12), pp. 1805-1812.

Philip, A.G.S. & Mills, P.C. (2000). Use of C-reactive protein in minimizing antibiotic exposure: experience with infants initially admitted to a well-baby nursery. *Pediatrics,* 106(1), p. 5.

Pierce, B.L., Ballard-Barbash, R., Bernstein, L., Baumgartner, R.N., Neuhouser, M.L., Wener, M.H., Baumgartner, K.B., Gilliland, F.D., Sorensen, B.E., McTiernan, A. & Ulrich, C.M. (2009). Elevated Biomarkers of Inflammation Are Associated With Reduced Survival Among Breast Cancer Patients. *Journal of Clinical Oncology,* 27(21), pp. 3437-3444.

Plavec, T., Celinsek, B., Dolinar, K., Pecar, J., Nemec, A. & Butinar, J. (2006). Haemostasis impairment in bitches with pyometra. *Acta Veterinaria-Beograd,* 56(5-6), pp. 529-540.

Portela-Gomes, G.M., Grimelius, L., Wilander, E. & Stridsberg, M. (2010). Granins and granin-related peptides in neuroendocrine tumours. *Regulatory Peptides,* 165(1), pp. 12-20.

Powanda, M.C., Wannemac, R.W. & Cockerel, G.L. (1972). Nitrogen-metabolism and protein-synthesis during pneumococcal sepsis in rats. *Infection and Immunity,* 6(3), pp. 266-71.

Priego, T., Granado, M., de Caceres, II, Martin, A.I., Villanua, M.A. & Lopez-Calderon, A. (2003). Endotoxin at low doses stimulates pituitary GH whereas it decreases IGF-I and IGF-binding protein-3 in rats. *Journal of Endocrinology,* 179(1), pp. 107-117.

Rau, S., Kohn, B., Richter, C., Fenske, N., Kuechenhoff, H., Hartmann, K., Haertle, S., Kaspers, B. & Hirschberger, J. (2007). Plasma interleukin-6 response is predictive for severity and mortality in canine systemic inflammatory response syndrome and sepsis. *Veterinary Clinical Pathology,* 36(3), pp. 253-260.

Reichler, I.M. (2009). Gonadectomy in cats and dogs: a review of risks and benefits. *Reproduction in Domestic Animals,* 44(Suppl 2), pp. 29-35.

Rodriguez-Tarduchy, G., Collins, M.K.L., Garcia, I. & Lopezrivas, A. (1992). Insulin-like growth factor-I inhibits apoptosis in IL-3 dependent hematopoietic-cells. *Journal of Immunology,* 149(2), pp. 535-540.

Rootwelt-Andersen, V. & Farstad, W. (2006). Treatment of pyometra in the bitch: a survey among Norwegian small animal practitioners. *European Journal of Companion Animal Practice,* 16(2), pp. 195-198.

Ros, L., Holst, B.S. & Hagman, R. (2014). A retrospective study of bitches with pyometra, medically treated with aglepristone. *Theriogenology,* 82(9), pp. 1281-1286.

Rosjo, H., Nygard, S., Kaukonen, K.-M., Karlsson, S., Stridsberg, M., Ruokonen, E., Pettila, V., Omland, T. & Grp, F.S. (2012). Prognostic value of chromogranin A in severe sepsis: data from the FINNSEPSIS study. *Intensive Care Medicine,* 38(5), pp. 820-829.

Ru, G., Terracini, B. & Glickman, L.T. (1998). Host related risk factors for canine osteosarcoma. *Veterinary Journal,* 156(1), pp. 31-39.

Rubio, A., Boyen, F., Tas, O., Kitshoff, A., Polis, I., Van Goethem, B. & de Rooster, H. (2014). Bacterial colonization of the ovarian bursa in dogs with clinically suspected pyometra and in controls. *Theriogenology,* 82(7), pp. 966-971.

Rupprecht, C.E., Hanlon, C.A. & Slate, D. (2006). Control and prevention of rabies in animals: paradigm shifts. *Developments in Biologicals,* 125, pp. 103-11.

Salman, M.D., New, J.G., Jr., Scarlett, J.M., Kass, P.H., Ruch-Gallie, R. & Hetts, S. (1998). Human and animal factors related to relinquishment of dogs and cats in 12 selected animal shelters in the United States. *Journal of Applied Animal Welfare Science,* 1(3), pp. 207-26.

Sandholm, M., Vasenius, H. & Kivisto, A.K. (1975). Pathogenesis of canine pyometra. *Journal of the American Veterinary Medical Association,* 167(11), pp. 1006-1010.

Sato, J., Yasuda, J., Muraoka, N., Sato, R., Tomizawa, N., Miyake, Y., Okada, K. & Naito, Y. (2002). High level of serum alkaline phosphatase activity and isoenzymes in cases of canine pyometra. *Journal of the Japan Veterinary Medical Association,* 55(6), pp. 367-370.

Schneider, R., Dorn, C.R. & Taylor, D.O.N. (1969). Factors influencing canine mammary cancer development and postsurgical survival. *Journal of the National Cancer Institute,* 43(6), pp. 1249-61.

Schreiber, G., Howlett, G., Nagashima, M., Millership, A., Martin, H., Urban, J. & Kotler, L. (1982). The acute phase response of plasma-protein synthesis during experimental inflammation. *Journal of Biological Chemistry,* 257(17), pp. 271-277.

Shike, H., Lauth, X., Westerman, M.E., Ostland, V.E., Carlberg, J.M., Van Olst, J.C., Shimizu, C., Bulet, P. & Burns, J.C. (2002). Bass hepcidin is a novel antimicrobial peptide induced by bacterial challenge. *European Journal of Biochemistry,* 269(8), pp. 2232-2237.

Slauterbeck, J.R., Pankratz, K., Xu, K.T., Bozeman, S.C. & Hardy, D.M. (2004). Canine ovariohysterectomy and orchiectomy increases the prevalence of ACL injury. *Clinical Orthopaedics and Related Research,* 429(429), pp. 301-5.

Smith, A.N. (2014). The Role of Neutering in Cancer Development. *Veterinary Clinics of North America-Small Animal Practice,* 44(5), pp. 965-975.

Sorenmo, K.U., Shofer, F.S. & Goldschmidt, M.H. (2000). Effect of spaying and timing of spaying on survival of dogs with mammary carcinoma. *Journal of Veterinary Internal Medicine,* 14(3), pp. 266-70.

Srithunyarat, T., Bystrom, E., Stridsberg, M., Olsson, U. & Pettersson, A. (2014). The correlation of chromogranin A in saliva and plasma in healthy dogs. In: *Proceedings of Scientific Proceedings Veterinary Programme of BSAVA Congres,*Birmingham, United Kingdom, pp. 645-645.

Stevenson, C.K., Kidney, B.A., Duke, T., Snead, E.C.R., Mainar-Jaime, R.C. & Jackson, M.L. (2007). Serial blood lactate concentrations in systemically ill dogs. *Veterinary Clinical Pathology,* 36(3), pp. 234-239.

Stocklin-Gautschi, N.M., Hassig, M., Reichler, I.M., Hubler, M. & Arnold, S. (2001). The relationship of urinary incontinence to early spaying in bitches. *Journal of Reproduction and Fertility. Supplement,* 57, pp. 233-6.

Strage, E.M., Lewitt, M.S., Hanson, J.M., Olsson, U., Norrvik, F., Lilliehook, I., Holst, B.S. & Fall, T. (2014). Relationship among Insulin Resistance, Growth Hormone, and Insulin-Like Growth Factor I Concentrations in Diestrous Swedish Elkhounds. *Journal of Veterinary Internal Medicine,* 28(2), pp. 419-428.

Strage, M., Lewitt, M., Lilliehook, I., Strom Holst, B. & Jones, B. (2011). Evaluation of an IGFBP-blocked ELISA for measuring IGF-I in canine serum. In: *Proceedings of European Society for Veterinary Clinical Pathology (ESVCP) 13th Annual Congress*, Dublin, Ireland, pp 587-587.

Stridsberg M, Pettersson A, Hagman R, Westin C & O, H. (2014). Chromogranins can be measured in samples from cats and dogs. *BMC Research Notes,* 7(1), p. 4.

Stridsberg, M., Eriksson, B., Oberg, K. & Janson, E.T. (2003). A comparison between three commercial kits for chromogranin A measurements. *Journal of Endocrinology,* 177(2), pp. 337-341.

Stridsberg, M., Eriksson, B., Öberg, K. & Janson, E. (2004). A panel of eleven region-specific radioimmunoassays against different parts of the human chromogranin A molecule. *Regulatory Peptides,* 117, pp. 219-227.

Stridsberg, M., Houge Angeletti, R. & Helle, K. (2000). Characterisation of N-terminal chromogranin A and chromogranin B in mammals by region-specific radioimmunoassays and chromatographic separation methods. *Journal of Endocrinology,* 165, pp. 703-714.

Taupenot, L., Harper, K.L. & O'Connor, D.T. (2003). Mechanisms of disease - the chromogranin-secretogranin family. *New England Journal of Medicine,* 348(12), pp. 1134-1149.

Taupenot, L., Mahata, S.K., Mahata, M., Parmer, R.J. & O'Connor, D.T. (2000). Interaction of the catecholamine release-inhibitory peptide catestatin (human chromogranin A(352-372)) with the chromaffin cell surface and Torpedo electroplax: implications for nicotinic cholinergic antagonism. *Regulatory Peptides,* 95(1-3), pp. 9-17.

Teunissen, G.H.B. (1952). The development of endometritis in the dog and the effect of oestradiol and progesterone on the uterus. *Acta Endocrinologica,* 9(4), pp. 407-20.

Theurl, M., Schgoer, W., Albrecht, K., Jeschke, J., Egger, M., Beer, A.G.E., Vasiljevic, D., Rong, S., Wolf, A.M., Bahlmann, F.H., Patsch, J.R., Wolf, D., Schratzberger, P., Mahata, S.K. & Kirchmair, R. (2010). The Neuropeptide Catestatin Acts As a Novel Angiogenic Cytokine via a Basic Fibroblast Growth Factor-Dependent Mechanism. *Circulation Research,* 107(11), pp. 1326-1335.

Thrusfield, M.V. (1985). Association between urinary-incontinence and spaying in bitches. *Veterinary Record,* 116(26), pp. 695-695.

Tillett, W.S. & Francis, T. (1930). Serological reactions in pneumonia with a non-protein somatic fraction of Pneumococcus. *The Journal of Experimental Medicine,* 52(4), pp. 561-71.

Timmins, A.C., Cotterill, A.M., Hughes, S.C.C., Holly, J.M.P., Ross, R.J.M., Blum, W. & Hinds, C.J. (1996). Critical illness is associated with low circulating concentrations of insulin-like growth factors-I and -II, alterations in insulin-like growth factor binding proteins, and induction of an insulin-like growth factor binding protein 3 protease. *Critical Care Medicine,* 24(9), pp. 1460-1466.

Trasch, K., Wehrend, A. & Bostedt, H. (2003). Follow-up examinations of bitches after conservative treatment of pyometra with the antigestagen aglepristone. *Journal of Veterinary Medicine Series a-Physiology Pathology Clinical Medicine,* 50(7), pp. 375-379.

Trevejo, R., Yang, M. & Lund, E.M. (2011). Epidemiology of surgical castration of dogs and cats in the United States. *Journal of the American Veterinary Medical Association,* 238(7), pp. 898-904.

Tvarijonaviciute, A., Eralp, O., Kocaturk, M., Yilmaz, Z. & Ceron, J.J. (2011). Adiponectin and IGF-1 are negative acute phase proteins in a dog model of acute endotoxaemia. *Veterinary Immunology and Immunopathology,* 140(1-2), pp. 147-151.

Wang, J.-Y., Zheng, Y.-Z., Yang, J., Lin, Y.-H., Dai, S.-Q., Zhang, G. & Liu, W.-L. (2012). Elevated levels of serum amyloid A indicate poor prognosis in patients with esophageal squamous cell carcinoma. *BMC Cancer,* 12, p. 8.

Waters, D.J., Kengeri, S.S., Clever, B., Booth, J.A., Maras, A.H., Schlittler, D.L. & Hayek, M.G. (2009). Exploring mechanisms of sex differences in longevity: lifetime ovary exposure and exceptional longevity in dogs. *Aging Cell,* 8(6), pp. 752-755.

Watts, J.R., Wright, P.J. & Whithear, K.C. (1996). Uterine, cervical and vaginal microflora of the normal bitch throughout the reproductive cycle. *The Journal of Small Animal Practice,* 37(2), pp. 54-60.

Weiss, S.L., Fitzgerald, J.C., Pappachan, J., Wheeler, D., Jaramillo-Bustamante, J.C., Salloo, A., Singhi, S.C., Erickson, S., Roy, J.A., Bush, J.L., Nadkarni, V.M., Thomas, N.J., Sepsis Prevalence Outcomes, T. & Pediat Acute Lung Injury Sepsis, I. (2015). Global Epidemiology of Pediatric Severe Sepsis: The Sepsis Prevalence, Outcomes, and Therapies Study. *American Journal of Respiratory and Critical Care Medicine,* 191(10), pp. 1147-1157.

Wetmore, J.B., Lovett, D.H., Hung, A.M., Cook-Wiens, G., Mahnken, J.D., Sen, S. & Johansen, K.L. (2008). Associations of interleukin-6, C-reactive protein and serum amyloid A with mortality in haemodialysis patients. *Nephrology,* 13(7), pp. 593-600.

Wheaton, L.G., Johnson, A.L., Parker, A.J. & Kneller, S.K. (1989). Results and complications of surgical-treatment of pyometra- a review of 80 cases. *Journal of the American Animal Hospital Association,* 25(5), pp. 563-568.

Whitehead, M.L. (2008). Risk of pyometra in bitches treated for mismating with low doses of oestradiol benzoate. *Veterinary Record,* 162(23), pp. 746-9.

Wilkinson, J.S. (1960). Spontaneous diabetes mellitus. *Veterinary Record,* 72, pp. 548-554.

Wilson, B.S. & Lloyd, R.V. (1984). Detection of chromogranin in neuroendocrine cells with a monoclonal-antibody. *American Journal of Pathology,* 115(3), pp. 458-468.

Yamamoto, S., Shida, T., Miyaji, S., Santsuka, H., Fujise, H., Mukawa, K., Furukawa, E., Nagae, T. & Naiki, M. (1993). Changes in serum C-reactive protein levels in dogs with various disorders and surgical traumas. *Veterinary Research Communications,* 17(2), pp. 85-93.

Yamashita, K., Fujinaga, T., Miyamoto, T., Hagio, M., Izumisawa, Y. & Kotani, T. (1994). Canine acute phase response- relationship between serum cytokine activity and acute phase protein in dogs. *Journal of Veterinary Medical Science,* 56(3), pp. 487-492.

Yentis, S.M., Soni, N. & Sheldon, J. (1995). C-reactive protein as an indicator of resolution of sepsis in the intensive-care unit. *Intensive Care Medicine,* 21(7), pp. 602-605.

Yule, T.D., Roth, M.B., Dreier, K., Johnson, A.F., PalmerDensmore, M., Simmons, K. & Fanton, R. (1997). Canine parvovirus vaccine elicits protection from the inflammatory and clinical consequences of the disease. *Vaccine,* 15(6-7), pp. 720-729.

Zhang, D., Lavaux, T., Sapin, R., Lavigne, T., Castelain, V., Aunis, D., Metz-Boutigue, M.-H. & Schneider, F. (2009). Serum concentration of chromogranin A at admission: an early biomarker of severity in critically ill patients. *Annals of Medicine,* 41(1), pp. 38-44.

Zhang, D., Lavaux, T., Voegeli, A.-C., Lavigne, T., Castelain, V., Meyer, N., Sapin, R., Aunis, D., Metz-Boutigue, M.-H. & Schneider, F. (2008). Prognostic value of chromogranin a at admission in critically ill patients: A cohort study in a medical intensive care unit. *Clinical Chemistry,* 54(9), pp. 1497-1503.

Zhang, G., Sun, X., Lv, H., Yang, X. & Kang, X. (2012). Serum amyloid A: a new potential serum marker correlated with the stage of breast cancer. *Oncology Letters,* 3(4), pp. 940-944.

Zink, M.C., Farhoody, P., Elser, S.E., Ruffini, L.D., Gibbons, T.A. & Rieger, R.H. (2014). Evaluation of the risk and age of onset of cancer and behavioral disorders in gonadectomized Vizslas. *Journal of the American Veterinary Medical Association,* 244(3), pp. 309-319.

Acknowledgements

The studies included in this thesis were performed at the Department of Clinical Sciences, Section of Small Animals, Swedish University of Agricultural Sciences (SLU), Uppsala. Financial support was obtained from the Royal Thai Scholarship (Thai Government Scholarship), the Agria Pet Insurance and the Swedish Kennel Club's Research Foundation, The Thure F. and Karin Forsberg's Foundation, Michael Forsgren's Foundation, and Fjällveterinärerna. The Faculty of Veterinary Medicine, Khon Kaen University where I am employed and work in Thailand, is grateful acknowledged for giving me motivation and allowing me to perform my Doctoral studies in such a worthy academic field.

This great achievement has been possible thanks to encouragement from many individuals. I greatly appreciate you all. I would like to express my gratitude to all of you who supported me and have been such good friends of mine during the past four years.

My sincere thanks to my main supervisor, **Associate professor Ragnvi Hagman,** without your support, great enthusiasm in the project and being such a brilliant great supervisor, I would not have achieved my goal - a PhD in small animal surgery. Moreover, also her family has welcomed me warmly. In addition, **Associate professor Bodil Ström Holst, Associate professor Ann Pettersson and Senior lecturer Odd Höglund**, my co-supervisors, without your support this thesis would not exist. I would like to thank you all for being such an incredible supervisor group, for devoting your time to my work, for sharing your knowledge and for helping me a lot in how to make my thesis as great as it could be.

Special thanks to all of my co-workers, **Professor Ulf Emanuelson**, **Professor Ulf Olsson**, **Associate professor Mats Stridsberg**, **Professor Agneta Egenvall**, **Emma Strage**, **Caroline Alneryd-Bull** and **Assistant professor Fredrik Södersten** for your great contributions and sharing your

time and knowledge contributing to my studies to reach the goal and getting all papers published before the dissertation. UE, UO and AE, you all deserve my sincerely thanks for kindly performing statistical analyses. UE, besides being a co-worker, you are so generous and such a capable intellect and have put so much effort into teaching me and explaining nicely that I can get a better understanding of the statistical analyses. I greatly appreciate your speedy response; you always got back to me directly even during your most hard-working times.

Assistant professor Nareupon Kampa who is my colleague and also a great brother. His encouragement and support has brought me such a good experience of being a PhD student. He is also the person who motivated me to study at a higher level, started to make contact with universities and found a great supervisor at SLU. My sincere thanks to my former Dean at Khon Kaen University, **Associate professor Suneerat Aiumlamai**: your encouragement, motivation and great support has brought me the brilliant possibility of reaching my ambition.

Our former Head of Department, **Deputy Dean Torkel Ekman** and our present Head of Department **Professor Björn Ekesten**, I greatly appreciate all your help and support. Without Torkel's rapid action the opportunity for me to study at SLU would not have been possible. Thank you **Professor Jens Häggström** and **Professor Anne-Sofie Lagerstedt**, present and former Head of the Section of Small Animals, for accepting my Doctoral studies in small animal surgery.

Associate professor Kerstin Hansson, thank for your help in finding a good project and supervisors. I appreciate your thoughtfulness and without your help, I would not have attained my doctoral studies here at SLU. KH, thank you also for kindly allowing me to participate in all types of work in diagnostic imaging. Additionally, I would like to say a word of thanks to all those who work at the section of diagnostic imaging who generously shared their expertise and knowledge with me. You gave me such a warm welcome, even though I was not staff or a PhD student at your section.

Moreover, I would like to say thank you to all staff at the University Animal Hospital, in particular at the small animal surgery unit for kindly allowing me to participate in all kinds of surgery I was interested in and also for helping in the collection samples. **Gun Larsson** especially has been most helpful. Moreover, the staff at the Clinical Pathology Laboratory, UDS, SLU, Uppsala, who kindly helped with the analyses, samples and information of the analyses, and particularly **Associate professor Inger Lilliehöök** who has been most helpful in helping in finding methods for analyses and providing clinical blood data.

My sincere thanks to **Associate professor Elisabeth Persson** for helping with uterine samples and guiding me in morphology and **Gunilla Ericson-Forslund** for training me in the lab work with uterine morphology. And I also would like to thank **Annika Rikberg** and **Karin Selin-Wretling** for providing chemical substances, instruments and preparing samples necessary for my studies.

Annika Nyström, Susanne Pettersson, Anette Forsberg, Mikael Rosenius, Veikko Niemi and **Marie Sundberg**, my sincere thanks to all of you for your help to process documents, add course points and clear the bills.

IT department, thanks for your help with installing the programme, solving the problems with my computer. I also would like to thank the SLU librarians for excellent help with literature references, finding books and journals and providing useful courses for searching data and handling documents.

P-nid, uncle Slil, aunty Tasanee, aunty Pa, P-jit (Sawangjit), and P-Lha (Ratriporn), I greatly appreciate your warm welcome and for being such good family here in Sweden.

To all my friends, in particularly **Tong, Dew, Bow, Ae, Supaporn, Ping, Tiger**, I express my sincere thanks to you for being such good company. **Iulia Karlsson**, I greatly appreciate your help in getting to know the city when I first came to Uppsala, and my pleasure to have a good time together with you in Canada, I really enjoyed the trip with you. **Anna Hillström and Malin Öhlund**, I thank you both for being such good friends in Sweden.

To all other colleagues here at Department of Clinical Sciences, in particularly my colleagues at the Section of Small Animals for inviting me to join the section party after work, meeting and also being good friends, you are all so generous and nice to me. I would like to express my sincere thanks to **Professor Jens Häggström** and **Senior lecturer Ingrid Ljungvall** for allowing me to participate in echocardiography when I first came here. **IL**, I greatly appreciate your kindness in dropping me off at my place at the beginning of my time here on a very dark and cold day.

Finally, I greatly thank my family, my father **(Chawalit Jitpean),** my mother **(Boonkwan Jitpean)**, and my uncle **Sawang Jitpean** for their love and support.