Cytokines as Diagnostic Biomarkers in Canine Pyometra and Sepsis

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2015

Acta Universitatis agriculturae Sueciae
2015:46

Cover: “Den vitruvianska hunden” (Karlsson I.)

ISSN 1652-6880
ISBN (print version) 978-91-576-8290-1
ISBN (electronic version) 978-91-576-8291-8

Print: SLU Service/Repro, Uppsala 2015

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Abstract

Sepsis is a syndrome with high morbidity, mortality and astronomical health care costs and it is challenging to diagnose both in humans and animals due to the lack of suitable diagnostic biomarkers. Although several types of proteins have been suggested as diagnostic biomarkers of sepsis, none of them were shown to be reliable for routine use in the clinical practice. Dogs with uterine bacterial infection called pyometra often develop sepsis and have been suggested as a natural model of sepsis. To investigate whether there is a pattern of biomarkers that can be useful to diagnose bacterial sepsis on early stages in addition to existing clinical criteria, we measured both local gene expression and serum levels of cytokines in dogs with pyometra and compared these levels with known inflammatory markers and blood clotting parameters.

Serum concentrations of keratinocyte-derived chemokine (KC)-like protein and the global clot strength were significantly increased both in dogs with pyometra compared to healthy dogs and in dogs with sepsis compared to dogs without sepsis in pyometra. Moreover, the expression levels of the chemokines interleukin (IL)-8 and C-X-C motif ligand 5 (CXCL5) mRNA were significantly higher in uteri from dogs with pyometra compared to healthy dogs and in cultured stromal endometrial cells derived from uteri of healthy dogs and cocultured with LPS or pathogenic *Escherichia coli* compared to unstimulated cells. Although serum concentrations of IL-8, high-mobility group box 1 (HMGB1), prostaglandin F2α, IL-2, IL-15, IL-18, interferon (IFN)-γ and monocyte-macrophage colony stimulating factor (MG-CSF) were not different between dogs with or without sepsis in the presence of pyometra, some of these cytokines correlated significantly with clinical parameters such as total white blood cell count (correlated with HMGB1) and KC-like (correlated with IL-8). Measurements of serum IL-10, CXCL10, tumor necrosis factor (TNF)-α, IL-6 and IL-4 will require a more sensitive method in dogs with pyometra.

Our findings suggest that KC-like, CXCL5 and IL-8 may be useful as early diagnostic biomarkers of sepsis in dogs with pyometra. Further investigation of these chemokines in sepsis may help to improve routines in sepsis diagnosis in dogs and possibly also humans.

Keywords: sepsis, SIRS, uterine bacterial infection, pyometra, dog/canine, inflammation, cytokines, chemokines.

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Till min mormor

The only reason for time is that everything doesn't happen at once.
/Albert Einstein

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 Karlsson I, Hagman R, Johannisson A, Wang L, Karlstam E, Wernersson S (2012). Cytokines as immunological markers for systemic inflammation in dogs with pyometra. *Reprod Domest Anim*. 2012; 47 Suppl 6:337-41.

II Karlsson I, Hagman R, Johannisson A, Wang L, Södersten F, Wernersson S. Serum KC-like chemokine concentrations are significantly increased in canine bacterial sepsis (manuscript).

III Karlsson I, Hagman R, Guo Y, Humblot P, Wang L, Wernersson S (2015). Pathogenic Escherichia coli and LPS enhance the expression of IL-8, CXCL5 and CXCL10 in canine endometrial stromal cells. *Theriogenology* (In Press: http://dx.doi.org/10.1016/j.theriogenology.2015.02.008).

IV Karlsson I, Wernersson S, Ambrosen A, Kindahl H, Södersten F, Wang L, Hagman R (2013). Increased concentrations of C-reactive protein but not high-mobility group box 1 in dogs with naturally occurring sepsis. *Vet Immunol Immunopathol*. 2013;156:64-72.

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The contribution of Iulia Karlsson to the papers included in this thesis was as follows:

I Minor contribution to planning of the work and collection of the samples. Participated in performing of the experiment. Major contribution to data analysis and writing of the manuscript.

II Participated in planning of the work and performing the experiment. Minor contribution to sample collection. Major contribution to data analysis and writing of the manuscript.

III Planned, performed and analysed results for the majority of the work. Major contribution to the writing of the manuscript.

IV Planned, performed and analysed results for the majority of the work. Minor contribution to sample collection. Major contribution to the writing of the manuscript.

Abbreviations

CD	Cluster of differentiation
CRP	C-reactive protein
CXCL	C-X-C motif ligand
CLP	Cecal ligation and puncture
PAMP	Pathogen-associated molecular pattern
DAMP	Damage-associated molecular pattern
ELR	Glutamic acid–leucine–arginine motif
PGM	Prostaglandin metabolite
ENA	Epithelial-derived neutrophil-activating protein
PLA-II	Group II phospholipase A2
KC	Keratinocyte-derived chemokine
IP	Interferon-gamma-inducible protein
SIRS	Systemic inflammatory response syndrome
GRO	Growth-regulated oncogene
NK	Natural killer
ELISA	Enzyme-linked immunosorbent assay
GM-CSF	Granulocyte-macrophage colony stimulating factor
HMGB1	High-mobility group box 1
IFN	Interferon
IL	Interleukin
LPS	Lipopolysaccharide
Th	T helper
TNF	Tumor necrosis factor

1 Introduction

The incidence, severity and mortality rates in sepsis in both humans and animals are rising worldwide despite an increasing body of knowledge on sepsis pathophysiology and numerous experimental and clinical studies investigating sepsis. Current experimental models of sepsis provide considerable knowledge but are not able to reflect the actual clinical scenario of sepsis syndrome onset and progression, and the findings obtained in these models are often not directly applicable for clinical patients with sepsis. The major problem in sepsis remains the weakness of the clinical diagnostic criteria that account for the delays in life-saving treatment initiation and erroneous choice of treatment strategy for a given patient. Many biological molecules have been tested for the ability to predict the onset of sepsis and distinguish between sterile and infection-caused inflammation in order to minimize the use of broad-spectrum antibiotics and improve both short-term and long-term life quality of the patients. However, none of the evaluated biomarkers can be recommended for routine clinical use in specific diagnosis of sepsis (Bloos, 2015). Cytokines constitute a large group of biological molecules that participate actively in the immune response towards an infection in the body. The search for a pattern of cytokines associated with exacerbation of infection and the onset of sepsis syndrome that could contribute to an early and specific diagnosis of sepsis is currently intense. In this thesis several cytokines are examined as diagnostic biomarkers in naturally occurring canine sepsis caused by uterine bacterial infection, i.e. pyometra.

1.1 Cytokines

1.1.1 Definition

Cytokines are small protein molecules (5-20 kDa) that are produced and released by a great variety of cells in the body with the main purpose of

providing communication between cells (Cameron & Kelvin, 2003). A broad range of cells, including immune cells like macrophages, B lymphocytes, T lymphocytes and mast cells, as well as endothelial cells, fibroblasts, and various stromal cells can produce and release cytokines. Some cytokines can be produced by a limited number of cell types, whereas others - by virtually all known cell types. A given cytokine may be produced by more than one type of cell, and every cytokine is recognized by one or more receptors.

The production and release of cytokines from immune cells and other cells are critical for an efficient and well-orchestrated response to inflammation and infection in the body, which is why cytokines are considered to be immunomodulating (Meide & Schellekens, 1996). Cytokines can work at both systemic and local levels.

Based on function, cell of origin, or target of action, cytokines can be classified as interleukins (ILs), interferons (IFNs), tumor necrosis factors (TNFs) and chemokines. Interleukins are made primarily by leukocytes and act primarily on other leukocytes. Tumor necrosis factors can facilitate apoptotic cell death. Chemokines mediate chemoattraction (chemotaxis) between cells and interferons can "interfere" with viral replication, activate certain immune cells and increase antigen presentation (De Andrea *et al.,* 2002).

Chemokines is a large and diverse group of cytokines and it can be further subdivided into C-X-C (CXC), C-C (CC), X-C (XC), and C-3X-C (CXXXC) motif chemokines depending on the arrangement of the first two invariant cysteine residues in the amino acid sequence. This means that many chemokines share structural properties, but their chemotactic effects were shown to be diverse (Charo & Ransohoff, 2006). The CXC chemokines comprise one of the largest chemokine groups and can be produced by a great variety of both stimulated and unstimulated cells. There are two kinds of CXC chemokines, ELR^+ and ELR^-, based on whether or not the N-terminal sequences contain glutamic acid–leucine–arginine (ELR) motif in front of the first cysteine (Laing & Secombes, 2004). One particular property of ELR^+ CXC chemokines is that they are specialized in attracting and activating neutrophils (Baggiolini *et al*. 1991; Huber *et al*., 1991), but also other immune cells such as basophils, eosinophils, NK cells and some T lymphocytes. ELR^- CXC chemokines, on the other hand, attract mainly lymphocytes and monocytes and have a limited chemoattracting effect on neutrophils (Laing & Secombes, 2004).

Cytokines are important in health and disease, and knowledge on their involvement in host responses to infection, inflammation, trauma, cancer, reproduction and sepsis is of a great importance.

1.1.2 Cytokines in bacterial infection

Some cytokines are produced constitutively, but most of them are produced as a result of induction. A wide variety of factors including bacteria, viruses, other microbes and even other cytokines can stimulate various cells to produce one, a few, or many cytokines, which can in turn activate or inactivate other cells and affect the inflammatory response through a chain reaction (Chen *et al.,* 2014).

In a bacterial infection, the first immune cells responding are monocytes and macrophages, which upon activation by bacteria or bacterial products respond almost immediately by production of cytokines such as TNF-α, IL-1 and IL-6 (Russel *et al.,* 2010) (Figure 1). These cytokines activate and amplify the inflammatory response towards the infection and facilitate production of other inflammatory cytokines and chemokines, including IL-8, IL-12, IL-15 and IL-18, with the main purpose to attract and activate other immune cells, such as neutrophils and natural killer cells (Cohen, 2002). On later stages of an immune response to bacterial infection cells of the adaptive immunity, i.e. T and B lymphocytes, are activated in the lymph nodes and migrate to the site of infection. Lymphocytes need also to communicate with each other and with other immune cells, which is why they also produce a number of cytokines such as IL-2, IL-4, IL-5, IL-6 and IFN-γ (Zhu & Paul, 2008). The pro-inflammatory immune response must be counter balanced by an anti-inflammatory response manifested by the release of cytokines such as IL-10 produced by many types of immune cells including macrophages, dendritic cells, and different types of lymphocytes (Cohen, 2002; Shubin *et al.*, 2011).

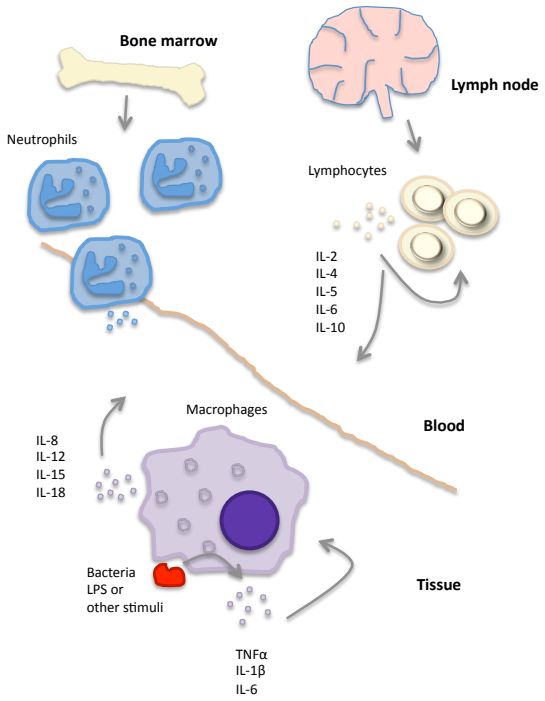

Figure 1. Cytokines and chemokines are released by several types of immune cells in response to bacterial infection and other stimuli. Tissue macrophages are one of the first cells that react to infection by producing and releasing cytokines. Macrophages initiate an immune response by recruiting and activating other immune cells such as neutrophils and lymphocytes. If the cell communication is impaired, lymphocytes, macrophages and neutrophils can loose some of their crucial functions, which in turn contributes to an exacerbated and unbalanced inflammatory reaction and impaired clearance of bacteria and bacterial products.

The fact that many cytokines are produced during a bacterial infection and that different patterns of cytokines are produced at different time points of infection in response to different stimuli makes it interesting for researchers and clinicians to measure levels of the cytokines and study these specific patterns. For instance, infections caused by different parts of *Helicobacter pylori* and different species of *Listeria* resulted in the induced mRNA expression of different cytokine patterns (Kuhn & Goebel 1994; Xiong *et al.,* 1994; Imanishi, 2000), and higher levels of type I interferons and TNF-α were detected both *in vitro* and *in vivo* during infection with different bacteria or their products (Degre, 1996). A recent report shows that serum concentrations of IL-2, IL-4, TNF-α and IFN-γ increased two-fold and concentrations of IL-6

and IL-10 increased more than 10-fold the normal levels in children with Gram negative nosocomial infections within 24 h after infection onset and before the increase of CRP levels, which is an acute phase protein (Chen *et al.,* 2013). Bacterial infections caused by *Escherichia coli*, *Streptococcus pneumonia*, *Chlamydophila pneumonia* or *Streptococcus agalactiae* were associated with significantly elevated serum concentrations of cytokines including IL-2, IL-6 and TNF-α in comparison with the levels observed in sera of patients with viral infections (Holub *et al.,* 2013).

Numerous studies have evaluated the ability of single cytokines to detect certain bacterial infections with high sensitivity in early stage of infection or assisting in infection prognoses (Chen *et al.,* 2013), including TNF-α and IFN-γ (Stoycheva & Murdjeva, 2005), IL-6 and IL-8 (Tavares *et al.,* 2005; Ventetuolo & Levy, 2008; Jacovides *et al.,* 2011), IL-12 and IL-18 (Sánchez-Hernández *et al.,* 2011), keratinocyte-derived chemokine (KC) (Huang *et al.,* 1992; Songlih *et al.,* 1992; Kim *et al.,* 2005), epithelial-derived neutrophil-activating protein (ENA)-78, also named CXCL5 (Nasu *et al.,* 2001; Mei *et al.,* 2010; Nouailles *et al.,* 2014), IL-17 (van de Veerdonk *et al.,* 2009), high-mobility group box 1 (HMGB1) (Scaffidi *et al.,* 2002; van Zoelen *et al.,* 2007; Zhou *et al.*, 2011; Allonso *et al.,* 2012; Yanai *et al.,* 2013), IL-10 (Couper *et al.,* 2008) and CXCL10, also named INF-γ-inducible protein (IP)-10 (Proost *et al.,* 2003; Chen *et al.,* 2009). Interestingly, CXCL10 was recently suggested for use as an alternative infection marker to IFN-γ, showing promising results in more than 20 clinical studies (Ruhwald *et al.,* 2012). CXCL10 is an ELR^- CXC chemokine produced by different kinds of cells including monocytes, endothelial cells and fibroblasts (Farber, 1997). CXCL10 shows relatively high diagnostic accuracy in infection and is also a reliable infection marker in young children and in individuals with low cluster of differentiation 4 ($CD4^+$) cell counts (T helper cells, monocytes, macrophages and dendritic cells) (Ruhwald *et al.,* 2012). CXCL10 has been suggested to have antimicrobial effect both for Gram positive and Gram negative bacteria (Yang *et al*., 2003; Egesten *et al*., 2007; Cole *et al*., 2001) and was shown to be upregulated in uteri from dogs with uterine bacterial infection (Hagman *et al*., 2009b).

Some of the cytokines that were shown to be useful in detecting or monitoring of immune response to bacterial infections may potentially be useful in the diagnosis of more severe immunological conditions caused by an infection such as sepsis. This may depend on their role, timing of action and specificity in sepsis pathogenesis, as well as to what extent they differ individually in patients depending on the concurrent conditions and diseases.

1.2 Sepsis

1.2.1 Definition

Sepsis, also called “blood poisoning” or septicemia, is defined as presence of systemic inflammatory response syndrome (SIRS) caused by an infection (Bone, 1992; Levy, 2003). Patients with two or more signs of SIRS and suspected or confirmed infection are diagnosed as having sepsis. Sepsis can further progress into severe sepsis and septic shock. Patients with diagnosed sepsis showing evidence of organ dysfunction (cardiovascular, renal, hepatic, or neurological) and those with evidence of inadequate tissue perfusion are classified as severe sepsis. Sepsis patients with inadequate tissue perfusion and persistently low blood pressure despite intravenous fluid administration are classified as having septic shock (Deutschman & Tracey, 2014). Most commonly sepsis is caused by Gram negative or Gram positive bacterial infections (Martin *et al.,* 2003), but mycobacterial, rickettsial, viral, fungal and protozoan infections can also lead to sepsis (King 2007, Martin, 2012). Both pathogenic bacteria and commensal microorganisms evading otherwise sterile tissues as a result of clinical manipulations such as surgery can cause infection that leads to sepsis.

Sepsis may rapidly lead to death or result in a severely compromised life quality when the diagnosis is delayed or the treatment is inefficient (Hotchkiss & Karl, 2003; Yende *et al.,* 2014). Being associated with astronomical health care costs, sepsis was listed as one of the most expensive condition in 2011 (Torio & Andrews, 2013).

1.2.2 Epidemiology of sepsis: incidence and outcome

The incidence and severity of sepsis in humans are increasing worldwide (Brun-Buisson *et al*., 1996; Angus *et al*., 2001; Danai & Martin, 2005; Harrison *et al*., 2006; Blanco *et al*., 2008; Esper & Martin, 2009), which is likely due to a combination of various factors, including increased awareness and tracking of disease conditions, an aging population, increased longevity of people with chronic diseases, the emergence and wide spread of antibiotic-resistant organisms and broader use of immunosuppressive and chemotherapeutic agents. It has been recently estimated that sepsis affects nearly 27 million people worldwide each year (Colón-Franco & Woodworth, 2014).

A decade ago, every third human patient with severe sepsis died (Angus *et al*., 2001), and today the acute mortality rate from sepsis, severe sepsis, and septic shock is in average 15%–20% (Kaukonen *et al.,* 2014). The risk of

mortality increases by 7.6% every hour for patients with septic shock (Kumar, 2006). Among sepsis survivors, 75% die within the next 5 years (Iwashyna *et al.,* 2010).

The risk of mortality increases with the increase of sepsis severity: 10-20% for sepsis without organ dysfunction or shock, 20-50% for severe sepsis and 40-80 for septic shock (Martin, 2012). It is therefore of vital importance to diagnose sepsis at as early stages as possible.

1.2.3 Immunological response during sepsis

It was recently highlighted that most of the clinical features of sepsis depend vaguely on the nature of the infection, and it appears that the immune response of each patient, and not the infecting microorganism, is the key to the understanding of sepsis pathogenesis (Faix, 2013; Kinasewitz *et al.,* 2004).

Initially during an infection, resident immune cells such as macrophages generate a pro-inflammatory state in response to pathogen-associated molecular patterns (PAMPs) from the infecting organism, and damage-associated molecular patterns (DAMPs) that are released by damaged host cells. Resident immune cells recognize these patterns mainly via Toll-like and lectin receptors and then release inflammatory mediators to attract other immune cells such as neutrophils and cells of adaptive immunity to the site of infection in order to eliminate the pathogen without harming the host (Kumar *et al.,* 2011). In most patients, the pro-inflammatory response is self-limiting, even in the absence of effective treatment, but in some patients the response becomes exaggerated, which leads to sepsis (Faix, 2013).

At the initial stage sepsis is characterized as a systemic hyperinflammation, meaning that the number of activated immune cells in the blood stream increases substantially, with the production and release of inflammatory mediators at a much higher rate than during a normal infection (Hotchkiss & Karl, 2003). As sepsis persists, a shift toward an anti-inflammatory immunosuppressive state usually follows (Natanson *et al.,* 1994; Oberholzer *et al.,* 2001). Studies on postoperative sepsis, however, suggest that immunosuppression can be a primary response in sepsis (Heidecke *et al.,* 1999; Weighardt *et al.,* 2000), and there are theories supporting the idea that hyperinflammation and immunosuppression in sepsis may occur simultaneously (Remick, 2007), which stresses the complexity of the inflammatory response in sepsis.

Factors leading to the onset of sepsis and affecting the overall character of the immunological response during sepsis are unknown but may be influenced by patient's age, nutritional status, concurrent diseases and pre-existing

immune dysfunctions and genetic factors (Hotchkiss & Karl, 2003). Polymorphisms or single base-pair alterations in cytokine genes are thought to be important genetic factors that may determine the levels of inflammatory and anti-inflammatory cytokines produced, and may influence whether individuals have marked hyperinflammatory or hypoinflammatory responses to infection (Chung & Waterer, 2011). In particular, the risk of death in sepsis has been linked to genetic polymorphisms for tumor necrosis factor (TNF)-α and TNF-β (Freeman & Buchman, 2000).

Activated CD4 T cells are programmed to secrete either T helper (Th)1 cytokines, including TNF-α, IFN-γ, and IL-2, or Th2 cytokines, such as IL-4 and IL-10 (Abbas *et al.,* 1996; Opal & DePalo, 2000). Th2 response was suggested to be beneficial for survival in sepsis (O'Sullivan *et al.,* 1995), but other studies have demonstrated that an increased level of IL-10 in patients with sepsis correlates positively with mortality (Gogos *et al.,* 2000). Whether CD4 T cells have Th1 or Th2 responses may depend on the virulence of the infecting organism, the size of the inoculum, the site of infection, and the patient's condition (Abbas *et al.,* 1996).

Studies on circulating lymphocyte count in patients with different stages of sepsis, and autopsy studies in persons who died of sepsis, showed that large numbers of cells of the adaptive immune system and gastrointestinal epithelial cells died by apoptosis during sepsis, which occurs primarily during the prolonged hypoimmune state (Hotchkiss *et al.,* 1999; Hotchkiss *et al.,* 2002). While there is virtually no loss of CD8 T cells, natural killer cells, or macrophages, the levels of B cells, CD4 T cells, and follicular dendritic cells decrease markedly during sepsis, which in turn leads to decreased antibody production, macrophage activation, and antigen presentation, respectively. Apoptosis of lymphocytes and gastrointestinal epithelial cells is normal for the body and facilitates a rapid cell turnover, which in sepsis accelerates to extreme levels and leads to unbalance. Apoptotic cells induce anergy, i.e. nonresponsiveness to pathogen, or facilitate the dominance of anti-inflammatory cytokines that impair the response to pathogens and increase the risk of lethal outcome (Voll *et al.,* 1997). Examination of spleens removed after death from patients with sepsis demonstrated that the more prolonged sepsis, the more profound was the loss of lymphocytes (Hotchkiss *et al.,* 2001).

Another cell type that has been shown to contribute to sepsis pathogenesis is the neutrophil (Remick *et al*., 2007). Both neutrophil migration and activity regulation can be impaired in septic patients. An elevated responsiveness of neutrophils to IL-8 and increased expression of IL-8 receptor CXCR2 can cause an exacerbated recruitment and activation of neutrophils at the site of inflammation. Together with a delay in apoptosis and overexpression of

adhesion integrins, neutrophils fail in diapedesis and instead accumulate along the surface of endothelium, causing serious damage to the vessel wall by releasing toxic amounts of active mediators that were originally produced for neutralization of bacteria. A decreased responsiveness of neutrophils to IL-8 can also occur in patients with sepsis, leading to impaired neutrophil recruitment and failure in bacterial clearance. The nonresponsiveness of neutrophils to the chemotactic and activating stimuli is a general characteristic of a hypoinflammatory sepsis and is commonly detected in patients with septic shock (Chishti et al., 2004).

Taken together, the findings on pathophysiology and immunology of sepsis available up to date indicate that both the diagnosis and treatment of sepsis during the hyperinflammatory phase may be beneficial and even life-saving (Natanson *et al.,* 1994).

1.2.4 Diagnosis

Despite the advanced technology and vast amount of knowledge currently available in the XXIst century, the diagnosis of sepsis remains primitive and unspecific. To diagnose sepsis, two or more SIRS criteria must be fulfilled, which include elevated heart and respiratory rates, abnormal number of leukocytes in the blood stream (leukopenia or leukocytosis) and fever or hypothermia, both in humans (Levy, 2003) and in animals such as dogs (Hauptman *et al.,* 1997), and an infection either suspected or clinically evident in a patient. In other words, it is enough, for instance, that the patient with a suspected infection has an elevated respiratory rate and fever, for sepsis to be diagnosed. Such criteria are vague and unspecific, because other conditions such as trauma or burns that lead to systemic inflammation can also cause elevated heart rate, respiratory rate, elevated number of leukocytes and even fever. Importantly, the symptoms and clinical manifestations vary considerably between patients and depend on type of pathogen, genetic factors, age, and nutritional factors as well as the health status of the patient (Hotchkiss & Karl, 2003). Moreover, some patients with sepsis, especially elderly, never develop fever (Gleckman & Hibert, 1982).

To verify systemic infection and to identify infecting pathogen, several sets of blood cultures using media for aerobic and anaerobic organisms are usually obtained, with at least one blood sample drawn through the skin and one blood sample drawn through a vascular access device that has been in place for more than 48 hours (Dellinger *et al.,* 2013). However, blood cultures usually require several days before they can be evaluated and are successful only in less than 50% of cases of late stages of sepsis, and almost completely unsuccessful in

early stages of sepsis both in humans (Previsdomini *et al.,* 2012) and in animals such as domestic dogs (Heilmann *et al.,* 2013). This makes blood cultures unreliable for a quick and precise diagnosis of sepsis. A novel method of pathogen detection in blood based on polymerase chain reaction (PCR) was developed recently that allows a faster retrieval of results and higher sensitivity and specificity compared to blood cultures (Chang *et al.,* 2013; Heilmann *et al.,* 2013). However, some clinical studies show that the PCR method has a high error frequency (Paolucci *et al.,* 2012), high cost, and the sensitivity varied greatly for different pathogens (Paolucci *et al.,* 2012; Chang *et al.,* 2013; Scvark *et al*., 2013), which is why it has not yet been recommended as a replacement for blood cultures.

As a consequence of the unspecific diagnostic criteria for sepsis, many patients, both human and animal, get an erroneous or delayed diagnosis in intensive care units. The false-negative diagnosis, i.e. when the patient that actually has sepsis does not satisfy the clinical criteria for SIRS at the time of clinical examination and is therefore diagnosed as not having sepsis, may lead to the progression of sepsis to more severe conditions or death. The effects of false-positive diagnosis in sepsis should not be underestimated either. When a patient with noninfectious inflammatory condition is falsely diagnosed as having sepsis, unnecessary massive doses of broad-spectrum antibiotics are administered. This leads to overuse of antibiotics and in turn an enriched pool of antibiotic-resistant pathogens, an increased number of patients with infections nonresponsive to antibiotic treatment, and consequently, a continuous increase of mortality in sepsis. The need for novel criteria that will allow for early and specific diagnosis of sepsis is thus urgent (Khair, 2010).

1.2.5 Biomarkers of sepsis

Biomarkers, or biological markers, have been defined as cellular, biochemical or molecular alterations measurable in biological media such as tissues, cells, or body fluids, that can be objectively evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacological response to a therapeutic intervention (Mayeux, 2004). Biomarkers can yield an understanding of the onset prediction, cause, diagnosis, progression, regression, or outcome of treatment of a given disease. A perfect biomarker would have a high diagnostic accuracy (sensitivity and specificity) especially on early stages of the disease, help to make rapid and correct bed-side therapeutic decisions and allow to monitor the patient's response to therapy at low cost (Scvark et al., 2013). Among the most important features of a sepsis biomarker in particular include the ability to early identify patients with sepsis

in a population with systemic inflammatory response syndrome, the ability to stratify severity of sepsis and reflect the responsiveness to therapy (Colón-Franco & Woodworth, 2014).

More than 180 biomarkers have been investigated in sepsis up to date and less than 20% of them were assessed specifically for use in the sepsis diagnosis (Pierrakos & Vincent, 2010; Reinhart *et al.,* 2012; Singer, 2013; Loonen *et al.,* 2014; Cho & Choi, 2014). Among them, only five were reported to have sensitivity and specificity above 90% (Pierrakos & Vincent, 2010). These five biomarkers include 1) integrin CD11b, 2) CD64, an activated polymorphonuclear neutrophil cell surface receptor with high affinity for the Fc part of the immunoglobulins, 3) T cell-activating cytokine IL-12, 4) CXCL10 and 5) the soluble fraction of group II phospholipase A2 (PLA2-II). PLA2-II was the only biomarker among those with the sensitivity and specificity above 90% that could distinguish between bacteremic and nonbacteremic infection in adults (Rintala *et al.,* 2001). CD64 was the only one that showed high accuracy in nonpediatric patients, i.e. in adults. However, the concentrations of PLA2-II were not assessed in patients with noninfectious inflammation, which makes it uncertain with respect to specificity to the presence of infection. Moreover, CD64 had low accuracy in distinguishing between viral and bacterial infections (Cardelli *et al.,* 2007; Nuutila *et al.,* 2007), which is not helpful for the choice of antimicrobial treatment in sepsis.

CRP and procalcitonin

Acute phase proteins, such as CRP and procalcitonin, are among the most extensively studied biomarkers in clinical sepsis, although their sensitivity and specificity for the sepsis diagnosis is typically below 90% (Pierrakos & Vincent, 2010). CRP is a protein produced in liver after the onset of inflammation and binds to dead or dying cells and some types of bacteria (Pepys *et al.,* 2003; Thompsom *et al.,* 1999). CRP concentrations in the blood are routinely measured in both human and veterinary clinics. Increased circulating concentrations of CRP were detected both in humans and animals during inflammation and infection (Epstein *et al.,* 1999; Clyne & Olshaker, 1999; Pomorska-Mól *et al.,* 2013; Nakamura *et al.,* 2008), and monitoring CRP concentrations in humans with sepsis allows evaluation of the efficiency of antimicrobial treatment (Schmit *et al.,* 2008; Póvoa *et al.,* 2011). However, CRP is released regardless of infectious or noninfectious origin of inflammation (Pepys & Baltz, 1983; Vigushin *et al*., 1993; Clyne & Olshaker, 1999; Au-Yong, 2012). Low concentrations of CRP cannot safely be used to exclude the presence of infection (Chan *et al.,* 2002), which makes CRP unreliable in sepsis diagnosis.

Procalcitonin, a precursor peptide for the hormone calcitonin, has been shown to be significantly increased during microbial infections, having particularly higher sensitivity and specificity than CRP for detection of bacterial infections (Simon *et al.,* 2004). Earlier studies have shown that procalcitonin could differentiate between sepsis and SIRS of noninfectious origin (Al-Nawas *et al.,* 1996; Karzai *et al.,* 1997; Brunkhorst *et al.,* 2000), differentiate sepsis patients with or without bacteremia (Chirouze *et al.,* 2002; Riedel *et al.,* 2011), correlate with severity of bacterial infection (Soreng & Levy, 2011) and predict mortality in humans with sepsis (Jain *et al.,* 2014). However, recent meta-analyses showed that a high heterogeneity of the reports could not be explained, and the suggested cutoff values for procalcitonin concentrations varied substantially between the studies (Tang *et al.,* 2007; Wacker *et al.,* 2013), as well as between surgical and medical patients (Clec'h *et al.,* 2006). Moreover, procalcitonin was shown to be elevated in a number of noninfectious disorders such as trauma (Becker *et al.,* 2008), which limits its specificity to infection and sepsis.

Complement system

Complement proteins have also been studied as biomarkers of sepsis, and the concentrations of complement peptide C5a were shown to be elevated in murine model of sepsis (Schreiber *et al.,* 2006), and in human patients with severe sepsis (Flierl *et al.,* 2008). However, complement proteins are participating in many other inflammatory processes in the body, and peptide C5a in particular have been used in diagnosis of autoimmune disorders (Yuan *et al.,* 2012).

Lactate

Serum lactate concentrations can reflect hypoxia and pathologic changes in tissue perfusion in severe sepsis and septic shock (Cho & Choi, 2014). Lactate clearance measurement is being widely used in septic patients to monitor therapy effectiveness (Jones, 2013), and it was also shown useful in outcome prognosis (Nguyen *et al.,* 2004). However, lactate cannot be used to differentiate patients with sepsis from those with noninfectious SIRS or to diagnose SIRS or sepsis on early stages, which makes its potential as sepsis biomarker limited (Nguyen *et al.,* 2004; Colón-Franco & Woodworth, 2014).

Cytokines and chemokines

Cytokines, one of the major groups of inflammatory mediators, are considered among the potential next-generation biomarkers for sepsis diagnosis (Russel & McCulloh, 2012). Analysis of cytokine production in

murine experimental model of sepsis and in human patients with sepsis show that both pro- and anti-inflammatory cytokines are elevated early in sepsis and the levels of cytokines may help to predict outcome (Burkovskiy *et al.,* 2013; Schulte *et al.,* 2013; Cabioglu *et al.,* 2002; Osuchowski *et al.,* 2006).

As pathogens or their toxins enter the blood stream, an unusually powerful systemic inflammatory reaction is provoked primarily by the production and release of toxic amounts of proinflammatory cytokines, including TNF-α and IL-1β. High levels of these cytokines cause an increased neutrophil–endothelial cell adhesion, overactivation of the clotting mechanism, and generation of microthrombi (Hotchkiss & Karl, 2003). Numerous other cytokines are then released at higher rates as a result of the exaggerated activation loop, which is usually called cytokine cascade or cytokine storm (Osterholm, 2005). In such massive pro-inflammatory reaction the importance of the negative feedback mechanism in the form of anti-inflammatory cytokine release increases consequently (Hotchkiss & Karl, 2003). During the immunosuppressive phase of sepsis the cytokine releases will markedly decrease, including TNF-α and IL-1β (Ertel *et al.,* 1995). Measurement of circulating concentrations of cytokines is therefore thought to be potentially useful in evaluating the stage of sepsis, tailoring the administration of anti-inflammatory agents and predicting outcome (Wang & Ma, 2008; de Pablo et al., 2011).

Circulating cytokines can be detected in biological fluids during different stages of sepsis. The presence of circulating cytokines, however, does not necessarily reflect the character and the time of their activity, and their absence in the blood or other fluids does not indicate an absence of cytokine production by activated cells (Cavaillon *et al.,* 1992). More than 20 different cytokines have been evaluated as sepsis biomarkers (Pierrakos & Vincent, 2010; Russel, 2012; Burkovskiy *et al*., 2013; Schulte *et al*., 2013), and several of them were studied both in human and animal sepsis (summarized in Table 1). For instance, IL-8 is the most studied cytokine in organ injury in sepsis in humans and animals (Faleiros *et al.*, 2009), and circulating plasma IL-8 concentrations were shown to be increased as a result of endotoxemia in human, primate and porcine models of sepsis (Kuhns *et al.,* 1995; VanZee *et al.,* 1991; Toft *et al.,* 2002). Serum and plasma concentrations of IL-8 were not only increased in patients with sepsis, but also correlated positively with the presence of multiple organ dysfunctions and were suggested as outcome predictors in severe sepsis in humans (Bozza *et al.,* 2007). Moreover, IL-8 concentrations were able to accurately predict the onset of sepsis in neonates (Ng & Lam, 2006), and IL-8 mRNA was one of the most highly upregulated cytokines in uteri from dogs with sepsis secondary to uterine bacterial infection (Hagman *et al.,* 2009b).

Table 1. Cytokines as sepsis biomarkers

Cytokine	Implication in sepsis	Host reference
TNFα	Increased in plasma/serum	Human (Munoz *et al*., 1991), dog (DeClue *et al*., 2012), rat (Ertel *et al*., 1991
	Plasma concentrations correlate with survival	Human (Casey *et al*., 1993; Blackwell & Christman, 1996)
	Early marker of endotoxin exposure	Dog (Otto, 2007)
	Outcome prognosis	Human (Oberholzer *et al*., 2005)
	Organ damage- and mortality-related	Human (Pinsky *et al.,* 1993)
IL-6	Increased in plasma/serum	Human (Munoz *et al*. 1991), dog (DeClue *et al*., 2012; Floras *et al*., 2014), rat (Ertel *et al*., 1991)
	Increased plasma concentrations correlate with survival	Human (Casey *et al*., 1993; Blackwell & Christman, 1996)
	Outcome prognosis	Human (Oberholzer *et al*., 2005; Novotny *et al*., 2012; Srisangthong *et al*., 2013), dog (Rau *et al*., 2007)
	Correlate with severity	Human (Martins *et al.,* 2003; Srisangthong *et al*., 2013)
	Monitoring immunomodulatory therapy efficacy	Dog (Hicks *et al*., 2012), mouse (Osuchowski *et al*., 2009)
IFN-γ	Increased in serum in septic shock	Human (Schulte *et al*., 2013)
	Neutralization increases resistance to septic shock	Mouse (Heinzel, 1990; Car *et al*., 1994)
IL-4	Low concentrations correlated with pneumonia onset	Human (Scott *et al*., 2002)
	Blockade restore lymphocyte function	Mouse (Scott *et al*., 2002)
IL-7	Lymphocyte survival and function	Human (Venet *et al*., 2012), mouse (Unsinger *et al.,* 2010)
	Improves survival	Mouse (Unsinger *et al.,* 2010)
	Increased in plasma	Human (Bozza *et al*., 2007)
	Increased local gene/mRNA expression	Dog (Hagman *et al*., 2009b), mouse (Unsinger *et al*., 2010)
IL-8/	Onset prediction	Human neonates (Ng & Lam, 2006)
	Organ dysfunction and outcome	Human (Bozza *et al*., 2007), horse

CXCL8	prediction	(Faleiros *et al.*, 2009)
	Increased in plasma/serum	Human (Kuhns *et al*., 1995), primate (VanZee *et al*., 1991), pig (Toft *et al*., 2002), dog (Floras *et al.,* 2014)
	Increased local gene expression	Dog (Hagman *et al*., 2009b)
IL-10	Incresed concentrations in serum and/or associated with outcome	Human (Kellum *et al*., 2007; Thijs and Hack, 1995; Gogos *et al*., 2000; Urbonas *et al*., 2012; Novotny *et al*., 2012), dog (Floras *et al*., 2014)
	Correlate with severity	Human (Wang *et al*., 2006; Collighan *et al*., 2004; Latifi *et al*., 2002)
IL-18	Increased in plasma and/or correlated with poor outcome	Human (Tschoeke *et al*., 2006; Grobmyer *et al*., 2000)
	Discriminate between Gram positive and Gram negative sepsis	Human (Tschoeke *et al*., 2006)
	Increased local gene expression	Dog (Hagman *et al*., 2009b)
KC/	Survival and bacterial clearance	Mouse (Jin *et al*., 2014)
KC-like/	Increased mRNA expression	Horse (Faleiros *et al*., 2009)
CXCL1/	Increased in serum at onset	Human neonates (Manoura *et al.*, 2010)
GROα	Increased in serum	Dog (Floras *et al*., 2014)
IP-10/ CXCL10	Increased gene/mRNA expression	Dog (Frangogiannis *et al*., 2000; Hagman *et al*., 2009b)
	Increased in serum/plasma and/or correlate with severity	Human (Punyadeera *et al*., 2010), dog (Floras *et al*., 2014)
HMGB1	Increased systemically in toxic shock	Rat (Degryse et al., 2001)
	Predict outcome and organ dysfunction	Human (Karlsson *et al*., 2008; Sundén-Cullberg *et al*., 2005; Gibot *et al*., 2007)

IL-8, also named CXCL8, is a chemokine with a strong chemoattracting and activating effects on many immune cells, especially neutrophils, and it is therefore thought to play an important role in sepsis pathogenesis (Wang *et al.,* 2010). IL-8 has been shown to be produced by a variety of cells, both immune and nonimmune, including monocytes, macrophages and endothelial cells (Baggiolini *et al.,* 1991). In mice and rats the genes encoding IL-8 and one of its receptors, CXCR1, are absent (Modi & Yoshimura, 1999). The murine keratinocyte-derived chemokine KC, also named CXCL1, is thought to be the

functional homolog of IL-8, and its lipopolysaccharide (LPS)-induced expression was similar to IL-8 (Singer & Sansonetti, 2004). Mouse KC, derived from both hematopoietic and resident cells, was shown to be essential for bacterial clearance and survival in mice with CLP-induced sepsis (Jin *et al*., 2014). Increased mRNA expression of KC was detected in several tissues in experimental sepsis in horses (Faleiros *et al*., 2009), and serum concentrations of KC, also named GRO-α in humans, were significantly increased at onset of sepsis in human neonates (Manoura *et al.*, 2010). In dogs, KC gene has not been identified, but mouse antibodies against KC detect specifically a protein called KC-like in canine body fluids. Concentrations of canine KC-like were significantly increased in supernatant from canine mononuclear cells stimulated with LPS *in vitro* (Levin *et al.,* 2014). Interestingly, a study on canine experimental LPS-induced endotoxemia showed that both serum concentrations of KC-like and IL-8 were significantly elevated 4 h after endotoxemia initiation (Floras *et al.,* 2014). The two canine chemokines that have the highest amino acid sequence similarity to mouse KC are CXCL5 and CXCL7 (Figure 2). The knowledge on the role of these chemokines in sepsis or bacterial infection in dogs is, however, limited.

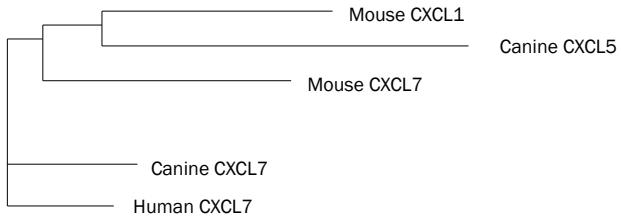

Figure 2. Phylogram tree showing the degree of amino acid sequence similarities between selected chemokines and species. Among all proteins known in dogs, CXCL5 and CXCL7 are most similar to mouse KC/CXCL1. The phylogram was generated using ClustalW2 at European Molecular Biology Laboratory (www.embl.org)

Human CXCL5, also known as ENA-78, was shown to act on the same receptor as mouse KC (Zlotnik & Yoshie, 2000). CXCL5 is an ELR^+ CXC chemokine that is preformed and stored in platelet granules under homeostatic conditions (Semple *et al.,* 2011). In inflammation, however, tissue-resident cells are thought to be the main source of circulating CXCL5, as shown in cultured human and bovine endometrial cells stimulated with LPS or bacteria (MacKintosh *et al.,* 2013; Fischer *et al.,* 2010; Nasu *et al.,* 2001; Bersinger *et al.,* 2008) and in *E. coli*-induced lung inflammation in mice (Mei *et al.,* 2010).

In mouse model of sepsis increased plasma CXCL5 concentrations were detected within 24 h after cecal ligation and puncture (CLP) (Zhang *et al.,* 2014). CXCL7, another ELR^+ CXC chemokine produced and released by platelets, is produced as a precursor peptide pro-platelet basic protein that is then cleaved to connective tissue activating peptide III, β-thromboglobulin and finally to CXCL7 (Majumdar *et al.*, 1991). CXCL7 has been shown to be a more sensitive marker for imaging of *E. coli* infection in rabbit cells *in vitro* compared to CXCL5 (Rennen *et al.,* 2004), but little is known about the levels of CXCL7 in human and veterinary patients with sepsis. On the contrary, an ERL^- CXC chemokine CXCL10 was extensively studied in sepsis in both humans and dogs. Serum concentrations of CXCL10 were significantly increased in dogs 4 hours after experimentally induced endotoxemia, and higher levels of CXCL10 mRNA were detected in the heart, lung, kidney, liver, and spleen after systemic endotoxin administration (Floras *et al*., 2014; Frangogiannis *et al*., 2000).

TNF-α and IL-6 are powerful inducers of coagulation and have been shown to be potentially useful as prognostic markers in sepsis (Oberholzer *et al.,* 2005). TNF-α concentrations were related to both organ damage and mortality (Pinsky *et al.,* 1993), and at the same time shown to be useful as an early marker for acute endotoxin exposure as shown in experimentally induced sepsis in dogs (Otto, 2007). On late stages of sepsis IL-6 correlated with disease severity (Martins *et al.,* 2003; Srisangthong *et al*., 2013) and was shown to have a prognostic value in both human and canine sepsis (Novotny *et al.,* 2012; Rau *et al.,* 2007; Srisangthong *et al*., 2013). Concentrations of IL-6 were shown to be predictive both for early mortality (<48 h) and for mortality after 28 days in humans with sepsis (Bozza *et al.,* 2007). Moreover, IL-6 could accurately direct immunomodulatory therapy in experimental sepsis in mice (Osuchowski *et al.,* 2009) and in dogs with severe staphylococcal pneumonia (Hicks *et al.,* 2012).

IFN-γ is another pro-inflammatory cytokine that together with TNF-α and IL-6 was shown to contribute to cytokine storm in sepsis (Huang *et al.,* 2005). The neutralization of IFN-γ results in increased resistance to septic shock induced by LPS in mice (Heinzel, 1990; Car *et al.,* 1994). IFN-γ is produced primarily by activated natural killer cells, Th1 and CD8 T cells, and its production is regulated by cytokines such as TNF-α and IL-18 (Schulte *et al.,* 2013). Although IFN-γ was not shown to correlate with sepsis severity and could not predict outcome, it could restore the macrophage function towards bacterial stimulation in macrophages isolated from mice with experimental sepsis (Flohé *et al.,* 2008), which indicates that a decrease in circulating IFN-γ

in patients with sepsis may be associated with the shift towards the immunosuppressive stage of the sepsis syndrome.

IL-7, a potent antiapoptotic cytokine that enhances immune effector cell function, was shown to be essential for lymphocyte survival in sepsis (Unsinger *et al.,* 2010) and could restore compromised lymphocyte functions in human patients with sepsis (Venet *et al.,* 2012). Circulating concentrations of IL-7 were more abundant in plasma from critically ill human patients with sepsis (Bozza *et al.,* 2007), and IL-7 expression was upregulated in uteri from dogs with sepsis caused by uterine bacterial infection (Hagman *et al*., 2009b). IL-7 receptor expression was also increased in an experimental septic mouse model (Unsinger *et al.,* 2010).

Concentrations of IL-10 were increased in patients with sepsis compared to healthy humans, and could predict the lethal outcome (Gogos *et al.,* 2000; Urbonas *et al.,* 2012; Novotny *et al.,* 2012). IL-10 was also shown to be at higher concentrations in patients with septic shock compared to patients with earlier stages of sepsis (Wang *et al.,* 2006; Collighan *et al.,* 2004). IL-10 is one of the most well described anti-inflammatory cytokines and it was shown to be an important regulator of inflammatory response and coagulation in sepsis (Cohen, 2010). Many different types of immune cells, including monocytes, Th2 and T regulatory cells, can produce IL-10 upon stimulation with a pathogen, but its expression is regulated tightly and normally minimal in the absence of inflammation (Li *et al.,* 2012). IL-10 is thought be one of the main cytokines that can indicate and control the onset of irreversible septic shock (Latifi *et al.,* 2002).

Recent findings show that HMGB1 may also have a potential as sepsis biomarker in humans. HMGB1 is a chromatin-binding nuclear factor that is normally present in nuclei of most cells and contributes to chromatin organization and transcriptional regulation (Bianchi, 2009). In healthy subjects, HMGB1 is undetectable in the extracellular environment. However, when cells respond to various danger signals, it can be released and readily detectable in the circulation acting as DAMP and a cytokine (Cho & Choi, 2014). HMGB1 can be released in different ways, including active release by immune cells such as monocytes and macrophages in response to endotoxin challenge, (Wang *et al*., 1999; Bianchi & Manfredi, 2014), and passive release by necrotic non-immune cells both in humans and animals (Bianchi, 2009). Depending on its redox state, HMBG1 can act as a chemoattractant and recruit and activate myeloid cells (Bianchi & Manfredi, 2014). Moreover, HMBG1 can form complexes with other chemokines and promote recruitment and activation of a great variety of immune cells (Schiraldi *et al.,* 2012). In sepsis, circulating concentrations of HMGB1 were detectable systemically in experimental toxic

shock in rats (Degryse *et al.,* 2001) and could predict organ dysfunction and outcome in human patients with severe sepsis and septic shock (Karlsson *et al.,* 2008; Sundén-Cullberg *et al.,* 2005; Gibot *et al.,* 2007).

Many cytokines seem to have a role in sepsis and their levels provide important information that may be useful in diagnosis of sepsis on different stages and in different species. However, the potential of all these cytokines to distinguish between a controlled local infection and an exacerbated systemic syndrome such as sepsis in a clinical setting for both humans and animals remains unclear. Moreover, little is known about the levels of sepsis-related cytokines in the circulation of animals with naturally occurring sepsis such as dogs with pyometra.

Currently none of the studied biomarkers have been shown to have a sufficient diagnostic strength to be routinely used for identification of patients with sepsis among those with systemic inflammation at early stages in the clinical setting (Pierrakos & Vincent, 2010; Hall *et al.,* 2011; Kibe *et al.,* 2011; Henriques-Camacho & Losa, 2014). The clinical setting of sepsis varies, and the complexity of sepsis and the comorbidities of patients at risk for sepsis make it unlikely that a single biomarker will fulfil all of the requirements. The emerging theory is that a panel of biomarkers may better diagnose sepsis among patients with systemic inflammation (Pierrakos & Vincent, 2010; Gibot *et al.,* 2012). With the recent availability of multiplex platforms allowing measuring of several immunological markers in each sample at once, it is now feasible that the concentration of panels of biomarkers can be measured both experimentally and routinely in the clinics. These new technologies may uncover an important knowledge for the improvement of sepsis diagnosis and monitoring of sepsis treatment efficiency.

1.2.6 Treatment strategies

There are no approved drugs that specifically target sepsis, and the treatment is limited primarily to support organ function via administration of intravenous fluids, antibiotics, and oxygen (Angus & van der Poll, 2013). Broad-spectrum antibiotics are used unless the pathogen is identified using blood culture (Marik, 2014; Dellinger *et al.,* 2008). Early therapeutic intervention to restore balance between oxygen delivery and oxygen demand improved survival among patients with severe sepsis (Rivers *et al.,* 2001). An intensive insulin therapy to maintain low blood glucose levels resulted in lower morbidity and mortality of sepsis patients, regardless of whether they had a history of diabetes (Van den Berghe *et al.,* 2001). The protective mechanism of insulin in sepsis is

unclear, but insulin was shown to have an antiapoptotic effect (Gao *et al*., 2002), and correcting hyperglycemia in patients with sepsis was shown to improve bacterial phagocytosis by neutrophils, which is impaired in patients with hyperglycemia. However, maintaining such low blood glucose levels (80 to 110 mg/dl) may put patients at risk for hypoglycemic brain injury.

Suggested sepsis treatment strategies include also administration of anti-inflammatory or immunostimulatory agents, depending on the phase of sepsis. Recombinant human activated protein C, an anticoagulant, is an anti-inflammatory agent preventing the generation of thrombin and has been proved effective in the treatment of sepsis and reduces the risk of death (Bernard *et al.,* 2001; Matthay, 2001). Activated protein C inhibits thrombin generation, and thus decreases inflammation by inhibiting platelet activation, neutrophil recruitment, and mast cell degranulation, blocking cell adhesion and the production of cytokines by monocytes. A major risk associated with activated protein C is hemorrhage, because activated protein C can cause serious life-threatening intracranial bleeding (Board, 2002). Currently, the use of activated protein C is approved only for use in severe sepsis patients who have multiple organ dysfunctions and the highest likelihood of death. Other suggested anti-inflammatory strategies include inhibition of cytokines such as TNF-α (Reinhart & Karzai, 2001) or targeting cytokine receptors by administration of IL-1 receptor antagonist (Zeni *et al.,* 1997). Immunostimulatory or immune-enhancing therapies include the administration of IFN-γ to restore macrophage activation (Docke *et al.,* 1997), and IL-12 to induce Th1 cells and restore resistance to bacterial challenge (O'Suillebhain *et al.,* 1996). Because the effective treatment strategies for different phases of sepsis can be diametrically opposite, the precise diagnosis of the phase is crucial for the choice and tailoring of the life-saving treatment in sepsis.

1.2.7 How sepsis is studied

Sepsis has been studied both clinically and experimentally for several decades. Most of the clinical studies have focused on patients with severe sepsis or septic shock (Mossie, 2013), correlating measured parameters with mortality rate as the main outcome. Data from patients at onset and early stages of sepsis remains limited, most likely due to the limitations of reliable parameters for early sepsis diagnosis.

Experimental models of sepsis comprise mainly of alteration of the endogenous protective barrier that allows bacterial translocation (cecal ligation and puncture) in inbred rodents and infusion of large doses of endotoxin or live bacteria into the blood stream of an otherwise healthy subject (Buras *et al.,*

2005). Unfortunately, none of the existing models can adequately reflect the clinical realities of sepsis (Fink & Heard, 1990; Nemzek *et al.,* 2008; Garrido *et al.,* 2004; Fink, 2014), which is the main reason for controversy between clinical and experimental findings. Important factors such as genetic heterogeneity and the high variability of patient's general condition, which comprise the biggest challenge in sepsis diagnosis and treatment, are omitted in animal models of sepsis. When large doses of endotoxin or bacteria are infused into an otherwise healthy individual, the immune system will react quickly and strongly, with exponential increase in levels of many circulating cytokines, such as TNF-α, which is most often not the case in patients with sepsis (Deitch, 1998). Moreover, experimental sepsis models rarely include supportive therapeutic interventions that are common in clinical practice and may influence the course of the sepsis syndrome. Altogether, this makes a large amount of experimental data not applicable to the clinical practice and sometimes misleading (Rittirsch *et al.,* 2007). To solve these problems, a model with natural onset and development of sepsis must be enrolled.

1.3 Pyometra – uterine bacterial infection

Pyometra is a disease caused by an opportunistic bacterial infection of the uterus (Hagman *et al.,* 2006a; Smith 2006) and is one of the most common bacterial diseases in dogs with incidence over 50% in adult intact dogs of certain breeds (Egenvall *et al.,* 2001). On average, nearly 20% of bitches of different breeds are expected to get pyometra before the age of 10 years (Jitpean *et al.,* 2012). Pyometra occurs less commonly in domestic cats and captive large felids, some livestock species, and laboratory animals such as rabbits and some strains of mice and rats (Kendziorski *et al.,* 2012). In humans, pyometra is an uncommon condition with rare but significant mortality resulting from spontaneous uterine perforation or rupture leading to sepsis. In the general population, the disease is estimated to account for about 0.04% of gynecological admissions; however, the incidence becomes increased to >13% in the elderly (Yildizhan *et al.,* 2006).

Pyometra diagnosis is based on patient history, physical examination findings, laboratory blood test results and diagnostic imaging using ultrasonography or radiology to demonstrate an enlarged, fluid-filled uterus (Hagman *et al.,* 2006a). Uterine enlargement and leukocytosis with neutrophilia and left shift are common findings relevant to the diagnosis of pyometra, but polyuria/polydipsia, anorexia, depression, vulvar discharge (in case of open cervix pyometra) are other classical signs that also are commonly

present (Hardy & Osborne, 1974; Jitpean *et al.,* 2014b). Bitches with a closed cervix pyometra and only a slight increase in uterine size may be difficult to diagnose, especially when leukocytosis is absent. A preliminary diagnosis of pyometra can be verified by identifying a pus-filled inflamed uterus during surgery in combination with postoperative macroscopic and histopathological investigation of the uterus and ovaries and bacterial culturing of the uterine content (Hagman, 2012).

Dogs with pyometra often develop sepsis, i.e. 6 of 10 cases display two or more clinical symptoms of SIRS at the time of admission to the animal hospital (Fransson *et al.,* 2007). Clinical diagnostic criteria for sepsis in dogs are as unspecific as in humans (as described in detail on Page 13) and include abnormal heart and respiratory rates, body temperature, blood leucocyte concentrations and high percentage of band neutrophils (Hauptman *et al.,* 1997), making it as difficult to diagnose sepsis in dog with pyometra as in human patients with severe infections. Because the inflammatory and coagulation changes that accompany severe infections and sepsis in dogs are similar to those in humans, dogs have been recognized as a more suitable animal species than rodents for studying of sepsis (Otto, 2007), and pyometra is lately studied as a natural model of sepsis in dogs (Conti-Patara *et al.,* 2012).

1.3.1 Etiology of pyometra: why and how it occurs

Exposure to estrogen in combination with high progesterone concentrations in the circulation has been shown experimentally to be one of the main triggering factors for pyometra initiation, and the incidence of pyometra rises with age (Niskanen & Thrusfield, 1998; Egenvall *et al.,* 2001; Kendziorski *et al.,* 2012). Genetic factors related to infiltration of leukocytes into the uterus play a major role in regulating sensitivity to estrogen-induced uterine inflammation and pyometra as shown in rats, dogs and mice (Gould *et al.,* 2005; Hagman *et al.,* 2011; Roper *et al.,* 1999; respectively). In sensitive strains of laboratory rats and mice, it is well established that chronic exposure to estradiol or the highly efficacious nonsteroidal estrogen can induce pyometra (Gardner & Allen, 1937; Gould *et al.,* 2005; Stone *et al.,* 1979).

Pyometra in dogs is mainly caused by a Gram negative bacteria, in particular *E. coli*, but other types of bacteria and even a combination of different bacterial species have also been detected (Hagman *et al.,* 2002).

1.3.2 Treatment of pyometra

The traditional therapy for pyometra is ovariohysterectomy, which is surgical removal of the uterus and ovaries. The main advantage of ovariohysterectomy is that it removes the source of the disease and is therefore most effective considering both cure and prevention of disease recurrence. When a dog with pyometra has a severely affected and unstable general condition, possibly due to the developed sepsis, an immediate surgical treatment can be life-threatening. This is why stabilization with treatment including intravenous fluids and antimicrobials is necessary, and hormonal treatment is administered in such cases prior to surgery to prevent death and serious complications. Therefore, a quick and accurate sepsis diagnosis in dogs with pyometra is crucial, and it may help to reduce treatment costs associated with pyometra and favor the improved life quality of the animals.

Because the surgery can be associated with high risk of mortality for some animals with concurrent diseases and results in permanent infertility, drug-based treatments of pyometra have emerged, including the administration of aglepristone with or without the additional administration of low doses of prostaglandins (Fieni *et al.,* 2014; Ros *et al.,* 2014). However, this is only prescribed in rare cases, and without surgical removal of the infection source in pyometra the risk for disease exacerbation and onset of sepsis remains high.

1.3.3 Biomarkers of sepsis in dogs with pyometra

Several biomarkers have been evaluated as diagnostic markers in canine sepsis (DeClue *et al*., 2011; Hagman *et al*., 2006b; Fransson *et al*., 2007). Increased CRP concentrations have been associated with sepsis in canine pyometra (Fransson *et al.,* 2007). However, CRP is a biomarker of inflammation caused by different kinds of stimuli, and its concentrations are elevated in a large number of noninfectious inflammatory conditions (Clyne & Olshaker, 1999). Concentrations of prostaglandin F2α metabolite were highly increased in bitches with endotoxemia (detectable endotoxin in the blood stream) caused by pyometra (Hagman *et al.,* 2006b). Serum amyloid A concentrations were significantly higher in dogs with sepsis compared to dogs without sepsis in pyometra, but the sensitivity and specificity for detecting sepsis was below 75% (Jitpean *et al.,* 2014a). Blood lactate and serum albumin concentrations could not differentiate between septic and nonseptic dogs with pyometra (Hagman *et al.,* 2009a; Conti-Patara *et al.,* 2012; Jitpean *et al.,* 2014a).

Cytokines as biomarkers of sepsis in dogs with pyometra

IL-8 and CXCL10 are among the most extensively studied chemokines of CXC chemokine group in dogs (Gangur *et al*., 2002). It has previously been shown that IL-8 and CXCL10 expression is highly upregulated in uteri from dogs with sepsis caused by pyometra (Hagman *et al*., 2009b). Other cytokines locally upregulated in dogs with pyometra and sepsis were IL-6, IL-7, IL-10, IL-15, IL-18 and TNF-α, but the knowledge on concentrations of these and other cytokines in the circulation of dogs with naturally occurring sepsis such as dogs with pyometra is limited.

2 Present investigations

2.1 Hypothesis and aims

The general hypothesis has been that a pattern of sepsis-associated circulating cytokines can be identified in dogs with naturally occurring sepsis that, in addition to current clinical diagnostic criteria, can be used to diagnose sepsis and contribute to improved specificity of sepsis diagnosis at early stages. The aim has been to investigate the potential usefulness of several cytokines as diagnostic markers for sepsis in dogs with pyometra and SIRS. More specific aims were to:

- Investigate the systemic concentrations of cytokines in pyometra and sepsis in dogs (**Paper I**)
- Investigate the systemic concentrations of KC-like protein compared to other cytokines in dogs with pyometra and sepsis (**Paper II**)
- Investigate the local effect of *E. coli* and LPS on the expression of chemokines in the canine endometrial cells *in vitro* (**Paper III**)
- Investigate the usefulness of measuring serum high-mobility group box 1 to differentiate between the presence or absence of sepsis in dogs with pyometra (**Paper IV**)

2.2 Results and Discussion

2.2.1 Paper I: Cytokines as immunological markers for systemic inflammation in dogs with pyometra

The main aim of this study (**Paper I**) was to investigate systemic concentrations of cytokines in sepsis caused by pyometra in dogs. Using a new commercially available canine multiplex cytokine assay, we measured

circulating concentrations of cytokines IFN-γ, IL-4, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18 and TNF-α simultaneously in serum samples from dogs with pyometra with signs of SIRS (P^+SIRS^+) or without (P^+SIRS^-) and compared with healthy controls. We could detect TNF-α, IL-7, IL-15, IL-18 and IL-8 in more than 90% and IL-10 in 28% of the samples, whereas only up to 3% of the samples had detectable concentrations of IL-4, IL-6 and IFN-γ. This is in agreement with other studies employing identical method for multiplexed cytokine detection in dogs, which showed that IL-4, IL-6, IFN-γ were among the cytokines under detection limit (Bastien *et al*., 2014; Zois *et al*., 2012). A possible explanation to the limited detection of these cytokines in circulation is that their concentrations may peak earlier (IL-6) or later (IL-4) during the inflammatory reaction and therefore be low at the time of admission and sampling, which would be in line with findings in human patients with sepsis (Kellum *et al*., 2007).

An earlier report showed an increased serum concentration of IL-10 in dogs with pyometra compared to healthy pregnant dogs (Maciel *et al*., 2014). Among samples that had detectable concentrations of IL-10 in the present study all were from dogs with pyometra and almost all of them had sepsis, indicating that an increased concentration of IL-10 may be one of the immunological indications of SIRS in pyometra. However, the differences of IL-10 concentrations between P^+SIRS^+ and P^+SIRS^- group were not significant. Thus, in order to define the role of IL-10 in distinguishing between dogs with or without SIRS in pyometra a larger number of subjects in each group and possibly also a more sensitive method for IL-10 measurement are needed. The concentrations of IL-7 were significantly higher in P^+SIRS^+ dogs compared to healthy controls, but did not differ between P^+SIRS^- and P^+SIRS^+ dogs, suggesting that IL-7 can be helpful in differentiating dogs with pyometra and sepsis from completely healthy dogs but is not a good marker to differentiate septic from nonseptic dogs with pyometra. Earlier studies have shown that IL-7 expression was upregulated in uteri from dogs with sepsis caused by pyometra (Hagman *et al*., 2009b), and plasma IL-7 concentrations were significantly elevated in critically ill human patients with sepsis, but not in patients with early stages of sepsis (Bozza *et al.,* 2007). This may indicate that there is a delay between the local production and the systemic release of IL-7 during sepsis and therefore suggesting a limited diagnostic value of IL-7 in early stages of sepsis.

The concentrations of IL-8 were higher in P^+SIRS^+ compared to P^+SIRS^- dogs, but lower in P^+SIRS^- dogs compared to healthy controls, which suggests that increased IL-8 concentrations could be a sign of sepsis in dogs with pyometra. There have been discrepancies regarding the usefulness of IL-8 in

sepsis diagnosis, with some reports showing that it can be both an early diagnostic and a reliable prognostic sepsis biomarker (Bozza *et al.,* 2007; Ng & Lam, 2006; Hack *et al*., 1992; Harbarth *et al*., 2001), but others also reporting that circulating IL-8 rapidly decreases in patients with sepsis (Söderquist *et al*., 1995). Therefore, further investigation on the role of IL-8 in canine infection and sepsis is needed.

The concentrations of TNF-α, IL-15 and IL-18 did not differ between the groups but IL-15 and IL-18 correlated significantly with concentrations of IL-7, suggesting a possible diagnostic value for IL-7, IL-15 and IL-18 in identifying systemic inflammation in dogs with pyometra. Moreover, two of the dogs from the $P^{+}SIRS^{-}$ group had extraordinarily high concentrations of IL-7, IL-15 and IL-18, suggesting a possible method artefact, or that these dogs may be falsely grouped as SIRS-negative according to conventional clinical criteria and possibly were at an early stage of sepsis. IL-18 and IL-15 are macrophage-derived cytokines that have been previously implicated in sepsis pathogenesis in both humans and dogs (Cohen, 2002; Hagman *et al*., 2009b). However, our data is consistent with a recent report showing that there was no difference in IL-15 and IL-18 concentrations in LPS-induced canine sepsis compared to placebo-treated healthy dogs (Floras *et al*., 2014). Taken together, these findings imply that although IL-15 and IL-18 may have an important role in sepsis pathogenesis, their potential as diagnostic biomarkers in canine sepsis remains elusive.

To assess whether increased concentrations of cytokines measured by multiplex assay reflected the inflammatory processes in dogs and were not the result of method artefacts, we measured concentrations of CRP using a sandwich enzyme-linked immunosorbent assay (ELISA) and compared with cytokine patterns. CRP concentrations were significantly higher in dogs with pyometra, with and without SIRS, compared with healthy controls, which is in line with studies showing an increased CRP in uterine infection in dogs (Enginler *et al.,* 2004). We found that concentrations of CRP were positively associated with IL-15, which indicated that inflammatory processes caused by pyometra were consequently evident using different methods. The association between CRP and IL-15 in canine pyometra is an interesting finding and needs to be further investigated.

Summary (Paper I):

- Multiplex cytokine assay allowed to simultaneously determine concentrations of 5 out of 9 cytokines in serum from dogs with pyometra and healthy dogs.

- Concentrations of IL-7 differed significantly between dogs with pyometra and healthy dogs.
- Concentrations of IL-8 were significantly higher in SIRS-positive compared to SIRS-negative dogs with pyometra.
- IL-18 correlated significantly with IL-15, and IL-7 correlated with IL-15 and IL-18.
- The results obtained using multiplex assay are in agreement with the inflammatory processes in the subjects detected independently of the multiplex assay.
- CRP, IL-7, IL-8, IL-15 and IL-18 may have a role in detection of SIRS caused by pyometra in dogs.

2.2.2 Paper II: Serum KC-like chemokine concentrations are significantly increased in canine bacterial sepsis

KC-like is a chemokine that has been recently shown to have increased systemic concentrations in experimental canine sepsis (Floras *et al*., 2014). In the present study we aimed to investigate systemic concentrations of KC-like compared to other cytokines in serum from dogs with naturally occurring sepsis, i.e. as a result of pyometra (**Paper II**). Special attention was paid to use samples that were not thawed prior to analyses and were not stored frozen for more than one month. A broadest possible spectrum of different circulating cytokines was measured simultaneously. We used dogs with pyometra as a natural model for sepsis as previously described and collected serum samples from totally 39 dogs, including dogs with pyometra (n=22) and healthy controls (n=17). Dogs with pyometra were further grouped into dogs with sepsis (n=18) and dogs without sepsis (n=4). The serum concentrations of a panel of cytokines, namely KC-like, GM-CSF, IL-2, IL-4, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, CXCL10 and TNF-α, were measured using multiplex analyses, serum concentrations of CRP were determined using an automated immunoturbidimetric assay, and hematological and serum biochemistry analyses were also performed to evaluate the overall clinical status of the dogs. Global clot strength was measured using thromboelastography.

Significantly higher serum concentrations of KC-like and higher global clot strength were detected in dogs with pyometra as compared to healthy dogs, and within the pyometra group, dogs with sepsis had a higher concentration of KC-like and higher global clot strength compared to dogs without sepsis. This is in line with recent studies showing increased circulating KC-like concentrations in canine experimental sepsis (Floras *et al.*, 2014), an overactivation of coagulation system in patients with sepsis (Levi, 2010; Schouten *et al*., 2008),

and elevated global clot strength in dogs with pyometra (Silins, 2013). We showed also that hemoglobin levels were significantly lower in all dogs with pyometra regardless of the presence or absence of sepsis compared to healthy controls, and correlated negatively with concentrations of KC-like. KC-like is a yet undescribed protein in dogs that has been recognized specifically in canine biological fluids by mouse antibodies against KC/CXCL1 (Garner *et al*., 2010; Zois *et al.*, 2012; Bastien *et al*. 2014) and has been detected in higher concentrations in serum from dogs with LPS-induced sepsis (Floras *et al*., 2014). KC, also known as GRO-α, is a neutrophil chemoattractant that was also implicated in human, experimental murine and equine sepsis (Faleiros *et al*., 2009; Manoura *et al.*, 2010; Jin *et* al., 2014). Interestingly, we found also that KC-like concentrations correlated positively with concentrations of CRP, hospitalization duration, number of monocytes, concentrations of IL-8, percentage band neutrophils and global clot strength. Our data suggest that KC-like is a suitable biomarker candidate for sepsis and uterine bacterial infection in dogs. Further studies are needed to identify and characterize canine KC-like as well as investigate its usefulness in sepsis diagnosis in dogs.

Summary (Paper II):

- Serum KC-like concentrations and global clot strength were significantly increased in dogs with naturally occurring sepsis
- Serum KC-like concentrations were associated with CRP, hospitalization length, number of monocytes, concentrations of IL-8, percentage band neutrophils, hemoglobin levels and global clot strength in dogs
- Serum KC-like could better differentiate between dogs with and without sepsis in pyometra compared to serum CRP

2.2.3 Paper III: Pathogenic *Escherichia coli* and LPS enhance the expression of IL-8, CXCL5 and CXCL10 in canine endometrial stromal cells

It was previously shown that dogs with pyometra and sepsis have higher mRNA levels of chemokines, including IL-8, in the uterus (Hagman *et al*., 2009b), and in Paper II we demonstrated that serum concentrations of an unidentified canine chemokine KC-like were significantly increased in dogs with pyometra and sepsis. The aim of this study (**Paper III**) was to investigate the local effect of *E. coli* and LPS on the expression of chemokines in the canine endometrial cells *in vitro* and to assess whether the stromal part of the endometrium express higher levels of chemokines in response to bacterial infection.

IL-8, CXCL5 and CXCL7 are ELR^+ CXC chemokines, which means that they are potent neutrophil chemoattractants. mRNA sequences of these chemokines have high sequence identity/similarity to mouse KC/CXCL1, (75.5%, 77.8% and 88.6%, respectively). IL-8 and CXCL5 were earlier shown to be produced at a higher rate in human and bovine endometrium exposed to LPS or bacteria (MacKintosh *et al.,* 2013; Fischer *et al.,* 2010; Nasu *et al.,* 2001), and CXCL7 has been shown to be suitable for imaging of *E. coli* infection in rabbit *in vitro* (Rennen *et al.,* 2004). In the present study we assessed mRNA levels of these chemokines in cultured endometrial stromal cells isolated from healthy dogs and exposed to either live pathogenic *E. coli* isolated from uterus of a dog with pyometra, or to LPS. The mRNA levels of chemokines were measured using quantitative real-time PCR and showed that IL-8 and CXCL5 were upregulated in endometrial stromal cells after 24 h of exposure to *E. coli* or LPS. The levels of CXCL7 were decreased or unaffected. Considering the main function known for these chemokines, our results suggest that, in response to presence of live Gram negative bacteria or bacterial fragments, canine stromal endometrial cells may play an important role in early neutrophil recruitment to the site of infection and inflammation.

CXCL10, or IP-10, is an ELR^- CXC chemokine and a T-cell attractant with an antimicrobial effect both for Gram positive and Gram negative bacteria. Canine CXCL10 mRNA sequence has 50% amino acid sequence identity to mouse KC. In the present study we found that mRNA levels of CXCL10 were higher both in canine endometrial stromal cells exposed to Gram negative bacteria *E. coli* and in cells exposed to LPS compared to nonstimulated cells. This is in agreement with another study showing that CXCL10 mRNA expression was induced in heart, lung, kidney, liver, and spleen tissues from dogs with induced endotoxemia (Frangogiannis *et al*., 2000) and may indicate that endometrial stromal cells participate in recruitment of T cells during a bacterial infection in the uterus.

To compare the experimental results with the real infection situation *in vivo*, we measured mRNA levels of IL-8, CXCL5, CXCL7 and CXCL10 in uteri from dogs with pyometra, and found that IL-8 and CXCL5, but not CXCL7 or CXCL10, were significantly higher in dogs with pyometra as compared to healthy dogs. Considering the high sequence similarity between canine IL-8, CXCL5 and mouse KC, and the fact that KC-like and not IL-8 was increased in serum from dogs with pyometra and sepsis (**Paper II**), our findings may indicate that CXCL5 may be the putative canine KC-like protein released from the uterus into the blood stream when pyometra in dogs exacerbates into sepsis. To unravel the differences and similarities between canine CXCL5 and KC-

like these chemokines must be further identified and described in dogs. However, further studies are needed to identify canine KC-like.

Our results show that the endometrial stromal cells are capable of releasing neutrophil-attracting chemokines in response to bacterial infection *in vitro*, which indicates that these cells may have an important role in neutrophil recruitment during bacterial infection of the uterus *in vivo*. Further studies are needed to clarify the role of chemokines in host response to bacterial infection in dogs and the possibility of using these chemokines as diagnostic parameters for bacterial infection in this species.

Summary (Paper III):

- Pathogenic uterine-derived *E. coli* and LPS induce increased CXCL5, CXCL10 and IL-8 levels in cultured canine endometrial stromal cells within 24 h.
- mRNA levels of CXCL5 and IL-8 are increased in pyometra-affected uteri from dogs.
- Endometrial stromal cells may have a role in recruitment of neutrophils during uterine bacterial infection.

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

HMGB1 has been shown to act as a proinflammatory cytokine when secreted by activated, damaged or killed cells and its systemic concentrations were increased in humans with sepsis and in canine model of endotoxemia (Gibot *et al*., 2007; Yu & Park, 2011). The aim of the present study (**Paper IV**) was to investigate the usefulness of systemic concentrations of HMGB1 to differentiate the presence and absence of sepsis in dogs with pyometra.

To find a sepsis-specific biochemical marker that could contribute to more accurate and early diagnosis of sepsis in dogs with pyometra, we measured serum concentrations of HMGB1 using sandwich ELISA in 23 healthy control dogs and 27 dogs with pyometra, 74% of which had sepsis. We also measured concentrations of the major acute phase protein CRP using an automated immunoturbidimetric assay, and an indicator for endotoxemia, prostaglandin F2α metabolite 15-keto-13,14-dihydro-PGF2α (PGM), using radioimmunoassay, to assess the relative contribution of HMGB1 to the detection of systemic inflammation and natural endotoxemia. We found that HMGB1 concentrations, in line with concentrations of CRP and PGM, were significantly increased in dogs with pyometra. However, HMGB1 concentrations did not differ significantly between dogs with sepsis and dogs

without sepsis with pyometra. Earlier studies on HMBG1 involvement in sepsis showed distinctive levels of HMGB1 in patients with severe sepsis, organ failure and septic shock (Gibot *et al*., 2007; Sundén-Cullberg *et al.*, 2005; Hatada *et al*., 2005) and in serum of LPS-treated mice (Wang *et al*., 1999), indicating that HMGB1 may be useful as a late marker of sepsis. Interestingly, although it was thought that HMGB1 is undetectable in extracellular environment of healthy subjects (Cho & Choi, 2014), we could detect circulating concentration of HMGB1 not only in dogs with pyometra but also in healthy controls.

The concentrations of CRP were significantly higher in dogs with sepsis compared to dogs without sepsis. PGM correlated with concentrations of CRP, but did not differ between dogs with and without sepsis in pyometra. Although serum HMGB1 was not correlated with either CRP or PGM concentrations, HMGB1 was correlated with the total white blood cell counts and was increased in dogs after surgery, suggesting an independent regulation and involvement of HMGB1 in inflammation. Moreover, serum HMGB1 but not CRP concentrations were significantly increased in dogs one day after surgical pyometra treatment, ovariohysterectomy. This is in agreement with another study showing that HMGB1 concentrations were higher on day one after surgery and thereafter decreased, and that the CRP concentrations increased first 3 days after ovariohysterectomy in dogs with pyometra (Ishida *et al*., 2011). The difference of CRP and HMGB1 concentration patterns in dogs indicate that HMGB1 and CRP may have different kinetics in the inflammation process caused by surgery and trauma compared to bacterial infection in dogs. Also, HMGB1 may have a different turnover than CRP, which makes it useful to further investigate the role of HMGB1 in sepsis.

Our results show that although HMGB1 may have an important role in inflammation in dogs with pyometra, its potential as a diagnostic biomarker for sepsis in dogs with pyometra is limited.

Summary (Paper IV):

- Serum concentrations of HMGB1 were detectable in both healthy intact female dogs and dogs with pyometra.
- HMGB1 concentrations were higher in serum from dogs with pyometra compared to healthy dogs but did not allow differentiation between the dogs with or without SIRS in pyometra.
- Serum HMGB1 in dogs with pyometra was associated with the well-known inflammatory marker CRP as well as with the inflammatory mediator PGM previously shown to be an indicator for endotoxemia.

- Serum concentrations of HMGB1, and not CRP, were significantly increased in dogs with pyometra after surgery.

3 Concluding remarks and future perspectives

Dogs with uterine bacterial infection pyometra are one of the most physiologically relevant models of naturally occurring sepsis currently studied, and canine inflammatory response characteristics are more similar to that of humans compared with mouse or rat. We have investigated several biomarkers that can improve the specificity of early sepsis diagnosis in clinical setting and found that KC-like, CXCL5 and IL-8 are promising candidates. Measured repeatedly and simultaneously in a large number of subjects, these chemokines may possibly provide a distinctive pattern reflecting different stages of bacterial infection and sepsis in dogs with pyometra and accounting for individual variations in the clinical symptoms. For instance, if at the time of admission a dog with pyometra has no fever and disturbances of heart- and respiratory rates, and an increased number of band neutrophils in the blood is the only clinically evident criteria for sepsis, an increased serum KC-like and/or tissue IL-8 may serve as an additional indicator on that sepsis is ongoing.

KC-like, unidentified and uncharacterized in dogs and yet specifically detected at protein level in canine immunoassays using mouse KC antibodies, seems to have high potential to discriminate between the presence and absence of sepsis in dogs with pyometra. Although CRP is a well-known marker of inflammation and one of the most well studied biomarkers in sepsis, it could not differentiate between the presence and absence of sepsis in dogs with pyometra in the same setting. CXCL5 (or ENA-78) is another chemokine that is poorly described in dogs, and at amino acid level it showed highest similarity to mouse KC sequence, also called CXCL1. Currently, it is not possible to measure circulating concentrations of CXCL5, ENA-78 or CXCL1 in dogs, but our data in cultured canine endometrial cells exposed to live pathogenic *E. coli* and in uteri from dogs with pyometra demonstrate that the

mRNA of CXCL5 is synthesized at a higher rate compared to nonstimulated cells and uteri from healthy dogs. Identification of KC-like or CXCL5 in dogs is therefore of high interest for bacterial infection and sepsis diagnosis in dogs with pyometra. Although mRNA expression of IL-8 was previously shown as one of the most elevated in uteri from dogs with pyometra compared to uteri from healthy dogs, the circulating protein concentrations of IL-8 in dogs were barely useful for the identification of dogs with pyometra or dogs with sepsis in pyometra. This may be dependent on the role of IL-8 in sepsis and the timing of measurement of circulating IL-8 in naturally occurring sepsis. The local synthesis and possibly also release of IL-8 in infected tissues is known to have a main purpose of attracting neutrophils to the site of infection. Whether IL-8 performs this function in collaboration with other neutrophil-attracting cytokines such as KC (KC-like) or CXCL5 and therefore has a limited role in sepsis pathogenesis or diagnosis in dogs is poorly understood.

We found that using multiplexing immunologic assays for measuring of several biomarkers in one sample at the same time may have its drawbacks, including a low or absent detection of certain cytokines such as CXCL10, IL-10, IL-4 and TNF-α, and high variability within the runs. Multiplexing methods available today may therefore have a limited usefulness in the routine clinical practice but can be helpful in initial screening for the potential biomarker patters for a certain disease including sepsis.

Other methods evaluating the activation, receptor expression and apoptosis rates of immune cells in sepsis can be of potential benefit for identification and evaluation of new sepsis biomarkers in dogs with pyometra. For instance, the measurement of integrin CD64 and chemokine receptor CXCR2 expression on circulating neutrophils, expression of apoptosis markers in neutrophils and leukocytes may be of potential use for improving the diagnosis of sepsis in dogs. However, both for humans and animals, it may be beneficial that the focus is shifted from comparing healthy subjects and patients with sepsis towards comparing patients with sepsis and those with non-infectious SIRS, as well as comparing patients with local infection and patients at onset of sepsis, because the biggest challenge for sepsis diagnosis remains the quick and robust differentiation of patients with bacteremia and/or endotoxemia among those with an exacerbated inflammation in the absence of infecting agent.

Dogs with pyometra and sepsis will, as well as human patients with severe infections and sepsis, benefit highly from an earlier and specific sepsis diagnosis and reduction of unnecessary antibiotic use. Therefore, the findings obtained using canine pyometra as a model of sepsis may be beneficial both for human and veterinary medicine.

4 Populärvetenskaplig sammanfattning

Sepsis (blodförgiftning) är ett alvarligt inflammatoriskt tillstånd som orsakas av en infektion. En sådan systemisk infektion leder till kraftigt sänkt blodtryck, massiv inflammation, vävnadsskada, organsvikt, chock och död hos både djur och människor. Det är viktigt att diagnostisera sepsis tidigt och exakt för att rätt och livräddande behandling ska kunna ges.

De kliniska kriterierna som används för att diagnosticera sepsis är enbart generella såsom kraftiga ändringar i hjärtfrekvens, andningsfrekvens, blodtryck, kroppstemperatur och antal neutrofiler eller vita blodkroppar. Dessa kliniska symptom kan även tyda på andra patologiska tillstånd förutom sepsis, vilket gör att många sepsisdiagnoser blir felaktiga eller försenade. Det finns därför ett stort behov av kompletterande diagnostiska kriterier för sepsis. Biokemiska markörer, d. v. s. proteiner som naturligt utsöndras i kroppen, som är detekterbara i blodomloppet i samband med utveckling av ett septiskt tillstånd, skulle kunna komplettera generella kliniska kriterier och bidra till en mer specificerad och robust sepsisdiagnos.

Tikar som inte kastreras i tid får ofta en allvarlig bakterieinfektion i livmodern som vanligtvis orsakas av bakterien *Escherichia coli* och leder till sjukdomen som kallas för pyometra. Varje år drabbas ca 10 000 tikar i Sverige av pyometra och sjukdomen kan snabbt utvecklas till sepsis om den inte behandlas. Tikar som diagnostiseras med pyometra har ofta haft sjukdomen ett tag och löper därför stor risk att utveckla sepsis. Närmare 6 av 10 tikar med pyometra utvecklar sepsis innan behandlingen är påbörjad. Hundar med pågående sepsis måste hjälpas med starka antibiotika innan livmodern får opereras bort. Det är viktigt därför att snabbt och exakt bedöma om sepsis är på gång i hundar med pyometra. Dessutom har hundarnas inflammatoriska och toxikologiska reaktioner flera likheter med motsvarande reaktioner hos människor, vilket gör att hundar med pyometra och naturlig sepsis är en

mycket värdefull modell för att få kunskap med relevans även för sepsis hos människor.

Vi har studerat femton olika inflammationsmarkörer hos hundar med pyometra som sedan utvecklat blodförgiftning och jämfört med hur dessa utrycks hos friska hundar. Halter av ett protein som heter KC-like var högre hos hundar med pyometra och sepsis jämfört med hos friska hundar. Mängden KC-like var också starkt kopplat till flera andra sepsis-relaterade faktorer så som blodkoaglets hållfasthet, antal monocyter i blodet, koncentrationen av ett känt inflammatoriskt akutfasprotein samt förlängd sjukhusvistelsetid. KC-like är ett kemokin, vilket betyder att det är ett signalprotein som attraherar andra celler till infektionskällan med hjälp av en kemisk gradient. KC-like är dock inte fullständigt identifierad hos hund och lånar därför namnet frånmotsvarande KC kemokin hos möss.

Vi har identifierad att ett annat kemokin hos hund som heter CXCL5 är mest lik KC-like i dess proteinsekvens. Vi mätte hur CXCL5 uttrycktes lokalt av livmoderceller vid en bakterieinfektion. Livmoderceller från friska hundar isolerades, odlades och infekterades med delar från döda bakterier (endotoxin) alternativt med levande bakterier (*Escherichia coli*) som tidigare isolerats från en tik med pyometra. En analys av cellernas genuttryck visade att både endotoxin och levande bakterier gav förhöjt uttryck av bland annat CXCL5. Dessutom kunde vi påvisa ett ökat uttryck av CXCL5 även i livmodervävnad från hundar med pyometra. För att kunna avgöra om hundens KC-like är samma sak som CXCL5 samt om KC-like eller CXCL5 kan fungera som kompletterande diagnostiska markörer för sepsis på hund måste dessa signalproteiner beskrivas och testas vidare på en större grupp tikar med pyometra och sepsis.

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Acknowledgements

The work presented in this thesis was carried out at the Department of Anatomy, Physiology and Biochemistry at the Swedish University of Agricultural Sciences. Funding was provided by the Michael Forsgrens Research Foundation, Thure F. and Karin Forsbergs Foundation, the Agria Insurance Co. and the Swedish Kennel Club's Research Foundation, and the Companion Animals Research Foundation at the Swedish University of Agricultural Sciences.

I want to express my sincere gratitude to all of you that in various ways contributed to the work or were otherwise helpful and supportive:

My supervisors:

Sara Wernersson, thank you for your very skilful and pedagogic guidance, your true wisdom and limitless expertise, your incredible patience and trust, and for always doing things of 100% quality. You allowed me to experience, learn and to grow and you were always there to support me. You have been the best supervisor one could ever imagine!

Ragnvi Hagman, thank you my for your valuable expertise, never-ending positive energy and enthusiasm, for always finding the right people at the right time and making the best out of all situations. For always nourishing my adventurous spirit and taking deadly cute photos of the dogs (and me) for the newsletters.

Liya Wang, thank you for an incredible expertise and limitless support in all kinds of scientific and pedagogic questions. For giving me all the courage it takes for testing things outside the box, and for showing a genuine interest in both my scientific and artistic achievements.

Present and past members of the group:

Ida, for trusting me with that IL-13 ELISA that I never done in my life before and making me discover many of my lab talents

Gunnar, for always being my inspiration although you probably didn't know it :)

Fabio, for supporting me at all times and being sincerely and endlessly kind to me!

Josefin, för att du visade mig mästarnivån på att klara av manga saker på kort tid, och för att du alltid staller upp med att förklara knepiga program

Mirjana, for being very kind and always supportive to me, and for being a true friend

Gianni, for being simply a great guy in everything and having a fantastic sense of humor

Anders, för att du alltid var där och erbjöd hjälp när man behövde det som allra mest

Helena W, för att du aldrig var rädd för svåra uppgifter och för att du är så fin människa

Elin, för att du alltid var så lugn och sansad och hade koll på allt man kunde tänka sig

Gabriela, for encouraging me to apply for this PhD position and coaching me for the interview :)

Carl-Fredrik, your dedication and curiosity for science is extra contagious

Aida, thank you for the nice chats at BMC, I still hope you will join me to a dance course one day :P

Helena Ö, for giving a new perspective into old things and bringing a new good culture into our group

Collaborators:

Anders Johannisson, for helping me organizing all those nervous hours of running the method that my thesis is largely based on

Yongzhi Guo, for giving me the crucial push in the right direction with my cell isolation method and being so kind to me

Patrice Humblot, for all your encouraging words and helpful advises

Supranee, for helping me with pyometra samples and being such a good company during the tough Canada trip

Aime Ambrosen, for being my student and making me proud

Fredrik Södersten, Erika Karlstam and **Hans Kindahl**, for all the valuable clinical data that made my publications possible

Present and past members of the department:

Lena Holm, för att du kam lära en göra världens bästa bilder på vävnadssnitt på fem minuter och för att du var med som prefekt alla mina år som doktorand

Carolina, för råd och stöd i alla krångliga administrative rutiner

Piotr, for fixing everything that is possible and not possible to fix in the lab and for valuable guidance in important technical questions

Ronnie, du var min förebild i laborationsundervisningen och inte minst under de tuffa veckor av avhandlingsskrivandet

Kiran, thank you for nice time with student labs and for knowing how to work with enzymes

Naresh, thank you for your kindness and help

Staffan, Magnus, Janne, Anna R, Marlene, Ren, Ingrid, Ellinor, Kerstin, Mona, Diana, My, Kristina, Ulrika, Mona, Anna W, Erik, Louise, Elena, Hanna, Jenny, Mirela, Nancy, Linnea, Johan, Anton, Jane and all present and past members of the department for your knowledge, support and friendly energy!

Other people at SLU:

Johanna L and Emelie Z for a truly nice time and lively discussions at SLU PhD student board

People at UU:

Lars, Mike, Jenny, Jocke and **Behdad** for good immunology seminars and great time on mast cell meetings, **Kerstin** and **Anna** for being my first scientific teachers in Sweden and great role models

My friends:

Sergey, Maya, Dasha, Denis, Patrik, Magnus, Csilla, Katia and many others, I am grateful to have met such wonderful and fantastic people like you in my life.

To my family:

Mama, **Papa i Anton**, esli vy docitali do etoy stranitsy, vam mojno uje toje davati kak minimum doktorskuu stepen'. Spasibo za vsio, berregite sebia.

Moey **Babulecke**, ty samaya zameciatelnaya i tvoe upriamstvo ocen' zarazitelno.

Daniel, tack för att du finns och att du pallade med mig alla de där tuffa veckorna.

Älskar dig.