Biomarker and Cytokine Measurements in Dogs with Endotoxemia

by

Alexandra Nicole Karrys Floras

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ABSTRACT

BIOMARKER AND CYTOKINE MEASUREMENTS IN DOGS WITH ENDOTOXEMIA

Alexandra Floras
University of Guelph, 2014

Advisor:
Dr. Marie K Holowaychuk

Rapid identification of sepsis enables prompt intervention and improved patient survival. Procalcitonin (PCT) is a biomarker used to diagnose sepsis in people; assays for its measurement have not been validated in dogs. In dogs, serum N-terminal pro-C-natriuretic peptide (NT-proCNP) concentration, at admission, differentiates naturally occurring sepsis from non-septic inflammation; however, its concentrations during the course of sepsis are unknown. This study investigated the validity of a commercially available enzyme-linked immunosorbent assay (ELISA) for the measurement of canine PCT, and determined kinetics of serum NT-proCNP and select cytokine concentrations in dogs with endotoxemia, a model of canine sepsis.

Serum samples from three dogs with sepsis and one healthy dog were examined. The PCT ELISA’s ability to detect recombinant and native canine PCT was investigated, coefficients of variability were calculated, and mass spectrometry of the standard solution was performed. Subsequently, 8 healthy adult Beagles were randomized to receive an intravenous bolus of lipopolysaccharide (LPS, 5 µg/kg) or saline placebo in a randomized crossover study. Serum was collected, and NT-proCNP, and 13 cytokines and chemokines were measured at 0, 1, 2, 4 and 24 hours.
The PCT ELISA generated inconsistent results. Intra- and inter-assay variability was 18.9-77.4%, and 56.1-79.5%, respectively. Mass spectrometry of the PCT ELISA standard solution did not confirm presence of PCT. Serum NT-proCNP concentrations did not differ significantly between LPS- and placebo-treated dogs at any time. When comparing serum cytokine concentrations, LPS-treated dogs had higher interleukin (IL)-6 IL-10, tumor necrosis factor-α and KC-like at 1, 2, and 4 hours; higher C-C motif chemokine ligand-2 at 1, 2, 4 and 24 hours; and higher IL-8 and C-X-C motif chemokine ligand-10 at 4 hours compared to placebo-treated dogs (p < 0.05). There were no significant differences in serum granulocyte macrophage-colony stimulating factor, interferon-γ, IL-2, IL-7, IL-15 or IL-18 concentration between LPS- and placebo-treated dogs.

These results do not support use of this commercial ELISA for detection of PCT in dogs. Serum NT-proCNP concentration did not change in response to LPS administration; however, certain serum cytokine and chemokine concentrations warrant further investigation of using cytokine profiles for the detection of sepsis in dogs.
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Thank you Geoffrey Wood, for sharing your laboratory and providing access to the Luminex® technology, and thank you Tami Harvey and Jonathan Liu for your technical assistance with running the multiplex assays. My gratitude is also extended to Gabrielle Monteith and William Sears for their assistance with statistical analyses.

Finally, I would like to thank the many people who helped support me throughout this DVSc program; my fellow residents and the ICU technicians for their moral support, as well as my family for their unwavering love and encouragement.
DECLARATION OF THE WORK PERFORMED

I declare that, with the exception of the items below, all work in this thesis was performed by me.

The thyroid lysate was extracted and the recombinant canine PCT was created by Henry Marr and colleagues at the North Carolina State University College of Veterinary Medicine.

Mass spectrometry was performed by the Mass Spectrometry Facility, Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, Ontario, Canada.

The pre-study complete blood counts and serum biochemistry profiles were performed at the Animal Health Laboratory of the University of Guelph. The care of the research dogs was overseen and provided by the Central Animal Facility of the University of Guelph. The endotoxin and placebo study phases were administered and overseen by Drs. Marie Holowaychuk and Vincent Gauthier. Blood collection was performed by Dr. Gauthier and technicians from the Department of Clinical Studies and Ontario Veterinary College Health Sciences Centre, with the assistance of summer students and volunteers.

The Luminex® Multiplex analyzer, used for the cytokine and chemokine analyses, was maintained and operated by Tami Harvey.
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<tr>
<td>CCL2</td>
<td>C-C motif chemokine ligand 2 (also referred to as monocyte chemotactic protein-1, MCP-1)</td>
<td></td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
<td></td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of variation</td>
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<td>C-X-C motif chemokine ligand 10 (also referred to as interferon gamma-induced protein-10, IP10)</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage-colony stimulating factor</td>
<td></td>
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<tr>
<td>ICU</td>
<td>Intensive care unit</td>
<td></td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
<td></td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
<td></td>
</tr>
<tr>
<td>KC-like</td>
<td>Keratinocyte-derived chemokine (also referred to as CXCL1)</td>
<td></td>
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<tr>
<td>LCMS/MS</td>
<td>Liquid chromatography-tandem mass spectrometry</td>
<td></td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
<td></td>
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<tr>
<td>NK</td>
<td>Natural killer</td>
<td></td>
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<tr>
<td>NT-proCNP</td>
<td>Amino-terminal pro-C-type natriuretic peptide</td>
<td></td>
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<tr>
<td>OD</td>
<td>Optical density</td>
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<td>PCT</td>
<td>Procalcitonin</td>
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<tr>
<td>rcPCT</td>
<td>Recombinant canine PCT</td>
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<td>SIRS</td>
<td>Systemic inflammatory response syndrome</td>
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<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
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CHAPTER 1

Literature Review

1.0 Introduction

Sepsis, the systemic inflammatory response to infection, is the most common cause of death in people hospitalized in intensive care units (ICUs). Sepsis also frequently results in significant morbidity and mortality among critically ill dogs, with only 50% surviving to hospital discharge. (Kenney et al 2010) Early diagnosis of infection is crucial to the appropriate management of sepsis, with early antibiotic administration consistently resulting in improved outcomes. (Dellinger et al 2013) However, the clinical signs of sepsis are not specific for infection, and confirmation using traditional diagnostic tests can take several days. In people, biomarkers are now routinely used to rapidly diagnose sepsis and justify antibiotic use. (Wacker et al 2013) In this review, the epidemiology and immunopathophysiology of sepsis will be discussed. Traditional diagnostic tests, including bacterial culture, antimicrobial susceptibility, cytologic evaluation of fluid or tissue samples, and serology will be reviewed, followed by a discussion surrounding the utility of biomarkers. Cytokines that have previously been evaluated in dogs, as well as the novel biomarkers procalcitonin (PCT) and amino-terminal pro-C-type natriuretic peptide (NT-proCNP), will be discussed in the context of their potential to aid in the diagnosis of sepsis. In addition, a model of endotoxemia in dogs will be reviewed as a controlled method for studying the inflammatory and early innate immune response to a bacterial component, and finally multiplex magnetic bead-based assays will be described as a potential means of biomarker investigation.
1.1 The Epidemiology and Immunopathophysiology of Sepsis

1.1.1 Definitions

Critically ill dogs often experience a pronounced and potentially detrimental systemic inflammatory response syndrome (SIRS), characterized by at least two of the following criteria: hypothermia or hyperthermia, tachycardia, tachypnea, leukopenia or leukocytosis, and elevated band neutrophil concentration (Table 1.1). (Hauptman et al 1997, De Laforcade 2007)

<table>
<thead>
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<th>Parameter</th>
<th>Values Consistent with SIRS</th>
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<td>&lt; 38°C or &gt; 39.2°C</td>
</tr>
<tr>
<td>Heart rate</td>
<td>&gt; 120 bpm</td>
</tr>
<tr>
<td>Respiratory rate</td>
<td>&gt; 20 breaths/min</td>
</tr>
<tr>
<td>Leukogram</td>
<td>WBC &lt; 6 or &gt; 16 x 10³/µL</td>
</tr>
<tr>
<td></td>
<td>&gt; 3% band neutrophils</td>
</tr>
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(Table 1.1: Systemic Inflammatory Response Syndrome (SIRS) Criteria in Dogs: Fulfillment of at least 2 of the 4 criteria constitutes a diagnosis of SIRS.

These clinical signs can result from a widespread inflammatory response to either an infectious or noninfectious insult. If untreated, this inflammation can lead to multiple organ failure and death. If no infection is detected, non-septic SIRS (NSIRS) is diagnosed; whereas, if the inflammatory response is because of a confirmed infection, sepsis is diagnosed. The cause of infection can be bacterial (gram-positive or -negative), viral, protozoal, or fungal. For the purpose of this review, only bacterial causes of sepsis will be considered. When sepsis is complicated by single or multiple organ dysfunction, as well as hypoperfusion or hypotension, it is termed severe sepsis. Septic shock occurs when the response to infection results in acute circulatory failure and persistent arterial hypotension despite intravascular fluids administered at
resuscitation rates.(De Laforcade 2007, Bone et al 1992)

1.1.2 The Epidemiology of Sepsis


Sepsis secondary to severe bacterial infections can be particularly devastating in veterinary patients, with 50-70% of dogs either succumbing to the disease or being euthanized prior to the initiation of therapy due to the projected poor prognosis and high cost of treatment.(Kenney et al 2010, Hauptman et al 1997, Bentley et al 2007, Hosgood et al 1988, Winkler et al 2000, Staatz et al 2002) Disappointingly, mortality rates have failed to improve over the past several decades, despite the availability of new therapies to support dogs afflicted with this condition.(Bentley et al 2007) The variable survival rates reported among studies could reflect the complexity and heterogeneity of sepsis. Furthermore, although many factors have been evaluated as contributors to outcome, reliable prognostic indicators remain elusive.

An estimated 6-10% of dogs admitted to small animal referral practice are diagnosed with sepsis.(Otto 2007) Genetic variation might predispose certain breeds, such as Rottweilers and Doberman Pinschers, to a more severe inflammatory response to sepsis resulting in increased morbidity and mortality from parvoviral enteritis in comparison to other breeds.(Nemzek et al 2007) The most common causes of sepsis include peritonitis secondary to a ruptured abdominal
viscous, uterine infections, abscesses, prostatitis, and osteomyelitis. (Hauptman et al 1997) The type and location of infection often determine the presenting clinical signs and outcome is frequently associated with the severity of sepsis and consequential organ dysfunction. (Kenney et al 2010) Dogs that are critically ill and are hospitalized in an intensive care unit (ICU) are at particularly high risk for mortality, either due to infection related to their primary disease process, or as a result of a hospital-acquired infection with potentially multi-drug resistant bacteria. (Staatz et al 2002) In people, gram-positive infections are more common that gram-negative infections. (Martin et al 2003) however, this difference has not been well characterized in veterinary medicine.

Because there is so much variability in causes of sepsis, and hence also in the manifestation and individual responses, many physical exam and routine biochemical parameters are neither sensitive nor specific for the diagnosis of this condition. (Otto 2007) Investigation into biomarkers able to aid in the diagnosis and treatment of sepsis could therefore be a step towards decreasing the morbidity and mortality associated with this devastating condition. In order to identify biomarkers that warrant examination, thorough understanding of the physiological response to inflammation and sepsis is essential.

1.1.3 The Immunopathophysiology of Sepsis

Although a complete review of the inflammatory pathways and early innate immune response during sepsis is beyond the scope of this review, the role of cytokines will be discussed to provide a context for biomarker development.

Proliferation of microorganisms within a nidus of infection can either result in invasion of the bloodstream by the microbe itself, or in situ proliferation with subsequent release of components into the bloodstream. These may consist of structural components such as
endotoxin or teichoic acid from gram-negative or gram-positive bacteria, respectively, or exotoxins that are synthesized by the pathogen. These substances lead to the activation of monocytes, macrophages, endothelial cells and neutrophils, resulting in the release of mediators that initiate and sustain SIRS and sepsis (Figure 1.1). (Parrillo 1993, Lewis et al 2012)

Figure 1.1: The immunopathophysiology of sepsis. TSST-1 = toxic shock syndrome toxin 1; Toxin A = Pseudomonas aeruginosa toxin A; TNF-α = tumor necrosis factor-α; IL = interleukin; PAF = platelet-activating factor; NO = nitric oxide; COX = cyclooxygenase; LOX = lipoxygenase; PGE = prostaglandin; LT = leukotriene; CNS = central nervous system. Adapted from Parrillo JE, N Engl J Med 1993; 328 (20): 1471-1477.
SIRS stems from the body’s response to injury or infection. The inflammation becomes deleterious when it persists, is exuberant, or accelerates to systemic effect, rather than remaining at the site of infection or injury. (Smith et al 2006) After infection, the innate immune system mounts an initial host response. Types of organisms (such as bacteria versus fungi) frequently have exclusive or preferred use of certain pattern recognition receptors, such as Toll-like receptors (TLRs) on immune system cells. (Basset et al 2003, Akira et al 2003, Lewis et al 2012) Interaction with TLRs is a common initiator of subsequent adaptive immune activation, which within days to weeks provides a host response with high specificity for a wide range of foreign antigens.

The innate immune response during inflammation and infection is mediated through the complex interaction of cytokines, neutrophils, monocytes, macrophages, and natural killer (NK) cells. The major pro-inflammatory mediators of SIRS include tumor necrosis factor (TNF)-α, interleukin (IL)-1, IL-6 and IL-8, which signal neutrophils, the first and predominant cells to respond to an insult. (Lewis et al 2012) However, during severe SIRS, neutrophils can experience decreased chemotaxis, decreased phagocytic ability, and alterations in their ability to release free oxygen radicals due to the down-regulation and increased internalization of the chemokine receptors (CXCR)-1 and -2, which bind IL-8, keratinocyte-derived chemokine (KC), and macrophage inflammatory protein (MIP)-2. (Tarlowe et al 2004, Bjornson et al 1993, Wagner et al 1999) The result is decreased intracellular killing of bacteria and an increased risk of sepsis via bacterial translocation from the gastrointestinal tract. (Faist 1996, Wagner et al 1999, Gosain et al 2005)

Upon stimulation, NK cells promote inflammation and monocyte activation through the release of interferon (IFN)-γ and IL-12. (Goldmann et al 2005) Both NK and NKT cells also
secrete IL-3 and granulocyte macrophage colony-stimulating factor (GM-CSF). Consequently, microbial substances and pro-inflammatory cytokines activate the major antigen presenting cells (APCs) of the innate immune system, resulting in the development of immune tolerance. (Stein et al 1984, Hotchkiss et al 2002) Macrophages are also primed and activated by IFN-γ, TNF-α, and bacterial products such as lipopolysaccharide (LPS), providing a further bridge between the innate and adaptive immune systems via antigen presentation and the production of cytokines that polarize T-cells. (Ma et al 2003) Macrophage cytokine release is normally controlled by negative feedback mechanisms mediated in part by prostaglandin E2 (PGE2). However, with severe inflammation, sensitivity to PGE2 might be diminished resulting in exuberant production of IL-6 and TNF-α, leading to immune dysregulation and bone marrow suppression. (Deitch 1992, Langrehr et al 1993, Schwacha et al 1998)

Innate immune system cells, such as APCs, direct the adaptive immune response to inflammation and infection through T cell activation. During the initial SIRS response, the T-helper (Th)1 phenotype of T-helper cells predominates, resulting in the production of IL-12, IFN-γ and TNF-α. Approximately 24-72 hours later, there is a shift to Th2 cell differentiation with a cytokine profile of enhanced IL-4, IL-5, IL-6, IL-10, IL-13, and GM-CSF secretion. (Smith et al 2006) Although this shift might protect against excessive inflammation during late SIRS, the latter Th2 profile has also been associated with decreased antigen presentation and immunosuppression. (Smith et al 2006)

A second adaptive response to control excessive inflammation, known as the compensatory anti-inflammatory response syndrome (CARS), occurs simultaneously with SIRS to decrease antigen presentation and reduce inflammatory cytotoxic cellular responses. (Bone 1996, O'Sullivan et al 1995, Lewis et al 2012) During this phase, an increase in anti-
inflammatory cytokines and mediators such as IL-10, PGE<sub>2</sub> and transforming growth factor (TGF)-β, as well as down-regulation of IFN-γ, limit the negative effects of inflammation on organ function. (Schwacha 2003) While CARS can be protective because it limits inflammation, this response can also lead to a decreased cell-mediated response, which can predispose patients with SIRS to further infection, sepsis, multiple organ dysfunction and death. (Pellegrini et al 2000, Aziz et al 2013) Overall, the balance between the inflammatory and anti-inflammatory pathways during SIRS and sepsis is coordinated by interaction of the immune and neuroendocrine systems. (Deutschman et al 2014) Dysfunction in either of these systems can lead to a disruption in homeostasis and can result in excessive, deleterious inflammation. (Deutschman et al 2014)

Meanwhile, complement activation is also required for effective innate host defense mechanisms through formation of the membrane attack complex (MAC) and the anaphylatoxins C3a, C4a and C5a that lead to lysis and death of target cells. However, these mediators can also cause endothelial cell activation and subsequent neutrophil recruitment that results in organ dysfunction. (Zilow et al 1990) For example, complement-mediated increased vascular permeability in the lung can result in acute respiratory distress syndrome (ARDS), which has a high morbidity and mortality. (Zilow et al 1990) Similarly, endothelial injury due to either complement or extracellular histones can cause hemodynamic instability due to decreased systemic vascular resistance, decreased cardiac output, and altered vascular integrity. (Schirmer et al 1989, Solomkin 1990, Xu et al 2009) The latter is associated with the development of edema and both macrovascular and microvascular complications, including large vessel thrombosis and disseminated intravascular coagulation (DIC) that can reduce perfusion to organ systems leading to multiple organ dysfunction syndrome (MODS). An overview of immune dysfunction leading to MODS during sepsis is presented in figure 1.2.
In light of the potentially devastating consequences of sepsis, identification of the disease process in its early stages might allow more rapid support and effective modification of the inflammatory response.
Figure 1.2: Immune dysfunction during sepsis.

TNF-α = tumor necrosis factor-α; IL = interleukin; PMN = polymorphonuclear cells; NK = natural killer; Th = T helper; PGE = prostaglandin. Adapted from Smith JW, et al. J Intensiv Care Med 2006.
1.2 Endotoxemia as a Model for Sepsis in Dogs

Models developed for the investigation of sepsis include: intravascular infusion of endotoxin or live bacteria, induction of bacterial peritonitis, cecal ligation and perforation, soft tissue infection, and inoculation with *Staphylococcus spp.* to produce pneumonia or meningitis. (Garrido *et al* 2004, Zanotti-Cavazzoni *et al* 2009, Freise *et al* 2001) Although models have limitations, they remain central to developing a better understanding of the pathophysiology of sepsis, as well as the benefits of medications, fluids, and immunomodulatory treatment modalities. Endotoxemia was the model used for the present study, and will therefore be the only model reviewed here.

1.2.1 Lipopolysaccharide (LPS)

Endotoxin, also known as lipopolysaccharide (LPS), is the predominant component of the cell wall of gram-negative bacterial organisms. It is composed of a hydrophobic lipid (lipid A) covalently bound to a hydrophilic core polysaccharide chain and a hydrophilic O-antigenic polysaccharide side chain (figure 1.3). (Raetz *et al* 2002) Variability in the length of the polysaccharide chain, the individual sugar molecules it contains, and the nature and size of the lipid chains of an LPS molecule all contribute to the ability of LPS to protect the bacteria against destruction by host bile salts and lipophilic antibiotics. (Raetz *et al* 2002)
Lipopolysaccharide is a molecule that can be stored as a purified and stable lyophilized product, allowing accurate dosing after reconstitution with saline. (Garrido et al 2004) It is commercially available, standardized, and safe to handle. (Fink et al 1990) These properties confer simplicity and consistency to endotoxemia as a model for sepsis.

### 1.2.2 Role of LPS in inflammation and sepsis

The LPS portion of the gram-negative bacterial cell membrane initiates the immunoinflammatory response by binding to cell surface TLRs. This class of receptors is responsible for the recognition of pathogenic organisms; each subset is able to identify distinct pathogen-associated molecular patterns (PAMPs). After infection with gram-negative bacteria, LPS is bound in circulation by LPS binding protein (LBP). Cell surface receptor CD14 then interacts with LBP to activate TLR4 and initiate a cascade of phospholipases and protein kinases that lead to activation of the transcription factor nuclear factor kappa B (NF-κB). The result is an

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**Figure 1.3: Structure of lipopolysaccharide.**

Cytokine-induced changes in both adhesion and migration can influence the chemotactic response of neutrophils in the presence of LPS. (Maeda et al 2010, Wagner et al 1999, Lynn et al 1991, Detmers et al 1994) The overall effect of LPS on granulocyte function appears to be dose-dependent and potentiated by other inflammatory mediators, including GM-CSF and TNF-α. (Maeda et al 2010) A summary of the effects of LPS on white blood cells, in particular neutrophils, is presented in figure 1.5.

Figure 1.4: Transcription of inflammatory mediator genes via LPS activation. LPS = lipopolysaccharide; TLR4 = toll-like receptor 4; PI3K = phosphoinositide 3-kinase; PDK = 3-phosphoinositide-dependent kinase; AKT = AKT8 virus oncogene cellular homolog; IκB = inhibitor of NF-κB; NF-κB = nuclear factor kappa B; IL = interleukin; TNF = tumor necrosis factor; P = phosphate moiety; PIP = phosphatidylinositol phosphate.
1.2.3 Cytokine profile after bolus LPS administration in dogs

Although LPS exposure produces neutrophils that are hyperadhesive, hyperoxidative and deficient in their ability to migrate, endotoxemia also results in rapid up-regulation of many additional cytokines, chemokines, and inflammatory mediators that contribute to altered neutrophil function (figure 1.5). One of the earliest effectors of inflammation is TNF-α; this

Figure 1.5: Effects of LPS on neutrophils. LPS = lipopolysaccharide; TNF-α = tumor necrosis factor-α; IL = interleukin; PAF = platelet activating factor; CSF = colony-stimulating factor; MIP = macrophage inflammatory protein; CINC = cytokine-induced neutrophil chemoattractant; PGE = prostaglandin; NO = nitric oxide. Adapted from Wagner JG and Roth RA. J Leukoc Biol 1999; 66 (1): 10-24.
cytokine is produced in large quantities by macrophages, appearing in plasma within 30 minutes of LPS administration. (Miyamoto et al 1996, Wagner et al 1999) Peak TNF-α concentration occurs after 1-2 hours, while a short half-life and inhibition by other circulating cytokines results in its disappearance 6 hours post-LPS administration. (Tracey et al 1993, Miyamoto et al 1996, Wagner et al 1999, Song et al 2012)

Secondary intermediate effectors are then synthesized in response to both TNF-α and LPS; these include the proinflammatory cytokines IL-1 and IL-6, chemokine IL-8, and the anti-inflammatory mediator IL-10. (Wagner et al 1999) In parallel with TNF-α, IL-1 increases in serum within 30-60 minutes of LPS administration, peaks after approximately 1.5 hours, and disappears within 6 hours of the onset of endotoxemia. (Miyamoto et al 1996) In contrast, although IL-6 concentrations are elevated within 1 hour of LPS administration and peak at 1.5-3 hours, this cytokine does not begin to disappear until 24 hours have elapsed. (Miyamoto et al 1996, Song et al 2012) Concurrently, neutrophil chemoattractant IL-8 begins to increase within 1 hour and peaks within 2-3 hours of LPS administration. (Martich et al 1991) In addition, activated macrophages produce granulocyte-colony stimulating factors (G-CSFs), resulting in peak activity approximately 2 hours post-LPS administration and contributing to the neutrophilia which can be observed at 4-6 hours. (Kuhns et al 1995) The anti-inflammatory mediator IL-10 increases in plasma within 1 hour of LPS administration and peaks by 3 hours, in a dose-dependent manner. (Wagner et al 1999, Ogasawara et al 2012) This cytokine is partially responsible for the short half-life of early inflammatory mediators, whose production is turned off by IL-10’s activation of cAMP pathways for at least 48 hours. (Wang et al 1994, Ogasawara et al 2012) The normal anti-inflammatory response is therefore augmented during endotoxemia in order to control the systemic inflammatory response.
1.2.4 Lipopolysaccharide administration as a model for sepsis in dogs

In people, elevated concentrations of serum endotoxin are associated with the development of sepsis and with an increased incidence of mortality; up to 75% of people with sepsis in an ICU setting have detectable levels of serum endotoxin.(Fink et al 1990, Parker et al 2001) However, different species have marked variation in their sensitivity and susceptibility to LPS; for instance dogs, cats and rodents are relatively resistant to endotoxin, requiring doses of approximately 2-5 mg/kg intravenously (IV) to produce circulatory collapse, whereas humans, other primates, rabbits and sheep are quite sensitive to the effects of endotoxin, experiencing the same clinical signs at doses of less than 1 mg/kg.(Fink et al 1990, Michie 1998) Consequently, much higher doses of LPS are required to model sepsis in dogs when compared to the natural process in people.

Intra-species variation might also result in differing experimental results; LPS responsiveness is reported to be markedly discrepant between individual dogs.(Ogasawara et al 2012) For instance, the inflammatory response to endotoxemia is altered by advanced age, known in humans as immunoaging;(Egorina et al 2005, Angus et al 2001, Ernoffson et al 1996, Osterud et al 1982) this could be one of the reasons why geriatric dogs are more likely to succumb to endotoxemia and sepsis.(Deitschel et al 2010) In dogs, an additional layer of complexity might reside in the reported immune dysfunction noted in breeds such as Rottweilers and Doberman Pinschers during critical illness and sepsis.(Nemzek et al 2007) All of these factors need to be considered when translating an experimental endotoxemia model to clinical trials in dogs.

Endotoxin dose selection is also essential when comparing and interpreting study results. At LPS bolus doses of 2-5 mg/kg IV, dogs experience a potentially fatal severe hypodynamic
state of shock, characterized by rapid decreases in arterial blood pressure, cardiac output, systemic vascular resistance and hepatic blood flow, in conjunction with an increase in serum lactate concentration. (Spapen et al 1999) This is in contrast to an initial hyperdynamic phase, which is more commonly observed during the development of naturally occurring sepsis. In order to mimic this hemodynamic variability, smaller boluses of 2 µg/kg IV have been administered to dogs. This dose is capable of reproducing the hyperdynamic phase typical of early sepsis, which is characterized by an increase in cardiac output to counterbalance low systemic vascular resistance in an attempt to maintain normotension. (Holowaychuk et al 2012, LeMay et al 1990) This sublethal dose also typically results in lethargy and mild gastrointestinal upset (vomiting and diarrhea) within 30 minutes of administration. Within 1 hour, tachypnea, tachycardia and hypotension can develop, to the point of hemodynamic instability requiring resuscitation with intravenous fluid therapy. Fever develops within the first 2-4 hours. However, these signs are transient, with the lethargy and vital signs normalizing after approximately 6 hours, and a complete return to normal activity level 24 hours after LPS administration. (Holowaychuk et al 2012, Song et al 2012) Infusions of 1-10 µg/kg/hr IV for 8-24 hours in healthy adult beagles produced a hyperdynamic phase and have been useful in demonstrating the importance of maintaining or improving hepatosplanchnic blood flow and intestinal tissue oxygenation during sepsis. (Sakaue et al 2004, Yu et al 2012) These low-dose infusions of LPS result in a rapid increase in cardiac index within 1 hour, which continues and peaks at 2 hours, then gradually decreases. (Sakaue et al 2004, Yu et al 2012) In addition, mean arterial and pulmonary artery pressures experience a nadir at 2 hours after the initiation of a low-dose LPS infusion, normalizing again at 8 hours. (Sakaue et al 2004, Yu et al 2012) Dose selection is therefore an integral aspect of using endotoxemia as a model for sepsis.
In relation to dose, the bacterial strain from which LPS is derived plays an important role in the degree of endotoxemia induced. Different types and strains of bacteria contain variable amounts of endotoxin, measured in endotoxin units. Because the size of the endotoxin molecule varies greatly due to polymorphisms within the O-chain, and because different degrees of acylation within the lipid A component affect its biological activity, the use of endotoxin units permits standardization of LPS dosing. (Mueller et al. 2004)

As with any model of disease, using endotoxemia as a model for naturally occurring sepsis has some limitations. First, LPS is not a component of gram-positive bacteria; therefore, this model might only reflect processes involved in gram-negative sepsis, despite similar immune responses and mortality rates occurring from either cause of infection. (Opal et al. 1999) Second, LPS represents only one component of gram-negative bacteria. It is likely that other cell wall and bacterial components also contribute to the SIRS response to infection. Finally, intravenous LPS administration does not produce a local infection nor does it induce a sustained immunologic response. As a result, the complex local and systemic pro- and anti-inflammatory cytokine responses produced are often exaggerated and not a true reflection of naturally occurring sepsis. (Friese et al. 2001)

While using LPS administration to model sepsis in dogs is a controlled and standardized method of reproducing the systemic challenge imposed by gram negative bacterial products without creating an infectious focus, the transient endotoxemia is unable to completely reproduce the complex and more prolonged physiological response to viable microbes during sepsis.
1.3 Traditional Diagnostic Tests Employed to Confirm Sepsis

Early diagnosis of infection is essential for the appropriate management of sepsis, as it allows rapid administration of appropriate antibiotics resulting in improved outcomes. (Dellinger et al 2012) Although the SIRS criteria are helpful for identifying inflammation in critically ill dogs, clinical signs such as fever and an elevated white blood cell count are not specific for infection. (Hauptman et al 1997) Additional diagnostic tests are, therefore, required to identify patients with sepsis.

1.3.1 Bacterial culture and antimicrobial susceptibility

According to the Surviving Sepsis Campaign guidelines, appropriate cultures should be obtained prior to the initiation of antimicrobial therapy, as long as obtaining the cultures does not delay the administration of medications by more than 45 minutes. (Dellinger et al 2013) Ideally, both aerobic and anaerobic samples are obtained. At least one sample should be a percutaneously drawn blood culture and all indwelling intravenous and urinary catheters in place for more than 48 hours should be sampled. (Dellinger et al 2013) Urine, wounds, cerebrospinal fluid (CSF), respiratory secretions, or any other fluids that could provide evidence of a bacterial source of infection should also be cultured. (Dellinger et al 2013) Cultures permit identification of the type of bacteria, while antimicrobial susceptibility profiles provide information that enables de-escalation of antimicrobial therapy upon obtaining the results. Quantitative (or semi-quantitative) cultures provide the additional benefit of potentially identifying the original bacterial source if various sites are compared. (Blot et al 1998)

While cultures are considered a gold standard for the identification of bacterial sepsis, timing of sample acquisition is essential. Depending on the organism and the antimicrobial used, sterilization of blood and fluid cultures can occur within hours of therapy, thereby decreasing the
sensitivity of this test. (Dellinger et al 2013) In addition, some organisms are fastidious or can cause disease even when present only in low numbers, requiring enrichment in liquid media prior to blood agar inoculation. Blood culture collection medium provides this enrichment; however, if this medium is not selected, then some organisms might not be identified. (Greene 2012) Ultimately, a culture requires a minimum of 24 hours of incubation in order to detect bacterial growth. After this period of time, preliminary results can provide additional diagnostic clues; however, confirmation of the bacterial species and its antimicrobial susceptibility profile is generally not available until 2-3 days after plating of the sample. This delay poses many challenges regarding appropriate antimicrobial selection and their timely administration in cases of severe sepsis and septic shock, where mortality is high.

1.3.2 Cytologic evaluation of fluid or tissue samples

Cytologic evaluation of fluid or tissue samples is a rapid and cost effective test that provides the benefit of immediately recognizing the presence of bacteria, or deducing its presence based on degenerative neutrophils. A Gram stain can be useful if a lot of bacteria are present to suggest whether a bacterial culture will be fruitful, help distinguish major classes of microbes, and provide additional information about the likely predominant organisms within a mixed infection, to aid making an appropriate empiric antibiotic selection. (Dellinger et al 2013, Greene 2012)

Nevertheless, like bacterial culture, cytologic evaluation is not 100% sensitive. A minimum concentration must be present for organisms to be seen in a smear. (Greene 2012) Consequently, false negative results are possible if low numbers of bacteria are present due to the type of sample (such as CSF) being examined. Alternatively, prior administration of effective antibiotics can produce a negative result. In addition, some bacteria cannot be readily identified
using a Gram stain. For instance, *Leptospira* and other spirochetes require the use of dark-field microscopy because they stain poorly and have both Gram positive and negative characteristics, and therefore can be missed during routine cytologic sample evaluation. (Greene 2012) Finally, many potential artifacts can cause either false positive or negative results by interfering with interpretation; mucus and proteinaceous matter can hide the presence of slender, weakly staining bacilli, and debris within the stain itself can look similar to Gram positive cocci. (Greene 2012)

### 1.3.3 Serology and molecular detection of organisms

For some organisms that are difficult to isolate or culture, such as *Leptospira* or tick-borne agents, identification of antigen using molecular techniques such as PCR, or a systemic antibody response using serology, can be beneficial in establishing a diagnosis. Nucleic acid amplification can also be used to differentiate toxicogenic from non-toxicogenic strains of *Escherichia coli* and *Clostridium perfringens* more accurately than any other method. (Greene 2012)

While PCR methods are extremely sensitive, they can be fraught with false positives due to sample contamination with DNA present in the laboratory environment; they are also expensive and are not always readily available within a reasonable turnaround time. In addition, the presence of microbial DNA does not confer viability of the organism. (Greene 2012) Serology overcomes this last hurdle because it measures a rise in antibody titer in response to an infection. Nevertheless, a true change in titer can only be appreciated if acute and convalescent titers are compared. (Greene 2012) The 2 weeks required between these sampling periods means that not all patients will survive long enough to confirm a diagnosis.

Ultimately, because cytological confirmation of infection is not always possible, and since culture results are hampered by a delay of at least 24 hours, clinical intuition is often used
in the emergency setting to determine whether to initiate empiric antimicrobial therapy. Consequently, a rapid and reliable surrogate diagnostic marker for infection is needed. This test would assist veterinarians in identifying patients with sepsis, thereby facilitating early initiation and discriminant use of antibiotics.

1.4 Biomarkers of Sepsis

A biological marker, or biomarker, refers to any objective measurement able to indicate a state of health or disease accurately and reproducibly. According to the National Institutes of Health (NIH) Biomarkers Definitions Working Group, a biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”. (Biomarkers 2001) A biomarker can be any substance, structure, or process that can be measured in the body, and can be physiological or biochemical at the cellular or molecular level. This broadly encompasses everything from vital signs and routine hematology or biochemistry testing, to specific and advanced laboratory tests. (Strimbu et al 2010)

Individual prognostic biomarkers are advantageous because they can guide and inform the decisions of clinicians and pet owners. Although population-based biomarkers should not be used for this same purpose, they offer the ability to classify and compare the severity of disease in dogs across multiple centers and different investigations for research purposes. Biomarkers can, therefore, strengthen both our understanding of disease, and improve clinical judgment and therapy. Unfortunately, few reliable biomarkers for sepsis in dogs exist at this time.
1.5 Procalcitonin

Procalcitonin (PCT) was first identified as a prohormone in the 1970s, when the hormone calcitonin (CT) was noted to be heterogeneous.(Roos et al 1974, Deftos et al 1975, Moya et al 1975, Goltzman et al 1978, Goodman et al 1979) The structure of PCT was determined nearly a decade later by Jacobs et al.(Jacobs et al 1981) Because the physiologic role of PCT was poorly understood, there was minimal scientific interest in further examining this protein until the early 1990s when elevations in PCT concentration were first identified during human sepsis using a monoclonal immunoradiometric assay for CT precursors.(Assicot et al 1993) Since then, studies in people suggest a potential role for PCT in modulating inflammation.(Schuetz et al 2011)

1.5.1 The PCT protein

In health, PCT is produced solely within thyroidal C-cells as a CT precursor. C-cells are stimulated by hypercalcemia, β-adrenergic stimulation, and increased concentrations of glucocorticoids, calcitonin gene-related peptides (CGRP), glucagon, and gastrin. Conversely, C-cells are suppressed by vitamin D and somatostatin.(Maruna et al 2000)

amino acids (a 25 amino acid hydrophobic signal peptide followed by the 116 amino acids of PCT) and CGRP. The preprocalcitonin transcript is likely the default pathway.\cite{Russwurm1999, Meisner2002} Once the signal peptide is cleaved, the 116 amino acid PCT product is a prohormone weighing approximately 13–14 kDa (figure 1.6).\cite{Russwurm1999, Giunti2010, Wanner2000, Jensen2006, Maruna2000, Becker2008, Meisner2009, Schneider2007}

Figure 1.6: Structure and processing of PCT. PCT, procalcitonin; CT, calcitonin; CCP-1, calcitonin carboxypeptide-1 (also known as katacalcin); PAM, peptidylglycine α-amidating monooxygenase. Adapted from Jin M and Khan AI. LabMedicine 2010; 41: 173-177.
1.5.2 Source and metabolism of PCT

Under the direction of the signal peptide sequence, PCT undergoes glycosylation within C-cells prior to entering a secretory pathway. (Russwurm et al. 1999) Translation proteolysis of the primary preprocalcitonin transcript results in the cleavage of the signal sequence from PCT by endopeptidase, leading to the formation of three peptides: the 57 amino acid amino-terminal fragment (N-PCT), the 32 amino acid central immature CT molecule, and the 21 amino acid carboxyterminus peptide I (CCP-I), previously referred to as katacalcin. (LeMoullec et al. 1984, Becker et al. 2004, Russwurm et al. 1999, Maruna et al. 2000)

Although the C-cells of the thyroid produce PCT constitutively, mature CT is the only post-translational fragment that is eventually secreted. (Russwurm et al. 1999) If PCT escapes normal intracellular proteolysis and is not converted to CT, then it can be released into the bloodstream where there are no plasma proteolytic enzymes available to degrade it. The half-life of human PCT in circulation is 25-30 hours. (Maruna et al. 2000) Deamidation resulting in activation of circulating PCT can then occur in the presence of inflammatory mediators and endotoxin, via peptidases located on both epithelial and endothelial cell surfaces. (Meisner 2002)

1.5.3 Similarity of PCT across species

The hydrophobic signal sequence of the preprocalcitonin molecule is highly conserved across various species, and enables the detection of PCT in plasma. Two cysteine residues within PCT form a disulfide bridge that is essential to CT receptor binding; these residues are therefore also highly conserved across species. (Meisner 2002, Russwurm et al. 1999) Beyond these key similarities, other regions of the PCT peptide show varying levels of homology between species; in comparison to the human amino acid sequence, similarity is 74% in rats and mice, 60% in sheep, 45% in chickens, and 43% in salmon. (Russwurm et al. 1999, Meisner 2002)
Upon sequencing canine PCT, Mol et al estimated approximately 67% homology to the human peptide, with large differences noted in the CCP-1 (or katacalcin) region; canine PCT was more closely related to the same prohormone in porcine, bovine and ovine species. (Mol et al 1991)

Commercially available PCT assays designed for humans were unable to differentiate dogs with septic and non-septic SIRS as classified based on bacterial culture results. (Giunti et al 2006) This was not surprising, as the antibodies used in these assays are specific to the C-terminal katacalcin region of human PCT. (Schneider et al 2007) This region of the PCT molecule is only 8 amino acids in the dog, but 21 amino acids in humans. (Mol et al 1991) An alternative test for PCT in dogs is therefore required for this biomarker to be useful in this species.

1.5.4 PCT as a marker and mediator of sepsis in humans

With the discovery of elevated serum PCT concentrations during sepsis, the question of the role of this prohormone was raised. (Assicot et al 1993) Suggestions that PCT inhibited the synthesis of PGE and thromboxane B₂ (TXB), and dampened the release of TNF-a and IL-6 in response to LPS stimulation, implied a potential immunomodulating role. (Russwurm et al 1999, Schmidt et al 1997) Additionally, infection in thyroidectomized patients still resulted in elevated PCT concentrations, suggesting that inflammatory and sepsis-mediated PCT was produced from a non-thyroidal source. (Nishikura 1999) A hypothesis that PCT follows classical hormone expression from the thyroid in health, but can also be stimulated to result in cytokine-like ubiquitous expression during sepsis led to its classification as a ‘hormokine’. (Muller et al 2001, Christ-Crain et al 2005) After over 20 years of research, it is now known that the source and regulation of PCT during inflammation and sepsis is very different from the presence and formation of this protein in health.
Currently, a trimodal pattern of PCT expression in neuroendocrine cells of the thyroid, monocytes, and parenchymal tissue, is suspected. (Linscheid et al 2004) Ubiquitous, non-thyroidal parenchymal tissues, such as adipose, release PCT when stimulated by adhered mononuclear cells. (Linscheid et al 2003, Linscheid et al 2004) Adhered and activated macrophages result in transient PCT elevations that rise within 3-5 hours of the onset of sepsis and disappear 18 hours later. (Monneret et al 1999, Linscheid et al 2004) Subsequently, sepsis-induced PCT likely binds to two groups of receptors: the calcitonin receptors (CR) and the calcitonin receptor-like receptors (CRLR), which differ from the receptors bound in health. (Meisner 2002) Despite years of research, the definitive physiologic role of PCT has not been determined; however, anti-inflammatory effects, (Schmidt et al 1997, Hoffmann et al 2001, Monneret et al 2000) pro-inflammatory effects, (Nylen et al 1998, Russwurm et al 1999) and pro-apoptotic effects have all been proposed. (Becker et al 2010)

Despite the knowledge gaps that remain with regards to the mechanism of action of PCT during sepsis, the kinetics of this hormokine in people have been well described. Procalcitonin rises within 4 hours of the diagnosis of naturally occurring sepsis, peaks at approximately 6 hours, and then plateaus over the following 8-24 hours. (Schneider et al 2007) These kinetics offer PCT an advantage as an early biomarker of sepsis when compared with C-reactive protein (CRP), another commonly used biomarker that does not begin to rise until 12-24 hours after the onset of sepsis, and remains elevated longer with a plateau between 20 to 72 hours. (Schneider et al 2007) In the absence of ongoing sepsis, PCT will normalize within 2-3 days of induction, making it potentially more useful in monitoring human patients than CRP, which remains elevated for 3-7 days. (Schneider et al 2007) In addition, the stability of PCT ensures that an early peak and fall is not missed, as is often the case with cytokines such as TNF-α and IL-6.
It is important to note that surgery, severe trauma, heat shock, burns, prolonged cardiogenic shock, severe SIRS and MODS have also been associated with elevations in PCT in people. (Meisner et al 2001, Chiesa et al 1998) Up-regulation of pro-inflammatory mediators in the absence of bacteria is likely the cause for this increase; however, in each of these patient categories, elimination kinetics of the hormokine show a rapid decline in PCT. Persistently elevated PCT concentrations can therefore be used to distinguish sepsis or septic complications from these other conditions. (Meisner 2002)

1.5.4.1 Available tests for PCT

Although a PCR was historically used to detect PCT, (Oberhoffer and Stonans 1999) currently variations of ELISA are the preferred method to quantify this prohormone. (Sauer et al 2012) An experimental chip sandwich ELISA has been developed to detect PCT in conjunction with other inflammatory markers, such as IL-6. (Sauer et al 2012) With a limit of detection of 0.31 µg/L and a coefficient of variation (CV) of 29%, this test utilizes two monoclonal anti-PCT antibodies to detect PCT in serum, plasma, and saliva, with serum samples providing the most accurate results. (Sauer et al 2012) In addition, several individual tests for PCT are commercially available and are already used within human hospitals across the world.

The BRAHMS PCT-Q® rapid assay is a semi-quantitative immunochromatographic test that provides a result within 30 minutes. Monoclonal mouse anti-katakalcin and polyclonal sheep anti-calcitonin antibodies capture PCT to result in a coloured band with an intensity that reflects the concentration of PCT in the sample. This semi-quantitative test is acceptable, because a PCT concentration of up to 0.1 µg/L is considered normal, whereas a greater than 10-fold increase (2-10 µg/L) is required for a diagnosis of severe sepsis. Although this is a rapid test that is useful at the bedside, the assay is unreliable in the presence of hemolysis. Conversely,
the BRAHMS PCT LIA® non-automated assay is a sandwich luminescence assay with two monoclonal antibodies; one antibody recognizes the C-terminal region of katacalcin (the anti-katacalcin antibody) and the other antibody recognizes the middle region of katacalcin (the anti-calcitonin antibody). This test requires a 1-hour incubation after which the intensity of light emitted is proportional to the sample PCT concentration. This test has a reported CV of 6-10%, a sensitivity of 95%, and a specificity of 100%. Recently, this manual test has been adapted to produce several automated assays, also by the manufacturer BRAHMS, that are capable of generating quantitative results for a PCT concentration within 30 minutes with the same degree of accuracy.a

The greatest challenge in evaluating the accuracy of these PCT ELISAs is that no gold standard differentiating infectious from non-infectious causes of inflammation exists. Although often used, disadvantages of bacterial culture as a gold standard include a lack of sensitivity and a 2-3 day delay prior to result reporting.(Muller et al 2006) Selection of a reference test therefore introduces potential bias to every PCT study; consequently, all of the analyses examining this relatively new test are inherently flawed to some degree.(Muller et al 2007) Nevertheless, serum PCT concentration elevations up to several thousand-fold have been consistently documented in humans with sepsis, helping to affirm the acceptance of the prohormone as a widely utilized test.

1.5.4.2 Diagnostic utility of PCT

In comparison to previously investigated biomarkers such as CRP, potential advantages of PCT as a diagnostic test for sepsis include: its absence in healthy individuals, rapid induction with the onset of sepsis, and relatively short half-life that makes daily monitoring to confirm the efficacy of therapy useful.(Schneider et al 2007) Various studies comparing the two proteins
have demonstrated conflicting results because of different cut-offs used and differences in disease severity; some have found CRP to be superior, (Simon et al 2004, Meisner et al 1999) or PCT to be superior, (Uzzan et al 2006, Uusitalo-Seppala et al 2011) or that they were equivalent. (Ugarte et al 1999, Suprin et al 2000, Bell et al 2003, Castelli et al 2004) Since PCT increases earlier in the onset of sepsis and has been found to be more specific for infection than CRP, the former is potentially a better indicator to detect the development of sepsis in the face of ongoing background inflammation in cases of major surgery, organ transplantation, cardiac arrest and other inflammatory conditions. (Castelli et al 2009, Jensen et al 2009, Schneider et al 2007) Similarly, when comparing PCT to cytokines, PCT is superior to both IL-6 and IL-8 in identifying critically ill patients with sepsis. (Rau et al 1997, Harbarth et al 2001, Tsantes et al 2013, Riedel and Carroll 2013) Additional biomarkers that could be valuable for the diagnosis of sepsis include soluble urokinase-type plasminogen activator receptor (suPAR) and proadrenomedullin (proADM). However, the diagnostic value of these novel biomarkers has not been as well defined when compared to either PCT or CRP. (Donadello et al 2012, Suberviola et al 2013)

Large clinical trials and meta-analyses have revealed inconsistent results, with the specificity of PCT declining in the context of immunocompromised patients and in the emergency department. (Bele et al 2011, Jones et al 2007, Uzzan et al 2006, Simon et al 2004) Some incongruity can be attributed to: ambiguous patient assignment from unreliable culture results due to contamination, or antibiotic use prior to obtaining the sample, small sample size studies where each serial PCT measurement is considered a single episode being compared to larger studies where a single patient is analyzed as one episode, and different assays with varying sensitivities used to determine different underlying illnesses. (Schneider et al 2007, Becker et al
Nevertheless a recent meta-analysis provided strong support for PCT as a reliable marker and diagnostic test in differentiating sepsis from non-septic SIRS in critically ill patients.(Wacker et al 2013)

Serum PCT concentration can also identify patients early in the development of septic complications from critical illness, trauma, or following abdominal surgery, and is superior to other biochemical parameters for this purpose.(Wanner et al 2000, Hoeboer et al 2013, Castelli et al 2009, Deng et al 2013) Readily available PCT tests can therefore be integrated into patient assessment and monitoring for dehiscence and sepsis in the emergent and post-operative setting, which are associated with high morbidity and mortality.(Rau et al 2004) Because earlier recognition of patients developing septic complications can lead to more aggressive treatment, PCT could be used to decrease the morbidity and mortality associated with such adverse events. Finally, the additional ability of serial PCT measurements to guide antimicrobial therapy, especially with respect to respiratory infections,(Nobre et al 2008, Christ-Crain et al 2004, Christ-Crain et al 2006, Briel et al 2005, Briel et al 2008, Stolz et al 2007, Schuetz et al 2009, Schuetz et al 2011, Charles et al 2009, Schroeder et al 2009, Jensen et al 2011) is one of the most interesting and applicable uses for this biomarker, but is beyond the scope of this review. However, it should be emphasized that using a hormokine (or other biomarker) as a diagnostic test is only one small portion of thorough patient assessment. Therapeutic decisions must be based on an overall clinical picture, including physical examination and other laboratory and diagnostic imaging findings.

1.5.5 Canine studies
Measurement of PCT is becoming part of the standard-of-care to rapidly diagnose sepsis, minimize morbidity and mortality, and decrease unnecessary antibiotic use in people.(Wacker et
An assay for PCT might have similar value in dogs as in people; however, extremely little is known about PCT in dogs due to the lack of a validated assay to measure canine PCT.

Procalcitonin mRNA expression in non-thyroidal tissue has been shown in dogs with sepsis and SIRS,(Kuzi et al 2008) but very little is known about serum PCT concentration in dogs because sequence differences with the human peptide preclude cross-reactivity of canine PCT with assays for human PCT.(Mol et al 1991, Giunti et al 2010) Accordingly, an existing ELISA for human PCT did not differentiate samples from dogs with sepsis versus non-septic SIRS.(Giunti et al 2010)

In dogs, a quantitative reverse transcription PCR (qRT-PCR) assay for PCT mRNA using whole blood was able to demonstrate significantly elevated gene expression of PCT in critically ill dogs in comparison to healthy controls.(Kuzi et al 2008) Although mRNA concentration was not associated with the type of disease (inflammatory, infectious, neoplastic, other disease), it was associated with clinical assessments of improvement or deterioration.(Kuzi et al 2008) Attempts to replicate this qRT-PCR and the results of this experiment have been unsuccessful, to date.(Holowaychuk, personal communication)

Extrathyroidal CALCA expression in dogs with SIRS has also been documented, with transcription predominantly in the lung, liver and spleen.(Giunti et al 2010) Multiple organ expression reflects the variety of diseases potentially underlying SIRS. However, this analysis of CALCA transcription was not quantitative, making it impossible to evaluate differential expression between dogs with sepsis and non-septic SIRS.

In collaboration with North Carolina State University, the gene for canine PCT was cloned, expressed, and the recombinant protein purified for use in generating a canine-specific quantitative PCT ELISA for clinical use. Nevertheless, developing an assay that demonstrates
appropriate sensitivity and specificity in differentiating sepsis from non-septic SIRS was not successful. (Holowaychuk, personal communication) Meanwhile, a commercially available canine PCT ELISA\textsuperscript{b} incorporating a polyclonal antibody to PCT has been marketed for the quantification of this canine peptide in a research setting; however, this kit has not been validated.

Based on the abundance of human medical literature regarding the clinical utility of measuring PCT, this protein remains a potential biomarker and therapeutic target warranting further investigation in septic dogs. (Holowaychuk \textit{et al} 2007, Silverstein \textit{et al} 2009) Although PCT showed promise in furthering our understanding, diagnosis, and management of sepsis, significantly more research and a reliable assay are required to better understand this hormokine.

\textbf{1.6 Amino-terminal pro-C-type natriuretic peptide}

Amino-terminal pro-C-type natriuretic peptide, a biomarker able to differentiate septic from non-septic causes of critical illness in people, is strongly associated with organ dysfunction and is a convincing predictor of mortality. (Hama \textit{et al} 1994, Bahrami \textit{et al} 2010, Koch \textit{et al} 2011) The concentration of this protein is also increased in dogs with sepsis compared to dogs with non-septic SIRS, and NT-proCNP shows promise as a biomarker in this species.

\textit{1.6.1 Source, activation and metabolism of CNP}

Natriuretic peptides are predominantly responsible for body fluid homeostasis and blood pressure control via the actions of the cardiac hormone atrial natriuretic peptide (ANP) and the neuropeptide brain natriuretic peptide (BNP). The third member of this group, C-type natriuretic peptide (CNP), has also been isolated from brain tissue. (Sudoh \textit{et al} 1990)

Initially, a 103 amino acid propeptide (proCNP) is synthesized. Cleavage of this precursor by the intracellular endoprotease furin results in two products: the biologically active
53 amino acid CNP peptide at the C-terminus (CNP-53), and the 50 amino acid N-terminus known as NT-proCNP. (Wu et al 2003, Prickett et al 2001) An unknown extracellular enzyme then cleaves CNP-53 to the 22 amino acid CNP-22, the predominant form found in plasma. (Stingo et al 1992) The 22 amino acids of CNP form a ring structure around a disulfide bond that is conserved within this family of peptides, resulting in a protein that weighs approximately 13 kDa, while NT-proCNP is a 5 kDa protein fragment (figure 1.7). (Minamino et al 1990, Suga et al 1992, Prickett et al 2001) Since CNP-53, CNP-22 and NT-proCNP are distributed mainly within the CNS, they were initially classified as neuropeptides. (Kojima et al 1990, Komatsu et al 1991)
Outside the CNS, bovine endothelial aortic cell culture stimulated with TGF-β induced CNP gene expression and produced transcripts detected by RT-PCR. (Suga et al 1992) Neither ANP nor BNP concentrations increased following stimulation, suggesting that TGF-β is both a potent and a specific stimulator of CNP production in endothelial cells. (Suga et al 1992) Similarly, LPS and TNF-α are also potent stimulators of CNP expression, suggesting a potential role for this cytokine during sepsis. (Hama et al 1994) Furthermore, inflammation can create a milieu where the monocyte/macrophage system becomes an additional source of CNP expression. (Kubo et al 2001) Other factors that influence the synthesis of CNP include...
Following its release, CNP acts as a local regulator of vascular endothelial function by binding and activating the ANP-B receptor, located within the CNS, as well as peripheral tissues such as blood vessels. Paracrine effects within the vasculature result in vasodilation via smooth muscle relaxation, as well as growth inhibition of smooth muscle cells. In addition, vascular endothelial cell proliferation is affected by the autocrine effects of CNP. Because CNP exerts its major effects within blood vessels, this cytokine can be considered a novel type of endothelium-derived relaxing factor (EDRF) and the central mediator of a ‘vascular natriuretic peptide system’. In addition, CNP inhibits microbial growth and modifies the pathogenicity of microorganisms. The multifaceted role of this peptide in the innate immune response to infection makes it particularly attractive as a possible biomarker for sepsis.

Due to its mainly paracrine activity, CNP is quickly internalized and degraded by lysosomes within surrounding tissues and then, like other natriuretic peptides, is renally excreted. Because NT-proCNP does not undergo the same degradation, this peptide is very stable in circulation and experiences a slower renal clearance rate.

1.6.2 NT-proCNP as a marker of sepsis

1.6.2.1 Available tests for CNP and NT-proCNP

The predominantly paracrine action of CNP, in addition to its rapid clearance, results in very low concentrations of this peptide in circulation. As a result, a preliminary extraction and
puriﬁcation process is generally required to concentrate a sample prior to radioimmunological
detection of CNP in people using a rabbit polyclonal anti-CNP antibody.(Del Ry and Maltinti
2005, Hama et al 1994) This necessary step requires relatively large sample volumes (2-3 mL)
and can increase analytic variability due to a lower than expected recovery yield.(Del Ry et al
2011)

In comparison to CNP, NT-proCNP is a smaller molecule with a longer half-life in
circulation, making it more readily detectable. In addition, NT-proCNP does not cross-react with
other natriuretic peptides, making it a more clinically useful biomarker than CNP.(Del Ry et al
2011, Prickett et al 2001, Palmer et al 2009) Since CNP and NT-proCNP are produced together
and secreted in equimolar amounts, NT-proCNP is a reliable surrogate for CNP that requires
only very small volumes of plasma (50-100 µL) for its quantiﬁcation.(Del Ry et al 2011)

A commercial sandwich ELISA has been developed and used to quantify NT-proCNP in
dog serum. This assay uses two highly puriﬁed sheep polyclonal antibodies; the first is directed
against amino acids 1-19 and the second is directed against amino acids 30-50 of human NT-
proCNP, a region that shares 96% homology with the canine peptide.6 (DeClue et al 2011)
Because there is no cross-reactivity with other natriuretic peptides, this NT-proCNP ELISA is
reported to be 100% speciﬁc.(Del Ry et al 2011) Depending on the study, CVs reported range
from 5-9% for intra-assay and 4-15% for inter-assay variability.(DeClue et al 2011, Del Ry et al
2011) This ELISA is simple to perform and readily available, permitting comparisons of results
across studies.

1.6.2.2 Diagnostic utility of CNP and NT-proCNP

Although still incompletely understood, the role of CNP as a vascular relaxing factor in
response to LPS and TNF-α suggests that this biomarker could contribute to the pathogenesis of
vasodilatory shock during sepsis. Patients with septic shock experience an approximately 10-fold increase in serum CNP, which is not seen with ANP or BNP concentrations, suggesting that the upregulation of CNP expression is unique among the family of natriuretic peptides in response to a septic insult. (Hama et al 1994, Koch et al 2011)

Due to a much lower clearance rate, circulating NT-proCNP concentrations are significantly higher than those of CNP, and are therefore easier to detect. Human patients experiencing severe multi-trauma, but not traumatic brain injury, have NT-proCNP concentrations that double or triple approximately 2-3 days prior to, and predict, the clinical onset of septic complications. (Bahrami et al 2010) Since one of the main sites of production and regulation of CNP is the CNS, it is possible that traumatic brain injury could disrupt the expression of this protein. The other main limitation of NT-proCNP as a biomarker for sepsis is that any pathology causing endothelial dysfunction could result in an increase in the synthesis of this peptide, while pathology causing decreased excretion could result in persistently elevated concentrations. Some conditions of note include causes of hypoxia, chronic kidney failure and congestive heart failure. (Wu et al 2003, Barr et al 1996, Cargill et al 1994, Del Ry et al 2005, Del Ry et al 2007, Del Ry et al 2008, Del Ry and Cabiati 2008, Nielsen et al 2008, Wei et al 1993) As a result, this biomarker must be used in conjunction with thorough patient assessment, and in light of other diagnostic tests.

**1.6.3 Use of NT-proCNP to diagnose sepsis in dogs**

The same commercial NT-proCNP ELISA used in the above human studies has also been investigated in dogs. (DeClue et al 2011) In a prospective clinical investigation, 112 dogs that presented to a teaching hospital were included if they met the SIRS criteria. (DeClue et al 2011) Dogs were classified as septic if they had evidence of an infection based on cytologic evaluation,
bacterial culture, histopathologic evaluation and/or serology, or as having NSIRS if there was no evidence of an infection based on these tests. (DeClue et al 2011) In this study, a cut-off value for NT-proCNP of 10.1 pmol/L had a 65.5% sensitivity and 89.2% specificity for differentiating dogs with naturally occurring sepsis from dogs with NSIRS, but serum NT-proCNP was unable to distinguish the NSIRS group from healthy dogs. (DeClue et al 2011) For reasons unknown, the subset of dogs with peritoneal sepsis had significantly lower serum NT-proCNP concentrations than dogs with other sources of sepsis. When this subgroup was removed from the analysis, the sensitivity of NT-proCNP to distinguish sepsis from NSIRS increased to 94%, while the specificity remained 89%. (DeClue et al 2011) No significant association between NT-proCNP concentration and survival was found. Although NT-proCNP might not be of prognostic value, its diagnostic ability could help improve outcomes by prompting more rapid identification of sepsis and more timely intervention with source control and antimicrobial therapy in dogs with nonperitoneal sources of sepsis.

To gain further understanding of the mechanism of NT-proCNP production in dogs, canine aortic endothelial cells were stimulated with various inflammatory mediators and bacterial products in a cell culture model. (Osterbur et al 2013) Significant elevations in NT-proCNP were measured after IL-1β, TNF-α and LPS administration in both a dose- and time-dependent manner, with peak production occurring at 24 hours. (Osterbur et al 2013) In contrast, significant NT-proCNP stimulation was not observed after administration of lipoteichoic acid peptidoglycan, IL-6, IL-10, IL-21, CXC chemokine ligand (CXCL)-8 (previously known as IL-8), IFN-γ, vascular endothelial growth factor (VEGF)-A or phosphate-buffered saline (PBS) control. (Osterbur et al 2013) These results are consistent with the previously defined role of
CNP in the innate immune system (Suga and Nakao 1992, Suga et al 1993) and support the use of IL-1β, TNF-α and LPS to further study the kinetics of NT-proCNP during sepsis.

To date, serum NT-proCNP has only been measured in dogs with naturally occurring sepsis upon hospital admission. In addition, other than the previously discussed cell culture model, no published studies investigating NT-proCNP concentrations in a controlled model of systemic inflammation or sepsis in dogs exist. Therefore, little is known about the serum concentration of this protein in dogs during the course of sepsis. Knowing how this biomarker changes after LPS administration in dogs could help clinicians interpret results and improve the clinical utility of this test.

1.7 Cytokines

The role of cytokines in sepsis and their potential utility as biomarkers for the diagnosis of this condition has been evaluated at length in humans. A complete synthesis of this topic is beyond the scope of this review. The focus will, therefore, be on research to date regarding cytokines and sepsis in dogs in veterinary medicine.

1.7.1 Tests used to measure cytokines

1.7.1.1 Cell kill bioassays

The primary cytokine quantified using cell kill bioassays is TNF-α due to its apoptotic effects; IL-6 is also occasionally measured. This methodology generally uses plasma to measure a cytokine’s activity. Sample handling is extremely important for these assays; whole blood samples must immediately be placed on ice and centrifuged, then the plasma must be immediately frozen in order to maintain cytokine activity. (DeClue et al 2008) During the assay, diluted plasma samples are added to mouse fibroblast cell lines that have been cultured in a 96-
well plate and incubated for approximately 24 hours. Surviving cell counts are then quantified using a colorimetric assay via standard curves established using murine recombinant TNF-α that convert the colorimetric absorbance to a cell count.(DeClue et al 2008, Sekut et al 1995, Baarsch et al 1991, Hogan et al 1991, Ruaux et al 1999) The fewer viable cells remaining, the more TNF-α activity there is in the sample.

Use of whole blood culture supernatant to quantify cytokine activity has been reported in dogs and other species.(Deitschel et al 2010, Sharp et al 2010, Figueiredo et al 2008, Damsgaard et al 2009) This methodology is more closely able to mimic in vivo physiologic conditions, including interactions with other elements such as leukocytes, other cytokines, growth factors, and mediators necessary for LPS activation.(Deitschel et al 2010, Damsgaard et al 2009, Mattsson et al 1994)

While cell kill bioassays have been widely used and validated, they can be cumbersome and have generally been replaced by ELISA analysis.

1.7.1.2 ELISA

Currently, single-protein ELISA analysis is commonly performed to quantify peptide concentrations in both research and clinical settings; in large laboratories this methodology is often automated.(Zangar et al 2006) For proteins present in low concentrations, sandwich ELISAs are used to increase sensitivity of assays. First, a capture antibody directed against one portion of the protein is applied to bind and concentrate the cytokine. Then, a second antibody directed against a different site on the protein is applied. This second antibody, known as the detection antibody, is tagged with either a fluorescent dye or an enzyme that can be quantified and used to measure the concentration of the protein of interest (figure 1.8). By using two high affinity antibodies, a sandwich ELISA is a very sensitive and specific assay.(Zangar et al 2006)
Canine-specific ELISA assays have been commercially developed for the measurement of several cytokines, including IL-6, IL-10, and IL-8. (Deitschel et al 2010, Dabrowski et al 2009, Mohamed et al 1997) These ELISAs can be performed using cell culture (including whole blood culture) supernatants, serum or plasma. (Deitschel et al 2010)

Many of these tests utilize the same kit designed to detect the cytokine in humans (e.g. many TNF-α ELISAs). Others have been adapted from human assays and their antibodies have been modified based on interspecies protein similarity (e.g. IL-6 and IL-10 ELISAs), while others still have been developed specifically for dogs (e.g. IL-8 ELISA). (Mohamed et al 1997)
Although commercial assays report detection limits, sensitivities, specificities, inter- and intra-assay CVs, and have been used repeatedly in veterinary studies, to the author’s knowledge, only the assays for IL-6 and IL-8 have published validation data for their use in dogs.(Rau et al 2007, Mohamed et al 1997)

1.7.2 Utility of cytokines for the diagnosis of sepsis and for prognosticating outcomes in dogs

Many cytokines and chemokines have been investigated as biomarkers for sepsis in dogs. Increases in serum TNF-α, measured via cell-kill bioassays, predict mortality in dogs with parvovirus,(Otto et al 1997) but not acute pancreatitis, pyometra, or naturally occurring sepsis.(Ruaux et al 1999, Fransson et al 2007, DeClue et al 2012) Measured alone, this cytokine is also unable to differentiate sepsis from NSIRS.(DeClue et al 2012) These findings could be the result of inappropriate sample selection because TNF-α concentrations are reported to be higher in peritoneal effusion than in serum in dogs with septic peritonitis,(Humm et al 2008) and because breed-related variation in TNF-α production exists.(Nemzek et al 2007)

Other cytokines previously measured via ELISA include IL-6, IL-10, and IL-8.(DeClue et al 2012) Although one study demonstrated that IL-6 plasma concentration was predictive of disease severity and mortality in dogs with SIRS and sepsis,(Rau et al 2007) more recently, plasma IL-6, IL-8 and IL-10 were unable to distinguish sepsis from NSIRS and were not predictive of survival to hospital discharge.(Fransson et al 2007, DeClue et al 2012)

While some of these cytokines show promise as biomarkers of sepsis, inconsistent results and the transient nature of some of these peptides have left the veterinary community searching for a superior biomarker. Consequently, new biomarker discovery continues, with the aid of novel, higher throughput testing.
1.8 Multiplex Magnetic Bead-Based Assays


1.8.1 Principles of multiplex assays

Multiplex assays can be used to quantify the concentration of cytokines and chemokines in serum, plasma, cell and tissue culture supernatants, cell and tissue lysates, lavage samples, and other sources suspended in the sample buffer provided with the kit. (Li et al 2013)

1.8.1.1 Components of the assay

The canine MILLIPLEX® multiplex assay is based on the Luminex® methodology. Magnetic microspheres are coded with two fluorescent dyes each, enabling the development of up to 100 distinctly coloured sets, each of which is coated with a specific capture antibody. The coated beads are mixed together and then added to the sample of interest. Biotinylated detection antibodies are then added and incubated with a reporter molecule, a streptavidin-phycoerythrin conjugate, which completes the reaction on the surface of each microsphere. Within the plate reader, one laser excites the internal dyes to identify the microspheres, while a second laser excites the reporter molecule, resulting in the fluorescence used to identify and quantify the cytokines in the sample. Figure 1.9 presents a summary of the assay procedure.
A single kit contains up to 13 sets of beads with species- and antigen-specific antibody pairs to the antigens of interest, permitting the simultaneous measurement of up to 13 different cytokines and chemokines. The magnetic beads enable thorough wash procedures between steps without the risk of bead loss.

1.8.1.2 Data interpretation

Analysis of the data is facilitated by the plate-reader’s companion software. A four-parameter logistic model, consisting of the upper and lower asymptotes, the mid-point of the curve, and the slope of the curve at the midpoint, is used to fit the standard data. This rigorous model is required because data interpretation relies heavily on the quality of the standard curve. Based on the standard curves created, the software provides the cytokine and chemokine concentrations within each sample.

1.8.2 Comparison of multiplex assays with traditional ELISAs

Although 96-well ELISAs are simple to perform, reproducible and widely used to measure cytokine activity, they are very inefficient when the simultaneous measurement of multiple proteins is desired. Assays for multiple cytokines can be performed side-by-side, however, each ELISA requires a minimum sample volume. Consequently, very large samples would be required to measure all of the biomarkers of interest. In contrast, multiplex assays require only 20-50 µL of sample to analyze up to 100 different analytes with broad concentration ranges. Analyzing all of the biomarkers at once reduces sample handling requirements, leading to decreased variability and greater sample stability. Finally, the principles used within the multiplex assay remain consistent with a traditional ELISA. As a result, findings from the multiplex can be
readily translated into the development of clinically useful ELISA assays that are suitable for daily bedside use. (Zangar et al 2006, Gonzalez et al 2008)

Nevertheless, no test is perfect and multiplex assays have their own disadvantages. Designed to detect multiple peptides at once, multiplex assays can be prone to measurement error due to reagent cross-reactivity. (Gonzalez et al 2008) For example, capture and detection antibodies can interact directly, leading to a signal whose intensity is independent of the antigen concentration. Alternatively, detection antibody for one biomarker can non-specifically bind others, or two antigens can interact together, causing a signal that measures both the target and the interfering protein. (Gonzalez et al 2008)

Because of these potential non-specific assay interactions, it is essential that the multiplex assay be optimized to maintain the high degree of specificity expected from an ELISA. Maximization of the signal-to-noise ratio for each assay is an integral part of maintaining the assay specificity; the concentrations of each detection antibody are adjusted simultaneously to ensure that a strong signal is maintained when antigen concentrations are in the upper range of the standard curve. (Gonzalez et al 2008) In addition, all possible cross-reactivity reactions must be assessed during multiplex development. This can be performed by first running the assay without an antigen, in order to determine whether capture and detection antibodies interact independently, and then by measuring each targeted protein individually with the application of all the detection antibodies, to determine whether other antibodies non-specifically bind the biomarker. (Gonzalez et al 2008)

Ultimately, most multiplex ELISA analyses experience minimal assay interference, potentially due to the low concentrations of the peptides being measured in serum. (Gonzalez et al 2008) As a result, these assays can be used to reliably measure multiple cytokines and

1.8.3 Use of multiplex assays in dogs

Multiplex assays have been embraced due to the large amount of data that can be obtained from small sample sizes. Often, these assays are used to confirm the induction of sepsis in a model by quantifying changes in IL-6, IL-1β, TNF-α and IL-10, such that the marker or therapy of interest can be assessed. (Solan et al 2012) In dogs, a canine-specific multiplex kit has been validated and used to confirm stimulation of the proinflammatory cytokines CXCL-1, IL-8, TNF-α and monocyte chemoattractant protein (MCP)-1 following LPS treatment of cell cultures. (Li et al 2013) In addition, multiplex assays can be used to identify new potential biomarkers.

To date, multiplex assays have been used primarily in studies of animal models of human disease; however, their use is beginning to appear in the veterinary literature as well. Serum myokines and inflammatory markers have been prospectively measured in endurance racing sled dogs using a multiplex assay; (Yazwinski et al 2013) this study identified IL-6 and MCP-1 as biomarkers of interest for future investigations of exertional rhabdomyolysis. (Yazwinski et al 2013) Interleukin-6 has also been shown to be significantly increased in dogs with congenital portosystemic shunts, shedding light on the role that inflammation might play in hepatic encephalopathy. (Kilpatrick et al 2014) Finally, the role of several cytokines has been determined in both immune mediated hemolytic anemia and thrombocytopenia. (Kjelgaard-
Hansen et al 2011, LeVine et al 2012) Although small, these studies demonstrate that multiplex assays could play an important role in biomarker discovery in many disease processes beyond sepsis.
Figure 1.9: Bio-Plex® cytokine assay workflow. Adapted from the full protocol available at BioRad.com.
1.9 Conclusion

Sepsis remains a complex and heterogeneous condition with a high mortality rate. Biomarker development has explored many possibilities to date; from transiently expressed cytokines, to the more stable peptides such as PCT and NT-proCNP, with some promising results. In people, PCT measurement is helping to identify and predict the development of sepsis and septic complications, and improve outcomes with more expeditious and judicious antibiotic use. Efforts to develop similarly clinically useful assays for dogs have been underway, but have thus far been unsuccessful. In contrast, the success of NT-proCNP as a biomarker for sepsis does appear to translate to hospitalized dogs. Still, a deeper understanding of the kinetics of this peptide is needed in order to refine its clinical use. Finally, a new biomarker could be awaiting discovery among the plethora of cytokines that can be tested with ELISA. Multiplex assays could therefore play a significant role in expediting the discovery and validation of these potential biomarkers.

It is evident that diagnostic biomarker development in dogs is still in its infancy, with many possible avenues to explore. But prior to going forward, we must ensure that the existing work is sound, and assay validation is essential in order to obtain reliable, reproducible results that can be translated into a clinical setting. With the long-term goal of rapidly diagnosing sepsis in dogs at the cage-side, we must first turn our focus towards the basic pathophysiology and molecular tools that will enable us to succeed.
1.10 Objectives and Hypotheses

This study consisted of two main parts, both aimed at identifying potential biomarkers for the diagnosis of sepsis, and assessing methods for their detection.

The purpose of the first portion of this study was to validate a commercial canine PCT ELISA with the goal to apply the assay to investigate PCT concentrations in dogs with LPS-induced systemic inflammation and naturally occurring sepsis. Because a gold standard for the quantification of canine PCT does not exist, alternate techniques were required to validate this ELISA. We hypothesized that the kit would allow for accurate and precise determination of canine PCT with intra- and inter-assay CVs of less than 10%.

The purpose of the second portion of this study was to determine the kinetics of serum NT-proCNP and multiplex-based cytokine concentrations over a 24-hour period in healthy dogs and dogs with endotoxemia after LPS administration. We hypothesized that dogs would have increased serum NT-proCNP one to four hours after LPS administration due to the induction of systemic inflammation, and that this would decrease by 24 hours. We also hypothesized that pro-inflammatory cytokines such as TNF-α and IL-6 would increase within hours of LPS administration and would normalize within 24 hours coinciding with resolution of systemic inflammation. Finally, we hypothesized that the multiplex assay would identify additional cytokines and chemokines that have not previously been investigated, but whose kinetics could aid in the diagnosis of sepsis in dogs.
1.11 Footnotes

a http://www.procalcitonin.com, Thermo Scientific, Rockford, IL, (February 26, 2014)

b Canine procalcitonin, PCT ELISA kit, EIAab Science Co Ltd, Wuhan, China

c NT-proCNP ELISA, Biomedica Medizinprodukte GmbH & Co KG, Vienna, Austria

d MILLIPLEX MAP for Luminex® xMAP® technology canine cytokine/chemokine magnetic bead panel immunoassay, EMD Millipore Corporation, Billerica, MA

e Bio-Plex® 200, Bio-Rad Laboratories, Mississauga, Canada

f Bio-Plex® Data-Pro software, Bio-Rad Laboratories, Mississauga, Canada
1.12 References


32. Canine procalcitonin, PCT ELISA kit, EIAab Science Co. Ltd., Wuhan, China


CHAPTER 2

Investigation of a Commercial ELISA for the Detection of Canine Procalcitonin

Abstract

**Background** - Rapid identification of sepsis enables prompt administration of antibiotics and is essential to improve patient survival. Procalcitonin (PCT) is a biomarker used to diagnose sepsis in people. Commercial assays to measure canine PCT peptide have not been validated.

**Objective** – To investigate the validity of a commercially available enzyme-linked immunosorbent assay (ELISA) marketed for the measurement of canine PCT.

**Animals** - Three dogs with sepsis, 1 healthy dog, 1 dog with thyroid carcinoma.

**Methods** – Experimental study. The ELISA’s ability to detect recombinant and native canine PCT was investigated and intra-assay and interassay coefficients of variability were calculated. Assay validation including mass spectrometry of the kit standard solution was performed.

**Results** – The ELISA did not consistently detect recombinant canine PCT. Thyroid lysate yielded a positive ELISA signal. Intra-assay variability ranged from 18.9 to 77.4%, while interassay variability ranged from 56.1 to 79.5%. Mass spectrometry of the standard solution provided with the evaluated ELISA kit did not indicate presence of PCT.

**Conclusions and clinical importance** - The results of this investigation do not support the use of this ELISA for the detection of PCT in dogs.
Portions of this chapter have been previously published and are included with permission.


### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>CV</td>
<td>coefficient of variation</td>
</tr>
<tr>
<td>LCMS/MS</td>
<td>liquid chromatography-tandem mass spectrometry</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PCT</td>
<td>procalcitonin</td>
</tr>
<tr>
<td>rePCT</td>
<td>recombinant canine PCT</td>
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<tr>
<td>SIRS</td>
<td>systemic inflammatory response syndrome</td>
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Introduction

Severe bacterial infections can result in marked morbidity and death in veterinary patients, with 50-70% of dogs with sepsis succumbing to their disease. (Kenney et al 2010) Early diagnosis of infection is essential for the appropriate management of sepsis, as it allows rapid administration of antibiotics resulting in improved outcomes. (Dellinger et al 2013) A rapid diagnostic marker for infection would assist veterinarians in determining whether antibiotic therapy is indicated, thereby facilitating early initiation and discriminate use of antibiotics.

Procalcitonin (PCT) is a 14 kDa prohormone identified as a biomarker for sepsis in people. Normally, PCT is produced only in the thyroid C-cells as a precursor to calcitonin; however, during sepsis this peptide is ubiquitously expressed. (Russwurm et al 1999) Although the biologic role of PCT is poorly understood, studies in people suggest a potential role in modulating inflammation. (Schuetz et al 2011) Advantages of PCT as a serum biomarker for sepsis include absence in healthy individuals, rapid induction with the onset of sepsis, and a moderately long half-life, making daily monitoring more practical compared to other biomarkers such as tumor necrosis factor-α and interleukin-6. (Schuetz et al 2011) In people, marked elevations in serum PCT are used to distinguish patients with severe bacterial, fungal, or parasitic infections from those with severe non-septic SIRS, to guide and shorten the course of antimicrobial treatment, and to prognosticate outcome. (Schuetz et al 2011) As such, PCT is becoming part of the standard-of-care to rapidly diagnose sepsis, minimize morbidity and mortality, and decrease unnecessary antibiotic use resulting in fewer adverse effects, lower health care costs, and decreased antimicrobial resistance. An assay for PCT might have similar value in dogs as in people.
Although PCT mRNA expression from nonthyroidal tissue has been shown in dogs with sepsis and SIRS, very little is known about serum PCT concentration in dogs attributable to the lack of a validated assay. (Giunti et al 2010, Kuzi et al 2008) Sequence differences with the human peptide (Mol et al 1991) could preclude cross-reactivity of canine PCT with assays for human PCT. Hence, an existing ELISA for human PCT did not differentiate samples from dogs with septic from nonseptic SIRS. (Giunti et al 2010) A commercially available canine PCT ELISA\(^a\) incorporating a polyclonal antibody to PCT has been marketed for the quantification of this peptide in research; however, this kit has not been validated. The purpose of this study was to validate a commercial canine PCT ELISA with the future goal to apply the assay to investigate the kinetics of PCT in dogs. Because a gold standard for the quantification of canine PCT does not exist, alternate techniques were required to validate this ELISA. We hypothesized that the kit would allow for accurate and precise determination of canine PCT with intra-assay and interassay coefficients of variation (CV) of less than 10%.

**Materials and Methods**

**Samples**

Thyroid lysate acquired post-mortem from a dog with a thyroid carcinoma and canine recombinant PCT (rcPCT) were tested with an ELISA for detection of native and rcPCT, respectively. Canine recombinant PCT was cloned by PCR-amplifying complementary DNA reverse transcribed from canine thyroid gland mRNA. Restriction enzyme\(^b\) sites *Nhel* and *SacI* were integrated into the forward and reverse primer, respectively, for cloning PCT with a HIS tag into the pET100/D TOPO expression vector.\(^c\) One-Shot BL-21 star competent *E. coli* cells\(^d\)
were transformed with the pET/PCT construct according to the manufacturer’s instructions, grown in lysogeny broth containing ampicillin, and induced with isopropyl β-D-1-thiogalactopyranoside. The rcPCT was purified by histidine tag affinity for nickel resin, dialyzed against phosphate-buffered saline, and then centrifuged in a Vivaspin column. The recombinant protein was analyzed by polyacrylamide gel electrophoresis and Coomassie staining (data not shown), and quantified by a Bradford Lowry assay. The thyroid lysate was diluted with the sample diluent provided in the ELISA kit to yield neat, 1:2 and 1:4 dilutions.

Serum from 3 dogs obtained within 24 hours of a diagnosis of sepsis (2 dogs with septic peritonitis and 1 dog with sepsis secondary to penetrating wounds) was pooled to establish a ‘septic’ sample. Sepsis was diagnosed if 2 of any of the following 4 criteria were present: body temperature < 38 or > 39.2°C (< 100.6 or > 102.6°F), heart rate > 120 beats per minute, respiratory rate > 20 breaths per minute, white blood cell count < 6 or > 16 x 10^3 cells/dL, or > 3% band neutrophils. Serum from a healthy dog undergoing elective surgery (determined on the basis of results of a normal physical examination, complete blood count, and serum biochemistry profile) was used as a ‘non septic’ sample. The serum collected was left over after routine biochemical evaluation in the hospital laboratory performed at the discretion of the attending veterinarian. All samples were stored at −80°C from the time of collection and thawed just prior to use.

**PCT ELISA**

The reagents were prepared and all incubations and washes were performed according to the manufacturer’s instructions. The standard solution provided was reconstituted with 1 mL of standard diluent to yield a stock solution if 1000 pg/mL. Serial dilutions of the stock standard
solution were then performed using standard diluent to yield 500, 250, 125, 62.5, 31.2 and 15.6 pg/mL solutions, with a final blank well containing only the standard diluent (0 pg/mL PCT). Assay diluent concentrates A and B (6 mL) were diluted in 6 mL of deionized water to yield 12 mL of each of the assay diluents. The stock detection A and B were briefly centrifuged and then diluted 1:100 with assay diluent A or B, as appropriate, to yield the detection reagent A or B. The 30X wash solution concentrate (20 mL) was diluted with 580 mL of deionized water to yield 600 mL of 1X wash solution.

Each dilution of the standard and the samples (100 µL) were added to a 96-well ELISA pre-coated with an antibody specific to PCT. The plate was covered with a plate sealer and incubated for 2 hours at 37°C. The liquid was removed from each well, then 100 µL of detection reagent A (a biotin-conjugated polyclonal antibody preparation specific for PCT) was added to each well. The plate was covered with a plate sealer and incubated for 1 hour at 37°C. The solution in each well was aspirated and washed with 400 µL of 1X wash buffer using a multi-channel pipette. After 1-2 minutes, the liquid was removed by inverting the plate, and the remaining liquid was removed by snapping the plate onto absorbent paper. The wash procedure was repeated 3 times. After the final wash, any remaining wash buffer was aspirated. Detection reagent B (100 µL, Avidin conjugated to horseradish peroxidase) was then added to each well. The plate was covered with a plate sealer and incubated for 30 minutes at 37°C. The aspiration and wash procedure previously described was repeated 5 times. Substrate solution (90 µL, TMB substrate) was then added to each well. The plate was covered with a plate sealer, protected from light, and incubated 15-25 minutes at 37°C. The stop solution (50 µL, sulfuric acid) was then added to each well, and the plate was gently tapped to ensure uniform mixing.
The optical density (OD) of the samples was then immediately determined using a microplate reader set to a wavelength of 450 nm. The OD of the blank well was subtracted from each sample OD. Log transformation of the OD values from the standard dilutions was performed to plot the standard curve, which was subsequently used to determine the PCT concentration in samples using the formula $10^{(\log_{10} \text{OD} - \text{intercept}) / \text{slope}}$.

Detection of canine recombinant and native PCT: Eight 1:4 dilutions of rcPCT, resulting in concentrations ranging from 22,938 pg/mL to 1.4 pg/mL, as well as neat, 1:2 and 1:4 dilutions of thyroid lysate were assayed as positive controls.

Intra-assay and interassay variation: Stored serum samples from dogs with and without sepsis were thawed, vortexed and centrifuged prior to being assayed in 5 repeat wells of the same ELISA kit to determine the intra-assay variability. The same samples were then divided into 4 aliquots, which were stored at −20°C until analyzed in 4 additional different assays to determine the interassay variability.

Mass spectrometry

The standard solution from two separate ELISA kits from different lots, and an aliquot of rcPCT were analyzed by liquid chromatography-tandem mass spectrometry (LCMS/MS) to identify peptides in the samples.

Database searching: All LCMS/MS spectra were analyzed by MASCOT. MASCOT was set up to search in the National Centre for Biotechnology Information database (selected for Canine lupus familiaris, 24,867 entries) assuming the digestion enzyme trypsin. MASCOT was searched with a fragment ion mass tolerance of 0.40 Da and a parent ion tolerance of 20 parts per million. Iodoacetamide derivation of cysteine was specified in MASCOT as a fixed modification.
Pyroglutamate formation at the N-terminus, S-carbamoylmethylcysteine cyclization of the N-terminus, deamidation of asparagine and glutamine, oxidation of methionine, and acetylation of the N-terminus, were specified in MASCOT as variable modifications.\textsuperscript{1}

Criteria for protein identification: Scaffold\textsuperscript{k} was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 90.0% probability by the Peptide Prophet algorithm. Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm. Proteins that contained similar peptides but could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.\textsuperscript{(Searle 2010)}

Statistical analysis

Intra-assay and interassay CVs were calculated by dividing the standard deviation (SD) by the mean of the 5 results obtained for each sample. All data are reported as mean ± SD. Graphs were generated using commercial software.\textsuperscript{1}

Results

PCT ELISA

Detection of rcPCT and native PCT: Analysis of dilutions of the standard solution supplied in the ELISA kit yielded a standard curve with $R^2 = 0.985$. Analysis of specific concentrations of rcPCT yielded random results with no apparent relationship to the amount of
rcPCT (Fig 2.1A). Assessment of different dilutions of thyroid lysate yielded PCT concentrations of 15 pg/mL, 19 pg/mL and 38 pg/mL, respectively (Fig 2.1B).

**Intra-assay variability:** To determine the precision of the ELISA, 5 replicates of two different serum samples were analyzed in the same ELISA. Replicate analysis of a serum sample from a dog without sepsis yielded PCT concentrations ranging from 0 to 213 pg/mL with a mean ± SD of 99 ± 77 pg/mL and a CV of 77.4%. Replicate analysis of a pooled serum sample from dogs with sepsis yielded PCT concentrations ranging from 218 to 380 pg/mL with a mean ± SD of 305 ± 58 pg/mL and a CV of 18.9% (Fig 2.2).

**Inter-assay variability:** To investigate repeatability of the ELISA across assays performed on different days, serum samples from dogs with and without sepsis were analyzed on 5 different days. Standard curves prepared for each assay had R² values > 0.96 (Fig 2.3). The sample from a dog without sepsis yielded PCT concentrations ranging from 28 to 120 pg/mL with a mean ± SD of 68 ± 38 pg/mL and a CV of 56.1%. The sample from dogs with sepsis yielded PCT concentrations ranging from 6 to 309 pg/mL with a mean ± SD of 160 ± 127 pg/mL and a CV of 79.5% (Fig 2.4).

**Mass spectrometry**

To verify that the rcPCT preparation indeed contained PCT, an aliquot was analyzed by LCMS/MS. Results indicated that with > 95% probability canine PCT and canine albumin were contained in the preparation. To investigate whether the standard provided in the commercial ELISA contained PCT, 2 samples of the standard solution submitted from two separate ELISA kits were also analyzed by MS/MS. Spectra for PCT were not detected in either of the 2 samples,
but both contained spectra for 2 or more peptides yielding a probability of 100% for canine albumin.

**Discussion**

The assay investigated in this study is marketed for the detection of both native and rcPCT in serum, tissue homogenates, or other biological fluids.\(^a\) Based on the investigation presented here, this commercially available canine PCT ELISA could not be validated and data generated do not support use of this assay to measure PCT in dogs.

In this study, different dilutions of rcPCT were not detected in a dose-dependent or consistent fashion. Possible reasons might be that the recombinant version of PCT was not in its native form and contained a polyhistidine tag, which is required for purification but could have interfered with immunodetection. Therefore, thyroid lysate, which should contain native PCT, was also assayed. A neat preparation of this lysate yielded a putative PCT concentration of 38 pg/mL. This result might reflect the actual concentration of PCT in canine thyroid tissue or another protein. Analysis of dilutions of a standard containing another protein in an ELISA incorporating an antibody to that protein might yield a standard curve with an acceptable slope, however, of an irrelevant substance.

Based on results of LCMS/MS analysis, the standard within the ELISA kit did not contain PCT. The 2 standard solutions analyzed were from 2 different lots, and had been shipped from the manufacturer on 2 different dates to decrease the possibility errors during lot preparation or of the destruction of the PCT during shipping. However, additional attempts to obtain alternative aliquots of the standard solution from the manufacturer were not made. Ideally,
LCMS/MS would also have been used to confirm the presence or absence of PCT in the pooled sample from the dogs with sepsis, the serum from the healthy dog, and the thyroid lysate. This was not performed because of the insufficient remaining sample volume.

The lack of PCT in the standard solution could indicate a problem with the standard only. It is possible that the capture and detection antibodies (detection reagents A and B) could still work despite a problem with the standard. Their potential utility is suggested by the relative linearity of the thyroid lysate sample dilutions, as well as the relatively higher concentrations noted in the serum from the dogs with sepsis when compared to healthy dog serum; however, this is challenging to determine because only a small number of samples were used. It therefore remains possible that the antibody pair could detect PCT.

Nevertheless, typically accepted intra-assay and interassay CVs for ELISAs are less than 10%\(^\text{a}\). According to the certificate of analysis supplied by the manufacturer for the commercial assay, the intra-assay CV is \(< 4.5\%\) and interassay CV is \(< 7.4\%\).\(^\text{a}\) However, the intra-assay and interassay CVs determined in this study were much higher, and of a magnitude typically considered unacceptable for ELISA technology.\(^\text{a}\) Hence, the ELISA assessed here yielded neither accurate nor precise results.

The high degree of variation could be the result of poor assay technique. Newly calibrated multi-channel pipettes were utilized to increase the uniformity of the steps and to decrease delays between the steps; however, manual error cannot be ruled out. All assays were performed by a single individual, which precluded an analysis of agreement between users. In the absence of an automated method, having multiple experienced technicians perform the assay for comparison would be beneficial in assessing the impact of human error on the assay. In addition, the inter- and intra-assay variability results for the non-septic and septic samples appear
to exhibit a pattern; because these wells were aligned on the plate, an edge or drift effect could have contributed to the high degree of variation. Finally, it is possible that the additional storage time and freeze-thaw cycle experienced by the day 2-5 samples could have resulted in the degradation of PCT. However, PCT is known to be stable at -20°C and -80°C, and serum can undergo 3 cycles of freezing at -80°C and thawing at room temperature without any significant loss of PCT concentration. (Hedelius et al 2013)

A major limitation of this study was that a suitable gold standard for measuring canine PCT was not available. Hence, the ELISA under investigation could not be compared to a known assay. However, absence of PCT in the standard solution as investigated with a highly sensitive method such as LCMS/MS precluded further attempts at validating this assay.

Substances yielding an optical signal in thyroid lysate and serum samples from septic and nonseptic dogs may consist of other proteins that bind nonspecifically to wells in the kit. Ideally, validation of an ELISA would entail assessment of precision and accuracy over multiple analyte concentrations within an assay, between different assays and between different laboratories. Within the time frame of this study, additional kits for the detection of canine PCT became available. To the authors’ knowledge, these kits are marketed for research purposes only and have not undergone industrial or governmental review. As a result, information regarding their components may be proprietary. Whether these newer kits are more suitable for the detection of canine PCT remains unknown. Nevertheless, the results of this investigation do not support the use of this ELISA for the detection of PCT in dogs.
Footnotes

a Canine procalcitonin, PCT ELISA kit, EIAab Science Co. Ltd., Wuhan, China

b *NheI* and *SacI* restriction endonucleases, New England BioLabs, Ipswich, MA

c Topo TA Kit, Invitrogen, Grand Island, NY

d One-Shot BL-21 star competent *E. coli*, Invitrogen, Grand Island, NY

e Sartorius Vivaspin sample concentrators, GE Life Sciences, Piscataway, NJ

f NuPage 4-12% Bis-Tris gels, Invitrogen, Grand Island, NY

g Pierce BCA protein assay, Thermo Scientific, Rockford, IL

h PowerWave XS2 with Gen5.0™ version 1.11 software, BioTek Instruments Inc, Winooski, VT

i Mass Spectrometry Facility, Advanced Protein Technology Centre, The Hospital for Sick Children, Toronto, Canada.

j MASCOT version 2.3.02, Matrix Science, London, UK

k Scaffold version 4.0.3, Proteome Software Inc., Portland, OR

l GraphPad Prism version 6.0a for Mac OS X, GraphPad Software, La Jolla, CA

References


**Figure 2.1.** (A) Specific concentrations of rcPCT detected by a commercial PCT ELISA. (B) PCT concentration in different dilutions of thyroid lysate as measured by commercial ELISA.
Figure 2.2. PCT as measured 5 times by commercial ELISA in a pooled sample from dogs with, and a single sample from a dog without, sepsis. Replicate 5 of the nonseptic sample had a concentration of 0 pg/mL.
Figure 2.3. Representative standard curve: raw data (A), transformed data (B).
Figure 2.4. PCT as measured on 5 different days by commercial ELISA in the same samples from dogs with and without sepsis.
CHAPTER 3

N-Terminal pro-C-Natriuretic Peptide and Cytokine Kinetics in Dogs with Endotoxemia

Abstract

**Background:** Serum N-terminal pro-C-natriuretic peptide (NT-proCNP) concentration at hospital admission has sufficient sensitivity and specificity to differentiate naturally-occurring sepsis from non-septic systemic inflammatory response syndrome (SIRS). However, little is known about serum NT-proCNP concentrations in dogs during the course of sepsis.

**Objective:** To determine serum NT-proCNP and cytokine kinetics in dogs with endotoxemia, a model of canine sepsis.

**Samples:** Eighty serum samples from dogs.

**Methods:** Eight healthy adult Beagles were randomized to receive *Escherichia coli* O127:B8 lipopolysaccharide (LPS, 5 µg/kg) or placebo (0.9% NaCl) as a single IV dose in a randomized crossover study. Serum collected at 0, 1, 2, 4 and 24 hours was stored at -80°C for batch analysis. Serum NT-proCNP was measured by ELISA and 13 cytokines and chemokines were measured by multiplex magnetic bead-based assay.

**Results:** Serum NT-proCNP concentrations did not differ significantly between LPS- and placebo-treated dogs at any time. When comparing serum cytokine concentrations, LPS-treated dogs had higher IL-6, IL-10, TNF-α and KC-like at 1, 2, and 4 hours; higher CCL2 at 1, 2, 4 and 24 hours; and higher IL-8 and CXCL10 at 4 hours compared to placebo-treated dogs (p < 0.05).
There were no differences in serum GM-CSF, IFN-γ, IL-2, IL-7, IL-15 or IL-18 between LPS- and placebo-treated dogs.

**Conclusions and Clinical Importance:** Serum NT-proCNP concentration does not change significantly in response to LPS administration in healthy dogs. Certain serum cytokine and chemokine concentrations are significantly increased within 1-4 hours after LPS administration and warrant further investigation as tools for the detection and management of sepsis in dogs.

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Abbreviations

CCL2  C-C motif chemokine ligand 2 (also referred to as monocyte chemotactic protein-1, MCP-1)

CRP  C-reactive protein

CV  coefficient of variation

CXCL10  C-X-C motif chemokine ligand 10 (also referred to as interferon gamma-induced protein-10, IP10)

GM-CSF  granulocyte macrophage-colony stimulating factor

IFN  interferon

IL  interleukin

KC-like  keratinocyte-derived chemokine (also referred to as CXCL1)

LPS  lipopolysaccharide

NK  natural killer

NT-proCNP  amino-terminal pro-C-type natriuretic peptide

OD  optical density

SIRS  systemic inflammatory response syndrome

TNF  tumor necrosis factor
Introduction

A biological marker, or biomarker, refers to any objective measurement used to indicate a state of health or disease accurately and reproducibly. Biomarkers can be used to promptly identify sepsis, thereby enabling rapid initiation of antibiotic therapy. (Wacker et al 2013) This feature is in contrast to bacterial culture and susceptibility testing, which require several days to complete. Cytokines and chemokines that have been investigated as biomarkers for sepsis in dogs include tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), interleukin-10 (IL-10), and interleukin-8 (IL-8, also known as C-X-C motif chemokine ligand-8 or IL-8). (DeClue et al 2012, Rau et al 2007, Fransson et al 2007) When measured using cell-kill bioassays, increases in serum TNF-α did not differentiate sepsis from non-septic systemic inflammatory response syndrome (SIRS). (DeClue et al 2012) In addition, although in one study IL-6 plasma concentration was predictive of disease severity and mortality in dogs with SIRS and sepsis, (Rau et al 2007) in other studies, plasma IL-6, IL-8 and IL-10 concentrations did not distinguish sepsis from non-septic SIRS. (DeClue et al 2012, Fransson et al 2007)

Amino-terminal pro-C-type natriuretic peptide (NT-proCNP) is a local regulator of vascular endothelial function that is expressed after stimulation by lipopolysaccharide (LPS) and TNF-α. It is a stable pro-peptide that differentiates sepsis from other non-septic causes of critical illness in people, (Hama et al 1994, Bahrami et al 2010, Koch et al 2011) and also is increased in dogs with naturally-occurring sepsis. (DeClue et al 2011) A study evaluating an enzyme-linked immunosorbent assay (ELISA) for the quantification of NT-proCNP indicated that a cut-off concentration of 10.1 pmol/L had 65.5% sensitivity and 89.2% specificity for differentiating dogs with naturally-occurring sepsis from dogs with non-septic inflammation. (DeClue et al
A recent study also identified the induction of this biomarker from canine aortic endothelial cells in cell culture after IL-1β, TNF-α and LPS stimulation.(Osterbur et al 2013) However, in clinical studies, NT-proCNP has only been measured in dogs with naturally-occurring sepsis at admission to the hospital, and to the authors’ knowledge, there are no published studies investigating blood concentrations of NT-proCNP in dogs with endotoxemia.

Although most biomarkers for sepsis, including chemokines and cytokines, are measured by individual ELISA, multiplex magnetic bead-based assays offer the advantage of concurrently quantifying a large number of cytokines and chemokines.(Ebong et al 1999, Remick et al 2002, Oberhoffer et al 1999, Dienstknecht et al 2004, Jarrar et al 2000, Kang et al 2004, Tateda et al 1996, Faulker et al 1995, Matsutani et al 2005) The simultaneous measurement of multiple biomarkers provides more information that might help to differentiate sepsis from non-septic inflammation. Sepsis is a complex and heterogeneous disease during which the extent of pro-inflammatory cytokine production per patient depends on the severity of inflammation incited by the infection and varies with age, sex, genetics, and other concurrent diseases.(Otto et al 1997, Ruaux et al 1999, Nemzek et al 2007, Gebhardt et al 2009) A multiplex assay enables the measurement of multiple cytokines and chemokines in a single blood sample. The purpose of the present study was to determine the kinetics of serum NT-proCNP and multiplex-based serum cytokine concentrations over a 24-hour period in healthy dogs and dogs with endotoxemia after LPS administration. We hypothesized that dogs would have increased serum NT-proCNP 1-4 hours after LPS administration as a consequence of the induction of systemic inflammation, and that NT-proCNP concentration would decrease by 24 hours. We also hypothesized that pro-inflammatory cytokines such as TNF-α and IL-6 would increase within hours of LPS administration and would normalize within 24 hours coincident with resolution of systemic inflammation.
inflammation. Finally, we hypothesized that the multiplex assay would identify additional cytokines and chemokines that have not been investigated previously, but whose kinetics could aid in the diagnosis of sepsis in dogs.

**Materials and Methods**

**Animals**

Eight adult purpose-bred Beagles deemed healthy based on normal physical examination, complete blood count and serum biochemistry profile were included in this survival study. All dogs were female (3 intact, 5 spayed) ranging in age from 16-44 months (median, 18 months). The median body weight was 8.85 kg (range, 7.8-11 kg). None of the dogs had received LPS as part of an experiment at any time before this study. Dogs were housed and handled according to the guidelines of the Canadian Council on Animal Care, the requirements of the Animals for Research Act Revised Statutes of Ontario, and institutional Animal Care Policy. This study was approved by the institution’s Animal Care Committee.

**Experimental design**

A randomized, placebo-controlled, crossover study was performed. Dogs were randomized to receive a bolus of either 5 µg/kg LPS, IV (*Escherichia coli* O127:B8)\(^a\) or an equal volume of placebo (0.9% NaCl)\(^b\) IV through a cephalic catheter.\(^c\) Thirty minutes later, each dog was resuscitated with IV fluids (40 mL/kg 0.9% NaCl). After a minimum 14-day washout period during which no treatments were administered beyond normal husbandry and care, the study was repeated with dogs receiving the alternate pre-fluid treatment (LPS or placebo).
Dogs were fasted for 12 hours before the study and allowed free access to water. At the initiation of the experiment, butorphanol\textsuperscript{d} (0.2 mg/kg IM) was given for sedation and analgesia and was repeated IV 2 and 4 hours after baseline. A 20-gauge catheter was placed in the cephalic vein to enable IV administration of placebo, LPS, butorphanol, and resuscitation fluids. After 6 hours, the cephalic catheter was removed and the dogs were returned to their housing and offered food.

**Sample collection and storage**

Blood samples were collected by jugular venipuncture at baseline (0), 1, 2, 4, and 24 hours after LPS or placebo administration. Blood samples were transferred into plastic Vacutainer tubes with no additive\textsuperscript{e} and allowed to clot for 15 minutes. The samples then were centrifuged at 700 x g for 15 minutes, and serum was removed and stored at -80°C for batch analysis of NT-proCNP, cytokine and chemokine concentrations. In addition, stored serum samples remaining after clinician-ordered serum biochemistry profiles from a clinically healthy dog, a dog with sepsis secondary to bite wounds, and a non-septic dog that was neutropenic 7 days after chemotherapy were used to confirm NT-proCNP kit intra- and inter-assay coefficients of variation (CV).

**NT-proCNP ELISA**

An NT-proCNP ELISA kit\textsuperscript{f} previously validated in dogs was obtained, and the assay was performed according to the manufacturer’s instructions.(DeClue et al 2011) This kit was reported to have a lower limit of detection of 0.55 pmol/L, and inter- and intra-assay coefficients of variation of 7-9 and 4-5%, respectively.(DeClue et al 2011) The kit control, as well as serum
samples from the above-mentioned individual dogs, each were assayed 5 times in one assay run to further evaluate intra-assay CV, and on 5 different dates to assess inter-assay variability. All study samples were assayed in duplicate, and 1 well was left empty as a blank reference. The absorbance was measured at 450 nm with a 620 nm reference; the measured optical density (OD) was (OD at 450 nm) - (OD at 620 nm). The OD of the blank well was subtracted from all sample measurements. Log transformation was performed to obtain a standard curve that was used to determine the NT-proCNP concentration of the samples using the formula $10^{\left(\log\text{OD} - \text{intercept}\right) / \text{slope}}$.

**Multiplex cytokine immunoassay kit**

An antibody-coated magnetic microsphere-based multiplex cytokine immunoassay kit designed for the simultaneous quantification of 13 cytokines, including several not previously investigated in dogs with sepsis, was used to determine cytokine kinetics. Those included in the kit were: IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, TNF-α, interferon-γ (IFN-γ), granulocyte macrophage-colony stimulating factor (GM-CSF), keratinocyte-derived chemokine (KC)-like, C-C motif chemokine ligand 2 (CCL2) and CXCL10. This multiplex kit gives an accurate measurement of serum cytokines when compared with individual ELISAs and has been used previously to measure serum cytokine concentrations in dogs. (Yazwinski et al 2013, Dossus et al 2009, Kjelgaard-Hansen et al 2011) All 80 samples from the LPS- or placebo-treated dogs were assayed once, and the assay standards and quality control sample each were assayed twice, for a total of 116 samples. The assay was performed according to the manufacturer’s instructions. Overnight incubation at 4°C was performed and a magnetic plate washer was utilized. The plates then were read using a multiplex plate reader with the companion software.
Statistical analyses

Intra- and inter-assay CVs for the NT-proCNP ELISA kit were calculated by dividing the standard deviation by the mean of the 5 results obtained for each sample. Duplicate NT-proCNP results were averaged and the mean value was used for statistical analysis. Single values were obtained for the quantification of cytokines. The NT-proCNP and cytokine concentrations over time were analyzed by a generalized linear mixed-model using the mixed procedure and a repeated measures test. Different error structures were tried, with the final model for auto-regression chosen based on the Akaike information criterion. The assumptions of the ANOVA were assessed by comprehensive residual analyses and the Shapiro-Wilk test was conducted to analyze for normal distribution. The residuals were plotted against the predicted values and explanatory variables (treatment, time, dogs) to identify outliers or unequal variance. If residual analyses suggested a need for data transformation, logarithmic transformation was done before data analysis. The level of significance was corrected using a Dunnett’s or Tukey’s test for multiple comparisons where appropriate. Significance was set at p < 0.05 for all variables. Data are presented as mean ± the standard deviation (SD). All analyses were performed using standard statistical software.\(^{1}\) Graphs were generated using commercially available software.\(^{2}\)

Results

Within 30 minutes of LPS administration, all LPS-treated dogs demonstrated lethargy and gastrointestinal upset characterized by diarrhea and hypersalivation. Rectal temperature was
significantly increased after 3 (p < 0.001) and 4 (p < 0.001) hours in LPS- versus placebo-treated dogs.

The intra-assay CV for the NT-proCNP kit ranged from 9.2-17.9% and the inter-assay CV ranged from 6.3-18.0%. There was no significant difference in NT-proCNP concentration at any time point when samples from placebo-treated dogs were compared to LPS-treated dogs (Figure 3.1).

When comparing serum cytokine concentrations in LPS-treated dogs to placebo-treated dogs, IL-6, IL-10, TNF-α and KC-like were significantly higher at 1, 2 and 4 hours (p < 0.05; Figure 3.2). In addition, CCL2 was higher at 1, 4 and 24 hours, whereas IL-8 and CXCL10 were higher at 4 hours (p < 0.05; Figure 3.2). There were no significant differences in serum GM-CSF, IFN-γ, IL-2, IL-7, IL-15 or IL-18 between LPS- and placebo-treated dogs (data not shown).

**Discussion**

Using an assay with inter- and intra-assay CVs ≤ 18%, the present study indicated that NT-proCNP was not increased in dogs given a single IV injection of a low dose of LPS but increases in several other cytokines and chemokines were observed in the same dogs. Potent simulators of NT-proCNP production from canine aortic endothelial cells include LPS, TNF-α and transforming growth factor-β.(Hama et al 1994, Suga et al 1993, Osterbur et al 2013) In addition, inflammation can create an environment in which NT-proCNP is produced by the monocyte/macrophage system, resulting in vasodilatation and the inhibition of microbial growth and pathogenicity.(Hama et al 1994, Kubo et al 2001, Veron et al 2008)
Although LPS induced NT-proCNP in a dose-dependent manner in a canine cell culture system, LPS at the dosage administered to dogs in the present study (5 µg/kg, IV) did not produce an increase in serum NT-proCNP. This LPS dosage was chosen because it produces clinical illness in healthy dogs without being fatal. Nevertheless, the absence of an increase in the concentration of NT-proCNP over time in the dogs with endotoxemia could have been because the dosage of LPS was too low, or because the duration of endotoxemia was too short. However, the measured increases in IL-6, IL-8, IL-10 and TNF-α confirmed induction of inflammation in the dogs given LPS. These pro-inflammatory cytokines are similarly increased in dogs with naturally-occurring sepsis.

Therefore, it is likely that the endotoxemia model used in this study, as in previous studies in other species, cannot fully reproduce the complexity of the in vivo interactions resulting in NT-proCNP expression during naturally-occurring sepsis.

Previous studies using cell-kill bioassay methods to measure cytokine concentrations in dogs after LPS administration have identified rapid changes in TNF-α concentration. TNF-α increased within 30 minutes after IV LPS administration and a large overlap in sustained peak concentrations was observed after approximately 2 hours in response to dosages ranging from 0.1-40 µg/kg, suggesting that its activity was not dose-dependent. In contrast, the increase in IL-6 after the IV administration of LPS over the same dosage range had a slower onset, peak activity that persisted >2 hours longer than TNF-α activity, and greater dose-dependency. (LeMay et al 1990) TNF-α and IL-6 kinetics seen in the present study are consistent with these previous results, which reflect the pro-inflammatory response to LPS administration.
Other chemokines, including CCL2, KC-like, and CXCL 10, were increased at ≥ 1 time point after LPS administration in the present study, suggesting their possible utility as biomarkers of systemic inflammation or sepsis. The multiplex assay has been previously used to measure IL-18, CCL2, IL-6, IL-15, IL-8 and TNF-α in dogs with immune-mediated hemolytic anemia,\(^1\)(Kjelgaard-Hansen \textit{et al} 2011) immune-mediated thrombocytopenia,\(^1\) and in endurance racing sled dogs that experience a sustained inflammatory response.(Yazwinski \textit{et al} 2013) To the authors’ knowledge, CCL2 has not yet been evaluated as a potential biomarker for sepsis in dogs. In murine models of pancreatitis and sepsis, CCL2 mediates the immediate pro-inflammatory response by recruiting circulating monocytes, T lymphocytes, natural killer (NK) cells and neutrophils to the site of infection or inflammation;(Shanmugam \textit{et al} 2010) this cytokine could play a similar role in dogs. KC-like is expressed by macrophages, neutrophils and epithelial cells, resulting in neutrophil chemoattractant activity and increased bacterial phagocytosis, thereby playing a key role in sepsis.(Shanmugam \textit{et al} 2010, Kelly-Scumpia \textit{et al} 2010) Similarly, CXCL10 is secreted by cells such as monocytes, endothelial cells and fibroblasts in response to IFN-γ.(Kelly-Scumpia \textit{et al} 2010) This cytokine plays a role in the chemoattraction of monocytes and macrophages, T-cells, NK cells and dendritic cells, and promotes T-cell adhesion to endothelial cells in order to resist bacterial and viral infections.(Kelly-Scumpia \textit{et al} 2010) As in mouse models of sepsis,(Kelly-Scumpia \textit{et al} 2010) CXCL10 also could prove to be a useful biomarker of sepsis in dogs.

Cytokines such as IL-6, IL-10, and TNF-α are associated with the innate immune response, which is the non-specific first line of host defense during infection, responsible for triggering a proinflammatory response after LPS administration. The cytokines that did not increase after LPS administration (GM-CSF, IFN-γ, IL-2, IL-7, IL-15 or IL-18) in the present
study typically play a role in the adaptive immune response, which is responsible for the elimination of pathogens in the late phase of infection. Consequently, the timing of sampling could have precluded these cytokines from becoming increased. For example, the lack of a significant increase in IL-15 is consistent with studies demonstrating an increase in this cytokine only when it was measured beyond 24 hours. (Inoue et al 2010) Similarly IL-2 and IL-7 are expected to be increased 24-72 hours or 48 hours, respectively, after bacterial infection. (O’Sullivan et al 1995, Unsinger et al 2012)

Limitations of the present study include sample collection at a limited number of time points after LPS or placebo administration. Additional time points might have yielded more information detailing the kinetics of NT-proCNP and cytokines; specifically, concentrations of IL-2, IL-7 and IL-15 that might have increased beyond 24 hours. Blood was sampled at only a small number of time points because of funding limitations. Similar constraints limited cytokine multiplex analysis to single replicates, although duplication of samples would have been preferred. Likewise, validation of the multiplex analysis using cytokine controls or mass spectrometry was not within the scope of this study. In addition, only female Beagle dogs were available through the Central Animal Facility at the University from which the dogs were acquired. Consequently, any effect of sex on NT-proCNP or cytokine kinetics could not be assessed; this could be important because sex does appear to play a significant role in human sepsis, with women being more resistant. (Dellinger et al 2012) Finally, the average NT-proCNP inter- and intra-assay CVs in this study (13.5% and 13.2%, respectively) were higher than anticipated based on those reported previously. (DeClue et al 2011) The lowest CVs (9.2% for intra-assay CV and 6.3% for inter-assay CV) were obtained for the control provided in the kit. The higher CVs were obtained from the samples with the lowest NT-proCNP concentrations.
Although these concentrations were not below the detection limit of the assay, they were towards the lower end of the standard curve and therefore more variable.

In conclusion, serum NT-proCNP concentrations did not differ significantly between healthy dogs and dogs with endotoxemia after LPS administration. Future studies using either higher dosages of LPS, or a low dose endotoxin infusion, or an alternative model that might better mimic naturally-occurring sepsis, could be useful for determining the kinetics of this protein biomarker during sepsis. Serum cytokines and chemokines including IL-6, IL-8, IL-10, TNF-α, KC-like, CCL2 and CXCL10 were significantly increased in dogs within 1-4 hours of LPS administration, and warrant further investigation as tools for the detection and management of sepsis in dogs. Considering the complexity and redundancy of the inflammatory response to sepsis, it is unlikely that a single biomarker will have adequate sensitivity and specificity for diagnosis of sepsis, but a multiplex assay could yield valuable diagnostic information if available to the clinician.
Footnotes

a Escherichia coli serotype 0127:B8, Sigma-Aldrich, St. Louis, MO

b 0.9% NaCl, Baxter, Mississauga, ON, Canada

c BD Insyte Autoguard catheter, BD Medical, Sandy, UT

d Torbugsic, Fort Dodge Animal Health, NY

e BD Vacutainer plus plastic plasma tubes, Becton Dickinson and Company, Franklin Lakes, NJ

f NT-proCNP ELISA, Biomedica Gruppe, Vienna, Austria

g MILLIPLEX MAP for Luminex® xMAP® technology canine cytokine/chemokine magnetic bead panel immunoassay, EMD Millipore Corporation, Billerica, MA

h Bio-Plex® 200, Bio-Rad Laboratories, Mississauga, Canada

i Bio-Plex® Data-Pro software, Bio-Rad Laboratories, Mississauga, Canada

j SAS v.9.2, SAS Institute Inc., Cary, NC

k Prism 5, GraphPad Software, La Jolla, CA

References


**Figure 3.1.** Serum NT-proCNP concentrations in dogs after LPS or placebo administration. Values are presented as mean ± SD.

**Figure 3.2.** Serum cytokine concentrations in dogs after LPS or placebo administration: IL-6 (A), IL-10 (B), TNF-α (C), KC-like (D), CCL2 (E), IL-8 (F), CXCL10 (G), GM-CSF (H), IFN-Υ (I), IL-2 (J), IL-7 (K), IL-15 (L) and IL-18 (M). Significant differences between LPS and placebo dogs are indicated with * (p < 0.05). Values are presented as group mean ± SD.
Potential biomarkers and cytokines that could be used for the diagnosis of sepsis were investigated in this thesis. The methods for their detection were assessed, and the kinetics of these biomarkers and cytokines in dogs with endotoxemia were determined.

The purpose of the first portion of this study was to validate a commercial canine PCT ELISA with the goal to apply the assay to investigate PCT concentrations in dogs with LPS-induced systemic inflammation and naturally occurring sepsis. Because a gold standard for the quantification of canine PCT does not exist, alternate techniques were required to validate this ELISA. We hypothesized that the kit would allow for accurate and precise determination of canine PCT with intra- and inter-assay CVs of less than 10%. This hypothesis was refuted; based on this investigation, the commercially available canine PCT ELISA examined could not be validated. First, different dilutions of rcPCT were not detected in a dose-dependent or consistent fashion. Second, the intra-assay and inter-assay CVs determined in this study were unacceptable for ELISA technology. Finally, mass spectrometry revealed that the standard within the ELISA kit did not contain PCT. Hence, the ELISA assessed here yielded neither accurate nor precise results and was subsequently not used to measure serum PCT concentrations in dogs with LPS-induced systemic inflammation or naturally-occurring sepsis.

The primary limitation of this portion of the study was that a suitable gold standard for measuring canine PCT was not available, precluding comparison of the ELISA under investigation to a known and validated assay. Nevertheless, absence of PCT in the kit standard solution as investigated with a highly sensitive method such as mass spectrometry made further
attempts at validating this assay irrelevant. Consequently, the results of this investigation do not support the use of this ELISA for the detection of PCT in dogs.

Since the completion of this portion of the study, additional kits for the measurement of PCT in dogs have become available. Examination of these new kit components should be performed prior to their application in a research setting. Continued attempts at developing a canine PCT ELISA that is accurate, precise, sensitive and specific are warranted, with the goal of determining whether this biomarker can differentiate dogs with naturally occurring sepsis from those with NSIRS.

The purpose of the second portion of this study was to determine the kinetics of serum NT-proCNP and multiplex-based cytokine concentrations over a 24-hour period in healthy dogs and in dogs with endotoxemia after LPS administration. In the model used, there was no significant difference in NT-proCNP concentration at any time point when samples from placebo-treated dogs were compared to LPS-treated dogs. Future studies using either a higher dose of LPS, or a low dose endotoxin infusion, should be considered to further assess this biomarker. Alternatively, a model that might better mimic naturally occurring sepsis, such as a Staphylococcus aureus pneumonia model, could be useful for determining the kinetics of NT-proCNP during sepsis. While more closely simulating naturally occurring sepsis, the latter model is more invasive and can be associated with a higher morbidity and potential mortality for the dogs enrolled in the study. If a more complete understanding of the behavior of NT-proCNP during sepsis can be achieved, adaptation of the 96-well ELISA to a point-of-care assay could enhance the veterinarian’s ability to rapidly diagnose sepsis in dogs.

When comparing serum cytokine concentrations in LPS-treated dogs to placebo-treated dogs, IL-6, IL-10, TNF-α and KC-like were significantly higher at 1, 2 and 4 hours, consistent
with the induction of inflammation. In addition, CCL2 was higher at 1, 4 and 24 hours, while IL-8 and CXCL10 were higher at 4 hours. There were no significant differences in serum GM-CSF, IFN-γ, IL-2, IL-7, IL-15 or IL-18 between LPS- and placebo-treated dogs at any time point. The kinetics of TNF-α and IL-6 determined in the present study are consistent with prior reports and our current understanding of their role in the inflammatory response in dogs. However, the kinetics of other cytokines and chemokines, including CCL2, KC-like, and CXCL 10, have not been as extensively characterized in dogs to date. The increases in these cytokines and chemokines observed at one or more time points after LPS administration in this study, and their known effects on white blood cells, together identify them as potentially useful biomarkers of systemic inflammation or sepsis.

Serum cytokines and chemokines including IL-6, IL-8, IL-10, TNF-α, KC-like, CCL2 and CXCL10 that were significantly increased in dogs within 1-4 hours of LPS administration, therefore, warrant further investigation as tools for the detection of sepsis in dogs. Future studies evaluating these cytokines and chemokines should focus on additional time points after LPS administration in order to further refine our understanding of their contributions and diagnostic utilities during inflammation and sepsis. In keeping with the current literature, and considering the complexity and redundancy of the inflammatory response to sepsis, it is unlikely that a single biomarker will have adequate sensitivity and specificity for the diagnosis of sepsis in dogs. In people, various additional biomarker combinations are being evaluated.(Faix 2013, Riedel and Carroll 2013) These include PCT, CRP, soluble triggering receptor expressed on myeloid cells-1 (sTREM-1), neutrophil CD64, and others.(Gibot et al 2012) As a result, a user-friendly multiplex assay for dogs could also yield valuable diagnostic information in this species if it were easily and rapidly accessible to the clinician.
In conclusion, the results of this investigation do not support the use of the evaluated commercial ELISA for the detection of PCT in dogs. Optimization of ELISA technology is complex; until a reliable assay is developed and validated, the question as to whether PCT can be used to diagnose dogs with sepsis will remain unanswered. Although serum NT-proCNP is a strong alternate biomarker candidate, based on its elevation in dogs with naturally occurring sepsis, a deeper understanding of the kinetics of this protein during inflammation is required to optimize its use. Finally, certain serum cytokine and chemokine concentrations that were significantly increased within 1-4 hours of LPS administration warrant further investigation as tools for the detection and management of sepsis in dogs. Multiplex assays present a novel opportunity to simultaneously assess various potential biomarkers, possibly enhancing the sensitivity and specificity of their ability to differentiate dogs with sepsis from dogs with NSIRS. However, interpreting multiple cytokine and chemokine concentrations at once infers a complexity that might be beyond our current understanding of the immunopathophysiology of sepsis. Significant further research will, therefore, be required before the objective interpretation of cytokine and chemokine panels for the diagnosis of sepsis in dogs becomes routine.
CHAPTER 5

 Appendices

 Portions of chapters 2 and 3 have been previously published and are included with permission.
