THE MECHANISM OF C-TYPE NATRIURETIC PEPTIDE PRODUCTION IN DOGS AND ITS USE AS A PROGNOSTIC INDICATOR IN CRITICALLY ILL DOGS

A Thesis
presented to
the Faculty of the Graduate School
at the University of Missouri-Columbia

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

by
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JULY 2012
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THE MECHANISM OF C-TYPE NATRIURETIC PEPTIDE PRODUCTION IN
DOGS AND ITS USE AS A PROGNOSTIC INDICATOR IN CRITICALLY ILL
DOGS

Presented by Kara Osterbur

A candidate for the degree of Master of Biomedical Sciences

And hereby certify that, in their opinion, it is worthy of acceptance.

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ACKNOWLEDGEMENTS

I would like to acknowledge Dr. Amy DeClue, without whom my thesis project would likely not exist. Her help and guidance has been invaluable during the course of my research, manuscript submissions, and thesis project completion. She is an excellent mentor, both clinically and academically, and I will be forever grateful for all that she has done for me.

I would also like to acknowledge the assistance provided by the members of my committee, Dr. F. A. Mann and Dr. Keiichi Kuroki. I appreciate their dedication to my success as a master’s student and veterinary resident.

I would also like to recognize Dr. Chee-Hoon Chang, Hong Liu, Dr. Do-Hyeon Yu, Dr. Juliana Amorim, and Allison Honaker for providing technical support.

Finally, I would like to acknowledge my resident mates, Dr. Sarah Deitschel, Dr. Adesola Odunayo, Dr. Meredith Thoen, Dr. Mary Jackson, Dr. Shaunita Sharpe, and Dr. Beth Tynan for their support, encouragement, and assistance through the course of this project.
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LIST OF ABBREVIATIONS

ACTH  Adrenocorticotropic hormone
AKI   Acute kidney injury
AKIN  Acute Kidney Injury Network
ALI   Acute lung injury
ALP   Alkaline phosphatase
ALT   Alanine transaminase
ANP   Atrial natriuretic peptide
APC   Activated Protein C
ARDS  Acute Respiratory Distress Syndrome
AUC   Area under the curve
BNP   Brain natriuretic peptide
CAoE  Canine aortic endothelial cells
CARS  Compensatory Anti-inflammatory Response Syndrome
CBC   Complete blood count
CI    Confidence interval
CIRCI Critical Illness Related Corticosteroid Insufficiency
CNP   C-Type natriuretic peptide
COH   Cost of hospitalization
CORTICUS Corticosteroid therapy of septic shock
CRRT  Continuous renal replacement therapy
CXCL  CXC chemokine ligand
DAMP  Danger-Associated Molecular Pattern
DIC   Disseminated intravascular coagulation
DOH   Duration of hospitalization
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>FDP</td>
<td>Fibrinogen degradation products</td>
</tr>
<tr>
<td>FiO₂</td>
<td>Fraction of inspired oxygen</td>
</tr>
<tr>
<td>GFR</td>
<td>Glomerular filtration rate</td>
</tr>
<tr>
<td>HMGB1</td>
<td>High Mobility Group Box 1</td>
</tr>
<tr>
<td>HPA</td>
<td>Hypothalamic-pituitary-adrenal</td>
</tr>
<tr>
<td>ICU</td>
<td>Intensive Care Unit</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ISTH</td>
<td>International Society of Thrombosis and Haemostasis</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LR</td>
<td>Likelihood ratio</td>
</tr>
<tr>
<td>LTA</td>
<td>Lipotechoic acid</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MODS</td>
<td>Multiple Organ Dysfunction Syndrome</td>
</tr>
<tr>
<td>MPT</td>
<td>Mitochondrial Permeability Transition</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NT-pCNP</td>
<td>Amino-terminal portion of pro-CNP</td>
</tr>
<tr>
<td>ODI</td>
<td>Organ dysfunction index</td>
</tr>
<tr>
<td>PAMP</td>
<td>Pathogen-Associated Molecular Pattern</td>
</tr>
<tr>
<td>PaO₂</td>
<td>Partial pressure of arterial oxygen</td>
</tr>
<tr>
<td>PARP</td>
<td>Poly-(ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PE</td>
<td>Phycoerythrin</td>
</tr>
<tr>
<td>PG</td>
<td>Peptidoglycan</td>
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<tr>
<td>PT</td>
<td>Prothrombin time</td>
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</table>
PTT  Partial thromboplastin time
SIRS  Systemic Inflammatory Response Syndrome
RIFLE  Risk Injury Failure
ROC  Receiver operating characteristic
SAE  Sepsis-Associated Encephalopathy
TLR  Toll like receptor
TNF  Tumor Necrosis Factor
VEGF  Vascular endothelial growth factor
### LIST OF ILLUSTRATIONS

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Introduction

The multiple organ dysfunction syndrome (MODS) is defined as “the presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention”.\(^1\) In people, MODS is most commonly a sequela to severe sepsis or septic shock, but it also develops secondary to trauma, neoplasia, or other causes of the systemic inflammatory response syndrome (SIRS). The exact incidence of MODS is difficult to estimate because there is no true consensus as to the definition of each individual organ system dysfunction;\(^2\) however, it has been estimated that 15\% of all people admitted to the intensive care unit (ICU) will develop MODS and that MODS is responsible for 80\% of all ICU deaths.\(^3,4\) The incidence of MODS in dogs with trauma is approximately 4\%, while dogs with sepsis have an incidence of 50\%; in both cases MODS is associated with a poor outcome.\(^5,6\) The following review will provide information regarding the history, pathophysiology and epidemiology of MODS and will then discuss how MODS manifests in each organ system.
MODS is a relatively new concept in both human and veterinary medicine and it has been described as an iatrogenic disorder. Application of advanced medical knowledge and technology has allowed people and animals to survive initial insults, which at one time would have been fatal, so that relatively long-term sequelae like MODS manifest. The first reports of individual forms of organ dysfunction were during World War II and the Vietnam War when improved resuscitation techniques allowed soldiers to survive the initial battlefield injury only to go on to die from renal failure or respiratory failure (i.e., Da Nang Lung or Vietnam Lung). In 1969, multiple organ system involvement was first reported in 8 people with acute gastric ulcerations and sepsis that developed a clinical syndrome associated with respiratory failure, hypotension, and icterus. Similarly in a 1973 retrospective study of 18 people with abdominal aortic aneurysms, 17 died from sequential organ failure starting with pancreatic and pulmonary failure which progressed to cardiac and upper gastrointestinal hemorrhage. In these patients, pulmonary failure was considered to be the primary cause of death. As life-support technology continued to improve, the incidence of organ dysfunction secondary to infectious and non-infectious diseases became increasingly more common.

In 1991, the American College of Chest Physicians and the Society of Critical Care Medicine held a consensus conference to develop definitions for clinical syndromes including MODS with the goal of improving disease detection,
allow early therapeutic intervention, and patient stratification in clinical trials.\textsuperscript{2} The syndrome of organ dysfunction/failure that had been described over the previous 20 years was officially termed “multiple organ dysfunction syndrome”. The term “failure” was excluded from the name because it implied an absolute presence or absence of function as opposed to a continuum. MODS was defined as the “presence of altered organ function in an acutely ill patient such that homeostasis cannot be maintained without intervention”.\textsuperscript{2} Further, MODS was described as a primary process in which the organ dysfunction could be directly attributable to the insult itself or as a secondary process in which the organ dysfunction was a consequence of the systemic response to a distant insult. Specific criteria for clinical identification of MODS were not described.\textsuperscript{2}

**Epidemiology**

In 1995, it was estimated that 9.3% of all human deaths in the United States were related to severe sepsis with a total healthcare cost of $16.7 billion dollars.\textsuperscript{17} The incidence of sepsis has increased over time. In a review of 750 million hospitalizations, there was an annualized increase in the episodes of sepsis from 82.7 episodes/100,000 hospital admissions in 1979 to 240.4 episodes/100,000 hospital admissions in 2000.\textsuperscript{18} Sepsis is the most common inciting cause of MODS in people and MODS is more common in people with sepsis compared to other forms of critical illness (75% versus 43%).\textsuperscript{19}

There is a limited amount of information regarding the epidemiology of MODS in animals. Sepsis is a major cause of MODS in animals. A multi-center
report of 114 dogs treated surgically for abdominal sepsis found 78% of dogs had dysfunction of one or more organ systems and 50% had dysfunction of 2 or more organ systems.\textsuperscript{5} The incidence of MODS in dogs with trauma is 4%.\textsuperscript{6}

**Pathophysiology**

The pathophysiology of MODS is complex, multifactorial, and poorly understood. Three models to explain the initiation of MODS have been proposed. The first is the “one-hit” model in which organ failure develops as the direct result of a massive initial insult, such as sepsis, polytrauma, or burn injury. The second model, or the “two-hit” model, describes a priming insult (first “hit”) which is followed by a subsequent insult (second “hit”). The subsequent insult may seem small, such as a catheter-related infection, and it induces enhanced inflammation and immune dysfunction. The third model is known as the “sustained-hit model.” This model describes a continuous insult such as ventilator-associated pneumonia which causes both the initial insult and sustains the dysfunction.\textsuperscript{20}

The current understanding of the pathophysiology leading to MODS involves intricate cross-talk among multiple cell populations, hormonal systems, metabolites, and neural signaling along with alterations in oxygen delivery, derangements in oxygen utilization, and modifications in cell phenotypes. This highly coordinated, yet dysregulated, adaptive mechanism might arise from primordial protective strategies. There are several proposed mechanisms for the development of MODS including (1) cell or tissue hypoxia, (2) microbial and inflammatory mediator induced cytotoxicity, (3) induction of cellular apoptosis, (4)
translocation of microbes or components of microbes from the gastrointestinal tract, and (5) downregulation of mitochondrial function. While MODS likely results from a complex combination of these factors and others yet to be identified, emerging evidence suggests that immune system dysregulation and subsequent mitochondrial dysfunction are thought to be the prevailing pathways.

Immune system dysregulation is the imbalance between pro-inflammatory and anti-inflammatory counter-regulatory mechanisms.\textsuperscript{21} The innate immune system is designed to rapidly respond to danger signals including pathogen-associated molecular patterns (PAMPs) and alarmins. PAMPs are a diverse set of microbial molecules that share a number of different recognizable biochemical features that alert the organism to the invading pathogen. Alarmins are similar to PAMPs; however, they are markers of endogenous cell damage. PAMPs and alarmins, collectively referred to as danger-associated molecular patterns (DAMPs), are identified by the innate and adaptive immune systems, most commonly via toll-like receptors, which then activate signaling pathways to incite inflammation.\textsuperscript{22}

Once activated, first responder cells of the innate immune system, predominantly macrophages, produce proinflammatory cytokines (i.e., tumor necrosis factor (TNF)-α, interleukin (IL)-1β). These early cytokines stimulate the synthesis of other inflammatory mediators and result in the activation of other leukocytes.\textsuperscript{23} Late inflammatory mediators [e.g, high-mobility-group box 1 (HMGB1), IL-6] provide ongoing inflammation as appropriate.\textsuperscript{23} Concurrently, anti-inflammatory cytokines (e.g, IL-10) are produced to help maintain immune
system balance. During SIRS or MODS, balance is lost resulting in prolonged, damaging inflammation or, conversely, immunoparalysis. Immunoparalysis is also referred to as the compensatory anti-inflammatory response syndrome (CARS), and this syndrome is characterized by suppressed immune responses which put the host at risk for infection and further injury.\textsuperscript{24}

Neutrophils are major contributors to the pathogenesis of innate immune dysregulation. Neutrophil priming by cytokines (e.g., TNF-\(\alpha\)) or other factors leads to alterations in cell surface protein expression, interaction with vascular endothelium, trafficking to various extravascular sites and production of superoxides.\textsuperscript{25} Neutrophils undergo downregulation of apoptotic pathways during inflammation resulting in relative neutrophil “immortality”. This sets up a scenario in which neutrophils infiltrate tissues, produce superoxides and induce tissue damage resulting in perpetuation of inflammation through various pathways, including release of the alarmin and HMGB1 from damaged cells.\textsuperscript{26}

In addition to direct tissue damage, neutrophils contribute to relative cellular dysfunction by activating mitochondrial dysfunction pathways. Superoxide from neutrophils along with nitric oxide production from vascular endothelium combine to form peroxynitrite. Peroxynitrite causes inhibition of several aspects of mitochondrial respiration and mitochondrial synthesis of ATP by activating the enzyme poly-(ADP-ribose) polymerase (PARP).\textsuperscript{27} Pharmacologic inhibition of PARP prevents mitochondrial dysfunction,\textsuperscript{28-30} and PARP-knock out mice are relatively resistant to the harmful effects of endotoxin.\textsuperscript{31,32} Additionally, oxidative stress and pro-inflammatory cytokine
signaling lead to uncoupling of oxidative phosphorylation via mitochondrial permeability transition (MPT). In MPT, a pore is opened in the inner mitochondrial membrane which allows an inappropriate proton gradient within the mitochondria and uncoupling of oxidation from phosphorylation. These acquired intrinsic derangements in cellular energy metabolism during MODS are referred to as cytopathic hypoxia. The concept of cytopathic hypoxia was developed to explain the disconnect between adequate oxygen delivery and poor utilization of oxygen at the tissue level.

When mitochondrial energy production is reduced because of cytopathic hypoxia, the result is cellular dysfunction and, in some cases, cell death. Mitochondrial dysfunction has been documented during sepsis-induced MODS in people with naturally developing sepsis and in experimental models. Pharmacologic inhibition of mitochondrial derangement prevents the development of MODS in experimental bacterial sepsis indicating that mitochondrial damage is a causative factor in the development of MODS and thus could be a therapeutic target.

While generally viewed as “bad” clinically, downregulation of mitochondrial function might be a cellular adaptive response to prolonged inflammation. In general, cell death (necrosis) is not a common finding in people with MODS. Instead, it appears that mitochondrial dysfunction causes a transient reduction in cellular activity that can return when the animal recovers. This phenomenon has been referred to as a cellular hibernatory-like state.
Individual Organ System Dysfunction

Several different forms of organ dysfunction have been recognized in people and animals during sepsis and other inflammatory states. The predominate organ systems involved in MODS that are identified and characterized clinically are the hepatic, respiratory, gastrointestinal, cardiovascular, coagulation, renal, central nervous, and endocrine systems. These forms of organ dysfunction are discussed in detail below.

Hepatic Dysfunction

Hepatic damage caused by sepsis or other forms of SIRS is typically divided into primary and secondary stages. In the primary stage, septic shock results in hepatic hypoperfusion leading to decreased protein synthesis, lactate clearance, gluconeogenesis, glycogenolysis, and hypoglycemia. Blood concentrations of aminotransferase increase as the result of hepatocellular leakage and coagulopathy may become clinically apparent. The secondary stage of hepatic dysfunction results from Kupffer cell activation and subsequent production of proinflammatory cytokines, chemokines, reactive oxygen species, and nitric oxide leading to further liver damage and dysfunction.

In the context of MODS, hepatic dysfunction is often defined as hyperbilirubinemia in the absence of preexisting liver disease, although other definitions such as increased concentrations of alanine transaminase (ALT) or alkaline phosphatase (ALP) in the blood or the presence of hepatic encephalopathy are sometimes used which makes it difficult to assess the overall
incidence of hepatic dysfunction in people and small animals.\textsuperscript{44} Hepatic dysfunction is an inconsistent predictor of mortality in people and dogs with MODS.\textsuperscript{44-50}

The incidence of hepatic dysfunction in people in the ICU approaches 11\%.\textsuperscript{44} Hepatic dysfunction appears to be more common in dogs and cats compared to people. The incidence of hepatic dysfunction in dogs with sepsis ranges from 33 to 72\%.\textsuperscript{50-52} Increased blood concentrations of ALP were the most common biochemical abnormality with either Gram-positive or Gram-negative bacteremia in one retrospective study of 140 dogs.\textsuperscript{53} Hyperbilirubinemia is common in cats with sepsis with reports ranging from 21 to 50\% of cats affected.\textsuperscript{54-57} Serum ALT concentrations are also commonly increased in cats with sepsis and are associated with prognosis.\textsuperscript{54-57} Increased ALP is an inconsistent finding in cats with sepsis.\textsuperscript{54-57}

Liver dysfunction is managed with supportive care including balanced crystalloid fluids given intravenously, S-adenosyl methionine, ursodeoxycholic acid, and vitamin K. If coagulation abnormalities are present, it may be necessary to give fresh frozen or frozen plasma. Hypoalbuminemia, if present, may be managed in hospital with colloidal and nutritional support, and some animals might benefit from human albumin transfusions, although the use of human albumin in dogs and cats is controversial.\textsuperscript{58} If hepatic encephalopathy is present, treatment consists of decreasing ammoniogenesis by feeding a low protein diet, providing gastrointestinal ulcer prophylaxis, administering oral antibiotics (e.g., neomycin, metronidazole) to decrease the numbers urease-producing bacteria in
the gut, and giving lactulose to speed transit through the gastrointestinal tract and decrease the amount of ammonia available for absorption into the bloodstream and, therefore, accessible to cross the blood brain barrier.

**Respiratory Dysfunction**

Acute Lung Injury (ALI) and Acute Respiratory Distress Syndrome (ARDS) are two manifestations of respiratory dysfunction in people and animals. ALI/ARDS can result from two different pathways: (1) direct pulmonary causes (e.g., bacterial or aspiration pneumonia, lung contusions, and inhalation injury) or (2) indirect causes (e.g., sepsis, pancreatitis, trauma, burns, and blood transfusions [transfusion-associated acute lung injury]).\(^{59-66}\) ALI/ARDS is characterized by neutrophil infiltration of the lung, alveolar-capillary barrier damage, pulmonary vascular leakage, and alveolar and systemic release of pro-inflammatory cytokines. Alveolar-capillary barrier damage and increased vascular permeability result in pulmonary edema while the production of cytokines perpetuates inflammation, promotes atelectasis, and causes structural damage to the type I alveolar pneumocytes.\(^{67,68}\) Type II pneumocytes begin to proliferate in an effort to repair the areas of denuded epithelium left by the damaged type I pneumocytes and eventually fibroblastic proliferation leads to narrowing and collapse of the pulmonary interstitium and alveoli. Fibrosis and collagen deposition takes place in the final stages of ARDS.\(^{69-71}\)

Gross necropsy findings in dogs with ARDS include diffusely firm, heavy and mottled lungs which often ooze fluid on cut surface. Histopathologic findings
include severe, diffuse, suppurative alveolitis and bronchiolitis, alveolar hemorrhage, neutrophil infiltration, hyaline membrane formation, proliferation of type II pneumocytes, and interstitial fibrosis.\textsuperscript{72} Lung pathology consistent with ARDS was common in a cohort of dogs with parvovirus and changes included diffuse accumulations of edema fluid, fibrin, macrophages, and neutrophils within the interalveolar septa and alveoli.\textsuperscript{64}

ARDS was first described in 1967 and termed “adult respiratory distress syndrome”. In 1994, the American-European Consensus Conference on ARDS defined ALI and ARDS as a syndrome of inflammation and increased permeability that is associated with a constellation of clinical, radiologic, and physiologic abnormalities that cannot be explained by, but may coexist with, left atrial or pulmonary hypertension. ARDS is a more severe form of ALI and over half of people who are diagnosed with ALI will progress to ARDS, which has a poorer prognosis.\textsuperscript{73} ARDS and ALI are diagnosed in people based on the following criteria: (1) acute onset, (2) bilateral infiltrates on thoracic radiographs, (3) pulmonary artery wedge pressure of <18 mmHg or no evidence of left atrial hypertension, and (4) a PaO$_2$/FiO$_2$ ratio of less than 300 for ALI and less than 200 for ARDS.\textsuperscript{74} Similar criteria have been established in veterinary medicine (Tables 1 and 2).\textsuperscript{75}

Mortality rates for ALI/ARDS in people are difficult to accurately estimate because of the variability of diseases that cause ALI/ARDS, but range from 15 to 80\% with no difference in overall mortality between direct and indirect causes of ARDS.\textsuperscript{59,76} Survival rates of ALI/ARDS in veterinary medicine is thought to be
lower than in human medicine, although it is difficult to estimate the true survival rate because of the influence of economic or philosophical confounding variables. Additionally, there is limited information available pertaining to ARDS-specific mechanical ventilation in small animals. Description of successful management of dogs with ALI/ARDS has been limited to case reports and case series.\textsuperscript{77,78} In a retrospective study of dogs and cats that required mechanical ventilation, 12/73 were diagnosed with ARDS and only 1 survived.\textsuperscript{79}

There is a growing amount of experimental and clinical literature on ALI/ARDS in veterinary medicine. Small animals develop ALI/ARDS following experimentally induced gram negative sepsis or endotoxemia.\textsuperscript{80-89} Endotoxin causes patchy alveolar congestion, perivascular and peribronchial edema and hemorrhage, thickened alveolar septae, multifocal acute alveolar epithelial necrosis, alveolar infiltration of neutrophils and macrophages, and endothelial neutrophil margination in cats;\textsuperscript{89,90} these changes are dose dependent in nature.\textsuperscript{90} Dogs are relatively resistant to endotoxin infusions compared to cats and other species;\textsuperscript{91} however, the development of ALI and apoptotic lung parenchyma, increases in bronchoalveolar lavage fluid neutrophil counts, and functional alterations of canine pulmonary macrophages have been documented following endotoxin administration.\textsuperscript{85,92-94}

In 1992, 19 dogs with ARDS were described in a case series. Bacterial pneumonia, sepsis, aspiration pneumonia, and shock were the most common causes identified. Thoracic radiographs ranged from normal to diffuse alveolar infiltrates in all lung lobes, and radiograph appearance worsened over time in
most dogs.\textsuperscript{72} Respiratory support consisted of oxygen supplementation (15/19) and/or mechanical ventilation (11/19).\textsuperscript{95} All dogs died; twelve dogs were euthanatized and 7 dogs developed cardiopulmonary arrest and died. In a study describing dogs undergoing celiotomy for various reasons including gastrointestinal surgery, splenectomy, and gastric dilatation-volvulus, 1.9\% (3/162) dogs developed ARDS following surgery. All 3 dogs died and had evidence of ARDS on necropsy.\textsuperscript{96}

Supplemental oxygen therapy is the mainstay of treatment for ALI/ARDS and can be provided through several routes including oxygen tent, nasal prongs, nasal catheters, and oxygen cage. However, supplemental oxygen is often not enough to support the animal; mechanical ventilation may be required. Mechanical ventilation is pursued if appropriate oxygenation or ventilation cannot be met with spontaneous ventilation alone, or if respiratory fatigue is likely to occur.

Institution of lung protective mechanical ventilation strategies has improved survival in people with ALI/ARDS.\textsuperscript{97-99} Traditional methods of mechanical ventilation often use high tidal volumes in order to achieve optimal arterial partial pressures of oxygen and carbon dioxide; however, volutrauma and subsequent perpetuation of tissue damage and inflammation may result.\textsuperscript{100-105} Lung protective ventilation strategies such as low tidal volume (6 ml/kg) ventilation increased survival and ventilator-free days when compared to the high tidal volume (12 ml/kg) ventilation in people.\textsuperscript{106} The Surviving Sepsis Campaign guidelines for people with ALI/ARDS recommend the use of low tidal volume
ventilation, positive end-expiratory pressure in order to avoid extensive lung collapse at end-expiration, permissive hypercapnia as needed to avoid high plateau pressures and tidal volumes, and conservative fluid therapy. Similar management strategies are recommended in dogs and cats with ALI/ARDS; however, they have not been rigorously tested. 

**Gastrointestinal Dysfunction**

The gastrointestinal tract serves as a nutrient absorber and barrier to the rest of the body, and these barrier functions are important in the prevention of bacteria and bacterial PAMPs such as endotoxin from leaving the intestines and entering the general circulation. Bacterial translocation is defined as the passage of gastrointestinal microflora through the intestinal wall toward local mesenteric lymph nodes and from there to other extranodal sites. Risk factors for bacterial translocation in people include intestinal obstruction, icterus, inflammatory bowel disease, neoplasia, emergency surgery, and lack of enteral nutrition. The gastrointestinal tract has several defense mechanisms in place to avoid bacterial translocation, including the mucus layer of the gastrointestinal lumen, luminal IgA, the epithelial barrier lining the gastrointestinal tract, and gastrointestinal and hepatic macrophages.

The principal mechanisms thought to be responsible for bacterial translocation are an alteration in the normal gastrointestinal flora which results in bacterial overgrowth, physical disruption of the gut mucosal barrier, ischemia, and impaired host defenses. Following a severe insult such as multiple trauma...
or cardiac arrest, the gut flora (including obligate anaerobes and *Lactobacillus*) is destroyed immediately and the number of intestinal pathogenic bacteria gradually increases.\textsuperscript{115} Destruction of gut flora is detrimental because these commensal organisms are an important defense against pathogenic bacteria colonization and thus help aid in the prevention of bacterial translocation.\textsuperscript{116} In addition to barrier dysfunction, sepsis also causes changes in gastrointestinal motility and absorption of nutrients. Endotoxin given intravenously to dogs causes a reduction in the number and strength of jejunal contractions, net absorption of water, electrolytes, and glucose from the jejunum\textsuperscript{117,118} colonic absorption of water and sodium\textsuperscript{119} and increased colonic motility and contractions.\textsuperscript{120} These changes can lead to diarrhea, dehydration, and electrolyte abnormalities.

Gastrointestinal dysfunction is described in people and animals as hypo or anorexia, inability to tolerate enteral feedings, decreased intestinal motility, hemorrhagic diarrhea, increased intestinal permeability, and bacterial translocation.\textsuperscript{121-123} The incidence of overall gastrointestinal dysfunction in people is difficult to gauge compared to other forms of organ dysfunction due to the lack of a clear definition;\textsuperscript{122,124} however, it is considered to be common.\textsuperscript{110,121}

Gastrointestinal dysfunction has been evaluated experimentally in dogs and cats. Dogs and cats with experimentally-induced endotoxemia have significantly increased gastrointestinal mucosal permeability when compared to control animals. Cats also exhibit significantly more jejunal epithelial necrosis and neutrophil infiltration.\textsuperscript{125,126} Endotoxin-induced jejunal mucosal barrier dysfunction was prevented when cats were pretreated with anti-TNF-\(\alpha\) antibodies prior to
endotoxin administration, suggesting that TNF-α may be an important mediator of endotoxin-induced mucosal epithelial barrier dysfunction.\textsuperscript{127}

Several treatment and management strategies aimed at the gastrointestinal system have been evaluated in critically ill people. Early enteral nutrition, selective decontamination of the digestive tract with oral and parenteral antibiotics, probiotics, immunonutrients such as glutamine, arginine, and omega-3 fatty acids, prokinetic drugs, and changing the route of nutritional support have all been associated with decreased morbidity and/or mortality.\textsuperscript{121,128-131} Enteral nutrition, when compared to total parenteral nutrition, prevents gastrointestinal mucosal atrophy, maintains immune competence, and preserves normal gut flora.\textsuperscript{113} Neither enteral nor parenteral nutrition is without complications. Enteral nutrition has disadvantages including diarrhea, nausea, and vomiting. Negative aspects of total parenteral nutrition include intestinal atrophy, decreased mucosal resistance to bacteria, catheter-related sepsis, hyperglycemia, liver dysfunction, and increased cost.\textsuperscript{132-134}

In veterinary species, enteral feeding is typically recommended unless the animal will not tolerate it in which case parenteral nutrition is recommended.\textsuperscript{135} In clinical trials and animals models of critical illness, enteral nutrition alone and enteral nutrition combined with parenteral nutrition is superior to total parenteral nutrition alone or no nutrition.\textsuperscript{112,113,136-143} However, total parenteral nutrition is superior to no nutritional intervention in regards to improved gut mucosal barrier function, decreased bacterial translocation, and/or survival rates.\textsuperscript{137,144}
Cardiovascular Dysfunction

The cause of cardiovascular dysfunction is multifactorial but is generally thought to be associated with the production of substances which lead to decreased cardiac contractility and mitochondrial damage. Endotoxin and inflammatory cytokines (e.g., IL-1β and TNF-α) and calcium leak from the sarcoplasmic reticulum, ultimately leading to a decrease in myocardial cell contractility. In dog, guinea pig, and human models, several cardiac abnormalities occur including decreased contractility, left ventricular dilation, and decreased left ventricular ejection fraction after exposure to Staphylococcus aureus and Escherichia coli, TNF-α, IL-1β, and IL-6. The pro-inflammatory complement protein C5a may also play a role in myocardial dysfunction by producing reactive oxygen species. Nitric oxide (NO) production leads to decreased cardiac contractility through down-regulating the beta-adrenergic myocardial receptors and by decreasing cytosolic calcium. Peroxynitrite is formed from NO and results in oxidative mitochondrial damage and decreased cardiac contractility. Finally, NO may be the cause of vasodilation that ultimately leads to septic shock.

Cardiovascular dysfunction is common in people with sepsis and is considered to be a major contributor to morbidity and mortality rates as high as 70%. Cardiovascular dysfunction is characterized by biventricular dilatation, decreased ejection fraction, hypotension often despite fluid therapy, and decreased response to catecholamines. When cardiovascular dysfunction is caused by sepsis, it is often referred to as septic shock, which is defined as
sepsis-induced hypotension despite adequate volume resuscitation, along with the presence of perfusion abnormalities that may include, but are not limited to, lactic acidosis, oliguria, or an acute alteration in mental status.⁰²

Critical illness-induced left ventricular dysfunction has been described in 16 dogs in which primary heart disease was not suspected and congestive heart failure was not present.¹⁵⁸ Four of these dogs survived to discharge and 1 of these dogs had an echocardiogram performed 2 years later. The percent fractional shortening at the time of time of admission to the ICU was low (21%) and 2 years later it was considered normal (34%).

In small animals, cardiovascular dysfunction has been defined as hypotension requiring vasopressor therapy and is associated with reduced survival.⁶,⁵⁰ In a cohort of dogs with gastrointestinal sepsis, 17% had cardiovascular dysfunction and only 10% of these dogs survived to discharge.⁵⁰ Of 33 dogs with various forms of sepsis, 7 had septic shock, 6 of which had persistent hypotension despite aggressive vasopressor therapy, and all of the dogs with hypotension died or were euthanatized due to grave prognosis.¹⁵⁹ In dogs with septic peritonitis, non-survivors received a greater number of vasopressors; 44% of non-survivors compared to 8% of survivors received 2 vasopressors and 7/44 dogs received more than two vasopressors; none of these dogs survived.¹⁶⁰ All of 10 dogs with septic shock and MODS due to babesiosis died, despite aggressive treatment.⁵¹

Literature regarding sepsis-induced cardiovascular dysfunction is cats is lacking. Relative bradycardia is a common and unique finding in cats with sepsis;
19/29 cats with severe sepsis were reported to have an inappropriately low heart rate, and this mechanism is suspected to be secondary to increased vagal tone or cytokine-associated myocardial dysfunction.\textsuperscript{55} The combination of bradycardia and hypothermia was a negative prognostic indicator in a case series of 12 cats with primary bacterial septic peritonitis;\textsuperscript{56} however, another study did not support this conclusion.\textsuperscript{55} Cats given lipopolysaccharide (LPS) intravenously develop a significant biphasic hypotensive response at 1.5 hours following injection and then again at 5 hours.\textsuperscript{89} Recovery of cardiovascular function is variable; 2/3 cats treated with dopamine for hypotension in a case series of cats with primary bacterial septic peritonitis survived,\textsuperscript{56} while only 3/31 critically ill cats who required catecholamines to maintain blood pressure while being mechanically ventilated survived to discharge.\textsuperscript{108}

The Surviving Sepsis Campaign guidelines for people recommend maintaining mean arterial blood pressure $\geq 65$ mmHg. Appropriate fluid resuscitation through the use of crystalloids, colloids, or blood products is of utmost importance for the treatment of non-cardiac causes of shock in order to maintain adequate tissue perfusion. When hypotension is no longer responsive to volume therapy, inotropic and vasopressor drugs should be used to effect. Dobutamine, a $\beta$-1 agonist, is recommended when myocardial dysfunction is present based on increased cardiac filling pressures and low cardiac output. If vasopressors are necessary, dopamine and norepinephrine are considered the first line drugs of choice.
Coagulation Dysfunction

Coagulation is a physiologic process intended to localize inflammation at the site of infection, prevent the spread of microorganisms, stop active hemorrhage, and promote wound healing.\(^{161}\) Disseminated intravascular coagulation (DIC) occurs when the appropriate physiologic response is exaggerated by the presence of pro-inflammatory cytokines such as IL-1\(\beta\), IL-6, and TNF-\(\alpha\). This pro-inflammatory response leads to fibrin formation and microvascular thrombosis through the up-regulation of procoagulant pathways, down-regulation of anticoagulant pathways, and suppression of fibrinolysis.\(^{162-164}\) Disseminated intravascular coagulation is magnified by a positive feedback loop that perpetuates the coagulation cascade.\(^{165}\) Animals with DIC may develop microvascular thrombosis or hemorrhage resulting from consumption and exhaustion of coagulation factors, or both simultaneously.\(^{163}\) Disseminated intravascular coagulation most commonly occurs in people with sepsis, trauma, and cancer.\(^{162}\)

In dogs, coagulation dysfunction has been defined as prolongation of prothrombin time (PT) or partial thromboplastin time (PTT) > 25% above the upper reference limit and/or a platelet count ≤ 100,000/µL,\(^{50}\) and it is a negative prognostic indicator in dogs with sepsis and trauma.\(^{6,50}\) Of dogs with sepsis, 60.5% had coagulation dysfunction and coagulation dysfunction was the most common disorder diagnosed in a recent multi-center retrospective veterinary study.\(^{50}\) Thrombocytopenia is the most commonly cited sign of coagulation dysfunction in people.\(^{166}\) Thrombocytopenia is an independent predictor of ICU
mortality,\textsuperscript{167,168} and sustained thrombocytopenia for more than 4 days or > 50% decrease in platelet count is associated with a 4- to 6-fold increase in mortality in people.\textsuperscript{167,169}

The diagnosis of DIC is not straightforward and is often made based on clinical assumptions and the combination of a variety of clinical parameters. A scoring system for the diagnosis of DIC in people has been proposed by the International Society of Thrombosis and Haemostasis (ISTH) and includes various coagulation indices such as platelet count, prothrombin and partial thromboplastin time, and fibrin degradation products,\textsuperscript{170} and the severity of DIC according to this scoring system correlates with mortality during sepsis.\textsuperscript{171} In veterinary medicine, similar diagnostic criteria are used,\textsuperscript{172} and a scoring system for the diagnosis of DIC in dogs has been proposed,\textsuperscript{173} although to date there is no diagnostic gold standard for DIC in dogs or cats (Table 3).\textsuperscript{174} D-dimers have been evaluated in cats for the diagnosis of DIC; however, D-dimers were neither sensitive nor specific for differentiating sick cats with DIC to sick cats without DIC.\textsuperscript{175} Antithrombin also does not appear to be a good diagnostic test for DIC in cats as only 2/7 cats with DIC had decreased antithrombin concentrations in one clinical study.\textsuperscript{176} Thromboelastography has gained popularity in recent times in veterinary medicine and has been evaluated in dogs with DIC. Thromboelastography allows for the differentiation of hypo-, normo-, and hypercoagulable states during DIC in dogs. Dogs with DIC are more likely to be hypercoagulable than hypocoagulable, and hypercoagulability is associated with a better outcome.\textsuperscript{174}
Experimental and clinical evidence of DIC is available in the literature for dogs and cats. Endotoxin administration to dogs results in decreased protein C, factors V and VIII, fibrinogen, and prolonged PT and PTT,\textsuperscript{177} and marked thrombocytopenia.\textsuperscript{178} Other coagulation abnormalities found in dogs with sepsis include increased D-dimer, fibrinogen degradation products (FDP), and Von Willebrand factor, and depletion of antithrombin and activated protein C.\textsuperscript{50,179-181} There is some evidence that decreased antithrombin concentrations are associated with decreased survival in dogs with critical illness including sepsis,\textsuperscript{182,183} while other studies show no correlation.\textsuperscript{179} Of 10 dogs with septic shock and MODS due to babesiosis, 9 had thrombocytopenia and none of these dogs survived.\textsuperscript{51}

Cats given LPS intravenously developed a significantly prolonged PT compared to placebo at 8 hours; PTT, antithrombin, fibrinogen, and D-dimer concentrations were not significantly different.\textsuperscript{89} A retrospective study evaluated 46 cats with DIC. The most common primary diseases were neoplasia, sepsis, and pancreatitis, and lethargy and anorexia were the most common presenting complaints. Coagulation abnormalities included a prolonged PT (26/34 cats) and PTT (33/33 cats), thrombocytopenia (12/24 cats), increased FDP (10/33 cats), and decreased fibrinogen (22/33 cats). The median PT of non-survivors was significantly prolonged compared the median PT of survivors. Treatments included blood products, unfractionated heparin, and vitamin K, and no treatment significantly affected outcome. Only 3/46 cats survived.\textsuperscript{184} A necropsy was performed on 24 of the cats, and microvascular thrombosis consistent with DIC
was histologically confirmed on 19/24 cats.\textsuperscript{184} There were 3 cats with evidence of DIC on histopathologic examination, but there were no coagulation parameters that met the criteria for DIC.\textsuperscript{184} Additional retrospective evaluations of DIC in cats have shown similar coagulation profiles.\textsuperscript{185,186}

The mainstay of treatment for DIC is correcting the underlying problem, which is often challenging. Fresh frozen plasma transfusions can be administered with the goal of replenishing consumed coagulation factors. However, plasma transfusion should be reserved for animals with a documented deficiency in coagulation factors and active bleeding, or if an invasive procedure such as surgery is planned.\textsuperscript{107,187} Platelet transfusion may also be necessary if the patient is bleeding, or is at risk for bleeding, due to thrombocytopenia.\textsuperscript{107}

Activated Protein C (APC) has also become a treatment option for people with sepsis and DIC. In normal coagulation, APC has anticoagulant, profibrinolytic, and anti-inflammatory activity and also decreases endothelial permeability.\textsuperscript{188,189} Currently, the use of APC is only recommended for adult people with sepsis-induced organ dysfunction associated with a high risk of death;\textsuperscript{107} however, there is more recent evidence that there is no survival benefit for adults with septic shock given APC.\textsuperscript{190} APC is an effective anticoagulant and antithrombotic agent in dogs; however, the doses required to achieve this effect are 15- to 20-fold higher than in humans, and APC is prohibitively expensive in veterinary medicine.\textsuperscript{191}
Renal Dysfunction

Renal dysfunction is referred to as acute kidney injury (AKI). Like many forms of organ dysfunction, AKI is caused by several different pathways. There are two main forms of AKI associated with MODS. One form involves a more traditional definition of kidney failure and is characterized by renal epithelial necrosis, and renal hypoperfusion and ischemia are often cited in the pathogenesis.\textsuperscript{192-195} The second form of AKI is specific to MODS and is not associated with necrosis; this is the most common form in people. A review of 6 studies looking at AKI caused by sepsis in humans found that only 22\% of patients with sepsis-induced AKI had histopathologic evidence of acute tubular necrosis. Similarly, only 37\% and 23\% of primate and rodent sepsis-induced AKI models, respectively, were consistent with acute tubular necrosis while a dog and sheep sepsis-induced AKI model had no evidence of acute tubular necrosis. In fact, the majority of animals or people in these studies were reported to have histopathologically normal kidneys.\textsuperscript{196} Instead of global hypoperfusion during sepsis, renal blood flow is adequate or increased which may explain the lack of acute tubular necrosis.\textsuperscript{197-199} It has been proposed that during sepsis-induced AKI, the efferent arteriole dilates to a greater degree than the afferent arteriole resulting in increased renal blood flow with a reduced glomerular filtration rate (GFR).\textsuperscript{200} Apoptosis caused by inflammatory cytokines (e.g., TNF-\textalpha) and endotoxin also appear to be a predominant mechanism of sepsis-induced AKI.\textsuperscript{201} In addition to causing apoptosis, TNF-\textalpha also causes tubular neutrophil infiltration and a reduction in GFR.\textsuperscript{202,203}
Acute kidney injury is a syndrome characterized by decreased glomerular filtration and is classified in people by the severity of azotemia, urine production, or GFR. Two similar classification schemes are used in human medicine: (1) The RIFLE criteria stratifies patients into three different levels of kidney injury ("Risk", "Injury", and "Failure") and two different clinical outcomes ("Loss" and "End-stage disease"); (2) The AKIN (Acute Kidney Injury Network) criteria divides patients into stages 1, 2, and 3 (Table 4). Sepsis-induced AKI is classified by the simultaneous presence of (a) the RIFLE or AKIN criteria for AKI and (b) the criteria for sepsis in the absence of non-sepsis-related causes of AKI, such as nephrotoxins. Sepsis-induced AKI is an important form of organ dysfunction in people because it markedly increases mortality. A multinational, multicenter human study found that AKI had a prevalence of 5 to 6% and only 40% of these people survived to dismissal. Septic shock was the most common cause of AKI in this study. Acute kidney injury occurs in up to 65% of people with septic shock. The prevalence of AKI in dogs is unknown; however, AKI is considered to decrease survival. In a population of dogs that underwent surgery for septic peritonitis, 12.3% met the criteria for renal dysfunction and only 14% of these dogs survived to discharge. Renal dysfunction was the most common organ dysfunction in a cohort of dogs with septic shock caused by babesiosis.

A recent veterinary study aimed to identify hospitalized dogs with AKI by creating a classification scheme based on the AKIN criteria. Dogs that were azotemic at admission were excluded. The investigators found 14.6% of dogs
met criteria for AKI during hospitalization and the survival rate was 45.8%. The survival rates between these two studies are markedly different, and the disparity can most likely be related to differences in patient population. The first study involved only patients with septic peritonitis while only approximately half of the patients with AKI in the second study had sepsis due to various causes.

Optimizing renal blood flow is important for the prevention and treatment of AKI and this can be achieved by maintaining cardiac output, intravascular volume, and renal perfusion pressure through the use of early goal-directed therapy. The Surviving Sepsis Campaign guidelines in people recommend maintaining a mean arterial pressure of ≥ 65 mmHg, central venous pressure of 8 to 12 mmHg, urine output ≥ 0.5 mL/kg/hr, and central venous oxygen saturation ≥ 70% by proper fluid resuscitation, blood transfusions, positive inotropes, and vasopressors as necessary. The use of low-dose dopamine infusions is no longer recommended to increase renal blood flow. When low-dose dopamine was compared to placebo in a large, randomized trial and meta-analysis, investigators found no difference in several outcome measures including peak serum creatinine, need for renal replacement, urine output, and survival to discharge. Hemodialysis, continuous renal replacement therapy (CRRT), and peritoneal dialysis are mainstays for treatment of acute renal failure in human medicine, and opportunities for these therapies are becoming more common in veterinary medicine. Basic supportive care treatments for small animals include balanced electrolyte solutions given intravenously, oral
phosphate binders, and gastrointestinal protectants including H-2 blockers and proton pump inhibitors.

**Central Nervous System Dysfunction**

Sepsis-associated encephalopathy (SAE) is an acute and reversible deterioration of mental status characterized by changes in consciousness, awareness, cognition, and behavior in people. The pathophysiology of SAE is not completely understood. Initially, the blood-brain barrier is intact and this protects the brain from systemic inflammation. Inflammatory mediators (e.g., IL-1β, TNF-α) stimulate the afferent fibers of the vagus nerve, which acts as a conduit to the central nervous system. Following stimulation of the vagus nerve, cerebral endothelial cells are then activated, resulting in breakdown of the blood-brain barrier. The activation of cerebral endothelial cells also causes alterations in the microcirculation and vascular tone leading to hemorrhagic and ischemic lesions. Additionally, reactive oxygen species are formed which compromise neuronal and microglial cell function and survival and eventually lead to apoptosis and edema. Finally, SAE is thought to decrease the vasodilatory response of the cerebrum leading to impairment of cerebral autoregulation of blood flow. Brain histopathology from patients with septic shock show a variety of lesions including cerebral edema, infarcts, microabscesses, intravascular thrombosis, and neuronal cell death.

Sepsis-associated encephalopathy is the most common form of encephalopathy in people with an incidence of 8 to 70% of people with sepsis in
the ICU. However, the recognition of SAE is often hindered by the use of sedatives for mechanical ventilation. The development of SAE in people with sepsis has long-term detrimental consequences including neurologic impairment, reduced cognitive scores in children, and physiological disorders. The incidence and long-term impact of this phenomenon in veterinary species is unknown.

**Adrenal Dysfunction**

Critical illness related corticosteroid insufficiency (CIRCI) involves reversible inhibition of adrenocorticotropin hormone (ACTH) secretion and adrenal cortical cell response to ACTH by pro-inflammatory mediators (e.g., TNF-α). Additionally, corticosteroid tissue resistance increases in acute inflammatory diseases such as sepsis. So while adequate amounts of cortisol are produced, corticosteroid receptor binding is impaired. Critical illness related corticosteroid insufficiency has also been associated with adrenocortical hemorrhage or infarction due to microvascular thrombosis; in this situation, adrenal insufficiency may be permanent.

Critical illness related corticosteroid insufficiency is defined as inadequate corticosteroid activity relative to illness severity and describes a dysfunction of any aspect of the hypothalamic-pituitary-adrenal (HPA) axis. Critical illness related corticosteroid insufficiency is a dynamic process that is characterized by basal serum cortisol concentrations that are often within or above the reference interval; however, following ACTH administration there is dampened cortisol
secretion. Critical illness related corticosteroid insufficiency has an approximate overall prevalence of 30% in critically ill people and the prevalence increases to approximately 60% in people with septic shock.

A delta (Δ) cortisol (difference between cortisol measured pre- and post-ACTH stimulation) of <9 µg/dL or a baseline total cortisol of <10 µg/dL is considered the best way to diagnose CIRCI in people. The typical electrolyte changes associated with hypoadrenocorticism (i.e., hyponatremia, hyperkalemia, and hypercalcemia) are not recognized in people with CIRCI. Although parameters have been provided in human medicine to objectively diagnose CIRCI, an ACTH stimulation test is not recommended because of the limitations of this test in critically ill patients. These limitations include overestimation of CIRCI because of decreased bound cortisol due to low concentrations of corticosteroid binding globulin and albumin in critical illness, and high inter-assay variations of total serum cortisol in patients with septic shock. The measurement of free cortisol is superior to total cortisol, however, the measurement of free cortisol is not widely available.

There are a handful of veterinary studies regarding CIRCI, and the majority have found that critically ill animals have similar adrenal dysfunction to people. In one study, 48% of dogs with sepsis had CIRCI, and dogs with a Δ-cortisol of <3 µg/dL were more likely to be hypotensive and less likely to survive. There is only one case report each of a dog and cat with septic shock and evidence of CIRCI whose shock was reversed with the use of hydrocortisone or dexamethasone, respectively. Both animals had complete recoveries.
Prospective clinical trials are needed to determine if treatment with corticosteroids improves outcome in animals suspected of having CIRCI. At the present time, there are no definitive guidelines for the diagnosis or treatment of CIRCI in veterinary medicine.

The Surviving Sepsis Campaign guidelines for people suggest that treatment should be considered in adults with septic and hypotension that is poorly responsive to fluid resuscitation and vasopressor therapy.\textsuperscript{107} This recommendation is not considered to be strong because of the conflicting results of two different multicenter clinical trials. In a French multicenter study, hydrocortisone and fludrocortisone administration resulted in a 30\% decrease in 28-day mortality; this benefit was limited to people with a poor ACTH stimulation test response.\textsuperscript{249} A second large multicenter trial known as the CORTICUS (Corticosteroid Therapy of Septic Shock) trial found no difference in 28-day mortality between patients who received hydrocortisone as compared to placebo regardless of ACTH stimulation response.\textsuperscript{250} However, hydrocortisone treatment resulted in a faster reversal of shock in all patients.\textsuperscript{250} Discrepancies between these two studies have been attributed to differences in inclusion criteria, patient demographics, timing of treatment, and the administration of fludrocortisone.\textsuperscript{107,238}
**Prognosis**

In people with MODS, the number of dysfunctional organ systems correlates with mortality in the ICU in people.\(^{251}\) People with severe sepsis and multiple organ dysfunction are 2.2 times more likely to die than patients with severe sepsis and single organ dysfunction,\(^ {252}\) and people with four or more dysfunctional organs are four times more likely to die than those with single organ dysfunction.\(^ {252}\) One multi-center study found mortality rates corresponding with 1, 2, 3, and more than 4 dysfunctional organ systems was 21.2%, 44.3%, 64.5%, and 76.2%, respectively, in critically ill people.\(^ {17}\) Children with MODS have worse functional outcomes, higher mortality, and longer stays in the ICU than children who do not have MODS.\(^ {253}\) Mortality rates associated with MODS in people are influenced by co-morbidities such as chronic kidney disease, cancer, and diabetes\(^ {17,254}\) and cumulative co-morbidities are associated with greater risk for organ dysfunction.\(^ {255}\)

MODS is also associated with non-survival in veterinary medicine. In dogs treated surgically for abdominal sepsis, survival was inversely proportional to the number of dysfunctional organ systems with a survival rate of 30% in dogs with MODS compared to a 75% survival rate in dogs without MODS.\(^ {5}\) Similar results were found in a report of canine babesiosis; 10% of dogs had MODS, and 55%, 90% and 100% of dogs with 2, 3, and 4 failed organs, respectively, died.\(^ {256}\) In dogs with trauma, the incidence of MODS was lower compared to dogs with abdominal sepsis, but all dogs with MODS died.\(^ {6}\)
Biomarkers for MODS

The lack of objective methods to identify MODS is just one of the challenges veterinarians face when treating critically ill animals. Biomarkers are important in both human and veterinary medicine as we attempt to diagnose diseases, determine prognosis, and stratify patients as part of clinical studies and clinical decision-making. Biomarkers for sepsis, SIRS, and MODS have been researched extensively.\textsuperscript{52,257-266} A study of human trauma patients found plasma concentrations of TNF-\(\alpha\), IL-6, CXC chemokine ligand (CXCL)-8, and IL-10 to be increased in patients who developed MODS. IL-1\(\beta\) was not associated with MODS. IL-6 was the most reliable predictor of MODS and mortality with an overall accuracy rate of 84.7% and 86.1% on day 1, respectively.\textsuperscript{264} Matrix metalloproteinase-9 (MMP-9) is released from polymorphonuclear leukocytes in the presence of endotoxin and pro-inflammatory mediators. Strong positive correlations of plasma MMP-9 to tissue MMP-9 in patients with MODS have been found in early MODS, suggesting that MMP-9 may be an early predictor of the severity of MODS.\textsuperscript{265} Biomarkers specific for MODS have not been evaluated in veterinary medicine.
CHAPTER 2
MECHANISM OF NT-pCNP PRODUCTION IN DOGS

Introduction

C-type natriuretic peptide (CNP) is a part of the natriuretic peptide family and has been evaluated as a biomarker for sepsis and other forms of critical illness in people and dogs.\(^{267-270}\) The amino-terminal portion of pro-CNP (NT-pCNP), which is secreted in equimolar amounts and is a more stable molecule than CNP, is significantly higher in dogs with non-peritoneal forms of sepsis compared to dogs with non-infectious causes of SIRS and healthy dogs. The use of NT-pCNP as a biomarker for diagnostic or prognostic purposes in dogs with MODS has not been investigated.

The natriuretic peptide family is comprised of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and CNP.\(^{271}\) Unlike ANP and BNP, CNP has venodilatory, cardiac inotropic and chronotropic, vascular smooth muscle anti-proliferative, and osteogenic effects, with relatively limited natriuretic and diuretic effects.\(^{271-277}\) Additionally, CNP plays a role in the innate immune response to infection through modulation of P-selectin,\(^{278}\) direct microbial growth inhibition, and virulence modification.\(^{279,280}\) The theory that CNP is involved in the response to infection is supported by the clinical usefulness of this biomarker.
in distinguishing sepsis from non-infectious causes of systemic inflammation in dogs and people\textsuperscript{267,269,281}.

C-type natriuretic peptide is expressed primarily by the vascular endothelium in response to various stimuli\textsuperscript{282,283}. CNP exerts its effects through autocrine and paracrine mechanisms, and undergoes rapid local degradation by neutral endopeptidase and uptake by clearance receptors with subsequent endocytosis and hydrolysis\textsuperscript{284}. The rapid local degradation of CNP has hindered assessment of CNP production in the past, but development of assays measuring NT-pCNP have offered a new approach to investigating CNP production. NT-pCNP and CNP are produced and secreted in equimolar amounts following intracellular proteolytic cleavage of pCNP\textsuperscript{284-287}. Compared to CNP, NT-pCNP is a larger molecule; NT-pCNP has a longer half-life in circulation and does not readily cross react with other natriuretic peptides making NT-pCNP a useful marker of CNP biosynthesis\textsuperscript{284,287,288}.

Although NT-pCNP has shown promise as a diagnostic biomarker for non-peritoneal sources of sepsis in dogs, the signaling pathways involved in CNP induction from canine endothelium are unknown. Our goal was to investigate regulation of CNP production from canine endothelium by stimulating canine aortic endothelial cells with various inflammatory mediators and pathogen associated molecular pattern (PAMP) motifs and then evaluating NT-pCNP production. Since NT-pCNP appears to be expressed primarily during infection, we hypothesized that the PAMP motifs would induce NT-pCNP secretion from
canine aortic endothelial cells to a significantly greater degree than inflammatory mediators.

Materials and Methods

*Cell culture*—Canine aortic endothelial cells (CAoE; Cell Applications, Inc.) were grown in Canine Endothelial Cell Growth Medium (Cell Applications, Inc.) in an atmosphere of 5% carbon dioxide at 37º Celsius (C) until confluence. Canine aortic endothelial cells in passages 7-11 were used. Cells were seeded in flasks and allowed to multiply until an appropriate cell number was reached and then 1x 10^6 cells were seeded in 24-well plates in a 225 µL volume. Cells were allowed to adhere for 24 hours and grow to confluence (~48 hours). Media exchanges were performed every 24 hours. Each cell culture experiment was repeated a minimum of three times.

*Exposure of CAoE to inflammatory mediators and PAMPs*—Lipopolysaccharide (LPS) from *Escherichia coli* 0127:B8 (Sigma-Aldrich), lipotechoic acid (LTA) from *Streptococcus faecalis* (Sigma-Aldrich), peptidoglycan (PG) from *Staphylococcus aureus* (Sigma-Aldrich), canine recombinant tumor necrosis factor-alpha (TNF-α, Thermo Scientific), interleukin-1 beta (IL-1β, Kingfisher Biotech, Inc.), IL-6 (Kingfisher Biotech, Inc.), IL-10 (Kingfisher Biotech, Inc), IL-21 (Kingfisher Biotech, Inc.), CXC chemokine ligand-8 (CXCL-8, Kingfisher Biotech, Inc.), interferon-gamma (IFN-γ, Kingfisher Biotech, Inc.), or vascular endothelial growth factor-A (VEGF-A, Kingfisher Biotech, Inc.) at various concentrations in phosphate-buffered saline (PBS) at a
volume of 25 µL were used to stimulate CAoE cells. Phosphate-buffered saline (25 µL) was used as a control. After addition of the stimulants, the plates were gently rocked for 3 minutes. The cell cultures were incubated and then centrifuged at 4°C and 500 x gravity (g) for 7 minutes. The supernatant was collected and placed into individual freezer resistant tubes. The tubes were coded so that the source of the sample was only known by the investigator performing the cell culture experiments (KO). Samples were stored at -80°C for batch analysis.

**Exposure of CAoE to PAMPs and serum--** Cells were prepared and stimulated with LPS (1000 ng/ml), LTA (1000 ng/ml), PG (1000 ng/ml), or PBS with or without 25 µL of pooled serum from healthy dogs. Cells were incubated for 24 hours and the supernatant collected.

**Cell lysate NT-pCNP--** Cells were prepared and stimulated with LPS (1000 ng/ml), TNF-α (20 ng/ml), and VEGF-A (100 ng/ml). After 24 hour incubation, one half of the wells were frozen, allowed to thaw and then physically disrupted. The supernatant was then collected and stored as for the other experiments. Concurrently, the supernatant was collected from the remaining samples without physical disruption, frozen, allowed to thaw and then stored as for the other experiments.

**Evaluation of CAoE viability after stimulation with inflammatory mediators and PAMPS--** Cells were prepared and stimulated with inflammatory mediators (20, 100 ng/mL), PAMPs (10,000, 100,000 ng/mL) or PBS. At 24 hours, a 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was used to
determine cell viability in each well as previously described.\textsuperscript{289,290} Absorption was measured at 630 nanometers and the optical density was compared among treatments.

\textbf{NT-pCNP assay--} NT-pCNP was measured using sandwich ELISA (Veterinary Diagnostic Institute, Simi Valley, CA) previously validated for canine NT-pCNP.\textsuperscript{281} This assay utilizes a highly purified polyclonal sheep antibody directed against amino acids 1-19 and 30-50 of human NT-pCNP (96% homologous with canine pCNP).\textsuperscript{281} The lower limit of detection of this assay is 0.55 pMol/L. All samples were stored for less than 6 months prior to analysis.\textsuperscript{270,281}

\textbf{Evaluation of TLR4 expression by CAoE cells in comparison to canine leukocytes--} Whole blood was collected from a healthy dog. Phycoerythrin (PE)-conjugated Anti-Human CD284 (TLR4, HTA125 clone, eBioscience Inc.) was incubated with 100 ul of whole blood or 1×10\textsuperscript{6} of canine endothelial cells (100 uL in the Flourescence Activated Cell Sorter [FACS] buffer: PBS solution containing 1% bovine serum albumin), for 30 minutes in the dark on ice. Matched isotype controls from the same manufacturer were used for negative controls of the antibody. Cells were then washed twice with FACS buffer and centrifuged at 400 × g for 5 minutes. For whole blood samples, red blood cells were lysed by adding 2 mL of ammonia chloride potassium lysis buffer solution (8.26 g NH\textsubscript{4}Cl, 1.0 g KHCO\textsubscript{3}, 0.037 g Na\textsubscript{2}EDTA in 1.0 L deionized distilled H\textsubscript{2}O, pH 7.2), and additionally washed twice after 10 minutes of incubation. Finally cells were re-suspended in 400 uL of FACS buffer and analyzed immediately. Flow
cytometry was performed at the University of Missouri Cell and Immunology Core Facility using a CyAn™ ADP (Beckman Coulter) flow cytometer, and analyzed with Summit™ software (Dako Colorado Inc.). Leukocyte components (neutrophils) were identified based on logical gating. A minimum of 10,000 events was recorded for each sample.

**Statistical Analysis**

Statistical analysis was performed using commercially available software (SigmaStat, Systat Software Inc.). The Mann-Whitney Rank Sum test was used to compare NT-pCNP production and CAoE viability among stimulants. Dose and time response curves were analyzed using a Kruskal-Wallis One Way Analysis of Variance on Ranks with post-hoc Dunn's multiple comparison procedure. Type I statistical error was limited to 5%. Data are presented as median and range unless otherwise indicated.

**Results**

*Exposure of CAoE to inflammatory mediators and PAMPs*-- For the initial evaluation of inflammatory mediator and PAMP stimulants, concentrations of 100 ng/mL or 100,000 ng/mL, respectively, were used. Lipopolysaccharide (P=0.024) was the only PAMP, and TNF-α (P=0.012) and IL-1β (P<0.001) were the only inflammatory mediators that resulted in significant production of NT-pCNP when compared to PBS at 24 hours (figure 1). Additionally, IL-1β resulted in significantly greater NT-pCNP secretion than LPS or TNF-α (P<0.001).
Lipoteichoic acid, PG, IL-6, IL-10, IL-21, CXCL-8, IFN-γ, and VEGF-A did not induce significant NT-pCNP production when compared to PBS. To insure that the lack of NT-pCNP production from these stimulants was not concentration or incubation time related, we repeated the experiment using 10 and 100 fold higher concentrations and incubated for 24 or 48 hours. However, increasing the stimulant concentration or incubation time did not result in significant NT-pCNP production (data not shown).

To further characterize NT-pCNP production from CAoE cells, dose and time response experiments were performed using the stimulants that induced significant NT-pCNP production in the initial evaluation. Stimulation of CAoE cells with LPS, TNF-α, and IL-1β resulted in NT-pCNP secretion that was dose dependent in nature (figure 2). The lowest concentration of IL-1β (P=0.004), TNF-α (P=0.002) and LPS (P=0.010) that stimulated significant NT-pCNP production were 10, 100, and 10,000 ng/mL, respectively. Induction of NT-pCNP was also time dependent for IL-1β (P=0.011), TNF-α (P=0.008) and LPS (P=0.005) with significant CNP production at 24 hours (figure 3).

*Exposure of CAoE to PAMPs and serum*—To evaluate if serum co-factors would increase PAMP-induced NT-pCNP production, the addition of healthy canine serum to the cell culture system was evaluated. The addition of canine serum to the culture media did not significantly alter LPS, LTA or PG-induced NT-pCNP production (data not shown).

*Cell lysate NT-pCNP*—We compared cell culture supernatant NT-pCNP concentrations to cell lysate NT-pCNP concentrations to evaluate the relative
ratio of intracellular to secreted NT-pCNP. For this experiment we tested one stimulant (VEGF-A) that did not induce significant NT-pCNP production to determine if NT-pCNP was produced but not secreted and moderate concentrations of 2 stimulants (TNF, LPS) that induced significant NT-pCNP production for evaluation. There was no significant difference in supernatant and cell lysate NT-pCNP concentrations for any of the stimulants (data not shown).

_Evaluation of CAoE viability after stimulation with inflammatory mediators and PAMPs—_ There were no significant differences in cell viability after the CAoE cells were incubated with each of the stimulants or PBS (data not shown).

_Evaluation of TLR4 expression by CAoE cells in comparison to canine leukocytes—_Toll like receptor 4 expression on CAoE cells was similar to the expression on neutrophils (figure 4).

**Discussion**

We found that LPS, TNF-α, and IL-1β, caused significant NT-pCNP secretion from canine aortic vascular endothelium in a cell culture model. IL-1β resulted in the greatest production of NT-pCNP. The effects of LPS, TNF-α, and IL-1β were both time and dose-dependent with maximal NT-pCNP secretion occurring when endothelial cells were stimulated with 10-1000 ng/mL of IL-1β or 100 ng/ml of TNF-α or LPS with a peak production time of 24 hours. We found that IL-6, IL-10, IL-21, CXCL-8, IFN-γ, and VEGF-A did not induce NT-pCNP secretion from CAoE cells within 48 hours over a range of concentrations.
Differences in NT-pCNP secretion among tested stimulants could not be explained by differences in CAoE cell viability.

Of the stimulants tested, IL-1β appears to be the predominant stimulatory signal for NT-pCNP production from canine endothelium with additional signaling from LPS and TNF-α. The role of IL-1β in the pathogenesis of inflammation in the dog is somewhat poorly understood. In the dog, IL-1β is produced in response to pathogen signaling in experimental models and there is some evidence that sterile inflammation may also trigger IL-1β production but little is known about IL-1β in naturally developing disease.\textsuperscript{291-296} It is possible that infection induces dramatic IL-1β production, in comparison to sterile forms of inflammation, resulting in CNP production predominately during infection. However, although important in the pathogenesis of sepsis, TNF-α is involved in other forms of inflammation including naturally developing non-infectious inflammation in dogs.\textsuperscript{180,296-298} For these reasons, we expected PAMPs to have the greatest stimulatory properties since serum NT-pCNP concentrations differentiate infectious causes of inflammation from non-infectious causes of inflammation in the dog.\textsuperscript{299}

One possible explanation for our unexpected results is that a cell line other than endothelial cells is responsible for significant NT-pCNP secretion during sepsis. While NT-pCNP is primarily produced by the endothelium, several cell types secrete NT-pCNP including lymphocytes, monocytes, macrophages, and peritoneal macrophages.\textsuperscript{300-302} In vivo, during naturally developing sepsis, one of these other cells lines may be the predominate source of NT-pCNP.
Additionally, there could be co-stimulatory molecules or signaling from other pathogen moieties that results in NT-pCNP production during infection.

We performed a set of experiments to see if the addition of co-factors, endothelial receptor expression or alterations in CNP secretion could explain the lack of NT-pCNP in the cell culture supernatant of some stimulants. We proposed that serum co-factors would promote CNP production since they are important for cellular activation in some situations including PAMP activation of cells. However, the addition of canine serum to LPS, LTA, and PG failed to result in additional NT-pCNP secretion. We also evaluated canine endothelial cell surface expression of toll like receptor-4 (TLR-4) since it is involved in PAMP signaling. Using flow cytometry, we were able to confirm that our canine endothelial cell line expressed TLR-4 in a similar fashion to canine neutrophils. Unfortunately, at the time this study was conducted, reliable antibodies specific for canine TLR-2 were not available. Thus, the lack of LTA and PG-induced NT-pCNP production could be ascribed to differences in TLR-2 expression between our cell line and in vivo endothelial cells. Additionally, some proteins require post-transcriptional processing or a second stimulus for secretion from the cell. To evaluate intracellular NT-pCNP was greater than secreted NT-pCNP, we physically disrupted the cells to release the cytosolic components. However, cell culture supernatant and cell lysate concentrations of NT-pCNP were equivalent.

Similar to our findings, LPS, TNF-α and IL-1β induce CNP secretion from bovine endothelial cells. However, from bovine endothelium, TNF-α and LPS were more potent stimulators of CNP production than IL-1β. Additionally,
the kinetics of CNP production is marginally different between cattle and dogs. Measurable CNP production begins as early as 6 hours from bovine endothelial cells, which is similar to our observations with canine endothelial cells. However, at 48 hours TNF-α and IL-1β stimulated NT-pCNP production from canine endothelial cells declined and LPS demonstrated a plateau effect, while from bovine endothelial cells LPS and IL-1β demonstrated a plateau effect and TNF-α continued to stimulate CNP production. These data indicate some mild differences in endothelial cell CNP production across species.

In conclusion, our investigation revealed that IL-1β, TNF-α, and LPS stimulate significant NT-pCNP secretion in a dose- and time-dependent manner from canine aortic endothelial cells. Of the stimulants tested, IL-1β appears to be the most potent inducer of NT-pCNP secretion from canine endothelium. These data provide some insight into the mechanisms of CNP induction in dogs. Additional research is needed to fully elucidate the mechanism of infection induced NT-pCNP production in vivo.
CHAPTER 3

NT-pCNP AS A PROGNOSTIC BIOMARKER FOR CRITICAL ILLNESS IN DOGS

Introduction

Dogs and cats are commonly affected by critical illnesses that result in the systemic inflammatory response syndrome (SIRS). There is a need for prognostic biomarkers to allow clinicians and pet owners to make informed decisions regarding treatment and for proper stratification in clinical trials. Several biomarkers have been evaluated as prognostic indicators in dogs and cats with various forms of critical illness; however, the optimal biomarker has yet to be identified.

C-type natriuretic peptide has been evaluated as a diagnostic biomarker for various conditions in humans and has been shown to have prognostic value in critically ill people. Serum NT-pCNP concentrations at admission and on day 3 of hospitalization are closely associated with both ICU and long-term survival; high serum concentrations indicated a poor prognosis. Additionally, serum NT-pCNP concentrations decrease between days 1 and 3 in the people that survive.

Serum NT-pCNP has been evaluated previously as a diagnostic biomarker for sepsis in dogs. Using a cut-off value of 10.1 pmol/L, NT-pCNP has a sensitivity and specificity of 94% and 89%, respectively, for diagnosing non-
peritoneal forms of sepsis in dogs.\textsuperscript{270} However, the usefulness of CNP for predicting prognosis in dogs has not been extensively evaluated. The goal of this study was to determine if single and/or serial NT-pCNP concentrations can be used as a prognostic indicator in critically ill dogs hospitalized for at least 48 hours with suspected sepsis based on a positive NT-pCNP test at the time of hospital admission. Our hypothesis was two-fold: we hypothesized that (1) dogs with higher NT-pCNP concentrations at admission would be less likely to survive to discharge than dogs with lower NT-pCNP concentrations, and (2) a reduction in NT-pCNP concentrations over time would be associated with a better prognosis.

Materials and Methods

\textit{Animals}-- Dogs that presented to the University of Missouri Veterinary Medical Teaching Hospital ICU between December of 2008 and November of 2011 were eligible for inclusion in this prospective observational study with client consent. The study was conducted in accordance with guidelines for clinical studies from the MU Animal Care and Use Committee. Each dog was required to be deemed critically ill by the attending veterinarian, hospitalized in the ICU for a minimum of 48 hours, and have a complete physical examination, complete blood count (CBC) and plasma biochemical profile performed. Additionally, all dogs were required to have a positive serum NT-pCNP test based on a previously established cut-off value (> 10.1 pmol/L) for inclusion.\textsuperscript{270} Dogs that
were less than 6 months of age were excluded. Patient management was at the discretion of the attending veterinarian.

*Sample collection*-- The medical records of each dog enrolled were reviewed and clinical parameters were recorded for each dog including CBC, plasma biochemical profile, duration of hospitalization, cost of hospitalization and survival to discharge. Blood samples for NT-pCNP analysis were collected into serum blood tubes within 24 hours of ICU admission. Additionally, in a smaller subset of dogs, blood was collected serially every 24 hours for the duration of hospitalization. Blood was centrifuged (1500 x g, 7 minutes) and serum harvested within one hour of sample collection. The serum was placed in an airtight, freezer-resistant plastic tube and stored at -80 °C for batch analysis. Tubes were coded so that the identity of the sample was only known by the investigator (i.e., the laboratory conducting the NT-pCNP assay was blinded).

*Disease severity scoring* – Dogs were assigned an organ dysfunction index (ODI) score using a modification of an established scoring system (table 5). Organ dysfunction index scores were determined by assigning one point for each form of organ dysfunction present for a total possible score of 5 points. Multiple organ dysfunction syndrome (MODS) was defined as an organ dysfunction index score of ≥2/5.

*NT-pCNP assay*-- Serum NT-pCNP was evaluated by a commercial laboratory (Veterinary Diagnostics Institute, Simi Valley, CA). The NT-pCNP assay was performed by a sandwich ELISA, which utilizes a highly purified polyclonal sheep antibody directed against amino acids 1–19 and 30–50 of
human NT-pCNP (96% homologous with canine pCNP [http://www.ncbi.nlm.nih.gov/genome/guide/dog; http://blast.ncbi.nlm.nih.gov/Blast.cgi]),\textsuperscript{270} As previously reported, interassay and intra-assay coefficient of variation and percent recovery for this assay using dog serum are 7–9%, 4–5%, and 91.3±5.9% using spiked canine serum samples.\textsuperscript{270} Day-to-day coefficient of variation for serum concentrations of NT-pCNP in healthy dogs is 9.8±2.5% over a 5-day period.\textsuperscript{270} The lower limit of detection of this assay is 0.55 pmol/L.

**Statistical Analysis**

Statistical analysis was performed using commercially available software (SigmaStat, Systat Software Inc, Chicago, IL). Normality was assessed using histogram plots. A Mann-Whitney rank sum test was used to compare NT-pCNP concentrations between survivors and non-survivors. A receiver operating characteristic (ROC) curve was used to determine the area under the curve (AUC) and select the optimum cut-off value that maximized the Youden’s J statistic (sensitivity +specificity-1) for sensitivity and specificity reporting. Spearman Rank Order Correlation was used to assess the relationship between serum NT-pCNP concentration, mortality, duration of hospitalization and the cost of hospitalization. Serial serum NT-pCNP concentrations in a subset of dogs were evaluated using descriptive statistics. Type I error was limited to 5%.
Results

Thirty-two dogs met our inclusion criteria and a wide variety of breeds and diseases were represented. Our study population consisted of 11 neutered males, 10 sexually intact males, 8 spayed females, and 3 intact females. The mean ± SD age was 5.7 ± 4.2 years (range, 7 months to 14.2 years) and mean ± SD weight was 23.4 kg ± 14.9 kg (range, 1.6 kg to 60 kg). Breeds represented included Labrador retriever [6], mixed breed [4], golden retriever [3], Shih Tzu [3], cocker spaniel [2], and Great Dane [2]. Critical illness was associated with the abdominal cavity (14), pulmonary/pleural space (7), urogenital (4), subcutaneous/skin (4), generalized systemic disease (2), and central nervous system (1). One dog with urogenital disease also had pulmonary disease and 1 dog with urogenital disease also had musculoskeletal disease contributing to critical illness. Eleven of 32 dogs met the criteria for MODS; thirteen dogs had an ODI score of 0, 9 dogs each had ODI scores of 1 and 2, and 1 dog each had an ODI score of 3 and 4. Thirty-one% of dogs had liver dysfunction (10/32), 25% had respiratory dysfunction (8/32) and 2/8 dogs required mechanical ventilation, 16% had cardiovascular dysfunction (5/32), 16% had coagulation dysfunction (5/32), and 16% had renal dysfunction (5/32). There was no significant relationship between serum NT-pCNP concentrations and ODI score (r = -0.002; P=0.99).

The median duration of hospitalization (DOH) was 7 days (range, 2 to 29 days). Dogs that died had a significantly shorter median DOH (3; 2 to 29) than dogs that survived to discharge (6; 2 to 29) (P=0.045). Duration of hospitalization
was not significantly correlated with NT-pCNP concentrations (r= 0.13, P=0.469). The median cost of hospitalization (COH) was $2945.23 ($873.61 to $15,286.71) and cost of hospitalization did not differ significantly between survivors ($3168.44, $873.61 to $15,286.54) and non-survivors ($2850.66, $984.22 to $9608.73; P= 0.546). There was no significant relationship between serum NT-pCNP and COH (r=0.05; P=0.789). Overall survival in this cohort was 62.5% (20/32). Serum NT-pCNP concentration was significantly lower in the group who survived (Figure 5; P=0.049). There was a moderate inverse relationship between NT-pCNP concentrations and survival (r= -0.357; P=0.045). Based on evaluation of a ROC curve, if a cut-off value of 16.9 pmol/L was used, the AUC for predicting which dogs will survive to discharge versus which dogs will not was 0.71 (95% confidence interval [CI], 52.0-90.5%). The sensitivity was 92% (95% CI, 61.5-99.8%) and specificity was 65% (95% CI, 40.8-84.6%) for differentiating the dogs that will survive to discharge versus the dog that will not (positive likelihood ratio [LR], negative LR; 2.6191, 0.1282) (figure 6). In order to obtain a specificity of 90% (95% CI, 68.3%-98.8%), the sensitivity was 25% (95% CI, 5.4%-57.2%) when using a cut-off value of 35.75 pmol/L (positive LR, negative LR; 2.500, 0.8333).

Serial NT-pCNP concentrations- In addition to evaluating NT-pCNP concentrations at admission, 15/32 dogs had serial serum NT-pCNP concentrations available for evaluation over at least the first 2-3 days of hospitalization (figure 7). Eleven of 15 dogs (78.6%) of these dogs survived to be discharged from the hospital. Three of 4 dogs that did not survive had increasing
serum NT-pCNP concentrations over time; the other had a decreasing NT-pCNP concentration. Of the survivors, dynamic change in NT-pCNP was variable with concentrations increasing (5/11), decreasing (5/11) or increasing and then decreasing (1/11). Four of the survivors with increasing concentrations over the first 3 days of hospitalization had additional samples collected for the duration of hospitalization. All four of these dogs went on to have serum NT-pCNP concentrations below the serum NT-pCNP concentration at admission prior to discharge.

Discussion

In our evaluation of dogs with critical illness, we found that serum NT-pCNP concentrations at admission are significantly lower in dogs that survive compared to dogs that do not survive. Serum NT-pCNP concentrations predicted survival in dogs with critical illness with a sensitivity of 92% and a specificity of 65% using a cut-off value of 16.9 pmol/L. To achieve a specificity of 90%, the sensitivity of serum NT-pCNP for predicting non-survival was only 25%. Evaluation of dynamic changes in serum NT-pCNP concentration over the first 3 days of hospitalization did not appear to be helpful in predicting outcome. Serum NT-pCNP concentrations were not associated with organ dysfunction index score, duration of hospitalization or cost of hospitalization. Overall, serum NT-pCNP concentrations at admission or performed serially is not a useful prognostic indicator for critical illness in dogs.
We have previously evaluated serum NT-pCNP concentrations as a prognostic indicator for dogs with sepsis and found that serum NT-pCNP concentrations did not significantly correlate with survival (P= 0.792).\textsuperscript{270} However, in the previous study, we included all dogs with sepsis regardless of NT-pCNP concentrations (i.e., dogs with positive and negative NT-pCNP concentrations) in the survival statistics, while in the current study, we only included dogs with positive NT-pCNP concentrations. By only evaluating dogs with a positive NT-pCNP concentration, we hoped to increase its usefulness as a prognostic biomarker for dogs because only dogs with activation of the pathway necessary for CNP induction were included. When only dogs with positive NT-pCNP tests were included, dogs who survived had significantly lower NT-pCNP concentrations at admission than dogs with higher concentrations.

Biomarkers able to predict prognosis for critically ill dogs are lacking and it is necessary to indentify objective biomarkers that will predict outcome in dogs with critical illness in order to assist veterinarians and clients in making treatment decisions as well as allow for appropriate stratification in clinical trials. Several prognostic biomarkers, such as C-reactive protein,\textsuperscript{258} IL-6,\textsuperscript{313} and calcium,\textsuperscript{315} have been evaluated previously in dogs with critical illness or sepsis, but a clinically useful prognostic biomarker has not been identified. NT-pCNP is a promising diagnostic biomarker for non-peritoneal forms of sepsis in dogs;\textsuperscript{270} however, with a specificity of only 65%, NT-pCNP is not a useful prognostic indicator. Using this cut-off value, 8/21 survivors would have been predicted to die and this test result may have swayed pet owners towards unnecessary
euthanasia. If a cut-off value of 35.75 pmol/L is used, the specificity of NT-pCNP as a prognostic biomarker is maximized at 90%, the sensitivity decreases to 25%. If this high cut-off value was used in order to maximize specificity, it would have incorrectly predicted survival in 9/11 dogs who actually died. Despite this optimal specificity, NT-pCNP is not a useful prognostic indicator. NT-pCNP concentrations have prognostic value in critically ill people. Serum NT-pCNP concentrations at admission and on day 3 were strong predictors for release from ICU and overall survival, and a decrease in serum NT-pCNP concentrations from admission to day 3 was associated with survival, whereas non-survivors had NT-pCNP concentrations that remained stably increased. We elected to evaluate serial serum NT-pCNP concentrations in order to maximize the clinical utility of NT-pCNP as a prognostic biomarker and to see if NT-pCNP behaves the same way prognostically in dogs as it does in people. We evaluated serial serum NT-pCNP concentrations in 15 dogs and found there was no relationship between serial NT-pCNP concentrations and survival. The dynamic change in serum NT-pCNP concentrations of surviving dogs and non-survivors were variable over the first 3 days. Increasing NT-pCNP concentrations were present in 3 of the 4 dogs with serial samples that did not survive, while 5 of the 11 dogs who survived also had increasing NT-pCNP concentrations. The ideal prognostic indicator in veterinary medicine would be able to predict prognosis at admission or soon thereafter. Pet owners need to be able to make informed decisions as soon as possible, especially with the increasing cost of veterinary care. For example, a prognostic biomarker that is only useful on day 3 would not be helpful to an
owner deciding whether or not to pursue emergency surgery for septic peritonitis. Further, in our study, 8/11 non-survivors died on or before day 3 which would make any biomarker not predictive of survival at admission unhelpful. The usefulness of biomarkers which require daily samples is often low in veterinary medicine because the veterinarian often has other clinical clues that a pet is improving or not.

The DOH was significantly shorter for the non-survivors compared to the survivors, and this reflects that dogs that died tended to do so early in the course of disease. The COH was not different between survivors and non-survivors, and this likely reflects the increased cost of monitoring, diagnostics, and treatments needed for the more critically ill patients (the non-survivors). An ODI score was assigned to each dog and 11/32 dogs met the criteria for MODS. Organ dysfunction index scores were not associated with survival and the DOH, COH, and ODI scores were not associated with serum NT-pCNP concentrations. There are several limitations to our study that should be discussed. Our sample size was small in part due to our exclusion criteria. We immediately eliminated dogs that were in the ICU for less than 48 hours. We did this in order to eliminate the dogs that were not treated. However, in doing so we also eliminated dogs that were euthanatized due to a grave prognosis or who died early in the course of their critical illness. These dogs may have had high (or low) serum NT-pCNP concentrations that could have altered our results. Finally, because we are a tertiary care facility, some dogs received treatment prior to presentation, which

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may have affected the serum NT-pCNP concentrations and therefore skewed our results.

In conclusion, we found that serum NT-pCNP concentrations at admission have a sensitivity and specificity of 92% and 65%, respectively, for predicting survival in critically ill dogs. Serial NT-pCNP concentrations were not associated with organ dysfunction index scores, survival, duration or cost of hospitalization, and organ dysfunction index scores were not correlated with survival. Although NT-pCNP is a clinically useful biomarker for the diagnosis of non-peritoneal forms of sepsis in dogs, it does not appear to be a promising prognostic biomarker.
CHAPTER 4
FUTURE DIRECTIONS

Sepsis, SIRS, and MODS are heavily studied areas of human medicine, and the veterinary literature regarding these subjects is growing. Still, there are many unanswered questions, and additional research is necessary so that we can increase our knowledge of the diagnostics and therapeutics necessary to treat these critical illnesses.

My research attempted to determine the mechanism by which NT-pCNP is produced by stimulating canine aortic endothelial cells with various PAMPs and inflammatory mediators and measuring the amount of NT-pCNP produced. The question still remains why IL-1β caused the greatest NT-pCNP production since dogs with sepsis have higher concentrations of NT-pCNP compared to dogs with non-infectious SIRS. Additional research is necessary to further characterize this mechanism.

It is possible that IL-1β plays a larger role in the pathogenesis of naturally developing sepsis in dogs than in dogs with non-infectious SIRS. Concentrations of IL-1β have not been evaluated and compared to each other in these two cohorts, and this research might help us to explain the results of our NT-pCNP mechanism study. Additionally, other cell types such as monocytes, macrophages, or neutrophils may be the predominant source of NT-pCNP in
sepsis. Our same experiment could be conducted using cell types other than canine aortic endothelial cells.

NT-pCNP is a good biomarker for naturally developing non-peritoneal forms of sepsis in dogs. Peritoneal forms of sepsis occur commonly and more investigations should be done on widening the biomarker capability of NT-pCNP. Dogs with peritoneal forms of sepsis usually have peritoneal effusion. It is possible that the NT-pCNP is compartmentalized in the peritoneal cavity and, therefore, the effusion would be a better sample to test NT-pCNP concentrations compared to peripheral blood.

NT-pCNP is not a useful prognostic indicator in dogs with critical illness. Our sample size, especially in the group with serial NT-pCNP concentrations, was very small, so larger clinical trials evaluating NT-pCNP as a prognostic indicator for critical illness are warranted. Additionally, we could have included dogs that were in the hospital for less than 48 hours in order to expand our sample population. If we did this, we could have excluded the dogs that were euthanatized for reasons other than a grave prognosis, but included the dogs that had severe enough illness to result in death or euthanasia because of a grave prognosis.
Table 1: Definition of VetALI/VetARDS: Veterinary Acute Lung Injury and Acute Respiratory Distress Syndrome

Must meet at least one each of the first 4 criteria; 5 is a recommended but optional measure

1. Acute onset (< 72 hours) of tachypnea and labored breathing at rest
2. Known risk factors (see Table 2)
3. Evidence of pulmonary capillary leak without increased pulmonary capillary pressure (any one or more of the following):
   a. Bilateral/diffuse infiltrates on thoracic radiographs (more than 1 quadrant/lobe)
   b. Bilateral dependent density gradient on CT
   c. Proteinaceous fluid within the conducting airways
   d. Increased extravascular lung water
4. Evidence of inefficient gas exchange (any one or more of the following):
   a. Hypoxemia without PEEP or CPAP and known FiO₂
      i. \( \text{PaO}_2/\text{FiO}_2 \) ratio
         1. \( \leq 300 \text{ mmHg for VetALI} \)
         2. \( \leq 200 \text{ mmHg for VetARDS} \)
      ii. Increased alveolar-arterial oxygen gradient
      iii. Venous admixture (noncardiac shunt)
   b. Increased ‘dead-space’ ventilation
5. Evidence of diffuse pulmonary inflammation
   a. Transtracheal wash/bronchoalveolar lavage sample neutrophilia
   b. Transtracheal wash/bronchoalveolar lavage biomarkers of inflammation
   c. Molecular imaging (PET)
CT, computed tomography; PEEP, positive end expiratory pressure; CPAP, continuous positive airway pressure; FiO₂, fraction inspired oxygen; PET, positron emission tomography
Table 2: Risk Factors for Veterinary Acute lung Injury and Acute Respiratory Distress Syndrome

1. Inflammation
2. Infection
3. Sepsis
4. Systemic inflammatory Response Syndrome
5. Severe trauma
   a. Long bone fracture
   b. Head injury
   c. Pulmonary contusion
6. Multiple transfusions
7. Smoke inhalation
8. Near-drowning
9. Aspiration of stomach contents
10. Drugs and toxins
Table 3: Model based scoring system for the diagnosis of DIC in dogs

Probability of DIC= 15.99 - 0.14 x fibrinogen – 2.52 x PT – 2.13 x PTT + 0.28 x (PTT - PT) + (5.41, when D-dimer > 0.5 mg/L)

The ROC area under the curve was 0.958 (95% CI; 0.874; 0.992) and the optimal diagnostic cut-off was assessed to be a P value (DICprob) of 0.401, with an observed diagnostic sensitivity and specificity of 90.9% (95% CI: 70.8–98.6) and 90.0% (95% CI: 76.3–97.1), respectively.
Table 4: RIFLE and AKIN Staging Systems for AKI\textsuperscript{205,206}

<table>
<thead>
<tr>
<th>RIFLE</th>
<th>Serum creatinine criteria</th>
<th>Urine output criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Risk (R)</td>
<td>$\geq 1.5$-fold increase from baseline serum creatinine or $\geq 25%$ decrease in GFR</td>
<td>$&lt; 0.5 \text{ ml/kg/h for } \geq 6 \text{ hours}$</td>
</tr>
<tr>
<td>Injury (I)</td>
<td>$\geq 2.0$-fold increase from baseline serum creatinine or $\geq 50%$ decrease in GFR</td>
<td>$&lt; 0.5 \text{ ml/kg/h for } \geq 12 \text{ hours}$</td>
</tr>
<tr>
<td>Failure (F)</td>
<td>$\geq 3.0$-fold increase from baseline serum creatinine or $\geq 75%$ decrease in GFR or an absolute serum creatinine $\geq 354 \mu\text{mol/L (4.0 mg/dL)}$ with an acute rise $\geq 44 \mu\text{mol/L (0.5 mg/dL)}$</td>
<td>$&lt; 0.3 \text{ ml/kg/h for } \geq 24 \text{ hours or anuria } \geq 12 \text{ hours}$</td>
</tr>
<tr>
<td>AKIN</td>
<td>Serum creatinine criteria</td>
<td>Urine output criteria</td>
</tr>
<tr>
<td>--------</td>
<td>------------------------------------------------------------------------------------------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>Stage 1</td>
<td>≥ 26.4 μmol/L (0.3 mg/dL) or ≥ 150-200% increase from baseline serum creatinine</td>
<td>&lt; 0.5 ml/kg/h for ≥ 6 hours</td>
</tr>
<tr>
<td>Stage 2</td>
<td>&gt; 200-300% increase from baseline serum creatinine</td>
<td>&lt; 0.5 ml/kg/h for ≥ 12 hours</td>
</tr>
<tr>
<td>Stage 3</td>
<td>≥ 300% increase from baseline serum creatinine or absolute serum creatinine ≥ 354 μmol/L (4.0 mg/dL) with an acute rise of ≥ 44 μmol/L (0.5 mg/dL)</td>
<td>&lt; 0.3 ml/kg/h for ≥ 24 hours or anuria for ≥ 12 hours</td>
</tr>
</tbody>
</table>
Table 5: Definitions for organ dysfunction (modified from original)\textsuperscript{50}

<table>
<thead>
<tr>
<th>Organ system</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Renal</td>
<td>Creatinine concentration &gt; 1.6 mg/dl with no evidence of postrenal azotemia</td>
</tr>
<tr>
<td>Cardiovascular</td>
<td>Hypotension severe enough to require vasopressor therapy within 24 hours of presentation.</td>
</tr>
<tr>
<td>Respiratory</td>
<td>Need for supplemental oxygen administration or mechanical ventilation within 24 hours of presentation; determined based on clinical assessment, blood gas analysis (alveolar-arterial gradient in partial pressure of oxygen &gt;10 mm Hg) and/or results of pulse oximetry (SpO2 &lt; 95)</td>
</tr>
<tr>
<td>Hepatic</td>
<td>Plasma or serum total bilirubin of &gt;0.5 mg/dL</td>
</tr>
<tr>
<td>Coagulation</td>
<td>PT or PTT &gt; 25% above the upper reference limit and/or platelet count of ≤ 100,000/µL</td>
</tr>
</tbody>
</table>
Figure 1: Comparison of lipopolysaccharide (LPS; 100,000 ng/mL), lipotechoic acid (LTA; 100,000 ng/mL), peptidoglycan (PG; 100,000 ng/mL), and canine recombinant TNF-α (100 ng/mL), IL-1β (100 ng/mL), IL-6 (100 ng/mL), IL-10 (100 ng/mL), IL-21 (100 ng/mL), CXCL-8 (100 ng/mL), IFN-γ (100 ng/mL), VEGF-A (100 ng/mL) or PBS stimulated NT-pCNP production from canine aortic endothelial cells at 24 hours. *P<0.05 compared to PBS. † P<0.05 compared to TNF-α and LPS.
Figure 2: Comparison of IL-1β (A), TNF-α (B) and LPS (C) -induced NT-pCNP production from canine aortic endothelial cells using various concentrations of each stimulant. Stimulation of CAoE cells with LPS, TNF-α, and IL-1β resulted in NT-pCNP secretion that was dose dependent in nature. *P<0.05 compared to 0 ng/ml.
Figure 3: Comparison of LPS (1000 ng/ml), TNF-α (100 ng/ml), and IL-1β (100 ng/ml) induced NT-pCNP production from canine aortic endothelial cells after 1, 6, 12, 24, or 48 hours of stimulation. NT-pCNP production was time dependent with maximal stimulation at 24 hours. * P<0.05 compared to 1 hour CNP production for the same stimulant.
Figure 4: Peripheral blood leukocytes and canine aortic endothelial cells were used in a flow cytometric assay to identify surface expression of TLR-4 (CD284). A forward/side scatter plot was used to identify neutrophils (A) and endothelial cells (C). Similar PE positivity is demonstrated on the histogram plot comparing PE-conjugated Anti-Human CD284 (red or green) and isotype control (solid black) for neutrophils (B) and canine aortic endothelial cells (D).
Figure 5: Comparison of serum NT-pCNP concentrations between dogs that survived to hospital discharge (survivors) and those that did not (non-survivors). Data are presented in a box and whisker plot format. The upper and lower edges of the box represent the 75th and 25th percentiles respectively, whereas the line within the box is the median value. Whiskers represent the largest and smallest values. Extreme outliers have been excluded. *P=0.049 compared to survivors.
Figure 6: Receiver operating characteristic curves comparing the diagnostic sensitivity and 1-specificity of serum NT-pCNP concentration for differentiating survivors from non-survivors (black line). AUC = 0.71.
Figure 7: Graphic representation of the 15 dogs with serial samples for days 1, 2 and if applicable, day 3. The non-survivors are represented by a black line while the survivors are represented by a dotted line.


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