Developing an Improved Laboratory Diagnostic Test for Early Detection of Sepsis in Dogs

Laura Brookes BVMS BSc

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Declaration

I declare that this thesis is my own account of my research and contains as its main content work which has not previously been submitted for a degree at any tertiary education institution.

Laura M Brookes

Dedication

This thesis is dedicated to the patients – dogs who contributed to this work, and those who may be helped by it in the future. Especially you, Denis!

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Abstract

Dogs with sepsis – the life-threatening tissue damage resulting from an inappropriate host response to infection – often suffer up to 50% mortality, largely due to a lack of accurate clinical and laboratory tests for rapid diagnosis and guiding management. Procalcitonin (PCT) is a biomarker used for rapid and accurate diagnosis and management of sepsis in humans, thereby improving outcomes. Commercial assays for canine PCT (cPCT) are available; although prior to this project they were not validated. Different antigenicity between recombinant and native PCT was one possible explanation for poor performance of canine PCT ELISAs. Chapter One identified a need for a monoclonal antibody confirmed to detect native canine PCT. This would enable development of a reliable ELISA for procalcitonin measurement in dogs with sepsis. This project aimed to produce monoclonal antibodies (Mabs) recognizing native cPCT to develop a rapid and specific cPCT diagnostic test for sepsis. Additionally, this project aimed to better understand the epidemiology of canine sepsis.

Chapter Two features an epidemiological study of sepsis in hospitalized dogs, contributing a large study to the currently limited data on clinical impact of canine sepsis. For 486 admissions of dogs, sepsis prevalence was 5.5% and incidence was 2.2%. The most common source of infection was peritonitis. Mortality was highest in the sepsis group at 35.1% ($p=0.0008$, χ^2 test; overall mortality 15.4%), compared with dogs who had non-infectious systemic inflammatory response syndrome (SIRS) or infection. All sepsis deaths occurred within four days of diagnosis. Cost and length of hospitalisation were also highest for sepsis.

Chapters Three and Four describe how native cPCT was extracted from canine thyroid glands, isolated via chromatography, identified with a commercial procalcitonin antibody (BioVendor, Asheville, NC, USA), confirmed by mass spectrometry (MS), and used to immunize mice although purity was low (≤42%). This produced two Mabs, that were assessed for PCT affinity using immunoprecipitation and MS. Unfortunately, neither monoclonal antibody demonstrated affinity to native or recombinant PCT, most likely due to insufficient purification or sameness between canine and murine PCT. However, the same screening confirmed the BioVendor polyclonal antibody does recognise native canine procalcitonin in septic dog plasma, contributing to its validation. Furthermore, native PCT was detected in septic dog plasma directly using mass spectrometry.

In Chapter Five, a general discussion gives an overview of the project findings and recommends future directions. Focus should be directed towards full validation of the existing antibody in the BioVendor ELISA, calibration, and reference intervals. A monoclonal antibody should be developed to native canine PCT to enable consistent quantitation of PCT. Larger epidemiological studies can provide information on the impact of sepsis in the wider canine population.

Abbreviations

2-ME – beta-mercaptoethanol

A280 – UV absorbance at 280 nm

ABTS – 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt

ACN – acetonitrile

APP – acute phase protein

BCA – bicinchoninic acid

BSA – bovine serum albumin (BSA)

BV – BioVendor (company): refers to the BioVendor polyclonal antibody when used alone, vs BV protein (crPCT from BioVendor)

CEC – cation exchange chromatography

cPCT – canine procalcitonin

CRP – C-reactive protien

crPCT – canine recombinant PCT

CT – calcitonin

CV – coefficient of variation

DTT – dithiothreitol (reducing agent)

E – glutamic acid

ECL – enhanced chemiluminescence

EDTA - ethylenediaminetetraacetic acid

ELISA - enzyme-linked immunosorbent assay

FBS – foetal bovine serum

HPLC – high-performance liquid chromatography, or high-pressure liquid chromatography (same process)

IEC – ion exchange chromatography

IgG – immunoglobulin G

IMS – immunoprecipitation mass spectrometry

KC – katacalcin

kDa – kilodaltons (1,000 daltons)

µ – micron

Mab – monoclonal antibody

MODS – multiple organ dysfunction syndrome

MW – molecular weight

MRM – multiple reaction monitoring

MS – mass spectrometry

N-PCT – N-terminal PCT (the amino-terminal fragment of PCT)

nm – nanometres

Pab – polyclonal antibody

PAGE – polyacrylamide gel electrophoresis

PAMP – pathogen-associated molecular pattern

PBS – phosphate-buffered saline

PCR – polymerase chain reaction

PCT – procalcitonin

PIC – protease inhibitor cocktail

prePCT – preprocalcitonin (the parent of procalcitonin)

PRR – pattern recognition receptor

psi – pound-force per square inch

PVDF – polyvinylidene fluoride

Q – glutamine

qr-PCR – quantitative real-time PCR

qSOFA – 'quick' SOFA

RP-HPLC – reversed-phase HPLC

rPCT – recombinant PCT

RUO – research use only

SAA – serum amyloid A

SDS – sodium dodecyl sulfate

SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis

SIRS – systemic inflammatory response syndrome

SNP – single nucleotide polymorphism

SOFA – sequential organ failure (score)

SPE – solid phase extraction

TBS – tris-buffered saline. TBS-T is with Tween-20.

TFA – trifluoroacetic acid

UV – ultraviolet

WB – Western blotting

w/v – weight for volume

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Publications arising from this thesis

At the time of writing, two chapters were in draft and review:

Epidemiology of sepsis in dogs admitted to a veterinary teaching hospital – In review, submitted to Frontiers in Veterinary Science.

Current status of canine procalcitonin assays: a possible sepsis biomarker in dogs – prepared for submission to Veterinary Clinical Pathology

Conferences attended

ASVP, Perth WA 2019. Presentation: Epidemiology of sepsis and SIRS in dogs admitted to an Australian tertiary veterinary hospital

ISACP XVIII Congress, Tokyo 2018. Presentation: Native canine procalcitonin purification and antibody affinity evaluation for sepsis diagnosis

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Day of Immunology (ASI), Perth WA 2017. Presentation: Sepsis diagnosis – finding a better test for sepsis in dogs

1 Chapter One

Introduction

1.1 Project premise

This thesis describes the significant impact of sepsis in dogs and outlines the process of investigating and developing a solution to this problem in the form of a laboratory assay for canine PCT as a potential rapid sepsis biomarker. Sepsis occurs when a generalized and severe inflammatory disease develops due to infection; and is associated with high mortality of up to 70% in dogs. In all species, sepsis is a severe condition involving systemic inflammation. In dogs, studies featuring septic peritonitis usually report a high mortality of 30 to 65% (Cortellini, 2015; Dayer et al., 2013; Dickinson, 2015; Li, 2016). Only one study (abstract only) reports on financial outcomes of sepsis in dogs: cost and length of stay in hospital associated with sepsis were both higher than for infection or non-infectious SIRS (Guenther-Yenke et al., 2007).

Sepsis can develop and worsen within hours, and this makes it hard to diagnose rapidly enough to allow effective treatment. Clinicians have no gold-standard diagnostic test for sepsis. A combination of general diagnostic tests, such as microbial culture and various blood analytes, must be interpreted in combination with clinical judgement to diagnose sepsis, manage each case optimally, and make predictions about outcomes. This is especially

challenging since the currently available tests are not specific or sensitive for sepsis or may not provide a timely result (i.e within 6 to 72 hours).

In humans, diagnostic tests for sepsis now include rapid biomarker assays – including point-of-care tests – that can improve management of sepsis. Examples include biomarkers for inflammation and infection such as C-reactive protein and procalcitonin (PCT), which are routinely used in neonatal and adult humans. Recent veterinary research has demonstrated similar properties of PCT in dogs with sepsis, to those observed in humans. Specifically, increased plasma PCT concentration has been measured in 20 dogs with sepsis (median 78.7 pg/ml, 39.1–164.7) relative to 52 healthy dogs (49.8 pg/ml, range 36.2–63.7) (Goggs et al. 2018). In a similar study using the same cPCT assay, PCT plasma concentration correlated with severity and prognosis (Troia, Giunti & Goggs, 2018).

Based on these advancements, this project examined new options for a rapid diagnostic test to improve sepsis outcomes in dogs; and focused on PCT as a prospective biomarker for this purpose. The aim for this project was to develop a quantitative ELISA using monoclonal antibodies (Mabs) to the native PCT protein of dogs, because this showed strong potential to act as a rapid sepsis biomarker that could improve outcomes for dogs with sepsis.

1.2 Definitions relating to sepsis

Sepsis is the life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al., 2016), also referred to as “sepsis-3”. In this 2016 article (Singer et al.), an international panel of experts developed this definition using data review, Delphi

processes, meetings, and voting as an update taking current sepsis knowledge into account. Previously to this, sepsis was defined as the systemic inflammatory response to an infection (Bone et al., 1992). This definition, lately re-coined “sepsis-2”, applied in all species (Singer et al., 2016). Organ dysfunction can be defined as reduced or abnormal function of a given organ or organ system; organ failure is a degree of dysfunction in an organ or organ system that requires clinical intervention in order to prevent failure of homeostasis (Deitch, 1992; Fujishima, 2016; Kumar et al., 2014). Infection can be defined as a pathogenic presence of an organism (bacterial, fungal, viral, parasitic, or protozoan) in host tissue. Section 1.3.1 discusses some of the diagnostic tests available for confirming infection. Currently, veterinary medicine recognizes the new definition of sepsis-3 but still utilizes the earlier paradigm of systemic inflammatory response syndrome (SIRS) plus infection for clinical diagnosis purposes.

1.2.1 SIRS and SOFA

In veterinary medicine, sepsis is diagnosed when infection is present concurrently with systemic inflammatory response syndrome (SIRS) equivalent to the sepsis-2 system in humans (Bone et al., 1992; Dunne, 2015; Sharp, 2015; Silverstein, 2015). Specifically, SIRS is diagnosed when two or more of four specific criteria are present at the same time: abnormal temperature, abnormal heart rate, abnormal respiratory rate, and abnormal leukocyte count (see

Table 1.1). These may be significantly increased or decreased from normal according to pre-determined cut-offs. There are multiple sets of cut-offs defined by different clinical

researchers. One set is highly sensitive and may result in over-diagnosis, since it was designed to include all patients at risk of sepsis (Hauptman et al., 1997). Conversely, Okano and colleagues (2002) used more restrictive SIRS cut-offs to screen for a poor prognosis. The cut-offs defined by de Laforcade and colleagues (2003) are a clinically useful compromise between these two extremes. The choice of the clinician or researcher to use certain SIRS criteria is an example of how different diagnostic approaches could easily alter sepsis diagnosis rates; or influence when during their disease course patients are identified as having sepsis. The SIRS score cut-off values are species-specific.

Table 1.1: Three sets of SIRS criteria used in dogs

	Hauptman et al. (1997)	**de Laforcade et al. (2003)**	**Okano et al. (2002)**
Temperature (ºC)	<38 or >39	<37.8 or >39.4	<37.8 or >39.7
Heart rate (beats/minute)	>120	>140	>160
Respiratory rate (breaths/minute)	>20	>20	>40
Leukocyte count (cells/µL)	<6,000 or >16,000 or >3% band neutrophils	<6,000 or >16,000 or >3% band neutrophils	<4,000 or >12,000 or >10% band neutrophils

Diagnosis of sepsis in humans is no longer directly comparable to veterinary medicine because the recent updated definition of sepsis (sepsis-3) recognises organ dysfunction in place of systemic inflammation. This was accompanied by the transition to use organ dysfunction scores instead of SIRS criteria (alongside infection) for diagnosis of sepsis in

humans (Singer et al., 2016). Organ dysfunction is diagnosed using the sequential organ failure (SOFA) score (Hayes et al., 2010; Kenney et al., 2010; Marshall et al., 1995; Vincent et al., 1996) This was deemed to be more specific for sepsis than systemic inflammation by an international panel of experts, although its benefit for sepsis diagnosis and management is debated on some points such as subsequent changes in incidence of sepsis (Dykes et al., 2019; Fullerton et al., 2017; Vincent et al., 2016). In dogs, a limited number of studies utilise the SOFA score and it is not regularly used for canine sepsis (Hayes et al., 2010; Kenney et al., 2010; Marshall et al., 1995; Vincent et al., 1996). For humans, there is also a limited version of the SOFA score, including blood pressure and mentation status, which can be applied with minimal diagnostics: qSOFA (Singer et al., 2016; Vincent et al., 1998; Vincent et al., 1996). This system is designed for screening for sepsis quickly in a human critical care setting; to date qSOFA has not been featured in any veterinary clinical research.

1.3 Benefits of rapid diagnosis

1.3.1 Rapid intervention improves survival

One of the primary findings in sepsis research is the 'need for speed', where rapid recognition, diagnosis, and treatment of sepsis are associated strongly with better patient outcomes. Early supportive care and treatment of the underlying infection are the only effective treatment for sepsis because efforts to develop specific pharmaceuticals or targeted treatments consistently fail (Bone, 1996; Marshall, 2014).

Severe or systemic infection, for example bacteraemia, can trigger widespread inflammation through pathogen-associated molecular patterns (PAMPs) stimulating pattern recognition receptor (PRR) signalling. Activation of PRRs on such a wide scale results in a pro-inflammatory cytokine storm and leukocyte activity that can perpetuate the damage to host tissues to a lethal degree within hours to days (Ackermann, 2017; Kumar et al., 2014; Lewis et al., 2012). In this way, sepsis can lead to shock and multiple organ dysfunction syndrome (MODS) or organ failure within hours, with mortality levels commonly up to 50% in dogs (Kenney et al., 2010) and 23.9% to 40% for septic shock in some human populations (Bauer et al. 2020; Li, L. et al. 2020).

In humans, sepsis mortality reduces considerably – up to 50% – when the only intervention is to commence appropriate supportive and infection-control therapies in a timely fashion as recommended in an international 'Surviving Sepsis' campaign (Dellinger et al., 2013; Funk et al., 2009; Kumar et al., 2006; Levy et al., 2014; Seymour et al., 2017; Uittenbogaard et al., 2014). All studies investigating the timeframe to intervention in sepsis, show that outcomes are improved with more rapid recognition and treatment, including in large multicentre trials such as Edusepsis and MONARCS (Ferrer, Artigas et al., 2008; Ferrer, Artigas et al. 2009; MacArthur et al. 2004). MacArthur and colleagues (2004) explored the MONARCS trial data to investigate the association between increased mortality and inadequate empirical therapy, and found that all patients benefited from empirical therapy although those who had initial ineffective or delayed empirical therapy had higher mortality.

This is reflected in canine studies where decreased time until effective treatment of sepsis resulted in reduced mortality rates, and sepsis recognition protocols reduced the time until treatment was administered (Abelson et al., 2013; Bush, 2016; Levy et al., 2010; Seymour et al., 2017). One study (Kenney et al., 2010) reported that mortality increased to 70% with multiple organs in dysfunction; and Dickinson (2015) reported a mortality rate of 94% in dogs with septic shock compared with 43% for sepsis overall. In contrast, dogs with open-cervix pyometra had very low mortality (Jitpean et al., 2017).

Disease progression, organ failure, and time to diagnosis of infection using microbial culture are all time-dependent: that is, they either take time to occur, or worsen over time. Furthermore, there is the opportunity to intervene when the disease is less severe (not yet progressed), damage is not as extensive, or using empirical therapies rather than targeted therapies based on microbial culture and sensitivity. Any assessment or diagnostic test that can identify or indicate sepsis or infection in the presence of SIRS is valuable because it can enable these earlier interventions, each of which is clearly associated with better patient outcomes. Rapid patient-side or in-house monitoring is also valuable to guide clinicians decisions in patient management – the next section describes other important clinical goals of antimicrobial stewardship and appropriate early reduction in antimicrobial dosing.

1.3.2 Antimicrobial stewardship

Antimicrobial stewardship is the organised and systematic effort to optimise the prescription of antimicrobials to ensure efficacy, reduce overuse, avoid antimicrobial resistance, and reduce patient exposure. Antimicrobial resistance is one of the foremost concerns of the medical field and poses significant health risk to humanity and animals (Guardabassi & Prescott, 2015; O'Brien & Gould, 2013; Prescott & Boerlin, 2016; Pulia et al., 2017; Roberts et al., 2009; Septimus & Kuper, 2009).

Antimicrobial stewardship can be difficult to observe in cases of suspected sepsis, i.e. prior to diagnosis, because the disease worsens rapidly and early intervention is essential for improved outcome. Control of the source infection with antimicrobial therapy is required for effective treatment (MacArthur et al., 2004; Micek et al., 2005). Empirical antimicrobial therapy is commonly applied in veterinary medicine (Guardabassi & Prescott, 2015), and blood cultures are performed rarely in general practice (more studies are needed to officially document diagnostic testing practices in a primary care setting); so antibiotic drugs are often employed contrary to the recommendations of antimicrobial stewardship programs. Furthermore, antibiotic drugs may be withdrawn too soon or continued longer than necessary because of the lack of rapid, affordable tests to indicate the infection status of the patient. Microbial culture may take up to 72 hours to return a result, and inflammatory blood biomarkers (e.g. leukocytes) are not specific for infection (Kumar et al., 2014; Tizard, 2017). Overuse or injudicious use of antimicrobials is a major driver of resistance (Malhotra-Kumar et al., 2007).

In humans, biomarkers that are more specific for infection guide the administration of antimicrobials (Bouadma et al., 2010; Pulia et al., 2017) although one biomarker alone is not sufficient in all cases (e.g. Albrich & Harbarth, 2015). Guidance of antimicrobial therapy using PCT, including early truncation of the course, resulted in reduction in antimicrobial usage without sacrificing patient safety in cases of respiratory tract infections including sepsis (Schuetz et al., 2011). The PRORATA trial found the same outcome in a study of 307 non-surgical ICU patients (Bouadma et al., 2010), and larger trials of more than 1,000 human patients have found a reduction in mortality with PCT-guided antimicrobial usage (Assink-de Jong et al., 2013; de Jong et al., 2016; Rhee, 2016). However, other studies were unable to show the same benefits although these were often low power or poor quality (Andriolo et al., 2017; Prkno et al., 2013; Shehabi & Seppelt, 2008). When appropriate use of antimicrobials results in reduced amounts administered, this reduces cost in a clinical setting in addition to a reduced pharmaceutical toxicity burden for the patient no matter the species (Cosgrove, 2006; Eubank et al., 2020).

1.4 Current diagnostic methods for canine sepsis

A wide variety of diagnostic tests and assays are currently used to detect, manage, and prognosticate sepsis. Several aspects of the disease are measured independently; it is then the role of the clinician to interpret the information in combination and the context of that case (Dunne, 2015; Kumar et al., 2014; Silverstein, 2015). For example, the clinical criteria used for SIRS diagnosis can be measured independently of organ function assessments requiring blood biochemistry, or additional clinical exams to screen for shock. Measuring

blood biomarkers of inflammation is becoming routine; and tests to confirm infection are often carried out, for example microbial culture and sensitivity. However, there is no single gold-standard or definitive test for sepsis. And the tests used will depend in many circumstances on the individual case, including the location of the infection and the severity of the disease, as well as financial considerations in many cases.

Bacterial culture is relatively insensitive, prone to false positives and false negatives (Campbell et al., 2003; Hall & Lyman, 2006; Hall et al., 2011; Laukemann et al., 2015), and results are hampered by an inherent delay of at least 24 hours. Biomarkers such as C-reactive protein (CRP) may not indicate significant inflammation for up to 24 hours after the onset of clinical signs (Meisner et al., 1999; Ryoo et al., 2019), and this along with cytokines and leukocyte counts are not specific for infection (Cerón et al., 2005; Christensen et al., 2014; Eckersall & Bell, 2010; Gebhardt et al., 2009; Greiner et al., 2008; S. Yamamoto et al., 1993). Veterinarians frequently make use of in-house cytology for infection diagnosis, although this also has a high risk of false negatives. Point-of-care tests for blood biomarkers or antigens, such as parvovirus, are also used regularly, along with imaging such as thoracic radiographs or abdominal ultrasound which may confirm effusions or abscess formation. Histopathology can also diagnose infection, although this would more often be an incidental diagnosis (McGavin & Zachary, 2007; Quintana, 2015; Sharp, 2015; Silverstein, 2015).

The most common reports of sepsis in dogs include at least fifty reports of peritonitis from a range of sources including gastrointestinal perforation, pancreatitis, or liver abscesses, a few of which are listed here (Abelson et al., 2013; Bush, 2016; Cortellini, 2015; Dayer et al.,

2013; Dickinson, 2015; Guieu et al., 2015; Kenney et al., 2010; Liu et al., 2012). However, sepsis can result in infection from any tissue (Camargo et al., 2020; Declue et al., 2012; Greiner et al., 2007; Karlsson et al., 2013). Based only on preliminary searches, all-cause sepsis studies in dogs are relatively rare, include a small cohort (often less than thirty dogs), or focus on biomarker measurement and not the sepsis epidemiology itself. There is currently no meta-analysis or review summarizing all epidemiological information on sepsis or infection in dogs. The most common infections in dogs may not translate to the most common infection sources in cases of sepsis. More epidemiological studies are needed on larger cohorts with all-cause sepsis to provide more in-depth information on infection rates and outcomes.

1.5 Biomarkers

A biomarker can be defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (Aronson, 2005). The ideal biomarker for a condition such as sepsis would have rapid and quantitative measurement; be accurate (sensitive and specific), directly indicative of disease severity, not confounded by comorbidities or interventions, and both practical and cost-effective to test. There are guidelines published by the FDA-NIH Biomarker Working Group to aid in efficient and productive research, development, and clinical translation of biomarkers (Group, 2016; Poste et al., 2015; Robb et al., 2016).

Many biomarkers are studied or used for sepsis diagnosis and management in dogs (Cerón et al., 2005; Eckersall & Bell, 2010; Karlsson et al., 2016; Köster et al., 2019; Lewis et al., 2012; Ok et al., 2015). These include pro-inflammatory cytokines and acute inflammation markers, which generally do not show ability to differentiate between sepsis and non-infectious SIRS (Declue et al., 2012; A. N. K. Floras et al., 2014), but may predict outcome (Rau et al., 2009). A full review of canine inflammatory biomarkers, focusing on those useful in sepsis diagnosis, is beyond the scope of this chapter – however, the many studies available demonstrate that despite dozens of candidates, very few potential biomarkers translate to a valuable clinical biomarker for rapid diagnosis and management of sepsis.

1.5.1 C-reactive protein

C-reactive protein (CRP) is a common biomarker of inflammation for several species, including dogs and humans. It is commonly measured in cases of sepsis in humans and increasingly also in dogs with the advent of CRP tests into routine blood panels; it is the only acute phase protein to be measured routinely in clinical settings apart from albumin. C-reactive protein is a major acute phase protein (APP) meaning that it is one of a group of proteins produced in high levels (an increase in plasma concentration greater than 25%) rapidly by the liver after stimulation by pro-inflammatory cytokines. Acute phase proteins have a variety of antimicrobial, protective, or pro-inflammatory effects and are part of the innate immune system (Tizard, R. 2017). The level of CRP, a relatively stable pentamer protein, may increase to over 50-fold within 24 hours, and decrease within 72 hours back to normal concentrations when inflammation is resolved (Kjelgaard-Hansen, 2010; Löfqvist et

al., 2018). Its function includes activation of complement, and acting as an opsonin (Du Clos, T. 2000). C-reactive protein is measured in plasma samples using immunoassays. The specificity of CRP for infectious cause of inflammation is poor, and it is unable to distinguish sepsis from non-infectious SIRS except for limited circumstances such as one study of arthritis (Hillström et al., 2016; Hindenberg et al., 2020). It does not predict outcome in dogs with SIRS although may trend downwards over time in survivors (Gebhardt et al., 2009; Gommeren et al., 2018; McClure et al., 2013). Overall, CRP is a good first-line diagnostic to rule in systemic inflammation; and is valuable after diagnosis of sepsis for monitoring of treatment efficacy and relapse (Christensen et al., 2015; Löfqvist et al., 2018; Nakamura et al., 2008; Sato et al., 2017; Viitanen et al., 2017; Yamamoto et al., 1994). However, biomarkers are still being sought, that are more specific for sepsis and more rapid in indicating onset and resolution of disease.

1.6 Procalcitonin

One widely studied blood biomarker for sepsis in humans is procalcitonin (PCT). Procalcitonin is a small protein (less than 15 kDa), which in health is a precursor protein produced in C cells of the thyroid, as one of five products of the CALCA gene. This gene produces calcitonin and katacalcin which regulate plasma calcium levels, along with calcitonin gene-related peptide which has vasodilating and antimicrobial functions (Le Moullec et al., 1984). In a state of health CALCA translates to the precursor pre-procalcitonin which is cleaved into PCT. This in turn is cleaved primarily into the biologically active hormone calcitonin. Proteolysis also liberates N-terminal PCT (N-PCT) from the N-

terminal end and katacalcin (KC) from the C-terminal end of PCT. Canine PCT shares about 65% homology with the human protein (Le Moullec et al., 1984; Mol et al., 1991; Wende et al., 2000), and is cleaved into the same products including the hormone calcitonin. This process is illustrated in a schematic: Figure 3.1.

1.6.1 PCT in sepsis

Uncleaved PCT is produced constitutively during inflammation in mammalian species by many extrathyroidal and extrahepatic tissues including the liver, lungs, fat, and leukocytes (Giunti et al., 2010; Kuzi et al., 2008; Meisner, 2002; Meisner et al., 2003; Morgenthaler et al., 2003; Russwurm et al., 2001; Russwurm et al., 1999). Although it has been classified as an APP by some, or a 'hormokine' (Muller & Becker, 2001; Nijsten et al., 2000; Whicher et al., 2001), it is also produced locally by inflamed and infected tissues such as in wound beds and urinary tract infections without being equivalently increased in the bloodstream (Bassim et al., 2008; Forsberg et al., 2008; Maruna et al., 2000). Stimulus of PCT secretion is initiated by pro-inflammatory cytokines including IL-6, IL-8, and TNF-a, and is therefore indirectly responsive to endotoxin and PAMPs (Bonelli et al., 2017; reviewed in Dong et al., 2019).

In sepsis in many species, PCT is secreted from many somatic cell types without further cleavage, in a much higher proportion to all other cleavage variants (Becker et al., 2004; F. Bonelli et al., 2015; Ercan et al., 2014; Meisner et al., 2003). Notably, calcitonin (CT) does not increase during sepsis (Assicot et al., 1993).

The function of constitutively expressed PCT is not known (in any species). Researchers have suggested several possible functions for PCT including endotoxin binding (Matera et al., 2012), analgesic (Maruna & Gürlich, 2003), and a mediator involved with macrophages or cytokine signalling such as TNF-suppression (Monneret et al., 2000) and having a potentially harmful or pro-inflammatory role (Baranowsky et al., 2021; Becker et al., 2010; Braithwaite, 1998; Dahaba & Metzler, 2009; Liappis et al., 2011). It is also suggested to affect vessel dynamics (Hoffmann et al., 2002; Sexton et al., 2008).

1.6.2 Human procalcitonin as a sepsis biomarker

Bohuon and colleagues (Bohuon, 2000) first raised monoclonal antibodies to PCT and its various cleavage products in the process of investigating it as a marker for medullary thyroid carcinoma. They found PCT was also markedly increased by as much as 100-fold in patients with sepsis (Assicot et al., 1993). Further investigation showed that plasma calcitonin did not also increase during sepsis (Becker et al., 2004; Becker et al., 2010; Holowaychuk & Martin, 2007; Muller et al., 2000). Therefore, PCT was speculated to be secreted into plasma as a result of infection or inflammation. Subsequently it was shown that PCT plasma concentrations increase in correlation with endotoxin administration (Dandona et al., 1994; Matera et al., 2012). Production or secretion is inhibited by interferon gamma, thus PCT is not increased in viral infections (Christ-Crain & Müller, 2005; Linscheid et al., 2003). Overall, many review articles have summarised extensive research showing PCT concentrations are increased in the bloodstream during inflammation and particularly when the cause is

bacterial infection (Reinhart et al., 2006; Tang et al., 2007; Trásy et al., 2016; Uzzan et al., 2006; Wacker et al., 2013). The reason for this constitutive, inflammatory expression of PCT (a precursor protein) into the plasma, and any function in inflammation, is not fully understood. Several possibilities have been researched and are discussed in Chapter Two. But PCT is not specific for infection. Over the previous two decades there has been disagreement on whether PCT is more sensitive or specific for sepsis than CRP (Tang et al., 2007; Wacker et al., 2013). Sensitivity and specificity vary depending on the disease cohort and context, how PCT is measured, and even which other biomarkers are measured in combination (Arkader et al., 2006; Han et al., 2015). Procalcitonin is generally considered to have moderate specificity for infection as the cause of inflammation, and good sensitivity (Russwurm et al., 1999; Singer et al., 2016). Procalcitonin has been shown to reduce antimicrobial usage or allow earlier discontinuation, with no reduction in survival (Agarwal & Schwartz, 2011; Bouadma et al., 2010; Schuetz, Bretscher, et al., 2017; Schuetz et al., 2011; Tang et al., 2009). A full review of the extensive PCT literature is beyond the scope of this chapter, and already reviewed in many publications, some of which are listed here (Christ-Crain & Müller, 2005; Davies, 2015; Jones et al., 2007; Liu et al., 2015; Pepper et al., 2019; Pierrakos & Vincent, 2010).

Rather than its specificity for infection, the main advantage of PCT in a clinical setting is its rapidity. Concentrations of PCT become increased comparatively more quickly than CRP usually within four to six hours. Importantly PCT concentrations often drop more quickly

than CRP or cytokines as patients recover while being slightly more stable than the quickly fluctuating concentrations of cytokines (Castelli et al., 2004; Dandona et al., 1994; Meisner, 2014); and resolves rapidly after resolution compared with CRP (Arkader et al., 2006; Casado-Flores et al., 2003; Meisner, 2014; Russwurm et al., 2001; Schuetz, Birkhahn, et al., 2017; Schuetz et al., 2013). Plasma concentrations of PCT may drop by half within eight to 24 hours, and the measure in the change between timepoints is referred to as delta-PCT or PCT-clearance (de Azevedo et al., 2015; Mat Nor & Md Ralib, 2014). Procalcitonin therefore occupies a niche as a dynamic biomarker (Molnár & Bogár, 2006; Trásy et al., 2016), where repeated measures assist in confirming or excluding sepsis, and guiding treatment of sepsis. Overall, PCT consistently performs strongly as a severity or prognostic indicator, including when guiding truncation of antimicrobial courses by quantitative or semi-quantitative tests (Christ-Crain & Müller, 2005; Tang et al., 2009); indicating severity of disease (Castelli et al., 2004); and combining synergistically with other biomarkers or calculators (Han et al., 2015; Huang et al., 2016; Kim et al., 2012; Ruiz-Rodríguez et al., 2012; Trásy et al., 2016). Despite its limitations, PCT is still one of the most researched inflammation and infection biomarkers in the sepsis research field; and one of the only ones, besides CRP, to be developed and enter into common usage in some parts of the world: reliable tests are now widely available in human medicine (Schuetz, Bretscher, et al., 2017; Wang et al., 2019). This shows that a rapid and specific biomarker that can aid in sepsis diagnosis and management is highly sought after by clinicians who diagnose and treat sepsis.

1.6.3 Types of quantitative assay for human PCT

There are several assays currently available to measure human PCT quantitatively and qualitatively in the clinical setting. These use monoclonal antibodies as the basis to detect and quantitate PCT (Bohuon, 2000; Meisner, 1997; Schuetz, Bretscher, et al., 2017). Immunoassays are valuable because of their sensitivity, specificity, accessibility, flexibility (e.g. binding to a bead or surface or modifying with labels), sample economy, and ease of use at the bedside in some cases.

The ELISA is one of the most common types of immunoassays and is considered the gold standard. There are several types of ELISA which each have advantages and disadvantages (Aydin, 2015; Clark et al., 1986; Tizard, 2017). The four main types are direct, indirect, sandwich, and competitive, although each uses a combination of antibody, antigen, and a detector, and relies on all of these components binding together in some way. Direct ELISA is the simplest type and is often the least sensitive and specific, whereas competitive and sandwich ELISAs use multiple or complex steps and are more often fully quantitative and highly sensitive and specific. While some ELISAs are quantitative, others are qualitative only (give a yes or no result), and others may be semiquantitative (no, low, high) such as some human PCT assays (Schuetz, Bretscher, et al., 2017). Each ELISA should have a control or a series of standards if it is quantitative, to allow interpretation of negative results and enable standardised quantification. All ELISAs involve some incubation or wait time and use buffers or specially designed diluents to ensure optimal antigen-antibody binding conditions.

Each ELISA must be fully validated for a given sample type and conditions (Arnold et al., 2019; Minic & Zivkovic, 2020). When developed, an ELISA must first be optimized to ensure buffer conditions and antigen and antibody concentrations are appropriate to allow accurate measurements and consistent functionality. The analytical validation process then involves experiments involving several repeated measurements of replicates of different known concentrations of analyte, to determine accuracy (sensitivity, specificity), linearity (range, lower limit of detection, spike/recovery), precision (intra- and inter-assay variability or coefficients of variation), and robustness. For example, coefficients of variation greater than 15% are considered unacceptable. An analytically validated ELISA can then undergo clinical validation studies, standardisation, calibration (Eckersall, 2019).

The antibody is the primary determinant of immunoassay performance – an antibody with poor affinity or high non-specific cross-reactivity would produce a worthless ELISA. Polyclonal and monoclonal antibodies are both routinely used in immunoassays, each having advantages and disadvantages (reviewed in Lipman et al., 2005). Polyclonal antibodies (Pabs) can be cheaper to produce and easier to obtain in a short timeframe. Their heterogenous nature (arising from a normal complement of unique plasma cells in an immune response of a healthy individual) makes them polyspecific to an antigen, often recognizing several epitopes. This may provide improved recognition of the antigen but can also increase non-specific binding. Furthermore, they are batch-specific. Monoclonal antibodies in contrast are highly consistent since they arise from a single immortal clonal line. Their antigen recognition is often highly specific which may be a strength if it enables

them to distinguish between subtly different conformations of their antigen – but by the same properties they can be more susceptible to varying experimental conditions.

In the case of human PCT, Mabs were raised to recombinant PCT (Althaus & Walter, 2002; Kremmer et al., 2012). Any recombinant protein is typically produced in an *E. coli* or similar bacterial vector, and as such will include a short sequence of amino acids in addition to the inherent amino acids of the protein (a purification tag), and is not expected to fold or have glycosylation or other modifications applied. For hPCT, certain peptides within the PCT sequence were selected to ensure recognition of specific epitopes reflecting certain cleavage variants (Ghillani et al., 1988; Ghillani et al., 1989; Meisner, 1997). This strong specificity is similar to that of class-specific antibodies (Herbst & Klein, 1995). However, Pabs with good antigen specificity often show slightly higher sensitivity than a monoclonal (Carr-Smith et al., 2016; Prontera et al., 2009), so Pabs should not be considered inferior *per se*. In many direct comparisons the overall performance is similar (Busby et al., 2016; Prontera et al., 2009). Ultimately the choice of monoclonal vs polyclonal antibody should be based on planned use. In the case of this project, sensitivity and specificity are required and consistency across batches is a priority, making Mabs the preferred type for canine PCT assays in clinical use.

1.6.4 Antibody validation

Validation of antibodies is essential but rarely performed. Many researchers have called for stringent standards of antibody characterization and validation, and blamed the reproducibility crisis on poor validation practices (Bradbury & Plückthun, 2015; Saper, 2005).

It is not appropriate to use an antibody without knowledge or assessment of its functionality, structure, or affinity. Researchers must prioritize comprehensive validation of individual antibodies. Sound characterization and guarantee of antibody affinity and performance will make antibody-based research reproducible (Berglund et al., 2008; Bradbury & Plückthun, 2015; Edfors et al., 2018; Uhlen et al., 2016). While it may increase initial cost, observing best practice and testing assumptions improves final research outcomes, as noted for other biomarkers (Christensen et al., 2015).

Several methods are available for antibody validation, and the more of these that can be done, the better the validation (Edfors et al., 2018; Uhlen et al., 2016). Five approaches have been recommended for validation of an individual antibody, using complementary methodologies (adapted in Edfors et al., 2018). These are described in Table 1.2. Further validation and calibrations should be performed on an immunoassay independently to those performed on the antibody itself (Eckersall, 2019; Lee & Hall, 2009; Valentin et al., 2011).

Table 1.2: Antibody validation methods, and examples of how they can be achieved.

	Method	**Example**
1	Orthogonal quantitation of the target protein using antibody-independent methods	Proteomics or another antibody-independent analysis e.g molecularly imprinted polymers
2	Independent antibody that recognizes a different epitope	Mab vs Pab, multiple companies' antibodies, multiple epitopes
3	Repeated measure with the original antibody after known increase of target protein level	Increase protein with recombinant methods, or confirmed increase in disease

4	Repeated measure with the original antibody after known decrease of target protein level	Gene knock-out models, or confirmed decrease or absence in healthy plasma
5	Mass spectrometry to analyze the antigen-antibody complex	Identify the antigen after capture by immunoprecipitation

1.6.5 Mass spectrometry

Mass spectrometry is another method to detect and quantitate PCT in biological samples. It detects molecules based on their mass and charge, or that of their product ions after fragmentation, and matches the precise measurements against known amino acid sequences to find a match – or calculates an amino acid sequence *de novo*. Mass spectrometry is highly sensitive and specific (Bilić et al., 2018; Yates et al., 2009). Sample clean-up (processing) is required prior to mass spectrometry of a protein, especially one from a complex biological matrix such as blood products or urine (Gummer et al., 2009; Gundry et al., 2010). But its main drawbacks, other than high-level sample preparation, include cost and inaccessibility. Quantitative analyses can be run using a type of mass spectrometry termed multiple reaction monitoring (MRM): quantitation can be relative (positive controls and known standards included in the run) or independent if a labelled peptide of some kind is also included (Liebler & Zimmerman, 2013). Mass spectrometry is a strong antibody-independent method for calibration (if quantitative) and validation in the context of veterinary sepsis biomarkers (Bilić et al., 2018; Eckersall, 2019; Mansor et al., 2013).

Molecular imprinted polymers (MIPs) are also referred to as synthetic antibodies. A template or 'mould' of a given target protein is made using small molecules that are polymerised under conditions such as UV light. The level of sensitivity and specificity of the interaction between MIP and target is similar to that of an antibody or enzyme-substrate (Dickert et al., 2000; Moreno-Bondi et al., 2008; Smolinska-Kempisty et al., 2016; Xu et al., 2020). The resulting chip can then be used (once or more) to detect the target protein. Despite the robust and sensitive nature of this assay type, it is not yet used in clinical applications and is infrequently featured in sepsis laboratory and clinical research.

1.7 Procalcitonin measurement in dogs

Calcitonin is highly conserved across many species including dogs (Chang et al., 2004). The PCT protein was first characterized in dogs in 1991 and found to be very similar to human PCT (Mol et al., 1991). See Figure 4.7 for alignment data. Despite significant similarities between mammalian species, it is not known if the function or any modified structure of constitutively expressed PCT is also conserved. Canine PCT has been detected or measured by PCR (gene expression or mRNA), immunoassays (which were only validated in some cases), and most recently by mass spectrometry and molecular imprinted polymers MIPs.

1.7.1 RNA or DNA

Elevations of PCT mRNA have been detected in tissues of dogs with sepsis and systemic inflammatory conditions (Giunti et al., 2010). Elevated expression of the CALCA gene (see section 1.6) was detected via qr-PCR in the majority of samples of lung, thyroid, liver and

spleen of nine dogs who had died with parvovirus or SIRS, thus PCT has been proposed as a veterinary inflammatory biomarker (Matur et al., 2017). Based on this, it was hoped that PCT may fill a similar role to that in human sepsis diagnosis and monitoring. This was supported by a subsequent study measuring mRNA with quantitative reverse-transcriptase PCR of PCT mRNA (Kuzi et al., 2008). This study of 17 dogs with inflammatory disease, 17 dogs with infectious, and other groups with neoplastic or other diseases, reported increased PCT mRNA for all disease groups compared with the healthy controls, but no correlation between quantity of mRNA and haematological measurements. Canine PCT was concluded to be an APP.

1.7.2 MS and MIPs

Mass spectrometry has been used to detect canine PCT but not for quantitative measurement (A. Floras et al., 2014; Goggs et al., 2018). The only cPCT assay currently published that is not an immunoassay, uses molecularly imprinted polymers (MIPs - also called synthetic antibodies) (Dickert et al., 2000; Smolinska-Kempisty et al., 2016; Xu et al., 2020), detected with surface plasmon resonance (Battaglia et al., 2021). The MIPs successfully detected recombinant PCT when spiked into canine plasma at concentrations from 25-1000 ng/mL (higher than the 50-500 pg/mL concentrations reported with the BV ELISA (Goggs et al., 2018)), with minimal interference from other plasma components. With the potential to re-use imprinted chips, this economical and stable method may become a robust and specific antibody-independent option for PCT quantitation, with a next milestone

being to confirm that the MIPs detect PCT in the native form; but would need further validation before use in a diagnostic laboratory.

1.7.3 Immunoassays

Two research groups have independently carried out analytical and preliminary clinical validation with an anti-cPCT ELISA by BioVendor (Asheville, NC, USA) (Battaglia et al., 2020; Goggs et al., 2018). This quantitative (sandwich) assay (designated BV throughout this thesis) uses a polyclonal raised to recombinant PCT. The standard of this ELISA was confirmed to contain cPCT (Goggs et al., 2018) but its affinity for native PCT and its quantitation of cPCT have not been confirmed with an antibody-independent method. Assay parameters included low coefficients of variation (≤4.5%) and high recovery of PCT spiked into kit dilution buffer. Another analytical validation study of the BV ELISA showed similar good performance with recovery ≥96% and all coefficients of variation ≤13% (Battaglia et al., 2020).

Several studies have now been performed using this assay. (Note, one recent study is not yet peer-reviewed but is noted in the Appendix 6.1). In the first study, plasma samples from 30 dogs with gastric dilation and volvulus (with SIRS) and 20 dogs with sepsis were tested with the BV ELISA. Canine PCT concentration was found to be significantly increased in plasma samples from dogs with sepsis (median 78.7 pg/mL, range 39.1-164.7) compared to healthy controls (49.8 pg/mL, range 36.2-63.7), but not compared to SIRS (60.3 pg/mL median, range 43.4-137.2) (Goggs et al., 2018). Conversely, a study of 18 dogs with septic

peritonitis compared to 19 dogs with non-infectious ascites showed no difference in plasma PCT concentration (Martiny & Goggs, 2019).

Plasma PCT concentrations became increased very rapidly – as soon as two hours – following intravenous administration of endotoxin to six dogs (Easley et al., 2020), which agrees with the rapid response noted to LPS infusion in several other species (Becker et al., 2004; Bonelli et al., 2017; Meisner et al., 2003; Zannoni et al., 2012). Increase in plasma cPCT was significantly correlated with organ dysfunction, and was higher in dogs with septic shock than sepsis without shock (Troia, Giunti, & Goggs, 2018): twelve normal dogs had median PCT plasma concentration of 41.6 pg/mL (21.5–88.7) compared to the 53 presenting with sepsis, 103.0 pg/mL (range 15.4–470.2); the 42 dogs with sepsis but no cardiovascular compromise (77 pg/mL, 18.2–470.2) had lower cPCT concentrations than those 11 with septic shock (243.1 pg/mL, 56.7–369.8). Dogs who survived sepsis had higher PCT clearance (decreased plasma concentration compared with a prior timepoint) at 24 hours, as observed in humans recovering from sepsis (Bouadma et al., 2010; Ruiz-Rodríguez et al., 2012; Schuetz, Birkhahn, et al., 2017; Troia, Giunti, & Goggs, 2018). Survivors of sepsis and gastric dilatation and volvulus (GDV) showed significantly lower plasma concentrations of PCT compared with non-survivors at 24 and 48 hour timepoints after diagnosis (Troia, Giunti, Calipa, et al., 2018; Troia, Giunti, & Goggs, 2018). Plasma PCT concentrations correlated highly with plasma lactate concentrations in the GDV patients (Troia, Giunti, Calipa, et al., 2018). Furthermore, PCT plasma concentrations showed no association with comorbidities or antimicrobial administration in 53 dogs presenting with sepsis (Troia, Giunti & Goggs,

2018). This indicates PCT could be a prognostic marker for sepsis and SIRS, and a direct comparison of its kinetics against C-reactive protein (CRP) would be valuable.

1.7.3.1 Limitations of the BV assay

The results obtained with the BV ELISA are promising; however, they not absolute because the BV assay itself still carries assumptions and limitations. The affinity of the BV ELISA for native canine PCT remains undetermined, as does the possibility of non-specific binding or interference from plasma components. Recent recommendations for validation of an individual antibody are not yet all applied to the BV polyclonal (Uhlen et al., 2016). Some recovery values were lower than expected in more than one study, leading researchers to consider that different affinity for isoforms of PCT could be affecting performance; although buffer conditions and other factors such as storage also could not be ruled out (Battaglia et al., 2020; Goggs et al., 2018).

Batch to batch variability of assays should be borne in mind when interpreting all cPCT studies that use Pab immunoassays. One possible example of batch variability is for a cPCT from another manufacturer that failed analytical validation, producing results with no apparent relationship to the standard dilution series during standard curve analysis (A. Floras et al., 2014; Rossi et al., 2014). Another publication using this ELISA reported a significant difference in PCT plasma concentrations between healthy subjects and those with babesia (Brkljačić et al., 2014), which appear plausible when considered in isolation: human PCT concentrations have been increased in malaria (Arora et al., 2015; Schuetz et al., 2013). Their results clustered into significantly different groups with healthy dogs showing

low PCT concentrations similar to other reported values for healthy dogs (see Table 1.3). While batch variability (these studies were carried out in different years) is one possible explanation, unreliable performance cannot be ruled out. For example, the assay may be measuring some other substance increased in inflammation or infection.

A third PCT assay, validated for human PCT, measured an increase in presumed plasma cPCT following intravenous endotoxin administration – findings which were expected, and later confirmed with the BV assay (Easley et al., 2020; Yilmaz et al., 2008) and seen in other species including pig and horse and humans (Becker et al., 2004; F Bonelli et al., 2015; Meisner et al., 2003; Zannoni et al., 2012). However, the assay used for the endotoxin measurements could not be independently validated during an attempt several years later, because it failed to recognize recombinant canine PCT (Battaglia et al., 2020).

The obvious solution to avoid uncertainty surrounding assay performance is to repeat analytical ELISA validation in each study; but the expense involved may be prohibitive to many researchers. Transition to monoclonal anti-cPCT antibodies offers a better solution to this challenge.

1.7.4 Lack or failure of validation

Prior to the BV ELISA publication reporting successful analytical validation (Goggs et al. 2018), several RUO ELISAs for canine PCT were commercially available but none were independently validated. All of these studies used various immunoassays that were described by the manufacturer as recognising canine PCT and made with a recombinant

antigen; except for one where the manufacturer stated that the immunoassay was for native cPCT, although did not specify the antigen used for its production or any information to validate or clarify this statement (Battaglia et al., 2020) although the immunogen source is not specified by the manufacturer.

The first attempt to perform analytical validation on a cPCT ELISA was unsuccessful (A. Floras et al., 2014). The assay produced a good standard curve using the supplied standard but gave unpredictable and random results for recombinant canine PCT (crPCT) dilution series, prompting mass spectrometry analysis of the recombinant protein and the standard (A. Floras et al., 2014). No PCT was detected in the standard - this prompted the uncommon step of mass spectrometry in future validation studies of other canine PCT ELISAs (Goggs et al., 2018). The same assay also had an unacceptably high intra-assay and inter-assay variability of up to 74%. Rossi and colleagues (2014) reported similarly disappointing results for this and another brand of canine PCT ELISA in the same year (Rossi et al., 2014).

Despite failed validation of this particular cPCT ELISA in 2014, three further studies published results using canine PCT ELISAs without showing validation (Andonova et al., 2017; Dunning et al., 2017; Gürbüz & Ulutaş, 2017). The results of these and earlier canine PCT studies often reflect human PCT outcomes and therefore seem plausible, but cannot be relied on. For example, rapid increase of cPCT within 30 minutes after endotoxin administration (Yilmaz et al., 2008) also occurs in humans (Dandona et al., 1994) and was later confirmed in 2020 with the BV assay (Easley et al., 2020). Another study showed realistic timing: concentrations of cPCT increased for up to 48 hours following subcutaneous

inoculation with *Pseudomonas*, before reduction within 24 after antimicrobial administration (Andonova et al., 2017). This study tested extract of feverfew (a plant, *Tanacetum parthenium*) as an anti-inflammatory agent without comparison to a registered anti-inflammatory pharmaceutical. A study measuring PCT after routine ovariohysterectomy did not observe an increase in cPCT past one day (Gürbüz & Ulutaş, 2017) – but this would not be expected since PCT concentrations are not significantly increased after sterile inflammation associated with minor surgery in humans (Meisner et al., 1998; Uzzan et al., 2006). Their conclusions should be interpreted with full understanding of the assay limitations.

A third brand of assay (TSZ, see Table 1.3) was subjected to analytical validation by an independent research group in 2020 (Battaglia et al., 2020). The assay achieved good experimental assay performance, but random and unexpected results with a dilution series of clinical samples: cPCT concentrations measured with this ELISA were different to results obtained in parallel with the BV assay (Battaglia et al., 2020). Also, the TSZ assay did not recognize recombinant PCT. In both studies reporting failed validation, differences between native and recombinant PCT (such as purification tags) were suggested as a possible explanation for the unexpected or negative results incongruent with the known concentrations in the samples (Battaglia et al., 2020; A. Floras et al., 2014).

1.7.4.1 Cross-species PCT measurement

The first report of canine PCT measurement used a semi-quantitative immunoassay for hPCT (Giunti et al., 2006). In this abstract-only report, a positive result based on increased human

PCT immunoreactivity was presumed to indicate cPCT in the tested plasma of dogs with sepsis and SIRS (Giunti et al., 2006). However, wide overlap between these and healthy patients – and no significant difference between blood culture results – mean that this low-powered pilot study was inconclusive. A second hPCT assay, this time quantitative, reported presumed cPCT results for sixteen dogs receiving saline or endotoxin intravenously. Healthy dogs who received saline infusion had a measured concentration of 1700 pg/mL while healthy dogs in other studies including those utilizing the BV assay typically measure PCT concentrations of less than 50 pg/mL in healthy dogs (Goggs et al., 2018).

Sixteen healthy dogs receiving 1 µg/kg endotoxin had plasma cPCT quantified at 1,700 to 3,400 pg/mL using the hPCT assay; but six dogs receiving twice as much endotoxin (2 µg/kg intravenously) only had plasma cPCT concentrations of 99.5-295.9 pg/mL when measured with the BV assay (Easley et al., 2020). Evidently comparison of cPCT between studies, and especially human and canine PCT assays, is invalid (see Table 1.3). The BV assay quantitation appears consistent over two years based on independent validations (Battaglia et al., 2020; Goggs et al., 2018) but is still subject to batch variability due to use of a Pab as discussed above. It remains unclear whether this type of variability is solely due to difference between species isoforms of PCT or other factors. This highlights the importance of caution in use or interpretation of assays used before cross-reactivity between species is not yet known, since this can influence assay performance (Caspi et al., 1984; Cerón et al., 2005; Kjelgaard-Hansen & Jacobsen, 2011).

Table 1.3: Summary of assays measuring canine procalcitonin. Includes analytical and preliminary clinical validation, using either blood-derived samples or urine. References where indicated: 1 (Goggs et al., 2018); 2 (Troia, Giunti, & Goggs, 2018); 3 (Battaglia et al., 2020)

Assay product information	**Assay type**	**Target species**	**Validation level (reference)**	**Studies where used**	**PCT concentrations measured in dogs**
PCT-Q BRAHMS Diagnostica, GmbH, Berlin	Semi-quantitative, Immuno-chromato-graphic	Human	None for dogs. Validated in humans	Healthy vs inflammatory conditions, abstract only (Giunti et al., 2006)	calibration range: 0.5-10 **ug/L** (ng/mL) Large overlap between healthy and sepsis (Giunti et al., 2006)
PCT-LIA (Lumi-test) BRAHMS Diagnostica, GmbH, Berlin	Quantitative, Immune-luminometric	Human	None for dogs. Validated in humans.	Kinetics (Yilmaz et al., 2008)	Approx. 150-300 nmol/L (~1.73-3.45 ng/mL) in dogs receiving endotoxin (Yilmaz et al., 2008)
Canine PCT ELISA BioVendor, Asheville, NC, USA	Quantitative, sandwich ELISA	Canine	Independent analytical validation and mass spectrometry	Initial analytical and clinical validation (Battaglia et al., 2020; Goggs et al., 2018; Troia, Giunti, & Goggs, 2018)	Calculated RI 5.8-91.1 pg/mL [1] 39.1-164.7 pg/mL [1] 15.4-470.2 pg/mL [2] 79.3–944.7 pg/mL [3]
PCT ELISA Kit Wuhan EIAab Science Co. Ltd., China	Quantitative, sandwich ELISA	Canine	Failed independent validation.	Analytical validation (A. Floras et al., 2014) and *Babesia* investigation (Brkljačić et al., 2014)	218-380 pg/mL (A. Floras et al., 2014) Approx 15-200 pg/mL (Brkljačić et al., 2014)
Canine PCT ELISA kit, Cusabio, China	Quantitative, sandwich ELISA	Canine	Nil	Pre- and post-surgery (Gürbüz & Ulutaş, 2017)	21.99 ± 2.47 pg/mL, in non-septic dogs post surgery (Gürbüz & Ulutaş, 2017)
CA1033, TSZ ELISA, Scientific LLC, BIOTANG, USA	Quantitative, sandwich ELISA	Canine	Good analytical performance; unable to detect recombinant (Battaglia et al., 2020)	Monitoring *Pseudomonas* treatment (Andonova et al., 2017). Not fully validated (Battaglia et al., 2020)	90-400 pg/mL, in dogs with subcutaneous *Pseudomonas* (Andonova et al., 2017)
Canine PCT ELISA, NovaTeinBio, Cambridge, MA, USA	Competitive ELISA	Canine	Failed independent validation	Analytical validation (Rossi et al., 2014)	Not reported (abstract, poster)
Independent	Not reported (abstract only)	Canine	Specially designed; validated but details not reported	UTI study (Dunning et al., 2017)	Not reported
Novel MIP assay (molecularly imprinted polymer)	Quantitative MIP assay	Canine	Independent analytical validation with canine recombinant PCT	Detection and quantitation of recombinant canine PCT in standards and plasma (Battaglia et al., 2021)	LOD of 15 ng/mL for recombinant cPCT spiked into plasma (Battaglia et al., 2021)

1.7.5 Effect of biological matrix on cPCT measurement

Canine PCT has been measured in some matrices other than plasma. One study attempted to measure cPCT concentration in abdominal effusion of dogs with septic peritonitis in comparison with non-infectious ascites, but cPCT showed no discrimination between the two (Martiny & Goggs, 2019): 38.4 pg/mL and 98.9 pg/mL respectively (medians). In this case, there was also no difference between cPCT concentration in plasma samples from the same groups of 18 and 19 dogs respectively: 99.6 pg/mL median for septic peritonitis plasma PCT concentration vs 93.5 pg/mL for the dogs with non-septic ascites. Therefore, the low PCT concentration in ascites fluid is likely a true reading; although low PCT concentration secreted into ascites is another possible explanation, along with inability of the BV ELISA to measure PCT concentration in this type of abdominal fluid for example due to interference by other proteins. More study is needed to discover the capacity of this assay and any others, to detect and measure PCT concentrations in non-plasma samples of biological fluids in dogs.

Another study, published only in abstract form using an independently manufactured and validated ELISA, concluded that cPCT is not a useful marker of urinary tract infection in dogs (Dunning et al., 2017). Canine cPCT was increased in urine from dogs with Enterococcus compared with a mixed bacterial load (Dunning et al., 2017). This is in contrast to hPCT which is useful for discriminating urinary tract infection from sterile disesae (Levine et al., 2018). Therefore, cPCT may not be a useful biomarker in urine; but assay limitations cannot be ruled out.

1.8 Conclusions

This introduction has demonstrated the clear need to improve outcomes for dogs with sepsis. Progress in human sepsis provides some direction for canine sepsis research, and the current research focus is therefore towards a biomarker – procalcitonin – which has the potential to enable rapid diagnosis and guide management in canine sepsis. Based on types of reliable PCT assays in human medicine, this project will aim to produce an immunoassay employing Mabs that have been fully validated via mass spectrometry.

Veterinary medicine has a great need for new and improved sepsis biomarkers, but researchers must be knowledgeable and transparent about the limitations of assays used; and publish negative results when relevant. This helps others avoid costly research into non-viable biomarkers. To date, PCT appears to have some similar properties in dogs to those reported in humans: rapid changes reflecting disease course, and good indication of survival and disease severity – assuming that the results reflect PCT and only PCT, which is yet to be confirmed. In contrast to human PCT, canine PCT sensitivity to distinguish infection or sepsis from the non-infectious counterpart appears poor.

While not the ultimate sepsis biomarker that the veterinary industry is hoping for, PCT does show early promise in limited aspects of sepsis diagnosis and management – including challenging areas such as prognosis and antimicrobial stewardship. Further research employing validated assays will continue to define PCT uses and limitations in canine sepsis diagnosis and management. In addition, focus on a monoclonal that is confirmed to detect native PCT specifically, is of primary focus.

1.9 Aims of the project

Sepsis has a significant clinical impact on dogs and the impact is greater when the disease is more severe or goes longer untreated. Rapid biomarkers specific for sepsis are not available for dogs, but PCT is used for this purpose in humans. There is a need for a Mab confirmed to detect native cPCT, for use in an ELISA.

1.9.1 Overall aim

The overall aim of this project is to develop a rapid laboratory test for improved diagnosis of sepsis in dogs.

1.9.2 Specific Aims

1. Critically review canine sepsis diagnosis, and the existing assays that measure canine procalcitonin and their performance (Chapter One)
2. Investigate the epidemiology of naturally occurring sepsis in a large canine population over three months (Chapter Two)
3. Investigate and develop a rapid laboratory assay for canine PCT, in the form of an ELISA using a monoclonal antibody detecting native canine procalcitonin (cnPCT) (Chapter Three and Chapter Four)

1.9.3 Hypotheses

The overall and specific aims of this thesis are based on some fundamental hypotheses:

1. Procalcitonin is a useful biomarker for diagnosis, management, and prognosis of sepsis in dogs.
2. A monoclonal antibody raised to the native form of PCT will detect native PCT in dog plasma samples with greater affinity than a polyclonal antibody raised to recombinant PCT.
3. Anti-canine PCT monoclonal antibodies can be produced using native canine PCT obtained from thyroid tissue.

1.9.4 Objectives

Chapter One will perform a critical review of the literature surrounding diagnosis of sepsis, biomarkers including C-reactive protein (CRP) and PCT, and then focus on the current research on existing cPCT assays including studies published during the project duration.

Chapter Two will perform a three-month, prospective observational study of the descriptive epidemiology of sepsis in hospitalised dogs, including those with sepsis, infection, and non-infectious systemic inflammatory response syndrome (SIRS).

Chapter Three will isolate native cPCT from canine thyroid tissue using typical extraction and chromatographic methods. Further objectives in this chapter include to evaluate and comprae various methods to detect and identify cPCT, and to develop a quantitative assay for native cPCT using mass spectrometry (MS).

Chapter Four will use native cPCT (from Chapter Three) to immunize mice to produce hybridomas and develop monoclonal antibodies, which will then be affinity-tested using a variety of methods including immunoprecipitation and MS.

The objective of Chapter Five is to integrate the findings from all previous chapters, to clearly indicate future directions and highlight new contributions to canine PCT research.

1.9.5 Structure of the thesis

Chapters reflect a publication format and thus each addresses a specific category of work within this project with a self-contained introduction, methods, results, and discussion of any subsidiary aims or hypotheses introduced in that chapter. Figure 1.1 shows the overall organization of chapters and their content.

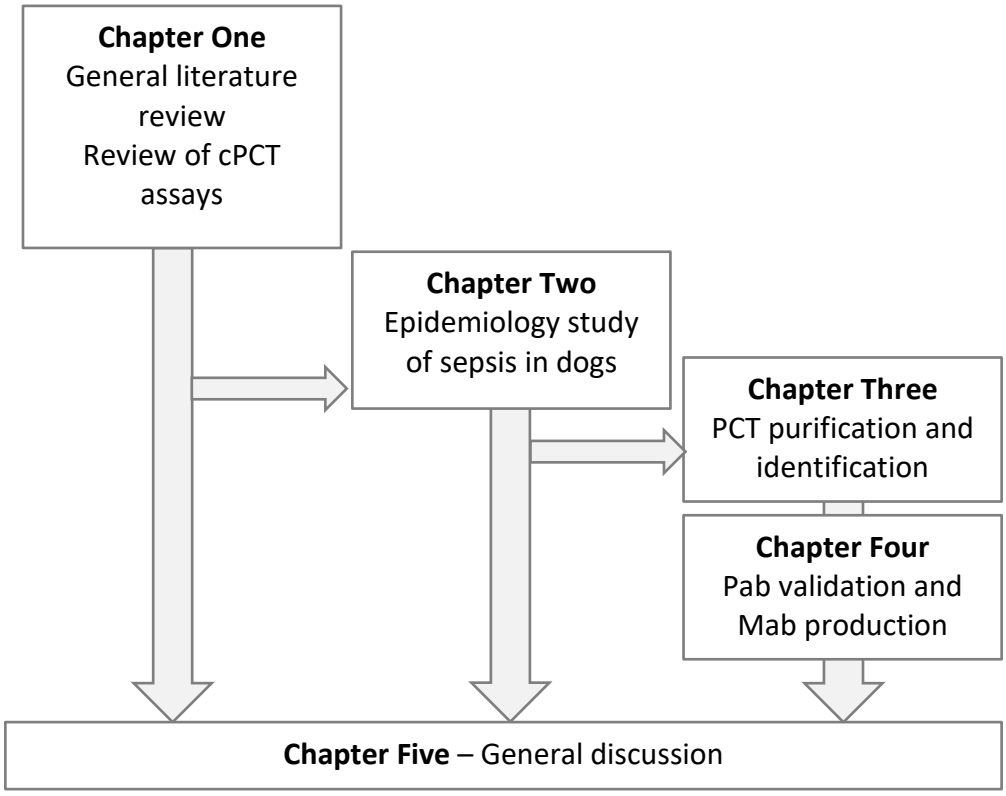


Figure 1.1. Layout of thesis chapters. One review chapter, an epidemiological study, and two experimental chapters related to monoclonal antibody development, are integrated in a final discussion.

2 Chapter Two

Epidemiology of sepsis in dogs admitted to a veterinary teaching hospital

2.1 Acknowledgement

Dr Claire Sharp is a co-author of this chapter's contents. CS contributed to design of the study, investigator shifts, data analysis, and editing the manuscript.

2.2 Abstract

This chapter describes the epidemiology of sepsis (the systemic inflammatory response syndrome (SIRS) with infection), in a prospective observational study of 486 owned dogs admitted to an urban veterinary teaching hospital over a three-month period. In total, 37 dogs (7.3%) had sepsis, 226 had non-infectious SIRS, and 26 had infection. Sepsis prevalence at admission was 5.5%, and 10 cases developed after admission (incidence 2.2%). Sepsis was most commonly bacterial (89.5%; *Escherichia coli* in 19% of cases). The most common sepsis sources were abdominal including peritonitis (13, 35.1%), soft tissue infection including abscesses (8, 21.6%), and pneumonia (6, 16.2%). Dogs with sepsis were two and a half times more likely to have a comorbidity such as neoplasia or diabetes mellitus (odds ratio 2.5, 95% CI: 1.4 to 4.7). Eleven septic dogs developed organ dysfunction, and four had multiple organ dysfunction syndrome with up to five organ systems affected. Mortality was highest in the sepsis group at 35.1% (p=0.0008, χ^2 test; overall mortality 15.4%), and sepsis mortality was higher in dogs with organ dysfunction (p=0.013; odds ratio 7.4, 95% CI: 1.5 – 35.3). Most

sepsis deaths occurred in the first 4 days after diagnosis. Sepsis cases incurred the highest median cost of care (AUD$4,778) and shared the highest median length of stay with the infection group (3 days). In conclusion, sepsis has a significant impact on dogs and early recognition of sepsis is vital for positive outcomes. Large, multi-center, prospective studies will be valuable to further explore sepsis outcomes and the impact of various diagnostics.

2.3 Introduction

The full impact of all-cause sepsis on canine populations is not well characterized due to limited studies. A single study published only in abstract form described some epidemiology of all-cause sepsis in dogs (Guenther-Yenke et al., 2007). This census-based study included 993 dogs. Those with sepsis had a longer ICU stay, longer hospital stay, and higher cost of care than dogs without sepsis. Mortality of dogs with sepsis was 55%. The remainder of veterinary sepsis literature consists of various-sized, prospective or retrospective case series or cohort studies focusing on individual manifestations of naturally-occurring sepsis such as septic peritonitis (Bush, 2016; Dayer et al., 2013; Dickinson, 2015; Kenney et al., 2010; Li, 2016; Liu et al., 2012; Turk et al., 1990), pyometra (Jitpean et al., 2017), pneumonia (Kogan et al., 2008), or individual pathogens (Greiner et al., 2008). Others investigate specific biomarkers in a limited cohort (Cortellini, 2015).

Sepsis epidemiology has been studied in some detail in humans and cats – two species comparable to dogs (Greiner et al., 2008; Otto, 2007; Paoloni & Khanna, 2008). These studies show sepsis has a high clinical impact. A single-centre study based at a university

teaching hospital in the United States described the epidemiology of all-cause sepsis in hospitalized cats over a 3-month period. That study reported a prevalence of community-acquired sepsis of 6.2 cases per 100 admissions, an incidence of hospital-acquired sepsis of 1.5 cases per 100 admissions, and a mortality rate of 33% (Babyak & Sharp, 2016). In humans, sepsis is estimated to affect up to 31.5 million humans worldwide annually (Fleischmann et al., 2016), and is one of the most frequent and costly causes of mortality and significant morbidity in hospitalized patients (Angus et al., 2001; Martin et al., 2003). Sepsis in humans is a common complication of pneumonia, blood stream infection, or abdominal infection, and is more likely to occur in those with pre-existing disease or immunocompromise, including the elderly and neonates or those on ventilators (Fleischmann-Struzek et al., 2018; Fleischmann et al., 2016; Martin, 2012). Rapid diagnosis and close monitoring enable early appropriate treatment and prognostication, and this approach alone achieves significantly better outcomes including reduced mortality of sepsis in humans (Eubank et al., 2020; Levy et al., 2010; Seymour et al., 2017). Where these have been studied in dogs, they are often similar. Due to many similarities in sepsis between dogs and humans, it is reasonable to predict that outcomes for dogs would similarly improve if diagnostic methods enabled more rapid diagnosis and informed management of sepsis.

A thorough epidemiologic understanding of sepsis facilitates resource allocation and guides interventional studies aimed at improving outcome by effective treatment of sepsis. It also establishes a clear baseline of the frequency of sepsis diagnosis, allowing changes to be observed if a new diagnostic method is used including novel biomarkers and clinical scoring systems such as the sequential organ failure (SOFA) score (Vincent et al., 1996).

The aim of this chapter was to investigate the epidemiology of naturally occurring sepsis in a large canine population by observing a large cohort of hospitalized dogs with sepsis, systemic inflammatory response syndrome (SIRS), and infection. It was hypothesized that the prevalence and incidence of sepsis in dogs presenting to a veterinary teaching hospital would be similar to that previously reported in cats and humans: that dogs with sepsis would have higher mortality, longer length of stay, and higher cost of care than those without sepsis; and that the impact of canine sepsis is significant, for example as reported in one canine population (Guenther-Yenke et al. 2007) sepsis affects 5% of dogs at some point during their hospitalization, and is associated with high costs (mean >$10,000 per admission) and high mortality (>30%).

2.4 Methods

2.4.1 Data collection

This was a prospective, observational, single-center study performed at a university teaching hospital that sees primary accessions, as well as secondary and tertiary referral cases. All dogs admitted for any reason from 1st May 2019 to 31st July 2019 were eligible for inclusion in the study. A three month period provided a sufficient sample size to achieve adequate statistical power, while being achievable within the larger project timeframe. Ethics approval was waived by the institutional animal ethics committee due to the strict observational census design (with no patient or client interaction for the sole purpose of this study, and no identification of individuals outside the study databank). Two investigators

(veterinarians) recorded all data for this study and conferred regularly to ensure consistent recording. Cases were managed by clinicians independently to this study. Daily at 7 a.m., an investigator performed a census of all dogs that were inpatients at that timepoint. Investigators physically identified dogs in cages or runs, then extracted their clinical data for the preceding 24-hour period from their paper medical records (daily treatment sheets) and electronic medical records, to satisfy a standardized data collection sheet designed for this study. Data extracted from these sources included signalment, date of admission, diagnoses, clinical exam results, and diagnostic test results. At the end of each patient's hospitalization investigators recorded outcome of hospitalization (natural death, euthanasia, or survived to discharge), and cost of care. Breed was recorded as mixed breed for any dog that was not clearly a purebred dog; for example, "Kelpie cross" was listed as mixed breed dog.

Investigators recorded 30-day outcome where possible (status of a patient 30 days post discharge: still alive, died naturally, or euthanized since discharge). To obtain this information, investigators searched for recheck records at the study institution, then if necessary, contacted the nominated regular veterinarian, and finally if necessary contacted the owner via their nominated email address with a one-off standard veterinary follow-up query.

All recorded data were entered into Research Electronic Data Capture (REDCap), and the following calculations were performed within REDCap (Harris et al., 2009). Age was calculated by the difference between date of birth and date of hospital admission to the

nearest 0.1 year. Length of stay was the number of days that the dog was in hospital, where anything less than 24 hours was recorded as zero days.

Dogs admitted to the hospital were excluded retrospectively only if they had insufficient signalment information (e.g. stray), insufficient clinical data to determine the presence of SIRS, were medical boarding, or if their hospitalization was short enough that they were not an admitted patient present at 7 a.m. on any day of the study. Cost of care at discharge was excluded for one surrendered animal since costs are billed differently in such circumstances. Dogs who were euthanized under financial considerations were included in the study because this is a true reflection of disease impact for owned dogs.

2.4.2 Definitions and patient categorization

In this study the term 'abdominal' is used to group anatomical sites including abdominal viscera and peritoneal cavity. The term 'soft tissue' groups skin, subcutaneous tissue and muscle. Systemic inflammatory response syndrome was defined using the criteria from Hauptman et al. (1997), outlined in Table 2.1. Briefly, SIRS was diagnosed if a dog had the tabulated abnormal results for any two of heart rate, respiratory rate, temperature, or leukogram, in a single clinical exam (CE). Infection was diagnosed based on laboratory testing or high clinical suspicion, such as malodorous or purulent wounds outlined in Table 2.1 (Cutting & White, 2004; Finfer et al., 2004; Sharp, 2015; Singer et al., 2016). Sepsis was diagnosed when SIRS was present concurrently with strongly suspected or confirmed infection, equivalent to sepsis-2 in human medicine (Bone et al., 1992; Sharp, 2015; Silverstein, 2015; Singer et al., 2016). Cases of sepsis diagnosed within 48 hours of admission

were considered community-acquired. Cases of sepsis first diagnosed from 48 hours after admission without prior proof of incubation were classified as hospital-acquired (Haque et al., 2018).

Table 2.1: Classification criteria of the systemic inflammatory response syndrome (SIRS) according to the criteria by Hauptman et al. (1997); and infection criteria.

Condition	Criteria for clinical diagnosis
SIRS	Abnormalities in any two of the following four criteria (Hauptman et al 1997): • Respiratory rate (>20 breaths/minute), • Heart rate (>120 beats/minute), • Temperature (<38°C or >39°C), or • Change in the leukon (<6,000 or >16,000 WBCs/µL; or >3% band neutrophils)
Infection - confirmed	• Cytology demonstrating intracellular bacteria from a normally sterile site • Culture of a pathogenic organism from normally sterile site • Histology demonstrating intracellular bacteria from a normally sterile site • Imaging consistent with infectious causes of pneumonia • Serology, PCR, or other diagnostic modality confirming presence of a pathogen in affected site, consistent with presenting disease
Infection – strong clinical suspicion (Finfer et al 2004; Cutting et al 2004)	• Increasing pain, friable granulation tissue, or development of a foul odour associated with a wound • Wound breakdown in a chronic or non-healing wound • Purulent exudate in a normally sterile site

Four disease categories were defined for the study, based on the above definitions: *sepsis* if SIRS and infection were both present in the same 24 hour period; *nSIRS* (non-infectious SIRS) if at least two SIRS criteria were met, but no infection; *infection*, if infection was

confirmed or strongly suspected but there were less than two SIRS criteria met; and *control*, if none of sepsis, SIRS, nor infection were present at any time during that 24 hour period. The control group (a default group) included all other diseases or lesions, and healthy dogs undergoing elective procedures.

Every admitted patient received a daily categorization throughout its admission (on each day that it was present at the 7 a.m. timepoint), based on these definitions, to enable tracking of daily changes and incidence of disease during admission. Dogs would typically have a minimum of two clinical exams per day, or more depending on their medical needs. Therefore, investigators screened clinical exam data for the full 24 hour period and prioritized the first complete exam performed during the relevant time period. This excluded CEs from the pre-operative exam in a healthy patient admitted for elective surgery because it did not represent the surgical-induced trauma that the patient was hospitalized for. In addition to the daily categorization, dogs were also placed into a single overall category (sepsis, nSIRS, infection, control) for analysis of hospitalization outcomes such as total cost. In a small percentage of cases more than one of the above disease categories was displayed during this time (e.g. SIRS at the time of surgery, then developed an infection without SIRS several days later). Dogs with sepsis at any time during their hospitalization were assigned to the sepsis group, regardless of any other diagnoses; dogs with infection and SIRS at different times were assigned into the infection group because of sample size (very few infection patients and many SIRS patients); and dogs with none of these conditions were categorized as control.

Organ dysfunction was diagnosed based on published criteria for dogs with septic peritonitis (Kenney et al., 2010) (Kenney et al 2010) (Table 2.2) based on abnormal clinical findings, or abnormal biomarker values compared with defined reference intervals. Multiple organ dysfunction syndrome (MODS) was diagnosed when more than one organ system was in dysfunction simultaneously. Records were made of any clinically significant comorbidities (pre-existing or concurrent medical conditions that may affect outcome). Comorbidities considered clinically significant included endocrine disease (e.g. diabetes mellitus), neoplasia, kidney or liver disease that were clinically manifest or receiving treatment, or immunosuppression. Risk factors noted included age younger than four months, unvaccinated status, or an indwelling medical device.

Table 2.2: Criteria and cut-off values for identification of organ dysfunction in dogs during this study, based on the criteria detailed by Kenney and colleagues (2010). Multiple organ dysfunction syndrome (MODS) was defined as dysfunction in 2 or more organs. (PT: prothrombin time. PTT: partial thromboplastin time.)

Organ	Criteria for dysfunction
Renal	Creatinine concentration ≥176.21 µmol/L (≥ 44.21 µmol/L from the preoperative or baseline value; or maximum normal reference range, if no baseline was available) • with no evidence of prerenal azotemia - excluded if clinical evidence of adequate hydration (including adequate IV fluids) • and no evidence of postrenal azotemia - excluded if no evidence of urinary obstruction.
Cardiovascular	Hypotension sufficiently severe to require vasopressor treatment
Respiratory	Supplemental oxygen administration or mechanical ventilation was required. • either arterial blood gas analysis (ie, alveolar-arterial gradient in partial pressure of oxygen > 10 mmHg) • or results of pulse oximetry (SpO_2< 95%) • or if results of clinical assessment indicated a need for oxygen supplementation

Hepatic	Highest measured value for total bilirubin concentration was > 8.55 µmol/L
Coagulation	PT or PTT was > 25% above the upper reference limit Or platelet count was ≤ 100,000/µL

2.4.3 Data management and statistical analysis

Study data were managed and stored using REDCap: a secure, web-based software platform (Harris et al., 2019; Harris et al., 2009). Preliminary validity checking was performed by confirming details (including diagnoses) of 10 randomly selected REDCap records, and showed 100% consistency. A dog's overall hospitalization category was the dependent variable for the majority of tests; its daily census category created a frequency for number of days the dog was affected by that disease, which was the dependent variable in analyses describing disease over time. Prevalence was defined as the proportion of dogs in a given disease category at the first timepoint of their hospitalization. Incidence was defined as the proportion of new cases diagnosed after admission, i.e. from the second timepoint onwards. Continuous independent variables were checked for normality by D'Agostino & Pearson normality tests; those with non-normal distribution included age, length of stay, and cost of care between hospitalization groups; here, the Kruskal-Wallis test was used with Dunn's multiple comparisons test. To compare frequencies between hospitalization disease categories (groups), including days with a specific disease or number of organs/pathogens/diagnoses, the χ^2 test (if >2 groups) or Fisher's exact test (two groups) were used. Survival was examined through Mann-Whitney U tests and Mantel-Cox analysis. Relationship between cost and length of hospital stay was analyzed by correlation and

weighted linear regression analyses. All statistical analyses were performed with commercially available software (GraphPad Prism). A p value of < 0.05 was considered statistically significant. Relationships (interactions) between multiple independent variables, including multiple admissions of the same patient, were not examined since the study was not designed for these analyses; models were not robust due to low sample size in some groups; and additional hospitalizations outside of the study institution (which were common due to referrals and overnight care discharge to the regular vet clinic) were not recorded.

2.5 Results

During the three-month study period a total of 510 unique dog admissions were observed. Twenty-four of these were excluded using the criteria outlined in methods above. The remaining 486 admissions were for 467 unique dogs, 34 of which were admitted twice and three were admitted thrice. The cumulative total number of days of hospitalization was 1,078 days. After categorization, the study included 37 dogs with sepsis, 226 with nSIRS, 26 with infection, and 197 control patients with neither SIRS nor infection.

Signalment for all groups, as well as the study population overall, are summarized in Table 2.3. Body weight and age distributions are shown in Figure 2.1. Unexpectedly, the nSIRS group had a significantly lower body weight (14kg) than the sepsis group (26kg; p=0.0226) and the infection group (30.55kg; p=0.0023). The majority of dogs were neutered males (42%), followed by spayed females (33.5%), intact males (14.2%) and intact females (10.3%). Mixed breed dogs were most common (150 dogs, 30.9%), with a total of 72 breeds

represented. There were no significant differences in age or sex among groups (Table 2.3 and Figure 2.1). In total, 117 dogs (24.1%) underwent surgery within the first 24 hours of their hospitalization.

Table 2.3: Signalment and outcome results for 486 admissions of dogs to a university teaching hospital over a three-month period, that were categorized as having either sepsis, non-infectious systemic inflammatory response syndrome (nSIRS), infection, or control (neither SIRS, nor infection). Where not otherwise indicated, counts are listed as number (%). Age, length of stay, and cost of care are listed as median (range). Length of stay includes values of 0 days, if the admission was <24 hours duration.

Variables	Sepsis	nSIRS	Infection	Control	All dogs
Number in group	37 (7.6%)	226 (46.5%)	26 (5.3%)	197 (40.5%)	486 (100%)
Age in years (range)	6.0 (0.2 – 13)	5.9 (0.1 – 16.2)	7.7 (0.7 – 12.6)	6.3 (0.2 – 18.7)	6.1 (0.1 – 18.7)
Sex (%)					
Male intact	6	37	2	24	69 (14%)
Male neutered	14	87	12	92	205 (42%)
Female speyed	9	79	12	63	163 (34%)
Female intact	8	23	0	19	50 (10%)
Body weight in kg (range)	26 (1.7 – 75)	14 (1.1 – 73.1)	30.55 (4.5 – 90)	16.85 (2.2 – 84)	16.4 (1.1 – 90)
Initial outcome					
Survived to discharge	25 (67.6%)	205 (90.7%)	25 (96.1%)	190 (96.5%)	445 (91.6%)
Died naturally	1 (2.7%)	3 (1.3%)	0	1 (0.5%)	5 (0.8%)
Euthanized	11 (29.7%)	18 (8.0%)	1 (3.9%)	6 (3.0%)	36 (7.4%)
All-cause mortality	12 (32.4%)	21 (9.3%)	1 (3.9%)	7 (3.6%)	40 (8.2%)
30-day outcome	total 25	total 205	total 25	total 190	total 445
Still alive	21 (84.0%)	129 (62.9%)	21 (84.0%)	129 (67.9%)	300 (67.4%)
Died since discharge	0	5 (2.4%)	0	1 (0.5%)	6 (1.3%)
Euthanized since discharge	1 (4.0%)	16 (7.9%)	3 (12.0%)	8 (4.2%)	28 (6.3%)
Lost to follow-up	3 (12.0%)	55 (26.8%)	1 (4.0%)	52 (27.4%)	111 (24.9%)
Overall mortality	13 (35.1%)	42 (18.6%)	4 (15.4%)	16 (18.1%)	75 (15.4%)
Comorbidity or infection risk	10 (27.0%)	33 (14.6%)	7 (26.9%)	21 (10.7%)	71 (14.6%)
Length of stay in days (range)	3 (1 – 34)	1 (0 – 16)	3 (1 – 15)	1 (0 – 9)	1 (0 – 34)
Cost of care in AUD (range)	4,778 (1,026 – 22,360)	3,004 (257-26,711)	4,050 (562-14,080)	2,757 (257-9,522)	3,084 (257 – 26,711)

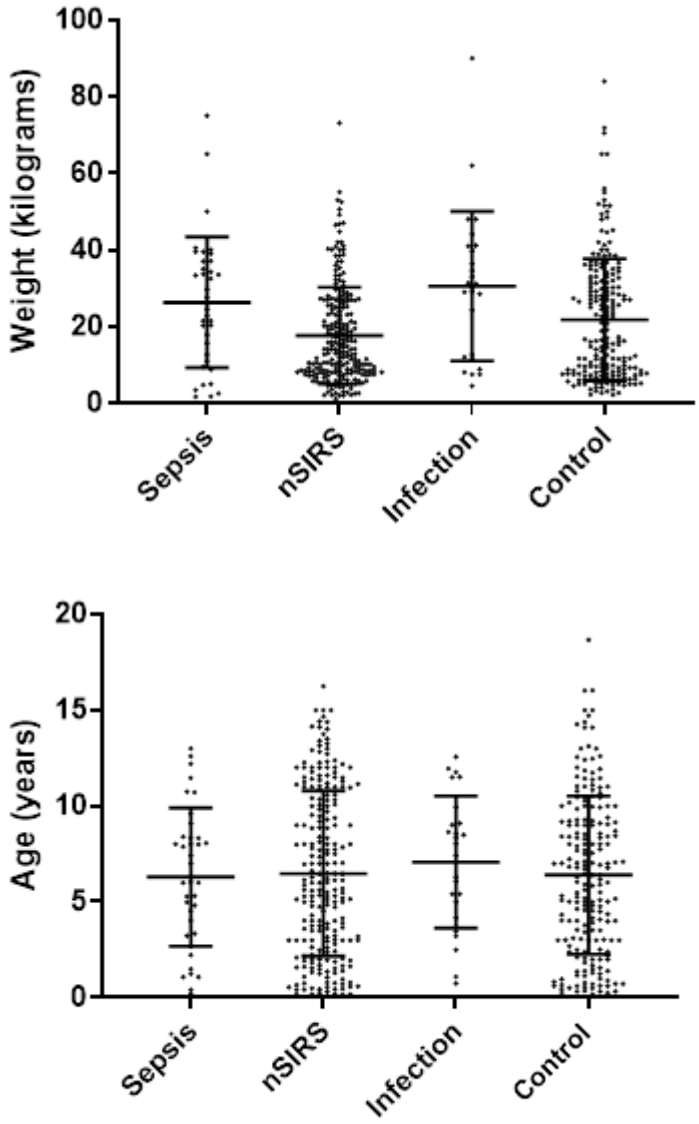


Figure 2.1: Body weight (top) and age (bottom) distribution of dogs from 486 admissions to a veterinary hospital during a three month period. Most groups (categorised by disease) are not normally distributed. Analysis: Kruskall-Wallis test, bars show mean ± SD. nSIRS group had a significantly lower body weight (14kg) than the sepsis group (26kg; p=0.0226) and the infection group (30.55kg; p=0.0023). No significant difference in age between groups.

2.5.1 Sepsis

Sepsis occurred in 37 of 486 dog admissions (7.3%). Of these, 27 were diagnosed at the first timepoint (prevalence 5.5 cases/100 admissions) and 10 patients were diagnosed with new cases of sepsis from 24 hours after admission (2.2% incidence, or incidence rate of 1.0 case per 100 dog-days). Seven of these (2.1%) were hospital-acquired cases and therefore possible nosocomial infections. Infection was diagnosed most frequently based on microbial culture (n=19, 51.3%), followed by cytology (14, 37.8%), diagnostic imaging (8, 21.6%), histopathology (2, 5.4%), serology (1, 2.7%), and PCR (1, 2.7%). In eight cases an infection was evident on more than one diagnostic modality. Six dogs (16%) were classified as having sepsis when their infection was diagnosed based on strong clinical evidence alone (e.g. malodorous and purulent discharge from a wound or abdominocentesis; SIRS, neutropenia and cardiovascular compromise together with a suspected infection) as described in the methods.

Infection was most frequently bacterial (89.5%), including hemolytic and non-hemolytic *Escherichia coli* (n=7, 18.9%), two each of *Enterococcus* and *Staphylococcus spp.*, (5.4%) one each of *Streptococcus spp.*, *Proteus mirabilis*, *Enterobacter sp.*, *Serratia sp.*, *Clostridium perfringens*, and unspeciated cocci (totaling 16.2% of total infections). Three cultures isolated two organisms each. There was one case of parvoviral enteritis and one case of disseminated aspergillus. The source of infection in dogs with sepsis included abdominal sepsis (13, 35.1%), soft tissue infection (8, 21.6%), pneumonia (6, 16.2%), lower urinary tract infection (3, 8.1%), implant-associated infection (3, 8.1%), pyometra (2, 5.4%) and systemic

infection (2, 5.4%). Of those with abdominal sepsis, nine were of gastrointestinal source (24.3%), three (8.1%) were associated with abdominal surgery such as gastropexy and enterotomy, and one (2.7%) was hepatobiliary. Sepsis cases with a soft tissue nidus included three dog bite wounds, two abscesses, two infected neoplastic masses, and one surgical wound (8.1%, 5.4%, 5.4%, and 2.7%, respectively). Frequency of clinical signs, categorized by body system, are listed for disease groups in Table 2.4. Specific clinical diagnoses of the dogs with sepsis are listed in Table 2.5.

Table 2.4: The frequency of major clinical signs or body systems affected, as observed in 486 admissions of dogs to a university teaching hospital over a three-month period, for four disease cohorts (sepsis, nSIRS, infection, and control). 'Non-specific' refers to clinical signs such as lethargy or anorexia that could not be attributed to a specific body system.

Clinical sign or body system affected	Disease Cohorts				Total Count (%)
	Sepsis	nSIRS	Infection	Control	
Gastrointestinal	17	67	6	54	144 (29.6%)
Musculoskeletal	3	42	7	56	108 (22.2%)
Neurological	6	38	4	34	81 (16.7%)
Dermatological, soft tissue	13	19	8	24	65 (13.4%)
Hepatobiliary	3	22	2	14	41 (8.4%)
Haematological	3	17	2	8	30 (6.2%)
Lower urinary tract	3	6	8	12	29 (6.0%)
Upper respiratory tract	3	13	0	13	29 (6.0%)
Neoplastic	1	13	3	10	27 (5.6%)
Toxicological	0	17	1	9	27 (5.6%)
Cardiovascular	3	14	1	5	23 (4.7%)
Lower respiratory tract	7	12	0	4	23 (4.7%)
Trauma	0	15	1	2	19 (3.9%)
Endocrine	1	12	2	4	19 (3.9%)
Non-specific	2	4	0	5	11 (2.3%)
Renal	1	5	0	3	9 (1.9%)
Immune-mediated, inflammatory	0	7	0	2	9 (1.9%)
Ophthalmological	0	1	0	4	5 (1.0%)
Reproductive	2	0	0	2	4 (0.8%)

Table 2.5: Frequency of clinical diagnoses, observed in 486 dogs admitted to a university teaching hospital over a three-month period. Some patients received multiple diagnoses. Diagnoses include presenting signs or interim diagnoses. (BOAS: brachycephalic obstructive airway syndrome. MVA: motor vehicle accident. TPLO: tibial plateau leveling osteotomy.)

Clinical sign or body system affected	Disease Cohorts				Total
	Sepsis	nSIRS	Infection	Control	
Wound, infected (surgical, fasciitis, bite)	10	6	4	3	23
Abscess	5				5
Septic peritonitis	5				5
Urinary tract infection	4		11		15
Pneumonia (including aspiration and submersion injury) and pleuritis	5			1	6
Paresis, paraparesis, paralysis	3	4	1	3	11
Gastroenteritis (including haemorrhagic, ulcerative, and parvoviral)	4	4	1	3	12
Surgery, soft tissue	2	22	4	31	59
Gastrointestinal foreign body (perforating or simple obstruction)	2	7		5	14
Immune-mediated haematologic diseases	2	2			4
Liver disease including hepatitis	2	1		3	6
Diabetes mellitus or diabetic ketoacidosis	2			2	4
Pyometra	2				2
Diarrhea, hematochezia, melena	1	18	3	18	40
Implant infection	1		3		4
Pancreatitis	1	13	1	3	18
Splenic mass, uncharacterized	1	5			6
Addison's, Cushings, or hypothyroidism	1	3	1	1	6
Surgery, BOAS	1	3		8	12
Neoplasia		14	2	10	26
Arthritis, septic			2		2
Toxicity, any		19	1	10	30
Vomiting, emesis		16	1	12	29
Trauma including polytrauma eg MVA		15	1	3	19
Heart disease or failure		9	1	1	11
Seizure		7	1	12	20
Spinal disease, intervertebral disk disease		10	2	8	20
Hemorrhage (any source or severity)		2	1	3	6
Constipation, obstipation, sand ingestion		1	1	3	5

Pain, primary disease or unknown origin	11	7	18
Surgery, TPLO	10	20	30
Fracture	6	4	10
Surgery, orthopedic (other than TPLO)	6	9	15
Anaphylaxis or bee sting	4	2	6
Elbow disease	3	7	10
Dyspnoea	3		3
Pyrexia of unknown origin	3	2	5

Of the 37 dogs with sepsis, 11 had dysfunction in at least one organ system. Four had MODS. Organ dysfunctions included hepatic (8, 21.6%), coagulopathy (4, 10.8%), renal (3, 8.1%), respiratory (3, 8.1%), and cardiovascular (3, 8.1%) (Figure 2.2).

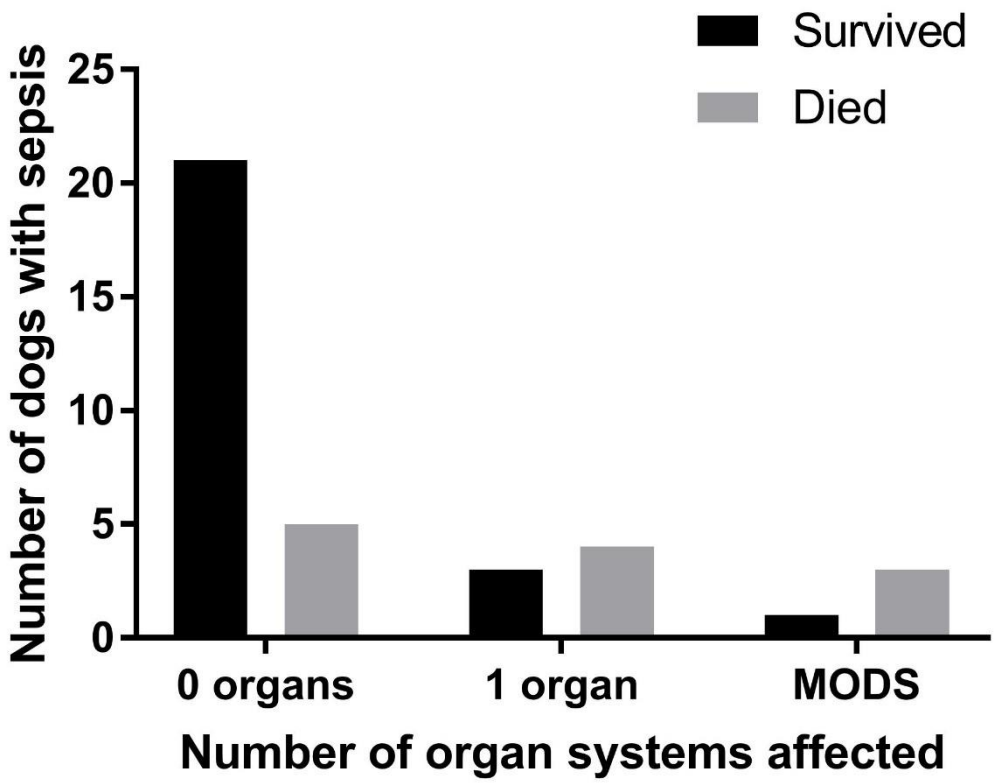


Figure 2.2: Dogs with sepsis but no organ dysfunction have lower mortality (19.2%) than those with a single organ in dysfunction (57%, p=0.0023) or multiple organ dysfunction syndrome (MODS; 75%, p<0.0001), during hospitalization in a university teaching hospital.

2.5.2 Non-infectious SIRS (nSIRS)

Many hospitalized dogs in the study were classified has having nSIRS (226 of 486, 46.5%). Of these dogs, 208 presented with or developed nSIRS by their first timepoint, giving a prevalence of 42.8 cases/100 admissions. A further 58 dogs developed nSIRS later bringing the incidence of nSIRS to 20.9% or an incidence rate of 6.7 per 100 dog-days. Development of nSIRS in the 24-hour post-operative period was common, accounting for 59 of 302 SIRS-days (19.5%). In these cases SIRS was commonly characterized by a hypothermia and tachypnoea associated with recovery from surgery under general anesthesia, despite adequate warming and analgesia.

The primary pathology in dogs with nSIRS was most often the gastrointestinal system, for example gastroenteritis (n=67, 29.6%). This was followed by musculoskeletal (42, 18.6%), neurological including seizure, neurotoxicities, and spinal disease (38, 16.8%), hepatobiliary diseases including non-infectious pancreatitis (22, 9.7%) and dermatological including non-infectious soft tissue injury such as trauma or surgical wounds (19, 8.4%). The most common specific clinical diagnoses are listed in Table 2.5.

2.5.3 Infection

Infection without SIRS accounted for the smallest number of patients in the study (26/486, 5.3%). Six dogs presented with infection or were diagnosed within 24 hours of admission, while a further 20 developed infection later during hospitalization. Therefore, infection prevalence was 1.2 per 100 admissions, and incidence was 4.2% with an incidence rate of

1.9 cases per 100 dog-days. The most frequent nidus of uncomplicated infection was the lower urinary tract (n=12, 46.2%), followed by skin and soft tissue (7, 26.9%). Less common sites of uncomplicated infection included surgical implants (3, 11.5%), the gastrointestinal tract (2, 7.7%), septic arthritis (1, 3.8%), and hepatobiliary infection (1, 3.8%). Infection was confirmed based on culture (n=18, 69.2%) and cytology (6, 23.1%) in most cases. Isolates cultured included *E. coli* (n=9, 34.6%), *Staphylococcus* spp. (5, 19.2%), *Streptococcus* spp. (4, 15.4%), *Proteus mirabilis* (2, 7.7%), *Pseudomonas sp.* (1, 3.8%), and *Enterococcus sp.* (1, 3.8%). Four cultures identified multiple isolates. Two dogs were classified as having infection based on strong clinical evidence alone. These included one parasitic infection with intraperitoneal tapeworm metacestodes identified during exploratory celiotomy; and another with purulent, malodorous wound discharge.

2.5.4 Co-morbid conditions and risk profiles

Of the 486 admissions, 71 (14.6%) were considered to have a comorbidity, or pre-existing disease that may predispose to sepsis developing or worsening. The presence of a comorbidity was two and a half times as common in the sepsis and infection groups collectively compared to the nSIRS and control groups (odds ratio [OR] 2.5; 95% CI: 1.4 to 4.7). The most common co-morbid conditions were neoplasia (n=28, 5.7%), diabetes mellitus (8, 1.6%), and liver disease such as portosystemic shunt or cirrhosis (10, 2.0%). The comorbidity or infection risk was often clinically relevant to the source of infection, for example a patient with a pacemaker developed an implant-associated infection, and a puppy less than 10 weeks of age contracted parvoviral gastroenteritis. Regarding the total

study population, dogs that did not survive to discharge were significantly older than dogs that survived to discharge (p=0.0042).

2.5.5 Outcomes of hospitalization

The outcomes for each disease group are summarized in Table 2.3. A total of 445 out of 486 admissions (91.6%) resulted in survival to discharge, while 36 (7.4 %) resulted in euthanasia, and four (0.8%) ended with a natural death (see Table 2.3). As such, overall all-cause mortality in this population of dogs during hospitalization was 8.2%. Of 24 cases where the reason for euthanasia was stated, only two were for financial reasons in the face of poor prognosis. By 30 days after admission a further 34 dogs had been euthanized (n=28, 6.3%) or died (6, 1.3%), but a total of 24.9% (111) were lost to follow-up. Dogs with sepsis had significantly higher overall mortality than dogs in other cohorts: 35.1% (p=0.0008) compared to 18.6% nSIRS, 15.4% infection, and 18.1% control (Figure 2.3). However, although their initial mortality was significantly higher (32.4% for sepsis, compared with nSIRS 9.3%, infection 3.9%, and control 3.6%; p<0.0001), there was no difference between sepsis and other groups at 30 days after discharge (see Table 2.3).

During hospitalization, septic cohort mortality was significantly higher in the 11 dogs with any organ dysfunction than without (p=0.013; OR 7.4, 95% CI: 1.5 – 35.3). Overall, 29% of septic dogs had organ failure, and their collective mortality rate was 63%. Dogs with sepsis but no organ dysfunction had lower mortality (19.2%) than those with a single organ in dysfunction (57%, p=0.0023) or MODS (75%, p<0.0001). Sepsis mortality rates did not differ significantly between community-acquired and hospital-acquired sepsis. Of the dogs with

sepsis, 27% died within one day of their diagnosis, and a further 10% had died by the fourth day since their diagnosis (see Figure 2.3). In other words, of those dogs who did not survive sepsis, 72% of them had died within the first 24 hours.

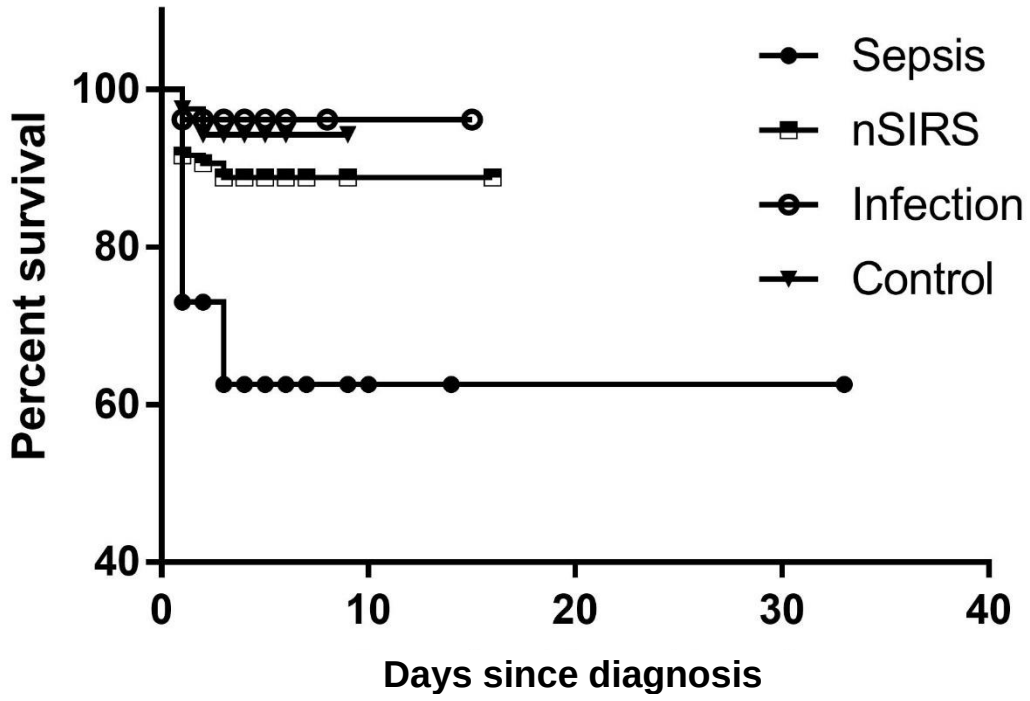


Figure 2.3: Survival of dogs during 486 admissions to a veterinary teaching hospital. Survival is counted from the day of onset of the disease (sepsis, non-inflammatory SIRS, or infection), to the time of death (including euthanasia) or discharge. For the control group, the onset of disease was counted from admission to hospital, and is included for rough comparison, since diagnosis did not always occur on the day of admission. The difference between curves is significant ($p<0.0001$, Mantel-Cox χ^2 test).

Dogs with sepsis had the longest two hospitalizations (34 days maximum – Figure 2.3). Median length of stay was 3 days for both the sepsis and infection groups. This was three times longer than the median length of stay for nSIRS or control dogs (1 day). The control group was hospitalized for a significantly shorter time compared with all other disease cohorts ($p \leq 0.0019$). Dogs in the septic cohort had the highest median cost of care (median AUD$4,778), followed by infection ($4.050), nSIRS ($3,004), and control ($2,757) (Figure 2.4). Dogs with sepsis also had the highest minimum cost of care (AUD$1,026). Cost was significantly higher in the sepsis group compared with the nSIRS group ($p=0.0078$) and the control group ($p=0.0002$). Cost of care of septic dogs was positively correlated with length of stay (0.8 r), but in a linear regression model the length of stay only accounted for maximum 40% of cost ($R^2 = 0.4$, see Figure 2.5).

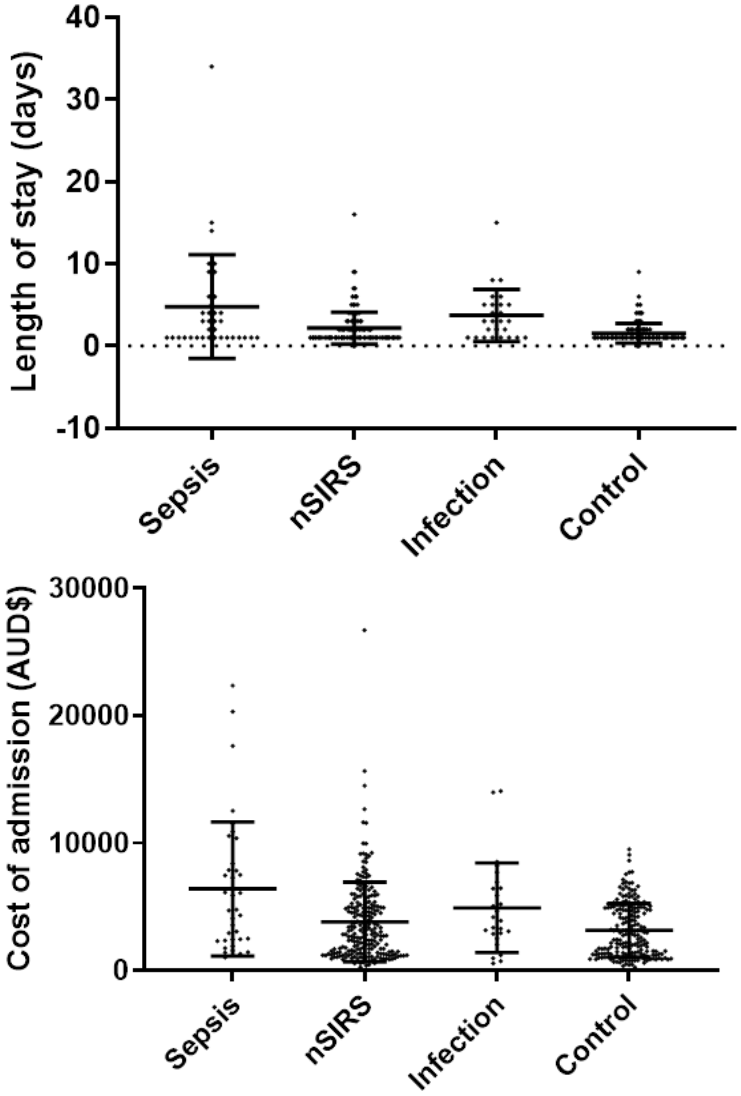


Figure 2.4: Dogs with sepsis had a higher length of stay and cost of care than other cohorts (sepsis, non-infectious SIRS (nSIRS), infection, and control) out of 486 dog hospitalizations to a veterinary teaching hospital. Length of stay (A) was significantly longer for dogs with sepsis, infection, and nSIRS, than the control group ($p<0.0001$); but nSIRS dogs also stayed significantly longer than dogs with infection ($p=0.0145$). The cost of care (B) for dogs with sepsis was significantly higher than for those with nSIRS ($p=0.0082$), and higher still compared to control dogs ($p=0.0002$). Length of stay <24 hours was rounded to 0 days.

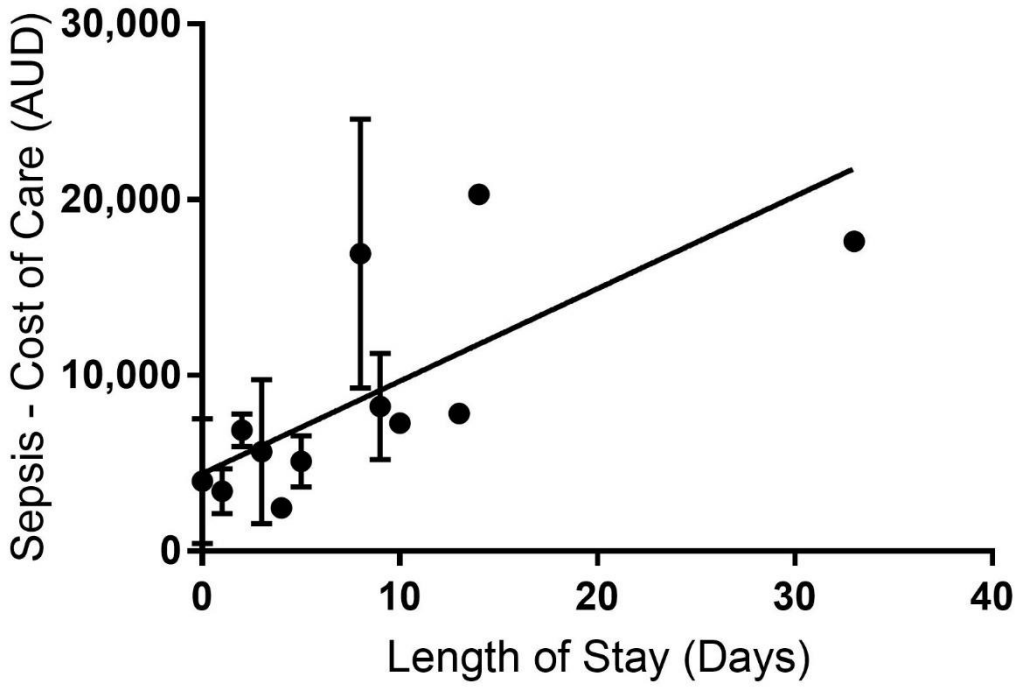


Figure 2.5: For dogs with sepsis, cost of care (COC) showed a positive correlation with length of stay in hospital (LOS) (r = 0.81). In a regression model, only 40% of the variation in COC could be predicted by the length of stay (R2 = 0.40). Bars show mean ± SD, some values do not have replicates.

2.6 Discussion

This prospective observational study achieved the aim to investigate the epidemiology of naturally occurring sepsis in a large canine population, reporting a high clinical impact of sepsis in a population of dogs admitted to a veterinary teaching hospital over three months. Consistent with our hypotheses, the prevalence of community-acquired sepsis in dogs (5.5

cases/100 admissions) was similar to that previously reported in cats (6.2 cases/100 hospital admissions), as was the incidence of hospital-acquired sepsis (2.1 dog cases/100 hospital admissions vs. 1.5 cat cases/100 hospital admissions) (Babyak & Sharp, 2016). These findings are consistent with previous epidemiologic studies of all-cause sepsis in dogs and cats (Babyak & Sharp, 2016; Guenther-Yenke et al., 2007). The results are also similar to those of a recent study that followed 42 dogs with confirmed sepsis (Camargo et al., 2020). The study population includes varied signalment, and both primary and referred cases, and therefore is likely representative of dogs presenting to veterinary hospitals in Australia.

The mortality rate of septic dogs in this study, 35.1% increasing with organ dysfunction, is comparable to the aforementioned all-cause sepsis studies in dogs and cats (Babyak & Sharp, 2016; Guenther-Yenke et al., 2007), and to those mortality rates recorded historically for humans with sepsis using the sepsis-2 paradigm, and before wide-scale improvements in sepsis outcomes through the Surviving Sepsis campaigns (Martin, 2012; Martin et al., 2003; Melville et al., 2015). Conversely, mortality is lower than some studies in dogs (Camargo et al., 2020; de Laforcade et al., 2003), which may reflect study design or SIRS criteria used, or local medical protocols. We used the Hauptman SIRS criteria (Hauptman et al., 1997). These enable maximal detection of patients with sepsis, but like other SIRS criteria can be overly sensitive due to being non-specific: elevations in heart rate, respiratory rate, or even pyrexia, can also present during stress, pain, anaesthetic recovery, and other non-inflammatory diseases. This likely led to false positives, since 46% of admissions were diagnosed with SIRS. Yet, any over-sensitivity was insufficient to mask the significantly higher mortality and other negative outcomes in the sepsis cohort compared with other

groups. Further study is needed to scrutinize and update the approach to screening dogs for sepsis using SIRS or other organ function scoring systems, since SIRS criteria vary and may not be the most appropriate (de Laforcade et al., 2003; Okano et al., 2002; Singer et al., 2016). For example, human medicine has now adopted SOFA (Vincent et al., 1996). In contrast to the over-sensitive SIRS criteria in this study, false negatives for sepsis could also have occurred if microbial cultures were unsuccessful or declined (Hall et al., 2011), or if clinical signs were not sufficient to support strong clinical suspicion of true infection.

Importantly, this study also showed that early intervention is a priority for dogs with sepsis. A total of 72% of deaths from sepsis occurred within the first 24 hours from diagnosis, and no further mortalities occurred between the fourth day and discharge. The first hours of sepsis are therefore a crucial time for recognition and effective treatment, and any delay in diagnosis will be associated with a higher risk of mortality. This is supported by the infection location, which in this study was most often the abdominal organs which may have delays before successful diagnosis or require surgery for full treatment. This is in contrast with another study that reported skin and soft tissue was the anatomical site in more than half of their 37 dogs with sepsis (Camargo et al., 2020). Early and adequate recognition of sepsis in dogs could lead to better outcomes, as with humans (Dellinger et al., 2013). Improved diagnostic tests are required to achieving this. While this chapter has shown that improved diagnostic tests are needed for sepsis, further research is indicated to explore current diagnostic tests and any relationship with time to diagnosis from presentation or onset of clinical signs.

This study confirms that age, organ dysfunction, predisposing risk factors, and comorbidities have an association with mortality and development of sepsis in dogs, although the sample size of each predisposing factor was too small to interrogate the relationship thoroughly. The mortality rates in dogs with sepsis were similar to that described by Kenney et al. (2010), where mortality of increased to 70% with multiple organ dysfunction; in this study, 29% of septic dogs had organ failure, and their collective mortality rate was 63%. This likely reflects use of the same definitions of organ failure, even though this study population included all-cause sepsis rather than only peritonitis. In contrast, another study using the sepsis-2 consensus definitions reported organ dysfunction in 73% of 37 sepsis cases (Bone et al., 1992; Camargo et al., 2020). These definitions of organ failure may not be the most appropriate, and future research should compare these with other organ function scores including APPLE such as was used in another epidemiology study in cats (Babyak & Sharp, 2016) and SOFA which is now recommended in humans (Singer et al., 2016; Vincent et al., 1998). Alternative scoring systems should also consider common complications of sepsis such as disseminated intravascular coagulation or acute respiratory distress syndrome. The higher mortality associated with sepsis compared with either infection or SIRS underscores the prognostic value of classifying a patient appropriately when they have sepsis, rather than an infection or SIRS alone.

Sepsis has significant long-term morbidity and mortality in humans; 81% survival one year after discharge in one study (Peltan et al., 2019; Shankar-Hari & Rubenfeld, 2016). Unexpectedly, 30-day mortality was no higher in dogs with sepsis than in dogs from other disease cohorts. Organ damage resulting from sepsis can cause ongoing adverse sequelae

for dogs (Lewis et al., 2012; Osterbur et al., 2014). However, the option of humane euthanasia in hospital for severely ill dogs undoubtedly reduces the number of dogs contributing to long-term mortality statistics. Alternatively, a 30-day time-point may be too early to allow sepsis-related morbidity and late mortality to present in dogs. Although this study reports on long-term survival of sepsis for the first time, a prospective study following dogs after discharge is needed to investigate this in depth. Based on human critical care research, survival to hospital discharge does not always correlate with positive long-term outcomes, and the reasons for this remain poorly understood (Mostel et al., 2019; Winters et al., 2010). Future investigations of sepsis epidemiology in dogs should include both short- and long-term outcome measures.

The longer duration of hospitalization associated with sepsis care contributes to higher cost. But these are complex outcomes; several other factors also likely influence both cost and length of stay in turn – including disease severity, need for surgery, owner finances, body weight (itself influenced by age and breed), and in some cases distance owner lives from the hospital (up to 5 hours away in some referred cases). High costs or a long hospitalization negatively influenced the outcome observed for some patients, for example by contributing to decisions to euthanize. Furthermore, abdominal and soft-tissue sites were the most common sources of sepsis in dogs. This may contribute to higher cost because source control often includes surgery and thus is more expensive than medical management alone. Further research is warranted to explore these relationships since cost is a major factor in disease management.

The most important limitation in this study is the observational census design, which restricts the type and quantity of data obtained with independently managed cases of owned dogs. Most analyses were affected by at least some missing values including declined diagnostic tests in some instances. Surveying patients at 7 a.m. inadvertently excluded some patients admitted later during the day that were then discharged, referred to regular care, or died before the next census time-point. At least one patient developed sepsis after the timepoint for that day but died prior to the next timepoint, meaning that case went uncounted. Another example of the limitations of a single-centre observational study is that repeat hospitalizations at other clinics either before or after the study institution were not recorded. A single-centre study limits the sample size and reflection on greater population.

2.7 Conclusion

In conclusion, this study confirms hospitalized dogs often have SIRS, some have sepsis, and fewer have uncomplicated infection. Sepsis in dogs is an important clinical entity associated with higher mortality, longer hospital stay, and higher cost, than non-infectious causes of SIRS or infection without SIRS. Preliminary data suggest that dogs surviving sepsis may not have increased long-term mortality – an unexpected finding that needs further study. Crucially, all sepsis deaths occurred within four days of sepsis diagnosis, and therefore this study recommends improved diagnostic tests for rapid recognition and dynamic monitoring of sepsis. Ongoing research is needed to further explore sepsis epidemiology in dogs.

3 Chapter Three

Canine procalcitonin purification and detection in thyroid tissue and plasma

3.1 Introduction

The main objective of this project is to develop and validate monoclonal antibodies that are confirmed to recognize the native form of canine PCT (cPCT) in dog plasma samples. In order to achieve this, native cPCT is required as an antigen. This chapter describes acquisition of this antigen, and preliminary investigation of its characteristics and antigenicity to existing cPCT Pabs.

3.1.1 PCT structure

Canine PCT (cPCT) post-translational structure is not fully described. Figure 3.1 summarises what is known to date. The molecular weight of cPCT is approximately 14.5 kDa for preprocalcitonin (pre-PCT) which includes the N-terminal sequence from translation. The molecular weight (MW) of only PCT in dogs is 11.5 kDa (Wende et al., 2000). Following production by the C cells of the thyroid, PCT is further cleaved into three daughter proteins (N-terminal PCT (NproCT), calcitonin (CT), and katacalcin (KC)) or some combination of these peptides (Becker et al., 2004). Due to being a small protein, PCT is unlikely to have complex or antigenically significant folding, but one author has suggested possible lipopolysaccharide (LPS)-binding sites on PCT as a result of certain basic folding of the protein similar to beta sheets (Matera et al., 2012).

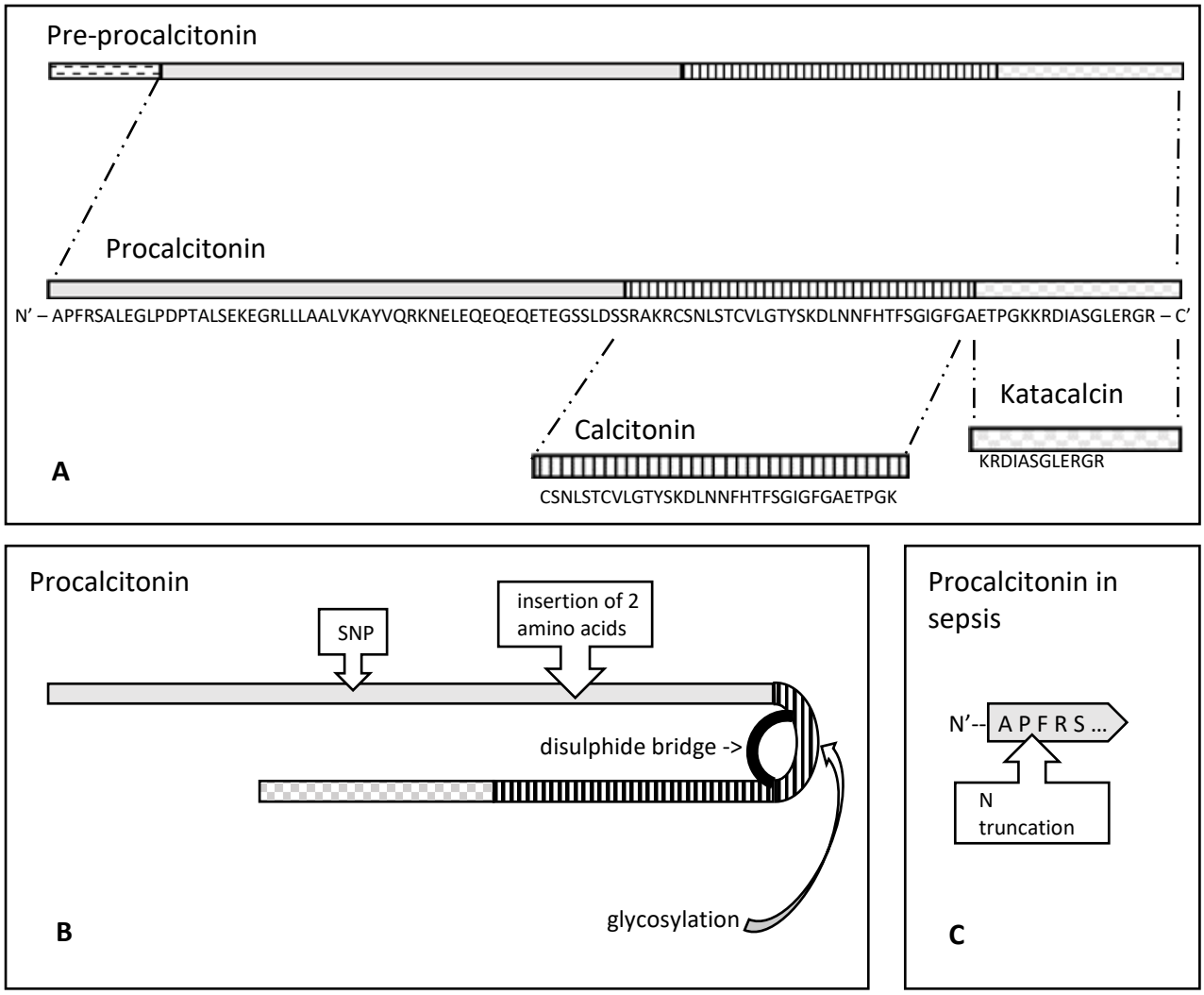


Figure 3.1: Procalcitonin structure can vary, with modifications following cleavage, in health and in disease. **A:** Cleavage of pre-procalcitonin into PCT, with its amino acid sequence. Normal cleavage products of PCT, with amino acid sequences: calcitonin (CT) and katacalcin (KC). **B**: Known mutations and post-translational modifications include an insertion, single nucleotide polymorphism (SNP), glycosylation, and a disulphide bridge. **C**: Pathological change: cleavage of the two amino-terminal amino acids in sepsis.

Native vs recombinant PCT antigenicity

Recombinant PCT (rPCT) is currently the antigen for most commercially available antibodies. Differences between native and recombinant cPCT have been shown or speculated to affect performance of canine PCT ELISAs (Battaglia et al., 2020; A. Floras et al., 2014). Mammalian PCT may have lower recognition by an anti-rPCT antibody, than rPCT derived from *E. coli* due to the absence of purification tags in the native mammalian isotope (Goggs et al., 2018), or interference by post-translational modifications or other structural variations. Glycosylation is one potential post-translational modification that may affect antibody recognition of PCT: the glycosylation of canine C-reactive protein (CRP), in contrast to human CRP that is not glycosylated, has accounted for the failure of some human CRP assays to recognise canine CRP (Caspi et al., 1984). Glycosylation was observed on human PCT when it was expressed in the presence of canine mitochondrial membranes (Jacobs et al. 1081) but was not present when PCT was isolated from the serum of humans with sepsis and analysed via mass spectrometry (Weglöhner et al., 2001).

For a monoclonal antibody (Mab) to have strong affinity for the PCT present in canine blood during sepsis, an antigen closest to that native form of PCT is desired. Therefore, the antigen source for a Mab should ideally be blood-derived PCT. Blood purification of inflammatory PCT can be low yield, especially when using an antibody with low affinity for the target protein (Kim et al., 2018); and a sufficient source of blood from dogs with sepsis was not available for this project. The second preference is tissue-derived PCT, then recombinant full-length PCT, and finally limited peptides. Therefore, native PCT from thyroid was the

antigen of choice over recombinant or peptide-restricted options for this project, although peptides were successfully used in human Mab generation (Ghillani et al., 1988; Meisner, 1997).

3.1.2 Identification of PCT

Definitive identification and efficient detection of PCT is required for effective purification. There is no known specific activity or enzymatic assay for PCT. There are commercially available antibodies raised against crPCT, but while it is assumed they detect native PCT, this has not been definitively confirmed.

3.1.3 Mass spectrometry – quantitative and qualitative

Mass spectrometry (MS) is an antibody-independent method of protein identification and quantitation. Using a multiple reaction monitoring (MRM) approach, the tryptic peptides of a select protein can be targeted in a highly sensitive and precise quantitation (Lange et al., 2008). If an isotopically labelled peptide is included as a reference, this quantitation can be absolute.

An MS-based method for quantitation of human PCT (hPCT) in clinical samples was described by Krastins et al (2013), but this method relies on affinity purification to target PCT and sufficiently enrich it prior to analysis. A separate study that measured and sequenced hPCT also used affinity enrichment (Weglöhner et al., 2001). A primary quantitative MS assay has not been described for cPCT, however two groups have published qualitative MS results for crPCT (A. Floras et al., 2014; Goggs et al., 2018). This study was

reviewed in detail in Chapter One – briefly, Goggs and colleagues (2018) confirmed that rPCT was present in the standard of the BioVendor-brand PCT ELISA kit, and concluded that positive antibody reactivity in clinical samples represents native PCT. However, this has not yet been confirmed with immunoprecipitation or other antibody-independent techniques.

3.1.4 Aims

The experiments described in this chapter aim to optimise a method to purify native cPCT from thyroid tissue, with the goal to use the protein as antigen for raising Mabs using hybridoma production. A second aim is to evaluate the immunoassays and MS methods available for detection of PCT. Finally, a quantitative MS assay will be developed with the aim to use this as a gold-standard benchmark for validation and calibration of other quantitative PCT assays. It is hypothesised that native canine PCT can be isolated to 90% purity (of a total estimated 0.1g per thyroid gland based on C-cell to thyroid follicle ratio in histology) from thyroid tissue using standard chromatographic methods (ion exchange and reversed-phase chromatography), based on the original experiments of Mol et al. (1991); that antibody raised against crPCT, or PCT from another species, cannot recognize native cPCT; and that multiple forms of PCT are detectable and quantifiable at low quantities (picograms) in canine biological samples using MS (including MRM).

3.2 Methods

3.2.1 Materials

All chemicals and reagents were obtained from Sigma (Millipore-Sigma, Merck, Burlington, MA, USA); unless otherwise specified. Equipment and cartridges for chromatography, SDS-PAGE, and Western blotting, was from Bio-Rad (Hercules, CA, USA). Near-infrared imaging reagents and equipment was from Li-Cor (Lincoln, NE, USA). Antibodies, ELISA kits, and crPCT were from either abcam (Cambridge, UK) or BioVendor (Asheville, NC, USA) as noted. Recombinantn canine PCT from these sources was typically 15 kDa in size.

3.2.2 Ethics

Dog cadavers were donated to the research institution for general research purposes: as such, no specific ethics approval was required by the institution's Animal Ethics Committee. The tissue utilised via this method was reported to the Ethics body on an annual basis. animal ethics approval and informed owner consent was obtained for collection of blood samples (whole blood, plasma, and serum) from dogs (#R2769/15), and samples were taken immediately after diagnosis after an acute presentation or onset of sepsis prior to any intervention or treatment.

3.2.3 Extraction and purification of PCT

Purification protocols were selected and optimised with the priority of preserving cPCT in its native form wherever possible. Sixteen thyroid glands were obtained from euthanised dogs of various signalment, free from thyroid disease. Thyroid tissue was collected between two

and 24 hours post-mortem. After removal, thyroids were immediately frozen individually in liquid nitrogen, and stored at -80ºC up to a maximum of twelve months until use.

Thawed thyroid tissue was handled and processed at 4ºC or on ice. Thyroid tissue was finely minced manually with a scalpel and rinsed in phosphate-buffered saline (PBS), and suspended with extraction buffer A-1 at a minimum ratio of 1:4 tissue:buffer (10 mM Tris, pH 7.6, 150 mM NaCl; with one tablet of protease inhibitor cocktail (PIC) per 100 mL, cat no. S8830). The tissue was mechanically homogenised using a Mini-Beadbeater (Bio Spec, Bartlesville, OK, USA) at 3,000 oscillations per minute for two minutes with 0.5 mm zirconia/silica lysis beads (Daintree Scientific, Tas, Australia), and then centrifuged at 5,000 x *g* at 4ºC for five minutes. The pellet was resuspended in additional extraction buffer and re-spun, and both supernatants were pooled.

All supernatant was chilled, and aliquots drawn and saved for BCA (bicinchoninic acid) protein assay (described below) before adding reagents (0.5 mM DTT, 0.5 mM EDTA) to form extraction buffer A-2. This extract was further diluted to 1:10 with A-2 before clarification via ultracentrifugation for 30 minutes at 30,000 x *g*. Aliquots were drawn from clarified extract (extract A) for subsequent analysis and purification. Portions of 5 mL of extract A were stored at 4ºC for a maximum of 48 hours before being used to test protein chromatography methods and the remainder was stored at -80ºC until the purification protocol was finalized.

3.2.3.1 Cation exchange chromatography (CEC or IEC)

Cation exchange chromatography was performed with a Bio-Scale Mini Macro-prep High S 5-mL cartridge (Bio-Rad) run on a BioLogic™ Low-Pressure liquid chromatography system. Extract A was diluted 1:1 with buffer B-1 (0.01 M sodium acetate, pH 4.8) which also primed the system. The pH of extract A was altered to 5.2 (pH of 4.8 resulted in some protein precipitation) before application to the column at 1 mL/min at 4°C. After the absorbance at 280 nm (A_{280}) returned to baseline, flow rate was adjusted to 1.5 mL/min and salinity was increased by a linear gradient from 0 to 100% of buffer B-2 (0.1 M sodium acetate with 0.5 M sodium chloride, pH 4.8) over 30 minutes, followed by a five-minute plateau of buffer B-2 at 100%. Conductivity (millisieverts) and absorbance (280 nm wavelength; A_{280}) were continuously monitored, and fractions were collected every minute.

3.2.3.2 Solid phase extraction (SPE)

Samples were cleaned and desalted by solid phase extraction (SPE) prior to high-pressure liquid chromatography (HPLC) or to prepare aqueous samples for analysis with MS. SPE was performed using solid phase extraction with Supelclean LC-18 SPE cartridges (3 mL, Sigma Merck) with a manual set-up i.e. filling and emptying the columns with the solutions manually using a pipette and suction guided by a timer to ensure appropriately slow flow rates. Cartridges were conditioned with acetonitrile (ACN), then equilibrated with S-1 buffer: 5% ACN to 95% TFA water (MS-grade water with 0.1% trifluoroacetic acid (TFA)). Sample was diluted 1:1 with S-1 buffer and applied to cartridges, washed with at least three bed volumes of S-1, then eluted in two stages: 1.5 mL of S-2 (70% ACN to 30% TFA water)

and then 2.5mL of S-3 (80% ACN to 20% TFA water). Eluted sample was then lyophilised to concentrate and store the sample prior to analysis.

3.2.3.3 Reversed-phase high-pressure liquid chromatography (RP-HPLC)

Desalted samples underwent RP-HPLC (designated 'C' in this thesis) in a Nova-Pak C18 column (60Å, 4 µm, 3.9 mm X 150 mm, Waters, Milford, MA, USA). Protocols were managed with a Shimadzu 10A High Performance system. Samples were reconstituted after SPE or lyophilisation, or diluted 1:1 with, buffer C-1 (200 mM ammonium acetate, 5% ACN) for loading onto the column. After the A_{280} returned to baseline for five minutes, buffer C-2 (200 mM ammonium acetate, 90% ACN) was added in a linear gradient over 20 minutes, maintained at 100% buffer C-2 for ten minutes, then returned to buffer C-1 to re-equilibrate. Buffer was run through at 0.5 mL per minute and pressure reached approximately 2000 psi during runs. Fractions were collected every two minutes, and protein concentration (absorbance at 230 and 280 nm) was monitored throughout.

3.2.4 Sample preparation and storage

All reagents were kept sterile where possible. Sterilised glassware was used; heat-stable buffers were autoclaved, and typically filtered through 4 µ nitrocellulose vacuum filter membrane. Protein solutions were centrifuged at 2,000 x *g* for five minutes prior to application to columns. Extract and fractions along with any temperature-sensitive buffers were routinely stored at 4°C and handled on ice or in a cold-room. Long-term storage was at -80°C for tissue or protein fractions, and -20°C for reagents and buffers.

3.2.5 cPCT quantitation and identification

Aliquots were drawn from each fraction for measurement of total protein (see Section 3.2.5.3). Canine PCT immunoreactivity (assumed equivalent to content of cPCT) was determined either by an anti-hPCT sandwich ELISA (abcam) or an anti-cPCT sandwich ELISA (BioVendor). Fractions underwent analysis via Western blot, either using surplus antibody from the ELISA kits mentioned above, or using a separate antibody preparation of anti-cPCT polyclonal raised in rabbit (BioVendor) when available. Select fractions demonstrating positive immunoreactivity were prepared and analysed by MS for protein identification.

Predicted or known biochemical properties of PCT were used to estimate presence of PCT in certain fractions and make empirical decisions for separation. Immunoreactivity of most fractions was measured using either of two antibodies (see Methods sections or figure legends) in an ELISA (see above paragraph) or on Western blots using antibodies previously described. Positive fractions are termed PCT-positive or having immunoreactivity for PCT throughout this thesis – but this did not absolutely confirm the presence of native PCT. Also, positive results could have indicated non-specific binding to other proteins. Therefore, a qualitative MS analysis was performed on PCT-positive fractions, to confirm whether PCT was present in the sample or not. Mass spectrometry could not be performed on all fractions due to cost limitations.

3.2.5.1 SDS-PAGE and Western Blotting

Polyacrylamide gel electrophoresis (PAGE), with or without SDS (sodium dodecyl sulfate) was carried out using Bio-Rad systems and pre-cast TGX gels (10-well, mini size, 4-20% gradient for resolution of a range of molecular weight proteins). Samples were prepared by mixing 1:1 with 2x Laemmli buffer, with 10% β-mercaptoethanol (2-ME); then heating to 95°C for five minutes. One of three ladders was used depending on downstream imaging (molecular weights are indicated in the figures): Dual protein plus or Western C (Bio-Rad), or Chameleon Duo (Li-Cor). In standard running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS), voltage was set to 50 for five minutes and then maintained at 200 volts for approximately 20 minutes or until the dye front reached the bottom of the gel. Native PAGE was performed in the same way but with omission of SDS, 2-ME, and heating.

Gels were either stained with a Coomassie-based stain or blotted with Trans-Blot Turbo Mini 0.2 µm Transfer Packs (Bio-Rad), using the pre-programmed TGX turbo protocol in a Trans-Blot Turbo transfer system (Bio-Rad). To confirm transfer, Coomassie was used to stain gels after blotting, or some blots were reversibly stained directly with Revert (Li-Cor). Nitrocellulose membrane (Bio-Rad) was used in earlier blots imaged with enhanced chemiluminescence (ECL), and PVDF (polyvinylidene fluoride) (Immobilon-PSQ, Merck Millipore) was used later in the project and for any blots to be imaged with near-infrared (NIR); membrane thickness was 0.2 µ (microns).

Immunoblotting was carried out according to standard protocols and the recommendations for Li-Cor systems with a tris-buffered saline (TBS) buffer system (TBS: 20 mM Tris, 150 mM

NaCl; with 0.1% Tween-20 becomes TBS-T). Membranes were blocked with milk (5% w/v non-fat powdered milk) for nitrocellulose, or Odyssey blocking buffer (Li-Cor) for PVDF and NIR, for minimum one hour at room temperature with shaking or 4°C overnight. More than one primary and secondary antibody was used in total throughout this project, and the details of the individual antibody is included in any relevant results and methods sections. Primary antibodies were either from abcam (rabbit anti-human CT polyclonal antibody, ab16697) or from BioVendor (rabbit anti-canine PCT polyclonal antibody, RD481488100) unless otherwise specified. Based on dot blot titrations, primary antibody was diluted to 1:3,000 (unless otherwise noted) in blocking buffer and applied to the membrane for 60 minutes at room temperature with rocking, before four five-minute rinses with tris-buffered saline with Tween 0.1% (TBS-T). Secondary antibody was from abcam: goat anti-rabbit IgG (H&L) HRP-conjugated; or goat anti-rabbit IgG (H&L) conjugated to Alexa Fluor® 680 (ab175773) – unless otherwise stated. In all cases, secondary antibody was diluted 1:10,000 (HRP-conjugated) or 1:20,000 (fluorescent) in blocking buffer based on preliminary dot blot titrations, and applied to the membranes for 45 minutes (in the dark if fluorescent) with shaking before four five-minute rinses in TBS-T. Blots for NIR were rinsed in TBS once and allowed to dry before imaging with an Odyssey Classic Imaging System (Li-Cor); blots for ECL were developed using a Clarity Western ECL substrate (Bio-Rad) and imaged on a Fusion FX imaging system (Vilber). Blot images were analyzed by the Li-Cor Image Studio Lite software. Gel PAGE images were acquired with a Gel Doc EZ Imager and Image Lab software (Bio-Rad). Molecular weight was calculated from SDS-PAGE gels with a 4-20% gradient (as above) using Rf values of a commercial protein ladder standard curve and rounded to the nearest 500

daltons (Da). Densitometry was calculated within the Image Lab and Image Studio Lite software. Blots and gels were not designed for quantitative analysis – purity was assessed only by band size (densitometry) relative to that lane. Because the gels were not quantitative, and featured multiple different purification fractions within one gel, loading controls were not relevant; even loading, running, and transfer of gels was confirmed by staining a gel and any post-blotted gels as described above.

3.2.5.2 ELISA

Quantitative PCT measurement was performed using either the anti-human PCT (hPCT) sandwich ELISA kit (ab221828, abcam) (Hestiantoro et al., 2018; Motie et al., 2018), or the anti-canine PCT sandwich ELISA kit (BioVendor) which was independently analytically validated twice, during and after this project (Battaglia et al., 2020; Goggs et al., 2018). Each ELISA was used according to the instructions of the manufacturer. Briefly, fractions were applied neat and standards were prepared and diluted according to the manufacturer's instructions for that batch. Bovine serum albumin (BSA) was used as a negative control, and fraction buffers were included as blanks in addition to the ELISA buffer provided with the kit. Positive controls were included with recombinant canine PCT protein (from abcam or BioVendor) at physiologic concentrations (approximately 50-500 pg/mL or higher as indicated). Antibodies were prepared strictly in accordance with kit instructions and absorbance measurement was carried out on a pre-stabilized Model 680 Microplate reader (Bio-Rad). Standards were used to calculate a standard curve and mean blank absorbance

was subtracted from all values before calculations were carried out. Due to limited resources, independent analytical validation of ELISAs was not performed.

3.2.5.3 Protein quantitation

Protein was quantitated with a Pierce Microplate BCA assay (BCA assay, ThermoFisher Scientific) using a BSA standard curve, according to the manufacturer's instructions. Briefly, samples were made fresh by a 1:1 mix of 150uL of experimental sample (or BSA standard curve dilution sample), and 150uL of 'working reagent'. Working reagent is 25:24:1 parts reagents A, B and C respectively. These fresh samples were applied into microplate wells before incubating at room temperature for one hour and reading absorbance at 562 nm. Some fractions were measured with a Nanodrop (2000/2000c, ThermoFisher) at 230 nm and 280 nm absorbance to estimate protein concentration first, to ensure results would fall within the quantitative range of the BCA assay and dilute when necessary. Unfortunately it was not possible to include recombinant canine PCT protein in positive control samples because of insufficient quantity of the protein available. Fluorescent methods of protein quantitation were not used, since these rely on the presence of tryptophan which is not included in the amino acid sequence of canine PCT.

3.2.5.4 Mass spectrometry

For protein identification (ID) by MS, fractions were desalted by SPE as described above, with the exception of C fractions which were already in a high-ACN matrix. Samples were lyophilised to dryness and up to 0.5 mg of total protein was submitted for MS, before

extraction of peptides with a standard protocol involving aklylation, denaturation, and digestion with trypsin (as described in Bringans et al., 2008). Mass spectrometry was performed by Proteomics International (Proteomics International Facility, Harry Perkins Institute of Medical Research, QEII Medical Centre, Nedlands, Australia), using a Shimadzu Prominence nano HPLC system (Shimadzu) coupled to a 5600 Q-TOF mass spectrometer (Sciex). Peptides (between 20 µg and 20 pg) were loaded onto an Agilent Zorbax 300SB-C18 column (3.5 µm, Agilent Technologies) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v) over 20 minutes, before electrospray ionisation. Data were processed using Mascot and the Swiss-Prot database, using protein search parameters: precursor and product (MS/MS) ion mass tolerance of ± 0.2 Da, peptide charge 2+ 3+ and 4+, monoisotopic mass, and tryptic peptides with maximum one missed cleavage. One microgram of BSA was included in each run as quality control. In addition, aliquots with confirmed negative cPCT by immunoreactivity were used as confirmatory negative control for MS.

3.2.5.5 Quantitative mass spectrometry

An antibody-independent, molecular-based method to quantitate PCT was needed to validate and calibrate PCT results obtained with quantitative PCT ELISA. An MRM based approach was selected to achieve this.

Standard curves were generated by serial dilution into aliquots of ammonium bicarbonate buffer, ranging from 2,000 to 20 pg/mL concentration of crPCT standard (BioVendor). Lyophilised, isotopically labelled peptide of a similar size but irrelevant to PCT

(STVEELHEPIPSLFR, Lys, Arg ^{13}C and ^{15}N metabolically labelled recombinant protein fragment), was used as a quality control, reconstituted in 0.1% TFA in 2% acetonitrile (ACN)/98% water v/v to create 1 nmol/mL aliquots stored at -80°C. Each sample was spiked with 400 fmol. Initial MRM runs tested undiluted aliquots of heavy labelled peptide and the target tryptic peptide of cPCT (LLLAALVK) and were able to repeatably detect both. Admixing the heavy labelled peptide (HLP or 'label') and the PCT was partially successful, where some batches showed both, but one batch did not detect label – subsequent re-testing of the label showed it was still viable. Plasma samples were either in lithium heparin or EDTA anticoagulant. Volumes of plasma varied from 0.1 to 0.5 mL and included replicates for most patients, thus it was decided to pool samples from several sepsis patients. Each 'healthy' or 'sepsis' sample was the equivalent of 400 µL of plasma. All samples were prepared as above on the day of submission for analysis, or immediately lyophilised and stored at -20°C for up to two weeks.

3.2.5.6 Analysis of MS samples and data

Protein fractions were analysed by nanoLC-MS, with an acquisition time of 18 minutes, at a flow rate of 0.3 µL/minute. The tryptic peptides were passed through a C18 LC column (Acclaim™ PepMap™ 100 C18 LC Column, 2 µm particle size x 150 mm, ThermoScientific) at 40°C, controlled with an UltiMate 3000 nanoflow UHPLC system (Thermo Scientific). Sample was loaded onto the column in 0.1% FA in 2% ACN, 98%H20 (v/v) and elution gradient of 10-50% was achieved with 0.1% FA in 2%H20, 98% ACN over 10 minutes.

Peptides were ionised by electrospray ionization on a 5500 QTRAP (SCIEX) with an IonSpray voltage (IS) of 2950 and a dwell time of 80 milliseconds. The declustering potential (DP) was 61.8, and a collision energy (CE) of 24 eV applied to target peptide LLLAALVK (endogenous PCT), and for STVEELHEPIPSLFR (label) was 73.8/29.4 for DP/CE respectively. Further settings are listed in appendix. Raw data were exported to Skyline software (MacCoss Lab, University of Washington) for analysis and visualization.

3.3 Results

In this series of experiments, the presence of cPCT was confirmed in partially purified fractions made from canine thyroid extract. Purity was a maximum of 42% based on densitometry. Affinity of PCT ELISAs was found to be different for PCT from different species. This chapter also reports the first detection of native PCT in a sample of plasma from a septic dog using MS.

3.3.1 Extraction and chromatography

Procalcitonin protein was extracted from canine thyroid tissue using an aqueous extraction buffer (A). Two types of extraction were trialled, because an acetone-based method has been previously published (Mol et al., 1991) but an aqueous and non-denaturing method was preferred for this project (Tolun et al., 2007; Yubin et al., 2000). Both aqueous (A) and acetone-based (Ac) extraction had similar characteristics in terms of protein bands visible on SDS-PAGE. Gross appearance of the two extracts (A and Ac) was very different as shown in Figure 3.2, reflecting different protein content or denatured proteins. Extract A had a higher yield of PCT per mL as measured by the BV ELISA (see Figure 3.3).

Figure 3.2: Aqueous extract (extract A, left) and acetone extract (extract Ac, right). Beads for mechanical disruption of cells are in base of the tube, and extract has not yet been centrifuged.

From Extract A, purification progressed to initial separation of high concentration protein solutions with cation exchange chromatography (B); and further enrichment of select B fractions with reversed-phase HPLC (C). Thus, for example, the final preferred fraction was ABC18. This process is mapped in Figure 3.3

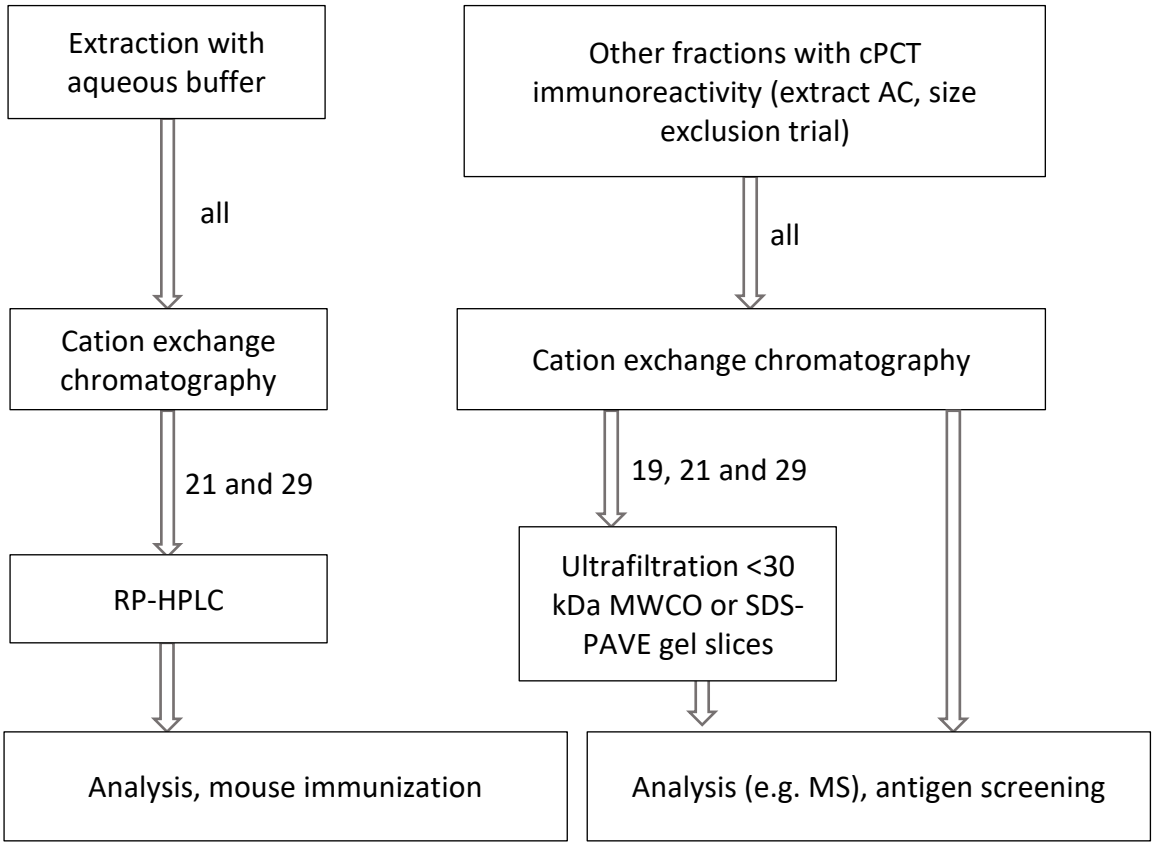


Figure 3.3: Outline of the purification process and the source of PCT for different purposes. MWCO (molecular weight cut-off).

The limited amount of PCT extracted via this primary protocol was insufficient for the needs of the project, so all remaining fractions with any PCT immunoreactivity were pooled. This included stored fractions from method development, excluding a small volume of denatured extract containing acetone. These underwent cation exchange chromatography (B), and then fraction 21 and 29 were pooled and concentrated with ultrafiltration (<30 kDa MWCO) to further enrich for small proteins (designated U).

Select PAGE gels and Western immunoblots are shown in Figure 3.4, demonstrating the different protein profile of fractions with PCT immunoreactivity, and the presence of cPCT at the expected molecular weight. Notably, the fraction ABC18 showed markedly fewer protein bands and therefore fewer contaminants than AB21 or AB29; although AB29 had a higher purity based on immunoreactivity than AB21, as noted above in

Table 3.1. Native PAGE gels were carried out to investigate native PCT migration. Proteins with PCT immunoreactivity did not migrate to the expected molecular weight under these conditions.

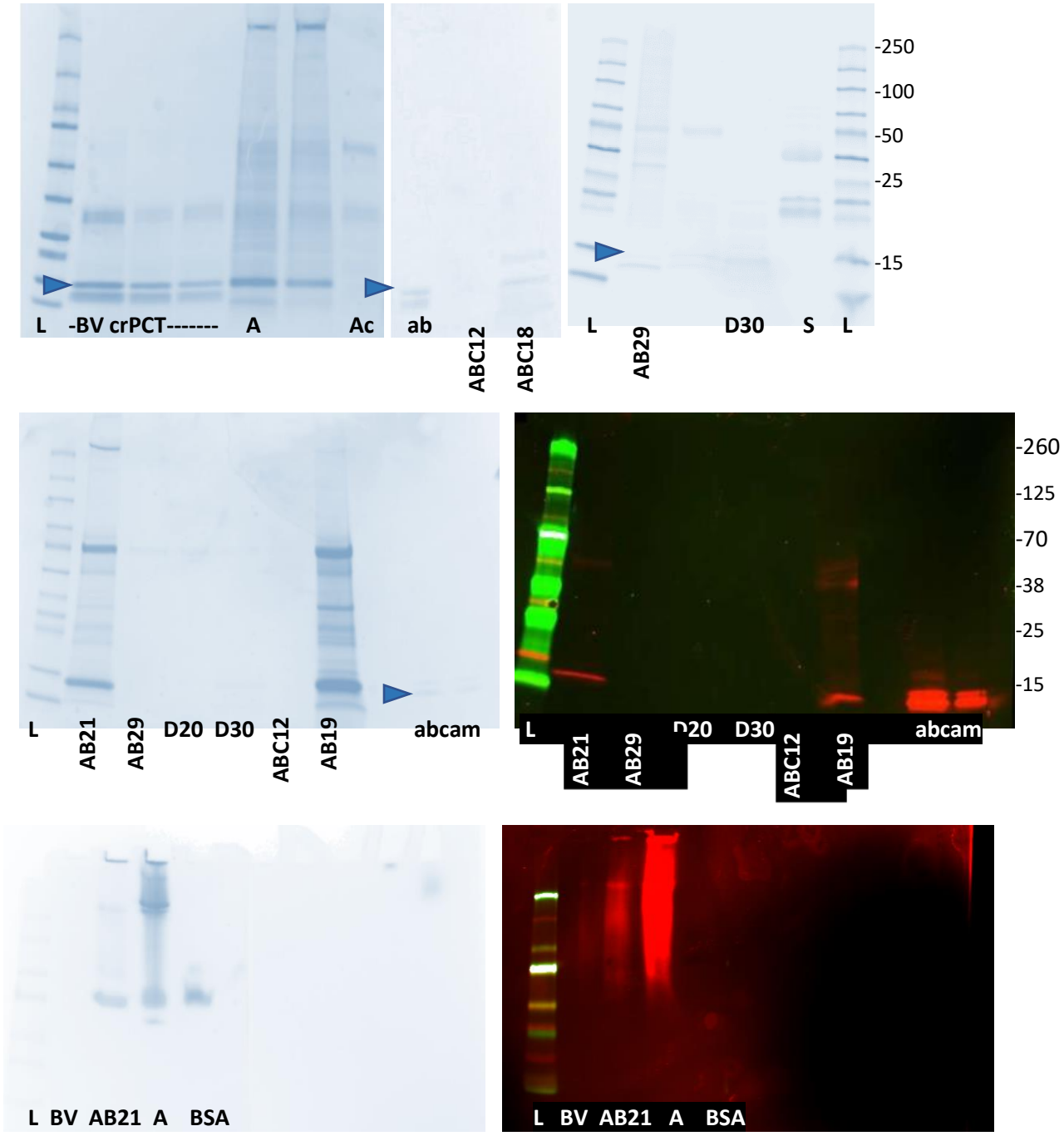


Figure 3.4: PCT purification from thyroid, with various fractions shown in SDS-PAGE gel and immunoblots. L=ladder. Arrowheads indicate 15 kDa, the molecular weight of the recombinant PCT protein (abcam or BioVendor). First row: select fractions including

recombinant PCT, two extracts (A), and several fractions (C#, B#, and D#). S=sepsis plasma. Second row: gel and blot pair. Strong staining bands on SDS-PAGE, correlating with immunoreactivity (red bands in the blot) can be seen in AB21 and AB19 (probed with BioVendor rabbit anti-canine PCT recombinant antibody). Two bottom images: native-PAGE gel and blot pair. The native PAGE and concurrent blot showed very poor isolation of proteins with many of the same fractions as pictured in the SDS-PAGE.

Haemoglobin and albumin were suspected contaminants based on their propensity to bind or adsorb to other proteins, and the molecular weight of the contaminant proteins. Contamination with brownish-red or pinkish pigment affected some columns and membranes (see Figure 3.5). For example, immunoprecipitation of plasma samples (presented in Chapter Four) often led to formation of a fluffy brownish-red plaque on the surface of the magnetic bead pellet that would not wash or elute off. This was presumed to be haemoglobin or protein and haem products deriving from it. Thus, ultrafiltration (<30 kDa) of plasma was performed to remove as much haemoglobin as possible.

Figure 3.5: Haemoglobin or other ferric contaminants in chromatography. Top left: pinkish-brown pigment binding to SPE column. Top right: B15 before (left) and after (right: insert and outer tube) ultrafiltration through a <30 kDa MWCO membrane. Bottom: Cation exchange colums with pigment (pigment is at the leftwards end).

Chromatography under native conditions was not efficient or reliable for complete isolation of PCT protein, as visible in Figure 3.6. Cation exchange chromatography produced three peaks, despite a single calculated isoelectric point for PCT (6.84). The RP-HPLC also showed two peaks. These peaks are shown in Figure 3.6. This cation exchange chromatography was run a total of five times, and gave repeatable results i.e. the PCT peaks occurred in the same fractions and the bulk of the protein trace was the same. Any minor variations appeared to be due to batch variations (different thyroid mixtures) but were overall very similar.

Size exclusion chromatography (designated D) was performed during method development (methods in 6.1.3) but showed a plateau of PCT immunoreactivity and no bands on SDS-PAGE even after concentration of the fractions.

A summary of the details of fraction selected for immunisation of mice is included at the beginning of Chapter Four.

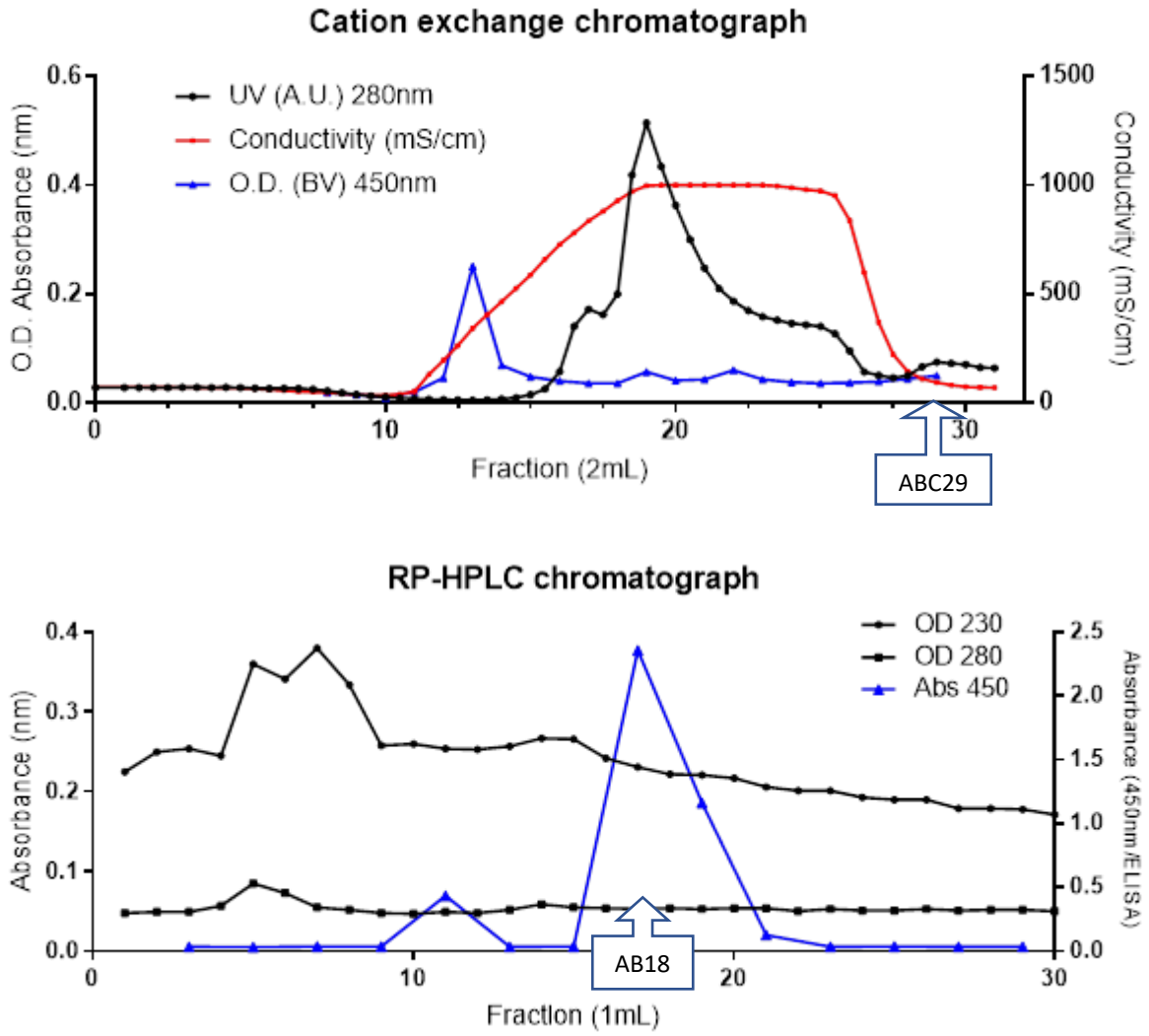


Figure 3.6: Chromatographs of cation exchange (top), and RP-HPLC separation (bottom) with PCT immunoreactivity (shown in blue triangles) as measured by the crPCT ELISA (BioVendor). Each of these chromatographs is carried out on the same sample (extract A, cleaned with SPE) to allow more direct comparison, but the peaks of PCT were consistent between extract A and other more refined fractions within each type of chromatography.

3.3.2 Purity and yield

The purity and yield of PCT in each major fraction are shown in

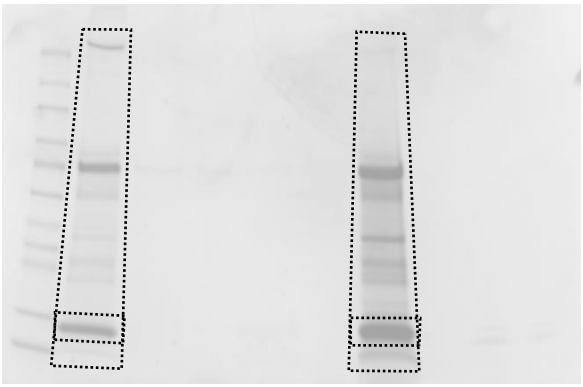
Table 3.1. No fraction had apparent PCT purity above 42%. The fraction with the highest purity (according to densitometry from SDS-PAGE, shown in Figure 3.1A:sub-part of Table 3.1A) was AB29 obtained by cation exchange chromatography, and ABC18 showed the second-highest purity despite being an enriched fraction of AB21. All fractions with PCT immunoreactivity according to the ELISA were confirmed to contain PCT with MS analysis. A negative control fraction with no immunoreactivity for PCT (C25) was found to have no PCT content with MS.

Table 3.1: Summary of the yield and purity of select fractions (F#) during the purification of PCT. Yield is fraction PCT divided by PCT content of Extract A. Purity is PCT concentration divided by total protein of the same fraction. A: extract A. B: cation exchange chromatography. D: size exclusion chromatography. C: reversed-phase HPLC. U: cation exchange chromatography (A) with ultrafiltration to isolate proteins less than 30 kDa.

	Protein quantitation by BCA assay				Quantitation via densitometry
F#	**TP mg/mL**	**PCT pg/mL**	**Yield**	**Purity**	**Purity**
A	0.35	1178.0	-	0.000337%	8.99%
AB21	0.076	879.43	0.75	0.0011 (3.2 fold)	42.01%
AB29	0.029	476.7	0.4	0.016% (47.5-fold)	30.27%

ABC18	0.054	490	0.42	0.00907 (26.9-fold)	31.14%

Figure 3.1A: a schematic of how the densitometry purification calculations were performed (methods 3.2.5). A relative quantitation was made between the band of presumptive PCT at ~14 kDa (according to Rf values calculated using the ladder as a standard curve), and the lane as a whole, as shown in the dotted lines below on an SDS-PAGE image. Left highlighted area: AB21 (42% of the total); Right highlighted area: AB19 (28% of the total).



3.3.3 Procalcitonin antibody affinity for different species PCT

Initially, this project utilised an anti-human PCT ELISA because independent researchers had confirmed good performance (Hestiantoro et al., 2018; Motie et al., 2018) compared with poor performance or no independent published studies for cPCT ELISAs (discussed in Chapter One). Part way through this project, an independent research group performed successful analytical validation of an anti-canine PCT ELISA, providing strong evidence that it would detect recombinant (and hopefully also native) canine PCT (Goggs et al., 2018). This ELISA was used for the remainder of the study. Due to this change, affinity to human and canine PCT was compared between the kits. The two ELISA kits, anti-human PCT (abcam) and canine PCT (BioVendor), were tested for ability to recognize crPCT (from abcam or BioVendor), human recombinant PCT (hrPCT, from abcam), and native PCT in thyroid lysate. (Figure 3.7). Both kits showed similar immunoreactivity to a fraction with native PCT content later confirmed by MS (insert Figure 3.8). Each ELISA shows superior recognition of the recombinant PCT from the same species: the human CT ELISA showed higher immunoreactivity to the human recombinant PCT than to the same calculated amount of crPCT. Likewise, the crPCT ELISA shows hardly any recognition of the hrPCT at equivalent concentrations. This antibody shows higher immunoreactivity to the pure recombinant protein than it did to the standard supplied with the kit, despite equivalent calculated PCT concentrations (Figure 3.7).

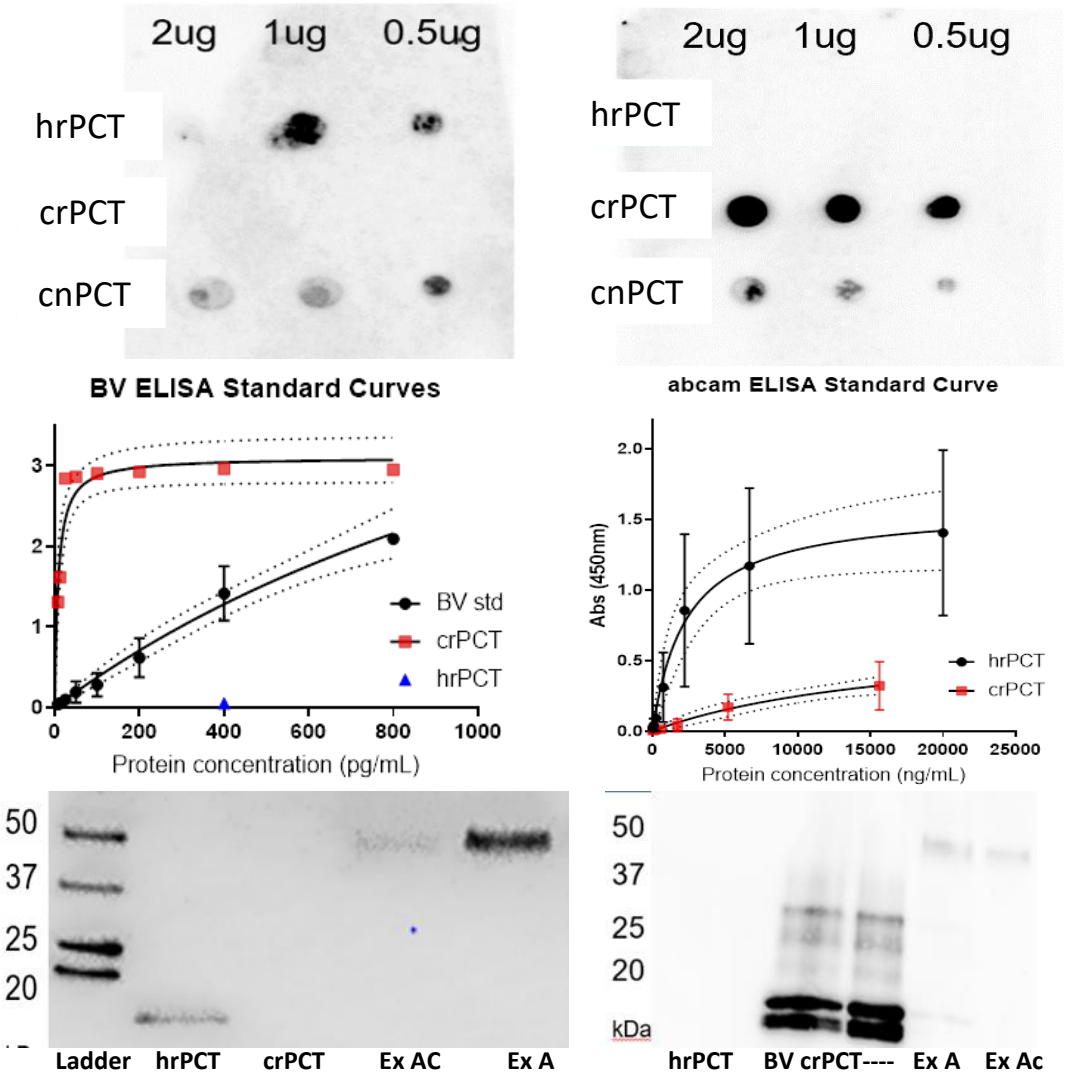


Figure 3.7: Recognition of cPCT and hPCT and native PCT by different antibody. The immunoreactivity of the canine to human (and vice versa) is low. LEFT COLUMN: a-canine PCT antibody (BioVendor). RIGHT COLUMN: a-human PCT antibody (abcam). Samples include recombinant proteins, PCT protein standard from a BioVendor ELISA kit (see methods; BV Std), aqueous thyroid extract (ex A) and an acetone-based extract (ex Ac).

3.3.4 MS protein ID

Fractions with PCT content (listed in Table 3.3) were analyzed by MS. Procalcitonin was identified in all samples with PCT immunoreactivity. Analysis of the tryptic peptides by tandem MS (MS/MS) provided amino acid sequence confirmation supporting the identity of PCT and other proteins within the protein fractions (Figure 3.8 and Table 3.2). For example, the peptide LLLAALVK (839.58 Da neutral weight) had eight fragment ion hits, for several charge states. This peptide was one detected in all samples analysed with a positive PCT detection, and each fraction with a positive match for PCT had a score above 50 (significant match). Several other proteins, including haemoglobin, albumin, and thyroglobulin, were also present in most fractions – some of these proteins from fraction AB29 along with their scores are shown in Table 3.4. Additional data on the MS samples is located in the appendix (section 6.1.4).

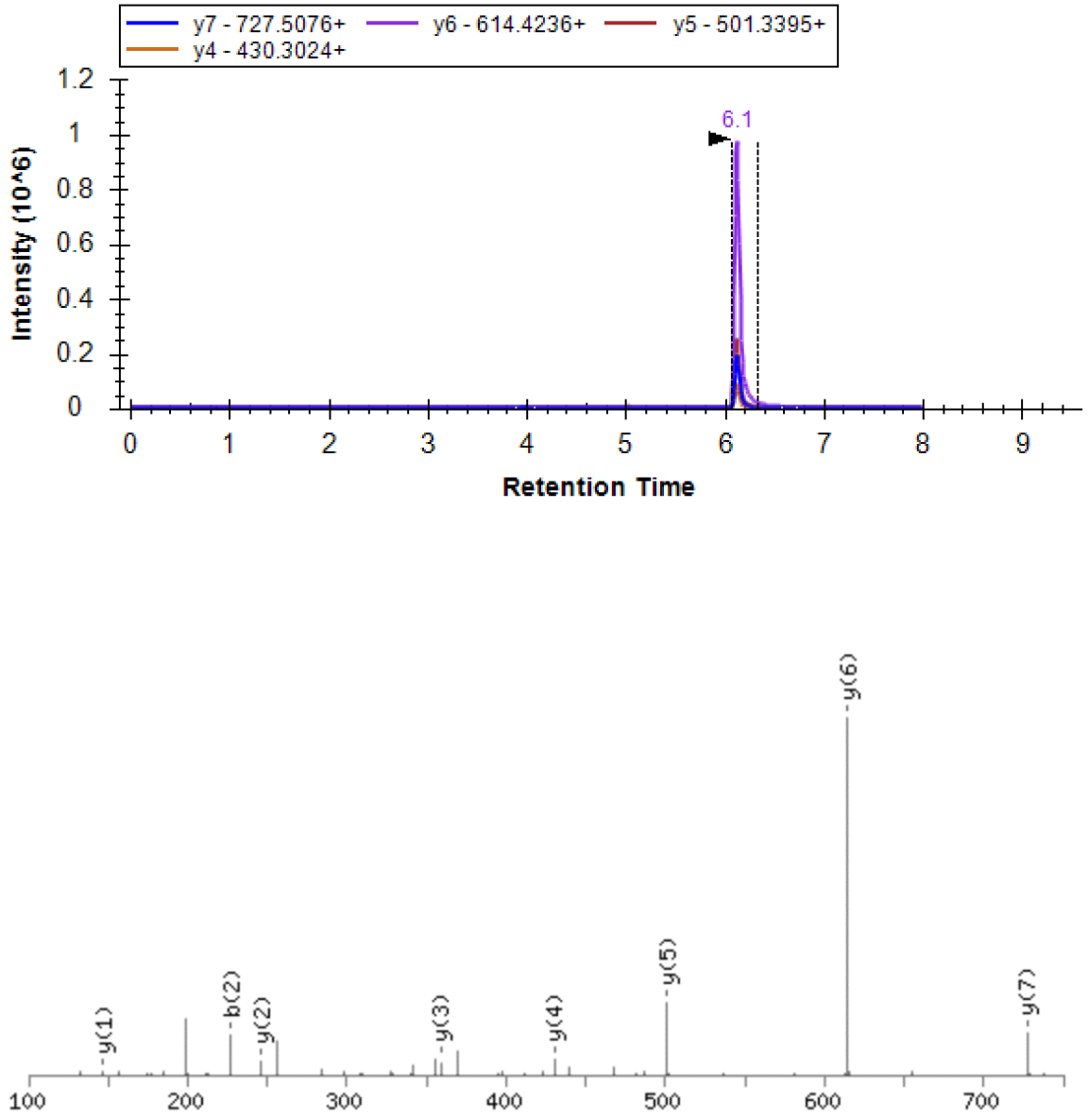


Figure 3.8: Top: Extracted ion chromatogram (m/z for peptide LLLAALVK). Bottom: MS/MS fragmentation. Product ion mass spectral annotations of b+ and y+ ions of PCT peptide LLLAALVK. Visualised with Skyline (MacCoss Lab) and Mascot search engine.

Table 3.2: Peptide sequence identification: order of the corresponding amino acids for the LLLAALVK peptide of PCT (Figure 3.9), based on matches of the neutral ion masses (bold).

#	b	b^{++}	Seq.	y	y^{++}	y*	y^{*++}	#
1	114.0913	57.5493	L					8
2	**227.1754**	114.0913	L	**727.5076**	364.2575	710.4811	355.7442	7
3	340.2595	170.6334	L	**614.4236**	307.7154	597.3970	299.2022	6
4	411.2966	206.1519	A	**501.3395**	251.1734	484.3130	242.6601	5
5	482.3337	241.6705	A	**430.3024**	215.6548	413.2758	207.1416	4
6	595.4178	298.2125	L	**359.2653**	180.1363	342.2387	171.6230	3
7	694.4862	347.7467	V	**246.1812**	123.5942	229.1547	115.0810	2
8			K	**147.1128**	74.0600	130.0863	65.5468	1

Table 3.3: Primary fractions with positive PCT identification. Each fraction (only final chromatography type included) lists its biochemical properties, immunoreactivity to the anti-canine PCT polyclonal antibody (BioVendor), and the results of the MS analysis including score. Identification of non-target proteins; albumin, haemoglobin, and thyroglobulin, are included.

	Biochemical properties	**Ab**	**MS (score, coverage)**
B15	Isoelectric point 5	Positive	113, 4 unique peptides, 61% coverage
B19	Isoelectric point 5.5	Positive	70, 3 unique peptides, 59% coverage
B21	Isoelectric point 6.5	Positive	197, 4 unique peptides, 61% coverage
B29	Isoelectric point >9	Positive	189, 4 unique peptides, 61% coverage
D20	MW 30-50 kDa	Positive	79, 3 unique peptides, 59% coverage
D30	MW <25 kDa	Positive	94, 1 unique peptide, 0.8% coverage
C12	Markedly hydrophilic	Positive	129, 3 unique peptides, 59% coverage
C18	Moderately hydrophilic	Positive	62, 4 unique peptides, 61% coverage

Table 3.4. A list of non-PCT protein hits from fraction AB29, including the protein name and species, its calculated MW (mass), and score. Queries matched is the number of individual peptide sequences matched to the Swiss-Prot database. A more complete list including irrelevant matches is included in the appendix (6.1.4).

Protein ID	Mass	Score	Queries matched
Apolipoprotein A-I OS=Canis lupus familiaris	30163	1062	25
Globin A2 OS=Canis lupus familiaris	16117	849	43
GLOBIN domain-containing protein OS=Canis lupus familiaris	16651	797	42
Globin A1 OS=Canis lupus familiaris	16183	733	40
GLOBIN domain-containing protein OS=Canis lupus familiaris	16717	681	39
hemoglobin subunit alpha OS=Vulpes vulpes	15382	547	76
GLOBIN domain-containing protein OS=Canis lupus familiaris	15339	519	76
Cationic trypsin OS=Bos taurus	25769	209	3
Adenylate kinase 2, mitochondrial OS=Vulpes vulpes	25690	96	2
Ig-like domain-containing protein OS=Canis lupus familiaris	11158	90	3
Pentaxin OS=Vulpes vulpes	27901	67	1
immunoglobulin lambda-1 light chain-like OS=Vulpes vulpes	24659	66	3

3.3.5 Identification of native canine PCT in septic dog plasma

Further MS analyses were conducted on six dog plasma samples, using MRM to quantitate the PCT present. One PCT peptide (LLLAALVK) was selected as the target peptide in these experiments because it was common to all the above samples, was an appropriate MW, and had a stable sequence in terms of trypsin digestion. A heavy-labelled peptide (HLP or ‘label’) was spiked into each sample as a control. Native cPCT was detected in one sample of plasma from a dog with severe sepsis (see Figure 3.9) but there was unfortunately no opportunity to repeat this analysis. Other clinical samples did not show native PCT content, and no PCT was ever detected in a clinical sample spiked with crPCT. These plasma samples were blotted with the BV anti-cPCT antibody with a positive band at the expected MW (see WB

results above; pooled as ‘sepsis’ or ‘normal’) but not run in the BV ELISA. Out of four batches, only one had successful analysis of the standards thus enabling creation of a standard curve. Analysis of standards in the same batch as the PCT-positive plasma sample was unsuccessful, and label spiked into the samples showed considerable variation (despite being added in equal amounts in all samples), so the native PCT in septic dog plasma could not be quantitated.

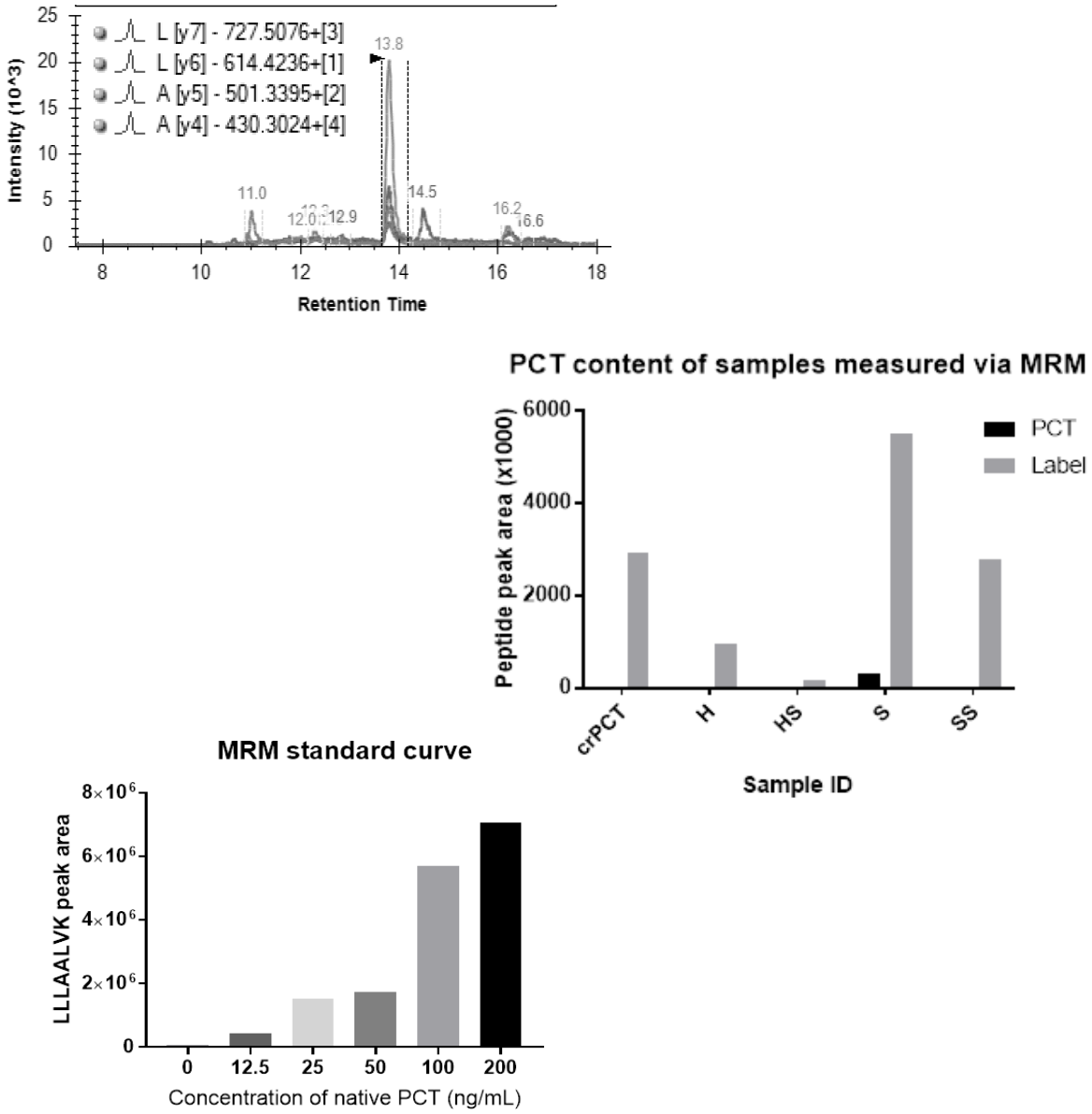


Figure 3.9: Top: MS/MS results including the unique product ions and the retention time of the target peptide (LLLAALVK). This is typical for samples where PCT was identified. Middle: Clinical samples and control samples. Non-spiked sepsis plasma (S) showed presence of PCT. Bottom: Successful standard curve. Only one of four batches produced a successful standard curve; analysis of clinical samples included in this batch failed.

3.4 Discussion

This chapter addressed the specific aims of purification of cPCT from thyroid in native form, and secondly to develop a quantitative assay. In addition, this chapter investigated the affinity of different PCT ELISAs.

3.4.1 Detection of native PCT in septic plasma sample

An interesting finding in this chapter was the detection of native PCT in septic dog plasma, although this is yet to be repeated. Additional samples expected to contain PCT returned a negative result. Because MS measurement involves matching molecular information to a protein sequence, and can be prone to assay failures and variation between results, it is more likely the positive result is a true result than the negative a false negative. Regardless, more study is required to confirm these findings. Several studies using ELISA have measured increased concentrations of plasma PCT during sepsis or SIRS in dogs (Battaglia et al., 2020; Easley et al., 2020; Goggs et al., 2018; Martiny & Goggs, 2019; Troia, Giunti, Calipa, et al., 2018; Troia, Giunti, & Goggs, 2018) (also see Appendix 6.1). Until now this has relied on the assumption that an unvalidated Pab detects native PCT as well as recombinant, and that the immunoreactivity is specific for PCT. This finding of PCT in septic dog plasma using MS therefore strongly supports this body of research, and their conclusion that PCT may be useful for sepsis management or diagnosis in dogs. Given that the current project's MS result was not repeatable for other samples, it cannot be inferred that every dog with sepsis has increased plasma PCT concentration. Recombinant cPCT spiked into samples was never detected – likely due to proteolytic enzymes or adsorption of proteins in the complex matrix

of plasma. Precipitation protocols to deplete high abundance or larger proteins may prevent potential masking of low-abundance, small PCT protein; different target peptides should also be tested in future (Feist & Hummon, 2015; Fricker, 2015).

Analysis of plasma samples in several studies is quantitated in the picogram range using the BV ELISA , detailed in Chapter One (Goggs et al., 2018). The MS assay appears equally sensitive, as hypothesized, and can detect both native and recombinant forms of PCT because it is able to match the peptides for each back to the same protein database entry. The MS analysis that detected the native PCT in plasma was not quantitative despite developments to this end. Antibody-independent quantitation of protein is valuable for validation and quantitation of ELISAs and similar immunoassays. However, recent development of a molecular imprinted polymer for rPCT offers an alternative to MS for this purpose (Battaglia et al., 2021) in research settings only.

In this project, no direct comparison was made of the PCT content in canine plasma from a dog with diagnosed sepsis, using ELISA (BV) and MS; each was only done separately. Unfortunately, the particular canine plasma samples available for the quantitative MS assay were not tested by BV ELISA due to insufficient resources. Plasma samples from dogs with sepsis were previously tested in the BV ELISA during this project and yielded a positive result; but these were no longer available for MS testing later in the project. This project has shown that ELISA methodology is superior to the MS approach for detection and measurement of canine PCT, namely in terms of logistics, availability, operator skill, and cost. The reliability of the results depends on the specific tests – but in the case of the BV

ELISA appears to perform better than MS (more reliable and perhaps more sensitive when sample preparation options are limited). In this case, the role of MS was to validate the ELISA further supporting its use – the MS methods described in this thesis are not recommended as the primary method for routine detection or measurement of canine PCT.

3.4.2 Native vs recombinant PCT affinity

Human and canine Pabs have a different affinity for PCT proteins of different origins, supporting one of the hypotheses that underpin this thesis. Cross-reactivity is present to PCT from a different source to that which raised the antibody (e.g. anti-crPCT antibody recognising native PCT) but is reduced compared with its source protein. Procalcitonin variants are similar enough that both native and recombinant could be effectively digested by trypsin and matched back to the database entry for PCT. Fractions with PCT-like immunoreactivity as detected by anti-human CT or anti-canine recombinant PCT antibodies, did in fact contain native PCT when analysed with MS. Thus, it appears that all forms of PCT (including from different species) have similar structure and will consistently show some cross-reactivity. A porcine anti-human CT antibody was successfully used to identify PCT in canine thyroid extract previously (Mol et al., 1991). Despite the encouraging results regarding affinity to multiple forms of PCT, Pabs used in this chapter also displayed non-specific binding or non-target protein recognition.

3.4.3 Purification

Purification of canine PCT in its native form was challenging and inefficient. This resulted in lower purity than expected, which could potentially impact Mab production. The purification difficulties were unexpected. Other researchers have successfully purified native cPCT and CT or its related proteins from other species (Barrett et al., 1968; Mol et al., 1991; Tenenhouse et al., 1965). This project used a greater amount of thyroid tissue than the study by Mol et al. (1991 – 5g), but the amount of thyroid is not specified in the other studies. These researchers used denaturing methods but noted that PCT was often low yield. In these cases, denatured vs native conformation was not a priority because protein use was generally limited to sequencing and characterization. Hilton and colleagues (1998) used a temporarily denaturing (acidic) extraction, and were able to demonstrate some biological activity in the resulting protein. Wimalawansa and colleagues (1993) showed high level purification of a protein related to PCT in its biologically active form using a tris-based buffer and affinity purification. Functional studies of CT typically extract recombinant protein under tris or phosphate-buffered saline system with protein protective additives and detergents (Tolun et al., 2007; Yubin et al., 2000).

In this study, several variations of methods preserving native structure were tried before the final purification method was chosen. Acetone, acid-denaturing, and detergents were avoided to preserve any post-translational modifications or any native characteristic that might be immunogenic, so that the antigen used for Mab production would be highly likely to recognise canine PCT if present in plasma from septic patients. This presumes that any

further modifications to PCT in sepsis are minor and do not alter its immunogenicity (e.g. Weglöhner et al., 2001).

The major challenges using the chosen methods included very small quantities of PCT, significant interference from other proteins, and lack of certainty surrounding the identification of PCT. For example, AB29 had very low reading from ELISA detecting canine PCT: lower than the other peaks detected in the same chromatograph, and minimally elevated from baseline. The peak was not further characterised because it fell at the completion of the chromatographic run and no further fractions were taken. And yet, this fraction had confirmed PCT content according to mass spectrometry and had relatively high purity according to densitometry (comparison of the putative PCT band with the entire bands (protein content) in the fraction.

Although denaturing methods could not be considered in this project due to the need for native PCT, they would likely have eliminated many of the issues encountered such as poor isolation (e.g via adsorption or protein binding) and contamination. There are other methods or approaches which may have benefited this project including immunodepletion of abundant proteins (e.g. albumin, thyroglobulin, haemoglobin). Also, sourcing PCT from different tissues may have allowed a greater yield. Concentrations of PCT in septic canine plasma is reported to be in pg/mL and thus is not a feasible source (Goggs et al. 2018; Weglöhner et al., 2001); but unlike physiological PCT or other APPs, constitutive PCT is also produced by several tissues of the body other than thyroid and liver, including lung, adipose tissue, monocytes and intestine, and these tissues harvested from euthanised dogs with

sepsis may have provided a greater yield (Jin M. & Khan A., 2010; Linscheid et al. 2003; Meisner, 2002; Meisner et al., 2003; Morgenthaler et al., 2003; Russwurm et al., 2001). Finally, antigen for Mab production could use denatured or recombinant PCT, and then use native PCT samples to cross-screen any resulting Mabs, ensuring they also recognise the native. Any immunogenic differences between denatured and native canine PCT are not characterised – since it's a small protein, any such differences may be minimal, but it should be assumed they exist until disproven.

True purity of the PCT in fractions could not be calculated with confidence. BV could not reliably quantitate PCT higher than 1008 picograms per mL due to its reported calibration range, confirmed by independent researchers (Battaglia et al., 2020; Goggs et al., 2018). As such, true fraction PCT content may be underestimated. Densitometry indicated purity of up to 42% in some fractions, based on narrow and distinct protein bands present at the expected molecular weight of PCT (11.5-12 kDa, up to 14.5kDa for prePCT). Thyroid tissue may contain all forms of PCT. Any or all of these could be detected by the Pabs since they display heterogeneity in epitope recognized (reviewed in Lipman et al., 2005). For example, Hilton et al. (1998) noted several fractions with CT immunoreactivity during a purification from rat tissues.

3.5 Conclusion

This chapter describes the partial purification of canine PCT in its native form. The primary fraction of interest, ABC18, was produced by cation exchange chromatography and RP-

HPLC, and prepared for inoculation into mice. Chapter Four will describe the process of Mab production using this antigen.

One remarkable result was PCT detected in a sample from a canine patient with sepsis. Unfortunately, this result could not be quantitated or repeated but it shows that the assay can detect the small amounts of PCT shown by ELISA to be present in the blood of dogs with sepsis. Method development was carried out for a quantitative MS using MRM. However, the assay could not be optimised during this project.

4 Chapter Four

Monoclonal antibody development and polyclonal antibody validation

4.1 Introduction

An ELISA or similar immunoassay using monoclonal antibodies to procalcitonin (PCT) is the recommended method to measure PCT in clinical samples. It would enable quantitative and qualitative measurement rapidly and with great accuracy, in addition to being relatively affordable and accessible to clinicians (Hosseini et al., 2018).

Several commercially available assays for PCT are available for research use only (listed in Table 1.3). They all use polyclonal antibodies (Pabs) which have various advantages and disadvantages compared with the monoclonal antibody (Mab) immunoassays used to measure human PCT (Lipman et al., 2005). For example, Pabs may be cheaper and quicker to produce, and may show good sensitivity and specificity since several unique antibodies may exist to different epitopes of the same target protein. However, this variety may also contribute to non-specific binding, and there can be variations between lots since each relies on the immune response of a unique animal making it inappropriate for routine clinical use. Production of a Mab that reliably detects recombinant and native cPCT is the next step in canine PCT clinical research.

Hybridomas are a common method of producing Mabs to a target protein. Hybridomas have some advantages over the other common production method of phage display, including

cheaper production, lower cross-reactivity, and less demanding laboratory protocols (Schmitz et al., 2000; Zhang, 2012). The immune system of a living animal responds to the presented antigen even if it is complex; thus, there is more scope for slightly lower purity of the antigen, or different matrix such as polyacrylamide gel like that obtained after gel electrophoresis of proteins. Conversely, the monoclonal response can take much longer since it is dependent on the normal functioning and timeframe of the adaptive immune system response.

In all available assays for canine PCT, the antigen used to raise the Pab is the recombinant PCT (rPCT) protein as produced in an *E. coli* vector (see 1.6). Such a protein would lack post-translational modifications that might occur in mammalian native PCT – for example, Jacobs and colleagues showed that PCT is glycosylated by mitochondrial membranes from canine pancreas (1981), and others showed that PCT in humans with sepsis is cleaved at the amino-terminal end (Weglöhner et al., 2001; Wrenger et al., 2000). Researchers have reported amyloid formation (Sletten et al., 1991) or that the protein may fold to form functional sites (Matera et al., 2012). For these reasons, the use of rPCT may not be optimal for producing Mabs with good recognition of native PCT present in sepsis. All three research groups who performed validation studies on cPCT ELISAs reported different performance or apparent affinity between variants of cPCT or speculated that difference between cPCT and rPCT could be the cause of the results (Battaglia et al., 2020; A. Floras et al., 2014; Goggs et al., 2018). Examples of this lack of equivalent antigenicity between recombinant and native proteins include C reactive protein (CRP) where a recombinant epitope (unglycosylated) may be completely biologically irrelevant (S Yamamoto et al., 1993).

Antibodies used in immunoassays are rarely validated to a high degree. Lack of reproducibility in research that uses antibodies has raised questions about the quality and validity of many antibodies assumed to be fit for purpose – this widespread problem has affected a considerable amount of research, Berglund and colleagues (2008) suggesting more than 50% of thousands of antibodies analysed bioinformatically did not have the expected affinity. Recent reviews have developed and recommended stringent validation standards for any antibody to ensure quality and reproducibility (Bradbury & Plückthun, 2015; Edfors et al., 2018; Uhlen et al., 2016). These validation steps are independent to any subsequent analytical or clinical validation of an immunoassay or other application of that antibody.

This project aims to develop an ELISA using Mabs with high affinity for the native form of cPCT. The previous chapter described purification of cPCT from thyroid tissue. This work described in this chapter aims to use the native, naturally-occurring cPCT protein as the antigen to produce hybridomas and thus Mabs. It was the intent that resulting antibodies would be validated to a high degree in accordance with recent recommendations, to verify their affinity for native PCT as well as recombinant PCT. It was hypothesised that an immunoassay using Mabs against native cPCT will detect cPCT in canine plasma with greater affinity than tests currently available.

4.2 Methods

4.2.1 Materials

BioVendor brand a-cPCT antibody was the Pab used for control. Dynabeads were from ThermoFisher. Non-denatured, native canine PCT (cnPCT) protein was obtained through purification from dog thyroid tissue as described in Chapter Three, and select immunoprecipitation protocols were repeated with plasma from dogs with sepsis. Reagents were from Sigma unless otherwise stated. Materials and reagents for PAGE and immunoblotting are the same as described in Chapter Three, with differences noted in the text as applicable. Mass spectrometry was outsourced to Proteomics International and the Mab was produced at the Monoclonal Antibody facility, both at Harry Perkins Institute in Perth, Western Australia.

4.2.2 Production of monoclonal antibodies

4.2.2.1 Immunisation of mice and screening of sera

The immunisation protocol was granted ethics approval (#AE134, Perkins Institute Animal Ethics Committee). Specialist work including immunisations, bleeds, splenic harvest, and the initial screening ELISA were performed by staff at the Monoclonal Antibody Lab (Harry Perkins North, WA, Australia). This monoclonal development, including hybridisation and clone isolation, was supported by a grant from the Murdoch University Vet Pet Trust, Caring for Pets. For each immunisation, four healthy AJ female mice each received intraperitoneal

injections of a mix of 20 µL of Advax (Vaxine) with either 50 µg protein in aqueous solution or 15 µg of protein in minced polyacrylamide gel.

Fractions used were ABC18 and ABC12 in combination for the first and final immunizations, ABC18 (from AB19, AB21 and AB29) in SDS-PAGE gel slice for the second and third immunizations, and AB29-U for the fourth. Figure 4.1A shows the immunization fractions as run on SDS-PAGE.

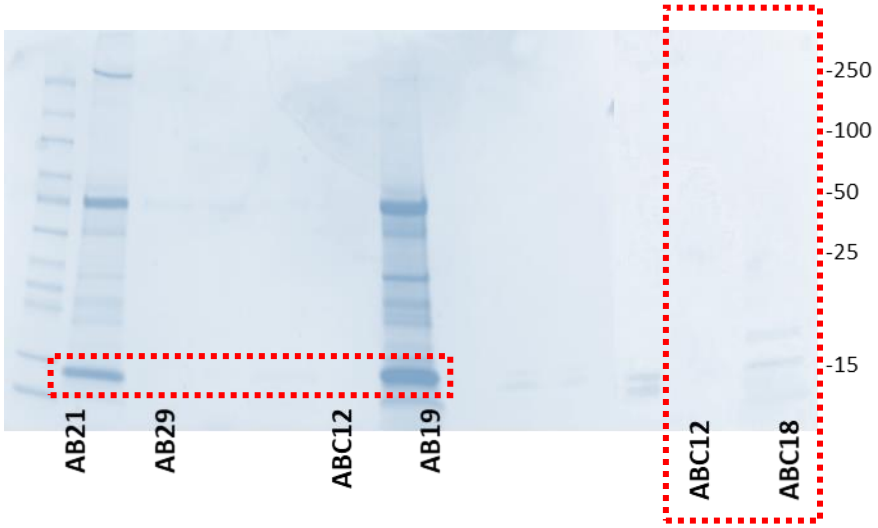


Figure 4.1A. Immunization fractions as run on SDS-PAGE. AB21, AB19, and ABC18 each have a band at the 14-15 kDa MW range and have a positive result on BV cPCT ELISA, with confirmed presence of PCT by a positive protein identification of a fraction using MS. Some immunizations used the entire fraction (AB29 and ABC12 and ABC18) while others used MW-restricted preparations such as gel-slices or fractions then ultrafiltrated to retain proteins less than 30 kDa (see Chapter 3).

4.2.2.2 Timeline

Three initial injections were performed at approximately four or five week intervals, followed by tail bleeding for ELISA screening of sera after ten days. Low titres dictated a subsequent booster, and the immunisation course was completed with a final aqueous boost ten days prior to splenic harvesting. Immediately before harvest, sera were drawn by tail bleed and re-screened via ELISA and Western blotting. The timing is shown diagrammatically in Figure 4.1.

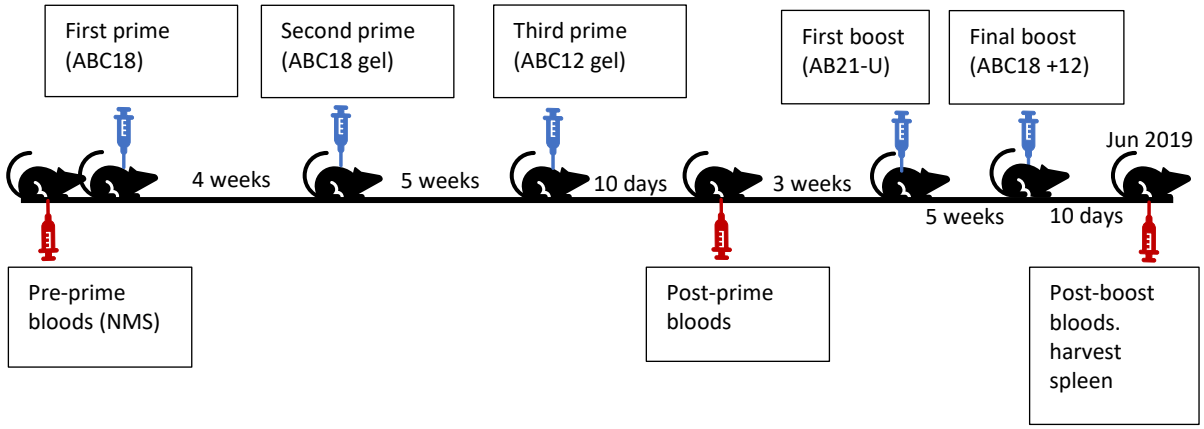


Figure 4.1: Immunisation timeline. The immunisation protocol followed a standard priming and boosting protocol with varying intervals allowing for antibody response, depending on the titre results. A variety of available PCT fractions were mixed in a best effort to boost the PCT component, since there was insufficient quantity of any maximal purity fraction to complete the course.

4.2.2.3 Hybridoma production and parental line screening

Fusion with myeloma lines and selection of hybridomas were performed according to standard protocols. Single cell suspensions from splenic pulp of Mouse 1 were mixed with 10^8 Sp2/O Ag-14 myeloma cells, fused using polyethylene glycol (PEG, 15,000 MW, Sigma) and added into 96 well plates (Nunc) in Hybridoma-SFM (Gibco)/5% FBS (Gibco). Immortal, fused hybrids were selected by use of HAT medium (Hypoxanthine, Aminopterin, Thymidine) according to standard protocols (Davis et al., 1982).

Once confluent, wells were screened via ELISA against two antigen sources: cnPCT protein in solution from aqueous fractions (either AB21 or 29; or AB21-U or 29-U). The specific ELISA

method is described later in this chapter. Five hybridomas positive to either of the native antigens or both, were expanded and supernatant collected for freezing and re-screening via ELISA and Western blotting. For both of these, the antigen used was the immunization fraction e.g. ABC18; and the hybridoma supernatant was used as the primary antibody mixed neat 1:2 with blocking buffer. Secondary antibody was anti-mouse IgG, Fc-region specific, HRP-conjugated antibody in the ELISA, and goat anti-mouse conjugated to Alexa Fluor 680 for the Western blotting. For Western blotting a 1:2 dilution into blocking buffer yielded no clear results, thus antibody was precipitated with 60% ammonium sulphate and the pellet de-salted by filtering through a <100 kDa MW cut-off (Milli-pore, Merck) before being re-suspended into 1 x TBS pH 7.6 at a concentration of 0.3 mg/mL as determined by BCA assay (described in Chapter Three). This preparation was diluted 1:2 with blocking buffer. The specific methods for blotting are the same as those described in Chapter Three unless otherwise noted.

4.2.2.4 Monoclonal antibody production and screening

Of the five parental lines, ELISA screening was repeated with the native antigens AB21, AB21-U, and the recombinant PCT (from BioVendor, designated BV). The two with highest sensitivity were selected for cloning. Their primary frozen stock was thawed and hybridomas cloned by limiting dilution. Once clonal, hybridomas were screened for IgG production by coating an ELISA plate with a gamma chain-specific goat anti-mouse antibody, before applying neat supernatant to incubate for 1h then washed out, and detecting enzymatically according to the same ELISA method as 4.2.2.3 above (probing with anti-mouse FC region,

HRP-conjugated antibody). From each of the two chosen parental lines, three clones (six in total) with high absorbances (>1.0 O.D. at 405 nm wavelength) and healthy growth were further selected for expansion, and supernatant collected, before selection of the two most stable clones for expansion into 1% foetal bovine serum (FBS) and final screening via ELISA and WB.

4.2.2.5 Purification of antibody

Each of the five parental lines was re-drawn from frozen stock and expanded to 1 L for purification of IgG by affinity chromatography on protein G-Sepharose 4B Fast Flow (poured in-house). Supernatant from clones was also purified after immunoassay screening. Supernatant was filtered under vacuum through 0.45 micron nitrocellulose membrane (GVS north America, Sanford ME), then was applied to the washed column at 4 mL/sec using a BioLogic DuoFlow system (BioRad) at 4°C. The column was then rinsed with PBS; antibody was eluted with 0.1 glycine pH 2.7, concentrated via ultrafiltration (50 kDa MWCO) and dialyzed through 10 kDa snakeskin dialysis tubing (ThermoScientific) stirred in 1x PBS at 4°C for at least 24h. Protein concentration and purity were determined by applying samples of eluent to chip-based electrophoresis (outsourced to AGRF, Nedlands, WA, Australia). Solutions were then normalised to 1 mg/mL. The majority of the purification was performed by Monoclonal Antibody Laboratory staff.

4.2.3 Screening, detection, and antibody validation

4.2.3.1 Indirect enzyme-linked immunosorbent assay (ELISA)

To screen antibodies, ELISA was performed according to the following protocol. Immunization antigen was diluted to 2 µg/mL in fresh carbonate coating buffer, pH 9.6 with 1% Hyclone (Cytiva). Each antigen was plated into a different row, when multiple were used in the same plate. Then 50uL was applied to a 96-well plate (Corning) and incubated overnight at 4°C. The plate was then washed three times by immersing in 1x PBS-T. To screen mouse vaccination titres, 100uL of a 1:100 serum dilution into PBS-1% Hyclone was added to the first well and serially diluted 1:2 across the plate in duplicate. To screen hybridoma production, 50 µL of supernatant was added neat to wells in duplicate. The plate was incubated at room temperature for 1 hour, before discarding the primary solution and washing thrice as above; then 50uL of HRP-conjugated goat anti-mouse IgG (ThermoFisher) diluted 1:1000 (or 1:5000 for sera) in loading solution was added to all wells. The plate was incubated for 45 minutes at room temperature before removing the secondary solution and washing as above, then the plate was rinsed with deionised water to remove Tween-20 residue. Substrate (ABTS, citric acid, H_2O_2) was used to develop colour by adding 50 µL to each well and incubating in the dark, and absorbances were read at 405 nm using a spectrophotometer (Viktor Plate Reader, PerkinElmer) at 15 minutes. Positive controls were normal mouse serum (NMS) for antibody and recombinant cPCT (BV) for antigen; negative controls were an irrelevant immunoglobulin for primary antibody, and plain cell culture media for antigen.

In subsequent ELISAs that tested antibody affinity, the same method was used but instead coating the plate with different antigens as noted in the results.

4.2.3.2 Western immunoblotting (WB)

General methods and materials of SDS-PAGE and immunoblotting were the same as that described in Chapter Three. Positive controls include crPCT (BV), native PCT in complex and purified preparations, and septic dog plasma with a presumed increased cnPCT content. Negative controls include BSA, canine tissue with no cPCT expression theoretically (healthy plasma).

4.2.3.3 Immunoprecipitation

For immunoprecipitation, coated magnetic beads (Protein G Dynabeads, ThermoFisher) were used to bind antibodies and create affinity chromatography columns according to the manufacturer's instructions. A given antibody was coated onto a 50 µL aliquot of beads, and 5 µg of antibody diluted in 200 µL of 1x PBS was applied before mixing the beads gently to suspend and rotating the tube for ten minutes at room temperature. Beads were then pelleted with a rare earth magnet before removing supernatant and washing (with resuspension) with 1x PBST. Antigen sample, between 500-1000 µL, was then added to the bead-antibody complex which was gently resuspended and rolled for ten minutes at room temperature. After pelleting the beads, supernatant was removed and reserved for analysis. Beads were washed as above three times in PBS-T then the suspended beads were transferred to a clean tube and pelleted before adding 20 µL of 0.1 glycine pH 2.7 to elute

antigen. Eluted proteins were either immediately neutralized with 0.5 µL of saturated tris and Laemmli sample loading buffer for SDS-PAGE; or sent for MS protein identification (Immunoprecipitation MS or IMS).

4.2.4 Antigenicity of canine PCT

The published sequence of canine preprocalcitonin was used in several online tools to predict the antigenicity of cPCT. The sequence was accessed via NCBI Protein[1] which is based on research by Mol and colleagues (1991), and supplemented by additional studies by Wende and colleagues (2000). Procalcitonin structure and potential antigenicity was analysed *in silico* with several online tools. Homology between species and sequences of PCT isomers or variants were analysed with Clustal Omega (Madeira et al., 2019) and visualized with Jmol (Cohlberg, 2021). Three-dimensional structure was predicted with Phyre2 (Kelley et al., 2015). Epitope prediction programs were BcePred b (Saha & Raghava, 2004), BepiPred-2.0 (Jespersen et al., 2017), IEDB T cell (Vita et al., 2019), Imed – b (accessed 2017) (Sanchez-Trincado et al., 2017).

4.3 Results

This chapter reports production of two Mabs, and furthers the validation of a commercially available antibody (BV) by confirming for the first time that it detects naturally-occurring PCT in biological samples using MS.

4.3.1 Polyclonal anti-canine PCT antibody binds native PCT

A commercially available polyclonal antibody (BV) was used throughout these experiments as a control for cPCT immunoreactivity and monoclonal validation. During

[1] https://www.ncbi.nlm.nih.gov/protein?cmd=retrieve

immunoprecipitation, this antibody bound PCT from a canine thyroid tissue extract (AB21, AB29). The elution from the protein G-Pab affinity column had protein hits from the CALCA gene including PCT and calcitonin gene-related peptide. Procalcitonin had a high score of 232, and four unique peptides from PCT were detected matching to a parent protein of 14 kDa consistent with preprocalcitonin as listed in the SwisProt database. This data is presented in 4.3.2.3.

4.3.2 Production of monoclonal antibodies

4.3.2.1 Sera screening

Each mouse responded to the antigen used for immunisation. An ELISA was used to measure titres, and immunoblotting showed the response specificity. Screening of post-prime and post-boost sera showed that titres overall were consistently lower than expected, and at a sub-optimal level (2 or above is considered optimal). The post-prime titres ranged from 0.88 to 1.24, after subtracting the normal mouse serum values as a blank. Screening of the final post-boost sera at the time of splenic harvest, showed that the titres had increased but were still lower than optimal with the highest being Mouse 1 with 1.91 absorbance. The ELISA results are shown in Figure 4.2.

Mouse 1 and Mouse 2 had the highest titre to the antigen of interest (1.91 and 1.74 respectively, after accounting for normal mouse serum control), and all mice showed very low reactivity to BV protein with a maximum of 0.049 absorbance. All four mice showed higher recognition to the same pooled fraction fresh than frozen which indicates that the

immunogenic component degraded with storage. On immunoblotting, each mouse showed a very similar profile including a distinct band at approximately 12 kDa, and in some cases a distinct band at approximately 28 kDa. No sera detected the BV protein (see Figure 4.2).

Mouse 1 was selected for hybridoma production because it had one of the highest titres to the antigen, a clear and relatively dark band at the approximate molecular weight of PCT, and comparatively few bands or smears at higher molecular weight (notably compared to Mouse 2). However, the difference between mice was minimal.

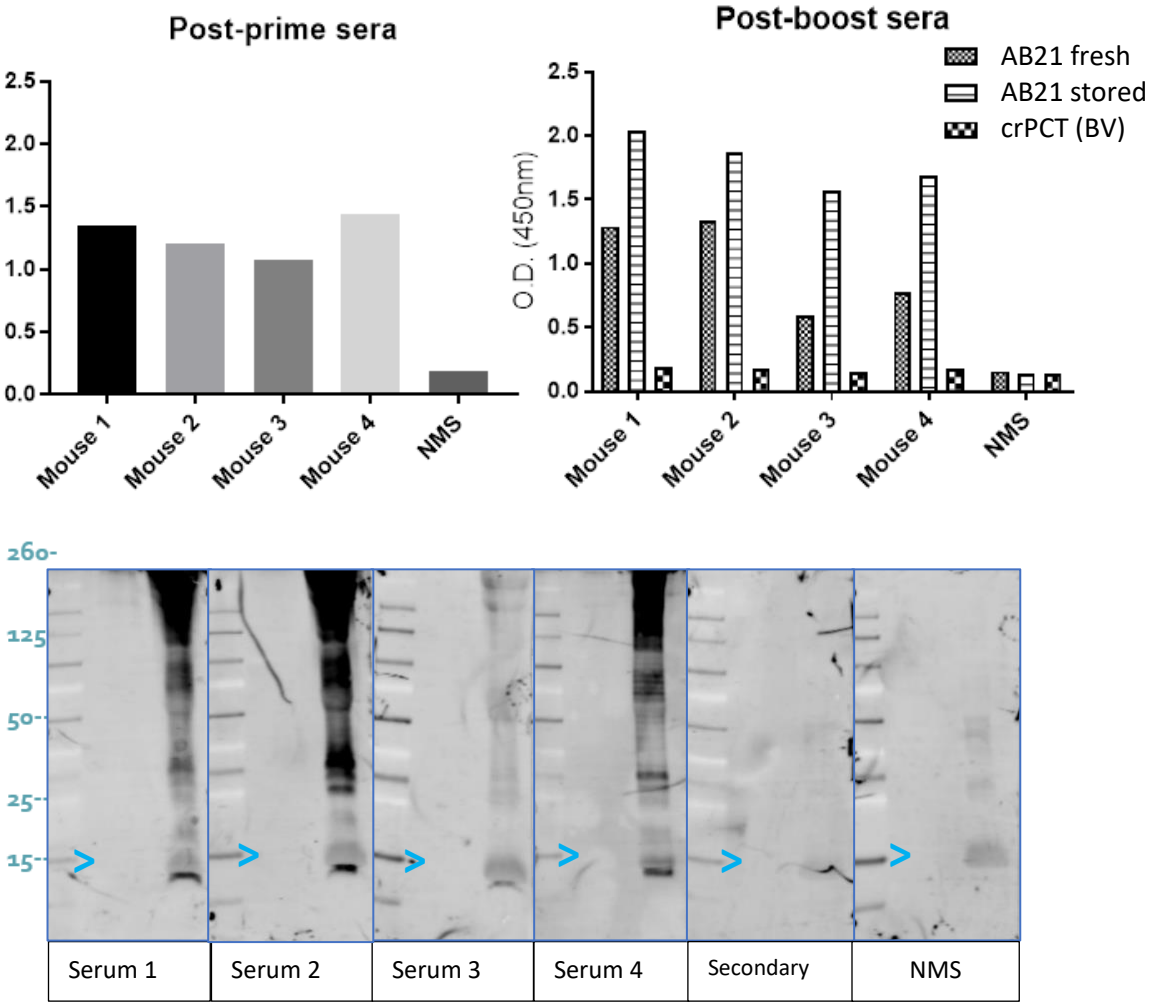


Figure 4.2: Production of antibody by four mice, to native PCT antigen preparations. Top: ELISA results of initial serum screening after immunization. All mice have elevated titre compared with non-immunized mice (NMS). The highest titres are seen in mouse 1 and mouse 4, and mouse 2 to some samples post-boosting. Bottom: immunoblots. From left to right each blot includes a ladder, an aliquot of BV, and then an aliquot containing extract A. Mouse 1 = serum 1 etc. Blue arrowheads: 15kDa. Mouse 2 seems to have most response but this includes the highest response for high MW proteins.

4.3.2.2 Parental screening

After successful fusion a total of four 96-well plates were established and showed good growth rates. Because the sera showed reactivity to such a wide range of molecular weights, initial screening of these parental lines used two antigen sources: AB21; and AB21-U which contained only proteins less than 30kDa. The parental lines in well 7F2 had the highest reactivity to the ultrafiltrated fraction (shown in Figure 4.3 A) and BV. After two months of stabilisation, all five parentals showed dual reactivity to native and recombinant PCT (Figure 4.3 B) although in some cases hardly above the non-immunised mouse control (NMS) and therefore of questionable significance.

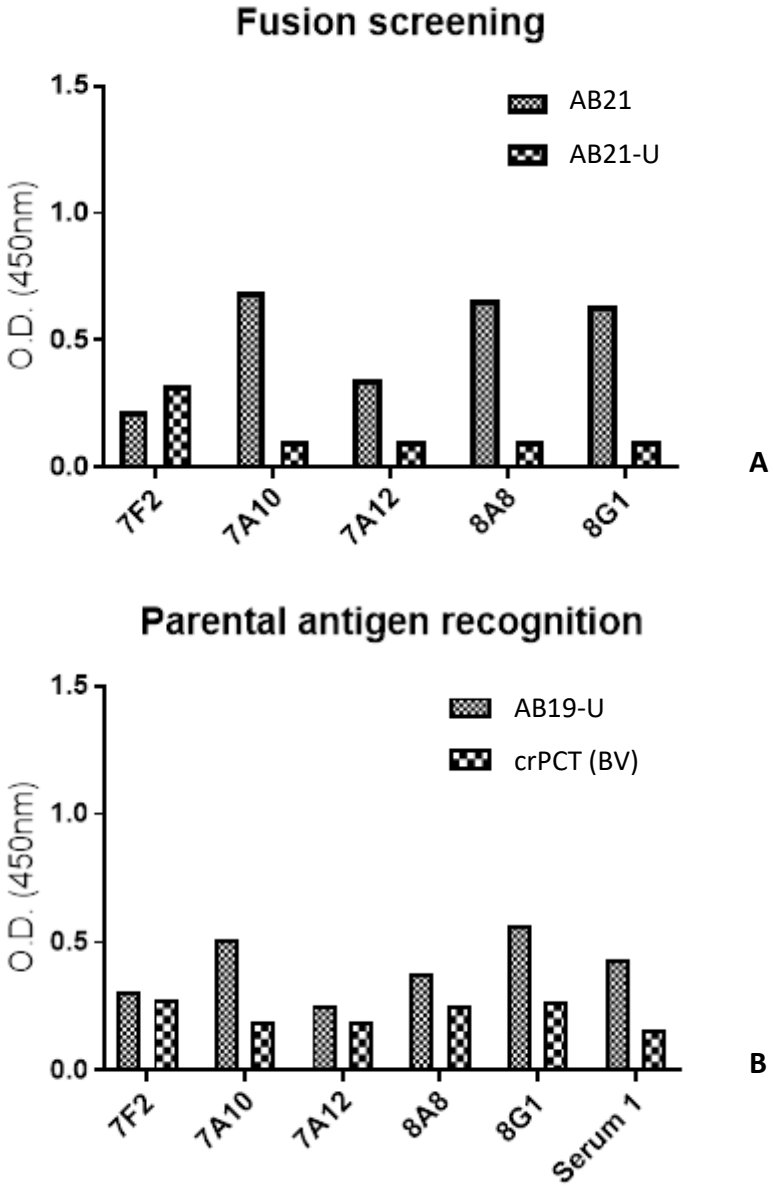


Figure 4.3: Parentals screening after fusion then after growth for 2 months. **A**: Initial ELISAs screening the hybridomas after fusion: 7F2 had the lowest recognition of the C21 fraction, but the highest recognition for the same protein source after enrichment for small proteins less than 30kDa (C21-U). **B**: After a period of growth for and purification: 7F2 and 8G1 have the best antigen recognition profiles but all have low absorbance values.

Based on the above results, the parentals were expanded for one month in 500 mL flasks and the supernatant purified using protein G affinity purification selecting for IgG. The results are shown in Figure 4.4 and indicated that 7A10, 8A8, and 8G1 were better candidates – although none were ideal. For example, 8A8 had the highest titre to recombinant PCT, but had disappointing results on immunoblotting. Two parentals, 7A10 and 8G1 were selected for cloning, since 8A8 was rejected based on its blot results. Some of these fractions showed markedly reduced recognition by the BV Pab after prolonged storage (18 months) than in previous blots.

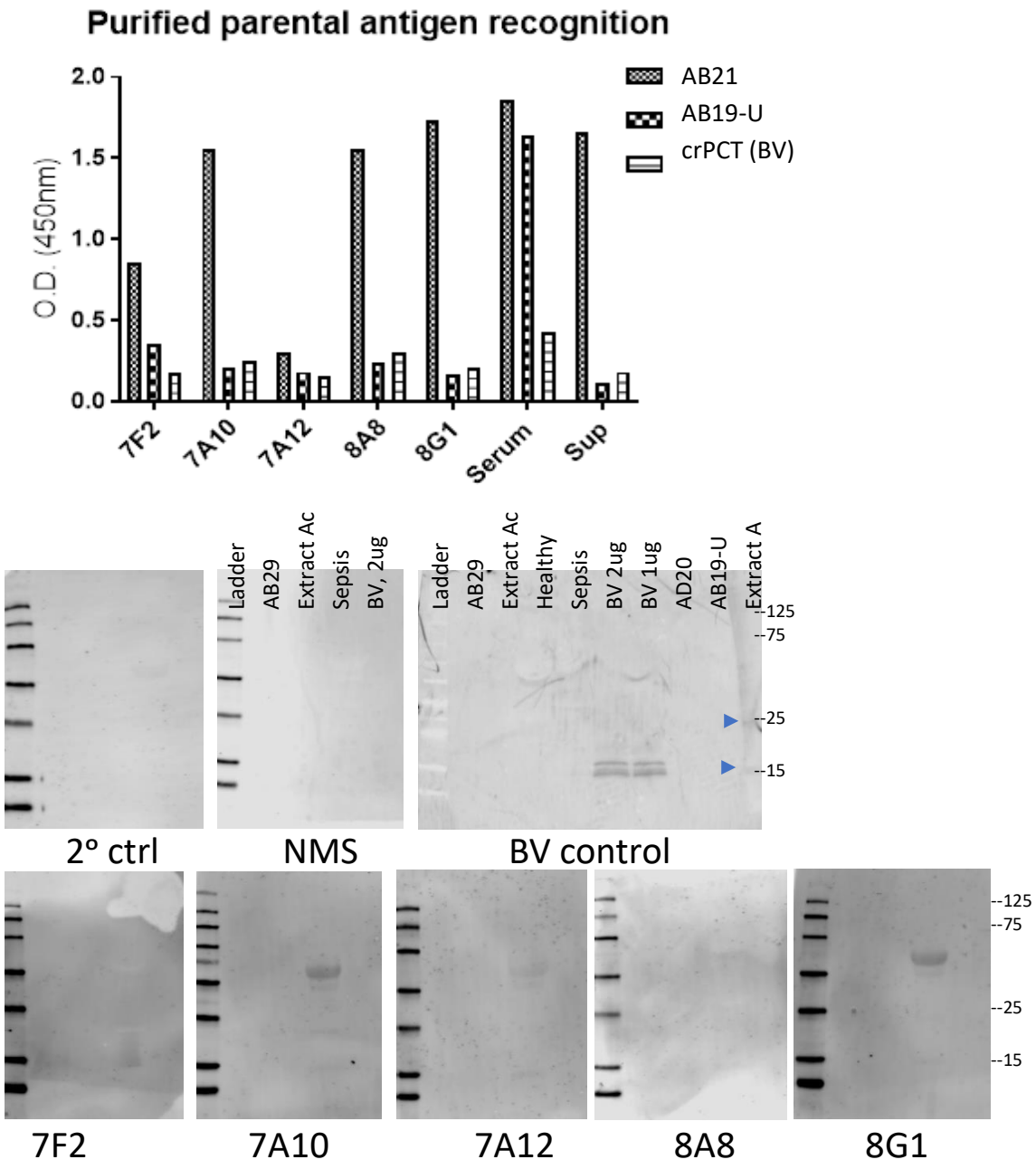


Figure 4.4: Antigen recognition of parentals after purification. **Top**: 7A10 and 8G1 had highest reactivity to AB21, low reactivity to AB21-U, and improved reactivity to recombinant PCT compared with prior screenings. **B**: All parental lines had similar antigen recognition profiles. 8A8 had poorest blotting performance. Blood samples are plasma. All small blots have the same lanes.

4.3.2.3 Monoclonal screening

Two parental lines were cloned successfully by limiting dilution to produce K9 (from 8G1) and 2E12 (from 7A10). These, along with the BV Pab as positive control, were the three antibodies used for the remainder of the antibody screening and assessment. ELISAs using these Mabs produced a profile of reactivity for each (shown in Figure 4.5). Only the polyclonal (BV) recognised the recombinant PCT protein (BV protein), with K9 showing negligible reactivity to this antigen, and 2E12 showing none. The BV antibody showed nearly equal immunoreactivity to the native fraction AB21. But it is unlikely that this was solely due to native PCT since it also showed high immunoreactivity to healthy dog plasma and haemolysate of healthy dog blood (a proxy for canine haemoglobin). The 2E12 monoclonal was the only antibody to have a higher reading for sepsis plasma than for plasma from healthy dogs.

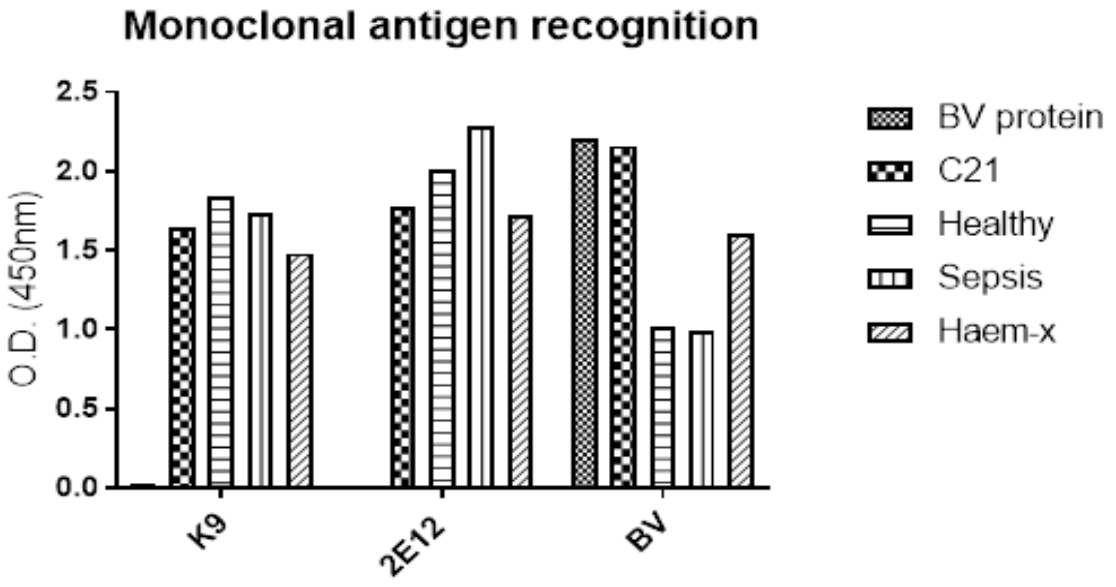


Figure 4.5: Antigen affinity profile for three antibodies including polyclonal (BV) and two novel monoclonals (K9 and 2E12). BV is the only Ab to have significant recognition of crPCT (BV protein). Haem-x = haemolysate. Healthy and sepsis samples are from canine plasma. C21 = AB21 (c for cation exchange).

The final antibody assessment was immunoprecipitation (IP), which allowed protein to be taken up from samples by affinity chromatography and then eluted for further analysis. This process showed that the BV Pab detected native PCT from thyroid tissue extracts. The Pab and K9 Mab both bound antigen from the native fraction (AB29) sufficient to be detected in SDS-PAGE after elution. For both antibodies, the native protein eluted was 12-14 kDa. The BV Pab showed good uptake of the crPCT protein but the K9 Mab did not (see Figure 4.6). In

Figure 4.6, the eluate from each antibody immunoprecipitation is shown on SDS-PAGE alongside aliquots from other parts of the procedure (wash, beads after washing etc.).

Figure 4.6: SDS-PAGE of immunoprecipitation products for two antibodies. Top antibody: a commercial polyclonal (BioVendor, BV). Bottom antibody: a novel monoclonal (K9). Each antibody was tested with two antigens: native PCT source (N) and BioVendor crPCT (BV). Only the BioVendor antibody shows specific binding and elution of enough protein to show

in the gel (see top gel, 'Elution' columns). Native cPCT antigen source: AB29. N=native, BV=crPCT by BioVendor, Beads=Protein G Dynabeads, after washing. "Beads only"=an aliquot of beads never subjected to immunoprecipitation. Arrowheads show 15 kDa.

The antigen of each antibody was conclusively determined by repeating the IP protocol with thyroid extract fractions, and submitting the eluted protein from each antibody for protein identification with IMS. The BV polyclonal was the only antibody that detected PCT or any protein from the same gene family. The BV protein also isolated other proteins from the fraction, including thyroglobulin (also detected with a high score by 2E12), haemoglobin and albumin (detected by all antibodies). However, it is not possible to determine if these were non-specific binding by the antibodies, or present in the samples due to contamination or protein aggregation. Haemoglobin was a contaminant in many fractions and also adsorbed to tubes, gel beds, and immunoprecipitation beads – so aggregation and adsorption is likely. The IMS results are summarized in Table 4.1.

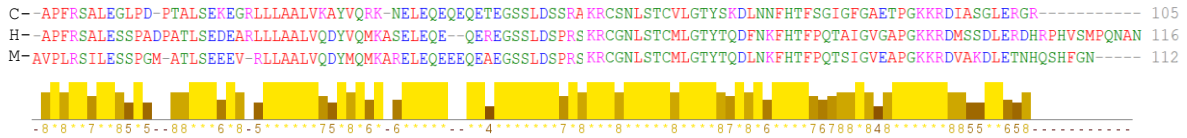
Table 4.1: Immunoprecipitation mass spectrometry results for protein bound by three antibodies: a-crPCT (BV), Mab K9, and Mab 2E12. Only the BV antibody detected PCT.

	Antibodies		
Protein hit	**K9**	**2E12**	**BV**
PCT	Not found	Not found	Score 232 14,042, 4 peptides Also CGRP (score 58)
Thyroglobulin	Not found	Present Score 184, single hit	Present Score 111, 84. Two hits

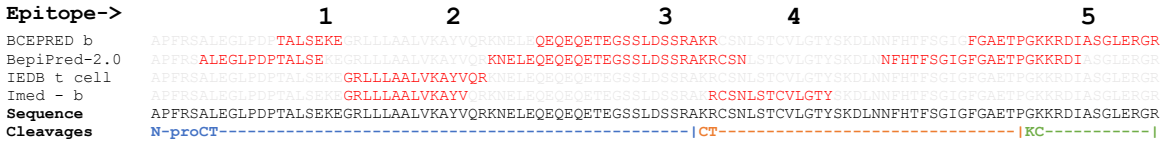
Haemoglobin/globin 12.5-16.5 kDa, usu 15.5	Abundant, 9 hits, several isomers	Abundant, 10 hits, several isomers	Abundant, >11 hits, several isomers
Albumin	Minor	Minor	Minor
Other hits from corresponding protein ID of B21	None	Alpha fetoprotein	None

4.3.3 Antigenicity of canine PCT

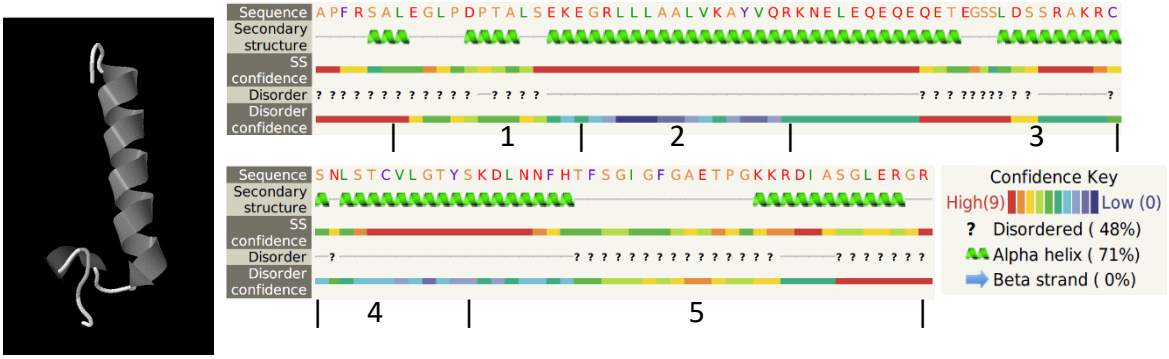
The antigenicity of cPCT in mice was investigated using basic comparative molecular biology and bioinformatics tools. There is moderately high homology between mouse and canine PCT, about 60%. As a small precursor protein, PCT structure is predicted to be primarily alpha helix. No significant sequence similarity was detected in a Blast comparing PCT with albumin or haemoglobin subunits. Finally, potential epitopes were indicated on almost every section of PCT (see Figure 4.7).



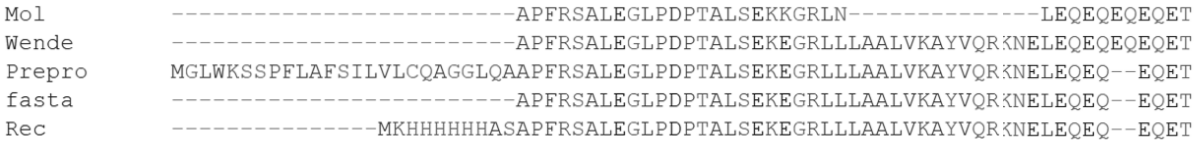
A



B



C



D

Figure 4.7: Conservation, structure, and predicted antigenicity of canine PCT. **A**: PCT is highly conserved between species (canine (C), human (H), and mouse (M)). **B**: Predicted epitopes vary, using five different online tools. **C**: Predicted structure based on homology modelling. The 3-D image inset only depicts CT. **D**: Comparison of sequences of canine PCT from different references (in methods).

4.4 Discussion

This chapter aimed to develop an anti-canine native PCT Mab that could be used in an immunoassay to diagnose sepsis rapidly in dogs, using native PCT purified from canine thyroids as described in Chapter Three.

4.4.1 Validation of a commercial polyclonal antibody

Importantly, this study confirms that a commercial Pab does bind native cPCT, as shown for the first time by immunoprecipitation and MS. This confirmation completes one of the validation steps recommended by Uhlen, Edfors and colleagues (Edfors et al., 2018; Uhlen et al., 2016), by independently and qualitatively confirming the affinity of the antibody for the native version of PCT present in canine tissues. However, this antibody also demonstrates non-specific binding to other proteins or substantial interference from abundant proteins. Haemoglobin and albumin were contaminants visualised or detected in ELISA, immunoblots, or MS. This may be due to the polyclonal nature of the antibody, given that there is no apparent homology between cPCT and these proteins, as determined by a BLAST. The finding of cross-reactivity between the Pab and native PCT supports the findings of Chapter Three in refuting the hypothesis that an antibody raised against crPCT, or PCT from another species, cannot recognise native canine PCT.

4.4.2 Monoclonal production

Monoclonal antibodies were produced but they did not recognize PCT, despite careful adaptations of the method to increase success. Therefore, this project was unable to

produce or optimise a novel quantitative immunoassay. When considering only the methods employed in this project, native PCT protein isolated from canine thyroid glands cannot be used to create anti-cPCT Mabs using mouse hybridoma methodology. Possibly these methods could be improved and refined to improve isolation and purification of PCT sufficient for mouse Mab production, but most known issues related to interference from other native proteins which would be difficult to overcome when using all native-friendly methods. Specialised methods such as immunoprecipitation may be required to adequately enhance these methods.

A likely cause for the lack of recognition of PCT by Mabs is insufficient purity of the antigen. Although the immunisation protocol and screening process both utilised a combination of canine thyroid fractions to maximise selection for PCT rather than other contaminants, low purity could still explain this failure. Contaminants may have been more abundant or more antigenic, or may have accelerated the degradation or adsorption of PCT. Monoclonal antibody production is less sensitive to low purity in the antigen source than phage display (Lipman et al., 2005; Schmitz et al., 2000). The disadvantages of using gel slices (denatured protein, smaller amounts of protein) were hoped to be counteracted by the benefits of size-restriction, and additional adjunct functionality of the gel matrix (Fuller et al., 1992; Greenfield et al., 2019; Pupo et al., 1999). But purity should still ideally be above 50%. The fractions used in this project were maximum 42% pure, and this may have been an over-estimate since it was based on densitometry. On the other hand, the BV ELISA would have been unable to measure protein concentrations higher than 1008 pg/mL (even if more than that was present) because it falls outside its calibration range. Proteins 10-15 kDa bound by

the BV Pab during Chapter Three were expected to be predominantly cPCT; however, IMS showed that several other proteins including haemoglobin subunits were also present in bands of this molecular weight, since the MW of a Hb monomer is 15-16 kDa.

A second possible reason is that canine PCT may be too similar to mouse PCT. Human PCT Mabs were successfully raised in mice using peptides (Ghillani et al., 1988; Herbst & Klein, 1995) but similar homology between mouse and canine does not guarantee sufficient antigenicity. Specific peptides that avoid the highly conserved CT region of the protein may be sufficiently antigenic, and the resulting Mabs could be screened against a native PCT source to identify Mabs that recognise both the peptides and full native protein in plasma samples.

A significant limitation during this study was the time taken to adequately purify antigen and develop and screen monoclonals, as well as the small amount of resources (only 16 thyroids). Quantity of native PCT, a low-abundance protein, was restricted; and production of an optimal Mab can take several months or years since it relies on a random biological process and growth of cell lines can take considerable time.

4.5 Conclusions

A commercially available anti-canine PCT Pab binds both recombinant and native canine PCT from thyroid tissue. This antibody can be used with confidence to quantitate cPCT in biological samples, bearing in mind that it may show batch variability as a polyclonal.

Development of Mabs to cPCT remains uncompleted and future researchers should focus on preparation of an improved source of antigen for this purpose.

5 Chapter Five

General Discussion

5.1 Thesis summary

The overarching aim of this project was to develop an improved assay for more rapid diagnosis of sepsis in dogs. The mortality of sepsis in dogs is often as high as 50%, and sepsis is associated with high cost and length of hospitalization yet is difficult to diagnose early enough to significantly improve outcomes. A rapid assay would lead to better outcomes for dogs with sepsis by providing veterinarians with a means to recognize and treat in the early phase of disease. Based on a review of the current literature of sepsis biomarkers and diagnostic methods, this thesis proposed the best way to improve sepsis diagnosis in dogs would be to focus on canine procalcitonin (PCT) as a rapid biomarker of inflammation caused by infection, as in humans (reviewed in Chapter One). Therefore, it was planned to develop a canine native PCT Mab, validated with MS, employed in an ELISA. Additional epidemiological data on canine sepsis was also identified as a need, leading to a study aiming to investigate the current impact of sepsis in a large study of dogs.

Chapter One also reviewed several studies reporting on existing canine PCT immunoassays. This comprehensive critique outlined the current progress in cPCT research, including some key findings published during the course of this project (Goggs et al., 2018), and provides a detailed background of the problems previously observed in some cPCT ELISAs that this

project was aiming to improve on. Based on the findings of this review, recommendations were made to improve the existing cPCT ELISA and direct future studies, for instance the importance of Mabs and calibration studies. This chapter concluded that PCT is a rapid indicator of sepsis and SIRS severity in dogs and could be used to determine prognosis.

Chapter Two consisted of a three months duration, large-scale epidemiological study of owned dogs admitted to a veterinary hospital. Dogs diagnosed with sepsis, non-infectious systemic inflammatory response syndrome (SIRS), and infection, were compared with each other and the remainder of the hospitalized dogs served as a control. This study confirmed previous studies that sepsis is associated with high mortality (35.1%, increasing above 50% with organ failure), and higher cost and length of hospitalisation compared with infection or non-infectious SIRS. This study showed for the first time that 72% of deaths from sepsis occur in the first 24 hours after diagnosis, highlighting a need for a rapid test for sepsis.

The rationale and process of obtaining native canine PCT to use as the antigen for a Mab, was outlined in Chapter Three. Purification was performed on thyroid glands of dogs using traditional chromatographic methods such as ion exchange and high-performance liquid chromatography. This process was guided by anti-canine PCT antibodies and confirmed with mass spectrometry – and the ability of each of these methods to detect different forms of PCT was also examined. The purification was not fully optimized (maximum 42%) but did yield several fractions with PCT immunoreactivity which were confirmed to contain PCT, and the antibodies were confirmed to have better affinity to the version of PCT to which they were raised compared with other species or conformations. Finally, a sample of plasma

from a dog with sepsis was confirmed to contain PCT, during development of a quantitative mass spectrometry assay.

Chapter Four described the process of Mab production, using the antigen preparation produced in Chapter Three. Hybridomas were developed and screened against native and recombinant antigen, and the resulting antibodies were characterized. While the two resulting monoclonals did not appear to detect PCT, the commercial polyclonal binds native PCT from thyroid tissue and plasma from a dog with sepsis. This contributes to validation of the BV Pab since it was confirmed with mass spectrometry identification of the eluted protein. Several possible causes for the failure of Mabs to recognize the target antigen were discussed and recommendations were made for future research, due to the importance of anti-PCT Mabs in canine PCT research.

5.2 Integrated findings and future directions

5.2.1 Epidemiology of canine sepsis

Sepsis is a curable, preventable condition that has a huge impact on patients through mortality and morbidity as well as hospitalization costs. These facts relating to human sepsis are less well documented for dogs, although existing sepsis research indicating that the immunopathology and burden of sepsis is similar between these species. Recognition of the need for rapid diagnosis of sepsis is a major driver in persistent research into canine PCT (Battaglia et al., 2020; Cerón et al., 2005; Eckersall & Bell, 2010; A. Floras et al., 2014; Goggs et al., 2018; Troia, Giunti, Calipa, et al., 2018; Troia, Giunti, & Goggs, 2018). This project

included a large prospective observational study of 486 admissions of dogs to a veterinary hospital and noted the mortality rate of sepsis was high and that death resulting from sepsis occurred within a maximum of four days following diagnosis, with the majority of deaths in the first 24 hours. This strongly underscores the need for rapid recognition of sepsis. Larger studies are recommended, including multi-center trials, inclusion of all admissions rather than a daily census, and longer timeframes with several months of follow-up. These more detailed studies will bring understanding of canine sepsis epidemiology further in line with that of human sepsis supporting the translation of clinical research between the two species, which is a priority (Otto, 2007). To this end, interventional studies in dogs should also investigate the new diagnostic paradigms based on the sequential organ failure (SOFA) score and qSOFA (Singer et al., 2016; Vincent et al., 1996) – the method now employed in human sepsis diagnosis. More comprehensive epidemiological studies would also allow full appreciation of the significant financial and medical impact of sepsis in dogs on a scale more applicable to large populations. Larger prospective studies could also compare interventions such as new antimicrobial stewardship programs.

5.2.2 Polyclonal antibody validation

A polyclonal anti-cPCT antibody, used as a positive control in this project, detected native cPCT in the plasma of a dog with sepsis. This was confirmed using mass spectrometry to identify proteins bound and then eluted from the antibody during immunoprecipitation. Additional experiments compared the affinity of this Pab and an anti-human PCT Pab to recombinant human and canine PCT, and found that both Pabs are able to detect each PCT

type. Therefore, this anti-canine PCT Pab detects native and recombinant cPCT, as well as hPCT, supporting the use of this Pab in further research including comparative research. This finding also lends considerable strength to the conclusions of research already performed using this antibody and the ELISA kit that employs it.

Limitations still apply to this Pab, including that it is a polyclonal and therefore different lots may demonstrate different affinity or performance. Therefore repeating analytical validation is still recommended for any study using it in research. In addition, although this Pab displays cross-reactivity to native cPCT and recombinant hPCT, it shows highest affinity for recombinant cPCT and may not correctly quantitate other forms. This study as well as another researcher (Goggs et al., 2018) reported that quantitation of PCT was different when using the standard vs the separate recombinant PCT. Finally, although this Pab was confirmed to bind native and recombinant PCT, it also binds other non-target proteins including haemoglobin and albumin which may have altered plasma concentration during sepsis and cause the ELISA to give misleading results that seem to fit the context.

This Pab could be subjected to more in-depth validation according to the new antibody recommendations (Edfors et al., 2018), which if it passes would increase confidence in reliability and reproducibility of results obtained using this Pab or its ELISA. Ideally a Mab would be developed in order to improve consistency and potentially also specificity. Until then, keeping its limitations in mind, this cPCT ELISA (BioVendor) is the only one currently available that is adequately validated, and therefore should be used for all future cPCT

research, until other assays can demonstrate equivalent or better performance and reliability.

5.2.3 Production of anti-cPCT monoclonal antibodies

Monoclonals that recognize native PCT remain the next recommended advancement for cPCT immunoassays, providing reproducibility and consistency for standardization, calibration, and comparison and translation of research; and potentially higher specificity for cPCT. A stable antibody conformation will also facilitate epitope mapping, so that Mabs can be selected which bind to regions of PCT that exclude other cleavage products or related proteins from the same gene. This has been the basis for successful production of discerning Mabs for hPCT (Ghillani et al., 1988; Herbst & Klein, 1995). It is essential that this antibody has high affinity to the native PCT present in tissue including plasma during sepsis in dogs – but also to a recombinant or similarly mass-produced variant, for purposes of quality control, calibration, and standardization (Eckersall, 2019).

In this project, the approach to achieve a native cPCT Mab was to raise the antibody with a purified native cPCT preparation. However, this was not effective. Despite the cPCT reaching apparent purity of 42% according to densitometry readings, the Mabs produced using this antigen did not recognize cPCT of either native or recombinant form. Based on this outcome, the actual purity of the cPCT fractions should be questioned. The purification process was challenging and affected by significant contamination, and this approach is not recommended for future efforts to obtain purified native cPCT. The purification was guided by PCT immunoreactivity of a recombinant cPCT Pab, which had not at that stage been

confirmed to bind native, and which later was confirmed also to bind non-target proteins – this paradoxical approach was necessary under the circumstances but would certainly have contributed to inefficient purification.

High similarity between canine and murine PCT could also have contributed to low antigenicity of native cPCT. Homology between PCT of these species is not theoretically so high as to preclude antigenicity of cPCT. (Mouse monoclonals to hPCT were produced despite 60% homology; canine-murine homology is about 70% with similar distribution of conserved sequences.) In practice, homology could have reduced antigenicity – structural studies of PCT are lacking and would be valuable for PCT research, especially if this led to better understanding of its function. High sameness between canine and murine PCT could explain why other attempts to create cPCT Mabs or immunoassays have failed (Floras, 2014 pers. comm.; Sharp, 2017), or are not yet commercially available. To counteract possible low antigenicity, a highly pure preparation of any type should be used to encourage a significant mouse immune response. For example, recombinant cPCT could be produced in bulk via an *E. coli* vector (currently the major source), or ideally a mammalian cell line such as HEK (mammalian) cells. Resulting Mabs should be screened with IMS, immunohistochemistry or other methods to confirm cross-reactivity with the native, and epitope mapping would confirm a common binding site on PCT variants. Peptides could be used similarly to restrict Mabs to those with the desired epitopes. This is crucial to ensure that PCT as a whole, and not a cleavage product, is the target of any immunoassay – and two antibodies (e.g. sandwich) may be necessary to achieve this. If homology between canine and murine PCT is altogether too high, another species or a phage display library may be required to produce

Mabs. By default, this approach would supply a Mab with a readily available and consistent standard. No quantitative PCR assay for cPCT has yet been developed, but this could be reconsidered if immunoassays continue to be challenging to develop.

Validation of an antibody is vital for its results to be interpreted with confidence and to ensure reproducible performance across studies – and potentially diagnostic assays. Current recommendations include an antibody-independent method of quantitatively and qualitatively measuring cPCT, for comparison with the antibody being validated. In the case of cPCT, a molecularly imprinted polymer assay was recently developed (Battaglia et al., 2021); along with MS assays such as those explored in this work, full validation of cPCT antibodies is now very achievable for any novel or existing cPCT antibody.

5.2.4 Canine PCT as a biomarker

Several studies using the BV ELISA discussed above have consistently shown that cPCT concentrations are increased in the plasma of dogs with sepsis compared to healthy dogs. During this study, native PCT was detected using mass spectrometry in plasma samples from two different dogs with sepsis – but the PCT could not be quantitated, and lack of PCT in other samples including those from healthy dogs cannot be ruled out as false negatives.

In human sepsis, PCT is relatively specific for bacterial infection compared with other causes of inflammation. As a biomarker, hPCT is rapid and used to indicate severity or prognosis, and also to guide length of antimicrobial courses (discussed in Chapter One). In dogs, cPCT does not show significant difference between SIRS and sepsis although its concentrations

become increased very rapidly after induction, and appear to reduce on improvement within the same timeframe as that observed in humans with sepsis. Related to this, cPCT concentrations compared with normal or a baseline have a strong correlation with severity of disease, and may be an effective means to predict survival of dogs with sepsis. A reliable and adequately rapid prognostic biomarker is not currently available to veterinarians.

Several studies have been performed with non-validated cPCT immunoassays, or ELISAs that subsequently failed validation. The results from these studies are generally in line with expected findings or were later confirmed; however, one priority for future cPCT research using the BV ELISA should be to replicate any such studies and obtain conclusive results. The primary direction for cPCT research is to transition to an immunoassay using a validated Mab, because consistent assay performance and reproducible results with a readily available and executable test will enable progress into more pre-clinical and clinical studies for a large number of clinical research groups internationally. Clinical research and larger cohorts will add significant understanding to how cPCT can be employed in diagnosis or management of canine sepsis. Ultimately, in order to be valuable in clinical scenarios such as sepsis, any cPCT assay must be more rapid than other existing inflammation biomarkers (such as CRP) or provide unique information. Canine PCT has potential as a rapid test for sepsis prognosis and antimicrobial stewardship.

5.3 Final statement

This thesis has clearly shown the need for a rapid, improved diagnostic test for sepsis in dogs, and described attempts to develop a novel assay for measurement of canine PCT – a valuable sepsis biomarker in people. This led to the discovery of native canine PCT protein in the plasma of a dog with sepsis; and confirmation that a commercially available Pab recognizes native PCT. Consequently, a canine PCT ELISA that is currently available can more confidently be used to pursue canine PCT preclinical research – and its existing findings are supported. Several avenues for improved diagnostic tests, or improvements to current tests, have been described which will significantly aid researchers in progress towards an improved diagnostic test for sepsis in dogs. In particular, although a Mab to native canine PCT was not developed during this project, several recommendations arise from this body of work to guide this crucial step in future.

6 Appendix

6.1 Canine PCT assay review – new publication

At the time of writing and submitting this thesis, a new publication was in pre-release via Researchsquare[2] which describes the measurement of cPCT with the BioVendor a-cPCT ELISA in dogs with pyometra before and after ovariohysterectomy. They reported that the concentration of cPCT in heparinised plasma was significantly reduced in 22 dogs with sepsis due to pyometra, after ovariohysterectomy; but not significantly different from healthy controls. The authors also reported that PCT concentrations were correlated with several other biomarkers or parameters commonly measured during inflammation including monocyte count and blood urea nitrogen. However, this article is not yet reviewed or published in a peer-reviewed journal – therefore, this preliminary report cannot be considered conclusive, and is reported here for completeness and not discussed within Chapter One as valid information.

6.1.1 Reference at the time of thesis submission

Ahn, S., Han, H., Park, J., Kim, S. K., Jung, D. I., & Yu, D. (2021). Comparison of Clinical and Inflammatory Parameters in Dogs with Pyometra Before and After Ovariohysterectomy.Protein purification

[2] https://assets.researchsquare.com/files/rs-143024/v1/4ffb812d-f29f-4ba1-b4cd-25d9911b5b49.pdf?c=1631870427

6.1.2 Extract Ac (acetone-based extraction)

During method development, one thyroid was processed to extract protein according to a method previously published (Mol et al., 1991). Briefly, lipid was removed from lyophilized thyroids by washing and rinsing in acetone; then homogenized in acetone with 20% 0.1M hydrochloric acid and 2% beta-mercaptoethanol, before centrifugation at 15 minutes at 4500 x *g* all at 4°C. Supernatant was collected from this preparation and after one resuspension of the pellet and solvent was removed to leave a concentrated extract. In this project, this extract (extract Ac) was resuspended in TBS for analysis in BCA assay, or buffer B1 for cation exchange chromatography.

6.1.3 Size exclusion chromatography methods and results

For size exclusion chromatography, Sephadex G-50 was hydrated and suspended in an excess of Buffer D1 (50mM Tris-HCl, 150mM sodium chloride, pH 7.6, 0.5mM EDTA, and protease inhibitor cocktail) and poured by hand to make a column 18cm tall and 2cm diameter. A total of 1mL of extract A was applied per run and passed through the column at a flow rate of 0.5mL per minute at 4°C. Fractions were collected every 2 minutes, and protein concentration was determined after the run by analyzing aliquots from each fraction via BCA assay.

The results of the size exclusion chromatography (designated 'D' in this thesis) are shown in Figure 6.1. The PCT immunoreactivity, as measured with the abcam ELISA and confirmed later with BV, showed no clear peaks indicative of PCT. Fractions 30-40, where smaller

molecular weight proteins should be, have several irregular readings not consistent with peaks useful in chromatography. The large molecular weight fractions (eight to twenty) are not expected to have PCT immunoreactivity, but were shown to contain PCT on MS analysis (Table 3.3).

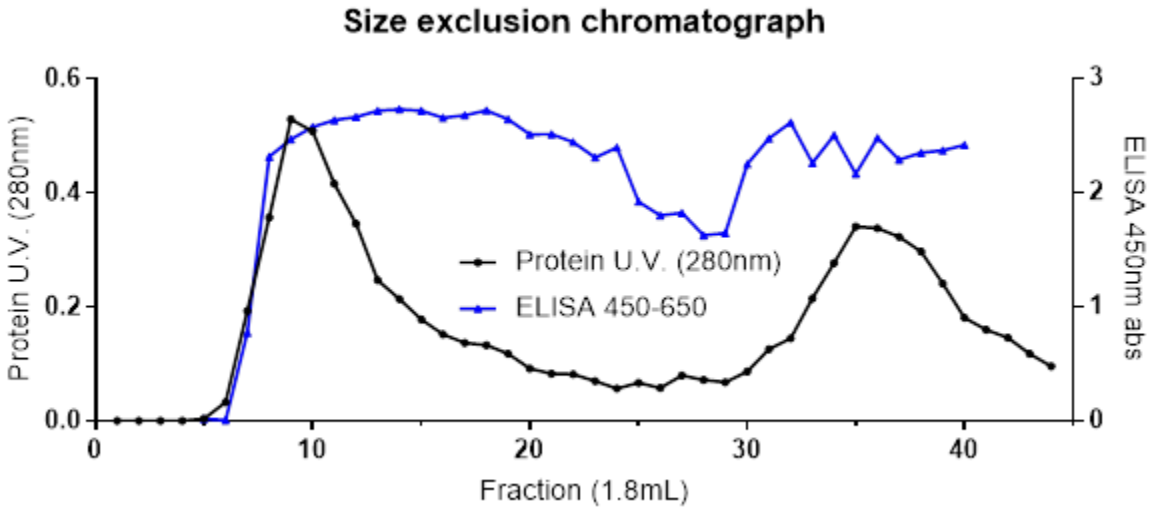


Figure 6.1: Size exclusion chromatograph of a total of 1mL of Extract A. Blue line (triangles) shows the absorbance at 280 nm after fractions were measured with an ELISA (BioVendor).

6.1.4 Mass spectrometry results

The mass spectrometry protein ID assays produced a large number of protein hits in each analysis (sometimes more than a hundred). Many of these were similar or equivalent hits of the same protein, for example several hits of albumin but each from a different species. This demonstrates how MS results were obtained: molecular identification of the amino acid sequence then matched against a database. Several proteins in the results list could then be ruled out as junk hits (because samples were not included from cat, camel etc). Remaining

protein hits included abundant proteins such as albumin, and haemoglobin which contaminated all samples despite efforts to rinse blood from thyroids during extraction.

In the MS protein ID results, PCT is represented as the CALCA gene or specifically as "calcitonin" or "calcitonin precursor". When present, its score was always significant to support a true positive and true match (score above 50 and peptides confirming sequence). The scores were always much lower than albumin, haemoglobin, thyroglobulin when present (the latter sometimes had scores >1,000). But PCT scores were equivalent to proteins identified in the sample which were known to be present such as trypsin (from the digest protocol preparing the samples). The example shown below here in Table 3.4 shows protein hits from an aliquot of AB29.

Table 3.4. Mass spectrometry analysis produces a large number of protein hits in addition to procalcitonin for fraction AB29 (189, 4 unique peptides, 61% coverage; section 3.3.4). This table highlights the vast quantity of results in the MS output. The major proteins from this table, including only those from Canis lupus familiaris and Vulpes vulpes, are included in Table 3.4 along with more data for each protein such as its score and calculated MW.

Protein hits (excluding AB29)
Apolipoprotein A-I OS=Canis lupus familiaris OX=9615 GN=APOA1 PE=3 SV=1
serum albumin OS=Vulpes vulpes OX=9627 GN=ALB PE=4 SV=1
Globin A2 OS=Canis lupus familiaris OX=9615 GN=GLNA2 PE=3 SV=1
GLOBIN domain-containing protein OS=Canis lupus familiaris OX=9615 GN=LOC480784 PE=3 SV=2
Globin A1 OS=Canis lupus familiaris OX=9615 GN=GLNA1 PE=3 SV=1
GLOBIN domain-containing protein OS=Canis lupus familiaris OX=9615 GN=LOC476825 PE=3 SV=1

hemoglobin subunit alpha OS=Vulpes vulpes OX=9627 GN=LOC112909065 PE=3 SV=1
GLOBIN domain-containing protein OS=Canis lupus familiaris OX=9615 GN=LOC100855558 PE=3 SV=1
Uncharacterized protein OS=Gulo gulo OX=48420 GN=BN2614_LOCUS2 PE=4 SV=1
Hemoglobin subunit beta OS=Macaca mulatta OX=9544 GN=HBB PE=1 SV=1
Hemoglobin subunit beta OS=Paguma larvata OX=9675 GN=HBB PE=1 SV=1
serum albumin OS=Leptonychotes weddellii OX=9713 GN=ALB PE=4 SV=1
IF rod domain-containing protein OS=Capra hircus OX=9925 GN=LOC102176726 PE=3 SV=1
serum albumin OS=Odobenus rosmarus divergens OX=9708 GN=ALB PE=4 SV=1
Serum albumin OS=Sus scrofa OX=9823 GN=ALB PE=1 SV=1
Alpha-fetoprotein OS=Myotis brandtii OX=109478 GN=D623_10008572 PE=4 SV=1
Hemoglobin subunit beta OS=Cheracebus torquatus OX=30592 GN=HBB PE=2 SV=3
Beta globin OS=Panthera tigris OX=9694 GN=HBB PE=3 SV=1
Uncharacterized protein OS=Mustela putorius furo OX=9669 GN=ALB PE=4 SV=1
GLOBIN domain-containing protein OS=Ictidomys tridecemlineatus OX=43179 GN=LOC101977640 PE=3 SV=1
Hemoglobin beta (Fragment) OS=Marmota caligata OX=93160 GN=HBB PE=2 SV=1
Hemoglobin subunit beta OS=Saimiri boliviensis boliviensis OX=39432 GN=HBB PE=3 SV=1
ALB OS=Cervus elaphus hippelaphus OX=46360 GN=Celaphus_00017423 PE=4 SV=1
Keratin, type II cuticular Hb6 OS=Fukomys damarensis OX=885580 GN=H920_07182 PE=3 SV=1
IF rod domain-containing protein OS=Loxodonta africana OX=9785 GN=LOC100665280 PE=3 SV=1
Beta-globin (Fragment) OS=Orycteropus afer OX=9818 GN=HBB-A PE=3 SV=1
Globin A1 OS=Oryctolagus cuniculus OX=9986 GN=HBB2 PE=3 SV=1
Hemoglobin subunit beta OS=Otospermophilus beecheyi OX=34862 PE=1 SV=1
Cationic trypsin OS=Bos taurus OX=9913 PE=1 SV=3
hemoglobin subunit alpha OS=Odobenus rosmarus divergens OX=9708 GN=HBA1 PE=3 SV=1
Albumin OS=Microtus fortis calamorum OX=311220 PE=2 SV=1
Serum albumin OS=Rattus norvegicus OX=10116 GN=Alb PE=1 SV=1
Uncharacterized protein OS=Camelus dromedarius OX=9838 GN=Cadr_00029170 PE=4 SV=1

Keratin 34 OS=Rattus norvegicus OX=10116 GN=Krt34 PE=2 SV=1
Beta-globin (Fragment) OS=Macaca hecki OX=90382 PE=3 SV=1
IF rod domain-containing protein OS=Cervus elaphus hippelaphus OX=46360 GN=Celaphus_00011456 PE=3 SV=1
IF rod domain-containing protein OS=Ictidomys tridecemlineatus OX=43179 GN=LOC101957527 PE=3 SV=2
Thioredoxin domain-containing protein OS=Felis catus OX=9685 GN=PRDX6 PE=4 SV=1
Hemoglobin beta (Fragment) OS=Ictidomys tridecemlineatus OX=43179 GN=HBB PE=2 SV=1
Hemoglobin subunit beta OS=Tupaia glis OX=9395 GN=HBB PE=1 SV=1
Apolipoprotein A-I isoform 1 preproprotein OS=Sus scrofa OX=9823 PE=4 SV=1
IF rod domain-containing protein OS=Ornithorhynchus anatinus OX=9258 GN=LOC100083755 PE=3 SV=1
Apolipoprotein A-I (Fragment) OS=Pongo pygmaeus OX=9600 PE=3 SV=1
Hemoglobin subunit alpha OS=Tamias striatus OX=45474 PE=1 SV=1
Epsilon-globin OS=Peromyscus maniculatus OX=10042 GN=HBE-T1 PE=3 SV=1
Keratin 32 OS=Gorilla gorilla gorilla OX=9595 PE=3 SV=1
serum albumin precursor OS=Mesocricetus auratus OX=10036 GN=Alb PE=4 SV=1
IF rod domain-containing protein OS=Papio anubis OX=9555 GN=KRT32 PE=3 SV=1
Hemoglobin subunit beta-S/F OS=Urocitellus townsendii OX=99861 PE=1 SV=1
keratin, type II cuticular Hb6 OS=Balaenoptera acutorostrata scammoni OX=310752 GN=LOC102999373 PE=3 SV=1
Hemoglobin subunit alpha 1 OS=Scalopus aquaticus OX=71119 GN=HBA1 PE=2 SV=1
Globin C1 OS=Erinaceus europaeus OX=9365 GN=GLNC1 PE=3 SV=1
IF rod domain-containing protein OS=Myotis lucifugus OX=59463 GN=LOC102442579 PE=3 SV=1
serum albumin isoform X1 OS=Tursiops truncatus OX=9739 GN=ALB PE=4 SV=1
Serum albumin OS=Equus caballus OX=9796 GN=ALB PE=4 SV=1
HBA protein OS=Ochotona princeps OX=9978 GN=HBA PE=3 SV=1
hemoglobin subunit delta OS=Tarsius syrichta OX=1868482 GN=LOC103254683 PE=3 SV=1
Hemoglobin subunit alpha-3 OS=Gorilla gorilla gorilla OX=9595 PE=1 SV=1
Hemoglobin subunit alpha OS=Suncus murinus OX=9378 GN=HBA PE=1 SV=1
GLOBIN domain-containing protein OS=Sarcophilus harrisii OX=9305 PE=3 SV=1

rho GDP-dissociation inhibitor 1 OS=Mesocricetus auratus OX=10036 GN=Arhgdia PE=4 SV=1
HHa7 protein (Fragment) OS=Homo sapiens OX=9606 GN=KRTHA7 PE=2 SV=1
Beta globin (Fragment) OS=Homo sapiens OX=9606 GN=HBB PE=3 SV=1
Adenylate kinase isoenzyme 1 OS=Fukomys damarensis OX=885580 GN=H920_19191 PE=3 SV=1
KRT81 OS=Cervus elaphus hippelaphus OX=46360 GN=Celaphus_00011462 PE=3 SV=1
Theta 1 globin (Predicted) OS=Rhinolophus ferrumequinum OX=59479 GN=HBQ1_3 PE=3 SV=1
GLOBIN domain-containing protein OS=Rhinopithecus bieti OX=61621 GN=HBD PE=3 SV=1
Uncharacterized protein OS=Aotus nancymaae OX=37293 GN=ALB PE=4 SV=1
phosphatidylethanolamine-binding protein 1 OS=Odobenus rosmarus divergens OX=9708 GN=PEBP1 PE=4 SV=1
Hemoglobin subunit alpha OS=Episoriculus fumidus OX=150090 GN=HBA PE=3 SV=1
Hemoglobin subunit epsilon OS=Bradypus tridactylus OX=9354 GN=HBE1 PE=2 SV=3
keratin, type I cytoskeletal 14-like OS=Delphinapterus leucas OX=9749 GN=LOC111166381 PE=3 SV=1
IF rod domain-containing protein OS=Nomascus leucogenys OX=61853 GN=KRT75 PE=3 SV=3
Adenylate kinase 2, mitochondrial OS=Vulpes vulpes OX=9627 GN=AK2 PE=3 SV=1
Glutathione S-transferase OS=Macaca mulatta OX=9544 GN=GSTM2 PE=2 SV=1
Uncharacterized protein (Fragment) OS=Neotoma lepida OX=56216 GN=A6R68_06206 PE=3 SV=1
Thioredoxin domain-containing protein OS=Monodelphis domestica OX=13616 GN=PRDX6 PE=4 SV=2
Ig-like domain-containing protein OS=Canis lupus familiaris OX=9615 PE=4 SV=2
Hemoglobin subunit beta OS=Tamias merriami OX=123787 PE=1 SV=1
Adult beta-globin OS=Hydropotes inermis OX=9883 PE=3 SV=1
keratin, type I cytoskeletal 39 OS=Dipodomys ordii OX=10020 GN=Krt39 PE=3 SV=1
Transthyretin OS=Vulpes vulpes OX=9627 GN=TTR PE=3 SV=1
Thioredoxin domain-containing protein OS=Neotoma lepida OX=56216 GN=A6R68_17182 PE=4 SV=1
Thioredoxin domain-containing protein OS=Pan troglodytes OX=9598 PE=4 SV=1

Mutant hemoglobin alpha 2 globin chain OS=Homo sapiens OX=9606 GN=HBA2 PE=3 SV=1
Globin B2 OS=Otolemur garnettii OX=30611 GN=GLNB2 PE=3 SV=1
Delta globin (Predicted) OS=Callithrix jacchus OX=9483 GN=HBD PE=3 SV=1
HBB protein OS=Ochotona rufescens OX=42346 GN=HBB PE=3 SV=1
GLOBIN domain-containing protein OS=Sus scrofa OX=9823 GN=LOC100515788 PE=1 SV=2
Olfactory receptor family 52 (Fragment) OS=Artibeus jamaicensis OX=9417 GN=OR52 PE=3 SV=1
Glutathione S-transferase OS=Cricetulus griseus OX=10029 GN=H671_1g2859 PE=3 SV=1
Globin B2 OS=Oryctolagus cuniculus OX=9986 GN=GLNB2 PE=3 SV=1
Hemoglobin subunit beta OS=Spalax ehrenbergi OX=30637 GN=HBB PE=1 SV=1
Transgelin OS=Fukomys damarensis OX=885580 GN=H920_07533 PE=3 SV=1
Pentaxin OS=Vulpes vulpes OX=9627 GN=CRP PE=3 SV=1
immunoglobulin lambda-1 light chain-like OS=Vulpes vulpes OX=9627 GN=LOC112907472 PE=4 SV=1
Malate dehydrogenase, mitochondrial OS=Felis catus OX=9685 GN=MDH2 PE=2 SV=1
Serum albumin OS=Oryctolagus cuniculus OX=9986 GN=ALB PE=1 SV=1
Hemoglobin subunit beta (Fragment) OS=Dasyurus viverrinus OX=9279 GN=HBB PE=2 SV=1
calcitonin receptor-stimulating peptide 2-like isoform X2 OS=Odobenus rosmarus divergens OX=9708 GN=LOC101368405 PE=4 SV=1
calcitonin receptor-stimulating peptide 2-like OS=Vulpes vulpes OX=9627 GN=LOC112927043 PE=4 SV=1
NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial OS=Vulpes vulpes OX=9627 GN=NDUFS4 PE=4 SV=1
Ig-like domain-containing protein OS=Propithecus coquereli OX=379532 PE=4 SV=1
Dihydropteridine reductase OS=Fukomys damarensis OX=885580 GN=H920_12925 PE=4 SV=1
dihydropteridine reductase isoform X2 OS=Vulpes vulpes OX=9627 GN=QDPR PE=4 SV=1
Calbindin-like protein OS=Cricetulus griseus OX=10029 GN=H671_2g6716 PE=4 SV=1
Uncharacterized protein OS=Sus scrofa OX=9823 GN=NDUFS4 PE=1 SV=1
Ribosomal protein L15 OS=Otolemur garnettii OX=30611 PE=3 SV=1

Peroxiredoxin-2 OS=Cricetulus griseus OX=10029 GN=H671_3g9966 PE=4 SV=1
Beta-globin (Fragment) OS=Saguinus oedipus OX=9490 PE=4 SV=1

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