Kinetics of the inflammatory response during experimental *Babesia rossi* infection of Beagle dogs

By

Brogan Kim Atkinson

Submitted in partial fulfilment of the requirements for the degree of Master of Veterinary Medicine (Small Animal Medicine) in the Department of Companion Animal Clinical Studies in the Faculty of Veterinary Science, University of Pretoria

Date submitted: March 2021

Acknowledgements

I would like to express my gratitude and appreciation to the following groups of people, without whose help, this project would not have been possible:

My fellow research group co-workers, Prof Amelia Goddard, Prof Johan Schoeman, Dr Estee Van Zyl and Dr Yolandi Rautenbach, for their assistance in project planning, sample collection and support throughout the project. Thank you for your support and help.

The Section of Clinical Pathology, Department of Companion Animal Clinical Studies in the Faculty of Veterinary Science, University of Pretoria for their friendly assistance, efficient sample processing and for providing storage space for the research samples.

Prof Peter Thompson from the Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria, thank you for your assistance in the statistical analysis of the data, your support and input in this project.

Prof Melvyn Quan from the Department of Veterinary Tropical diseases, Faculty of Veterinary Science, University of Pretoria, thank your patience, assistance and advisory input.

The staff of the University of Pretoria Biomedical Research Centre (UPBRC) of the Faculty of Veterinary Science, University of Pretoria for their friendly assistance, outstanding animal care and excellent service.

Prof Leisewitz and the National Research Foundation (NRF grant: NRF CPRR160425163064) for their assistance with funding for this project.

My supervisor team of Professor Andrew Leisewitz and Dr Varaidzo Mukorera. Prof Leisewitz, thank you for all your help with protocol drafting, encouragement and completion of this dissertation. I couldn’t have done this project without you and I am grateful for all your help. Dr Varaidzo Mukorera, thank you for all your support and understanding. You were always willing to offer your assistance and I truly appreciate your support.

UNIVERSITY OF PRETORIA

FACULTY OF VETERINARY SCIENCE

DECLARATION OF ORIGINALITY

This document must be signed and submitted with every essay, report, project, assignment, mini-dissertation, dissertation and/or thesis

Full names of student: Brogan Kim Atkinson

Student number: 27102093

Declaration:

1. I understand what plagiarism is and am aware of the University's policy in this regard.
2. I declare that this dissertation is my own original work. Where other people's work has been used (either from a printed source, Internet or any other source), this has been properly acknowledged and referenced in accordance with departmental requirements.
3. I have not used work previously produced by another student or any other person to hand in as my own.
4. I have not allowed, and will not allow, anyone to copy my work with the intention of passing it off as his or her own work.

Signature of student:

Signature of supervisor: ……… ………...................

Table of Contents

Acknowledgements……2

Declaration of originality……3

List of tables……7

List of figures……7

List of abbreviations……8

Summary……11

Chapter 1

Literature review……14

Babesiosis……14

Canine babesiosis……14

Canine babesiosis in South Africa……17

The evolution of the inflammatory response in babesiosis……19

Parasitaemia……29

Conclusion……30

Project justification……31

Chapter 2

Aims……32

Objectives……32

Hypotheses……32

Benefits arising from the study……34

Chapter 3

Materials and methods……35

Model systems and justification of the model……35

Experimental design……37

Experimental procedures……42

Sample collection schedule and volumes……42

Observation/Analytical procedures……45

Cytokine analysis……46

Data management and analysis……52

Project management……53

Experimental animals……53

Staff……54

Facilities and laboratories……56

Equipment and supplies……56

Chapter 4

Results……57

Clinical parameters……66

Clinicopathological parameters……68

Cytokine kinetics……72

Correlations……77

Chapter 5

Discussion……..78

Study limitations………95

Conclusion……96

References……98

Addendums

Appendix A: University of Pretoria Animal Ethics Approval Certificate……………………………….109

Appendix B: Data collection forms…………………………………………………………………………………110

Appendix C: Sample collection schedule…………………………………………………………………………112

Appendix D: Cytokine plate map…………………………………………………………………………………….114

Appendix E: Sample storage sheet…………………………………………………………………………………..115

Appendix F: Presentations and publications arising from this study…………………………………..116

Presentations……116

List of tables:

Table 1: Sample collection schedule

Table 2: Immunoassay sensitivity

Table 3: Immunoassay precision

Table 4: Clinical data indicating disease progression

Table 5: Clinicopathological data indicating disease progression

Table 6: Category *a* cytokines: Cytokines that rose during the infection and fell after treatment

Table 7: Category *b* cytokines: Cytokines that rose and remained high even after treatment

Table 8: Category *c* cytokines: Cytokines that rose dramatically after treatment

Table 9: Category *d* cytokines: Cytokines that showed no distinct pattern of change

Table 10: Correlation summary

List of figures:

Figure 1: Study design

Figure 2: Standard preparation

Figure 3: Standard concentrations

Figure 4: Clinical parameters

Figure 5: Clinicopathological parameters

Figure 6: Category *a* cytokines: Cytokines that rose during the infection and fell after treatment

Figure 7: Category *b* cytokines: Cytokines that rose and remained high even after treatment

Figure 8: Category *c* cytokines: Cytokines that rose dramatically after treatment

Figure 9: Category *d* cytokines: Cytokines that showed no distinct pattern of change

List of abbreviations

% – Percentage

/L – Per litre

< – Less than

> – More than

β – Beta

°C – Degrees Celsius

µm – Micrometer

µL – Microlitre

B. – Babesia

Bpm – breaths per minute (respiration) or beats per minute (heart rate)

BrEMA 1 – *Babesia rossi* erythrocyte membrane antigen 1

CBC – Complete blood count

CPDA-1 – Citrate Phosphate Dextrose Adenine

CRP – C-reactive protein

dL - Decilitre

DMSO – Dimethylsulphoxide

EDTA – Ethylenediaminetetraacetic acid

g – Grams

GM-CSF – Granulocyte-macrophage colony-stimulating factor

H0 – Null hypothesis

H1 – Alternative hypothesis

HD – High dose

ICAM-1 – Intercellular adhesion molecule 1

IFNγ – Interferon gamma

IL – Interleukin

IP-10 – Interferon gamma-induced protein 10

KC-like – Keratinocyte Chemotactic-like

Kg – Kilogram

L – Litre

LD – Low dose

MCP-1 – Monocyte chemoattractant protein-1

mg – Milligrams

mL – Millilitre

mm – Millimetre

mmHg – Millimetre of mercury

Mmol – Millimoles

$NaHCO_3$ – Sodium bicarbonate

NAOH – Sodium hydroxide

NK cells – Natural killer cells

OVAH – Onderstepoort Veterinary Academic Hospital

PAMPs – Pathogen associated molecular patterns

PCR – Polymerase chain reaction

pg – Picograms

PRRs – Pattern recognition receptors

RBC – Red blood cells

RI – Reference interval

RLB – Reverse line blotting

RNA – Ribonucleic acid

SIRS – Systemic inflammatory response syndrome

TGFβ – Transforming growth factor beta

TNFα – Tumour necrosis factor alpha

TPR – Temperature, pulse, respiration

UPBRC – University of Pretoria Research Centre

VCAM-1 – Vascular adhesion molecule 1

WCC – White cell count

Summary

Kinetics of the inflammatory response during experimental *Babesia rossi* infection of Beagle dogs

By

Brogan Kim Atkinson

Promoter: Prof Andrew Leisewitz

Co-promoter: Dr Varaidzo Mukorera

Department: Companion Animal Clinical Studies

Degree: Master of Veterinary Medicine (Small Animal Medicine)

Background: *Babesia rossi*, the most virulent canine *Babesia* parasite, causes severe clinical disease and death in dogs in sub-Saharan Africa. Complications and multiple organ dysfunction seen with babesiosis are likely caused by the effects of an unfocused, imbalanced and excessive inflammatory response. The aim of this study was to investigate markers of inflammation and cytokine kinetics from the point of inoculation with *B. rossi* throughout the course of clinical disease and determine if infectious dose influenced rate and severity of disease progression.

Hypothesis: Dogs infected with a higher inoculum dose would experience more severe clinical disease and systemic inflammation over a shorter disease course. Measurable differences would be found between the baseline, low dose and high dose groups for clinical, clinicopathological and cytokine variables.

Materials and Methods: This experimental study was performed on 6 healthy sterilised male beagle dogs. One dog was splenectomised and used to raise a viable parasite inoculum. Three dogs were given a high infectious dose (HD group) and 2 dogs a low infectious dose (LD group) of parasite. Appetite, habitus, clinical examination, glucose, lactate and CBC was performed daily and EDTA plasma was stored at -80°C. C-reactive protein and albumin were determined every second day. Cytokines were quantified on the stored plasma using a canine specific cytokine magnetic bead panel (Milliplex©). Dogs were monitored and parasitaemia was determined daily until predetermined endpoints for treatment were reached. The dogs in the high dose group were treated at 96 hours and those in the low dose group were treated at 108 hours post infection.

Results: The infection was allowed to run its course for 4 days prior to intervention. The HD group was treated at 96 hours and the LD group was treated 12 hours later, at 108 hours. No significant difference was noted for baseline data between the LD and HD groups for any variable. Post inoculation, initial parasitaemia occurred at 24 hours in the HD group and 72 hours in the LD group. The rate of increase in parasitaemia in the HD group was considerably faster than that seen in the LD group. The mean temperature peaked 36-hours earlier in the HD group. The pyrexia persisted for at least 24 hours after treatment in both groups. In addition to the difference seen in vital parameters between the two groups, the HD group also demonstrated a more pronounced decline in habitus and appetite during the course of the infection. The red cell count showed a significant decline from 96 hours in the HD group, worsening after treatment with the lowest count at 120 hours. The dogs in the HD group required multiple blood transfusions before the red cell count stabilised and started to improved. Although there was a drop in the red cell counts of the LD group after treatment, it resulted in tolerable clinical levels of anaemia which did not require transfusions. The C- reactive protein also peaked 36-hours earlier in the HD group. A neutropenia was seen in both groups, but the nadir was earlier and more dramatic in the HD group. Thirteen cytokines were evaluated in total and the results are divided into 4 groups by pattern of change.

The categories for the kinetic patterns identified include:

a. Cytokines that rose during the infection and fell after treatment: IFNγ and KC-like. Both these cytokines peaked earlier in the high dose group and declined rapidly after treatment.

b. Cytokines that rose and remained high even after treatment: MCPI-1, IL-6, IL-8 and IL-10. Both MCP-1 and IL-6 gradually increased during infection in the HD group with minimal changes in the LD group. After treatment, these cytokines increased drastically in the HD group, with only 1 dog in the LD group showing an increase at 192 hours. IL-8 had a different kinetic pattern between HD and LD groups, not only a delay in the increase. The anti-inflammatory cytokine IL-10 increased progressively during parasite replication in both groups.

c. Cytokines that rose dramatically after treatment: GM-CSF, TNFα, IL-2 and IL-7 were all markedly increased after treatment in the HD group with moderate increases seen in one dog the LD group.

d. Cytokines that showed no distinct pattern of change: IL-15, IL-18 and IP-10.

Conclusion: Our findings suggest that the initiation of inflammation occurs before the onset of clinical disease with a possible imbalance in the pro- and anti-inflammatory cytokine concentrations during parasite replication. Infectious dose influenced the course of inflammation as well as the course and

severity of disease. Treatment of the infection did not result in the resolution of inflammation. In fact, many markers of inflammation and cytokines were significantly increased following treatment. This is in agreement with the hypothesis that severe inflammation and complications are associated with an unfocused, imbalanced and pronounced inflammatory response that may even be perpetuated by chemotherapeutic intervention.

Keywords: *Babesia rossi,* cytokines, inflammation, parasitaemia, experimental

Chapter 1: Literature review

Babesiosis

Babesiosis is one of the most common infections of vertebrates worldwide and is gaining increasing interest as an emerging zoonosis in humans (Homer et al., 2000). It is a disease caused by intracellular *Babesia* protozoa that are transmitted by arthropod vectors to vertebrate hosts, invading and proliferating within erythrocytes (Homer et al., 2000). Human babesiosis is caused by a number of *Babesia* species and the inciting protozoa are dependent on the geographic localisation of the vectors and original vertebrate hosts as well as their proximity to humans (most commonly *Babesia microti* in North America and *Babesia divergens* in Europe) (Dammin et al., 1981, Homer et al., 2000). In order for *Babesia* parasites to survive in an environment they require specific invertebrate vectors (primarily ixodid ticks) to transmit them to a vertebrate host thus completing the life cycle (Telford III et al., 1993, Homer et al., 2000, Jalovecka et al., 2019). Although some hosts have adapted to these parasites, demonstrating minimal parasitaemia and clinical signs with wild type infections, any potential host for the tick may act as a potential host for the *Babesia.* Clinical manifestations of infection may be severe in poorly adapted hosts as seen in hamsters and humans infected with *B. microti* or sub-clinical in well adapted hosts such as the white-footed mouse (Benach et al., 1978, Morters et al., 2020, Penzhorn et al., 2017). *Babesia* parasites undergo three stages of reproduction during their life cycle, these include gamogony (sexual) within the tick gastro-intestinal tract, sporogony (asexual) within the salivary glands of the tick and finally merogony (asexual) within the vertebrate host (Homer et al., 2000, Uilenberg, 2006, Jalovecka et al., 2019). In addition to being a potential zoonoses, this infection, particularly with certain *Babesia spp.* (e.g. *Babesia rossi*), bears a striking resemblance to the disease caused by *Plasmodium falciparum* in humans (Krause et al., 2007, Reyers et al., 1998). Studying the pathogenesis of *Babesia* infections may prove to be a valuable approach for understanding some aspects of the pathophysiology of *Plasmodium falciparum* malaria because of their similarity. As *Babesia spp.* infect an array of vertebrate hosts, they may serve as disease models for both zoonotic *Babesia* and malarial infections (Krause et al., 2007).

Canine babesiosis

Aetiology

Canine babesiosis is caused by a limited number of parasites that can be grouped into either large or small *Babesia* species. Previously there were only two recognised species namely the large *B. canis* and the small *B. gibsoni* but with advances in molecular diagnostics additional species have been

identified (Boozer and Macintire, 2003, Carret et al., 1999, Zahler et al., 1998). The large babesias include *B. canis, B. rossi and B. vogeli* and the small are *B. gibsoni*, *B. conradae, B. negevi and B. vulpes* (Boozer and Macintire, 2003, Schoeman, 2009, Zahler et al., 1998, Kjemtrup and Conrad, 2006, Baneth et al., 2019, Baneth et al., 2020). In South Africa only two species are found, namely *Babesia rossi* and *Babesia vogeli*, with the former being the most abundant and clinically significant (Matjila et al., 2004, Matjila et al., 2008). The severity of clinical signs in canine babesiosis varies from subclinical disease with a low parasitaemia to severe life threating illness with very high parasitaemias, and is dependent on the infecting species as well as the age and immune status of the dog (Schoeman, 2009, Schetters et al., 1997, Morters et al., 2020, Leisewitz et al., 2019b). *Babesia rossi*, the most virulent canine *Babesia* parasite, is a cause of severe clinical disease and mortality in dogs in sub-Saharan Africa (Penzhorn, 2011, Schetters et al., 1997). This particular protozoan is transmitted by the ixodid tick *Haemaphysalis elliptica* (Matjila et al., 2008, Lewis et al., 1996, Walker, 1991, Apanaskevich et al., 2007). The high incidence of *B. rossi* infections correlates with the high prevalence of vectors found on infected dogs in dogs in South Africa (Matjila et al., 2008). Babesiosis in South Africa is often a severe, acute disease leading to systemic inflammation and possible multiple organ dysfunction (Leisewitz et al., 2001, Jacobson and Clark, 1994, Welzl et al., 2001). The reason for the virulence in this species has been postulated to be due to unique features such as the expression of a polymorphic phosphoprotein *B. rossi* erythrocyte membrane antigen 1 (BrEMA 1), on the cytoplasmic membrane of infected erythrocytes (Matjila et al., 2009). However the most likely reason for the virulence of this genus is a lack of evolutionary adaptation of dogs to the parasite (Penzhorn, 2011). Most dog breeds found in South Africa are not indigenous to Africa and thus have not had sufficient time to develop endemic resistance to the parasite through a process of natural selection (Penzhorn, 2011). With the need to intensively treat many clinical cases, natural selection may be prevented resulting in a persistence of virulence in the domestic dog in South Africa (Penzhorn, 2011).

<u>Immunology</u>

All mammals are able to develop immunity to *Babesia* parasites, either following infection of a sufficient duration or immunisation with vaccines (Homer et al., 2000, Lewis et al., 1995, Schetters et al., 1994). Both the cellular and humoral immune responses are involved in the immunity against *Babesia spp* (Homer et al., 2000). Depending on the phase of the infection, the importance of the various components differs. During initial infection, when the sporozoites are transiently free in the plasma, immunoglobin G antibodies could bind to and neutralise the free parasites, preventing them from entering erythrocytes (Homer et al., 2000). Seroconversion to infection takes place from approximately 7 to 21 days post infection (Brandao et al., 2003, Schetters et al., 1994). For antibody titres to be protective they should be ≥ 320:1 (Brandao et al., 2003). With infections caused by *Babesia*

canis that were allowed to resolve without treatment, titres remained protective for up to six months post initial infection (Brandao et al., 2003). For those dogs treated with imidocarb dipropionate, sterilizing the infection, demonstrated a disruption of the antigenic stimulation and subsequently a rapid decline in the protective antibody titres. At six months these dogs were not able to prevent reinfection (Brandao et al., 2003). Given the virulence of *Babesia rossi* infections, clinicians treat the infection as soon as it is diagnosed thus preventing an adequate immune response. Explaining why dogs may be infected multiple times in one season (Brandao et al., 2003, Penzhorn et al., 1995). Vaccination of dogs against *B. canis* has shown a reduction in clinical sequela that appears to be unrelated to level of parasitaemia. It appears these vaccines are anti-disease rather than anti-parasite (Schetters et al., 2006, Schetters et al., 1994). Protection was seen from 3 weeks after the booster vaccination (initial vaccination with a booster 3 weeks later) and was protective for up at 6 months (Schetters et al., 2006). Vaccination against *Babesia rossi* proved more challenging and immunity was at best only partial with the use of monovalent homologous vaccines (Lewis et al., 1995, Schetters et al., 2007). The use of a bivalent vaccine utilising antigens from culture supernatants of both *B. rossi* and *B. canis* provided protection (Schetters et al., 2007). Although anaemia still occurred, no parasitaemia was noted and the dogs were able to recover without treatment when challenged (Schetters et al., 2007). Following infection with the parasite, clinical disease progresses as parasites become intraerythrocytic and the parasitaemia begins to rise (Homer et al., 2000). It is during the parasitaemia phase that cell mediated immunity becomes important as cells of the innate immune system limit parasite replication. Although poorly understood, this inhibition is most likely due to the production of soluble factors such as gamma interferon (IFNγ) by natural killer (NK) cells and tumour necrosis factor alpha (TNFα), nitric oxide and reactive oxygen species by macrophages (Homer et al., 2000, Aguilar-Delfin et al., 2003, Shoda et al., 2000). The final stage of infection is the resolution phase and is characterised by intraerythrocytic parasite degradation, splenic clearance and a drop in parasitaemia. This is mediated by T lymphocytes, specifically CD4+ T helper cells (Homer et al., 2000, Igarashi et al., 1999). During the resolution phase, *Babesia microti* parasites were shown to die within intact red cells, indicating that a soluble mediator was most likely responsible for the degeneration (Clark et al., 1975). One soluble mediator appears to be IFNγ produced by CD4+ T lymphocytes (Igarashi et al., 1999). Additional mediators that have also been shown to inhibit parasite growth or mediate death include nitric oxide, TNFα and reactive oxygen species (Rosenblatt-Bin et al., 1996, Clark, 1979, Johnson et al., 1996). The spleen also plays a central role in the immune reaction to babesiosis, demonstrated in mice where the adoptive transfer of splenic cells from immune animals resulted in protection to infectious challenge (Meeusen et al., 1984).

Canine babesiosis in South Africa

Clinical presentation

In South Africa two *Babesia* species infect domestic dogs, namely *B. rossi* and *B. vogeli* (Matjila et al., 2008, Matjila et al., 2004). Of the two species *B. vogeli* is the less pathogenic, causing mild disease in adult dogs but can occasionally run a more severe course in puppies (Schoeman, 2009, Irwin and Hutchinson, 1991). *Babesia rossi,* on the other is a markedly virulent species, which causes severe clinical disease in the domestic dog and even with treatment mortality rates reach 12% or more depending on accompanying complications (Schoeman, 2009, Leisewitz et al., 2019b). The severity of clinical disease at presentation can range from lethargy and anorexia to collapse. Most dogs present with pale mucous membranes, pyrexia, variable degrees of tachycardia, tachypnoea and palpable splenomegaly (Schoeman, 2009). A small proportion of dogs can present with congested mucous membranes and haemoconcentration. The pathophysiology of the haemoconcentration is poorly understood and these dogs have a poorer prognosis for survival (Leisewitz et al., 2019b). A number of prognostic markers and relevant cut off values associated with death were recently published by Leisewitz *et al* including cortisol (>388 nmol/L), urea (>14 mmol/L), lactate (>4.1 mmol/L), total bilirubin (>14.7 µmol/L) and thyroxine (<4.8 nmol/L) (Leisewitz et al., 2019b). All in all, *B. rossi* is a highly virulent pathogen infecting domestic dogs in South Africa, leading to clinical disease often requiring intensive veterinary treatment.

Treatment

Treatment is aimed at eliminating the parasite, limiting the degree of anaemia and managing any complications that arise after killing of the parasites. There are a number of drugs available for the treatment of *Babesia* parasites. The most commonly used treatment in South Africa is diminazene aceturate dosed at 3.5 mg/kg intramuscularly or subcutaneously (Baneth, 2018, Collett, 2000). Due to its narrow safety margin and pharmacokinetics diminazene aceturate should not be repeated within a 21 day period (Miller et al., 2005). In a survey by Collet, diminazene was associated with the highest incidence of treatment failure with recurrence of the infection and in these cases multi-drug regimens are required to clear infection (Collett, 2000). Imidocarb dipropionate given at a dose of 6.6 mg/kg repeated after a 14-day interval has been shown to successfully clear the parasite in the cases of *B. canis, B. rossi* and *B. vogeli* (Baneth, 2018, Collett, 2000). Of the three treatments, imidocarb was associated with the highest number of side effects in the survey by Collett (Collett, 2000). It is sometimes used as a follow up protocol in cases of treatment failure with diminazene aceturate (Collett, 2000). The final drug, which has fallen out of favour in recent years (and in fact is no longer available), is trypan blue given at 10 mg/kg slowly intravenously (Collett, 2000, Schoeman, 2009). It is

not advisable to sterilise the infection, as this may hinder the development of long-lasting immunity, diminazene aceturate and trypan blue are two drugs which do not sterilise the infection (Schoeman, 2009).

Supportive treatment is instituted depending on the severity of clinical signs, anaemia and occurrence of complications. This treatment is generally tailored to individual patient requirements. In the case of severe life threatening anaemia, which worsens after treatment, blood transfusions are given as required (Schoeman, 2009). Cross matching is required in patients that have received blood previously. Fluid therapy, electrolyte (particularly potassium), glucose, and oxygen supplementation, immunosuppressive therapy (glucocorticoids) as well as other supportive treatments, given as needed.

Pathogenesis and complications

Babesiosis is a complex multi-systemic disease that can be classified as either uncomplicated or complicated (Jacobson and Clark, 1994, Jacobson, 2006, Leisewitz et al., 2019b). Uncomplicated disease occurs when the clinical signs can be attributed directly to the haemolytic anaemia caused by the parasite (Jacobson, 2006). Complicated babesiosis occurs when the pathology noted cannot be directly attributed purely to the anaemia or when the anaemia becomes severe enough to perpetuate organ dysfunction (Leisewitz et al., 2019b). The pathogenesis of the anaemia is incompletely understood. Intravascular and extravascular haemolysis appears to play a central role as evidenced by the increase haemoglobinuria and bilirubin commonly seen during the course of infections (Leisewitz et al., 2019b). Immune mediated destruction of red blood cells has been proposed as a possible contributor to the anaemia in some cases of canine babesiosis, and a large proportion of cases in South Africa have been found to be Coomb's positive (Reyers et al., 1998). Despite appropriate erythropoietin levels for the degree of anaemia there appears to be an inadequate bone marrow response with reticulocyte counts $<100 \times 10^9/L$ in up to 70% of dogs (Leisewitz et al., 2019b). A possible mechanism for this inadequate regeneration is dyserythropoiesis and erythroid hypoplasia induced by the inflammatory response to infection as seen in human malaria patients (Leisewitz et al., 2019b, Chang and Stevenson, 2004, Menendez et al., 2000). Complications include severe anaemia, acute renal failure, cerebral babesiosis, coagulopathy, icterus and hepatopathy, immune-mediated haemolytic anaemia, acute respiratory distress syndrome, haemoconcentration, pancreatitis and rhabdomyolysis (Jacobson, 2006, Jacobson and Lobetti, 1996, Welzl et al., 2001, Mohr et al., 2000, Basson, 1965, Botha, 1964, Malherbe and Parkin, 1951, Purchase, 1947). The complications and multiple organ dysfunction seen in severe complicated *Babesia rossi* infections are likely caused by

the effects of an unfocused and excessive inflammatory response, rather than by the parasite itself (Jacobson and Clark, 1994).

The evolution of the inflammatory response in babesiosis

Babesia rossi infection, like *Plasmodium falciparum* malaria in humans, results in a protozoal sepsis with a severe systemic inflammatory response (Bone et al., 1992, Jacobson et al., 2002, Clark et al., 2006). It is suggested that the pathology results from excessive production of pro-inflammatory cytokines and a disturbance in the balance of pro-inflammatory and anti-inflammatory cytokine secretion (Goddard et al., 2016, Galán et al., 2018, Zygner et al., 2014, Brown et al., 2015, Leisewitz et al., 2019a). Cytokines and chemokines are a group of endogenous inflammatory and immunomodulating proteins that play a key role in the host response to infection (Ouyang et al., 2011, Chaudhry et al., 2013, Ahmed, 2002). Pro-inflammatory cytokines and chemokines such as TNFα, IFNγ, IL-1β, IL-2, IL-6, IL-8, IL-12, IL-18 and MCP-1 are necessary to initiate an effective inflammatory response and the immunomodulating cytokines, IL-4, IL-10 and TGFβ, are required to control and down regulate the inflammatory response (Goddard et al., 2016, Blackwell and Christman, 1996, Gogos et al., 2000). Pro-inflammatory cytokines play a number of roles in systemic inflammation, they are endogenous pyrogens, they trigger the production of secondary mediators and cytokines from cells such as macrophages and mesenchymal cells, they promote the release of acute phase proteins and attract inflammatory cells to regions of tissue damage (Chaudhry et al., 2013). A number of key cytokines have been identified to either significantly increase or decrease in canine babesiosis, namely IL-6, IL-8, IL-10, MCP-1 and TNFα (Goddard et al., 2016, Galán et al., 2018, Zygner et al., 2014, Leisewitz et al., 2019a). The cytokines evaluated in this study are reviewed below.

Tumour necrosis factor alpha is considered an initiator cytokine of the pro-inflammatory response as it triggers the release of other cytokines such as IL-6 and IL-8 (Blackwell and Christman, 1996). TNFα is one factor mediating endothelial glycocalyx shedding during sepsis, leading to a disruption in the barrier function of the endothelium (Schmidt et al., 2012, Ince et al., 2016). This cytokine is predominantly produced by monocyte/macrophages and plays a prominent role in the development and progression of systemic inflammation in both infectious and non-infectious conditions (Blackwell and Christman, 1996, Jaffer et al., 2010, Idriss and Naismith, 2000). In models of sepsis TNFα reaches peak concentrations within 60-90 minutes of exposure to endotoxin, this rapid rise places TNFα in a prime position to trigger the release other inflammatory mediators (Blackwell and Christman, 1996, Jaffer et al., 2010). This was the first parasite-induced cytokine to be evaluated in malaria and is produced by macrophages in response to parasitized erythrocytes, hemozoin and some glycolipids (Malaguarnera and Musumeci, 2002, Kwiatkowski et al., 1990). TNFα inhibits malaria in mice and *in*

vitro Plasmodium falciparum in the presence of sufficient leukocytes and provided these leukocytes are able to produce distal anti-parasite substances such as nitric oxide (Clark et al., 2006). A strong positive correlation between TNFα and increased risk of a fatal outcome has been identified in falciparum malaria (Day et al., 1999). Although TNFα is protective against parasites, excessive and prolonged production of TNFα may potentiate pathology and even contribute to the anaemia seen in falciparum malaria (Clark et al., 2006, Malaguarnera and Musumeci, 2002). In an experimental infection of dogs with *B. gibsoni*, TNFα showed marked increase in the two dogs in which infection was established (Brown et al., 2015). TNFα also increases in both *B. canis* and *B. rossi* infections (Zygner et al., 2014, Vaughan-Scott et al., 2009). In addition to being increased in *B. canis* infection, TNFα was associated with hypotension and renal failure (Zygner et al., 2014). Leisewitz et al found that TNFα was significantly higher in dogs infected with *B. rossi* than healthy controls, and in complicated versus uncomplicated cases and in non-survivors (Leisewitz et al., 2019a).

Interleukin 10 on the other hand, down-regulates the cell-mediated inflammatory response by inhibiting the synthesis of pro-inflammatory cytokines (Couper et al., 2008). IL-10 is a vital cytokine in the anti-inflammatory response and is secreted by T helper 2 lymphocytes, B lymphocytes and monocytes (Chaudhry et al., 2013). The production of pro-inflammatory cytokines such as TNFα, IL-1, IL-6, IL-8, IL-12 and GM-CSF from monocyte/macrophages, neutrophils and NK cells is supressed by IL-10 (Opal and DePalo, 2000). This cytokine is an important immunoregulator during infection with a wide range of pathogens and modulates excessive T helper 1 and cytotoxic T lymphocyte responses which contribute to the pathology seen in severe inflammation (Couper et al., 2008). Excessive IL-10 production on the other hand may limit an effective pro-inflammatory response allowing pathogens to escape immune destruction (Couper et al., 2008). A high IL-10:TNFα ratio has been associated with death and continuous overproduction of IL-10 has led to profound immunosuppression in sepsis (Chaudhry et al., 2013). Excessive production of IL-10 is a major risk factor for severe sepsis and death (Chaudhry et al., 2013). IL-10 was increased in patients with falciparum malaria and high levels correlated with increased disease severity, in one study (Lyke et al., 2004, Berg et al., 2014). Increased concentrations of IL-10 were seen in dogs experimentally infected with *B. gibsoni (Brown et al., 2015)*. Dogs infected with *B. rossi* demonstrated an increase in IL-10 concentrations and in the study by Leisewitz et al the higher concentrations correlated with increased risk of death (Goddard et al., 2016, Leisewitz et al., 2019a). High IL-10 concentrations may contribute to disease pathology by resulting in a state of immunosuppression in these dogs but the high concentrations may also be the result of the severe disease and an attempt by the body to control widespread inflammation.

Interleukin 6 plays a central role in host defence during inflammation caused by infection and has a variety of biological functions including induction of acute phase proteins (C-reactive protein and

serum amyloid A) production by hepatocytes, stimulation of adrenocorticotropic hormone and secretion of cortisol, suppression of the hypothalamic-pituitary-thyroid axis, activation of B and T lymphocytes as well as modulation of haematopoiesis (Blackwell and Christman, 1996, Song and Kellum, 2005). This cytokine is secreted by endothelial cells, monocytes/macrophages and fibroblasts (Akdis et al., 2011). The production of IL-6 is induced by tissue damage and the binding of pathogen associated molecular patterns (PAMPs) to pattern recognition receptors (PRRs) on cells of the innate immune system (Chaudhry et al., 2013). In human studies IL-6 has a positive correlation with severity of sepsis and increased risk of death (Chaudhry et al., 2013). In human malaria patients, IL-6 increases with high parasite numbers, disease severity and increased risk of mortality (Day et al., 1999). In the study by Day et al IL-6 and IL-10 were positively correlated reflecting the counter-regulatory balance between pro and anti-inflammatory cytokines in most malaria patients (Day et al., 1999). A high IL-6:IL-10 ratio was associated with increased risk of death, demonstrating a possible imbalance of these cytokines in malaria patients that die (Day et al., 1999). This cytokine also appears to be involved in myocardial dysfunction during sepsis in people (Clark et al., 2006). In a *B. gibsoni* experimental study by Brown et al, IL-6 concentrations showed pronounced increases in the two dogs in which infection was established (Brown et al., 2015). *Babesia rossi* infection, like human sepsis, produces increased IL-6 concentrations which correlate positively with disease severity and death (Leisewitz et al., 2019a, Goddard et al., 2016). In canine babesiosis, IL-6 is thought to suppress triiodothyronine production through decreased activity of type 1 and 2 deiodinase activity (Zygner et al., 2015). IL-6 stimulates the release of corticotropin releasing hormone and adrenocorticotropic hormone, thus stimulating the release of cortisol possibly contributing to the high cortisol levels identified in canine babesiosis (Zygner et al., 2015, Schoeman et al., 2007). In the study by Goddard et al, IL-6 was significantly higher in dogs that were sick for <48 hours prior to presentation, suggesting this may be an important cytokine early on the in the inflammatory response to *B. rossi* but may be replaced by other pro-inflammatory cytokines later in the disease course (Goddard et al., 2016).

Interleukin 8 is a chemokine produced by monocytes/macrophages, neutrophils, lymphocytes epithelial cells and endothelial cells amongst others (Chaudhry et al., 2013, Akdis et al., 2011). The functions of IL-8 include chemo-attraction of neutrophils to sites of inflammation, activation and degranulation of neutrophils and basophils, as well as inhibiting leukocyte adhesion to cytokine-activated endothelial cells (Blackwell and Christman, 1996, Remick, 2005, Gimbrone et al., 1989). Circulating concentrations are increased during sepsis in humans and have even been shown to correlate with the stages of sepsis, even in subtle septic processes (Livaditi et al., 2006). In human patients with falciparum malaria, IL-8 concentrations increased and higher levels were identified in symptomatic versus asymptomatic patients as well as in younger patients (Lourembam et al., 2013).

The chemokine is significantly associated with disease severity and was markedly higher in patients co-infected with HIV and those that died during falciparum malaria (Berg et al., 2014). *Babesia rossi* infection, unlike infections with other *Babesia spp.* affecting canines, is associated with a decreased concentration of IL-8 (Goddard et al., 2016, Galán et al., 2018, Brown et al., 2015). This surprising finding may be due to the peak concentration occurring earlier or later in the disease course than what has previously been investigated.

Monocyte chemoattractant protein-1 (MCP-1/CCL2) is one of the key chemokines that regulate migration and infiltration of monocytes/macrophages from the blood stream across the vascular endothelium which is required for routine immunological surveillance of tissues and response to inflammation (Deshmane et al., 2009). MCP-1 is produced by a number of cells including endothelial cells, fibroblasts, epithelial cells as well as monocytes/macrophages which form a major source of this cytokine (Deshmane et al., 2009). Increased levels of MCP-1 have been associated with many human conditions, such as HIV, thrombus formation, cardiovascular disease, neoplasia and a number of inflammatory conditions (Deshmane et al., 2009). This chemokine is one of the most studied in human medicine and has been associated with both pathological conditions such as rheumatoid arthritis and multiple sclerosis as well as playing a key role in immune modulation and clearing of viral infections (Deshmane et al., 2009). The recruitment of monocytes/macrophages to regions of inflammation is important for clearing of pathogens through phagocytosis and production of a number of inflammatory mediators (Deshmane et al., 2009). MCP-1 was increased in human malaria patients when compared to healthy controls and significantly higher levels were found in patients that died in hospital (Berg et al., 2014). In canine babesiosis caused by *B. rossi* MCP-1 concentrations were increased in infected dogs and the degree of this increase was positively correlated with increased risk of death (Goddard et al., 2016, Leisewitz et al., 2019a). Complicated cases of canine babesiosis in dogs infected with *B. canis* had higher levels of MCP-1 on day 1 and day 7 after presentation than uncomplicated cases (Galán et al., 2018).

Keratinocyte-derived chemoattractant (KC/CXCL1/KC-like) is another cytokine involved in inflammation and its expression plays a role in neutrophil chemoattraction and migration (Bozic et al., 1995). In one study the major source of KC was TNF-stimulated endothelial cells (Girbl et al., 2018). KC recruits and activates neutrophils in sepsis by providing chemotactic cues for neutrophils to move to regions of inflammation followed by activation of these neutrophils to release reactive oxygen radicals and various proteases (Jin et al., 2014, Sawant et al., 2016). This is supported by the finding that KC has been identified as a key mediator of neutrophil migration into sites of infection in experimental Lyme arthritis and carditis (Ritzman et al., 2010). KC has been shown to increase in Canine Visceral Leishmaniasis and was able to differentiate between dogs with different clinical scores

(Solca et al., 2016). Dogs with higher clinical scores in that study also demonstrated high parasite loads (Solca et al., 2016). Increased concentrations of KC-like were seen in dogs experimentally infected with *B. gibsoni* (Brown et al., 2015). In *Babesia canis* infection, KC-like was significantly increased during the first 7 days after diagnosis and treatment (Galán et al., 2018). In addition to this, concentrations were also significantly higher in dogs that developed complicated babesiosis during the study period (Galán et al., 2018).

Interferon gamma (IFNγ) is a cytokine that plays an essential role in the immune response to viral and intracellular bacterial infections (Chaudhry et al., 2013). This is the main cytokine defining T helper 1 lymphocyte responses (Chaudhry et al., 2013). Cellular sources of IFNγ include NK cells, macrophages, T helper 1 lymphocytes, cytotoxic T lymphocytes and B lymphocytes (Akdis et al., 2011). Interferon gamma is one of the most important cytokines influencing macrophage response in the immune system (Schroder et al., 2004). IFNγ-activated macrophages release a number of pro-inflammatory substances such as cytokines (TNFα, TGFβ, IL-1 and IL-6) as well as reactive oxygen and nitrogen radicals (Malaguarnera and Musumeci, 2002). It also coordinates leukocyte chemotaxis, influences the development of many other cell types, has a role in B lymphocyte immunoglobulin production and class switching, and promotes NK cell function (Schroder et al., 2004). IFNγ is increased in falciparum malaria particularly in complicated cases (Lourembam et al., 2013). It is thought to limit the initial phases of parasite replication but excessive production may actually promote more severe disease (Lourembam et al., 2013, Malaguarnera and Musumeci, 2002). In the experimental infection of dogs with *B. gibsoni,* IFNγ did not reach detectable concentrations at any time during the study (Brown et al., 2015). Given the importance of this cytokine in clearing intracellular pathogens and its clinical relevance in malaria, more research into its kinetics during babesiosis is needed. A correlation between IFNγ and disease severity was not identified in natural *B. rossi* infections in one study (Leisewitz et al., 2019a).

Interferon-gamma-inducible protein 10 (IP-10) is a potent chemokine with functions including chemoattraction of activated T lymphocytes to sites of inflammation, contributions to capillary injury and inhibiting angiogenesis (Frangogiannis et al., 2000, Taub et al., 1993, Sgadari et al., 1996, Angiolillo et al., 1995). This chemokine was discovered when monocytes treated with INFγ showed marked induction of the genes coding for this cytokine (Farber, 1997). Under the influence of INFγ, IP-10 is produced from monocytes, endothelial cells, fibroblasts and keratinocytes to variable degrees (Luster and Ravetch, 1987). IP-10 acts as a chemoattractant for T lymphocytes predominantly but also monocytes and NK cells as well as enhancing NK cell activity (Farber, 1997). An IP-10 murine analogue (Crg-2) was induced in multiple organs in experimental infection of mice with protozoa, one being

Plasmodium yoelii (Amichay et al., 1996). In an experimental infection of dogs with *B. gibsoni* IP-10 did not reach detectable levels in the dogs at any point during the infection (Brown et al., 2015).

Interleukin-2 is a cytokine primarily affecting T lymphocytes. This cytokine is secreted from activated $CD4^+$ and $CD8^+$ T lymphocytes, dendritic cells and NK cells (Akdis et al., 2011). It promotes clonal expansion of antigen activated T lymphocytes and is pivotal in the production of T regulatory lymphocytes (Malek, 2003, Gaffen and Liu, 2004). Additional effects include increased B lymphocyte proliferation and antibody production (Akdis et al., 2011, Gaffen and Liu, 2004). IL-2 also acts as a growth factor for NK cells together with IL-15, promoting their differentiation and proliferation (Gaffen and Liu, 2004, Akdis et al., 2011). In malaria IL-2 was increased in *Plasmodium falciparum* positive cases but the concentrations were lower in complicated cases when compared to those without complications (Lourembam et al., 2013). IL-2 concentrations were increased in dogs experimentally infected with *B. gibsoni* (Brown et al., 2015). In *B. rossi* infection IL-2 appears to be associated with the acute phase of the disease as higher levels were seen in dogs presented earlier in the course of infection (Goddard et al., 2016).

Interleukin-7 is key to the development of T and B lymphocytes as well as modulation of mature T lymphocyte activity (Fry and Mackall, 2002). This trophic cytokine is capable of markedly expanding both T and B lymphocyte populations, it is involved in VDJ recombination and even plays a role in the positive selection of lymphocytes during lymphopoiesis (Hofmeister et al., 1999, Akdis et al., 2011). Cellular sources of IL-7 include epithelial cells, dendritic cells, B lymphocytes, monocytes/macrophages and keratinocytes (Akdis et al., 2011). The effect on mature T lymphocytes includes co-stimulation and enhancement their propagation, increasing their cytokine production particularly IFNγ, IL-2 and to a lesser degree IL-4 (Fry and Mackall, 2002). Another role of IL-7 is the inhibition of mature T lymphocyte programmed cell death, prolonging survival and playing a critical role in the development of memory T lymphocytes (Fry and Mackall, 2002). IL-7 also increase the cytolytic activity of $CD8^+$ T lymphocytes and induces monocytes to produce inflammatory mediators (Fry and Mackall, 2002, Akdis et al., 2011). In dogs with pyometra, IL-7 was identified as a possible marker for systemic inflammatory response and may be used to identify dogs that are more severely affected (Karlsson et al., 2012). Increased concentrations of IL-7 were seen in dogs experimentally infected with *B. gibsoni* (Brown et al., 2015). IL-7 concentrations were significantly increased in dogs infected with *B. canis* and these levels were higher in dogs with complicated disease on day 1 after diagnosis thereby acting as a possible predictor for the risk of complications (Galán et al., 2018). In contrast to this finding, the study by Leisewitz et al where cytokines were evaluated after natural infection of dogs with *B. rossi,* no difference was found in IL-7 levels between infected dogs and healthy controls (Leisewitz et al., 2019a).

Interleukin-15 is essential for the maturation, activation, proliferation and persistence of natural killer cells (NK cells) (Perera et al., 2012, Akdis et al., 2011). It also plays a role in the activation of many cells of the innate immune system as well as the development of cytotoxic T lymphocytes and the maintenance of memory cytotoxic T lymphocytes (Perera et al., 2012). It is produced by monocytes, activated T helper lymphocytes, keratinocytes and skeletal muscle (Akdis et al., 2011). In human *Plasmodium falciparum* infections, IL-15 upregulation may enhance early innate immune system activation potentially providing a protective response (Bouyou-Akotet et al., 2004). Increased concentrations of IL-15 were seen in experimental infection of dogs with *B. gibsoni* (Brown et al., 2015).

Another cytokine important to the innate and adaptive immune responses is IL-18 and it is produced by a number of cells such as macrophages, Kupffer cells, keratinocytes, dendritic cells amongst others (Akdis et al., 2011). IL-18 induces the production of INFγ from T helper 1 lymphocytes, activated macrophages and natural killer cells, especially under the influence of IL-12 (Kojima et al., 2004, Lourembam et al., 2013). This cytokine also stimulates T lymphocyte and NK cell maturation, cytokine production, endothelial marker expression (ICAM-1 and VCAM-1) and promotes cytotoxicity of immune cells (Gracie et al., 2003). Concentrations of IL-18 were increased in falciparum malaria particularly during the early stages, however levels varied in the intermediate to late phases depending on complications (Kojima et al., 2004). In human studies, IL-18 concentrations also increase in a number of other inflammatory conditions and sepsis (Chaudhry et al., 2013). Significant concentrations of IL-18 were found in asymptomatic cases of canine leishmaniasis (Manna et al., 2006). IL-18 demonstrated increased concentrations during experimental infection of dogs with *B. gibsoni* (Brown et al., 2015). It was also increased during the acute phases of canine babesiosis in dogs infected with *B. rossi* (Goddard et al., 2016).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a key director of every level of granulocyte and macrophage cell line development, with a role in inflammation and autoimmunity (Hamilton, 2002). It is secreted by macrophages, T-cells, mast cells, endothelial cells, and fibroblasts, and stimulates the stem cells of the monocyte, neutrophil, eosinophil and basophil lineages (Chaudhry et al., 2013). Increases in GM-CSF in human sepsis is variable and has even been trialled as a treatment to counter the immunosuppression associated with sepsis (Chaudhry et al., 2013). Inflammation and immune-mediated disease are known contributors to *B. rossi* complications, GM-CSF could be considered as a potential influencer in these processes. Increased concentrations of GM-CSF were seen in dogs experimentally infected with *B. gibsoni* (Brown et al., 2015). This cytokine may play a role in the acute phases of *B. rossi* infection as concentrations were increased in dogs presenting earlier in the course of the disease (Goddard et al., 2016).

The presence of a 'cytokine storm' in babesiosis may play a key role in the pathogenesis and the severity of disease. For some time, the 'cytokine storm' theory has been proposed to underly the pathophysiology of a number of systemic inflammatory conditions such as protozoal, bacterial and viral infections as well as events following trauma (Clark et al., 2008b). This theory proposes that systemic illness and the course of the disease is not necessarily the result of microbial toxins themselves but rather excessive production of pro-inflammatory cytokines in response to antigens (Clark et al., 1997). TNFα given to human cancer patients as a possible treatment resulted in dose dependent malaise, pyrexia, headaches, hypotension, myalgia, thrombocytopenia, nausea, vomiting and diarrhoea, all clinical symptoms associated with early malaria and viral infections such as influenza (Clark et al., 2008a). The theory of excessive cytokine production contributing to disease pathogenesis was further supported by a number of studies during which neutralizing antibodies to human TNFα given to malaria patients actually resulted in decreased duration of pyrexia (Clark et al., 1997). IFNγ is another cytokine implicated in the excessive inflammatory response to *Plasmodium falciparum* (Clark et al., 1997). Anti-inflammatory cytokines such as IL-10 also play a critical role in the pathogenesis of disease because imbalances between anti- and pro-inflammatory cytokines are often associated with poor disease outcome (Clark et al., 2008a). The sickness induced by cytokines is thought to be an evolutionary mechanism to promote survival, resulting in a syndrome which reduces the desire to seek out food, encourages individuals to find shelter for rest and reduces metabolic demand to conserve energy long enough to overcome a diseased condition (Clark et al., 2008b). Although this sickness behaviour may be beneficial, excessive expression of the mediators responsible for this may lead to cumulative pathology such as those seen in severe systemic inflammatory response (Clark et al., 2008b). Even the anaemia seen in inflammatory conditions may be seen as a survival advantage by conserving energy but if the suppression is prolonged and excessive this may lead to reduction in the oxygen carrying capacity of the blood (Clark et al., 2008b). TNFα is a key contributor to the dyserythropoiesis and has been shown to suppress nuclear elements governing the division of erythropoietic cells in the bone marrow (Clark et al., 2008b). There is an abundance of research on TNFα and the effect it has on systemic illness but other cytokines such as IL-1, IL-6, IL-10, IL-4 and many more are known to contribute with more research being published (Clark et al., 2006). Clark proposed that the reason parasitaemia in mice reach much higher levels than those seen in the intended hosts for *Babesia,* malaria and trypanosomes, is that mice are more tolerant of the cytokine induced illness and require a higher parasite load to trigger illness (Clark, 2000). Although the 'cytokine storm' has become a popular term used to explain the pathophysiology of numerous inflammatory conditions it is important to remember that the triggers for these diverse conditions will differ and subsequently the kinetic profiles of the cytokines and chemokines involved will differ (Clark, 2007). In

falciparum malaria functional tissue hypoxia with sustained dependence on anaerobic metabolism is a key mechanism in the death of patients (Clark et al., 2006). Inflammatory cytokines are capable of suppressing the bone marrow, negatively impacting red cell deformity thus promoting their early removal by the spleen and encouraging sequestration of parasitised erythrocytes in specific sites, contributing to this tissue hypoxia (Clark et al., 2006). Although less research has been done in canine babesiosis, the disease course seen in *B. rossi* infections bears a striking resemblance to that seen in falciparum malaria, leading one to hypothesize a similar 'cytokine storm' may be an essential mechanism in this disease. This is supported by studies demonstrating excessive cytokine production and their correlation to disease severity, the occurrence of complications and negative outcomes in *B. rossi* infections (Goddard et al., 2016, Leisewitz et al., 2019a). Many more cytokine changes may not have been identified in canine babesiosis which could also contribute to this inflammatory milieux. All studies to date have been based on samples collected at a single time point (at presentation) and aspects of the progression of this highly inflammatory condition have likely been missed.

Infections, such as those caused by *B. rossi*, trigger an acute phase response. This is a nonspecific complex systemic early-defence system serving as an integral part of the innate immune response. It involves physical as well as molecular barriers and responses that serve to prevent infection, clear potential pathogens, initiate inflammatory processes and contribute to resolution and healing processes (Cray et al., 2009). Acute phase proteins, blood proteins produced by hepatocytes, are an integral part of the acute phase response and are grouped as either positive or negative based on whether they increase or decrease in inflammation (Cray et al., 2009). Albumin represents the major negative acute phase protein decreasing during inflammation. Decreases may be the result of either selective loss of albumin due to renal or gastrointestinal permeability changes or a decrease in hepatic synthesis (Kaneko et al., 2008, Cray et al., 2009). Mild hypoalbuminemia has been reported before in babesiosis (Sudhakara Reddy et al., 2016, Leisewitz et al., 2019b). The acute pan-systemic sepsis-like state of the disease more than likely results in a drop in albumin as a result of a negative acute phase response and a leaky endothelium (Cray et al., 2009, Ince et al., 2016, Adembri et al., 2011, Conroy et al., 2010). Albumin can act as a major source of amino-acids in the body when necessary (Ceron et al., 2005). This redirected pool of amino-acids may be used to generate positive acute phase proteins and other important mediators of inflammation (Paltrinieri, 2008, Cray et al., 2009). In addition to other functions, albumin also binds to drugs and this can have important pharmaco-kinetic implications in clinical therapy because variations in the plasma levels of acute phase proteins during inflammation can alter the free plasma concentration of drugs (Ceron et al., 2005, Belpaire et al., 1987).

C-reactive protein (CRP) is classified as a major positive acute phase protein due to the magnitude of its response (10-to-100-fold increase from normal) as well as the speed with which it increases

following an acute stimulus (Yamamoto et al., 1992, Caspi et al., 1984, Ceron et al., 2005). Serum levels of CRP closely reflect the extent of tissue damage and inflammation following trauma, as well as some infective and non-infective conditions (Caspi et al., 1984). CRP increases in response to pro-inflammatory cytokines such as IL-6 and IL-1β (Black et al., 2004). The main biological functions of CRP include bacterial binding with promotion of complement binding and phagocytosis, induction of cytokines, inhibition of chemotaxis and modulation of neutrophil function (Ebersole and Cappelli, 2000, Ceron et al., 2005, Ballou and Lozanski, 1992). In a previous study by Koster et al. there was insufficient evidence for CRP as a prognostic marker for mortality or morbidity in dogs suffering from canine babesiosis caused by *B. rossi* in South Africa (Koster et al., 2009). However measurement of the C-reactive protein concentration was found to be valuable in assessing of severity of babesiosis caused by *Babesia canis*, and in following the response to anti-babesia treatment in another study where they differentiated complicated from uncomplicated cases (Matijatko et al., 2002). A time course evaluation of the effect of infection with *B. rossi* and treatment on CRP concentrations may shed further light on this.

Babesiosis has been associated with an inflammatory leukogram (Scheepers, 2013, Reyers et al., 1998, Leisewitz et al., 2019b) and can cause a moderate to severe monocytosis, especially in dogs with more severe anaemia (Weltan et al., 2008, Reyers et al., 1998). This is not surprising because activated monocytes phagocytose parasitized erythrocytes in circulation as well as in the spleen in canine babesiosis (Maegraith et al., 1957, Jacobson and Clark, 1994, Weltan et al., 2008, Henning et al., 2020). Interestingly, the babesiosis associated inflammatory leukogram is characterised mainly by a left shift (increased band neutrophil count) without concurrent mature neutrophilia (Scheepers, 2013, Weltan et al., 2008). In fact, some *B. rossi* infected dogs even had a degenerative left shift in one study, which was defined as an increased band neutrophil count with normal or low mature neutrophil count (Scheepers, 2013). These findings could indicate excessive neutrophil destruction or utilisation, an early stage of bone marrow response, impaired bone marrow response, or previous depletion of the storage pool of neutrophils from the bone marrow (Scheepers, 2013). Dogs with complicated babesiosis are more likely to have higher white blood cell counts, band neutrophils and monocytes than dogs with uncomplicated babesiosis, however there is a large degree overlap in these values (Weltan et al., 2008). Assessing these leukocyte counts in conjunction with other known markers of inflammation may help differentiate complicated from uncomplicated cases.

Hyperlactataemia has been identified as a prognostic indicator in canine babesiosis (Nel et al., 2004) and malaria (Maitland, 2006, Possemiers et al., 2021). With serial measurements identifying persistent hyperlactataemia having the strongest predictive value for outcome (Nel et al., 2004, Aramburo et al., 2018). Elevations in serum lactate during sepsis have been attributed to a combination of tissue

hypoxia and a hypermetabolic state (Mizock and Falk, 1992). In states of sepsis, such as babesiosis, a hypermetabolic state has been noted where increased glycolytic flux, transamination from alanine and down regulation of pyruvate dehydrogenase may contribute to elevations in lactate (Mizock and Falk, 1992). Hypoxia in severe babesiosis is related to the decreased oxygen carrying capacity of blood due to haemolytic anaemia and hypotension leading to hypoperfusion of tissues with subsequent anaerobic glycolysis and hyperlactataemia (Lobetti, 2005, Jacobson et al., 2000). Hypoglycaemia is a common finding in babesiosis but tends to occur together with hyperlactataemia (Nel et al., 2004, Keller et al., 2004). Lactate was found to be an adequate predictor of outcome alone when assessed at the time of presentation for clinical disease (Nel et al., 2004). Blood glucose concentrations should be measured in all *B. rossi* infected dogs particularly in collapsed animals, puppies and patients with severe anaemia, vomiting or icterus as dogs with neurological signs may be erroneously diagnosed with cerebral babesiosis instead of neuroglycopenia (Keller et al., 2004).

Parasitaemia

Higher parasitaemia at the time of presentation is significantly associated with disease severity and eventual mortality in canine *B. rossi* infections (Bohm et al., 2006, Leisewitz et al., 2019a). Peripheral capillary parasite numbers are higher when compared to central venous samples from patients infected with *B. rossi* at the same time point, making peripheral blood smear samples more sensitive diagnostic tools (Bohm et al., 2006). Although these findings have been identified at time of presentation of clinically affected dogs, the influence of infectious dose on rate and severity of disease course as well as increase in parasitaemia over time has not been investigated in canine babesiosis caused by *B. rossi*. High infectious dose of *B. microti* and *B. hylomysci* reduced the length of the prepatent period and increased the peak parasitaemia in experimentally infected mice (Gray and Phillips, 1983). Although the duration of *B. microti* parasitaemia was not influenced, higher infectious doses of *B. hylomysci* resulted in the death of the infected mice (Gray and Phillips, 1983). Higher infectious doses of *B. rossi* parasites may shorten the prepatent period, increase the rate of complications and even reduce the ability of the host to survive as seen with *B. hylomysci* infections in mice (Gray and Phillips, 1983).

Conclusion

In conclusion, babesiosis is a disease caused by intracellular *Babesia* protozoa transmitted by arthropod vectors, is characterised by haemolysis and a variety of severe complications (Schetters et al., 2009b). Babesiosis in dogs is caused by the species *B. rossi*, *B. canis*, *B. vogeli*, *B. gibsoni, B. vulpes* and *B. conradae* of which *B. rossi* and *B. vogeli* are found in South Africa (Schoeman, 2009, Matjila et al., 2004, Parnell et al., 2008, Uilenberg, 2006). *Babesia rossi* is the predominant parasite infecting dogs in South Africa (Schoeman, 2009, Jacobson, 2006). It is also the most virulent *Babesia* species, causing severe clinical disease and even death in many domestic dogs in South Africa (Penzhorn, 2011). In recent years, there has been increasing concern about the global spread of parasitic arthropods and the pathogens they transmit across the planet, which is influenced by environmental and climatic changes, enhanced international commerce, increased global transport, human and animal population dynamics, and emerging drug resistance among both vectors and pathogens (Otranto et al., 2009, Knols and Takken, 2007). Babesiosis, a known disease of livestock, dogs and more recently an emerging disease in humans, is caused by related intra-erythrocytic protozoa and has a similar pathogenesis and clinical course to malaria (Krause et al., 2007). Malaria, caused by *Plasmodium falciparum,* is a significant cause of morbidity and mortality in humans accounting for hundreds of thousands of deaths every year in the world, most of whom are children in Africa (Fernandes et al., 2008, Murphy and Breman, 2001, White et al., 2014). The pathophysiology of malaria has been extensively studied in humans but many questions remain, especially regarding fulminant disease with severe malarial anaemia, cerebral malaria, respiratory distress and hypoglycaemia being the main complications of *Plasmodium falciparum* infection (Fernandes et al., 2008, Murphy and Breman, 2001, White et al., 2014). Recent studies of cytokine concentrations in these two diseases have demonstrated similarities in the associated inflammatory response that may provide insights into malarial pathophysiology (Krause et al., 2007, A. Clark L. S. Jacobson, 1998). The zoonotic potential of the canine *Babesia* parasites is low and probably insignificant (Otranto et al., 2009, Gray et al., 2010). Advantages of the study of host response during canine babesiosis is that infection might serve as a disease model for both babesia and malaria infections (Krause et al., 2007, Gray et al., 2010) whilst also providing a model for parasite-induced haemolysis.

It is suggested that the complications associated with *B. rossi* canine babesiosis result from an excessive production of pro-inflammatory cytokines as well as a disturbance in the balance between pro- and anti-inflammatory cytokine secretion (Goddard et al., 2016, Galán et al., 2018, Zygner et al., 2014, Brown et al., 2015, Leisewitz et al., 2019a). The idea of a 'cytokine storm' playing a critical role in the pathogenesis of this infection requires further investigation and although we have some understanding of the pathophysiology of the inflammatory response triggered by *B. rossi*, most of the

existing research was performed on dogs presenting at variable stages of disease with varying degrees of disease severity and very little is actually known about the initial stages of host response to infection or the effect that treatment has on progression of the inflammatory response.

Project justification

Babesia rossi infections are one of the most common reasons for canine patient presentation to the outpatient clinic at Onderstepoort Veterinary Academic Hospital. Not only is the morbidity and mortality of this disease high but it also puts significant financial strain on the owners and the clinicians who are often expected to treat these patients with limited budget. The identification of underlying pathophysiology which may act as therapeutic targets and the identification of prognostic markers to better advise clients of the potential outcome of treatment, would be invaluable tools in a clinician's arsenal when combatting this infectious disease. Evaluation of cytokines and markers of clinical disease over the course of experimental infection has been investigated in both *B. canis* and *B. gibsoni* in dogs, this however has never been studied in *B. rossi* (Brown et al., 2015, Schetters et al., 2009a). As *Babesia rossi* is the most virulent canine *Babesia sp.,* an investigation into the progression of the inflammatory response from the time of parasite inoculation to the onset of clinical disease and after treatment may provide a better understanding of the underlying pathophysiology influencing the outcome of this disease. Clinical studies investigate disease in cases with large variation in breed, sex, age, background health, co-infections, time since infection parasite strain and infectious dose. This results in scientifically 'noisy' clinical studies which may limiting their usefulness. Establishing an experimental model where these factors are controlled for, would greatly enhance our ability to study this disease and potentially allow the establishment of a model system to test novel treatments.

Chapter 2: Aims, objectives and hypotheses

Aims

In this prospective longitudinal observational study, we aimed to investigate changes in markers of inflammation (cytokine concentrations, neutrophil count, monocyte count, CRP and albumin) and important indicators of disease severity (habitus, appetite, vital parameters, blood pressure, haematocrit (Ht), red cell count (RCC), lactate, glucose) over time in an experimental *Babesia rossi* infection of beagle dogs. We also aimed to investigate the influence of infectious dose on disease progression by infecting two different groups of dogs with a high and a low dose parasite inoculum, respectively. In addition to the former, we also wanted to evaluate the influence treatment would have on all measured variables. Finally, we aimed to identify any significant correlations existing between the markers of inflammation and indicators of disease severity.

Objectives

1. To compare the severity, onset and progression of disease based on simple clinical (temperature, pulse, respiratory rate and blood pressure) and clinicopathological variables (red blood cell count, blood glucose and blood lactate) between a high infectious and a low infectious dose groups from baseline control data collected before infection.
2. To compare cytokine concentrations, serum albumin, C-reactive protein, neutrophil count and monocyte count over time between the two groups of dogs as well as between each group and the baseline control data.
3. To compare the rate of change in measured variables including cytokine concentrations, serum albumin, C-reactive protein, neutrophil count and monocyte count over time between the two groups of dogs from the baseline control data.
4. To correlate cytokine kinetics with changes in the markers of inflammation (serum albumin, C-reactive protein, neutrophil count and monocyte count) between the two groups of dogs from baseline control data.
5. Determine whether cytokine kinetics and changes in markers of inflammation (serum albumin, C-reactive protein, neutrophil count and monocyte count) will be reflected in the clinical and clinicopathological variables that are known to associate with disease severity.
6. Determine whether the progression of parasitaemia will differ between the two groups over time and whether the parasitaemia will correlate with any of the markers of inflammation.

Hypotheses

1. H0: There will be no significant difference in the severity, onset and progression of disease based on simple clinical (habitus, appetite, TPR, mucous membrane colour, blood pressure) and clinicopathological variables (red blood cell count, blood glucose and blood lactate) between the low and high infectious challenge groups.

   H1: There will be a significant difference in the severity, onset and progression of disease based on simple clinical (habitus, appetite, TPR, mucous membrane colour, blood pressure) and clinicopathological variables (haematocrit, blood glucose and blood lactate) between the low and high infectious challenge groups.
2. H0: There will be no significant difference in the cytokine concentrations, serum albumin, C-reactive protein and the complete blood count (CBC) variables over time between the low and high infectious challenge groups.

   H1: There will be a significant difference in the cytokine concentrations, serum albumin, C-reactive protein and the complete blood count (CBC) variables over time in the low and high infectious challenge groups.
3. H0: The rate of change in the measured variables over time between the low and high infectious challenge groups will not differ significantly.

   H1: The rate of change in the measured variables over time between the low and high infectious challenge groups will differ significantly.
4. H0: There will be no correlation in the cytokine kinetics and changes in the other markers of inflammation (serum albumin, C-reactive protein and components of the CBC) between the low and high infectious challenge groups.

   H1: There will be significant correlation in the cytokine kinetics and changes in the other markers of inflammation (serum albumin, C-reactive protein and components of the CBC) between the low and high infectious challenge groups.
5. H0: Cytokine kinetics and changes in markers of inflammation (serum albumin, C-reactive protein and components of the CBC) will not be reflected in the clinical and clinicopathological variables that associate with disease severity.

   H1: Cytokine kinetics and changes in markers of inflammation (serum albumin, C-reactive protein and components of the CBC) will be reflected in the clinical and clinicopathological variables that associate with disease severity.
6. H0: The progression of parasitaemia will not differ between the two groups over time and will not correlate with any of the markers of inflammation.

H1: The progression of parasitaemia will differ between the two groups over time and will correlate with some of the markers of inflammation.

Benefits arising from this study

This time course study of markers of inflammation in experimental *Babesia rossi* infection will improve our understanding of the evolution of the systemic inflammatory response associated with canine babesiosis. This data will add to the growing database established by the Babesia research group of the Faculty of Veterinary Science at the University of Pretoria and will assist with our ongoing endeavours as a group to optimise our approach to the treatment of this disease. We will compare this canine disease with other important blood-borne protozoal infections affecting humans and animals, in an attempt to better grasp the pathophysiology of this group of infections. In addition to this we will establish an experimental *B. rossi* infection to potentially pave the way for a disease model for studying blood-borne protozoal disease and haemolysis. This pilot study may provide the groundwork for larger scale and more in-depth investigations.

Chapter 3: Materials and methods

Model system and justification of the model

Study design

This was a prospective longitudinal observational study that included six purpose bred sterilised male beagle dogs. One dog was splenectomised and used to raise a viable parasite inoculum from cryopreserved wild type *Babesia rossi*. The remaining five dogs were experimentally infected with either a high or low dose *Babesia rossi* parasite inoculum. The five dogs finally infected with the *Babesia rossi* inoculum acted as their own baseline controls, with samples collected from each dog at 2 separate time points prior to infection.

Study setting

The beagles were housed at the University of Pretoria Biomedical Research Centre (UPBRC) from eight weeks of age until the end of the experimental period, thereafter the five surviving beagles were rehomed as pets. The clinical examinations and sample collection were performed on sight at the UPBRC. The complete blood count and biochemistry analysis was performed at Clinical Pathology Laboratory of the OVAH and the cytokine analysis was performed at the Department of Veterinary Tropical Diseases.

Study population

There were six purpose bred intact male beagle dogs. All dogs were permanently identified by unique microchip implantation (Back Home®, Virbac, South Africa)

Inclusion criteria for splenectomised dog:

- The dog was clinically healthy, clinical variables (temperature, pulse, respiration rate, abdominal palpation, capillary refill time and mucous membrane colour, haematology and biochemistry) were within normal.
- The dog was free from *Babesia rossi* or any other regional blood-borne parasitic infections (Confirmed by polymerase chain reaction and reverse line blotting (PCR-RLB) prior to experimental infection).
- All vaccinations and deworming were current.

Exclusion criteria for dog to be splenectomised:

- Co-infection with other blood parasitic infections namely *Theileria sp., Anaplasma sp.* and *Ehrlichia sp.* (Evaluated by PCR prior to experimental infection).

- The presence of comorbid disease.

Inclusion criteria for experimentally infected dogs:

- They were clinically healthy, clinical variables (temperature, pulse, respiration rate, abdominal palpation, capillary refill time and mucous membrane colour, haematology and biochemistry) were within normal limits.
- They were free from *Babesia rossi* or any other regional blood-borne parasitic infections (Confirmed by PCR-RLB prior to experimental infection).
- All vaccinations and deworming were current.

Exclusion criteria for experimentally infected dogs:

- Co-infection with other blood parasitic infections (Evaluated by PCR prior to experimental infection).
- The presence of comorbid disease.
- The splenectomised dog was excluded from the data collection.

The dogs acted as their own controls and were sampled as follows:

All 5 remaining dogs were sampled at 2 separate time points prior to their experimental infection.

<u>Sample method</u>

The five dogs meeting the necessary inclusion criteria were included in the study and randomly assigned to either a low infectious dose (LD) group or high infectious dose (HD).

<u>Sample size</u>

The 5 spleen-whole dogs were subject to sample collection for baseline control data.

These dogs were then randomly divided into 2 groups:
LD group:

- Dogs experimentally infected with a low dose (10^4 infected red blood cells) of *Babesia rossi* parasite inoculum
- 2 dogs were allocated to this group.

HD group:

- Dogs experimentally infected with a high dose (10^8 infected red blood cells) of *Babesia rossi* parasite inoculum
- 3 dogs were allocated to this group.

The high and low infectious doses were originally based on doses previously used in an experimental *B. canis* infection (Schetters et al., 2009a). We decided to use the mid-range dose from the previous study for the low dose group and a higher dose for the high dose group in this study because unlike the *B. canis* experimental infection, our parasites had been cryopreserved and viability of all the parasites were not necessarily guaranteed (Schetters et al., 2009a). The higher doses were selected to account for possible parasite loss during cryopreservation and improve the chances of establishing a viable infection in the current experimental model.

Experimental design

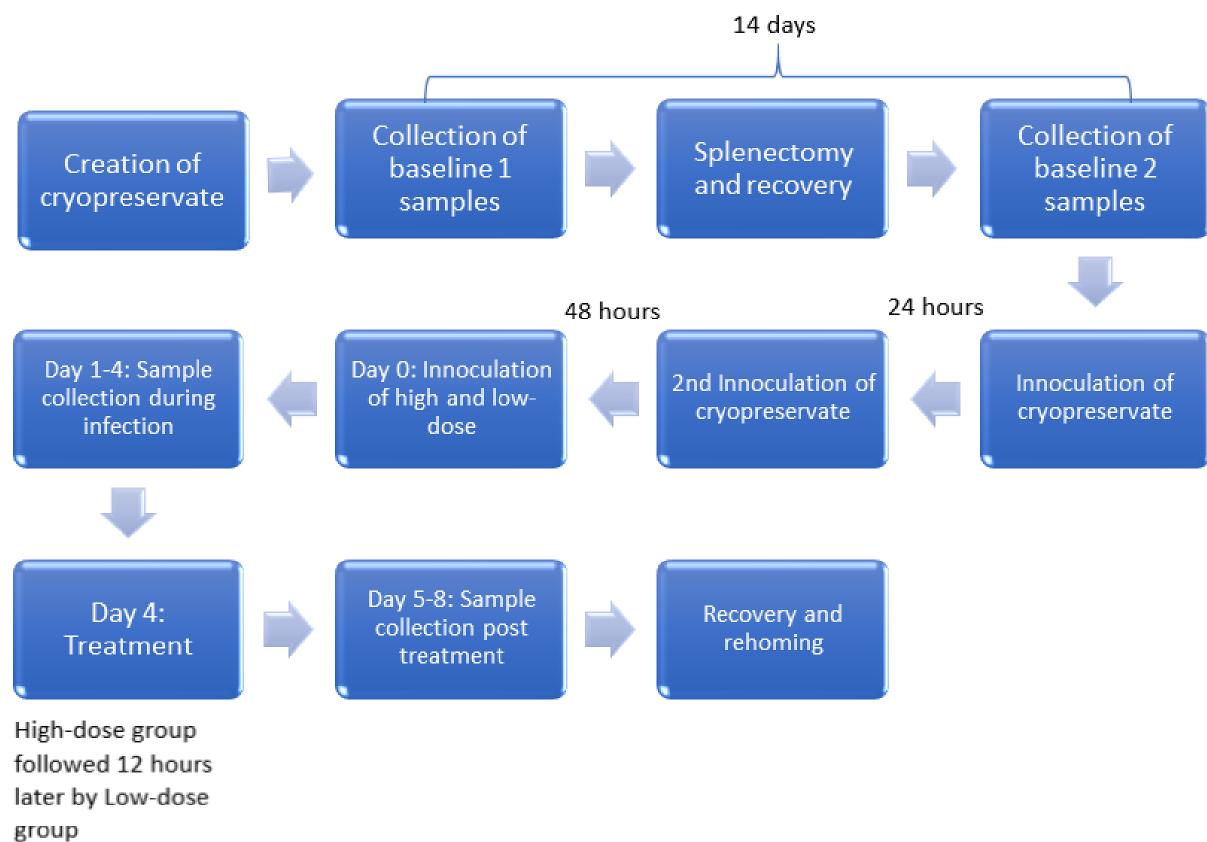


Figure 10: Study design

Phase 1:

Creation of a pure wild-type *Babesia rossi* parasite cryopreservate from a naturally infected dog presented to the Outpatients clinic of the Onderstepoort Veterinary Academic Hospital for veterinary care. The mono-infection and confirmation of the speciation of the parasite was determined by PCR-RLB.

Creation of *B. rossi* cryopreservate

Equipment, materials and facilities:

- Blood infected with parasites
- Dimethylsulphoxide (DMSO) (Kyron Laboratories, RSA)
- Heparin Vacutainer Brand Tubes (Beckton Dickinson Vacutainer Systems, UK)
- Sterile 2 mL or 4 mL cryotubes (Corning incorporated, USA) and labels for cryotubes
- Ice and racks to contain tubes
- 50 mL Erlenmeyer flask
- Pipettes (including micropipettes) and pipette tips
- Syringes
- Microscope slides
- Cover slips (24 x 24 mm)
- -80 ^{o}C deep freezers

Procedure

Blood was collected in EDTA Vacutainer Brand Tubes (Beckton Dickinson Vacutainer Systems, UK) from a *B. rossi* infected dog. The blood had a parasitaemia of approximately 10 %. The blood was stored at 4°C for 2 hours prior to further processing. A 10% DMSO and blood mixture was made using 8 mL of blood transferred from the collection tubes into a 50 mL flask and kept on melting ice. Slowly 0.88 mL of DMSO was added drop by drop, at a rate of about 10 seconds between drops, using a 1 mL pipette, while gently swirling the blood in the flask (whilst keeping the flask in melting ice water). The contents were gently mixed by pipetting them up and down several times. The blood was then dispensed into 2 mL cryotubes, kept in a rack on the laboratory bench, also in melting ice water. The cryotubes were then shortly transferred to and stored in a -80°C freezer.

Just prior to infection of the splenectomised dog, the stored samples were thawed in a water bath adjusted to 37°C and kept under water until just thawed. The contents were transferred to a syringe and inoculated intravenously within 15 minutes.

<u>Phase 2: Determination of baseline data and creation of infectious inoculum</u>

All 5 spleen-whole dogs had their clinical variables (temperature, pulse, respiration, habitus/mental status, mucous membrane colour, capillary refill time, thoracic auscultation, abdominal palpation, blood pressure and hydration status) evaluated and recorded. Blood was collected using a vacutainer system into a serum and EDTA tube for the determination of cytokine concentrations, albumin concentration, C-reactive protein concentration and a complete blood count (for neutrophil and monocyte counts). A small amount of fresh whole blood was used directly from the needle for the

determination of the blood lactate and glucose concentrations using handheld readers (specified later). Blood was collected directly into a heparinized syringe for the determination of venous blood gas – second lactate measurement. The clinical examinations and sample collections were done once a day between 8 and 10 am before feeding, on two separate days prior to the initiation of the experimental infection.

The randomly selected dog was splenectomised, by a specialist surgeon employed at the Onderstepoort Veterinary Academic Hospital (OVAH) and conducted in the UPBRC theatre. The splenectomised dog was then infected with the cryopreserved wild-type infected blood 2 weeks post-surgery, once he had recovered completely. 2 mL of cryopreservate was given to the splenectomised dog intravenously, repeated 24 hours later.

The parasitaemia of the splenectomised dog was determined manually twice daily on central venous blood collected 12-hourly, starting 12 hours after initially inoculum dose.

The parasitaemia throughout the study was determined as follows (Bohm et al., 2006):

- Blood smears were stained with Kyro-quick (Kyron Laboratories, Benrose, South Africa), a Romanowski stain, and scored at 1000 times magnification with the aid of a digital image analysis program (Optimas 6 for Win 95/ NT 4.0, Media Cybernetics, distributed by Carl Zeiss Ltd., Randburg, South Africa).
- Digital photographs of 3 sections namely: the red cell area, the feather edge and the sides (straight lateral margins) of the smears were transferred to a computer screen and magnified. The images were saved and printed out. The red blood cells were counted using markers over counted cells to ensure accurate counting.
- Free parasites were not counted.
- Unparasitized RBC and parasitized RBC were counted in each field, with a different marker for parasitized vs unparasitized.
- At least 650 RBC were counted in each of three fields. A total of at least 1950 RBC were examined per smear. Each oil immersion field was completely counted to avoid artificially increased parasitaemia had only portions been examined.
- The counting and scoring of all slides were done by the author and the slides were archived.
- The results were expressed as a percent parasitized red blood cells.
- A score of <0.05% was given if no parasitized RBC were detected in the designated areas but were observed somewhere else on the smear.
- A score of 0% was assigned if no parasitized RBC were detected on the smear after 15 minutes of scanning the slide without counting.

Once a parasitaemia above >0.05% was identified, citrated whole blood was collected from the splenectomised dog and the parasitaemia was calculated. When the percentage parasitaemia was calculated, it was multiplied by the RBC count obtained from a complete blood count analysis.

- Number of parasitized RBC (per mL) = % parasitized RBC X RBC count (per mL)

Dilutions to obtain desired inoculum doses were done as follows:

- A culture medium was utilised for the dilution of the parasitized blood:
  - Culture Media RPMI 1640 (ThermoFischer Scientific, USA)
  - Filtered water (1 L)
  - 25 millimolar Hepes
  - $NaHCO_3$ (Sodium bicarbonate) 2.1 g/L
  - Sodium pyruvate (1 millimolar/L)
  - Gentamycin (100 mg/mL)

  The RPMI (powder form) was dissolved in 600 mL of ultra-pure water. 2.1 g of NaHCO3 was added to the above suspension. The pH was adjusted to 7.3, using NaOH 5M. 10 mL of sodium pyruvate was then added, in sterile conditions to avoid contamination of the stock solution. Ultra-pure water was added up to 1 Litre and the solution was filtered, using 0.22 µm pore filter units and a peristaltic pump. Gentamicin 10 mg/L was added after filtration. The solution was aliquoted into adequate volumes and stored at 4°C.
- Desired inoculum doses were 10^4 and 10^8 parasitized RBC, for the LD and HD groups respectively.
- The dilution was done as follows:
  - 17mL of blood was collected from the splenectomised donor dog into 3mL Citrate Phosphate Dextrose Adenine (CPDA-1) anticoagulant, with a final concentration of 0.15 ml of CPDA-1 per ml of blood admixture.
  - An additional 1 mL of blood was collected separately directly into and EDTA Vacutainer Brand Tubes (Beckton Dickinson Vacutainer Systems, UK) for CBC determination.
  - Percent parasitaemia on day of blood collection:
    - 33 parasitized red blood cells/1950 red blood cells counted = 1.69 % parasitaemia
  - Percent parasitaemia (in decimal form) x red blood cell count (determined from CBC) 0.0169 x (6.15 x 10^9/L) = 0.1041 x 10^9 parasitized red blood cells/mL x 10
    = 1.041 x 10^8 parasitized red blood cells/mL.

- To account for citrate dilution:
  (1.041 x 10^8 parasitized red blood cells/L x 17 mL of blood)/ 20mL total fluid volume = 0.88465 x 10^8 parasitized red blood cells/mL.
- To get a high dose inoculum of 10^8 parasitized red blood cells:
  1 x 10^8 parasitized red blood cells/ (0.88465 x 10^8 parasitized red blood cells/mL) = 1.14 mL of total Citrated blood provided the high dose of 10^8 parasitized red blood cells.
- The low dose was achieved with serial dilutions as follow:
- Three serial dilutions of:
  - 1 mL of parasitized blood (Infectious dose of 8.8465 x 10^7/mL) with 9 mL diluent
  - 1 mL of parasitized blood (Infectious dose of 8.8465 x 10^6/mL) with 9 mL diluent
  - 1 mL of parasitized blood (Infectious dose of 8.8465 x 10^5/mL) with 9 mL diluent
- Then a serial final dilution of:
  - 1 mL of parasitized blood (Infectious dose of 8.8465 x 10^4/mL) with 7.8465 mL diluent.

- This provided a final low dose concentration of 10^4 parasitized red blood cells/mL.
- Before the low dose infectious inoculum was given, 1 mL of the sample was centrifuged, and a smear of the pellet was used to confirm presence of parasitized RBC. Once parasitaemia was confirmed 1mL of this solution was used as the low dose.

Phase 3: Infection of the experimental dogs

The splenectomised dog was drug cured with diminazene aceturate (3.5mg/kg SC dosed once) straight after blood collection for the experimental infections.

Infection of the experimental dogs with fresh whole blood diluted to achieve the 2 parasite doses required, namely 10^4 and 10^8 parasitized RBC. The dogs infected were infected as follows:

- Low dose group 1 (2 dogs): 1.14 mL containing 10^4 *Babesia rossi* parasitized RBC was given intravenously.
- High dose group 2 (3 dogs): 1 mL containing 10^8 *Babesia rossi* parasitized RBC was given intravenously.

Blood was collected once daily between 8 and 10 am from all 5 dogs for the duration of the experiment (see Table 1). Following the unexpected death of one dog in the high dose group on day 4, the remaining high dose dogs were sampled and treated intensively. For ethical reasons and concerns over the wellbeing on the remaining low dose group dogs, they were sampled 12 hours later (for the

second time that day) and then treated. All the dogs were drug cured with diminazene aceturate at 3.5mg/kg subcutaneously once off and received additional supportive therapy as needed (including blood transfusions as needed). The remaining dogs (including the splenectomised dogs) recovered well and were rehomed as pets following a minimum of 2 weeks of recovery time

The endpoints (point of treatment) set before the study included (any one criterium determined the point of treatment):

Humane endpoints:

- A haematocrit (packed cell volume) of between 10 and 15%.
- A dog with a habitus scores of 1+.
- A dog showing nervous signs (such as seizure activity) whether due to neuroglycopenia or not.
- A dog with clinical evidence of lung pathology in which there is arterial blood gas evidence of acute respiratory distress syndrome (arterial pO_2<60mmHg, normal > 80mmHg at our altitude).
- A dog that becomes oliguric (defined as a urine production of < 1 mL/Kg/hour) with a serum creatinine > 200mmol/L (normal <140 mmol/L).
- A dog that demonstrates haemoconcentration (defined as a PCV >55%) in the face of obvious haemolysis (macroscopically evident haemoglobinuria and or haemoglobinaemia).

Experimental endpoint:

- Any dog that lives to 20 days post infection will be treated on the 20th day post infection.

All dogs were drug cured (besides the single mortality) and provided with supportive treatment needed. The HD group was treated on day four in the morning (at 96 hours). Due to the unexpected death of one dog in the HD group, for ethical reasons to avoid any further losses, the LD group was then treated 12 hours later (at 108 hours) even though they had not reached the same degree of disease severity as the HD group.

Duration of the study was from the 28 February 2019 to 8 March 2019.

Experimental procedures

Sample collection schedule and volumes

All sample collection as well as the blood smear evaluation was performed by the main investigator and assisted by supervisors and co-workers. Clinical examinations and all blood samples were

collected from the jugular vein with 21G vacutainer needles (Precision Glide^{TM}, UK) between 8:00am and 10:100 am daily.

For this experiment the blood collection schedule was as follows:

Table 1: Sample collection schedule

Parameter to be measured	**Schedule**	**Test to be run**
Habitus, appetite, temperature, pulse, respiratory rate, mucous membrane colour, blood pressure	Daily on all dogs	General health status and wellbeing of all dogs.
Blood smear	Daily on small volume of peripheral blood from the ears of all dogs and from the EDTA sample below.	Parasitaemia determination.
0.5 mL of whole blood in EDTA tube from jugular collection	Daily for CBC from all dogs from day 0 until endpoints were reached and infected dogs were treated.	Complete blood count and cytokine concentration determination.
3 mL of whole blood in serum tube from jugular collection	Every second day from all dogs from day 0 (i.e., day 0 and then 2/4/6 etc) until endpoints were reached and infected dogs were treated.	Biochemistry: Total serum protein, glucose, albumin, C-reactive protein.
0.5 mL of whole blood in heparinized syringe from jugular collection	Every second day from all dogs from day 0 (i.e., day 0 and then 2/4/6 etc) until endpoints were reached and infected dogs were treated.	Venous blood gas and lactate.
Small amount of fresh whole blood remaining in the needle after collection.	Daily on all dogs.	Lactate and glucose from handheld readers.

For the full collection schedule please refer to Appendix C.

Haematology: Venous samples for CBC were collected atraumatically into EDTA Vacutainer Brand Tubes (Beckton Dickinson Vacutainer Systems, UK) from the jugular and were run on an ADVIA 2120 (Siemens, Munich, Germany). A differential count was performed manually by an experienced laboratory technologist. An automated haematology was performed (ADVIA 2120i, Siemens, Germany) within 1 hour of blood collection. The remaining volume of blood was then centrifuged and aliquoted within 30 minutes of collection for storage of EDTA plasma at -80°C.

Serum biochemistry samples were collected in Serum Vacutainer Brand Tubes (Beckton Dickinson Vacutainer Systems, UK), and the CRP (using canine specific immunoturbidimetric CRP method[h], Gentian, Norway), albumin (using a colorimetric assay with bromocresal green, Roche Diagnostics, Switzerland) were determined run on the Cobas Integra 400 plus (Roche, Switzerland). This sample was allowed to clot for 10 minutes, samples were run, and the remaining serum volumes were aliquoted within 30 minutes of collection for storage at -80°C.

Glucose was analysed using fresh whole blood with the point of care AlphaTRAK 2 (Zoetis, USA) (Cohen et al., 2009, Kang et al., 2016).

Blood gas samples were collected anaerobically into a commercially prepared heparinized syringe (BD A-Line, arterial blood collection syringe, Becton, Dickinson and Company, UK) using a 21G needle from the jugular. Lactate was obtained directly from a fresh blood sample using a Lactate Pro 2 (Arkray Global Business Incorporated, Japan) handheld lactate reader and from the venous blood gas analysis, analysed within 20 minutes (Rapidpoint 405, Siemens).

Once all samples had been collected and the experiment had come to an end, the stored plasma samples were thawed at room temperature and used to analyse GM-CSF, IFN-y, IL-2, IL-6, IL-7, IL-8, IL-15, IL-10, IL-18, TNF-a, IP-10, KC-like and MCP-1 concentrations. These were analyzed using fluorescent-coded magnetic beads (MagPlex-C; MILLIPLEX. MAP Kit, Canine Cytokine Magnetic Bead Panel, 96-Well Plate Assay, CCYTO-90K, Millipore, Billerica, MA), based on the Luminex xMAP technology (Luminex 200, Luminex Corporation, Austin, TX).

All serum and plasma not utilized in initial tests remains stored at -80°C and is logged in the OVAH biobank (See Appendix E).

Blood pressure was measured using the Vet HDO® MDPro and the HDO management software (S + B medVET GmbH, Germany). The protocol for blood pressure measurement was standardised as follows (Brown et al., 2007):

- The environment was isolated, quiet, and away from other animals. The same environment was used every day and the dogs were trained and conditioned for 4 weeks prior to the onset of the experiment.
- No sedation was used, and the dogs were allowed 5-10 minutes to relax and become accustomed to the environment.
- Each dog was gently restrained in right lateral recumbency.
- The cuff width was approximately 40% of the circumference of the cuff site and the cuff size was recorded at every measurement.
- The cuff was placed around the tail base.
- All blood pressure measurements were performed by the same person every day.
- The dogs were calm and relatively motionless as they were all well-conditioned to the process prior to the start of the experimental period.
- The first measurement was discarded. Five consecutive and consistent (<20% variability in systolic values) were recorded and the average of the values was calculated to obtain the blood pressure measurement.
- Written records were kept daily of the readings.

<u>Observational/analytical procedures</u>

The following data was collected every day for all the dogs in the study until endpoints were reached and the dogs were treated:

- Habitus and mental status (scored 1+ - 4+)
    - Habitus: 1+ Lethargic and non-responsive; 2+ Lethargic but responsive; 3+ Alert and responsive; 4+ Bright alert and responsive
- Appetite (scored 1+ - 4+)
    - Appetite: 1+ Refusal to eat; 2+ Eats when hand or syringe fed; 3+ Eating unassisted but inadequate intake; 4+ Eating very well
- Temperature
- Pulse (rate, rhythm, and quality)
- Respiratory rate and type
- Mucous membrane colour and capillary refill time
- Thoracic auscultation
- Abdominal palpation

- Hydration status
- Blood smear examination
- Blood pressure
- Haematology (CBC)
- EDTA plasma was stored for cytokine determination at the end of the experiment
- Blood glucose
- Blood lactate

A data collection sheet (Appendix B) was drawn up to record this daily information.

The following was only collected every second day for all dogs:

- A serum sample for biochemistry (Albumin and C-reactive protein)
- Venous blood gas

Cytokine Analysis

The stored plasma samples were thawed at room temperature and used to determine GM-CSF, IFN-y, IL-2, IL-6, IL-7, IL-8, IL-15, IL-10, IL-18, TNF-a, IP 10, KC and MCP-1 concentrations. These cytokines were analyzed in duplicate by fluorescent-coded magnetic beads (MILLIPLEX® MAP Kit, Canine Cytokine Magnetic Bead Panel, 96-Well Plate Assay, CCYTO-90K, Millipore, Billerica, MA, USA), based on the xMAP® technology (Bio-Plex®-MAGPIX™ 200, Bio-Rad Laboratories, Inc., California, USA). Two quality controls were included in the plate as internal quality controls. The assay was performed according to the manufacturer's instructions.

Preparation of the plasma samples:

- EDTA plasma samples were utilized. Samples were thawed, mixed well by vortexing and then centrifuged prior to use to remove any particulate material.

Preparation of reagents for immunoassay:

- The vial containing the premixed beads was sonicated for 30 seconds and then vortexed for 1 minute to ensure thorough mixing as contents prior to use.
- Quality control 1 and 2 were reconstituted with 250 µL of deionized water, inverted several times and vortexed. These were then allowed to sit for 10 minutes.
- The 10X Wash Buffer was brought to room temperature and mixed well. 60 mL of Wash Buffer was diluted with 540 mL of deionized water.

- 1 mL of deionized water was added to the lyophilized Serum Matrix, mixed well and given at least 10 minutes to reconstitute completely. 2 mL of Assay Buffer was added to this solution and mixed well.

Preparation of Canine Cytokine Panel Standards

- The Canine Cytokine Panel Standard was reconstituted with 250 µL of deionized water, inverted, vortexed for 10 seconds and then left to stand for 10 minutes before vortexing it a second time. This was Standard 7.
- 6 Polypropylene microfuge tubes were labelled Standard 1 to 6 respectively and 150 µL of Assay Buffer was added to each tube. A four-fold dilution was then performed by adding 50 µL of Standard 7 to the Standard 6 tube and mixed thoroughly. This process was repeated by adding 50 µL of Standard 6 to the Standard 5 tube and so on until all 7 standards were prepared.

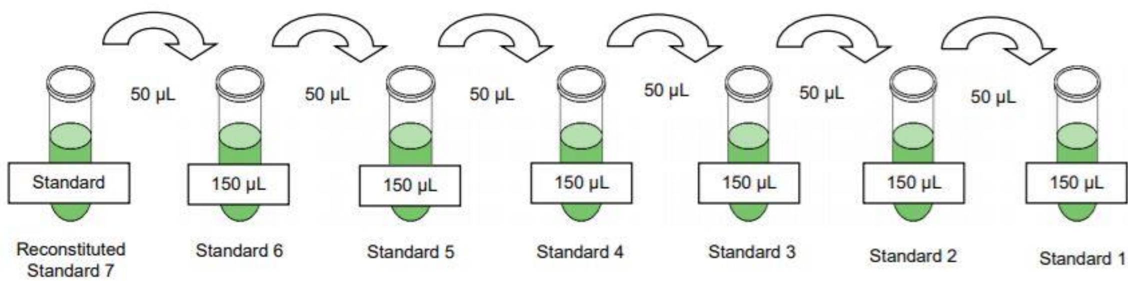


Figure 2: Standard preparation

- After the dilutions the tubes contained the following concentrations for constructing the standard curves:

Standard Tube #	IFN-γ (pg/mL)	All Other Analytes (pg/mL)
1	2.44	12.2
2	9.77	48.8
3	39.1	195
4	156	781
5	625	3,125
6	2,500	12,500
7	10,000	50,000

Figure 3: Standard concentrations

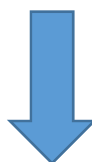
The immunoassay procedure:

- All reagents were warmed to room temperature (20-25°C).
- A sample well map was created, please see appendix D for detailed map.
- Flow diagram of the immunoassay procedure:

200 µL of Assay buffer added to each well

Sealed and placed in the plate shaker for 10 minutes at room temperature.

Assay buffer was decanted, and residue removed.



- Added 25 µL Standard and Control to appropriate wells
- Added 25 µL Assay Buffer to background and sample wells
- Added 25 µL appropriate matrix solution to background, standards, and control wells
- Add 25 µL Samples to sample wells
- Add 25 µL Beads to each well

The plate was incubated overnight at 4°C on a plate shaker.

Well contents were removed, and the plate was washed twice with Wash Buffer using an automatic plate washer (Bio-Plex Pro™ Wash Station, Laboratories, Inc., California, USA).

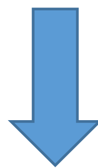
Added 25 µL Detection Antibodies per well

The plate was sealed and incubated on a plate shaker for 1 hour at room temperature.

Contents were not aspirated.

Added 25 µL Streptavidin-Phycoerythrin per well

The plate was sealed and incubated on a plate shaker for 30 minutes at room temperature.



Well contents were removed, and the plate was washed twice with Wash Buffer using an automatic plate washer.

- Added 150 µL Sheath Fluid per well
- The plate was then read on the Luminex®

- All equipment was set to manufacturers specifications.
- A standard curve was constructed using the concentrations obtained in from the standards in the wells and sample concentrations were calculated accordingly.
- The minimum detectable concentrations of the cytokines provided by the manufacturer were regarded as the detection limits in this study and measurable values below the detection limit were assigned a value equal to the minimum detectable concentration for the respective cytokine and those with no measurable values were set as zero.
- Sensitivity of the assay:

Table 2: Immunoassay sensitivity

Analyte	**Minimum detectable concentrations**
GM-CSF	9.2 pg/mL
IFNγ	13.6 pg/mL
IP-10	3.2 pg/mL
IL-2	3.5 pg/mL
IL-6	3.7 pg/mL
IL-7	7.5 pg/mL
IL-8	21.7 pg/mL
IL-10	8.5 pg/mL
IL-15	9.0 pg/mL
IL-18	5.8 pg/mL
KC-like	5.3 pg/mL
MCP-1	21.0 pg/mL
TNFα	6.1 pg/mL

- Standard curve range for all the reagents: 12.2 – 50 000 pg/mL

- Immunoassay precision:

Table 3: Immunoassay precision

Analyte	Intra-Assay precision (CV%)	Inter-Assay precision (CV%)
GM-CSF	<5	<15
IFNγ	<5	<15
IP-10	<5	<15
KC-like	<5	<15
IL-2	<5	<17
IL-6	<5	<15
IL-7	<5	<15
IL-8	<5	<15
IL-10	<5	<15
IL-15	<5	<17
IL-18	<5	<15
MCP-1	<5	<15
TNFα	<5	<15

Data management and analysis

Data management

Paper data capture sheets were physically completed for each animal daily. The data was copied into data capture sheets set up in Microsoft Excel and were stored on the primary investigator's laptop, a back-up was copied onto a flash drive, another back-up was stored online in a shared Google drive for all co-investigators to have access to daily. All physical documentation was scanned and stored on the Google drive.

Statistics

For the analysis, variables that are known or suspected to be non-normally distributed, i.e., right-skewed, were log-transformed; these were parasitemia, the leukocyte counts, CRP, GM-CSF, IFNγ, KC-like, all the interleukins, MCP-1 and TNFα. The other variables were not transformed. Variables were then compared between the HD group and the LD group at each time point as well as between each time point and the mean baseline value within each group using linear mixed models, with animal

identity as random effect and the Bonferroni adjustment for multiple comparisons. Linear mixed models were used to compare the continuous and ordinal variables. Pairwise correlations between variables were assessed using Spearman's rank correlation. Significance was assessed at $P<0.05$. Statistical analysis was done using Stata 15 (StataCorp, College Station, TX, U.S.A.).

Project Management

Experimental animals

The recommended minimum cage sizes for accommodation of canids for scientific purposes is provided for in the South African National Standards for the use and care of animals for scientific purposes (SANS 10386: 2008). These conditions are adhered to in the OVARU. Animals were fed a breed and age specific commercial food according to weight. Animals are sensitive to human interactions and these dogs were well socialized to a spectrum of people caring and interacting with them daily. They were housed in pairs at night and together during the day. A designated area which included space for sleeping and eating was provided in each pen. Sleeping enclosures were entirely indoors. Daytime areas were a combination of indoor and outdoor facilities (which they chose freely themselves). Adequate protection was provided from outside elements such as wind, cold, rain and solar radiation. Allowance was made for above ground sleeping and bedding in the pen, and this was served with a short access ramp. Each pen had a mixture of artificial and natural light and the photoperiod was the natural day-night cycle. Temperature and humidity were controlled in the range of 15 to 30°C and in the range of 30 to 70% respectively. Environmental enrichment was provided in the form of chew toys (ropes, balls, rings) and a splash pool. After each clinical training session, animals were treated with a small amount of treat-food. Several times a week each animal was given a raw-hide chew bone or cattle hoof. The presence of frequent (several times a day) human interaction allowed for the observation of any abnormal behaviour. The UP based primary investigator (and the other named key persons from UP) were all highly qualified and experienced veterinary clinicians which enhanced the level of daily health care of the animals.

The beagles were purchased at 8 weeks of age from a commercial breeder (Studvet Beagles, Onderstepoort, South Africa) and moved to UPBRC for housing where they were kept up to and for the duration of the experimental infection and their recovery (at approximately 6 months of age). The trained staff at the UPBRC were responsible for the day-to-day care and feeding. In the month preceding the study the dogs were trained and socialised by all co-workers involved in the project. Dr Quixie Sonntag (Special interest in small animal behaviour) was consulted on the best training and socialisation techniques for the growing dogs. In the months leading up to the trial date the dogs were specially trained and adapted, using positive reinforcement, to sit for blood collection, lie down for

blood pressure measurement. All six beagle dogs were vaccinated completely and dewormed regularly during their stay at the UPBRC. They were all neutered at approximately five months of age to avoid any possible aggressive behaviour as they were housed and socialised together. All co-workers were involved in the daily training, socialisation, sample collection, veterinary supervision, and emergency care of all experimental animals.

Staff

Dr Brogan Atkinson[1]

Principle investigator responsible for:

- Project design and drafting of the protocol
- Participate in the training and socialisation of beagle dogs
- Collection of data from beagle dogs and submission of samples to the laboratory
- Ensured that all the samples collected were of sufficient quality and quantity and are correctly submitted for analysis
- Clinical examination, clinical disease severity scoring and blood collection
- Storage of the remainder of the samples once the analyses had been performed
- Completion of data collection spreadsheets
- Parasitaemia determination
- Performing the cytokine immunoassay
- Analysis and interpretation of data
- Drafting of the dissertation and manuscript

Prof Andrew Leisewitz[2]

Project leader and supervisor of research responsible for:

- Project design
- Funding through a grant from the National Research Foundation (CPRR160425163064) held by Prof Andrew Leisewitz
- Supervision of the principal investigator throughout the research process, including assistance in the drafting of the dissertation and manuscript that will arise from the research

[1] MMedVet (Small Animal Medicine) candidate, Section of Small Animal Medicine, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria
[2] Professor in Small Animal Medicine, Section of Small Animal Medicine, Department of Companion Animal Studies, Faculty of Veterinary Science, University of Pretoria

- Training of the dogs and habituation to laboratory procedures for several weeks before the experiment
- Data collection
- Analysis of data
- Co-author on article

Prof Peter Thompson[3]

Co-worker, responsible for:

- Statistical analysis and interpretation of data
- Drafting of the manuscript
- Co-author on article

Dr Estee Van Zyl[4]

Co-worker, responsible for:

- Data sample collection
- Completion of data collection spreadsheets

Prof Amelia Goddard[5]

Co-worker, responsible for:

- Data collection
- Facilitation of sample processing in the laboratory and sample storage
- Drafting of the manuscript
- Co-author on the paper

Prof Johan Schoeman[6]

Co-worker, responsible for:

- Data collection

[3] Professor in Veterinary Epidemiology, Department of Production Animal Studies, Faculty of Veterinary Science, University of Pretoria
[4] MMedVet (Small Animal Medicine), Fourways Veterinary Hospital
[5] Professor in Clinical Pathology, Section of Clinical Pathology, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria
[6] Professor in Small Animal Medicine, Section of Small Animal Medicine, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria

- Drafting of the manuscript
- Co-author on the paper

Dr Yolandi Rautenbach[7]

Co-worker, responsible for:

- Data collection
- Facilitation of sample processing in the laboratory and sample storage

Dr Varaidzo Mukorera[8]

Academic co-supervisor, responsible for:

- Assistance in drafting of dissertation and manuscript
- Co-author on the publication

UPBRC Staff, responsible for:

- Day to day care of the beagle dogs
- Training of dogs for the experimental procedures
- Rehoming of the dogs after the study

Facilities and laboratories

- All dogs were housed at the UPBRC for the duration of the study.
- The clinical examination and sample collection were performed in a designated area of the UPBRC.
- The Clinical Pathology laboratory was used to evaluate blood smears, determine parasitaemia as well as perform analysis on the serum and anti-coagulated EDTA samples.
- PCR-RLB and cytokine determination will be performed by the technicians of the Dept. of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria.

Equipment and supplies

All equipment used was provided by the Faculty of Veterinary Science, University of Pretoria.

[7] Senior lecturer in Clinical Pathology, Section of Clinical Pathology, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria

[8] Senior lecturer in Small Animal Medicine, Section of Small Animal Medicine, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria

Chapter 4: Results

The demographic characteristics of the experimental group of dogs were as follows: All dogs were 6-month-old sterilised male beagles. Vaccinations, deworming and ectoparasite control was current for each dog. All 6 dogs tested negative for regional blood borne diseases namely *Babesia, Ehrlichia, Anaplasma* and *Theileria* based on PCR-RLB testing done prior to the initiation of the experimental study. The dog selected for splenectomy was not included in the data set. Each dog acted as their own baseline, with samples collected at two separate occasions prior to infection. The mean of these two baseline data sets was used as the control against which changes were compared over time. The HD group was treated on day four in the morning (at 96 hours). Due to the unexpected death of one dog in the HD group, for ethical reasons to avoid any further losses, the LD group was then treated 12 hours later (at 108 hours) even though they had not reached the same degree of disease severity as the HD group.

The results for key variables are presented in Tables 4 – 9, providing the daily comparisons between the groups as well as between each group and the baseline. A set of box plot graphs (Figures 4 – 9) were used to present the data as trends over the duration of the experimental period. Each parameter is briefly discussed. Important correlations identified during the study are presented in table 10.

The infection ran its course for 4 days prior to intervention. The HD group was treated on day four in the morning (at 96 hours) and the LD group was then treated 12 hours later (at 108 hours). No significant difference was noted between the low-dose (LD) and high dose (HD) group for any variable for the baseline data.

Table 4: Clinical data evaluated during disease progression from inoculation to recovery

	Baseline	24 hours	48 hours	72 hours	96 hours	108 hours	120 hours	144 hours	168 hours	192 hours
Habitus:		**Units: (1 – 4) [a]**								
LD mean/median (Range)	4/4 (4 – 4)	4/4 (4 – 4)	4/4 (4 – 4)	4/4 (4 – 4)	3/3 (3 – 3)	3.5/3.5 (3 – 4)	4/4 (4 – 4)	4/4 (4 – 4)	3/3 (3 – 3)	3.5/3.5 (3 – 4)
HD mean/median (Range)	4/4 (4 – 4)	4/4 (4 – 4)	3.33/3 (3 – 4)	2 (2 – 2)	1.67/2 (1 – 2)	NA	2/2 (2 – 2)	2/2 (2 – 2)	3/3 (3 – 3)	3/3 (3 – 3)
P value: LD vs HD LD vs Base HD vs Base	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	0.0172* 1.0000 0.0082*	0.0000* 1.0000 0.0000*	0.0000* 0.0003* 0.0000*	0.0000* 0.5731 NA	0.0000* 1.0000 0.0000*	0.0000* 1.0000 0.0000*	1.0000 0.0003* 0.0000*	0.3184 0.5731 0.0000*
Appetite:		**Units: (1 – 4) [b]**								
LD mean/median (Range)	4/4 (4 – 4)	4/4 (4 – 4)	4/4 (4 – 4)	4/4 (4 – 4)	3/4 (3 – 3)	2/2 (1 – 3)	2.5/2.5 (2 – 3)	3/3 (3 – 3)	3/3 (3 – 3)	3/3 (3 – 3)
HD mean/median (Range)	4/4 (4 – 4)	4/4 (4 – 4)	3.33/3 (3 – 4)	3/3 (2 – 4)	1/1 (1 – 1)	NA	1/1 (1 – 1)	1/1 (1 – 1)	2/2 (1 – 3)	3/3 (3 – 3)
P value: LD vs HD LD vs Base HD vs Base	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	0.7364 1.0000 0.8190	0.0729 1.0000 0.0486*	0.0000* 0.2575 0.0000*	NA 0.0000* NA	0.0024* 0.0043* 0.0000*	0.0000* 0.2575 0.0000*	0.1431 0.2575 0.0000*	1.0000 0.2575 0.1312
Temperature:	**Units °C**									
LD mean (Range)	37.9 (37.6–38.3)	38 (37.8–38.2)	38.6 (38.6–38.6)	38.3 (37.9–38.7)	39.45 (39–39.9)	39.9 (39.6–40.2)	39.75 (39.6–39.9)	39.3 (39–39.6)	38.65 (38.5–38.8)	38.5 (38.4–38.6)
HD mean (Range)	38 (37.7–38.1)	37.73 (37.1–38.1)	38.97 (38.7–39.2)	39.4 (39.2–39.6)	38.93 (36.4–40.7)	NA	39.5 (39.3–39.7)	38.65 (38.5–38.8)	38.6 (38.6–38.6)	38.3 (38.1–38.5)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	1.0000 1.0000 1.0000	1.0000 1.0000 0.3976	0.2676 1.0000 0.0197*	1.0000 0.0911 0.4832	NA 0.0051* NA	1.0000 0.0143* 0.0324*	1.0000 0.2068 1.0000	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000
Pulse:		**Units: bpm**								
LD mean (Range)	116 (112 – 120)	142 (136 – 148)	130 (128 – 132)	138 (128 – 148)	124 (120 – 128)	128 (116 – 140)	158 (156 – 160)	162 (136 – 188)	124 (116 – 132)	132 (128 – 136)
HD mean (Range)	130 bpm (120 – 135)	133 bpm (128 – 140)	112 (96 – 124)	92 (88 – 96)	143 (128 – 168)	NA	164 (160 – 168)	138 (136 – 140)	128 (128 – 128)	130 (128 – 132)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	1.0000 0.00631 1.0000	0.3650 1.0000 0.2719	0.0000* 0.2428 0.0000*	0.3010 1.0000 1.0000	NA 1.0000 NA	1.0000 0.0000* 0.0007*	0.0897 0.0000* 1.0000	1.0000 1.0000 1.0000	1.0000 0.7671 1.0000

	Baseline	24 hours	48 hours	72 hours	96 hours	108 hours	120 hours	144 hours	168 hours	192 hours
Respiration:		**Units: bpm**								
LD mean	32	22	28	30	24	26	20	40	24	30
(Range)	(32 – 32)	(20 – 24)	(24 – 32)	(24 – 36)	(20 – 28)	(20 – 32)	(16 – 24)	(32 – 48)	(16 – 32)	(20 – 40)
HD mean	29	21	27	23	43	NA	30	20	22	22
(Range)	(24 – 38)	(20 – 24)	(24 – 32)	(20 – 28)	(40 – 48)		(24 – 36)	(20 – 20)	(20 – 24)	(20 – 24)
P value: LD vs HD	1.0000	1.0000	1.0000	1.0000	0.0004*	NA	0.4160	0.0006*	1.0000	1.0000
LD vs Base	NA	0.7315	1.0000	1.0000	1.0000	1.0000	0.2524	1.0000	1.0000	1.0000
HD vs Base	NA	1.0000	1.0000	1.0000	0.0081*	NA	1.0000	1.0000	1.0000	1.0000
Systolic blood pressure:		**Units: mmHg**								
LD mean	136	130	141	133	140	137	142	121	129	123
(Range)	(135 – 137)	(126 – 134)	(129 – 154)	(126 – 141)	(126 – 155)	(136 – 138)	(129 – 155)	(118 – 123)	(123 – 135)	(118 – 128)
HD mean	124	140	137	156	137	NA	134	132	137	132
(Range)	(122 – 125)	(131 – 146)	(130 – 149)	(145 – 161)	(109 – 162)		(131 – 137)	(127 – 136)	(136 – 137)	(129 – 134)
P value: LD vs HD	1.0000	1.0000	1.0000	0.0568	1.0000	NA	1.0000	1.0000	1.0000	1.0000
LD vs Base	NA	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	0.9082	1.0000	1.0000
HD vs Base	NA	0.1237	0.4730	0.0000*	0.4996	NA	1.0000	1.0000	1.0000	1.0000
Diastolic blood pressure:		**Units: mmHg**								
LD mean	63	66	65	73	78	68	71	66	68	67
(Range)	(63 – 63)	(63 – 68)	(63 – 67)	(66 – 80)	(68 – 88)	(57 – 80)	(61 – 81)	(63 – 69)	(65 – 70)	(65 – 68)
HD mean	65	74	72	76	74	NA	73	65	77	71
(Range)	(63 – 67)	(71 – 76)	(68 – 74)	(74 – 77)	(64 – 83)		(67 – 79)	(65 – 65)	(73 – 81)	(71 – 71)
P value: LD vs HD	1.0000	0.8011	1.0000	1.0000	1.0000	NA	1.0000	1.0000	1.0000	1.0000
LD vs Base	NA	1.0000	1.0000	0.2596	0.0034*	1.0000	0.9945	1.0000	1.0000	1.0000
HD vs Base	NA	0.1042	0.7464	0.0132*	0.0978	NA	0.8259	1.0000	0.0440*	1.0000
MAP:		**Units: mmHg**								
LD mean	89	89	91	94	100	92	96	86	90	87
(Range)	(89 – 89)	(86 – 91)	(86 – 96)	(87 – 101)	(89 – 112)	(84 – 101)	(85 – 107)	(83 – 88)	(86 – 93)	(84 – 89)
HD mean	86	98	95	105	97	NA	94	89	98	93
(Range)	(74 – 87)	(92 – 100)	(91 – 102)	(101 – 110)	(80 – 111)		(92 – 96)	(87 – 90)	(96 – 101)	(92 – 94)
P value: LD vs HD	1.0000	0.9777	1.0000	0.4789	1.0000	NA	1.0000	1.0000	1.0000	1.0000
LD vs Base	NA	1.0000	1.0000	1.0000	0.2399	1.0000	1.0000	1.0000	1.0000	1.0000
HD vs Base	NA	0.0357*	0.3337	0.0000*	0.0840	NA	1.0000	1.0000	0.1641	1.0000

[a] Habitus: 1+ Lethargic and non-responsive; 2+ Lethargic but responsive; 3+ Alert and responsive; 4+ Bright alert and responsive. [b] Appetite: 1+ Refusal to eat; 2+ Eats when hand or syringe fed; 3+ Eating unassisted but inadequate intake; 4+ Eating very well. * Significant difference when applying linear mixed models, P<0.05.

Table 5: Haematological and biochemical data evaluated during disease progression from inoculation to recovery

	Baseline	24 hours	48 hours	72 hours	96 hours	108 hours	120 hours	144 hours	168 hours	192 hours
Parasitaemia:		**Units: %**								
LD mean (Range)	0	0	0	0.075 (0.05–0.1)	3.88 (3.15–4.61)	5.76 (4.71–6.81)	0.047 (0.044–0.051)	0.0015 (0.001–0.002)	0	0
HD mean (Range)	0	0.05 (0.05–0.05)	0.916 (0.69–1.06)	10.877 (6.74–14.81)	46.763 (34.95–59.8)	NA	0.072 (0.059–0.085)	0.0005 (0–0.001)	0	0
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	0.0000* 1.0000 0.0000*	0.0000* 1.0000 0.0000*	0.0000* 0.0000* 0.0000*	0.0000* 0.0000* 0.0000*	NA 0.0000* NA	1.0000 0.0000* 0.0000*	0.0000* 0.0000* 0.0001*	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000
Haematocrit:		**Units: L/L**								
LD mean (Range)	0.38 (0.37–0.39)	0.38 (0.39–0.36)	0.41 (0.4–0.41)	0.38 (0.36–0.4)	0.39 (0.37–0.4)	0.39 (0.3–0.42)	0.28 (0.26–0.29)	0.25 (0.24–0.25)	0.25 (0.24–0.26)	0.28 (0.27–0.29)
HD mean (Range)	0.36 (0.35–0.38)	0.38 (0.33–0.42)	0.4 (0.38–0.42)	0.34 (0.31–0.36)	0.22 (0.12–0.28)	NA	0.17 (0.14–0.19)	0.2 (0.17–0.23)	0.26 (0.22–0.29)	0.25 (0.23–0.26)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	0.8980 1.0000 1.0000	0.0000* 0.0000* 1.0000	NA 1.0000 NA	0.0008* 0.0032* 0.0000*	1.0000 0.0000* 0.0000*	1.0000 0.0001* 0.0002*	1.0000 0.0063* 0.0000*
Red cell count:		**Units: x 10^{12}/L**								
LD mean (Range)	5.61x10^{12}/L (5.44– 5.77)	5.57 (5.35–5.78)	5.93 (5.83–6.02)	5.59 (5.27–5.91)	5.66 (5.52–5.8)	5.65 (5.24–6.05)	4.05 (3.88–4.21)	3.62 (3.52–3.72)	3.54 (3.42–3.65)	4.1 (3.91–4.28)
HD mean (Range)	5.44x10^{12}/L (5.11–5.64)	5.77 (4.93–6.25)	5.95 (5.68–6.21)	4.94 (4.67–5.23)	3.02 (1.67–3.96)	NA	2.37 (2.04–2.7)	2.92 (2.41–3.43)	3.68 (3.23–4.12)	3.55 (3.36–3.73)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	0.6876 1.0000 1.0000	0.0000* 1.0000 0.0000*	NA 1.0000 NA	0.0002* 0.0011* 0.0000*	0.7210 0.0000* 0.0000*	1.0000 0.0000* 0.0000*	1.0000 0.0018* 0.0000*
Segmented neutrophils:		**Units: x 10^{9}/L**								
LD mean (Range)	4.68 (2.39–6.98)	3.56 (2.99– 4.13)	3.1 (2.55–3.65)	2.96 (2.13–3.78)	2.71 (1.63–3.78)	1.49 (1.17–1.81)	2.49 (1.62–3.36)	1.9 (1.28–2.51)	2.92 (1.49–4.35)	4.2 (3.874.53)
HD mean (Range)	3.96 (3.33–5.06)	2.44 (2.21–2.74)	2.78 (2.4–3.18)	1.79 (1.36–2.44)	1.57 (1.12–1.88)	NA	2.64 (1.73–3.54)	7.23 (6.16–8.3)	17.64 (12.21–23.07)	27.35 (23.75–30.94)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	1.0000 1.0000 0.0480*	1.0000 1.0000 0.5077	0.5006 1.0000 0.0000*	0.5514 0.1718 0.0000*	NA 0.0000* NA	1.0000 0.0640 0.3342	0.0000* 0.0003* 0.0055*	0.0000* 0.2503 0.0000*	0.0000* 1.0000 0.0000*

	Baseline	24 hours	48 hours	72 hours	96 hours	108 hours	120 hours	144 hours	168 hours	192 hours
Band neutrophils:		**Units: x 10^9/L**								
LD mean (Range)	0.11 (0–0.21)	0	0.1 (0–0.19)	0.06 (0.05–0.06)	0.14 (0.1–0.17)	0.24 (0.07–0.4)	0.17 (0.17–0.17)	0	0	0.16 (0.09–0.23)
HD mean (Range)	0.14 (0.13–0.17)	0.04 (0–0.08)	0.15 (0.08–0.14)	0.14 (0.11–0.18)	0.37 (0.16–0.8)	NA	1.81 (0.88–2.74)	0.67 (0.39–0.95)	2.77 (2.28–3.25)	6.45 (4.32–8.57)
P value: LD vs HD	0.4287	0.2775	0.3313	1.0000	1.0000	NA	0.0031*	0.0000*	0.0000*	0.0000*
LD vs Base	NA	0.1670	1.0000	1.0000	1.0000	0.4133	0.4809	0.1670	0.1670	0.7975
HD vs Base	NA	0.0524	1.0000	1.0000	1.0000	NA	0.0002*	0.1138	0.0000*	0.0000*
Monocytes:		**Units: x 10^9/L**								
LD mean (Range)	0.55 (0.39–075)	0.3 (0.28–0.32)	0.5 (0.47–0.53)	0.5 (0.27–0.72)	0.32 (0.22–0.41)	0.32 (0.15–0.48)	0.27 (0.17–0.37)	0.54 (0.51–0.56)	0.61 (0.53–0.68)	0.65 (0.6–0.7)
HD mean (Range)	0.52 x 10^9/L (0.46–0.65)	0.28 (0.2–0.32)	0.27 (0.2–0.34)	0.42 (0.3–0.61)	0.58 (0.36–0.71)	NA	0.73 (0.54–0.91)	1.86 (1.27–2.45)	3.13 (2.69–3.57)	3.04 (1.8–4.28)
P value: LD vs HD	1.0000	1.0000	0.1109	1.0000	0.1550	NA	0.0028*	0.0002*	0.0000*	0.0000*
LD vs Base	NA	0.3928	1.0000	1.0000	0.4063	0.1202	0.0534	1.0000	1.0000	1.0000
HD vs Base	NA	0.0436*	0.0236*	1.0000	1.0000	NA	1.0000	0.0000*	0.0000*	0.0000*
Glucose:		**Units: mmol/L**								
LD mean (Range)	6.7 (6.4–6.9)	6.8 (6.4–7.1)	7 (6.8–6.2)	6.8 (6.6–6.9)	NA	6.8 (6.2–7.4)	7.3 (6.7–7.9)	7.2 (6.8–7.5)	7.5 (7.4–7.6)	7.8 mmol/L (7.6–8)
HD mean (Range)	6.7 (6.6–6.8)	6.5 (6.2–6.7)	6.4 (6.4–6.4)	6.1 (5.8–6.3)	5.4 (5.2–5.7)	NA	7.7 (7.6–7.7)	7.9 (7.1–8.6)	8.2 (8.1–8.3)	8.2 mmol/L (8.1–8.3)
P value: LD vs HD	1.0000	1.0000	0.3127	0.1965	NA	NA	1.0000	0.2395	0.2395	1.0000
LD vs Base	NA	1.0000	1.0000	1.0000	NA	1.0000	0.2608	1.0000	0.0250*	0.0003*
HD vs Base	NA	1.0000	1.0000	0.1628	0.0000*	NA	0.0016*	0.0000*	0.0000*	0.0000*
Lactate:		**Units: mmol/L**								
LD mean (Range)	1.2 (1.2–1.2)	1.1 (0.9–1.3)	1.3 (1.2–1.4)	1.2 (1.1–1.2)	2 (1.7–2.4)	1.6 (1.5–1.7)	1.3 (1–1.5)	1.4 (1.4–1.4)	1.2 (0.8–1.5)	1 (1–1)
HD mean (Range)	1.1 (1–1.5)	1.1 (1–1.3)	1.4 (1.3–1.6)	1.9 (1.2–2.4)	NA	NA	1.2 (1–1.3)	1 (1–1)	0.8 (0.8–0.8)	0.9 (0.8–0.9)
P value: LD vs HD	1.0000	1.0000	1.0000	0.0020*	NA	NA	1.0000	0.5814	0.9736	1.0000
LD vs Base	NA	1.0000	1.0000	1.0000	0.0017*	1.0000	1.0000	1.0000	1.0000	1.0000
HD vs Base	NA	1.0000	1.0000	0.0004*	NA	NA	1.0000	1.0000	1.0000	1.0000

	Baseline	24 hours	48 hours	72 hours	96 hours	108 hours	120 hours	144 hours	168 hours	192 hours
Lactate Venous blood gas:		**Units: mmol/L**								
LD mean (Range)	NA	0.83 (0.57–1.08)	1.2 (1.04–1.36)	0.81 (0.71–0.9)	NA	1.24 (1.17–1.31)	NA	0.71 (0.58–0.84)	NA	0.66 (0.6–0.71)
HD mean (Range)	NA	0.84 (0.61–1.03)	1.32 (1.2–1.54)	1.27 (1.18–1.39)	5.07 (3.05–7.5)	NA	0.55 (0.49–0.6)	0.6 (0.54–0.66)	NA	0.61 (0.55–0.67)
P value: LD vs HD	NA	1.0000	1.0000	1.0000	NA	NA	NA	1.0000	NA	1.0000
LD vs Base	NA	NA	0.1266	1.0000	NA	1.0000	NA	1.0000	NA	1.0000
HD vs Base	NA	NA	1.0000	1.0000	0.0000*	NA	1.0000	1.0000	NA	1.0000
Albumin:		**Units: g/L**								
LD mean (Range)	30.93 (30.8–31.05)	32.85 (32.6–33.1)	NA	32.9 (32.6–33.1)	NA	32.3 (31.5–33.1)	NA	26.7 (26–27.4)	NA	28.34 (28.3–28.37)
HD mean (Range)	32.25 (31.5–33.25)	34.13 (32.9–35.3)	NA	29.47 (27.8–31)	20.03 (11–25.3)	NA	18.2 (16.3–20.1)	20.8 (19–22.6)	NA	25.01 (24.35–25.66)
P value: LD vs HD	1.0000	1.0000	NA	0.8053	NA	NA	NA	0.0298*	NA	0.6139
LD vs Base	NA	1.0000	NA	1.0000	NA	1.0000	NA	0.5630	NA	1.0000
HD vs Base	NA	1.0000	NA	1.0000	0.0000*	NA	0.0000*	0.0000*	NA	0.0004*
C- reactive protein:		**Units: mg/L**								
LD mean (Range)	25 (10–40)	10 (10–10)	NA	13 (11–15)	NA	178 (160–197)	NA	150 (131–168)	NA	55 (53–57)
HD mean (Range)	14.33 (10–21.5)	20 (15–26)	NA	150 (135–163)	125 (92–160)	NA	82 (81–82)	59 (54–64)	NA	21 (19–23)
P value: LD vs HD	0.6119	0.0201*	NA	0.0000*	NA	NA	NA	0.0007*	NA	0.0003*
LD vs Base	NA	0.0350*	NA	0.7487	NA	0.0000*	NA	0.0000*	NA	0.0001*
HD vs Base	NA		NA	0.0000*	0.0000*	NA	0.0000*	0.0000*	NA	0.7213

* Significant difference when applying linear mixed models, P<0.05.

Table 6: Category *a* cytokines: Cytokines that rose during the infection and fell after treatment

	Baseline	24 hours	48 hours	72 hours	96 hours	144 hours	192 hours
IFNγ:	**Units pg/mL**						
LD mean (Range)	0	6.8 (0–13.6)	0	7.42 (0–14.83)	128.3 (50.28–206.32)	16.2 (13.6–18.79)	13.62 (0–27.24)
HD mean (Range)	10.96 (0–32.885)	33.32 (13.6–63.54)	77.08 (19.48–124.28)	9.22 (0–14.07)	16.07 (0–24.11)	14.4 (13.6–15.2)	18.05 (13.6–22.49)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	0.2711 1.0000 0.0725	0.0001* 1.0000 0.0024*	1.0000 1.0000 1.0000	0.0709 0.0000* 1.0000	1.0000 0.0403* 0.8149	1.0000 1.0000 0.5031
KC-like:	**Units pg/mL**						
LD mean (Range)	27.36 (19.58–35.14)	40.45 (36.18–44.71)	30.07 (17.88–42.25)	49.05 (44.6–53.49)	152.37 (104.96–199.77)	46.09 (33.52–58.66)	21.83 (16.14–27.52)
HD mean (Range)	23.87 (18.19–31.38)	84.97 (52–140.06)	87.58 (67.16–104.96)	141.4 (103.74–183.18)	766.48 (625.08–913.78)	11.01 (8.13–13.89)	5.02 (4.2–5.84)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	0.0496* 0.7634 0.0000*	0.0000* 1.0000 0.0000*	0.0001* 0.0838 0.0000*	0.0000* 0.0000* 0.0000*	0.0000* 0.2726 0.0038*	0.0000* 1.0000 0.0000*

* Significant difference when applying linear mixed models, P<0.05.

Table 7: Category *b* cytokines: Cytokines that rose and remained high even after treatment

	Baseline	24 hours	48 hours	72 hours	96 hours	144 hours	192 hours
MCP-1:	**Units pg/mL**						
LD mean (Range)	156.19 (123.49–188.9)	174.05 (103.86–244.23)	144.31 (56.05–232.56)	212.77 (196.48–229.06)	360.59 (253.52–467.65)	195.97 (98.85–293.09)	541.64 (70.61–1012.66)
HD mean (Range)	67.1 (64.67–68.86)	244.06 (225.92–264.24)	282.53 (241.14–321.89)	336.13 (258.86–404.03)	1217.07 (523.22–2271.2)	49402.62 (201.93–98603.3)	113660 (74.19–113660)
P value: LD vs HD	0.3352	1.0000	1.0000	1.0000	1.0000	0.0777	0.2315
LD vs Base	NA	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
HD vs Base	NA	1.0000	1.0000	1.0000	0.0501	0.0008*	0.0011*
IL-6:	**Units pg/mL**						
LD mean (Range)	20.42 (18.49–22.36)	51.21 (17.32–85.09)	43.89 (7.14–80.63)	39.69 (14.27–65.1)	39.16 (15.81–62.51)	93.75 (16.32–171.18)	500.38 (13.06–987.69)
HD mean (Range)	8.95 (4.09–15.29)	18.88 (8.5–29.58)	24.33 (14.96–34.98)	22.49 (20.46–25.9)	280.67 (40.22–644.35)	17999.95 (39.47– 5960.43)	30369.21 (10.56–60727.85)
P value: LD vs HD	1.0000	1.0000	1.0000	1.0000	1.0000	0.2260	1.0000
LD vs Base	NA	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
HD vs Base	NA	1.0000	1.0000	1.0000	0.0485*	0.0001*	0.0005*
IL-8:	**Units pg/mL**						
LD mean (Range)	87.02 (71.8–102.25)	256.96 (211.61–302.31)	218.71 (103.39–334.03)	101.11 (78.62–123.59)	70.27 (48.04–92.5)	214.17 (103.39–324.95)	519.26 (193.33 – 845.19)
HD mean (Range)	154.91 (139.24–165.98)	125.58 (76.02–176)	157.68 (144.41–164.83)	44.78 (37.03–55.44)	867.64 (271.26–1817.67)	245.34 (53.25–437.43)	248.71 (123.03 – 374.39)
P value: LD vs HD	1.0000	0.6495	1.0000	0.5221	0.0000*	1.0000	1.0000
LD vs Base	NA	0.1344	0.8359	1.0000	1.0000	0.8973	0.0033*
HD vs Base	NA	1.0000	1.0000	0.0039*	0.0006*	1.0000	1.0000
IL-10:	**Units pg/mL**						
LD mean (Range)	42.65 (12.1–73.19)	36.2 (16.1–56.29)	51.85 (27.29– 6.41)	124.03 (81.89–166.16)	288.42 (229.09–347.74)	126.24 (87.43–165.05)	204.61 (8.5–400.72)
HD mean (Range)	16.57 (16.05–23.34)	98.13 (36.57–211.81)	131.01 (109.22–147.73)	253.56 (182.15–384.13)	680.69 (150.44– 1429.07)	121.15 (44.27–198.02)	110.26 (8.5–212.01)
P value: LD vs HD	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
LD vs Base	NA	1.0000	1.0000	1.0000	0.0741	1.0000	1.0000
HD vs Base	NA	0.2943	0.0194*	0.0006*	0.0000*	0.2066	1.0000

* Significant difference when applying linear mixed models, $P<0.05$.

Table 8: Category *c* cytokines: Cytokines that rose dramatically after treatment

	Baseline	24 hours	48 hours	72 hours	96 hours	144 hours	192 hours
GM-CSF:	**Units pg/mL**						
LD mean (Range)	35.07 (34.06–36.09)	78.89 (33.23–124.54)	60.68 (14.13–107.23)	53.87 (24.78–82.95)	39.78 (11.25–68.31)	177.71 (22.96–332.46)	1228.75 (18.95–2438.55)
HD mean (Range)	9.2 (9.2–9.2)	22.53 (0–58.38)	15.64 (0–37.71)	9.1 (0–27.3)	22.36 (11.25–32.07)	4629.59 (38.37–9220.8)	4620.24 (19.67–9220.8)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	0.8472 1.0000 1.0000	1.0000 1.0000 1.0000	0.3367 1.0000 1.0000	1.0000 1.0000 1.0000	1.0000 1.0000 0.0001*	1.0000 0.7879 0.0006*
TNFα:	**Units pg/mL**						
LD mean (Range)	12.6 (12.28–12.93)	36.38 (8.74–64.02)	32.15 (6.1–58.2)	24.58 (6.97–42.18)	23.34 (6.1–40.63)	76.88 (7.96–145.8)	422.07 (6.77–837.37)
HD mean (Range)	6.1 (6.1–6.1)	9.55 (6.1–14.19)	9.46 (6.1–12.61)	10.62 (6.1–13.72)	22.95 (13.08–37.97)	6306.60 (13.77–12599.51)	7699.37 (6.1–15392.63)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	1.0000 1.0000 0.6373	0.6519 1.0000 0.0003*	1.0000 1.0000 0.0008*
IL-2:	**Units pg/mL**						
LD mean (Range)	21.24 (18.67–23.82)	65.94 (12.19–119.68)	53.62 (0–107.23)	41.11 (7.13–75.08)	31.68 (0–63.35)	135.89 (7.65 264.13)	1052.81 (3.5–2102.12)
HD mean (Range)	7.64 (0–19.42)	20.34 (0–37.66)	14.43 (3.5–29.09)	5.44 (0–12.81)	5.48 (3.5–9.44)	71563.81 (29.33–143098.3)	177399 (7.91–354790.1)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	0.3286 1.0000 0.0002*	0.8005 1.0000 0.0003*
IL-7:	**Units pg/mL**						
LD mean (Range)	20.23 (15.64–24.82)	55.79 (18.26–93.31)	45.83 (7.5–84.15)	38.08 (12.89–63.26)	29.12 (7.5–50.74)	148.96 (16.14–281.77)	967.68 (22.91–1912.44)
HD mean (Range)	20.92 (7.5–47.77)	20.03 (7.5–32.12)	12.4 (7.5–17.84)	7.84 (3.39–13.57)	15.28 (7.83–23.08)	22636.76 (23.42–45250.1)	29754.71 (11.77–59497.64)
P value: LD vs HD LD vs Base HD vs Base	1.0000 NA NA	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	1.0000 1.0000 1.0000	0.5223 1.0000 0.0021*	1.0000 0.7685 0.0042*

* Significant difference when applying linear mixed models, P<0.05.

Table 9: Category *d* cytokines: Cytokines that showed no distinct pattern of change

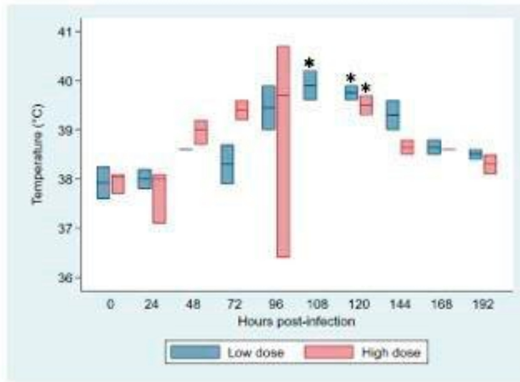
	Baseline	24 hours	48 hours	72 hours	96 hours	144 hours	192 hours
IL-15:	**Units pg/mL**						
LD mean (Range)	63.73 (49.71–77.75)	221.32 (68.19–374.45)	187.07 (21.42–352.71)	142.83 (45.11–240.55)	120.62 (23.29–217.95)	529.53 (63.16–995.9)	3043.38 (64–6022.76)
HD mean (Range)	22.61 (13.86–28.73)	51.02 (10.32–116.44)	37.7 (24.79–69.46)	20.94 (9–43.5)	31.26 (15.23 – 49.96)	64.28 (0–128.56)	23.36 (0–46.72)
P value: LD vs HD	1.0000	1.0000	1.0000	0.6899	1.0000	0.0484*	0.0007*
LD vs Base	NA	1.0000	1.0000	1.0000	1.0000	1.0000	0.2715
HD vs Base	NA	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
IL-18:	**Units pg/mL**						
LD mean (Range)	20.18 (19.99–20.38)	54.65 (21.99–87.3)	42.66 (9.9–75.41)	31.22 (16.56–51.88)	28.95 (10.86–47.03)	140.81 (20.47–261.14)	792.75 (18.86–1566.64)
HD mean (Range)	9.42 (8.33–10.33)	20.6 (7.61–43.92)	14.6 (7.42–26.28)	9.34 (5.56–16.36)	11.43 (7.23–16.96)	87.87 (0–175.73)	7.59 (0–15.17)
P value: LD vs HD	1.0000	1.0000	1.0000	1.0000	1.0000	0.5816	0.0016*
LD vs Base	NA	1.0000	1.0000	1.0000	1.0000	1.0000	0.2681
HD vs Base	NA	1.0000	1.0000	1.0000	1.0000	1.0000	1.0000
IP-10:	**Units pg/mL**						
LD mean (Range)	1.84 (0–3.67)	1.94 (0–3.88)	2.34 (0–4.67)	5.16 (3.35–6.97)	16.78 (14.33–19.22)	11.63 (6.11–17.14)	14.59 (6.11–17.14)
HD mean (Range)	4.26 (0–12.77)	14.25 (10.68–17.51)	14.91 (13.66–15.81)	11.23 (10.96–11.52)	10.7 (8.65–13.07)	0	0
P value: LD vs HD	1.0000	0.0010*	0.0007*	0.4243	0.4238	0.0073*	0.0003*
LD vs Base	NA	1.0000	1.0000	1.0000	0.0003*	0.0691	0.0039*
HD vs Base	NA	0.0067*	0.0028*	0.1915	0.3125	1.0000	1.0000

* Significant difference when applying linear mixed models, P<0.05.

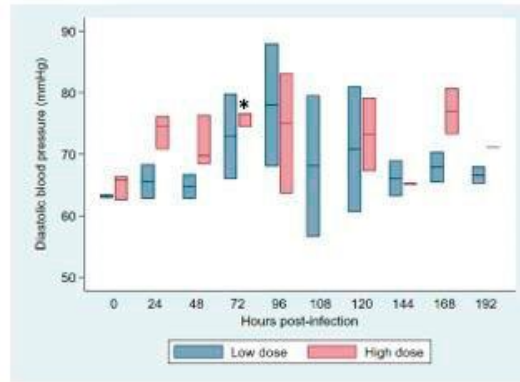
Clinical parameters

Significant differences were found at various time points for all clinical parameters. In the HD group there was a statistically significant decrease in habitus from 72 hours with all three dogs demonstrating lethargy and at 96 hours the dog who later died from this group was acutely collapsed. On the other hand, only a very mild decline in habitus was seen in the LD group at 96 and 108 hours (Table 4). In a similar pattern, appetite was mildly decreased in the LD group at 96 hours with one dog refusing to eat at 108 hours and the other requiring hand feeding at 124 hours, thereafter appetites improved in this group (Table 4). The HD group demonstrated a mild decline in appetite at as early as 48 hours post inoculation, which progressed to anorexia at 96 hours and persisted for at least another 48 hours in this group (Tablet 4). The mean temperature peaked 36 hours earlier in HD group than the LD group. For two of the dogs in the HD group, their temperatures continued to increase to above 40°C at 96 hours but the dog that died, demonstrated a severe drop in temperature at this time point. Pyrexia persisted for at least 24 hours after treatment in the remaining dogs in both groups (Table 4 and Figure 4A). When evaluating heart rate changes over time there were significant increases in heart rate in the LD group after treatment, at 120 and 144 hours. In the HD group the heart rate declined significantly at 72 hours. Thereafter, heart rate progressively increased from 96 hours reaching significance after treatment at 120 hours in this group (Table 4 and Figure 4B). The respiratory rate increased significantly in the HD group compared to the baseline and LD group at 96 hours. At 144 hours, after treatment, the LD group demonstrated a significantly higher respiratory rate than the HD group (Table 4 and Figure 4C). The infectious dose appeared to influence the time to onset of changes in clinical parameters. There was a tendency for these changes to occur between 36 to 48 hours earlier in the HD group when compared to the LD group. It is possible the degree of the change in the LD group would have been similar to that seen in the HD group, if infection was permitted to progress to the same degree of severity. When evaluating the clinical parameters, infectious dose appears to affect the time to onset of changes and not necessarily the severity of these changes.

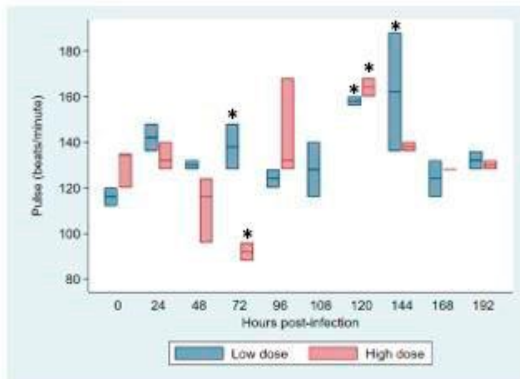
In the HD group, mean arterial blood pressure increased above baseline at 24 hours. This was followed by significant increases in diastolic, systolic and mean arterial pressures at 72 hours. Another spike in diastolic pressure was then noted at 168 hours in this group. The LD group on the other hand only had a mild increase in diastolic blood pressure above baseline at 96 hours (Table 4 and Figure 4 D – F). The impact of infectious dose on blood pressure was unclear, with was only the HD group demonstrating a transient hypertension. The only the dog that demonstrated mild hypotension in this study was the dogs that collapsed at 96 hours in the HD group.



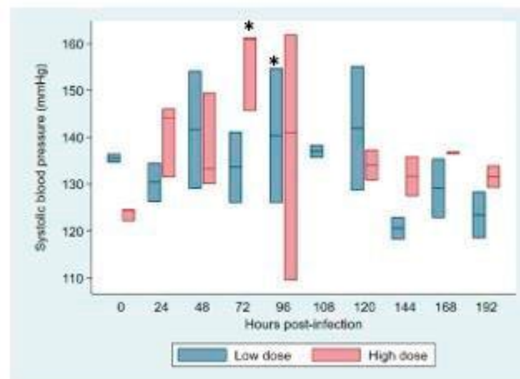
Graph A: Temperature



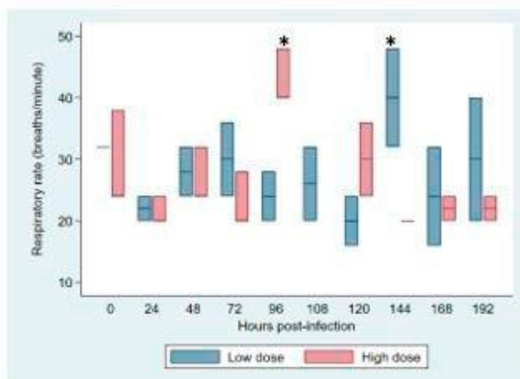
Graph D: Diastolic blood pressure



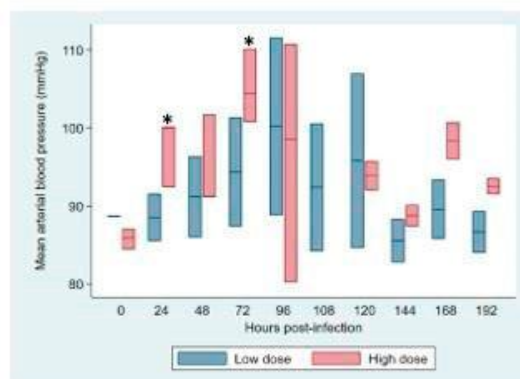
Graph B: Pulse



Graph E: Systolic blood pressure



Graph C: Respiratory rate



Graph F: Mean Arterial blood pressure

Figure 4: Clinical data evaluated during disease progression from inoculation to recovery. Values represented for each parameter include the range and mean (as indicated by the line within the box). * Indicate time points with significant differences.

Clinicopathological parameters

In the HD group, there was a rapid and profound increase in the parasitaemia from 24 hours post-inoculation with the peak, up to 59%, just before treatment at 96 hours. Parasitaemia in this group dropped to undetectable levels within 48-hours of treatment, at 168 hours. The parasitaemia in the LD group increased more gradually and remained statistically lower than the HD group at every time point during infection. Parasites were only identified at 72 hours in the LD group, peaking at 108 hours and dropping to undetectable levels 36-hours after treatment, at 168 hours (Table 5 and Figure 5A). The RCC and Ht started showing significant decline from 96 hours in the HD group, worsening after treatment until reaching a nadir at 120 hours. From there the RCC and Ht progressively increased for the remainder of the study but both were still below baseline levels at the conclusion of the study. The HD group received multiple blood transfusions and the severity of the anaemia after 120 hours was likely underestimated. In the LD group the RCC and Ht only started declining after treatment at 120 hours, reaching a nadir at 168 hours. Although the drop in RCC and Ht were significant in this group, they only resulted in mild clinical anaemia in all dogs and no blood transfusions were necessary (Table 5 and Figure 5B).

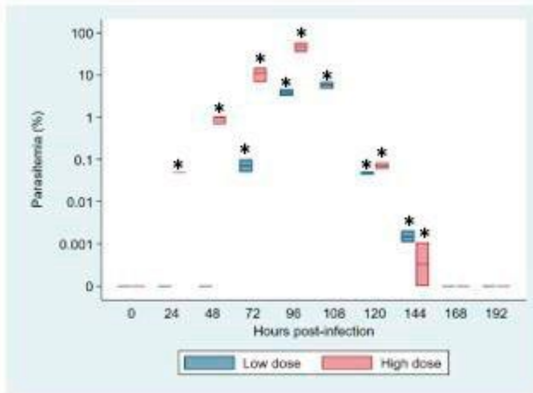
No dog became hypoglycaemic (<3.3 mmol/L) during this experimental investigation. There was however a significant reduction in glucose concentrations below baseline at 96 hours in the HD group, but concentrations did not drop below the reference limit (<3.3 mmol/L). After treatment, there were significant increases in glucose levels above baseline in the HD group, between 120 to 192 hours, peaking at 168 hours. The LD group had increased glucose concentrations above baseline at 168 and 192 hours (Table 5 and Figure 5H). When lactate was measured using the handheld Lactate Pro 2 (Table 5 and Figure 5I), there was a peak in lactate above baseline at 72 and 96 hours in the HD and LD groups respectively, a 24-hour difference. When utilizing the venous blood gas values, the lactate increased significantly from 48 hours but only exceeded the reference interval (>2.5 mmol/L) at 96 hours in the HD group (5.07 mmol/L, Range 3.05 – 7.5; RI 0 - 2), returning to normal limits within 24 hours. There was a large discrepancy in the results between these two techniques.

The acute phase proteins we investigated included albumin (Table 5 and Figure 5G) and CRP (Table 5 and Figure 5F). There were statistically significant decreases in albumin concentrations from 96 to 192 hours in the HD group with a nadir at 120 hours. Hypoalbuminaemia below the RI was seen from 96 until 192 hours in this group. In the LD group a hypoalbuminaemia was seen at 144 hours which did not reach significance. C-reactive protein started increasing above baseline from 24 hours, peaked at 72 hours and then gradually declined from there but remained significantly increased for the remainder of the study in the HD group. The LD group showed a drastic increase in CRP above baseline

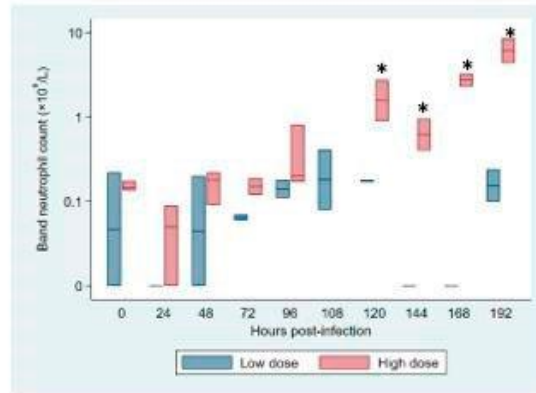
at 108 hours, declining thereafter but remaining significantly increased for the remainder of the study. The CRP peaked 36-hours earlier in the HD group.

Significant progressive segmented neutropenia was seen from 72 hours in HD group and 96 hours in the LD group. The nadir for the HD group was at 96 hours and the LD group was 108 hours, just prior to treatment for both. After treatment there was a gradual recovery of the neutrophil count to normal at 192 hours in the LD group. In the HD group on the other hand demonstrated a marked increase in neutrophil numbers after treatment, reaching neutrophilia (neutrophil count above the RI: 3 – 11.5 x 10^9/L) at 168 and 192 hours (Table 5 and Figure 5C). A significant left shift neutrophilia (band neutrophil count above RI: 0 – 0.5 x 10^9/L) was seen in the HD group from 120 to 192 hours (Table 5 and Figure 5D). At no point during the study did the LD group band neutrophil count exceed the RI or increase significantly above baseline values. The other inflammatory cell line evaluated was monocyte count. A statistically significant reduction in monocyte count compared to the baseline was seen in the HD group at 48 hours. Following treatment, the monocyte counts were increased from 120 to 192 hours in the HD group, exceeding the RI (0.15 – 1.35 x 10^9/L) from 144 hours onwards. Although not statistically significant, the LD group had a decline in in monocyte counts 108 and 120 hours, just over 48 hours after the decrease seen in the HD group (Table 5 and Figure 5E). Lymphocyte counts remained within the reference intervals throughout the study for both groups and no significant difference was found for either group at any time point.

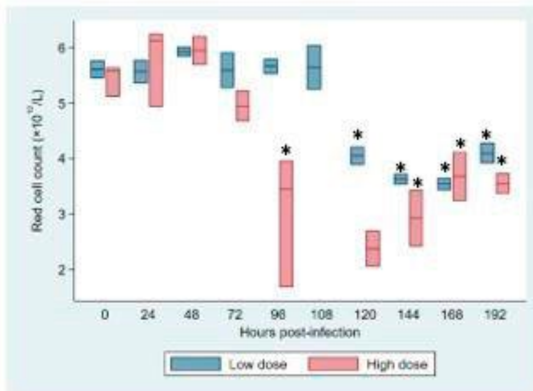
The infectious dose, again, appeared to influence the onset of alterations in the clinicopathological variables. Although some changes were more severe in the HD group, perhaps if the disease in the LD group would have been allowed to progress further, these parameters would have reached similar degrees of change.



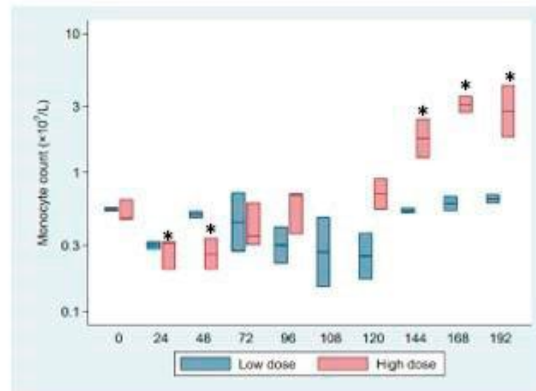
Graph A: Parasitaemia percentage



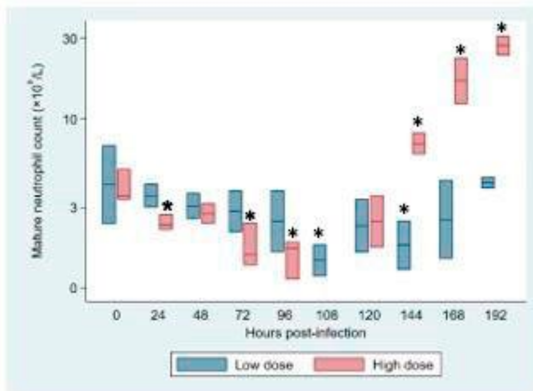
Graph D: Band neutrophil count



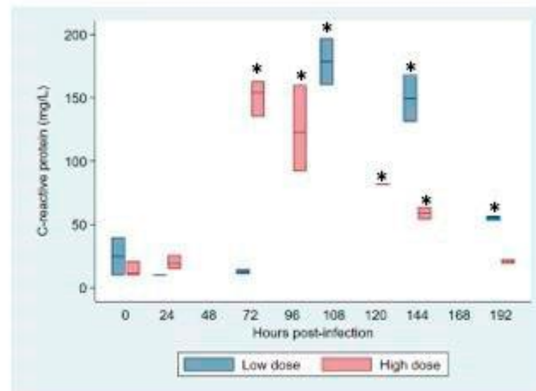
Graph B: Red cell count



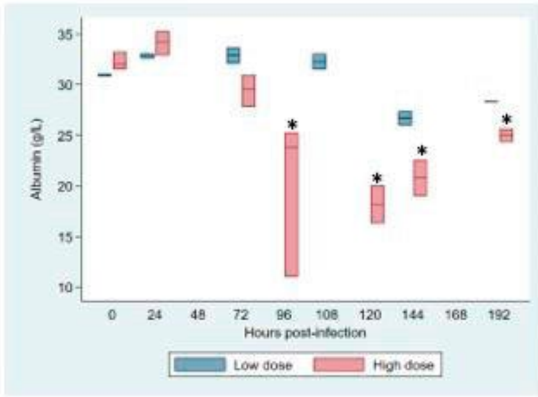
Graph E: Monocyte count



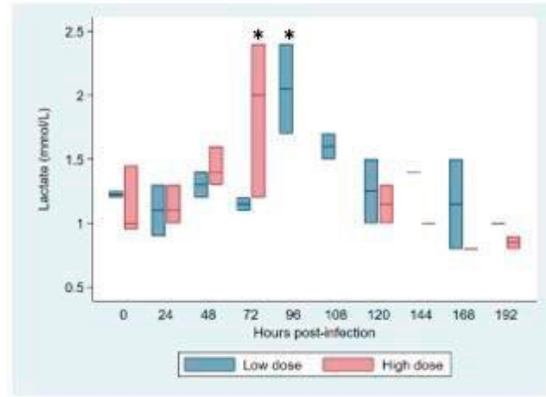
Graph C: Segmented/Mature neutrophil count



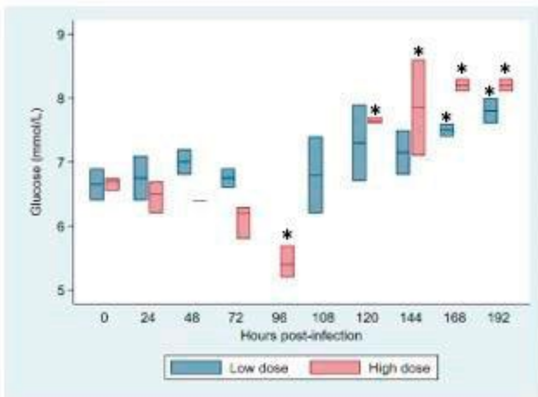
Graph F: C-reactive protein concentrations



Graph G: Albumin concentrations



Graph I: Lactate concentrations



Graph H: Glucose concentrations

Figure 5: Clinicopathological parameters evaluated during disease progression from inoculation to recovery. Values represented for each parameter include the range and mean (as indicated by the line within the box). * Indicate time points with significant differences.

Cytokine kinetics

Thirteen cytokines were evaluated in total, and the results were divided into 4 groups by pattern of change. The categories for the kinetic patterns identified include:

a. Cytokines that rose during infection and fell after treatment

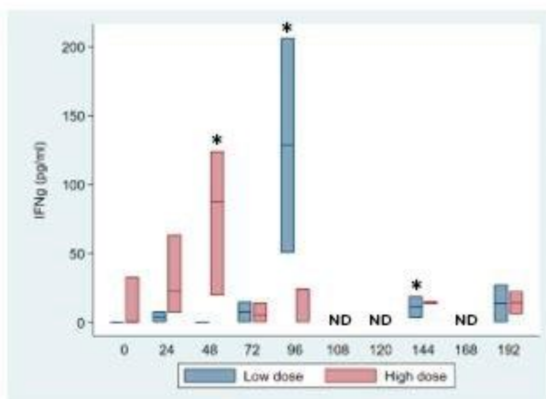
b. Cytokines that rose during infection and remained high even after treatment

c. Cytokines that rose dramatically after treatment

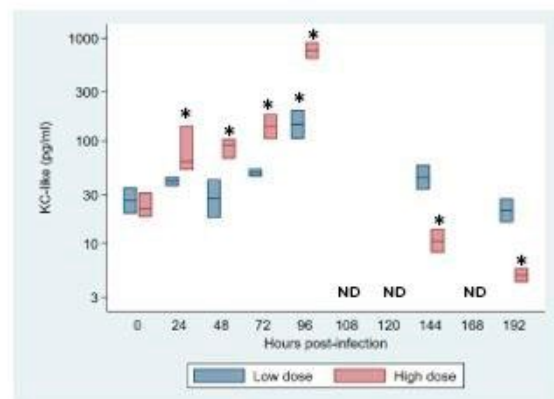
d. Cytokines that showed no distinct pattern of change

a. Cytokines that rose during infection and fell after treatment

The cytokines which fall into this category were IFNγ and KC-like. Concentrations of IFNγ in the HD group had peaked at 48 hours and concentrations in the LD group peaked at 96 hours, a 48-hour difference (Table 6 and Figure 6A). The HD group also showed a progressively significant increase in the KC-like concentrations from 24 hours until treatment at 96 hours with a sharp drop to sub-baseline levels at 144 and 192 hours. The LD group only had a significant increase in KC-like concentrations at 96 hours (Table 6 and Figure 6B). Infectious dose appeared to influence the onset of increases noted in this group of cytokines with a delay seen in the LD group. Concentrations in IFNγ started to increase during the period of gradual parasitaemia expansion in both groups, suddenly dropping off when the parasitaemia climbed almost exponentially in the HD group. A similar drop was not identified in the LD group but nor was such a drastic increase in parasite numbers.



Graph A: IFN gamma concentrations

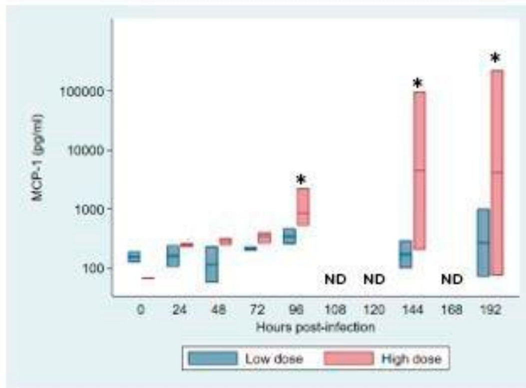


Graph B: KC-like concentrations

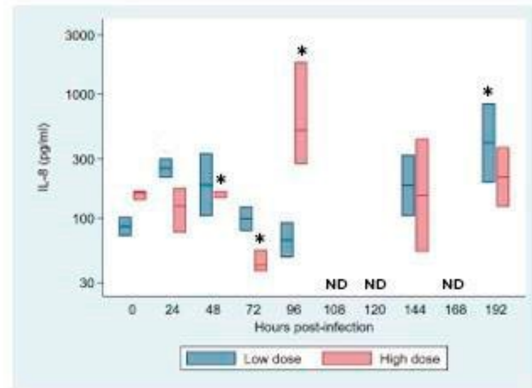
Figure 6: Category *a* cytokines: Cytokines that rose during the infection and fell after treatment. Values represented for each parameter include the range and mean (as indicated by the line within the box). * Indicate time points with significant differences. ND: Concentrations not determine at these time points.

b. Cytokines that rose during infection and remained high even after treatment

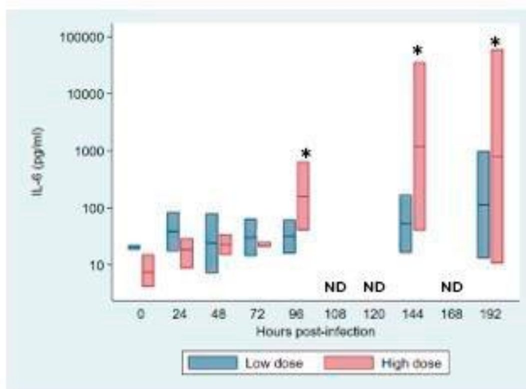
In category *b* we identified the following cytokines MCP-1, IL-6, IL-8, and IL-10. The chemokine MCP-1 was increased above baseline in the HD group from 24 hours, gradually increasing until treatment whereafter the concentrations spiked to very high levels in one dog. The LD group did not have a significant increase in MCP-1 throughout the study, but one dog in this group did show a noticeable increase at 192 hours (Table 7 and Figure 7A). IL-6 concentrations were moderately increased at 96 hours in the HD group. After treatment the concentrations were severely increased in one dog and moderately so in the other dog in the HD group (Table 7 and Figure 7B). IL-6 in the LD group did not increase significantly at any point but, like changes seen in MCP-1 concentrations, one dog in this group had a noticeable increase at 192 hours. Interestingly the dog that died in the HD group had the highest concentrations of MCP-1 and IL-6, by far, then any other dog in that group at 96 hours. The only dog to develop icterus after treatment had the highest concentrations of MCP-1 and IL-6 during that period. In the LD group, IL-8 concentrations increased at 24 hours with a gradual decline until treatment, thereafter, increasing up to significance at 192 hours. The HD group on the other hand showed a trough in the IL-8 concentrations at 72 hours, with a marked increase at 96 hours when parasitaemia reach massive levels. After treatment IL-8 remained mildly elevated in one dog in the HD group (Table 7 and Figure 7C). IL-10 increased from 24 hours in the HD group, with significant difference from baseline at 48, 72 and 96 hours. The peak concentration was seen at 96 hours, decreasing after treatment but remaining increased. The LD group showed no significant increase in IL-10 but both dogs demonstrated a progressive increase in concentrations from 72 hours after inoculation until treatment, the concentration remained increased in one dog after treatment (Table 7 and Figure 7D). Similar kinetic profiles were seen between the HD and LD groups for MCP-1, IL-6 and IL-10, varying in onset but not necessarily severity. IL-8 concentrations however appeared to follow different kinetic pathways between the HD and LD groups with the LD group having higher concentrations for the first 72 hours after inoculation and showing no increase during infection.



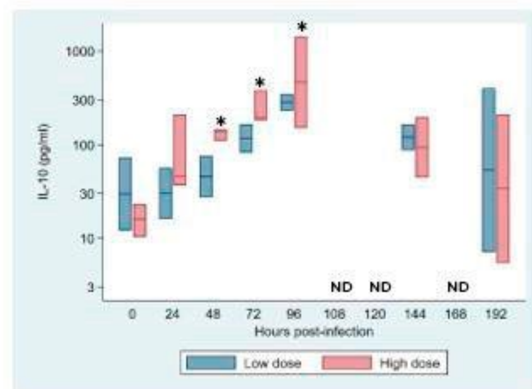
Graph A: MCP-1 concentrations



Graph C: IL-8 concentrations



Graph B: IL-6 concentrations

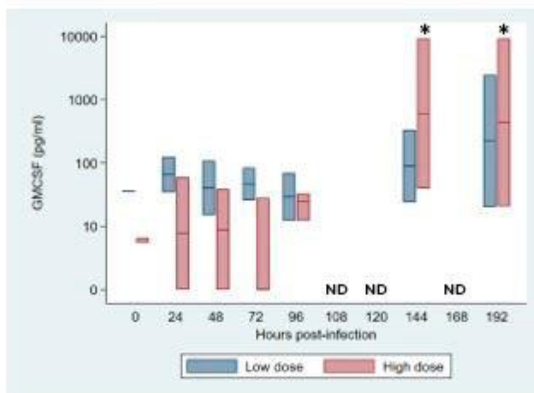


Graph D: IL-10 concentrations

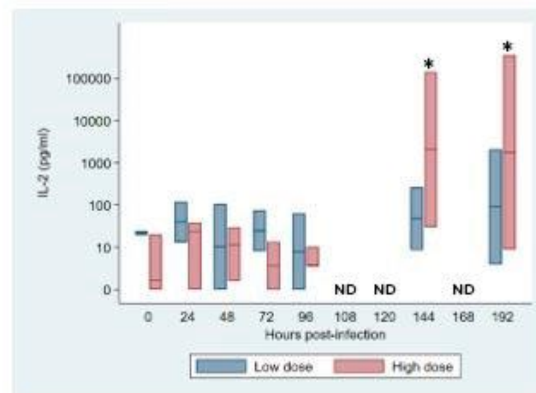
Figure 7: Category *b* cytokines: Cytokines that rose and remained high even after treatment. Values represented for each parameter include the range and mean (as indicated by the line within the box). * Indicate time points with significant differences. ND: Concentrations not determine at these time points.

c. Cytokines that rose dramatically after treatment

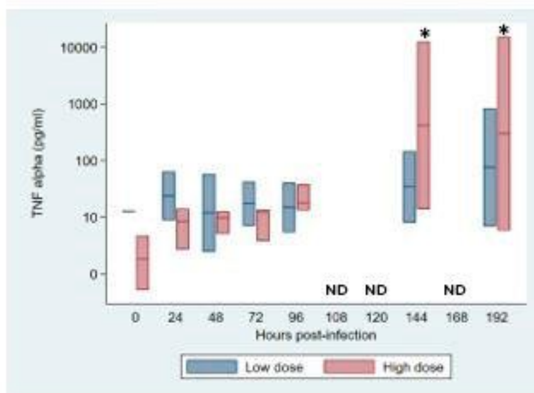
The next category of cytokines, all had very similar patterns of changes with dramatic increases after treatment. GM-CSF, TNFα, IL-2 and IL-7 were all markedly increased after treatment in the HD group (Table 8 and Figure 8A – D). This was specifically seen in one dog with severe increases in all four of these cytokines after treatment, at 144 and 192 hours. Although not statistically significant, a dog from the LD group showed a similar profile after treatment with marked increases in all 4 cytokines but not nearly to the degree seen in the HD group. Infectious dose did not appear to influence cytokines in this category. What did appear to have a marked influence was the treatment itself and the level of parasitaemia at the time of treatment. Both dogs in each group with the highest parasitaemia's showed the most drastic increases in cytokine concentrations after treatment.



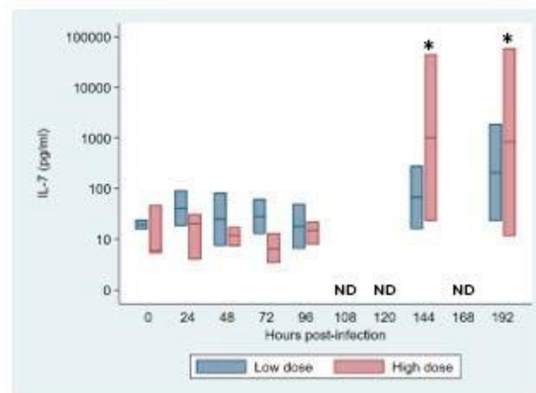
Graph A: GM-CSF concentrations



Graph C: IL-2 concentrations



Graph B: TNF alpha concentrations

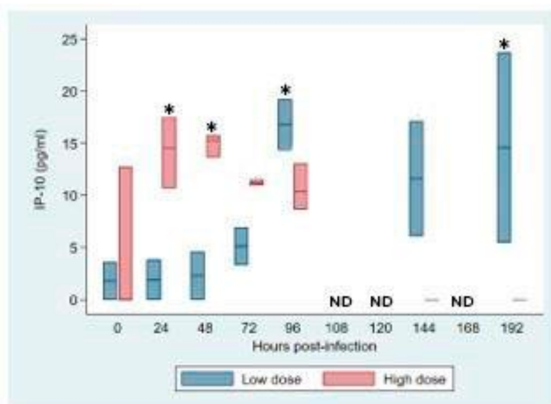


Graph D: IL-7 concentrations

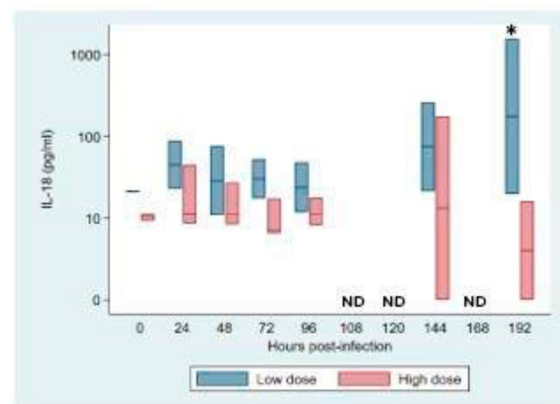
Figure 8: Category *c* cytokines: Cytokines that rose dramatically after treatment. Values represented for each parameter include the range and mean (as indicated by the line within the box). * Indicate time points with significant differences. ND: Concentrations not determine at these time points.

d. Cytokines that showed no distinct pattern of change

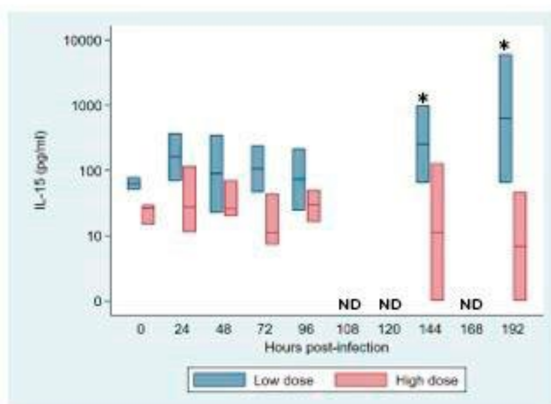
The last three cytokines reported only showed minor changes in the cytokine concentrations. IL-15 and IL-18 were both increased from 24 hours in one dog in the LD group (Table 9 and Figure 9B – C). Although the increases were persistent prior to treatment; they did not reach significance until after treatment at 192 hours in the LD group. Finally, IP-10 showed very mild changes during the study period (Table 9 and Figure 9A). In the HD group, IP-10 was significantly increased at 24 and 48 hours. This cytokine only increased at 96 hours in the LD group and remained increased after treatment. The role of infectious dose in this group is unclear as the LD group had consistently higher concentrations of IL-15 and IL-18, and IP-10 concentrations after treatment were only increased in the LD group.



Graph A: IP-10 concentrations



Graph C: IL-18 concentrations



Graph B: IL-15 concentrations

Figure 9: Category *d* cytokines: Cytokines that showed no distinct pattern of change. Values represented for each parameter include the range and mean (as indicated by the line within the box). * Indicate time points with significant differences. ND: Concentrations not determine at these time points.

Correlations

Some parameters demonstrated strong significant correlations during the course of the study, which are summarised in table 10.

Table 10: Clinical, haematological, biochemical and cytokine variables demonstrating strong correlations during the course of infection and after treatment

MCP-1	IL-6	0.7924	<0.001*
	IL-10	0.8284	<0.001*
KC-like	Parasitaemia	0.8881	<0.001*
	Segmented neutrophils	-0.8169	<0.001*
TNFα	IL-6	0.9253	<0.001*
	GM-CSF	0.8114	<0.001*
	IL-2	0.8097	<0.001*
	IL-7	0.8061	<0.001*
IL-2	IL-7	0.8724	<0.001*
RCC	Albumin	0.8931	<0.001*
IL-15	IL-18	0.9809	<0.001*
CRP	Temperature	0.7216	0.003*
	Parasitaemia	0.6462	0.056
Parasitaemia	Glucose	-0.6245	0.006*
	Lactate	0.6344	0.006*
	KC-like	0.8881	<0.001*
	IL-10	0.6735	0.009*
	Segmented neutrophils	-0.6742	<0.001*
MCP-1	IL-6	0.7924	<0.001*
	IL-10	0.8284	<0.001*

* Significant correlations

Chapter 5: Discussion

Our study was the first canine model to continuously evaluate the kinetics of markers of inflammation from inoculation to treatment and recovery, in dogs experimentally infected with *Babesia rossi*. The results of this study illustrate the potential role cytokines play in initiating and perpetuating inflammation in this disease. Additionally, this study has also demonstrated that a pronounced inflammatory response continues and even worsens despite killing of the parasite with appropriate treatment. The influence of inoculum dose was also demonstrated, with a high infectious dose increasing the rate at which disease developed as well as resulting in a more fulminant form of the disease. All the findings agreed with our original hypotheses, providing more in-depth insights into the pathogenesis of this haemolytic disease, and additionally shedding light on the influence of treatment on the progression of the inflammatory response.

As with any veterinary species, the detection of disease in our patients often starts with a decline in habitus and appetite. Although habitus and appetite are not very specific, they are important indicators of the presence of disease. In our study there was a notable decline in both these parameters and it was associated with infectious dose, level of parasitaemia and disease severity. Habitus and appetite improved with resolution of systemic inflammation, parasitaemia and anaemia in our study. Although this was an anticipated finding it did highlight, yet again, the importance of habitus and appetite in the monitoring of disease progression. Of all the variables evaluated, clinical parameters such as vital signs, form an easily accessible set of quantitative diagnostic evaluations for the clinician to utilise in monitoring disease progression. Given the inflammatory response associated with canine babesiosis, especially that caused by *B. rossi*, these parameters may be used to identify systemic inflammatory response syndrome (SIRS) in this subset of patients. Three of the four criteria required to identify the presence of SIRS are clinical parameters which form part of the assessment of vital signs. These include heart rate >140 bpm, respiratory rate >40 bpm and temperature >39.2°C or <37.2°C (Ettinger et al., 2017, Hauptman et al., 1997, Brady and Otto, 2001, Okano et al., 2002). SIRS has been described in canine babesiosis with alteration in temperature being one of the most common criteria met (Schetters et al., 2009a, Welzl et al., 2001, Matijatko et al., 2010). In our study temperature tended to increase as the parasitaemia increased and gradually dropped back to normal after treatment following the decline in parasitaemia. This is similar to findings by Schetters et al in the experimental infection of dogs with *B. canis* (Schetters et al., 2009a). The inoculum dose influenced onset of pyrexia with the HD group exceeding the SIRS threshold 24 hours earlier than the LD group. Unlike the study by Schetters et al, the correlation between temperature and parasitaemia did not reach significance, but temperature and CRP on the other hand were moderately correlated,

indicating that the rise in temperature likely mirrors an increase in systemic inflammation. One dog in the high dose group showed a notable drop in temperature of 2.8^{o}C at 96 hours, which was followed by collapse and eventual death. In a recent study of dogs naturally infected with *B. rossi*, rectal temperatures were significantly lower in dogs that presented collapsed and those that eventually died (Leisewitz et al., 2019b). As a whole temperature was a good indicator of the onset and progression of inflammation. Dogs presenting with a low rectal temperature or demonstrating a marked drop in temperature over the course of management may also indicate a guarded prognosis. Not only could temperature act as a proxy for the degree of systemic inflammation but may also be used as a monitoring and prognostic tool in canine *B. rossi* infections. Heart rate is a quick and easy clinical parameter to determine but its interpretation may be complicated by confounding factors such as stress and anxiety. Despite acclimatisation to the procedures performed during the experimental study, dogs in the LD group had increased heart rates during the first three days of the study, at times even exceeding the SIRS criteria cut-off. As these dogs were otherwise clinically healthy and had very low levels of parasitaemia detectable only at 72 hours, it was unlikely the tachycardia was due to disease progression or inflammation. The tachycardia was probably due to the procedural handling of these dogs and the associated stress. By 96 hours their heart rates normalised, (likely due to acclimation to the procedures) and then proceeded to peak at 120 hours coinciding with the greatest drop in red cell count in this group. The impact of the stress on heart rate should be kept in mind when evaluating canine patients. If dogs are clearly distressed or anxious during handling in an unfamiliar environment, heart rates should be interpreted with caution. The dogs in the HD group showed a similar spike in heart rates initially but appeared to acclimate more rapidly. At 72 hours, there was a significant decline in heart rates in all three dogs in the HD group. This was an unexpected finding as there was an increase in other indicators of systemic inflammation and we would have expected their heart rates to be increasing at this point. A possible explanation may be found in the abrupt increase in blood pressure noted at the same time point in this group, resulting in a physiological baroreceptor activation and a decline in their heart rates (La Rovere et al., 2001). Acute hypertension associated with sepsis has been identified in humans in recent years, particularly during the acute phases of disease and may increase with severity of clinical disease (Pandey et al., 2014, Saleh, 2014). The cause of this hypertension is unknown but neuro-endocrine involvement is thought to play a role and it would be unsurprising if similar mechanisms were not at work in severe canine babesiosis (Saleh, 2014, Pandey et al., 2014). After 72 hours, the heart rate in the HD groups increased more consistently with the reduction in red cell count and increasing inflammation. In our study respiratory rate appeared to be the least reliable of the SIRS criteria, only the HD group at the peak of their parasitaemia exceeded the 40 bpm SIRS cut-off. Changes in respiratory rate are most likely

multifactorial in canine babesiosis with SIRS, compensation for metabolic acidosis caused in part by hyperlactataemia as well as stress/anxiety contributing to the tachypnoea. For this reason, it may be a less reliable indicator of disease progression.

Although less readily available, blood pressure is an easy-to-use tool in the assessment of critically ill patients. During an experimental infection of dogs with *B. canis*, the mean arterial blood pressure, measure using a non-invasive oscillometric blood pressure meter, declined several days after inoculation (Schetters et al., 2009a). The timing of the drop in blood pressure was correlated with increased infectious dose. The highest infectious dose demonstrated a decline in blood pressure first, followed by the intermediate and lastly the low dose (Schetters et al., 2009a). In another study evaluating blood pressure changes in dogs naturally infected with *B. rossi*, hypotension was the most common change seen in these dogs, with more severe hypotension with increased disease severity (Jacobson et al., 2000). Contrary to previous studies, we did not find progressive hypotension after infection. There was a transient significant increase in blood pressure noted at 72 hours in the HD group resulting in mild hypertension. As mentioned previously, similar findings of acute hypertension have been identified in human patients with sepsis and although poorly understood, it may be the effect of the neuroendocrine and sympathetic nervous systems involved in this response in septic patients (Pandey et al., 2014). One such influence is hypothalamic-pituitary-adrenal axis activation in septic patients, a finding also known to occur in canine babesiosis (Bendel et al., 2008, Pandey et al., 2014, Schoeman et al., 2007, Leisewitz et al., 2019b). The increased release of cortisol in these patients may contribute to the increase in blood pressure, a common consequence of increased levels of circulating cortisol (Whitworth et al., 2005). Another mechanism that may play a role is cerebral ischemia leading to a loss of cerebrovascular autoregulation which is commonly identified in human sepsis (Saleh, 2014, Jacobson, 2006, Schramm et al., 2012, Pardini, 2013). This may play a role in canine babesiosis with severe anaemia and cerebral vascular endothelial pathology seen in cerebral babesiosis (Pardini, 2013). The acute hypertension seen during sepsis may be a neuro-protective response to preserve cerebral blood flow (Saleh, 2014). In addition to these mechanisms, another factor unique to haemolytic disease such as canine babesiosis, especially that caused by *B. rossi*, is the presence of cell-free haemoglobin. Cell-free haemoglobin, released during intravascular haemolysis, binds to and consumes endothelial derived nitric oxide interfering with vascular haemostasis and contributing to vasoconstriction (Gladwin et al., 2004). In this study a marked increase in cell-free haemoglobin was identified from 72 hours onwards in the HD group and this may have contributed to the transient hypertension noted in this group. Several other factors likely play a role in this response and further research is needed in both humans and small animals. The blood pressure values in the dog that died in the HD group were the lowest measured and although it was only a mild hypotension,

this would agree with previous findings that hypotension worsened with disease severity (Schetters et al., 2009a, Jacobson et al., 2000). Our study is the first to investigate changes from the point of infection to the onset of clinical disease, it is possible the transient hypertension followed by normotension occur early in natural infection and may have been missed due to the timing of previous studies. The absence of hypotension may be due to the timing of treatment, if the infection had been left to progress beyond our predetermined endpoints, perhaps we would have identified hypotension in more dogs. Alternatively, hypotension may be a terminal event in the course of *B. rossi* infection. It does however appear in this experiment that obvious changes in blood pressure are not consistent with disease progression.

High levels of parasitaemia are characteristic of *B. rossi* infection and higher levels of parasitaemia are correlated with increased risk of complications and death (Bohm et al., 2006, Leisewitz et al., 2019a). Other *Babesia spp.* such as *B. vogeli* may have very low parasitaemia levels and even subclinical infections (Irwin and Hutchinson, 1991). Venous parasitaemia's of up to 30% have been recorded in natural *B. rossi* infections (Bohm et al., 2006) and this high parasitaemia is one of the factors thought to contribute to its virulence (Schetters et al., 2009b). Circulatory collapse is a severe clinical complication which has been associated with higher levels of parasitaemia (Bohm et al., 2006). However, parasitaemia is unlikely to be the sole trigger of circulatory collapse because dogs with low parasitaemia may also develop circulatory compromise (Bohm et al., 2006). The only dog that developed circulatory compromise in our study was in the high dose group but did not have the highest parasitaemia within this group. Although parasitaemia does appear to play a role, other individual patient factors are probably involved. The virulence of *B. rossi* may be due, in part, to an inability of the immune system to effectively control replication of the parasite. Control of parasitaemia does not appear to correlate with resolution of clinical signs in *B. canis* infections. A such, there is likely to be more factors over and above uncontrolled *B. rossi* replication that contribute to the virulence of this species in poorly adapted domestic dogs (Schetters et al., 2009b). Higher levels of parasitaemia in *B. rossi* infections may be associated with a difference in the type of host immune response to that triggered by different *Babesia* species. One such difference is the lower levels of fibrinogen identified in *B. rossi* compared to *B. canis* infections, which may result in reduced coating of parasitised erythrocytes and decreased erythrocyte sequestration (Schetters et al., 1997). As with natural and previous experimental *B. rossi* infections, our study demonstrated a progressive rise in parasitaemia requiring chemotherapeutic intervention. Higher inoculum dose reduced the length of the prepatent period as well as increased the peak parasitaemia with levels of parasitaemia reaching up to 59% within 4 days in the HD group. The LD group demonstrated a more gradual rise in parasitaemia's, more closely mirroring natural infection. The immune system may be overwhelmed

and unable to mount an effective and timeous response to the *B. rossi* parasites when faced with very high doses or alternatively, it is possible that these parasites actively suppress an immune response more effectively at higher doses. The concept of an ineffective immune response may be supported by the positive correlation identified between parasitaemia and IL-10, a prominent anti-inflammatory cytokine. Immune evasion by protozoa is a well-known phenomenon and has been demonstrated in *Plasmodium, Trypanosoma* and *Leishamania* among others (Morrot, 2020). In *Leishamania* infections, the parasites promote an immunosuppressive cytokine profile with high levels of IL-10, allowing them unrestricted replication (Morrot, 2020). A similar interaction may take place in *B. rossi* infections. The negative correlation we identified between levels of parasitaemia and segmented neutrophil count may also point to a deficient innate immune response to the *B. rossi* parasites in dogs. The timing of treatment relative to parasitaemia levels also appears to have a marked influence on the degree of cytokine response after treatment, with cytokines such as, MCP-1, IL-6, TNFα, GM-CSF, IL-2 and IL-7 all demonstrating increases after parasites were damaged/killed by the treatment in the HD group with mild to moderate increases in the LD group. The biggest difference between these groups at the time of treatment was the level of parasitaemia and it appears that higher parasitaemia at the time of treatment may result in a more severe, unregulated pro-inflammatory response after treatment. The damage/killing of the parasites probably releases soluble parasite factors (such as PAMPs) which appear to be efficient in stimulating a profound immune response (potentially through PRRs on the cells of the innate immune system). Although this response may increase the rate of *B. rossi* parasite clearance, it is probably unnecessary once the parasites are damaged by the treatment and may instead result in widespread 'innocent bystander' injury to host tissues.

Anaemia is a well-known haematological abnormality in dogs infected with *B. rossi*. In a recent study on natural *B. rossi* infections, approximately 84% of the dogs had a haematocrit below the reference interval at presentation (Leisewitz et al., 2019b). Although anaemia is not a reliable predictor of death in canine babesiosis, it is an important abnormality for disease management. Severe anaemia requires treatment to avoid systemic complications of hypoxia and eventual death (Leisewitz et al., 2019b). Clinicopathological measurements used to determine the presence of anaemia in infected dogs in this current study were haematocrit and red cell count. Haematocrit is easy and rapid to measure in most clinical settings as it does not require the use of a haematology analyser. For this reason, it is the most used measurement in everyday practice. Red cell count on the other hand is more accurate but less accessible. In the current study the LD group only developed mild anaemia after treatment and no blood transfusions were necessary. The absence of anaemia during active infection in this group was likely due to the insufficient time allowed for the anaemia to develop before treatment was instituted. The HD group on the other hand demonstrated a significant anaemia at 96 hours after inoculation

which worsened considerably after treatment. The anaemia seen at 96 hours was a direct consequence of the infectious process and although the anaemia seen during canine babesiosis is incompletely understood, haemolysis appears to play a key role (Jacobson and Clark, 1994, Schoeman, 2009, Leisewitz et al., 2019b). Intravascular and extravascular haemolysis appear to be involved. Haemoglobinaemia and haemoglobinuria, identified in this and previous studies, point to the significant contributions made by intravascular haemolysis (Lobetti et al., 1996, Defauw et al., 2012, Leisewitz et al., 2019b). Erythrophagocytosis constituting the extravascular component to the haemolysis, takes place in both the spleen and liver during canine babesiosis (Jacobson and Clark, 1994, Maegraith et al., 1957). Hyperbilirubinemia has been identified in many *B. rossi* infection cases and significant pre-hepatic component is suspected (Leisewitz et al., 2019b). Similar to previous experience a marked drop in haematocrit and red cell count occurred after treatment in both the LD and HD groups (Jacobson et al., 1996). The post treatment drop in haematocrit is thought to be due to a continued inflammatory host response with progressive erythrocyte destruction and sequestration until all parasites are eliminated and the disease process is brought under control (Jacobson et al., 1996). It is possible that this process is exacerbated by damage to the parasites caused by treatment leading to the releases of soluble antigens which in turn trigger an even more profound pro-inflammatory immune response. Treatment of the dogs in this study was significantly associated with a drop in red cell count as well as increased levels of mature and band neutrophils, monocytes and several pro-inflammatory cytokines namely IL-6, MCP-1, GM-CSF, TNFα, IL-2 and IL-7. Treatment appears to exacerbate the inflammatory response and worsen the anaemia in dogs infected with *B. rossi*. The anaemia was also associated with an inadequate reticulocyte response. Although there may have been insufficient time for the bone marrow to mount an appropriate regenerative response, the inflammatory cytokine milieu may play a role in suppressing effective erythropoiesis as seen in malaria (Haldar and Mohandas, 2009).

Our study is the first to evaluate leukocyte changes over the course of *B. rossi* infection, with particular focus on the cell lines involved in inflammation. White cell count (WCC) is the fourth and final criteria used to identify SIRS with a WCC of more than 19.5 x 10^9/L or less than 5 x 10^9/L or more than 5% band cells being consistent with SIRS (Ettinger et al., 2017, Hauptman et al., 1997, Brady and Otto, 2001, Okano et al., 2002). In our study the HD group developed a leukopenia below this cut-off from 24 hours post inoculation already. The band cell percentage was greater than 5% in 2 dogs from the HD group at 96 hours and was consistently above this level for all dogs in this group after treatment. One dog in the LD group had a leukopenia from 48 hours but it was only at 108 hours that both dogs from this group demonstrated a leukopenia below 5 x 10^9/L. Only one dog in this group had a band cell count greater than 5% at 108 hours. It appears this component of SIRS is a prominent finding in more

severe canine babesiosis and not only persisted but progressed following treatment of the HD group, demonstrating another influence of inoculum dose. Leukocyte counts may act as markers of more severe clinical disease and further research into their use as monitoring and prognostic tools in canine babesiosis may be warranted. The neutrophil, a primary role player in the innate immune response, is important in the development of the adaptive immune response and clearance of not only extracellular but also intracellular pathogens (Appelberg, 2007). A previous study evaluating haematological changes after treatment of *B. rossi* infections found that although the median segmented neutrophil count in *B. rossi* infections remained within the reference interval for 6 days, a large percentage of these dogs actually presented with a neutropenia (Scheepers, 2013). In our study the HD group developed a significant segmented neutropenia from 72 hours until the point of treatment, thereafter the segmented neutrophil counts increased progressively reaching levels of moderate neutrophilia. The LD group also developed a segmented neutropenia before treatment, 36 hours after the HD group. Unlike the HD group, the LD group did not develop a neutrophilia after treatment, but the segmented neutrophil counts did return to normal limits. In the study by Scheepers et al there was a higher proportion of dogs with a neutrophilia in the group that received blood transfusions (Scheepers, 2013). In the current study, the HD group was the only group to receive blood transfusions and may be one explanation for the presence of the neutrophilia after treatment. A left shift neutrophilia was not detected in 4 out of the 5 dogs prior to treatment but after treatment a progressive band neutrophilia was seen in the HD group. Band neutrophilia in *B. rossi* infections has been associated with lower haematocrits at presentation and dogs receiving blood transfusions as part of their treatment (Scheepers, 2013). A band neutrophil count of > 0.5 x 10^9/L at presentation carries an odds ratio for death of 5.9 (Leisewitz et al., 2019b). Interestingly the only dog with a band neutrophil count above this level prior to treatment in our study, with a count of 0.8 x 10^9/L, was the only dog that died. The left shift noted in the HD group may have been, in part, due to the transfusions received but the overall increase in systemic inflammation trigger by the death of the parasites probably also played a role. There was a strong negative correlation between segmented neutrophil counts and KC-like, a cytokine with a major role in neutrophil migration and activation (Ritzman et al., 2010). IL-8, another important cytokine in the migration and activation of neutrophils, had a peak concentration at 96 hours, coinciding with the segmented neutrophil nadir in the HD group (Harada et al., 1994). The migration of neutrophils, under the influence of cytokine cues, out of circulation to various sites of tissue inflammation may contribute to the circulating neutropenia seen in *B. rossi* infections. GM-CSF provides a stimulatory cue to the bone marrow to initiate granulopoiesis, and this cytokine was only increased after treatment, particularly in the HD group (Hamilton, 2002). In *Plasmodium falciparum* malaria, a similar segmented neutropenia has been identified in acute

infections (Dale and Wolff, 1973). Mechanisms for the segmented neutropenia identified included a shift in the circulating pool to the marginated pool and premature release of immature granulocytes from the bone marrow (Dale and Wolff, 1973). Similar changes may be taking place in *B. rossi* infections. In a recent study on the myeloperoxidase index (MPXI) in neutrophils of dogs infected with *B. rossi*, the MPXI was unexpectedly increased and one possible cause discussed was an increase in toxic change from accelerated maturation and premature neutrophil release from the bone marrow in these dogs, painting a similar picture to that seen in malaria (Celliers et al., 2020, Dale and Wolff, 1973).

Monocyte counts in *B. rossi* do not generally exhibit obvious changes although mild monocytosis after treatment has been identified (Scheepers, 2013). The HD group had a mild monocytosis after treatment, but the monocyte count remained within the reference interval for the entire study for the LD group. The monocytosis seen in the HD group may have been in response to the increased demand for phagocytic cells to remove parasites, parasitised erythrocytes, and cellular debris after treatment. It should be noted that a recent pathological study of the lungs of babesia infected dogs demonstrated monocyte/macrophage accumulation in the pulmonary interstitium as the dominant change (Martin, 2020). The same was demonstrated in the spleen (Henning et al., 2020). A drastic increase in MCP-1 after treatment in the HD group may indicate an increased demand for monocyte/macrophage activity during this period. MCP-1 is chemokine that regulates migration and infiltration of monocytes/macrophages (Deshmane et al., 2009). Marked increases in GM-CSF may have provided the bone marrow stimulation necessary to increase production of monocytes after treatment (Hamilton, 2002).

Acute phase proteins are frequently used in the detection and monitoring of systemic inflammation. The two acute phase proteins evaluated in our study were albumin and CRP. Albumin is a negative acute phase protein and decreases during inflammation but there are several other factors that may influence its decline in canine babesiosis. Given the systemic inflammatory response seen in canine babesiosis two major contributors to a decline in albumin concentrations would be the negative acute phase response and a leaky endothelium with extravasation of albumin (Cray et al., 2009, Ince et al., 2016). Hypoalbuminaemia was identified in the HD group at 96 hours and progressed for 24 hours after treatment, before gradually improving. The LD group only showed hypoalbuminaemia transiently, 24 hours after treatment. The decreased albumin concentrations in both groups after treatment are an indication that one or both above-mentioned mechanisms persist and may progress shortly after treatment suggesting ongoing systemic inflammation and its associated consequences for some time after treatment. As a sole marker of inflammation, albumin is not an ideal candidate because of the influence of several factors on its concentrations, but it may be useful when monitoring

the resolution of inflammation and endothelial integrity in these patients. Hypoalbuminaemia has commonly been described in canine babesiosis caused by *B. rossi* (Leisewitz et al., 2019b).

CRP is a useful and sensitive marker of inflammation in diseases such as sepsis, pneumonia, neoplasia, and immune mediated conditions in the dog (Nakamura et al., 2008, Viitanen et al., 2014, Gebhardt et al., 2009). Though non-specific for cause, CRP is an acute phase protein that has demonstrated consistent increases in canine babesiosis (Matijatko et al., 2007, Schetters et al., 2009a, Koster et al., 2009). In one study on CRP in natural *B. canis* infection, CRP had its peak concentration at presentation and declined progressively following treatment (Matijatko et al., 2007). In a *B. canis* experimental infection, CRP increased before the presence of a detectable parasitaemia (Schetters et al., 2009a). The onset of CRP increase was inoculum dose dependent with the highest dose group showing increased concentrations first (Schetters et al., 2009a). Although CRP increases in dogs naturally infected with *B. rossi*, no correlation could be identified between concentrations at presentation or at 24-hours or with survival or duration of hospital stay (Koster et al., 2009). The inoculum dose in our study influenced the onset of increase in CRP concentrations with the HD group showing a significant increase 36 hours earlier than the LD group. In both groups treatment resulted in a progressive decline in CRP concentrations. There was a moderate correlation between CRP concentration and temperature. Pyrexia may act as a proxy for an increase in CRP concentrations and systemic inflammation, as the two mirrored each other. CRP increased dramatically with the onset of moderate parasitaemia but it did not appear to increase in proportion to the degree of parasitaemia. As seen in the *B. canis* experimental study by Schetters et al, CRP concentrations appeared to reach a ceiling and remain relatively stable at this level despite increasing parasitaemia (Schetters et al., 2009a). Although infectious dose has an impact on the onset of the initial increase in CRP concentrations, the degree of parasitaemia beyond a certain point does not strongly influence these concentrations. Circulating CRP levels remained high even after parasitaemia was undetectable. The delay in decline of CRP concentrations was most likely due to the half-life (which is approximately 161 hours in dogs, with significant inter-individual variation) rather than continued production (Kuribayashi et al., 2015). Unlike the findings of increased CRP prior to detectable parasitaemia in experimental *B. canis* infection, low levels of parasitaemia were detectable prior to significant increases in CRP concentrations in both groups. As a prognostic marker in canine babesiosis, CRP performs poorly. There was no significant correlation with parasitaemia, degree of anaemia or death. Similar to findings identified in malaria patients in sub-Saharan Africa (Sarfo et al., 2018). Perhaps a more useful application of CRP would be as a sensitive marker in the exclusion of babesiosis as a cause of disease in canine patients, as a monitoring tool to ensure the resolution of the babesiosis after treatment and may even help to exclude possible active co-infections such as ehrlichiosis should concentrations

remain high despite anti-babesial treatment. Co-infections with *Ehrlichia canis* may occur in up to 2% of dogs (Rautenbach et al., 2018). As with *Babesia*, active infection with *Ehrlichia* is associated with high CRP concentrations but returns to baseline in subclinical disease (Rautenbach et al., 2018, Mylonakis et al., 2011). This would therefore limit the usefulness of CRP to assessment of cases with an active *Ehrlichia* infection. Despite lacking obvious clinical utility, it is clear that CRP increases significantly in babesia infections and it underscores the inflammatory nature of this infection.

Glucose and lactate were evaluated in our study as they have been identified as prognostic markers in previous research on *B. rossi* infections (Nel et al., 2004, Leisewitz et al., 2019b, Keller et al., 2004). Most clinics have handheld readers available to assess these parameters rapidly and in a cost-effective way. In a previous study on natural *B. rossi* infections, hypoglycaemia (<3.3mmol/L or 59.4 mg/dL) and hyperlactataemia (>22.5 mmol/L) at presentation as well as persistent hyperlactataemia (>22.5 mmol/L) or a failure of lactate concentrations to drop more than 50% within 16 hours of treatment was positively correlated to increased risk of mortality (Nel et al., 2004). Similar findings supporting the prognostic implications of hyperlactataemia and hypoglycaemia have been identified in several studies (Leisewitz et al., 2019a, Keller et al., 2004, Jacobson, 2006). Hyperglycaemia is also a frequent finding in *B. rossi* infections but is generally not severe and is not predictive of disease severity (Keller et al., 2004, Leisewitz et al., 2019b). No dog in our study developed hypoglycaemia but a mild significant hyperglycaemia was identified in the HD group after treatment. The mild hyperglycaemia was likely a consequence of a neuro-endocrine stress response with associated stress hyperglycaemia (McCowen et al., 2001). Two techniques were used to determine lactate concentrations in this subset of dogs, a handheld lactate reader and venous blood gas. Given the large discrepancy between the 2 techniques utilised for the measurement of lactate, the Lactate Pro 2 proved to be unreliable when compared to the validated venous blood gas measurement. Lactate concentrations were only significantly increased above the reference interval in the HD group at 96 hours. However, these levels did not exceed the previously identified prognostic cut-off of >22.5 mmol/L (Nel et al., 2004). As with malaria, the hyperlactataemia is probably a multifactorial entity with both increased production and reduced clearance playing a role (Possemiers et al., 2021). Anaemia, hypotension, disturbances in tissue perfusion, liver and renal dysfunction and a hypermetabolic state are all possibly contributing to the increased lactate levels in *B. rossi* infection (Mizock and Falk, 1992, Nel et al., 2004, Jacobson et al., 2000, Possemiers et al., 2021). The hyperlactatemia is one contributor to the metabolic acidosis identified in canine babesiosis (Leisewitz et al., 2001).

Cytokines are a group of proteins secreted by cells of the immune system which act as key signalling molecules in the inflammatory response. A number of cytokine changes have been identified in *B. rossi* infections, but these have only been evaluated in dogs at presentation, providing us with a single

snap shot in time of the ongoing processes in this complicated and dynamic disease (Goddard et al., 2016, Leisewitz et al., 2019a). Cytokines shown to significantly increase during *B. rossi* infections include IL-6, IL-10, MCP-1 and TNFα, and their concentrations tended to be higher in more complicated cases (Goddard et al., 2016, Leisewitz et al., 2019a). Only IL-6 and IL-10 concentrations were significantly higher in dogs that died compared to survivors (Goddard et al., 2016). Decreased concentrations of IL-8 were consistently identified in natural *B. rossi* infections, in contrast to findings in *B. canis* infections (Goddard et al., 2016, Leisewitz et al., 2019a, Galán et al., 2018). Significant changes were identified in all thirteen cytokines under investigation in this study. Changes were identified during at least one time point during the study period for each of the cytokines. The cytokines demonstrating similar kinetics were grouped together into one of four categories, namely:

a. Cytokines that rose during infection and fell after treatment
b. Cytokines that rose and remained high even after treatment
c. Cytokines that rose dramatically after treatment
d. Cytokines that showed no distinct pattern of change

a) Cytokines that rose during infection and fell after treatment

The first category included the cytokines IFNγ and KC-like, both of which increased with the start of infection and declined consistently after treatment. The release of these two cytokines appears to be stimulated by an increase in the parasite biomass and be released by the host response in an attempt to control of the parasites. They also appear to have relatively short half-lives. In falciparum malaria, IFNγ is considered an important mediator in the protective innate immune response during the blood stage and initial parasite replication (Malaguarnera and Musumeci, 2002). IFNγ also appears to be involved in the protective immunity by the CD4+ response induced by re-infection of mice with *B. microti* (Igarashi et al., 1999). This cytokine increased early in the course of the experimental infection, coinciding with the initial increase in parasitaemia in both groups. The concentrations did however decline abruptly once parasitaemia exceeded 5% in HD group. Unlike the HD group, concentrations only dropped following treatment in the LD group. IFNγ may be important in the initial immune response to *B. rossi* infection, suppressing early replication of the parasite as seen in falciparum malaria (Malaguarnera and Musumeci, 2002). The sudden decline in IFNγ concentrations in the HD group coincided with a very drastic increase in parasitaemia in this group bringing into question whether the severe increase in parasitaemia was the cause or effect of this abrupt drop in IFNγ concentrations? Also, would we have seen a similar drop in concentrations in the LD group if the infection was permitted to progress beyond 108 hours? The unnatural levels of parasitaemia seen in

the HD group may have induced a state of immune exhaustion. High concentrations of IL-10 may also have contributed to the sudden decline in IFNγ, suppressing its secretion and contributing to the resultant unregulated parasite replication (D'Andrea et al., 1993, Toliver-Kinsky et al., 2002, Fiorentino et al., 1991). Suppression of IFNγ secretion may be one mechanism employed by the parasite to limit constraints placed on its replication. The other cytokine in this group was KC-like. This cytokine increases in both *B. gibsoni* and *B. canis* infections and was even able to discriminate between complicated and uncomplicated *B. canis* cases, with significantly higher concentrations seen in complicated disease (Galán et al., 2018). In our study, KC-like increased progressively during infection and declined following treatment, correlating strongly to parasitaemia. The concentrations were also consistently higher in the HD group than the LD group prior to treatment. A strong negative correlation was also identified between segmented neutrophil count and KC-like. KC-like triggers neutrophil tissue migration and activation (Jin et al., 2014, Sawant et al., 2016). The increased concentration of KC-like seen in canine babesiosis may contribute to the segmented neutropenia seen because of increased tissue migration. High KC-like concentrations may contribute to an increased probability for the development of complications because of its enhancing effect on neutrophil activation. The release of reactive oxygen species and neutrophil extracellular traps by KC-like activated neutrophils may form an important mechanism by which host tissue is damaged in an otherwise haemolytic disease (Jin et al., 2014).

b) Cytokines that rose and remained high even after treatment

The next category of cytokines discussed will be those that increased during infection and remained high after treatment. This group of cytokines may represent additional attempts by the host immune system to control parasite replication and maintain a balanced inflammatory response. Persistent increases in these cytokines after drug parasite clearance could have a role in the ‘run-away’ inflammation that persists after removal of the initial trigger as seen in malaria and sepsis (Clark et al., 2008b, Clark, 2007). Two pro-inflammatory cytokines in this category, MCP-1 and IL-6, had similar kinetics and a strong positive correlation during the study period. Both displayed progressive increases from the point of inoculation to treatment in HD group. After chemotherapeutic intervention, one dog in the HD group showed severely increased concentrations of both cytokines. Interestingly this was the only dog in the study to develop a complication associated with the *B. rossi* infection after treatment, namely icterus. No statistically significant increases were noted in the LD group throughout the study period, but concentrations of MCP-1 had started to increase 24 hours prior to treatment in this group. After treatment one dog in the LD group also showed a notable increase in concentrations of both these cytokines. In previous studies on natural *B. rossi* infections MCP-1 and IL-6 were

increased in infected dogs and higher levels were associated with increased risk of mortality (Goddard et al., 2016, Leisewitz et al., 2019a). The concentrations of MCP-1 and IL-6 in the dog that died were considerably higher than all the other dogs in our study, a finding that is in agreement with those previous studies. Higher concentrations were also noted in complicated cases of *B. canis* infections (Galán et al., 2018). Release of MCP-1 from several cell types is induced by oxidative stress, other inflammatory cytokines and certain growth factors (Deshmane et al., 2009). MCP-1 is important in the recruitment of monocytes/macrophages to sites of inflammation and their activation leading to release of additional inflammatory mediators from these cells as well as enhancing their phagocytic activity (Deshmane et al., 2009). Tissue macrophages are the dominant cell present in the lungs of dogs that died of *B. rossi* infection in which acute lung injury and acute respiratory distress syndrome were present (Martin, 2020). MCP-1 would be a vital host defence mechanism in the immune response to babesia parasites by amplifying inflammatory signals and enhancing phagocytosis of parasites and damaged erythrocytes. Persistently high levels in *B. rossi* infections could however contribute to an unregulated redundant inflammatory response and increase the risk of complications such as acute lung injury (Martin, 2020). Tissue damage and binding of PAMPs to PRRs on cells of the innate immune system trigger the production of IL-6 (Chaudhry et al., 2013). Another potent stimulator of IL-6 production is TNFα, and a very strong positive correlation was detected between IL-6 and TNFα in this study. The role of IL-6 in septic conditions is poorly understood, although it does play a role in many pro-inflammatory activities such as enhanced production of acute phase proteins like C-reactive protein from hepatocytes (well known to be raised in *B. rossi* infection (Koster et al., 2009)), activation of lymphocytes, acting as a pyrogen as well as suppressing the production of TNFα and IL-1β (Blackwell and Christman, 1996, Song and Kellum, 2005). This cytokine may therefor promote inflammation in the early stages of infection but potentially assist in the resolution of this inflammation by suppressing the production of key pro-inflammatory cytokines in the later stages. IL-6 has been identified as one mechanism linking inflammation with thrombosis in sepsis (Blackwell and Christman, 1996, Song and Kellum, 2005). Widespread coagulation and the formation of microthrombi are well-defined pathologies in canine babesiosis, particularly in *B. rossi* infections, and increases in IL-6 may be an important trigger for this (Goddard et al., 2013). Widespread coagulopathy seen in canine babesiosis may contribute to an increased risk of complications such as cerebral babesiosis, myocardial dysfunction and death (Dvir et al., 2004, Goddard et al., 2013, Pardini, 2013). IL-6 is also shown to play an important role in the acute endocrine response to infection which is well described and typical of this disease (Schoeman et al., 2007, Karga et al., 2000)

In previous studies on the cytokine changes in canine babesiosis caused by *B. rossi*, IL-8 has shown significantly decreased concentrations in infected dogs at presentation when compared to healthy

control dogs (Goddard et al., 2016, Leisewitz et al., 2019a). In contrast to these findings, IL-8 was increased in *B. canis* infections and even showed a progressive rise for at least 7 days after treatment (Galán et al., 2018). Our study identified that IL-8 decreased significantly in the earlier stages of infection but shortly before treatment, when parasitaemia was very high, there was a considerable increase in IL-8 concentrations noted in the HD-group. It also remained moderately increased for at least 4 days after treatment in 2 dogs, one from each group. The nadir of the mature neutrophil count correlated with peak IL-8 concentrations in the HD group, suggesting a possible contribution to the neutropenia by enhancing migration out of circulation to sites of inflammation. IL-8 is a leukocyte chemotactic factor with a primary role in neutrophil recruitment and activation in acute inflammation (Harada et al., 1994). The production of IL-8 by several cells including monocytes, T lymphocytes, endothelial cells and hepatocytes, occurs under the influence of inflammatory mediators such as IL-1, TNFα, viral or bacterial antigens and cellular stress (Harada et al., 1994, Hoffmann et al., 2002). IL-8 concentrations are increased in falciparum malaria patients and human sepsis where high concentrations may even predict increased risk of mortality (Livaditi et al., 2006, Lourembam et al., 2013). The decline in IL-8 production in *B. rossi* infections is poorly understood. This cytokine is not generally constitutively produced but rather requires stimulation by inflammatory molecules such as the cytokines TNFα and IL1 to trigger its production (Harada et al., 1994). It is possible that in the early stages of infection before parasitaemia and haemolysis become severe there may be insufficient stimulation (Harada et al., 1994). Although TNFα concentrations were significantly higher in *B. rossi* infected dogs in one study, the increases were only mild when compared to the normal dogs (Leisewitz et al., 2019a). In our study, TNFα concentrations only increased after treatment and this may be one factor leading to reduced IL-8 concentrations seen in the initial stages of infection. Suppression of IL-8 and the cytokines that stimulate its production (TNFα and IL-1) during infection may also be due to high concentrations of the anti-inflammatory cytokine IL-10 (Couper et al., 2008). Again, the persistence of high levels of an inflammatory cytokine like IL-8 after treatment, especially one involved in neutrophil recruitment and activation, is potentially superfluous and could contribute to unnecessary tissue damage in this disease.

The final cytokine in this group was IL-10, a prominent anti-inflammatory cytokine. Concentrations of IL-10 progressively increased over the course of the experimental infection and decreased after treatment but did remain significantly increased for at least 4 days thereafter. High levels of IL-10 have been noted in natural *B. rossi* infections (Goddard et al., 2016, Leisewitz et al., 2019a). IL-10 is essential in the regulation of the inflammatory response and plays a key role in preventing excessive inflammation and promoting resolution of inflammation once the inciting pathogen has been eliminated (Couper et al., 2008). Although the anti-inflammatory effects of IL-10 are critical, excessive,

or inappropriately timed production of IL-10 may prevent an effective immune response to an organism, allowing persistence or even unregulated replication in the host (Couper et al., 2008). This has been seen in *Leishmania spp.* and *Plasmodium spp.* at times leading to fulminant fatal infections or chronic persistent infections (Couper et al., 2008). Human septic patients with continuous over production of IL-10 and high IL-10:TNFα ratio develop marked immunosuppression and have a higher risk of mortality (Chaudhry et al., 2013). A strong positive correlation was seen between IL-10 and parasitaemia as well as between IL-10 and MCP-1 in our study. The role of IL-10 in the pathogenesis of *B. rossi* infections may not be limited to counter regulation of the pro-inflammatory response but high concentrations may also have a permissive effect on the replication of these parasites.

c) Cytokines that rose dramatically after treatment

The third category of cytokines were those that did not increase significantly until after treatment. This group included GM-CSF, TNFα, IL-2 and IL-7. All four of these cytokines increased in one dog from the HD and LD groups respectively in this study, and the change seen in the dog from the HD group was considerable. The myelopoietic activity of GM-CSF is well described promoting the production of granulocytes and macrophages from bone marrow precursors (Hamilton, 2002). The function of GM-CSF is not limited to bone marrow stimulation alone, it also enhances the functions of mature granulocytes and macrophages and may be a prime modulator of inflammation (Hamilton, 2002). Its production is triggered by other inflammatory cytokines such as IL-1, TNFα and IFNγ, and a strong positive correlation was noted between GM-CSF and TNFα in this study (Hamilton, 1993, Hamilton, 2002). Increases in GM-CSF concentrations stimulate the survival, proliferation, adhesion and activation of mature macrophages and granulocytes in addition to increasing their production by the bone marrow (Hamilton, 1993, Hamilton, 2002). Increased levels of GM-CSF have been identified in *B. rossi* infections, particularly in dogs presented earlier in the course of disease (Goddard et al., 2016). The rise in GM-CSF concentrations noted in our study may act as a double-edged sword, on the one side replenishment of neutrophil counts is essential but on the other, excessive production, adhesion and activation of granulocytes and macrophages after the parasites are eliminated may contribute to wide-spread tissue damage.

The initiator pro-inflammatory cytokine TNFα is one of the most studied cytokines in human medicine and is an important mediator in the protection against microbial infections (Idriss and Naismith, 2000). TNFα can however contribute to pathology in cases where there is disproportionate and dysregulated immune response to an infection by the host (Idriss and Naismith, 2000). One mechanism by which

TNFα contributes to the pathology of infection is its ability to induce cellular necrosis and apoptosis (Idriss and Naismith, 2000). This is a necessary tool to eliminate intracellular microbes and neoplastic cells but unregulated, it would lead to bystander cellular destruction (Idriss and Naismith, 2000). TNFα has a role in the suppression of *Plasmodium falciparum* parasites but may have injurious effects such as fever, malaise, shock and augmentation of anaemia in a proportion of patients (Clark et al., 2006, Malaguarnera and Musumeci, 2002). It is also a potent stimulator for the production of other pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8, serving as co-ordinator of the inflammatory response (Blackwell and Christman, 1996). High concentrations were found in natural *B. rossi* infections with higher levels being associated with increased risk of complications and death (Leisewitz et al., 2019a). Concentrations of TNFα were only increased after treatment in this study and reached remarkably high levels in one dog in the HD group. There were also strong positive correlations identified between TNFα and IL-6, GM-CSF, IL-2, and IL-7 in our study. As mentioned earlier, a high IL-10:TNFα ratio is associated with increased risk of mortality in human septic patients as these patients are in a state of profound immunosuppression (Chaudhry et al., 2013). Interestingly our study demonstrated a high IL-10 to TNFα ratio during the course of infection, and this may indicate the predominance of an immunosuppressive cytokine milieu during *B. rossi* infection thereby inhibiting the hosts ability to effectively control parasite replication. The pronounced production of TNFα following treatment suggests a pro-inflammatory retaliation by the host, indicative of an inappropriate dysregulated immune response. It seems likely that the dogs in this study may be in a state of immunosuppression with concurrent hyper-inflammation, which has been described in human sepsis (Yadav and Cartin-Ceba, 2016, Lyn-Kew and Standiford, 2008, Kox et al., 2000).

Both IL-2 and IL-7 act on lymphocytes (Gaffen and Liu, 2004, Hofmeister et al., 1999). These two cytokines had a strong positive correlation with each other in this study. IL-2 influences the function of a few cell phenotypes, notably enhancing proliferation and survival of T lymphocytes (Malek, 2003, Gaffen and Liu, 2004). It also plays a critical role in the development and expansion of T regulatory cells which are vital to the prevention of autoimmunity (Malek, 2003, Gaffen and Liu, 2004). Additional effects of IL-2 are the expansion and activation of NK cells as well as promotion of the their production of cytokines such as TNFα and GM-CSF (Gaffen and Liu, 2004). Antibody secretion by B-lymphocytes is also augmented by IL-2 (Gaffen and Liu, 2004). IL-2 did not increase when *B. rossi* infected dogs were compared to healthy control dogs but there appeared to be higher concentrations in infected dogs presented within 48 hours of clinical illness (Goddard et al., 2016, Leisewitz et al., 2019a). Although IL-7 also exerts its effects on lymphocytes, this appears to be at a different level than IL-2. It acts as a tropic factor for and supports VDJ recombination in immature B and T lymphocytes (Hofmeister et al.,

1999). During infections IL-7 is vital in the proliferation and maintenance of antigen-specific T lymphocytes and enhances memory T lymphocyte differentiation (Fry and Mackall, 2002). Increases in IL-7 were not noted in previous studies on *B. rossi* (Leisewitz et al., 2019a). Both cytokines only displayed significant increases after treatment. Treatment and subsequent lysis of the parasites may have released soluble antigens, triggering the adaptive immune response and stimulating the proliferation and activation of lymphocytes under the influence of these cytokines. These cytokines likely play a role in the clearance of remaining parasites, limiting autoimmunity in this inflammatory condition and encouraging the development of immunity to the *Babesia* parasites.

d) Cytokines that showed no distinct pattern of change

In this category there are three cytokines that did not show a distinct pattern or only demonstrated changes in one dog. A strong positive correlation was identified between IL-15 and IL-18 in this study. Both these cytokines only increased significantly in one dog in the LD group after treatment. The functions of IL-15 are extensive, including activation and persistence of cells involved in the innate and adaptive immune responses such as NK cells as well as development of cytotoxic T lymphocytes and survival of memory T helper lymphocytes (Perera et al., 2012, Akdis et al., 2011). Higher levels of IL-15 were identified in uninfected women in a falciparum malaria endemic region and it may have a role in enhancing early protective innate immune responses (Bouyou-Akotet et al., 2004). In this study concentrations of IL-15 were persistently higher in the LD group. It is possible that a lower inoculum dose allows more time for the host to initiate a stronger protective innate immune response and dogs with higher IL-15 production during the acute phase may be better able to control initial parasite replication. As IL-15 also plays a role in the production of memory T helper cells, it may be important in the development of immunity to future re-infections (Perera et al., 2012). The increased concentrations seen after treatment is likely a consequence of the pro-inflammatory environment triggered by damage to the parasites and the widespread activation of inflammatory cells such as monocytes which produce IL-15 (Perera et al., 2012). IL-15 is also a key regulator to the innate and adaptive immune responses (Gracie et al., 2003). Together with IL-12, IL-15 is well known for its ability to induce the production of IFNγ but this is only one of many functions identified (Gracie et al., 2003). It enhances T and NK cell cytotoxicity, inflammatory cytokine release (IFNγ and IL-6) from monocyte/macrophage lineage cells and even amplifies neutrophil activation (Gracie et al., 2003). There have been mixed findings on its levels and correlation to disease severity in malaria, with one study suggesting an immunoregulatory role while another finding an association with severe disease (Kojima et al., 2004, Malaguarnera et al., 2002). Its role in canine babesiosis warrants further

investigation. IL-18 concentrations correlated positively with severity of falciparum malaria in at least one study suggesting it may promote exaggerated inflammation and worsen disease severity if excessive concentrations are present (Kojima et al., 2004). A trend in the increase of IL-18 concentrations early on in disease course of *B. rossi* infection was identified in one study (Goddard et al., 2016). Again, concentrations of IL-18 were consistently higher in the LD group and increased significantly after treatment. Suggesting this cytokine may play a role in the exacerbating the inflammatory response after treatment in patients treated early in the disease course.

The last cytokine in this group was IP-10, its production is induced by IFNγ and it is a chemokine involved in the attraction of T lymphocytes, monocytes, and NK cells to sites of inflammation and augmenting their transmigration through the endothelium (Taub et al., 1993, Frangogiannis et al., 2000). IP-10 was identified as a possible biomarker for cerebral malaria and its mortality rate in falciparum malaria (Jain et al., 2008). In the current study IP-10 concentrations were mildly but significantly increased in the HD group from 24 hours onwards, decreasing to undetectable limits after treatment. The LD group on the other hand had a delayed increase in IP-10 concentrations, about 72 hours later then HD group, reaching peak levels just before treatment but unlike the HD group concentrations remained high after treatment. During infection IP-10 may have a role in co-ordinating leukocyte transmigration and enhance the vascular injury seen during *B. rossi* infections (Taub et al., 1993, Jacobson and Clark, 1994).

Study limitations

The main limitation of this study was the small sample size. This was an experimental pilot study and the number of dogs utilised was only 6. Every attempt was made to exclude any confounding or influencing factors. All dogs were the same age, sex, and breed. The dogs were neutered, eliminating the influence of sex hormones. All vaccination and deworming protocols were identical for the dogs. They were housed in an isolated environment and exposed to similar indoor and outdoor facilities. Diet, training, experimental procedure, sample collection and human interaction was consistent for all dogs. Due to the small sample size, it is possible that significant differences between the groups may have been missed (type 2 error). Another consequence of the low number of animals in this study was the exclusion of a control group.

As an intravenous injection was used to infect the dogs in this study, the effect of the tick vector and local immunity on the prepatent period could not be investigated. The doses utilised in our study were

also likely far higher than those seen in natural infections and would have influenced the progression of the disease.

Unfortunately, due to ethical considerations the infection in the LD group was not permitted to progress to the same point as the HD group preventing further comparisons.

We utilised two different techniques to measure lactate and the handheld Lactate Pro 2 results correlated poorly to those measured by venous blood gas analysis. Unfortunately, the Lactate Pro 2 has not been validated in dogs. This shortcoming was offset by the use of the venous blood gas analysis but we failed to obtain baseline venous blood gas samples from the dogs in this study which prevented comparison to baseline data and instead we utilised the 24-hour sample as a baseline. Fortunately, this did not have a large impact on the findings of this study as we were still able to accurately identify clinically significant increases in lactate above the reference interval.

Conclusion

Our study has found that not only was onset influenced by the infectious dose, but the dynamics of the inflammatory response may also be affected in *B. rossi* infections. In the *B. canis* experimental study, they found that infectious dose affected the onset of the systemic response but not the dynamics of this response (Schetters et al., 2009a). The changes seen in most of the variables when comparing the LD and HD groups in our study appeared similar, with differences being noted in the timing of onset rather than degree of change. If the infection in the LD group had progressed beyond the predetermined endpoint, it is likely these variables would have reached similar degrees of change to those seen in the HD group. There were however several exceptions, where the kinetic patterns differed during the course of the infection such as IL-8 which had an initial peak 24 hours after infection in the LD group and concentrations of IL-8 in this group even exceeded those seen in HD group after treatment. In addition to IL-8 there was also a trend toward higher concentrations of IL-15 and IL-18 in the LD group throughout the study. Concentrations in the LD group exceeded those of the HD group after treatment in an additonal three variables namely CRP, KC-like and IP-10. These findings suggest that not only will infectious dose influence the onset of inflammation, but it may influence the kinetics and nature of the inflammatory response to *B. rossi* infection. Moreover, the level of parasitaemia at the time of treatment played a critical role in the systemic inflammatory response seen thereafter and may have a major influence in the development of complications after treatment.

Our study highlighted the influence of chemotherapeutic damage to the parasites on the progression of the resultant inflammatory response. As opposed to initiating the resolution of the inflammatory

response, treatment may have augmented the inflammation by perpetuating the production of a number of pro-inflammatory cytokines and proliferation of inflammatory cells. The pronounced inflammatory response after treatment appears redundant and may contribute to widespread host tissue damage.

We also identified one possible mechanism by which *B. rossi* parasites may replicate uncontrollably in the canine host, a feature which may add to the virulence of this species. An imbalance in the anti- and pro-inflammatory cytokine milieu during infection, particularly high concentrations of IL-10 and low concentrations of key pro-inflammatory cytokines such as TNFα and IFNγ, may promote a state of immunosuppression preventing the host from initiating an effective immune response to the invading organisms.

It is clear from the findings of our study that *B. rossi* infection and treatment triggers a classical 'cytokine storm' in which the host's response promotes inflammation and tissue damage beyond that induced by the parasite itself (Clark et al., 2008a, Clark, 2007). Of note was the progression of this 'cytokine storm' and associated inflammation after treatment, despite reduction in the parasite biomass. In addition to the 'cytokine storm' there was an indication of an imbalance between pro and anti-inflammatory cytokines during the later stages of active infection, particularly a high IL-10 to TNFα ratio and low IFNγ concentrations (Chaudhry et al., 2013). These alterations may lead to a state of immunosuppression and reduced host ability to quell parasite replication. The biphasic theory of hyperinflammation and immunosuppression is characterised by an initial pro-inflammatory phase upon the initiation of inflammation by various triggers followed by a period of immune exhaustion and suppression (Yadav and Cartin-Ceba, 2016). Increasing evidence has shown that the models of pro and anti-inflammatory responses are not necessarily sequential and that a large amount of overlap and integration of these responses takes place during systemic inflammation. The balance between these opposing responses influences the outcome of conditions such as sepsis, malaria and severe trauma (Yadav and Cartin-Ceba, 2016, Lyn-Kew and Standiford, 2008). It is possible that during *B. rossi* infection a state of hyperinflammation is intermingled with immunosuppression and eventual immune exhaustion leading to a failure to control parasite replication and disease progression.

References

A. CLARK L. S. JACOBSON, I. 1998. Do babesiosis and malaria share a common disease process. *Annals of Tropical Medicine And Parasitology,* 92**,** 483-488.

ADEMBRI, C., SGAMBATI, E., VITALI, L., SELMI, V., MARGHERI, M., TANI, A., BONACCINI, L., NOSI, D., CALDINI, A. L., FORMIGLI, L. & DE GAUDIO, A. R. 2011. Sepsis induces albuminuria and alterations in the glomerular filtration barrier: a morphofunctional study in the rat. *Crit Care,* 15**,** R277.

AGUILAR-DELFIN, I., WETTSTEIN, P. J. & PERSING, D. H. 2003. Resistance to acute babesiosis is associated with interleukin-12- and gamma interferon-mediated responses and requires macrophages and natural killer cells. *Infect Immun,* 71**,** 2002-8.

AHMED, J. S. 2002. The role of cytokines in immunity and immunopathogenesis of pirolasmoses. *Parasitol Res,* 88**,** S48-50.

AKDIS, M., BURGLER, S., CRAMERI, R., EIWEGGER, T., FUJITA, H., GOMEZ, E., KLUNKER, S., MEYER, N., O'MAHONY, L., PALOMARES, O., RHYNER, C., OUAKED, N., SCHAFFARTZIK, A., VAN DE VEEN, W., ZELLER, S., ZIMMERMANN, M. & AKDIS, C. A. 2011. Interleukins, from 1 to 37, and interferon-gamma: receptors, functions, and roles in diseases. *J Allergy Clin Immunol,* 127**,** 701-21 e1-70.

AMICHAY, D., GAZZINELLI, R. T., KARUPIAH, G., MOENCH, T. R., SHER, A. & FARBER, J. M. 1996. Genes for chemokines MuMig and Crg-2 are induced in protozoan and viral infections in response to IFN-gamma with patterns of tissue expression that suggest nonredundant roles in vivo. *J Immunol,* 157**,** 4511-20.

ANGIOLILLO, A. L., SGADARI, C., TAUB, D. D., LIAO, F., FARBER, J. M., MAHESHWARI, S., KLEINMAN, H. K., REAMAN, G. H. & TOSATO, G. 1995. Human interferon-inducible protein 10 is a potent inhibitor of angiogenesis in vivo. *J Exp Med,* 182**,** 155-62.

APANASKEVICH, D. A., HORAK, I. G. & CAMICAS, J. L. 2007. Redescription of Haemaphysalis (Rhipistoma) elliptica (Koch, 1844), an old taxon of the *Haemaphysalis (Rhipistoma) leachi* group from East and southern Africa, and of *Haemaphysalis (Rhipistoma) leachi* (Audouin, 1826) (Ixodida, Ixodidae). *Onderstepoort J Vet Res,* 74**,** 181-208.

APPELBERG, R. 2007. Neutrophils and intracellular pathogens: beyond phagocytosis and killing. *Trends Microbiol,* 15**,** 87-92.

ARAMBURO, A., TODD, J., GEORGE, E. C., KIGULI, S., OLUPOT-OLUPOT, P., OPOKA, R. O., ENGORU, C., AKECH, S. O., NYEKO, R., MTOVE, G., GIBB, D. M., BABIKER, A. G. & MAITLAND, K. 2018. Lactate clearance as a prognostic marker of mortality in severely ill febrile children in East Africa. *BMC Med,* 16**,** 37.

BALLOU, S. P. & LOZANSKI, G. 1992. Induction of inflammatory cytokine release from cultured human monocytes by C-reactive protein. *Cytokine,* 4**,** 361-8.

BANETH, G. 2018. Antiprotozoal treatment of canine babesiosis. *Vet Parasitol,* 254**,** 58-63.

BANETH, G., CARDOSO, L., BRILHANTE-SIMOES, P. & SCHNITTGER, L. 2019. Establishment of *Babesia vulpes* n. sp. (Apicomplexa: Babesiidae), a piroplasmid species pathogenic for domestic dogs. *Parasit Vectors,* 12**,** 129.

BANETH, G., NACHUM-BIALA, Y., BIRKENHEUER, A. J., SCHREEG, M. E., PRINCE, H., FLORIN-CHRISTENSEN, M., SCHNITTGER, L. & AROCH, I. 2020. A new piroplasmid species infecting dogs: morphological and molecular characterization and pathogeny of *Babesia negevi* n. sp. *Parasit Vectors,* 13**,** 130.

BASSON, J. 1965. Canine babesiosis: a report on the pathology of three cases with special reference to the'cerebral'form. *Journal of the South African Veterinary Association,* 36**,** 333-341.

BELPAIRE, F. M., DE RICK, A., DELLO, C., FRAEYMAN, N. & BOGAERT, M. G. 1987. Alpha 1-acid glycoprotein and serum binding of drugs in healthy and diseased dogs. *J Vet Pharmacol Ther,* 10**,** 43-8.

BENACH, J. L., WHITE, D. J. & MCGOVERN, J. P. 1978. Babesiosis in Long Island. Host-parasite relationships of rodent- and human-derived *Babesia microti* isolates in hamsters. *Am J Trop Med Hyg,* 27**,** 1073-8.
BENDEL, S., KARLSSON, S., PETTILA, V., LOISA, P., VARPULA, M., RUOKONEN, E. & FINNSEPSIS STUDY, G. 2008. Free cortisol in sepsis and septic shock. *Anesth Analg,* 106**,** 1813-9.
BERG, A., PATEL, S., GONCA, M., DAVID, C., OTTERDAL, K., UELAND, T., DALEN, I., KVALOY, J. T., MOLLNES, T. E., AUKRUST, P. & LANGELAND, N. 2014. Cytokine network in adults with falciparum Malaria and HIV-1: increased IL-8 and IP-10 levels are associated with disease severity. *PLoS One,* 9**,** e114480.
BLACK, S., KUSHNER, I. & SAMOLS, D. 2004. C-reactive Protein. *J Biol Chem,* 279**,** 48487-90.
BLACKWELL, T. S. & CHRISTMAN, J. W. 1996. Sepsis and cytokines: current status. *Br J Anaesth,* 77**,** 110-7.
BOHM, M., LEISEWITZ, A. L., THOMPSON, P. N. & SCHOEMAN, J. P. 2006. Capillary and venous *Babesia canis rossi* parasitaemias and their association with outcome of infection and circulatory compromise. *Vet Parasitol,* 141**,** 18-29.
BONE, R. C., BALK, R. A., CERRA, F. B., DELLINGER, R. P., FEIN, A. M., KNAUS, W. A., SCHEIN, R. M. H. & SIBBALD, W. J. 1992. Definitions for Sepsis and Organ Failure and Guidelines for the Use of Innovative Therapies in Sepsis. *Chest,* 101**,** 1644-1655.
BOOZER, A. L. & MACINTIRE, D. K. 2003. Canine babesiosis. *Vet Clin North Am Small Anim Pract,* 33**,** 885-904, viii.
BOTHA, H. 1964. The cerebral form of babesiosis in dogs. *Journal of the South African Veterinary Association,* 35**,** 27-28.
BOUYOU-AKOTET, M. K., KOMBILA, M., KREMSNER, P. G. & MAVOUNGOU, E. 2004. Cytokine profiles in peripheral, placental and cord blood in pregnant women from an area endemic for *Plasmodium falciparum*. *Eur Cytokine Netw,* 15**,** 120-5.
BOZIC, C. R., KOLAKOWSKI, L. F., JR., GERARD, N. P., GARCIA-RODRIGUEZ, C., VON UEXKULL-GULDENBAND, C., CONKLYN, M. J., BRESLOW, R., SHOWELL, H. J. & GERARD, C. 1995. Expression and biologic characterization of the murine chemokine KC. *J Immunol,* 154**,** 6048-57.
BRADY, C. A. & OTTO, C. M. 2001. Systemic inflammatory response syndrome, sepsis, and multiple organ dysfunction. *Vet Clin North Am Small Anim Pract,* 31**,** 1147-62, v-vi.
BRANDAO, L. P., HAGIWARA, M. K. & MYIASHIRO, S. I. 2003. Humoral immunity and reinfection resistance in dogs experimentally inoculated with *Babesia canis* and either treated or untreated with imidocarb dipropionate. *Vet Parasitol,* 114**,** 253-65.
BROWN, A. L., SHIEL, R. E. & IRWIN, P. J. 2015. Clinical, haematological, cytokine and acute phase protein changes during experimental *Babesia gibsoni* infection of beagle puppies. *Experimental Parasitology,* 157**,** 185-196.
BROWN, S., ATKINS, C., BAGLEY, R., CARR, A., COWGILL, L., DAVIDSON, M., EGNER, B., ELLIOTT, J., HENIK, R., LABATO, M., LITTMAN, M., POLZIN, D., ROSS, L., SNYDER, P., STEPIEN, R. & AMERICAN COLLEGE OF VETERINARY INTERNAL, M. 2007. Guidelines for the identification, evaluation, and management of systemic hypertension in dogs and cats. *J Vet Intern Med,* 21**,** 542-58.
CARRET, C., WALAS, F., CARCY, B., GRANDE, N., PRECIGOUT, E., MOUBRI, K., SCHETTERS, T. P. & GORENFLOT, A. 1999. *Babesia canis canis, Babesia canis vogeli, Babesia canis rossi*: differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. *J Eukaryot Microbiol,* 46**,** 298-303.
CASPI, D., BALTZ, M. L., SNEL, F., GRUYS, E., NIV, D., BATT, R. M., MUNN, E. A., BUTTRESS, N. & PEPYS, M. B. 1984. Isolation and characterization of C-reactive protein from the dog. *Immunology,* 53**,** 307-13.
CELLIERS, A., RAUTENBACH, Y., HOOIJBERG, E., CHRISTOPHER, M. & GODDARD, A. 2020. Neutrophil Myeloperoxidase Index in Dogs With Babesiosis Caused by *Babesia rossi*. *Front Vet Sci,* 7**,** 72.

CERON, J. J., ECKERSALL, P. D. & MARTYNEZ-SUBIELA, S. 2005. Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Vet Clin Pathol,* 34**,** 85-99.
CHANG, K. H. & STEVENSON, M. M. 2004. Malarial anaemia: mechanisms and implications of insufficient erythropoiesis during blood-stage malaria. *Int J Parasitol,* 34**,** 1501-16.
CHAUDHRY, H., ZHOU, J., ZHONG, Y., ALI, M. M., MCGUIRE, F., NAGARKATTI, P. S. & NAGARKATTI, M. 2013. Role of cytokines as a double-edged sword in sepsis. *In Vivo,* 27**,** 669-84.
CLARK, I. A. 1979. Resistance to *Babesia spp*. and *Plasmodium sp*. in mice pretreated with an extract of Coxiella burnetii. *Infect Immun,* 24**,** 319-25.
CLARK, I. A. 2000. More on Babesia, NO and malaria. *Parasitol Today,* 16**,** 264-5.
CLARK, I. A. 2007. The advent of the cytokine storm. *Immunol Cell Biol,* 85**,** 271-3.
CLARK, I. A., AL YAMAN, F. M. & JACOBSON, L. S. 1997. The biological basis of malarial disease. *Int J Parasitol,* 27**,** 1237-49.
CLARK, I. A., ALLEVA, L. M., BUDD, A. C. & COWDEN, W. B. 2008a. Understanding the role of inflammatory cytokines in malaria and related diseases. *Travel Med Infect Dis,* 6**,** 67-81.
CLARK, I. A., BUDD, A. C. & ALLEVA, L. M. 2008b. Sickness behaviour pushed too far--the basis of the syndrome seen in severe protozoal, bacterial and viral diseases and post-trauma. *Malar J,* 7**,** 208.
CLARK, I. A., BUDD, A. C., ALLEVA, L. M. & COWDEN, W. B. 2006. Human malarial disease: a consequence of inflammatory cytokine release. *Malar J,* 5**,** 85.
CLARK, I. A., RICHMOND, J. E., WILLS, E. J. & ALLISON, A. C. 1975. Immunity to intra-erythrocytic protoza. *Lancet,* 2**,** 1128-9.
COHEN, T. A., NELSON, R. W., KASS, P. H., CHRISTOPHER, M. M. & FELDMAN, E. C. 2009. Evaluation of six portable blood glucose meters for measuring blood glucose concentration in dogs. *J Am Vet Med Assoc,* 235**,** 276-80.
COLLETT, M. G. 2000. Survey of canine babesiosis in South Africa. *J S Afr Vet Assoc,* 71**,** 180-6.
CONROY, A. L., PHIRI, H., HAWKES, M., GLOVER, S., MALLEWA, M., SEYDEL, K. B., TAYLOR, T. E., MOLYNEUX, M. E. & KAIN, K. C. 2010. Endothelium-based biomarkers are associated with cerebral malaria in Malawian children: a retrospective case-control study. *PLoS One,* 5**,** e15291.
COUPER, K. N., BLOUNT, D. G. & RILEY, E. M. 2008. IL-10: the master regulator of immunity to infection. *J Immunol,* 180**,** 5771-7.
CRAY, C., ZAIAS, J. & ALTMAN, N. H. 2009. Acute phase response in animals: a review. *Comp Med,* 59**,** 517-26.
D'ANDREA, A., ASTE-AMEZAGA, M., VALIANTE, N. M., MA, X., KUBIN, M. & TRINCHIERI, G. 1993. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med,* 178**,** 1041-8.
DALE, D. C. & WOLFF, S. M. 1973. Studies of the neutropenia of acute malaria. *Blood,* 41**,** 197-206.
DAMMIN, G. J., SPIELMAN, A., BENACH, J. L. & PIESMAN, J. 1981. The rising incidence of clinical *Babesia microti* infection. *Hum Pathol,* 12**,** 398-400.
DAY, N. P., HIEN, T. T., SCHOLLAARDT, T., LOC, P. P., CHUONG, L. V., CHAU, T. T., MAI, N. T., PHU, N. H., SINH, D. X., WHITE, N. J. & HO, M. 1999. The prognostic and pathophysiologic role of pro- and antiinflammatory cytokines in severe malaria. *J Infect Dis,* 180**,** 1288-97.
DEFAUW, P., SCHOEMAN, J. P., SMETS, P., GODDARD, A., MEYER, E., LIEBENBERG, C. & DAMINET, S. 2012. Assessment of renal dysfunction using urinary markers in canine babesiosis caused by *Babesia rossi*. *Vet Parasitol,* 190**,** 326-32.
DESHMANE, S. L., KREMLEV, S., AMINI, S. & SAWAYA, B. E. 2009. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res,* 29**,** 313-26.
DVIR, E., LOBETTI, R. G., JACOBSON, L. S., PEARSON, J. & BECKER, P. J. 2004. Electrocardiographic changes and cardiac pathology in canine babesiosis. *J Vet Cardiol,* 6**,** 15-23.

EBERSOLE, J. L. & CAPPELLI, D. 2000. Acute-phase reactants in infections and inflammatory diseases. *Periodontol 2000,* 23**,** 19-49.
ETTINGER, S. J., FELDMAN, E. C. & CÔTÉ, E. 2017. *Textbook of veterinary internal medicine : diseases of the dog and the cat,* St. Louis, Missouri, Elsevier.
FARBER, J. M. 1997. Mig and IP-10: CXC chemokines that target lymphocytes. *J Leukoc Biol,* 61**,** 246-57.
FERNANDES, A. A., CARVALHO, L. J., ZANINI, G. M., VENTURA, A. M., SOUZA, J. M., COTIAS, P. M., SILVA-FILHO, I. L. & DANIEL-RIBEIRO, C. T. 2008. Similar cytokine responses and degrees of anemia in patients with *Plasmodium falciparum* and *Plasmodium vivax* infections in the Brazilian Amazon region. *Clin Vaccine Immunol,* 15**,** 650-8.
FIORENTINO, D. F., ZLOTNIK, A., MOSMANN, T. R., HOWARD, M. & O'GARRA, A. 1991. IL-10 inhibits cytokine production by activated macrophages. *J Immunol,* 147**,** 3815-22.
FRANGOGIANNIS, N. G., MENDOZA, L. H., SMITH, C. W., MICHAEL, L. H. & ENTMAN, M. L. 2000. Induction of the synthesis of the C-X-C chemokine interferon-gamma-inducible protein-10 in experimental canine endotoxemia. *Cell Tissue Res,* 302**,** 365-76.
FRY, T. J. & MACKALL, C. L. 2002. Interleukin-7: from bench to clinic. *Blood,* 99**,** 3892-904.
GAFFEN, S. L. & LIU, K. D. 2004. Overview of interleukin-2 function, production and clinical applications. *Cytokine,* 28**,** 109-23.
GALÁN, A., MAYER, I., RAFAJ, R. B., BENDELJA, K. O., SUŠIĆ, V., CERÓN, J. J. N., MRLJAK, V. & DUMLER, J. S. 2018. MCP-1, KC-like and IL-8 as critical mediators of pathogenesis caused by Babesia canis. *PLOS ONE,* 13**,** e0190474.
GEBHARDT, C., HIRSCHBERGER, J., RAU, S., ARNDT, G., KRAINER, K., SCHWEIGERT, F. J., BRUNNBERG, L., KASPERS, B. & KOHN, B. 2009. Use of C-reactive protein to predict outcome in dogs with systemic inflammatory response syndrome or sepsis. *J Vet Emerg Crit Care (San Antonio),* 19**,** 450-8.
GIMBRONE, M. A., JR., OBIN, M. S., BROCK, A. F., LUIS, E. A., HASS, P. E., HEBERT, C. A., YIP, Y. K., LEUNG, D. W., LOWE, D. G., KOHR, W. J. & ET AL. 1989. Endothelial interleukin-8: a novel inhibitor of leukocyte-endothelial interactions. *Science,* 246**,** 1601-3.
GIRBL, T., LENN, T., PEREZ, L., ROLAS, L., BARKAWAY, A., THIRIOT, A., DEL FRESNO, C., LYNAM, E., HUB, E., THELEN, M., GRAHAM, G., ALON, R., SANCHO, D., VON ANDRIAN, U. H., VOISIN, M. B., ROT, A. & NOURSHARGH, S. 2018. Distinct Compartmentalization of the Chemokines CXCL1 and CXCL2 and the Atypical Receptor ACKR1 Determine Discrete Stages of Neutrophil Diapedesis. *Immunity,* 49**,** 1062-1076 e6.
GLADWIN, M. T., CRAWFORD, J. H. & PATEL, R. P. 2004. The biochemistry of nitric oxide, nitrite, and hemoglobin: role in blood flow regulation. *Free Radic Biol Med,* 36**,** 707-17.
GODDARD, A., LEISEWITZ, A. L., KJELGAARD-HANSEN, M., KRISTENSEN, A. T. & SCHOEMAN, J. P. 2016. Excessive Pro-Inflammatory Serum Cytokine Concentrations in Virulent Canine Babesiosis. *PLoS One,* 11**,** e0150113.
GODDARD, A., WIINBERG, B., SCHOEMAN, J. P., KRISTENSEN, A. T. & KJELGAARD-HANSEN, M. 2013. Mortality in virulent canine babesiosis is associated with a consumptive coagulopathy. *Vet J,* 196**,** 213-7.
GOGOS, C. A., DROSOU, E., BASSARIS, H. P. & SKOUTELIS, A. 2000. Pro- versus anti-inflammatory cytokine profile in patients with severe sepsis: a marker for prognosis and future therapeutic options. *J Infect Dis,* 181**,** 176-80.
GRACIE, J. A., ROBERTSON, S. E. & MCINNES, I. B. 2003. Interleukin-18. *J Leukoc Biol,* 73**,** 213-24.
GRAY, G. D. & PHILLIPS, R. S. 1983. Influence of infective dose, degree of host anaemia and multiparasitism of erythrocytes on *Babesia microti* and *Babesia hylomysci* parasitaemias in mice. *Int J Parasitol,* 13**,** 399-404.
GRAY, J., ZINTL, A., HILDEBRANDT, A., HUNFELD, K. P. & WEISS, L. 2010. Zoonotic babesiosis: overview of the disease and novel aspects of pathogen identity. *Ticks Tick Borne Dis,* 1**,** 3-10.

HALDAR, K. & MOHANDAS, N. 2009. Malaria, erythrocytic infection, and anemia. *Hematology Am Soc Hematol Educ Program*, 87-93.
HAMILTON, J. A. 1993. Colony stimulating factors, cytokines and monocyte-macrophages--some controversies. *Immunol Today,* 14, 18-24.
HAMILTON, J. A. 2002. GM-CSF in inflammation and autoimmunity. *Trends Immunol,* 23, 403-8.
HARADA, A., SEKIDO, N., AKAHOSHI, T., WADA, T., MUKAIDA, N. & MATSUSHIMA, K. 1994. Essential involvement of interleukin-8 (IL-8) in acute inflammation. *J Leukoc Biol,* 56, 559-64.
HAUPTMAN, J. G., WALSHAW, R. & OLIVIER, N. B. 1997. Evaluation of the sensitivity and specificity of diagnostic criteria for sepsis in dogs. *Vet Surg,* 26, 393-7.
HENNING, A., CLIFT, S. J. & LEISEWITZ, A. L. 2020. The pathology of the spleen in lethal canine babesiosis caused by *Babesia rossi*. *Parasite Immunol,* 42, e12706.
HOFFMANN, E., DITTRICH-BREIHOLZ, O., HOLTMANN, H. & KRACHT, M. 2002. Multiple control of interleukin-8 gene expression. *J Leukoc Biol,* 72, 847-55.
HOFMEISTER, R., KHALED, A. R., BENBERNOU, N., RAJNAVOLGYI, E., MUEGGE, K. & DURUM, S. K. 1999. Interleukin-7: physiological roles and mechanisms of action. *Cytokine Growth Factor Rev,* 10, 41-60.
HOMER, M. J., AGUILAR-DELFIN, I., TELFORD, S. R., 3RD, KRAUSE, P. J. & PERSING, D. H. 2000. Babesiosis. *Clin Microbiol Rev,* 13, 451-69.
IDRISS, H. T. & NAISMITH, J. H. 2000. TNF alpha and the TNF receptor superfamily: structure-function relationship(s). *Microsc Res Tech,* 50, 184-95.
IGARASHI, I., SUZUKI, R., WAKI, S., TAGAWA, Y., SENG, S., TUM, S., OMATA, Y., SAITO, A., NAGASAWA, H., IWAKURA, Y., SUZUKI, N., MIKAMI, T. & TOYODA, Y. 1999. Roles of CD4(+) T cells and gamma interferon in protective immunity against *Babesia microti* infection in mice. *Infect Immun,* 67, 4143-8.
INCE, C., MAYEUX, P. R., NGUYEN, T., GOMEZ, H., KELLUM, J. A., OSPINA-TASCON, G. A., HERNANDEZ, G., MURRAY, P., DE BACKER, D. & WORKGROUP, A. X. 2016. The Endothelium in Sepsis. *Shock,* 45, 259-70.
IRWIN, P. J. & HUTCHINSON, G. W. 1991. Clinical and pathological findings of Babesia infection in dogs. *Aust Vet J,* 68, 204-9.
JACOBSON, L. S. 2006. The South African form of severe and complicated canine babesiosis: clinical advances 1994-2004. *Vet Parasitol,* 138, 126-39.
JACOBSON, L. S. & CLARK, I. A. 1994. The pathophysiology of canine babesiosis: new approaches to an old puzzle. *J S Afr Vet Assoc,* 65, 134-45.
JACOBSON, L. S. & LOBETTI, R. G. 1996. Rhabdomyolysis as a complication of canine babesiosis. *The Journal of small animal practice,* 37, 286-91.
JACOBSON, L. S., LOBETTI, R. G., BECKER, P., REYERS, F. & VAUGHAN-SCOTT, T. 2002. Nitric oxide metabolites in naturally occurring canine babesiosis. *Vet Parasitol,* 104, 27-41.
JACOBSON, L. S., LOBETTI, R. G. & VAUGHAN-SCOTT, T. 2000. Blood pressure changes in dogs with babesiosis. *J S Afr Vet Assoc,* 71, 14-20.
JACOBSON, L. S., REYERS, F., BERRY, W. L. & VILJOEN, E. 1996. Changes in haematocrit after treatment of uncomplicated canine babesiosis: a comparison between diminazene and trypan blue, and an evaluation of the influence of parasitaemia. *J S Afr Vet Assoc,* 67, 77-82.
JAFFER, U., WADE, R. G. & GOURLAY, T. 2010. Cytokines in the systemic inflammatory response syndrome: a review. *HSR Proc Intensive Care Cardiovasc Anesth,* 2, 161-75.
JAIN, V., ARMAH, H. B., TONGREN, J. E., NED, R. M., WILSON, N. O., CRAWFORD, S., JOEL, P. K., SINGH, M. P., NAGPAL, A. C., DASH, A. P., UDHAYAKUMAR, V., SINGH, N. & STILES, J. K. 2008. Plasma IP-10, apoptotic and angiogenic factors associated with fatal cerebral malaria in India. *Malar J,* 7, 83.
JALOVECKA, M., SOJKA, D., ASCENCIO, M. & SCHNITTGER, L. 2019. Babesia Life Cycle - When Phylogeny Meets Biology. *Trends Parasitol,* 35, 356-368.

JIN, L., BATRA, S., DOUDA, D. N., PALANIYAR, N. & JEYASEELAN, S. 2014. CXCL1 contributes to host defense in polymicrobial sepsis via modulating T cell and neutrophil functions. *J Immunol,* 193**,** 3549-58.
JOHNSON, W. C., CLUFF, C. W., GOFF, W. L. & WYATT, C. R. 1996. Reactive oxygen and nitrogen intermediates and products from polyamine degradation are Babesiacidal in vitro. *Ann N Y Acad Sci,* 791**,** 136-47.
KANEKO, J. J., HARVEY, J. W. & BRUSS, M. 2008. *Clinical biochemistry of domestic animals,* Amsterdam ;, Academic Press/Elsevier.
KANG, M. H., KIM, D. H., JEONG, I. S., CHOI, G. C. & PARK, H. M. 2016. Evaluation of four portable blood glucose meters in diabetic and non-diabetic dogs and cats. *Vet Q,* 36**,** 2-9.
KARGA, H., PAPAIOANNOU, P., VENETSANOU, K., PAPANDROULAKI, F., KARALOIZOS, L., PAPAIOANNOU, G. & PAPAPETROU, P. 2000. The role of cytokines and cortisol in the non-thyroidal illness syndrome following acute myocardial infarction. *Eur J Endocrinol,* 142**,** 236-42.
KARLSSON, I., HAGMAN, R., JOHANNISSON, A., WANG, L., KARLSTAM, E. & WERNERSSON, S. 2012. Cytokines as immunological markers for systemic inflammation in dogs with pyometra. *Reprod Domest Anim,* 47 Suppl 6**,** 337-41.
KELLER, N., JACOBSON, L. S., NEL, M., DE CLERQ, M., THOMPSON, P. N. & SCHOEMAN, J. P. 2004. Prevalence and risk factors of hypoglycemia in virulent canine babesiosis. *J Vet Intern Med,* 18**,** 265-70.
KJEMTRUP, A. M. & CONRAD, P. A. 2006. A review of the small canine piroplasms from California: Babesia conradae in the literature. *Vet Parasitol,* 138**,** 112-7.
KNOLS, B. G. & TAKKEN, W. 2007. Alarm bells ringing: more of the same, and new and novel diseases and pests. *Emerging pests and vector-borne diseases in Europe***,** 13-19.
KOJIMA, S., NAGAMINE, Y., HAYANO, M., LOOAREESUWAN, S. & NAKANISHI, K. 2004. A potential role of interleukin 18 in severe falciparum malaria. *Acta Trop,* 89**,** 279-84.
KOSTER, L. S., VAN SCHOOR, M., GODDARD, A., THOMPSON, P. N., MATJILA, P. T. & KJELGAARD-HANSEN, M. 2009. C-reactive protein in canine babesiosis caused by *Babesia rossi* and its association with outcome. *J S Afr Vet Assoc,* 80**,** 87-91.
KOX, W. J., VOLK, T., KOX, S. N. & VOLK, H. D. 2000. Immunomodulatory therapies in sepsis. *Intensive Care Med,* 26 Suppl 1**,** S124-8.
KRAUSE, P. J., DAILY, J., TELFORD, S. R., VANNIER, E., LANTOS, P. & SPIELMAN, A. 2007. Shared features in the pathobiology of babesiosis and malaria. *Trends Parasitol,* 23**,** 605-10.
KURIBAYASHI, T., SEITA, T., MOMOTANI, E., YAMAZAKI, S., HAGIMORI, K. & YAMAMOTO, S. 2015. Elimination Half-Lives of Acute Phase Proteins in Rats and Beagle Dogs During Acute Inflammation. *Inflammation,* 38**,** 1401-5.
KWIATKOWSKI, D., HILL, A. V., SAMBOU, I., TWUMASI, P., CASTRACANE, J., MANOGUE, K. R., CERAMI, A., BREWSTER, D. R. & GREENWOOD, B. M. 1990. TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *Lancet,* 336**,** 1201-4.
LA ROVERE, M. T., GNEMMI, M. & VACCARINI, C. 2001. Baroreflex sensitivity. *Ital Heart J Suppl,* 2**,** 472-7.
LEISEWITZ, A., GODDARD, A., DE GIER, J., VAN ENGELSHOVEN, J., CLIFT, S., THOMPSON, P. & SCHOEMAN, J. P. 2019a. Disease severity and blood cytokine concentrations in dogs with natural *Babesia rossi* infection. *Parasite Immunol,* 41**,** e12630.
LEISEWITZ, A. L., GODDARD, A., CLIFT, S., THOMPSON, P. N., DE GIER, J., VAN ENGELSHOVEN, J. & SCHOEMAN, J. P. 2019b. A clinical and pathological description of 320 cases of naturally acquired *Babesia rossi* infection in dogs. *Vet Parasitol,* 271**,** 22-30.
LEISEWITZ, A. L., JACOBSON, L. S., DE MORAIS, H. S. & REYERS, F. 2001. The mixed acid-base disturbances of severe canine babesiosis. *J Vet Intern Med,* 15**,** 445-52.

LEWIS, B. D., PENZHORN, B. L., LOPEZ-REBOLLAR, L. M. & DE WAAL, D. T. 1996. Isolation of a South African vector-specific strain of *Babesia canis*. *Vet Parasitol,* 63**,** 9-16.
LEWIS, B. D., PENZHORN, B. L. & LOPEZ REBOLLAR, L. M. 1995. Immune responses to South African *Babesia canis* and the development of a preliminary vaccine. *J S Afr Vet Assoc,* 66**,** 61-5.
LIVADITI, O., KOTANIDOU, A., PSARRA, A., DIMOPOULOU, I., SOTIROPOULOU, C., AUGUSTATOU, K., PAPASTERIADES, C., ARMAGANIDIS, A., ROUSSOS, C., ORFANOS, S. E. & DOUZINAS, E. E. 2006. Neutrophil CD64 expression and serum IL-8: sensitive early markers of severity and outcome in sepsis. *Cytokine,* 36**,** 283-90.
LOBETTI, R. G. 2005. Cardiac involvement in canine babesiosis. *J S Afr Vet Assoc,* 76**,** 4-8.
LOBETTI, R. G., REYERS, F. & NESBIT, J. W. 1996. The comparative role of haemoglobinaemia and hypoxia in the development of canine babesial nephropathy. *J S Afr Vet Assoc,* 67**,** 188-98.
LOUREMBAM, S. D., SAWIAN, C. E. & BARUAH, S. 2013. Dysregulation of cytokines expression in complicated falciparum malaria with increased TGF-beta and IFN-gamma and decreased IL-2 and IL-12. *Cytokine,* 64**,** 503-8.
LUSTER, A. D. & RAVETCH, J. V. 1987. Biochemical characterization of a gamma interferon-inducible cytokine (IP-10). *J Exp Med,* 166**,** 1084-97.
LYKE, K. E., BURGES, R., CISSOKO, Y., SANGARE, L., DAO, M., DIARRA, I., KONE, A., HARLEY, R., PLOWE, C. V., DOUMBO, O. K. & SZTEIN, M. B. 2004. Serum levels of the proinflammatory cytokines interleukin-1 beta (IL-1beta), IL-6, IL-8, IL-10, tumor necrosis factor alpha, and IL-12(p70) in Malian children with severe *Plasmodium falciparum* malaria and matched uncomplicated malaria or healthy controls. *Infect Immun,* 72**,** 5630-7.
LYN-KEW, K. & STANDIFORD, T. J. 2008. Immunosuppression in sepsis. *Curr Pharm Des,* 14**,** 1870-81.
MAEGRAITH, B., GILLES, H. M. & DEVAKUL, K. 1957. Pathological Processes in Babesia cam's Infections. *Zeitschrift fur Tropenmedizin und Parasitologie,* 8**,** 485-514.
MAITLAND, K. 2006. Severe malaria: lessons learned from the management of critical illness in children. *Trends Parasitol,* 22**,** 457-62.
MALAGUARNERA, L. & MUSUMECI, S. 2002. The immune response to *Plasmodium falciparum* malaria. *Lancet Infect Dis,* 2**,** 472-8.
MALAGUARNERA, L., PIGNATELLI, S., MUSUMECI, M., SIMPORE, J. & MUSUMECI, S. 2002. Plasma levels of interleukin-18 and interleukin-12 in *Plasmodium falciparum* malaria. *Parasite Immunol,* 24**,** 489-92.
MALEK, T. R. 2003. The main function of IL-2 is to promote the development of T regulatory cells. *J Leukoc Biol,* 74**,** 961-5.
MALHERBE, W. & PARKIN, B. 1951. Atypical symptomatology in *Babesia canis* infection. *Journal of the South African Veterinary Association,* 22**,** 25-36.
MANNA, L., REALE, S., VIOLA, E., VITALE, F., FOGLIA MANZILLO, V., PAVONE, L. M., CARACAPPA, S. & GRAVINO, A. E. 2006. Leishmania DNA load and cytokine expression levels in asymptomatic naturally infected dogs. *Vet Parasitol,* 142**,** 271-80.
MARTIN, C. A. 2020. *Pathology of complicated Babesia rossi-associated acute lung injury and respiratory distress syndrome in dogs.* University of Pretoria.
MATIJATKO, V., KIŠ, I., TORTI, M., BRKLJAČIĆ, M., RAFAJ, R. B., ŽVORC, Z. & MRLJAK, V. 2010. Systemic inflammatory response syndrome and multiple organ dysfunction syndrome in canine babesiosis. *Veterinarski arhiv,* 80**,** 611-26.
MATIJATKO, V., KUČER, N., RAFAJ, R. B., FORŠEK, J., KIŠ, I., POTOČNJAK, D., RAZDOROV, G. & MRLJAK, V. CRP concentration in dogs with uncomplicated and complicated babesiosis. 3rd European colloquium on acute phase proteins, 2002.
MATIJATKO, V., MRLJAK, V., KIS, I., KUCER, N., FORSEK, J., ZIVICNJAK, T., ROMIC, Z., SIMEC, Z. & CERON, J. J. 2007. Evidence of an acute phase response in dogs naturally infected with *Babesia canis*. *Vet Parasitol,* 144**,** 242-50.
MATJILA, P. T., CARCY, B., LEISEWITZ, A. L., SCHETTERS, T., JONGEJAN, F., GORENFLOT, A. & PENZHORN, B. L. 2009. Preliminary Evaluation of the BrEMA1 Gene as a Tool for Associating

Babesia rossi Genotypes and Clinical Manifestation of Canine Babesiosis. *Journal of clinical microbiology.,* 47**,** 3586.
MATJILA, P. T., LEISEWITZ, A. L., JONGEJAN, F. & PENZHORN, B. L. 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. *Vet Parasitol,* 155**,** 152-7.
MATJILA, P. T., PENZHORN, B. L., BEKKER, C. P., NIJHOF, A. M. & JONGEJAN, F. 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. *Vet Parasitol,* 122**,** 119-25.
MCCOWEN, K. C., MALHOTRA, A. & BISTRIAN, B. R. 2001. Stress-induced hyperglycemia. *Crit Care Clin,* 17**,** 107-24.
MEEUSEN, E., LLOYD, S. & SOULSBY, E. J. 1984. *Babesia microti* in mice. Adoptive transfer of immunity with serum and cells. *Aust J Exp Biol Med Sci,* 62 (Pt 5)**,** 551-66.
MENENDEZ, C., FLEMING, A. F. & ALONSO, P. L. 2000. Malaria-related anaemia. *Parasitol Today,* 16**,** 469-76.
MILLER, D. M., SWAN, G. E., LOBETTI, R. G. & JACOBSON, L. S. 2005. The pharmacokinetics of diminazene aceturate after intramuscular administration in healthy dogs. *J S Afr Vet Assoc,* 76**,** 146-50.
MIZOCK, B. A. & FALK, J. L. 1992. Lactic acidosis in critical illness. *Crit Care Med,* 20**,** 80-93.
MOHR, A. J., LOBETTI, R. G. & VAN DER LUGT, J. J. 2000. Acute pancreatitis: a newly recognised potential complication of canine babesiosis. *J S Afr Vet Assoc,* 71**,** 232-9.
MORROT, A. 2020. Immune Evasion Strategies in Protozoan-Host Interactions. *Frontiers in Immunology,* 11.
MORTERS, M. K., ARCHER, J., MA, D., MATTHEE, O., GODDARD, A., LEISEWITZ, A. L., MATJILA, P. T., WOOD, J. L. N. & SCHOEMAN, J. P. 2020. Long-term follow-up of owned, free-roaming dogs in South Africa naturally exposed to *Babesia rossi*. *Int J Parasitol,* 50**,** 103-110.
MURPHY, S. C. & BREMAN, J. G. 2001. Gaps in the childhood malaria burden in Africa: cerebral malaria, neurological sequelae, anemia, respiratory distress, hypoglycemia, and complications of pregnancy. *Am J Trop Med Hyg,* 64**,** 57-67.
MYLONAKIS, M. E., CERON, J. J., LEONTIDES, L., SIARKOU, V. I., MARTINEZ, S., TVARIJONAVICIUTE, A., KOUTINAS, A. F. & HARRUS, S. 2011. Serum acute phase proteins as clinical phase indicators and outcome predictors in naturally occurring canine monocytic ehrlichiosis. *J Vet Intern Med,* 25**,** 811-7.
NAKAMURA, M., TAKAHASHI, M., OHNO, K., KOSHINO, A., NAKASHIMA, K., SETOGUCHI, A., FUJINO, Y. & TSUJIMOTO, H. 2008. C-reactive protein concentration in dogs with various diseases. *J Vet Med Sci,* 70**,** 127-31.
NEL, M., LOBETTI, R. G., KELLER, N. & THOMPSON, P. N. 2004. Prognostic value of blood lactate, blood glucose, and hematocrit in canine babesiosis. *J Vet Intern Med,* 18**,** 471-6.
OKANO, S., YOSHIDA, M., FUKUSHIMA, U., HIGUCHI, S., TAKASE, K. & HAGIO, M. 2002. Usefulness of systemic inflammatory response syndrome criteria as an index for prognosis judgement. *Vet Rec,* 150**,** 245-6.
OPAL, S. M. & DEPALO, V. A. 2000. Anti-inflammatory cytokines. *Chest,* 117**,** 1162-1172.
OTRANTO, D., DANTAS-TORRES, F. & BREITSCHWERDT, E. B. 2009. Managing canine vector-borne diseases of zoonotic concern: part one. *Trends Parasitol,* 25**,** 157-63.
OUYANG, W., RUTZ, S., CRELLIN, N. K., VALDEZ, P. A. & HYMOWITZ, S. G. 2011. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu Rev Immunol,* 29**,** 71-109.
PALTRINIERI, S. 2008. The feline acute phase reaction. *Vet J,* 177**,** 26-35.
PANDEY, N. R., BIAN, Y. Y. & SHOU, S. T. 2014. Significance of blood pressure variability in patients with sepsis. *World J Emerg Med,* 5**,** 42-7.
PARDINI, A. D. 2013. *The pathology and pathogenesis of canine cerebral babesiosis.* University of Pretoria.

PARNELL, N. K., GUPTILL, L. & SOLANO-GALLEGO, L. 2008. Protozoal Infections. *In:* MORGAN, R. V. (ed.) *Handbook of Small Animal Practice.* Saint Louis: W.B. Saunders.
PENZHORN, B. L. 2011. Why is Southern African canine babesiosis so virulent? An evolutionary perspective. *parasites & vectors* [Online], 4.
PENZHORN, B. L., LEWIS, B. D., DE WAAL, D. T. & LOPEZ REBOLLAR, L. M. 1995. Sterilisation of *Babesia canis* infections by imidocarb alone or in combination with diminazene. *J S Afr Vet Assoc,* 66**,** 157-9.
PENZHORN, B. L., VORSTER, I., HARRISON-WHITE, R. F. & OOSTHUIZEN, M. C. 2017. Black-backed jackals (Canis mesomelas) are natural hosts of *Babesia rossi*, the virulent causative agent of canine babesiosis in sub-Saharan Africa. *Parasit Vectors,* 10**,** 124.
PERERA, P. Y., LICHY, J. H., WALDMANN, T. A. & PERERA, L. P. 2012. The role of interleukin-15 in inflammation and immune responses to infection: implications for its therapeutic use. *Microbes Infect,* 14**,** 247-61.
POSSEMIERS, H., VANDERMOSTEN, L. & VAN DEN STEEN, P. E. 2021. Etiology of lactic acidosis in malaria. *PLoS Pathog,* 17**,** e1009122.
PURCHASE, H. 1947. Cerebral babesiosis in dogs. *The Veterinary Record,* 59**,** 269-269.
RAUTENBACH, Y., SCHOEMAN, J. & GODDARD, A. 2018. Prevalence of canine Babesia and Ehrlichia co-infection and the predictive value of haematology. *Onderstepoort J Vet Res,* 85**,** e1-e5.
REMICK, D. G. 2005. Interleukin-8. *Crit Care Med,* 33**,** S466-7.
REYERS, F., LEISEWITZ, A. L., LOBETTI, R. G., MILNER, R. J. & JACOBSON, L. S. 1998. Canine babesiosis in South Africa: more than one disease. Does this serve as a model for falciparum malaria. *Annals of Tropical Medicine and Parasitology,* 92**,** 503-511.
RITZMAN, A. M., HUGHES-HANKS, J. M., BLAHO, V. A., WAX, L. E., MITCHELL, W. J. & BROWN, C. R. 2010. The chemokine receptor CXCR2 ligand KC (CXCL1) mediates neutrophil recruitment and is critical for development of experimental Lyme arthritis and carditis. *Infect Immun,* 78**,** 4593-600.
ROSENBLATT-BIN, H., KLEIN, A. & SREDNI, B. 1996. Antibabesial effect of the immunomodulator AS101 in mice: role of increased production of nitric oxide. *Parasite Immunol,* 18**,** 297-306.
SALEH, M. 2014. Sepsis-related hypertensive response: friend or foe? *BMJ Case Rep,* 2014.
SARFO, B. O., HAHN, A., SCHWARZ, N. G., JAEGER, A., SARPONG, N., MARKS, F., ADU-SARKODIE, Y., TAMMINGA, T. & MAY, J. 2018. The usefulness of C-reactive protein in predicting malaria parasitemia in a sub-Saharan African region. *PLoS One,* 13**,** e0201693.
SAWANT, K. V., POLURI, K. M., DUTTA, A. K., SEPURU, K. M., TROSHKINA, A., GAROFALO, R. P. & RAJARATHNAM, K. 2016. Chemokine CXCL1 mediated neutrophil recruitment: Role of glycosaminoglycan interactions. *Sci Rep,* 6**,** 33123.
SCHEEPERS, E. 2013. *The haematological kinetics of canine babesiosis in South Africa.*
SCHETTERS, T. P., KLEUSKENS, J. A., SCHOLTES, N. C., PASMAN, J. W. & BOS, H. J. 1994. Vaccination of dogs against *Babesia canis* infection using antigens from culture supernatants with emphasis on clinical babesiosis. *Vet Parasitol,* 52**,** 219-33.
SCHETTERS, T. P., KLEUSKENS, J. A., SCHOLTES, N. C., VAN DE CROMMERT, J., KRIJNEN, E., MOUBRI, K., GORENFLOT, A. & VERMEULEN, A. N. 2006. Onset and duration of immunity against *Babesia canis* infection in dogs vaccinated with antigens from culture supernatants. *Vet Parasitol,* 138**,** 140-6.
SCHETTERS, T. P., KLEUSKENS, J. A., VAN DE CROMMERT, J., DE LEEUW, P. W., FINIZIO, A. L. & GORENFLOT, A. 2009a. Systemic inflammatory responses in dogs experimentally infected with *Babesia canis*; a haematological study. *Vet Parasitol,* 162**,** 7-15.
SCHETTERS, T. P., MOUBRI, K. & COOKE, B. M. 2009b. Comparison of *Babesia rossi* and *Babesia canis* isolates with emphasis on effects of vaccination with soluble parasite antigens: a review. *J S Afr Vet Assoc,* 80**,** 75-8.
SCHETTERS, T. P., MOUBRI, K., PRECIGOUT, E., KLEUSKENS, J., SCHOLTES, N. C. & GORENFLOT, A. 1997. Different Babesia canis isolates, different diseases. *Parasitology,* 115 (Pt 5)**,** 485-93.

SCHETTERS, T. P., STRYDOM, T., CRAFFORD, D., KLEUSKENS, J. A., VAN DE CROMMERT, J. & VERMEULEN, A. N. 2007. Immunity against *Babesia rossi* infection in dogs vaccinated with antigens from culture supernatants. *Vet Parasitol,* 144**,** 10-9.

SCHMIDT, E. P., YANG, Y., JANSSEN, W. J., GANDJEVA, A., PEREZ, M. J., BARTHEL, L., ZEMANS, R. L., BOWMAN, J. C., KOYANAGI, D. E., YUNT, Z. X., SMITH, L. P., CHENG, S. S., OVERDIER, K. H., THOMPSON, K. R., GERACI, M. W., DOUGLAS, I. S., PEARSE, D. B. & TUDER, R. M. 2012. The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. *Nat Med,* 18**,** 1217-23.

SCHOEMAN, J. P. 2009. Canine babesiosis. *Onderstepoort J Vet Res,* 76**,** 59-66.

SCHOEMAN, J. P., REES, P. & HERRTAGE, M. E. 2007. Endocrine predictors of mortality in canine babesiosis caused by *Babesia canis rossi*. *Vet Parasitol,* 148**,** 75-82.

SCHRAMM, P., KLEIN, K. U., FALKENBERG, L., BERRES, M., CLOSHEN, D., WERHAHN, K. J., DAVID, M., WERNER, C. & ENGELHARD, K. 2012. Impaired cerebrovascular autoregulation in patients with severe sepsis and sepsis-associated delirium. *Crit Care,* 16**,** R181.

SCHRODER, K., HERTZOG, P. J., RAVASI, T. & HUME, D. A. 2004. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol,* 75**,** 163-89.

SGADARI, C., ANGIOLILLO, A. L., CHERNEY, B. W., PIKE, S. E., FARBER, J. M., KONIARIS, L. G., VANGURI, P., BURD, P. R., SHEIKH, N., GUPTA, G., TERUYA-FELDSTEIN, J. & TOSATO, G. 1996. Interferon-inducible protein-10 identified as a mediator of tumor necrosis in vivo. *Proc Natl Acad Sci U S A,* 93**,** 13791-6.

SHODA, L. K., PALMER, G. H., FLORIN-CHRISTENSEN, J., FLORIN-CHRISTENSEN, M., GODSON, D. L. & BROWN, W. C. 2000. Babesia bovis-stimulated macrophages express interleukin-1beta, interleukin-12, tumor necrosis factor alpha, and nitric oxide and inhibit parasite replication in vitro. *Infect Immun,* 68**,** 5139-45.

SOLCA, M. S., ANDRADE, B. B., ABBEHUSEN, M. M., TEIXEIRA, C. R., KHOURI, R., VALENZUELA, J. G., KAMHAWI, S., BOZZA, P. T., FRAGA, D. B., BORGES, V. M., VERAS, P. S. & BRODSKYN, C. I. 2016. Circulating Biomarkers of Immune Activation, Oxidative Stress and Inflammation Characterize Severe Canine Visceral Leishmaniasis. *Sci Rep,* 6**,** 32619.

SONG, M. & KELLUM, J. A. 2005. Interleukin-6. *Critical care medicine,* 33**,** 463-5.

SUDHAKARA REDDY, B., SIVAJOTHI, S., VARAPRASAD REDDY, L. S. S. & SOLMON RAJU, K. G. 2016. Clinical and laboratory findings of Babesia infection in dogs. *Journal of Parasitic Diseases,* 40**,** 268-272.

TAUB, D. D., LLOYD, A. R., CONLON, K., WANG, J. M., ORTALDO, J. R., HARADA, A., MATSUSHIMA, K., KELVIN, D. J. & OPPENHEIM, J. J. 1993. Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J Exp Med,* 177**,** 1809-14.

TELFORD III, S. R., GORENFLOT, A., BRASSEUR, P. & SPIELMAN, A. 1993. Babesial infections in humans and wildlife. *Parasitic protozoa,* 5**,** 1-47.

TOLIVER-KINSKY, T. E., VARMA, T. K., LIN, C. Y., HERNDON, D. N. & SHERWOOD, E. R. 2002. Interferon-gamma production is suppressed in thermally injured mice: decreased production of regulatory cytokines and corresponding receptors. *Shock,* 18**,** 322-30.

UILENBERG, G. 2006. Babesia--a historical overview. *Vet Parasitol,* 138**,** 3-10.

VAUGHAN-SCOTT, T., UNIVERSITY OF PRETORIA. DEPARTMENT OF COMPANION ANIMAL CLINICAL, S., JACOBSON, L. S. J. L. S. S. & REYERS, F. R. F. C.-S. 2009. *Serum concentrations of tumour necrosis factor in dogs naturally infected with Babesia canis and its relation to severity of disease.* University of Pretoria.

VIITANEN, S. J., LAURILA, H. P., LILJA-MAULA, L. I., MELAMIES, M. A., RANTALA, M. & RAJAMAKI, M. M. 2014. Serum C-reactive protein as a diagnostic biomarker in dogs with bacterial respiratory diseases. *J Vet Intern Med,* 28**,** 84-91.

WALKER, J. B. 1991. A review of the ixodid ticks (Acari, Ixodidae) occurring in southern Africa. *Onderstepoort J Vet Res,* 58**,** 81-105.

WELTAN, S. M., LEISEWITZ, A. L. & GODDARD, A. 2008. A case-controlled retrospective study of the causes and implications of moderate to severe leukocytosis in dogs in South Africa. *Vet Clin Pathol,* 37**,** 164-72.

WELZL, C., LEISEWITZ, A. L., JACOBSON, L. S., VAUGHAN-SCOTT, T. & MYBURGH, E. 2001. Systemic inflammatory response syndrome and multiple-organ damage/dysfunction in complicated canine babesiosis. *J S Afr Vet Assoc,* 72**,** 158-62.

WHITE, N. J., PUKRITTAYAKAMEE, S., HIEN, T. T., FAIZ, M. A., MOKUOLU, O. A. & DONDORP, A. M. 2014. Malaria. *Lancet,* 383**,** 723-35.

WHITWORTH, J. A., WILLIAMSON, P. M., MANGOS, G. & KELLY, J. J. 2005. Cardiovascular consequences of cortisol excess. *Vasc Health Risk Manag,* 1**,** 291-9.

YADAV, H. & CARTIN-CEBA, R. 2016. Balance between Hyperinflammation and Immunosuppression in Sepsis. *Semin Respir Crit Care Med,* 37**,** 42-50.

YAMAMOTO, S., TAGATA, K., NAGAHATA, H., ISHIKAWA, Y., MORIMATSU, M. & NAIKI, M. 1992. Isolation of canine C-reactive protein and characterization of its properties. *Vet Immunol Immunopathol,* 30**,** 329-39.

ZAHLER, M., SCHEIN, E., RINDER, H. & GOTHE, R. 1998. Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. *Parasitol Res,* 84**,** 544-8.

ZYGNER, W., GOJSKA-ZYGNER, O., BASKA, P. & DLUGOSZ, E. 2014. Increased concentration of serum TNF alpha and its correlations with arterial blood pressure and indices of renal damage in dogs infected with Babesia canis. *Parasitol Res,* 113**,** 1499-503.

ZYGNER, W., GOJSKA-ZYGNER, O., BASKA, P. & DLUGOSZ, E. 2015. Low T3 syndrome in canine babesiosis associated with increased serum IL-6 concentration and azotaemia. *Vet Parasitol,* 211**,** 23-7.

Addendums

Appendix A: University of Pretoria Animal Ethics Approval Certificate

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

Faculty of Veterinary Science
Animal Ethics Committee

15 October 2019

Approval Certificate
New Application

AEC Reference No.: REC048-19
Title: A time course study of markers of inflammation and cytokine concentrations during experimental Babesia rossi infection of beagle dogs.
Researcher: Dr BK Atkinson
Student's Supervisor: Prof AL Leisewitz

Dear Dr BK Atkinson,

The **New Application** as supported by documents received between 2019-07-29 and 2019-10-08 for your research, was approved by the Animal Ethics Committee on its quorate meeting of 2019-10-08.

Please note the following about your ethics approval:

1. The use of species is approved:

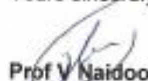
Species	Number
Dogs (Canine)	
Samples (retrospective study utilizing samples collected) V003-18 Blood smear	14
Whole blood in EDTA	14
Whole blood in serum tube	7

2. Ethics Approval is valid for 1 year and needs to be renewed annually by 2020-10-15.
3. Please remember to use your protocol number (REC048-19) on any documents or correspondence with the AEC regarding your research.
4. Please note that the AEC may ask further questions, seek additional information, require further modification, monitor the conduct of your research, or suspend or withdraw ethics approval.
5.

Ethics approval is subject to the following:

- The ethics approval is conditional on the research being conducted as stipulated by the details of all documents submitted to the Committee. In the event that a further need arises to change who the investigators are, the methods or any other aspect, such changes must be submitted as an Amendment for approval by the Committee.

We wish you the best with your research.
Yours sincerely



Prof V Naidoo
CHAIRMAN: UP-Animal Ethics Committee

Room 6-13, Arnold Theiler Building, Onderstepoort
Private Bag X04, Onderstepoort 0110, South Africa
Tel +27 12 529 8483
Fax +27 12 529 8321
Email aec@up.ac.za
www.up.ac.za

Fakulteit Veeartsenykunde
Lefapha la Diseanse tša Bongakadiruiwa

Appendix B: Data Collection Sheet

DEPARTMENT OF COMPANION ANIMAL

CLINICAL STUDIES

FACULTY OF VETERINARY SCIENCE

UNIVERSITY OF PRETORIA

Patient Number:	**Group Allocation:**

Parameter	**Result**
Habitus (1+ - 4+)[9]	
Temperature	
Pulse	
Respiratory rate	
Mucous membrane colour (Normal/Pale/Icteric/Congested)	
CRT (<1 sec/1-2 sec/>2 sec)	
Femoral pulse strength (Strong/Weak/Absent)	
Appetite (1+ - 4+)[10]	
Stool consistency (Normal/Diarrhoea/Firm)	

Blood Pressure:

	Systolic	**Diastolic**	**MAP**
Reading 1			
Reading 2			
Reading 3			
Reading 4			
Reading 5			
Average			

[9] Habitus: 1+ Lethargic and non-responsive; 2+ Lethargic but responsive; 3+ Alert and responsive; 4+ Bright alert and responsive.
[10] Appetite: 1+ Refusal to eat; 2+ Eats when hand or syringe fed; 3+ Eating unassisted but inadequate intake; 4+ Eating very well.

Urinalysis:

Colour	SG	pH	Protein	Glc	Bilirubin	Ketones	Hb/RBC

Sediment evaluation:

Blood smear evaluation

RBC	Leukocytes	Thrombocytes	Parasitaemia

Point of care (AlphaTRAK 2) Glucose:

Blood Collection:

Completed		Volume:

Appendix C: Sample collection schedule

DEPARTMENT OF COMPANION ANIMAL

CLINICAL STUDIES

FACULTY OF VETERINARY SCIENCE

UNIVERSITY OF PRETORIA

Patient Number:		**Group Allocation:**		
Day	Samples and volumes to be collected	Collected	Date of collection	Total blood collected per day
1	1. Whole blood EDTA 0.5 mL 2. Whole blood serum 3 mL 3. Whole blood heparinized 11 mL 4. Whole blood sodium citrate 4 mL 5. Whole blood RNALater 2.5 mL			21 mL
2	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL			11.5 mL
3	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL 3. Whole blood serum 3 mL 4. Whole blood sodium citrate 4 m L 5. Whole blood RNALater 2.5 mL			21 mL
4	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL			11.5 mL
5	1. Whole blood EDTA 0.5 ml 2. Whole blood heparinized 11 mL 3. Whole blood serum 3 mL 4. Whole blood sodium citrate 4 mL 5. Whole blood RNALater 2.5 mL			21 mL
6	1. Whole blood EDTA 0.5ml 2. Whole blood heparinized 11 mL			11.5 mL
7	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL 3. Whole blood serum 3 mL 4. Whole blood sodium citrate 4 mL 5. Whole blood RNALater 2.5 mL			21 mL
8	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL			11.5 mL
9	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL 3. Whole blood serum 3 mL 4. Whole blood sodium citrate 4 mL 5. Whole blood RNALater 2.5 mL			21 mL
10	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL			11.5 mL
11	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL			21 mL

	3. Whole blood serum 3 mL 4. Whole blood sodium citrate 4 mL 5. Whole blood RNALater 2.5 mL			
12	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL			11.5 mL
13	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL 3. Whole blood serum 3 mL 4. Whole blood sodium citrate 4 mL 5. Whole blood RNALater 2.5 mL			21 mL
14	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL			11.5 mL
15	1. Whole blood EDTA 0.5 mL 2. Whole blood heparinized 11 mL 3. Whole blood serum 3 mL 4. Whole blood sodium citrate 4 mL 5. Whole blood RNALater 2.5 mL			21 mL

Appendix D: Cytokine plate map

	1	2	3	4	5	6	7	8	9	10	11	12
A	0 pg/mL Standard Background	Standard 4	QC-1 Control	HD1 Base 1	LD2 Base 2	LD1 D1	HD 3 D1	HD 2 D2	HD 1 D3	LD2 D4	LD1 D6	LD1 D8
B	0 pg/mL Standard Background	Standard 4	QC-1 Control	HD1 Base 1	LD2 Base 2	LD1 D1	HD 3 D1	HD 2 D2	HD 1 D3	LD2 D4	LD1 D6	LD1 D8
C	Standard 1	Standard 5	QC-2 Control	HD2 Base 1	HD1 Base 2	LD2 D1	LD1 D2	HD 3 D2	HD 2 D3	HD 1 D4	LD2 D6	LD2 D8
D	Standard 1	Standard 5	QC-2 Control	HD2 Base 1	HD1 Base 2	LD2 D1	LD1 D2	HD 3 D2	HD 2 D3	HD 1 D4	LD2 D6	LD2 D8
E	Standard 2	Standard 6	LD1 Base 1	HD3 Base 1	HD2 Base 2	HD 1 D1	LD2 D2	LD1 D3	HD 3 D3	HD 2 D4	HD 1 D6	HD 1 D8
F	Standard 2	Standard 6	LD1 Base 1	HD3 Base 1	HD2 Base 2	HD 1 D1	LD2 D2	LD1 D3	HD 3 D3	HD 2 D4	HD 1 D6	HD 1 D8
G	Standard 3	Standard 7 Reconstituted	LD2 Base 1	LD1 Base 2	HD3 Base 2	HD 2 D1	HD 1 D2	LD2 D3	LD1 D4	HD 3 D4	HD 2 D6	HD 2 D8
H	Standard 3	Standard 7 Reconstituted	LD2 Base 1	LD1 Base 2	HD3 Base 2	HD 2 D1	HD 1 D2	LD2 D3	LD1 D4	HD 3 D4	HD 2 D6	HD 2 D8

Appendix E: Sample Storage Sheet

UNIVERSITEIT VAN PRETORIA
UNIVERSITY OF PRETORIA
YUNIBESITHI YA PRETORIA

DEPARTMENT OF COMPANION ANIMAL

CLINICAL STUDIES

FACULTY OF VETERINARY SCIENCE

UNIVERSITY OF PRETORIA

Patient Number:	Group Allocation:

Date	Sample ID	Volume	Sample type: Plasma/serum	Temperature	Location

Appendix F: Presentations and publications arising from this study

The following presentations and publications have resulted from this study:

Presentations

Event	Date	Title	Type
ACVIM 2020 online congress	10 June 2020	Markers of Inflammation and Cytokine Concentrations During Experimental Babesia rossi Infection of Beagle Dogs	Abstract poster presentation
Third International Babesiosis Meeting	24-25 April 2021	Markers of inflammation and cytokine concentrations during experimental Babesia rossi infection of beagle dogs	Abstract presentation