SERUM CONCENTRATIONS OF TUMOUR NECROSIS FACTOR IN DOGS NATURALLY INFECTED WITH *BABESIA CANIS* AND ITS RELATION TO SEVERITY OF DISEASE

by

Tarquin Vaughan-Scott

Submitted in partial fulfilment of the requirements
for the degree M.Med.Vet.(Med.)
in the Faculty of Veterinary Science, University of Pretoria

Cape Town
2001

Table of Contents

Résumé

Canine babesiosis, caused by the tick-borne protozoan *Babesia canis rossi*, is an economically important and potentially fatal disease of dogs in South Africa. The host's response to many infectious diseases is mediated (at least in part) by intercellular messengers called cytokines. One of the most important cytokines released is tumour necrosis factor (TNF).

A study was designed to measure serum concentrations of TNF in dogs naturally infected with canine babesiosis and to relate TNF concentrations to clinical severity, mortality, rectal temperature and parasitaemia.

There was a statistically significant difference in TNF concentrations between groups of differing disease severity, with a general trend of increasing mean log(TNF) with increasing severity of disease. A noteworthy finding was that dogs with hypoglycaemia had very high TNF (mean 15.03 ng/ml compared to a mean of 2.32 ng/ml for other sick dogs without hypoglycaemia). When TNF values were compared between survival and non-survival groups, there was no significant difference. The rectal temperature of the dogs in this study did not show any statistically significant association with TNF concentrations. When parasitaemia and TNF were examined within groups of infected dogs, there was no significant relationship. However, when the sample size was increased by pooling all infected dogs and treating them as a single group, there was a highly significant positive correlation ($p = 0.003$) between parasitaemia and serum TNF concentrations.

The results of this study were encouraging and indicate that canine babesiosis may share a similar pathophysiology with human malaria in terms of TNF being associated with disease severity. One of the most significant findings in this study was the presence of very high TNF values in two of three dogs with hypoglycaemia. Hypoglycaemia has not been previously recorded in dogs with babesiosis and is a potentially important finding particularly in view of the hypoglycaemia associated with malaria in humans. Malarial hypoglycaemia is correlated with a higher mortality in humans, especially in pregnant women and children. If the findings of this study can be

confirmed and expanded, they may lend further support to the use of canine babesiosis as a model for some of the problems encountered in human malaria research.

Acknowledgements

1. Dr Linda Jacobson, my supervisor, provided invaluable guidance, advice, physical assistance and encouragement during all stages of the study.

2. Prof Fred Reyers, my co-supervisor, provided invaluable assistance, advice and encouragement in the writing up of this dissertation.

3. The Department of Veterinary Tropical Diseases, Onderstepoort Veterinary Faculty, in particular Trudie Goosen who assisted in the tissue culture and bioassays required for this study.

4. The Department of Pharmacology, Onderstepoort Veterinary Faculty, in particular Dr Jonathan Taylor, who made funds available for the support of this study.

5. The Research Committee of the Onderstepoort Veterinary Faculty who financially supported this study.

6. Dr Daniel Polakow of the Department of Mathematics, University of Cape Town, who gave excellent statistical advice for this study.

7. Dr Ian Clark of the Division of Biochemistry and Molecular Biology, School of Life Sciences, Australian National University, for the donation of the WEHI 164 cells used in the bioassays techniques in this study.

8. Prof. Keith Zucker, Division of Transplant Surgery, University of Miami, who very kindly donated the recombinant canine tumour necrosis factor used in this study.

9. Prof. Remo Lobetti, for his assistance in sample collection and patient evaluation during this study.

Index of Tables

Index of Figures

List of Abbreviations

ALP = alkaline phosphatase

ALT = alanine transaminase

ANOVA = analysis of variance

CNS = central nervous system

EDTA = ethylenediaminetetraacetic acid

ELISA = enzyme-linked immunosorbent assay

IFN-γ = interferon-gamma

IL-1 = interleukin-1

IL-6 = interleukin-6

MODS = multiple organ dysfunction syndrome

mRNA = messenger ribose nucleic acid

mTNF = membrane expressed tumor necrosis factor

MTS = 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide

NCPO = non-cardiogenic pulmonary oedema

NF-κβ = nuclear factor kappa beta

OVAH = Onderstepoort Veterinary Academic Hospital

PaO_2 = partial pressure of oxygen within the pulmonary artery

PAO_2 = partial pressure of oxygen within the alveoli

PO = pulmonary oedema

rcTNF = recombinant canine TNF

SIRS = systemic inflammatory response syndrome

sTNF = soluble tumor necrosis factor

sTNFR = soluble tumor necrosis factor receptors

TNF = tumor necrosis factor

$TNFR_I$ = TNF receptor I

$TNFR_{II}$ = TNF receptor II

1 LITERATURE REVIEW

1.1 CANINE BABESIOSIS

1.1.1 Introduction

Canine babesiosis, caused by the tick-borne protozoan *Babesia canis rossi*, is an economically important and potentially fatal disease of dogs in South Africa[45]. Mild or uncomplicated forms of the disease (involving only haematological involvement and no other organ pathology) are effectively treated with anti-babesial drugs and where required, supportive therapy[46]. Complicated forms of the disease are difficult to treat, with a high mortality rate[45,46]. A major shortfall in the treatment of these complicated forms is a lack of understanding of the underlying pathophysiology[45,46]. It has been proposed that the clinical manifestations of the complicated forms are due to the host's response to the infection and not the direct action of the *Babesia canis* parasite[45]. This derives from findings in complicated malaria caused by *Plasmodium falciparum* in humans, where disease severity has been related to inflammatory factors (mainly cytokines) produced by the host, rather than the direct action of the parasite[12,18,65]. A similar argument has been proposed to explain the pathophysiology of septic shock in both man and animals[6]. The serum concentration of inflammatory mediators has also been shown to be related to malarial parasitaemia[11,41,53,73,88].

The most important cytokine released by the host during these inflammatory conditions is tumour necrosis factor (TNF)[78,79]. This cytokine is produced mainly by monocytes and has widespread effects on almost all cells of the body. The effects are both beneficial and detrimental as TNF can protect certain cells but has also been associated with septic shock and cerebral malaria[11,16,35,36,41,49,54,73,82,88,91].

Considering the remarkable similarities between the complicated forms of malaria in humans and babesiosis in dogs, proposals have been put forward that they share a similar pathophysiology. Furthermore canine babesiosis may be a good experimental model for complicated (especially cerebral) malaria[45,61,62,65]. As with canine babesiosis, the underlying pathophysiology of complicated malaria has not been completely defined, but cytokines do play an important role[65].

1.1.2 Definitions and Clinical Descriptions of Canine Babesiosis

Canine babesiosis is divided clinically into two entities: uncomplicated and complicated babesiosis[45,46,62]. Uncomplicated babesiosis is characterised by haemolysis and anaemia[46]. Complicated babesiosis involves other organs in addition to the haematological system[46,62].

The following is a description of the complications seen at the Onderstepoort Veterinary Academic Hospital (OVAH). The definitions are based on WHO criteria for severe falciparum malaria[95] and a classification of complicated babesiosis previously proposed[45]:

Cerebral Babesiosis:

Cerebral babesiosis is defined as central nervous system (CNS) signs not attributable to any other causes, in a dog with babesiosis. These CNS signs include acute behaviour changes, in-coordination, hindquarter paresis, muscle tremors, nystagmus, anisocoria and changes in mentation (stupor, coma and seizures)[45]. Histopathological changes commonly seen in the brain of such cases, at autopsy, are sequestration of parasitised erythrocytes in cerebral capillary beds and cerebral haemorrhages[3].

***Acute renal failure*:**

Acute renal failure is an infrequent complication of babesiosis. This complication is recognised by azotaemia and oliguria or anuria that is unresponsive to fluid therapy[45].

***Non-Cardiogenic Pulmonary Oedema (NCPO)*:**

NCPO is the formation of fluid within the lung parenchyma[26] and is a serious complication of babesiosis[45]. Clinical signs observed in these dogs include dyspnoea, moist cough and blood- tinged nasal discharge[45].

***Hypoglycaemia*:**

Hypoglycaemia is a well recognised entity in human malaria patients but has not previously been documented in canine babesiosis[45]. CNS signs of hypoglycaemia are variable and depend on the extent and rate of the glucose decrease[22].

***Red babesiosis (haemoconcentration)*:**

Red babesiosis is the paradoxical occurrence of an elevated haematocrit during a haemolytic episode in a dog with babesiosis[45]. It is postulated that haemoconcentration is the result of loss of plasma and hence blood volume from the intravascular space[45]. Red cells remain within the vascular compartment and haemoconcentration results.

***Liver Disease*:**

Liver disease occurs fairly commonly in dogs with babesiosis[45]. Histopathological examination of affected livers has shown congestion and centrilobular necrosis[45].

1.2 PATHOPHYSIOLOGY OF CANINE BABESIOSIS

1.2.1 The Systemic Inflammatory Response Syndrome/ Multiple Organ Dysfunction Syndrome Paradigm

Sepsis can be defined as a systemic inflammatory response that occurs in reaction to an invading microorganism[9]. A pathologic or excessive inflammatory response has been coined the "systemic inflammatory response syndrome" or SIRS[9]. SIRS and its related organ failures (also called the multiple organ dysfunction syndrome or MODS) are a major cause of death in human intensive care units, and thus have been the subject of intensive research[25]. A characteristic of SIRS and MODS is that organs that fail are generally not involved in the initial insult but are damaged by the excessive systemic inflammatory response generated by the host[25].

A classic example of SIRS and MODS is gram-negative bacterial infection where the bacteria release endotoxin. Many patients with this type of infection develop multiple organ failure far removed from the initial site of infection and also develop septic shock[50]. Current knowledge indicates that endotoxin itself is relatively harmless, but the response that it initiates from the host is devastating. Of the many theories proposed to explain the pathogenesis of this type of response, the mediator theory is most widely accepted[79]. This proposes that substances such as bacterial endotoxin are able to "trigger" the immune system to release mediators or cytokines that have a dual beneficial and harmful effect. The key player in this response is the monocyte which is stimulated to produce cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), interleukin-6 (IL-6), interferon-γ (IFN-γ) and others[79]. TNF is considered to be the most important cytokine released during this response[25,67].

Obviously TNF must have a beneficial role to play, otherwise evolution would have deleted this harmful factor from our immunological repertoire and accordingly TNF has been shown to have anti-bacterial and anti-protozoal properties[6]. It is also beneficial, if the response is localised to a focal area, as occurs in bacterial peritonitis where elevated serum TNF concentrations have been correlated with increased survival[27,81]. However, TNF has a dual role in that it has also been correlated with sepsis-related organ failures[23,78]. Furthermore the administration of anti-TNF antibody and anti-TNF drugs prior to the administration of endotoxin can completely prevent all the harmful consequences of sepsis[72]. It is this duality that led Bone[8] to propose that TNF is harmful only as a result of an "immunological dissonance". This proposes that a systemic inflammatory response will be associated with a coexisting anti-inflammatory response that serves to control and limit the inflammation. If there is an excess of pro-inflammatory mediators or a deficiency of anti-inflammatory mediators then an "immunological dissonance" results with widespread organ damage. There are many disease syndromes that fit the pattern of initial "trigger" that provokes a SIRS- and MODS-like response. Examples are malaria, heatstroke, pancreatitis, trauma, snakebite, neoplasia and babesiosis[45,79]. Indeed, the clinical signs of most of the above syndromes are often so similar as to be indistinguishable[17]. Table 1 shows the marked similarities between sepsis, babesiosis and malaria in humans, and sepsis and babesiosis in dogs.

1.2.2 Tumour Necrosis Factor and Complicated Malaria

The clinical signs of complicated malaria caused by *Plasmodium falciparum* are strikingly similar to sepsis-related MODS[15,17](Table 1). This prompted the proposal that the underlying pathophysiology may be similar and may involve cytokines such as TNF[15].

Since TNF plays a major role in sepsis-related MODS[23,78], it would be logical to hypothesise that TNF would also be associated with malaria related-MODS. Numerous studies have shown that this is indeed the case[11,16,35,36,41,49,54,73,88,91]. Most of the TNF studies relating to malaria have concentrated on the cerebral form caused by *P.falciparum,* which is clinically important as it is associated with high mortality rates. There are clearly elevated serum TNF concentrations in these patients when compared to non-cerebral malaria cases[35,54].

1.2.3 Tumour Necrosis Factor and Human Babesiosis

Human babesiosis, caused mainly by *B.microti* and *B.divergens,* displays clinical signs very similar to malaria and sepsis[17]. These clinical signs range from fever and malaise to severe multiple organ failure (Table 1). A case report of human babesiosis showed, as expected, very high concentrations of TNF, IL-6, IL-2 and IFN-γ during the acute phase of infection[89].

1.2.4 Tumour Necrosis factor and Complicated Canine Babesiosis

If sepsis, malaria and human babesiosis share a common pathophysiology, then other diseases that have a similar clinical picture could share the same cytokine-mediated pathophysiology. Canine babesiosis is a very similar disease, based on the clinical picture (Table 1), and may meet these criteria. It has been proposed that canine babesiosis is an example of a disease in which SIRS plays a major role and may share the common pathophysiology of sepsis and malaria[17,45]. It is therefore reasonable to expect that TNF should also play a role in canine babesiosis.

There is only one recorded study in which TNF concentrations have been evaluated in dogs with babesiosis[85]. The study showed that TNF concentrations were not elevated

during experimentally induced *Babesia canis* infection. This, however, does not exclude a role for TNF in cases of canine babesiosis, due to the following:

a) Complicated babesiosis was not observed in any of the experimental dogs and these are the cases in which TNF would most likely be expected to play a role
b) The TNF assay was performed on a single sample that had been pooled from all the serum samples taken from dogs infected. This would have had the effect of diluting out TNF activity in samples in which it was high.
c) The subspecies of parasite used in this study was probably *B. canis canis* and not *B. canis rossi*, as is found in South Africa. It would therefore not cause the type of severe disease seen in South Africa (see point 1.2.6 for a discussion on strain pathogenicity).

1.2.5 Endotoxin, Malaria Toxin and Babesia Toxin

It is now well known that endotoxin (which is a relatively harmless substance in isolation) mediates its effects, at least in part, by stimulating TNF production[6,55]. Therefore, endotoxin should only be seen as the trigger that initiates a cascade of events that causes cytokine production and resultant inflammatory disease. This concept has also been applied to malaria, initially by Clark[15]. It has been found that *P.falciparum* parasites release a soluble toxin during schizogony and the toxin is a potent inducer of TNF production[4,52,53]. Considerable effort has been expended in trying to isolate the malarial toxin as it is a potential target for vaccine development. The actual source of the toxin has recently been challenged, as it has been found that *Mycoplasma* contamination of *Plasmodium* cultures can result in the production of "mycoplasmal toxins" that have a similar effect to endotoxin, namely the stimulation of TNF production[83]. This has cast doubt on much of the previous plasmodial-toxin work. Subsequent studies have shown that the host's cytokine response to *Plasmodium* infections is not similar to the response to endotoxin, even though the end result may be the elevation of serum TNF levels[86]. The response is mediated via lymphocytes and not monocytes, as is the classical response to endotoxin. This indicates that the malarial toxin may not be comparable to endotoxin from a biochemical point of view. However, the end result of malarial infection is the elevation of TNF levels.

Considering the similarities in the pathology and host response (fever, clinical signs, organ pathology) between *Plasmodium* and *Babesia* parasites, it is possible that the *Babesia* parasite also releases a *Babesia* toxin or triggers a host response similar to *Plasmodial* organisms.

1.2.6 The Parasite's Ability to Induce TNF Production Relates to Severity of Disease

If TNF is the mediator of disease, as has been proposed, then it is possible that different sub-species of parasite (or different isolates of the same sub-species) with varying pathogenicity, would have varying abilities to induce TNF production. In support of this concept it is important to recognise that there are different sub-species of *B.canis* parasites[93]. There is also evidence in the malaria and *Babesia* literature that different isolates of parasites relate to the clinical severity of disease[2,93]

Uilenberg et al[93] showed that there are three sub-species of *B.canis* that vary in their pathogenicity:

- ***Babesia canis canis***: is transmitted by *Dermacentor* ticks in Europe and is pathogenic in splenectomised dogs and moderately pathogenic in intact dogs. Some intact dogs can self-cure after experimental infection.
- ***Babesia canis vogeli***: is transmitted by *Rhipicephalus sanguineus* ticks in tropical and subtropical regions. It is considered a non-pathogenic strain and causes very mild disease in intact and splenectomised dogs.
- ***Babesia canis rossi***: is transmitted by *Haemophysalis leachi* in Southern Africa[57] and is highly pathogenic.

Pathogenicity may also be isolate specific. A study showed that isolates of the same sub-species can have varying pathogenicity when 119 different isolates of *Babesia canis canis* were tested for pathogenicity by inoculation into Beagles[65]. Four of the isolates caused severe cerebral babesiosis which was repeatable in more than 50 successive infections. Another study in South Africa using the Thomas isolate of *Babesia canis rossi* showed that all dogs infected required treatment due to the developement of severe anaemia, but complications did not occur[56]. The virulence of *Babesia bovis* has also been shown to be isolate specific[92].

What are the mechanisms underlying isolate pathogenicity? The previous discussion indicates that TNF is a key factor in the development of disease and therefore it may be the isolate's ability to induce TNF production that relates to its pathogenicity. Studies with human malaria have shown that this is the case[1,2,19]. Phenotypes of virulent malaria parasites have been shown to differ from avirulent parasites, and different isolates have been shown to induce different levels of TNF production *in vivo*. One study showed that parasites isolated from patients with cerebral malaria, were able to stimulate higher concentrations of TNF production than parasites from non-cerebral cases[1]. There have been no such studies performed in canine babesiosis.

1.2.7 Experimental Administration of TNF to Humans and Dogs

If TNF is central to the pathophysiology of endotoxic shock, complicated malaria and complicated babesiosis, then administration of TNF to experimental subjects should cause a similar clinical picture. TNF has been evaluated for the treatment of neoplasia in humans[6,16]. Administration caused nausea with vomition, fever, rigors, headache, myalgia, hypotension, thrombocytopaenia and lung oedema. These are very similar findings to endotoxic shock and complicated malaria[16,69].

TNF has also been administered to dogs to evaluate its role in endotoxic shock[28,29,33,58,66,68,70,71,94,96]. Clinical signs observed after administration include fever, weakness, malaise, vomition, hypotension, decreased cardiac output, leukopaenia, lung oedema, metabolic acidosis and acute renal failure. Most of these features are also observed in dogs with complicated babesiosis (Table 1)[45].

1.2.8 Further Evidence for the Involvement of Cytokines: the Malarial and Babesial Fever Response

Fever has been defined as a state of elevated core temperature which is part of the defensive response of the host to invading microorganisms[60]. Fever must play a beneficial role in the host's defence mechanisms to be conserved evolutionarily. With regard to protozoal disease, it has been shown to retard *Plasmodium* growth[52]. Fever could, therefore, be seen as an advantageous response with respect to the host.

The febrile response is a complex physiological reaction mediated by cytokines, the most important of which are TNF, IL-1, IL-6 and IFN-γ[60]. These substances are known as endogenous pyrogens. The presence of fever therefore is strongly indicative of an elevation in circulating cytokines in the host[53,55,60]. Current theory indicates that TNF and interferon-γ are the most important pyrogens. Furthermore TNF levels have been shown to be directly related to body temperature in dogs and humans with endotoxaemia and malaria respectively[52,55].

A study in dogs, in which endotoxin was injected *in vivo*, showed that the fever response only occurred above a certain threshold of blood TNF concentration in an all-or-none type response[55]. However is it is also important to note that blood cytokine concentrations are relatively short lived and therefore may be normal at the time of blood sampling[7]. Fever is a common clinical sign in dogs with babesiosis and this presents further evidence that cytokines may play a role in the pathogenesis of this disease.

1.2.9 Tumour Necrosis Factor and Parasitaemia

As discussed previously, the malaria parasite has been shown to stimulate the production of TNF[1,2,52]. It has been proposed that, in malaria patients, as parasitaemia rises, so does the production of TNF[52]. This could occur due to an increased number of parasites that release proportionately increased levels of "malaria toxin", which would then provide a greater stimulus for TNF production. A number of studies have shown a direct correlation between serum TNF levels and parasitaemia in people with malaria, that is, the higher the parasitaemia, the higher the TNF level[11,41,53,73,88,91]. There are no reported studies of correlations between TNF and parasitaemia in dogs infected with *Babesia canis*.

1.3 THE HISTORY AND BIOLOGY OF TUMOUR NECROSIS FACTOR

1.3.1 Introduction

In order for a host to mount an effective defensive response, the immune system must be mobilised and co-ordinated to repel the invading organism. The immune system provides the host with a system of intercellular messengers to cause widespread activation of defence mechanisms, which allows even focal insults to cause systemic activation. TNF is a classic example of one of these intercellular messengers (or cytokines).

The name tumour necrosis factor indicates its historical origin and does not reflect its function. TNF was initially described in 1975 by Carswell, who demonstrated that the *in vivo* administration of bacterial endotoxin, resulted in a protein factor in blood that was capable of inducing tumour cell necrosis[13]. Subsequently a large amount of research has been performed, which has characterised TNF in minute detail.

The following is a summary of its production, release into plasma, cellular receptors and biological half-life.

1.3.2 Production of Tumor Necrosis Factor

Considering its role as a mediator in the immune response, it is not surprising that a large number of substances are able to "trigger" the production of TNF. It is produced by many types of cells including macrophages, lymphocytes, mast cells, keratinocytes, microglia, smooth muscle cells and tumour cells[69]. The macrophage is considered the most important source of TNF and is discussed further.

Various triggering agents are able to interact with the macrophage cell membrane and result in the production of TNF[6]. The most intensively-studied substance is bacterial endotoxin which binds to the cell surface receptor CD14[6]. Other trigger factors include bacterial exotoxins, lipoproteins produced by *Treponema* and *Borrelia sp*[6] and a heat stable soluble malarial toxin[4] (although, as discussed previously, the presence of a malarial toxin has been challenged[83]).

After cell-surface receptor binding by the triggering agents mentioned above, various cytosolic secondary messengers are activated including the transcription factor, nuclear factor kappa beta (NF-κβ)[63]. NF-κβ is liberated from its inhibitory factor, Iκβ, and is allowed to move into the cell nucleus. It then initiates mRNA-mediated protein synthesis and TNF production. TNF is initially produced as a large 26kDa protein that is inserted into the cell membrane of the cell that produces it[38,69]. At this stage the TNF molecule has an extracellular and an intracellular domain and is known as the membrane expressed form of TNF (mTNF)[38,69,82]. mTNF is biologically active behaves very differently from soluble TNF [38].

The extracellular domain is then cleaved from the cell by membrane-associated metalloproteinases (possibly serine proteinase-3 or other zinc-related metalloproteinases) which results in the extracellular domain being shed as a 17kDa protein known as soluble TNF (sTNF)[38,39,69,77,82]. sTNF is initially a single molecule which is biologically inactive as it cannot interact with its cellular receptors[5]. However three of these single sTNF molecules soon aggregate to an active trimer configuration[5]. The trimer configuration is essential to activate the corresponding cellular receptor.

1.3.3 Tumour Necrosis Factor receptors

Like many mediators TNF must bind to target cell-surface receptors to initiate a response. Investigation thus far has shown that there are two types of TNF receptors[5,38,39].

TNF receptor I ($TNFR_I$) is a 55 kDa molecule that interacts mainly with sTNF[38]. The cellular effects of $TNFR_I$ activation include cytotoxicity, gene induction, fibroblast proliferation, IL-6 and PG-E_2 synthesis and activation of NF-κβ[69]. Most reports have indicated that sTNF and $TNFR_I$ are involved in most of the detrimental effects of TNF (this will be discussed later as cerebral malaria and possibly babesiosis may be exceptions to this generality)[6,59].TNF receptor II ($TNFR_{II}$) is a 75 kDa molecule that interacts mainly with mTNF[38]. Its functions include granulocyte-monocyte colony simulating factor synthesis and lymphocyte proliferation[69]. Selective interaction of sTNF and mTNF can be explained by receptor binding affinity. $TNFR_{II}$ has a very low

affinity for sTNF but a very high affinity for mTNF, and $TNFR_I$ binds with high affinity to sTNF.

Another important aspect of TNF receptors is that they can become solubilised and shed into circulation[82]. These soluble receptors (sTNFR) can bind TNF in circulation and inactivate it. The bond however is not permanent and sTNFR can also dissociate over time[21]. This has been seen as part of the host anti-inflammatory mechanism (a deficiency of these soluble receptors would result in immunological dissonance as described previously) and there can be very high concentrations in patients with sepsis[78]. Some studies have shown increased mortality in patients that have low capacity to produce sTNFR (this will allow more TNF to be biologically active)[77]. The soluble receptors are excreted via the kidney and therefore a reduced glomerular filtration rate can elevate serum concentrations[21].

1.3.4 Tumor Necrosis Factor Metabolism and Kinetics

Once TNF has bound to the cellular receptor it is rapidly internalised and degraded. When injected into rabbits the serum half-life of TNF is 6-7 minutes[7]. A study, in which TNF was radiolabelled, showed the following organs to be involved with accumulation and degradation - liver (31%), skin (30%), gastrointestinal tract (8,8%), kidney (7,8%), lung (1,8%), spleen (0,9%) and other organs (20,2%) [7]. The high uptake and degradation of TNF by the liver and skin does not imply that these organs are preferentially damaged by TNF. The TNF trimer is also labile and degrades to the monomer with a half-life of 15-20 hours[21].

The short half life has important implications if the substance is to be measured from *in vivo* samples, as serum concentrations may be low at the time of blood sampling[72,77]. Even if serum concentrations are normal, TNF release and binding to the receptor may have occurred with all the subsequent downstream events. This is a severe limitation of one-off blood sampling.

1.3.5 Which Form of Tumor Necrosis Factor and Which Receptors are Involved in the Pathophysiology of Sepsis and Malaria?

From the previous discussion it becomes obvious that TNF has a complex interaction with its target cells and there are a number of possibilities regarding which component has a pathogenic effect.

Most investigators believe that sTNF and $TNFR_I$ play a major role in the organ pathology associated with sepsis. However a number of studies have not been clear on this point. This is because serum sTNF concentrations are sometimes normal in the face of severe SIRS and MODS[72]. One interpretation is that serum concentrations do not reflect what is happening at the tissue level and sTNF concentrations may be very high in the affected tissue compartment[72]. Another school of thought is that mTNF plays a more important role (which is not measured in serum samples as it is cell-associated)[77].

One study, concerning patients with sepsis, showed a poor correlation between TNF and MODS scores, but a very good correlation between mTNF concentrations (induced *in vitro*) and MODS scores[77]. Other studies have shown very good correlations between sTNF and MODS scores[23,78].

Several studies have shown good correlation between sTNF and the presence of cerebral malaria[11,36,49]. A study of murine cerebral malaria showed that mTNF was critical in the development of the syndrome and $TNFR_{II}$ knockout-mice (mice that have been bred so they lack TNF receptor II) did not develop nervous signs[59].

This issue has not been resolved but it does indicate that before the role that TNF plays in a syndrome can be evaluated, the methodology used to measure it must be critically analysed.

1.4 DETERMINATION OF SERUM TUMOR NECROSIS FACTOR CONCENTRATIONS

1.4.1 Introduction

There are two categories of tests available for the assay of TNF:

- Antibody-linked testing eg enzyme-linked immunosorbent assays (ELISA)[24]
- Bioassays that rely on the sensitivity of specific cell cultures towards TNF[21,31,76,97].

Each of these groups has advantages and disadvantages (Table 2).

Antibody-Based Assays

Antibody-based assays have a number of advantages over bioassays (Table 2). The most important is the ability of some of these assays to measure sTNFR-bound TNF. This is, however, a point of contention, as some ELISAs utilise antibody that binds at the same epitope as the sTNFR[21]. This can give false low results as binding can occur *in vitro*[21].

A major disadvantage of using antibody-based assays for canine TNF is that specific canine anti-TNF antibody is not commercially available[55,97]. Human and murine antibodies are freely available, but it was shown in a comparative study, that murine monoclonal anti-human TNF antibody does not cross-react with canine TNF[76]. Anti-murine TNF antibody has been shown to neutralise canine TNF[97]. However there are no reported studies utilising an anti-murine TNF antibody for the detection of canine TNF. Some investigators have used human TNF ELISA assays for the detection of canine TNF without reporting whether or how the assays were validated[14]. This essentially rules out the use of antibody-linked testing for the detection of canine TNF until further studies have been conducted.

Bioassays

Bioassays rely on the sensitivity and specificity of various cell lines to the cytotoxic effects of TNF[64]. There are many cell lines available for the detection of TNF such as WEHI-164 mouse fibrosarcoma cells[31,32], L929 fibroblast cells[87], and PK15 porcine cells[76] amongst others. These cell lines all show varying sensitivities to TNF and

varying sensitivities to inter-species TNF. It is therefore important to select a cell line that has a proven sensitivity to the TNF of the species under scrutiny.

The WEHI-164, subclone 13, cell line has a proven superior sensitivity (when compared with other cell lines) to human and canine TNF and has been recommended over other cell lines[32]. Concerning canine TNF; the same group of workers that produced recombinant canine TNF showed the WEHI-164 subclone 13 bioassay to be sensitive for canine TNF[99].This cell line has also been used in numerous other studies for the measurement of canine TNF[20,34,55,74,80,98].

Bioassays, however, do have certain disadvantages. They only detect biologically active TNF and therefore will not detect sTNFR-bound TNF or TNF monomers[21]. One study showed a poor diagnostic accuracy when sTNFR were added *in vitro*[21]. The effect of TNF degrading to monomers has not been evaluated.

The species of origin of TNF used to generate a standard curve also introduces a variable. This has not been formally investigated so it is difficult to predict if it would be problematic, but it would be ideal to use a recombinant canine TNF to generate the standard curve as opposed to recombinant human or murine TNF, which is a common compromise in published studies.

The first reported study of canine TNF utilised a bioassay for the detection of TNF in serum[55]. The study was performed several years ago but the recommendations made then are still valid today considering the absence of canine anti-TNF antibody. The authors recommended that bioassays be utilised until specific anti-canine TNF monoclonal antibodies are available. Subsequent studies on canine TNF have used this approach[20,34,40,42,43,67,74,80,90,97,98].

1.4.2 Bioassay Methodology and Unique Problems Associated with Canine Babesiosis

The TNF bioassay is based on the number of cells killed by a fluid sample (such as serum) containing TNF[31]. Cells are cultured in a tissue culture plate and then the sample in question is placed in each well of the plate. After an incubation period, the number of cells that are still alive within the well is inversely related to the TNF

concentration of the added sample. The living cells are revealed with a dye that only interacts with viable cells such as MTS (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide). This is a substance that is taken up by the cell and converted by the mitochondria from a yellow to an "active" brown colour. The amount of "active" dye produced is therefore directly related to the number of cells that are still viable in the well and inversely related to the amount of TNF in the sample. The amount of "active" dye that is present in the well is then analysed by measuring the amount of light absorbed photometrically at 490nm.

Canine babesiosis presents problems that could interfere with this type of assay. Due to the haemolysis associated with the disease, serum samples can be severely discoloured due to the presence of haemoglobin and/or bilirubin. These colour changes could to interfere with the photometric measurement.

1.4.3 Storage of Serum samples

TNF is a protein and is therefore susceptible to environmental degradation. However TNF has been found to be very stable in serum samples stored at -70C°, and is still active after 11 freeze-thaw cycles[31].

1.5 Tables

Table 1 Features of *Plasmodium sp* and *Babesia sp* infections and TNF and endotoxin administration in humans and dogs[17,89]

FEATURE	Human babesiosis	Canine babesiosis	Human malaria	Human TNF Administration	Canine TNF Administration	Human Endotoxin Administration	Canine Endotoxin Administration
Fever	+	+	+	+	?	+	+
Thrombocytopaenia	+	+	+	+	?	+	+
Coagulopathy	+	+	+	+	+	+	+
Hypotension	?	+	+	+	+	+	+
Gastrointestinal involvement	+	+	+	+	+	+	+
Renal dysfunction	+	+	+	+	+	+	+
Pulmonary oedema	+	+	+	+	+	+	+
Hyperlactataemia	?	+	+	+	+	+	+
High TNF	+	?	+			+	+

Table 2 A comparison of the advantages and disadvantages of antibody-based and bioassays for the detection of TNF[21,69]

ANTIBODY-BASED TESTING	BIOASSAYS
Advantages	Disadvantages
Detects both sTNFR-bound and free TNF	Only detects unbound biologically active TNF
Detects TNF monomers and trimers	Only detects biologically active trimers
Species-specific TNF supplied as standard	Ideally requires a species-specific standard
Easy to perform	Technically difficult
Repeatable	Not as repeatable as cell cultures are susceptible to external conditions
Disadvantages	Advantages
Species specific	Not species specific
Expensive	Less expensive

2 PROBLEMS

2.1 Even though complicated canine babesiosis is still a significant cause of morbidity and mortality in South Africa, there are no effective treatments. An improved understanding of the underlying pathophysiology may lead to a better therapeutic approach.

2.2 Complicated malaria is a major cause of human death. There are important limitations in existing experimental animal models, restricting their usefulness in investigating the pathophysiology and treatment of this disease. Complicated canine babesiosis may be a good model for this investigation, if the underlying pathophysiology can be shown to be similar.

2.3 There is ample evidence that TNF plays a significant role in human falciparum malaria but only circumstantial evidence for this in canine babesiosis.

3 OBJECTIVES

3.1 To gain a better understanding of the pathophysiology of canine babesiosis by measuring serum TNF concentrations in dogs naturally infected with *Babesia canis rossi*.

3.2 To determine whether serum TNF concentrations are associated with disease severity.

3.3 To determine whether TNF concentrations are associated with the degree of parasitaemia.

4 RESEARCH QUESTIONS

Regarding canine babesiosis:

4.1 Do dogs with severe or complicated disease have higher serum TNF concentrations than dogs with mild and uncomplicated disease and dogs without babesiosis?

4.3 Do dogs that die have higher serum TNF concentrations than dogs that survive?

4.4 Is there a relationship between rectal temperatures and serum TNF concentrations?

4.5 Is there a relationship between parasitaemia and serum TNF concentrations?

5 MATERIALS AND METHODS

5.1 INTRODUCTION

Three groups of dogs formed the sample populations that were used in this study:

The first group was composed of dogs presented to the Outpatients Department of the Onderstepoort Veterinary Academic Hospital (OVAH) with clinical babesiosis. The dogs all originated from the same area North of Pretoria, due to the policy of the hospital, that is, dogs from other areas are not allowed admission to the hospital without first seeing their local veterinarian.

The second group of dogs were healthy dogs admitted for ovariohysterectomy or castration operations. These dogs also originated from the abovementioned area.

The third group of dogs originated from Biocon research laboratory, which is situated in the same area and has a number of Beagle dogs that are kept in isolation for research purposes. These dogs are housed in parasite-free conditions and are regularly screened for internal and external parasites, thus ensuring that they are free from parasite-related disease.

5.2 EXPERIMENTAL DESIGN

5.2.1 Selection Criteria and Experimental Groups

General Selection Criteria

All dogs had to weigh over 5 kg to be considered for inclusion (this was to prevent drawing a relatively large volume of blood from an already compromised patient). No animal with a history of another acute inflammatory or infectious disease in the preceding 6 weeks was considered. It was not possible to exclude canine ehrlichiosis effectively but all animals with known concurrent *Ehrlichia canis* infections (morulae detected on bloodsmear examination) were excluded.

Specific Selection Criteria

The following criteria were used to categorise cases into the following groups:

Group I: Mild uncomplicated babesiosis

- acute onset (< 7 days) of lethargy, inappetence/anorexia
- *B. canis* parasites identified on thin capillary bloodsmear examination
- mild to moderate anaemia (haematocrit 20-36 %)
- no clinical signs of acute disease other than the above, as judged by the attending clinician

Group II: Severe uncomplicated babesiosis

- acute onset (< 7 days) of lethargy, inappetence/anorexia
- *B. canis* parasites identified on thin capillary bloodsmear examination
- mild to moderate anaemia (haematocrit < 15 %)
- no clinical signs of acute disease other than the above, as judged by the attending clinician

Group III: Complicated babesiosis

- acute onset (< 7 days) of clinical signs
- *B. canis* parasites identified on thin capillary bloodsmear examination
- additional signs observed, or laboratory findings that have been described as complicated babesiosis[45] and include one or more of the following:

Cerebral Babesiosis

Clinical signs characteristic of cerebral babesiosis described previously. Animals with CNS signs limited to stupor or semi-coma, that also had severe anaemia (<15%), were not included as it is difficult to distinguish weakness due to hypoxia from stupor due to CNS involvement in these cases.

Acute renal failure

Acute renal failure was diagnosed if urine production was < 1ml/kg/hour and serum creatinine > 133 µmol/l and these abnormalities were not resolved by adequate intravenous fluid therapy. Adequate fluid therapy was defined as correction of fluid deficits and was dependent on the supervising clinician. Urine production was monitored by catheterization and a closed-circuit urine collection system.

Pulmonary Oedema (PO)

A diagnosis of PO was made if patients fulfilled one or more of the following criteria which have been adapted from human studies and validated for use in canine patients:
Chest radiographs- bilateral diffuse interstitial and/or alveolar patterns[26].
Arterial blood gas - an arterial partial pressure of oxygen <60mmHg that is refractory to treatment or a PAO_2-PaO_2 of >15 when breathing room air[26,75]. PAO_2 is the partial pressure of oxygen within the alveoli and PaO_2 is the partial pressure of oxygen within the pulmonary artery. The difference between the two values is seen as a good measure of the ability of oxygen to diffuse across the alveolar membrane into the pulmonary artery[26,75].

Hypoglycaemia

Hypoglycaemia was recorded if plasma glucose was less than or equal to 2.2 mmol/l.

Red babesiosis

Red babesiosis was recorded if the patient's haematocrit was greater than 45%, *in conjunction with* congested mucous membranes and signs of severe haemolysis (haemoglobinuria and/or grossly visible haemoglobinaemia).

Liver Disease:

Liver disease was recorded if there was clinically visible icterus *in conjunction with* significant elevation (over two times top-normal value[a]) of both serum alanine transaminase (ALT) and alkaline phosphatase (ALP) activity.

Group IV: Healthy aparasitaemic animals from an endemic area

- clinically normal
- bloodsmear negative for *B. canis*
- all parameters investigated (as for patients) within normal canine reference range[b]

Group V: Healthy experimental dogs from a parasite-free-environment

- clinically normal
- bloodsmear negative for *B. canis*
- all parameters investigated (as for patients) within canine reference range

5.2.2 Sample Size

The expected range of TNF concentrations in diseased dogs was expected to be 0-240 ng/ml and it was decided that a TNF level difference of 50 ng/ml between the groups would be considered significant (these figures were derived from a review of the literature in which TNF has been measured in dogs in a variety of conditions, and extrapolating expected values). A desired sample size of 11 dogs in each group was calculated based on a 5% level of significance and a 90% power of the test based on tables in "Sample Size in Clinical and Laboratory Research" by C.H. Goldsmith, 1978.

---

[a] Top-normal is 40 U/l for ALT and 190 U/l for ALP.

[b] Reference range supplied by the Section of Clinical Pathology, Department of Medicine, Faculty of Veterinary Science, University of Pretoria

The actual sample sizes were 10 dogs in the mild uncomplicated group, 9 dogs in the severe uncomplicated group, 12 dogs in the complicated group and 10 dogs in both control groups. The number of samples in each group was limited by financial and time constraints. These constraints did not allow more samples to be obtained during the study period.

5.3 OBSERVATIONS AND CLINICAL MEASUREMENTS

5.3.1 Procedures Performed on Subject Animals

Clinical Evaluation

A general clinical examination was performed and the results recorded by the principal investigator, T. Vaughan-Scott or a co-investigator R. Lobetti (both investigators were experienced small animal clinicians associated with the section of Small Animal Medicine[a]. The results of the clinical examination of Case 11 are attached as Appendix I to demonstrate the data recorded during this procedure. A peripheral blood smear was stained and examined microscopically to detect the presence of *Babesia canis* parasites and exclude other pathogens (eg. *Ehrlichia canis*)

Sample Collection and Handling

Blood samples were drawn (approximately 3ml into each of 3 tubes) from the cephalic or jugular vein. Blood was collected in EDTA, plain and fluoride Vacutainer tubes and taken immediately to the Clinical Pathology Laboratory. If a blood gas analysis was deemed necessary by the attending clinician, an arterial blood sample (0.5-1ml) was taken from the femoral artery into a pre-heparinised syringe. Excess air was expelled form the syringe and it was immediately sealed and transported to the Clinical Pathology Laboratory for blood-gas analysis.

[a] Department of Medicine, Onderstepoort Veterinary Academic Hospital, Faculty of Veterinary Science, University of Pretoria.

5.3.2 Laboratory Measurements

The following clinicopathological parameters were measured to allow classification of animals as discussed above:

- full blood count - EDTA blood collection tube
- serum biochemistry (creatinine, ALT, ALP) – plain blood collection tube
- plasma glucose – fluoride blood collection tube
- blood-gas analysis (if deemed necessary by attending clinician)

Full blood count was conducted on a Cell Dyn 3500 analyzer (Abbott Laboratories, Abbott Park, Illinois, USA).

Biochemistry consisted of serum creatinine, alanine transaminase and alkaline phosphatase and was determined on a Technicon RA 1000 system (Technicon Instruments Corporation, Tarrytown, USA) using the following methodology:

Creatinine- Based on the Jaffe alkaline picrate reaction: Technicon Method # SM4-0141D91 kinetic modification for the RA-1000 of the method described by Rossignol, B; Rossignol, D & PetitClerk, C (1984) "Improvement of creatinine measurement on RA-1000." Clinical Biochemistry, 17; 203-204.

Alanine Transaminase (ALT)- Optimized, kinetic assay based on the procedure of Wroblewski and LaDue: Technicon Method # SM4-0134D91 modification for the RA-1000 of the method described by Bergmeyer, HU; Scheibe, P & Wahlefeld, AW (1978) "Optimization of methods for aspartate aminotransferase and alanine aminotransferase." Clinical Chemistry, 24; 58-73 and The International Federation of Clinical Chemistry (IFCC) (1978) "Provisional recommendations on IFCC methods for the measurement of catalytic concentrations of enzymes." Clinical Chemistry, 24; 720-721.

Alkaline Phosphatase (ALP)- Kinetic assay based on the p-nitro phenyl phosphate substrate procedure of Bowers & McComb: Technicon Method # SM4-0132D91 modification for the RA-1000 of the method described, using AMP buffer, by Bessey, OA; Lowry, OH & Brock, MJ (1946) "A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum." Journal of Biological Chemistry, 164; 321-329.

Glucose- Based on the hexokinase procedure for glucose determination:

Technicon Method # SM4-0143D91 modification for the RA-1000 of the method described by Léon, LP; Chu, DK; Stasw, RO & Snyder, LR (1977) "Advances in Automated Analysis Technicon International Congress. 1976 Volume 1." Tarrytown, NY. Mediad. Inc, 152-156.

Blood-gas analysis- performed with a blood gas and lactate analyzer (Stat 7 analyzer, Nova biomedical, Waltham, Massachusetts, USA) .

Storage of serum - Serum for further analyses was placed into plastic, air-tight storage tubes and stored at –70°C. Serum for each dog was separated into 0.5ml aliquots so that thawing and refreezing could be minimised for each sample. At least two such 0.5 ml samples were stored for each dog.

Parasitaemia – blood collected in the EDTA collection tube was also used to determine percent parasitaemia. The blood was used to make a blood smear which was air-dried and stained with CAMs Quick(C.A. Milsch, Krugersdorp,South Africa). The smear was then examined using a 100X oil immersion lens. Red blood cells were counted in several fields to determine the approximate number of cells per field. The results of this calculation were used to approximate the number of fields required to evaluate 8000 red blood cells and then that number of fields was used to calculate parasitaemia. Parasitaemia was expressed as the percentage of red blood cells containing *Babesia* parasites.

5.3.3 Determination of Serum Tumour Necrosis Factor Concentrations

Assessment of the effects of serum discolouration on the TNF bioassay

Canine babesiosis is a disease characterised by severe haemolysis, which can result in severely discoloured serum samples. The TNF assay used in this study is based on a colour change and a difference in light absorbance. Therefore there is the potential that haemolysis could interfere with the assay and result in inaccurate results. To evaluate the effects of haemolysis on light absorbance, a crude solution of haemoglobin was prepared by haemolysing 2 ml of canine blood (haemolysis was achieved by freezing and thawing the sample, followed by centrifugation). The serum containing the haemoglobin was then separated from the cells. A sample of the serum was placed into a well of a 96-well tissue culture plate and serially diluted with saline at a ratio of 1:2 to make a further 3 dilutions. These dilutions were considered to be within the range

expected in severe haemolysis. The light absorbance was then read in duplicate at 490nm and the results were averaged (Table 3).

Absorbance was considerable, with readings ranging from 0.848 to 2.876. To give some perspective to these values, control samples were included on the same plate. These consisted of culture medium alone (light absorbance of 0.514) and medium plus 40 000 WEHI cells/well (light absorbance of 0.969). It was therefore concluded that haemoglobin could interfere with the proposed bioassay at high concentrations as it had a light absorbance reading significantly above that of culture medium plus cells.

To counter this problem it was necessary to use a modified technique of washing wells after incubation of the samples with the WEHI cells, to remove haemoglobin. This was achieved by gently removing 100µl of medium per well after the cells had settled (the total volume of the well was 200µl), and then adding 100µl of fresh medium. Cells were then allowed time to settle again (about 5 minutes) and the process was repeated once. Light absorbance readings were then repeated and the results compared favourably with the controls. Cell counts were also performed on washed wells and control wells that were not washed, to ensure cells were not removed during the process. The results showed that cells were not removed during the washing process. It was concluded that this procedure was therefore effective for removing interfering pigments from the well without removing cells.

A similar evaluation was performed to evaluate the effect of bilirubin on light absorbance. Light absorbance was measured using grossly yellow serum samples from four separate dogs with severe jaundice (Table 4). Sample readings were duplicated and the results are the average of both duplicates. The bilirubin did not interfere significantly with light absorbance with the most icteric sample showing a value of 0.923. This value was not within a significant range of absorbance that would cause interference with the assay.

Bioassay

The complete bioassay method is attached as Appendix II. What follows is a summary: WEHI-164 cells[a] were propagated in RPMI 1640 with 25mM HEPES and L-glutamine (Gibco BRL, Grand Island, New York), plus 50 µg of gentamicin (Gibco BRL) per ml and 10% foetal bovine serum (Gibco BRL). Cells were incubated at 37°C with 5% CO_2 for 72 hours and were then scraped off for further propagation or tests. TNF was assayed in 96-well flat-bottomed plates (See Figure 1 for plate layout). Cells were made up to a concentration of 400 000 cells/ml (20µl of Actinomycin D (1mg/ml)(Sigma) was then added per 10ml of the cell solution) and 100 µl of this solution was added to the test wells resulting in 40 000 cells per well.

20µl of serum sample and 80µl of culture medium was then added to each test well resulting in a 1:10 dilution. Samples were performed in duplicate. A duplicate dilution series of recombinant canine TNF[b] (rcTNF) ranging in final concentration from 100 ng/ml to 3,374 ng/ml served as a standard reference range.

Plates were then incubated overnight at 37°C with 5% CO_2. The following day the light absorbance of each well was evaluated at 490 nm. This was to determine if any samples contained haemoglobin at a level that would interfere with the assay. If any well had a light absorbance of greater than 0,250 then all wells on the plate were treated with the washing process previously. Forty microlitres of MTS/PMS (Sigma) was then added to each well and the plate was incubated for a further 4 hours. Final light absorbance readings were then performed at 490 nm.

A standard curve of TNF concentration (ng/ml) was produced using the results from the rcTNF standards. Sample TNF concentrations were calculated using regression analysis. All standards and samples were assayed in duplicate. The average was used to calculate final results expressed as nanograms of TNF per millilitre.

---

[a] American Type Culture Collection (ATCC CRL 1751) kindly donated by Ian Clark, Division of Biochemistry and Molecular Biology, Australian National University, Canberra, Australia.

[b] Kindly donated by Keith Zucker, Division of Transplant Surgery, University of Miami School of Medicine, Miami, Florida, USA

5.4 DATA ANALYSIS

5.4.1 General

Dr. Daniel Polakow, a statistician at the Department of Mathematics and Statistics, University of Cape Town, performed the statistical analyses.

5.4.2 Serum TNF concentrations in relation to study group

When the frequency distribution of the raw TNF values were studied, it was found to be skewed to the right (Figure 2). The raw TNF distribution was tested against a null normal distribution to see whether it differs significantly from normality. The Kolmogorov-Smirnov goodness-of-fit test was used for this procedure. As TNF was to be compared between the different study groups, it was necessary to transform the TNF variable into a distribution that came closer to approximating the normal distribution, allowing the use of parametric statistical procedures.

The raw TNF distribution was log transformed and again tested against a null normal distribution using the same test (Table 5). The results from the Kolmogorov-Smirnov test indicated that the distribution of log(TNF) did not differ significantly from a normal distribution. The histogram of log(TNF) (Figure 3) showed that the frequency distribution of log(TNF) approximates normality[a].

It was then necessary to enter into a formal test of the mean differences in TNF levels between the five groups. The techniques used were the analysis of variance (ANOVA), the LSD test and the Duncan test. The differences between groups were also assessed using non-parametric analogies (i.e. the Kruskal-Wallis test).

Evaluating the overall TNF results it was necessary to assess whether or not the different groupings have statistically different TNF levels. To clearly assess the statistical methods used to differentiate between groups (Groups I-III) two questions should be considered:

---

[a] When utilising parametric procedures (Pearson r, ANOVA, multiple-contrast tests) the logged variable log(TNF) is used. This treatment conforms to how TNF has been analysed in other studies[88].

Firstly, should parametric or non-parametric testing methodologies be used? It is understood that parametric statistical tests are superior (more likely to detect differences and less likely to commit Type I error) to non-parametric tests, but only if the critical assumption underlying such tests are met, namely, the condition of normality of the response variable. Given that we have such knowledge, as determined through the Kolmogorov-Smirnov test on the log transformed variable (TNF), it would not be prudent to resort to non-parametric testing where the probability of a Type I error is an immediate concern.

Secondly is there any *a priori* basis for expecting TNF levels to differ between groups and how? If there is no reason to expect differences, then one ought to simply consider two-tailed test methodology. If however, we have good reason (theoretical or other) to believe in the directionality of the response of TNF as a nominal function of the groupings (where more severe cases ought to be associated with higher TNF levels), then we are justified in using one-tailed testing.

5.4.3 TNF concentrations in relation to survival

The Mann-Whitney U test was used to compare TNF concentrations between dogs that survived and did not survive.

5.4.4 TNF concentrations in relation to rectal temperature

The relationship between rectal temperature and serum TNF concentrations was evaluated using three correlation statistics (Pearson correlation r, Spearman r_s and Kendall's Tau τ). The Kruskal-Wallis test was also used to determine whether temperatures differed between groups I (mild babesiosis), II (severe babesiosis) and III (complicated babesiosis).

5.4.5 TNF concentrations in relation to parasitaemia

The relationship between serum TNF concentrations and parasitaemia was evaluated using three correlation statistics (Pearson correlation r, Spearman r_s and Kendalls Tau

τ). The two way ANOVA was also used to determine the relationship between TNF concentrations and parasitaemia within each group and overall.

5.5 TABLES

Table 3 Light absorbance of haemolysed serum samples

Sample	Light Absorbance
Serum sample 1	2.876
Dilution 1	1.385
Dilution 2	1.043
Dilution 3	0.848

Optical density measured at 490nm

Table 4 Light absorbance of jaundiced serum samples

Sample	Light Absorbance
Icteric sample 1	0.923
Icteric sample 2	0.769
Icteric sample 3	0.688
Icteric sample 4	0.701

Optical density measured at 490nm

Table 5 TNF and log-transformed TNF tested for normality using the Kolmogorov-Smirnov goodness of fit test

Variable	d_{max}	χ^2 *statistic*	p-value
TNF	0.255	77.393	0.005
Log(TNF)	0.083	2.034	0.157

5.6 FIGURES

	1	2	3	4	5	6	→
A	Blank	Blank	Sample 1	Sample 1			
B	Medium	Medium	Sample 2	Sample 2			
C	Cells only	Cells only	Sample 3	Sample 3			
D	Standard 1	Standard 1	↓	↓			
E	Standard 2	Standard 2					
F	Standard 3	Standard 3					
G	Standard 4	Standard 4					
H	Standard 5	Standard 5					

Figure 1 Plate Diagram showing the layout of the wells

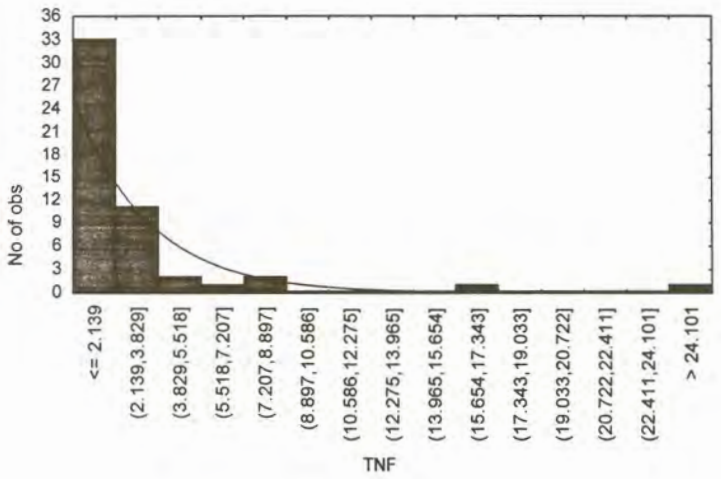

Figure 2 Frequency Distribution of TNF concentrations (ng/ml)

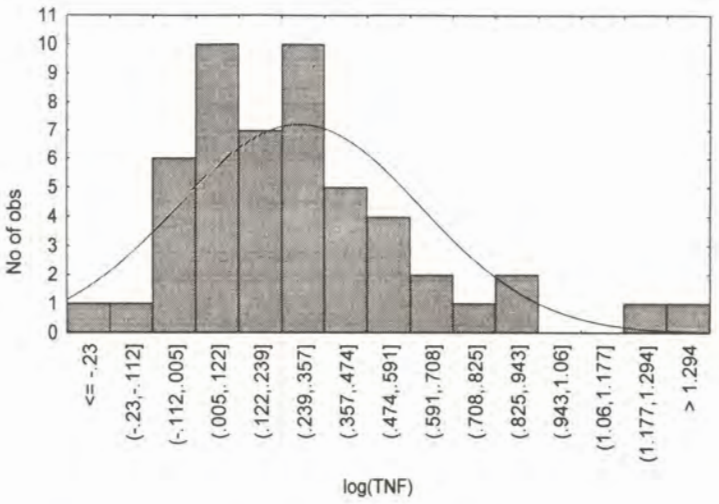

Figure 3 Frequency distribution of log TNF concentrations (ng/ml)

6 RESULTS

6.1 STUDY GROUPS

During the period of investigation, 31 dogs that were presented to the OVAH with babesiosis were suitable for inclusion into the study. The dogs were categorised into Groups I (mild uncomplicated babesiosis), II (severe uncomplicated babesiosis) and III (complicated babesiosis) according to the criteria discussed previously. This resulted in 10 dogs in Group I, 9 dogs in Group II and 12 dogs in Group III (Table 6). The remaining dogs included in the study were selected from the OVAH, Department of Surgery Clinic (10 dogs for Group IV - Endemic controls) and from Biocon research laboratory (10 dogs for Group V – Parasite free controls).

6.2 GENERAL OVERVIEW

In all studies concerning canine TNF that were reviewed, baseline TNF concentrations in healthy dogs have not been reported as an absolute concentration[20,34,55,67,74,80,97]. Results have either been reported as U/ml[20,34,55,74] (with a Unit being the concentration of TNF that causes 50% activity when compared to a standard concentration range of TNF), or as optical densities recorded during the bioassay[67,97]. The results of this study indicate that a normal concentration of TNF in healthy parasite-free dogs (Mean [TNF] of Group V) is 1.38 ng/ml (Standard Deviation 0.60 ng/ml, Standard Error 0.19, n = 10, 95% confidence interval = 1.38 ± 1.2).

The results indicate that 3 dogs in Group I, 4 dogs in Group II and 5 dogs in Group III had elevated TNF concentrations when compared to normal values (0.18-2.58 ng/ml).

An interesting and note-worthy finding was that two of three dogs with co-existing hypoglycaemia had markedly high TNF concentrations (more than ten times normal and much higher than any other dog in the study).

Two dogs in Group IV also had significantly elevated TNF values. These elevated values were surprising as these were outwardly healthy animals with no clinical or laboratory evidence of disease.

Most dogs that died were in Group III (n=7), with a single death in Group II. Of the dogs that died, only three had significantly elevated TNF concentrations. Three of the dogs that did not survive (cases 22,26,31) were euthanased *in extremis* by the attending clinician on ethical grounds. These dogs were still included in the non-survival group as it was considered that they would not have survived if medical treatment was continued.

6.3 SERUM TUMOUR NECROSIS FACTOR CONCENTRATIONS IN DOGS WITH BABESIOSIS

The TNF concentrations and survival data for the study groups are shown in Table 6. Box-plots of TNF (Figure 4) and log(TNF) (Figure 5) reveal that, in general, the means of TNF (and log(TNF)) levels increased with the severity of babesiosis.

The two control groups appeared to have approximately similar or slightly decreased average log(TNF) levels relative to Group I (mild uncomplicated) and Group II (severe uncomplicated). Summary statistics (means, valid sample size and standard deviations) for log(TNF) for all five experimental groups (and pooled) are presented in Table 7.
It is important to note the degree of variation in TNF about the means. Here, it can be seen that the variation of Group III is greater than Groups I and II (graphically depicted in Figure 6 as a scatter plot). The control groups demonstrated levels of variation that were comparable with Groups I (mild uncomplicated) and II (severe uncomplicated).

Two-tailed ANOVA testing showed a p-value of 0.089, with a one-tailed approach showing a p-value of 0.045 (at the 0.05 alpha level of significance). ANOVA test results are displayed in Table 8.

Results from the LSD test are presented in Table 9, with statistically significant results appearing in boldface. Here, Group II (severe uncomplicated) is seen to differ significantly from Group V (parasite-free control), and Group III (complicated) is seen to differ significantly from Group V (parasite-free control). No other statistically significant differences were found.

Results from the Duncan test are presented in Table 10. Here, Group II (severe uncomplicated) is again seen to differ significantly from Group V (parasite-free control), and Group III (complicated) is also seen to differ significantly from Group V

(parasite-free control). No other statistically significant differences were found. The results from the Duncan test and from the LSD test were in agreement with one another with the Duncan model applying slightly stricter criteria.

The Kruskal-Wallis test showed no significant differences between groups (Kruskal-Wallis, H = 7.548, N = 51, P = 0.110) (Table 11).

6.4 SERUM TUMOUR NECROSIS FACTOR CONCENTRATIONS IN DOGS THAT SURVIVED AND DID NOT SURVIVE

Survival data is presented in Table 6. Most dogs that did not survive (7 of 8 mortalities) were in Group III (complicated), with the remaining death in Group II. Results from the statistical contrast of TNF levels by survivorship (Group I through Group III) revealed no significant difference (Mann-Whitney U test, Rank Sum of survivors = 378, Rank Sum of death/euthanased individuals = 118, U = 82, P = 0.652). The average TNF levels (and degree of variation) about the surviving/dying group are represented graphically in Figure 7.

6.5 SERUM TUMOUR NECROSIS FACTOR CONCENTRATIONS IN RELATION TO RECTAL TEMPERATURE

TNF was correlated with rectal temperature (Table 12) with the results of Pearson's correlation r, Spearman r_s and Kendalls Tau τ presented in Table 13. These was no statistically significant relationship between TNF and temperature. The graphical relationship between log(TNF) and temperature is presented in Figure 8. In addition, the Kruskal-Wallis test reveals no statistically significant temperature difference between groups (Kruskal-Wallis, H = 1.295, P = 0.523) (Table 14).

It is evident however, that complicated babesiosis (and elevated TNF levels) may be associated with a low rectal temperature with cases 21,23 and 31 illustrating this point.

A box-plot of temperature within Groups I through III is presented in Figure 9.

6.6 SERUM TUMOUR NECROSIS FACTOR CONCENTRATIONS IN RELATION TO PARASITAEMIA

Some cases had a zero parasitaemia even though they were confirmed as positive babesiosis cases on peripheral capillary blood-smears. This may be due to parasites sequestering in peripheral blood vessels but not appearing frequently in central venous blood (where parasitaemia was calculated).

TNF was correlated with parasitaemia (Table 15) with the results of Pearson correlation r, Spearman r_s and Kendalls Tau τ presented in Table 15. There is no statistically significant or rigorous relationship between TNF concentrations and parasitaemia when the data is evaluated within groups (Table 16).

When the data is pooled and all cases are treated as a single group, the results of Pearsons correlation showed a significant positive relationship between the two parameters (Pearson's r = 0.55, p = 0.003, N = 31), indicating that higher levels of parasitaemia were associated with higher levels of TNF. Figure 10 shows the results graphically and it can be observed that there is an outlier result (at Log(TNF) = 1.2 and parasitaemia = 2.4, Case 27) that may have influenced the statistical relationship between TNF and parasitaemia. The statistical evaluation was re-run with the outlier removed and showed a p = 0.07. This is still very close to the 0.05 level of significance. A two-way ANOVA was then conducted to see whether the variation in log TNF was better apportioned when both parasitaemia and study group were treated simultaneously. There was no statistically significant interaction between parasitaemia and study group (p = 0.421) in the two-way ANOVA, and the model was consequently rerun without including the interaction term. The results of the two-way ANOVA, excluding the interaction term, showed that parasitaemia emerged as the dominant covariate, whereas study group displayed no statistically significant effect on partitioning the variation of log TNF in the presence of parasitaemia. (Table 17).

6.7 TABLES

Table 6 Serum TNF concentrations, survival data and specific complications for dogs in the study groups

Group I Mild uncomplicated babesiosis		**Group II** Severe uncomplicated babesiosis		**Group III** Complicated babesiosis		**Group IV** Healthy dogs from endemic area		**Group V** Healthy dogs from parasite-free environment	
Case Number	**[TNF] (ng/ml)**	**Case Number**	**[TNF] (ng/ml)**	**Case Number**	**[TNF] (ng/ml)**	**Case Number**	**[TNF] (ng/ml)**	**Case Number**	**[TNF] (ng/ml)**
1	0.94	11	**3.54**	15 H	**25.79**	36	**3.09**	46	2.03
2	0.85	12 ☠	**7.46**	21 H,P	**2.96**	37	1.64	47	2.49
3	1.96	13	**3.04**	22 ☠,A,P,C	0.86	38	2.02	48	1.04
4	**3.19**	14	2.48	23 P	**5.74**	39	1.41	49	1.88
5	2.16	16	1.36	24 P,R	1.85	40	1.23	50	1.10
6	**2.86**	17	**4.36**	25 R	1.32	41	1.00	51	1.45
7	1.46	18	2.23	26 ☠,H	**16.34**	42	1.27	52	0.91
8	**2.70**	19	1.79	27 ☠,A,P,R	1.02	43	**7.40**	53	0.45
9	1.11	20	1.76	28 ☠,C	1.11	44	1.99	54	1.09
10	0.67			29 ☠,C,R	1.42	45	1.66	55	1.31
				30 ☠,A,C,R	1.01				
				31 ☠,C	**4.67**				

Results above normal range (1.38 ± 1.2 ng/ml) appear in bold face

☠ = Dogs that did not survive

H = Hypoglycaemia

A = Acute Renal Failure

P = Pulmonary Oedema

C = Cerebral Babesiosis

R = Red Babesiosis

L = Liver Disease

Table 7 Average TNF values and standard deviations of groups I to V

Group	Mean of log(TNF)	Sample Size	Standard Deviation of log(TNF)
I - mild uncomplicated	0.196	10	0.242
II – severe uncomplicated	0.435	9	0.229
III – complicated	0.421	12	0.495
IV – healthy (endemic)	0.275	10	0.249
V – healthy (laboratory)	0.096	10	0.210
Pooled	0.287	51	0.331

Table 8 ANOVA test comparing mean log TNF concentrations of groups I to V

Effect	Df effect	MS effect	Df Error	MS Error	F	P-value (two-tailed)	P-value (one-tailed)
Groupings	4	0.216	46	0.100	2.156	0.089	0.045

Table 9 LSD Test comparing mean log TNF concentrations (ng/ml) between groups I to V

	Group I Mild uncomplicated babesiosis	**Group II** Severe uncomplicated babesiosis	**Group III** Complicated babesiosis	**Group IV** Healthy dogs from endemic area	**Group V** Healthy dogs from parasite free area
Group I		0.106	0.102	0.577	0.486
Group II			0.921	0.276	**0.024**
Group III				0.285	**0.021**
Group IV					0.213
Group V					

Statistically significant results appear in bold-face.

Table 10 Duncan Test comparing mean log TNF concentrations (ng/ml) between different groups

	Group I Mild uncomplicated babesiosis	**Group II** Severe uncomplicated babesiosis	**Group III** Complicated babesiosis	**Group IV** Healthy dogs from endemic area	**Group V** Healthy dogs from parasite free area
Group I		0.127	0.136	0.575	0.484
Group II			0.921	0.289	**0.035**
Group III				0.304	**0.038**
Group IV					0.238
Group V					

Statistically significant results appear in bold-face.

Table 11 Kruskal-Wallis test comparing mean log TNF concentrations (ng/ml) between groups I to V. (Kruskal-Wallis test: $H_{(4,\ N=51)} = 7.548306\ p = 0.1096$)

Nonparametric Statistics Dependent: TNF			
	Code	**Valid N**	**Sum of Ranks**
Group I	1	10	232.00
Group II	2	9	322.00
Group III	3	12	334.00
Group IV	4	10	261.00
Group V	5	10	177.00

Table 12 Rectal temperature data of dogs in Groups I to III

Group I Mild uncomplicated babesiosis		**Group II** Severe uncomplicated babesiosis		**Group III** Complicated babesiosis	
Case Number	**Rectal temperature (°C)**	**Case Number**	**Rectal temperature (°C)**	**Case Number**	**Rectal temperature (°C)**
1	39.5	11	39.9	15	40.2
2	NR	12	40.2	21	37.2
3	NR	13	40.4	22	40.5
4	39.9	14	39.5	23	36.6
5	38.6	16	38.9	24	40.3
6	41	17	NR	25	41
7	NR	18	40.5	26	40
8	41.4	19	40.5	27	NR
9	40.1	20	40.5	28	39.4
10	NR			29	39.6
				30	40
				31	35
Mean (standard deviation)	40.08 (1.015)		40.05 (0.586)		39.07 (1.919)

NR = Not recorded due to unforeseen circumstances

Table 13 Results from statistical procedures comparing serum TNF concentrations and rectal temperature

Correlation of log(TNF) and temperature	Correlation coefficient	P-value	Valid N
Pearson *r*	- 0.171	0.414	25
Spearman r_s	- 0.131	0.533	25
Kendall τ	- 0.092	0.521	25

Table 14 Kruskal-Wallis test comparing temperatures between groups I, II and III (Kruskal-Wallis test: H(2, N=25) = 1.295092 p = 0.5233)

Nonparametric Statistics Dependant: TEMPERATURE			
	Code	**Valid N**	**Sum of Ranks**
Group I	1	6	84.50
Group II	2	8	118.00
Group III	3	11	122.50

Table 15 Percentage parasitaemia values for groups I to III

Group I Mild uncomplicated babesiosis		**Group II** Severe uncomplicated babesiosis		**Group III** Complicated babesiosis	
Case Number	**Parasitaemia**	**Case Number**	**Parasitaemia**	**Case Number**	**Parasitaemia**
1	0.2748	11	0.5077	15	0.7752
2	0.0446	12	0.0357	21	0.6427
3	0	13	0.4065	22	0.1526
4	0.2125	14	0.0248	23	0.7696
5	0.1481	16	0.0126	24	0.2533
6	0.7204	17	0	25	2.426
7	0.0348	18	0.2083	26	0.6328
8	1.2882	19	0.2738	27	1.3267
9	0.05126	20	0.7353	28	NA
10	0			29	NA
				30	NA
				31	NA
Mean(Standard deviation)	0.27(0.415)		0.25(0.261)		0.87(0.722)

NA= not available due to unforeseen circumstances

Table 16 Results from statistical procedures correlating serum TNF concentrations and parasitaemia within groups I to II and pooled data (groups I to III treated as one group)

Correlation of log(TNF) and parasitaemia	**Correlation coefficient**	**P-value**	**Valid N**
Group I Mild uncomplicated babesiosis			
Pearson *r*	0.55	0.103	10
Spearman r_s	0.584	0.765	10
Kendall τ	0.405	0.103	10
Group II Severe uncomplicated babesiosis			
Pearson *r*	-0.25	0.513	9
Spearman r_s	-0.167	0.668	9
Kendall τ	-0.111	0.677	9
Group III Complicated babesiosis			
Pearson *r*	0.66	0.657	8
Spearman r_s	0.524	0.182	8
Kendall τ	0.357	0.216	8
Pooled data (Groups I-III)			
Pearson *r*	**0.55**	0.003	27
Spearman r_s	0.261	0.189	27
Kendall τ	0.177	0.194	27

Statistically significant results appear in bold-face.

Table 17 ANOVA Test comparing mean log TNF concentrations and parasitaemia of pooled data

Effect	Df effect	MS effect	Df Error	MS Error	F	P-value (two-tailed)	P-value (one-tailed)
Parasitaemia	1	0.857	23	0.098	8.751	0.007	0.004
Group	2	0.151			1.543	0.235	0.118

6.8 FIGURES

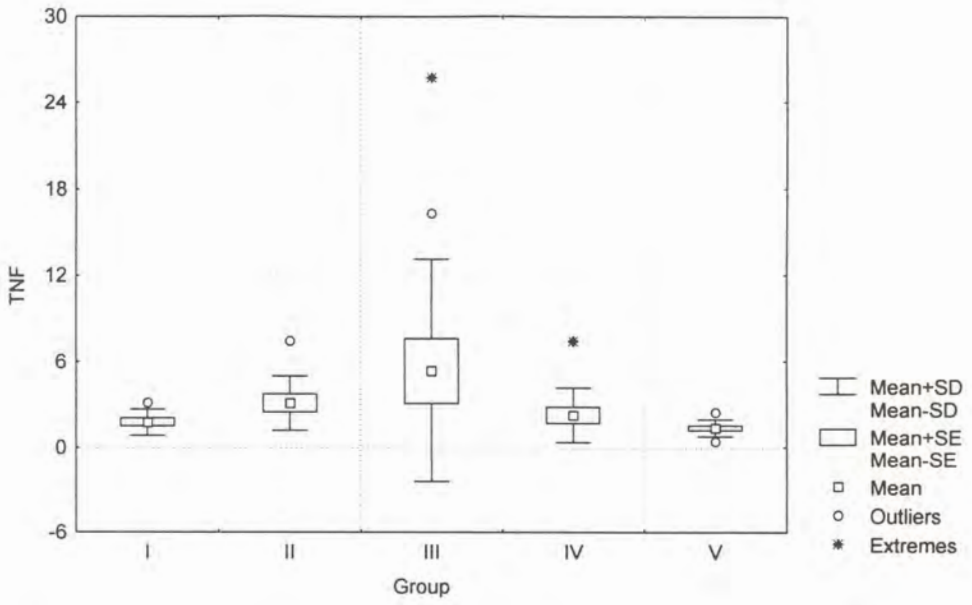

Figure 4 Mean and standard deviation TNF values for groups I-V

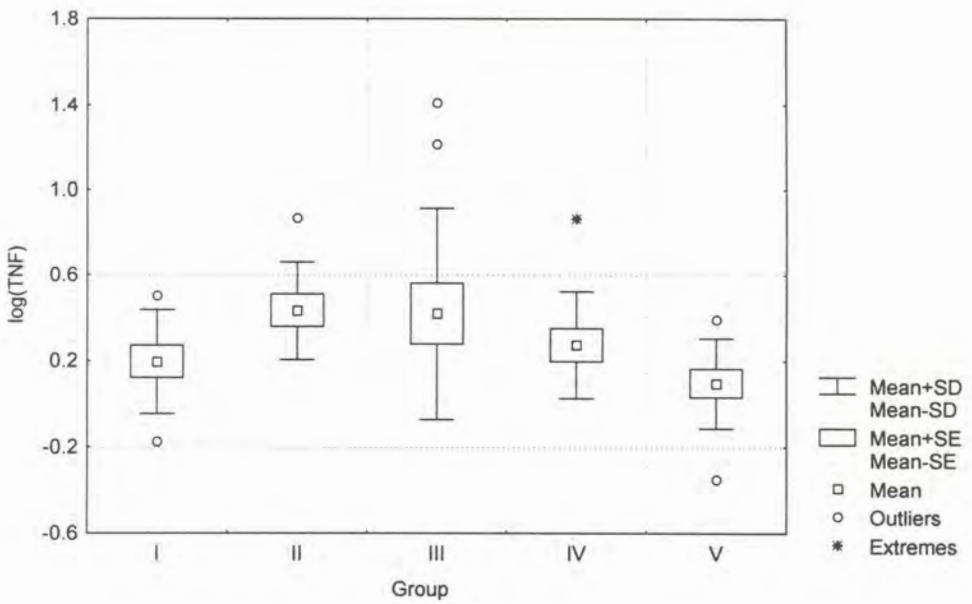

Figure 5 Mean and standard deviation log TNF values for groups I-V

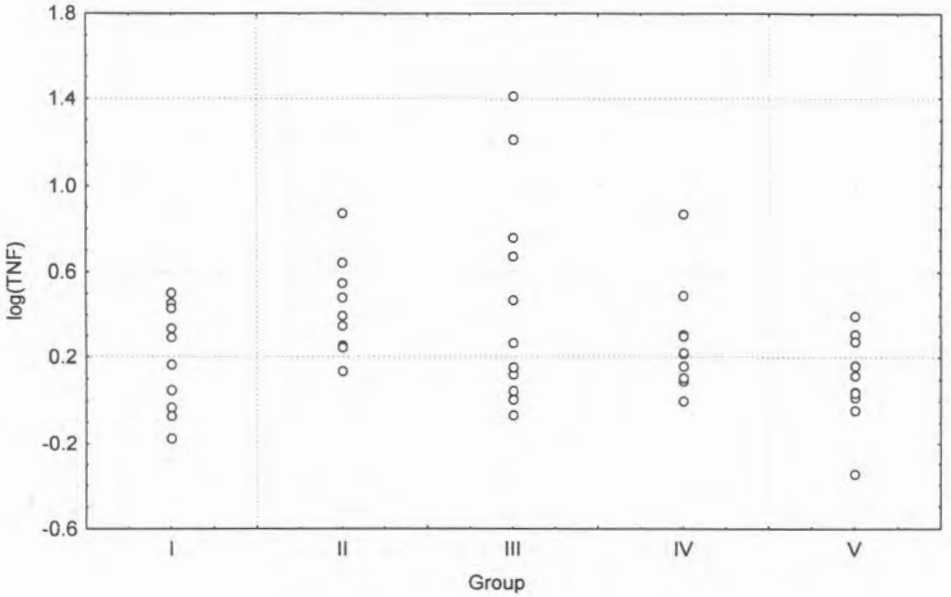

Figure 6 Log TNF values for groups I-V

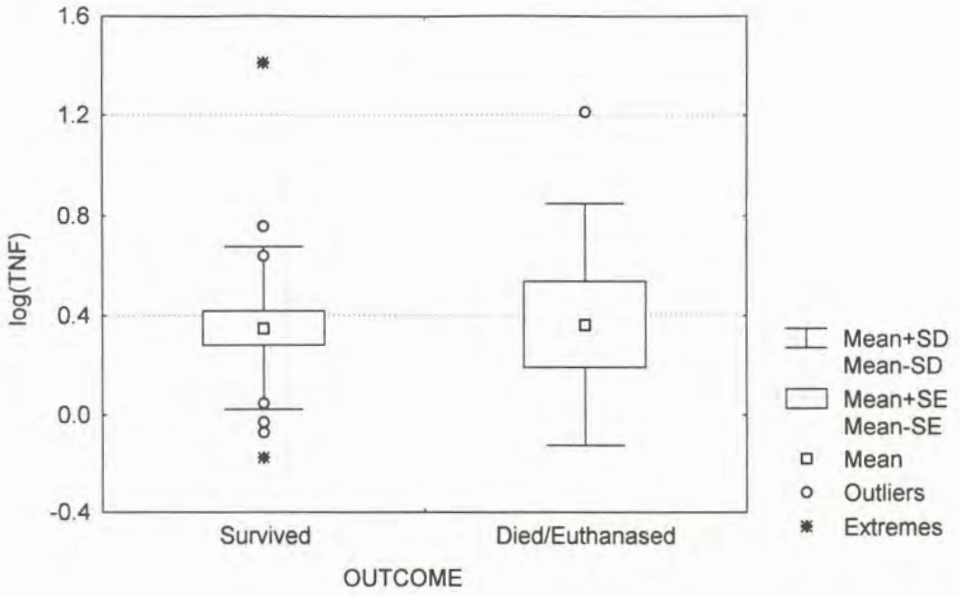

Figure 7 Average TNF values and degree of variation of survival group and non-survival group within Groups I, II and III

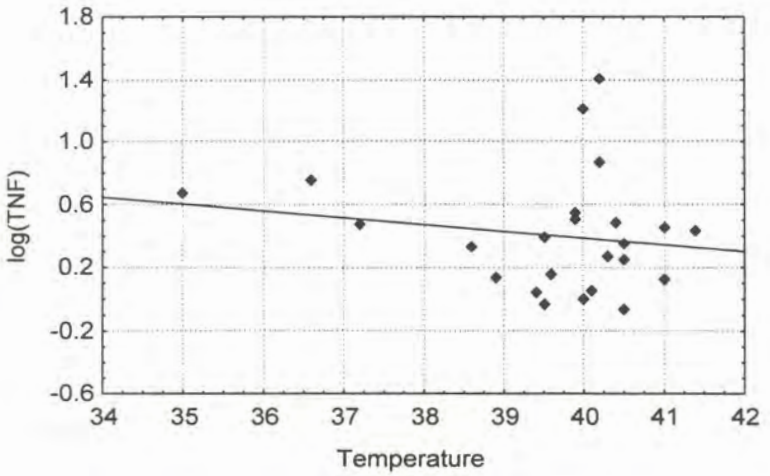

Figure 8 Relationship of log TNF to rectal temperature

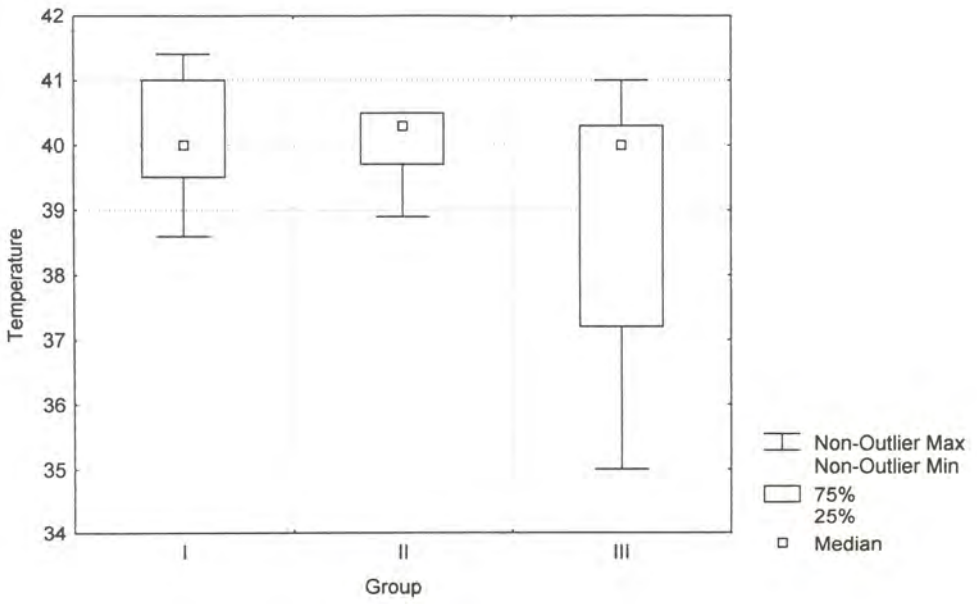

Figure 9 Median and percentiles of temperature within groups I to III

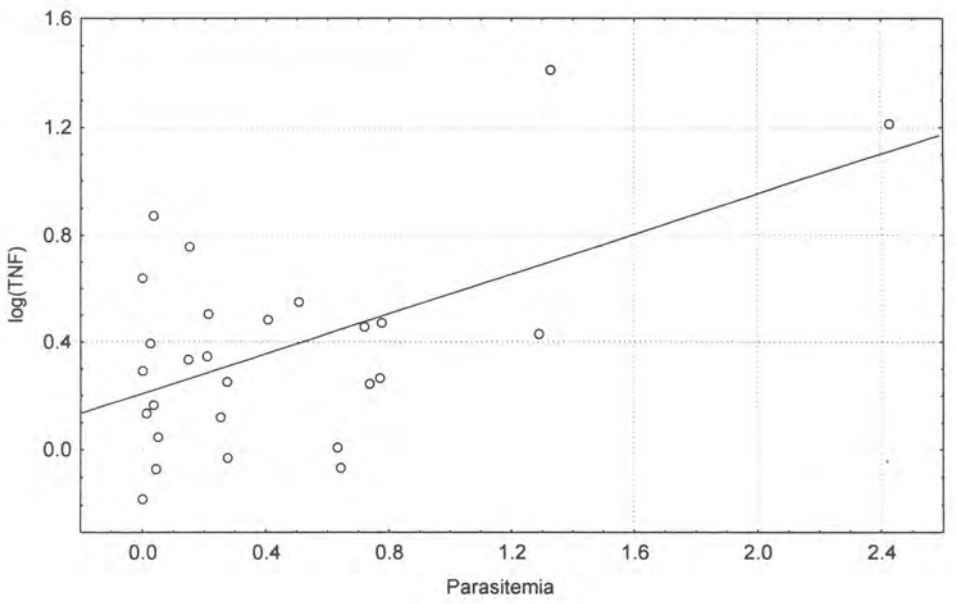

Figure 10 Relationship of log TNF to parasitaemia

7 DISCUSSION

7.1 GROUPING PROCEDURE

Assessment of the grouping criteria revealed that they were not ideal, especially the criteria used to detect the presence of liver disease. One dog (Case 12) that had severe liver pathology on post-mortem examination (and a high serum TNF concentration) was not included in Group III, even though there were high serum liver enzyme activities (ALT=77, ALP=1008), since it was not clinically icteric. Post-mortem examination of this case showed moderate icterus and histopathological examination of multiple organs revealed hepatosis, cholestasis, cardiac echymoses, nephrosis, ascites, hydrothorax and hydropericardium. This indicated that case 12 had pathology that could have placed it into the complicated group. Case 15 also had elevated ALT levels and was clinically icteric but the ALP was normal, therefore disqualifying it from liver disease. Another case (case 21) also had markedly elevated ALT activity (and ultrasonographic evidence of liver disease), but was also not clinically icteric and had a normal ALP and therefore was not classified as having liver disease. The case did have hypoglycaemia however, and was therefore classified as Group III. The conclusion is that liver disease needs to be evaluated with more sensitive and specific tests in dogs with babesiosis or the classification of liver disease should be modified to include certain serum biochemical evidence only (ALT or ALP) and not the presence of icterus. The other criteria used to group dogs appeared to be acceptable and allowed the classification of dogs into groups which reflected varying severity of disease and which appeared to reflect clinical severity when the mortalities were considered, with 7 of 8 dogs that died being classified as complicated babesiosis (Group III) and the remaining mortality (case 12 as discussed above) in the severe babesiosis group (Group II).

7.2 TUMOUR NECROSIS FACTOR BIOASSAY

Serum TNF concentrations in the dogs were successfully assayed using a bioassay technique. The bioassay method relies on a colour change to determine the concentration of TNF, which proved to be problematic in this situation, as the serum samples from canine babesiosis cases are often grossly discoloured due to haemolysis or severe icterus. The preliminary assays performed showed that haemoglobin concentrations in the range expected in haemolysed samples, could potentially interfere

with the assay. A washing technique was therefore developed that removed the discolouration from the samples but did not affect the results.

Previous canine TNF bioassays have had to rely on human TNF for use as a standard[55]. This has led some authors to use the term "TNF-like activity" when reporting the TNF concentration[55]. This study made use of recombinant canine TNF, which allows the use of the term TNF, and not "TNF-like activity", and also potentially gave more creditable results. In all the studies concerning canine TNF reviewed, baseline TNF concentrations in healthy dogs have not been reported as an absolute concentration[34,55,67,74,80,97]. The results of this study indicate that a normal reference range of TNF in healthy parasite-free dogs (derived from Group V) is 1.38 ± 1.2 ng/ml at the 95% confidence limit.

7.3 SERUM TUMOUR NECROSIS FACTOR CONCENTRATIONS IN RELATION TO PARASITAEMIA AND DISEASE SEVERITY

The main objective of this study was to determine whether serum TNF concentrations are elevated in dogs with babesiosis and to determine if TNF plays a role in the pathophysiology of the disease. Human malaria is often referred to in this study for two reasons: firstly it has been found that TNF plays a significant role in the pathophysiology of malaria and secondly it has been proposed that canine babesiosis may be useful as a model for the investigation of human malaria. This proposal will be strengthened if it could be shown that TNF plays a role in the pathophysiology of both diseases.

The very high TNF in dogs with hypoglycaemia was probably of significance. These dogs (Cases 15,21,26) had high values, with cases 15 and 26 having exceptionally high values (the mean TNF values of hypoglycaemic dogs was 15.03 ng/ml compared to a mean of 2.32 ng/ml for other sick dogs without hypoglycaemia). Hypoglycaemia has not been previously reported in dogs with babesiosis (other than in a conference proceedings[44]) and is a potentially important finding in view of the hypoglycaemia associated with malaria in humans. Malarial hypoglycaemia is correlated with a higher mortality in humans, especially in pregnant women and children[48,51,84]. It has also been proposed that it may be caused by high TNF levels[51], although one study showed no

correlation between TNF and hypoglycaemia in mice[30]. Other proposed mechanisms for hypoglycaemia include anti-malarial drugs such as quinine[30], hyperinsulinaemia[30], starvation[48] or alterations in glucose metabolism and kinetics[48]. The underlying cause of hypoglycaemia in this study remains undetermined, but all blood samples were obtained before treatment, thus excluding the effects of drugs. The treatment of hypoglycaemia associated with human malaria does not increase survival, indicating that it is an indicator of a severe physiological disturbance that results in mortality[48]. Dogs with babesial hypoglycaemia also reflect this situation with hypoglycaemic dogs having a lower mean haematocrit, higher mortality rate, higher mean lactate, higher mean parasitaemia and more severe acidosis[44]. It is possible that the hypoglycaemia of babesiosis in dogs is similar to malarial hypoglycaemia and may share a common pathophysiology. Therefore dogs with babesia-related hypoglycaemia may be useful as an experimental model for the investigation of malarial hypoglycaemia.

Cases 36 and 43, in Group IV, had significantly elevated TNF values (especially Case 43). These dogs were clinically and biochemically normal and the elevated TNF values were unexpected. It is difficult to speculate on the cause of these elevated values, but possibly these dogs had an undetected disease or these individuals had an above average basal TNF value.

The TNF results showed there was a statistically significant difference in TNF concentrations between the different groups of sick dogs, with a general trend of increasing mean log(TNF) with increasing severity of disease (Figure 4). More specifically there was a difference between Groups II and V and Groups III and V, thus indicating that dogs with severe and complicated babesiosis have higher serum TNF concentrations than parasite-free dogs. Statistical significance was achieved when the ANOVA test was converted from a two-tailed to a one-tailed test (p-value 0.089 with the two-tail test and 0.045 with the one-tailed test). This suggests that the interpretation should be treated with some caution, however the *a-priori* expectation of elevation in TNF is quite reasonable, justifying the use of a one-tailed approach.

As discussed, case 12 had clinical and post-mortem evidence of complicated babesiosis, but was not included in Group III as it did not meet that group's criteria. To determine the influence of this case on the results, the statistical analysis was performed with this

case moved to Group III. The ANOVA test showed an increased significance between groups with the new grouping values (p-value 0.079 for the two-tailed test and a p-value of 0.039 for the one-tailed test). The LSD and Duncan tests showed significant differences between Groups III and IV but not Groups II and IV.

These results are generally in line with the situation in malaria patients, in which TNF has been associated with disease severity, and suggest that TNF may also be associated with severity of disease in canine babesiosis. These results should not be taken to mean that TNF is the only or direct cause of the pathology associated with babesiosis or malaria, as patients with malaria caused by *Plasmodium vivax* (mild disease) can have much higher levels of TNF than patients with malaria caused by *Plasmodium falciparum* (severe complicated disease)[47,53]. TNF probably plays a role as an intermediary in the production of other harmful substances such as nitric oxide or free oxygen radicals or the interaction between parasitised erthrocytes and the blood vessel wall (sequestration)[53].

7.4 SERUM TUMOUR NECROSIS FACTOR CONCENTRATIONS IN RELATION TO SURVIVAL

When TNF values were compared between survival and non-survival groups, there was no statistically significant association. This result does not rule out a role of TNF in mortality. It has been proposed that, due to the short serum half-life of TNF, the time of blood sampling may not reflect the peak TNF value[72]. An inability to measure TNF in affected compartments may also play a role[72].

7.5 SERUM TUMOUR NECROSIS FACTOR CONCENTRATIONS IN RELATION TO FEVER

The most characteristic feature of malaria is short paroxysms of fever occurring at intervals of 48 hours[47]. The fevers are caused by ruptured malaria-infected red blood cells and an associated elevation of TNF[10,37,52,53], which occurs approximately 45 minutes before the onset of fever[47]. Fever occurs at a certain threshold of parasitaemia (the so-called fever threshold) and parasitaemia then oscillates around this threshold in 48 hour cycles[53]. Initial TNF studies in a canine endotoxaemia model showed that fever is an all-or-none response, with fever only occurring above a certain TNF concentration

threshold[55]. If one couples this to the fact that fever only occurs above a certain parasitaemia threshold, then the conclusion could be drawn that TNF concentrations rise in parallel with parasitaemia levels. One human malaria study showed a significant correlation between plasma TNF levels and body temperature, with higher temperatures associated with higher TNF concentrations[10]. The rectal temperature of the dogs in this study did not show any statistically significant association with TNF concentrations. The results could once indicate the limitations of once-off serum sampling. TNF has a very short half-life *in vivo* and may not have been at a high level at time of sampling or other unmeasured endogenous pyrogens may have been playing a role[7,60,72,77]. Future studies to evaluate the role of TNF in canine babesiosis should consider blood sampling at frequent intervals so as to try and avoid these potential problems.

7.6 SERUM TUMOUR NECROSIS FACTOR CONCENTRATIONS IN RELATION TO PARASITAEMIA

Parasitaemia levels have been shown to be associated with TNF concentrations in a number of malaria studies[11,41,73,88,91]. The theory is that the parasite releases a toxin[52] or ruptured red cells shower the circulation with "toxic" substances[53]. When parasitaemia and TNF were correlated with each other within groups (I, II and III), there was no significant relationship. However, when the sample size was increased by pooling all cases and treating them as a single group, there was a highly significant positive correlation (Pearsons $p = 0.003$) between parasitaemia and serum TNF concentrations. Even with the outlier value removed (as discussed in the results section), the p value still approached the level of significance. This implies that a similar mechanism to malaria may be in operation and that the *babesia* parasite may have the capability to stimulate TNF production.

8 CONCLUSION

The results of this study were encouraging and indicate that canine babesiosis may share a similar pathophysiology to human malaria in terms of TNF being associated with disease severity. Sample size and frequency of sample collection may be areas worth investigating in future studies. One of the most significant findings in this study was the result of very high TNF values in some dogs with hypoglycaemia. This is an important complication in human malaria patients and a better understanding of the underlying pathophysiology may allow more successful treatment. If this finding can be confirmed in further studies, then it may lend further support to the use of canine babesiosis as a model for some of the problems encountered in human malaria research.

9 REFERENCES

1. Allan R, Beattie P, Bate C, Boele Van Hensbroek M, Morris-Jones S, Greenwood B, Kwiatkowski D 1995 Strain variation in tumor necrosis induction by parasites from children with acute falciparum malaria. *Infection and Immunity* 63: 1173-1175
2. Allan R, Rowe A, Kwiatkowski D 1993 *Plasmodium falciparum* varies in its ability to induce tumor necrosis factor. *Infection and Immunity* 61: 4772-4776
3. Basson P, Pienaar 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
4. Bate C, Taverne J, Playfair J H L 1989 Soluble malarial antigens are toxic and induce the production of tumor necrosis factor *in vivo*. *Immunology* 66: 600-605
5. Bazzoni F, Beutler B 1995 How do tumor necrosis factor receptors work? *Journal of Inflammation* 45: 221-238
6. Beutler B, Grau G 1993 Tumor necrosis factor in the pathogenesis of infectious disease. *Critical Care Medicine* 21: S423-S435
7. Beutler B, Milsark I, Cerami A 1985 Cachectin/tumor necrosis factor: production, distribution, and metabolic fate *in vivo*. *The Journal of Immunology* 135: 3972-3977
8. Bone R 1996 Immunological dissonance: a continuing evolution in our understanding of the systemic inflammatory response syndrome (SIRS) and the multiple organ dysfunction syndrome (MODS). *Annals of Internal Medicine* 125: 680-687
9. Bone R, Balk R, Cerra F, Dellinger R, Fein A, Knaus W, Schein R, Sibbald W 1990 Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM consensus conference committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 101: 1-65
10. Brandts C, Ndjave M, Graninger W, Kremsner P 2001 Effects of paracetamol on parasite clearance time in *Plasmodium falciparum* malaria. *Lancet* 350: 704-709
11. Butcher G, Garland T, Ajdukiewicz A, Clark I 1990 Serum tumor necrosis factor associated with malaria in patients in the Solomon Islands. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 84: 658-661

12. Caro H N, Sheikh N A, Taverne J, Playfair J H L, Rademacher T W 1996 Structural similarities among malaria toxins, insulin second messengers, and bacterial endotoxin. *Infection and Immunity* 64: 3438-3441
13. Carswell E A, Old L J, Kassel R L, Green S, Fiore N, Williamson B 1975 An endotoxin induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Science* 72: 3666-3670
14. Chang S, Hsu H, Perng R, Shiao G, Lin C 1991 Significance of biochemical markers in early detection of canine lung allograft rejection. *Transplantation* 51: 579-584
15. Clark I 1982 Correlation between susceptibility to malaria and babesia parasites and to endotoxicity. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 76: 4-7
16. Clark I, Chaudri G, Cowden W 1989 Roles of tumour necrosis factor in the illness and pathology of malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 83: 436-440
17. Clark I, Jacobson L 1998 Do babesiosis and malaria share a common disease process? *Annals of Tropical Medicine and Parasitology* 92: 483-488
18. Clark I, Rockett K 1994 The cytokine theory of human cerebral malaria. *Parasitology Today* 10: 410-411
19. Contamin H, Fandeur T, Rogier C, Bonnefoy S, Konate L, Trape J, Mercereau-Puijalon O 1996 Different genetic characteristics of *Plasmodium falciparum* isolates collected during successive clinical malaria episodes in Senegalese children. *American Journal of Tropical Medicine and Hygiene* 54: 632-643
20. Coran A, Drongowski R, Paik J, Remick M 1992 Ibuprofen intervention in canine septic shock: reduction of pathophysiology without decreased cytokines. *Journal of Surgical Research* 53: 272-279
21. Corti A, Poiesi C, Merli S, Cassani G 1994 Tumor Necrosis Factor (TNF)α quantification by ELISA and bioassay: effects of TNFα-soluble TNF receptor (p55) complex dissociation during assay incubations. *Journal of Immunological Methods* 177: 191-198
22. Cuddon P 1996 Metabolic encephalopathies. *Veterinary Clinics of North America: Small Animal Practice* 26: 893-923

23. Damas P, Canivet J, De Groot D, Vrindts Y, Albert A, Franchimont P, Lamy M 1997 Sepsis and serum cytokine concentrations. *Critical Care Medicine* 25: 405-412

24. de Kossodo S, Houba V, Grau G, WHO Collaborative Study Group 1995 Assaying tumor necrosis factor concentrations in human serum. A WHO International Collaborative study. *Journal of Immunological Methods* 182: 107-114

25. Deitch E 1997 Multiple organ failure pathophysiology and potential future therapy. *Annals of Surgery* 216: 117-134

26. Drobatz K, Concannon K 1994 Noncardiogenic pulmonary edema. *Compendium on Continuing Education for the Practising Veterinarian* 16: 333-345

27. Echtenacher B, Hultner L, Mannel D 1996 Cellular and molecular mechanisms of TNF protection in septic peritonitis. *Journal of Inflammation* 47: 85-89

28. Eichacker P, Hoffman W, Farese A, Banks S, Kuo G, MacVittie T, Natanson C 1991 TNF but not IL-1 in dogs causes lethal lung injury and multiple organ dysfunction similar to human sepsis. *Journal of Applied Physiology* 71: 1979-1989

29. Eichenholz P, Eichacker P, Hoffman W, Banks S, Parrillo J, Danner R, Natanson C 1992 Tumor necrosis factor challenges in canines: pattern of cardiovascular dysfunction. *American Journal of Physiology* 263: H668-H675

30. Elased K, Taverne J, Playfair J H L 1996 Malaria, blood glucose, and the role of tumour necrosis factor (TNF) in mice. *Clinical and Experimental Immunology* 105: 443-449

31. Eskandari M, Nguyen D, Kunkel S, Remick D 1990 WEHI 164 subclone 13 assay for TNF: sensitivity, specificity and reliability. *Immunological Investigations* 19: 69-79

32. Espevik T, Nissen-Meyer J 1986 A highly sensitive cell line, WEHI 164 clone 13, for measuring cytotoxic factor/tumor necrosis factor from human monocytes. *Journal of Immunological Methods* 95: 99-105

33. Evans D, Jacobs D, Rounds J, Wilmore D 1993 The effects of tumor necrosis factor after removal of the gut. *Journal of Surgical Research* 54: 39-45

34. Field G, Conn C, McClanahan T, Nao B, Kluger M, Gallagher K 1994 Tumor necrosis factor and interleukin-6 are not elevated in venous blood from ischemic canine myocardium. *Proceedings of the Society for Experimental Biology and Medicine* 206: 384-391

35. Grau G, Piguet P, Vassali P, Lambert P 1989 Tumor necrosis factor and other cytokines in cerebral malaria: experimental and clinical data. *Immunological Reviews* 112: 49-70

36. Grau G, Taylor T, Molyneux M, Wirima J, Vassali P, Hommel M, Lambert P 1989 Tumor necrosis factor and disease severity in children with falciparum malaria. *The New England Journal of Medicine* 320: 1586-1591

37. Greenwood B 1996 Fever and malaria. *Lancet* 348: 280-281

38. Grell M 1996 Tumor necrosis factor (TNF) receptors in cellular signalling of soluble and membrane-expressed TNF. *Journal of Inflammation* 47: 8-17

39. Grell M, Douni E, Wajant H, Lohden M, Clauss M, Maxeiner B, Georgopolous S, Lesslauer W, Kollais G, Pfizenmaier K, Scheurich P 1995 The transmembrane form of tumor necrosis factor is the prime activating ligand of the 80 kDa tumor necrosis factor receptor. *Cell* 83: 793-802

40. Hay C, Chu Q, Budsberg S, Clayton M, Johnson K 1997 Synovial fluid interleukin 6, tumour necrosis factor, and nitric oxide values in dogs with osteoarthritis secondary to cranial cruciate ligament rupture. *American Journal of Veterinary Research* 58: 1027-1032

41. Hemmer C, Kern P, Holst F, Radtke K, Egbring R, Biehaus A, Nawroth P, Dietrich M 1991 Activation of the host response in human *Plasmodium falciparum* malaria: relation of parasitaemia to tumor necrosis factor/cachectin, thrombin-antithrombin III, and protein C levels . *The American Journal of Medicine* 91: 37-44

42. Hoffman W, Danner R, Quezado M, Banks S, Elin R, Hosseini J, Natanson C 1996 Role of endotoxaemia in cardiovascular dysfunction and lethality: virulent and nonvirulent *Escherichia coli* challenges in a canine model of septic shock. *Infection and Immunity* 64: 406-412

43. Hoffman W, Pollack M, Banks S, Koev C, Solomon M, Danner R, Koles N, Guelde G, Yatsiv I, Mouginis T, Elin R, Hosseini J, Bacher J, Porter J, Natanson C 1994 Distinct functional activities in canine septic shock of monoclonal antibodies specific for the O polysaccharide and core regions of *Escherachia coli* lipopolysaccharide. *The Journal of Infectious Diseases* 169: 553-561

44. Jacobson, L. 2000 Recently described complications of canine babesiosis. *Proceedings of the South African Veterinary Association Congress*:200-205

45. Jacobson L, Clark I 1994 The pathophysiology of canine babesiosis: new approaches to an old puzzle. *Journal of the South African Veterinary Association* 65: 134-145

46. Jacobson L, Swan G 1995 Supportive treatment of canine babesiosis. *Journal of the South African Veterinary Association* 66: 95-105

47. Karunaweera N, Grau G, Gamage P, Carter R, Mendis K 1992 Dynamics of fever and serum levels of tumour necrosis factor are closely associated during clinical paroxysms in *Plasmodium vivax* malaria. *Proceedings of the National Academy of Science* 89: 3200-3203

48. Kawo N, Msengi A, Swai A, Chuwa L, Alberti K, McLarty D 1990 Specificity of hypoglycaemia for cerebral malaria in children. *Lancet* 336: 454-457

49. Kern P, Hemmer C, Van Damme J, Gruss H, Dietrich M 1989 Elevated tumor necrosis factor alpha and interleukin-6 serum levels as markers for complicated *Plasmodium falciparum* malaria. *The American Journal of Medicine* 87: 139-143

50. Kollef M, Eisenberg P 1997 A rapid qualitative assay to detect circulating endotoxin can predict the development of multiorgan dysfunction. *Chest* 112: 173-180

51. Krishna S, Waller D, ter Kuile F, Kwiatkowski D, Crawley J, Craddock C, Nosten F, Chapmen D, Brewster D, Holloway P 1994 Lactic acidosis and hypoglycaemia in children with severe malaria: pathophysiological and prognostic significance. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 88: 67-73

52. Kwiatkowski D 1995 Malarial toxins and the regulation of parasite density. *Parasitology Today* 11: 206-212

53. Kwiatkowski D, Bate C, Scragg P, Beattie P, Udalova I, Knight J 1997 The malarial fever response - pathogenesis, polymorphisms and prospects for intervention. *Annals of Tropical Medicine and Parasitology* 91: 533-542

54. Kwiatkowski D, Hill A, Sambou I, Twumasi P, Castracane J, Manogue K, Cerami A, Brewster D, Greenwood B 1990 TNF concentration in fatal cerebral, non-fatal cerebral, and uncomplicated *Plasmodium falciparum* malaria. *The Lancet* 336: 1201-1204

55. LeMay D, LeMay L, Kluger M, D'Alecy L 1990 Plasma profiles of IL-6 and TNF with fever inducing doses of lipopolysaccharide in dogs. *American Journal of Physiology* 259: R126-R132

56. Lewis B, Penzhorn B, Lopez Rebollar L 1995 Immune responses to South African *Babesia canis* and the development of a preliminary vaccine. *Journal of the South African Veterinary Association* 66: 61-65

57. Lewis B, Penzhorn B, Lopez-Rebollar L, De Waal D 1996 Isolation of a South African vector specific strain of *Babesia canis*. *Veterinary Parasitology* 63: 9-16

58. Lodato R, Feig B, Akimaru K, Soma G, Klostergaard J 1995 Hemodynamic evaluation of recombinant human tumor necrosis factor (TNF)-alpha, TNF-SAM2 and liposomal TNF-SAM2 in an anaesthetized dog model. *Journal of Immunotherapy with Emphasis on Tumor Immunology* 17: 19-29

59. Lucas R, Juillard P, Decoster E, Redard M, Burger D, Yves D, Giroud C, Monso-Hinard C, De Kesel T, Buurman W, Moore M, Dayer J, Fiers W, Bluethmann H, Grau G 1997 Crucial role of tumor necrosis factor (TNF) receptor 2 and membrane-bound TNF in experimental cerebral malaria. *European Journal of Immunology* 27: 1719-1725

60. Mackowaik P 1998 Concepts of fever. *Archives of Internal Medicine* 158: 1870-1879

61. Maegraith B, Gilles H M, Devakul K 1957 Pathological processess in *Babesia canis* infections. *Zeitschrift fur Tropenmedizin und Parasitologie* 8: 485-514

62. Malherbe W, Parkin B 1951 Atypical symptomatology in *Babesia canis* infection. *Journal of the South African Veterinary Medical Association* 22: 25-61

63. May M, Ghosh S 1998 Signal transduction through NF-κβ. *Immunology Today* 19: 80-88

64. Meager A, Leung H, Woolley J 1989 Assays for tumour necrosis factor and related cytokines. *Journal of Immunological Methods* 116: 1-17

65. Mendis K, Carter R 1995 Clinical disease and pathogenesis in malaria. *Parasitology Today* 11: PTI1-PTI16

66. Mitaka C, Hirata Y, Ichikawa K, Yokoyama K, Emori T, Kanno K, Amaha K 1994 Effects of TNF-alpha on haemodynamic changes and circulating endothelium-derived vasoactive factors in dogs. *American Journal of Physiology* 267: H1530-H1536

67. Miyamoto T, Fujinaga T, Yamashita K, Hagio M 1996 Changes of serum cytokine activities and other parameters in dogs with experimentally induced endotoxic shock. *Japanese Journal of Veterinary Research* 44: 107-118

68. Moore A, Theilen G, Newll A, Madewell B, Rudolf A 1991 Preclinical study of sequential tumor necrosis factor and interleukin 2 in the treatment of spontaneous canine neoplasms. *Cancer Research* 51: 233-238

69. Myers M, Murtaugh M 1995 Biology of tumor necrosis factor. In Myers M, Murtaugh M (ed.) *Cytokines in Animal Health and Disease*. Marcel Dekker,Inc., New York: 121-151

70. Nakatsuji K, Kii Y, Fujitani B, Ito T 1990 General pharmacology of recombinant human tumor necrosis factor. 1st communication: effects on cardiovascular, gastrointestinal, renal and blood functions. *Arzneimittel-Forschung* 40: 218-225

71. Natanson C, Eichenholz P, Danner R, Eichacker P, Hoffman W, Kuo G, Banks S, MacVittie T, Parrillo J 1989 Endotoxin and tumor necrosis factor challenges in dogs simulate the cardiovascular profile of human septic shock. *Journal of Experimental Medicine* 169: 823-832

72. Natanson C, Hoffman W, Suffredini A, Eichacker P, Danner R 1994 Selected treatment strategies for septic shock based on proposed mechanisms of pathogenesis. *Annals of Internal Medicine* 120: 771-783

73. Nyakundi J, Warn P, Newton C, Mumo J, Jepthah-Ochala J 1994 Serum tumour necrosis factor in children suffering from *Plasmodium falciparum* infection in Kilifi district, Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 88: 667-670

74. Otto C, Drobatz K, Soter C 1997 Endotoxaemia and tumor necrosis factor activity in dogs with naturally occurring parvoviral enteritis. *Journal of Veterinary Internal Medicine* 11: 65-70

75. Parent C, King L, Van Winkle T, Walker L 1996 Respiratory function and treatment in dogs with acute respiratory distress syndrome: 19 cases (1985-1993). *Journal of the American Veterinary Medical Association* 208: 1428-1433

76. Pauli U, Bertoni G, Duerr M, Peterhans E 1994 A bioassay for the detection of tumor necrosis factor from eight different species: evaluation of neutralisation rates of a monoclonal antibody against human TNF-α. *Journal of Immunological Methods* 171: 263-265

77. Pellegrini J, Puyana J, Lapchak P, Kodys K, Miller-Grazanio C 1996 A membrane TNF-α/TNFR ratio correlates to MODS score and mortality. *Shock* 6: 389-396

78. Presterl E, Staudinger T, Petterman M, Lassnigg A, Burgmann H, Winkler S, Frass M, Graninger W 1997 Cytokine profile and correlation to the APACHE III and MPM II scores in patients with sepsis. *American Journal of Respiratory and Critical Care Medicine* 156: 825-832

79. Purvis D, Kirby R 1994 Systemic inflammatory response syndrome: septic shock. *Veterinary Clinics of North America: Small Animal Practice* 24: 1225-1247

80. Quezado M, Natanson C, Banks S, Alling D, Koev C, Danner R, Elin R, Hosseini J, Parker T, Levine D, Rubin A, Hoffman W 1995 Therapeutic trial of reconstituted human high-density lipoprotein in a canine model of gram-negative septic shock. *The Journal of Pharmacology and Experimental Therapeutics* 272: 604-611

81. Riche F, Panis Y, Laisne M, Briard C, Cholley B, Bernard-Poenaru O, Graulet A, Gueris J, Valleur P 1996 High tumor necrosis factor level is associated with increased survival in patients with abdominal septic shock: A prospective study in 59 patients. *Surgery* 120: 801-807

82. Rink L, Kirchner H 1996 Recent progress in the tumor necrosis factor-α field. *International Archives of Allergy and Immunology* 111: 199-209

83. Rowe J A, Scragg I G, Kwiatkowski D, Ferguson D, Carucci D J, Newbold C I 1998 Implications of mycoplasma contamination in *Plasmodium falciparum* cultures and methods for its detection and eradication. *Molecular and Biochemical Parasitology* 92: 177-180

84. Saeed B, Atabani G, Nawwaf A, Nassr A 1990 Hypoglycaemia in pregnant women with malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 84: 349-350

85. Schetters T, Kleuskens J, Scholtes N, Pasman J, Bos H 1994 Vaccination of dogs against *Babesia canis* infection using antigens from culture supernatants with emphasis on clinical babesiosis. *Veterinary Parasitology* 52: 219-233

86. Scragg I G, Hensmann M, Bate C, Kwiatkowski D 1999 Early cytokine induction by *Plasmodium falciparum* is not a classical endotoxin-like process. *European Journal of Immunology* 29: 2636-2644

87. Sekut L, Han B, Baer P, Verghese M, Silverstein R, Clifton L, Dennis S, Numerick M, Connolly K 1995 A canine model for determination of the therapeutic index of cytokine inhibitors. *Laboratory Animal Science* 45: 647-651

88. Shaffer N, Grau G, Hedberg K, Davachi F, Lyamba B, Hightower A, Breman J, Nguyen-Dinh P 1991 Tumor necrosis factor and severe malaria. *The Journal of Infectious Diseases* 163: 96-101

89. Shaio M, Lin P 1998 A case study of cytokine profiles in acute human babesiosis. *American Journal of Tropical Medicine and Hygiene* 58: 335-337

90. Straubinger R, Straubinger A, Summers B, Erb H, Harter L, Appel M 1998 *Borrelia burgdorferi* induces the production and release of proinflammatory cytokines in canine synovial explant cultures . *Infection and Immunity* 66: 247-258

91. Tanaka M, Ferreira-da-Cruz M, Alecrim M, Mascarenas L, Ribeiro C 1995 Tumor necrosis factor alpha interferon gamma and macrophage stimulating factor in relation to severity of *Plasmodium falciparum* malaria in the Brazilian Amazon. *Tropical and Geographical Medicine* 47: 1-4

92. Timms P, Stewart N, De Vos A 1990 Study of virulence and vector transmission of *Babesia bovis* by use of cloned parasite lines. *Infection and Immunity* 58: 2171-2176

93. Uilenberg G, Franssen F, Perie N, Spanjer A 1989 Three groups of *Babesia canis* distinguished and a proposal for nomenclature. *The Veterinary Quarterly* 11: 33-40

94. van Lanschot J, Mealy K, Jacobs D, Evans D, Wilmore D 1997 Splenectomy attenuates the innapropriate diuresis associated with tumor necrosis factor administation. *Surgery, Gynecology and Obstetrics* 172: 293-297

95. Warrell D, Molyneux M, Beattie P 1990 Severe and complicated malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 84: 1-65

96. Wilcox P, Wakai Y, Walley K, Cooper D, Road J 1994 Tumor necrosis factor alpha decreases in vivo diaphragm contractility in dogs. *American Journal of Respiratory and Critical Care Medicine* 150: 1368-1373

97. Yamashita K, Fujinaga T, Hagio M, Miyamoto T, Izumisawa Y, Kotani T 1994 Bioassay for interleukin-1, interleukin-6, and tumor necrosis factor-like activities in canine sera. *Journal of Veterinary Medical Science* 56: 103-107

98. Yamashita K, Fujinaga T, Miyamoto T, Izumisawa Y, Kotani T 1994 Canine acute phase response: relationship between serum cytokine activity and acute phase protein in dogs. *Journal of Veterinary Medical Science* 56: 487-492

99. Zucker K, Lu P, Fuller R, Asthana D, Esquenazi V, Miller J 1994 Cloning and expression of the cDNA for canine tumor necrosis factor-α in *E. Coli*. *Lymphokine and Cytokine Research* 13: 191-196

10 APPENDICES

10.1 APPENDIX I: RESULTS OF THE GENERAL CLINICAL EXAMINATION OF CASE 11

Signalment			
Age	2 years	**Sex**	Female
Breed	Pitbull	**Weight**	29.4 kg
History			
Chief complaint	Listless, not eating for 2 days	**Urination**	Unknown
Duration	2 days	**Weight loss**	Unknown
Previous diseases	None	**Diet**	Not recorded
Appetite	Not eating	**Stamina**	Very listless
Vomition	Yes	**Urine colour**	Dark red
Diarrhoea	No	**Treatments**	None
Respiration	Normal		
Clinical Findings			
Temperature	39.9	**Neck**	Normal
Pulse	147	**Cardiovascular**	Normal
Respiration	34	**Abdominal cavity**	Normal
Mucous membranes	Very pale	**Urogenital**	Normal
Lymph nodes	Normal	**Locomotory**	Normal
Skin	Normal	**Nervous system**	Normal
Head	Normal		

10.2 APPENDIX II: DESCRIPTION OF THE TUMOUR NECROSIS FACTOR BIOASSAY

1. WEHI-164 cells were propagated in RPMI 640 with 25mM HEPES and L-glutamine (Gibco BRL, Grand Island, New York), 50 µg of gentamicin (Gibco BRL) per ml and 10% foetal bovine serum (Gibco BRL). Cells were incubated at 37°C with 5% CO_2 for 72 hours and were then scraped off for further propagation or tests.
2. TNF was assayed in 96-well flat-bottomed plates (See Figure 1 for plate layout).
3. The wells (displayed in Figure 1) that were required for the assay were set up as follows: blank wells consisted of 200µl water only, the negative control well consisted of 200µl of cell culture medium and the positive control well was 200µl cell culture medium with cells.
4. To set up the standard curve wells, the following procedure was followed: a starting solution consisting of 200 ng/ml recombinant canine TNF was made up and used for the serial dilutions for the standard curve wells. The serial dilutions were achieved by placing 175µl of the TNF solution into the well labelled Standard 1. 100µl of cell culture medium was placed into wells Standard 2 to Standard 5. Serial dilution was then performed by transferring 75µl from wells Standard 1 to Standard 2, and repeating this until well Standard 5 was reached. 75µl was removed from Standard 5 and discarded. The resultant TNF concentrations of the standard wells are displayed in Table 18. Only five standard wells were used as the concentrations achieved approximated the linear region of the standard curve.
5. Sample wells were set up as follows: 80µl cell culture medium was placed into each sample well. WEHI 164 cells were harvested from a culture flask and diluted to 400 000 cells/ml with cell culture medium. 20µl of Actinomycin D (1mg/ml)(Sigma) was then added per 10ml of the cell solution. 100µl of the cell solution was added to all wells except the blanks and the negative control wells. This resulted in approximately 40 000 cells per well.
6. 20µl of serum sample was then added to each sample well. The total volume of the sample wells was now 200µl.
7. The tissue culture plate was then incubated at 37°C with 5% CO_2 overnight (at least 12 hours).

8. The following morning the light absorbance of each well was read at 490 nm. This initial reading was to determine if there was haemoglobin discolouration of the wells containing the serum samples. If the absorbance of any sample well exceeded 0.25, then all wells (samples plus controls) were processed using the following washing technique: 100µl of medium was gently removed per well. 100µl of fresh cell culture medium was then added. The cells were then allowed five minutes to settle and the process was repeated once.
9. 40µl of MTS/PMS(Sigma) was then added to each well (except blanks).
10. The tissue culture plate was then incubated at 37°C with 5% CO_2 for 4 hours.
11. Light absorbance was then read at 490 nm for final results.

10.3 TABLES

Table 18 Tumour necrosis factor concentrations of the standard curve

Standard Number	[TNF] ng/ml
1	100
2	42.857
3	18.367
4	7.872
5	3.374