A Preliminary Study to Investigate the Serum Urea: Creatinine Ratio in Canine Babesiosis in South Africa.

by

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RESUME´

In order to investigate the increased serum urea: creatinine ratio encountered in canine babesiosis in South Africa, packed cell volume (PCV), serum haemoglobin, serum total bilirubin, plasma ammonia, serum urea, serum creatinine, calculated serum urea: creatinine ratio, serum cystatin-C, total serum protein (TSP) and urine analysis were performed in this study. The model consisted of 28 canine babesiosis patients, 25 in group 1, the anaemic group with PCV < 20%, and 3 in group 3, the haemoconcentrated group with PCV > 50%. 13 canine babesiosis negative dogs made up group 2, the control group. Unpublished human malaria data from 363 patients was also analysed and the urea: creatinine ratio was calculated in order to determine whether elevated serum urea: creatinine ratios also occur in this disease.

The serum urea: creatinine ratio was > 20 in 23/25 anaemic patients, 3/13 control patients and 2/3 haemoconcentrated patients. The mean and median serum urea: creatinine ratio for the anaemic babesia group was 46.32 and 41.36, for the haemoconcentrated babesia group 25.10 and 20.85, for the malaria patients 20.64 and 18.75, and for the control group 15.91 and 15.36 respectively. Mean and median serum urea: creatinine ratio was significantly elevated and the distribution varied significantly between the anaemic babesiosis group compared with the control group (p=0.000003). Serum urea: creatinine ratio was elevated in canine babesiosis patients, especially when they are anaemic. The serum urea: creatinine ratio was also found to be elevated in the human malaria patients.

Serum urea was found to be elevated in 17/25 anaemic patients, 0/13 control patients and 3/3 haemoconcentrated patients. Mean and median values for serum urea were 12.83 mmol/l and 11.8 mmol/l and 5.09 mmol/l and 4.3 mmol/l for the anaemic group and the control group respectively. Mean and median serum urea was significantly elevated and the distribution varied significantly between the anaemic group compared with the control group (p=0.00003). Serum creatinine was elevated in 0/25 anaemic patients 0/13 control patients and 2/3 haemoconcentrated patients. Mean and median values for serum creatinine were 72.08 µmol/l and 67 µmol/l, and 78.69 µmol/l and 75 µmol/l for the anaemic group and the control group respectively. Mean, median and

distribution of serum creatinine did not vary significantly between the control group and the anaemic babesia group (p=0.274488). Serum cystatin-C was elevated in 0/25 anaemic patients, 0/13 control patients and 2/3 haemoconcentrated patients. The two haemoconcentrated patients with elevated serum urea and serum creatinine also had elevated serum cystatin-C. One of these two patients was available for post mortem and had significant renal lesions on histopathology. These two patients also had a urine specific gravity (SG) < 1.030 (1.020 for both cases). Mean and median values for serum cystatin-C were 0.71 mg/l and 0.7 mg/l, and 0.67 mg/l and 0.7 mg/l for the anaemic group and the control group respectively. Mean, median and distribution of serum cystatin-C did not vary significantly between the control group and the anaemic babesia group, (p=0.450172). Using the Spearman-rank test for correlation coefficients serum creatinine but not serum urea had a significant positive correlation with serum cystatin-C; and serum urea had a significant positive correlation with the serum urea: creatinine ratio. Log serum creatinine was significantly correlated to log serum cystatin-C, (r^2=0.52, p<0.001). Log serum urea was also significantly correlated to log serum cystatin-C, (r^2=0.14, p=0.024), however, this correlation was relatively poor compared to that of serum creatinine with serum cystatin-C. Log serum urea: creatinine ratio was not significantly correlated with log serum cystatin-C, (r^2=0.014, p=0.485). Given its resistance to interference from the elevated plasma and serum pigments found in canine babesiosis, an elevated serum cystatin-C was taken to indicate significant renal disease. Therefore we can deduce that the increased serum urea: creatinine ratio encountered in canine babesiosis is a result of disproportionately elevated serum urea concentrations, which is of non-renal origin. A similar event appears to be present in human falciparum malaria patients. The cause of this phenomenon in either disease is unknown; but various forms of prerenal azotaemia, hyperureagenesis and laboratory perturbations may play a role. Serum creatinine was a better measure of significant renal disease than serum urea in this study. There also appeared to be no added benefit of measuring serum cystatin-C.

Mean and median values for PCV were 11.64% and 11%, and 46.08% and 48% for the anaemic group and the control group respectively. As expected the mean

and median PCV was significantly lower and the distribution varied significantly between the control group and the anaemic babesia group (p=0.000001). The mean and median values for PCV in the haemoconcentrated group were 54.33% and 54% respectively. Serum haemoglobin was > 1.6 g/l in 8/25 anaemic patients, 0/13 control patients and 3/3 haemoconcentrated patients. Mean and median values for serum haemoglobin were 1.72 g/l and 1.3 g/l, and 0.88 g/l and 0.8 g/l for the anaemic group and the control group respectively. Mean and median serum haemoglobin was significantly elevated and distribution of serum haemoglobin varied significantly between the control group and the anaemic babesia group (p=0.002364). Serum total bilirubin was > 15 µmol/l in 11/25 anaemic patients, 0/3 haemoconcentrated patients and 0/13 control patients. Mean and median values for serum total bilirubin were 22.42 µmol/l and 10.6 µmol/l, and 10.63 µmol/l and 11.0 µmol/l for the anaemic group and the control group respectively. The median and the distribution of serum total bilirubin did not vary significantly between the control group and the anaemic babesia group (p=0.355888). TSP was elevated in 5/25 anaemic patients, was below the normal laboratory reference range in 5/25 anaemic patients, was elevated in 1/13 control patients, and was normal in all the haemoconcentrated cases. Mean and median values for TSP were 58.60 g/l and 54 g/l, and 60.92 g/l and 60.0 g/l for the anaemic group and the control group respectively. The mean, median and distribution of TSP did not vary significantly between the control group and the anaemic babesia group (p=0.130661).Plasma ammonia was elevated in 16/25 anaemic patients, 0/13 control patients and 2/3 haemoconcentrated patients. Mean and median values for plasma ammonia were 78.77 µmol/l and 53.8 mol/l, and 23.18 µmol/l and 21.5 µmol/l for the anaemic group and the control group respectively. Mean and median plasma ammonia was significantly elevated and the distribution of plasma ammonia varied significantly between the control group and the anaemic babesia group (p=0.009562).

Using the Spearman-rank test for correlation coefficients PCV had a significant negative correlation with plasma ammonia, serum urea and serum urea: creatinine ratio. Serum haemoglobin and serum bilirubin had a significant positive correlation with plasma ammonia, serum urea and serum urea:

creatinine ratio. Plasma ammonia also had a significant positive correlation with serum urea and serum urea: creatinine ratio. These correlations may be explained by substrate loading as a result of haemolysis. I speculate that various protein by-products of haemolysis may cause the hyperammonaemia encountered via deamination of these proteins. This in turn may lead to ureagenesis, the physiological process of ammonia clearance. This ureagenesis may be excessive and therefore may account for the elevated serum urea encountered in patients with normal serum creatinine and serum cystatin-C concentrations. Alternatively the presence of an elevated serum haemoglobin concentration with its high intrinsic absorptive capacity in the 300 nm to 500 nm wave length range, may positively bias the measurement of serum urea and plasma ammonia concentrations. Serum urea is measured at 340 nm and plasma ammonia is measured at 365 nm. Thus a non-physiological process could also explain the above correlations.

Urine analysis of the canine babesiosis patients showed mild evidence of renal disease as portrayed by proteinuria, renal tubular epithelium (RTE) celluria and granular casts. However, these findings, as well as the presence of an elevated serum urea, haemoglobinaemia and haemoglobinuria, bilirubinaemia and bilirubinuria, were present in both patients with mild renal disease and patients with overt renal disease. Therefore the benefit of single serum urea concentrations and random urine sediment analysis in canine babesiosis is questioned. Serial urine analysis monitoring was not investigated.

This study concluded that serum urea is often elevated due to non-renal factors in canine babesiosis patients. This causes an elevated serum urea: creatinine ratio in this disease, with a similar event appearing to occur in human malaria patients. The cause of these elevated ratios remains undetermined, but is likely to be as a result of hyperureagenesis or laboratory error. The measurement of serum creatinine, urine SG and hourly urine production is advocated to detect significant renal disease in these patients. There appeared to be no added benefit derived from the measurement of serum cystatin-C concentrations in canine babesiosis patients in this preliminary study.

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ABBREVIATIONS

ALP: Alkaline phosphatase

ARDS: Acute respiratory distress syndrome

CD14: Cluster of differentiation receptor 14

DCT: Distal convoluted tubule

DIC: Disseminated intravascular coagulation

EDTA: Ethylenediaminetetraacetic acid

FcK: Fractional clearance of potassium

FcNa: Fractional clearance of sodium

GFR: Glomerular filtration rate

GGT: Gamma-glutamyl transpeptidase

Hb: Haemoglobin

ICAM: Intracellular adhesion molecule

IL: Interleukin

iNOS: Inducible nitric oxide synthetase

MHC: Major histocompatibility complex

MODS: Multiple organ dysfunction syndrome

NO: Nitric oxide

OVAH: Onderstepoort Veterinary Academic Hospital

PCR: Polymerase chain reaction

PCT: Proximal convoluted tubule

PCV: Packed cell volume

RBC: Red blood cell

RTE: Renal tubular epithelium

SG: Specific gravity

SIRS: Systemic inflammatory response syndrome

T-cell: Thymic lymphocyte

Th_1: Type 1 helper T cell

Th_2: Type 2 helper T cell

TNF: Tumour necrosis factor

TSP: Total serum protein

CHAPTER 1
INTRODUCTION

Canine Babesiosis in South Africa

Canine babesiosis in South Africa is caused by the haemoprotozoans *Babesia canis rossi* and, recently recognized, *B. c. vogelli.* These parasites are transmitted by the ticks *Haemaphysalis leachi* and, most likely, *Rhipicephalus sanguineus* respectively[68]. *Babesia canis rossi* is notorious in causing the most severe forms of the disease seen in the dog worldwide[59]. This is an important disease in South Africa and represented 12% of sick dogs presented to the OVAH over a 6 year period. A third of these patients required intensive treatment[91]. The disease can be divided into uncomplicated and complicated forms. In its uncomplicated form patients have mild to moderate anaemia, fever, anorexia and depression and splenomegaly[61]. The PCV of complicated patients may be vary from severely anaemic or haemoconcentrated. They may have clinical or biochemical evidence of organ dysfunction or failure, and/or haematological evidence of immune mediated haemolytic anaemia, and/or mixed acid-base abnormalities. The organ dysfunction or failure encountered is typically CNS, renal or pulmonary but may also include skin necrosis, pancreatitis and myositis[1,3,45,57,61,63,81,86,103].

Canine babesiosis shares similarities with human malaria

Babesia c. rossi has been proposed as model for falciparum malaria in humans because these diseases share many similarities[16,86,90]. An appreciation of the similarities between these two diseases has prompted those interested in canine babesiosis to pursue similar lines of investigation as have been followed by malariologists. It is believed that there are aspects of this canine disease that may be helpful as an animal model for falciparum malaria in humans[42,44].

Canine babesiosis, like human malaria, is not a single disease

With the advent of molecular techniques it has become clear that there are several canine babesia parasites. To date, two of the over 100 babesia species identified have been shown to affect the dog, namely *B. canis* and *B. gibsoni*. *B. canis*, the large babesia, has three distinct subtypes, namely *B. c. canis*, *B. c.*

rossi and *B. c. vogelli*. *Babesia gibsoni* was thought to represent the small babesia that affected the dog. Genetic sequencing has determined that there are at least three strains of small babesia that affect the dog, the classical Asian *B. gibsoni*, a Californian *B. gibsoni* closely related to Theileria and an European *B. microti* "like" organism, *Theileria annae*[13,52,107,108,109]. This classification is not complete and further work is currently in progress. It is becoming clear, however, that there are marked differences in the distribution, vector specificity, virulence, prognosis, and treatment response between the various recognised babesia species and subtypes that infect the dog[110]. Lewis[58] demonstrated the genetic diversity between babesia species when he showed a lack of cross protection by the French *B. c. canis* vaccine for any other strain of canine babesiosis. The diversity in virulence between babesia species that affect the dog becomes clear when comparing the mild disease seen in the United States, caused by *B. c. vogeli* which is transmitted by *Rhipicephalus sanguineus,* to the severe and often complicated forms of the disease seen in South Africa, caused by *B. c. rossi* which is transmitted by *Haemophysalis leachi*[59]. As mentioned previously it has recently been shown that both diseases do occur in South Africa. Different species of the falciparum parasite that infect humans show distinct variation in the type of disease seen. For example, *Plasmodium malariae* causes membranoproliferative glomerular nephritis whereas *P. falciparum* may cause acute tubular necrosis or post-infection acute glomerulonephritis[25]. It is likely that babesia parasite-specific clinical manifestations are likely to emerge as more information becomes available on the various species of babesia parasites that infect dogs. It has become clear that the host response to the presence of the babesia parasite is highly variable between individual patients and between different breeds of dogs. Fighting breeds may be more susceptible to developing the haemoconcentrated form of canine babesiosis in South Africa[86]. This phenomenon may be genetically predetermined. An example of this can also be found in human malaria where the absence of the Fy^a or Fy^b erythrocyte plasmodium receptor renders most West Africans resistant to *P. vivax*[104]. Human malarial disease phenotype has been shown in numerous studies to be influenced by host genotype. The effect of the single nucleotide polymorphism in the tumour necrosis factor gene promoter region is a classic example[71]. Similar mechanisms are likely in dogs if

one considers the wide breed scope in this species and the variation of disease phenotype seen with canine babesiosis, some of which appears to segregate with breed. A sub-clinical or asymptomatic carrier state has been found to occur in canine babesiosis. In a study in an endemic area in France, 33% of asymptomatic dogs tested positive by way of blood culture for *B. c. canis*[110]. The incidence of asymptomatic carriers of babesiosis in endemic areas in South Africa is unknown but is presumed to be low. The importance of the asymptomatic carrier state is also unknown. Adachi[2] reported that lymphocyte blastogenesis was found to be deficient in dogs with sub-clinical infections of *B. gibsoni*, indicating that there may well be biologically significant effects of a sub-clinical carrier state. A nested polymerase chain reaction (PCR), which amplifies *Babesia* small subunit rRNA, has been developed[6] which detects parasitaemias of 0.0001%. The approximate limit of detectible parasitaemia by light microscope is 0.001%. The North Carolina State University has developed a seminested PCR with a limit of detection of 0.000001% that differentiates between *B. gibsoni, B. c. vogeli, B. c. rossi,* and *B. c. canis*[8]. Future studies on babesiosis should therefore include analysis of the 18S rRNA to determine exactly which species is responsible for the disease being reported. This would allow for the study of virulence, symptomatology, prognosis, and treatment requirements for specific species. This investigation did not include such an analysis and the babesia species represented are referred to as canine babesiosis in South Africa, most probably representing *B. c. rossi*.

Co-infections with other tick borne diseases

Ehrlichiosis, anaplasmosis, mycoplasmosis, borreliosis and bartonellosis are all tick borne diseases sharing common vectors with canine babesiosis. These parasites or the babesia parasite itself may induce immuno-suppression as well as parasite specific lesions in organs such as the kidney[54]. Other concomitant infections or parasites may alter host responses to babesia parasites by inducing a T helper lymphocyte type 1 (Th_1) or a T helper lymphocyte type 2 (Th_2) cytokine profile bias. These responses may either be beneficial or detrimental to the host[17]. A full discussion on these diseases is, however, beyond the scope of this study.

Patho-mechanisms of haemolysis in canine babesiosis

Haemolytic anaemia is a hallmark feature of all canine babesia infections irrespective of species[61]. The causes of haemolysis have been studied in *B. gibsoni* where circulating anti-erythrocyte antibodies, IgG bound erythrocytes, erythrocyte oxidation, erythrocyte phagocytosis, increased osmotic fragility, and a haemolytic factor have been implicated in the pathogenesis[1,3,76,77,78,79,81].

Wosniak[106] suggested that the presence of haemoglobinuria, diffuse erythrophagocytosis, and haemosiderosis indicates that both intravascular and extravascular haemolysis occurs. Reyers[86] reported that 90% of severely anaemic dogs infected with babesia in South Africa were Coombs positive. In a review on canine babesiosis in South Africa over 40% of canine babesiosis infected dogs demonstrated macroscopic agglutination, once again indicating that immune mechanisms play a role in the haemolysis observed[42]. Other likely and speculative causes of haemolysis and/or anaemia extrapolated from malaria studies include haemophagocytic syndrome, decreased red blood cell (RBC) deformability, indiscriminate erythrophagocytosis, complement mediated erythrolysis and opsonisation of RBC, decreased RBC production related to parasite mediated dyserythropoeitinaemia, and disseminated intravascular coagulation (DIC) related haemorrhag[21,47,97].

Renal disease in canine babesiosis

Canine babesiosis patients seen at the Onderstepoort Veterinary Academic Hospital (OVAH) with renal failure are severely azotaemic with both serum urea and creatinine raised. In addition they are frequently oliguric or anuric, with any residual urine being darkly pigmented[61].

The variable findings of proteinuria, renal tubular casts and RTE cells in urine sediment indicate a degree of renal insult but do not reflect or predict renal failure. In a study on the presence and degree of renal damage in canine babesiosis in South Africa, Lobetti[63] investigated serum urea, serum creatinine, serum sodium, serum potassium, fractional clearance of sodium (FcNa), fractional clearance of potassium (FcK), urinary enzyme activity of gamma

glutamyl transferase (GGT) and alkaline phosphatase (ALP), urine protein: creatinine ratio, and urine analysis in three groups of canine babesiosis: uncomplicated mildly anaemic; uncomplicated severely anaemic and complicated cases. That study demonstrated that minimal intrinsic renal impairment without overt renal failure was common in canine babesiosis. When renal failure does occur in canine babesiosis patients in South Africa it is usually fatal[63,103]. Renal failure is reported to occur in less than 3% of South African canine babesiosis infections and appears to be more common in the haemoconcentrated form of the disease[42]. In a study by Reyers[86] mean serum creatinine concentrations were elevated in a haemoconcentrated group, but were normal in the moderately anaemic and severely anaemic groups. In the Reyers study 44% of the haemoconcentrated dogs that died had elevated serum creatinine concentrations, compared to only 14% of those that survived.

In a Spanish study of 58 dogs infected with *B. microti*-"like" organisms[14], 36% of patients were found to be azotaemic. During the following seven days 22% of these dogs died. Dogs with azotaemia were ten times more likely to die than non-azotaemic patients. High urine protein: creatinine ratios suggested a glomerular component to this disease. In a Bangkok study on 112 human falciparum malaria patients[105], 90.2% required haemodialysis. Of these patients 83% were oliguric at presentation. In total 10.7% of the patients died; however 67% of the mortalities were from the cerebral form of malaria and not renal failure. In another study of 64 children hospitalised with malaria in Yemen[92], 43.8% of patients died from malaria associated severe acute renal failure where peritoneal dialysis, but not haemodialysis, was available as supportive treatment. Human malaria patients with renal failure appear to respond well to haemodialysis. Haemodialysis, therefore, may be useful to help reduce the high mortality seen in canine babesiosis patients with renal failure. Other possible reasons for a poor outcome in canine babesiosis patients with renal failure include a differing disease pathogenesis and severity and stage of the disease at the time of diagnosis when compared to human malaria patients.

A pathological study of 30 experimentally infected cases of fatal babesiosis in 1957 by Maegraith[67] is probably still the best source for the description of renal

pathology available for canine babesiosis to date. Given the severity of signs he describes, the disease he studied was most likely caused by *B. c. rossi*.

- Macroscopically Maegraith found the kidney medulla to be normal to severely congested and the cortex to be normal to severely pale. 3/30 cases showed gross congestion. The most consistent macroscopic finding was the great prominence of the vessels at the cortico-medullary junction, associated with local vascular congestion. The most severe macroscopic changes were seen in cases with anuria or haemoglobinuria.
- Microscopically free and intraerythrocytic parasites were seen in the capillaries and venules in all cases, however, the degree of pathology did not correlate to the intensity of local parasite concentration. The glomeruli of some anuric cases appeared ischaemic and showed retraction and sometimes irregularly scattered congestion, their capsules often contained debris and coagulated material. The vascular endothelium of these glomeruli was unaffected.
- There was also irregular to gross medullary congestion with degenerative tubular changes in all cases. Some cellular debris was found in the tubular lumen. Small amounts of haemoglobin were detected in the proximal convoluted tubule (PCT) and distal convoluted tubule (DCT) cells. Tubular haemoglobin casts were common and were found in both anuric and non-anuric cases.

Other pathological reports on renal failure lesions in canine babesiosis have concentrated on the presence of pigments within kidney[40,69]. The term "haemoglobinuric nephrosis" was condoned and used. The finding of haemoglobinuria, however, is not synonymous with kidney damage. In a study examining the effect of haemoglobin, anaemic hypoxia or both on the kidney, Lobetti showed that haemoglobin was not significantly nephrotoxic, nor does it contribute to the mild renal damage seen with hypoxia. The hypoxic group in his study had more casts and RTE cells in the urine, greater enzymuria (also suggestive of RTE cell pathology), and a decreased glomerular filtration rate (GFR) compared to the other two groups. Haemoglobinuria did not appear to have any effect on urine enzyme activity.

Histologically the majority of the animals in all three groups had mild single cell tubular necrosis with granular casts found only in the hypoxic group. Electron microscopic evaluation showed minimal changes in all three groups[65]. Lobetti also reported that the potentially nephrotoxic combination of methhaemoglobinuria and acidic urine was often present in canine babesiosis patients in South Africa[64]. As previously mentioned renal failure is more commonly seen in haemoconcentrated forms of the disease in South Africa. This form of the disease is distinctly different to the anaemic form of the disease. This is possibly due to variation in inflammatory cytokine profiles between the two forms of the disease[42]. This hypothesis would be based on studies of the pathogenesis of severe forms of *P. falciparum*[55]. Given the diversity of host responses to canine babesiosis, as well as the interparasite and intraparasite diversity of virulence, the pathological reports on renal disease in canine babesiosis would be expected to be diverse as well.

Renal disease in malaria

The pathomechanisms of the parasite virulence and subsequent host responses, regarding the kidney, are diverse within human malaria disease. Age, race and treatment appear to be major role players in the host diversity encountered. A complete summary of renal disease in human malaria is beyond the scope of this review. Some interesting findings will, however, be summarised.

In a study on 78 patients in Ghana, Burchard[12] detected glomerular as well as tubular patterns of proteinuria in 85% of children with uncomplicated *P. falciparum* malaria. Plasma concentrations of cystatin-C were found to be elevated in 17% of these children. Electrophoretic studies have shown both glomerular and tubular patterns of proteinuria in vivax and falciparum malaria infections[28]. Simao[93] has reported that renal failure was common in severe adult falciparum malaria. In another study Day[19] found that elevated plasma cytokines, namely interleukin 6 (IL6), interleukin 10 (IL10), tumour necrosis factor alpha (TNF-α), and interferon gamma (IFN-γ), and the imbalance between pro- and anti-inflammatory cytokines in severe falciparum malaria

were associated with hyperparasitaemia, jaundice, shock, renal failure and mortality. TNF- α levels were shown to have a strong association with acute renal failure adult falciparum malaria patients. In yet another study Glycosylphosphatidylinositol moieties were proposed to act like endotoxin[29]. These moieties are linked covalently to falciparum malarial parasite surface antigens and signal TNF release by monocytes via the cluster of differentiation 14 (CD14) receptor. This is believed to be the initiator of other cytokine cascades and inflammatory mediators. This process is thought to contribute to acute renal failure as well as changes in blood volume status through vascular tone and permeability. Duvic[26] reported that obstruction of capillaries and post capillary venules by infected red blood cells as well as hypovolaemia, catecholamine/renin-angiotensin interactions, complement activation and rhabdomyolysis were the main pathomechanisms involved in causing acute renal failure during severe malaria. Sitprija[95] emphasized the role of blood hyperviscosity and hypovolaemia in the pathogenesis of acute renal failure in falciparum malaria. White[104] also suggested that hypotensive shock is a major cause of renal failure in human falciparum malaria patients. Kilbourn[50,51] suggested that hypotensive shock is common and appears to be parasite initiated but cytokine mediated. TNF and/or IL-1 induced inducible nitric oxide synthetase (iNOS) may play a major role. Rui-Mei[88,89] showed that adhesion molecules, including intracellular adhesion molecule 1 (ICAM-1) and major histocompatability complex (MHC) class I and II antigens play an important role in the pathogenesis in nephritis caused by the murine malarial parasite *P. berghei*. In a study by Clarke[15], iNOS was thought to be a synergistic product of elevated cytokines and generalized hypoxia or localized hypoxia through parasite sequestration. iNOS has been implicated in renal failure, acute respiratory distress syndrome (ARDS), and hypoglycaemia in falciparum malaria but not vivax malaria. iNOS induction and endothial injury with resultant vasodilation and hypovolaemia are thought to cause the ischaemic tubular necrosis seen.

A South African study[44] investigating the role of nitric oxide (NO) in canine babesiosis did not lead to any definite conclusions; however, some evidence of NO involvement was found.

Macrophage and thymic lymphocyte (T-cell) mediated rhabdomyolysis with resultant myoglobinuria is also thought to contribute to acute renal failure in many cases of falciparum malaria[94]. Perhaps some or all of these mechanisms play a role in canine babesiosis.

Disproportionate elevation in serum urea in canine babesiosis

In 1986 Lyman[66] described the determination of serum urea and creatinine as "standard fare" in the assessment of renal function. He suggested that they were best viewed in concert, observing their absolute values as well as their relation to each other. He posed the following questions. Are the values real? Are there extra-renal aetiologies for the abnormal levels? What is the serum urea: creatinine ratio? We now find ourselves asking the same questions about these metabolites in canine babesia patients. In a retrospective study by Medaille[72] on 4799 canine data sets, 27.5% of unselected paired results of serum urea and serum creatinine concentrations had an elevated serum urea: creatinine ratio. Changes in creatinine associated with breed and muscle mass, although significant, did not account for all the changes and it was speculated that non renal influences were present. Azotaemia, in the form of elevated serum urea concentrations, is common in canine babesiosis in South Africa. In a recent retrospective study of approximately 400 *B. canis* cases, the mean and median serum urea concentrations were approximately double the normal laboratory serum concentrations, whilst mean and median serum creatinine concentrations fell within normal laboratory values[20]. Although it was not evaluated, this phenomenon would have presented an increased serum urea: creatinine ratio. Reyers[86] also reported high serum concentrations for serum urea relative to creatinine in canine babesiosis in South Africa. Whenever a marked increase in the serum urea: creatinine ratio is found, traditional wisdom would have us link this finding to acute renal azotaemia, prerenal azotaemia, or a disrupted urinary tract with retro or intra-peritoneal leakage[31,32]. Speculation has it that this phenomenon in canine babesiosis may be caused by a falsely raised serum urea, a form of prerenal azotaemia, or by serum substances interfering with the analysis of serum creatinine[7,9,22,33,41,85,98,101]. This speculation may have had its foundation from increased serum urea on creatinine ratios found in cases of

intestinal haemorrhage, excessive protein loading or increased protein catabolism, where hyperureagenesis is common[82,96]. It is hypothesised that ammonia loading occurs in canine babesiosis as a result of haemolysis, blood transfusions and gastrointestinal haemorrhage[85]. This could lead to a non-renal related elevation in serum urea concentrations via hyperureagenesis and could cause the increased serum urea: creatinine ratio apparent in canine babesiosis[20]. In a human study[31] on 19 intensive care patients that developed a high serum urea: creatinine ratios, 11 patients died. The high mortality was attributed to severe illness, especially infection, decreased renal function and a hypercatabolic state. Hypovolaemia was present in 9 of these patients, 8 had congestive heart failure, 6 hypovolaemic shock, 2 GIT haemorrhage, 2 received high dose steroids, 14 had documented infections of which 7 had sepsis. All 19 had one of the above described complications and 16/19 had two or more complications present. These cases are not unlike the SIRS and MODS cases described in canine babesiosis[103]. Anorexia and cachexia can cause unexpectedly low creatinine and serum urea values[32]. In this setting both analytes may be affected to variable degrees causing low serum urea and creatinine values in the presence of a decreased GFR, however, the deamination of proteins for energy in a catabolic state may lead to increased serum urea concentrations. The effects of catabolism in canine babesiosis patients have not been investigated.

In a human study Sklar[96] showed that 24 hour urinary urea nitrogen determination could be used to separate hyperureagenesis from renal hypoperfusion in patients with high serum urea: creatinine ratios. In that study on 27 patients 37% had hyperureagenesis and 63% had renal hypoperfusion.

Although overt clinical evidence of rhabdomyolysis is rare in canine babesiosis, it has been shown that biochemical evidence of myopathy (as evidenced by a significantly raised creatine kinase activity) is common in severe cases[103]. Biochemical evidence of muscle damage is commonly reported in bovine babesiosis and in human malaria[94]. This fact as well as the reporting of two cases of rhabdomyolysis in South African canine babesiosis[43] has led to speculation that increased muscle catabolism may account for the observed disproportionate increase in serum urea relative to serum creatinine in canine babesiosis.

Serum urea is also subject to other perturbations. Elevations in serum urea may be caused by a recent protein meal and tubular reabsorption during low tubular urine flow rates. A recent protein meal is an unlikely event in canine babesiosis patients because anorexia is an almost universal finding in this acute disease. Decreases in serum urea are seen due to decreased production in chronic liver disease, decreased protein intake, non-renal excretion in vomitus, and losses in faeces during diarrhoea[32].

A normal[72] serum urea: creatinine ratio is 10-15. Brady[10] proposed that in humans a serum urea: creatinine ratio in an azotaemic patient of ≥ 20 indicates prerenal azotaemia, whereas a serum urea: creatinine ratio of < 20 in an azotaemic patient indicates intrinsic renal disease. This would indicate that serum urea is more likely to increase due to prerenal factors than serum creatinine, where as both parameters are equally likely to increase due to renal disease.

Possible substrates for hyperurea-genesis in canine babesiosis

The globin portion of haemoglobin represents 95% of the soluble protein of RBC, reflecting the importance of the RBC for oxygen transport and acid base buffering[4]. The RBC, however, also needs energy to reduce methaemoglobin, protect the numerous sulph-hydryl groups found in erythrocyte enzymes, protect membrane components and haemoglobin, maintain a high potassium and low sodium gradient relative to plasma, regulate cell size, shape and deformability and to modulate the uptake and delivery of oxygen by haemoglobin[4]. Numerous reductases, mutases, catalases, phosphatases, dehydrogenases, peroxidases, transferases, kinases, isomerases, dismutases, epimerases, transketolases and transaldolases are required for these processes[4]. Together with the membrane protein proteases these enzymes contribute to the cytoplasmic proteins[4]. Erythrocyte membrane proteins may also contribute to the total metabolisable protein load during haemolysis. They consist of proteins of the erythrocyte membrane including major and minor integral proteins and peripheral proteins[83]. Glycoporin A is an example of an integral protein, whereas spectrin and actin are examples of peripheral proteins[83]. These proteins are important in

transmembrane signalling and transportation as well as in the cytoskeletal structure of the erythrocyte membrane[83].

The fate of haemoglobin during haemolysis

Haemoglobin is made up of a haem prosthetic group, protoporphyrin IX chelating a ferrous ion, and the apoprotein, globin. The globin moiety consists of a tetramer of two dissimilar polypeptide chains α and β each chain containing approximately 140 amino acids[35].

A plethora of pathogenic mechanisms in canine babesiosis results in one of two fates for the targeted erythrocyte: either intravascular haemolysis or erythrophagocytosis. Haemoglobin from either fate ends up in the hepatic Kupffer cells or circulating, splenic or bone marrow macrophages. The porphyrin portion is converted by the macrophages, through a series of stages, into the bile pigment bilirubin, which is re-released into the blood to be secreted by the liver, after conjugation, into bile or by the kidney into urine[37].

Haemolytic jaundice is thought to represent excessive bilirubin preload on the liver which cannot adequately respond via increased conjugation and excretion[39].

The globin portion of haemoglobin is split into its constitutive amino acids within the tissue macrophages and then released back into the blood. These amino acids are utilized by tissues as building blocks for proteins or they may be deaminated, mainly in the liver, and utilized as an energy source. In a human study[80], upper gastrointestinal bleeding was shown to be an ammoniagenic event related to the complete absence of isoleucine and the increased presence of high amounts of leucine and valine. Isoleucine is an amino acid of high biological value, where as leucine and valine are amino acids of poor biological[80]. Deamination takes place via aminotransferases, the most common reaction is the transfer of an amino group from the amino acid to α-ketoglutaric acid to form glutamic acid and an α-keto acid[38]. Glutamic acid can transfer the amino group to other substances or release it as ammonia[38]. Essentially all the released ammonia is converted to urea in the liver[39]. Two ammonia molecules plus one carbon dioxide molecule combine to form one urea molecule and water[39]. Failure of the urea cycle to adequately metabolise ammonia may cause

a state of hyperammonaemia to develop[39]. It is unknown whether hypocapnoea, which can be severe in canine babesiosis patients in South Africa, would cause any rate limitation on this reaction[39,57]. According to Maegraith[67] the erythrocyte count may fall by 1 x 10^{12}/litre of blood/ day of babesia infection. This represents a daily loss of approximately 12.5% of the patients RBC. The normal percentage loss of RBC per day is 100% divided by 110 days (the normal RBC survival time) or 0.9%. This means that during severe haemolysis 14 times the normal daily haemoglobin load on the body can occur.

Serum substances likely to interfere with biochemical tests in canine babesiosis

Of particular importance in canine babesiosis is the interference caused by haemoglobin and haemoglobin breakdown products, especially bilirubin, on the biochemical analysis of serum creatinine concentrations when the kinetic Jaffé alkaline picrate or the indirect enzymatic colourimetric test is used. These chemicals may cause a negative bias on the measurement of serum creatinine concentrations[7,41,98,101]. This negative bias may help explain the discrepancy encountered between serum urea and creatinine levels in canine babesiosis patients, where intravascular haemolysis and icterus is common[61,65,99].

In a study comparing the effects of interference on the kinetic Jaffé reaction and an enzymatic colourimetric test for creatinine concentration in dogs, serum bilirubin caused a negative bias on both tests whilst serum haemoglobin had little or no influence on the tests[41]. The values of bilirubin and haemoglobin ranged from 0-1000 µmol/l (normal < 6.8 µmol/l) and 0-4 g/l (normal = 0 g/l) respectively. In a study where haemoglobin was infused into an experimental model, a decline in creatinine levels with increasing haemoglobin levels was noticed[65]. It should also be noted that high levels of serum haemoglobin can cause a positive bias in the assay of serum bilirubin[22].

The current method of creatinine determination at the Section of Clinical Pathology, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria is based on the kinetic modification of the Jaffé alkaline picrate reaction[100].

Test interference related to wavelength spectra

The current method of serum urea determination at the Section of Clinical Pathology, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria is based on the enzymatic method of Talke, which is a modification of the method described by Tiffany[100]. This method has its absorption spectrum at 340 nm and may be interfered with by the elevated serum haemoglobin concentrations encountered in canine babesiosis due to its high intrinsic absorptive capacity at 300-500 nm[100]. Similarly plasma ammonia is measured spectrophotometrically at 365 nm, using Boehringer-Mannheim reagent and a mercury vapour lamp based on the method described by Da Fonseca-Wollheim[18], and is also subject to be interfered with by the elevated serum haemoglobin concentrations. The increased absorption afforded by the elevated serum haemoglobin concentrations may cause erroneously high serum urea and plasma ammonia values to be reported[18].

Cystatin-C an alternative measure of renal function

Cystatin-C is a low-molecular mass cysteine protease inhibitor consisting of a nonglycosylated polypeptide chain containing 120 amino acid residues[5,48]. In humans it is constantly produced by all nucleated cells, is freely filtered by the glomerulus, metabolised by the luminal tubular epithelium, and is without significant unmetabolised tubular reabsorption[84]. Cystatin-C has been proposed as a more sensitive marker for reduced glomerular filtration rate than creatinine, and thus an earlier detector of renal disease in humans[36,49,84]. Limited studies have shown cystatin-C may hold similar promise for as an early detector of renal disease in dogs[5,48,102]. In a recent study in humans in which cystatin-C was compared with creatinine in falciparum malaria, it was deduced that renal disease appears to have been underestimated using creatinine as a common screening test[36]. Because there appears to be substantial common disease mechanisms between canine babesiosis and human falciparum malaria, it is possible that a similar situation exists in canine babesiosis[16,86,103]. Serum haemoglobin, bilirubin, and triglycerides do not interfere with the cystatin-C

assay[56]. Serum and plasma haemoglobin concentrations up to 2 g/l, serum bilirubin concentrations up to 300 µmol/*l*, and serum triglyceride concentrations up to 8.5 mmol/l were tested. Cystatin-C may, therefore, prove to be the more accurate and sensitive test for renal disease compared with serum urea and creatinine in the clinical setting of canine babesiosis.

The human test for cystatin-C has been validated for canine use[5]; Western blot analysis demonstrated considerable cross-reactivity of the polyclonal antihuman cystatin-C antibody with canine cystatin-C, thus substantiating other preliminary studies[48,102] evaluating canine cystatin-C like immunoreactivity in dogs using the test designed for humans. In one study[5] it also appeared that cystatin-C was less affected by prerenal azotaemia than creatinine.

CHAPTER 2

PROBLEM

From the above review, the following is evident.

- Renal failure is not common in anaemic *B. canis* patients.
- Renal pathology is common in anaemic *B. canis* patients.
- Renal failure is relatively more common in the haemoconcentrated form of *B. canis* infection in dogs.
- Haemoglobinaemia and haemoglobinuria are common in *B. canis* patients but are not synonymous with renal pathology or renal failure.
- Serum urea has been observed to be disproportionately elevated relative to serum creatinine in anaemic canine babesiosis patients. The cause of this phenomenon is not established.
- The potential exists for prerenal causes of azotaemia (such as gastric ulceration, tissue catabolism and hypovolaemic shock) to cause the observed disproportionate elevation in serum urea relative to serum creatinine in anaemic *B. canis* infected dogs.
- The potential exits for hyperureagenesis, as a result of haemolysis driven substrate loading, to cause the observed disproportionate elevation in serum urea relative to serum creatinine in anaemic *B. canis* infected dogs.
- The potential exists for serum haemoglobin to positively bias spectrophotometric determinations of plasma ammonia and serum urea concentrations due to its high intrinsic absorbance of light in anaemic *B. canis* patients.
- Finally, the potential exists for the elevated concentrations of serum haemoglobin and/or serum bilirubin to interfere with the measurement of serum creatinine concentrations causing a negative bias for creatinine and possibly explaining the observed disproportionate elevation in serum urea relative to serum creatinine in anaemic *B. canis* infected dogs.
- Renal failure is common in severe human malaria patients.
- There is extensive research published on the pathogenesis of renal failure in human malaria and pro-inflammatory cytokines seem to be

an important factor in the pathogenesis of this organ failure No similar research exists for canine babesiosis

- To date, changes in serum urea: creatinine ratios have not been reported in human malaria infections.
- Serum cystatin-C as an emerging test of renal function, is thought to be more sensitive than serum creatinine and less prone to interference by serum haemoglobin and serum bilirubin than creatinine.

CHAPTER 3

OBJECTIVES

HYPOTHESIS

The hypothesis is proposed that:

- The elevated serum urea: creatinine ratio that occurs in anaemic *B. canis* patients is not of renal origin but rather as a result of falsely elevated serum urea concentrations.
- Serum creatinine alone is a better measure of renal disease in anaemic *B. canis* patients in than serum urea.
- Furthermore, due to the similarities in pathomechanism between the two diseases, and the fact that serum cystatin-C has been found to be a useful indicator of renal disease in human malaria studies, serum cystatin-C will prove to be a useful indicator of renal disease in canine babesiosis.

Research Questions

- Is the increased serum urea: creatinine ratio encountered in severely anaemic canine babesiosis patients of renal origin or not?
- Can a plausible explanation be proposed for the elevated serum urea: creatinine ratio encountered in severely anaemic canine babesiosis patients?
- Which parameter, serum urea or serum creatinine, is least influenced by the increased serum haemoglobin and/or serum total bilirubin concentrations and therefore best indicates renal disease in severely anaemic canine babesiosis patients?
- Is there any benefit in the measurement of serum cystatin-C above that of serum urea or serum creatinine in severely anaemic or haemoconcentrated cases in canine babesiosis patients?
- Is ammonia elevated in canine babesiosis?

Study objectives

1. To report whether the concentrations of serum urea, serum creatinine and serum cystatin-C indicate renal disease in severely anaemic or haemoconcentrated canine babesiosis patients.
2. To determine whether an elevated serum urea: creatinine ratio is encountered in anaemic *B. canis* patients and whether the increased ratio originates from elevated serum urea or depressed serum creatinine concentrations or both, using serum cystatin-C as a reference.

3. To establish whether this increased ratio was of renal origin or not, and to compare serum creatinine with serum urea as a measure of renal function in *B. canis* patients.
4. To determine the usefulness or otherwise of serum cystatin-C concentrations as an indicator of renal disease in canine babesiosis.
5. To determine whether hyperammonaemia is present in *B. canis* patients as a possible indicator of hyperureagenesis.

CHAPTER 4

MATERIALS AND METHODS

Model and Exclusions

Group 1: Severely anaemic canine babesiosis group.

Inclusion criteria:

- Canine patients positive for babesiosis on peripheral thin blood smear using the diff quick staining technique.
- Haematocrit ≤ 20%.
- > 5 kg body mass.

Exclusion criteria:

- Haematocrit > 20%.
- Suggestive or positive *Ehrlichia canis:*
    - Morula or the absence of the expected inflammatory white cell response on thin blood smear using the diff quick staining technique.
    - Peripheral lymphadenopathy.
    - Purulent nasal discharge or epistaxis.
- Any other known systemic disease.
- Macroscopic serum lipaemia after whole blood centrifugation.
- < 6 hours fasting.

Group 2: Control group.

Inclusion criteria:

- Canine patients negative for canine babesiosis on peripheral thin blood smear using the diff quick staining technique.
- > 5 kg body mass.
- Normal resident departmental dogs used in student training at the OVAH.

Exclusion criteria:

- Positive for *B. canis* on peripheral thin blood smear using the diff quick staining technique.
- Suggestive or positive *E. canis:*
    - Morula or thrombocytopenia, leukopenia and/ or nonregenerative anaemia on peripheral thin blood smear using the diff quick staining technique.
    - Peripheral lymphadenopathy.
    - Purulent nasal discharge or epistaxis.

- Any other known systemic disease.
- Macroscopic evident serum haemoglobinaemia and/or jaundice after whole blood centrifugation and/or macroscopic evident haemoglobinuria.
- Macroscopic serum lipaemia after whole blood centrifugation.
- < 6 hours fasting.

Group 3: Haemoconcentrated canine babesiosis group

Inclusion criteria:

- Canine patients positive for babesiosis on peripheral thin blood smear stain.
- Haematocrit ≥ 50%.
- > 5 kg body mass.

Exclusion criteria:

- Haematocrit < 50%.
- Suggestive or positive E. canis
    - Morula or the absence of the expected inflammatory white cell response on peripheral thin blood smear using the diff quick staining technique.
    - Peripheral lymphadenopathy.
    - Purulent nasal discharge or epistaxis.
- Any other known systemic disease.
- Macroscopically evident serum lipaemia after whole blood centrifugation.
- < 6 hours fasting.

Experimental design

Principal analyses to test the hypotheses

- The time frame for samples collection was from November 2003 to September 2004
- Patients were accepted into the study only after the inclusion/ exclusion criteria were met and the owners or departmental consent was obtained.
- 1 Serum and 1 EDTA anticoagulated blood sample was collected from the jugular vein into appropriate 3 ml vacuum tubes. The samples for ammonia were analyzed within 30 minutes of collection. The patients selected had a peripheral blood smear, haematocrit, total serum protein (by refractometer), in saline agglutination test and

urine analysis (urine collected by cystocentesis) analysed. All samples were collected prior to any treatment.

- The values of serum urea, serum creatinine, serum haemoglobin, serum total bilirubin, serum cystatin-C and plasma ammonia generated from group 2 were used to establish a reference range against which to compare group 1 (anaemic patients) and group 3 (haemoconcentrated patients), where applicable.
- Serum urea, creatinine, cystatin-C, haemoglobin, bilirubin, plasma ammonia, TSP concentrations, haematocrit values, serum urea: creatinine ratios and urine analysis, were appropriately analysed, measured or calculated for all cases and tabulated. Urea and creatinine were converted to mg/dl for the calculation of the serum urea: creatinine ratio in order to ensure equivalent units (mathematical laws require that equivalent units are used for ratio determinations).
- The urea: creatinine ratio for the human malaria patients was calculated from data supplied by Dr Arjen M Dondorp, Wellcome-Mahidol University, Oxford, department Tropical Medicine.
- All normal findings as well as deviations from normality were reported for the measured parameters. All diseased patients' urine analysis findings were also reported and compared to the biochemical results. One case was available for post mortem and these findings are also reported.
- The data was analysed for significant differences between the groups using the Wilcoxon-rank sum test for difference in medians. Correlations between the various serum parameters were assessed using the Spearman-rank correlation coefficient. Scatter plots were plotted on the logarithmic scale in order to more closely approximate the normal distribution. Two-tailed tests were used and the significance level was set at $\alpha = 0.05$. The statistical software package used was NCSS 2004 (NCSS, Kaysville, Utah, USA).
- Histopathology was performed on the single patient included in this study that died, as a result of the canine babesia infection.

Laboratory measurements

Serum Cystatin-C

Serum cystatin-C concentration was measured using the particle-enhanced turbidimetric immunoassay (Diagnostech, Honeydew Dako Cytomation, Cystatin C PET kit, Denmark), designed for the determination of human cystatin-C[48]. Normal values were set at < 1.7 mg/l according to the control group and the findings of Wehnerl[102].

Serum haemoglobin assay

Serum haemoglobin concentration was measured using an adaptation of the Drabkin's cyanmethhaemoglobin method[7] for the Bayer-Technicon RA-XT (Bayer (Pty) Ltd Isando, SA). Samples were considered to have significantly elevated serum haemoglobin if the measured concentration was > 1.6 g/l (altered according to the control group).

Serum Urea

Serum urea concentrations were measured using the Technicon Method[100] which is a modification of the enzymatic method of Talke and Schubert for the RA-1000 analyser (Bayer (Pty) Ltd Isando, SA). Normal values were 3.6-8.9 mmol/l.

Serum Creatinine

Serum creatinine concentrations were measured using the Technicon Method[87] which is a kinetic modification of the Jaffé alkaline picrate reaction for the RA-1000 analyser (Bayer (Pty) Ltd Isando, SA)**.** Normal values were < 133 µmol/l.

Serum Bilirubin

Serum total bilirubin concentrations were measured using the Technicon Method[24] which is a modification of the Van den Bergh diazo reaction for the RA-1000 analyser (Bayer (Pty) Ltd Isando, SA). Normal values < 15 µmol/l (altered according to the control group).

Plasma ammonia

Plasma ammonia concentrations were measured using an enzymatic, spectrophotometeric test based on an improvement of the method described by Da Fonseca-Wollheim for the

Technicon RA-XT analyzer (Bayer (Pty) Ltd Isando, SA). The reagent kit used is Roche Diagnostics(KAT Medical, Calicom Trading, Ansfere)[18]. Normal values were <40 µmol/l.

PCV

PCV was measured as a percentage of packed red blood cells to serum once centrifuged. Normal values for non-parasitised patients were 37-55%. Parasitised patients were expected to have a pathologically lowered PCV as a result of haemolysis and any PCV ≥ 50% was considered haemoconcentrated.

TSP

TSP was measured by refractometer (American Optical, Scientific Instrument division, Buffalo, USA). Normal values were 50-70 g/l.

Urine analysis

The following comprised the urine analysis:

- Urine SG using a refractometer (American Optical, Scientific Instrument division, Buffalo, USA).
- Urine chemistry dip stick (Lenstrip 8 Dipsticks, Benmore Diagnostics, Sandton, RSA).
- Urine sediment using the Sterheimer Mahlbein staining technique after centrifugation.

Special attention was paid to the following:

- Urine SG
  - < 1.030 inadequately concentrated if the patient is truly azotaemic.
  - ≥ 1.030 adequately concentrated.
- Urine colour
  - Clear: no macroscopically detectible bilirubin or haemoglobinuria.
  - Yellow normal urine colour.
  - Dark yellow: possible presence of macroscopically detectible bilirubinuria.
  - Red: Macroscopically detectible mild haemoglobinuria.
  - Dark red/ black: Macroscopically detectible severe haemoglobinuria

- Urine protein (chemistry dip stick)
    - 1+, 2+ or 3+ according to the urine chemistry strip reaction.
- Urine bilirubin (chemistry dip stick)
    - 1+, 2+ or 3+ according to the urine chemistry strip reaction.
- Urine blood/ haemoglobin (chemistry dip stick)
    - 1+, 2+, 3+ or 4+ according to the urine chemistry dip stick reading.
- Presence of casts
    - Hyaline casts denoting proteinuria.
    - Fine and coarse granular casts denoting increasing degrees of tubular lesions
    - Granular casts containing RTE cells donating a higher degree of tubular lesion.
    - Haemoglobin casts denoting degrees of haemoglobinuria.
- Urine RTE cells
    - 1+ 1 every 2-3 High Powered Field (HPF) (40 X 10)
    - 2+ 1-2 per HPF
    - 3+ 2-4 per HPF
    - 4+ > 5 per HPF
- Presence of crystals
    - 1+, 2+, 3+ or 4+ haemoglobin crystals denoting degrees of haemoglobinuria.
    - 1+, 2+, 3+ or 4+ bilirubin crystals denoting degrees of bilirubinuria.

CHAPTER 5

RESULTS

Overview of the model, tables and graphs.

Twenty eight canine babesiosis patients treated at the OVAH, and 13 babesiosis negative dogs (group 2), as a control group, were used for this study. The babesiosis patients were divided into 2 groups according to their PCV. The anaemic group consisted of patients with a PCV ≤ 20% (n=25) (Group 1), and the haemoconcentrated group consisted of patients with a PCV ≥ 50% (n=3) (Group 3).

Table 1 is a summary of the biochemical data and the calculated urea: creatinine ratio for the three canine groups. Urine analysis data for these cases are displayed in numerical order in **table 3**. **Table 2** is a summary of the descriptive statistics for the canine data in table 1 and human malaria data in table 5. **Table 5** is found in the appendix and consists of the paired samples of serum urea and serum creatinine from human malaria patients as supplied by Dr Arjen M Dondorp via a personal communication. Table 5 also contains the urea: creatinine ratio for the supplied data. **Table 4** is the Spearman-rank correlation matrix for the canine data (Groups 1, 2 and 3). **Plot 1-9** are box plots of the biochemical results for groups 1 and 2; and the serum urea creatinine ratio for groups 1, 2 and the human malaria data. **Plot 10, 11 and 12** are scatter graphs of the linear regression between log cystatin-C and log creatinine, log cystatin-C and log urea and log urea: creatinine ratio and log cystatin-C respectively.

Raw data and basic calculations

Table 1 is a summary of all the canine raw data and the calculated serum urea: creatinine ratio; but excludes the urine analysis data which is found in table 3.

Table 1: Table of canine biochemical results and the babesia serum urea: creatinine ratio

Group 1	Cystatin-C mg/l	Bil-T µmol/l	Urea mmol/l	Creat µmol/l	Ammonia µmol/l	Serum Hb g/l	PCV %	TSP g/l	Urea mg/dl	Creat mg/dl	U: C ratio
1	1.3	25.7	7.3	61	37.7	1.7	10	76	20.45	0.69	29.63
2	1.2	11.4	6.8	53	64.6	1.3	16	52	19.05	0.60	31.77
3	0.7	85.7	27.2	71	387.8	5.1	7	50	76.19	0.80	94.86
4	0.0	10.3	5.1	39	43.1	1.3	10	57	14.29	0.44	32.38
5	0.8	9.4	8.7	67	0.0	1.3	10	52	24.37	0.76	32.15
6	0.5	19.6	11.7	50	48.5	1.3	10	54	32.77	0.57	57.94
7	1.1	8.4	9.6	103	59.3	1.3	13	80	26.89	1.17	23.08
8	1.03	45.2	16.2	100	95.0	0.9	15	45	45.38	1.13	40.11
9	0.8	29.2	16.4	86	70.0	1.2	11	66	45.94	0.97	47.22
10	0.0	33.4	7.3	22	204.7	3.0	13	40	20.45	0.25	82.16

11	0.6	4.7	3.9	46	43.1	1.1	13	53	10.92	0.52	20.99
12	0.7	32.5	32.8	88	226.2	4.1	11	66	91.88	1.00	92.29
13	1.0	6.3	8.3	119	21.5	2.0	13	100	23.25	1.35	17.27
14	0.0	9.4	9.1	57	59.2	1.7	11	58	25.49	0.64	39.53
15	0.66	16.6	13.8	111	161.6	3.1	6	60	38.66	1.26	30.79
16	0.8	94.8	21.0	82	107.7	1.1	10	72	58.82	0.93	63.41
17	0.6	19.7	16.7	100	53.8	0.9	13	48	46.78	1.13	41.35
18	0.0	40.8	14.4	69	140.0	2.2	10	54	40.34	0.78	51.68
19	0.6	10.6	11.8	60	0.0	0.7	10	58	33.05	0.68	48.70
20	1.4	8.1	13.8	104	37.7	1.6	14	80	38.66	1.18	32.86
21	0.9	5.1	12.8	63	0.0	1.2	14	44	35.85	0.71	50.31
22	0.9	4.3	6.0	85	16.2	0.9	18	50	16.81	0.96	17.48
23	0.6	9.7	11.4	58	0.0	1.0	15	44	31.93	0.66	48.67
24	0.9	9.7	14.7	53	21.5	1.5	10	54	41.18	0.60	68.68
25	0.54	9.9	13.9	55	70.0	1.6	8	52	38.94	0.62	62.58
Group 2	**Cystatin-C mg/l**	**Bil-T µmol/l**	**Urea mmol/l**	**Creat µmol/l**	**Ammonia µmol/l**	**Serum Hb g/l**	**PCV %**	**TSP g/l**	**Urea mg/dl**	**Creat mg/dl**	**U: C ratio**
1	0.77	9.3	3.0	56	21.5	0.5	48	68	8.40	0.63	13.27
2	0.68	11.6	3.6	74	5.4	0.7	48	64	10.08	0.84	12.05
3	0.75	14.4	3.7	82	32.3	1.6	44	58	10.36	0.93	11.17
4	0.67	11.0	3.9	75	16.1	0.7	49	60	10.92	0.84	12.88
5	0.74	11.9	4.1	59	37.7	0.0	39	72	11.48	0.67	17.21
6	0.73	11.2	4.2	74	32.3	1.4	52	64	11.76	0.84	14.05
7	0.64	9.0	4.3	67	21.5	1.1	44	60	12.04	0.76	15.89
8	0.62	8.9	4.7	65	26.9	0.8	34	64	13.17	0.74	17.90
9	0.66	9.1	4.9	79	21.5	0.8	50	58	13.73	0.89	15.36
10	0.63	8.5	5.2	114	10.8	0.9	48	56	14.57	1.28	11.29
11	0.55	8.5	7.1	86	32.3	1.6	51	50	19.89	0.97	20.44
12	0.48	13.3	8.5	104	37.7	1.1	46	54	23.81	1.18	20.24
13	0.75	11.5	8.9	88	5.4	0.3	46	64	24.93	1.00	25.04
Group 3	**Cystatin-C mg/l**	**Bil-T µmol/l**	**Urea mmol/l**	**Creat µmol/l**	**Ammonia µmol/l**	**Serum Hb g/l**	**PCV %**	**TSP g/l**	**Urea mg/dl**	**Creat mg/dl**	**U: C ratio**
1	2.6	10.6	14.4	171	43.1	5.6	55	65	40.34	1.93	20.85
2	0.6	9.0	13.0	86	26.9	2.1	54	60	36.41	0.97	37.43
3	5.7	10.1	41.8	608	172.3	2.8	54	60	117.09	6.88	17.02

Descriptive stats

Table 2 is a summary of the descriptive stats for all the chemistry data in this study, canine and human cases, as well as the p value for the Wilcoxon-rank sum test for differences in medians between group 1 and groups 2.

Table 2 Summary of descriptive statistics.

Parameter	Group	Number	Mean	Median	Standard Deviation	95% Confidence Interval	P value#
Cystatin-C mg/l	Group 1	25	0.71	0.7	0.39	0.54 - 0.87	0.45
	Group 2	13	0.67	0.7	8.51^{-02}	0.62 - 0.72	
	Group 3*	3	2.97	2.6	2.57	-3.42 - 9.35	
BiliT µmol/l	Group 1	25	22.42	10.6	23.50	12.72 - 32.12	0.36
	Group 2	13	10.63	11.0	1.91	9.47 - 11.79	
	Group 3*	3	9.90	10.1	0.82	7.87 - 11.93	
Urea mmol/l	Group 1	25	12.83	11.8	6.68	10.07 - 15.58	<0.01
	Group 2	13	5.09	4.3	1.89	3.94 - 6.22	
	Group 3*	3	23.07	14.4	16.24	-17.27 - 63.41	
Creatinine µmol/l	Group 1	25	72.08	67.0	24.64	61.91 - 82.25	0.27
	Group 2	13	78.69	75.0	16.64	68.64 - 88.75	
	Group 3*	3	288.33	171.0	280.08	407.43 - 984.10	
Urea: Creat Ratio Calculated in traditional units mg/l	Group1	25	46.32	41.4	21.68	37.37 - 55.26	<0.01
	Group2	13	15.91	15.4	4.15	13.40 - 18.42	
	Group 3*	3	25.10	20.9	10.85	-1.84 - 52.05	
	Human malaria group	363	20.64	18.8	9.47	19.66 - 21.61	

Parameter	Group	Number	Mean	Median	Standard Deviation	95% Confidence Interval	P value[#]
Ammonia μmol/l	Group 1	25	78.77	53.8	88.66	42.17 - 115.37	<0.01
	Group 2	13	23.18	21.5	11.29	16.36 - 30.01	
	Group 3*	3	80.77	43.1	79.68	117.18 - 278.71	
Serum Hb g/l	Group 1	25	1.72	1.3	1.06	1.29 - 2.16	<0.01
	Group 2	13	0.88	0.8	0.48	0.60 - 1.17	
	Group 3*	3	3.50	2.8	1.85	-1.10 - 78.10	
PCV %	Group 1	25	11.64	11.0	2.83	10.47 - 12.81	<0.01
	Group 2	13	46.08	48.0	4.99	43.06 - 49.09	
	Group 3*	3	54.33	54.0	0.58	52.90 - 55.77	
TSP g/l	Group 1	25	58.60	54.0	13.95	52.84 - 64.36	0.13
	Group 2	13	60.92	60.0	5.92	57.34- 64.50	
	Group 3*	3	61.67	60.0	2.89	54.50 - 68.84	

*The numbers in group 3 were too small (n=3) for meaningful comparison between the groups.

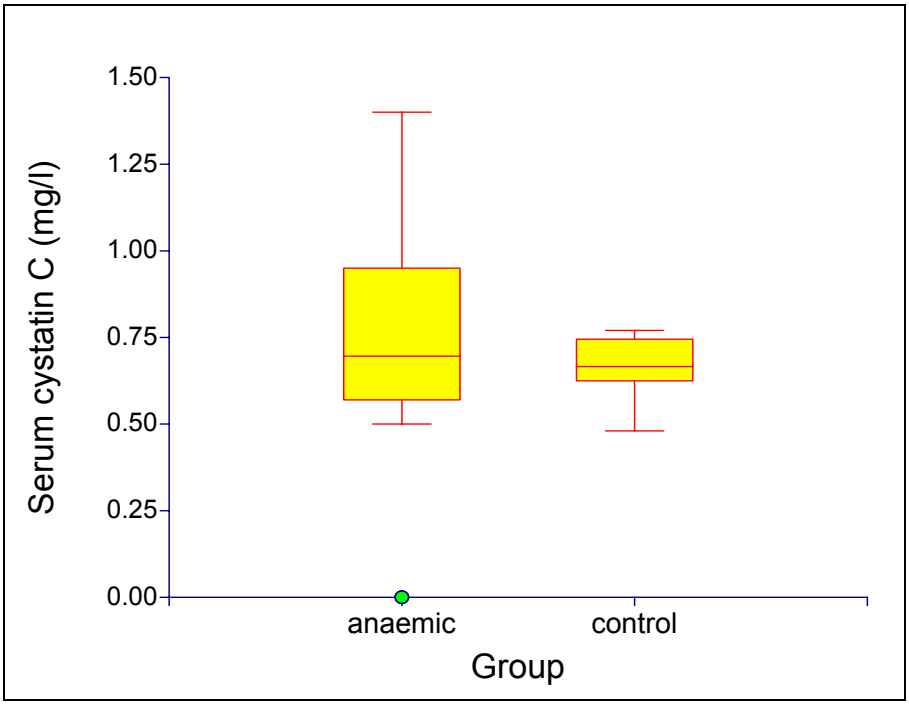
[#] P value for the Wilcoxon-rank sum test for difference in medians as applied to group 1 and group 2.

Summary of statistics results and box plots

Serum cystatin-C

Serum cystatin-C was > 1.7 mg/l in 0/25 anaemic patients, 0/13 control patients and 2/3 haemoconcentrated patients. Mean and median values for serum cystatin-C were 0.71 mg/l and 0.7 mg/l, and 0.67 mg/l and 0.7 mg/l for the anaemic group and the control group respectively. The mean, median and distribution of serum cystatin-C did not vary significantly between the control group and the anaemic babesia group (p=0.450172). The 2/3 haemoconcentrated patients with elevated cystatin-C also had elevated serum urea and serum creatinine concentrations. Both of these patients died. One of them was available for post mortem. The post mortem description of the renal lesions paralleled the degree of serum biochemical abnormalities. The post mortem findings are described in detail below.

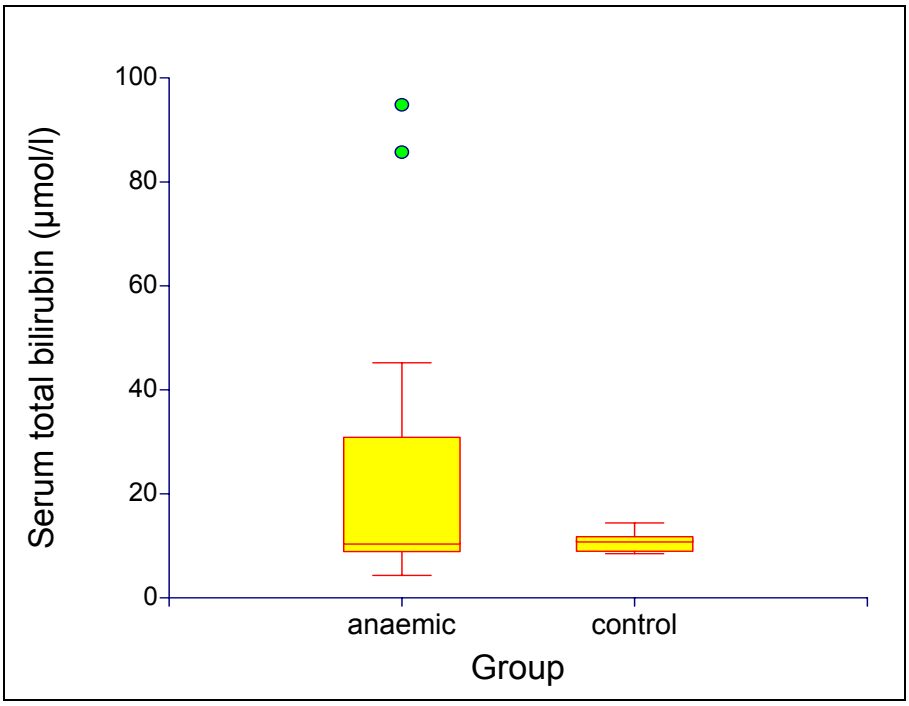
Plot 1: Serum cystatin-C concentrations in groups 1 and 2. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes) and 10th and 90th percentiles (T-bars). The upper normal limit for serum cystatin-C was 1.7 mg/l.



Serum total bilirubin

Serum total bilirubin was > 15 µmol/l in 11/25 anaemic patients, 0/3 haemoconcentrated patients and 0/13 control patients. Mean and median values for serum total bilirubin were 22.42 µmol/l and 10.6 µmol/l, and 10.63 µmol/l and 11.0 µmol/l for the anaemic group and the control group respectively. The distribution and the median serum total bilirubin did not vary significantly between the control group and the anaemic babesia group (p=0.355888)

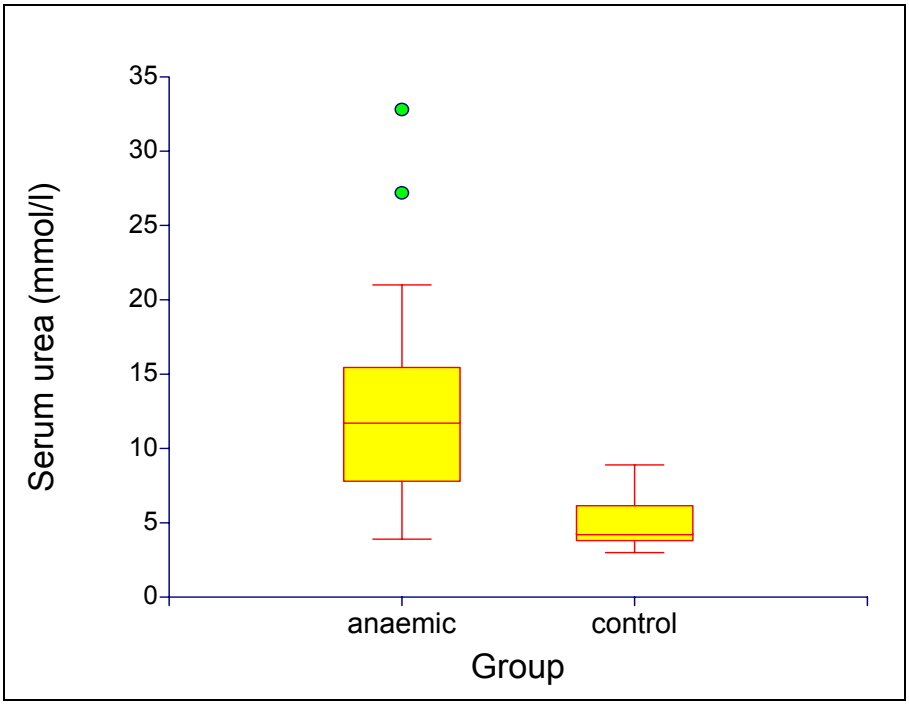
Plot 2: Serum total bilirubin concentrations in groups 1 and 2. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes) and 10th and 90th percentiles (T-bars). The green dots represent outliers. The upper normal limit for serum total bilirubin was ≤15 µmol/l.



Serum urea

Serum urea was elevated in 17/25 anaemic patients, 0/13 control patients, 3/3 haemoconcentrated patients and 135/363 human malaria patients. Mean and median values for serum urea were 12.83 mmol/l and 11.8 mmol/l and 5.09 mmol/l and 4.3 mmol/l for the anaemic group and the control group respectively. The mean, median and distribution of serum urea was significantly elevated in the anaemic babesia group compared with the control group (p=0.000030).

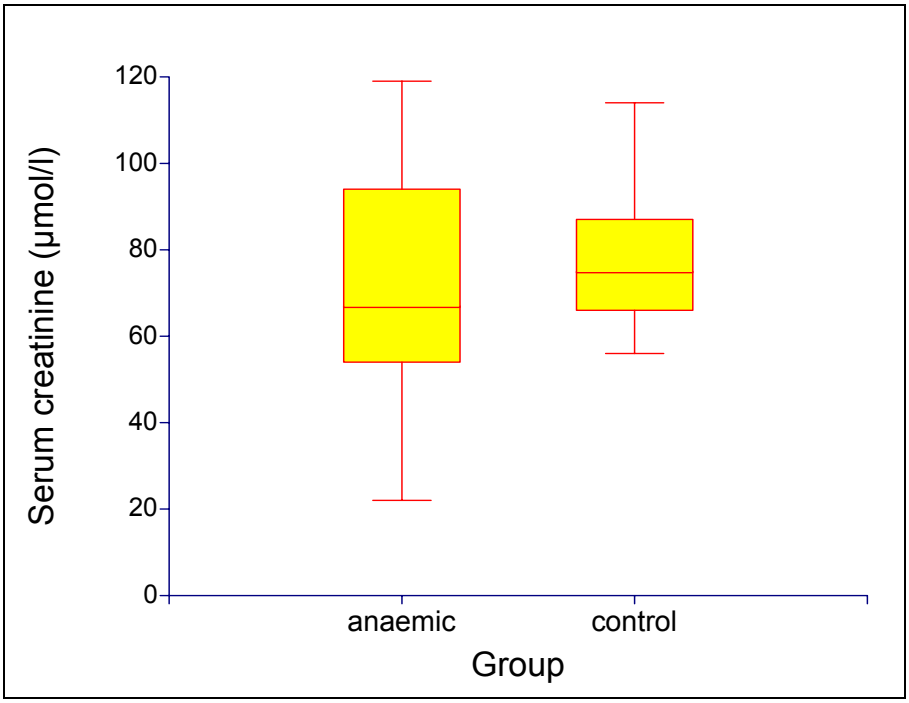
Plot 3: Serum urea concentrations in anaemic babesia patients and healthy control patients. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes) and 10th and 90th percentiles (T-bars). The green dots represent outliers. The normal range for serum urea was 3.6-8.9 mmol/l.



Serum creatinine

Serum creatinine was elevated in 0/25 anaemic patients 0/13 control patients, 2/3 haemoconcentrated and 67/363 human malaria patients. It is note worthy that the same babesia patients with elevated serum creatinine also had elevated serum cystatin-C and serum urea concentrations. Mean and median values for serum creatinine were 72.08 µmol/l and 67 µmol/l, and 78.69 µmol/l and 75 µmol/l for the anaemic group and the control group respectively. The mean, median and distribution of serum creatinine did not vary significantly between the control group and the anaemic babesia group (p=0.274488).

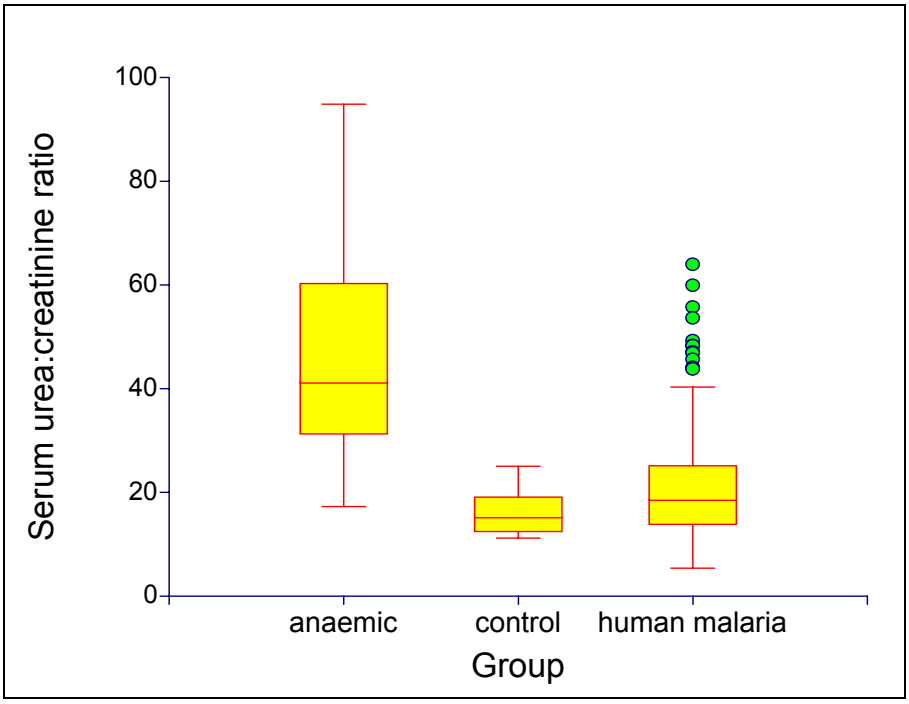
Plot 4: Serum creatinine concentrations in anaemic babesia patients and healthy control patient. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes) and 10th and 90th percentiles (T-bars). The normal range for serum creatinine was 40-133 µmol/l.



Serum Urea: Creatinine Ratio

The serum urea: creatinine ratio was elevated in 23/25 anaemic, 3/13 control, 2/3 haemoconcentrated and 162/363 human malaria patients. The mean and median serum urea: creatinine ratio for the anaemic babesia group was 46.32 and 41.36, for the haemoconcentrated babesia group 25.10 and 20.85, for the malaria patients 20.64 and 18.75, and for the control group 15.91 and 15.36 respectively. The mean and median of the urea: creatinine ratio was significantly elevated in the anaemic babesiosis group compared with the control group and the distribution between these two groups was significantly different ($p<0.000003$). The human malaria data was included in the box plot for visual comparison only.

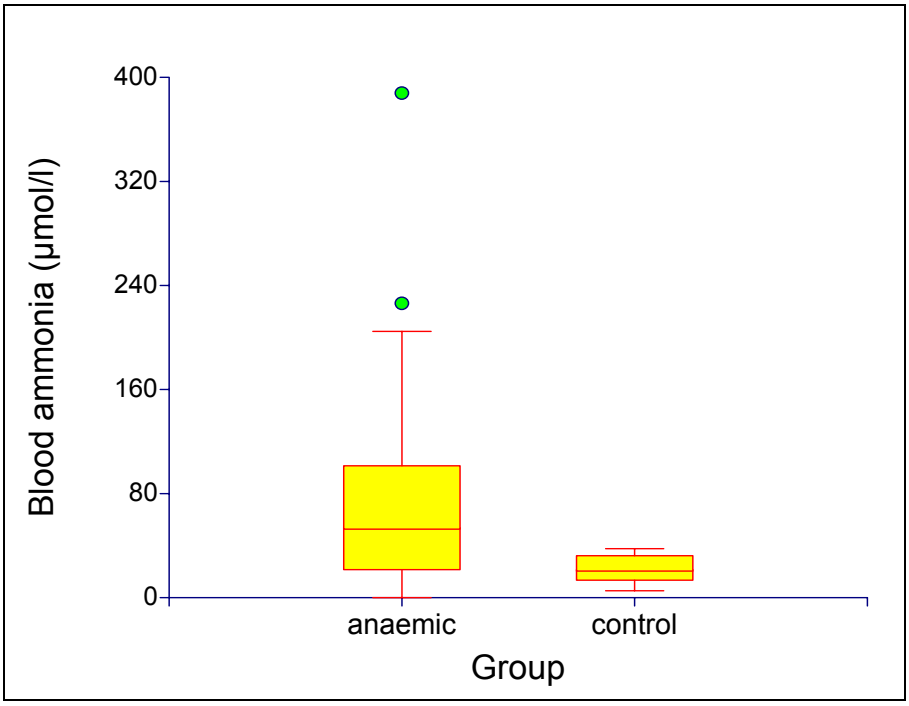
Plot 5: Serum urea: creatinine ratio in anaemic babesia patients, healthy control dogs and human malaria patients. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes) and 10th and 90th percentiles (T-bars). The green dots represent outliers. The normal range for the serum urea: creatinine ratio was 10-15.



Plasma ammonia

Plasma ammonia was elevated in 16/25 anaemic patients, 0/13 control patients and 2/3 haemoconcentrated patients. Mean and median values for plasma ammonia were 78.77 µmol/l and 53.8 mol/l, and 23.18 µmol/l and 21.5 µmol/l for the anaemic group and the control group respectively. The mean, median and distribution of plasma ammonia varied significantly between the control group and the anaemic babesia group (p=0.009562).

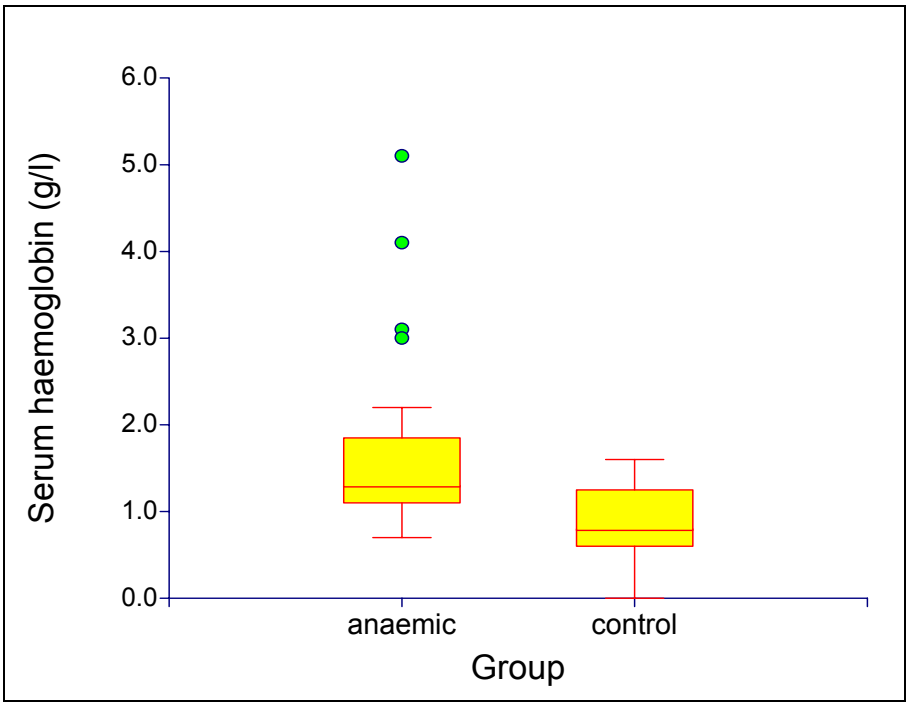
Plot 6: Plasma ammonia concentrations in groups 1 and 2. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes) and 10th and 90th percentiles (T-bars). The green dots represent outliers. The normal range for plasma ammonia was 3-40 µmol/l.



Serum haemoglobin

Serum haemoglobin was > 1.6 g/l in 8/25 anaemic patients, 0/13 control patients and 3/3 haemoconcentrated patients. Mean and median values for serum haemoglobin were 1.72 g/l and 1.3 g/l, and 0.88 g/l and 0.8 g/l for the anaemic group and the control group respectively. The mean, median and distribution of serum haemoglobin varied significantly between the control group and the anaemic babesia group (p=0.002364).

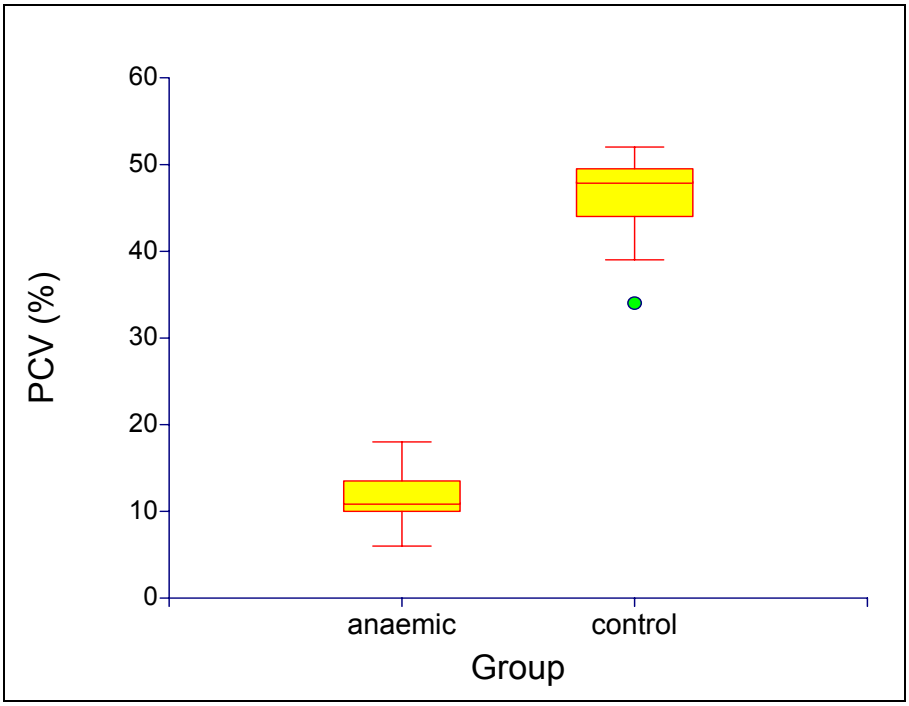
Plot 7: Serum haemoglobin concentrations in groups 1 and 2. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes) and 10th and 90th percentiles (T-bars). The green dots represent outliers. The upper normal limit for serum haemoglobin was 1.6 g/l.



Packed Cell Volume

PCV was by definition in this study < 20% in 25/25 patients in the anaemic group, by definition > 50% in 3/3 patients in the haemoconcentrated group and was found to range from 34% to 52% in the control group. Although the control group had some overlap with the haemoconcentrated group, the PCV is expected to decrease in canine babesiosis patients due to haemolysis. Therefore a babesia patient with a PCV ≥ 50% was considered haemoconcentrated. Mean and median values for PCV were 11.64% and 11%, and 46.08% and 48% for the anaemic group and the control group respectively. The mean, median and distribution of PCV varied significantly between the control group and the anaemic babesia group ($p<0.000001$).

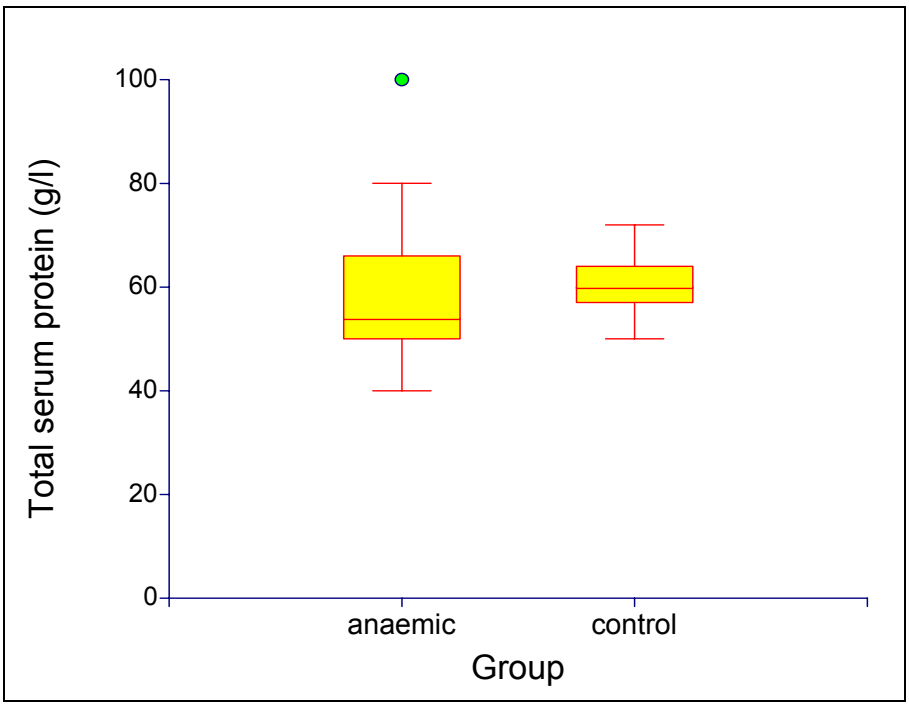
Plot 8: PCV in groups 1 and 2. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes) and 10th and 90th percentiles (T-bars). The green dots represent outliers. The normal range for PCV was 37%-55%.



Total Serum Protein

TSP was elevated in 5/25 anaemic patients, was below the normal laboratory reference range in 5/25 anaemic patients, was elevated in 1/13 control patients, and was normal in all the haemoconcentrated cases. Mean and median values for TSP were 58.60 g/l and 54 g/l, and 60.92 g/l and 60.0 g/l for the anaemic group and the control group respectively. The mean, median and distribution of TSP did not vary significantly between the control group and the anaemic babesia group (p=0.130661).

Plot 9: TSP in groups 1 and 2. Data are shown as median (horizontal line within box), 25th and 75th percentiles (horizontal ends of boxes) and 10th and 90th percentiles (T-bars). The green dot represents an outlier. The normal range for TSP was 50-80 g/l. Group 1 = anaemic babesiosis group. Group 2 = healthy control group.



Urine analysis:

All twenty eight babesiosis cases had urine available for analysis. The urine SG was ≥ 1.030 in 19/25 cases and < 1.030 in 6/25 cases in group 1. Three out of six patients with urine SG < 1.030 in group 1 had normal serum urea, serum creatinine and serum cystatin-C concentrations; and 3/6 cases in group 1 with urine SG < 1.030 had elevated serum urea but normal serum creatinine and serum cystatin-C concentrations. 1/3 cases in group 3 had a urine SG > 1.030 (1.050). This case had an elevated serum urea but normal serum creatinine and serum cystatin-C concentrations. Two out of three cases in group 3 had urine SG < 1.030. Both of theses cases had elevated serum urea, serum creatinine and serum cystatin-C concentrations. Both of these cases died, one of which was available for post mortem. The post mortem revealed significant renal lesions and is described below.

Twenty out of twenty five cases in group 1 and 3/3 cases in group 3 had haemoglobin crystals in their urine ranging from 1+ to 4+ and 2+ to 4+ respectively. Twenty three out of twenty five cases in group 1 and 3/3 cases in group 3 had haemoglobin positive reactions on urinary chemistry strip tests ranging from 1+ to 4+ and consistently 4+ respectively.

Twenty out of twenty five cases in group 1 and 1/3 cases in group 3 had positive reactions on urinary chemistry strip test for bilirubin ranging from 1+ to 3+ and equal to 3+ respectively. Nine cases, all in group 1, had bilirubin crystals in their urine. Of these cases only 4/9 had a serum bilirubin ≥ 15 µmol/l and 5/9 had a serum total bilirubin < 15 µmol/l.

Twenty two out of twenty five cases in group 1 and 2/3 cases in group 3 had positive reactions on urinary dip stick for protein ranging from 1+ to 3+ and equal to 3+ respectively. Hyaline casts were found in 7/25 cases in group 1 and 0/3 cases in group 3. All the cases with hyaline casts had a 3+ proteinuria on chemistry strip test. Fine and course granular casts were found in 12/25 cases in group 1 and 1/3 cases in group 3. The two cases in group 3 with proteinuria were also the 2 cases with elevated serum urea, serum creatinine and serum cystatin-C concentrations.

RTE cells were found in 18/25 cases in group1 and 2/3 cases in group 3. Once again the two cases in group 3 with RTE cells were the cases with elevated serum urea, serum creatinine and serum cystatin-C concentrations.

Table 3: Summary of urine analysis results.

GRP 1	Urine SG	Urine colour	Urine pH	Urine Protein	Urine Bilirubin	Urine Bld/Hb	Casts	Urine RTE cells	Crystals
1	1.028	Dark golden	5	2+	2+	4+ Hb	*	2+	1+ Hb
2	1.050	Very Dark Golden	6	3+	3+	4+ Hb	4+ Hyalin some fine granular casts.	3+	2+ Bili 2+ Hb
3	1.040	Dark Red/ Black	6	*	*	4+ HB	1+ Hb	2+	4+ Hb
4	1.026	Dark Yellow	5	3+	3+	1+ Hb	*	1+	Few Hb/Bili
5	1.030	Dark Yellow	7	3+	3+	4+ Hb	2+ Hyaline and course granular casts	1+	3+ Hb
6	1.047	Dark Red	6	3+	2+	4+ Hb	Few Hb	2+	2+ Hb
7	1.035	Dark Red	7	3+	3+	4+ Hb	1+ Waxy, 1+ Hyalin 1+ Granular	2+	2+ Hb
8	1.038	Golden Yellow	6	3+	3+	4+ Hb	3+ Hyalin	1+	1+ Hb
9	1.050	Very dark yellow	6	2+	3+	3+ Hb	1+ Coarse granular	2+	1+ Hb 2+ Bili
10	1.025	Dark Red/Black	6	*	*	4+ Hb	1+ Hb 1+ Coarse granular		3+ Hb
11	1.035	Gold Yellow	7	1+	*		3+ Coarse granular	1+	*
12	1.022	Black	6	3+	*	4+ Hb	1+ Coarse granular casts	1+	3+ Hb
13	1.048	Clear	6	3+	3+	4+ Hb	3+ Hyalin casts, 3+ fine granular casts, few coarse granular casts.	2+	1+ Hb
14	1.044	Red	6	3+	3+	4+ Hb	1+ Coarse granular, Hb	1+	4+ Hb Few Bili
15	1.016	Red	6	3+	2+	4+ Hb	2+ Dense coarse granular	2+	2+ Hb
16	1.040	Dark Yellow Red Tinged	6	2+	3+	4+ Hb	Few Hb	*	4+ Hb Few Bili

17	1.050	Dark golden	6	3+	3+	4+ Hb	4+ Hb	*	Hb and Bili
18	1.050	Red	6	3+	3+	4+ Hb	Few Hb	1+	3+ Hb Few Bilirubin
19	1.044	Dark Gold Yellow	7	3+	3+	4+ Hb	1+ Course Granular	*	3+ Hb
20	1.032	Dark Gold Yellow	6	2+	3+	4+ Hb	3+ Hb casts	*	*
21	1.050	V.Dark Yellow Red Tinged	7	3+	3+	4+ Hb	4+ Hyalin	2+	V.Few Hb
22	1.038	Yellow	7	1+	1+	1+ Hb	4+ Hb	*	Few Bili.
23	1.050	Dark Golden	6	3+	3+	4+ Hb	3+ Hyalin V.few Hb	3+	1+ Hb 2+ Bili
24	1.045	Dark Yellow	5	2+	2+	4+ Hb	3+ Coarse granular	1+	*
25	1.020	Yellow	*	*	*	*	1+ Hb casts	*	*
GRP 3	**Urine SG**	**Urine colour**	**Urine pH**	**Urine Protein**	**Urine Bilirubin**	**Urine Bld/Hb**	**Casts**	**Urine RTE cells**	**Crystals**
1	1.020	Black	6	3+	*	4+ Hb	3+ Hb	1+	3+ Hb
2	1.050	Black	6	*	*	4+ Hb	1+ Hb	*	4+ Hb
3	1.020	Dark Yellow	6	3+	3+	4+ Hb	3+ Coarse granular	3+	2+ Hb

* Empty cells indicate a negative result for that parameter. Hb= haemoglobin Bili= bilirubin

To summarise:

- The significant differences between the two groups were:
  - PCV which was significantly lower, by definition in the anaemic group relative to the haemoconcentrated group.
  - The presence of elevated serum creatinine and serum cystatin-C which occurred only in the haemoconcentrated group.
  - Urine SG which was < 1.030 (1.020 for both cases) in the two cases in group 3 where serum creatinine and serum cystatin-C was elevated; and was ≥ 1.030 (1.050) for the single case where serum creatinine and serum cystatin-C was normal.
  - Interestingly hyaline casts were only reported in group 1 despite the presence of 3+ proteinuria on dipstick in 2/3 cases in group 3.
- Abnormal parameters found to be present in both groups were:
  - Elevated serum urea.
  - Elevated plasma ammonia.
  - Elevated serum and urinary haemoglobin.

- Elevated serum and urinary bilirubin.
- Presence of urinary granular casts.
- Presence of proteinuria.
- Presence of RTE cells.

Histopathology Report from the Single Haemoconcentrated Patient that Died

There was only one case in this study that was available for post-mortem. The patient was a 12-month-old Irish terrier that died of the haemoconcentrated form of canine babesiosis. Histopathology:

- There was marked globular glomerular protein leakage.
- There was scattered tubular epithelial necrosis in the cortex and medulla.
- There was single tubular cell hyperplasia and hypertrophy.
- There was a monocytic leukostasis associated with the vasa rectae.
- There were many cortical and medullary pale eosinophilic protein tubular casts.
- There were also a few cortical bile pigment casts and globular protein casts, possibly haemoglobin.

The renal pathology described is that of renal inflammation with glomerular protein leakage and tubular epithelial necrosis. The renal pathology was reflected in the biochemical results. The concentrations of serum urea, serum creatinine and serum cystatin-C, were all elevated at 41.8 mmol/l, 608 µmol/l and 5.7 mg/l respectively. All three of these parameters were well above their normal reference ranges. PCV was 54% placing this case in the haemoconcentrated group. Serum haemoglobin and plasma ammonia concentrations were also elevated at 2.8 g/l and 172.3 µmol/l respectively. The urea: creatinine ratio was mildly increased at 17.02.

CORRELATIONS

Table 4: Spearman-rank correlation coefficients for all canine data (n=41).

	CystatinC	BilT	Urea	Creat	U: C Ratio	Ammonia	SerumHb	PCV
BilT	-0.0950 (P=0.5546)							
Urea	0.1657 (P=0.3005)	0.3678 (P=0.0180)						
Creat	0.3941 (P=0.0108)	-0.0224 (P=0.8892)	0.3302 (P=0.0350)					
U: C Ratio	-0.1881 (P=0.2390)	0.3912 (P=0.0114)	0.7369 (P=0.0001)	-0.2981 (P=0.0583)				
Ammonia	-0.0455 (P=0.7775)	0.5649 (P=0.0001)	0.5168 (P=0.0005)	0.0771 (P=0.6319)	0.4413 (P=0.0039)			
SerumHb	0.0507 (P=0.7529)	0.1670 (P=0.2967)	0.4798 (P=0.0015)	0.1509 (P=0.3462)	0.3797 (P=0.0143)	0.5786 (P=0.0001)		
PCV	0.1631 (P=0.3084)	-0.3291 (P=0.0356)	-0.4262 (P=0.0055)	0.3617 (P=0.0201)	-0.7161 (P=0.0001)	-0.3690 (P=0.0176)	-0.2762 (P=0.0804)	
TSP	0.4082 (P=0.0081)	-0.0274 (P=0.8648)	-0.1036 (P=0.5192)	0.3368 (P=0.0313)	-0.3449 (P=0.0272)	0.0072 (P=0.9645)	-0.0087 (P=0.9569)	0.1460 (P=0.3622)

According to the Spearman-rank correlations:

- Cystatin-C was significantly correlated with serum creatinine 0.39 ($p<0.01$) but not serum urea 0.17 ($p=0.30$) or the serum urea: creatinine ratio -0.19 ($p=0.24$).
- Serum total bilirubin was significantly correlated with serum urea 0.37 ($p=0.02$), serum urea: creatinine ratio 0.39 ($p<0.01$) and plasma ammonia 0.56 ($p<0.01$), and was negatively correlated with PCV -0.33 ($p=0.04$).
- Other than its positive correlation with serum total bilirubin; serum urea was also significantly correlated with serum creatinine 0.33 ($p=0.04$), serum urea: creatinine ratio 0.74 ($p<0.01$), plasma ammonia 0.52 ($p<0.01$) and serum haemoglobin 0.48 ($p<0.01$), and was negatively correlated to PCV -0.43 ($p<0.01$).
- Other than its positive correlations with serum cystatin-C and serum urea; serum creatinine was also significantly correlated with PCV 0.36 ($p=0.02$) and TSP 0.34 ($p=0.03$).
- Other than its positive correlations with serum urea and serum total bilirubin; serum urea: creatinine ratio was also significantly correlated with plasma ammonia 0.44

($p<0.01$) and serum haemoglobin 0.38 ($p<0.01$), and was significantly negatively correlated with PCV -0.72 ($p<0.01$) and TSP -0.34 ($p=0.03$).

- Other than its positive correlations with serum total bilirubin, serum urea and serum urea: creatinine ratio; plasma ammonia was also significantly correlated with serum haemoglobin 0.58 ($p<0.01$) and was significantly negatively correlated with PCV 0.37 ($p=0.02$).
- Other than its positive correlations with serum urea, serum urea: creatinine ratio and plasma ammonia; serum haemoglobin was not significantly correlated to any other parameters measured.
- Other than its positive correlation with serum creatinine and its negative correlations with serum total bilirubin, serum urea, serum urea: creatinine ratio and plasma ammonia; PCV was not significantly correlated to any other measured parameter.
- Other than its positive correlations with serum cystatin-C and serum creatinine and its negative correlation with serum urea: creatinine ratio; TSP was not significantly correlated to any other parameters measured.

To summarise in a potential physiological order in which changes to parameters caused by babesiosis may occur:

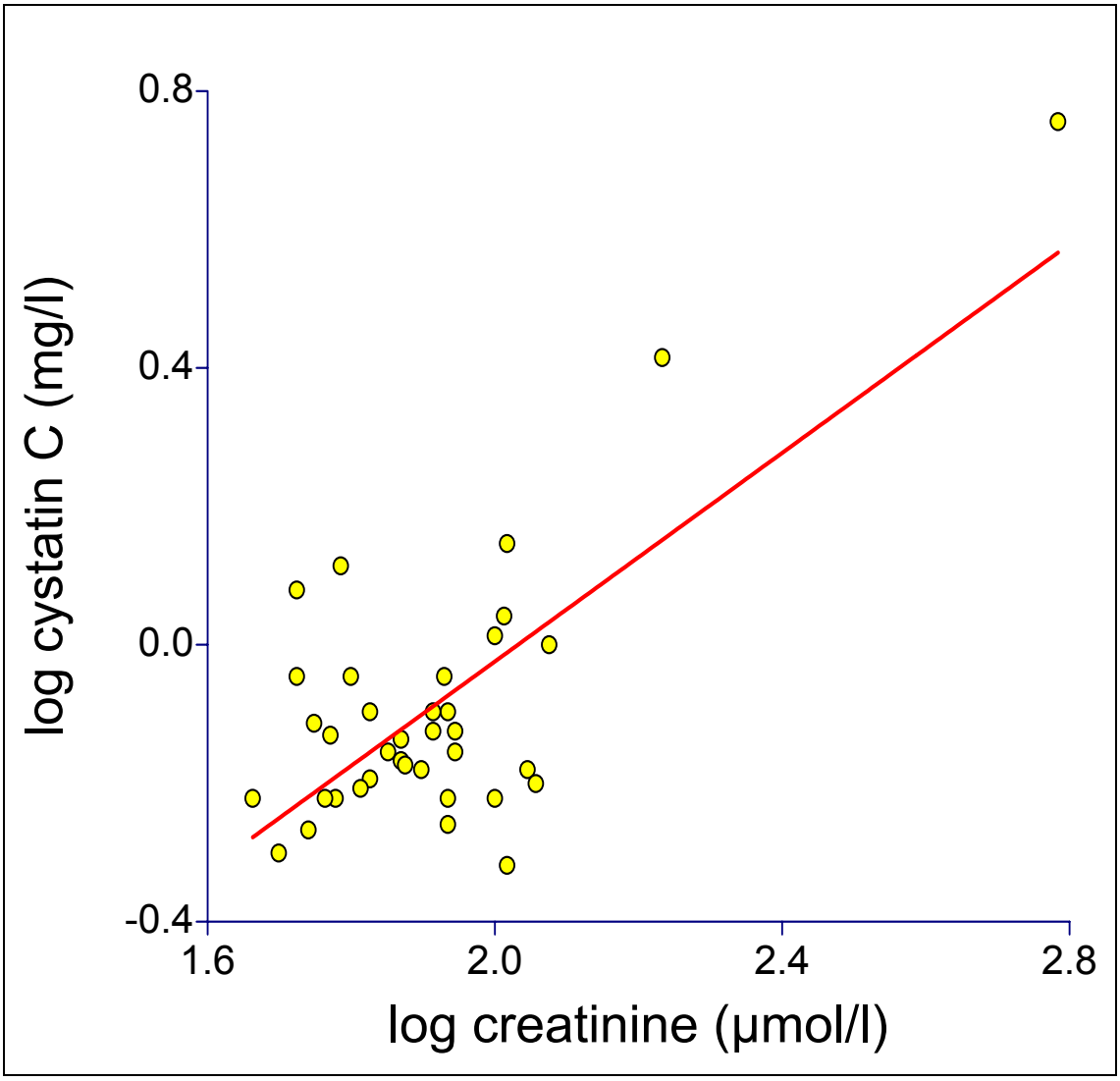
- PCV has a significant positive correlation with:
  - Serum creatinine.
- PCV has a significant negative correlation with:
  - Serum total bilirubin.
  - Plasma ammonia.
  - Serum urea.
  - Serum urea: creatinine ratio.
- Serum haemoglobin has a significant positive correlation with:
  - Plasma ammonia.
  - Serum urea.
  - Serum urea: creatinine ratio.
- Serum total bilirubin has a significant positive correlation with:
  - Plasma ammonia.
  - Serum urea.
  - Serum urea: creatinine ratio.

- Plasma ammonia has a significant positive correlation with:
    - Serum urea.
    - Serum urea: creatinine ratio.
- Serum urea has a significant positive correlation with:
    - Serum urea: creatinine ratio.
    - Serum creatinine.
- Serum creatinine has a significant positive correlation with:
    - Serum cystatin-C.
    - TSP

Log scatter plots

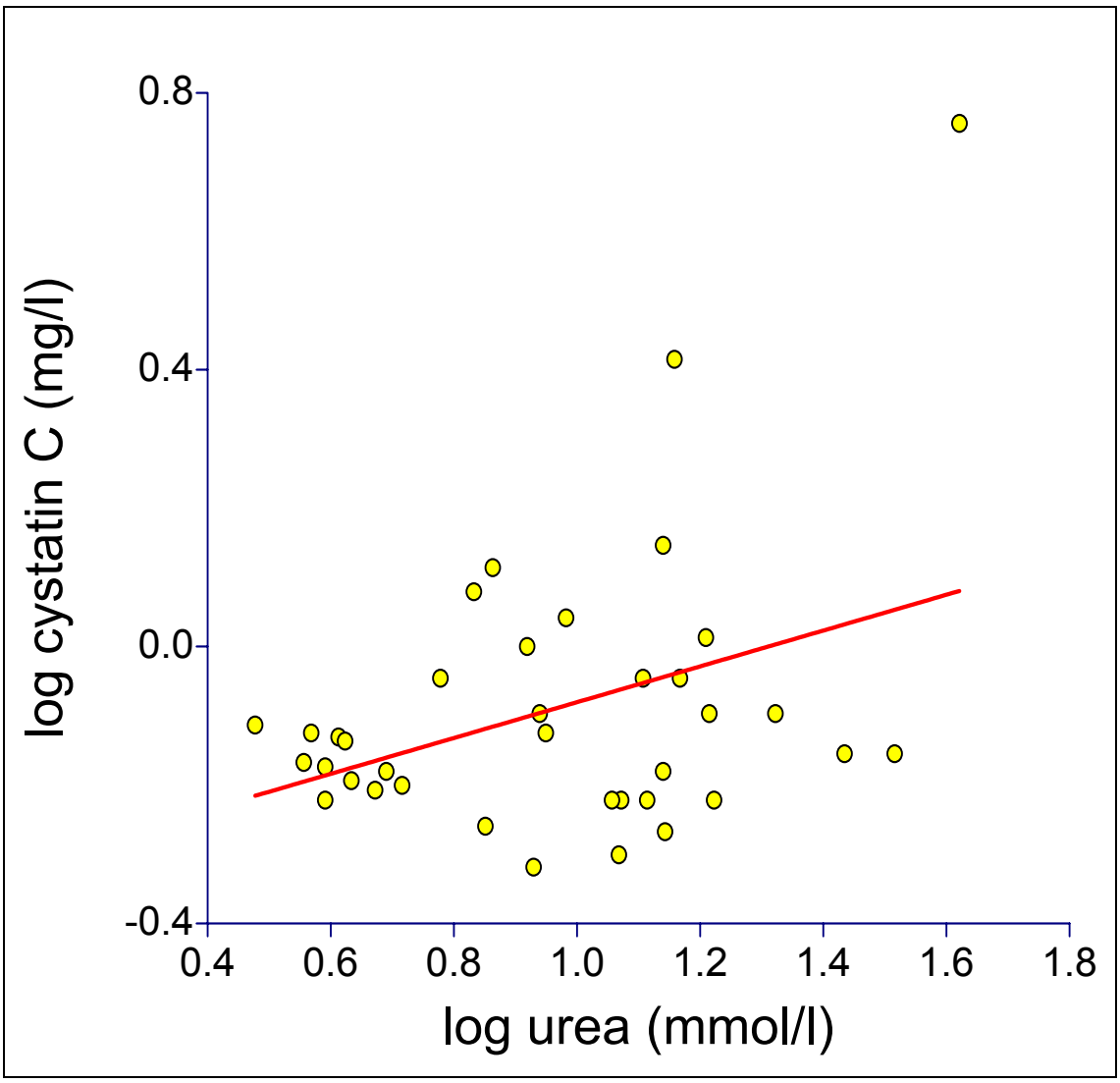
The scatter graph for log serum cystatin-C on log serum creatinine (plot 10) demonstrated a significant correlation between these two parameters r^2= 0.52 (p=<0.001).

Plot 10: Scatter graph of log cystatin-C on log creatinine of all dogs. Each of the yellow dots represents one of the 41 dogs in the study. The red line is a best fit line as determined by linear regression analysis of these cases.



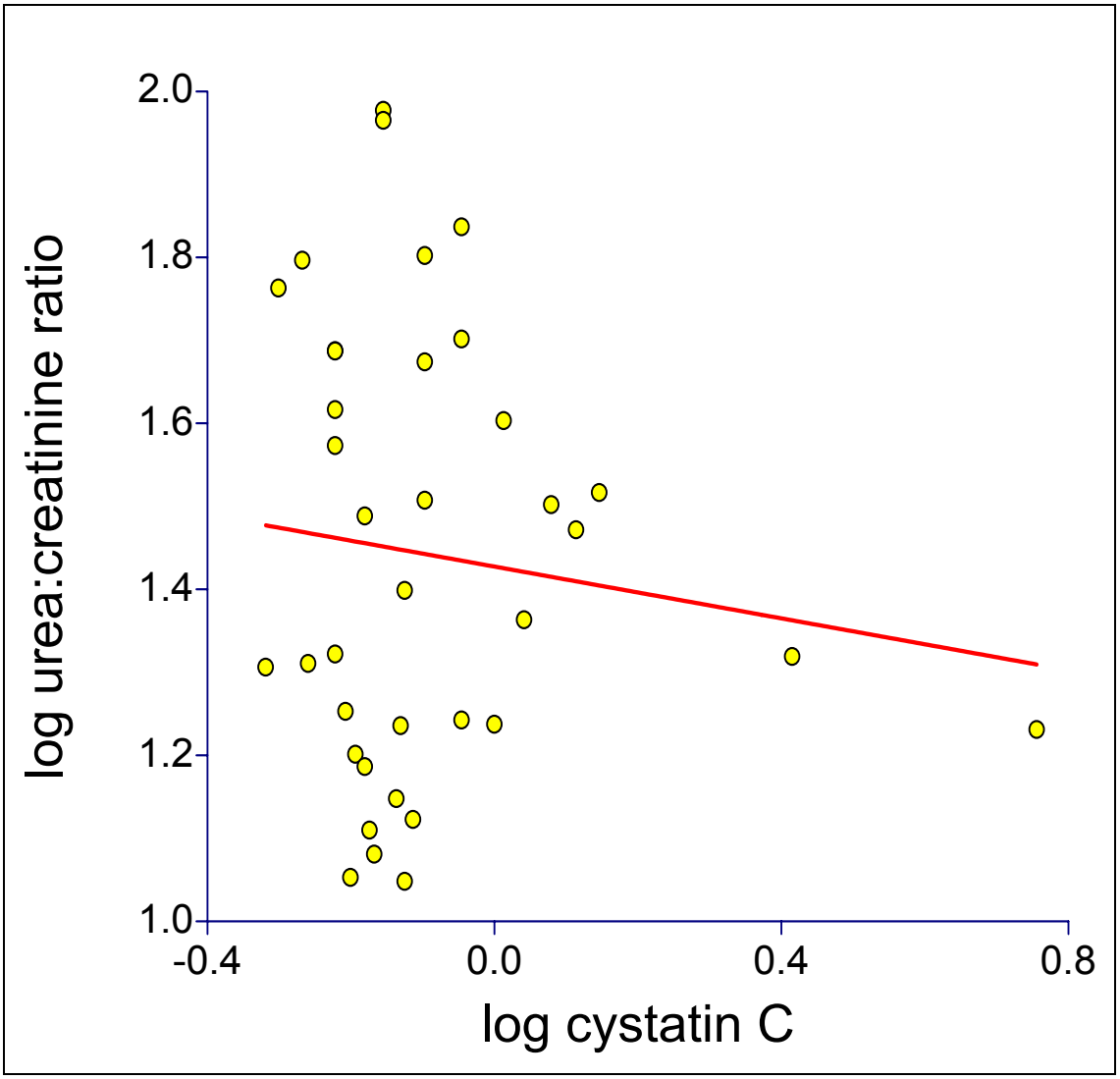
The scatter graph (plot 11)for log serum cystatin-C on log serum urea demonstrates a poor, but significant correlation between these two parameters r2= 0.14 (p=0.024).

Plot 11: Scatter graph of log cystatin-C on log urea of all dogs. Each of the yellow dots represents one of the 41 dogs in the study. The red line is a best fit line as determined by linear regression analysis of these cases.



The scatter graph (plot 12) for log serum urea: creatinine ratio on log serum cystatin-C demonstrates an insignificant correlation between the two parameters r2= 0.0140 (p= 0.485)

Plot 12: Scatter graph of log urea: creatinine ratio on serum cystatin-C of all dogs. Each of the yellow dots represents one of the 41 dogs in the study. The red line is a best fit line as determined by linear regression analysis of these cases.



CHAPTER 6
DISCUSSION
AND
CONCLUSIONS

This study confirmed that serum urea is disproportionately increased relative to serum creatinine in anaemic canine babesiosis patients. The mean and median serum urea was elevated in the anaemic patients in this study whereas mean and median serum creatinine was within the normal laboratory reference range. It is therefore not surprising that the mean and median serum urea: creatinine ratio was found to be elevated in the anaemic patients in this study, with a number of patients showing markedly increased ratios. These findings agree with the retrospective study by de Scally[20], where mean and median serum urea values were approximately double the laboratory normal values, whilst mean and median serum creatinine values were at the top end of the laboratory normal range. This also agrees with the findings of Reyers[86]. The serum urea: creatinine ratio was also found to be elevated in a significant number of the human malaria patients in the data analysed in this study. Unfortunately, as with canine babesiosis, no malaria studies have investigated the cause of this phenomenon. I propose that similar mechanisms may play a role with respect to this observation in these two diseases.

In the de Scally[20] study it was hypothesised that some of these patients with high serum urea and low or normal serum creatinine concentrations may have had renal azotaemia. The reasoning was that serum creatinine may have been negatively biased due to interference with the laboratory measurement of serum creatinine concentrations by the blood pigments encountered in canine babesiosis. In this current study, however, mean and median serum cystatin-C concentrations in the anaemic patients were within the normal laboratory reference range. Serum cystatin-C is considered to be free from similar interference[48]. In fact the log serum creatinine had a high correlation to log serum cystatin-C concentration whereas the log serum urea and the log serum urea: creatinine ratio had a poor correlation to log serum cystatin-C. Using the Spearman-rank correlation coefficients serum cystatin-C was significantly positively correlated with serum creatinine but not serum urea or the serum urea: creatinine ratio. In this current study cystatin-C was not elevated in any patient where serum creatinine was not elevated. This is unlike a *P. falciparum* study where serum cystatin-C was found to be a more sensitive marker of renal disease than serum creatinine[36]. It is possible that serum cystatin-C will prove its worth in larger studies with anaemic canine babesiosis patients that do in fact have significant renal disease.

Apart from elevated serum urea concentrations the only other potential evidence of renal disease in the anaemic canine babesiosis patients in this study was the RTE celluria, urinary

granular casts, proteinuria and a few cases where urinary SG was not concentrated above 1.030. The 2 haemoconcentrated cases with elevated serum urea, serum creatinine and serum cystatin-C had a urine SG of 1.020 which is inadequately concentrated. This combination indicates significant renal disease in these two patients. This was backed up by the post mortem finding in one of these patients. None of the anaemic patients with a urine SG of < 1.020 had elevated serum creatinine or serum cystatin-C. Never-the-less we speculate that if any of these patients did have significant renal disease, then they would have had elevated serum creatinine and/ or serum cystatin-C concentrations to go with their inadequately concentrated urine. There was evidence of elevated serum urea, haemoglobinaemia and haemoglobinuria, bilirubinaemia and bilirubinuria, granular casts, proteinuria and RTE celluria in the cases with elevated serum creatinine and serum cystatin-C and in the cases where these parameters were normal. I therefore speculate that the random presence of these parameters in the urine of canine babesiosis patients is not highly significant. Our findings in this current study indicate that minimal renal disease is common in anaemic canine babesiosis patients in South Africa. This concurs with the findings of Lobetti,[63] who found that mild as apposed to overt renal disease was common in canine babesiosis in South Africa; and that haemoglobinuria on its own or in the presence of hypoxia is not nephrotoxic in the dog[60].

It is now evident that an elevated serum urea concentration in a canine babesiosis patient in the absence of an elevated serum creatinine concentration is not likely to be of renal origin. In this current study serum creatinine remained normal in anaemic patients who have minimal babesiosis associated renal disease. Serum creatinine was, however, significantly raised in those patients that did have babesiosis associated renal disease. Serum creatinine is thus a better indicator of significant renal disease in canine babesiosis than serum urea. From this study it also appears that there is no additional benefit to be derived from the measurement of serum cystatin-C in canine babesiosis.

It can also be noted, despite the small numbers involved, that significant renal disease appears to occur more commonly in haemoconcentrated patients than it does in anaemic babesiosis patients. According to the Spearman-rank correlation coefficient PCV was significantly positively correlated with serum creatinine. However larger studies are necessary before any significant conclusions can be drawn.

The elevated serum urea concentrations encountered are therefore either a form of pre-renal azotaemia or a laboratory error in the measurement of serum urea. Potential causes of prerenal azotaemia include gastric ulceration, dehydration, hypotensive shock, cardiac disease, rhabdomyolysis and hyperureagenesis from substrate loading[32,34,66,43,72,73,80,82,85,96]. All the patients in this study were sampled prior to treatment. However, in clinical practice parental or enteral feeding, cortisone induced gastric ulceration, and blood or serum transfusions may contribute to protein loading and hyperureagenesis in isolated patients.

A GIT form of canine babesiosis has been described in South Africa[70]. A study by Mohr[75] showed that pancreatic enzymes were elevated in isolated patients with canine babesiosis. Mohr[75] also speculated that pancreatitis may represent the previously reported "gut" form of canine babesiosis. Gastric ulceration has not been conclusively proven to be regular feature of canine babesiosis in South Africa. However, gastric ulceration has been reported as a major cause of increased urea: creatinine ratios in dogs[82]. Although not a feature of this study due to the pre-treatment samples used, many canine babesiosis patients are placed on high doses of corticosteroids, to treat aggressive immune mediated haemolysis, which could increase the risk of gastric ulceration[46]. It is therefore possible that gastric ulceration plays a role in the elevated urea: creatinine ratios encountered in isolated canine babesiosis patients.

Studies in malaria have shown that total body water may be depleted in this disease, explaining why the elevated vasopressin concentrations in hyponatraemic children may be appropriate[30]. Leisewitz[57] indicated that a similar event may occur in canine babesiosis. Serum urea may increase before serum creatinine in a state of dehydration[31]. Pathologically altered PCV as well as the potential for decreased albumin in canine babesiosis renders these tests inadequate to assess dehydration in this disease. Albumin may be decreased in canine babesiosis as a negative acute phase protein as well as through decreased absorption, decreased production, increased loss and increased consumption. Possible pathomechanisms include GIT disease, glomerular disease, liver disease, vasculitis, catabolism. Dehydration, therefore, is a possible cause of the elevated serum urea: creatinine ratio encountered in severely anaemic canine babesiosis patients in South Africa; and it may not be detected by conventional tests.

Hypotensive shock is thought to be a major cause of renal failure in severe human falciparum malaria patients[104]. Florid cytokine encounters in severe falciparum malaria are commonly

associated with renal failure as a result of hypotension[50,51]. Inflammatory cytokines have been shown to play a role in the pathogenesis of canine babesiosis[103]. Jacobson[45] found 5/10 complicated canine babesiosis patients and 2/10 severe uncomplicated cases to be hypotensive in a South African study. Maegraith, in his pathological report on canine babesiosis found the kidney medulla to be normal to severely congested and the cortex to be normal to severely pale[67], which may be consistent with hypotension. Hypotensive shock has been documented in one case of *B. canis* in the USA[34]. Canine babesiosis patients may also present in hyperdynamic shock, a state which may progress to decompensated, hypotensive shock[42]. Hypotensive shock therefore may account for isolated increases in the serum urea: creatinine ratios encountered in the anaemic group in this study. However, as blood pressure and other parameters of hypotensive or hyperdynamic shock were not measured no further comment can be made in this regard about the subjects in this current study.

In a human study on causes of an increased urea: creatinine ratio, ischaemic cardiac disease was found to be major contributor[31]. Lobetti[62] found macroscpic and histopathological evidence of cardiac disease in the form of sero-haemorrhagic pericardial effusions, macroscopic epicardial and endocardial haemorrhages, inflammatory cell infiltrates, intercellular haemorrhage and necrosis of myocardial fibres, and fibrin thrombi in multiple cardiac blood vessels in 3 of 4 canine babesiosis patients with elevated cardiac troponin I (cTnI) concentrations. In the study 4/9 cases or 44.5% in the severely anaemic group (PCV<15%) had elevated cardiotroponin I concentrations. Dvir[27] also showed that a variety of electrocardiographic (ECG) changes do occur with canine babesiosis patients. The ECG changes included sinoatrial blocks or sinus arrest, ventricular premature complexes, low R wave amplitudes, prominent Q waves, axis deviations, prolonged QRS complexes, ST wave depression and coving, large T waves and notched R waves. Of these ECG changes the only one associated with cardiac pathology was the low R wave amplitude recordings that were associated with pericardial effusions. There was, however, a significant correlation between ventricular premature contractions (VPC's) and cTnI levels in the Lobetti[62] study. From the above studies we can deduce that cardiac pathology does occur to varying degrees in canine babesiosis patients. Cardiac disease therefore may account for the increased urea: creatinine ratio in isolated cases in canine babesiosis.

The reporting of two cases of rhabdomyolysis in South African canine babesiosis has led to speculation that increased muscle catabolism may account for the disproportionate increase in

serum urea concentrations compared to serum creatinine concentrations observed[43]. Further studies would be needed to determine the incidence of rhabdomyolysis in this disease before any significance could be placed on these findings.

The serum concentrations of a major product of haemolysis, namely haemoglobin, and a major by-product of haem breakdown, namely bilirubin, were elevated in the anaemic babesia group; although only haemoglobin displayed a significant difference in its distribution pattern from the control group. Haemolysis may produce substrates in the form of proteins that would require deamination and lead to a state of hyperammonaemia. These proteins could potentially come from the various erythrocyte components and comprise membrane proteins, including transport and structural proteins, the globin potion of haemoglobin and intracytoplasmic enzymes. Ammonia is cleared from the body through the process of ureagenesis[38,39]. Thus hyperammonaemia may lead to a state of hyperureagenesis. The rate of haemolysis is likely to be influenced by a plethora of host-parasite variables, such as immunity, genetic predisposition, coinfections and pathogenicity, as well as by the timing of the laboratory evaluation of the disease process; and is likely to directly influence the rate of hyperureagenesis in these patients. Mean and median plasma ammonia concentrations were significantly increased and the distribution for plasma ammonia was significantly different in the anaemic babesia group compared to the control group. There are, however, other conditions that may contribute to the state of hyperammonaemia in canine babesiosis. Metabolic acidosis is a common feature in canine babesiosis and occurs as a result of tissue hypoxia and resultant hyperlactataemia. In this situation the kidney assumes an important role in bicarbonate and ammonia production. Ammonia is deaminated from glutamine via glutaminase. Although this contribution is important physiologically it may not significantly alter the serum biochemically as the waste products are excreted in the urine. Concentrations of both plasma ammonia and serum urea may increase during a severe catabolic state due to the deamination process involved in using tissue proteins as an energy source. A catabolic state is likely in anaemic canine babesiosis patients because of decreased appetite, tissue hypoxia, metabolic acidosis, pyrexia and the increased work of breathing. Substantial catabolism of endogenous proteins was found in critically ill dogs in a study by Michel[73]. This study demonstrated an elevated twenty four hour urinary urea nitrogen concentration, which reflected the degree of hyperureagenesis in these patients.

Significantly increased ammonia values may indicate severe hepatic function loss. Hepatic encephalopathy has been reported in *Plasmodium falciparum* malaria[53]. A common test of liver function is the ammonia tolerance test. In the absence of a functional liver, ammonia cannot be converted to urea via the urea cycle. Under these circumstances serum urea would be expected to be low and plasma ammonia elevated. Traditional hepatic enzymology may be useful to indicate the degree of hepatic insult encountered. In a retrospective study 33% of 480 jaundiced canine babesiosis patients raised ALT activity and 22% of these patients had raised ALP activity[74]. The author hypothesized that some degree of hepatic insult was likely in this disease[74]. Maegraith[67] also reported on the pathology of the liver in canine babesiosis. There were varying degrees of hepatocellular atrophy, fatty degeneration or necrosis, which was essentially centrilobular in distribution. In a few cases there was gross hepatocellular destruction extending to all but the immediately peripheral cells. The hepatic lobular architecture was thus commonly disturbed. This pathology was associated with congestion to dilation of the sinusoids and central veins. In some cases swollen Kupffer cells were found containing parasitised and unparasitised erythrocytes, free parasites and haemosiderin. As mentioned previously the increased concentrations of serum urea rules out severe hepatic functional loss because urea production is compromised in hepatic causes of hyperammonaemia. This is not to say that some degree of liver disease is not present. An in depth discussion of hepatic pathology does, however, fall beyond the scope of this study. Suffice to say that substrate loading of rate limited metabolic and transportation pathways, such as bile conjugation and excretion, and hyperureagenesis from ammonia, as a result of haemolysis and/or catabolism are a more likely explanation of the biochemical changes described above for the anaemic babesiosis group.

There is, however, one more potentially important oversight; both serum urea and plasma ammonia were measured by enzymatic UV methods. These two methods have their absorbance spectra at wavelength 340nm and 365 nm respectively. This falls within the 300-500nm range of high intrinsic absorbance that serum haemoglobin displays[7]. Therefore it may be that a portion, large or small, of the elevated concentrations of serum urea and plasma ammonia encountered in the anaemic babesiosis group is as a result of laboratory error in which serum haemoglobin causes a positive bias on their measurements. An alternative would have been to measure plasma ammonia by the Du Pont aca ammonia method, which has been reported to be free of interference by haemoglobin and bilirubin[23]. Unfortunately this is with hindsight as plasma ammonia cannot be remeasured. Further more, in his dissertation,

Lobetti[60] described a method for precipitating haemoglobin in urine. This simple method could be adapted for serum to further this study in the future by reanalysing the serum urea concentrations and comparing them to the original values. These methods, however, were not included in this study and therefore we are unable to account for the non-renal causes of increased serum urea concentrations in the anaemic babesiosis group at a higher level. At best we can speculate that there is potentially a significant positive bias caused by serum haemoglobin concentrations on the measurement of serum urea and plasma ammonia concentrations. There is, however, also the potential for hyperureagenesis or other prerenal events to cause the elevated urea: creatinine ratios encountered. Possible causes of hyperureagenesis as a result of protein substrate loading include haemolysis, catabolism, gastric ulceration and/ or rhabdomyolysis. Possible causes of prerenal events include cardiac disease, hypotensive shock and hypovolaemia. As explained previously all of these are reported in canine babesiosis.

Finally if we look at the matrix of Spearman-rank correlation coefficients in physiological order we see that PCV is significantly negatively correlated with serum total bilirubin, plasma ammonia, serum urea and the serum urea: creatinine ratio. Serum haemoglobin and serum total bilirubin are both significantly positively correlated with plasma ammonia, serum urea and serum urea: creatinine ratio. Plasma ammonia in turn is significantly positively correlated with serum urea and the serum urea: creatinine ratio. Unfortunately this would be the picture whether hyperureagenesis or laboratory error, as described previously, was present.

CONCLUSIONS

The hypotheses postulated for this trial were that:

- The elevated urea: creatinine ratio that occurs in anaemic canine babesiosis patients is not of renal origin but as a result of falsely elevated serum urea concentrations, and, therefore, that serum creatinine is a better measure of renal disease in anaemic *B. canis* patients in South Africa than serum urea.

This study showed that the serum urea: creatinine ratio is elevated in anaemic *B. canis* patients. It has also demonstrated that this increased ratio is not of renal origin but as a result of non-renal elevations in serum urea concentrations. It has demonstrated that an increased serum urea: creatinine ratio also occurs in human malaria patients.

- Due to the similarities between the two diseases and the fact that serum cystatin-C has been found to be a useful indicator of renal disease in human malaria studies; that serum cystatin-C will prove to be a useful indicator of renal disease in canine babesiosis.

This study has showed that the correlation between serum creatinine and serum cystatin-C is highly significant in canine babesiosis, where as the correlation between serum urea and serum cystatin-C is not and, therefore, that serum creatinine is a better measure of renal disease in this circumstance than serum urea. It has failed to show that any additional benefit is derived from the measurement of serum cystatin-C concentrations in this disease.

- Other findings include the following:

    1. There has been a small contribution to increasing the divide between anaemic and haemoconcentrated patients in canine babesiosis demonstrating the relatively higher incidence of significant renal disease in the haemoconcentrated form of the disease.

    2. The study has demonstrated that significant hyperammonaemia is common in canine babesiosis. When coupled to the elevated serum urea concentrations encountered it is clear that this hyperammonaemia is unlikely to be caused by

hepatic disease. Further classification of this phenomenon in this study would be speculative, but plasma ammonia may prove to be a useful parameter to investigate in future studies.

3. This study has shown that elevated serum urea, haemoglobinaemia and haemoglobinuria, bilirubinaemia and bilirubinuria, urinary granular casts, proteinuria and RTE celluria are present in canine babesiosis patients with mild renal disease and in canine babesiosis patients with overt renal disease. The usefulness of single serum urea concentrations and single urine sediment analysis in these patients is therefore placed under question.

4. Unfortunately this study failed to highlight the pathogenesis of the increased serum urea: creatinine ratio, which therefore remains speculative.

How should we investigate renal disease in anaemic *B. canis* patients in South Africa?

Serum creatinine has proved to be a better indicator of renal disease in canine babesiosis than serum urea. Serum creatinine, however, is limited in its sensitivity to +/- 70% decrease in the GFR before it becomes elevated. Unfortunately, it appears from this study that there is no additional benefit to be derived from the measurement of serum cystatin-C concentrations in this disease. Larger studies with a greater spectrum of renal lesions may yet show additional benefit from the measurement of serum cystatin-C concentrations as it has in human studies. Other measures of GFR, such as scintigraphy, may be too invasive for these ill patients to be considered beneficial. Other methods such as creatinine clearance are likely to be subject to the same errors as the direct measurement of serum creatinine [11]. Urine analysis is another important method of detecting renal disease. Unfortunately RTE cells, hyaline casts, granular casts, bilirubinuria and haemoglobinuria were encountered in canine babesiosis patients with both mild and severe renal disease and therefore urine sediment analysis may not be helpful in this disease. Urine SG in concert with serum creatinine concentrations, however, remains an important deciding factor regarding significance of renal disease encountered. Perhaps the best way of detecting significant renal disease in canine babesiosis would be to measure hourly urine production in an adequately hydrated patient. Once the cause of the elevated serum urea: creatinine ratio in canine babesiosis is better defined additional benefit may be derived from determining this ratio. This remains to be seen.

Serial monitoring of serum creatinine, urine SG and rate of urine production together with the insight to potential laboratory errors and nonrenal causes of azotaemia, should be sufficient to diagnose significant renal disease in *B. canis* patients. Serum urea alone should definitely not be used as an indicator of renal azotaemia.

What does an increased urea: creatinine ratio mean in canine babesiosis in South Africa?

If a study could show that the elevated urea: creatinine ratio in canine babesiosis in South Africa was related to laboratory errors in the measurement of serum urea, then this would be equivalent to saying that serum haemoglobin concentrations are elevated and would say nothing more than a direct measurement of serum haemoglobin. In the absence of laboratory error in the measurement of serum urea concentrations, the elevated ratio may indicate hyperureagenesis, possibly as a result of haemolysis, or some other pathomechanism of ammonia loading such as severe tissue catabolism. Catabolism itself may be a consequence of haemolysis as a result of acute hypoxia. This hyperureagenesis may exceed the kidneys urea clearance ability resulting in the elevated serum urea: creatinine ratios encountered. Perhaps the next step in the process would be the measurement of serum urea and urinary urea concentrations after the removal of the blood pigments from these media. Urinary urea concentrations have been shown to be valuable in differentiating prerenal azotaemia from hypereureagenesis in humans[96].

SYNOPSIS

We can therefore conclude that serum urea is often elevated due to non-renal factors in canine babesiosis patients, which causes an elevated serum urea: creatinine ratio. A similar event appears to occur in human malaria patients. The cause of these elevated ratios remains undetermined, but may be as a result of hyperureagenesis or laboratory error. Plasma ammonia concentrations were found to be elevated in this study. The cause of these elevated concentrations is undetermined but may be as a result of protein deamination or laboratory error. A random urine sediment analysis is of questionable value in canine babesiosis patients. However, the measurement of serum creatinine, urine SG and hourly urine production is advocated to detect significant renal disease in these patients. There appears to be no added

benefit to be derived from the measurement of serum cystatin-C concentrations in canine babesiosis.

APPENDICES

Table 5 shows human malaria data in traditional and SI units as well as the urea: creatinine ratio.

Table 5: Human Malaria Data.

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
0.65	3.50	57.46	1.25	5.38
1.40	7.60	123.76	2.71	5.43
0.82	5.50	72.49	1.96	6.71
1.00	7.00	88.40	2.50	7.00
1.30	9.30	114.92	3.32	7.15
1.40	10.40	123.76	3.71	7.43
8.45	64.10	746.98	22.88	7.59
1.40	10.70	123.76	3.82	7.64
1.90	14.70	167.96	5.25	7.74
1.00	8.00	88.40	2.86	8.00
1.10	9.20	97.24	3.28	8.36
0.95	8.00	83.98	2.86	8.42
1.60	13.50	141.44	4.82	8.44
1.15	10.00	101.66	3.57	8.70
0.99	8.90	87.52	3.18	8.99
0.80	7.30	70.72	2.61	9.13
2.10	19.40	185.64	6.93	9.24
1.10	10.20	97.24	3.64	9.27
0.80	7.50	70.72	2.68	9.38
3.10	29.50	274.04	10.53	9.52
1.20	11.50	106.08	4.11	9.58
1.24	12.10	109.62	4.32	9.76
1.40	13.70	123.76	4.89	9.79
0.96	9.40	84.86	3.36	9.79
1.10	10.80	97.24	3.86	9.82
1.28	12.70	113.15	4.53	9.92
1.10	11.00	97.24	3.93	10.00
1.25	12.50	110.50	4.46	10.00
1.35	13.60	119.34	4.86	10.07
1.14	11.50	100.78	4.11	10.09
1.30	13.20	114.92	4.71	10.15

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
1.37	14.00	121.11	5.00	10.22
1.99	20.50	175.92	7.32	10.30
1.20	12.50	106.08	4.46	10.42
1.20	12.50	106.08	4.46	10.42
1.12	11.70	99.01	4.18	10.45
1.10	11.50	97.24	4.11	10.45
1.20	12.80	106.08	4.57	10.67
1.20	13.00	106.08	4.64	10.83
1.30	14.20	114.92	5.07	10.92
1.05	11.50	92.82	4.11	10.95
1.17	12.90	103.43	4.61	11.03
0.95	10.50	83.98	3.75	11.05
0.97	10.80	85.75	3.86	11.13
1.30	14.50	114.92	5.18	11.15
1.60	18.10	141.44	6.46	11.31
1.75	19.80	154.70	7.07	11.31
1.40	16.00	123.76	5.71	11.43
0.95	11.00	83.98	3.93	11.58
1.25	14.50	110.50	5.18	11.60
1.05	12.20	92.82	4.36	11.62
0.80	9.30	70.72	3.32	11.63
1.10	12.80	97.24	4.57	11.64
0.85	10.00	75.14	3.57	11.76
0.85	10.00	75.14	3.57	11.76
1.30	15.50	114.92	5.53	11.92
0.55	6.60	48.62	2.36	12.00
1.25	15.00	110.50	5.36	12.00
0.83	10.00	73.37	3.57	12.05
1.20	14.50	106.08	5.18	12.08
1.05	12.70	92.82	4.53	12.10
1.15	14.00	101.66	5.00	12.17
0.95	11.60	83.98	4.14	12.21
2.15	26.50	190.06	9.46	12.33
0.90	11.20	79.56	4.00	12.44
0.97	12.20	85.75	4.36	12.58
0.97	12.20	85.75	4.36	12.58
1.15	14.50	101.66	5.18	12.61

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
0.75	9.50	66.30	3.39	12.67
1.20	15.20	106.08	5.43	12.67
1.20	15.30	106.08	5.46	12.75
2.10	26.80	185.64	9.57	12.76
0.90	11.50	79.56	4.11	12.78
1.25	16.00	110.50	5.71	12.80
0.90	11.70	79.56	4.18	13.00
1.20	15.60	106.08	5.57	13.00
1.30	17.00	114.92	6.07	13.08
1.30	17.00	114.92	6.07	13.08
1.04	13.70	91.94	4.89	13.17
1.10	14.50	97.24	5.18	13.18
1.40	18.50	123.76	6.60	13.21
0.90	12.00	79.56	4.28	13.33
0.75	10.00	66.30	3.57	13.33
1.20	16.00	106.08	5.71	13.33
1.20	16.20	106.08	5.78	13.50
1.20	16.20	106.08	5.78	13.50
1.50	20.40	132.60	7.28	13.60
1.10	15.00	97.24	5.36	13.64
1.10	15.00	97.24	5.36	13.64
1.05	14.50	92.82	5.18	13.81
1.30	18.00	114.92	6.43	13.85
0.82	11.40	72.49	4.07	13.90
0.75	10.50	66.30	3.75	14.00
1.00	14.00	88.40	5.00	14.00
3.10	44.00	274.04	15.71	14.19
1.00	14.20	88.40	5.07	14.20
7.60	108.00	671.84	38.56	14.21
1.15	16.50	101.66	5.89	14.35
0.75	10.80	66.30	3.86	14.40
1.40	20.30	123.76	7.25	14.50
2.00	29.20	176.80	10.42	14.60
0.82	12.00	72.49	4.28	14.63
0.75	11.00	66.30	3.93	14.67
0.85	12.50	75.14	4.46	14.71
1.10	16.20	97.24	5.78	14.73

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
1.40	20.80	123.76	7.43	14.86
0.85	12.70	75.14	4.53	14.94
0.95	14.20	83.98	5.07	14.95
0.80	12.00	70.72	4.28	15.00
6.10	91.50	539.24	32.67	15.00
1.20	18.00	106.08	6.43	15.00
1.00	15.00	88.40	5.36	15.00
1.20	18.00	106.08	6.43	15.00
2.75	41.50	243.10	14.82	15.09
1.90	28.70	167.96	10.25	15.11
0.94	14.20	83.10	5.07	15.11
1.25	19.00	110.50	6.78	15.20
0.85	13.00	75.14	4.64	15.29
1.13	17.30	99.89	6.18	15.31
1.10	17.00	97.24	6.07	15.45
1.30	20.20	114.92	7.21	15.54
0.90	14.00	79.56	5.00	15.56
0.75	11.70	66.30	4.18	15.60
1.25	19.50	110.50	6.96	15.60
0.80	12.50	70.72	4.46	15.63
1.15	18.00	101.66	6.43	15.65
1.20	18.80	106.08	6.71	15.67
2.91	45.60	257.24	16.28	15.67
0.85	13.50	75.14	4.82	15.88
0.72	11.50	63.65	4.11	15.97
1.20	19.20	106.08	6.85	16.00
1.00	16.00	88.40	5.71	16.00
1.34	21.50	118.46	7.68	16.04
1.05	17.00	92.82	6.07	16.19
1.05	17.00	92.82	6.07	16.19
1.30	21.30	114.92	7.60	16.38
0.75	12.30	66.30	4.39	16.40
1.05	17.30	92.82	6.18	16.48
6.31	104.00	557.80	37.13	16.48
1.00	16.50	88.40	5.89	16.50
1.30	21.50	114.92	7.68	16.54
1.53	25.50	135.25	9.10	16.67

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
1.40	23.50	123.76	8.39	16.79
1.00	16.80	88.40	6.00	16.80
1.35	22.80	119.34	8.14	16.89
1.15	19.50	101.66	6.96	16.96
1.00	17.00	88.40	6.07	17.00
0.97	16.50	85.75	5.89	17.01
0.85	14.50	75.14	5.18	17.06
1.20	20.50	106.08	7.32	17.08
1.25	21.50	110.50	7.68	17.20
1.25	21.50	110.50	7.68	17.20
0.90	15.50	79.56	5.53	17.22
1.10	19.00	97.24	6.78	17.27
0.70	12.10	61.88	4.32	17.29
1.25	21.70	110.50	7.75	17.36
1.15	20.00	101.66	7.14	17.39
0.66	11.50	58.34	4.11	17.42
1.10	19.20	97.24	6.85	17.45
1.80	31.50	159.12	11.25	17.50
1.00	17.50	88.40	6.25	17.50
1.00	17.50	88.40	6.25	17.50
1.25	22.00	110.50	7.85	17.60
1.05	18.50	92.82	6.60	17.62
0.85	15.00	75.14	5.36	17.65
0.65	11.50	57.46	4.11	17.69
0.65	11.50	57.46	4.11	17.69
0.80	14.30	70.72	5.11	17.88
0.95	17.00	83.98	6.07	17.89
1.00	18.00	88.40	6.43	18.00
4.05	73.00	358.02	26.06	18.02
5.85	105.50	517.14	37.66	18.03
1.06	19.20	93.70	6.85	18.11
1.10	20.00	97.24	7.14	18.18
0.95	17.30	83.98	6.18	18.21
1.15	21.00	101.66	7.50	18.26
1.37	25.20	121.11	9.00	18.39
1.05	19.50	92.82	6.96	18.57
3.90	72.50	344.76	25.88	18.59

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
0.67	12.50	59.23	4.46	18.66
1.05	19.60	92.82	7.00	18.67
1.20	22.50	106.08	8.03	18.75
1.00	18.80	88.40	6.71	18.80
0.84	15.80	74.26	5.64	18.81
0.90	17.00	79.56	6.07	18.89
1.00	19.00	88.40	6.78	19.00
1.33	25.30	117.57	9.03	19.02
1.76	33.50	155.58	11.96	19.03
9.00	171.50	795.60	61.23	19.06
0.86	16.40	76.02	5.85	19.07
4.40	84.20	388.96	30.06	19.14
1.20	23.00	106.08	8.21	19.17
1.00	19.20	88.40	6.85	19.20
6.00	115.40	530.40	41.20	19.23
1.35	26.20	119.34	9.35	19.41
0.85	16.50	75.14	5.89	19.41
0.75	14.60	66.30	5.21	19.47
0.75	14.60	66.30	5.21	19.47
1.00	19.50	88.40	6.96	19.50
1.60	31.20	141.44	11.14	19.50
0.58	11.50	51.27	4.11	19.83
0.30	6.00	26.52	2.14	20.00
1.00	20.00	88.40	7.14	20.00
0.75	15.00	66.30	5.36	20.00
1.45	29.00	128.18	10.35	20.00
1.15	23.00	101.66	8.21	20.00
1.40	28.00	123.76	10.00	20.00
1.30	26.20	114.92	9.35	20.15
0.95	19.20	83.98	6.85	20.21
0.87	17.60	76.91	6.28	20.23
0.84	17.00	74.26	6.07	20.24
1.30	26.50	114.92	9.46	20.38
0.95	19.40	83.98	6.93	20.42
1.10	22.50	97.24	8.03	20.45
1.10	22.50	97.24	8.03	20.45
1.50	30.80	132.60	11.00	20.53

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
1.45	29.80	128.18	10.64	20.55
0.85	17.50	75.14	6.25	20.59
0.80	16.50	70.72	5.89	20.63
1.05	21.80	92.82	7.78	20.76
0.65	13.50	57.46	4.82	20.77
8.20	170.50	724.88	60.87	20.79
0.75	15.60	66.30	5.57	20.80
0.95	20.00	83.98	7.14	21.05
0.95	20.00	83.98	7.14	21.05
1.30	28.00	114.92	10.00	21.54
0.70	15.10	61.88	5.39	21.57
0.88	19.00	77.79	6.78	21.59
0.76	16.60	67.18	5.93	21.84
0.80	17.50	70.72	6.25	21.88
1.05	23.00	92.82	8.21	21.90
0.66	14.60	58.34	5.21	22.12
1.40	31.00	123.76	11.07	22.14
0.85	19.00	75.14	6.78	22.35
1.05	23.50	92.82	8.39	22.38
1.00	22.50	88.40	8.03	22.50
0.80	18.00	70.72	6.43	22.50
0.86	19.40	76.02	6.93	22.56
1.35	30.50	119.34	10.89	22.59
1.25	28.30	110.50	10.10	22.64
1.10	25.00	97.24	8.93	22.73
2.30	52.30	203.32	18.67	22.74
1.55	35.50	137.02	12.67	22.90
1.10	25.30	97.24	9.03	23.00
1.50	34.50	132.60	12.32	23.00
1.50	34.50	132.60	12.32	23.00
0.86	19.80	76.02	7.07	23.02
0.76	17.50	67.18	6.25	23.03
2.30	53.00	203.32	18.92	23.04
1.10	25.70	97.24	9.17	23.36
0.85	20.00	75.14	7.14	23.53
1.70	40.00	150.28	14.28	23.53
1.40	33.00	123.76	11.78	23.57

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
0.92	21.80	81.33	7.78	23.70
1.10	26.20	97.24	9.35	23.82
9.50	226.50	839.80	80.86	23.84
1.15	27.50	101.66	9.82	23.91
1.70	41.50	150.28	14.82	24.41
0.95	23.20	83.98	8.28	24.42
1.00	24.50	88.40	8.75	24.50
0.90	22.20	79.56	7.93	24.67
1.15	28.40	101.66	10.14	24.70
1.10	27.20	97.24	9.71	24.73
1.05	26.00	92.82	9.28	24.76
1.35	33.50	119.34	11.96	24.81
1.15	28.60	101.66	10.21	24.87
1.10	27.50	97.24	9.82	25.00
1.10	27.50	97.24	9.82	25.00
1.00	25.00	88.40	8.93	25.00
3.20	80.20	282.88	28.63	25.06
1.50	37.60	132.60	13.42	25.07
0.75	18.80	66.30	6.71	25.07
0.86	21.60	76.02	7.71	25.12
2.30	58.30	203.32	20.81	25.35
1.20	30.50	106.08	10.89	25.42
0.75	19.10	66.30	6.82	25.47
0.78	20.00	68.95	7.14	25.64
0.85	21.80	75.14	7.78	25.65
1.30	33.50	114.92	11.96	25.77
1.75	45.20	154.70	16.14	25.83
1.40	36.50	123.76	13.03	26.07
0.90	23.50	79.56	8.39	26.11
1.05	27.50	92.82	9.82	26.19
1.25	32.90	110.50	11.75	26.32
1.10	29.00	97.24	10.35	26.36
1.00	26.50	88.40	9.46	26.50
1.15	30.50	101.66	10.89	26.52
1.45	38.50	128.18	13.74	26.55
1.60	42.50	141.44	15.17	26.56
3.68	97.80	325.31	34.91	26.58

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
2.56	68.50	226.30	24.45	26.76
1.70	45.50	150.28	16.24	26.76
1.00	27.00	88.40	9.64	27.00
0.70	19.00	61.88	6.78	27.14
1.30	35.50	114.92	12.67	27.31
0.70	19.20	61.88	6.85	27.43
1.20	33.00	106.08	11.78	27.50
0.81	22.60	71.60	8.07	27.90
1.00	28.00	88.40	10.00	28.00
0.80	22.50	70.72	8.03	28.13
1.25	35.20	110.50	12.57	28.16
0.65	18.50	57.46	6.60	28.46
1.05	30.00	92.82	10.71	28.57
3.40	97.50	300.56	34.81	28.68
0.85	24.50	75.14	8.75	28.82
1.50	44.00	132.60	15.71	29.33
1.60	47.00	141.44	16.78	29.38
1.50	44.50	132.60	15.89	29.67
1.60	48.50	141.44	17.31	30.31
1.10	33.50	97.24	11.96	30.45
2.30	70.30	203.32	25.10	30.57
1.15	35.20	101.66	12.57	30.61
2.10	64.30	185.64	22.96	30.62
0.75	23.00	66.30	8.21	30.67
0.86	26.40	76.02	9.42	30.70
1.25	38.50	110.50	13.74	30.80
1.25	38.50	110.50	13.74	30.80
1.05	32.40	92.82	11.57	30.86
0.55	17.00	48.62	6.07	30.91
1.10	34.10	97.24	12.17	31.00
1.95	60.50	172.38	21.60	31.03
0.76	23.60	67.18	8.43	31.05
1.74	54.20	153.82	19.35	31.15
1.65	51.50	145.86	18.39	31.21
2.70	84.50	238.68	30.17	31.30
1.35	43.80	119.34	15.64	32.44
1.20	39.50	106.08	14.10	32.92

CREAT mg/dl	BUN mg/dl	Creat ummol/l	Urea mmol/l	U: C ratio
1.05	35.00	92.82	12.50	33.33
1.90	63.80	167.96	22.78	33.58
0.75	25.20	66.30	9.00	33.60
3.57	120.70	315.59	43.09	33.81
0.76	25.70	67.18	9.17	33.82
1.35	47.00	119.34	16.78	34.81
2.20	76.80	194.48	27.42	34.91
1.30	45.50	114.92	16.24	35.00
1.20	42.50	106.08	15.17	35.42
1.45	52.50	128.18	18.74	36.21
1.25	45.40	110.50	16.21	36.32
3.80	138.30	335.92	49.37	36.39
4.20	153.00	371.28	54.62	36.43
1.20	45.00	106.08	16.07	37.50
1.65	62.50	145.86	22.31	37.88
0.75	28.50	66.30	10.17	38.00
2.90	110.40	256.36	39.41	38.07
2.25	86.00	198.90	30.70	38.22
2.10	80.80	185.64	28.85	38.48
0.85	33.40	75.14	11.92	39.29
1.45	57.50	128.18	20.53	39.66
2.00	79.60	176.80	28.42	39.80
1.30	52.40	114.92	18.71	40.31
1.82	79.70	160.89	28.45	43.79
1.65	72.40	145.86	25.85	43.88
1.56	68.80	137.90	24.56	44.10
0.60	27.40	53.04	9.78	45.67
1.35	63.30	119.34	22.60	46.89
1.10	51.80	97.24	18.49	47.09
1.45	70.00	128.18	24.99	48.28
0.45	21.50	39.34	7.68	48.31
2.00	98.50	176.80	35.16	49.25
1.60	85.80	141.44	30.63	53.63
1.60	89.20	141.44	31.84	55.75
1.59	95.30	140.56	34.02	59.94
0.53	33.90	46.85	12.10	63.96

REFERENCES

1. Adachi K, Makimura S 1992 Changes in anti-erythrocyte membrane antibody levels of dogs experimentally infected with *Babesia gibsoni*. *Journal Veterinary Medical Science* 54: 1221-1223
2. Adachi K, Ueno C, Makimura S 1993 Immunosuppression in dogs naturally infected with *Babesia gibsoni*. *Journal of Veterinary Medical Science* 55:503-505
3. Adachi K, Yoshimoto A, Hasegawa T et al. 1992 Antierythrocyte membrane antibodies detected in the sera of dogs naturally infected with *Babesia gibsoni*. *Journal of Veterinary Medical Science* 54:1081-1084
4. Agar NS, Board PG 1983 Red cell metabolism. *In Red blood cells of domestic mammals*. Agar NS and Board PG (eds). Elsevier Science Publishers B.V. Pg 1-36
5. Almy FS, Christopher MM, King DP, Brown SA 2002 Evaluation of Cystatin-C as an Endogenous Marker of Glomerular Filtration Rate in Dogs. *Journal of Veterinary Internal Medicine* 16: 45-51
6. Ano H, Makimura S, Harasawa R 2001 Detection of *Babesia* species from infected dog blood by polymerase chain reaction. *Journal of Veterinary Medical Science* 63:111-113
7. Bauer JD 1980 Laboratory Investigations of Haemoglobin. In Sonnienwirth AC and Jarett L (eds) *Gradwohl's Clinical Laboratory Methods and Diagnosis Eighth edition*. CV Mosby Company, London 1: 809-902
8. Birkenheuer AJ, Levy MG, Breitschwerdt EB 2003 Development and evaluation of a seminested PCR for the detection and differentiation of *Babesia gibsoni* (Asian genotype) and *Babesia canis* DNA in canine blood samples. *Journal of Clinical Microbiology* 41: 4172-4177.
9. Blank D W, Kroll M H, Ruddel M E, Elin R J 1985 Hemoglobin interference from in vivo hemolysis. *Clinical Chemistry* 31: 1566-1569
10. Brady H R, Brenner B M 1994 Acute Renal Failure. In *Harrison's Principles of Internal Medicine 13th Edition.* Isselbacher KJ, Braunwald E, Wilson JD et al. (eds). McGraw-Hill Inc. Pg 1265-1274
11. Braun JP, Lefebvre HP, Watson AD 2003 Creatinine in the dog: a

review. *Veterinary Clinical Pathology* 32: 162-179

12. Burchard GD, Ehrhardt S, Mockenhaupt FP et al. 2003 Renal dysfunction in children with uncomplicated, Plasmodium falciparum malaria in Tamale Ghana. *Annuals of Tropical Medicine and Parasitology* 97: 345-350
13. Camacho AT, Pallas E, Gestal JJ et al. 2003 *Ixodes hexagonus* is the main candidate as vector of *Theileria annae* in northwest Spain. *Veterinary Parasitology* 112: 157-163
14. Camacho AT, Pallas E, Gestal JJ et al. 2001 Infection of dogs in North West Spain with a *Babesia microti*-like agent. *Veterinary Record* 149: 552-555
15. Clark IA, Cowden WB 1999 Why is the pathology of falciparum worse than that of Vivax malaria. *Parasitology Today* 15: 458-461
16. Clark I A, Jacobson L S 1998 Do babesiosis and malaria share a common disease process? *Annuals of Tropical Medicine and Parasitology* 92: 483-488
17. Cox FEG 2001 Concomitant infections, parasites and immune responses. *Parasitology* 122: 23-38
18. Da Fonseca-Wollheim, F 1973. Direct plasma ammonia determination without deproteinisation. Improved enzymatic ammonia test. *Zeitschrift fur Klinische Chemie und Klinische Biochemie* 11: 421 and 426
19. Day NP, Hien TT, Schollaardt T, Loc PP, Chuong LV, Chau TT, Mai NT, Phu NH, Sinh DX, White NJ, Ho M 1999 The prognostic and pathophysiologic role of pro- and anti-inflammatory cytokines in severe malaria. *Journal of Infectious Diseases* 180: 1288-1297
20. de Scally MP, Lobetti RG, Reyers F et al. 2004 Are urea and creatinine values reliable indicators of azotaemia in canine babesiosis? *Journal of the South African Veterinary Association* 75: 121-124
21. Dondorp AM, Nyanoti M, Kager PA et al. 2002 The role of reduced red cell deformability in the pathogenesis of severe falciparum malaria and its restoration by blood transfusion. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 96: 282-286
22. Dorner J L, Hoffmann W E, Filipov M M 1981 Effect of in *vitro*

Hemolysis on Values for Certain Porcine Serum Constituents. *Veterinary Clinical Pathology* XII: 15-19

23. Doumas BT, Hause LL, Sciacca RD 1979 Performance of the Du Pont aca ammonia method. *Clinical Chemistry* 25: 175-178
24. Doumas B, Kwok-Cheung P P, Perry B W, Jendrzejcak B, McComb R B, Schaffer R, Hause L L 1985 Candidate reference method for determination of total bilirubin in serum: Development and validation. *Clinical Chemistr*. 31: 1779-1789
25. Duvic C, Nedelec G, Debord T et al. 1999 Important parasitic nephropathies: update from the recent literature. *Nephrologie* 20: 65-74
26. Duvic C, Rabar D, Didelot F 2000 Acute renal failure during severe malaria: physiopathology and therapeutic management. Apropos of 2 cases. *la Medicina Tropical* 60: 267-270
27. Dvir, E., R. G. Lobetti, L. S. Jacobson, J. Pearson, and P. J. Becker. 2004. Electrocardiographic changes and cardiac pathology in canine babesiosis. *Jouranl of Veterinary Cardiology* 6:15-23
28. Ehrich JH, Horstmann RD 1985 Origin of proteinuria in human malaria. *Tropical Medicine and Parasitology* 36: 39-42
29. Eiam-Ong S, Sitprija V 1998 Falciparum malaria and the kidney: a model of inflammation. *American Journal of Kidney Diseases* 32: 361-375
30. English MC, Waruiru C, Lightowler C et al. 1996 Hyponatraemia and dehydration in severe malaria. *Archives of Diseases in Childhood* 74: 201-205
31. Feinfeld DA, Bargouthi H, Niaz Q et al. 2002 Massive and disproportionate elevation of blood urea nitrogen in acute azotaemia. *International Journal Urology Nephrology* 34: 143-145
32. Finco D R 1997 Evaluation of Renal Functions. In Osborne C A, Finco D R (ed) *Canine and Feline Nephrology and Urology*. Williams & Wilkins, Philadelphia: 216-229
33. Franzini C, Morelli AM, Cattozzo G 1991 Use of a synthetic soluble bilirubin derivative to assess interference in creatinine measurements. *Clinical Chemistry* 37: 236-238

34. Freeman MJ, Kirby BM, Panciera DL et al. 1994 Hypotensive shock syndrome associated with acute *Babesia canis* infection in a dog. *Journal of the American Veterinary Medical Association* 204: 94-96
35. Garrick MD, Garrick LM 1983 Haemoglobins and globin genes. *In Red blood cells of domestic mammals.* Agar NS and Board PG (eds). Elsevier Science Publishers B.V. Pg 1-36
36. Gunther A, Burchard G D, Slevogt H, Abel W, Grobusch M P 2002 Renal dysfunction in *falciparum malaria* is detected more often when assessed by serum concentration of cystatin-C instead of creatinine. *Tropical Medicine and International Health* 7: 931-934
37. Guyton MD, Hall JE 1996 Red Blood Cells, Anaemia, and Polycythemia. Guyton MD, Hall JE (eds) *In Text Book of Medical Physiology 9th edition*. W.B Saunders Company Chapter 32: 425-433.
38. Guyton MD, Hall JE 1996 Protein Metabolism. Guyton MD, Hall JE (eds) In *Text Book of Medical Physiology 9th edition*. W.B Saunders Company Chapter 69: 877-882.
39. Guyton MD, Hall JE 1996 The Liver as an Organ. Guyton MD, Hall JE (eds) *In Text Book of Medical Physiology 9th edition.* W.B Saunders Company Chapter 70: 883-888.
40. Hildebrandt PK 1981 The organ and vascular pathology of babesiosis. In Ristic MJ, Koeier JP (eds) *Babesiosis.* Academic Press, New York: 459-473
41. Jacobs R M, Lumsden J H, Taylor J A, Grift E 1991 Effects of Interferents on the Kinetic Jaffe' Reaction and an Enzymatic Colorimetric Test for Creatinine Concentration Determination in Cats, Cows, Dogs and Horses. *Canine Journal of Veterinary Research* 55: 150-154
42. Jacobson LS, Clarka IA 1994 The pathophysiology of canine babesiosis: new approaches to an old puzzle. *Journal of the South African Veterinary Association* 65: 134-145
43. Jacobson LS, Lobetti RG 1996 Rhabdomyolysis as a complication of canine babesiosis. *Journal of Small Animal Practice* 37: 286-291
44. Jacobson LS, Lobetti RG, Becker P et al. 2002 Nitric oxide metabolites in naturally occurring canine babesiosis. *Veterinary*

Parasitology 104: 27-41

45. Jacobson L, Lobetti RG, Vaughn-Scott T 1999 Hypotension: A Common Event In Canine Babesiosis. *In Proceedings of a Symposium on Canine Babesiosis and Ehrlichiosis*. Lobetti RG editor. Pg: 50-54
46. Jacobson L S, Swan G E 1995 Supportive treatment of canine babesiosis. *Journal of the South African Veterinary Association* 66: 95-105
47. Jakeman GN, Saul A, Hogarth WL, Collins WE 1999 Anaemia of acute malaria infections in non-immune patients primarily from destruction of uninfected erythrocytes. *Parasitology* 119: 127-133
48. Jensen A L, Bomholt M, Moe L 2001 Preliminary Evaluation of a Particle-enhanced Turbidimetric Immunoassay (PETIA) for the Determination of Cystatin-C-like Immunoreactivity in Dogs. *Veterinary Clinical Pathology* 30: 86-90
49. Jung K, Jung M 1995 Cystatin-C: A Promising Marker of Glomerular Filtration rate to Replace Creatinine. *Nephron* 370-371 (letter)
50. Kilbourn RG, Gross SS, Lodato RF et al. 1993 NG-methyl-l-arginine inhibits tumour necrosis factor-induced hypotension: implications for the involvement of nitric oxide. *Proceedings of the National Academy of Science*, USA 87: 3629-3632
51. Kilbourn RG, Gross SS, Lodato RF et al. 1992 Inhibition of interleukin-1-alpha-induced nitric oxide synthase in vascular smooth muscle and full reversal of interleukin-1-alpha-induced hypotension by N-amino-1-arginine. *Journal of the National Cancer Institute* 84: 1008-1016
52. Kjemtrup AM, Kocan AA, Whitworth L et al. 2000 There are at least three genetically distinct piroplasms from dogs. *International Journal of Parasitology* 30: 1501-1505
53. Kochar DK, Agarwal P, Kochar SK et al. 2003 Hepatocyte dysfunction and hepatic encephalopathy in Plasmodium falciparum malaria. *Quarterly Journal of Medicine* 96: 505-512
54. Kordik SK, Breitschwerdt EB, Hegarty BC et al. 1999 Coinfection with multiple tickborne pathogens in a Walker hound kennel in North Carolina. *Journal of Clinical Microbiology* 37: 2631-2638

55. Kwiatkowski, D., A. V. Hill, I. Sambou, P. Twumasi, J. Castracane, K. R. Manogue, A. Cerami, D. R. Brewster, and B. M. Greenwood. 1990. TNF concentration in fatal cerebral, non-fatal cerebral and uncomplicated Plasmodium falciparum malaria. *Lancet* 336: 1201-1204
56. Kyhse-Andersen J, Schmidt C, Nordin G, Andersson B, Nilsson-Ehle P, Lindström V et al.. 1994 Serum cystatin-C, determined by a rapid, automated particle-enhanced turbidimetric method, is a better marker than serum creatinine for glomerular filtration rate. *Clinical Chemistry* 40: 1921-1926
57. Leisewitz AL, Jacobson LS, de Morais HS et al. 2001 The mixed acid-base disturbances of severe canine babesiosis. *Journal of Veterinary Internal Medicin* 15: 445-452
58. Lewis BD, Penzhorn BL, Lopez Rebollar LM 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
59. Lindsay Boozer A, Macintire DK 2003 Canine Babesiosis. *Veterinary Clinics of North America Small Animal Practise* 33: 885-904
60. Lobetti RG 1994 The comparative role of haemoglobinaemia and hypoxia in the development of nephropathy in the dog. *Thesis in Library University of Pretoria Onderstepoort South Africa* Pg: 27
61. Lobetti RG 1998 Canine babesiosis. *Compendium on Continuing Education for the Practicing Veterinarian* 20: 418-430
62. Lobetti RG, Dvir E, Pearson J 2002 Cardiac troponins in canine babesiosis. *Journal of Veterinary Internal Medicine* 16: 63-68.
63. Lobetti R G, Jacobson L S 2001Renal involvement in dogs with babesiosis. *Journal of the South African Veterinary Association* 72: 23-28
64. Lobetti RG, Reyers F 1996 Met-haemoglobinuria in naturally occurring *Babesia canis* infection. *Journal of the South African Veterinary Association* 67: 88-90
65. Lobetti R G, Reyers F, Nesbit J W 1996 The comparative role of haemoglobinuria and hypoxia in the development of canine babesial nephropathy. *Journal of the South African Veterinary Association* 67:

188-198

66. Lyman JL 1986 Blood urea nitrogen and creatinine. *Emergency Medicine Clinics of North America* 4: 223-233
67. Maegraith B, Gilles HM, Devakul K 1957 Pathological Processes in *Babesia canis* Infections. *Tropin Medixzin und Parasitologie* 4: 485-514
68. Matjila, P. T., B. L. Penzhorn, C. P. Bekker, A. M. Nijhof, and F. Jongejan. 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. *Veterinary Parasitology* 122:119-25
69. Malherbe W 1966 Clinico-pathological studies of *Babesia canis* infection in dogs. The influence of the infection on kidney function. *Journal of the South African Veterinary Association* 37 :261-264
70. Malherbe WD, Parkin BS 1951 Atypical symptomatology in *Babesia canis* infection. *Journal of the South African Veterinary Association* 22: 25-36
71. McGuire, W., A. V. Hill, C. E. Allsopp, B. M. Greenwood, and D. Kwiatkowski. 1994. Variation in the TNF-alpha promoter region associated with susceptibility to cerebral malaria. *Nature* 371: 508-510
72. Medaille C, Trumel C, Concordet D et al. 2004 Comparison of plasma/ serum urea and creatinine concentration in the dog: a 5-year retrospective study in a commercial veterinary clinical pathology laboratory. *Journal Veterinary Medicine .A. Physiology, Pathology, Clinical Medicine* 51: 119-123
73. Michel KE, King LG, Ostro E 1997 Measurement of urinary urea nitrogen content as an estimate of the amount of total urinary nitrogen loss in dogs in intensive care units. *Journal of the American Veterinary Medical Association* 210: 356-359
74. Miller D 1999 The Yellow Patient. *In Proceedings of a Symposium on Canine Babesiosis and Ehrlichiosis*. Lobetti RG editor. 95-97
75. Mohr AJ, Lobetti RG, van der Lugt JJ 2000 Acute pancreatitis: a newly recognised potential complication of canine babesiosis. *Journal of the South African Veterinary Association* 71: 232-239
76. Morita T, Saeki H, Imai S et al. 1996 Erythrocyte oxidation in artificial *Babesia gibsoni* infection. *Veterinary Parasitology* 63: 1-7

77. Murase T, Ueda T, Yamato O et al. 1996 Oxidative damage and enhanced erythrophagocytosis in canine erythrocytes infected with *Babesia gibsoni*. *Journal of Veterinary Medical Science* 58: 259-261
78. Onishi T, Suzuki S, Horie M et al. 1993 Serum hemolytic activity of *Babesia gibsoni*-infected dogs: the difference in the activity between self and nonself red blood cells. *Journal of Veterinary Medical Science* 55: 203-206
79. Onishi T, Ueda K, Horie M et al. 1990 Serum hemolytic activity in dog infected with *Babesia gibsoni*. *Journal of Parasitology* 76: 564-567
80. Olde Damink SW, Dejong CH, Deutz NE et al. 1999 Upper gastrointestinal bleeding: an ammoniagenic and catabolic event due to the total absence of isoleucine in the haemoglobin molecule. *Medical Hypotheses* 52: 515-519
81. Otsuka Y, Yamasaki M Yamato O et al. 2002 The effect of macrophages on the erythrocyte oxidative damage and the pathogenesis of anaemia in *Babesia gibsoni*-infected dogs with low parasitaemia. *Journal of Veterinary Medical Science* 64: 221-226
82. Prause LC, Grauer GF 1998 Association of gastrointestinal hemorrhage with increased blood urea nitrogen and BUN/creatinine ratio in dogs: a literature review and retrospective study. *Veterinary Clinical Pathology* 27: 107-111
83. Ralston GB 1983 The membrane of the red blood cell. *In Red blood cells of domestic mammals.* Agar NS and Board PG (eds). Elsevier Science Publishers B.V. Pg 1-36
84. Randers E, Erlandsen E J 1999 Serum cystatin C as an endogenous marker for renal function- a review. *Clinical Chemistry Laboratory Medicine* 37: 389-395
85. Reyers F 1992 Is azotaemia in canine babesiosis an indication of renal disease? *Proceedings of the 9th Faculty Day, University of Pretoria, Faculty of Veterinary Science,* 1 October: 17
86. Reyers F, Leisewitz AL, Lobetti RG et al. 1998 Canine Babesiosis in South Africa: more than one disease. Does this serve as a model for falciparum malaria? *Annuals of Tropical Medicine and Parasitology*

92:503-511

87. Rossignol B, Rossignol D, PetitClerk C 1984 Improvement of creatinine measurement on RA-1000. *Clinical Biochemistry* 17: 203-204
88. Rui-Mei L, Kara AU, Sinniah R 1998 Upregulation of major histocompatibility complex (MHC) antigen in nephritis associated with murine malaria infection. *Journal of Pathology* 185: 212-218
89. Rui-Mei L, Kara AU, Sinniah R 1998 In situ analysis of adhesion molecule expression in kidneys infected with murine malaria. *Journal of Pathology* 185: 219-225
90. Schetters TP, Eling WM 1999 Can Babesia infections be used as a model for cerebral malaria? *Parasitology today* 15: 492-497
91. Shakespeare A 1995 The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. *JS Journal of the South African Veterinary Association Vet Assoc* 66:247-250
92. Sheiban AK 1999 Prognosis of malaria associated severe acute renal failure in children. *Renal Failure* 21: 63-66
93. Simao C, Stone R, Almeida M 2003 Kidney failure associated with Plasmodium falciparum infection. *Acta Medica Portuguesa* 16: 93-95
94. Sinniah R, Lye W 2000 Acute renal failure from myoglobinuria secondary to myositis from severe falciparum malaria. *American Journal Nephrology* 20: 339-343
95. Sitprija V, Vongsthongsri M, Poshyachinda V et al. 1977 Renal failure in malaria: a pathophysiological study. *Nephron* 18: 277-287.
96. Sklar AH, Riesenberg LA, Rehman UR et al. 1996 Prerenal azotaemia: differentiation of hyperureagenesis from renal hypoperfusion using urinary urea nitrogen data. *International Journal of Artificial Organs* 19: 164-169
97. Slovut DP, Benedetti E, Matas AJ 1996 Babesiosis and hemophagocytic syndrome in an asplenic renal transplant recipient. *Transplantation* 62: 537-539
98. Soldin S J, Henderson L, and Hill J G. 1978 The effect of bilirubin and ketones on reaction rate methods for the measurement of creatinine.

Clinical Biochemistry 11: 82-86

99. Taboada J, Merchant S R 1991 Babesiosis of companion animals and man. *Veterinary Clinics of North America Small Animal Practice* 21: 103-123
100. Tiffany T O, Jansen J M, Burtis C A, Overton J B, Scott C D 1972 Enzymatic kinetic rate and endpoint analyses of substrate, by use of a GEMSAEC fast analyser. *Clinical Chemistry* 18: 829
101. Weber JA and van Zanten AP 1991 Interferences in current methods for measurements of creatinine. *Clinical Chemistry*, 37: 695-700
102. Wehner A, HÖchel J, Finnah A, Bandt C, Hartmann H, Hirschberger J 2002 Evaluation of cystatin-C as an endogenous marker of renal function in dogs. Abstract. In *Proceedings 12th ECVIM.CA/ESVIM congress Munich* Pg: 160
103. Welzl C, Leisewitz A, Jacobson L et al. 2001 Systemic inflammatory response syndrome and multiple organ damage/dysfunction in complicated canine babesiosis. *Journal of the South African Veterinary Association* 72: 158-162
104. White NJ, Breman JG 1994 Malaria and Babesia. In *Harrison's Principles of Internal Medicine 13th Edition*. Isselbacher KJ, Braunwald E, Wilson JD et al. (eds). McGraw-Hill Inc. Pg 887-896
105. Wilairatana P, Westerlund EK, Aursudkij B et al. 1999 Treatment of malarial acute renal failure by haemodialysis. *American Journal of Tropical Medicine and Hygiene* 60: 233-237
106. Wosniak EJ, Barr BC, Thomford JW et al. 1997 Clinical, anatomic, and immunopathologic characterization of *Babesia gibsoni* infection in the domestic dog (Canis familiaris). *Journal of Parasitology* 83: 692-699
107. Zahler M, Rinder H, Gothe R 2000 Genotypic status of *Babesia microti* within the piroplasms. *Parasitology and Research* 86: 642-646
108. Zahler M, RinderH, Schein E 2000 Detection of a new pathogenic *Babesia microti*-like species in dogs. *Veterinary Parasitology* 89: 241-248
109. Zahler M, Rinder H, Zweygarth E et al. 2000 "*Babesia gibsoni*" of dogs from North America and Asia belong to different species.

Parasitology 120:365-369

110. Zahler M, Schein E, Rinder H et al. 1998 Characteristic genotypes discriminate between *Babesia canis* isolates of differing vector specificity and pathogenicity to dogs. *Parasitology and Research* 84: 544-548