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EVALUATION OF KIDNEY INJURY IN CANINE BABESIOSIS CAUSED BY *BABESIA ROSSI*

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LIST OF ABBREVIATIONS

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AKI	acute kidney injury
AKIN	Acute Kidney Injury Network
AP	alkaline phosphatase
ARDS	acute respiratory distress syndrome
ARF	acute renal failure
ATN	acute tubular necrosis
CKD	chronic kidney disease
CRP	C-reactive protein
ELISA	enzyme-linked immunosorbent assay
GFR	glomerular filtration rate
GGT	γ-glutamyl transferase
h	hours
HMW	high molecular weight
IGFBP7	insulin-like growth factor-binding protein 7
IgG	immunoglobulin G
IMHA	immune-mediated hemolytic anemia
IRIS	International Renal Interest Society
kDa	kilodaltons
KDIGO	Kidney Disease: Improving Global Outcomes
KIM-1	kidney injury molecule-1
LM	light microscopy
LMW	low molecular weight
m	month
MW	molecular weight
NAG	N-acetyl-β-D-glucosaminidase
NGAL	neutrophil gelatinase-associated lipocalin
OVAH	Onderstepoort Veterinary Academic Hospital
PAS	periodic acid-Schiff
PCR	polymerase chain reaction
RA	renal azotemia
RBP	retinol-binding protein
RLB	reverse line blot
RRT	renal replacement therapy
sCr	serum creatinine
SIRS	systemic inflammatory response syndrome
sSDMA	serum symmetric dimethylarginine
TEM	transmission electron microscopy
THP	Tamm-Horsfall protein
TIMP-2	tissue inhibitor of metalloproteinases-2
uCr	urinary creatinine
UO	urine output
uOsmol	urine osmolality
UPC	urinary protein to creatinine ratio
USG	urine specific gravity
UTI	urinary tract infection
XLHN	X-linked hereditary nephropathy

CHAPTER 1

GENERAL INTRODUCTION

The main objective of this thesis is to characterize kidney injury in canine babesiosis caused by *Babesia rossi*. Both routine biomarkers and urinary kidney injury biomarkers will be used to evaluate *Babesia*-induced kidney injury.

Chapter 1 will first describe the etiology, epidemiology, and clinical presentation of canine babesiosis. Secondly, the concept of acute kidney injury (AKI) will be introduced. In the third section, an overview of the current literature on *Babesia*-induced kidney injury will be provided. Next, limitations of the traditional evaluation of AKI caused by canine babesiosis will be presented. In the last section, the application of urinary kidney injury biomarkers to improve the detection of AKI will be introduced.

1. Canine babesiosis

1.1 Introduction

Canine babesiosis is a protozoal tick-borne disease of worldwide significance, caused by different *Babesia* species. The genus *Babesia* belongs to the phylum Apicomplexa in the order of the Piroplasmida. Within this order, the genera *Babesia* and *Theileria* are the 2 main piroplasms that can infect domestic animals. Both parasites have a mammalian intra-erythrocytic stage in their life cycle (Uilenberg, 2006; Irwin, 2009; Solano-Gallego and Baneth, 2011). They are commonly grouped together as the “piroplasmoses”, based on their pear-shaped form after multiplication. Historically, both genera have been separated based on differences in their development in the vertebrate host and tick vector (Uilenberg, 2006).

Although intra-erythrocytic parasitemia, leading to hemolytic anemia, is the main clinical manifestation of this parasitic disease, babesiosis can involve multiple organs. Therefore, severity of canine babesiosis can range from mild hemolytic to severe systemic disease manifestations that can be life-threatening (Lobetti, 1998; Jacobson and Clark, 1994).

1.2 Etiology

Historically, canine *Babesia* species were divided into 2 species based on their morphological appearance. Large *Babesia* parasites were designated *B. canis*, while small *Babesia* parasites were considered to be *B. gibsoni* (Boozer and Macintire, 2003; Uilenberg, 2006). Based on differences in vector specificity, pathogenicity, and lack of cross-immunity, the large *B. canis* species was further divided into 3 subspecies, *B. canis canis*, *B. canis vogeli*, and *B. canis rossi* (Uilenberg et al., 1989). Yet, subsequent molecular analyses found that the 3 subspecies were in fact separate species, namely *B. canis*, *B. vogeli*, and *B. rossi* (Zahler et al., 1998; Carret et al., 1999). Molecular studies also revealed the existence of multiple small *Babesia* species affecting dogs (Kjemtrup et al., 2000).

1.3 Epidemiology

1.3.1 Life cycle and transmission

Dogs and other vertebrate hosts are infected when *Babesia* sporozoites are injected through the saliva of infected ticks during their blood meal (Figure 1.1). In the vertebrate host, *Babesia* sporozoites directly invade erythrocytes, and develop into trophozoites. Asexual reproduction occurs in the vertebrate erythrocytes. Trophozoites divide by binary fission into merozoites. After erythrolysis, merozoites can invade new erythrocytes, where new merogonies occur. Some specific stages of the parasite (pre-gametocytes) survive in the tick after ingesting *Babesia*-infected erythrocytes during a blood meal. It is unknown whether sexual differentiation (gametocyte formation) begins in the vertebrate host or only in the tick. Gamogony (gamete differentiation and zygote formation) occurs in the intestinal cells of the tick. Asexual division of ookinetes into kinetes occurs in many tick organs, while final differentiation into sporozoites occurs in the salivary glands of the tick. Further sporozoite differentiation only starts when the tick attaches to its vertebrate host. Sporozoites will then be injected into the host during the next blood meal (Hunfeld et al., 2008; Chauvin et al., 2009).

In contrast to *Babesia* parasites, that directly infect erythrocytes once injected into the mammalian host, *Theileria* parasites first invade lymphocytes prior to their erythrocytic stage. The invasion of different organs in the tick vector is the other difference between the genus *Babesia* and *Theileria*. In *Babesia* infections, multiple organs of the tick are invaded, including the ovaries, resulting in transovarial transmission of the infection to the next generation of ticks through tick eggs. Contrarily, in *Theileria* infections, there is, besides the salivary glands, no invasion of other organs in the tick vector. *Theileria* transmission takes place solely by injection of infected saliva (transstadial transmission) (Uilenberg, 2006; Chauvin et al., 2009).

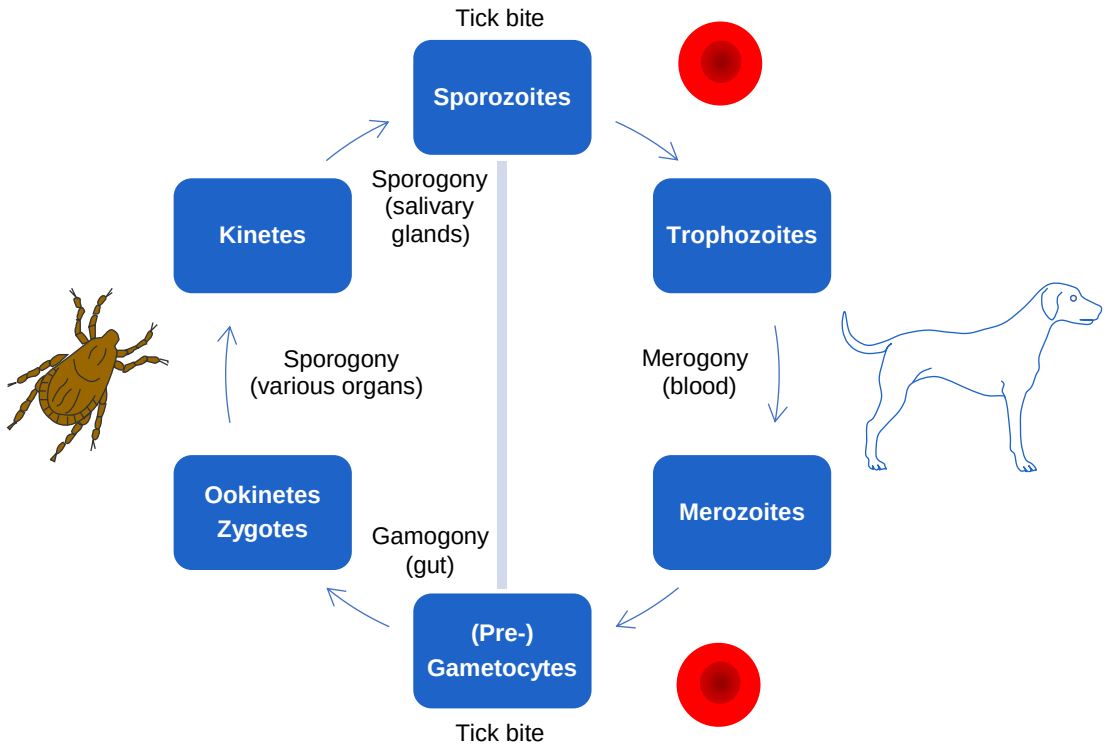

Figure 1.1. Life cycle of *Babesia* spp., based on Hunfeld et al. (2008) and Chauvin et al. (2009), created with Motifolio.

As mentioned before, the natural transmission of *Babesia* parasites to the vertebrate host occurs during tick bites. Transstadial and -ovarial transmission can occur in *Babesia* species. Notably, some ticks remain infected and infective for several generations without the need for new feeding on infected hosts (Chauvin et al., 2009). Final sporogony in the salivary gland of the tick takes at least 48 hours (h) (Schein et al., 1979). This led to the general assumption that daily removal of ticks and the use of acaricides that kill ticks during attachment could prevent disease transmission (Boozer and Macintire, 2003; Varloud et al., 2018). However, a recent study showed that early transmission of *B. canis* can occur within 8 h after tick attachment, provided the tick had a history of feeding on a previous host (Varloud et al., 2018). Minimal transmission times cannot be determined, because the history of individual ticks in natural infections is unknown. *Babesia* species have different tick vector specificities (Zahler et al., 1998). An overview of the main *Babesia* species infecting dogs and their specific tick vectors and associated geographic distribution is given in Table 1.1.

Table 1.1. Geographic distribution and vectors of the main *Babesia* species in dogs, adapted from Uilenberg et al. (1989) and Matijatko et al. (2012).

Species	Distribution	Tick vector	Other References
B. rossi	Southern Africa	*Haemaphysalis elliptica* (formerly *H. leachi*)	(Matjila et al., 2004)
B. canis	Europe, Asia	*Dermacentor reticulatus*	(Bourdoiseau, 2006) (Solano-Gallego et al., 2016)
B. vogeli	Worldwide (mostly tropical, subtropical regions)	*Rhipicephalus sanguineus*	(Irwin, 2009)
B. gibsoni	Worldwide (mostly Asia)	*Haemaphysalis* spp. *Rhipicephalus* spp.	(Irwin, 2009) (Solano-Gallego and Baneth, 2011)
B. microti-like	Southern Europe (mostly northwest Spain)	*Ixodes hexagonus*?	(Solano-Gallego et al., 2016) (Miró et al., 2015)

Transmission of babesiosis can also occur by blood transfusion (Taboada and Merchant, 1991), while dog bites seem to be an important mode of transmission for *B. gibsoni* in the USA (Taboada and Merchant, 1991; Yeagley et al., 2009). Vertical transmission between mother and pups is considered rare, although a few cases have been reported in different *Babesia* species (Mierzejewska et al., 2014; Földvári et al., 2016).

1.3.2 Geographic distribution

Presence of competent tick vectors is the most important factor of the geographic distribution of *Babesia* spp. infections (Table 1.1) (Solano-Gallego et al., 2016). Several vector-borne diseases, including canine babesiosis, are considered to be emerging diseases worldwide. Emergence of these infections is multifactorial in origin, and includes a broader distribution of competent vectors in the environment due to climate change, pet travel, animal trade, and changes in landscape use and other human activities (Dautel et al., 2006; Beugnet and Marié, 2009; Chomel, 2011).

1.3.2.1 Africa

Until 2004, *B. rossi* was the only known *Babesia* species infecting dogs in South Africa (Uilenberg et al., 1989; Matjila et al., 2004). Based on a molecular study using polymerase chain reaction (PCR) and reverse line blot (RLB), dogs infected with *B. vogeli* were also detected in South Africa (Matjila et al., 2004). Based on 2 studies (Matjila et al., 2004; Matjila et al., 2008), *B. rossi* was the most common species infecting dogs in all sampled areas throughout South Africa, except for the area around Bloemfontein (Free State), where *B. vogeli* infections were more common. In the area around Onderstepoort (Pretoria), where most of the South African *Babesia* studies were carried out, *B. rossi* was much more prevalent than *B. vogeli*. It can therefore be assumed that most dogs in previous studies, originating from the area around Onderstepoort, were infected with *B. rossi*, even though molecular analysis was not performed (Jacobson, 2006).

The 2 main *Babesia* species infecting dogs throughout Africa are *B. rossi* and *B. vogeli*. *Babesia rossi* infections have only been reported in sub-Saharan countries (Penzhorn et al., 2017), including South Africa (Matjila et al., 2004), Nigeria (Sasaki et al., 2007; Kamani et al., 2013), Sudan (Oyamada et al., 2005), Uganda (Proboste et al., 2015), and Zambia (Nalubamba et al., 2015). In contrast to the sub-Saharan localization of *B. rossi*, *B. vogeli*-infected dogs have been documented throughout the entire African continent (Matjila et al., 2004; Oyamada et al., 2005; Sasaki et al., 2007; M'ghirbi and Bouattour, 2008; Salem and Farag, 2014; Cardoso et al., 2016). A single case of an autochthonous *B. canis* infection has also been reported in a Nigerian dog (Kamani et al., 2010). Lastly, few studies also confirmed the presence of *B. gibsoni* in African dogs from Zambia and Angola (Nalubamba et al., 2015; Cardoso et al., 2016).

1.3.2.2 Europe

In most of Europe, canine babesiosis is predominantly caused by *B. canis* (Matijatko et al., 2012). *Babesia canis* is highly endemic in central European countries, including France (Bourdoiseau, 2006), Croatia (Beck et al., 2009), Italy (Solano-Gallego et al., 2008; Cassini et al., 2009), Hungary (Földvári et al., 2005), Romania (Ionita et al., 2012), and Poland (Cacciò et al., 2002; Zygner et al., 2011).

Distribution of canine babesiosis caused by *B. canis* is expanding in Europe, as autochthonous cases continue to be reported increasingly in previously non-endemic countries (Chomel, 2011). For example, an autochthonous outbreak of *B. canis* infection in 23 dogs was reported in 2 different areas of The Netherlands in 2004 (Matjila et al., 2005) and one autochthonous case was even reported in Norway (Øines et al., 2010). Most recently, an outbreak of *B. canis* infections was documented in Essex, UK (Swainsbury et al., 2016; Wright, 2018). Ticks removed from infected dogs were confirmed by PCR to be *Dermacentor reticulatus* infected with *B. canis* (Phipps et al., 2016). Other *B. canis*-infected *D. reticulatus* ticks were subsequently collected from the field in the same area in the UK (de Marco et al., 2017). The establishment of canine babesiosis caused by *B. canis* in previously non-endemic areas can be explained by the expanding distribution of the *D. reticulatus* tick vector, as a remarkable spread of these ticks has been observed in large areas of north-western and central Europe (Dautel et al., 2006; Beugnet and Marié, 2009; Claerebout et al., 2013; Földvári et al., 2016).

Babesia vogeli is mostly reported in Southern Europe, around the Mediterranean basin (Solano-Gallego et al., 2008; Beck et al., 2009; René-Martellet et al., 2015; Estrada-Peña et al., 2017).

Geographic distribution and prevalence data of small *Babesia* species is limited, because most observations come from individual case reports (Solano-Gallego et al., 2016). Occasionally, cases of *B. gibsoni* infection in dogs have been reported throughout Europe (Criado-Fornelio et al., 2003; Hartelt et al., 2007; Trotta et al., 2009). In Croatia, a prevalence of 0.7% for *B. gibsoni* was observed in asymptomatic dogs based on molecular analysis (Beck et al., 2009).

A small *Babesia* species, *B. microti*-like, was discovered in north-western Spain (Galicia) (Camacho et al., 2001), where it appears to be highly endemic (García, 2006). This same species has also been described as *B. annae* (Camacho et al., 2005), *Theileria annae* (García, 2006), *B.* 'Spanish isolate' (Yeagley et al., 2009), *B.* cf. *microti* (Hodžić et al., 2017), and also as *B. vulpes*, based on its natural host reservoir, the red fox (*Vulpes vulpes*) (Baneth et al., 2015). Since its first description in Galicia, isolated cases have been observed in several other European countries (Beck et al., 2009; Simões et al., 2011; Falkenö et al., 2013; René-Martellet et al., 2015).

1.3.2.3 Belgium

Three recent studies documented the presence of the competent tick vector of *B. canis*, *D. reticulatus*, at multiple locations in Belgium (Cochez et al., 2012; Claerebout et al., 2013; Obsomer et al., 2013), with the species now being reported throughout Belgium, based on a comprehensive review of several recent datasets (Rubel et al., 2016).

As a consequence, several autochthonous *Babesia* infections have been reported in Belgium. The first 3 autochthonous cases of canine babesiosis in Belgium were described in 1999 (Losson et al., 1999), and another case was reported in 2008 (Van de Maele et al., 2008). A questionnaire-based survey was performed in several western European countries to investigate the number of clinical cases of canine babesiosis diagnosed in 2010. Of the 38 cases diagnosed in Belgium, 22 cases were reported in the area around Mons (Halos et al., 2014). This area was already reported by local veterinarians to be a focus of canine babesiosis (Losson et al., 1999). In 2016, another autochthonous case was diagnosed, in which the infecting species was confirmed by PCR to be *B. canis* (*personal communication, Pieter Defauw*).

1.3.3 Global importance of babesiosis and other vector-borne diseases

The incidence of many tick-borne diseases in dogs and humans is increasing worldwide (Beugnet and Marié, 2009; Chomel, 2011; Dantas-Torres et al., 2012; Wikel, 2018). Aside from *Babesia*, multiple other pathogens are the cause of emerging tick-borne diseases in humans, including *Borrelia*, *Anaplasma*, *Ehrlichia*, *Rickettsia*, and several viruses (Hildebrandt et al., 2013; Steere et al., 2016; Ismail and McBride, 2017; Mansfield et al., 2017; Wikel, 2018).

The importance of babesiosis in veterinary medicine is illustrated by the fact that bovine babesiosis is economically the most important arthropod-borne disease of cattle worldwide (Uilenberg, 1995; Schnittger et al., 2012). Although it is likely that many cases were not diagnosed due to a lack of medical awareness, an increasing number of human *Babesia* infections have been reported in the last decades (Schnittger et al., 2012; Hildebrandt et al., 2013). It is difficult to quantify the number of cases of human babesiosis, because many cases may have been either overlooked or not reported (Hildebrandt et al., 2013). The most common form of human babesiosis occurs in North America and is caused by the rodent species *B. microti*, while the cattle species *B. divergens* is the main cause of human babesiosis in Europe (Vannier and Krause, 2012; Hildebrandt et al., 2013). However, several additional *Babesia* species have been reported to infect humans worldwide (Gray et al., 2010). Isolated cases of human babesiosis caused by uncharacterized species have also been reported in Africa (Bush et al., 1990; El-Bahnasawy and Morsy, 2008).

The genera *Babesia* and *Plasmodium* are 2 phylogenetically related intra-erythrocytic parasites belonging to the same phylum of Apicomplexa (Lau, 2009). Several *Plasmodium* species can cause malarial infections in humans and most cases are caused by either *P. falciparum* or *P. vivax* (White et al., 2014). Based on the World malaria report of 2017, malaria caused an estimated 216 million cases and 445000 deaths in 2016 worldwide. Most of these cases (90%) and deaths (91%) occurred in the WHO African region, with the majority of the global malaria burden occurring in sub-Saharan Africa (WHO, 2017). The pathogenesis and the clinical presentations of babesiosis share many features with falciparum malaria in humans. Therefore, it has been suggested that babesiosis occurring in several vertebrate animal species, more

specifically also canine babesiosis caused by *B. rossi* in South Africa, could serve as disease models for falciparum malaria (Clark and Jacobson, 1998; Reyers et al., 1998; Schetters and Eling, 1999; Krause et al., 2007).

1.4 Pathogenesis

Two principal mechanisms of pathogenesis were postulated in canine babesiosis based on a study of Reyers et al. (1998). One group of severely anemic dogs developed a hemolytic disease, while the second group consisted of non-anemic dogs who developed an acute systemic inflammatory response.

The pathogenesis of anemia is not completely understood, but both intra- and extravascular hemolysis, which are multifactorial in origin, play a principal role (Jacobson and Clark, 1994; Jacobson, 2006). An immune-mediated component was suggested (Reyers et al., 1998), and secondary immune-mediated hemolytic anemia (IMHA) is a recognized complication of canine babesiosis (Jacobson and Clark, 1994).

The development of an excessive systemic inflammatory response syndrome (SIRS) to the infection is considered to be the main cause of a variety of different complications that can occur in canine babesiosis (Jacobson and Clark, 1994; Welzl et al., 2001). A more detailed pathogenesis is described elsewhere (see Chapter 1, 3.2 Pathogenesis of *Babesia*-induced kidney injury). The pathogenicity of *Babesia* is determined by several factors including the species and strain involved, but also depends on host factors, such as age and immunological response against the parasite (Taboada and Merchant, 1991). Large *Babesia* species can be categorized according to pathogenicity*. Babesia rossi* is the most virulent of all large *Babesia* species. The least virulent species is *B. vogeli*, while *B. canis* is of intermediate pathogenicity (Uilenberg et al., 1989; Zahler et al., 1998; Penzhorn, 2011).

In Europe, a wide range of disease severity is seen between infections caused by the same *B. canis* species (Matijatko et al., 2012). For example, the mortality rate of *B. canis* throughout Europe varies from 1.5 to 24% (Martinod et al., 1986; Máthé et al., 2006). Differences in the parasite, the host, and the host-parasite interplay are

suggested to explain these differences (Matijatko et al., 2012). Genetic variation between different isolates of *B. canis* has been identified (Zahler et al., 1998), supporting the hypothesis of differences in virulence between different strains of *B. canis* (Matijatko et al., 2012).

Due to a lack of *in vitro* cultivation techniques of *Babesia*, experimental investigations of potential virulence factors was limited. Recently however, whole genome sequencing and characterization of a virulent field isolate of *B. canis* was performed and a dataset of potential virulence factors, with a focus on proteins secreted by *B. canis* during infection, was provided (Eichenberger et al., 2017).

1.5 Clinical presentation

The classical presentation of canine babesiosis is characterized by a febrile (sudden and often high fever, anorexia, depression) and hemolytic syndrome (clinical signs of anemia, icterus, splenomegaly, hemoglobinuria, bilirubinuria) (Taboada and Merchant, 1991; Bourdoiseau, 2006). Historically, canine babesiosis caused by *B. rossi* was classified in 2 main categories of uncomplicated and complicated disease.

1.5.1 Uncomplicated babesiosis

Uncomplicated babesiosis was diagnosed when all clinical signs could be explained by acute hemolysis. This category was further classified into mild, moderate, and severe uncomplicated disease based on the severity of anemia. These dogs typically present with signs of fever, anorexia, lethargy, pale mucous membranes, and splenomegaly. Progression to severe, life-threatening anemia is possible (Jacobson and Clark, 1994).

1.5.2 Complicated babesiosis

Dogs were considered to have complicated babesiosis when presented with clinical manifestations that could not be directly related to hemolysis alone (Jacobson and Clark, 1994). However, it was suggested to merge both categories into one group of severe disease, as in malaria, because of a significant clinical overlap between severe uncomplicated and complicated babesiosis (Jacobson, 2006; WHO, 2014).

Such dogs present with a variety of different organ complications (Jacobson and Clark, 1994; Welzl et al., 2001; Dvir et al., 2004; Jacobson, 2006). One or more organs can be affected simultaneously (Welzl et al., 2001). Complications in dogs with severe babesiosis include AKI, hepatopathy/icterus, IMHA, hemoconcentration (‘red biliary’), coagulopathy/disseminated intravascular coagulation (DIC), acid-base and electrolyte disturbances, hypotension/shock, arrhythmias (mainly sinoatrial blocks or sinus arrest, and ventricular premature complexes), acute respiratory distress syndrome (ARDS), cerebral babesiosis, hypoglycemia, and acute pancreatitis (Figure 1.2).

Figure 1.2. Overview of the reported complications in canine babesiosis, based on Jacobson and Clark (1994) and Jacobson (2006).
AKI, acute kidney injury; IMHA, immune-mediated hemolytic anemia; ARDS, acute respiratory distress syndrome; DIC, disseminated intravascular coagulation.

The most common organ involved in a group of 84 dogs with *B. rossi*-induced complicated babesiosis was the liver, while renal involvement was the second most common (Welzl et al., 2001). Kidneys were the most common organs affected in a group of 33 dogs with SIRS and multiple organ dysfunction syndrome (MODS) due to *B. canis* infection (Matijatko et al., 2010).

In studies where only dogs with complicated babesiosis are included, mortality rates are considerably higher. The mortality rate was 45% in *B. rossi*-infected dogs with complicated babesiosis (Welzl et al., 2001). In the latter study, outcome was not significantly affected by whether only one or multiple organs were involved. However, outcome was significantly affected by specific organ involvements. For example, dogs with renal involvement had a 5 times higher risk of death compared to all other complications (Welzl et al., 2001). Mortality rate was 67% in *B. canis*-infected dogs diagnosed with SIRS and MODS (Matijatko et al., 2010). In the latter study, dogs with 2 organs involved had a mortality rate of 55%, while mortality increased to 95% in dogs with 3 organs involved.

1.6 Diagnosis

Diagnosis of acute babesiosis is usually based on the classical clinical presentation combined with detection of the intraerythrocytic parasites on stained blood smears. Large *Babesia* species are usually seen as paired 2.4 x 5 µm-sized pyriform bodies, although some erythrocytes can contain more parasites (Figure 1.3) (Schoeman, 2009). Microscopic detection in blood smears is reasonably sensitive during acute infections, with a moderate to high parasitemia (Solano-Gallego and Baneth, 2011). Diagnosis based on blood smear evaluation can be more difficult in chronic or subclinical infections where parasitemia is often less severe (Boozer and Macintire, 2003; Schoeman, 2009). Blood smears containing capillary blood can increase parasite detection (Boozer and Macintire, 2003). Capillary parasitemia was confirmed to be higher than venous parasitemia in a study with *B. rossi*-infected dogs (Böhm et al., 2006).

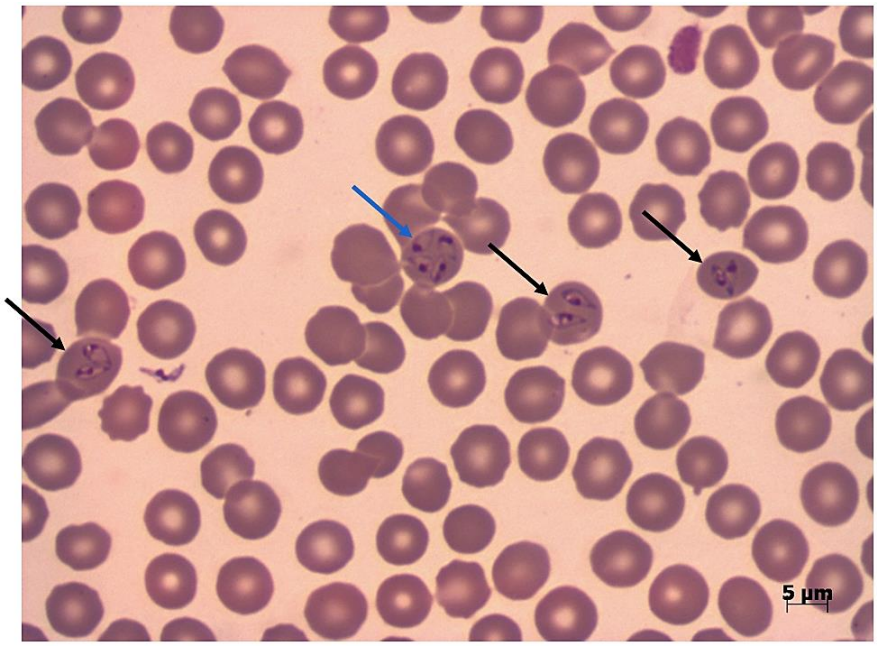

Figure 1.3. Blood smear of a dog, demonstrating multiple intraerythrocytic *B. rossi* parasites. Three red blood cells contain paired pyriform bodies (black arrows), while 1 red blood cell contains 4 bodies (blue arrow).

The most common serological test used to diagnose babesiosis is the indirect fluorescent antibody test (IFAT). However, during early infection, such serologic testing can be negative. In those cases, convalescent antibody titers are recommended after 4 weeks to confirm seroconversion and thus an acute infection (Taboada and Merchant, 1991; Solano-Gallego et al., 2016). In endemic areas, diagnosis should not be based on serology alone, because clinically normal dogs can be seropositive (Schoeman, 2009).

Molecular techniques provide additional information compared to serology. Detection by PCR is more sensitive than detection by blood smear evaluation and a positive PCR can be considered evidence of an active infection (Solano-Gallego et al., 2016). A more reliable identification of the *Babesia* species involved is another advantage of molecular techniques (Solano-Gallego and Baneth, 2011).

1.7 Treatment

Specific treatment to eliminate the parasite with antibabesial drugs, administration of blood transfusions in case of severe anemia, and supportive treatment especially in dogs with complicated babesiosis, are the main components of therapy (Jacobson and Swan, 1995). Diminazene aceturate or imidocarb dipropionate are the most commonly used and effective drugs to eliminate large *Babesia* species (Schoeman, 2009; Ayoob et al., 2010). In South Africa, diminazene aceturate is the most commonly used antibabesial drug to treat *B. rossi*-infected dogs (Collett, 2000). The combination of atovaquone and azithromycine is the first described therapy for *B. gibsoni* infections that can either eliminate the infection or greatly reduce parasitemia (Birkenheuer et al., 2004). Although this combination therapy was confirmed to be efficient during acute infections with *B. gibsoni*, relapse was common, and selection of drug-resistant variants after treatment was suspected (Sakuma et al., 2009). The combination of clindamycin, metronidazole, and doxyxycline has been suggested as an alternative option to treat dogs with *B. gibsoni* infections if the first-line treatment with atovaquone and azithromycine fails (Suzuki et al., 2007; Sakuma et al., 2009). In a recent study comparing imidocarb dipropionate, atovaquone/azithromycine, and buparvaquone/azithromycine, imidocarb showed the lowest efficacy against *B. microti*-like infection, while the combination of either atovaquone/azithromycine or buparvaquone/azithromycine showed the highest efficacy, but was still unable to fully eliminate the infection as shown by PCR (Checa et al., 2017).

2. Acute kidney injury

2.1 Renal physiology

Blood supply to the kidney is provided by the renal artery, which branches multiple times and eventually forms the afferent arterioles, that lead to the glomerular capillaries. The endings of the glomerular capillaries fuse together to form the efferent arterioles. The functional unit of the kidney, the nephron, is formed by the glomerulus and its associated renal tubular segments (Figures 1.4 and 1.5). The glomerulus is a network of capillaries where blood filtration occurs, leading to the formation of the glomerular filtrate. Fluid filtered from the glomerular capillaries enters the Bowman's space, flows into the proximal tubules, and consequently passes the loop of Henle, the distal tubules, the connecting tubules, and the collecting ducts. Finally, the larger ducts empty into the renal pelvis. This passage of the glomerular filtrate through the tubules converts the filtered fluid into urine (Verlander, 2007; Hall, 2011).

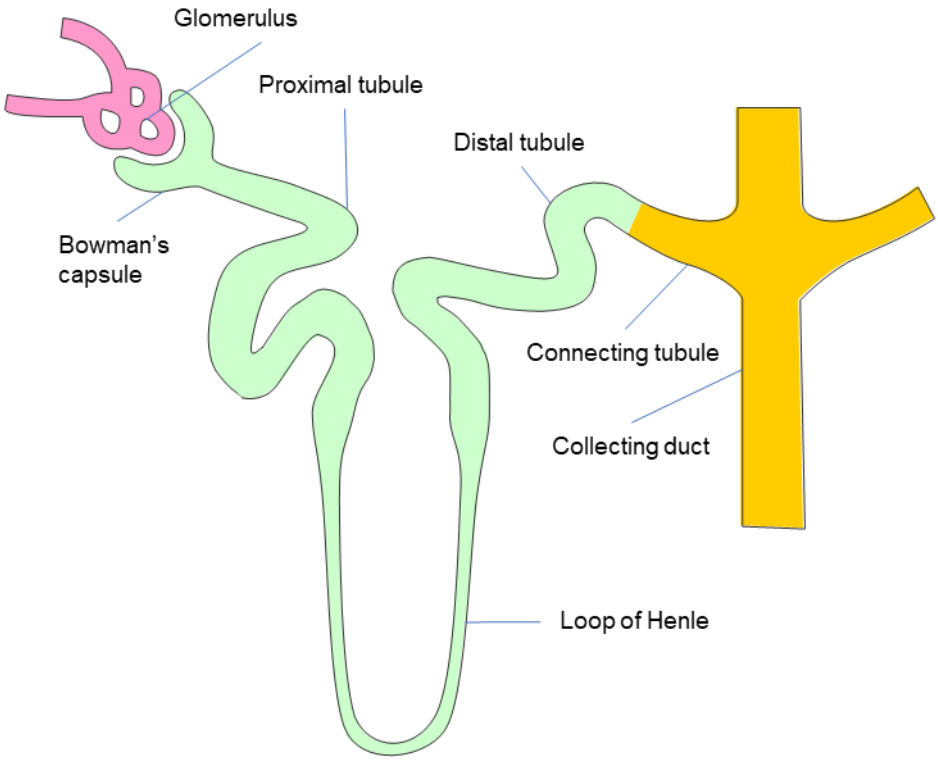

Figure 1.4. Anatomy of the nephron, based on Hall (2011), created with Motifolio.

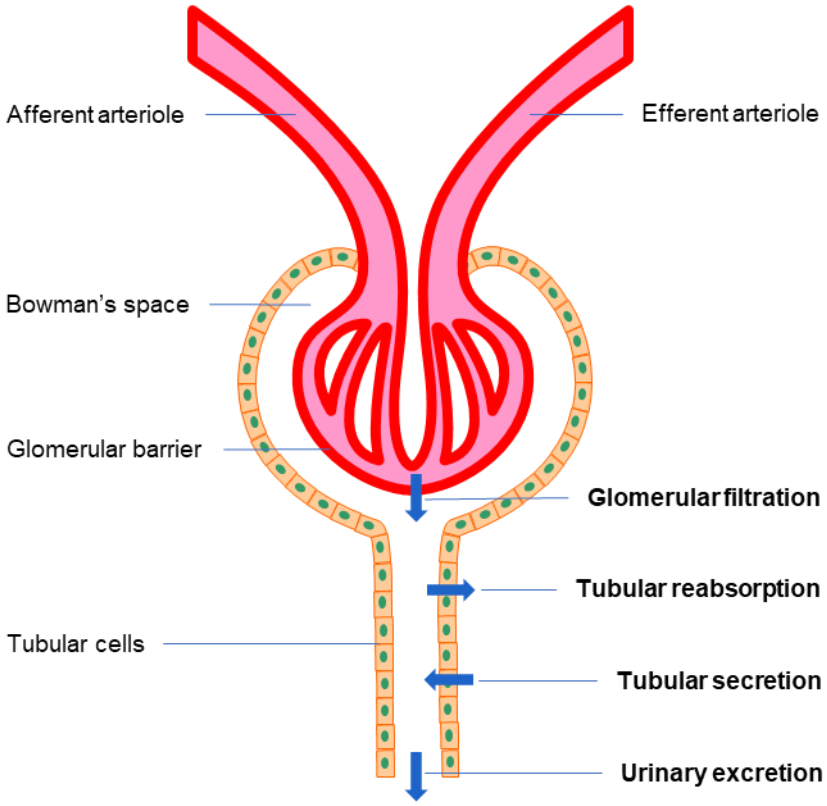

Figure 1.5. Anatomy and basic functional mechanisms at the level of the glomerulus and associated tubule, based on Hall (2011), created with Motifolio.

Formation of urine, leading to urinary excretion, results from 3 basic functional mechanisms at the level of the glomerulus and tubules: glomerular filtration, tubular reabsorption, and tubular secretion (Figure 1.5). A substance-specific combined relative rate of these 3 mechanisms occurs for each substance in plasma, resulting in a substance-specific urinary excretion rate. First, a large amount of fluid and molecules is filtered through the glomerular capillary wall, which consists of 3 major layers: a layer of fenestrated capillary endothelial cells, the glomerular basement membrane, and a layer of epithelial cells (podocytes). These layers form the glomerular barrier that functions as a selective filtration barrier, based on the size and electrical charge of the molecules. The glomerular barrier prevents the filtration of all cellular blood components and of plasma proteins that are at least as large as albumin (i.e., molecular weight (MW) of 69 kDa or higher), while water and solutes are freely filtered (Verlander, 2007; Hall, 2011). Plasma proteins with a MW <40 kDa (i.e., low (L)MW proteins) are also freely filtered through the glomerular barrier (D'Amico and Bazzi, 2003). The rate by which the glomerular filtration fluid is formed (i.e., the glomerular

filtration rate (GFR)), is a clinically important renal functional parameter. The GFR is determined by the mean net filtration pressure, the permeability of the glomerular barrier, and the surface area available for filtration (Verlander, 2007).

More recently, 2 additional layers of the glomerular barrier have been discovered (Salmon et al., 2009; Arkill et al., 2014). On the vascular side of the glomerular barrier, a gel-like layer of proteoglycans, glycosaminoglycans, and plasma proteins, covers the endothelial layer. This glycocalyx layer contributes to the regulation of the glomerular barrier permeability (Weinbaum et al., 2007; Satchell, 2013). On the urinary side, a subpodocyte space is present under the podocyte cell bodies.

The majority of filtered water and most other molecules are selectively reabsorbed by the renal tubules into the blood, while some substances are being secreted from the peritubular capillaries into the renal tubules (Figure 1.5). For most substances, glomerular filtration and tubular reabsorption rates are much higher than the excretion rates. For example, electrolytes such as sodium and chloride are freely filtered and highly reabsorbed, resulting in a low urinary excretion, while waste products, such as urea and creatinine, have a relatively high urinary excretion. Depending on the physiological needs, each of these mechanisms can be adjusted accordingly. For most substances that are actively reabsorbed in the renal tubules, there is a limit to the reabsorption rate. Consequently, when the filtered load exceeds the capacity of the reabsorption transport mechanism, urinary excretion will increase (Hall, 2011).

Besides the regulation of the water and electrolyte balances by renal excretion, the kidneys contribute to multiple other homeostatic regulations, such as the excretion of metabolic waste products, the regulation of arterial pressure, the acid-base balance, and the production of hormones (Verlander, 2007; Hall, 2011).

2.2 Concept and definition of acute kidney injury

Acute kidney injury encompasses a spectrum of diseases associated with kidney injury of sudden onset. It represents a continuum of kidney injury ranging from mild, clinically inapparent injury to severe acute renal failure (ARF). During ARF, a generalized failure of the kidneys to meet the excretory, metabolic, and endocrine demands occurs. Acute renal failure is characterized by rapid hemodynamic, filtration, tubulointerstitial, or outflow injury to the kidneys leading to an accumulation of uremic toxins and dysregulation of fluid, electrolyte, and acid-base balance. However, ARF only represents a subset of patients with the most severe form of AKI. Pre- and postrenal conditions are also included in the spectrum of diseases associated with AKI. These can be either independent or combined with intrinsic kidney injury depending on their origin, extent, and duration (Cowgill and Langston, 2011).

The concept of AKI was introduced in human medicine when it was recognized that renal dysfunction was not only considered significant when it reached the stage of failure (Kellum et al., 2007). In 2004, a first consensus definition of AKI was reached. Before 2004, a multitude of ARF definitions was used in human literature. Most of these definitions were based on serum creatinine (sCr), with or without urine output (UO). The consensus classification scheme of 2004 (RIFLE; Risk of renal dysfunction, Injury to the kidney, Failure of kidney function, Loss of kidney function, End-stage kidney disease) included sCr, GFR, and UO criteria (Bellomo et al., 2004). In 2007, an updated definition and classification of AKI was suggested by the AKI Network (AKIN) (Mehta et al., 2007). Compared to the RIFLE criteria, several modifications were proposed by the AKIN, including the removal of GFR criteria and outcome stages (Loss of kidney function and End-stage kidney disease). Also, a rise in sCr was assessed within 48 h, and AKI patients receiving renal replacement therapy (RRT) were immediately allocated to the highest stage (i.e., stage 3) (Mehta et al., 2007). In 2012, the most recent AKI classification guidelines were introduced by the Kidney Disease: Improving Global Outcomes (KDIGO) foundation, combining RIFLE- and AKIN-criteria in order to improve the sensitivity of the AKI diagnosis (Khwaja, 2012; Thomas et al., 2015). Although the current consensus definitions do not differentiate AKI based on its etiology, AKI was often classified into prerenal, intrinsic renal, and postrenal AKI

(Lameire et al., 2012; Makris and Spanou, 2016). According to this classification, a decrease in GFR is caused by renal hypoperfusion in prerenal AKI. Urinary excretory obstruction is the cause of postrenal AKI, and intrinsic AKI is the category where a decreased GFR is caused by intrarenal disease, such as acute tubular necrosis (ATN). However, this differentiation of prerenal versus intrinsic AKI has been discussed and questioned as being artificial, because a continuum and coexistence of renal hypoperfusion and intrinsic injury is present in most AKI patients (Belcher and Parikh, 2011; Lameire et al., 2012; Makris and Spanou, 2016).

Although there is no consensus definition, the term transient AKI is also sometimes used and is characterized by a limited duration of decrease in GFR. A reversible form of AKI lasting for less than 3 days is the most commonly used definition of transient AKI (Makris and Spanou, 2016). In this context, intrinsic AKI can be defined as a longer lasting form of AKI (Vanmassenhove et al., 2015).

2.3 Defining acute kidney injury in dogs

Until recently, exact definitions of AKI were not established in veterinary medicine. Similar to the human classification of AKI based on its etiology, azotemia (i.e., serum creatinine above reference interval) was traditionally classified in veterinary medicine as being prerenal, renal, and/or postrenal in origin (Stockham and Scott, 2008). Prerenal azotemia is defined as a decrease in GFR due to any process causing a reduced renal blood flow, while renal azotemia is defined as a decreased GFR caused by any renal disease. Postrenal azotemia is caused by a defective excretion of creatinine distal to the nephron. In dogs with prerenal azotemia, USG is expected to be >1.030, while dogs with renal azotemia have an expected USG beween 1.007 and 1.013. Azotemic dogs with a USG between 1.014 and 1.030 either have renal azotemia and/or prerenal azotemia combined with an extrarenal disease resulting in an impaired urinary concentrating ability. Prerenal and renal causes of azotemia can often be simultaneously present (Stockham and Scott, 2008). Acute renal failure was traditionally defined as an abrupt decrease in renal function resulting in renal azotemia (Vaden et al., 1997). Urine sediment findings such as the presence of marked

cylindruria and renal tubular epithelial cells are indicative of acute tubular injury (Vaden et al., 1997; Lobetti and Jacobson, 2001).

The International Renal Interest Society (IRIS) proposed a grading system to categorize the severity of AKI in dogs and cats. It defines AKI grades based on sCr (Table 1.2), and further subgrading based on UO (non-oliguria or oliguria/anuria) and the requirement for RRT. This AKI grading system was developed by Cowgill, adopted by the IRIS in 2013, and revised in 2016 (Braun, 2016). It was introduced to provide an instrument for earlier recognition of AKI, standardizing its classification, therapeutic stratification, and outcome assessment in dogs and cats (Braun, 2016). By analogy with the transient versus intrinsic AKI definitions in human medicine, volume-responsive AKI in dogs is defined as a decrease in sCr to baseline over 48 h of adequate fluid therapy or an increase in UO to >1 ml/kg/h over 6 h, while intrinsic AKI is defined as persistent azotemia for longer than 48 h (Braun, 2016; Troìa et al., 2018).

Although the fractional excretion of electrolytes can help to distinguish prerenal from renal azotemia, its use has been inconsistent and limited in veterinary medicine (Lobetti and Jacobson, 2001; Waldrop, 2008; Cowgill and Langston, 2011). Recently, measurement of the fractional excretion of several electrolytes, including sodium and chloride, has shown promising results to differentiate volume-responsive from intrinstic AKI in dogs (Troìa et al., 2018).

Table 1.2. International Renal Interest Society (IRIS) acute kidney injury (AKI) grading criteria in dogs and cats, based on Braun (2016).

AKI Grade	Serum creatinine	Clinical description
Grade I	<1.6 mg/dL <140 µmol/L	Non-azotemic AKI: a. Documented AKI: historical, clinical, laboratory, imaging evidence of AKI, oliguria/anuria, volume responsiveness and/or b. Progressive non-azotemic increase in serum creatinine (≥0.3 mg/dL or ≥26.4 µmol/L within 48 h) c. Measured oliguria (<1 ml/kg/h) or anuria over 6 h
Grade II	1.7 – 2.5 mg/dL 141 – 220 µmol/L	Mild azotemic AKI: a. Documented AKI and static or progressive azotemia b. Progressive azotemic increase in serum creatinine (≥0.3 mg/dL or ≥26.4 µmol/L within 48 h) or volume responsiveness c. Measured oliguria (<1 ml/kg/h) or anuria over 6 h
Grade III **Grade IV** **Grade V**	2.6 – 5.0 mg/dL 221 – 439 µmol/L 5.1 – 10.0 mg/dL 440 – 880 µmol/L >10.0 mg/dL >880 µmol/L	Moderate to severe azotemic AKI: Documented AKI and increasing severities of azotemia and functional renal failure

Volume responsiveness is defined as an increase in UO to >1 ml/kg/h over 6 h, and/or a decrease in serum creatinine to baseline over 48 h. Laboratory evidence of AKI includes biomarkers such as sSDMA, glucosuria, cylindruria, inflammatory sediment, and microalbuminuria. AKI, acute kidney injury; h, hour; sSDMA, serum symmetric dimethylarginine; UO, urine output.

Although the concept of AKI has been introduced to replace all previously used terms, the old terminology is still being used throughout this doctoral thesis for two reasons. Firstly, most veterinary publications that are referred to in this thesis used the old nomenclature. Consequently, replacing the old nomenclature by the term “AKI” would result in incomplete or sometimes even incorrect references to literature. Secondly, incomplete data and/or unavailable follow-up in some of the research performed in this doctoral thesis, made it practically impossible to accurately apply the current IRIS AKI grading criteria.

2.4 Trending of serum creatinine

Serum creatinine has a low intraindividual variability, resulting in minimal variation of sCr over time within the same individual dog (Ruaux et al., 2012; Hokamp and Nabity, 2016; Kopke et al., 2018). As a consequence of this characteristic, trending of sCr over time, even when increasing concentrations were still within the reference range, was found to be more sensitive in detecting decreases in GFR compared to the use of reference ranges (Nabity et al., 2015). By trending sCr using the critical difference or reference change value, early diagnosis of a decreased GFR might be improved in non-azotemic dogs (Pagitz et al., 2007). Therefore, a progressive non-azotemic increase of sCr has been incorporated in the AKI grading system for dogs and cats (AKI grade I) (Braun, 2016).

Multiple studies in human medicine demonstrated small increases in sCr to be independently associated with an increased mortality (Thomas et al., 2015). In one of these studies, adults with an increase in sCr of at least 0.3 mg/dL had a fourfold increase in the odds of hospital death (Chertow et al., 2005). Therefore, a rise in sCr of at least 0.3 mg/dL (26.4 µmol/L) was included in the AKIN and KDIGO classification schemes of AKI (Mehta et al., 2007; Khwaja, 2012). The calculated critical difference of sCr in healthy dogs, using both a colorimetric Jaffe method and an enzymatic method, was 0.26 mg/dL (23.4 µmol/L) and 0.27 mg/dL (23.4 µmol/L), respectively (Pagitz et al., 2007). In the IRIS AKI grading system, an increase in sCr of at least 0.3 mg/dL (26.4 µmol/L) within 48 h is a proposed criterion used to diagnose AKI grades I and II (Braun, 2016).

3. *Babesia*-induced kidney injury in dogs

3.1 Occurrence of kidney injury

Kidney injury is a recognized complication of canine babesiosis, which has been described in most *B.* species affecting dogs (Lobetti and Jacobson, 2001; Camacho et al., 2004; Máthé et al., 2006; Solano-Gallego et al., 2008; Ullal et al., 2018). The reported occurrence of azotemia, AKI, and ARF in dogs with babesiosis is highly variable between studies (Table 1.3), which can be explained by differences in the infecting species, the presence of sampling biases, and the variation in criteria to define AKI and ARF. Most of these studies did not differentiate prerenal from renal azotemia. Few studies did attempt to exclude dogs with prerenal azotemia, but uniformity to define renal azotemia is lacking. When only studies without apparent sampling bias are included, the reported occurrence of azotemia in canine babesiosis ranges from 0 to 36% (Lobetti and Jacobson, 2001; Camacho et al., 2004; Furlanello et al., 2005; Ruiz de Gopegui et al., 2007; Zygner and Wedrychowicz, 2009). The occurrence of AKI seems to depend on the Babesia species involved (Máthé et al., 2007).

The most often cited reference of *B. rossi*-induced AKI, states that ARF is an uncommon complication, because it was only diagnosed in 3/134 dogs (2.2%) (Jacobson and Clark, 1994). Based on this reference, many review articles state that ARF and AKI are uncommon complications of *B. rossi* infections (Lobetti, 1998; Boozer and Macintire, 2003; Ayoob et al., 2010; Köster et al., 2015a). However, it is of key importance to mention that this information was based on unpublished observations of one of the authors and that ARF was not defined. In the only *B. rossi* study without apparent sampling bias (Lobetti and Jacobson, 2001), azotemia was diagnosed in 4/30 dogs (13%). In the latter study, other findings consistent with AKI, such as the presence of enzymuria, proteinuria, and renal tubular epithelial cells in urine sediment, were relatively mild. The authors concluded that milder forms of AKI are much more common than overt ARF. An older study in *B. rossi*-infected dogs also found evidence of kidney injury both in dogs with uncomplicated and complicated disease, based on urine sediment findings (i.e., presence of granular and hemoglobin casts, renal tubular epithelial cells) (Moore and Williams, 1979).

Table 1.3. Occurrence of azotemia, AKI, and ARF in canine babesiosis.

Species	Country	Population sample	AKI Definition	AKI Occurrence	Reference
*B. rossi**	SA	Undefined	ARF (undefined)	3/134 (2.2%)	(Jacobson and Clark, 1994)
*B. rossi**	SA	Hospitalized dogs	Azotemia	127/662 (19%)	(Reyers et al., 1998)
*B. rossi**	SA	Complicated cases	Azotemia without clinical dehydration	21/56 (38%)	(Welzl et al., 2001)
*B. rossi**	SA	Representative	Azotemia	4/30 (13%)	(Lobetti and Jacobson, 2001)
*B. canis**	Hungary	Representative	Azotemia without increased PCV	19/61 (31%)	(Máthé et al., 2006)
*B. canis**	Croatia	Dogs with septic shock	Azotemia	9/10 (90%)	(Matijatko et al., 2009)
*B. canis**	Croatia	Dogs with MODS	Azotemia	30/33 (91%)	(Matijatko et al., 2010)
B. canis	Croatia	Representative	Azotemia	5/35 (14%)	(Crnogaj et al., 2010)
B. canis	Croatia	Representative	Azotemia	5/40 (13%)	(Crnogaj et al., 2017)
B. canis	Poland	Representative	Azotemia	68/230 (30%)	(Zygner et al., 2011) (Zygner et al., 2012)
Large *B.* sp.*	Italy	Representative	Azotemia	4/23 (17%)	(Furlanello et al., 2005)
B. canis	Italy	Representative	Azotemia	3/27 (11%)	(Solano-Gallego et al., 2008)
B. vogeli			Azotemia	2/5	(Solano-Gallego et al., 2008)
Large *B.* sp.*	Spain	Representative	Azotemia	0/45 (0%)	(Ruiz de Gopegui et al., 2007)
Large *B.* sp.*	Spain	Representative	Azotemia and/or oliguria not resolved by rehydration	8/72 (11%)	(Fraga et al., 2011)
B. microti-like**	Spain	Samples submitted to diagnostic laboratory	Azotemia	21/58 (36%)	(Camacho et al., 2004)
B. microti-like	Spain	Representative	Azotemia	7/71 (10%)	(Miró et al., 2015)

AKI, acute kidney injury; ARF, acute renal failure; SA, South Africa; PCV, packed cell volume; MODS, multiple organ dysfunction syndrome.

Azotemia was defined as serum creatinine above reference interval.

A population sample was considered representative when no sampling bias was identified.

*No PCR was performed to confirm the identity of the species involved. If possible, the most likely species based on available geographic PCR studies is provided. **PCR was performed in some of the submitted samples.

In a population of 16 dogs with uncomplicated disease caused by *B. vogeli*, which is considered the least pathogenic large *Babesia* species, mean sCr concentration (1.78 mg/dL) was higher than the reference range (0.5–1.4 mg/dL) (Pekmezci et al., 2015). Mean serum cystatin C, another functional renal biomarker, was significantly higher in the *B. vogeli*-infected dogs compared to healthy control dogs.

Comparing experimental infections of *B. rossi* with *B. canis* revealed differences in pathogenesis and clinical disease presentation (Schetters et al., 1997). However, disease severity is not only dependent on the infecting *Babesia* species (Matijatko et al., 2012). Throughout Europe, differences in disease severity within the same *Babesia* species (*B. canis*) have been observed, supporting the hypothesis of differences in virulence between strains of *B. canis* (Matijatko et al., 2012). These differences are likely to have an impact on the severity of *Babesia*-induced AKI, and could therefore explain the geographical variation in the occurrence of *B. canis*-induced azotemia.

In studies focusing on dogs with complicated forms of babesiosis, the occurrence of azotemia is often much higher. In a study of 56 dogs with complicated *B. rossi* infection, 21/56 dogs (38%) were azotemic without having clinical signs of dehydration (Welzl et al., 2001). Azotemia was detected in 9 out of 10 *B. canis*-infected dogs (90%) presenting with septic shock, which was defined as the presence of SIRS, MODS, and refractory hypotension (Matijatko et al., 2009). Of the 33 *B. canis*-infected dogs that were diagnosed with MODS, 30 dogs (91%) were azotemic (Matijatko et al., 2010).

Acute kidney injury is also a common complication in adults with severe falciparum malaria, where it occurs in about 40% of the patients (White et al., 2014). Malarial AKI in adults contributes to a high mortality rate that can reach 75% in the absence of RRT (Trang et al., 1992). Malaria is an important contributor to the burden of AKI in rural areas of low-to-middle-income countries (Hoste et al., 2018). On the other hand, in a study from the Netherlands, AKI was also diagnosed according to the KDIGO criteria in 38% of the patient that presented with imported and severe falciparum malaria (Koopmans et al., 2015).

3.2 Pathogenesis of *Babesia*-induced kidney injury

Although the clinical manifestations of complicated babesiosis are highly variable, there is increasing evidence of a uniform underlying pathophysiological mechanism. An excessive pro-inflammatory host response and subsequent development of MODS (including AKI), rather than direct effects of the parasite,

provide a unifying mechanism that links apparently unrelated complications in canine babesiosis (Jacobson and Clark, 1994; Welzl et al., 2001; Lobetti, 2005; Matijatko et al., 2009; Köster et al., 2015b; Goddard et al., 2016). Supportive of this mechanism, several studies showed that dogs with babesiosis caused by *B. rossi* and *B. canis* often present with SIRS and MODS (Welzl et al., 2001; Matijatko et al., 2009; Matijatko et al., 2010; Köster et al., 2015b). Shared pro-inflammatory mechanisms in the pathogenesis of severe human falciparum malaria and canine babesiosis have also been suggested (Jacobson and Clark, 1994; Clark and Jacobson, 1998; Reyers et al., 1998; Krause et al., 2007). An imbalance between pro- and anti-inflammatory responses in these septic conditions could be responsible for progression to MODS (Loisa et al., 2003; Jacobson, 2006; Goddard et al., 2016). Concentrations of the pro-inflammatory cytokine interleukin-6 (IL-6) and the pro-inflammatory chemokine monocyte chemoattractant protein-1 (MCP-1) were significantly higher in *B. rossi*-infected dogs that did not survive (Goddard et al., 2016). These findings are supportive of the hypothesis that an excessive pro-inflammatory host response is associated with disease severity. Another study demonstrated a strong and significant positive correlation between IL-6 and sCr in dogs with babesiosis caused by *B. canis* (Zygner et al., 2015). Whether this association between a pro-inflammatory cytokine and a biomarker of renal dysfunction is causally related or merely represents a link due to disease severity, was not determined.

Evidence of endothelial activation associated with a pro-inflammatory response was also documented in *B. canis*-infected dogs (Barić Rafaj et al., 2013). In this study, increased serum concentrations of soluble intercellular adhesion molecule-1 (ICAM-1) were seen in dogs with babesiosis compared to healthy dogs. Overexpression of ICAM-1 could also lead to increased adherence of parasitized red blood cells to the endothelium, resulting in the blockade of the microcirculation and locally increased pro-inflammatory cytokine concentrations (Schetters and Eling, 1999; Barić Rafaj et al., 2013). In a follow-up study from the same group, several other biomarkers of endothelial activation were also increased in *B. canis*-infected dogs compared to healthy control dogs (Kuleš et al., 2017). Differences in these biomarker concentrations were also detected between dogs with uncomplicated and complicated babesiosis. Endothelial dysfunction and changes in the microvasculature are considered to be important mechanisms in the development of sepsis-induced AKI (Gómez and Kellum,

2016). Therefore, vascular disturbances could represent an important pathomechanism in the development of *Babesia*-induced AKI as well.

Systemic hypotension was a common complication in dogs with complicated babesiosis caused by *B. rossi* (Jacobson et al., 2000). In a study that focused on dogs diagnosed with septic shock, defined by the presence of SIRS, MODS, and refractory hypotension, the most frequently affected organ was the kidney (Matijatko et al., 2009). The presence of refractory hypotension, in combination with pro-inflammatory mediators, was considered to be the main cause of AKI in the latter study.More recent indications of hypotension-induced AKI were provided by research on *B. canis*-infected dogs in Poland. Lower systolic, diastolic and mean arterial pressures were present in azotemic *Babesia*-infected dogs compared to their non-azotemic counterparts (Zygner and Gójska-Zygner, 2014). Increased serum levels of tumor necrosis factor alpha (TNF-α), a pro-inflammatory cytokine involved in the production of nitric oxide that can induce vasodilation and hypotension, were present in *Babesia*-infected dogs (Zygner et al., 2014). A strongly negative correlation between TNF-α and blood pressure and a strongly positive correlation between TNF-α and sCr were observed in the latter study. Lastly, significantly higher serum aldosterone levels and lower blood pressures were found in *B. canis*-infected dogs compared to healthy control dogs (Gójska-Zygner and Zygner, 2015). A strongly positive correlation was present between serum aldosterone and sCr. An inadequate mechanism of secondary hyperaldosteronism in response to hypotension was suggested to explain these findings.

Next to inappropriate pro-inflammatory host responses to the infection and hypotension-induced AKI, several other potential mechanisms for AKI have been suggested in canine babesiosis. Hemolysis resulting in hemoglobinuric nephropathy remains a controversial concept in canine babesiosis (Lobetti and Reyers, 1996). Historically, a hemoglobinuria-induced nephropathy was considered the most likely cause of AKI in babesiosis (Maegraith et al., 1957; Malherbe, 1966). An experimental study evaluated the effect of infused hemoglobin, induced anemia, and the combination of both, on kidney injury and dysfunction in 6 healthy young adult dogs (Lobetti et al., 1996). Based on enzymuria, urine sediment, and GFR, evidence of kidney injury was mild but present in all groups, and relatively more severe in the anemic group. In all groups, mild to moderate histological changes (i.e., degeneration and single-cell necrosis of the renal tubular epithelial cells) were found. Synergistic

effects of combined hemoglobinemia and anemia were not seen. Large individual differences of the kidneys to handle the infused hemoglobin were also observed. Although the authors concluded that hemoglobinuria by itself was not toxic to the kidney based on the absence of severe changes, they speculated that synergistic effects of hemoglobin with other mediators of AKI, such as inflammatory mediators or toxins, could be present (Lobetti et al., 1996).

Another hypothesis is that nephrotoxicity could occur after conversion of hemoglobin to methemoglobin. Significant methemoglobinuria was found in dogs with *B. rossi* infection, while methemoglobinemia was minimal, suggesting that oxidation of hemoglobin occurred either in the kidneys or urinary bladder (Lobetti and Reyers, 1996).

Rhabdomyolysis, a poorly documented, but probably underdiagnosed complication of canine babesiosis, has also been suggested as a cause of AKI due to tubular toxicity of myoglobin or its breakdown products (Jacobson and Lobetti, 1996).

Based on the experimental study of Lobetti et al. (1996) and histological findings from Máthé et al. (2007), tissue hypoxia is now considered to be more important than hemoglobinuria in the development of AKI in canine babesiosis. Many factors potentially contribute to the development of renal tissue hypoxia in babesiosis, such as the presence of systemic hypotension and renal vasoconstriction, anemia, altered hemoglobin function, autoagglutination, sequestration of parasitized red blood cells in capillaries, presence of microthrombi due to DIC, pulmonary edema, and myocardial dysfunction (Jacobson and Clark, 1994; Jacobson, 2006; Máthé et al., 2007; Ayoob et al., 2010).

3.3 Renal histological findings

Few studies examined renal histopathological changes induced by canine babesiosis. Macroscopic renal pathological findings of 2 dogs with *B. rossi*-induced complicated babesiosis included bilateral renal cortical petechiae in both dogs and an infarction of the posterior left renal cortex in one dog (Moore and Williams, 1979).

Degeneration, and in severe cases even necrosis, of the renal tubular epithelium, and presence of hemoglobin casts and droplets in the renal tubular epithelium were the main features of early histological examinations in the South African form of canine babesiosis, suggesting a hemoglobinuric nephropathy (Maegraith et al., 1957; Malherbe, 1966; Hildebrandt, 1981).

Light microscopic findings from dogs infected with a large *Babesia* species in Australia included degeneration of the proximal tubular epithelial cells, proteinaceous material in the tubular lumen, and parasitized red blood cells in capillaries of the renal cortex and medulla (Irwin and Hutchinson, 1991). No renal histological abnormalities were seen in one dog in Italy infected with a large *Babesia* sp. (Bonfanti et al., 2004), while another dog in Croatia that presented with complicated babesiosis showed ATN during necropsy (Torti et al., 2009).

The largest study that used both light microscopy and electron microscopy in 8 dogs to evaluate renal histological changes in canine babesiosis caused by large *Babesia* species was performed in Hungary (Máthé et al., 2007). The species involved was most likely *B. canis*, but no PCR was performed to confirm this. While glomerular lesions were mild, tubular lesions were prominent. These consisted mainly of degenerative changes and necrosis of the proximal renal tubular cells. Tubular hemoglobin casts were only seen in a few dogs. Currently, there are no publications reporting the use of immunofluorescence to examine kidney biopsies in dogs with large *Babesia* spp.

Renal histological findings in *B. gibsoni*-infected dogs show distinctly different features compared to infections caused by large *Babesia* species. A membranoproliferative glomerulonephritis with IgM antibody deposits in renal glomeruli was diagnosed in 3 out of 4 dogs experimentally infected with *B. gibsoni* (Wozniak et al., 1997). A dog that was naturally infected with *B. gibsoni* was diagnosed with an azotemic protein-losing nephropathy (Slade et al., 2011). Light and transmission electron microscopic renal changes in this dog were also consistent with a membranoproliferative glomerulonephritis, with immune deposits mainly located in the mesangium.

4. Limitations of the traditional evaluation of kidney injury in canine babesiosis

Traditional evaluation of kidney injury and dysfunction in dogs is based on blood (sCr and serum urea or blood urea nitrogen (BUN)) and urine tests (urine specific gravity (USG) and urinary protein to creatinine ratio (UPC)) (Hokamp and Nabity, 2016). Serum creatinine is the most frequently used endogenous biomarker in humans and dogs to indirectly estimate GFR (Braun et al., 2003).

However, all these traditional biomarkers have important limitations in the evaluation of kidney injury and dysfunction in dogs (Hokamp and Nabity, 2016). Additionally, the presence of hemolysis in canine babesiosis further complicates an accurate assessment of AKI using these biomarkers (de Scally et al., 2004).

4.1 Serum creatinine

Serum creatinine is an insensitive biomarker of reduced renal function (Braun et al., 2003). It is generally accepted that sCr will only increase above the reference range when at least 75% of the nephrons are nonfunctional (Braun et al., 2003; Hokamp and Nabity, 2016). Renal hypoperfusion and intrinsic renal causes will often coexist in many patients with AKI (Belcher and Parikh, 2011), leading to a decrease in GFR and eventually to an increased sCr. Using a research population-specific cutoff, increased sCr concentrations were seen after an average reduction in GFR of 48% (range, 39–68%), which demonstrates that population-specific reference ranges can improve the sensitivity of sCr to detect reduced renal function in dogs (Nabity et al., 2015).

Serum creatinine is characterized by a high individuality (i.e., low intra- but high inter-individual variability) (Ruaux et al., 2012; Kopke et al., 2018). Therefore, wide population-based reference ranges are necessary to account for this biological variability in sCr amongst individual dogs, which is one of the main reasons of its poor sensitivity to detect decreases in GFR (Hokamp and Nabity, 2016). Inherent to its origin, the sCr concentration is dependent on muscle mass and body weight. Large

breed dogs and dogs with a higher body weight have higher creatinine concentrations (Braun et al., 2003). Renal function could therefore be overestimated in cachectic, geriatric, very young, and in small-breed dogs (Hokamp and Nabity, 2016). Several other factors, such as recent feeding, a meat-based diet, biological (circadian and seasonal) rhythms, physical activity, drugs, and hydration status, can also alter sCr levels (Braun et al., 2003).

Several studies demonstrate a low and therefore acceptable analytical variability of sCr (Ulleberg et al., 2011; Ruaux et al., 2012; Kopke et al., 2018), while other studies show the presence of significant differences in the same sample of the same laboratory and even higher differences between laboratories (Braun et al., 2008; Ulleberg et al., 2011). Analytical variability is also much higher when small bench top chemistry analyzers are used (Braun et al., 2008). This highlights the importance of a strict quality assurance program (Hokamp and Nabity, 2016). Lastly, interferences from non-creatinine chromogens result in an analytical error when Jaffe-based methods are used to measure sCr (Braun et al., 2003; Delanghe and Speeckaert, 2011).

Glomerular filtration rate is not accurately estimated by sCr in patients with AKI, because they are not in a steady state situation (Star, 1998). After a change in GFR occurs, it can take several days for sCr to reach a new steady state (Mehta and Chertow, 2003; Makris and Spanou, 2016). Serum creatinine concentrations may not substantially increase until 48-72 h after the initial insult to the kidney, delaying diagnosis of AKI (Coca et al., 2008).

Next to these general limitations of sCr, several babesiosis-specific limitations of sCr further complicate its use in the diagnosis of *Babesia*-induced AKI. A summary of the babesiosis-specific factors that could affect sCr concentrations is given in Figure 1.6.

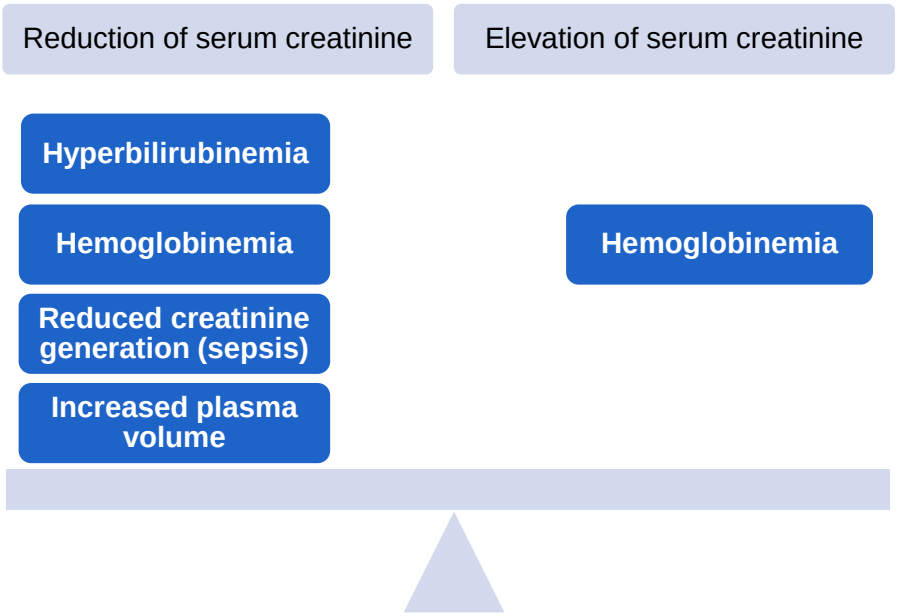

Figure 1.6. Babesiosis-specific, GFR-independent factors that could affect serum creatinine concentrations in canine babesiosis. While the presence of hyperbilirubinemia, reduced creatinine generation, and increased plasma volume can result in a lower sCr, the effect of hemoglobinemia (lower or higher sCr) is dependent on the analytical method used to measure sCr. GFR, glomerular filtration rate; sCr, serum creatinine.

Babesiosis is associated with hemoglobinemia and hyperbilirubinemia due to infection-induced hemolysis. Elevated serum free hemoglobin and serum bilirubin can interfere with the 2 main laboratory techniques used in dogs to measure sCr, the colorimetric Jaffe reaction and enzymatic methods (Braun et al., 2003; de Scally et al., 2004). Interference by these hemolysis-induced products could lead to an underestimation of sCr concentrations (de Scally et al., 2004). However, the interference is affected by the concentration of analytes and interferents, laboratory techniques, and the equipment used (Jacobson, 2006).

Significant interferences of serum bilirubin at concentrations occurring in canine babesiosis (de Scally et al., 2004; Eichenberger et al., 2016), with a consistent underestimation of sCr, measured by both the Jaffe reaction and enzymatic methods, have been observed in several studies in dogs and humans (Figure 1.6) (Jacobs et al., 1991; Weber and van Zanten, 1991; Jacobs et al., 1992).

In an experimental study where hemoglobin was infused in dogs for 4 consecutive days, decreasing sCr concentrations were observed during the trial (Lobetti et al., 1996). This led to the hypothesis that serum free hemoglobin could negatively bias the measurement of sCr (Figure 1.6) (de Scally et al., 2004). The reported concentrations of serum free hemoglobin in dogs with *B. rossi* infection range from 0.7 to 5.1 g/L, with a median of 1.3 g/L (de Scally et al., 2006). At these concentrations, a significant underestimation of sCr due to hemoglobin interference was observed in one interference study in dogs (Leard et al., 1990). However, 2 other interference studies in dogs showed no significant hemoglobin interference of sCr, measured by both the Jaffe reaction and enzymatic methods, again at concentrations occurring in canine babesiosis (Jacobs et al., 1991; Jacobs et al., 1992). Hemoglobin interference was also stated to be negligible at concentrations ≤16 g/L (Braun et al., 2003). In most interference studies in human research, the effect of hemoglobin on sCr measurements is negligible as well (Weber and van Zanten, 1991; Srisawasdi et al., 2010). Still, decreases in creatinine have been reported due to enzymatic hemolysis interferences (Weber and van Zanten, 1991), while increases in creatinine have been reported with hemolysis interference in the Jaffe reaction (Figure 1.6) (Thomas et al., 2015). In conclusion, at concentrations occurring in canine babesiosis, the effect of serum free hemoglobin on sCr measurements seems to be less pronounced, but also less consistent and less predictable, compared to serum bilirubin interference. The effects of hemolysis should therefore be determined on each analyzer and for each analytical method used (O'Neill and Feldman, 1989).

Reduced creatinine production during sepsis and critical illness, which has been documented in experimental research and in human critical care settings, is an additional limitation when relying on sCr to evaluate renal dysfunction (Doi et al., 2009; Wilson et al., 2012). Reduced creatinine production during sepsis could interfere with an early diagnosis of sepsis-induced AKI (Figure 1.6) (Doi et al., 2009). Decreasing plasma creatinine concentrations were observed after experimentally infecting dogs with *B. canis* (Schetters et al., 2009). The authors suggested this finding to be due to an increased plasma volume after infection (Figure 1.6), which could represent a compensatory response to infection-induced hypotension. A reduction in creatinine production could also explain this trend.

In conclusion, characterization of babesiosis-induced AKI based on elevated sCr is hampered in many ways as depicted schematically in Figure 1.6. Based on sCr, diagnosis of milder forms of AKI is difficult, especially in the setting of canine babesiosis.

4.2 Serum urea

While it is generally acknowledged that serum urea is not a specific functional renal biomarker, specificity of serum urea is even more limited in the setting of canine babesiosis (de Scally et al., 2006). A study of de Scally et al. (2006) documented that in canine babesiosis serum urea is often disproportionally increased in comparison to sCr and serum cystatin C, another GFR biomarker. Serum urea was frequently elevated, despite the presence of normal sCr and serum cystatin C levels. The authors hypothesized that serum urea was most likely elevated due to non-renal causes (de Scally et al., 2006). Many potential extra-renal causes of increased serum urea concentrations have been suggested in canine babesiosis. Ammonia loading as a result of hemolysis, blood transfusions, and gastro-intestinal bleeding have been hypothesized as causes of hyperureagenesis (de Scally et al., 2004; de Scally et al., 2006). However, dogs with experimentally induced hemoglobinemia did not have a corresponding increase in serum urea as was seen in dogs with babesiosis (Lobetti, 2012). Therefore, ammonia loading as a result of severe hemoglobinemia was considered an unlikely cause of disproportionate increases of serum urea in canine babesiosis. Based on these results, there was also no evidence of hemolysis-related interference in the laboratory analysis of serum urea. Further studies are needed to clarify the contributing non-renal factors of increased serum urea concentrations in canine babesiosis (Lobetti, 2012). In conclusion, based on these findings serum urea should never be used in the assessment of renal dysfunction in dogs with babesiosis (de Scally et al., 2006).

4.3 Urine specific gravity and urine osmolality

Estimation of urinary concentration by USG is frequently performed in veterinary and human medicine (Watson, 1998; Imran et al., 2010). However, because USG is dependent on the MW of the particles in solution, it can be disproportionately increased in the presence of large molecules, such as hemoglobin (Chadha et al., 2001; Imran et al., 2010). Several studies in humans already documented an overestimation of urinary concentration based on USG in the presence of proteinuria and hemoglobinuria (Voinescu et al., 2002; Imran et al., 2010). The presence of severe proteinuria and hemoglobinuria, which is common is canine babesiosis due to hemolysis (Lobetti et al., 1996; Lobetti and Jacobson, 2001), could therefore lead to an overestimated urinary concentration based on USG.

It has been recommended in human medicine that urinary concentration should not be estimated based on USG if these large molecules are present (Voinescu et al., 2002; Imran et al., 2010). In such situations, measurement of urine osmolality (uOsmol), the gold standard method of estimating urinary concentration, should be performed, since uOsmol is only dependent on the number of particles present, and unlike USG, is not affected by their MW (Chadha et al., 2001). The concentration of urinary electrolytes, such as sodium and chloride, has a significant impact on uOsmol measurements (Voinescu et al., 2002). Depending on the renal tubular function, decreased or increased concentrations of urinary electrolytes could be present in dogs with AKI (Waldrop, 2008). The unpredictability of this variable influence complicates the relationship between USG and uOsmol during AKI (Souza et al., 2015).

4.4 Urinary protein to creatinine ratio

Hemoglobinuria is expected to be present in all dogs with babesiosis, irrespective of disease severity (Lobetti and Jacobson, 2001), complicating the clinical assessment of proteinuria. Based on the pathogenesis of hemolysis in canine babesiosis, UPC measurements cannot differentiate between proteinuria of renal and prerenal origin (Lees et al., 2005). Differentiation between glomerular and tubular renal proteinuria, which is primarily based on the magnitude of UPC, is also not possible in canine babesiosis due to the presence of hemoglobinuria. In conclusion, the assessment to localize the origin of proteinuria as well as to estimate the magnitude of the renal proteinuria fraction in the total UPC value is significantly hampered in canine babesiosis.

There is a lack of knowledge in current canine babesiosis literature regarding the overall impact of these babesiosis-specific limitations in the evaluation of renal function. It can be hypothesized that the overall effect of these limitations could result in an underestimation of renal dysfunction when evaluation is primarily based on sCr and USG. However, the overall impact for an individual dog remains hard to predict.

Considering all these general and babesiosis-specific limitations in the traditional evaluation of AKI, more sensitive and specific methods are needed to evaluate AKI in canine babesiosis.

5. Urinary kidney injury biomarkers

5.1 Introduction

Urinary biomarkers have the potential to diagnose AKI at an early stage. Moreover, they have the capacity to quantify and localize the initial site of injury (Price, 2002). Urine is a very promising and easily accessible sample to identify biomarkers that could diagnose AKI early, because of the proximity to the kidneys (Rosner, 2009). Recognizing AKI at an early stage using sensitive biomarkers allows earlier therapeutic intervention compared to the traditional renal biomarkers (Hokamp and Nabity, 2016). Ultimately, using these biomarkers could improve the outcome of AKI, because therapeutic interventions can be most effective at an early stage (Coca et al., 2008; Hokamp and Nabity, 2016). Using these biomarkers to recognize AKI in an earlier stage could potentially have a higher clinical impact in veterinary medicine compared to human medicine. In humans, high risk patient should always receive optimal care, irrespective of biomarker concentrations. While the same holds true in the setting of veterinary medicine, financial limitations will often have a relatively higher impact on these clinical decisions. Therefore, implementation of these biomarkers in the global clinical decision-making process might be more relevant in veterinary medicine.

In addition to an earlier diagnosis of AKI, candidate biomarkers may serve multiple purposes, including differentiation of AKI subtypes (prerenal versus intrinsic renal AKI) and of AKI from chronic kidney disease (CKD), identification of AKI etiologies, prediction and quantification of AKI severity, monitor the course of AKI and the response to interventions, and aid in prognostication (i.e., recovery, need for RRT, mortality). The ideal clinically applicable biomarker has several important characteristics. Following easy and noninvasive sampling, its measurement should be easily and reliably performed either as a bedside test or in a standard clinical laboratory. Results should be rapidly available. In the setting of AKI, it should provide more information than the traditionally used biomarkers, have a high sensitivity and specificity for AKI (also in the presence of other diseases), and have cut-off values to

distinguish normal from abnormal (Nguyen and Devarajan, 2008; Makris and Spanou, 2016).

The function of specific nephron segments can be assessed by urinary quantification of proteins that are either filtered through the glomerular barrier and/or reabsorbed by tubular cells. All proteins that pass the glomerular barrier are present in negligible concentrations in the urine because of their efficient reabsorption by the proximal tubular cells. Dysfunction of specific segments can result in the presence of proteinuria by altering the glomerular filtration and/or tubular reabsorption (Figure 1.5) (D'Amico and Bazzi, 2003; Hall, 2011).

In normal conditions, the intact glomerular barrier is impermeable to high molecular weight (HMW) proteins due to their size. During glomerular dysfunction/injury, these HMW proteins can reach the tubular lumen because of an increased permeability of the glomerular barrier. When the reabsorptive capacity of the tubular cells is exceeded, they will appear in the urine (D'Amico and Bazzi, 2003).

Low MW proteins, which are freely filtered through the glomerular barrier, are completely reabsorbed by the proximal tubular cells in physiologic conditions. Presence of LMW proteins in urine can therefore indicate tubular dysfunction due to their decreased reabsorption. Excessive amounts of filtered proteins can also be toxic to the tubular cells by several mechanisms and lead to a further reduction of the reabsorptive capacity of these cells (D'Amico and Bazzi, 2003; Grauer, 2005).

Proteins that are either upregulated, secreted and/or leaked into the urine by tubular cells as a response to injury, can also be quantified in urine (Figure 1.5). Presence of specific urinary proteins can therefore suggest tubular dysfunction/injury not only due to a decrease in reabsorption, but also due to direct tubular injury (Emeigh Hart, 2005; De Loor et al., 2013; Hokamp and Nabity, 2016).

It is however important to realize that LMW proteinuria can also be present in the absence of tubular dysfunction/injury due to competition for tubular reabsorption, reducing the specificity of LMW proteinuria as a biomarker of tubular dysfunction/injury (Nejat et al., 2012). Saturation of the normal tubular reabsorptive capacity can occur either due to an increased systemic concentration of a specific LMW protein leading to an increased filtered load being presented to the tubular cells, and/or due to

competition with other filtered proteins (Thielemans et al., 1994; Hall, 2011; Nejat et al., 2012).

5.2 Glomerular injury biomarkers

5.2.1. Urinary immunoglobulin G

Immunoglobulins are large glycoproteins that are involved in the humoral immune response. The MW of immunoglobulin G (IgG) is 150 kilodaltons (kDa) (De Loor et al., 2013; Hokamp and Nabity, 2016). Being a HMW protein, it cannot pass an intact glomerular barrier. When glomerular dysfunction/injury is present, IgG may pass through the glomerular barrier into urine, resulting in urinary IgG (uIgG) to be a biomarker of glomerular injury (D'Amico and Bazzi, 2003).

Dogs with various diseases associated with AKI, including snake envenomation (Hrovat et al., 2013) and pyometra (Zaragoza et al., 2004; Maddens et al., 2010; Maddens et al., 2011), have increased uIgG. Also in leptospirosis, an important cause of AKI in dogs, uIgG was detected based on Western blotting analysis (Zaragoza et al., 2003b). However, the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern in canine leptospirosis indicated, as expected in a disease causing interstitial nephritis, that mainly LMW urinary proteins were present. Still, a glomerular involvement was suggested in some dogs with leptospirosis based on the presence of uIgG (Zaragoza et al., 2003b). Increased uIgG concentrations have also been documented in dogs with CKD (Hokamp et al., 2016), X-linked hereditary nephropathy (XLHN) (Nabity et al., 2012), hypercortisolism (Smets et al., 2012), and leishmaniosis (Zaragoza et al., 2003a; Pardo-Marín et al., 2017).

5.2.2. Urinary C-reactive protein

C-reactive protein (CRP) is a positive acute phase protein, produced by hepatocytes, with a MW of 110 to 144 kDa (Hokamp and Nabity, 2016). Glomerular injury can lead to the urinary presence of this HMW protein (D'Amico and Bazzi, 2003).

Glomerular injury based on increased urinary CRP (uCRP) has been documented in dogs with various diseases associated with both AKI and CKD, including leptospirosis (Oliveira et al., 2010), snake envenomation (Hrovat et al., 2013), heatstroke (Segev et al., 2015), pyometra (Maddens et al., 2010; Maddens et al., 2011), and leishmaniosis (Martínez-Subiela et al., 2013; Pardo-Marín et al., 2017). In contrast, uCRP was not significantly different between dogs with CKD and healthy control dogs in one study (Smets et al., 2010). All healthy dogs had undetectable concentrations of uCRP, while only 3/10 dogs with CKD had detectable uCRP concentrations.

5.2.3. Other glomerular injury biomarkers

Additional urinary biomarkers of glomerular injury, also under investigation in dogs but beyond the scope of this introduction, include other HMW and intermediate MW proteins such as immunoglobulin A (IgA), immunoglobulin M (IgM), albumin, and transferrin (De Loor et al., 2013; Hokamp and Nabity, 2016).

5.3 Tubular injury biomarkers

5.3.1. Urinary retinol-binding protein

Retinol-binding protein (RBP) is a transport protein for vitamin A (retinol) in plasma, acting as a negative acute phase protein, and has a MW of 21 kDa (Kanai et al., 1968; Blaner, 1989; Louw et al., 1992). This lipocalin protein has a primarily hepatic synthesis and coupling to retinol also occurs in the liver. Being a LMW protein, it can freely pass through the glomerular barrier (Blaner, 1989; Christensen et al., 1999; Monaco, 2000; D'Amico and Bazzi, 2003). However, in circulation, RBP is bound to another protein, transthyretin (Blaner, 1989). This RBP-transthyretin complex prevents glomerular filtration of RBP (Monaco, 2000). Not all of the circulating RBP is complexed to transthyretin, resulting in glomerular filtration of the non-complexed fraction. After filtration, RBP is efficiently reabsorbed by the proximal tubular cells (Christensen et al., 1999). Therefore, presence of urinary RBP (uRBP) indicates proximal tubular injury (D'Amico and Bazzi, 2003; Raila et al., 2003b).

Although uRBP has been studied more extensively in dogs with CKD, a few studies in dogs with diseases associated with AKI have already been published. Dogs with snake envenomation (Hrovat et al., 2013), heatstroke (Segev et al., 2015), and pyometra (Maddens et al., 2010; Maddens et al., 2011) had increased uRBP (Ahn and Hyun, 2013) concentrations present. In dogs with CKD, increased uRBP concentrations have been documented in several studies (Raila et al., 2003a; Raila et al., 2010; Smets et al., 2010; Hokamp et al., 2016). In dogs with CKD due to XLHN, a progressive increase in uRBP was seen parallel with the progression of CKD (Nabity et al., 2012). Dogs with hypercortisolism also had increased concentrations of uRBP (Smets et al., 2012).

5.3.2. Urinary neutrophil gelatinase-associated lipocalin

Neutrophil gelatinase-associated lipocalin (NGAL), also known as lipocalin-2 (LCN2), is a 25 kDa LMW protein belonging to the lipocalins, a family of carrier proteins (Kjeldsen et al., 1993; Flower, 1996). Initially isolated from activated neutrophils, its presence has also been demonstrated in many normal tissues, including the kidneys (Bundgaard et al., 1994; Kjeldsen et al., 1994; Friedl et al., 1999). Expression of NGAL is upregulated in inflammatory and neoplastic conditions (Nielsen et al., 1996; Friedl et al., 1999). In response to renal tubular injury, NGAL expression, production, and release from renal tubular cells is rapidly and markedly increased (Mori and Nakao, 2007; Bolignano et al., 2008). Being produced by activated neutrophils, renal tubular cells, and other epithelial cells during injury and other pathological conditions, NGAL has many functions, including anti-bacterial and renoprotective properties (Schmidt-Ott et al., 2007). As a LMW protein, it is freely filtered through the glomerular barrier, and efficiently reabsorbed in the proximal tubular cells of healthy kidneys (D'Amico and Bazzi, 2003; Mori et al., 2005; Schmidt-Ott et al., 2007). Impairment of tubular NGAL reabsorption combined with NGAL release from injured renal tubular cells, results in increased urinary NGAL (uNGAL) concentrations during AKI (Kuwabara et al., 2009). Overall, uNGAL originates from both systemic (extra-renal) sources and locally produced renal NGAL (Schmidt-Ott et al., 2007). Moreover, uNGAL can also orginate from neutrophils infiltrating the kidney during AKI (Mårtensson and Bellomo, 2014). Similar to humans, uNGAL exists in 3 molecular forms in dogs (a monomer, a dimer, and a heterodimer complex), originating from different locations (Hsu et al., 2014a). The existence of these different molecular forms from different origins complicates the interpretation of uNGAL, since the total uNGAL concentration represents a mixture of these forms (Mårtensson and Bellomo, 2014). Furthermore, it has been shown in urine from humans that the configuration of the specific antibody used in the immunoassays has an impact on the performance of these assays, because of differences in the recognition of the different NGAL molecular forms (Cai et al., 2010).

In humans, NGAL is one of the most intensively investigated candidate biomarkers of tubular injury and AKI during the last decade (Haase et al., 2010). Although initially considered a very promising AKI biomarker, its performance has been

variable and inconsistent across studies, in part due to its unpredictable specificity in heterogeneous critically ill populations (Mårtensson and Bellomo, 2014). In contrast, relatively few studies are available in dogs. The first canine study investigating NGAL showed that dogs with XLHN, a progressive glomerular disease, had almost significantly increased uNGAL concentrations compared to unaffected dogs, already early in the disease process (Nabity et al., 2012). Based on 3 experimental studies with gentamicin-induced AKI, uNGAL was considered to be a sensitive and early candidate biomarker of AKI in dogs (Kai et al., 2013; Zhou et al., 2014; Palm et al., 2016). However, in another gentamicin-induced AKI study, uNGAL was not superior compared with the traditional biomarkers of renal function (Sasaki et al., 2014). Two other initial studies evaluating uNGAL as an AKI biomarker in dogs, concluded uNGAL to be a sensitive and specific biomarker of AKI, and able to diagnose AKI at an early non-azotemic stage (Lee et al., 2012; Segev et al., 2013). Several other studies confirmed increased plasma, serum, and/or uNGAL concentrations in dogs with several naturally occurring causes of AKI, confirming its promising nature as a biomarker of AKI (Hsu et al., 2014b; Steinbach et al., 2014; Segev et al., 2015).

In dogs with CKD, increased concentrations of plasma, serum, and/or uNGAL have also been detected (Ahn and Hyun, 2013; Segev et al., 2013; Hsu et al., 2014b; Steinbach et al., 2014; Cobrin et al., 2016). Moreover, increased uNGAL has been documented in non-renal urinary conditions, such as non-azotemic urinary tract infections and non-infectious pyuria (Daure et al., 2013; Segev et al., 2013; Proverbio et al., 2015). However, dogs with AKI and CKD had significantly higher uNGAL concentrations compared to dogs with lower urinary tract disorders (Segev et al., 2013). Increased serum and uNGAL concentrations were also detected in dogs with neoplasia (lymphoma and carcinoma) (Cobrin et al., 2016). In a study of dogs with sepsis requiring emergency celiotomy, both serum and uNGAL were significantly increased compared to control dogs that underwent surgery for intervertebral disc disease (Cortellini et al., 2015). Neither serum nor uNGAL were associated with development of AKI over the course of hospitalization, which was defined as an increase in sCr of 0.3 mg/dL (26.4 µmol/L) within 48 h, questioning the specificity of NGAL for AKI in this population.

5.3.3. Other tubular injury biomarkers

Other biomarkers of tubular injury already being investigated in dogs include additional LMW proteins (β_2-microglobulin, α_1-microglobulin), tubular enzymes (N-acetyl-β-D-glucosaminidase (NAG), γ-glutamyl transferase (GGT), alkaline phosphatase (AP), alanine aminopeptidase (AAP), lactate dehydrogenase (LDH)), inflammatory proteins (interleukin-2 (IL-2), interleukin-8 (IL-8), MCP-1, keratinocyte-derived chemokine (KC), granulocyte macrophage colony stimulating factor (GMCSF)), and other urinary proteins (Tamm-Horsfall protein (THP), clusterin, cystatin C, kidney injury molecule-1 (KIM-1)) (De Loor et al., 2013; Hokamp and Nabity, 2016).

5.4 Kidney injury biomarkers and classification of AKI

The previously discussed AKI classification systems in humans (RIFLE, AKIN, KDIGO) did not include kidney injury biomarkers. Nevertheless, these biomarkers could be combined together with functional renal biomarkers to classify AKI. A systemic approach to the diagnosis of AKI was proposed by the ADQI (Acute Dialysis Quality Initiative) in 2013, combining both functional and injury biomarkers to allocate patients into a specific category (Figure 1.7) (McCullough et al., 2013). A decrease in GFR will often be preceded by kidney injury, which can be detected by an increase in kidney injury biomarkers. Although the combination of kidney injury biomarker increases without functional renal biomarker increases was associated with worse clinical outcomes in humans (Hoste and Vandenberghe, 2017), it is still unclear whether this association was caused by unrecognized kidney injury or merely represented a higher disease severity in this subset of patients (Vanmassenhove et al., 2015; Vanmassenhove et al., 2017).

In order to ensure consistency in the terminology used throughout this doctoral thesis, biomarkers of GFR (i.e., sCr, sSDMA, and serum cystatin C) are named functional biomarkers, while the investigated glomerular and tubular biomarkers are consistently referred to as kidney injury biomarkers. Although this dichotomization in biomarker terminology is performed for ease of understanding, it is important to realize that while they can occur independently, functional impairment and structural injury often occur simultaneously in the same patient (Figure 1.7). Moreover, kidney injury can lead to loss of function, while the reverse sequential order holds true as well.

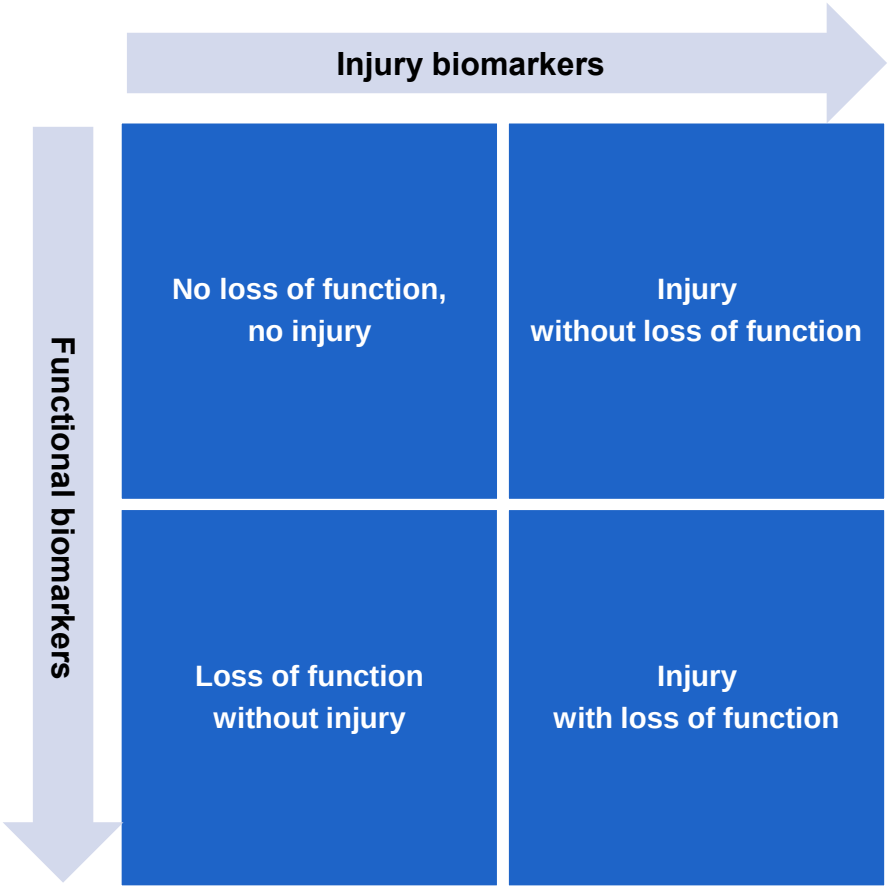

Figure 1.7. Combining functional and injury biomarkers to evaluate patients with acute kidney injury (AKI), proposed by the Acute Dialysis Quality Initiative (ADQI) and adapted from McCullough et al. (2013) and Zarbock et al. (2018).

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CHAPTER 2

SCIENTIFIC AIMS

Canine babesiosis is an emerging disease of worldwide significance. Although AKI is a recognized complication of canine babesiosis, its occurrence varies widely in current literature. While overt ARF is considered to be an uncommon complication of *B. rossi* infections based on traditionally used diagnostic tests, non-azotemic manifestations of AKI are considered to occur more often (Jacobson and Clark, 1994; Lobetti and Jacobson, 2001). With the introduction of the concept of AKI in human and in veterinary medicine, the importance of milder forms of AKI was emphasized (Kellum et al., 2007). Detection of AKI at early stages allows earlier therapeutic intervention and might eventually improve outcome (Hokamp and Nabity, 2016). Diagnosis of milder forms of AKI by routinely used biomarkers is however hampered in many ways, especially in the setting of canine babesiosis.

Therefore, the general scientific aim of this doctoral thesis was to characterize AKI in canine babesiosis caused by *B. rossi* using more sensitive biomarkers of kidney injury complementary to, and in comparison with, the traditionally used insensitive functional biomarkers. To overcome the general as well as babesiosis-specific limitations of the traditionally used tests to diagnose kidney injury and dysfunction, a selected number of diagnostic methods, mainly consisting of urinary kidney injury biomarkers, were applied in this setting.

Specific objectives of this doctoral thesis were:

1. To assess kidney injury in dogs that presented with uncomplicated babesiosis using a selected panel of sensitive urinary biomarkers of glomerular and tubular injury (i.e., urinary immunoglobulin G (uIgG), urinary C-reactive protein (uCRP), and urinary retinol-binding protein (uRBP), respectively).
2. To investigate the stability of IgG, CRP, and RBP in canine urine after long-term storage at -72°C.
3. To determine the occurrence of renal azotemia in a large population of dogs with babesiosis caused by *B. rossi*, using uOsmol instead of USG to evaluate their renal concentration ability.
4. To characterize *B. rossi*-induced AKI at presentation, during hospitalization, and 1 month after treatment, in a population of dogs with uncomplicated and complicated babesiosis, using a more expanded panel of urinary, plasma, and serum biomarkers of kidney injury and dysfunction.

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CHAPTER 3

KIDNEY INJURY IN UNCOMPLICATED CANINE BABESIOSIS

ASSESSMENT OF KIDNEY INJURY USING URINARY BIOMARKERS IN UNCOMPLICATED CANINE BABESIOSIS CAUSED BY *BABESIA ROSSI*

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Summary

Kidney injury is deemed a common, yet poorly documented, complication in canine babesiosis. Serum urea and creatinine are insensitive and non-specific biomarkers of early renal dysfunction and their measurements are influenced by hemolysis caused by babesiosis.

The aim of this study was to use urinary kidney injury biomarkers to assess the localization and degree of kidney injury in dogs with *Babesia rossi* infection. Urinary immunoglobulin G (uIgG) and urinary C-reactive protein (uCRP) were measured as biomarkers of glomerular kidney injury, while urinary retinol-binding protein (uRBP) was used as a biomarker of tubular kidney injury. Eighteen dogs presenting with uncomplicated babesiosis were included and compared with 8 clinically healthy dogs. Previously validated commercial enzyme-linked immunosorbent assay (ELISA) kits were used for the measurement of uIgG, uCRP, and uRBP. Results were related to urinary creatinine concentrations.

Dogs with babesiosis had significantly higher concentrations of all 3 measured urinary kidney injury biomarkers compared to healthy dogs. Except for urinary protein to creatinine ratio (UPC), traditional renal biomarkers (urine specific gravity (USG), serum urea and creatinine (sCr)) were not significantly different between dogs with babesiosis and healthy dogs. All 3 urinary biomarkers were positively correlated with each other and with UPC.

These data support the presence of both glomerular and tubular kidney injury in dogs suffering from uncomplicated *B. rossi* infection. Urinary biomarkers were superior to USG, serum urea and sCr concentrations for the early detection of kidney injury in dogs with babesiosis.

Introduction

Canine babesiosis is a tick-borne disease with worldwide significance, caused by an intra-erythrocytic protozoan. *Babesia* species have historically been divided into large (*Babesia canis*) and small (*Babesia gibsoni*) piroplasms (Taboada and Merchant, 1991). Three distinct species of large piroplasms can be identified in canine babesiosis: *B. canis, B. rossi*, *and B. vogeli* (Carret et al., 1999). Canine babesiosis in South Africa is most frequently caused by the more virulent *B. rossi*, although *B. vogeli* has also been identified recently (Matjila et al., 2004). In one study, 12% of all sick dogs presented to the Onderstepoort Veterinary Academic Hospital in South Africa were diagnosed with babesiosis (Shakespeare, 1995), making it an important and frequently diagnosed disease in this country. Moreover, canine babesiosis is considered an important emerging disease in Europe, chiefly due to increased transport of pets and climate change (Beugnet and Marié, 2009).

The clinical severity of canine babesiosis is variable, and is determined by the *Babesia* species and the immune response of the host. *Babesia rossi* is the most virulent form of the disease and infections are frequently fatal, while *B. vogeli* often only causes mild to moderate clinical disease (Uilenberg et al., 1989). Two main pathophysiologic mechanisms are thought to be responsible for the clinical signs: a hemolytic anemia, primarily of immune-mediated origin, and a severe systemic inflammatory response (Reyers et al., 1998). The presence of a systemic inflammatory response syndrome or multiple-organ dysfunction syndrome did not significantly affect the outcome in cases of complicated canine babesiosis (Welzl et al., 2001). However, the involvement of specific organ systems was found to influence outcome. The study concluded that renal involvement, defined as an increase of admission sCr (>150 µmol/L) in the absence of clinical dehydration, resulted in a 5 times increased risk of death.

True acute renal failure (ARF) is a severe complication of infection by several species of *Babesia*, with a prevalence varying from 2.2% to 36% (Jacobson and Clark, 1994; Camacho et al., 2004; Garcia, 2006; Máthé et al., 2006). However, prerenal azotemia could not be excluded in some of these cases (Garcia, 2006). Minimal kidney

injury is identified more often than overt ARF, making ‘renal involvement’ a more suitable description for this complication (Lobetti and Jacobson, 2001).

Many potential causes of acute kidney injury (AKI) in babesiosis have been proposed. Hemoglobinemia was proposed to be the main cause of kidney injury in babesiosis, however a study on healthy dogs, experimentally infused with hemoglobin, showed that a significant nephropathy was not induced (Lobetti et al., 1996). Moreover, histological changes typical for hemoglobinuric nephropathy were rarely observed in canine babesiosis (Máthé et al., 2007). Instead, the histopathological lesions were most consistent with hypoxic or toxic injury. Renal hypoxia, caused by anemia and systemic hypotension, seems to be the primary cause of kidney injury as opposed to hemoglobinuria (Lobetti et al., 1996; Máthé et al., 2007). It has also been suggested that met-hemoglobinuria, reported in canine babesiosis, may be another cause of kidney injury (Lobetti and Reyers, 1996). Moreover, cytokine-induced kidney injury due to a systemic inflammatory response is another likely mechanism (Jacobson and Clark, 1994; Reyers et al., 1998).

Serum urea and sCr will only increase when more than 75% of the nephrons are nonfunctional, making them insensitive biomarkers for early detection of renal dysfunction (Braun et al., 2003). In addition, serum urea concentration can be elevated due to extra-renal causes such as hemolysis and rhabdomyolysis, both of which occur in babesiosis. Thus, serum urea should not be used in the diagnosis of AKI caused by babesiosis (Jacobson and Lobetti, 1996; de Scally et al., 2004; de Scally et al., 2006). Furthermore, serum hemoglobin and bilirubin can interfere with the chemical analysis of sCr, leading to an underestimation of sCr concentration in several methods of sCr measurement (de Scally et al., 2004). Proteinuria, caused by hemolysis in babesiosis, induces a false increase in specific gravity (Chadha et al., 2001), which makes the measurement of USG a less reliable diagnostic method for renal function in babesiosis.

There is enough evidence to suggest that AKI is a serious complication associated with canine babesiosis that has been shown to influence outcome (Lobetti and Jacobson, 2001; Welzl et al., 2001). Therefore, sensitive biomarkers are necessary for the early diagnosis of AKI in canine babesiosis. Urinary biomarkers may allow this and additionally have the capacity to quantify and localize the site of kidney injury (Price, 2002). Immunoglobulin G, involved in the humoral immune response, and

CRP, a major acute phase protein in dogs, are 2 high molecular weight (HMW) proteins, to which an intact glomerular barrier is impermeable (D'Amico and Bazzi, 2003). Glomerular injury consequently leads to the urinary presence of these HMW proteins. Urinary IgG and/or uCRP detected glomerular kidney injury in dogs with *Escherichia coli* pyometra, chronic kidney disease (CKD), and hypercortisolism (Maddens et al., 2010a; Smets et al., 2010b; Smets et al., 2011). Retinol binding protein is filtered through the renal glomeruli because of its low molecular weight (LMW) and is reabsorbed in the proximal tubules under physiological circumstances (Christensen et al., 1999). Therefore, increased uRBP indicates tubular kidney injury, as previously reported in dogs with *E. coli* pyometra, CKD, and hypercortisolism (Maddens et al., 2010a; Smets et al., 2010b; Smets et al., 2011).

The aim of the current study was to assess kidney injury in dogs with babesiosis, caused by *B. rossi*, using urinary biomarkers of both glomerular (uIgG and uCRP) and proximal tubular kidney injury (uRBP). The potential interference of hemoglobinuria with these urinary biomarker analyses was additionally considered.

Material and methods

Animals

The study was approved by the Animal Use and Care Committee of the Faculty of Veterinary Science, University of Pretoria. A total of 26 dogs were enrolled in the study and divided into 2 groups.

Group 1 (B) included 18 dogs that were prospectively sampled after they presented with babesiosis to the Onderstepoort Veterinary Academic Hospital (OVAH), University of Pretoria in South Africa during 2010. The identity of the *Babesia* sp. responsible for the infection was confirmed by polymerase chain reaction (PCR) and reverse line blot (RLB). PCR and RLB was also used to exclude co-infection with *Ehrlichia canis*. Only dogs that presented with uncomplicated babesiosis caused by *B. rossi* were included in this study. Uncomplicated babesiosis was defined as a clinical presentation attributable to hemolytic anemia only (Jacobson and Clark, 1994). A

thorough anamnesis, physical examination, complete blood count, basic biochemistry profile (incl. serum urea, sCr, glucose, alanine aminotransferase, alkaline phosphatase, total serum protein, albumin, globulin, and electrolytes (sodium, potassium, total and ionized calcium)), and a urinalysis, including bacterial culture, was performed to exclude presence of concurrent diseases.

Group 2 (H) included 8 clinically healthy control dogs of comparable age and body weight, that also presented to the OVAH during the same period. The control dogs were considered healthy based on a thorough anamnesis, physical examination, complete blood count, basic biochemistry profile, and a urinalysis, including bacterial culture (negative culture and a UPC <0.5). PCR and RLB was performed to exclude infection with babesiosis and/or ehrlichiosis.

An additional group included 4 clinically healthy dogs of which a urine sample, collected by cystocentesis, was used for hemoglobin interference testing. These dogs were considered healthy based on a thorough anamnesis, physical examination, and a urinalysis, including bacterial culture (negative culture, no hemoglobinuria, and a UPC <0.5).

Sampling regimen

Blood and urine samples were collected at admission in all B and H dogs. All urine samples were collected by cystocentesis. Urinalysis consisted of a dipstick analysis (Combur 9 Test®, Roche Diagnostics, Germany), microscopic sediment analysis, USG, UPC, and a bacterial culture. After urine collection, quick centrifugation (3 minutes at 447 x g) was performed and the supernatant was divided into aliquots of 0.5 ml and stored at -80 °C. Frozen urine samples were transported for analysis to Belgium on dry ice. Upon arrival, samples were stored at -80 °C until analysis. All analyses of urinary biomarkers were performed within 6 months (m) of sample collection. To the author's knowledge, only one report on stability of urinary biomarkers during storage is published in veterinary medicine (Smets et al., 2010a). In that study, uRBP concentration was not different after storage for 12 m at -80 °C compared to fresh samples. Storage information about uCRP and uIgG are lacking in veterinary medicine.

Laboratory methods

All urine samples were analyzed with commercially available canine- or human-specific sandwich ELISAs (Immunology Consultants Laboratory, Newberg, USA) to determine the concentrations of either uIgG, uCRP, or uRBP, respectively. All assays were previously validated for use with canine urine in our laboratory (Maddens et al., 2010b). For each immunoassay, the absorbance was measured at a wavelength of 450 nm, using an ELISA plate reader (Multiskan MS®, Labsystems Thermo Fisher Scientific, Waltham, USA). A 4-parameter logistic curve fitting program (Deltasoft JV®, Biometallics Incorporated, Princeton, USA) was used to generate the standard curve and calculate the concentrations of uIgG, uCRP, and uRBP. Finally, results were indexed to urinary creatinine concentrations and expressed as ratios (/uCr).

Hemoglobin interference testing

To determine whether the presence of hemoglobinuria affects the urinary biomarker analyses, urinary biomarkers were measured on urine samples of 4 healthy dogs, to which different concentrations (0 g/L, +1 g/L, +4 g/L, +12 g/L, +24 g/L) of a human hemoglobin standard (Sigma-Aldrich, St. Louis, MO, USA) were added. These concentrations were based on previously published urinary hemoglobin concentrations in dogs with babesiosis (Lobetti and Reyers, 1996; Lobetti and Jacobson, 2001).

Statistical analysis

Two commercial software programs were used for data analysis (SPSS 16, SPSS Inc., Chicago, USA; GraphPad Prism 5, GraphPad Software Inc., CA, USA). The non-parametric Mann-Whitney *U*-test was used to compare group B with group H dogs. The non-parametric Wilcoxon signed-rank test was used as a paired difference test for hemoglobin interference testing. Correlations between the different urinary biomarkers and between these biomarkers and other variables (serum urea, sCr, USG, UPC, and serum albumin) were calculated using the non-parametric Spearman's correlation coefficient. Differences were considered statistically significant at $P<0.05$.

Results

Study population

All dogs in group B were diagnosed with uncomplicated babesiosis, caused by *B. rossi*. Median age in group B was 3.25 years (y) (range: 0.6–11 y), which was not significantly different from the median age of group H (3 y; range: 0.5–8 y) (*P*=0.48). There was no significant difference between the median body weight in group B (18.5 kg; range: 3.2–60 kg) and group H (22.5 kg; range: 6–40 kg) (*P*=0.87). In group B, 3 dogs were mixed breed, while pure-bred dogs included 3 Boerboel, 2 Rottweiler, 2 Dachshund, and one Cocker Spaniel, Pekingese, Great Dane, Staffordshire Bull Terrier, Toy Pomeranian, Chow Chow, Fox Terrier, and Jack Russell Terrier. There were 7 neutered and 4 intact females, and 3 neutered and 2 intact male dogs in group B, while one dog in both sexes was of unknown neuter status. Group H consisted of 2 mixed breed dogs, 2 Boerboel, 2 Bouvier des Flandres, one German Shepherd dog, and one Beagle. In group H, there were 5 intact and 2 neutered females, and one intact male dog.

Hematology and biochemistry results of groups B and H are shown in Table 3.1. Anemia ranged from mild to severe in group B. None of the dogs in group B had a leucocytosis, while all were moderately to severely thrombocytopenic. Sixteen of 18 dogs in group B (89%) had hypoalbuminemia, while 8 (44%) were hypoproteinemic. Serum albumin was significantly lower in group B than in group H (*P*=0.001), while total serum protein and globulin were not significantly different between both groups (*P*=0.075 and *P*=0.37, respectively).

Table 3.1. Clinicopathologic findings (hematology and biochemistry results) in 8 healthy dogs and 18 dogs with babesiosis (expressed as median and range).

Variable (unit)	RI	H	B
Hemoglobin (g/L)	120–180	171 (125–198)	89 (37–145)
RBC (x 10^{12} /L)	5.5–8.5	7.3 (5.3–8.3)	3.8 (1.6–6.3)
Packed cell volume (L/L)	0.37–0.55	0.51 (0.38–0.60)	0.27 (0.12–0.45)
WBC (x 10^{9} /L)	6–15	8 (7–20)	6 (2–14)
Platelet count (x 10^{9} /L)	200–500	302 (32–401)	35 (8–64)
Total serum protein (g/L)	53–75	60 (50–68)	53 (41–81)
Serum albumin (g/L)	27–35	35 (24–39)	25 (17–29)
Serum globulin (g/L)	20–37	24 (18–39)	30 (16–62)
Serum urea (mmol/L)	3.6–8.9	5.2 (4.0–7.7)	6.0 (2.6–47.4)
sCr (µmol/L)	40–133	92 (48–103)	68 (39–215)
Glucose (mmol/L)	3.3–5.5	4.5 (3.1–5.4)	4.6 (1.7–5.4)
Alanine aminotransferase (U/L)	9–73	28 (2–51)	25 (17–49)
Alkaline phosphatase (U/L)	65–311	38 (15–110)	98 (43–370)
Sodium (mmol/L)	140–155	145 (142–148)	142 (137–147)
Potassium (mmol/L)	3.6–5.1	4.7 (4.0–5.2)	3.9 (3.4–4.7)
Calcium (total) (mmol/L)	2.2–2.9	2.6 (2.5–2.9)	2.2 (2.0–2.5)

RI, reference interval; H, healthy dogs; B, dogs with babesiosis; RBC, red blood cell count; WBC, white blood cell count; sCr: serum creatinine.

Urine color varied from yellow to dark brown in group B, and was yellow in group H. Urine appearance varied from clear to turbid in both groups. The urinary pH ranged from 6 to 7 in group B, and from 5 to 9 in group H. Urinary glucose, ketone and urobilinogen measurements were negative in both groups. Bilirubinuria ranged from negative to 3+ in group B and was negative in group H. Hemoglobinuria was severe (4+) (range: 0–4+) in 17 of 18 dogs in group B, and was mild (1+) in one dog. In group H, hemoglobinuria varied from negative to 3+. Bilirubin crystalluria was present in 7 of 18 dogs in group B (39%). Micro-organisms were not observed during microscopic examination, and bacterial urine cultures were negative in all dogs.

Traditional renal biomarkers

Results of traditional renal biomarkers (serum urea, sCr, USG, and UPC) are presented for both groups in Table 3.2. Serum urea, sCr, and USG did not differ significantly between group B and group H (*P*=0.33, *P*=0.22, and *P*=0.37, respectively), while UPC was significantly higher in group B (*P*<0.001). Only one of 18 dogs in group B had a UPC <0.5. The USG of one of the dogs in group B was not included, because fluid therapy was initiated before sample collection.

Table 3.2. Results of traditional renal biomarkers and urinary kidney injury biomarkers in 8 healthy dogs and 18 dogs with babesiosis (expressed as median and range).

Variable (unit)	H	B	*P* value
Serum urea (mmol/L)	5.2 (4.0–7.7)	6.0 (2.6–47.4)	0.33
sCr (µmol/L)	92 (48–103)	68 (39–215)	0.22
USG	1.031 (1.014–1.048)	1.036 (1.012–1.065)	0.37
UPC	0.1 (0.05–0.35)	1.6 (0.23–5.53)	<0.001
uIgG/uCr (mg/g)	1.27 (0.52–3.23)	226.71 (11.32–2296.35)	<0.001
uCRP/uCr (mg/g)	BDL (n=8)	0.02 (BDL–0.81)	0.011
		BDL (n=6); BQL (n=2)	
uRBP/uCr (mg/g)	0.05 (BDL–0.16)	10.84 (0.91–58.23)	<0.001
	BDL (n=2); BQL (n=1)		

H, healthy dogs; B, dogs with babesiosis; sCr, serum creatinine; USG: urine specific gravity; UPC: urinary protein to creatinine ratio; uIgG: urinary immunoglobulin G; uCr: urinary creatinine; uCRP: urinary C-reactive protein; BDL: below detection limit; BQL: below quantification limit; uRBP: urinary retinol binding protein.

Upon sediment evaluation, granular casts were present in 5 dogs of group B (28%) and renal tubular epithelial (RTE) cells were present in 4 dogs of group B (22%). In contrast, urinary casts and RTE cells were absent in the urine sediment of all the dogs in group H.

Hemoglobin interference testing

Results of urinary biomarker analysis with different concentrations of added hemoglobin are shown in Table 3.3. No significant differences were found when the samples without added hemoglobin were compared to the samples with different hemoglobin concentrations (*P* value ranging from 0.13 to 0.63).

Table 3.3. Results of urinary kidney injury biomarkers in 4 healthy dogs with different concentrations of added human hemoglobin (expressed as median and range).

Variable (unit)	No added Hb	+ 1 g/L Hb	+ 4 g/L Hb	+ 12 g/L Hb	+ 24 g/L Hb
uIgG/uCr (mg/g)	0.54 (0.22–1.06)	0.52 (0.19–0.95) (*P*=0.13)	0.45 (0.16–0.94) (*P*=0.13)	0.52 (0.17–0.93) (*P*=0.13)	0.53(0.19–0.98) (*P*=0.13)
uCRP/uCr (mg/g)	BDL (n=4)	BDL (n=4)	BDL (n=4)	BDL (n=4)	BDL (n=4)
uRBP/uCr (mg/g)	0.06 (0.04–0.08)	0.06 (0.04–0.09) (*P*=0.63)	0.05 (0.03–0.08) (*P*=0.63)	0.07 (0.05–0.11) (*P*=0.25)	0.08(0.07–0.13) (*P*=0.13)

Hb, hemoglobin; uIgG: urinary immunoglobulin G; uCr: urinary creatinine; uCRP: urinary C-reactive protein; BDL: below detection limit; uRBP: urinary retinol binding protein.

Urinary biomarkers of kidney injury

Results of urinary biomarker analysis and comparison between groups B and H are shown in Table 3.2. Significantly higher values were found in group B compared to group H for all 3 urinary kidney injury biomarkers (uIgG, uCRP, and uRBP; *P*<0.001, *P*=0.011, and *P*<0.001, respectively).

Correlations between urinary biomarkers, traditional renal biomarkers, and other variables are listed in Table 3.4. All urinary kidney injury biomarkers, including UPC, were positively correlated with each other (*P*<0.001). A negative correlation was present between serum albumin and all 3 urinary kidney injury biomarkers (*P*<0.001). Serum urea was positively correlated with uCRP (*P*=0.042), while none of the 3 urinary kidney injury biomarkers were correlated with sCr nor USG.

Table 3.4. Correlations between urinary kidney injury biomarkers, traditional renal biomarkers and serum albumin.

	uIgG/uCr	uCRP/uCr	uRBP/uCr
uIgG/uCr	1	0.82 (*P*<0.001)	0.91 (*P*<0.001)
uCRP/uCr	0.82 (*P*<0.001)	1	0.75 (*P*<0.001)
uRBP/uCr	0.91 (*P*<0.001)	0.75 (*P*<0.001)	1
sCr	-0.37 (*P*=0.060)	-0.20 (*P*=0.34)	-0.37 (*P*=0.065)
Serum urea	0.17 (*P*=0.41)	0.40 (*P*=0.042)	0.24 (*P*=0.23)
USG	0.19 (*P*=0.35)	0.25 (*P*=0.21)	0.28 (*P*=0.16)
UPC	0.93 (*P*<0.001)	0.84 (*P*<0.001)	0.89 (*P*<0.001)
Serum albumin	-0.74 (*P*<0.001)	-0.66 (*P*<0.001)	-0.73 (*P*<0.001)

uIgG, urinary immunoglobulin G; uCr, urinary creatinine; uCRP, urinary C-reactive protein; uRBP, urinary retinol binding protein; sCr, serum creatinine; USG, urine specific gravity; UPC, urinary protein to creatinine ratio.

Outcome

Sixteen of 18 dogs in group B (89%) made a full recovery. One dog presented with uncomplicated babesiosis, but developed ARF and died 2 days after presentation. Another dog developed a mesenteric thrombus 2 weeks after presentation and died of septic peritonitis.

Discussion

Concentrations of all 3 urinary kidney injury biomarkers (uIgG, uCRP, and uRBP) were significantly higher in group B compared to group H, indicating the presence of both glomerular and tubular kidney injury in naturally occurring uncomplicated canine babesiosis, caused by *B. rossi*. The magnitude of increase in the median uRBP and uIgG in group B was similar (about 200 fold) for these proximal tubular and glomerular biomarkers, suggesting a similar degree of kidney injury at both levels of the nephron in dogs affected with uncomplicated babesiosis.

The potential interference of hemoglobin with the urinary biomarker analyses was excluded by performing interference testing. Previously published urinary hemoglobin concentrations in dogs with babesiosis ranged from 0.2 to 11.6 g/L (Lobetti and Reyers, 1996; Lobetti and Jacobson, 2001). No significant interferences were found on these, and even higher (24 g/L) concentrations.

Traditional renal biomarkers (with the exception of UPC) were not significantly different between both groups, demonstrating the earlier detection of kidney injury when using urinary kidney injury biomarkers. Granular casts and RTE cells, possibly indicative of tubular kidney injury (Lobetti and Jacobson, 2001), were only present in the urine of a minority of dogs in group B. Based on the pathogenesis of babesiosis, the observed proteinuria could be either of prerenal (hemoglobinemia, myoglobinemia) or renal origin (Lees et al., 2005). Severe hemoglobinuria was present in almost all the dogs in group B, demonstrating a major prerenal component in the observed proteinuria. Therefore, differentiation between glomerular and tubular proteinuria, which is primarily based on the magnitude of proteinuria, is difficult to make in babesiosis based on the UPC. Nevertheless, the results of this study strongly suggest that both glomerular and tubular kidney injury occur in dogs with uncomplicated babesiosis.

Increased amounts of serum CRP, together with other acute phase proteins, have been demonstrated in canine babesiosis, confirming the presence of an acute inflammatory response (Ulutas et al., 2005; Matijatko et al., 2007; Köster et al., 2009). The latter study failed to find an association between serum CRP concentration and outcome in canine babesiosis (Köster et al., 2009). Urinary CRP was previously measured in dogs with *E. coli* pyometra, Leptospirosis, and CKD (Maddens et al., 2010a; Oliveira et al., 2010; Smets et al., 2010b). The glomerular barrier must be damaged before HMW proteins such as CRP, irrespective of their circulating levels, can be detected in urine (D'Amico and Bazzi, 2003). The extent to which variations in serum CRP concentrations influence uCRP concentrations in canine babesiosis, remains to be determined.

Urinary IgG concentrations were also significantly higher in group B, demonstrating the transglomerular passage of yet another HMW protein. Theoretically, uIgG is a better and more selective biomarker of the severity of injury to the glomerular capillary wall than the overall degree of proteinuria, because increasing amounts of this HMW protein cross the glomerular barrier with an increasing severity of glomerular lesions (D'Amico and Bazzi, 2003).

Tubular kidney injury was assessed by measuring uRBP, a LMW protein. The unbound fraction of this negative acute phase protein is freely filtered through the glomeruli. Under physiological circumstances, this filtered RBP is reabsorbed by the proximal tubular cells, but tubular injury increases uRBP (Raila et al., 2003a; Raila et al., 2003b). Comparison of uRBP results from the dogs in group B with the reported results from dogs with different stages of CKD (Smets et al., 2010b) showed that the median uRBP of dogs with CKD was 5 times higher than in dogs with babesiosis, suggesting more severe tubular injury in dogs with CKD. In another study (Raila et al., 2010), uRBP could not detect a reduced plasma creatinine clearance in apparently healthy dogs, leading to the conclusion that uRBP had no diagnostic value in detecting mildly decreased glomerular filtration rate (GFR) in non-azotemic dogs, however the GFR measurement is questioned, because a limited sampling technique was used. Comparison of uRBP concentrations between dogs with babesiosis, which represents an acute systemic inflammatory disease, and dogs with chronic diseases (such as CKD) has to be performed with caution.

The studied urinary protein biomarkers were positively correlated even though they represent kidney injury at different levels of the nephron. Babesiosis might indeed cause direct injury at both the glomerular and tubular level. Another explanation could be a close interaction of these proteins in the nephron, resulting in an overload of filtered proteins that is harmful to tubular cells, as described in humans (D'Amico and Bazzi, 2003; Vinge et al., 2010). In the process of reabsorption by the proximal tubular cells, HMW proteins compete not only amongst each other, but also with LMW proteins. If this reabsorption mechanism becomes saturated due to large amounts of filtered HMW proteins, filtered proteins of all sizes appear in the urine. An overload of filtered proteins, due to increasing severity of glomerular disease, induces progressive injury to the epithelial cells of the proximal tubules as well as activation of cytokines and growth factors that induces interstitial infiltration and fibrosis. Therefore, urinary

LMW proteins, which escaped tubular reabsorption, are theoretically superior biomarkers of the severity of tubulo-interstitial injury than the overall amount of proteinuria (D'Amico and Bazzi, 2003; Vinge et al., 2010). Next to the presence of tubular injury, saturation of the tubular capacity for reabsorption could provide an alternative explanation for the observed increased urinary concentration of LMW proteins (Nejat et al., 2012). Proteinuria-induced increases of uRBP by competition for reabsorption might therefore decrease the specificity of uRBP for tubular kidney injury.

A recent histological study, examining kidneys from 8 dogs that suffered from a fatal *B. canis* infection, demonstrated mainly degenerative changes in the proximal renal tubules, with occasional complete necrosis of the proximal tubules, while no significant glomerular changes were reported (Máthé et al., 2007). This absence of glomerular pathology is in apparent contradiction with the evidence of glomerular injury in the current study. One possible explanation for this might be the difference in virulence between the different *Babesia* species. Another argument is that the severe clinical presentation in the histological study may have resulted in death or euthanasia before significant glomerular injury could have occurred. Although an association between the same urinary kidney injury biomarkers and histological lesions has recently been suggested for canine *E. coli* pyometra (Maddens et al., 2011), the specificity of these urinary biomarkers for histological changes needs further investigation.

Conclusion

Based on the urinary biomarker results of this study, the presence of both glomerular and tubular kidney injury was detected in naturally occurring uncomplicated canine babesiosis, caused by *B. rossi*. Earlier detection of kidney injury was possible using the urinary biomarkers uIgG, uRBP, and uCRP compared to USG, serum urea and sCr. Further studies are needed to assess the reversibility of kidney injury induced by *B. rossi* after treatment, to assess the ability of these urinary biomarkers to predict the risk for renal azotemia, and to evaluate the correlation between changes in renal histology and urinary biomarkers in canine babesiosis.

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CHAPTER 4

STABILITY OF KIDNEY INJURY BIOMARKERS AFTER STORAGE

STABILITY OF GLOMERULAR AND TUBULAR KIDNEY INJURY BIOMARKERS IN CANINE URINE AFTER 4 YEARS OF STORAGE

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Summary

Urinary biomarkers are sensitive indicators of early stage kidney injury, consequently, research in this area is expanding in both human and veterinary medicine. However, studies investigating the impact of pre-analytical factors, such as storage conditions, on urinary biomarker concentrations are largely lacking in veterinary medicine.

We evaluated the stability of several kidney injury biomarkers in canine urine after storage for 4 years at -72 °C. Urine samples were collected from 26 dogs; 18 dogs with babesiosis and 8 healthy dogs. Concentrations of urinary immunoglobulin G (uIgG), urinary C-reactive protein (uCRP), and urinary retinol-binding protein (uRBP) were measured, using validated commercial immunoassays, at the start of the study and 4 years later. To investigate the effect of long-term storage, absolute and relative differences between both measurements were compared. Additionally, dogs with babesiosis were compared with the healthy controls at both time points.

Storage caused significant absolute and relative decreases in concentrations of all 3 biomarkers. Significant differences between dogs with babesiosis and healthy dogs were found in uIgG and uRBP at both time points, however the difference in uCRP between both groups lost significance after storage. Because the main goal of these urinary biomarkers is to detect early stage kidney injury, the statistically significant decrease in their concentrations will be clinically relevant when a mild degree of kidney injury is present.

Our data indicate that the investigated urinary biomarkers show significant decay after 4 years of storage at -72 °C, adversely affecting their diagnostic utility.

Introduction

The use of urinary biomarkers to detect kidney injury at an early stage is gaining interest in both human and veterinary medicine (Maddens et al., 2010; Maddens et al., 2011; De Loor et al., 2013; Hrovat et al., 2013; Wasung et al., 2015), given that identification of early kidney injury allows earlier therapeutic intervention (Price, 2002). The latter is hampered by the traditionally used diagnostic biomarkers of decreased renal function, serum creatinine and urea, as their concentration will only increase above the upper limit of the reference interval when more than 75% of the nephrons are nonfunctional, making them insensitive biomarkers for the detection of early stage kidney injury (Finco, 1995; Braun et al., 2003). In contrast, urinary biomarkers are generally accepted to be sensitive indicators of glomerular or tubular kidney injury, and additionally have the capacity to quantify as well as localize this damage (Price, 2002; De Loor et al., 2013). In research settings, enzyme-linked immunosorbent assays (ELISA) are often used to detect and quantify biomarker proteins. Immunoassays of several glomerular and tubular biomarkers of kidney injury (Immunology Consultants Laboratory, Portland, OR, USA), including IgG and CRP, respectively, as well as RBP, have been previously validated in our laboratory for their use in canine urine (Maddens et al., 2010).

Consideration of pre-analytical factors, such as sample handling and storage conditions, is important in order to perform reliable biomarker research. Because of prospective sampling, shipping and performing analyses in batches, immediate analysis of fresh urine samples is impractical in most research settings. Therefore, storage of frozen samples for long periods (several months (m) to years (y)) is common. However, studies evaluating the stability of urinary kidney injury biomarkers during long-term storage are very scarce in veterinary medicine. Even in human medicine, relatively few studies have evaluated the impact of pre-analytical factors on urinary proteins (Hepburn et al., 2015; De Loor et al., 2016). To our knowledge, only one urinary biomarker study, investigating the stability of RBP, albumin, and N-acetyl-β-D-glucosaminidase (NAG) in canine urine during storage at both -20 °C and -80 °C, has been published to date (Smets et al., 2010). In the latter study, biomarker stability up to one y of storage was investigated. Studies evaluating stability of other urinary

kidney injury biomarkers after long-term storage (i.e., >1 y) are lacking in veterinary medicine. Therefore, we evaluated the stability of several kidney injury biomarkers in canine urine after long-term storage (4 y) at -72 °C.

Material and methods

Twenty-six dogs that were presented at the Onderstepoort Veterinary Academic Hospital (OVAH), University of Pretoria, South Africa, were prospectively included after the approval of the Animal Use and Care Committee of the Faculty of Veterinary Science, University of Pretoria. The study group consisted of 18 dogs diagnosed with babesiosis, caused by *Babesia rossi*, a protozoal infection known to cause a variable degree of kidney injury (Lobetti and Jacobson, 2001; Defauw et al., 2012), and 8 clinically healthy control dogs of comparable age and body weight. In this way, a broad range of urinary biomarker concentrations could be expected, making assessment of a proportional bias possible. At admission, urine collection was performed by cystocentesis in all dogs. Routine urinalysis included a dipstick analysis (Combur 9 Test, Roche Diagnostics, Mannheim, Germany), microscopic sediment analysis, evaluation of urine specific gravity by refractometry, urinary protein to creatinine ratio, and bacterial culture. Quick centrifugation was performed after urine collection at 447 x *g* for 3 minutes. Subsequently, the supernatant was divided into multiple aliquots of 0.5 mL and stored at -80 °C. No preservatives were added to the collected urine samples before storage. Frozen urine samples were shipped on dry ice to Belgium. Upon arrival, samples were still frozen and initially stored at -80 °C until first analysis. The first urinary biomarker analysis (T_0) was performed within 6 m of sample collection. These urinary biomarker results were already reported in a previous publication (Defauw et al., 2012), which documented the presence of glomerular and tubular kidney injury in dogs with uncomplicated babesiosis. In order to determine the long-term stability of these biomarkers, the same urine samples, stored at -72 °C, were analyzed again 4 y later ($T_{4\ years}$). All samples analyzed at T_0 underwent 1 or 2 freeze-thaw cycles; samples at $T_{4\ years}$ had 1 additional freeze-thaw cycle. Concentrations of uIgG, uCRP, and uRBP were measured at T_0 and $T_{4\ years}$ using commercially available canine- or human-specific ELISAs (Immunology Consultants Laboratory, Portland, OR,

USA). All immunoassays were previously validated for their use in canine urine, used in accordance with the manufacturer's instructions, and performed as described previously (Maddens et al., 2010). Inter-assay coefficient of variation (CV) was 7.7% for uIgG, 10.5% for uCRP, and 5.5% for uRBP (Maddens et al., 2010). For each assay, the absorbance was measured at a wavelength of 450 nm with 650 nm as a reference (Multiskan MS, Labsystems Thermo Fisher Scientific, Waltham, MA, USA). A 4-parameter logistic curve fitting program was used to generate the standard curve and to calculate the concentrations of each urinary biomarker after correction for the urine dilutions used (Deltasoft JV, Biometallics Incorporated, Princeton, NJ, USA). Biomarker concentrations at T_0 and $T_{4\ years}$ were indexed to urinary creatinine concentrations obtained at T_0 and expressed as ratios (/uCr), to compensate for variations in urinary concentration.

A commercial software program was used for data analysis (SAS version 6.4, SAS Institute Inc., Cary, NC, USA). To investigate the effect of storage, the absolute difference between the measurements at T_0 and $T_{4\ years}$ was calculated, and we tested whether this difference was significantly different from zero using the Wilcoxon signed-rank test. The absolute difference was calculated twice, using biomarker concentrations that were unadjusted and adjusted to uCr, respectively. The same statistical test was done for the relative difference between the measurements at T_0 and $T_{4\ years}$ (unadjusted to uCr), which was defined as the absolute difference between the measurements at T_0 and $T_{4\ years}$ divided by the measurement at T_0. Samples with a measurement of zero at T_0 (i.e., biomarker concentration values that were either below detection limit (BDL) or below quantification limit (BQL)) were discarded as no decrease can be evaluated with such samples after storage. The results are also presented by Bland-Altman plots. To investigate whether the changes in urinary biomarker concentrations after long-term storage would alter the study results of a previous publication, concluding that dogs with babesiosis had significantly higher uIgG, uCRP, and uRBP compared with healthy dogs (Defauw et al., 2012), dogs with babesiosis were compared with healthy control dogs separately for the results obtained at T_0 and $T_{4\ years}$, using the Wilcoxon rank-sum test. These calculations were performed using biomarker concentrations adjusted to uCr. Differences were considered statistically significant at $P<0.05$.

Results

Absolute and relative differences between T_0 and $T_{4\ years}$ were statistically significant for all 3 measured biomarkers (Figure 4.1). For the calculations unadjusted to uCr, absolute uIgG decreased significantly from T_0 to $T_{4\ years}$ (P=0.017), with a mean decrease (± the standard deviation) of 240 mg/L (± 478). Relative uIgG also decreased significantly (P<0.001), with a mean percent reduction of 38% (± 19) of uIgG after 4 y of storage at -72 °C. Absolute uCRP decreased significantly from T_0 to $T_{4\ years}$ (P=0.041), with a mean decrease of 0.12 mg/L (± 0.29). Relative uCRP also decreased significantly (P<0.001), with a mean percent reduction of 80% (± 24). Finally, absolute uRBP decreased significantly from T_0 to $T_{4\ years}$ (P=0.006), with a mean decrease of 17.80 mg/L (± 30.17). Relative uRBP also decreased significantly (P<0.001), with a mean percent reduction of 66% (± 9). For the calculations adjusted to uCr, absolute uIgG showed a mean decrease of 113 mg/g (± 196) (P=0.007). Absolute uCRP showed a mean decrease of 0.08 mg/g (± 0.19) (P=0.049). Absolute uRBP showed a mean decrease of 8.60 mg/g (± 11.93) (P=0.001). The same conclusion, namely significantly higher biomarker concentrations in dogs with babesiosis compared with healthy dogs as in our original paper (Defauw et al., 2012), was made for uIgG and uRBP after 4 y of -72 °C storage (P<0.001) (Table 4.1). In contrast, for uCRP, although significantly different at T_0 (P=0.012), the difference between diseased and healthy dogs no longer remained significant after 4 y of -72 °C storage (P=0.051).

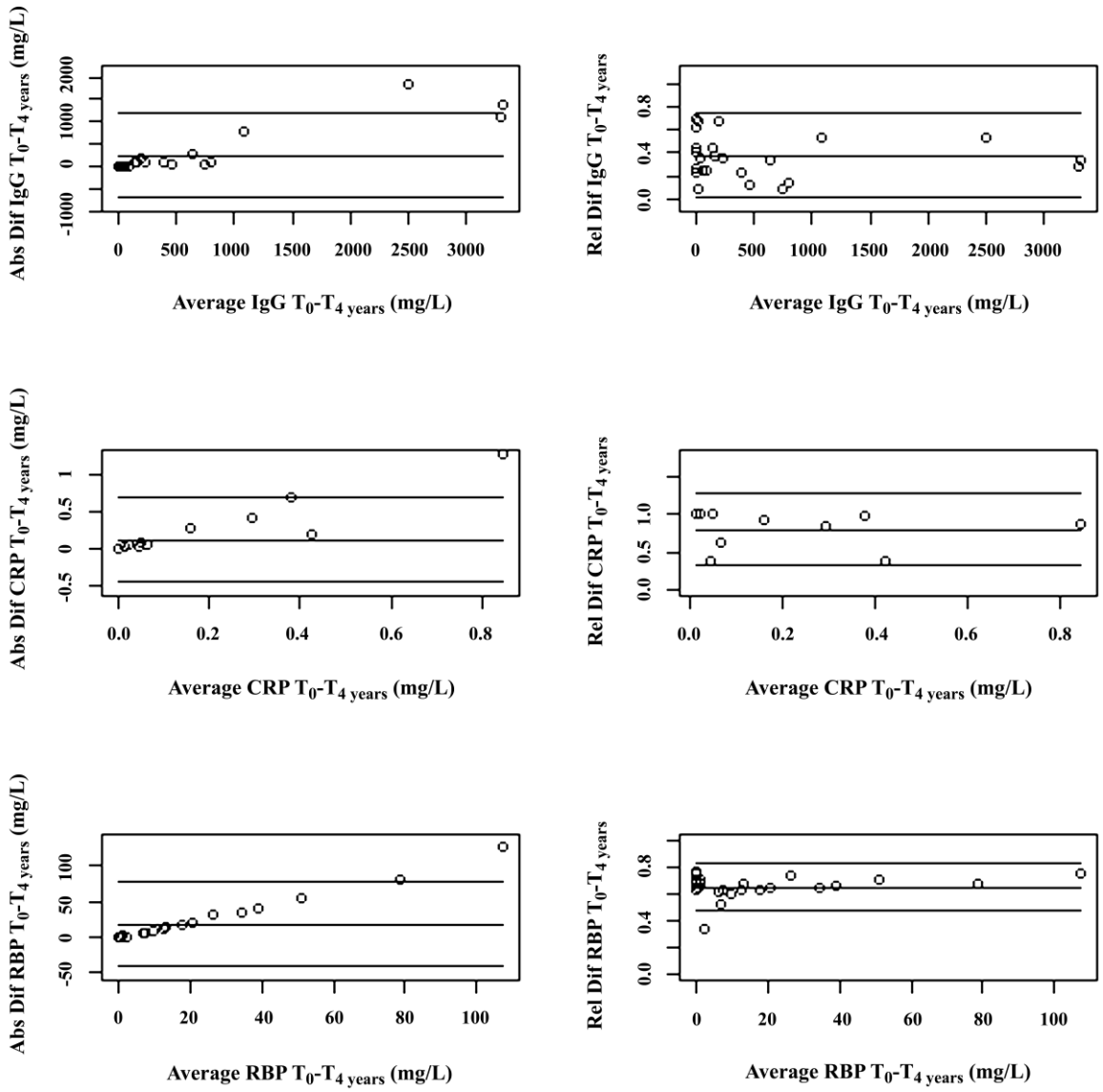

Figure 4.1. Absolute (Abs) and relative (Rel) differences (Dif) in urinary biomarker concentrations (IgG (immunoglobulin G), CRP (C-reactive protein), and RBP (retinol-binding protein)), unadjusted to urinary creatinine, after storage for 4 years at -72 °C, presented by Bland-Altman plots. The middle horizontal lines represent the absolute and relative mean differences between T0 and T4 years. The upper and lower horizontal lines represent the 95% limits of agreement (mean difference ± 1.96 x standard deviation of the differences).

Table 4.1. Urinary kidney injury biomarker concentrations in 8 healthy dogs and 18 dogs with babesiosis, performed at T0 and T4 years (expressed as median and range).

	H T_0	B T_0	*P* T_0	H $T_{4\ years}$	B $T_{4\ years}$	*P* $T_{4\ years}$
uIgG/uCr	1.27 (0.52–3.23)	227 (11–2296)	<0.001	0.86 (0.19–1.72)	122 (4–1631)	<0.001
uCRP/uCr	BDL (8)	0.02 (BDL–0.81)	0.012	BDL (8)	BQL (BDL–0.19)	0.051
		BDL (6), BQL (2)			BDL (8), BQL (3)	
uRBP/uCr	0.05 (BDL–0.16)	10.84 (0.91–58.23)	<0.001	0.01 (BDL–0.05)	4.19 (0.26–14.64)	<0.001
	BDL (2), BQL (1)			BDL (1)		

H, healthy dogs; B, dogs with babesiosis; uIgG, urinary immunoglobulin G; uCr, urinary creatinine; uCRP, urinary C-reactive protein; BDL, below detection limit; BQL, below quantification limit; uRBP, urinary retinol binding protein.

Discussion

Statistically significant decreases in all 3 measured urinary biomarker concentrations were observed after 4 y of -72 °C storage. The absolute decrease was highest for uIgG, intermediate for uRBP, and lowest for uCRP. This ranking can at least partly be attributed to the inherent differences in absolute concentrations of each biomarker in dogs with babesiosis. However, when evaluating the relative decreases, these were ranked vice versa, being highest for uCRP, and lowest for uIgG. Finally, the variation of the latter difference was highest for uCRP, and lowest for uRBP. The Bland-Altman plots illustrate the consistent bias that exists between the measurements at T_0 and $T_{4\ years}$ (i.e., consistently lower values at $T_{4\ years}$). Additionally, no significant proportional bias could be identified for any of the biomarkers as similar absolute and relative mean differences were observed for low versus high average concentrations. The decrease, found for all biomarkers, cannot be explained by assay variation based on their previously described inter-assay CV (Maddens et al., 2010), and based on the consistent decrease of all measured values. Although the same conclusions were made for uIgG and uRBP at T_0 and $T_{4\ years}$ (i.e., significantly higher concentrations in dogs with babesiosis compared with healthy controls), conflicting conclusions were made for uCRP at both time points. A loss in statistical significance occurred only for uCRP after long-term storage, because the difference between the diseased and the healthy population at T_0 was much smaller for uCRP compared with uIgG and uRBP. The higher relative decrease in uCRP compared with the other 2 biomarkers, further contributes to the latter finding. In populations in which kidney injury is subtle, the

statistically significant decrease in concentrations of all 3 biomarkers after long-term storage will be clinically relevant. Therefore, our data strongly indicate that long-term storage before analysis should be avoided, because the main goal of urinary biomarkers is to identify kidney injury at an early stage. This is mainly relevant for biobanking and research settings, in which long-term storage is commonly performed (Remer et al., 2014; Schuh et al., 2016).

Of the 3 biomarkers evaluated, only uRBP has been tested for stability in canine urine before (Smets et al., 2010). In the latter study from our group, no significant changes in uRBP were found after storage for up to one y at -80 °C. However, our results indicate that after much longer storage, stability of uRBP is severely affected. In humans, several studies investigated the stability of uIgG and uRBP during frozen storage (Kofoed-Enevoldsen et al., 1991; Mao et al., 1996; Tencer et al., 1997; Klasen et al., 1999; Schultz et al., 2000). Results of these studies are apparently conflicting yet hard to compare given differences in storage conditions and analytical methods. Urinary IgG, measured by ELISA, was not stable after 9 weeks of -20 °C storage in undiluted urine (Kofoed-Enevoldsen et al., 1991). When measured by immunoturbidimetry, a significant decrease in uIgG was found after 6 m of -20 °C (Tencer et al., 1997). Another study reported that uIgG, measured by nephelometry, decreased significantly during storage at -20 °C, but remained stable during 2 y of -70 °C storage (Klasen et al., 1999). When stored at -70 °C for longer than 4 m, uRBP, measured by ELISA, started to decrease significantly in one study (Mao et al., 1996). However, another study reported no significant changes in uRBP, although also measured by ELISA, when stored at -70 °C for 8 m (Schultz et al., 2000).

The main limitation of the study is the absence of biomarker analyses at intermediate time points to document the decay of these biomarkers over time in order to determine the maximum recommended storage time. Biomarker concentrations at T_0 and $T_{4\ years}$ were indexed to uCr concentrations obtained at T0, assuming that uCr remained stable during storage. Long-term stability studies showed that uCr is very stable in human urine (Remer et al., 2014). Although similar long-term studies have not been performed in canine urine, one study in dogs did show uCr concentrations to be stable at -20 °C for at least 3 m (Rossi et al., 2012). Another limitation of the current study is that an additional freeze-thaw cycle occurred at $T_{4\ years}$ compared with T_0, which might have influenced our results. One study evaluating the effect of multiple

freeze-thaw cycles on several kidney injury biomarkers in human urine (Schuh et al., 2015), showed a statistically significant, but clinically insignificant, decrease in concentration of all biomarkers after 3 freeze-thaw cycles. The authors concluded that reusing urine samples undergoing up to 3 freeze-thaw cycles resulted in only a minimal decrease of the tested biomarkers, so retesting could be performed with excellent stability (Schuh et al., 2015). The effect of multiple freeze-thaw cycles was also assessed in one study on kidney injury biomarkers in canine urine (Nabity et al., 2012). This study showed a statistically significant, but clinically insignificant, increase in urinary NAG activity after 4 freeze-thaw cycles; urinary neutrophil gelatinase-associated lipocalin concentrations (uNGAL) were not significantly affected by up to 4 freeze-thaw cycles. Although it should be emphasized that the effect of multiple freeze-thaw cycles has never been evaluated for canine uIgG, uCRP, and uRBP, we presume that one additional freeze-thaw cycle is highly unlikely to be a relevant confounding factor. Our results also only apply to urine samples stored at -72 °C. At -20 °C, long-term stability of urinary proteins is expected to be shorter. Indeed, because several human studies have demonstrated that urinary proteins are significantly underestimated after freezing at -20 °C (Mao et al., 1996; Berg et al., 1998; Klasen et al., 1999; Schultz et al., 2000), measurement of urinary proteins on specimens stored at -70 °C is recommended whenever analysis of fresh urinary samples is not feasible (MacNeil et al., 1991; Klasen et al., 1999; Schultz et al., 2000).

Many studies investigating kidney injury biomarkers in human and canine urine fail to mention the time interval between urine collection and biomarker analysis, although storage temperature is usually specified. Our results emphasize the importance of reporting these pre-analytical factors.

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CHAPTER 5

RENAL AZOTEMIA IN DOGS WITH *BABESIA ROSSI* INFECTION

RENAL AZOTEMIA AND ASSOCIATED CLINICAL AND LABORATORY FINDINGS IN DOGS WITH *BABESIA ROSSI* INFECTION

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Summary

Occurrence of acute kidney injury in canine babesiosis is not well documented. Furthermore, interpretation of urine specific gravity (USG) to assess renal concentrating ability is hampered by the presence of hemoglobinuria in this disease.

This cross-sectional study aimed to test the hypothesis that renal azotemia (RA) is underdiagnosed according to current canine babesiosis literature by determining its occurrence at presentation, using urine osmolality (uOsmol) instead of USG to measure urinary concentration. The second objective was to examine potential associations between the presence of RA and selected clinical and laboratory variables at presentation. Medical records available from 3 previously performed prospective data collections were reviewed retrospectively. Client-owned dogs that were diagnosed with babesiosis caused by *Babesia rossi,* were included if a complete blood count, biochemistry profile, and urinalysis was performed at admission. Urine osmolality was measured to identify dogs with RA. Differences between dogs with RA and dogs without RA were assessed by nonparametric statistics.

One hundred and fifty-two dogs were included, of which 26 (17%) were azotemic at admission. The occurrence of RA was 14% (21/152), hence 81% (21/26) of all azotemic dogs were diagnosed with RA. In contrast, when diagnosis of RA was based on an admission USG <1.030, only 23% (6/26) of the azotemic dogs would have been considered to have RA. Several signalment and clinicopathological findings were found to be associated with the presence of RA, including older age, and the presence of collapse, hypoglycemia, and hyperphosphatemia. Lastly, survival at discharge was significantly lower in dogs diagnosed with RA at presentation.

Our results clarified that RA is more common than previously reported in *B. rossi*. This study also demonstrated that USG determination is not a reliable method to evaluate renal concentrating ability in dogs with babesiosis. Thus, uOsmol should be part of the diagnostic work-up of dogs infected with *B. rossi* to avoid misclassification of dogs with RA as having prerenal azotemia. If uOsmol cannot be measured, clinicians should realize that most azotemic dogs with babesiosis caused by *B. rossi* have RA.

Introduction

Canine babesiosis is an intra-erythrocytic protozoan disease of worldwide clinical importance, which can be caused by several large and small *Babesia* species (Irwin, 2010). The most prevalent canine *Babesia* species in South Africa is *B. rossi*, although infections with *B. vogeli* have also been detected in dogs in South Africa (Matjila et al., 2004). *Babesia rossi* infections are associated with a variety of complications, which are thought to be consequences of systemic inflammatory response syndrome (SIRS) that is present in the majority of clinical infections (Welzl et al., 2001; Köster et al., 2015). Although acute kidney injury (AKI) is a recognized complication of canine babesiosis (Lobetti and Jacobson, 2001; Welzl et al., 2001), the occurrence of AKI and acute renal failure (ARF) varies widely in literature pertaining to canine babesiosis, mostly due to highly variable study populations, the presence of sampling biases, and different criteria used to define AKI and ARF. Solely based on studies where no sampling bias was identified, the reported occurrence of azotemia ranges from 0 to 36% when all *Babesia* species are included (Lobetti and Jacobson, 2001; Camacho et al., 2004; Furlanello et al., 2005; Ruiz de Gopegui et al., 2007; Zygner and Wedrychowicz, 2009). For *B. rossi* infections specifically, 13% of all dogs were azotemic in one study (Lobetti and Jacobson, 2001).

The biomarkers traditionally used to detect decreased renal function, serum creatinine (sCr) and urea, are generally insensitive biomarkers for early detection of kidney injury and dysfunction (Braun et al., 2003). Moreover, the presence of hemolysis in babesiosis leads to several additional limitations in the traditional clinical evaluation of renal function. Because of non-renal causes of an increased serum urea in babesiosis, serum urea has several limitations when used to assess renal function in this disease (de Scally et al., 2006). The presence of elevated serum bilirubin and free hemoglobin can lead to interference and consequently an underestimation of sCr concentrations (de Scally et al., 2004). This interference can occur when sCr is measured using the Jaffe reaction or enzymatic methods, which are the 2 most commonly used analytical techniques for the measurement of sCr in dogs (Braun et al., 2003; de Scally et al., 2004). Additionally, a previous study documented decreasing plasma creatinine concentrations after experimental *B. canis* infections, which was

suggested to be caused by an increased plasma volume after infection (Schetters et al., 2009). Lastly, USG can be disproportionately increased in the presence of large molecules such as hemoglobin (Chadha et al., 2001; Imran et al., 2010). Evaluation of USG could therefore lead to an overestimation of urinary concentration in canine babesiosis, caused by hemolysis-induced prerenal proteinuria.

Based on these babesiosis-specific limitations to evaluate renal function when using sCr and USG, we hypothesize that RA may be underdiagnosed in the current literature on canine babesiosis. Therefore, our primary objective was to determine the occurrence of RA at presentation in canine babesiosis caused by *B. rossi*, using the gold standard method of estimating urinary concentration (i.e., uOsmol). Our second objective was to explore potential associations between the presence of RA and selected clinical and laboratory variables at presentation.

Material and Methods

Study population

In this cross-sectional study, 297 medical records from client-owned dogs with babesiosis that presented to the Onderstepoort Veterinary Academic Hospital (OVAH), University of Pretoria, South Africa, were reviewed retrospectively. All records were available from 3 previously performed prospective data collections in 2006 (cohort 1, 89 dogs), 2011-2013 (cohort 2, 100 dogs), and 2013-2015 (cohort 3, 108 dogs). Several studies were published originating from the first 2 cohorts (Schoeman and Herrtage, 2007; Schoeman et al., 2007b; Rees and Schoeman, 2008; Schoeman and Herrtage, 2008; Goddard et al., 2015; Köster et al., 2015; Goddard et al., 2016). These studies were all approved by the University of Pretoria's Animal Ethics Committee (V V070-05; V055-11; V034-14).

All dogs that presented with babesiosis during the study periods were evaluated for inclusion, irrespective of disease severity. Diagnosis of babesiosis was initially made by demonstration of the intra-erythrocytic parasite on stained blood smears, and the species was later confirmed as *B. rossi* by polymerase chain reaction (PCR) and

reverse line blot (RLB) in all dogs. PCR and RLB were also used to exclude dogs with *B. vogeli* and/or concurrent *Ehrlichia canis* infection. Dogs were also excluded if any obvious wounds or trauma was evident on physical examination, or when concurrent neoplastic, inflammatory, cardiac disease, or other infections were diagnosed. Based on the initial inclusion during the previously performed prospective studies (exclusion when known concurrent disease was identified) and based on a thorough retrospective revision of all medical records, dogs with suspected chronic kidney disease (CKD) were excluded. Whenever a chronic history of weight loss, partial anorexia and/or polyuria/polydipsia was mentioned in the history, dogs were excluded from this study. Dogs known to have been treated with anti-inflammatory medication within 4 weeks prior to presentation were also excluded.

Sample collection and laboratory methods

Blood and urine samples were collected prior to any therapy, especially fluid therapy, was initiated. A complete medical record, including history, results of physical examination, complete blood count (Cell-Dyn 3700, Abbott Laboratories, Abbott Park, IL, USA; ADVIA 2120, Siemens, Munich, Germany) and serum biochemistry was available at admission for all dogs. Blood pressure was determined by an oscillometric technique. Blood lactate was measured with a hand-held point-of-care lactate device (Lactate Pro, Arkray, Kyoto, Japan). Different biochemistry analyzers were used for cohort 1 (NExCT/VetEX Alfa Wassermann, Bayer, Isando, South Africa) and cohort 2/3 (Cobas Integra 400 plus, Roche Diagnostics, Mannheim, Germany). Serum creatinine was determined using a modified Jaffe method in all dogs, but the reference interval of sCr was different between cohort 1 (40–133 µmol/L) and cohort 2/3 (59–109 µmol/L). Serum hemoglobin was only measured in cohort 1, using the same biochemistry analyzer. Serum cortisol (Radioimmunoassay cortisol, Coat-A-Count, Diagnostic Products Corporation, CA, USA) and total thyroxine (Radioimmunoassay canine T4, Coat-A-Count, Diagnostic Products Corporation, CA, USA) assays were performed using kits previously validated for dogs (Schoeman et al., 2007b). Dogs were only included in this study if urinalysis was performed at presentation. Most urine samples were collected by cystocentesis, but occasionally samples were obtained by free catch or by catheterization. Urinalysis consisted of a dipstick analysis, microscopic

sediment analysis, and USG which was measured by refractometry. In 2016-2017, stored urine samples were used to measure uOsmol by freezing point depression (Micro-Osmometer Autocal Type 13/13DR, Roebling, Berlin, Germany). Measuring uOsmol with this freezing point osmometer is based on the principle that a solution of 1 Osmol/kg freezes at a temperature 1.86 °C lower than that of pure water (Chadha et al., 2001). Before running the samples, osmolality of a calibration standard solution was measured in triplicate every day. All samples were measured in duplicate or triplicate, and the mean uOsmol and intra-assay coefficient of variation were calculated. The calculated mean values of all uOsmol measurements from an individual dog were used to determine the presence or absence of RA, since the individual coefficient of variation values were all <1% and the global intra-assay coefficient of variation was 0.19%.

Diagnosis of RA

Dogs were divided into 2 groups, based on the presence or absence of RA at admission. Renal azotemia was defined as the presence of azotemia (i.e., sCr concentration above the reference interval) combined with a uOsmol <1110 mOsmol/kg (i.e., the equivalent of USG <1.030). The conversion of USG <1.030 to uOsmol <1110 mOsmol/kg was based on a previously described conversion formula (Ayoub et al., 2013). Azotemic dogs with inappropriately concentrated urine (i.e., USG <1.030) either have intrinsic renal insufficiency (i.e., RA) or an underlying urine concentrating defect (Cowgill and Langston, 2011). Based on the study design, most diseases and medications leading to an impaired urine concentrating ability could be excluded. Because of the negative bias of severe hyperbilirubinemia on sCr concentrations when measured using the Jaffe reaction (de Scally et al., 2004), dogs with severe hyperbilirubinemia and a sCr concentration close to the upper reference value were excluded to avoid potential misclassification of azotemia. Hyperbilirubinemic dogs were excluded only if the total bilirubin concentration exceeded 85 µmol/L, which is the concentration at which significant interference (>±10% of the initial sCr concentration) may occur according to the manual of the chemistry analyzers (Glick et al., 1986), in combination with a sCr concentration close (±10%) to the upper reference limit.

Clinical assessment

Presence or absence of SIRS was based on canine-specific criteria, as previously described and applied in canine babesiosis (Okano et al., 2002; Köster et al., 2015). Defining uncomplicated disease and complications were based on previously published criteria for complicated babesiosis and severe falciparum malaria (Jacobson and Clark, 1994; Jacobson, 2006; WHO, 2014). Dogs were considered to be collapsed at presentation when they were unable to walk unaided. Hypotension was defined as a mean arterial pressure (MAP) <80 mmHg. Severe anemia was diagnosed when the hematocrit was <0.15. Hemoconcentration was defined as a hematocrit >0.37 in combination with signs of intravascular hemolysis (macroscopic hemoglobinuria and/or hemoglobinemia). Secondary immune-mediated hemolytic anemia (IMHA) was diagnosed based on a positive in-saline agglutination test. Hypoglycemia was diagnosed when blood glucose was <3.3 mmol/L. Jaundice was diagnosed when severe hyperbilirubinemia was present (total bilirubin >50 µmol/L). Cerebral babesiosis was defined as the presence of neurological signs that could not be attributed to any other cause, such as hypoglycemia. Acute respiratory distress syndrome (ARDS) was suspected clinically based on the presence of dyspnea and variably based on arterial blood gas analysis, radiological evidence of pulmonary edema, and exclusion of other causes of pulmonary edema. All dogs received standard antibabesial treatment with diminazene aceturate (Berenil RTU, Intervet, Kempton Park, South Africa) at 3.5 mg/kg, and supportive care such as packed red blood cells and intravenous fluid therapy as needed. Any complications were treated accordingly at the discretion of the attending clinician. Outcome categories were survival (i.e., short-term survival until discharge) or death/euthanasia due to poor prognosis with or without accompanying financial limitations.

Statistical analysis

A commercial software program was used for all statistical analyses (SAS version 9.4, SAS Institute Inc., Cary, NC, USA). Differences between dogs with RA and dogs without RA were assessed by the Wilcoxon rank-sum test for continuous and ordinal categorical variables, while the Fisher's exact test was used for binary variables. Clinical variables that were compared included age, body weight, duration

of illness, body temperature, heart rate, respiratory rate, and mean arterial pressure. Hematological and biochemical variables included the hematocrit, white blood cell count, segmented and band neutrophil count, platelet count, sCr and urea, glucose, total bilirubin, cortisol, total thyroxine, blood lactate, and serum hemoglobin. Variables from the urinalysis included pH, protein, bilirubin, blood/hemoglobin, color, turbidity, USG, and uOsmol. Binary or ordinal variables originating from clinical, clinicopathological data and the presence or absence of specific complications were also compared. These variables included the presence of collapse, hypotension, severe anemia, absence of anemia, presence of SIRS, hyperphosphatemia, hyperlactatemia, IMHA, hemoconcentration, jaundice, hypoglycemia, cerebral babesiosis, ARDS, the presence of ≥1 other complication, and outcome at discharge. Level of significance was set at 5% (*P* value <0.05).

Results

Study population and occurrence of azotemia and RA

Medical records from 297 dogs infected with *B. rossi* were evaluated for inclusion. One dog with severe hyperbilirubinemia (total bilirubin of 163 µmol/L) and a sCr concentration close to the upper reference limit (sCr of 104 µmol/L) was excluded to avoid potential misclassification of azotemia. Azotemia was present at admission in 15% (44/296) of the initial dog population.

Since urinalysis was not performed at admission in 144 dogs, only 152 dogs were finally included in the study. Azotemia was present at admission in 17% (26/152) of the final study population. The occurrence of RA at presentation was 14% (21/152) among all dogs included, hence present in 81% (21/26) of the azotemic dogs. In contrast, when RA was defined as the presence of azotemia combined with a USG <1.030, only 4% (6/152) of all dogs included and only 23% (6/26) of azotemic dogs would have been considered to have RA. The relationship between USG and uOsmol for each dog with RA and without RA is visualized in Figure 5.1.

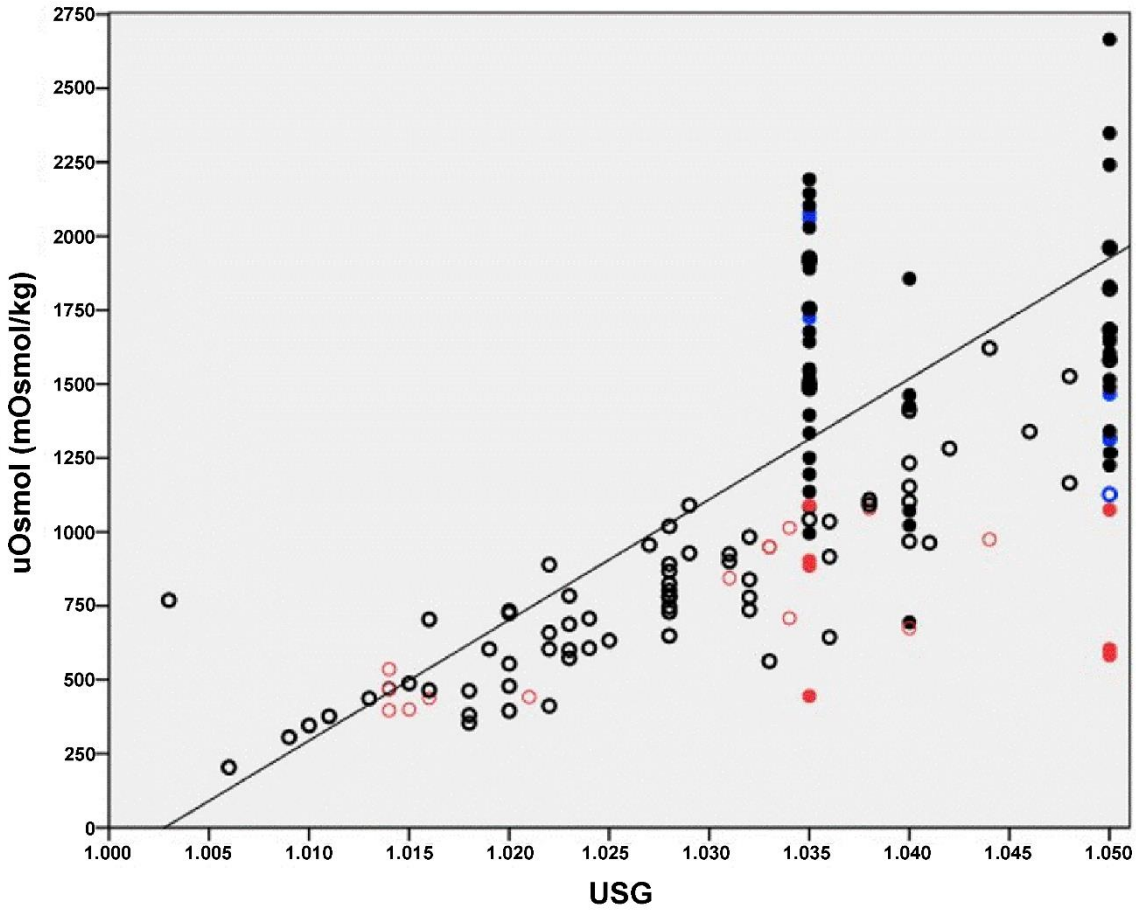

Figure 5.1. Scatterplot of urine specific gravity (USG) versus urine osmolality (uOsmol) from dogs with renal azotemia and dogs without renal azotemia at presentation. All 146 dogs that had both USG and uOsmol measured are represented. Data points from all individual dogs are visualized as circles and dots. Black colored data points represent 120 non-azotemic dogs, red colored data points represent 21 azotemic dogs with renal azotemia, and blue colored data points represent 5 azotemic dogs without renal azotemia. Circles represent dogs with an exact USG measurement value, while dots represent dogs with USG values higher than the highest calibrated point on the specific gravity scale of each refractometer used (USG >1.035, USG >1.040, and USG >1.050, respectively). The solid black line represents the univariate regression line for the association between USG and uOsmol from Ayoub et al. (2013) (uOsmol = -40.890 + (40.777 x USG)).

Comparison between dogs with and without renal azotemia

In the group of 21 dogs with RA, there were 9 Boerboel (43%), 3 Rottweiler (14%), and 2 mixed breed dogs (10%), while 16 Boerboel (12%), 2 Rottweiler (2%), and 28 mixed breed dogs (21%) were present in the 131 dogs without RA. Several other dog breeds were represented by a single dog in the RA group. Data from dogs with and without RA are summarized and compared in Tables 5.1-5.4. Dogs with RA were significantly older than dogs without RA. In Figure 5.2, age distribution is presented in histograms, demonstrating a right-skewed distribution in dogs without RA (Figure 5.2 A), in contrast to a bimodal distribution in dogs with RA (Figure 5.2 B). Dogs with RA had a significantly higher body weight and lower body temperature. Presence of collapse was significantly associated with the presence of RA, while gender, duration of illness, and mean arterial pressure were not. Most hematological parameters were not significantly different between both groups, except for band neutrophil count, which was significantly higher in dogs with RA. Serum urea, total bilirubin, serum hemoglobin, and lactate concentrations were significantly higher in dogs with RA. Hyperphosphatemia and hypoglycemia were significantly more common in dogs presenting with RA, while the presence of SIRS was not. Significantly higher median serum cortisol and lower median total thyroxine concentrations were seen in dogs with RA. Visually, urine was significantly darker and more turbid in dogs with RA, while USG, urinary pH, protein, bilirubin, and hemoglobin concentrations were not significantly different between both groups. All complications, except for IMHA and jaundice, occurred more frequently in dogs with RA. Survival at discharge was significantly lower in dogs with RA (Tables 5.3 and 5.4).

Table 5.1. Clinical data from dogs with renal azotemia and dogs without renal azotemia at presentation. All variables are continuous and expressed as median (range). Unless specified otherwise, data were available for all dogs.

Variable (unit)	RA (n=21)	No RA (n=131)	*P* value
Age (months)	37 (8–120)	19 (3–144)	0.016
Body weight (kg)	30.6 (9.8–65) (n=19)	14.5 (3–55) (n=124)	<0.001
Duration of illness (days)	2 (1–7) (n=19)	3 (1–21) (n=121)	0.14
Body temperature (°C)	39.0 (<32.0–40.2)	39.6 (32.9–41.2)	0.036
Heart rate (beats/min)	130 (24–160)	132 (28–>200) (n=130)	0.30
RR (breaths/min)	41 (9–84) (n=20)	48 (15–128) (n=121)	0.46
MAP (mmHg)	117 (67–139) (n=14)	105 (68–201) (n=79)	0.59

RA, renal azotemia; RR, respiratory rate; MAP, mean arterial pressure.

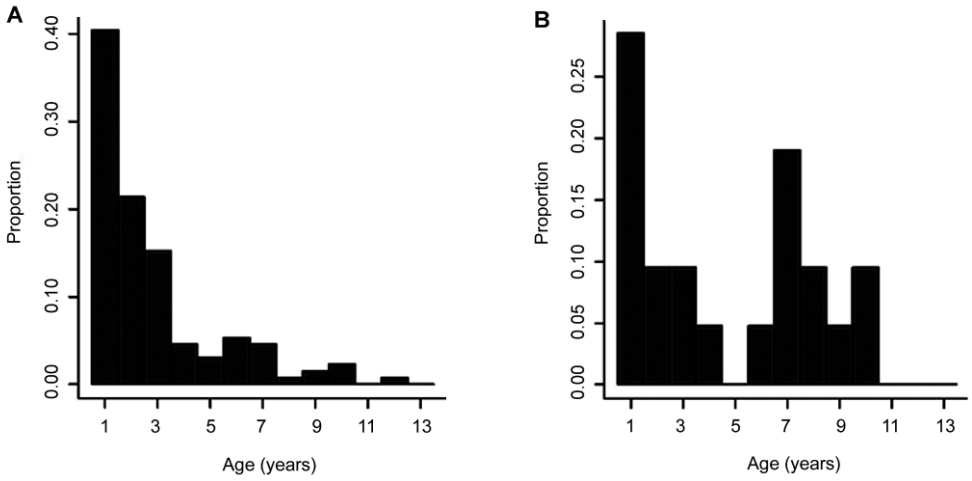

Figure 5.2. Age distribution of 131 dogs without renal azotemia (**A**) and 21 dogs with renal azotemia (**B**).

Table 5.2. Clinicopathological findings from dogs with renal azotemia and dogs without renal azotemia at presentation. All variables are continuous and expressed as median (range). Unless specified otherwise, data were available for all dogs.

Variable (unit)	RI	RA (n=21)	No RA (n=131)	*P* value
Hematocrit (L/L)	0.37–0.55	0.21 (0.05–0.60)	0.16 (0.04–0.53)	0.31
WBC (x 10^9 /L)	6–15	7.7 (1.1–104)	6.7 (1.1–33.1)	0.14
Segmented neutrophil count (x 10^9 /L)	3–11.5	4.8 (0.04–67.3)	4.4 (0.7–20.5)	0.38
Band neutrophil count (x 10^9 /L)	0–0.5	1.1 (0–21.8)	0.5 (0–9.1)	0.013
Platelet count (x 10^9 /L)	200–500	25 (0–147)	27 (0–636)	0.90
sCr (µmol/L) (cohort 1)	40–133	203 (142–527) (n=7)	72 (41–298) (n=35)	
sCr (µmol/L) (cohort 2, 3)	59–109	161 (125–865) (n=14)	59 (23–131) (n=96)	
Serum urea (mmol/L)	2.3–8.9	28.4 (8.9–104) (n=15)	8.2 (3.0–34.6) (n=89)	<0.001
Glucose (mmol/L)	3.3–5.5	4.2 (0.4–9.9)	4.6 (0.5–22.2) (n=130)	0.045
Total bilirubin (µmol/L)	1.0–6.8	42.5 (5.5–465) (n=13)	9.8 (0.4–408) (n=101)	0.039
Cortisol (nmol/L)	10–160	425 (67–690) (n=12)	122 (<5.5–861) (n=73)	0.002
Total thyroxine (nmol/L)	15–45	3.3 (<1.16–15.1) (n=12)	11.9 (<1.16–34.1) (n=74)	0.005
Blood lactate (mmol/L)	≤2.5	5.9 (1.3–13.2) (n=13)	2.8 (<0.8–23.9) (n=91)	0.026
Serum hemoglobin (g/L)		0.5 (0–1.3) (n=7)	0 (0–0.6) (n=35)	0.003
Urinary pH		6 (5–7) (n=19)	6 (5–8) (n=128)	0.057
Urine specific gravity		1.036 (1.014–>1.050)	1.040(1.003–>1.050) (n=129)	0.37
Urine osmolality (mOsmol/kg)		708 (397–1092)	1090 (204–2665) (n=127)	

RI, reference interval; RA, renal azotemia, WBC, white blood cell count, sCr, serum creatinine.

Cohort 1: data collections from 2006. Cohort 2: data collections from 2011-2013. Cohort 3: data collections from 2013-2015.

Table 5.3. Urinalysis results from dogs with renal azotemia and dogs without renal azotemia at presentation.

Variable	RA	No RA	*P* value
Urinary protein			0.78
0	1/20 (5%)	10/128 (8%)	
1+	4/20 (20%)	22/128 (17%)	
2+	3/20 (15%)	24/128 (19%)	
3+	12/20 (60%)	72/128 (56%)	
Urinary bilirubin			0.81
0	3/20 (15%)	13/127 (10%)	
1+	2/20 (10%)	15/127 (12%)	
2+	4/20 (20%)	38/127 (30%)	
3+	11/20 (55%)	61/127 (48%)	

Urinary blood/hemoglobin			0.052
0	0/20 (0%)	15/124 (12%)	
1+	0/20 (0%)	6/124 (5%)	
2+	0/20 (0%)	7/124 (6%)	
3+	3/20 (15%)	14/124 (11%)	
4+	17/20 (85%)	82/124 (66%)	
Urine color			0.009
Light yellow	0/19 (0%)	5/129 (4%)	
(Dark) yellow	6/19 (32%)	70/129 (54%)	
Amber / red	1/19 (5%)	21/129 (16%)	
Dark red / brown	10/19 (53%)	23/129 (18%)	
Dark brown / black	2/19 (10%)	10/129 (8%)	
Urine turbidity			<0.001
Clear or slightly turbid	0/11 (0%)	76/111 (68%)	
Moderate or severely turbid	11/11 (100%)	35/111 (32%)	

RA, renal azotemia.

Table 5.4. Binary and ordinal data from dogs with renal azotemia and dogs without renal azotemia at presentation.

Variable	RA	No RA	*P* value
Collapsed	11/21 (52%)	29/128 (23%)	0.007
Hypotensive (MAP <80 mmHg)	2/14 (14%)	5/79 (6%)	0.28
Presence of severe anemia (HcT <0.15%)	9/21 (43%)	55/131 (42%)	1.0
Absence of anemia (HcT ≥0.37%)	5/21 (24%)	15/131 (11%)	0.16
Presence of SIRS	17/21 (81%)	89/124 (72%)	0.44
Hyperphosphatemia	12/13 (92%)	16/81 (20%)	<0.001
Hyperlactatemia (lactate >2.5 mmol/L)	9/13 (69%)	50/91 (55%)	0.38
In-saline agglutination test positive	2/20 (10%)	22/129 (17%)	0.53
Hemoconcentration	4/21 (19%)	4/131 (3%)	0.013
Jaundice	6/13 (46%)	20/101 (20%)	0.071
Hypoglycemia			0.002
Absent (glucose ≥3.3 mmol/L)	11/21 (52%)	106/130 (82%)	
Mild (glucose <3.3, >2.2 mmol/L)	4/21 (19%)	13/130 (10%)	
Severe (glucose <2.2 mmol/L)	6/21 (29%)	11/130 (8%)	
Cerebral babesiosis	4/21 (19%)	1/131 (1%)	0.001
ARDS	6/21 (29%)	3/131 (2%)	<0.001
Presence of ≥1 other complication	20/21 (95%)	82/131 (63%)	0.002
Outcome at discharge (death/euthanasia)	12/21 (57%)	8/129 (6%)	<0.001

RA, renal azotemia; MAP, mean arterial pressure; HcT, hematocrit; SIRS, systemic inflammatory response syndrome; ARDS, acute respiratory distress syndrome.

Discussion

In our study, occurrence of RA at presentation was 14%, demonstrating that RA is a relatively frequent complication in dogs presenting with *B. rossi* infection. The hypothesis of a suspected underdiagnosis of RA in canine babesiosis, based on the traditional method of measuring urinary concentration (i.e., USG), was confirmed. By measuring uOsmol, our results showed that USG led to an overestimation of the renal concentrating ability in most dogs with babesiosis.

The overwhelming majority of dogs with babesiosis presented to the OVAH are first opinion cases, making the study population a representative sample of the general population in the area around Onderstepoort. One study without apparent sampling bias (Lobetti and Jacobson, 2001), reported azotemia in 13% of the dogs presented with *B. rossi*, which is slightly lower but still comparable to the 17% azotemic dogs found in this study. Previous studies on *B. rossi* infections that made a clear distinction between prerenal and renal azotemia are not available. However, the most often cited reference diagnosed ARF in only 2.2% (Jacobson and Clark, 1994), which was based on unpublished observations and without clearly defining ARF. This low number appears to be an underestimation, because the present study documented an almost 7 times higher occurrence of RA. Interestingly, the majority of azotemic dogs in our study (81%) had RA (prerenal azotemia was diagnosed in only 19% of azotemic dogs). Therefore, when uOsmol cannot be measured, the majority of azotemic dogs should be considered as suspected RA.

When USG was used to classify azotemia as prerenal or renal in origin, 71% (15/21) of dogs with RA were misclassified as having prerenal azotemia. A systematic overestimation of urinary concentration by USG was not only present in the azotemic dogs with babesiosis, but also in a majority of the non-azotemic dogs with babesiosis (Figure 5.1). These findings document that USG determination is not a reliable method to evaluate renal concentrating ability in our population of dogs with babesiosis. However, USG values below the cut-off of 1.030 were still clinically useful, because all azotemic dogs with a USG <1.030 were correctly identified to have RA. Presence of large molecules, such as hemoglobin, artefactually increases USG (Imran et al., 2010). This influence is clearly documented in human literature, where USG has been shown

to overestimate uOsmol in the presence of proteinuria and hemoglobinuria (Voinescu et al., 2002; Imran et al., 2010). To our knowledge, these interferences have not been reproduced in dogs. Proteinuria and hemoglobinuria did not significantly affect the correlation between USG and uOsmol in one study in dogs (Ayoub et al., 2013). However, the small number of dogs with hemoglobinuria and the fact that the majority of dogs only had trace to moderate hemoglobinuria, could have limited the power to detect a significant effect of hemoglobinuria in that study (Ayoub et al., 2013). Although we suspect that the presence of large amounts of urinary hemoglobin and its degradation products are the main cause of the documented discrepancy between USG and uOsmol in this population of azotemic dogs with babesiosis, this remains a hypothesis since we did not reliably quantify and correlate urinary hemoglobin. Urinary dipstick analysis did not show significant differences in hemoglobin concentrations between dogs with and without RA. However, since most urine samples had the maximum amount of hemoglobin concentration measurable by dipstick analysis (4+), more exact quantification of urinary hemoglobin using a validated method is needed to reveal its role in the discrepancy between USG and uOsmol, and to identify potential differences in urinary hemoglobin concentrations between dogs with and without RA. Our findings also raise questions regarding the reliability of USG measurements in other hemolytic diseases, such as IMHA.

Several factors were associated with a diagnosis of RA. Although these statistical associations do not prove causality, they provide potentially interesting information regarding the pathogenesis of AKI in babesiosis and could help clinicians to differentiate between prerenal and renal azotemia. The Boerboel and Rottweiler are popular breeds in South Africa (Mellanby et al., 2011), yet they were proportionally overrepresented in the RA group, compared to the group without RA, which might suggest a breed susceptibility for AKI. Another study documented that the majority of *B. canis*-infected Rottweiler dogs also presented with complicated disease (Máthé et al., 2006). The higher body weight in dogs with RA could be explained by the overrepresentation of these 2 large breeds in the RA group. However, it is also possible that large breed dogs and dogs with a higher body weight had higher baseline sCr values, making them more likely to have a sCr concentration above the cut-off value used to define azotemia.

Increasing age was significantly associated with RA. Figure 5.2 illustrates the presence of a population of older dogs with RA, while this subset of dogs is not represented in the group of dogs without RA. Acute renal failure was previously reported to be more common in older dogs with *B. rossi* infection (Jacobson and Clark, 1994). Older age was also associated with the risk of azotemia in a study with *B. microti*-like-infected dogs (Camacho et al., 2004). Several studies in humans with malaria, a disease that shows many similarities with canine babesiosis (Reyers et al., 1998; Jacobson, 2006), documented that AKI is more common in adults than children (Mishra and Das, 2008; Wassmer et al., 2015). Pre-existing kidney disease or an age-related decrease in glomerular filtration rate could explain this finding (Cianciolo et al., 2016). Pre-existing CKD could lead to a higher susceptibility to develop AKI when challenged with babesiosis. Partly due to the retrospective nature of this study, it cannot be excluded that some dogs with pre-existing CKD were included. Especially dogs with early CKD, which are often without overt clinical signs, would likely not have been detected. However, this problem of potential pre-existing disease will be present in almost any clinical study including client-owned dogs. On the other hand, an age-dependent host response to the infection has been suggested before. One study hypothesized that older dogs with *B. rossi* infection seemed more likely to develop an acute overwhelming inflammatory response to the infection (Reyers et al., 1998). In humans with malaria and dogs with *B. canis*, older age was also a risk factor to develop multiple organ involvement (Máthé et al., 2006; Wassmer et al., 2015). Based on these data, it could be suggested that older dogs are more likely to develop AKI, not only because of possible pre-existing CKD, but potentially also because they would more strongly react to the infection with an acute overwhelming inflammatory response. This hypothesis of age-dependent host response has not directly been documented in canine babesiosis, but differences in host immune status and age-dependent changes in the vascular system response to infection-induced inflammation are also suggested in severe falciparum malaria (Wassmer et al., 2015).

Collapse at presentation was significantly more common in dogs with RA, which is consistent with several studies documenting collapse to be an important indicator of severe disease (Leisewitz et al., 2001; Böhm et al., 2006; Jacobson, 2006). Mean arterial pressure and presence of hypotension was not different between both groups. This contrasts to findings in *B. canis*-infected dogs, where significantly lower diastolic, systolic, and mean arterial pressures were seen in azotemic dogs (Zygner and Gójska-Zygner, 2014). It is possible that hypotension is not a major contributor to AKI in *B. rossi*, however presence of a type II error cannot be excluded.

The only hematological parameter that differed significantly between both groups was the band neutrophil count. The higher band neutrophil count in dogs with RA reinforces the hypothesis that SIRS is an important contributor to complications in babesiosis (Jacobson and Clark, 1994; Reyers et al., 1998; Welzl et al., 2001; Köster et al., 2015). The lack of a significant difference in the presence of SIRS between both groups is likely caused by the problematic application of SIRS criteria in this context (Okano et al., 2002). Three of the 4 SIRS criteria (rectal temperature, heart rate, and respiratory rate) could have been influenced by uremia and anemia. Anemia was not associated with RA, which is consistent with current literature (Zygner and Wedrychowicz, 2009).

Total bilirubin concentrations were significantly higher in dogs with RA, but severe hyperbilirubinemia (jaundice) was not significantly more common in dogs with RA. Research in malaria showed direct bilirubin concentrations and icterus to be independently associated with AKI (Saravu et al., 2014). Anemia caused by hemolysis (prehepatic icterus) is unlikely to be a major cause of the association between RA and total bilirubin, since anemia was not associated with RA. Hemolysis resulting in hemoglobinuric nephropathy is a controversial concept in canine babesiosis (Lobetti and Reyers, 1996). An experimental study was unable to document significant kidney injury after inducing severe hemoglobinemia (Lobetti et al., 1996). The conclusion was that hemoglobin by itself was not toxic to the kidney, but synergistic effects with other mediators of AKI could not be excluded. A recent study in malaria-induced AKI suggested that plasma cell-free hemoglobin and associated oxidative stress biomarkers contribute to the pathogenesis of AKI (Plewes et al., 2017). Serum hemoglobin was significantly higher in dogs with RA in our study, but exact quantification of urinary hemoglobin is needed to further investigate its potential role in

AKI. Hepatic dysfunction, leading to bile cast nephropathy and hepato-renal syndrome, is considered to contribute to AKI in malaria (Silva et al., 2017). It is possible that hepatic dysfunction is linked to hypoglycemia, because icterus has been associated with hypoglycemia in dogs with *B. rossi* infection before (Keller et al., 2004). It remains to be determined whether hepatic dysfunction (leading to a hepatic icterus) is a direct contributor to AKI in canine babesiosis. Lastly, acute pancreatitis, another more recently described complication of canine babesiosis (Möhr et al., 2000; Jacobson, 2006), could also contribute to the development of jaundice (posthepatic icterus).

Higher serum cortisol and lower total thyroxine concentrations were seen in dogs with RA. These findings are consistent with studies in both babesiosis and parvovirus infection (Schoeman et al., 2007a; Schoeman et al., 2007b), that documented mortality and disease severity to be significantly associated with the same endocrine changes. These prognostic endocrine biomarkers and RA are most likely not causally related, but rather associated because of an increased disease severity and mortality in this subset of dogs with babesiosis.

Urine was darker and more turbid in dogs with RA compared to dogs without RA. Hemoglobin or its degradation products are the most likely causes of dark and turbid urine in dogs with babesiosis. This is unlikely to be caused by bilirubinuria, since no difference between groups was found in urinary bilirubin concentrations. As discussed above, the role of hemoglobinuria in the development of RA in babesiosis remains speculative.

The presence of RA was associated with a worse outcome in this study. Multivariate analysis was not performed, because of the limited number of dogs in the group with RA. Hence, we could not establish RA as an independent variable in this study, *inter alia* because RA was significantly associated with most other complications, such as ARDS, cerebral babesiosis, and hypoglycemia, which could all influence outcome (Welzl et al., 2001; Nel et al., 2004). However, a previous study in dogs with a *B. rossi* infection showed that the presence of azotemia, without clinical signs of dehydration, was associated with a higher risk of death compared to all other organ complications combined (Welzl et al., 2001).

Since uOsmol is not readily available in most clinical situations and USG is unreliable in this specific population, identifying multiple factors such as older age, the presence of collapse, hyperphosphatemia, and more turbid or darker urine, could help clinicians to differentiate between prerenal and renal azotemia. Evaluation of these factors should always be combined with routine clinical parameters, such as the severity of azotemia and presence or absence of glucosuria, cylindruria, and renal tubular epithelial cells, indicative of AKI. Application of novel kidney injury biomarkers, such as neutrophil gelatinase-associated lipocalin (Segev et al., 2013), could also be helpful in this context.

Since all data collections in this study were available from 3 previously performed prospective studies, many limitations inherent to retrospective studies were either absent or present to a lesser extent. Case inclusion and documentation of the medical records were standardized procedures during the previously performed prospective data collections, reducing inherent retrospective limitations. The use of different instruments (hematology and biochemistry analyzers, refractometers) throughout the study is a limitation inherent to the inclusion of data originating from 3 different cohorts. Although no sampling bias was present for the initial population of 297 dogs, urinalysis at admission was required for final inclusion in this study, which still could have led to both an under- or overestimation of RA in the final population of 152 dogs. In 2 of the cohorts (189/297 dogs), an attempt to collect urine at admission was made whenever a palpable urinary bladder was present, irrespective of the clinical condition of the dog. In the third cohort (108/297 dogs), urine collection was more likely to be performed in dogs that were hospitalized, potentially creating an overestimation of RA.

The occurrence of RA in 14% of the dogs was evaluated at admission only. It is highly likely that additional dogs have developed RA after presentation or during hospitalization. Importantly, RA includes dogs in advanced stages of AKI only. Because AKI represents a continuum of disease severity, ranging from very mild to severe kidney injury, our study design, which was based on a traditionally used cut-off value of urinary concentration to define RA, has obvious limitations and is unable to detect the majority of dogs with milder forms of AKI. Dogs with prerenal, fluid-responsive azotemia can also have AKI, but were categorized in the group of dogs without RA in this study. Lastly, dogs with mild azotemia might have been misclassified

as non-azotemic because of hemolysis-induced underestimation of sCr concentrations or an increased plasma volume after infection (Braun et al., 2003; de Scally et al., 2004; Schetters et al., 2009). Considering that 81% of azotemic dogs in this study had RA, and taking into account that dogs with prerenal azotemia can also sustain AKI, any dog that presents with azotemia should be considered to have AKI. Occurrence of RA in 14% is clearly an underestimation of AKI in general, also because several studies have already documented that milder forms of AKI are very common in dogs with *B. rossi* infection (Lobetti and Jacobson, 2001; Defauw et al., 2012). But overall, identifying those dogs that are most severely affected (i.e., dogs with RA) has proven its worth in many clinical situations in order to optimize individual management.

Urinary sodium was not measured in this study. Dogs with prerenal azotemia but normal tubular function will retain sodium, leading to low urinary sodium concentrations. In contrast, dogs with intrinsic AKI will have high urinary sodium concentrations due to tubular dysfunction (Waldrop, 2008). As a consequence, potential differences in urinary sodium concentrations between dogs with and without RA could have impacted the measurements of uOsmol. Another limitation regarding the osmolality was its measurement after long-term storage of urine samples at -80 °C. Samples were stored from 1 to 11 years before analysis. Although uOsmol measured by freezing point depression was shown to remain stable in human urine stored at -22 °C for a very long time (at least 15 years) (Remer et al., 2014), no data on long-term stability are available in dogs. A recently published research study in dogs did document a minor decrease in uOsmol during 90 days of storage at -80 °C (Reinhart et al., 2016). However, only 5 urine samples were evaluated in that study and the maximum change in uOsmol over time was very small (less than 5%). Hence, we cannot exclude that some dogs with a uOsmol close to 1110 mOsmol/kg might have been misclassified.

Conclusion

Our study demonstrated that RA is a relatively frequently occurring complication in canine babesiosis caused by B. *rossi*. Moreover, RA was associated with a higher mortality. Furthermore, compared to the gold standard method of urinary concentration (i.e., uOsmol), USG overestimated renal concentrating ability in the majority of azotemic dogs with babesiosis, which could lead to them being misclassified as having prerenal azotemia. The reliability of USG to measure urinary concentration should also be considered and investigated in other hemolytic diseases, because severe hemoglobinuria is the most likely cause of the documented discrepancy between USG and uOsmol.

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CHAPTER 6

ACUTE KIDNEY INJURY IN UNCOMPLICATED AND COMPLICATED CANINE BABESIOSIS

EVALUATION OF ACUTE KIDNEY INJURY IN DOGS WITH UNCOMPLICATED AND COMPLICATED *BABESIA ROSSI* INFECTION

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Summary

Dogs with babesiosis can present with multiple complications, including acute kidney injury (AKI). The objective of this study was to characterize AKI in dogs with babesiosis caused by *Babesia rossi* at presentation and after treatment. Thirty-five client-owned dogs with *B. rossi*-infection and 10 control dogs were included in this prospective observational study. Blood and urine were collected in *Babesia*-infected dogs at presentation (T0, n=35), after 24 hours (T24h, n=11), and after 1 month (T1m, n=9). The following urinary kidney injury biomarkers were assessed: urinary protein to creatinine ratio (UPC), urinary glomerular injury biomarkers (immunoglobulin G (uIgG) and C-reactive protein (uCRP)), and urinary tubular injury biomarkers (retinol-binding protein (uRBP) and neutrophil gelatinase-associated lipocalin (uNGAL)). Serum functional renal biomarkers were creatinine (sCr) and symmetric dimethylarginine (sSDMA). Post-mortem kidney biopsies were analyzed by light and transmission electron microscopy.

At T0, all kidney injury biomarkers were significantly higher in *Babesia*-infected dogs compared to healthy controls ($P<0.001$), while functional renal biomarkers were not significantly different ($P>0.05$). At T24h, all urinary tubular injury biomarkers and UPC decreased significantly ($P<0.01$), while glomerular injury biomarkers did not ($P=0.084$). At T1m, all urinary kidney injury biomarkers decreased to values not significantly different from healthy controls ($P>0.5$). Significant changes in functional renal biomarkers were not seen after treatment ($P>0.05$). Dogs with complicated babesiosis had significantly higher glomerular injury biomarkers, UPC, and sSDMA compared to uncomplicated cases ($P<0.05$), while all tubular injury biomarkers and sCr were not significantly different ($P>0.1$).

Dogs with babesiosis caused by *B. rossi* showed transient kidney injury, which was detected by all kidney injury biomarkers, but remained undetected by functional biomarkers.

Introduction

Babesia rossi is an intra-erythrocytic protozoan, capable of causing life-threatening disease in infected dogs (Jacobson, 2006). In South Africa, severity of this disease ranges from mild to peracutely fatal (Jacobson and Clark, 1994). *Babesia rossi*-infected dogs can present with a variety of complications, including AKI, hypotension, hemoconcentration, immune-mediated hemolytic anemia (IMHA), hepatopathy, hypoglycemia, pancreatitis, cerebral babesiosis, and acute respiratory distress syndrome (ARDS) (Jacobson and Clark, 1994; Welzl et al., 2001; Keller et al., 2004; Jacobson, 2006; Defauw et al., 2012; Köster et al., 2015). There is accumulating evidence that an excessive pro-inflammatory host response is a major cause of these *B. rossi*-associated complications (Welzl et al., 2001; Köster et al., 2015; Goddard et al., 2016).

Several studies documented that dogs infected with *B. rossi* often present with mild, non-azotemic manifestations of AKI, while overt acute renal failure (ARF) is less common (Jacobson and Clark, 1994; Lobetti and Jacobson, 2001; Welzl et al., 2001; Defauw et al., 2012). In a recently published study, renal azotemia was however diagnosed in 14% of dogs with babesiosis caused by *B. rossi*, documenting that renal azotemia is a relatively frequent complication (Defauw et al., 2018). Grading of AKI was introduced by the International Renal Interest Society (IRIS) in order to emphasize that AKI can vary from mild to severe kidney injury with overt ARF (http://www.iris-kidney.com/pdf/4_ldc-revised-grading-of-acute-kidney-injury.pdf). Diagnosis of such milder forms of AKI is difficult, especially in the setting of canine babesiosis. At least 75% of the nephrons must be nonfunctional before the routine functional renal biomarker sCr will increase above the upper limit of the reference interval (Braun et al., 2003; Hokamp and Nabity, 2016). In canine babesiosis, traditional assessment of renal function based on sCr is hampered in several additional ways. The presence of hemolysis, leading to elevated serum bilirubin and free hemoglobin, can result in interference with the most commonly used laboratory techniques measuring sCr (Braun et al., 2003; de Scally et al., 2004). Moreover, after experimentally infecting dogs with *B. canis*, decreasing plasma creatinine concentrations were seen, likely due to an increased plasma volume after infection (Schetters et al., 2009). Alternatively, a

reduced creatinine production in sepsis and in critical illness could also explain this trend (Doi et al., 2009; Wilson et al., 2012).

Considering these general as well as babesiosis-specific limitations, characterization of babesiosis-induced AKI based on sCr is severely hampered. More sensitive alternative biomarkers are needed to evaluate AKI in canine babesiosis. Novel urinary biomarkers can detect kidney injury at an early stage and can quantify and localize injury to a specific origin (i.e., glomerular versus tubular region) (De Loor et al., 2013; Hokamp and Nabity, 2016). Early recognition of AKI using these sensitive urinary biomarkers allows earlier therapeutic intervention (De Loor et al., 2013; Hokamp and Nabity, 2016). Symmetric dimethylarginine is a new functional renal biomarker that correlates strongly with glomerular filtration rate (GFR) in dogs with chronic kidney disease (CKD) (Nabity et al., 2015). In a recent study, plasma SDMA was also able to identify dogs with azotemic AKI (Dahlem et al., 2017). In humans, follow-up of renal function after an episode of AKI is advised at least in high risk patients, because patients surviving an AKI episode have an independently increased risk to develop CKD (Coca et al., 2012; Rimes-Stigare et al., 2015; Heung et al., 2016; Vanmassenhove et al., 2018). Kidney injury biomarkers have the potential to predict renal recovery from AKI in humans (Koraishy and Coca, 2014).

The main objective of this study was to characterize AKI in dogs with babesiosis caused by *B. rossi* at presentation, during hospitalization, and at follow-up 1 month (m) after treatment, comparing routine and novel biomarkers of kidney injury and dysfunction. As a secondary aim, the same biomarkers were compared between dogs with and without complicated babesiosis to determine the effect of complicated disease on kidney injury and dysfunction.

Materials and Methods

Study Population and Design

Client-owned dogs presenting with babesiosis to the Onderstepoort Veterinary Academic Hospital (OVAH, University of Pretoria, South Africa) were prospectively evaluated for inclusion in this observational study between December 2015 and January 2017. Initial diagnosis of babesiosis was made based on presence of compatible clinical signs and detection of large *Babesia* species on a stained blood smear. The causative agent was later confirmed as *B. rossi* based on polymerase chain reaction (PCR) and reverse line blot (RLB) (Matjila et al., 2008). During the same period, clinically healthy client-owned control dogs of comparable age and weight presenting to the OVAH for vaccination, routine sterilization, or blood donation, were also recruited. The study was approved by the Animal Ethics Committee of the University of Pretoria (protocol numbers V034-14 and V098-15) and informed owner consent was obtained.

In the dogs with babesiosis, a thorough medical history, physical examination, complete blood count, biochemistry profile (sCr, serum urea, glucose, total protein, albumin, globulin, alanine aminotransferase, alkaline phosphatase, total bilirubin, and phosphate) and a complete routine urinalysis (including UPC and aerobic bacterial culture) was performed at presentation (T0), also to exclude concurrent diseases. Blood pressure was measured in all dogs with babesiosis using oscillometry. Blood and urine samples were collected before any therapy, especially fluid therapy, was initiated. Control dogs were considered healthy based on the absence of clinically relevant abnormalities in the medical history, physical examination, complete blood count, biochemistry profile, and a complete routine urinalysis (including a UPC <0.5). Urine was collected by cystocentesis in all dogs.

In both groups, dogs with a urinary tract infection (UTI) at T0 were excluded based on bacterial growth detection in urine within 3 days of incubation. Dogs with suspected pre-existing CKD were excluded as best as possible based on a thorough history and physical examination. Dogs were also excluded if wounds or trauma were evident, if concurrent neoplastic, inflammatory, cardiac disease, or other infections

were diagnosed, or if they were known to have been treated with anti-inflammatory medication within 4 weeks prior to presentation. PCR/RLB batch analysis for babesiosis and ehrlichiosis was performed in both groups. Dogs with babesiosis were included only after confirmation of *B. rossi* infection and after exclusion of *B. vogeli* and/or concurrent *Ehrlichia canis* infection. Control dogs that tested positive for any *Babesia* species or *Ehrlichia canis* were excluded.

In dogs with babesiosis, follow-up blood and urine samples were obtained 24 hours (h) after presentation (T24h) and 1 m after treatment (T1m) whenever possible. Follow-up samples at T24h were only collected in dogs that were hospitalized because of disease severity. At T24h, sCr was measured and a complete urinalysis was performed. A complete blood count, biochemistry profile and complete urinalysis as well as PCR/RLB for babesiosis and ehrlichiosis was repeated at T1m.

All infected dogs received antibabesial treatment with diminazene aceturate (Berenil RTU, Intervet, Kempton Park, South Africa) at 3.5 mg/kg, and standard supportive care such as packed red blood cells and intravenous fluid therapy as needed. Any complications were treated as needed by the attending clinician. Dogs were diagnosed with complicated babesiosis when at least one of the following complications were diagnosed either at presentation or during hospitalization. These criteria were based on previous publications defining complicated babesiosis and severe falciparum malaria (Jacobson and Clark, 1994; Jacobson, 2006; WHO, 2014). Dogs were considered to be collapsed at presentation when they were unable to walk unaided. Hypotension was defined as a mean arterial pressure <80 mmHg. Severe anemia was diagnosed when the hematocrit was <0.15. Hemoconcentration was defined as a hematocrit >0.37 in combination with signs of intravascular hemolysis (macroscopic hemoglobinuria and/or hemoglobinemia). Secondary IMHA was diagnosed based on a positive in-saline agglutination test. Diagnosis and grading of AKI was based on IRIS criteria (http://www.iris-kidney.com/pdf/4_ldc-revised-grading-of-acute-kidney-injury.pdf). Grade I AKI was defined as a progressive increase in sCr (of at least 26.4 µmol/L) within the non-azotemic range and within 48 h. Hypoglycemia was diagnosed when blood glucose was <3.3 mmol/L. Jaundice was diagnosed when severe hyperbilirubinemia was present (total bilirubin >50 µmol/L). Cerebral babesiosis was defined as presence of neurological signs not attributable to another cause, such as hypoglycemia. Clinical diagnosis of ARDS was based on presence of dyspnea and

variably on arterial blood gas analysis, radiological evidence of pulmonary edema, and exclusion of other causes of pulmonary edema. Outcome categories were short-term survival (i.e., survival until discharge) or death/euthanasia due to poor prognosis (as deemed by the attending clinician), and long-term survival (i.e., survival based on telephone contact with owners 4 to 5 weeks after presentation).

Sample Handling and Laboratory Analyses

Automated analyzers were used for complete blood count (ADVIA 2120, Siemens, Munich, Germany) and serum biochemistry analysis (Cobas Integra 400 plus, Roche Diagnostics, Mannheim, Germany). Serum creatinine was determined using a modified Jaffe method.

A complete urinalysis, including dipstick (Combur 9 Test®, Roche Diagnostics, Germany), urine specific gravity (USG), microscopic sediment analysis within 60 minutes, UPC, and aerobic bacterial culture, was performed. After urine collection, 0.5 ml of uncentrifuged urine was submitted for aerobic bacterial culture. After centrifugation of remaining urine (3 minutes at 447 x *g*), the supernatant was used for dipstick analysis, measurement of USG by refractometry (Atago MASTER-SUR/NM, Atago Co., Tokyo, Japan), and measurement of UPC. Remaining supernatant was divided into aliquots of 0.5-1 ml, followed by storage at -80 °C. Remaining plasma and serum of all samples were also stored at -80 °C. PCR/RLB batch analysis was performed on stored packed blood cell pellets. Urine osmolality (uOsmol) was measured as a batch by freezing point depression within 10 m after collection (Micro-Osmometer Autocal Type 13/13DR, Roebling, Berlin, Germany). Osmolality of a calibration standard solution was measured in triplicate every day before sample analysis. All samples were measured in duplicate or triplicate, and the mean uOsmol and intra-assay coefficient of variation were calculated. Calculated mean uOsmol values from an individual dog at one time point were used, since individual coefficient of variation values were all <1% and global intra-assay coefficient of variation was 0.16%. Frozen samples were transported on dry ice from South Africa to Belgium for renal biomarker analysis. Upon arrival in Belgium, samples were still frozen and stored at -72 °C until biomarker analysis.

Kidney injury biomarkers, measured by commercial immunoassays at T0, T24h, and T1m, included uIgG, uCRP, uRBP, uNGAL, and plasma NGAL (pNGAL). Measurement of uIgG and uCRP was performed with canine sandwich ELISA kits (Immunology Consultants Laboratory, Newberg, USA), while uRBP was measured with a human sandwich ELISA kit (Immunology Consultants Laboratory, Newberg, USA). Validation of the IgG, CRP, and RBP assays for use in canine urine was previously performed in our laboratory (Maddens et al., 2010). Measurement of uNGAL and pNGAL was performed with a validated canine sandwich ELISA kit (BioPorto Diagnostics A/S, Hellerup, Denmark). Quantification of uIgG, uCRP, uRBP, uNGAL and pNGAL by immunoassays was performed as described (Defauw et al., 2012). Finally, urinary biomarker concentrations were normalized to urinary creatinine concentrations (/uCr). By dividing absolute uNGAL concentrations by pNGAL concentrations, the u/pNGAL concentration ratio was calculated (Uttenthal et al., 2012). The purpose of uNGAL to pNGAL normalization was to aid in the differentiation between kidney injury and non-renal increases of NGAL. All analyses of kidney injury biomarkers were performed within 6 m of sample collection, except 1 sample for uIgG and 4 samples for uRBP that required re-analysis with another dilution. Serum SDMA (IDEXX SDMA^{TM} Test) was measured as a novel functional renal biomarker at T0, T24h, and T1m. Serum samples were transported on dry ice to a reference diagnostic laboratory (IDEXX Laboratories, Inc., Ludwigsburg, Germany) for batch analysis. All sSDMA samples were analyzed within 24 m after collection.

Renal Samples, Light Microscopy, and Transmission Electron Microscopy

Post-mortem kidney biopsies were obtained whenever possible. Tissue samples were fixed in 10% buffered formalin for light microscopy (LM), and in 3% buffered glutaraldehyde for transmission electron microscopy (TEM). Samples were processed into paraffin wax blocks for LM and resin-embedded blocks for TEM. For LM, sections were stained with hematoxylin and eosin, periodic acid-Schiff (PAS), Masson's trichrome, and periodic acid methenamine silver, according to standard procedures (Aresu et al., 2017). All sections for LM and TEM were examined by a board-certified veterinary pathologist (LA).

Statistical Analysis

As the normal distribution assumption did not hold for some of the biomarkers, Mann-Whitney U tests were used to compare renal biomarkers between dogs with and without babesiosis, and between dogs with complicated and uncomplicated babesiosis. Renal biomarkers in *B. rossi*-infected dogs were compared between T0, T24h, and T1m using Wilcoxon signed-rank tests for paired samples. *P* values <0.05 were considered significant. Data were visualized through box plots. All analyses were performed using R (R version 3.3.2 Copyright © 2016, The R Foundation for Statistical Computing).

Results

Study Population and Clinicopathological Findings

Forty-three dogs with babesiosis were evaluated for inclusion. Eight dogs were excluded for the following reasons: based on PCR/RLB analysis, 4 dogs tested positive for *Ehrlichia canis*, one dog was infected with *B. vogeli,* and one dog was repetitively negative by PCR/RLB for *Babesia* species; 2 dogs had a UTI at presentation. For practical reasons, aerobic bacterial culture was not performed in urine of 3 dogs. These 3 dogs were included, because a UTI was considered unlikely, based on the absence of compatible clinical signs and of pyuria and bacteriuria on sediment analysis.

Finally, 35 of the 43 dogs with *B. rossi* infection were included. Ten dogs served as healthy controls. There was no significant age difference between dogs with babesiosis (median, 2.5 years (y); range: 2 m–12.5 y) and controls (2 y; 4 m–9.5 y) (*P*=0.47). Body weight also did not differ significantly between *Babesia*-infected dogs (22 kg; 2.1–48 kg) and controls (28.45 kg; 4.9–61 kg) (*P*=0.20).

In the babesiosis group, there were 9 mixed breed dogs, 2 American pit bull terrier, 2 Boerboel, 2 Jack Russell terrier, 2 Labrador retriever, 2 Pekingese, 2 Weimaraner, and a single dog from other breeds. The controls consisted of 3 German shepherd, 2 mixed breed dogs, and a single dog from other breeds. Hematology and biochemistry results of both groups are summarized in Table 6.1.

Table 6.1. Clinicopathological findings in 10 healthy control dogs and 35 dogs with babesiosis at presentation. All variables are expressed as median (range).

Variable (unit)	RI	H	B
Hematocrit (L/L)	0.37–0.55	0.47 (0.41–0.57)	0.23 (0.06–0.63)
WBC (x 10^9 /L)	6–15	8.75 (7.01–12.4)	6.75 (1.93–22.15)
Segmented neutrophil count (x 10^9 /L)	3–11.5	5.77 (2.94–8.93)	4.32 (1.17–15.28)
Band neutrophil count (x 10^9 /L)	0–0.5	0 (0–0.12)	0.16 (0–2.13)
Platelet count (x 10^9 /L)	200–500	281 (142–467)	11 (0–166)
Total serum protein (g/L)	56–73	55 (48–68)	56 (42–81)
Serum albumin (g/L)	28–41	35 (33–38)	25 (14–36)
sCr (µmol/L)	59–109	82 (47–104)	63 (25–132)
Serum urea (mmol/L)	2.3–8.9	5.6 (2.8–8.3)	7.4 (3.0–27.4)
Glucose (mmol/L)	3.3–5.5	5.6 (5.1–6.8)	5.1 (0.3–9.8)
Alanine aminotransferase (U/L)	9–73	35 (22–75)	30 (10–124)
Alkaline phosphatase (U/L)	20–165	58 (16–229)	124 (23–631)
Total bilirubin (µmol/L)	1–6.8	ND	8.6 (1.8–248.7)
Inorganic phosphorus (mmol/L)	0.7–2.1	ND	1.43 (0.73–3.55)

RI, reference interval; H, healthy control dogs; B, dogs with babesiosis; WBC, white blood cell count; sCr, serum creatinine; ND, not done.

In the dogs with babesiosis, urine ranged from clear to very turbid and urine color ranged from yellow to dark brown. In the urinary dipstick analysis of *Babesia*-infected dogs, pH ranged from 5 to 8, proteinuria from 1+ to 3+, bilirubinuria from negative to 3+, hemoglobinuria from negative to 4+ (4+ in 30/35 dogs), while glucose and ketones were always negative. Bilirubin crystalluria was present in 9 dogs with babesiosis. Rare to large amounts of granular casts were seen in 23 *Babesia*-infected dogs, while rare hyaline casts were seen in 5 *Babesia*-infected dogs. Microscopic hematuria (i.e., >5 red blood cells/high power field) was seen in 6 *Babesia*-infected dogs, while pyuria (i.e., >5 white blood cells/high power field) was absent in all dogs.

AKI Biomarkers at T0

In Table 6.2, results of routine renal biomarkers (sCr, serum urea, USG, uOsmol, and UPC) at T0 are compared between healthy controls and dogs with babesiosis. In Figure 6.1 and Table 6.3, results of kidney injury biomarkers (uIgG/uCr, uCRP/uCr, uRBP/uCr, uNGAL/uCr, pNGAL, u/pNGAL) and sSDMA at T0 are

presented and compared between both groups. All kidney injury biomarkers, including UPC, were significantly higher in *Babesia*-infected dogs compared to healthy controls, while sCr, serum urea, USG, uOsmol, and sSDMA did not significantly differ between both groups at T0. However, when healthy controls were compared with the subgroup of dogs with uncomplicated babesiosis, sCr was significantly lower in the dogs with uncomplicated disease (*P*=0.009).

Based on the reference interval (0–14 µg/dL for adult dogs, 0–16 µg/dL for dogs up to 12 m), 3/10 healthy dogs (30%) and 16/35 dogs with babesiosis (46%) showed increased sSDMA concentrations. On PE, 7/35 dogs with babesiosis had clinical signs of dehydration, of which 4 had increased sSDMA concentrations.

Follow-up at T24h and T1m

Twenty-four of the 35 dogs with babesiosis were hospitalized. At T24h, follow-up blood and urine samples were collected in 11 dogs with babesiosis, and follow-up samples were available from 9 *Babesia*-treated dogs at T1m. Seven of these dogs had follow-up samples collected at both T24h and T1m. Because of a UTI, one dog at T24h and 2 dogs at T1m were excluded from urinary kidney injury biomarker analyses. One of the 9 dogs had a faintly positive PCR/RLB test for a *Babesia* species at T1m. With exception of sSDMA, which was borderline increased (15 µg/dL), all renal biomarkers in this dog were normalized at T1m. Short-term survival was 89% (31/35 dogs). Two dogs died spontaneously within 8 h after admission, while 2 dogs were euthanized during hospitalization due to poor prognosis. As for long-term survival, all dogs discharged from the hospital survived, but 2 dogs were lost to follow-up.

In Table 6.2, routine renal biomarkers results are compared between T0, T24h, and T1m. In Figure 6.1 and Table 6.3, results of kidney injury biomarkers and sSDMA are compared between T0, T24h, and T1m. Results of biomarker comparison between healthy controls and *Babesia*-treated dogs at T1m are also shown in Figure 6.1, Table 6.2 and 6.3.

Table 6.2. Results of routine renal biomarkers in healthy control dogs (n=10) and in dogs with babesiosis at presentation (n=35), 24 hours after presentation (n=11), and 1 month after treatment (n=9). All variables are expressed as median (range).

Variable (unit)	H	B T0	B T24h	B T1m	P^a	P^b	P^c	P^d
sCr (µmol/L)	82	63	67	76	0.093	0.18	0.57	0.54
	(47–104)	(25–132)	(25–129)	(46–110)				
Serum urea (mmol/L)	5.6	7.4			0.096			
	(2.8–8.3)	(3.0–27.4)						
USG	1.039	1.042	1.032	1.023	0.13	0.057	0.008	0.39
	(1.017–1.050)	(1.015–>1.060)	(1.022–1.045)	(1.018–1.051)				
uOsmol (mOsmol/kg)	1254	1315	1062	927	0.45	0.15	0.098	0.35
	(579–2099)	(430–2470)	(683–1704)	(676–2211)				
UPC	0.14	2.63	1.02	0.11	<0.001	0.002	0.016	0.56
	(0.08–0.36)	(0.34–15.42)	(0.22–4.02)	(0.07–0.29)				

H, healthy control dogs; B T0, dogs with babesiosis at presentation; B T24h, dogs with babesiosis 24 hours after presentation; B T1m, dogs with babesiosis 1 month after treatment; sCr, serum creatinine; USG, urine specific gravity; uOsmol, urine osmolality; UPC, urinary protein to creatinine ratio.

[a]*P* values for the comparison between healthy control dogs and dogs with babesiosis at T0

[b]*P* values for the comparison between dogs with babesiosis at T0 and T24h

[c]*P* values for the comparison between dogs with babesiosis at T0 and T1m

[d]*P* values for the comparison between healthy control dogs and dogs with babesiosis at T1m

Table 6.3. Kidney injury biomarkers and serum SDMA in healthy control dogs (n=10) and dogs with babesiosis at presentation (n=35), 24 hours after presentation (n=11), and 1 month after treatment (n=9). All variables are expressed as median (range).

Variable (unit)	H	B T0	B T24h	B T1m	P^a	P^b	P^c	P^d
uIgG/uCr (mg/g)	1.93 (0.55–11.32)	435.43 (6.95–11544.30)	190.68 (4.62–1362.46)	1.29 (0.78–10.21)	<0.001	0.084	0.016	0.88
uCRP/uCr (mg/g)	BDL (n=10)	0.13 (BDL–17.63) BDL(n=2);BQL(n=2)	0.14 (BDL(n=1)–0.43)	BDL (n=10)	<0.001	0.084	0.016	1.0
uRBP/uCr (mg/g)	0.012 (BDL–0.034) BDL(n=2);BQL(n=2)	0.92 (0.26–7.71)	0.74 (0.020–1.16)	BQL (BQL(n=4)–0.32)	<0.001	0.006	0.016	0.73
uNGAL/uCr (µg/g)	0.76 (0.21–7.08)	377.57 (43.01–2645.43)	246.82 (29.60–782.87)	0.30 (0.08–28.06)	<0.001	0.002	0.016	0.53
pNGAL (ng/mL)	6.09 (2.08–12.32)	10.71 (5.95–121.77)	15.38 (6.17–29.68)	12.09 (3.52–15.41)	<0.001	0.12	0.30	0.028
u/pNGAL	0.21 (0.024–3.40)	54.77 (19.98–208.54)	31.82 (1.98–68.13)	0.28 (0.032–1.51)	<0.001	0.008	0.063	0.76
sSDMA (µg/dL)	14 (10–24)	14 (8–45)	17 (11–20)	12 (9–15)	0.68	0.88	0.078	0.12

H, healthy control dogs; B T0, dogs with babesiosis at presentation; B T24h, dogs with babesiosis 24 hours after presentation; B T1m, dogs with babesiosis 1 month after treatment; uIgG, urinary immunoglobulin G; uCr, urinary creatinine; uCRP, urinary C-reactive protein; BDL, below detection limit; BQL, below quantification limit; uRBP, urinary retinol-binding protein; uNGAL, urinary neutrophil gelatinase-associated lipocalin; pNGAL, plasma neutrophil gelatinase-associated lipocalin; u/pNGAL, urinary to plasma neutrophil gelatinase-associated lipocalin ratio; sSDMA, serum symmetric dimethylarginine.

[a]*P* values for the comparison between healthy control dogs and dogs with babesiosis at T0

[b]*P* values for the comparison between dogs with babesiosis at T0 and T24h

[c]*P* values for the comparison between dogs with babesiosis at T0 and T1m

[d]*P* values for the comparison between healthy control dogs and dogs with babesiosis at T1m

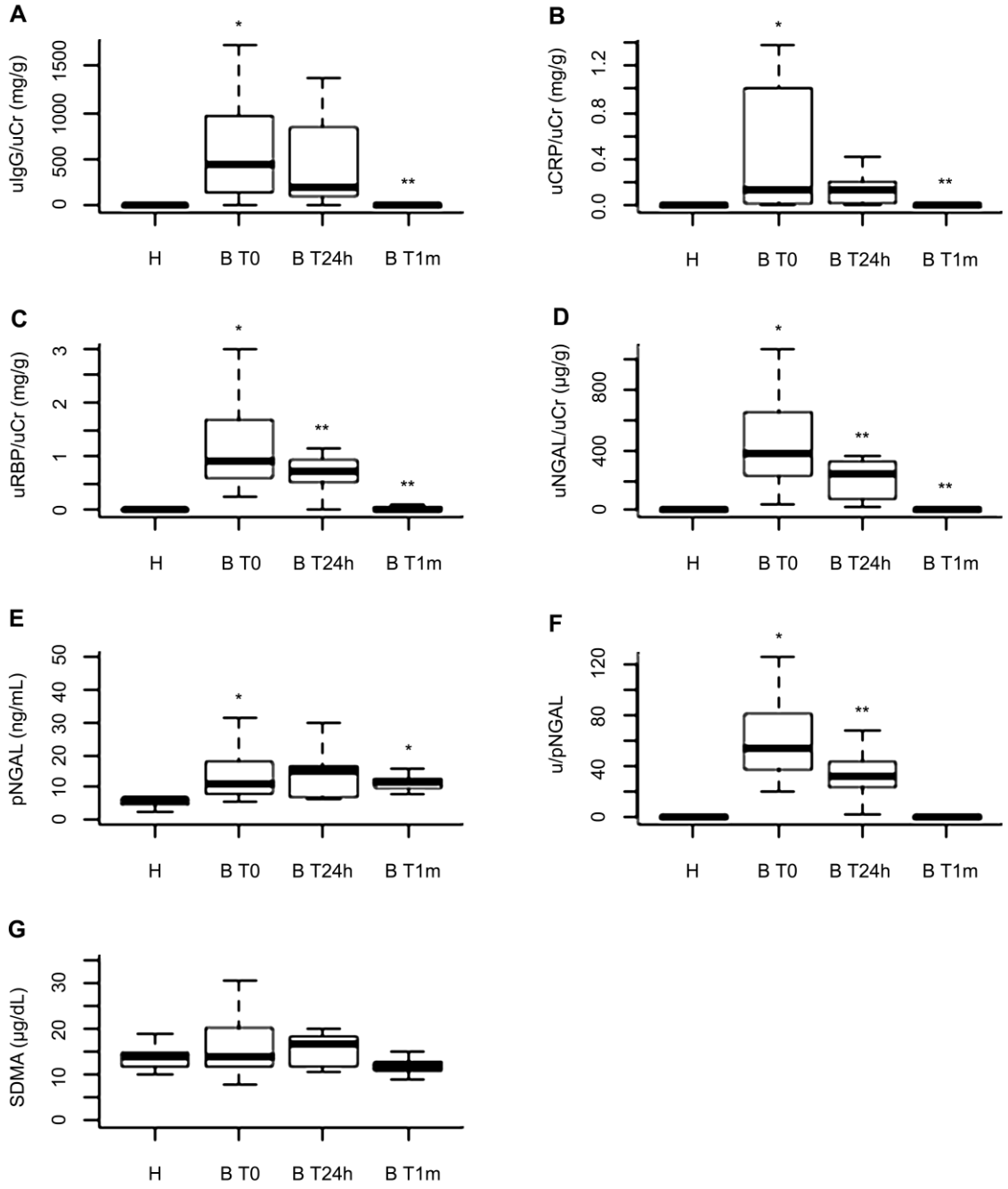

Figure 6.1. Kidney injury biomarkers (**A**, uIgG/uCr; **B**, uCRP/uCr; **C**, uRBP/uCr; **D**, uNGAL/uCr; **E**, pNGAL; **F**, u/pNGAL) and serum SDMA (**G**) in healthy control dogs (H; n=10) and dogs with babesiosis at presentation (B T0; n=35), 24 hours after presentation (B T24h; n=11), and 1 month after treatment (B T1m; n=9). Data are presented as boxes and whiskers. Each box includes the interquartile range and the line within each box represents the median. The whiskers represent the range, extending to a maximum of 1.5 times the interquartile range. Outliers are not shown. * Significantly different (P<0.05) from healthy control dogs. ** Significantly different (P<0.05) from dogs with babesiosis at presentation.

At T24h, UPC, uRBP/uCr, uNGAL/uCr, and u/pNGAL decreased significantly (Tables 6.2 and 6.3, Figure 6.1). At T1m, decreases were significant for USG and all urinary kidney injury biomarkers (Tables 6.2 and 6.3, Figure 6.1). Significant changes in sCr, sSDMA, pNGAL, and uOsmol were not seen during follow-up at T24h and T1m. At T1m, all renal biomarkers had values not significantly different from the healthy controls, except for pNGAL which was still significantly higher in the *Babesia*-treated dogs. At T24h, 6/11 dogs with babesiosis (55%) had increased sSDMA concentrations, while at T1m 2/9 dogs with babesiosis (22%) had a borderline increased sSDMA (15 µg/dL).

Uncomplicated and complicated babesiosis at presentation and during hospitalization

Ten dogs were diagnosed with uncomplicated babesiosis, while at least one complication occurred in each of the remaining 25 dogs. At T0, 7 dogs were collapsed, 4 dogs were hypotensive, 9 dogs were severely anemic, 3 dogs were diagnosed with hemoconcentration, while 4 dogs presented with secondary IMHA. Based on sCr, 3 dogs were mildly azotemic and diagnosed with AKI grade II. Two of these dogs had prerenal azotemia based on presence of concentrated urine (USG ≥1.030; uOsmol ≥1110 mOsmol/kg). The other dog was diagnosed with renal azotemia (USG 1.018; uOsmol 430 mOsmol/kg). Four dogs were hypoglycemic, while jaundice was diagnosed in 5 dogs. Cerebral babesiosis was diagnosed in 2 dogs.

At T24h, sCr was available in 13 dogs and 5 additional cases of AKI were diagnosed (3 and 2 dogs with AKI grade I and II, respectively). Finally, one dog was diagnosed with ARDS during hospitalization. Of the 9 dogs with follow-up available at T1m, 2 dogs were presented with AKI grade II at T0, while 2 dogs developed AKI grade I and one dog AKI grade II during hospitalization. In these 5 dogs diagnosed with AKI at T0 or T24h, no evidence of kidney injury or dysfunction was detected at T1m, except for one dog with a borderline increased sSDMA (15 µg/dL).

AKI Biomarkers in Uncomplicated and Complicated Babesiosis at T0

In Table 6.4, results of routine renal biomarkers, kidney injury biomarkers, and sSDMA at T0 are compared between the subgroups of dogs with uncomplicated and complicated disease. Dogs with complicated babesiosis had significantly higher uIgG/uCr, uCRP/uCr, UPC, serum urea, and sSDMA, while uRBP/uCr, uNGAL/uCr, pNGAL, u/pNGAL, USG, uOsmol, and sCr did not differ significantly between groups. Increased sSDMA concentrations were seen in 14/25 dogs with complicated disease (56%), while 2/10 dogs with uncomplicated babesiosis (20%) had increased sSDMA concentrations. Finally, sSDMA was increased in 2/3 dogs with an elevated sCr at T0.

Table 6.4. Results of routine renal biomarkers, kidney injury biomarkers, and serum SDMA in 10 dogs with uncomplicated and 25 dogs with complicated babesiosis at T0. All variables are expressed as median (range).

Variable (unit)	Uncomplicated disease	Complicated disease	*P* value
sCr (µmol/L)	53 (32–83)	70 (25–132)	0.27
Serum urea (mmol/L)	4.8 (3.0–8.2)	8.6 (3.4–27.4)	0.007
USG	1.045 (1.035–>1.060)	1.040 (1.015–>1.060)	0.30
uOsmol (mOsmol/kg)	1509 (1199–2470)	1236 (430–2118)	0.054
UPC	1.24 (0.34–3.79)	3.18 (0.51–15.42)	0.008
uIgG/uCr (mg/g)	174.40 (6.95–3373.12)	705.53 (38.86–11544.30)	0.025
uCRP/uCr (mg/g)	0.014 (BDL–1.07)	0.47 (BDL–17.63)	0.001
	BDL (n=1); BQL (n=2)	BDL (n=1)	
uRBP/uCr (mg/g)	0.83 (0.29–2.07)	0.92 (0.26–7.71)	0.51
uNGAL/uCr (µg/g)	322.32 (43.01–602.81)	401.38 (77.44–2645.43)	0.11
pNGAL (ng/mL)	13.08 (7.46–31.25)	10.50 (5.95–121.77)	0.73
u/pNGAL	55.23 (21.52–110.00)	50.99 (19.98–208.54)	0.90
sSDMA (µg/dL)	13 (8–23)	17 (10–45)	0.038

sCr, serum creatinine; USG, urine specific gravity; uOsmol, urine osmolality; UPC, urinary protein to creatinine ratio; uIgG, urinary immunoglobulin G; uCr, urinary creatinine; uCRP, urinary C-reactive protein; BDL, below detection limit; BQL, below quantification limit; uRBP, urinary retinol-binding protein; uNGAL, urinary neutrophil gelatinase-associated lipocalin; pNGAL, plasma neutrophil gelatinase-associated lipocalin; u/pNGAL, urinary to plasma neutrophil gelatinase-associated lipocalin ratio; sSDMA, serum symmetric dimethylarginine.

Histological and Ultrastructural Renal Findings in Dogs with Complicated Babesiosis

Within 6 h after spontaneous death, kidney samples were obtained from 2 dogs with complicated babesiosis. Dog 1 was a 2 m old male intact Irish wolfhound, presenting with peracute cerebral babesiosis. The first clinical signs (lethargy and vomiting) were noticed a couple of h before presentation. Serum creatinine was 34 µmol/L, sSDMA was 12 µg/dL, and UPC was 2.76. Urine sediment showed mild microscopic hematuria and absence of casts. Kidney injury biomarkers were as followed: uIgG/uCr: 126.03 mg/g, uCRP/uCr: 0.21 mg/g, uRBP/uCr: 0.26 mg/g, uNGAL/uCr: 77.44 µg/g, pNGAL: 8.57 ng/mL, u/pNGAL: 19.98. A moderate increase of mesangial matrix was seen at glomerular level, but the most significant lesions were evident at tubular level. Multifocally, tubules were necrotic and intratubular protein casts (PAS positive) were identified. Many tubules showed degenerating epithelial cells, characterized by cellular swelling and cytoplasmic rarefaction. Focal tubular epithelial cells showed loss of brush border. The histological diagnosis was compatible with a multifocal, moderate ATN and degeneration further confirmed by TEM.

Dog 2 was a 10 y old male intact mixed breed dog, presenting with a 4 day history of anorexia and lethargy. The dog was collapsed, hypotensive, and severely hypoglycemic at presentation. Serum creatinine was 101 µmol/L, sSDMA was 20 µg/dL, and UPC was 7.73. Urine was dark brown and turbid, sediment showed a large amount of granular casts. Kidney injury biomarkers were as followed: uIgG/uCr: 1708.90 mg/g, uCRP/uCr: 10.55 mg/g, uRBP/uCr: 7.71 mg/g, uNGAL/uCr: 2354.03 µg/g, pNGAL: 121.77 ng/mL, u/pNGAL: 34.02. A moderate increase of mesangial matrix was observed, but no other glomerular or tubular alterations were evident both at LM and TEM.

Discussion

In this population of dogs with babesiosis caused by *B. rossi*, our selected kidney injury biomarkers were all able to detect kidney injury at an earlier stage compared to both serum functional renal biomarkers (i.e., sCr and sSDMA). Earlier recognition of AKI could ultimately lead to an improved outcome by allowing prompt therapeutic interventions and appropriate monitoring. Moreover, there was no evidence of active ongoing kidney injury at T1m, suggesting a transient nature of kidney injury in most dogs after successful treatment. All infected dogs, irrespective of disease severity (uncomplicated versus complicated babesiosis), suffered comparable kidney injury based on tubular injury biomarker concentrations. However, sSDMA was significantly higher in dogs with complicated babesiosis, suggesting that renal function should be monitored more closely in this subgroup of critically ill dogs.

All kidney injury biomarkers were significantly higher in *Babesia*-infected dogs compared to healthy controls, while neither of the functional renal biomarkers differed significantly. Increased urinary concentrations of glomerular and tubular injury biomarkers (i.e., uIgG, uCRP and uRBP) in dogs with uncomplicated *B. rossi* infection have been documented in a previous study from our group (Defauw et al., 2012). Similar findings were observed in 2 recently published papers on *B. canis* infections in Europe, where glomerular and tubular injury were also detected based on increased urinary kidney injury biomarkers (Winiarczyk et al., 2017; Kuleš et al., 2018). Another study involving *B. rossi* demonstrated, based on urine sediment analysis, UPC and urinary enzyme concentrations, that milder manifestations of kidney injury are much more common than overt ARF (Lobetti and Jacobson, 2001). Based on these studies, *B. rossi*-induced AKI manifests itself primarily by kidney injury rather than by loss of function. However, an important limitation is that the functional renal biomarkers used in all these studies are either insensitive (sCr) or not fully validated for use in canine AKI (sSDMA) (Hokamp and Nabity, 2016; Dahlem et al., 2017). Indeed, sSDMA correlates well to GFR in dogs with CKD (Nabity et al., 2015), but the relation between sSDMA and GFR in dogs with AKI remains unknown (Dahlem et al., 2017). A recent study demonstrated that plasma SDMA was an excellent biomarker to identify azotemic AKI in dogs, but dogs with non-azotemic AKI grade I were not evaluated (Dahlem et al., 2017).

In the current study NGAL was used for the first time in *Babesia*-infected dogs as a sensitive biomarker of tubular injury (Segev et al., 2013; Nabity et al., 2015). In a previous study, uNGAL was also increased in dogs with AKI Grade I, showing its potential as a sensitive biomarker of milder forms of AKI (Segev et al., 2013). However, in human medicine (Mårtensson and Bellomo, 2014) and more recently also in veterinary medicine (Cortellini et al., 2015; Cobrin et al., 2016), the specificity of NGAL for AKI has been questioned. During systemic inflammation, pNGAL and uNGAL can increase due to increased synthesis from non-renal tissues and increased release from circulating neutrophils (Mårtensson and Bellomo, 2014). The u/pNGAL ratio was calculated in the current study to help in the differentiation between kidney injury and non-renal causes of increased uNGAL, because relatively higher concentrations of uNGAL compared to pNGAL are expected during kidney injury (Uttenthal et al., 2012). To the author's knowledge, this ratio has never been used in dogs before, but it has been applied in several other species, including humans (Bagshaw et al., 2010; Nielsen et al., 2010; Kaucsár et al., 2016). The highly increased u/pNGAL ratio at T0 is supportive of kidney injury-specific increases of uNGAL in *B. rossi*-infected dogs. Why NGAL appears to be more specific for kidney injury in the current study compared to other studies (Cortellini et al., 2015; Cobrin et al., 2016), may be explained by the fact that all dogs in our study were infected with the same organism which causes a disease in which neutrophilia is uncommon (Scheepers et al., 2011), thus reducing the impact of NGAL originating from circulating neutrophils (Mårtensson and Bellomo, 2014). However, the u/pNGAL ratio does not correct for variations in the urinary flow rate. Moreover, it is important to realize that competition for tubular reabsorption between NGAL and other filtered proteins could result in increased uNGAL and u/pNGAL ratio as well, independent of the presence of tubular injury (Nejat et al., 2012). Consequently, the limited specificity of uNGAL and other LMW proteins for tubular injury remains a difficult issue to interprete and overcome.

Several studies in humans demonstrated an increased risk for CKD and long-term mortality after an AKI episode (Coca et al., 2009; Heung et al., 2016; Vanmassenhove et al., 2018). This increased risk was documented even after milder forms of AKI with apparent fast recovery (Coca et al., 2009; Heung et al., 2016). Using specific biomarkers to detect active ongoing kidney injury might help to predict which dogs are at risk of developing CKD (Cowgill et al., 2016). Therefore, the concentration of renal biomarkers was followed over time in a subset of *B. rossi*-infected dogs to assess the reversibility of kidney injury and dysfunction.

At T24h, all urinary biomarkers of tubular injury (i.e., uRBP and uNGAL) and u/pNGAL decreased significantly more rapidly compared to those of glomerular injury (i.e., uIgG and uCRP), whose decreases only became significant by T1m. This finding indicates that ongoing tubular injury quickly abates, while uIgG and uCRP concentrations take longer to decrease likely due to ongoing glomerular injury and persistently increased serum concentrations of these proteins. Several studies demonstrated increased serum CRP concentrations in canine babesiosis, and in 2 of these only a mild decrease was seen after 24 h (Matijatko et al., 2007; Köster et al., 2009). Even though glomerular barrier injury must occur before uIgG and uCRP can be detected (D'Amico and Bazzi, 2003), the extent to which variations in their serum concentrations influence these urinary concentrations is unknown. With exception of pNGAL, all kidney injury biomarkers were not significantly different at T1m compared to healthy controls, suggesting that there was no evidence of active ongoing kidney injury in these dogs 1 m after *Babesia*-induced AKI. This finding argues for a transient nature of the kidney insult caused by acute *B. rossi* infections. The reason for increased pNGAL concentrations at T1m is unclear. It is very unlikely that tubular injury was still ongoing, considering the low uNGAL concentrations at that time. Therefore, non-renal causes of increased pNGAL are likely, such as the presence of an unrelated inflammatory condition. Based on sCr and sSDMA, there was no evidence of decreased renal function at T1m. However, sensitivity for decreases in GFR is limited for both of these functional biomarkers, especially for sCr (Hokamp and Nabity, 2016). Moreover, 2 dogs had a borderline increased sSDMA at T1m (15 µg/dL). One of these dogs had a sCr of 110 µmol/L and highly concentrated urine, but the other dog (sCr of 93 µmol/L) had a relatively low urinary concentration at T1m (USG 1.022; uOsmol 776 mOsmol/kg). Based on the normalization of all kidney injury biomarkers, there was no

evidence of active kidney injury in these 2 dogs at T1m. Whether the dog with relatively low urinary concentration had early-stage CKD, is uncertain. Interestingly, this dog was the only dog that had a positive PCR/RLB test for a *Babesia* species at T1m. Although diminazene aceturate is considered to be very effective in achieving clinical recovery, relapses after treatment are often reported, either due to reinfection and/or treatment failure in eliminating the parasite (Penzhorn et al., 1995; Collett, 2000; Ayoob et al., 2010). Although there was no evidence of active kidney injury based on the investigated kidney injury biomarkers in this small number of dogs included at T1m, a long-term follow-up study, including a larger numbers dogs followed over a longer time period, would be necessary to fully clarify if there is an increased risk for the development of CKD related to early-life *Babesia*-induced AKI. It would also be interesting to investigate the long-term effects of subclinical *B. rossi* infection on kidney injury and function, considering that the standard recommended dose of diminazene aceturate (i.e., 3.5 mg/kg) often does not fully eliminate the infection (Penzhorn et al., 1995).

In dogs with complicated disease, both glomerular injury biomarkers were significantly higher, while none of the tubular injury biomarkers differed significantly between uncomplicated and complicated cases. More severe glomerular injury and/or higher systemic concentrations of IgG and CRP in complicated cases could explain these findings. Significantly higher sSDMA concentrations in complicated cases could be explained by a decreased GFR caused by renal hypoperfusion and/or intrinsic AKI (Choi et al., 2017; Dahlem et al., 2017). Renal hypoperfusion, for example due to dehydration, is unlikely to be the main reason of higher sSDMA concentrations in most complicated cases when urinary concentrating ability is taken into account, as a higher uOsmol would be expected in that situation. However, uOsmol was close to being significantly lower in dogs with complicated babesiosis. Overall, these findings confirm that all dogs with babesiosis caused by *B. rossi*, irrespective of disease severity, suffer kidney injury based on the urinary biomarker results, while sSDMA and uOsmol concentrations suggest that the infection also induces significant loss of renal function in many dogs with complicated babesiosis.

Moderate to severe ATN and degeneration, without significant morphological lesions at glomerular level, were identified in dog 1. These findings are consistent with the sparse literature available on renal histological changes in canine babesiosis

caused by large species which document mainly tubular lesions (Maegraith et al., 1957; Hildebrandt, 1981; Irwin and Hutchinson, 1991; Máthé et al., 2007). Unexpectedly, mild morphological changes were found in dog 2, whose clinical presentation and presence of highly elevated kidney injury biomarkers were suggestive of clinically significant AKI. Animals in experimental studies and people with hypoxic and/or septic AKI also often lack histological ATN, either because of its absence or because of mild, focally distributed lesions (Langenberg et al., 2008; Heyman et al., 2010; Takasu et al., 2013). It is well documented in human nephropathology that renal tissue injury is often limited and focal when a clinical diagnosis of AKI/ATN is made (Heyman et al., 2010). Consequently, kidney injury biomarkers could lack accuracy for diagnosing histological ATN, which has already been documented in humans (Moledina et al., 2017). Considering only 2 dogs had histological evaluations performed in this study, additional research is necessary to clarify the link between kidney injury biomarkers and histological findings.

Considering that degeneration and necrosis of the tubular cells are amongst the most common histological changes seen in kidneys of dogs infected with large *Babesia* spp. (Maegraith et al., 1957; Irwin and Hutchinson, 1991; Máthé et al., 2007), higher concentrations of tubular injury biomarkers were anticipated in dogs with complicated disease. Rather unexpectedly, these biomarkers did not differ significantly between uncomplicated and complicated cases, while the functional renal biomarker sSDMA was significantly higher in complicated cases. These findings could potentially be explained by concurrent presence of multiple mechanisms leading to *Babesia*-induced AKI, that variably influenced biomarker concentrations. Increasing severity of AKI, as defined by IRIS AKI grading, was also not reflected by increasing uNGAL concentrations in a previous study in dogs (Segev et al., 2013). Other potential explanations for these unexpected findings include a possible influence of the criteria defining uncomplicated versus complicated disease (see Chapter 7, 8. Clinical classification of canine babesiosis), and the limited information available for sSDMA as a functional renal biomarker in canine AKI (Dahlem et al., 2017). The presence of a type II error to detect differences in tubular injury biomarker concentrations between uncomplicated and complicated cases can also not be excluded.

For practical reasons, follow-up at T24h and T1m was lacking for many cases. Some complications such as AKI grade I, as well as statistically significant differences might have been missed by this low number of dogs with follow-up. However, confirmation of full clinical recovery was obtained by telephone contact for all discharged dogs. Only dogs hospitalized because of disease severity had follow-up samples at T24h, leading to a sampling bias. Additional AKI diagnoses were made in 5/13 dogs with sCr follow-up at T24h, which is likely an overestimation due to this bias.

Conclusion

Dogs with babesiosis caused by *B. rossi* showed transient kidney injury, which was detected by all kidney injury biomarkers, yet remained undetected by routine and novel functional biomarkers. Nonetheless, our results also suggest that dogs with complicated babesiosis are more likely to present with significant loss of renal function. Therefore, adequate supportive therapy and monitoring of renal function during and after treatment is strongly advised for all dogs with babesiosis caused by *B. rossi*, even when no evidence of AKI is present based on routine sCr measurements.

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CHAPTER 7

GENERAL DISCUSSION

The main aim of this doctoral thesis (**Chapter 2**) was to characterize *B. rossi*-induced AKI in dogs, using urinary kidney injury biomarkers in comparison to the traditional evaluation of AKI. First, several urinary glomerular and tubular injury biomarkers were evaluated prospectively in a population of dogs with uncomplicated babesiosis (**Chapter 3**). The stability of these urinary kidney injury biomarkers after long-term frozen storage of diagnostic samples, which is an important pre-analytical factor to consider in biomarker research, was investigated in **Chapter 4**. In **Chapter 5**, dogs with more severe stages of AKI (i.e., dogs with RA) were identified retrospectively in a large population of dogs with babesiosis caused by *B. rossi*, and subsequently compared to dogs without RA. Finally, the initial panel of urinary kidney injury biomarkers was expanded with other urinary, plasma, and serum biomarkers of kidney injury and dysfunction, to provide a more complete prospective evaluation of AKI in a larger population of dogs with both uncomplicated and complicated babesiosis (**Chapter 6**).

In the following sections, the main findings of this doctoral thesis will be discussed in the context of canine babesiosis and in the broader context of AKI.

1. Occurrence of *Babesia rossi*-induced acute kidney injury in dogs

In the first study of this doctoral thesis (**Chapter 3**), several urinary kidney injury biomarkers (uIgG, uCRP, and uRBP) were able to detect kidney injury, both at glomerular and tubular level, in a population of dogs with apparently uncomplicated babesiosis. When this population of dogs was expanded to include not only dogs with uncomplicated disease, but also dogs with complicated babesiosis (**Chapter 6**), all investigated kidney injury biomarkers (uIgG, uCRP, uRBP, uNGAL, pNGAL, u/pNGAL) documented the presence of kidney injury. In contrast to the kidney injury biomarkers, functional biomarkers (sCr in Chapter 3, sCr and sSDMA in Chapter 6) were unable to document AKI in any of these studies. These findings indicate that *B. rossi*-induced kidney injury is present in all infected dogs, irrespective of disease severity, and manifests itself primarily in kidney injury rather than in significant loss of renal function. When combining functional and injury biomarkers to evaluate AKI (McCullough et al., 2013), results of this doctoral thesis show that most dogs with babesiosis caused by *B. rossi* can be allocated to the group of so-called subclinical AKI defined in human nephrology as increased injury biomarkers without loss of function based on functional biomarkers (Figure 7.1, yellow frame) (Ronco et al., 2012; Zarbock et al., 2018). The KDIGO definition of AKI only captures a decrease in renal function and does not detect kidney injury directly. A decrease in GFR is often preceded by injury to the kidneys, which can be demonstrated by increased kidney injury biomarkers (i.e., subclinical AKI) (Hoste and Vandenberghe, 2017).

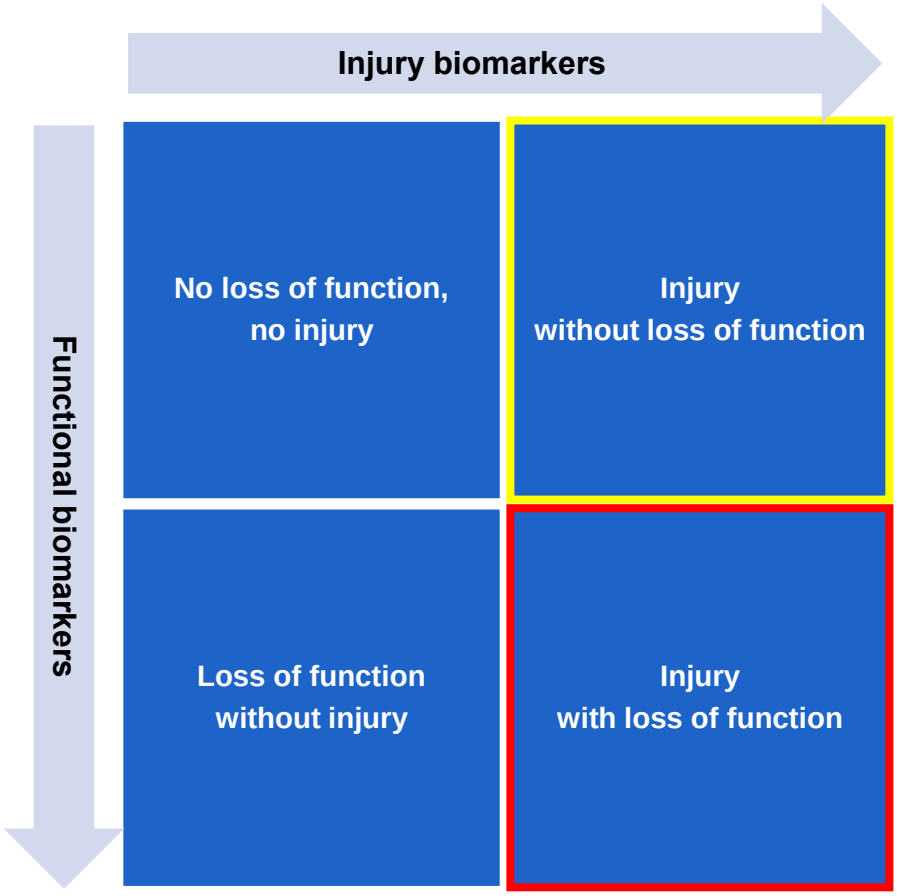

Figure 7.1. Combining functional and injury biomarkers to evaluate acute kidney injury (AKI) in dogs with babesiosis, adapted from McCullough et al. (2013) and Zarbock et al. (2018). The majority of dogs presented with *B. rossi* infection can be allocated to the 'injury without loss of function' quadrant (yellow frame), but many dogs with complicated babesiosis can be allocated to the 'injury with loss of function' quadrant (red frame).

In **Chapter 5**, a population of 296 dogs with babesiosis caused by *B. rossi* was evaluated for AKI based on admission sCr concentrations. To the author's knowledge, this is the largest population of dogs with babesiosis ever being evaluated for AKI, with all blood samples collected prior to any therapy. In this study, azotemia was present in 15% of the 296 dogs. Using uOsmol to assess renal concentrating ability in dogs with azotemia, RA was diagnosed in 14% of the 152 dogs where both blood and urine samples were available at admission. Kidney injury and functional biomarkers were

compared between dogs with uncomplicated and complicated babesiosis in **Chapter 6**. The tubular injury biomarkers did not differ significantly between both groups. However, one functional biomarker (sSDMA) was significantly higher in the dogs with complicated babesiosis. Compared to the occurrence of azotemia at presentation in Chapter 5 (15%), relatively few azotemic dogs were included in Chapter 6 (3/35, 9%). In spite of that, sSDMA results still suggested loss of function in the dogs with complicated disease. In conclusion, all dogs with babesiosis caused by *B. rossi* suffered kidney injury, irrespective of disease severity. Additionally, these results suggest that many dogs with complicated babesiosis also presented with loss of renal function (Figure 7.1, red frame). The presence of a unifying pro-inflammatory pathophysiological mechanism linking apparently unrelated complications in dogs with babesiosis, most likely explains the loss of renal function in many dogs with complicated disease (Jacobson and Clark, 1994; Welzl et al., 2001; Matijatko et al., 2009; Köster et al., 2015b; Goddard et al., 2016).

Based on a single unpublished observation (Jacobson and Clark, 1994), many review articles state that ARF and AKI are uncommon complications of *B. rossi* infections (Lobetti, 1998; Boozer and Macintire, 2003; Ayoob et al., 2010; Köster et al., 2015a). Evidence of *B. rossi*-induced kidney injury has been previously reported based on proteinuria and urine sediment findings (Moore and Williams, 1979; Lobetti and Jacobson, 2001). The authors of the most recent study concluded that milder forms of kidney injury are much more common than overt ARF (Lobetti and Jacobson, 2001). Based on the findings in this doctoral thesis, both subclinical AKI and more advanced stages of AKI (defined as increased injury biomarkers with loss of function, and RA) are frequently occurring complications in canine babesiosis caused by *B. rossi*.

2. Comparison of *Babesia*-induced acute kidney injury between different *Babesia* species based on urinary kidney injury biomarkers

Based on the traditional evaluation of AKI, the variable occurrence of azotemia, as well as of AKI and ARF in different canine *Babesia* species was summarized and discussed in **Chapter 1**. Except for a single study in dogs with *B. rossi* infection that measured the concentrations of 2 urinary tubular enzymes (AP and GGT) (Lobetti and Jacobson, 2001), previous research on urinary kidney injury biomarkers was lacking in the setting of canine babesiosis at the beginning of this doctoral thesis research. Recently, 3 other studies have evaluated the same - measured with the same commercial ELISA kits as assessed in the studies of this doctoral thesis (Immunology Consultants Laboratory, Newberg, USA) - and additional kidney injury biomarkers in dogs infected with other *Babesia* species (Sungpradit et al., 2016; Winiarczyk et al., 2017; Kuleš et al., 2018).

The same panel of urinary kidney injury biomarkers (uIgG, uCRP, and uRBP) was evaluated in dogs from Thailand infected with *B. vogeli* (Sungpradit et al., 2016). Concentrations of these 3 urinary kidney injury biomarkers were not significantly different between dogs with babesiosis and healthy control dogs. Consequently, there was no evidence of glomerular or tubular injury in these *B. vogeli*-infected dogs. Nevertheless, an important limitation of the latter study was the low number of *B.*-infected dogs included (n=6), creating a higher risk for a type II error (i.e., a higher risk of failing to detect the actual presence of glomerular or tubular injury in *B. vogeli*-infected dogs).

The first study in *B. canis*-infected dogs conducted in Poland investigated uIgG and uRBP, and additionally measured urinary THP (uTHP) as a biomarker of distal tubular injury (Winiarczyk et al., 2017). In accordance with the observations in this doctoral thesis, uIgG and uRBP concentrations were significantly higher in dogs with babesiosis compared to the healthy control dogs. Additionally, uTHP concentrations were also significantly increased.

A very recent study in *B. canis*-infected dogs was conducted in Croatia and also measured uIgG and uRBP (Kuleš et al., 2018). Additional urinary kidney injury biomarkers were measured in this study (i.e., uromodulin, KIM-1, albumin, and NAG), as well as sSDMA. In accordance with the *B. rossi* studies from this doctoral thesis and the latter *B. canis* study, significant increases of the glomerular and tubular kidney injury biomarkers were also observed in these *B. canis*-infected dogs. Unexpectedly, non-azotemic *Babesia*-infected dogs with a UPC <0.5 had significantly lower sSDMA concentrations compared to the healthy control dogs, which contrasts with the results of this doctoral thesis, where sSDMA did not differ significantly between healthy and *Babesia*-infected dogs. The reason for this apparent contradiction between both studies is unclear. The authors from the latter study suggested hyperfiltration in babesiosis as a possible explanation, but this hypothesis has not been examined yet (Kuleš et al., 2018). Another reason contributing to the difference in sSDMA concentrations between the *B. canis* study and the *B. rossi* study, in which even significantly higher sSDMA levels were found in dogs with complicated babesiosis, could be the use of different SDMA immunoassays. Initial validation of sSDMA as a biomarker of GFR in canine CKD was performed by measuring SDMA with the gold standard method of liquid chromatography-mass spectrometry (Nabity et al., 2015). The IDEXX SDMA™ Test used to measure sSDMA in the study from this doctoral thesis, is a new high-throughput immunoassay, validated for healthy dogs and dogs with CKD. Accuracy of this immunoassay was confirmed by comparison to the gold standard method of liquid chromatography-mass spectrometry (Relford et al., 2016). Moreover, although plasma SDMA was recently shown to be an excellent biomarker to identify azotemic AKI in dogs (Dahlem et al., 2017), validation of SDMA in the setting of canine AKI remains to be performed, leading to an unknown relationship between SDMA and GFR in canine AKI.

The same urinary kidney injury biomarkers (uIgG and uRBP) using the same commercial ELISA kits were measured in the prospective biomarker studies from **Chapter 3** and **Chapter 6** and in the latter 3 *Babesia* studies (Sungpradit et al., 2016; Winiarczyk et al., 2017; Kuleš et al., 2018). Therefore, comparison of biomarker concentrations between different *Babesia* species is tempting. Urinary IgG concentrations were highest in *B. rossi*-infected dogs, intermediate in *B. canis*-infected dogs, and lowest in *B. vogeli*-infected dogs. Higher uIgG concentrations could be

explained by higher systemic concentrations of IgG and/or more severe glomerular injury. When UPC was compared, *B. rossi*- and *B. canis*-infected dogs had similar levels of proteinuria, while UPC was much lower in *B. vogeli*-infected dogs. These comparative findings between species are in accordance with the reported differences in virulence of the large *Babesia* species (Uilenberg et al., 1989; Zahler et al., 1998). Importantly, differences between lot numbers of commercial ELISA kits can influence biomarker concentrations, which was suspected when uRBP results of both of our prospective biomarker studies were compared (as discussed below). Therefore, the results from this doctoral thesis indicate that direct comparison of absolute uRBP concentrations between studies should be avoided. As a final remark, urinary biomarker concentrations were divided by urinary creatinine concentrations (uCr) throughout this doctoral thesis research and also in the latter 3 *Babesia* studies, to compensate for variations in urinary flow rate. For this calculation to be correct, uCr excretion should be constant. However, this assumption is not correct when renal function is changing rapidly, as was demonstrated in a human AKI setting (Waikar et al., 2010). Consequently, normalization of urinary biomarkers to uCr has been questioned in AKI, although there is no consensus yet if and/or when normalization to uCr should be performed (Ralib et al., 2012). Urinary biomarker concentrations in this doctoral thesis were consistently reported after normalization to uCr, because the majority of urinary biomarker studies in dogs up to date report normalized biomarker concentrations only. This made comparison between studies more straightforward.

In conclusion, *Babesia*-induced AKI is not only limited to the *B. rossi* species, because similar findings were demonstrated in 2 parallel European studies on *B. canis*. Although extrapolation between *Babesia* species has to be done cautiously, the findings in this doctoral thesis are therefore likely to be highly relevant in the European context of *B. canis* infections as well. On the other hand, results also illustrate the differences in pathogenicity between species, considering the study of dogs infected with *B. vogeli* (Sungpradit et al., 2016). No evidence of subclinical AKI was found in these dogs (Figure 7.1; no loss of function and no injury), which is consistent with *B. vogeli* being the least virulent large *Babesia* species (Uilenberg et al., 1989; Zahler et al., 1998).

3. Limitations of serum creatinine and urine specific gravity in dogs with babesiosis

Unlike the kidney injury biomarkers, the routine functional biomarker sCr was unable to detect AKI in a population of dogs with uncomplicated babesiosis (**Chapter 3**) as well as in a population of dogs with both uncomplicated and complicated disease (**Chapter 6**). Additionally, sSDMA levels suggested a significant loss of renal function in dogs with complicated babesiosis compared to uncomplicated disease, which remained undetected by sCr (Chapter 6). On the other hand, sCr was capable to identify those dogs whose renal function was most severely affected (i.e., dogs with RA, **Chapter 5**), which emphasizes the critical importance of sCr in the evaluation of AKI. Intriguingly, dogs with uncomplicated babesiosis had significantly lower sCr concentrations compared to the healthy control dogs (**Chapter 6**). Although not statistically evaluated, sCr concentrations were also substantially lower in *B.*-infected dogs compared to the healthy control dogs in a recent *B. canis* study (Winiarczyk et al., 2017). These findings are in accordance with a previous study (Schetters et al., 2009), where plasma creatinine concentrations decreased in dogs after experimentally being infected with *B. canis*. Several GFR-independent factors, including reduced creatinine formation during sepsis, increased plasma volume, and analytical interferences due to hyperbilirubinemia and hemoglobinemia, could explain this remarkable finding and were summarized in Chapter 1. Another explanation could be the development of hyperfiltration after infection, which was hypothesized but not further examined by Kuleš et al. (2018). Augmented renal clearance is a recently recognized phenomenon in critically ill human patients, which is hypothesized to develop due to an increased glomerular filtration (Hobbs et al., 2015; Jacobs et al., 2018). In conclusion, all these findings further confirm the limitations of sCr-based evaluation of AKI in canine babesiosis.

Measurement of USG is a simple, rapid, and inexpensive method to estimate uOsmol and is frequently used in veterinary and human medicine for several clinical purposes (Watson, 1998; Imran et al., 2010). In the context of AKI, it is often used to assess the renal concentrating ability in azotemic dogs (Cowgill and Langston, 2011). Human literature clearly documents that USG measured by refractometry correlates poorly to uOsmol in the presence of hemoglobinuria and proteinuria (Voinescu et al., 2002; Imran et al., 2010). This resulted in the recommendation not to rely on USG in these particular conditions, but to measure uOsmol instead (Imran et al., 2010). The influence of proteinuria on USG and proposed correction formula are often mentioned in veterinary textbooks (Stockham and Scott, 2008; Fry, 2011). A more recent veterinary textbook states that USG can increase “slightly” when large amounts of protein are present (Skeldon and Ristić, 2016). To the author’s knowledge, interference caused by hemoglobinuria has not yet been reproduced in dogs. The correlation between USG and uOsmol in dogs was not significantly affected by hemoglobinuria in one study (Ayoub et al., 2013). In **Chapter 5**, USG was compared to uOsmol in dogs with babesiosis, and a systematic overestimation of urinary concentration by USG was found. Although hemoglobinuria is the most likely cause of this discrepancy between USG and uOsmol, this hypothesis was not yet tested. Interpretation of urinary concentration by USG led to a misclassification of 71% of the dogs with RA as having prerenal azotemia. This misleading clinical information might have important consequences as it could potentially result in different treatment decisions and in a delayed diagnosis of the most severe forms of AKI. These findings in this doctoral thesis further question the reliability of USG measurements in other hemolytic diseases. For example, in dogs with IMHA, AKI is an important complication associated with mortality (Piek, 2011). Failure to take the influence of hemoglobinuria on USG into account could result in a similar misinterpretation of renal concentrating ability in the face of azotemia. In conclusion, the discrepancy between USG and uOsmol found in Chapter 5 confirms that USG is not a reliable routine biomarker to evaluate renal concentrating ability in dogs with babesiosis.

4. Specificity of kidney injury biomarkers for acute kidney injury

Sensitivity of several kidney injury biomarkers for AKI was illustrated in this doctoral thesis by demonstrating their elevated urinary concentrations in dogs with uncomplicated babesiosis. However, the specificity of these biomarkers - especially of NGAL - for AKI, has been questioned, both in human and veterinary medicine (Mårtensson and Bellomo, 2014; Cortellini et al., 2015; Vanmassenhove et al., 2015; Cobrin et al., 2016). Except for the calculation of the urinary to plasma NGAL concentration ratio in Chapter 6, in an attempt to account for the influence of the NGAL from systemic origin in the total uNGAL concentration, this important aspect of biomarker research was not investigated in this doctoral thesis.

Saturation of the normal tubular reabsorptive capacity due to an increased systemic overload of an LMW protein (such as NGAL) or due to competition for tubular reabsorption with other filtered proteins (such as albumin) can result in LMW proteinuria in the absence of tubular injury (see Chapter 1, 5.1 Introduction) (Hall, 2011; Nejat et al., 2012). Moreover, (micro)albuminuria can occur due to an increased capillary permeability of the glomerular barrier (i.e., glomerular leakage) during systemic inflammation (Gosling et al., 2003; Nejat et al., 2012). These mechanisms are likely to be present in any systemic inflammatory disease and will result in LMW proteinuria due to an overload of filtered proteins, independent of tubular injury. Increasing glomerular leakage could result in HMW proteinuria as well. A practical consequence in the setting of AKI is that it will be difficult to discriminate how much of the increase in urinary biomarker concentration is the result of tubular injury and how much is caused by competition for reabsorption (Nejat et al., 2012). This will undoubtedly result in a reduced specificity and performance for AKI diagnosis in any inflammatory disease setting, including canine babesiosis.

Because both systemic and urinary NGAL concentrations were measured in Chapter 6, the impact of systemically originating NGAL in canine babesiosis can be assessed to a certain extent. The same commercial ELISA kit was used to measure NGAL in dogs with babesiosis from Chapter 6 and in septic dogs from the study of Cortellini et al. (2015). Similar median concentrations for both systemic and urinary NGAL were found when healthy control dogs from both studies were compared.

Although comparison of kidney injury biomarker concentrations between different studies has to be performed cautiously, the median systemic NGAL concentration was 5 to 6 times higher in dogs with bacterial sepsis compared to dogs with babesiosis, while the median uNGAL concentration was very similar. The relative impact of systemically orginating NGAL on the total uNGAL concentration therefore seems less pronounced in dogs with babesiosis, which is suggestive of more kidney injury-specific increases of uNGAL in dogs with babesiosis compared to septic dogs. Based on these comparative data, an important fraction of the total uNGAL concentration detected in dogs with babesiosis probably orginated from injured tubular cells. In conclusion, the increased uNGAL in dogs with babesiosis is highly likely the result of tubular injury, leading to an increased release and reduced reabsorption of NGAL, combined with mechanisms independent of tubular injury. These mechanisms consist of filtration of systemic NGAL and reduced reabsorption of NGAL, as glomerular leakage results in competition for reabsorption between NGAL and leaked proteins.

To ensure consistency in the terminology used throughout this doctoral thesis, the investigated urinary glomerular and tubular biomarkers are consistently referred to as urinary kidney injury biomarkers. However, these biomarkers can represent both injury and dysfunction at a specific nephron segment. It is likely that the increased concentrations of urinary biomarkers in this doctoral thesis represent both injury and dysfunction at the glomerular and tubular level of the nephron.

Increased systemic and glomerular capillary permeability during systemic inflammation can lead to glomerular leakage and thus result in proteinuria (Gosling et al., 2003; Paisley et al., 2003; Nejat et al., 2012). This mechanism will also occur in canine babesiosis due to the systemic inflammatory nature of this disease (Jacobson and Clark, 1994; Goddard et al., 2016; Kuleš et al., 2016). Previous research in canine babesiosis already linked inflammation with endothelial dysfunction (Barić Rafaj et al., 2013; Kuleš et al., 2017). While increased urinary glomerular biomarker concentrations (i.e., uIgG and uCRP) can be considered as clear evidence of glomerular dysfunction caused by leakage of the glomerular barrier, actual presence of glomerular injury is less evident to substantiate in our population of *B. rossi*-infected dogs. Histological research reports either mild or absent glomerular lesions in dogs infected with large *Babesia* species (Irwin and Hutchinson, 1991; Máthé et al., 2007). Similar glomerular histological findings were present in 2 *B. rossi*-infected dogs in Chapter 6. Significant

alterations to the glomerular barrier-associated glycocalyx layer, that contributes to the barrier permeability, has been documented in sepsis (Adembri et al., 2011). Because injury to this layer is considered to contribute to the microcirculatory changes occurring in sepsis-induced AKI (Gómez and Kellum, 2016), glomerular injury could potentially be present at the glycocalyx layer of dogs with babesiosis.

Increased urinary tubular biomarker concentrations (i.e., uRBP and uNGAL) likely represent both tubular injury and dysfunction in our population of *B. rossi*-infected dogs. As discussed above, LMW proteinuria in dogs with babesiosis likely results from direct tubular injury combined with a reduced functional tubular reabsorption. Presence of structural injury to the tubular cells is also evident based on the available histopathological literature in dogs infected with large *Babesia* species (Irwin and Hutchinson, 1991; Máthé et al., 2007), and based on the histological findings in one of the 2 *B. rossi*-infected dogs in Chapter 6 where kidney biopsies were obtained.

Acute kidney stress is a term that was recently introduced in human medicine to describe the clinical phase that may lead to AKI. Whether acute kidney stress is a condition of early injury or represents a pre-injury phase of increased susceptibility preceding AKI, is debatable (Katz and Ronco, 2016). In the initial phase of illness, stressed tubular cells are not necessarily injured, but progression of the injurious process could lead to sublethal or lethal tubular injury (Ronco et al., 2017). Acute kidney stress might therefore be an appropriate concept for many dogs with babesiosis caused by *B. rossi*. This term could possibly be used next to, or instead of, the concept of subclinical AKI.

5. Clinical relevance of increased kidney injury biomarkers

Based on the findings in **Chapter 3** and **Chapter 6**, most dogs with babesiosis were diagnosed with subclinical AKI. In human research, the clinical relevance of subclinical AKI was demonstrated since 2011 in several studies that showed a worse outcome (i.e., increased mortality and/or requirement for RRT) in patients with increased kidney injury biomarker levels but in the absence of increased sCr (Haase et al., 2011; Ronco et al., 2012; Coca et al., 2014; Albert et al., 2018). In 2 of these studies, urinary biomarkers were independently associated with a worse outcome (Coca et al., 2014; Albert et al., 2018). However, it remains unclear whether increased injury biomarkers, in the absence of loss of function based on functional biomarkers, actually detect unrecognized AKI or represent a higher disease severity in these patients (Vanmassenhove et al., 2015; Vanmassenhove et al., 2017). Studies specifically investigating the outcome of dogs with subclinical AKI are not available yet, thus the clinical relevance of subclinical AKI in dogs is currently unknown. Nonetheless, the presence of subclinical AKI in dogs has been documented before in a collaboration study with our group (Segev et al., 2015). Based on increased concentrations of uCRP, uRBP, and uNGAL, glomerular and tubular kidney injury were diagnosed in all 30 dogs presented with heatstroke, even though 11 of these dogs (37%) were not azotemic at presentation. However, outcome was not evaluated separately for those dogs with subclinical AKI.

Many research papers investigating the use of novel kidney injury biomarkers for the diagnosis of AKI in humans have been published in the last decade (Vanmassenhove et al., 2013). Initial enthusiasm to use these new biomarkers in the early diagnosis of AKI, was based on their excellent performance in specific populations, such as in children undergoing cardiac surgery (Mishra et al., 2005; Vanmassenhove et al., 2017). Nonetheless, results of subsequent studies investigating the performance of these biomarkers to diagnose AKI in various clinical settings, showed either far more variable and often even disappointing diagnostic performances, especially in heterogeneous populations such as in the general adult intensive care setting (Lameire et al., 2011; Vanmassenhove et al., 2013; Prowle and Rosner, 2017; Vanmassenhove et al., 2017). However, it is important to realize that in

these studies their performance is evaluated against sCr, which is an inadequate gold standard in this setting for several reasons (Waikar et al., 2012). Most importantly, sCr lacks the accuracy for a diagnosis of structural kidney injury (Moledina and Parikh, 2018). Serum creatinine is a measure of glomerular filtration and does not directly reflect tubular injury. However, most kidney injury biomarkers identify tubular injury, which is the most common underlying pathology of AKI (Waikar et al., 2012; Prowle and Rosner, 2017). Evaluating the performance of biomarkers of structural injury by comparing them to a functional biomarker is like comparing apples with oranges (Endre and Pickering, 2013). Although injury and functional biomarkers are related (Figure 7.1), it would be unfair to evaluate biomarker performance solely based on this comparison. Besides comparing them to sCr, biomarkers could also be evaluated against other endpoints, such as the outcome (Waikar et al., 2012). A worse outcome has been demonstrated in people with increased kidney injury biomarkers but without increased sCr (i.e., defined as subclinical AKI) (Haase et al., 2011; Coca et al., 2014; Albert et al., 2018). Therefore, the importance of such subclinical AKI is increasingly being recognized, resulting in kidney injury biomarkers now being included in new AKI definitions (McCullough et al., 2013; Zarbock et al., 2018). Nevertheless, it is also very important to recognize that most of these biomarkers are not only associated with kidney injury, but also with several non-renal conditions that could be the underlying cause of AKI (Vanmassenhove et al., 2013; Vanmassenhove et al., 2017). This is most notably the case in sepsis, where both serum- and uNGAL are influenced by the severity of illness, independent of the presence of AKI, emphasizing that the concept of subclinical AKI should be used with caution (Vanmassenhove et al., 2015). Recently, significant progress has been made in the evaluation of several new kidney injury biomarkers in dogs (Hokamp and Nabity, 2016). Similar to human studies, performance of uNGAL to diagnose AKI in dogs has been investigated by receiver operator characteristics (ROC) analysis (Segev et al., 2013). When uNGAL results from **Chapter 6** are compared to the cut-off value to diagnose azotemic AKI (uNGAL/uCr of 120 µg/g), reported by Segev et al. (2013), the majority of *B. rossi*-infected dogs should have been azotemic, which was clearly not the case. This comparison illustrates that, even if the same immunoassay is used, kidney injury biomarker thresholds to diagnose AKI cannot be extrapolated from one study to another. This holds true particularly for uNGAL, where systemically produced NGAL will variably impact uNGAL concentrations depending on the clinical setting and

disease severity (Mårtensson and Bellomo, 2014; Vanmassenhove et al., 2015). Although uNGAL concentrations rise proportionally to the severity and duration of kidney injury, defining an appropriate biomarker threshold for AKI diagnosis and determination of acceptable levels of increased biomarker concentrations may be different in each clinical setting, precluding the use of one generalized cut-off value to distinguish normal from abnormal (Haase et al., 2009; Nickolas et al., 2012; Kashani et al., 2017). The use of a single uNGAL cut-off value for AKI diagnosis might be appropriate in some specific populations such as in children without co-morbidities after cardiopulmonary bypass (Mishra et al., 2005). However, use of uNGAL cut-off values or reference ranges will be severely hampered in most clinical situations with a more heterogeneous population, due to a variable fraction of systemically produced NGAL in the total uNGAL concentration.

An increased risk for CKD and long-term mortality after an episode of AKI has been demonstrated in several human studies (Coca et al., 2009; Leung et al., 2013; Heung et al., 2016). This increased risk was also documented after apparent fast recovery from relatively mild forms of AKI (Coca et al., 2009; Heung et al., 2016). Persistently elevated kidney injury biomarkers have been detected in humans after apparent recovery from AKI, suggesting that subclinical kidney injury was likely still ongoing, which indicates that measuring these novel biomarkers might help to identify patients who will progress to CKD (Cooper et al., 2016). Several urinary kidney injury biomarkers measured at the time of kidney injury, were also able to predict long-term mortality after AKI in humans (Coca et al., 2014). Thus, an additional purpose of these kidney injury biomarkers might be to predict recovery and long-term outcome after AKI (Vanmassenhove et al., 2018). Also in dogs, it has recently been hypothesized that detecting ongoing injury using kidney injury biomarkers might help to predict which dogs are at risk of CKD after apparent recovery from AKI (Cowgill et al., 2016). In this context, urinary kidney injury biomarkers in **Chapter 6** were measured again 1 month (m) after presentation with *Babesia*-induced AKI. All urinary kidney injury biomarkers decreased to values not significantly different from the healthy control dogs. Thus, no evidence of active ongoing kidney injury was detected, suggesting a transient nature of kidney injury in most dogs with babesiosis caused by *B. rossi*. However, considering the low number of dogs included for a relatively short follow-up period, additional

studies including more dogs over a longer follow-up period should be performed to clarify if *Babesia*-induced AKI could have any negative effect on long-term outcomes.

In conclusion, kidney injury biomarkers should not replace the traditional approach in clinical decision making in AKI, but should rather be used as an extra tool in the clinical decision process if their added value can be demonstrated (Lameire et al., 2011; Vanmassenhove et al., 2017). Absence of a single cut-off value for biomarkers such as NGAL to distinguish “AKI” from “no AKI” throughout different populations, absence of a consensus whether or not to report urinary biomarker concentrations normalized to uCr or not, and absence of in-depth knowledge of the influence of non-renal conditions, currently limits the clinical use of kidney injury biomarkers. Even though the clinical relevance of subclinical AKI and clinically acceptable levels of increased kidney injury biomarkers remain to be determined in the context of canine babesiosis and also in other settings of AKI in veterinary medicine, results from several human studies demonstrate a worse renal outcome in patients with subclinical AKI (Haase et al., 2011; Ronco et al., 2012; Albert et al., 2018). Dogs with babesiosis can be considered an at risk population to develop azotemic AKI, because subclinical AKI was identified in the majority of *B. rossi*-infected dogs. Therefore, some clinical implications can be concluded from the biomarker findings in this doctoral thesis. Provision of adequate supportive therapy, avoidance of any nephrotoxic drug or intervention, and monitoring of renal function is strongly advised for all dogs with babesiosis caused by *B. rossi*, even when routine sCr evaluations cannot document any evidence of AKI. These recommendations are especially critical for dogs with other recognized predispositions to kidney injury, such as old age and dehydration.

6. Comparative medicine: acute kidney injury in dogs and humans

Although comparison with the extensive biomarker research from the human AKI setting is tempting, significant differences exist between the AKI setting in humans and dogs. These differences will undoubtedly affect the indications and performance of kidney injury biomarkers.

A major difference is the time of onset when AKI generally occurs. While most dogs present with community-acquired AKI, in humans AKI is commonly hospital-acquired (Eatroff et al., 2012; Segev et al., 2013; Braun, 2016; Moledina and Parikh, 2018). Indeed, AKI is a frequently occurring complication in the general human intensive care setting and also after cardiac surgery that required cardiopulmonary bypass (Hoste and Schurgers, 2008; Mao et al., 2013). Community-based AKI in humans is also common, still most AKI research focusses on hospitalized patients (Hsu et al., 2007). Consequently, the main focus of recent human AKI biomarker research (i.e., diagnosing AKI early, before sCr rises) might be less relevant in the canine AKI setting because AKI often develops outside the hospital setting in dogs. However, hospital-acquired AKI may well be underdiagnosed in dogs. Based on increasing sCr concentrations in dogs that were initially non-azotemic when admitted to the intensive care unit, 15% of the dogs were diagnosed with AKI, and these dogs were also less likely to survive (Thoen and Kerl, 2011). Similar to a recent study in humans (De Loor et al., 2017), the latter study also highlighted the importance of serial sCr measurements.

The relative occurrence of the different causes of prerenal azotemia might not be the same for dogs and humans. As an example, dehydration is probably the single most common cause of prerenal azotemia in dogs, whilst other causes leading to hemodynamic reductions in renal blood flow, such as cardio- and hepatorenal syndrome and use of renin-angiotensin aldosterone system (RAAS) inhibitors (Moledina and Parikh, 2018), might be more common in humans.

Many limitations of sCr, as discussed in Chapter 1, will be very similar in the human and canine AKI settings, but several additional obstacles are present in veterinary medicine. To classify patients by the RIFLE, AKIN, and KDIGO classification systems, baseline sCr should be available (Bellomo et al., 2004; Mehta et al., 2007; Khwaja, 2012). While the true baseline sCr is also often not exactly known in humans, methods of ‘looking back’ to obtain a baseline value or to calculate estimated baseline sCr and even estimated GFR, are available (Makris and Spanou, 2016). In dogs however, baseline renal function prior to hospitalization and AKI is rarely known (Thoen and Kerl, 2011). Obtaining a reliable baseline renal function will be very difficult in dogs for several reasons. Due to differences between dog breeds, variability of baseline sCr is likely to be more pronounced in dogs compared to a more uniform human setting.

The high interindividual variability of sCr in dogs, the absence of a previous sCr measurement, and the occurrence of mostly community-acquired AKI will prevent the use of methods and calculations available in human medicine to obtain a baseline renal function. Consequently, validation of novel kidney injury biomarkers in comparison to sCr will be even more hampered in dogs than in humans. While other techniques to measure or estimate GFR in the non-steady state situation of AKI, such as functional magnetic resonance imaging, real-time measurement of GFR, and the use of radioisotopes, are available in human medicine (Zhou et al., 2016; Solomon and Goldstein 2017), these techniques are currently either unavailable or less accessible for dogs. For example, although radioisotopes can be used to estimate GFR in dogs (Barthez et al., 1998), the required expertise and necessary equipment limit their use in veterinary medicine. Last but not least, more limited financial possibilities in veterinary medicine make cost-effectiveness analysis before implementation of new biomarkers in clinical practice an even more important critical aspect to consider.

All the above-mentioned limitations make research and application of kidney injury biomarkers far more challenging in the canine AKI setting, yet evaluation of the urinary concentration to differentiate prerenal from renal azotemia seems more straightforward in dogs. Again, this could be explained by the different occurrence of hospital- and community-acquired AKI in dogs versus humans. While hospitalized patients are typically already receiving fluid therapy and medication that influence urinary concentration, evaluation of urinary concentration in dogs with community-acquired AKI will usually be possible in the absence of these influencing factors. Hence, measuring USG and uOsmol will often be more useful in dogs than in humans. This translates to the traditionally used definition of renal failure in veterinary medicine (i.e., concurrent azotemia and inappropriately low USG), while the use of USG to assess renal function is not common in human AKI settings (Cowgill and Langston, 2011; Thoen and Kerl, 2011).

In conclusion, differences between the human and canine AKI settings, with the time of onset (hospital- versus community-acquired AKI, respectively) being the major contributing factor, have a significant impact on the performance and the clinical relevance of kidney injury biomarkers for various indications. However, with the steadily expanding and continuously improving medical care being provided to dogs, both AKI settings will likely become increasingly comparable to each other in the future.

7. Urinary biomarker immunoassay validation including storage time and hemoglobinuria

Validation of immunoassays is a critical step before these can be used reliably in clinical and research settings (Valentin et al., 2011). For the immunoassays used in **Chapter 3** and **Chapter 6**, species-specific validation in urine and/or plasma was already performed for all urinary and plasma biomarkers used, either in our laboratory (Maddens et al., 2010) or by the manufacturer of the ELISA kit (BioPorto Diagnostics A/S, Hellerup, Denmark). Although sSDMA is already validated for use in dogs (Nabity et al., 2015), proper validation in the setting of AKI is not currently available (Dahlem et al., 2017). Therefore, sSDMA results (Chapter 6) have to be interpreted cautiously.

The same urinary kidney injury biomarkers, using the same commercial ELISA kits, were measured in very similar clinical settings in both of our prospective biomarker studies. Therefore, direct comparison of these data was performed (Table 7.1).

Table 7.1. Comparison between urinary kidney injury biomarker results of Chapter 3 and 6.

	Chapter 3		Chapter 6	
Biomarker (unit)	H (n=8)	B uncomplicated (n=18)	H (n=10)	B uncomplicated (n=10)
uIgG/uCr (mg/g)	1.27 (0.52–3.23)	226.71 (11.32–2296.35)	1.93 (0.55–11.32)	174.40 (6.95–3373.12)
uCRP/uCr (mg/g)	BDL (n=8)	0.02 (BDL–0.81) BDL (n=6) BQL (n=2)	BDL (n=10)	0.01 (BDL–1.07) BDL (n=1) BQL (n=2)
uRBP/uCr (mg/g)	0.05 (BDL–0.16) BDL (n=2) BQL (n=1)	10.84 (0.91–58.23)	0.01 (BDL–0.03) BDL (n=2) BQL (n=2)	0.83 (0.29–2.07)

H, healthy control dogs; B uncomplicated, dogs with uncomplicated babesiosis; uIgG/uCr, urinary immunoglobulin G-to-creatinine ratio; uCRP/uCr, urinary C-reactive protein-to-creatinine ratio; uRBP/uCr, urinary retinol binding protein-to-creatinine ratio; BDL, below detection limit; BQL, below quantification limit.

With the exception of uRBP, similar results were obtained between the healthy control dogs of both studies, and between the dogs with uncomplicated babesiosis of both studies. Compared with the uRBP results of the *Babesia*-infected dogs in Chapter 3, lower concentrations of uRBP were measured in the dogs with babesiosis from Chapter 6. The manufacturer (Immunology Consultants Laboratory, Newberg, USA) changed the anti-human RBP antibody in the kit of the lot number used in Chapter 6, compared with the kits of previous lot numbers used in Chapter 3 and 4. Although the human RBP immunoassay used was validated for use in dogs (Maddens et al., 2010), it is likely that these changes in anti-human RBP antibodies are the reason of the comparative differences in uRBP concentrations between Chapters 3 and 6. Changes in the anti-human RBP antibodies probably resulted in a lower specificity for canine urinary RBP, resulting in overall lower uRBP concentrations in Chapter 6. Urinary RBP concentrations should not be interpreted as absolute values, but always as relative values, because of species-dependent cross-reactivity and specificity (van Hoek et al., 2008; Maddens et al., 2010). The initial validation remains valid, provided that uRBP concentrations are always interpreted as relative values and thus direct comparison of absolute uRBP concentrations between different studies is avoided when this human RBP immunoassay is used in canine urine.

To perform reliable biomarker research, several pre-analytical factors, such as stability of the biomarker during storage, have to be taken into account. In many research settings, analyses are not performed immediately after collection, but in batch after storage (Parikh et al., 2014; Remer et al., 2014). Despite the importance of this pre-analytical factor, the influence of long-term frozen storage on urinary kidney injury biomarker concentrations is limited for most biomarkers in human research (Nauta et al., 2012), and almost non-existing in veterinary medicine. However, stability of uNGAL and pNGAL during storage at -80 °C is well documented in humans. Using a human NGAL ELISA kit from the same manufacturer (BioPorto Diagnostics A/S, Hellerup, Denmark) as the dog NGAL kit in Chapter 6, stability of NGAL has been documented for at least 11 m in plasma (Pedersen et al., 2010), and up to 5 years (y) in urine (Schuh et al., 2016). One study evaluated the stability of several kidney injury biomarkers in canine urine during frozen storage (Smets et al., 2010). After 12 m of storage at -80 °C, uRBP concentrations were not significantly different from freshly analyzed samples (Smets et al., 2010). All urine and plasma samples in Chapters 3 and 6 were stored at

-80 °C and -72 °C respectively, and analyzed within 6 m of sample collection, because stability data in dogs are lacking for the other kidney injury biomarkers measured in this doctoral thesis.

To increase the knowledge on biomarker stability, urinary kidney injury biomarkers were measured again after 4 y of storage at -72 °C in **Chapter 4**. Significant decreases in the concentrations of all biomarkers (uIgG, uCRP, uRBP) were observed after such long-term storage. Rather than only a decay of these urinary biomarkers during long-term storage, an alternative explanation for these concentration differences could be lot-to-lot variations. These variations are a problem that could influence the accuracy of measurements, as reported for uNGAL in humans (Pedersen et al., 2010). However, differences between lot numbers are unlikely to have significantly influenced our results because of several reasons. Specifically for uRBP, the change in anti-human RBP antibody only occurred in Chapter 6. In Chapter 4, the same antibody was used at the start of the study and 4 y later. Secondly, the comparative data between Chapters 3 and 6 (Table 7.1) demonstrate that similar concentrations were obtained for uIgG and uCRP, making significant differences of the immunoassays over time less likely. Lastly, a consistent decrease of all measured values was observed for all biomarkers in Chapter 4, likely representing a decay over time.

Interference testing is most commonly performed for hemolysis, icterus, and lipemia (Dimeski, 2008). Although high concentrations of hemoglobin and bilirubin, which are commonly found in canine babesiosis (Lobetti and Jacobson, 2001; de Scally et al., 2004), could theoretically interfere with the quantification of reactions using spectral methods (Pesce and Michael, 1992), immunoassays are generally unaffected by both interferences, unlike other analyses using spectral methods (Tate and Ward, 2004). However, to test whether hemoglobinuria interfered with the measurement of our urinary kidney injury biomarkers by ELISA, interference testing was performed in **Chapter 3**. Different added concentrations of hemoglobin, based on previously reported urinary hemoglobin concentrations in dogs with babesiosis caused by *B. rossi* (Lobetti and Reyers, 1996; Lobetti and Jacobson, 2001), were tested. No significant interference was detected for uIgG, uCRP, and uRBP at all these concentrations. Although severe hemolysis in blood samples interfered with the measurement of human pNGAL (Pedersen et al., 2010), no significant interference of

added hemoglobin was found in canine urine and plasma in our laboratory (*unpublished data*). Yet, significant interferences of hemoglobin, bilirubin, and lipids were found for several other assays of canine kidney injury biomarkers in a recent study (Kuleš et al., 2018), emphasizing the importance of interference testing. The influence of hyperlipidemia, which was not assessed in this doctoral thesis, is more likely to interfere with some immunoassays, especially those based on nephelometry and turbidimetry (Tate and Ward, 2004). These techniques were not used in this doctoral thesis. A final reason why significant interferences in our urinary kidney injury biomarker measurements are unlikely, is the high dilution factor necessary to measure biomarker concentrations in most of the urine samples of dogs with babesiosis. Indeed, sample dilution is one of the main methods in immunoassays to eliminate the effects of interferents such as hemoglobin and lipids (Pesce and Michael, 1992).

In the recent study of Kuleš et al. (2018), sSDMA showed significant interferences when hemoglobin, bilirubin, or lipids were added. In contrast, the IDEXX SDMA™ Test that was used to measure sSDMA in **Chapter 6**, is reported to be unaffected by mild to moderate hemolysis or any degree of lipemia or icterus (Relford et al., 2016). Severe hemolysis was not seen in any of the analyzed samples in Chapter 6. The reason why significant interferences were found in the study of Kuleš et al. (2018) remains uncertain, but differences between both immunoassays should be considered.

8. Clinical classification of canine babesiosis

The initial classification of canine babesiosis into uncomplicated versus complicated disease was based on clinical experience in *B. rossi*-infected dogs and concurred with the classification of human malaria (Jacobson and Clark, 1994). In 2006, revisions of this classification system were suggested (Jacobson, 2006). Although most clinical studies in canine babesiosis based their classification of uncomplicated versus complicated/severe babesiosis on these latter publications, variable definitions of specific organ complications are used (Jacobson et al., 2000; Welzl et al., 2001; Máthé et al., 2006; Matijatko et al., 2009; Fraga et al., 2011; Goddard et al., 2013; Kuleš et al., 2016; Crnogaj et al., 2017). Differences between definitions are common, especially for renal and hepatic complications, making direct comparison between studies difficult.

The relatively recently introduced concept of AKI in veterinary medicine (Cowgill and Langston, 2011), together with the findings in this doctoral thesis clearly warrant a revised definition of the “acute renal failure/renal involvement” complication in canine babesiosis. However, it remains difficult to classify dogs with subclinical AKI into either uncomplicated or complicated disease. The introduction of the AKI guidelines for dogs by the IRIS in 2013 also resulted in different definitions used to diagnose AKI in **Chapter 3** versus **Chapter 6**. While the definition of “persistently elevated sCr despite appropriate fluid therapy” was still used to exclude dogs with complicated babesiosis due to AKI in Chapter 3, definition of AKI in Chapter 6 was based on the recent IRIS criteria (Braun, 2016). Consequently, dogs with prerenal azotemia and dogs with AKI grade I (defined as a progressive non-azotemic increase in sCr of at least 26.4 µmol/L within 48 h) were diagnosed with AKI and complicated disease in Chapter 6, while remaining uncomplicated cases in Chapter 3. Retrospectively, at least 3 dogs diagnosed with uncomplicated disease in Chapter 3, would have been classified as complicated cases based on the revised definition of AKI. These dogs presented with mild azotemia, which was presumed at that time to be prerenal in origin. Based on the discrepancy between USG and uOsmol observed in Chapter 5, it is even possible that these dogs actually presented with RA. Lastly, some complications could have been missed because follow-up was not available for most dogs in Chapter 3. In conclusion,

these findings demonstrate the continued difficulties in the clinical classification of canine babesiosis.

In human falciparum malaria, similar definitions are available to distinguish uncomplicated from severe malaria (WHO, 2014). In addition to these more extensive definitions, several simple bedside observations, such as the level of prostration and respiratory distress, are being used to identify children and adults with severe malaria (WHO, 2014). Similar simple measures, such as being collapsed at presentation (i.e., being unable to walk unaided), are currently being used in the clinical assessment of dogs presented with babesiosis (Jacobson, 2006). Although these bedside clinical observations are easy to implement and are invaluable in the initial clinical assessment, more extensive diagnostic work-up is preferred, especially for epidemiological and research purposes. In uncomplicated babesiosis, treatment with an effective antibabesial drug is usually sufficient, without the need of supportive therapy. However, dogs with complicated babesiosis require aggressive supportive care (Jacobson and Swan, 1995; Ayoob et al., 2010). Identifying those dogs at highest risk of dying and thus most in need of intensive monitoring and treatment is therefore of the utmost importance (Jacobson, 2006). Presence of RA was associated with a worse outcome in Chapter 5, while presence of azotemia without clinical signs of dehydration was also associated with a higher risk of death compared to all other complications combined (Welzl et al., 2001). Therefore, correctly identifying dogs with complicated babesiosis due to AKI is an important clinical assessment necessary to make in any dog presented with a *B. rossi* infection.

9. Future perspectives

An excessive systemic inflammatory response is thought to be one of the main causes of *Babesia*-induced kidney injury (Jacobson and Clark, 1994). Thus, it is likely that many of the kidney injury biomarker findings in this doctoral thesis are not specific for canine babesiosis, but rather represent observations that are very similar to those in other systemic inflammatory diseases. Additional potential mechanisms of AKI, such as hemolysis-induced kidney injury due to hemoglobinuria and anemia-induced renal tissue hypoxia (Lobetti et al., 1996; Máthé et al., 2007), could be shared with other hemolytic diseases, such as IMHA. Nonetheless, the sequestration of parasitized red blood cells in capillaries could represent a *Babesia*-specific contribution to tissue hypoxia and changes in renal microvasculature. The comparison of systemic and uNGAL concentrations between dogs with babesiosis (Chapter 6) and dogs with bacterial sepsis (Cortellini et al., 2015) is suggestive for a relatively more kidney injury-specific increase of uNGAL in canine babesiosis versus sepsis. To investigate the relative impact of the potential mechanisms of *Babesia*-induced kidney injury on the kidney injury biomarkers, biomarker concentrations could be compared between dogs with babesiosis, dogs with SIRS/sepsis, and dogs with idiopathic IMHA. The latter suggested study design could help to clarify how unique the kidney injury biomarker findings in this doctoral thesis are compared to other inflammatory and hemolytic diseases. Such a study design would also aid in the understanding of the pathogenesis of *Babesia*-induced kidney injury.

Future studies should assess the impact of the presence and the severity of concurrent diseases on the specificity of the kidney injury biomarkers for AKI. The influence of glomerular leakage during systemic inflammatory diseases should be taken into account. Lack of kidney injury biomarker accuracy for histological ATN has also been documented in humans (Moledina et al., 2017). While the limited histological evaluation in **Chapter 6** documented an intriguingly poor correlation between kidney injury biomarkers and renal histopathological findings in one *Babesia*-infected dog, further research is necessary in dogs to clarify the link between these biomarkers and histopathological findings.

Considering their variable specificity for structural injury and their variable diagnostic performance for early AKI diagnosis, candidate biomarkers could serve many other purposes in the setting of AKI. Purpose-specific studies are necessary to evaluate the performance of kidney injury biomarkers in various clinical settings. Ultimately, a purpose-specific setting, that should be investigated in canine babesiosis-induced AKI, is whether application of a (panel of) biomarker(s) could help in the clinical decision-making process to either hospitalize a *Babesia*-infected dog for more intensive treatment and renal monitoring or to treat the dog on an outpatient basis. If application of the investigated biomarker(s) in this setting would result in a higher survival rate and/or an improved renal recovery, an evidence-based clinical application of these biomarkers would be available for the clinician. To conduct these purpose-specific performance studies and to be applicable in clinical practice, large randomized clinical trials and the availability of bedside tests to measure these biomarkers are needed. The combination biomarker test of tissue inhibitor of metalloproteinases-2 (TIMP-2) and insulin-like growth factor-binding protein 7 (IGFBP7) (NephroCheck®) and NGAL are the only AKI biomarker tests currently available for humans in clinical practice (Vandenberghe et al., 2017). Even in human medicine, it still needs to be proven whether adding kidney injury biomarkers to the decision-making process can eventually improve outcome (Lameire et al., 2011; Schiffl and Lang, 2012; Prowle and Rosner, 2017; Vanmassenhove et al., 2017). Two recent single-center randomized clinical trials (PrevAKI, BigpAK) showed however that high-risk patients, identified by increased urinary biomarkers ([TIMP-2]x[IGFBP7] >0.3), had a significantly decreased occurrence and severity of peri-operative AKI after implementation of a supportive care “bundle”, as suggested by the KDIGO guidelines, compared with the standard intensive care (Meersch et al., 2017; Göcze et al., 2018). The latter kidney injury biomarker-guided pilot studies show promising results for implementing this biomarker panel in the clinical decision-making process. Both studies essentially documented an improved outcome by use of the KDIGO care bundle. However, it remains unknown whether a biomarker-guided approach to identify high-risk patients actually contributes to an improved outcome (Singh and Kilambi, 2018). Evaluation of other outcome parameters, such as survival and long-term renal recovery, are also needed in this setting. In the context of veterinary research, it could be even more challenging to demonstrate the added value of these biomarkers. However, considering that the negative predictive value of kidney injury biomarkers for identification of human AKI is

generally better than the positive predictive value, a more realistic approach could be to implement biomarkers in the decision-making process to rule out AKI (Prowle and Rosner, 2017). To decide whether the *Babesia*-infected dog should be hospitalized or not for AKI purposes, a negative result of a biomarker (panel) with a high negative predictive value could help in the global clinical decision to treat the dog on an outpatient basis, thus avoiding unnecessary and costly hospitalization care.

In **Chapter 5**, one of the main findings was the unreliability of USG in the assessment of urinary concentration in dogs with babesiosis and the consequences on the classification of azotemia. Future studies are necessary to elucidate the potential causes of this observed discrepancy between USG and uOsmol. The influence of severe hemoglobinuria and its degradation products on the agreement between USG and uOsmol should therefore be assessed, because hemoglobinuria is the most likely explanation of this observation in dogs with babesiosis. The influence of urinary electrolyte changes on the relationship between USG and uOsmol should also be investigated in the setting of canine AKI.

A secondary objective in **Chapter 5** was to explore associations between the presence of RA and selected clinical and laboratory variables. Although several statistical associations were found, these do not prove causality. However, results obtained by such an exploratory approach can be used to generate clearly defined hypotheses and thus help to perform proper hypothesis-based studies. Such studies can help in the understanding of the pathogenesis and ultimately in the treatment of *Babesia*-induced AKI. For example, several associations between hemoglobin and RA were found in Chapter 5. In an experimental study where dogs were included as an *in vivo* animal model, hemoglobinuria resulted in renal tubular injury mediated by free heme-triggered oxidative cell damage (Deuel et al., 2016). In falciparum malaria, the hypothesis of hemoglobin-mediated oxidative stress causing AKI was tested recently (Plewes et al., 2017). Cell-free hemoglobin and related oxidative stress biomarkers were associated with AKI in the latter study. Acetaminophen can inhibit cell-free hemoglobin-mediated oxidation and could therefore be renoprotective medication in the setting of falciparum malaria (Boutaud et al., 2010; Plewes et al., 2018). Evidence that acetaminophen improved renal function and reduced the risk of developing AKI in falciparum malaria, was very recently documented in a clinical trial (Plewes et al., 2018). These findings could be very relevant for other hemolytic diseases associated

with AKI, such as canine babesiosis, where hemoglobin-induced AKI (hemoglobinuric nephropathy) has been suggested before (Maegraith et al., 1957; Malherbe, 1966; Lobetti and Reyers, 1996; Lobetti et al., 1996). Therefore, further studies investigating the role of cell-free hemoglobin-mediated oxidative stress in *Babesia*-induced AKI in dogs are warranted.

10. General conclusions

Several kidney injury biomarkers were used in this doctoral thesis to characterize *B. rossi*-induced AKI, because traditionally used biomarkers have multiple general and babesiosis-specific limitations in the evaluation of AKI. Using urinary kidney injury biomarkers, the presence of subclinical AKI was first demonstrated in a group of dogs with uncomplicated babesiosis.

In the second part of this doctoral thesis, we investigated the stability of the previously measured urinary kidney injury biomarkers after 4 y of storage at -72 °C. A significant decay of all measured biomarkers was observed, emphasizing the importance to consider and report such pre-analytical factors in biomarker research.

In the third part of this doctoral thesis, occurrence of RA was assessed retrospectively in a large population of dogs with babesiosis caused by *B. rossi*. Occurrence of RA at presentation was 14%. This study also documented the unreliability of USG in the evaluation of renal concentrating ability in azotemic dogs with babesiosis.

In the last part of this doctoral thesis, dogs with uncomplicated and complicated *B. rossi* infection were evaluated at presentation and after treatment using a more extensive panel of biomarkers of kidney injury and dysfunction. Although *B. rossi*-induced AKI was primarily characterized as a transient manifestation of kidney injury, dogs with complicated babesiosis should be closely monitored for significant loss of renal function.

When all major findings of this doctoral thesis are merged, the overall conclusion is that subclinical and more advanced stages of AKI are frequently occurring complications in canine babesiosis caused by *B. rossi*. Use of traditional biomarkers to evaluate renal function (i.e., sCr and USG) results in an underestimation of *Babesia*-induced AKI. The clinical relevance of subclinical AKI is currently unknown in veterinary medicine. Nevertheless, provision of adequate supportive care and proactive elimination of any recognized risk factor of kidney injury should be advised in all dogs with babesiosis caused by *B. rossi*, irrespective of sCr, to prevent progressive AKI.

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SUMMARY

Canine babesiosis is an emerging protozoal vector-borne disease of worldwide significance. *Babesia rossi* is the most prevalent *Babesia* species infecting South African dogs, and is considered to be the most virulent of all canine *Babesia* species. Hemolytic anemia, which is caused by an intra-erythrocytic parasitemia, is the main clinical manifestation of this infectious disease. Life-threatening disease can develop due to a variety of different organ complications, including acute kidney injury (AKI).

Chapter 1 starts by describing the etiology of babesiosis and life cycle of *Babesia* spp. in dogs. All research in this doctoral thesis was performed in *B. rossi*-infected dogs in South Africa, therefore Chapter 1 is focused on the clinical setting of *B. rossi* infections in South Africa. However, the increasing relevance of this tick-borne disease is also highlighted in the European context. After introducing the concept of AKI, an overview of the current knowledge of *Babesia*-induced kidney injury is given. After summarizing the general and babesiosis-specific limitations of the traditionally used biomarkers of AKI, the general introduction concludes by providing the principles of urinary kidney injury biomarkers. Their application could help to improve detection and characterization of babesiosis-induced AKI in dogs.

The scientific aims of this doctoral thesis are described in **Chapter 2**. To overcome the limitations in the traditional evaluation of AKI, the main objective was to characterize AKI in canine babesiosis caused by *B. rossi*, using sensitive biomarkers of kidney injury next to, and in comparison with, the traditionally used diagnostic methods. Therefore, several urinary kidney injury biomarkers were first evaluated in *B. rossi*-infected dogs that presented with uncomplicated babesiosis. Stability of the same urinary biomarkers after long-term storage was also investigated. Occurrence of renal azotemia was then retrospectively determined in a large population of dogs with babesiosis caused by *B. rossi*. Lastly, to characterize *Babesia*-induced kidney injury at presentation and during follow-up, an expanded panel of different urinary, plasma and serum biomarkers of kidney injury and dysfunction was prospectively applied in a population of dogs with uncomplicated and complicated babesiosis.

In **Chapter 3**, kidney injury was assessed in 18 dogs that presented with uncomplicated babesiosis caused by *B. rossi*. Traditional biomarkers (serum urea, serum creatinine (sCr), urine specific gravity (USG), and urinary protein to creatinine ratio (UPC)) and several urinary kidney injury biomarkers (urinary immunoglobulin G (uIgG), urinary C-reactive protein (uCRP), and urinary retinol-binding protein (uRBP)), measured by validated immunoassays, were compared between the *Babesia*-infected dogs and 8 healthy control dogs. Potential interference by hemoglobinuria was also considered in the analyses of these urinary biomarkers. Different concentrations of a human hemoglobin standard were therefore added to urine samples of 4 healthy dogs. No significant interference was found when samples without added hemoglobin were compared to samples with different hemoglobin concentrations. All measured urinary kidney injury biomarkers were significantly higher in the *Babesia*-infected dogs compared to the healthy control dogs. In contrast, UPC was the only traditional biomarker significantly higher in the *Babesia*-infected dogs, while serum urea, sCr, and USG were not significantly different between both groups.

The investigated urinary biomarkers detected both glomerular and tubular kidney injury in this population of dogs with apparently uncomplicated babesiosis. Urinary biomarker analysis resulted in an earlier detection of kidney injury compared to the traditionally used biomarkers.

Reliable biomarker research is only possible when the influence of preanalytical factors, such as storage conditions, is known. In **Chapter 4**, stability of the previously measured urinary kidney injury biomarkers (uIgG, uCRP, and uRBP) was assessed after 4 years of storage at -72 °C. The same urine samples that were used to measure urinary biomarkers in Chapter 2, were analyzed again 4 years later. Long-term storage resulted in a significant decrease in the concentrations of all 3 biomarkers.

The main indication of these urinary biomarkers is detection of milder forms of kidney injury. In populations where kidney injury is subtle, their statistically significant decay during long-term storage could become clinically relevant. Long-term storage, which is common in research settings, should therefore be avoided.

In **Chapter 5**, occurrence of renal azotemia was determined in a population of 152 dogs that presented with babesiosis caused by *B. rossi*. Dogs were only included in this retrospective study if a complete blood count, biochemistry profile, and urinalysis was performed at admission, before any therapy was initiated. Interpretation of USG is hampered in babesiosis due to the presence of hemoglobinuria. Therefore, urine osmolality was used instead of USG to assess renal concentrating ability. Renal azotemia was defined as the presence of azotemia (i.e., sCr concentration above the reference interval) in combination with an inappropriately low urinary concentration (i.e., urine osmolality <1110 mOsmol/kg). Azotemia was diagnosed at presentation in 17% (26/152) of the *Babesia*-infected dogs. Occurrence of renal azotemia at admission was 14% (21/152) among all dogs included, hence present in 81% (21/26) of the azotemic dogs. In contrast, when renal azotemia was defined as the presence of azotemia combined with a USG <1.030, only 4% (6/152) of all dogs included and 23% (6/26) of azotemic dogs would have been considered to have renal azotemia. A secondary aim of this study was to examine potential associations between the presence of renal azotemia and selected clinical and laboratory variables. Several variables were associated with the presence of renal azotemia, including older age, the presence of collapse, hypoglycemia, hyperphosphatemia, and a worse survival.

Results of this study confirmed the initial hypothesis that renal azotemia is a relatively common complication, that was underdiagnosed in the literature on canine babesiosis. The unreliability of USG in the evaluation of renal concentrating ability in azotemic dogs with babesiosis was also demonstrated. Based on USG interpretation, 71% (15/21) of dogs with renal azotemia were misclassified as having prerenal azotemia, due to a systematic overestimation of urinary concentration by USG.

In **Chapter 6**, dogs with uncomplicated and complicated *B. rossi* infection were prospectively enrolled to characterize *Babesia*-induced kidney injury. In total, 35 dogs with babesiosis were included and compared to 10 healthy control dogs. Traditional biomarkers (sCr and UPC), several kidney injury biomarkers (uIgG, uCRP, uRBP, urinary and plasma neutrophil gelatinase-associated lipocalin (uNGAL, pNGAL)), and a novel functional renal biomarker (serum symmetric dimethylarginine (sSDMA)) were measured in all dogs at presentation, in 11 *Babesia*-infected dogs also after 24 hours, and in 9 *Babesia*-infected dogs also after 1 month. All renal biomarkers were also compared between the 10 dogs with uncomplicated and the 25 dogs with complicated

babesiosis. All kidney injury biomarkers were significantly higher in the *Babesia*-infected dogs compared to the healthy control dogs, while both functional biomarkers (sCr and sSDMA) were not significantly different. The concentrations of the urinary kidney injury biomarkers started to decrease after 24 hours, and this decrease was significant for all after 1 month, reaching values not significantly different from healthy control dogs. Significant changes in functional renal biomarkers and pNGAL were not seen after treatment. Lastly, dogs with complicated babesiosis had significantly higher urinary glomerular injury biomarkers (uIgG and uCRP), UPC, and sSDMA compared to uncomplicated cases, while tubular injury biomarkers (uRBP, uNGAL, and pNGAL) and sCr were not significantly different.

The last study in this doctoral thesis demonstrated that all dogs with babesiosis caused by *B. rossi*, irrespective of disease severity, presented with evidence of kidney injury. This was detected by all kidney injury biomarkers, but remained undetected by functional biomarkers. Based on the kidney injury biomarker results, a transient nature of the kidney injury was observed. Lastly, results also suggested that dogs with complicated babesiosis were more likely to present with significant loss of renal function.

In **Chapter 7**, the main findings of this doctoral thesis are discussed. Application of urinary kidney injury biomarkers and urine osmolality documented that both subclinical AKI (defined as increased concentrations of kidney injury biomarkers without loss of function based on functional biomarkers) and more advanced stages of AKI (i.e., increased concentrations of kidney injury biomarkers with loss of function based on functional biomarkers, and renal azotemia) are frequently occurring complications in canine babesiosis caused by *B. rossi*. Traditional evaluation of renal function clearly results in an underestimation of *Babesia*-induced AKI. Although the clinical relevance of subclinical AKI remains to be determined, adequate supportive care and proactive elimination of any recognized risk factor of kidney injury should be pursued in any dog with babesiosis caused by *B. rossi*, irrespective of sCr, in order to prevent progressive AKI.

SAMENVATTING

Babesiose is een opkomende, vector-overdraagbare protozoaire ziekte van wereldwijd belang bij de hond. *Babesia rossi* is de meest voorkomende *Babesia* soort die Zuid-Afrikaanse honden infecteert, en wordt beschouwd als de meest virulente van alle *Babesia* species bij de hond. De voornaamste klinische presentatie van deze infectieziekte is een hemolytische anemie, die veroorzaakt wordt door een intra-erythrocytaire parasitemie. Een levensbedreigende ziekte kan ontstaan ten gevolge van verschillende orgaancomplicaties, waaronder acute nierschade (ANS).

Hoofdstuk 1 start met de beschrijving van de etiologie van babesiose en de levenscyclus van *Babesia* spp. bij de hond. Omdat het onderzoek in dit proefschrift werd uitgevoerd bij *B. rossi*-geïnfecteerde honden in Zuid-Afrika, ligt de focus op de klinische situatie van *B. rossi* infecties in Zuid-Afrika. Er wordt echter ook aandacht geschonken aan het toenemende belang van deze teken-overdraagbare ziekte in Europa. Na de introductie van het concept ANS, wordt vervolgens een overzicht geschetst van de huidige kennis over nierschade veroorzaakt door *Babesia*. Na het bespreken van de algemene en babesiose-specifieke beperkingen van de traditionele biomerkers voor ANS, eindigt de algemene introductie met de principes van urinaire biomerkers voor nierschade. Toepassing van deze gevoeligere biomerkers zou namelijk het detecteren en omschrijven van babesiose-geïnduceerde ANS bij de hond kunnen bevorderen.

In **Hoofdstuk 2** worden de wetenschappelijke doelstellingen van dit proefschrift omschreven. Om de beperkingen van de traditionele evaluatie van ANS te omzeilen, was de hoofddoelstelling om ANS bij honden met babesiose, veroorzaakt door *B. rossi*, te karakteriseren door gebruik te maken van gevoelige biomerkers voor nierschade naast, en in vergelijking met, de traditionele diagnostische methoden. Daarvoor werden eerst verschillende urinaire biomerkers voor nierschade geëvalueerd bij *B. rossi*-geïnfecteerde honden die aangeboden werden met een ongecompliceerde vorm van babesiose. De stabiliteit van dezelfde urinaire biomerkers werd ook onderzocht na langdurige bewaring. Vervolgens werd het voorkomen van renale azotemie retrospectief onderzocht bij een grote populatie van honden met babesiose. Om *Babesia*-geïnduceerde nierschade prospectief te karakteriseren tijdens presentatie en opvolging, werd tenslotte gebruik gemaakt van een meer uitgebreide set van verschillende urinaire, plasma en serum biomerkers voor nierschade en -dysfunctie in

een populatie van honden met zowel ongecompliceerde als gecompliceerde babesiose.

In **Hoofdstuk 3** werd nierschade beoordeeld bij 18 honden die aangeboden werden met ongecompliceerde babesiose veroorzaakt door *B. rossi*. Traditionele biomerkers (serum ureum, serum creatinine (sCr), urinair soortelijk gewicht (USG), en urinair eiwit op creatinine ratio (UPC)) en verschillende urinaire biomerkers voor nierschade (urinair immunoglobuline G (uIgG), C-reactief proteïne (uCRP), en retinol-bindingsproteïne (uRBP)), gemeten door middel van gevalideerde immunoassays, werden vergeleken tussen deze *Babesia*-geïnfecteerde honden en 8 gezonde honden. Bij de analyses van de urinaire biomerkers werd een mogelijke interferentie door hemoglobinurie ook in overweging genomen. Daarvoor werden verschillende concentraties van een humane hemoglobine standaard toegevoegd aan urinestalen van 4 gezonde honden. Bij vergelijking van de stalen zonder en met hemoglobine werd geen significante interferentie gedetecteerd. Alle onderzochte urinaire biomerkers voor nierschade vertoonden significant hogere concentraties bij de *Babesia*-geïnfecteerde honden in vergelijking met de gezonde honden. De enige traditionele biomerker die significant hoger was bij de *Babesia*-geïnfecteerde honden was de UPC, terwijl serum ureum, sCr en USG niet significant verschillend waren tussen beide groepen.

De onderzochte urinaire biomerkers detecteerden zowel glomerulaire als tubulaire nierschade in deze groep van honden met ogenschijnlijk ongecompliceerde babesiose. Deze urinaire biomerkers zorgden dus voor een vroegere detectie van nierschade in vergelijking met de traditionele biomerkers.

Betrouwbaar biomerker onderzoek is enkel mogelijk als de invloed van pre-analytische factoren, zoals de bewaringsomstandigheden, gekend is. In **Hoofdstuk 4** werd de stabiliteit van de eerder gemeten urinaire biomerkers voor nierschade (uIgG, uCRP, en uRBP) bepaald na bewaring gedurende 4 jaar op -72 °C. Dezelfde urinestalen, die gebruikt werden om de urinaire biomerkers in Hoofdstuk 2 te meten, werden 4 jaar later opnieuw geanalyseerd. Langdurige bewaring zorgde voor een significante daling in de concentratie van alle biomerkers.

De hoofdindicatie van deze urinaire biomerkers is de detectie van mildere vormen van nierschade. In populaties waar nierschade beperkt is, zou dit statistisch significante verval na langdurige bewaring klinisch relevant kunnen worden.

Langdurige bewaring gebeurt echter vaak in onderzoeksomstandigheden en zou daarom vermeden moeten worden.

In **Hoofdstuk 5** werd het voorkomen van renale azotemie bepaald bij 152 honden die aangeboden werden met babesiose veroorzaakt door *B. rossi*. Honden werden alleen geïncludeerd in deze retrospectieve studie indien er een volledig hematologisch en biochemisch bloedonderzoek, alsook een urineonderzoek uitgevoerd werd bij presentatie, vooraleer er enige behandeling werd opgestart. De interpretatie van het USG wordt bij babesiose bemoeilijkt door de aanwezigheid van hemoglobinurie. Daarom werd gebruik gemaakt van de urine osmolaliteit in plaats van het USG om het concentrerend vermogen van de nieren na te gaan. Renale azotemie werd gedefinieerd als de aanwezigheid van azotemie (i.e., sCr concentratie boven het referentie-interval) in combinatie met een onaangepast lage urinaire concentratie (i.e., urine osmolaliteit <1110 mOsmol/kg). Op het moment van aanbieden werd een azotemie vastgesteld bij 17% (26/152) van alle *Babesia*-geïnfecteerde honden. Het voorkomen van een renale azotemie was bij presentatie 14% (21/152) bij alle honden, en was bijgevolg dus aanwezig bij 81% (21/26) van de azotemische honden. Als renale azotemie echter gedefinieerd werd als de aanwezigheid van azotemie samen met een USG <1.030, werd slechts 4% (6/152) van alle honden en 23% (6/26) van de azotemische honden beschouwd een renale azotemie te hebben. Een bijkomende doelstelling van deze studie was om mogelijke associaties tussen de aanwezigheid van renale azotemie en bepaalde klinische en labovariabelen te onderzoeken. Verschillende variabelen waren geassocieerd met de aanwezigheid van renale azotemie, waaronder een oudere leeftijd, de aanwezigheid van collaps, hypoglycemie, hyperfosfatemie, en een slechtere overleving.

Resultaten van deze studie bevestigden de initiële hypothese dat renale azotemie een relatief vaak voorkomende complicatie is, die ondergediagnosticeerd was in de literatuur over babesiose bij de hond. Ook werd de onbetrouwbaarheid van het USG bij de evaluatie van het concentrerend vermogen van de nieren bij azotemische honden met babesiose aangetoond. Gebaseerd op het USG, werd 71% (15/21) van de honden met een renale azotemie verkeerdelijk gediagnosticeerd met een prerenale azotemie, ten gevolge van een systematische overschatting van de urinaire concentratie door het USG.

In **Hoofdstuk 6** werden honden met ongecompliceerde en gecompliceerde *B. rossi* infecties op een prospectieve manier geïncludeerd om *Babesia*-geïnduceerde nierschade verder te karakteriseren. In totaal werden 35 honden met babesiose geïncludeerd en vergeleken met 10 gezonde honden. Traditionele biomerkers (sCr en UPC), verschillende biomerkers voor nierschade (uIgG, uCRP, uRBP, urinair en plasma neutrofiel gelatinase-geassocieerd lipocaline (uNGAL, pNGAL)), en een nieuwe functionele renale biomerker (serum symmetrisch dimethylarginine (sSDMA)) werden bij alle honden gemeten bij presentatie, bij 11 *Babesia*-geïnfecteerde honden ook na 24 uur, en bij 9 *Babesia*-geïnfecteerde honden ook na 1 maand. Alle renale biomerkers werden ook vergeleken tussen de 10 honden met ongecompliceerde en de 25 honden met gecompliceerde babesiose. Alle biomerkers voor nierschade waren significant hoger bij de *Babesia*-geïnfecteerde honden in vergelijking met de gezonde honden, terwijl beide functionele biomerkers (sCr en sSDMA) niet significant verschillend waren. De concentraties van de urinaire biomerkers voor nierschade begonnen te dalen na 24 uur, en die daling was significant voor al deze merkers na 1 maand, waarbij waarden bekomen werden die niet meer significant verschillend waren van de gezonde honden. Significante veranderingen werden niet vastgesteld na behandeling bij de functionele renale biomerkers en bij pNGAL. Tenslotte hadden honden met gecompliceerde babesiose significant hogere concentraties van de urinaire biomerkers voor glomerulaire nierschade (uIgG en uCRP), UPC, en sSDMA in vergelijking met de ongecompliceerde gevallen, terwijl de biomerkers voor tubulaire nierschade (uRBP, uNGAL, en pNGAL) en sCr niet significant verschillend waren.

In de laatste studie van dit proefschrift werd aangetoond dat alle honden met babesiose veroorzaakt door *B. rossi*, ongeacht de ernst van de ziekte, nierschade vertoonden bij presentatie. Dit werd aangetoond met alle biomerkers voor nierschade, maar bleef onopgemerkt met de functionele biomerkers. Op basis van de resultaten van de biomerkers voor nierschade bleek de opgelopen nierschade wel van voorbijgaande aard te zijn. Tenslotte suggereerden de resultaten ook dat honden met gecompliceerde babesiose meer kans hadden om aangeboden te worden met een significant verminderde nierfunctie.

In **Hoofdstuk 7** worden de voornaamste bevindingen van dit proefschrift besproken. Door gebruik te maken van urinaire biomerkers voor nierschade en van urine osmolaliteit, werd aangetoond dat zowel subklinische ANS (gedefinieerd als gestegen concentraties van biomerkers voor nierschade zonder verlies van functie gebaseerd op functionele biomerkers) als meer gevorderde stadia van ANS (i.e., gestegen concentraties van biomerkers voor nierschade met verlies van functie gebaseerd op functionele biomerkers, en renale azotemie) frequent voorkomende complicaties zijn bij honden met babesiose veroorzaakt door *B. rossi*. De traditionele evaluatie van de nierfunctie zorgt duidelijk voor een onderschatting van de *Babesia*-geïnduceerde ANS. Hoewel de klinische relevantie van subklinische ANS nog niet gekend is, dient een gepaste ondersteunende behandeling en een proactieve eliminatie van alle gekende risicofactoren voor nierschade nagestreefd te worden bij elke hond met babesiose veroorzaakt door *B. rossi*, onafhankelijk van het sCr, en dit om progressie van ANS te voorkomen.

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CURRICULUM VITAE

Pieter Defauw werd geboren op 20 augustus 1984 te Kortrijk. In 2002 behaalde hij zijn diploma algemeen secundair onderwijs Latijn-Wiskunde aan het Sint-Amandscollege te Kortrijk. Nadien startte hij met de studie Diergeneeskunde aan de Universiteit Gent. Hij behaalde in 2008 met grootste onderscheiding het diploma van Dierenarts in de afstudeerrichting Gezelschapsdieren.

Onmiddellijk na het afstuderen als dierenarts volgde hij een roterend internship aan de Vakgroep Kleine Huisdieren van de faculteit Diergeneeskunde aan de Universiteit Gent, meteen gevolgd door een vier jaar durende specialisatie opleiding (residency) in de interne geneeskunde voor kleine huisdieren aan dezelfde vakgroep. Deze opleiding werd met succes afgerond in 2015 na het behalen van de titel van erkend Europees dierenarts-specialist (*Diplomate of the European College of Veterinary Internal Medicine – Companion Animals*).

Sinds 2013 werkt Pieter Defauw als assistent aan dezelfde vakgroep op de dienst interne geneeskunde onder leiding van Prof. dr. Sylvie Daminet. Vanaf 2015 combineert hij het klinische werk in de interne geneeskunde en de begeleiding van studenten Diergeneeskunde, samen met een doctoraatsstudie naar nierschade bij honden met babesiose. Dit doctoraatsonderzoek gebeurt onder leiding van Prof. dr. Sylvie Daminet, samen met Prof. dr. Evelyne Meyer van de Vakgroep Farmacologie, Toxicologie en Biochemie van de Universiteit Gent, en in samenwerking met Prof. dr. Johan Schoeman van de Universiteit van Pretoria, Zuid-Afrika. In 2018 vervolledigde hij het trainingsprogramma van de *Doctoral School of Life Sciences and Medicine* van de Universiteit Gent.

Pieter Defauw is auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften en presenteerde ook verschillende abstracts op internationale veterinaire congressen. Tenslotte geeft hij regelmatig bijscholingen aan praktiserende dierenartsen.

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