Evaluation of acute kidney injury in dogs with complicated or uncomplicated *Babesia rossi* infection

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Abbreviations:

AKI, acute kidney injury; 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; IgG, immunoglobulin G; IMHA, immune-mediated hemolytic anemia; IRIS, International Renal Interest Society; LM, light microscopy; NGAL, neutrophil gelatinase-associated lipocalin; OVAH, Onderstepoort Veterinary Academic Hospital; PAS, periodic acid-Schiff; RBP, retinol-binding protein; RLB, reverse line blot; sCr, serum creatinine; SDMA, symmetric dimethylarginine; TEM, transmission electron microscopy; uCr, urinary creatinine; UPC, urinary protein to creatinine ratio; USG, urine specific gravity; uOsmol, urine osmolality; UTI, urinary tract infection

Abstract

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 (T_0, n=35), after 24 hours (T_{24h}, n=11), and after 1 month (T_{1m}, 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 T_0, 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 T_{24h}, all urinary tubular injury biomarkers and UPC decreased significantly (P<0.01), while glomerular injury biomarkers did not (P=0.084). At T_{1m}, 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. All infected dogs, irrespective of disease severity, suffered comparable kidney injury based on tubular injury biomarker concentrations, while loss of function was seen more often in dogs with complicated babesiosis based on sSDMA results.

1. Introduction

Babesia rossi is an intra-erythrocytic protozoan, capable of causing life-threatening disease in infected dogs (Jacobson, 2006). *Babesia* species have different tick vectors, with *B. rossi* in sub-Saharan Africa being transmitted by *Haemaphysalis elliptica* (and presumably *Haemaphysalis leachi*) (Uilenberg et al., 1989; Apanaskevich et al., 2007; Penzhorn et al., 2017). Only the adult life stage of the tick is capable of transmitting *B. rossi* (Lewis et al., 1996). Because domestic dogs are not native to sub-Saharan Africa and *B. rossi* and its tick vector only occur in this region (Mitchell, 2015), it has been suggested that indigenous African canids are the reservoir hosts of *B. rossi* (Penzhorn, 2011). Evidence suggests that these indigenous African canids are able to cope with *B. rossi* infection and become asymptomatic carriers of this parasite (Penzhorn, 2011). Black-backed jackals were subsequently confirmed to be natural hosts of *B. rossi* in South Africa (Penzhorn et al., 2017). After introduction of domestic dogs into sub-Saharan Africa, *Haemaphysalis* ticks could not only infest their natural hosts, but also this newly introduced and highly susceptible domestic dog population, in whom the parasite causes disease because they would have had insufficient time to co-evolve with the *B. rossi* parasite (Penzhorn, 2011; Mitchell, 2015). 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 acute kidney injury (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 (Cowgill, 2016). 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 serum creatinine (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 *Babesia 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 (SDMA) 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 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.

2. Materials and methods

2.1. 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 urinary protein to creatinine ratio (UPC) and aerobic bacterial culture) was performed at presentation (T_0), 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 T_0 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 *Babesia vogeli* and/or concurrent *Ehrlichia canis* infection. Control dogs that tested positive for any *Babesia* species or *E. canis* were excluded.

In dogs with babesiosis, follow-up blood and urine samples were obtained 24 hours after presentation (T_{24h}) and 1 month after treatment (T_{1m}) whenever possible. Follow-up samples at T_{24h} were only collected in dogs that were hospitalized because of disease severity. At T_{24h},

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 T_{1m}.

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 (Cowgill, 2016). 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 4-5 weeks after presentation).

2.2. 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 T_0, T_{24h}, and T_{1m}, included urinary immunoglobulin G (uIgG), urinary C-reactive protein (uCRP), urinary retinol-binding protein (uRBP), urinary and plasma neutrophil gelatinase-associated lipocalin (uNGAL and pNGAL). Measurement of uIgG and uCRP was performed with canine sandwich enzyme-linked immunosorbent assay (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 months of sample collection, except 1 sample for uIgG and 4 samples for uRBP that required re-analysis with another dilution. Serum SDMA (IDEXX SDMA™ Test) was measured as a novel functional renal biomarker at T_0, T_{24h}, and T_{1m}. 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 months after collection.

2.3. 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).

2.4. 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 T_0, T_{24h}, and T_{1m} 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).

3. Results

3.1. 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 *E. 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.

Table 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.

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; range, 2 months to 12.5 years) and controls (2 years; 4 months to 9.5 years) (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 1.

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.

3.2. AKI biomarkers at T_0

In Table 2, results of routine renal biomarkers (sCr, serum urea, USG, uOsmol, and UPC) at T_0 are compared between healthy controls and dogs with babesiosis. In Fig. 1 and Table 3, results of kidney injury biomarkers (uIgG/uCr, uCRP/uCr, uRBP/uCr, uNGAL/uCr, pNGAL, u/pNGAL) and sSDMA at T_0 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 T_0. 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 physical examination, 7/35 dogs with babesiosis had clinical signs of dehydration, of which 4 had increased sSDMA concentrations.

3.3. Follow-up at T_{24h} and T_{1m}

Twenty-four of the 35 dogs with babesiosis were hospitalized. At T_{24h}, 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 T_{1m}. Seven of these dogs had follow-up samples collected at both T_{24h} and T_{1m}. Because of a UTI, one dog at T_{24h} and 2 dogs at T_{1m} 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 T_{1m}. With exception of sSDMA, which was borderline increased (15 μg/dL), all renal biomarkers in this dog were normalized at T_{1m}. Short-term survival was 89% (31/35 dogs). Two dogs died spontaneously within 8 hours 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.

Table 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 T_0	B T_{24h}	B T_{1m}	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	5.6	7.4			0.096			
(mmol/L)	(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	1254	1315	1062	927	0.45	0.15	0.098	0.35
(mOsmol/kg)	(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 T_0: dogs with babesiosis at presentation; B T_{24h}: dogs with babesiosis 24 hours after presentation; B T_{1m}: 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 T_0

[b]P values for the comparison between dogs with babesiosis at T_0 and T_{24h}

[c]P values for the comparison between dogs with babesiosis at T_0 and T_{1m}

[d]P values for the comparison between healthy control dogs and dogs with babesiosis at T_{1m}
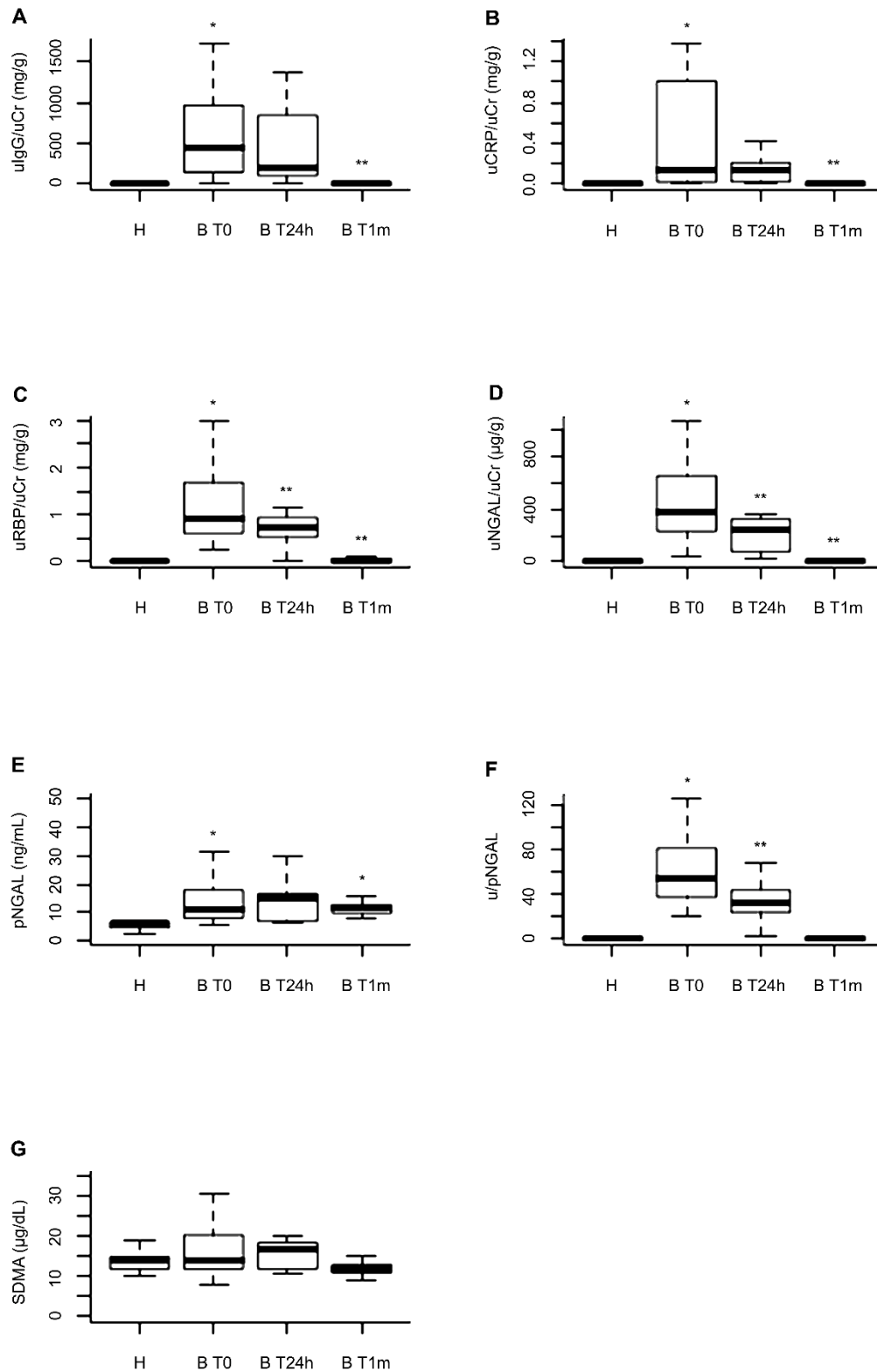
Fig. 1. Kidney injury biomarkers (A: uIgG/uCr; B: uCRP/uCr; C: uRBP/uCr; D: uNGAL/uCr; E: pNGAL; F: u/pNGAL) and serum SDMA (G) in healthy control dogs (H, n=10) and dogs with babesiosis at presentation (B T_0, n=35), 24 hours after presentation (B T_{24}h, n=11), and 1 month after treatment (B T_1m, 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.

Table 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 T_0	B T_{24h}	B T_{1m}	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 T_0, dogs with babesiosis at presentation; B T_{24h}, dogs with babesiosis 24 hours after presentation; B T_{1m}, 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 T_0

[b]P values for the comparison between dogs with babesiosis at T_0 and T_{24h}

[c]P values for the comparison between dogs with babesiosis at T_0 and T_{1m}

[d]P values for the comparison between healthy control dogs and dogs with babesiosis at T_{1m}

In Table 2, routine renal biomarkers results are compared between T_0, T_{24h}, and T_{1m}. In Fig. 1 and Table 3, results of kidney injury biomarkers and sSDMA are compared between T_0, T_{24h}, and T_{1m}. Results of biomarker comparison between healthy controls and *Babesia*-treated dogs at T_{1m} are also shown in Fig. 1, Tables 2 and 3.

At T_{24h}, UPC, uRBP/uCr, uNGAL/uCr, and u/pNGAL decreased significantly (Tables 2 and 3, Fig. 1). At T_{1m}, decreases were significant for USG and all urinary kidney injury biomarkers (Tables 2 and 3, Fig.1). Significant changes in sCr, sSDMA, pNGAL, and uOsmol were not seen during follow-up at T_{24h} and T_{1m}. At T_{1m}, 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 T_{24h}, 6/11 dogs with babesiosis (55%) had increased sSDMA concentrations, while at T_{1m} 2/9 dogs with babesiosis (22%) had a borderline increased sSDMA (15 μg/dL).

3.4. 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 T_0, 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 T_{24h}, 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 T_{1m}, 2 dogs were presented with AKI grade II at T_0, while 2 dogs developed AKI grade I and one dog AKI grade II during hospitalization. In these 5 dogs diagnosed with AKI at T_0 or T_{24h}, no evidence of kidney injury or dysfunction was detected at T_{1m}, except for one dog with a borderline increased sSDMA (15 μg/dL).

3.5. AKI biomarkers in uncomplicated and complicated babesiosis at T_0

In Table 4, results of routine renal biomarkers, kidney injury biomarkers, and sSDMA at T_0 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 T_0.

3.6. Histological and ultrastructural renal findings in dogs with complicated babesiosis

Within 6 hours after spontaneous death, kidney samples were obtained from 2 dogs with complicated babesiosis. Dog 1 was a 2-month-old male intact Irish wolfhound, presenting with peracute cerebral babesiosis. The first clinical signs (lethargy and vomiting) were noticed a couple of hours 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

Table 4

Results of routine renal biomarkers, kidney injury biomarkers, and serum SDMA in 10 dogs with uncomplicated and 25 dogs with complicated babesiosis at T_0. 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.

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 acute tubular necrosis (ATN) and degeneration further confirmed by TEM.

Dog 2 was a 10-year-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.

4. 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 T_{1m}, 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 from 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. 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). During systemic inflammation, pNGAL and uNGAL can also increase due to increased synthesis from non-renal tissues and increased release from circulating neutrophils (Mårtensson and Bellomo, 2014). Because both systemic and urinary NGAL concentrations were measured in this study, the impact of systemically originating NGAL in canine babesiosis can be assessed to a certain extent. The u/pNGAL ratio was also calculated in the current study to aid 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 authors' 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 u/pNGAL ratio does however not correct for variations in the urinary flow rate. When NGAL concentrations in the current study were compared to NGAL concentrations reported in a study with septic dogs (Cortellini et al., 2015), the median systemic NGAL concentration was 5-6 times higher in dogs with bacterial sepsis compared to dogs with babesiosis, while the median uNGAL concentration was very similar in both studies. 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. The highly increased u/pNGAL ratio at T_0 is also 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).

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 T_{24h}, 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 T_{1m}. 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 hours (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 T_{1m} compared to healthy controls, suggesting that there was no evidence of active ongoing kidney injury in these dogs 1 month 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 T_{1m} 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 T_{1m}. 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 T_{1m} (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 T_{1m} (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 T_{1m}. Whether the dog with relatively low urinary concentration had early-stage CKD, is uncertain. Although there was no evidence of active kidney injury based on the investigated kidney injury biomarkers in this small number of dogs included at T_{1m}, 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.

In dogs with complicated disease, both of the 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, 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 T_{24h} and T_{1m} 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 T_{24h}, leading to a sampling bias. Additional AKI diagnoses were made in 5/13 dogs with sCr follow-up at T_{24h}, which is likely to be an overestimation due to this bias.

5. Conclusions

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.

Declarations of interest

None.

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