Evaluation of C-reactive protein and its kinetics as a prognostic indicator in canine leptospirosis

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ABSTRACT

Objective: to evaluate C-reactive protein (CRP) in dogs with Acute Kidney Injury (AKI) due to leptospirosis at presentation and during hospitalisation, to compare it at presentation in dogs with AKI of another origin and to study its correlation with markers of inflammation, azotaemia and survival.

Methods: prospective observational study in 41 dogs with AKI secondary to leptospirosis and 15 control dogs with AKI of another origin. CRP was measured at presentation in both groups and daily for 7 days in a subgroup of 28 dogs with leptospirosis. The association of CRP with neutrophil count, albumin, urea, creatinine and survival was studied.

Results: CRP was increased at presentation in all dogs with leptospirosis, but not significantly different from control dogs (p=0.088). It was associated with markers of inflammation (neutrophil count, P=0.003; albumin, P=0.005), but not with azotaemia (creatinine, P=0.983; urea, P=0.744). CRP decreased gradually from d0-d4, with significantly lower concentrations for survivors than non-survivors. A spike in CRP was associated with a secondary infection in 2 dogs. Initial CRP was only weakly predictive of outcome (AUC=0.69, P=0.047), but its average concentration from d0-d2 was a strong predictor (AUC=0.88, P<0.001). In contrast, absolute and relative changes in CRP during hospitalisation and creatinine at presentation were not predictive of survival.

Clinical significance: a serial assessment of CRP may improve outcome prediction in dogs with leptospirosis compared to a single measurement at presentation or to markers of renal function. The course of CRP may alert the clinician for possible inflammatory or infectious complications.

Keywords: leptospirosis, acute-phase protein, CRP, dogs, acute kidney injury

INTRODUCTION

C-reactive protein (CRP) is a major acute phase protein in humans, dogs and pigs (Caspi *et al.* 1987, Murata *et al.* 2004, Pepys and Hirschfield 2003). Inflammatory, toxic or traumatic tissue insults and stress cause an increase in proinflammatory cytokines, especially interleukin-1, interleukin-6 and tumor necrosis factor-α (Murata *et al.* 2004). These cytokines induce the hepatic production of acute phase proteins such as CRP as part of the early, non-specific immune response (Ceron *et al.* 2005, Whicher and Westacott 1992). C-reactive protein is therefore very unspecific, but it represents one of the earliest markers of systemic inflammation and can be increased before clinical signs are visible (Ceron *et al.* 2005). It has been shown to be increased in a wide spectrum of diseases including infectious diseases (Gebhardt *et al.* 2009, Kocaturk *et al.* 2010, Mylonakis *et al.* 2011), immune-mediated disorders (Griebsch *et al.* 2009, Mitchell *et al.* 2009, Ohno *et al.* 2006) and neoplasia (Chase *et al.* 2012, Mischke *et al.* 2007), and it is used as a general marker of inflammation. Its concentration has also been shown to be useful in the surveillance of treatment success and for postoperative monitoring (Dabrowski *et al.* 2009, Nielsen *et al.* 2007). For some diseases, such as acute abdomen syndrome in dogs (Galezowski *et al.* 2010) or acute kidney injury (AKI) in humans, (Xie *et al.* 2011) CRP has value as a prognostic marker.

Leptospirosis is a bacterial infection that results in a multisystemic inflammatory reaction affecting the kidneys, liver, lung, pancreas, heart, muscles, joints, central nervous system, eyes, vessels, and haemostasis (Major *et* al. 2014, Sykes *et al.* 2011). C-reactive protein raises dramatically after infection in humans suffering from leptospirosis and normalisation is achieved approximately seven days later (Crouzet *et al.* 2011). The same study also showed that CRP concentration was associated with the severity of infection. In dogs with leptospirosis, CRP has been shown to be increased as well (Caspi *et al.* 1987, Mastrorilli *et al.* 2007) but its kinetics during the disease process has not been assessed and a direct association with survival has not been shown.

46 The aims of this study were therefore 1) to describe the concentration of CRP in dogs with AKI due to
47 leptospirosis at presentation and its time course during hospitalisation; 2) to evaluate the association
48 of CRP with other markers of inflammation and with the degree of renal injury; and 3) to evaluate the
49 association of CRP kinetics with survival. Since all dogs with leptospirosis had evidence of AKI, a control
50 group of dogs with AKI due to other causes was included for comparison of CRP at presentation.

51 Our main hypotheses were 1) that dogs with leptospirosis have an elevated CRP concentration at
52 presentation, higher than sick control dogs; 2) that CRP concentration is associated with the level of
53 azotaemia, with other markers of inflammation, and with survival; 3) that CRP concentration decreases
54 within 5 days of treatment initiation; and 4) that its serial evaluation in leptospirosis could improve
55 survival prediction compared to a single measurement at presentation.

MATERIALS AND METHODS

Case selection and clinical characterisation

This prospective study was approved by the [redacted] and it adhered strictly to national, and institutional guidelines. It included client-owned dogs diagnosed with AKI between May 2012 and December 2014 at the [redacted]. Acute kidney injury was defined by the combination of historical, clinical, laboratory, and imaging evidence, with at least two of the following criteria (International Renal Interest Society, IRIS, 2013): 1) presence of renal azotaemia with a serum creatinine ≥140 μmol/L persisting at least 24h after correction of prerenal factors; 2) increase in serum creatinine ≥26 μmol/L during a 48h interval in the absence of prerenal factors; 3) persistent pathological oligoanuria (<1 mL/kg/h over 6h) after volume repletion; and 4) evidence of tubular injury with renal glucosuria or granular casts on urinalysis. Dogs with evidence of chronic kidney disease (CKD) on ultrasonographic examination, such as small or irregular kidneys, or with a history of CKD were excluded from the study.

History was obtained from owners and referring veterinarians. Clinical examination, blood pressure measurement, complete blood work (complete blood count, full chemistry profile, and coagulation profile), urinalysis with culture (if the dog was not anuric on presentation), microagglutination test (MAT) for leptospirosis, and diagnostic imaging (thoracic radiographs and abdominal ultrasound) were performed on all dogs at presentation. Serum MAT was performed by the [redacted] with a panel of 12 locally prevalent *Leptospira* serovars Australis, Autumnalis, Bataviae, Bratislava, Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, Pomona, Pyrogenes, Sejroe, and Tarasovi. Sera were screened at a dilution of 1:100 and serial 2-fold dilutions were performed on positive samples up to a dilution of 1:3200.

Dogs diagnosed with AKI due to leptospirosis were included in the main study group (L) and dogs with AKI from other aetiologies formed the control group (nL). A diagnosis of acute leptospirosis was based

on compatible clinical and clinicopathologic findings confirmed by seroconversion with ≥4-fold MAT titre increase in paired samples 1-3 weeks apart, at least 4 weeks post-vaccination or by a positive urine, blood or tissue RT-PCR. When no alternative aetiology could be identified, PCR was not done or negative, and when early clinical deterioration precluded confirmation with seroconversion, a strong clinical suspicion alone or combined to a positive single MAT titre ≥1:800 or to a positive immunodiffusion rapid test (Test-it™ Leptospira Canine IgM Lateral Flow Assay, LifeAssay Diagnostics (Pty) Ltd) were considered diagnostic (Fraune *et al.* 2013, Gloor *et al.* 2017). A strong clinical suspicion was defined as ≥3 of the 4 main organ manifestations of leptospirosis defined below. Dogs with no rise in titre within 1-3 weeks and those where another cause of AKI could be identified were defined as the control group nL.

Organ manifestations of leptospirosis were assessed for each case of AKI at presentation. The AKI was graded according to the IRIS system (IRIS guidelines, 2013). Hepatic involvement was assessed based on serum bilirubin, alanine aminotransferase (ALAT), and aspartate aminotransferase (ASAT). Normal or slightly elevated liver values were considered as no hepatic involvement (grade 0); bilirubin concentration between 10-30 µmol/l with at least twofold increased ALAT or ASAT as grade 1; and bilirubin concentration >30 µmol/l with at least twofold increased ALAT or ASAT as grade 2. Pulmonary involvement was graded based on clinical and radiographic parameters after correction of possible fluid overload. Grade 0 included cases with no respiratory signs of dyspnoea and no radiographic abnormalities; grade 1, cases with no respiratory signs but radiographic abnormalities; grade 2, cases with clinical signs of respiratory impairment and radiographic abnormalities; and grade 3 cases with severe signs of respiratory distress resulting in death. Laboratory evidence of disseminated intravascular coagulation (DIC) included thrombocytopenia, coagulation times (prothrombin time or activated partial thromboplastin time) prolonged by at least 25%, and a reduced fibrinogen. Dogs were classified in DIC if at least 3/4 parameters were abnormal.

All dogs and their indwelling catheters were examined daily during hospitalisation for signs of inflammation. If an unexpected course of disease was noticed (especially development fever >39.4°C or inappropriate response to treatment), an aerobic blood culture was submitted.

CRP measurements

At presentation (d0), blood was sampled in all dogs from a peripheral or a central vein with a 23G or 21G needle and collected in a serum tube. It was left one hour for clotting at room temperature before centrifugation (10 min, 3000g, 4°C). Routine biochemical tests were run and the remaining serum was immediately frozen and stored for maximum 1 year at -80°C until batched analysis. C-reactive protein was assayed using a solid-phase sandwich immunoassay (Phase canine CRP Assay, Tridelta Development Ltd, Maynooth, Ireland), according to the manufacturer's instructions (Kjelgaard-Hansen *et al.* 2003). Samples were diluted 1:500 and measured in duplicates. Samples with a concentration >62.8 mg/l were diluted up to 1:5000 to obtain concentrations within the range of the standard curve. Since all samples were batched for analysis, the clinicians were unaware of the results when making clinical decisions.

When clinically acceptable, dogs weighing more than 8 kg were sampled daily for at least 7 days (d0-d6). Dogs weighing less than 8 kg were excluded from serial measurements to avoid excessive blood sampling.

Statistical analysis

Since some data sets were not normally distributed, they are presented as median (interquartile range, IQR) and analysed using non-parametric methods. Statistical significance was set as a P-value <0.05. Analyses were performed with the NCSS commercial statistical software (NCSS 9.0.15. NCSS, LLC. Kaysville, Utah, USA).

The reproducibility of the CRP measurements was evaluated using their coefficient of variation (CV) for duplicate measurements, calculated with the within-subject standard deviation method: CV (%) = 100 x (standard deviation / mean), where standard deviation = $\sqrt{[\Sigma(x_1-x_2)^2/2n]}$.

The CRP concentration at presentation was compared between dogs with AKI due to leptospirosis (L) and controls (nL) and between survivors and non-survivors, using a Mann-Whitney U Test. Outcome was defined as 30d post-discharge survival, and non-survival was differentiated between death and euthanasia. The main reasons for euthanasia were recorded. Markers of inflammation (body temperature, white blood cell count, neutrophil count, albumin) and level of azotaemia (creatinine, urea) were assessed for possible associations with CRP at presentation in the group L by calculating their Pearson's correlation coefficient (r). The not normally distributed CRP data were log-transformed to conform to normality. The strength of the relationship was qualified as weak for r: 0.0–0.3; moderate for r: 0.3–0.6; strong for r: 0.6–0.9; and very strong for r: 0.9–1.0.

The kinetics of CRP was characterized by its absolute and relative changes and by its time-average concentration (TAC-CRP) during the treatment. The TAC-CRP was calculated using the trapezoidal method to estimate the areas under the CRP time curve divided by the duration of the corresponding segment, to give the average CRP over that time and thus the average exposure to inflammation. Following time intervals were assessed: d0-2, d0-4, and d0-6 to identify the most optimal sampling period for prognostic evaluation. For example, the TAC-CRP (d0-2) was calculated as: TAC-CRP (d0-2) = $[t_{0\text{-}1} \times (CRP_0 + CRP_1)/2 + t_{1\text{-}2} \times (CRP_1 + CRP_2)/2] / t_{0\text{-}2}$, where CRP_0, CRP_1, CRP_2 represent CRP on d_0, d_1, and d_2, respectively, and $t_{0\text{-}1}$, $t_{1\text{-}2}$, and $t_{0\text{-}2}$ the duration of the corresponding time segments.

The predictive value of outcome for numerical parameters was determined with a receiver-operating characteristic (ROC) curve analysis. The area under this curve (AUC) indicated the strength of the predictive value for defined parameters, including CRP at presentation, TAC-CRP, and azotaemia. The AUC is presented with its 95% confidence interval, and its predictive value was qualified as weak (0.6-0.7), moderate (0.7-0.8), strong (0.8-0.9), and very strong (0.9-1.0). For CRP at presentation, the

optimal cutoff predicting outcome was defined as the value with the highest sum of sensitivity and specificity.

A power analysis was performed to determine the sample sizes necessary to reliably detect a CRP at presentation twice as high in dogs with leptospirosis than in controls, to detect a CRP at presentation twice as high in non-survivors than in survivors, and to detect a strong outcome predictive value to CRP at presentation (ROC AUC >0.8). Basis for these calculations were previously published data of CRP in canine leptospirosis (Mastrorilli *et al.* 2007, Oliveira *et al.* 2010) and data at our institution on expected AKI population (75% of dogs diagnosed with leptospirosis) and expected leptospirosis survival (70%). The expected means ± standard deviations for CRP used for these analyses were 100 ± 55 mg/L for dogs with leptospirosis and 60 ± 30 mg/L for survivors. A case to control ratio of 3:1 was used for the calculation based on the expected canine AKI population at our institution. Sample sizes were calculated as 26, 38, and 39 dogs with leptospirosis for the 3 mentioned goals, respectively, to achieve 90% power to reject the null hypothesis of equal means with a significance level alpha of 0.05, using a two-sided two-sample unequal-variance t-test (PASS 13 software, Kaysville UT, USA). Available data were insufficient to estimate the sample size necessary to evaluate the prognostic value of CRP kinetics on outcome. The study was therefore designed to enrol at least 40 dogs with leptospirosis and 14 controls for the one-time evaluation of CRP at presentation. When satisfying the additional body weight criteria, dogs with leptospirosis were enrolled for serial sampling. Taking in account a lower power due to a smaller group size, only descriptive statistics and evaluation of outcome prediction were performed for this part of the study.

RESULTS

Dogs and disease

Fourty-one dogs were diagnosed with AKI due to leptospirosis between 2012 and 2014 and they were enrolled in the main study group L. This population consisted of 6 mixed-breed dogs (15%) and 35 pure-bred dogs (85%), including 10 Labrador Retrievers (24%), 5 Golden Retrievers (12%), and 1 dog (2%) each from 20 other breeds. Thirty-one dogs (76%) were male (21 entire and 10 castrated) and 10 (24%) were female (5 entire and 5 spayed). Their median age was 3.9 years (IQR, 1.6- 7.8) and their median body weight 25.7 kg (IQR, 19.2 - 30.0).

The diagnosis of leptospirosis was based on double MAT serology with seroconversion (n=30, 73%); positive RT-PCR on liver and kidney (n=1, 2%); a strong clinical suspicion and a positive single MAT titre (n=7, 17%); a strong clinical suspicion and a positive immunodiffusion rapid test (n=1, 2%); and on a strong clinical suspicion alone (n=2, 5%). Initial MAT serology showed the highest titres for serogroups Australis (19/37, 51%), Pomona (4/37, 11%), and Autumnalis (4/37, 11%). The last MAT serology performed a median of 8.0 days later (IQR, 7.0-9.8) showed positivity for serogroups Australis (33/37, 89%), Pomona (14/37, 38%), and Grippotyphosa (10/37, 27%). For dogs with more than one MAT, seroconversion was observed for serogroups Australis (26/30, 87%), Pomona (23/30, 77%), and Autumnalis (16/30, 53%).

At presentation, the severity of AKI was classified as grade 1 in 1 dog, grade 3 in 6 dogs, grade 4 in 24 dogs, and grade 5 in 10 dogs. Pulmonary manifestation was diagnosed in 34 cases (83%; grade 1 in 28 dogs, grade 2 in 1 dog, grade 3 in 5 dogs), liver involvement in 10 cases (24%; grade 1 and 2 in 5 dogs each), and DIC in 7 cases (17%). Clinical and laboratory data of the dogs at presentation are summarized in Table 1.

Fifteen dogs diagnosed with AKI due to other aetiologies than leptospirosis were included in the control group nL, consisting of 3 mixed-breed dogs (20%) and 12 pure-bred dogs (80%), with 2 Golden Retrievers (13%) and 1 dog each (7%) from 10 other breeds. Seven dogs (47%) were male (4 entire and

3 castrated) and 8 (53%) were female (2 entire and 6 spayed). Their median age was 4.2 years (IQR, 1.4- 7.1) and their median body weight 24.0 kg (IQR, 19.9 - 42.5). The cause of AKI in these dogs was identified as maleic acid intoxication (n=2), use of non-steroidal anti-inflammatory agents (NSAIA, n=1), lymphoma (n=1), and trauma (n=1). The aetiology remained unidentified in 10 dogs. The absence of leptospirosis in this group was confirmed with double serology without seroconversion (n=7, 47%) or positive identification of an alternative diagnosis based on renal histopathology (n=4, 27%) or history (n=4, 27%). At presentation, the severity of AKI was classified as grade 1 in 1 dog, grade 3 in 6 dogs, grade 4 in 5 dogs, and grade 5 in 3 dogs (Table 1).

Pre-referral treatments included intravenous crystalloids (L: 28/41, nL: 5/15), antibiotics (penicillins, fluoroquinolones, metronidazole and tetracyclines; L: 33/41, nL: 6/15), gastric acid inhibitors (omeprazole, ranitidine; L: 12/41, nL: 2/15), steroids (L: 3/41, nL: 3/15), NSAIA (L: 6/41, nL: 4/15), antiemetics (maropitant, ondansetron and metoclopramide; L: 24/41, nL: 3/15) and opioids (L: 6/41, nL: 0/15). During hospitalisation, the dogs were treated with a standardized protocol basis consisting of fluids, antibiotics (amoxicillin - clavulanic acid), antiemetics (maropitant, ondansetron, and/or metoclopramide), gastric acid inhibitors (omeprazole, ranitidine), and opioids (buprenorphine, butorphanol, methadone), adjusted at the clinician's discretion. Haemodialysis was performed in 28/41 L-dogs (68%) and 6/15 nL-dogs (40%).

Twenty-eight dogs with leptospirosis survived (68%), 3 died (7%), and 10 were euthanized (24%). Eight dogs were euthanized in extremis due to acute worsening of severe pulmonary haemorrhages and 2 dogs due to non-recovery after 3 weeks of renal replacement support. In the control group nL, 12 dogs survived (80%), 1 dog died (7%), and 2 dogs were euthanized (13%) due to non-recovery despite 3 weeks of therapy.

C-reactive protein

Serum CRP was measured at presentation in all dogs (n=56) and serially during hospitalisation in 28/41 dogs with leptospirosis. The other 13 dogs with leptospirosis were not sampled after presentation because of their small size not allowing repeated sampling (n=7) or because of early euthanasia or death (n=6). Serial measurements could be performed during ≥7 days in 26/28 dogs. For two dogs, sampling was stopped earlier: one dog was euthanized on d4 due to severe pulmonary haemorrhages and one dog was discharged on d6 following recovery.

All 289 samples were measured in duplicates and the CV of these measurements was 13.4%; 9.9% for the highest tertile (CRP >57.3 mg/L, n=96), 15.4% for the mid tertile (CRP 25.4 – 57.3 mg/L, n=97), and 14.4% for the lowest tertile (CRP <25.4 mg/L, n=96).

Serum CRP was increased at presentation in all dogs with AKI due to leptospirosis, with a median of 74.4 mg/L (IQR, 44.9–120.4; reference range, 0-10.5). The control group showed a lower proportion of dogs with elevated CRP (12/15 dogs, 80%; P=0.016). With a median of 42.3 mg/L (IQR, 15.5–109.1), it was not statistically different from dogs with leptospirosis (P=0.088). Two of the 3 control dogs with a normal CRP concentration were diagnosed with maleic acid nephrotoxicosis and the aetiology of AKI in the third dog remained unclear. In dogs with leptospirosis, log-transformed CRP concentration was not associated with temperature (r=0.23, P=0.152), and moderately with other markers of inflammation, including white blood cell count (r=0.35, P=0.030), segmented neutrophil count (r=0.46, P=0.003), and serum albumin (r=-0.43, P=0.005) (Figure 1). No obvious association could be recognized between the main organ manifestations of leptospirosis, their severity, and serum CRP (Table 2). In dogs with leptospirosis, CRP was not associated either with the level of azotaemia at presentation, measured as serum creatinine (r=0.00, P=0.983) or serum urea (r=-0.05, P=0.744) and it was not significantly different between survivors (median 65.2 mg/L; IQR, 43.4 – 104.2) and non-survivors (median 110.1 mg/L; IQR, 73.5 – 174.6; P=0.059) in this population.

Kinetics of CRP and outcome analysis

In the 28 dogs with serial sampling, median CRP concentration decreased gradually from d0 (91.5 mg/L; IQR, 47.3 – 136.1) to d4 (22.8 mg/L; IQR, 18.8 – 38.6), where it reached a plateau for the rest of the sampling period (Figure 2). Median absolute decrease from d0 to d4 was 59.2 mg/L (IQR, 21.5 – 98.1) and median relative decrease was 66.6% (IQR, 53.1 – 81.7). Per protocol, a blood culture was performed in 3 dogs with leptospirosis showing a new episode of fever (n=2) or inappropriate treatment response (n=1). *Klebsiella pneumoniae* was cultured from the 2 dogs with fever and the culture from the third dog was negative. In these 3 cases, CRP spiked at the time of performing blood culture after an expected decrease the days before. This type of time course of CRP was not observed in any other dog in this study.

In this subgroup of dogs with serial CRP measurements, survivors had a significantly lower CRP than non-survivors at presentation (P=0.013) and on each sampling day thereafter until d5 (P<0.005, Figure 3). However, the absolute and relative decreases in CRP from d0 to d4 were not different between survivors and non-survivors (P=0.339 and 0.585, respectively).

In the ROC curve analysis including all dogs with leptospirosis, serum CRP at presentation was significantly, but only weakly, predictive of outcome (AUC=0.69, 95% CI: 0.46-0.83, P=0.047, Figure 4A). With a cutoff of 106 mg/L at presentation, CRP had a sensitivity of 79% (95% CI, 60-90) and a low specificity of 62% (95% CI, 36-82) to predict outcome; even with a high cutoff of 180 mg/L CRP at presentation was only 90% sensitive for predicting death. With repetitive measurements however, the TAC-CRP (d0-2) was strongly predictive of outcome (AUC=0.88, 95% CI: 0.67-0.96, P<0.001, Figure 4A). Adding more days of observation improved only minimally the predictive value of CRP kinetics (d0-4: AUC=0.92, 95% CI: 0.72-0.98, P<0.001; and d0-6: AUC=0.92, 95% CI: 0.71-0.98, P<0.001). In comparison, the most commonly used parameter to grade AKI severity, serum creatinine was not predictive of outcome (AUC=0.61, 95% CI: 0.41-0.75, P=0.212, Figure 4B) and serum urea showed a moderate predictive value (AUC=0.72, 95% CI: 0.52-0.84, P=0.008, Figure 4B).

Discussion

Inflammation is increasingly recognized as a contributor to the clinical manifestation of the uraemic syndrome (Jankowska *et al.* 2017). As evidence supporting this concept is evolving in small animals (Nentwig *et al.* 2016), the diagnostic and prognostic value of markers of inflammation should be evaluated. Screening with serial CRP measurements could further enable the early detection of inflammatory complications and thus improve the outcome of AKI, if the expected time course of its normalisation is known. To the authors' knowledge, this is the first study to investigate CRP kinetics in dogs with AKI due to leptospirosis. A single evaluation of CRP at presentation was weakly but inconsistently predictive of survival, depending on the subgroup tested. This possibly reflects the degree of inhomogeneity in the groups with inclusion of different-sized dogs, AKI of different origins, or a type II error caused by an insufficient group size. To overcome some of the limitations of one-point measurements, we hypothesized that a serial assessment of CRP during hospitalisation would be more predictive of the regression of inflammation and thus of the disease outcome.

We expected survivors to have a more rapid decrease of CRP than non-survivors, as shown in humans (Crouzet *et al.* 2011). This was however not the case in our study. In the subgroup of dogs with repeated sampling, CRP in survivors was significantly lower than in non-survivors, but the actual decrease of CRP over time was almost identical. The TAC-CRP during the first 3-7 days of hospitalisation however showed a strong to very strong predictive value. Using the described formula, this parameter can easily be computed on any pocket calculator. This difference in prognostic values possibly indicates that the average intensity of inflammation rather than its actual improvement rate is of clinical relevance. The low statistical power for the repeated measures of CRP however warrants a cautious interpretation of these results that need to be confirmed in a larger population of dogs. Interestingly, CRP at presentation and its TAC from d0-2 were more strongly predictive of outcome than the commonly used serum creatinine that is the basis of the IRIS AKI grading system (2013). A previous study by Mastrorilli (2007) did not show any association between CRP and survival, possibly due to a smaller number of dogs with leptospirosis (n=20).

We did not observe a significant difference between leptospirosis and other aetiologies regarding CRP at presentation. This finding potentially supports the concept of uraemic inflammation, where uraemia is considered a pro-inflammatory syndrome, independently of its underlying aetiology (Jankowska *et al.* 2017, Maissen-Villiger *et al.* 2016, Nentwig *et al.* 2016). However, this view mostly reflects the micro-inflammation of CKD and few data are available for AKI *(*Segev *et al.* 2015*).* It is likely that animals with severe AKI of any aetiology are more prone to develop overt inflammation. In addition to the cause of AKI and to the renal parenchymal lesions, gastrointestinal breakdown of mucosal integrity, erosions, ulcerations, and secondary aspiration pneumonia likely contribute to systemic inflammation.

In support of this, dogs with kidney disease have been shown to have increased CRP, with chronic activation of an acute phase response as a likely trigger (Raila *et al.* 2011). Interestingly, the authors showed a strong correlation between CRP and glomerular filtration rate, creatinine, and proteinuria in these dogs with mostly CKD. The clear lack of correlation between CRP and azotaemia observed in our study probably indicates a fundamental difference in AKI versus CKD kinetics, most biological parameters being not in steady-state in AKI with production and clearance varying over time. Furthermore, the organism is typically more severely affected both by the cause of AKI and by its multi-organ consequences, many of them triggering an acute phase response with increased CRP production. In a previous study including dogs with chronic and acute kidney diseases, the pattern and intensity of mRNA expression of the pro- and anti-inflammatory cytokines IL-1α, IL-1β, and TGF-β were similar between AKI and CKD (Nentwig *et al.* 2016). Whether this reflects a difference between cytokine expression and the actual concentrations of the acute-phase products remains to be elucidated.

Furthermore, the lack of difference regarding CRP at presentation between leptospirosis and nL group should be interpreted cautiously because of the small and heterogeneous control group, including aetiologies previously shown to affect CRP such as lymphoma and trauma (Mimoz *et al.* 1998, Mischke et al. 2007, Chase *et al.* 2012). The small number of dogs with non-inflammatory aetiologies does not support a separate analysis, but the 3 dogs with confirmed nephrotoxicosis had a CRP <20 mg/L at presentation.

Even though the present study was not designed to evaluate the causes and the mechanisms of inflammation in dogs with leptospirosis, the rapid decrease of serum CRP with initiation of therapy seems to indicate a major role of leptospires themselves. Although no obvious association was recognized between organ manifestation and CRP, this may be due to the limited case number. A potential confounding factor in dogs with leptospirosis is the presence of liver involvement, as CRP is synthesized in the liver and may therefore be falsely low in affected dogs. However, this is unlikely to be relevant in the present study as leptospirosis mainly causes intrahepatic cholestasis and liver failure was not observed in the 10 affected dogs.

The evaluation of markers of disease severity such as CRP is hampered by individual variations in the clinical course of the disease, with dogs presented at different stages of the infection. These variations result from differences in the size of the inoculum, the route of inoculation, the infecting strains of leptospires, the general health, and the immune status of the dogs. In addition, most dogs in this study were treated with a variety of drugs prior to presentation. Even though some of these drugs, including steroids and NSAIA, may decrease the inflammation, they have been shown to not directly influence CRP (Borer *et al.* 2003, Martinez-Subiela *et al.* 2004). During hospitalisation, the treatment was standardized, although the individual requirements of the dogs resulted in some differences.

It should be emphasized that a statistical difference between two groups does not necessarily imply a good diagnostic performance of the discriminating variable for an individual animal. The diagnostic value should first be re-assessed prospectively in an independent population. Therefore, even though the kinetics of CRP shows promise for the prognostic evaluation of dogs with AKI and leptospirosis, its true value and clinical implications can only be assessed after appropriate validation. However, in the meantime, the present study offers valuable information on what to expect and, in cases with unexpected course, caution and proper clinical re-evaluation are warranted. A high CRP at presentation or a prolonged high elevation during therapy should trigger additional diagnostics and a thorough search for inflammatory complications such as pancreatitis, aspiration pneumonia or sepsis. Even a very good prognostic indicator should only be used with caution for clinical decision-making.

The indication of a poor prognosis is not an indication for early euthanasia, but rather for a thorough clinical characterisation and a pro-active therapeutic approach. The inclusion of strong prognostic indicators in the diagnostic workup should therefore be considered essential in the risk-evaluation process of a critical patient. If confirmed in subsequent studies, these data could suggest a major role of inflammation in the pathophysiology of acute leptospirosis, raising the questions of its causes and of the potential for specific treatments to modulate inflammatory pathways in severe forms of the disease.

The main limitations of our study include the small and heterogeneous control group of dogs with AKI due to diseases other than leptospirosis, the low number of non-inflammatory conditions in this group, the lack of control on the treatment prior to referral, and the difficulty to assess the reasons for a negative outcome. The use of naturally-infected dogs implies both the main disadvantage of individual variations in the disease manifestations, severity and time course, and the main advantage of the clinical relevance and therefore the applicability of the observed results to clinical situations. Due to the low power in parts of the study concerning repeated measures and outcome prediction, its conclusions should be interpreted with caution and re-evaluated in a larger population.

In summary, we showed that dogs with leptospirosis had an elevated CRP at presentation. On the contrary to our hypothesis, no significant difference was found when compared to the AKI controls. At presentation, CRP was clearly associated with other markers of inflammation, but not with the level of renal dysfunction and inconsistently with survival. We further showed that CRP decreases within a few days of the initiation of therapy in dogs with AKI due to leptospirosis. Its serial measurement and the calculation of its average concentration over the first 3 days of hospitalisation potentially could represent a better tool for survival prediction, when compared to single measurements at presentation or to currently recommended parameters such as serum creatinine.

In conclusion, there is some evidence that serial CRP measurement has a prognostic value as part of the general assessment of dogs with leptospirosis and this should be re-evaluated using an independent population of affected dogs. A higher CRP at presentation may indicate a more severe

clinical course of the disease and a higher risk of death. It may justify therefore a more pro-active therapeutic approach. Similarly, an unusual delay in the normalisation of CRP may indicate a secondary inflammatory or infectious complication and justify a re-evaluation with additional tests such as abdominal ultrasound and blood culture.

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462 **Figure 1**: Correlation of log-transformed CRP at presentation with other markers of inflammation (A,
463 segmented neutrophil count; B, albumin) and markers of renal function (C, urea; D, creatinine) in 41
464 dogs with AKI due to leptospirosis.

465 The correlation was moderate with markers of inflammation (segmented neutrophil count, r=0.46,
466 P=0.003; serum albumin, r=-0.43, P=0.005) and non-significant with azotaemia (creatinine, r=0.00,
467 P=0.983; urea, r=-0.05, P=0.744).

468

469 **Figure 2**: Time course of CRP concentration during hospitalisation (d0-d7) in 28 dogs with leptospirosis.
470 The data are presented as a box plot, with the horizontal bar representing the median; the edges of
471 the box, the interquartile range; and the whiskers, the range or the data with exception of the
472 statistical outliers presented as separate dots. The day of presentation was defined as d0.

473

474 **Figure 3**: Time course of CRP concentration during hospitalisation in 28 dogs with leptospirosis
475 stratified as survivors (black diamonds, full line) and non-survivors (black circles, dotted line). The data
476 are presented as median (diamonds and circles) and IQR (error bars). The day of hospitalisation was
477 defined using the day of presentation as d0.

478

479 **Figure 4**: ROC curve representing the predictive value of outcome in dogs with leptospirosis for A) CRP
480 concentration at presentation (d0) and TAC-CRP (d0-2), and B) urea and creatinine concentrations at
481 presentation (d0).

482 The ROC curve analysis indicated a significant but weak predictive value for CRP at presentation
483 (AUC=0.69, P=0.047); TAC-CRP (d0-2) was strongly predictive (AUC=0.88, P<0.001) and serum urea
484 concentration was moderately predictive of outcome (AUC=0.72, P=0.008). Serum creatinine
485 concentration (AUC=0.61, P=0.212) was however not predictive of outcome.

Table 1: Clinical and clinicopathological parameters from the 41 dogs with AKI due to leptospirosis (group L) included in the study and from 15 control dogs with AKI from other aetiologies (group nL)

Table 2: Serum CRP concentration as a function of the main organ manifestations of leptospirosis

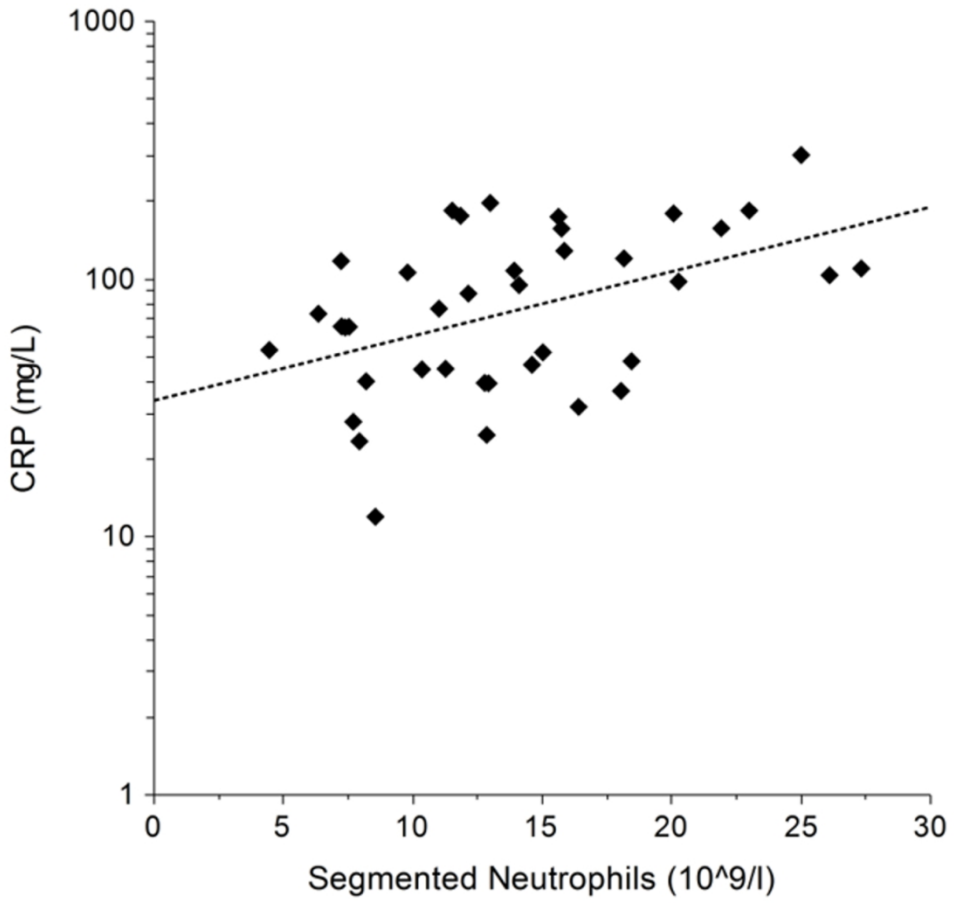

Figure 1A Correlation of log-transformed CRP at presentation with other markers of inflammation (A, segmented neutrophil count; B, albumin) and markers of renal function (C, urea; D, creatinine) in 41 dogs with AKI due to leptospirosis.
The correlation was moderate with markers of inflammation (segmented neutrophil count, r=0.46, P=0.003; serum albumin, r=-0.43, P=0.005) and non-significant with azotaemia (creatinine, r=0.00, P=0.983; urea, r=-0.05, P=0.744).

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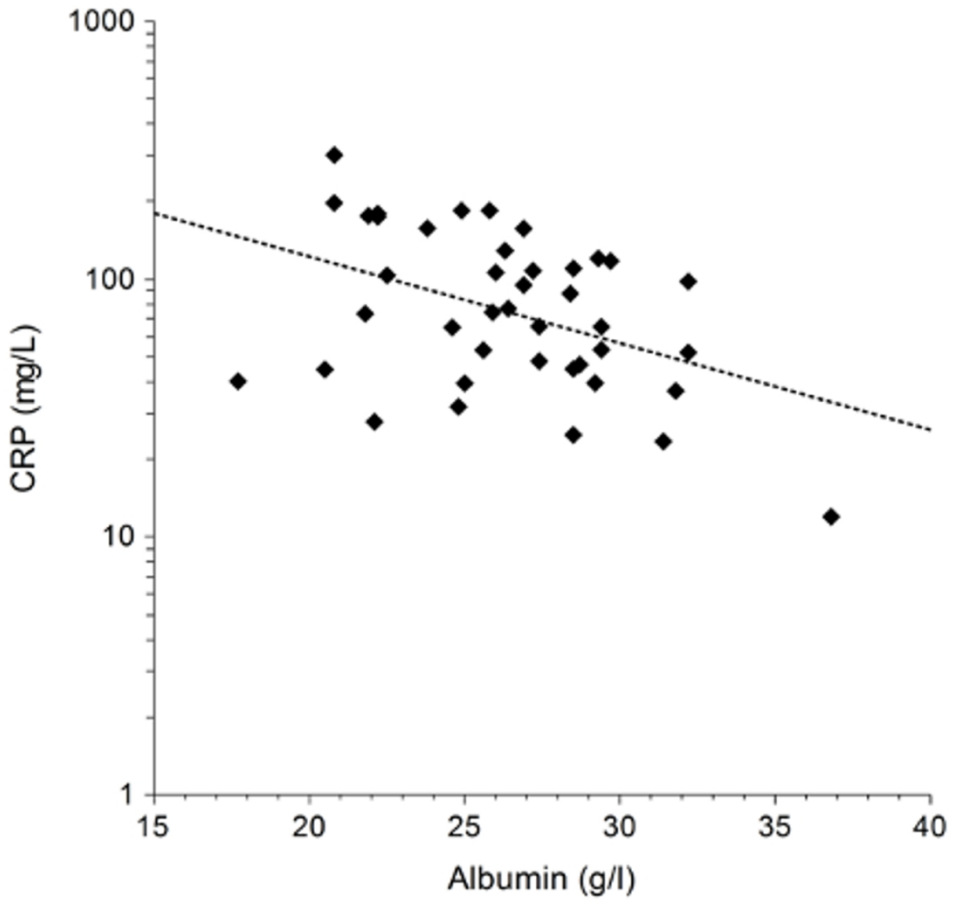

Figure 1B Correlation of log-transformed CRP at presentation with other markers of inflammation (A, segmented neutrophil count; B, albumin) and markers of renal function (C, urea; D, creatinine) in 41 dogs with AKI due to leptospirosis.
The correlation was moderate with markers of inflammation (segmented neutrophil count, r=0.46, P=0.003; serum albumin, r=-0.43, P=0.005) and non-significant with azotaemia (creatinine, r=0.00, P=0.983; urea, r=-0.05, P=0.744).

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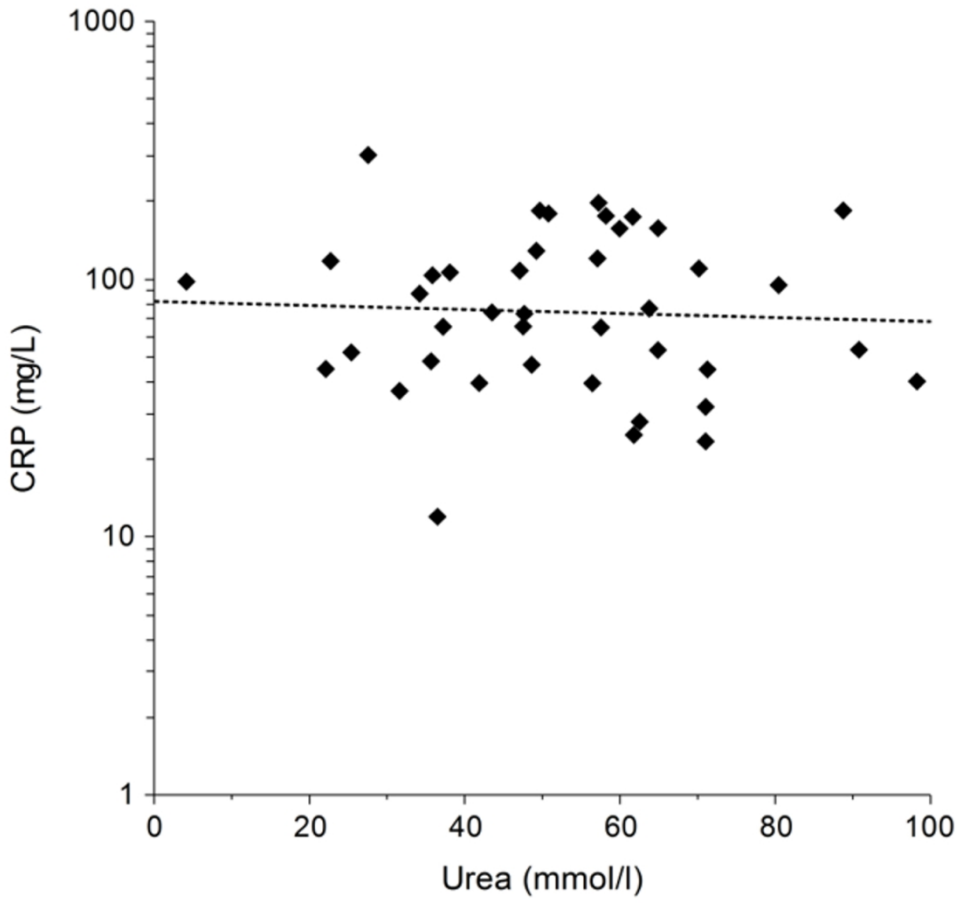

Figure 1C Correlation of log-transformed CRP at presentation with other markers of inflammation (A, segmented neutrophil count; B, albumin) and markers of renal function (C, urea; D, creatinine) in 41 dogs with AKI due to leptospirosis.
The correlation was moderate with markers of inflammation (segmented neutrophil count, r=0.46, P=0.003; serum albumin, r=-0.43, P=0.005) and non-significant with azotaemia (creatinine, r=0.00, P=0.983; urea, r=-0.05, P=0.744).

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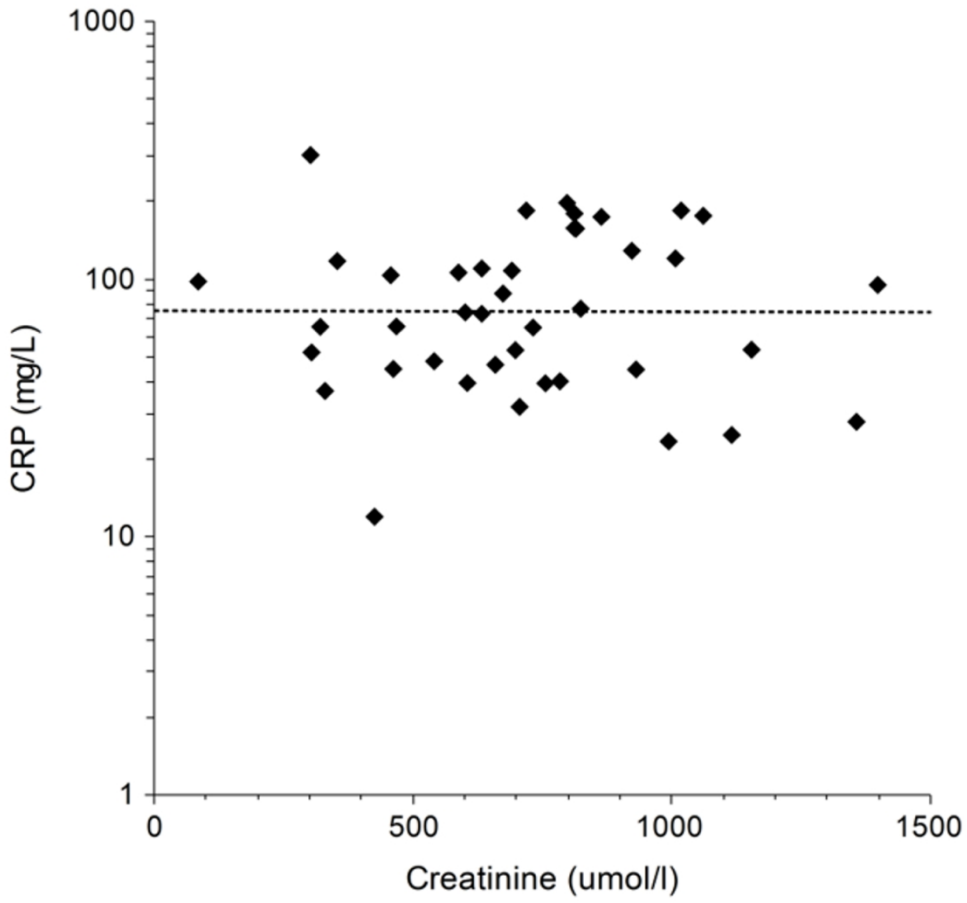

Figure 1D Correlation of log-transformed CRP at presentation with other markers of inflammation (A, segmented neutrophil count; B, albumin) and markers of renal function (C, urea; D, creatinine) in 41 dogs with AKI due to leptospirosis.
The correlation was moderate with markers of inflammation (segmented neutrophil count, r=0.46, P=0.003; serum albumin, r=-0.43, P=0.005) and non-significant with azotaemia (creatinine, r=0.00, P=0.983; urea, r=-0.05, P=0.744).

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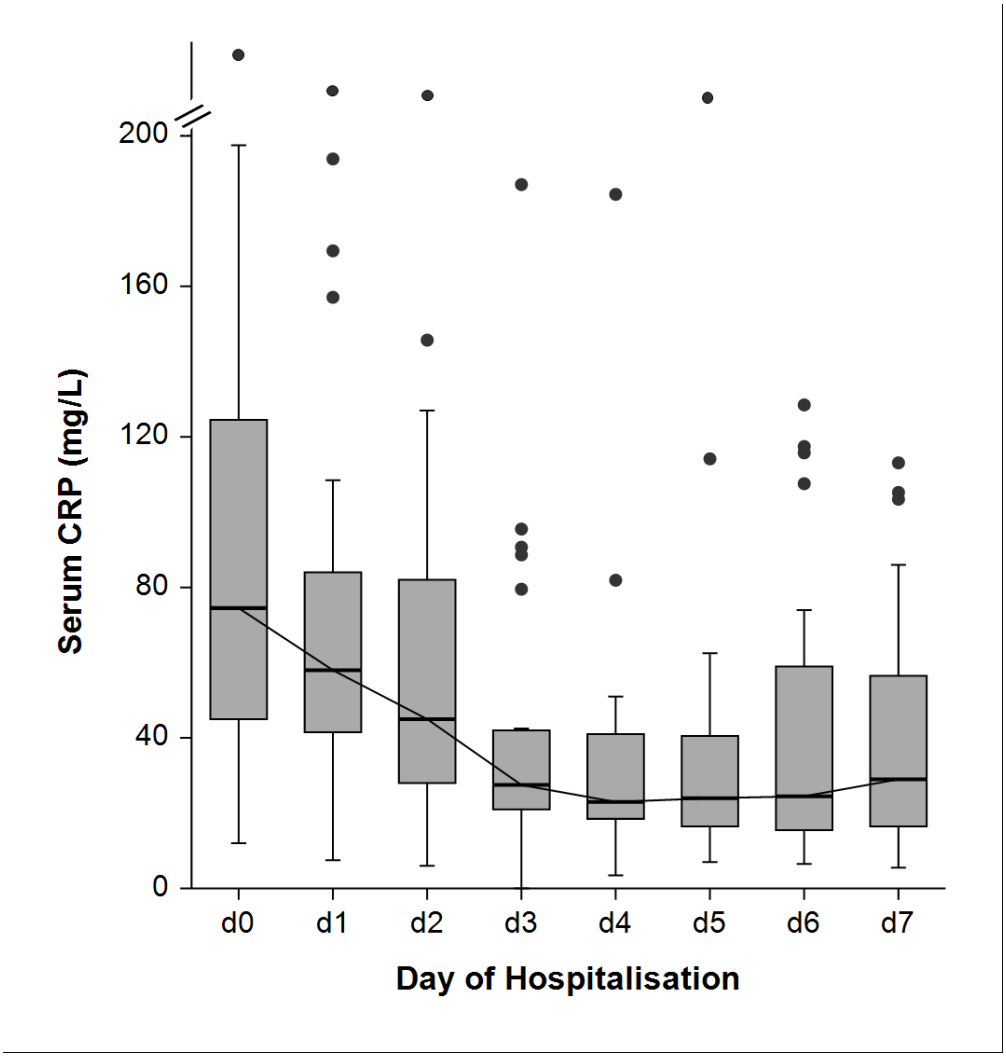

Figure 2 Time course of CRP concentration during hospitalisation (d0-d7) in 28 dogs with leptospirosis. The data are presented as a box plot, with the horizontal bar representing the median; the edges of the box, the interquartile range; and the whiskers, the range or the data with exception of the statistical outliers presented as separate dots. The day of presentation was defined as d0.

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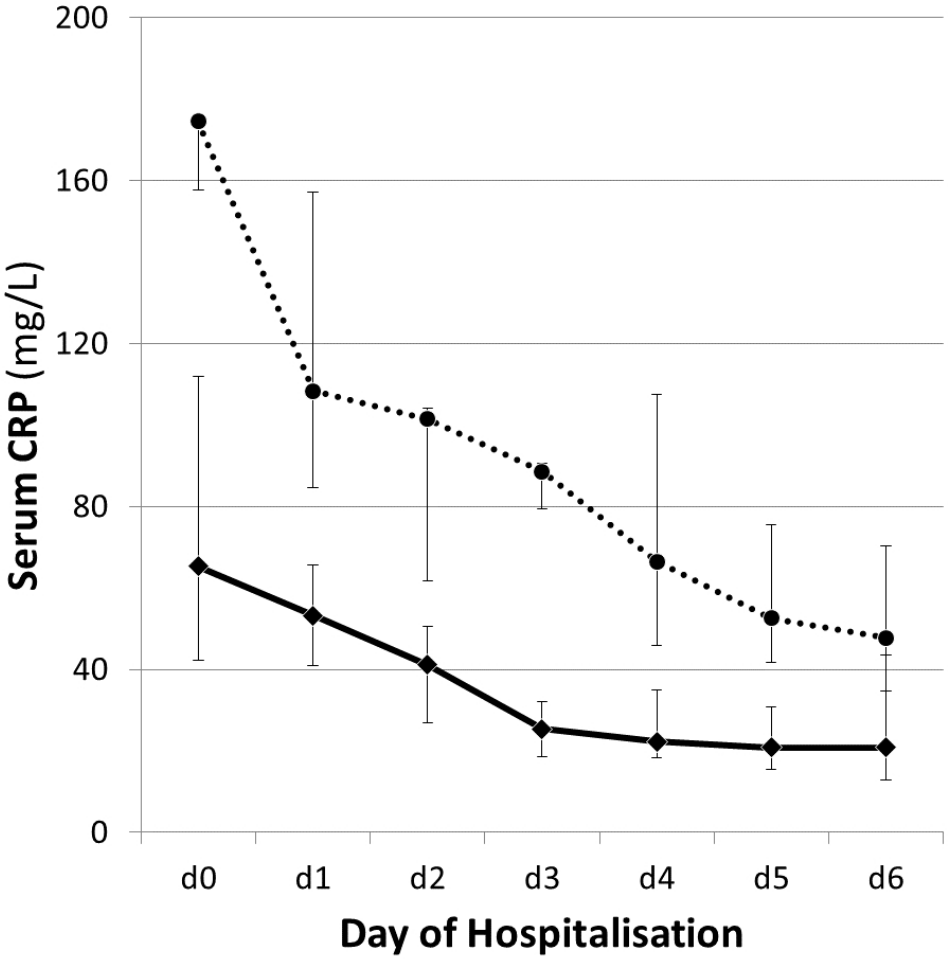

FIgure 3 Time course of CRP concentration during hospitalisation in 28 dogs with leptospirosis stratified as survivors (black diamonds, full line) and non-survivors (black circles, dotted line). The data are presented as median (diamonds and circles) and IQR (error bars). The day of hospitalisation was defined using the day of presentation as d0.

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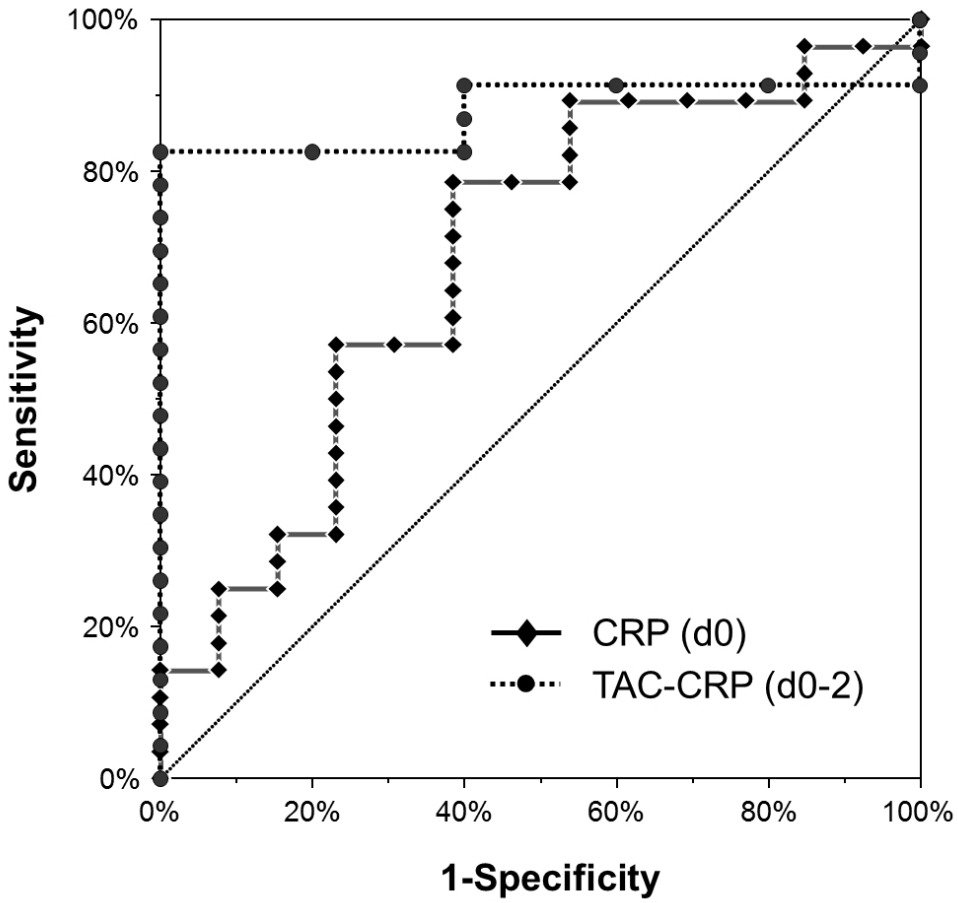

Figure 4A ROC curve representing the predictive value of outcome in dogs with leptospirosis for A) CRP concentration at presentation (d0) and TAC-CRP (d0-2), and B) urea and creatinine concentrations at presentation (d0).
The ROC curve analysis indicated a significant but weak predictive value for CRP at presentation (AUC=0.69, P=0.047); TAC-CRP (d0-2) was strongly predictive (AUC=0.88, P<0.001) and serum urea concentration was moderately predictive of outcome (AUC=0.72, P=0.008). Serum creatinine concentration (AUC=0.61, P=0.212) was however not predictive of outcome.

85x80mm (300 x 300 DPI)

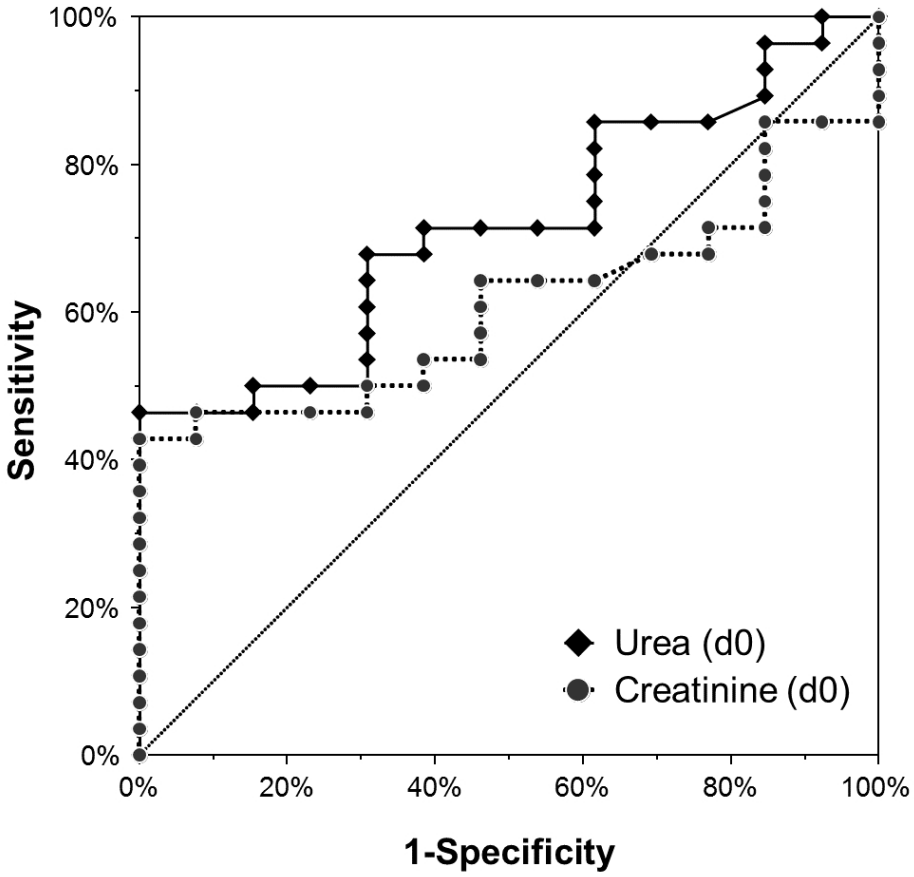

Figure 4B ROC curve representing the predictive value of outcome in dogs with leptospirosis for A) CRP concentration at presentation (d0) and TAC-CRP (d0-2), and B) urea and creatinine concentrations at presentation (d0).
The ROC curve analysis indicated a significant but weak predictive value for CRP at presentation (AUC=0.69, P=0.047); TAC-CRP (d0-2) was strongly predictive (AUC=0.88, P<0.001) and serum urea concentration was moderately predictive of outcome (AUC=0.72, P=0.008). Serum creatinine concentration (AUC=0.61, P=0.212) was however not predictive of outcome.

89x83mm (300 x 300 DPI)

Table 1: **Clinical and clinicopathological parameters from 41 dogs with leptospirosis and 15 control dogs with AKI from other aetiologies included in the study**

Parameter	Ref. range	**Leptospirosis** n=41	**Controls** n=15
Body temperature (°C)	38.0 – 39.0	37.7 (37.3 – 37.9)	37.7 (37.5 – 38.2)
Haematocrit (l/l)	0.39 – 0.57	0.35 (0.32 – 0.38)	0.42 (0.36 – 0.53)
WBC (10^9/l)	6.0 – 12.0	17.0 (13.3 – 20.4)	16.6 (8.1 – 19.9)
Seg. neutrophils (10^9/l)	3.0 – 11.5	12.9 (9.2 – 17.2)	12.4 (5.6 – 16.0)
Platelet count (10^9/l)	150 – 400	163 (93 - 206)	160 (92 - 226)
Urea (mmol/l)	3.5 – 11.1	50.8 (37.2 – 63.8)	34.4 (23.9 – 46.7)
Creatinine (µmol/l)	53 – 120	706 (541 - 864)	516 (350 - 850)
Phosphorus (mmol/l)	0.93 – 1.93	3.72 (2.89 – 5.12)	2.89 (2.00 – 3.68)
Bilirubin (µmol/l)	0.6 – 4.3	5.0 (3.8 – 8.3)	4.0 (2.9 – 5.7)
ALP (IU)	10 – 128	110 (78 - 171)	112 (42 - 156)
ASAT (IU)	20 – 73	60 (43 - 129)	71 (25 - 214)
ALAT (IU)	24 – 124	63 (41 - 109)	52 (38 - 122)
Renal involvement		41/41 (100%)	15/15 (100%)
Liver involvement		10/41 (24%)	3/15 (20%)
Coagulopathy		7/41 (17%)	2/15 (13%)
Pulmonary involvement		34/41 (83%)	2/15 (13%)

Numerical data are presented as median (interquartile range) and proportion with their absolute and percent values.

WBC, white blood cell count; seg., segmented; ALP, alkaline phosphatase; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase.

Table 2: **Serum CRP concentration as a function of the main organ manifestations of leptospirosis**

Organ manifestation	**Grade**	**n**	**CRP** (mg/L)	
			median	IQR
Renal	1	1	98.0	-
	2	-	-	-
	3	6	58.7	30.7 – 164.0
	4	24	75.7	47.0 – 145.5
	5	10	74.2	27.2 – 140.7
Pulmonary	0	7	87.9	23.5 – 98.0
	1	28	70.0	45.3 – 150.2
	2	1	40.2	-
	3	5	107.9	52.8 – 153.9
Hepatic	0	31	65.6	40.2 – 128.9
	1	5	73.5	37.3 – 86.2
	2	5	110.1	76.3 – 177.5
Coagulopathy	0	34	69.6	44.9 – 122.5
	1	7	107.9	40.2 – 157.3
Number of organs involved	1	6	76.7	20.6 – 147.0
	2	24	87.5	49.1 – 150.4
	3	6	42.5	36.6 – 53.3
	4	5	110.1	91.2 – 177.5

Data are presented descriptively as median and interquartile range (IQR).