



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

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1 ABSTRACT

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

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

9 Results: CRP was increased at presentation in all dogs with leptospirosis, but not significantly different
10 from control dogs ($p=0.088$). It was associated with markers of inflammation (neutrophil count,
11 $P=0.003$; albumin, $P=0.005$), but not with azotaemia (creatinine, $P=0.983$; urea, $P=0.744$). CRP
12 decreased gradually from d0-d4, with significantly lower concentrations for survivors than non-
13 survivors. A spike in CRP was associated with a secondary infection in 2 dogs. Initial CRP was only
14 weakly predictive of outcome ($AUC=0.69$, $P=0.047$), but its average concentration from d0-d2 was a
15 strong predictor ($AUC=0.88$, $P<0.001$). In contrast, absolute and relative changes in CRP during
16 hospitalisation and creatinine at presentation were not predictive of survival.

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

20

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

22 INTRODUCTION

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

38 Leptospirosis is a bacterial infection that results in a multisystemic inflammatory reaction affecting the
39 kidneys, liver, lung, pancreas, heart, muscles, joints, central nervous system, eyes, vessels, and
40 haemostasis (Major *et al.* 2014, Sykes *et al.* 2011). C-reactive protein raises dramatically after infection
41 in humans suffering from leptospirosis and normalisation is achieved approximately seven days later
42 (Crouzet *et al.* 2011). The same study also showed that CRP concentration was associated with the
43 severity of infection. In dogs with leptospirosis, CRP has been shown to be increased as well (Caspi *et*
44 *al.* 1987, Mastrorilli *et al.* 2007) but its kinetics during the disease process has not been assessed and
45 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.

56 MATERIALS AND METHODS

57 *Case selection and clinical characterisation*

58 This prospective study was approved by the [REDACTED]
59 and it adhered strictly to national, and institutional guidelines. It included client-owned dogs diagnosed
60 with AKI between May 2012 and December 2014 at the [REDACTED].

61 Acute kidney injury was defined by the combination of historical, clinical, laboratory, and imaging
62 evidence, with at least two of the following criteria (International Renal Interest Society, IRIS, 2013):

63 1) presence of renal azotaemia with a serum creatinine ≥ 140 $\mu\text{mol/L}$ persisting at least 24h after
64 correction of prerenal factors; 2) increase in serum creatinine ≥ 26 $\mu\text{mol/L}$ during a 48h interval in the
65 absence of prerenal factors; 3) persistent pathological oligoanuria (< 1 mL/kg/h over 6h) after volume
66 repletion; and 4) evidence of tubular injury with renal glucosuria or granular casts on urinalysis. Dogs
67 with evidence of chronic kidney disease (CKD) on ultrasonographic examination, such as small or
68 irregular kidneys, or with a history of CKD were excluded from the study.

69 History was obtained from owners and referring veterinarians. Clinical examination, blood pressure
70 measurement, complete blood work (complete blood count, full chemistry profile, and coagulation
71 profile), urinalysis with culture (if the dog was not anuric on presentation), microagglutination test
72 (MAT) for leptospirosis, and diagnostic imaging (thoracic radiographs and abdominal ultrasound) were
73 performed on all dogs at presentation. Serum MAT was performed by the [REDACTED]

74 [REDACTED]
75 with a panel of 12 locally prevalent *Leptospira* serovars Australis, Autumnalis, Bataviae, Bratislava,
76 Canicola, Grippotyphosa, Hardjo, Icterohaemorrhagiae, Pomona, Pyrogenes, Sejroe, and Tarasovi. Sera
77 were screened at a dilution of 1:100 and serial 2-fold dilutions were performed on positive samples up
78 to a dilution of 1:3200.

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

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

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

105 All dogs and their indwelling catheters were examined daily during hospitalisation for signs of
106 inflammation. If an unexpected course of disease was noticed (especially development fever $>39.4^{\circ}\text{C}$
107 or inappropriate response to treatment), an aerobic blood culture was submitted.

108

109 ***CRP measurements***

110 At presentation (d0), blood was sampled in all dogs from a peripheral or a central vein with a 23G or
111 21G needle and collected in a serum tube. It was left one hour for clotting at room temperature before
112 centrifugation (10 min, 3000g, 4°C). Routine biochemical tests were run and the remaining serum was
113 immediately frozen and stored for maximum 1 year at -80°C until batched analysis. C-reactive protein
114 was assayed using a solid-phase sandwich immunoassay (Phase canine CRP Assay, Tridelta
115 Development Ltd, Maynooth, Ireland), according to the manufacturer's instructions (Kjelgaard-Hansen
116 *et al.* 2003). Samples were diluted 1:500 and measured in duplicates. Samples with a concentration
117 $>62.8\text{ mg/l}$ were diluted up to 1:5000 to obtain concentrations within the range of the standard curve.
118 Since all samples were batched for analysis, the clinicians were unaware of the results when making
119 clinical decisions.

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

123

124 ***Statistical analysis***

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

129 The reproducibility of the CRP measurements was evaluated using their coefficient of variation (CV)
130 for duplicate measurements, calculated with the within-subject standard deviation method: $CV (\%) =$
131 $100 \times (\text{standard deviation} / \text{mean})$, where standard deviation = $\sqrt{[\sum(x_1-x_2)^2/2n]}$.

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

141 The kinetics of CRP was characterized by its absolute and relative changes and by its time-average
142 concentration (TAC-CRP) during the treatment. The TAC-CRP was calculated using the trapezoidal
143 method to estimate the areas under the CRP time curve divided by the duration of the corresponding
144 segment, to give the average CRP over that time and thus the average exposure to inflammation.
145 Following time intervals were assessed: d0-2, d0-4, and d0-6 to identify the most optimal sampling
146 period for prognostic evaluation. For example, the TAC-CRP (d0-2) was calculated as: $TAC-CRP (d0-2)$
147 $= [t_{0-1} \times (CRP_0 + CRP_1)/2 + t_{1-2} \times (CRP_1 + CRP_2)/2] / t_{0-2}$, where CRP_0 , CRP_1 , CRP_2 represent CRP on d_0 , d_1 ,
148 and d_2 , respectively, and t_{0-1} , t_{1-2} , and t_{0-2} the duration of the corresponding time segments.

149 The predictive value of outcome for numerical parameters was determined with a receiver-operating
150 characteristic (ROC) curve analysis. The area under this curve (AUC) indicated the strength of the
151 predictive value for defined parameters, including CRP at presentation, TAC-CRP, and azotaemia. The
152 AUC is presented with its 95% confidence interval, and its predictive value was qualified as weak (0.6-
153 0.7), moderate (0.7-0.8), strong (0.8-0.9), and very strong (0.9-1.0). For CRP at presentation, the

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

156 A power analysis was performed to determine the sample sizes necessary to reliably detect a CRP at
157 presentation twice as high in dogs with leptospirosis than in controls, to detect a CRP at presentation
158 twice as high in non-survivors than in survivors, and to detect a strong outcome predictive value to
159 CRP at presentation (ROC AUC >0.8). Basis for these calculations were previously published data of CRP
160 in canine leptospirosis (Mastrorilli *et al.* 2007, Oliveira *et al.* 2010) and data at our institution on
161 expected AKI population (75% of dogs diagnosed with leptospirosis) and expected leptospirosis
162 survival (70%). The expected means \pm standard deviations for CRP used for these analyses were $100 \pm$
163 55 mg/L for dogs with leptospirosis and 60 ± 30 mg/L for survivors. A case to control ratio of 3:1 was
164 used for the calculation based on the expected canine AKI population at our institution. Sample sizes
165 were calculated as 26, 38, and 39 dogs with leptospirosis for the 3 mentioned goals, respectively, to
166 achieve 90% power to reject the null hypothesis of equal means with a significance level alpha of 0.05,
167 using a two-sided two-sample unequal-variance t-test (PASS 13 software, Kaysville UT, USA). Available
168 data were insufficient to estimate the sample size necessary to evaluate the prognostic value of CRP
169 kinetics on outcome. The study was therefore designed to enrol at least 40 dogs with leptospirosis and
170 14 controls for the one-time evaluation of CRP at presentation. When satisfying the additional body
171 weight criteria, dogs with leptospirosis were enrolled for serial sampling. Taking in account a lower
172 power due to a smaller group size, only descriptive statistics and evaluation of outcome prediction
173 were performed for this part of the study.

174 **RESULTS**175 ***Dogs and disease***

176 Forty-one dogs were diagnosed with AKI due to leptospirosis **between 2012 and 2014** and they were
177 enrolled in the main study group L. This population consisted of 6 mixed-breed dogs (15%) and 35
178 pure-bred dogs (85%), including 10 Labrador Retrievers (24%), 5 Golden Retrievers (12%), and 1 dog
179 (2%) each from 20 other breeds. Thirty-one dogs (76%) were male (21 entire and 10 castrated) and 10
180 (24%) were female (5 entire and 5 spayed). Their median age was 3.9 years (IQR, 1.6- 7.8) and their
181 median body weight 25.7 kg (IQR, 19.2 - 30.0).

182 The diagnosis of leptospirosis was based on double MAT serology with seroconversion (n=30, 73%);
183 positive RT-PCR on liver and kidney (n=1, 2%); a strong clinical suspicion and a positive single MAT titre
184 (n=7, 17%); a strong clinical suspicion and a positive immunodiffusion rapid test (n=1, 2%); and on a
185 strong clinical suspicion alone (n=2, 5%). Initial MAT serology showed the highest titres for serogroups
186 Australis (19/37, 51%), Pomona (4/37, 11%), and Autumnalis (4/37, 11%). The last MAT serology
187 performed a median of 8.0 days later (IQR, 7.0-9.8) showed positivity for serogroups Australis (33/37,
188 89%), Pomona (14/37, 38%), and Grippotyphosa (10/37, 27%). For dogs with more than one MAT,
189 seroconversion was observed for serogroups Australis (26/30, 87%), Pomona (23/30, 77%), and
190 Autumnalis (16/30, 53%).

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

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

199 3 castrated) and 8 (53%) were female (2 entire and 6 spayed). Their median age was 4.2 years (IQR,
200 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
201 identified as maleic acid intoxication (n=2), use of non-steroidal anti-inflammatory agents (NSAIA, n=1),
202 lymphoma (n=1), and trauma (n=1). The aetiology remained unidentified in 10 dogs. The absence of
203 leptospirosis in this group was confirmed with double serology without seroconversion (n=7, 47%) or
204 positive identification of an alternative diagnosis based on renal histopathology (n=4, 27%) or history
205 (n=4, 27%). At presentation, the severity of AKI was classified as grade 1 in 1 dog, grade 3 in 6 dogs,
206 grade 4 in 5 dogs, and grade 5 in 3 dogs (Table 1).

207 Pre-referral treatments included intravenous crystalloids (L: 28/41, nL: 5/15), antibiotics (penicillins,
208 fluoroquinolones, metronidazole and tetracyclines; L: 33/41, nL: 6/15), gastric acid inhibitors
209 (omeprazole, ranitidine; L: 12/41, nL: 2/15), steroids (L: 3/41, nL: 3/15), NSAIA (L: 6/41, nL: 4/15),
210 antiemetics (maropitant, ondansetron and metoclopramide; L: 24/41, nL: 3/15) and opioids (L: 6/41,
211 nL: 0/15). During hospitalisation, the dogs were treated with a standardized protocol basis consisting
212 of fluids, antibiotics (amoxicillin - clavulanic acid), antiemetics (maropitant, ondansetron, and/or
213 metoclopramide), gastric acid inhibitors (omeprazole, ranitidine), and opioids (buprenorphine,
214 butorphanol, methadone), adjusted at the clinician's discretion. Haemodialysis was performed in
215 28/41 L-dogs (68%) and 6/15 nL-dogs (40%).

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

221

222 ***C-reactive protein***

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

229 All 289 samples were measured in duplicates and the CV of these measurements was 13.4%; 9.9% for
230 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
231 14.4% for the lowest tertile (CRP <25.4 mg/L, n=96).

232 Serum CRP was increased at presentation in all dogs with AKI due to leptospirosis, with a median of
233 74.4 mg/L (IQR, 44.9–120.4; reference range, 0–10.5). The control group showed a lower proportion of
234 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
235 was not statistically different from dogs with leptospirosis ($P=0.088$). Two of the 3 control dogs with a
236 normal CRP concentration were diagnosed with maleic acid nephrotoxicosis and the aetiology of AKI
237 in the third dog remained unclear. In dogs with leptospirosis, log-transformed CRP concentration was
238 not associated with temperature ($r=0.23$, $P=0.152$), and moderately with other markers of
239 inflammation, including white blood cell count ($r=0.35$, $P=0.030$), segmented neutrophil count ($r=0.46$,
240 $P=0.003$), and serum albumin ($r=-0.43$, $P=0.005$) (Figure 1). No obvious association could be recognized
241 between the main organ manifestations of leptospirosis, their severity, and serum CRP (Table 2). In
242 dogs with leptospirosis, CRP was not associated either with the level of azotaemia at presentation,
243 measured as serum creatinine ($r=0.00$, $P=0.983$) or serum urea ($r=-0.05$, $P=0.744$) and it was not
244 significantly different between survivors (median 65.2 mg/L; IQR, 43.4 – 104.2) and non-survivors
245 (median 110.1 mg/L; IQR, 73.5 – 174.6; $P=0.059$) in this population.

246

247 ***Kinetics of CRP and outcome analysis***

248 In the 28 dogs with serial sampling, median CRP concentration decreased gradually from d0 (91.5 mg/L;
249 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
250 sampling period (Figure 2). Median absolute decrease from d0 to d4 was 59.2 mg/L (IQR, 21.5 – 98.1)
251 and median relative decrease was 66.6% (IQR, 53.1 – 81.7). Per protocol, a blood culture was
252 performed in 3 dogs with leptospirosis showing a new episode of fever (n=2) or inappropriate
253 treatment response (n=1). *Klebsiella pneumoniae* was cultured from the 2 dogs with fever and the
254 culture from the third dog was negative. In these 3 cases, CRP spiked at the time of performing blood
255 culture after an expected decrease the days before. This type of time course of CRP was not observed
256 in any other dog in this study.

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

261 In the ROC curve analysis including all dogs with leptospirosis, serum CRP at presentation was
262 significantly, but only weakly, predictive of outcome (AUC=0.69, 95% CI: 0.46-0.83, P=0.047, Figure
263 4A). With a cutoff of 106 mg/L at presentation, CRP had a sensitivity of 79% (95% CI, 60-90) and a low
264 specificity of 62% (95% CI, 36-82) to predict outcome; even with a high cutoff of 180 mg/L CRP at
265 presentation was only 90% sensitive for predicting death. With repetitive measurements however, the
266 TAC-CRP (d0-2) was strongly predictive of outcome (AUC=0.88, 95% CI: 0.67-0.96, P<0.001, Figure 4A).
267 Adding more days of observation improved only minimally the predictive value of CRP kinetics (d0-4:
268 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
269 comparison, the most commonly used parameter to grade AKI severity, serum creatinine was not
270 predictive of outcome (AUC=0.61, 95% CI: 0.41-0.75, P=0.212, Figure 4B) and serum urea showed a
271 moderate predictive value (AUC=0.72, 95% CI: 0.52-0.84, P=0.008, Figure 4B).

272 **Discussion**

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

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

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

306 In support of this, dogs with kidney disease have been shown to have increased CRP, with chronic
307 activation of an acute phase response as a likely trigger (Raila *et al.* 2011). Interestingly, the authors
308 showed a strong correlation between CRP and glomerular filtration rate, creatinine, and proteinuria in
309 these dogs with mostly CKD. The clear lack of correlation between CRP and azotaemia observed in our
310 study probably indicates a fundamental difference in AKI versus CKD kinetics, most biological
311 parameters being not in steady-state in AKI with production and clearance varying over time.
312 Furthermore, the organism is typically more severely affected both by the cause of AKI and by its multi-
313 organ consequences, many of them triggering an acute phase response with increased CRP production.

314 In a previous study including dogs with chronic and acute kidney diseases, the pattern and intensity of
315 mRNA expression of the pro- and anti-inflammatory cytokines IL-1 α , IL-1 β , and TGF- β were similar
316 between AKI and CKD (Nentwig *et al.* 2016). Whether this reflects a difference between cytokine
317 expression and the actual concentrations of the acute-phase products remains to be elucidated.

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

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

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

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

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

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

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

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

376 clinical course of the disease and a higher risk of death. It may justify therefore a more pro-active
377 therapeutic approach. Similarly, an unusual delay in the normalisation of CRP may indicate a secondary
378 inflammatory or infectious complication and justify a re-evaluation with additional tests such as
379 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.

486

487 **Table 1:** Clinical and clinicopathological parameters from the 41 dogs with AKI due to leptospirosis

488 (group L) included in the study and from 15 control dogs with AKI from other aetiologies (group nL)

489

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

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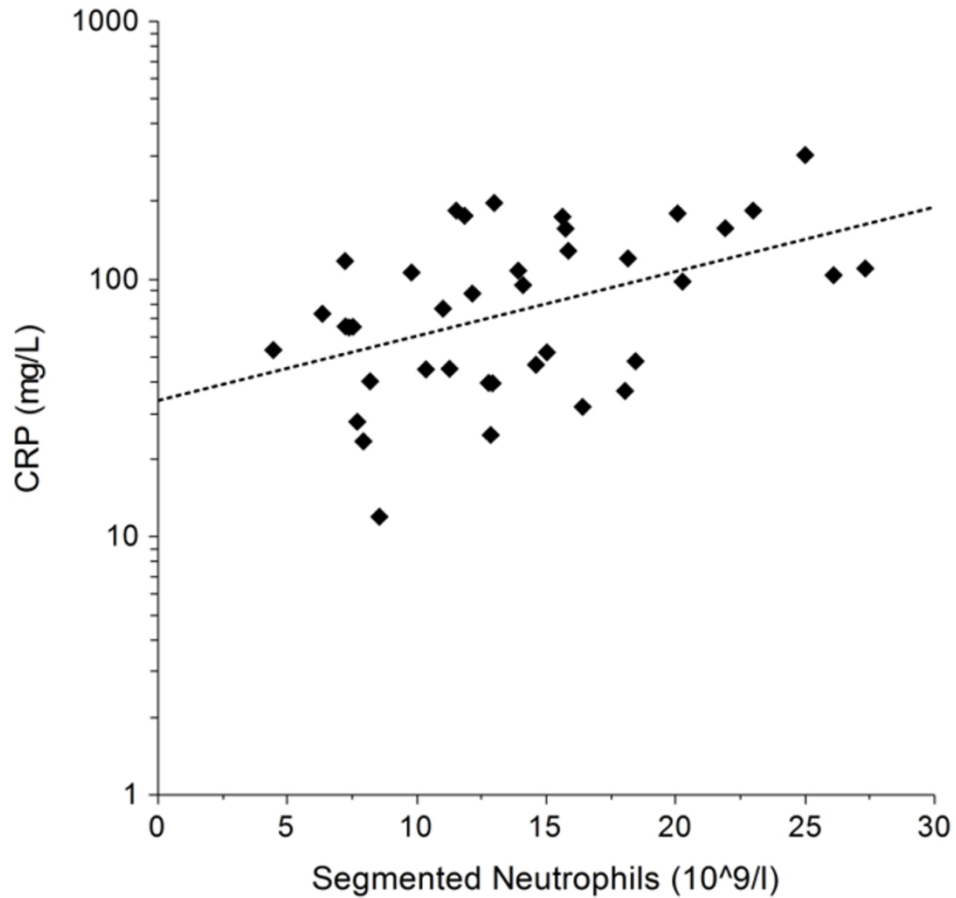


Figure 1A Correlation of log-transformed CRP at presentation with other markers of inflammation (A, segmented neutrophil count; B, albumin) and markers of renal function (C, urea; D, creatinine) in 41 dogs with AKI due to leptospirosis.

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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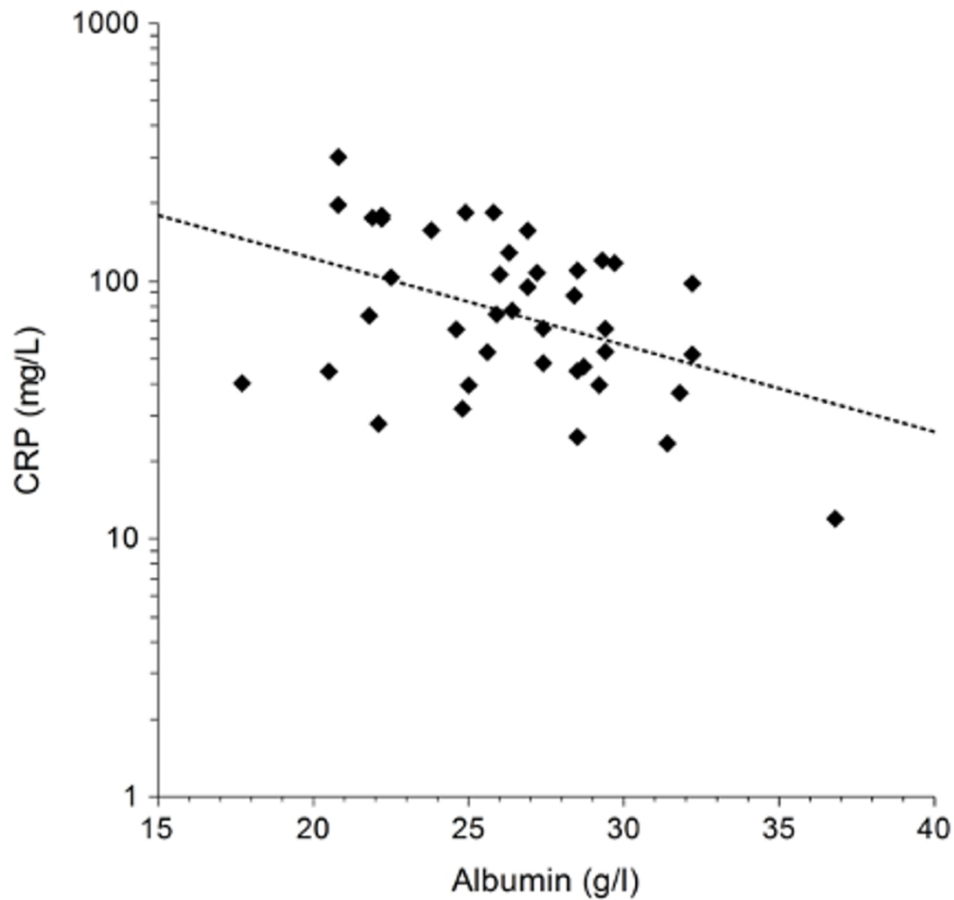


Figure 1B Correlation of log-transformed CRP at presentation with other markers of inflammation (A, segmented neutrophil count; B, albumin) and markers of renal function (C, urea; D, creatinine) in 41 dogs with AKI due to leptospirosis.

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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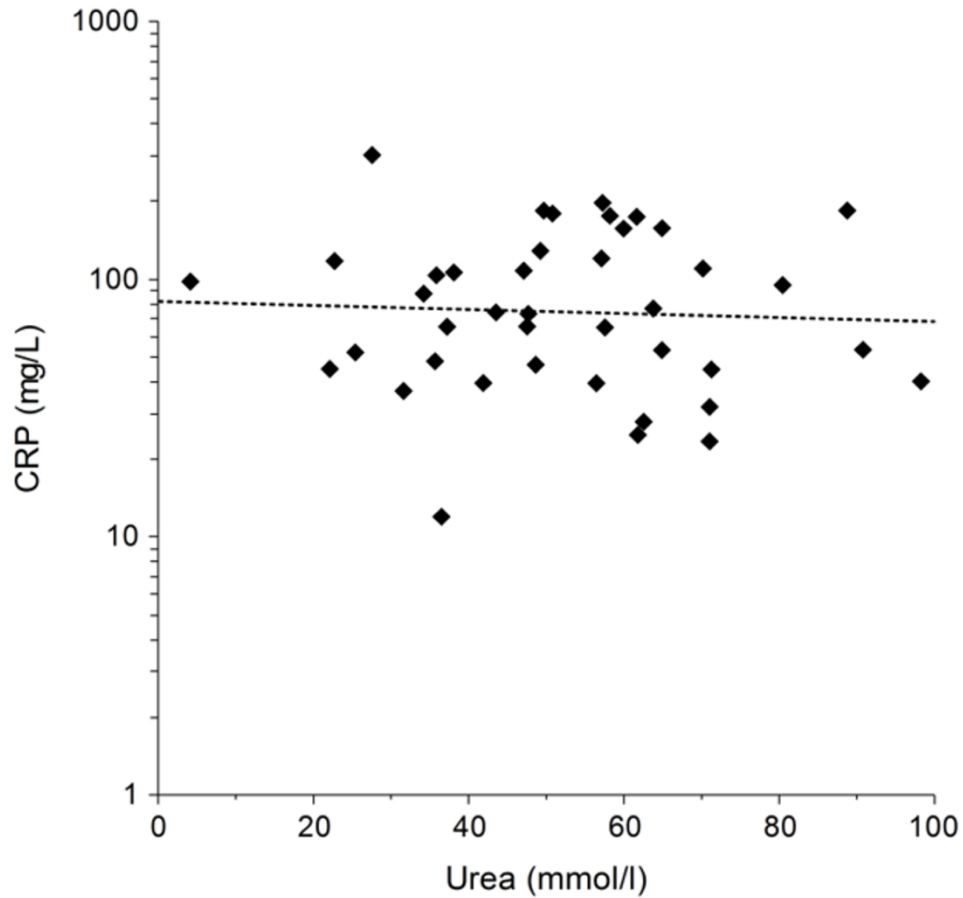


Figure 1C Correlation of log-transformed CRP at presentation with other markers of inflammation (A, segmented neutrophil count; B, albumin) and markers of renal function (C, urea; D, creatinine) in 41 dogs with AKI due to leptospirosis.

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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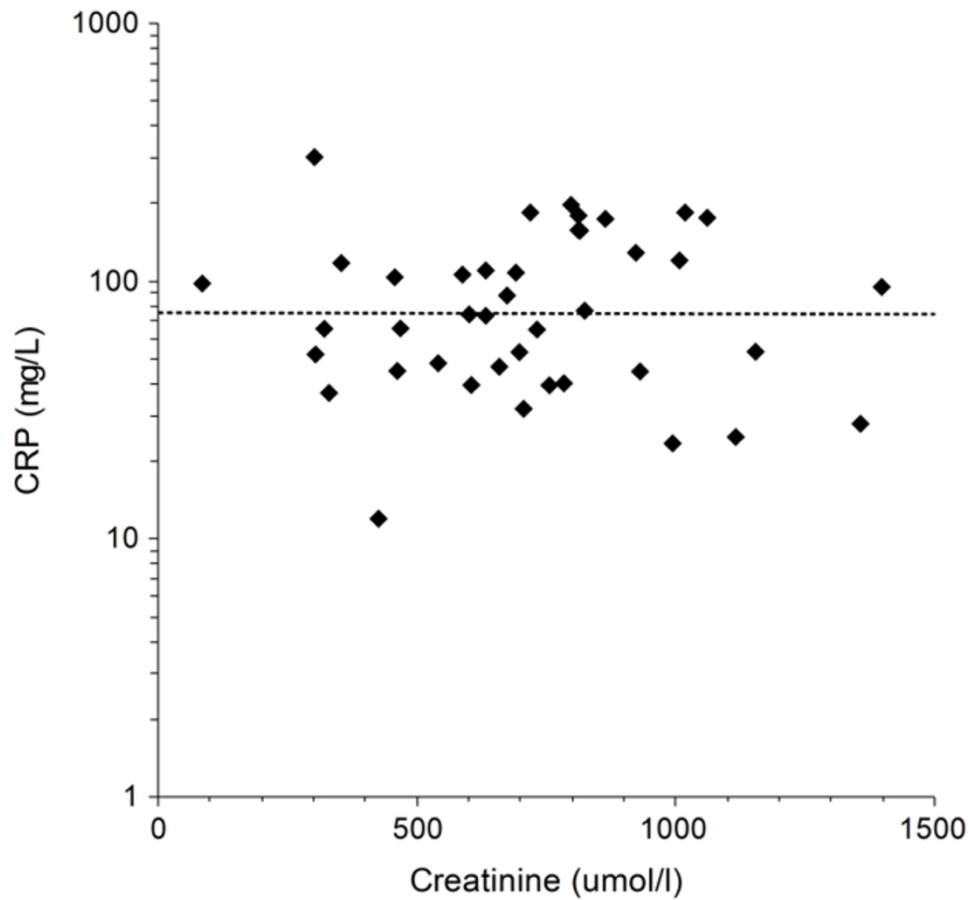


Figure 1D Correlation of log-transformed CRP at presentation with other markers of inflammation (A, segmented neutrophil count; B, albumin) and markers of renal function (C, urea; D, creatinine) in 41 dogs with AKI due to leptospirosis.

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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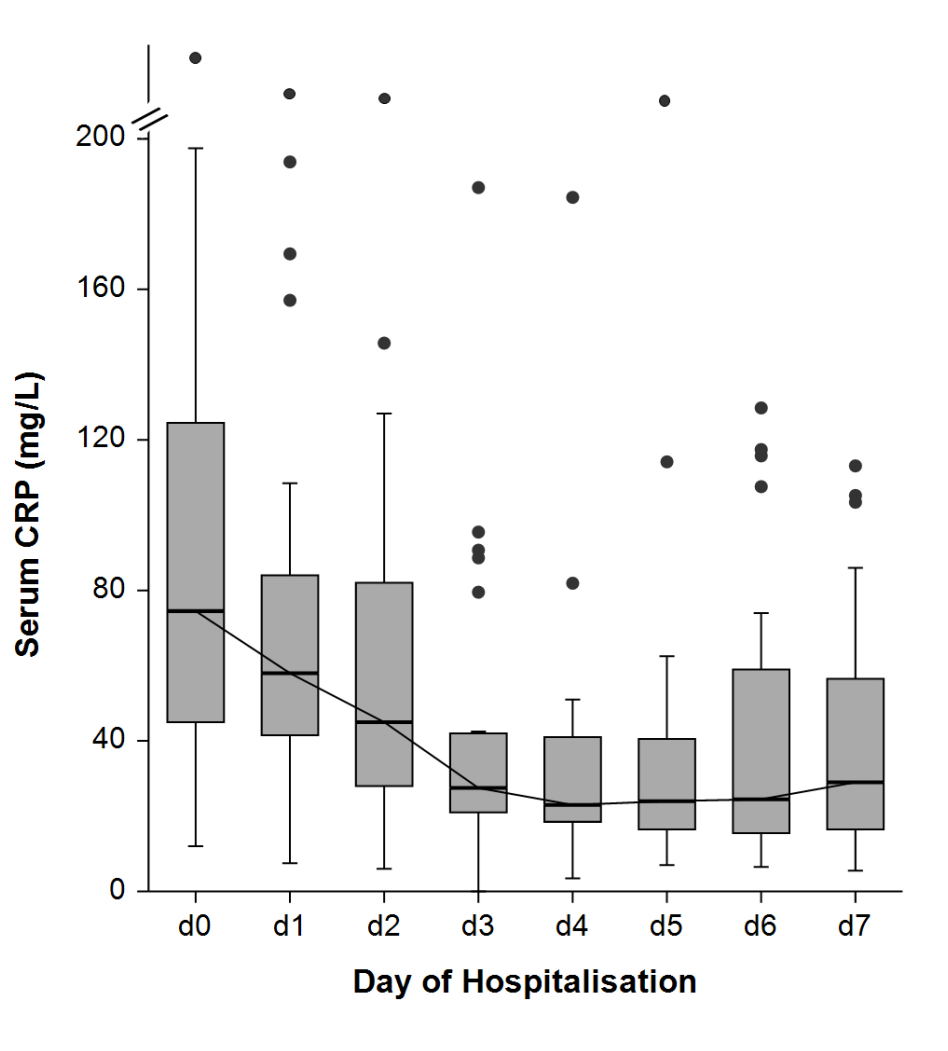


Figure 2 Time course of CRP concentration during hospitalisation (d0-d7) in 28 dogs with leptospirosis. The data are presented as a box plot, with the horizontal bar representing the median; the edges of the box, the interquartile range; and the whiskers, the range or the data with exception of the statistical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

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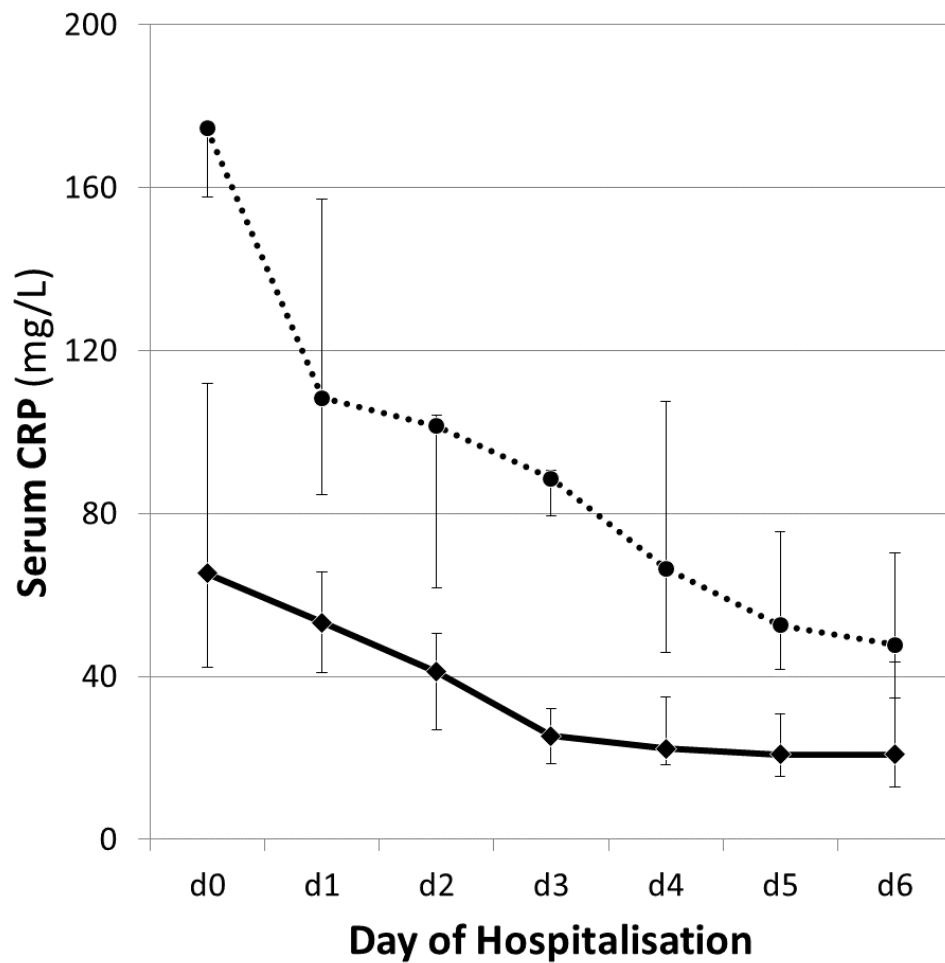


Figure 3 Time course of CRP concentration during hospitalisation in 28 dogs with leptospirosis stratified as survivors (black diamonds, full line) and non-survivors (black circles, dotted line). The data are presented as median (diamonds and circles) and IQR (error bars). The day of hospitalisation was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

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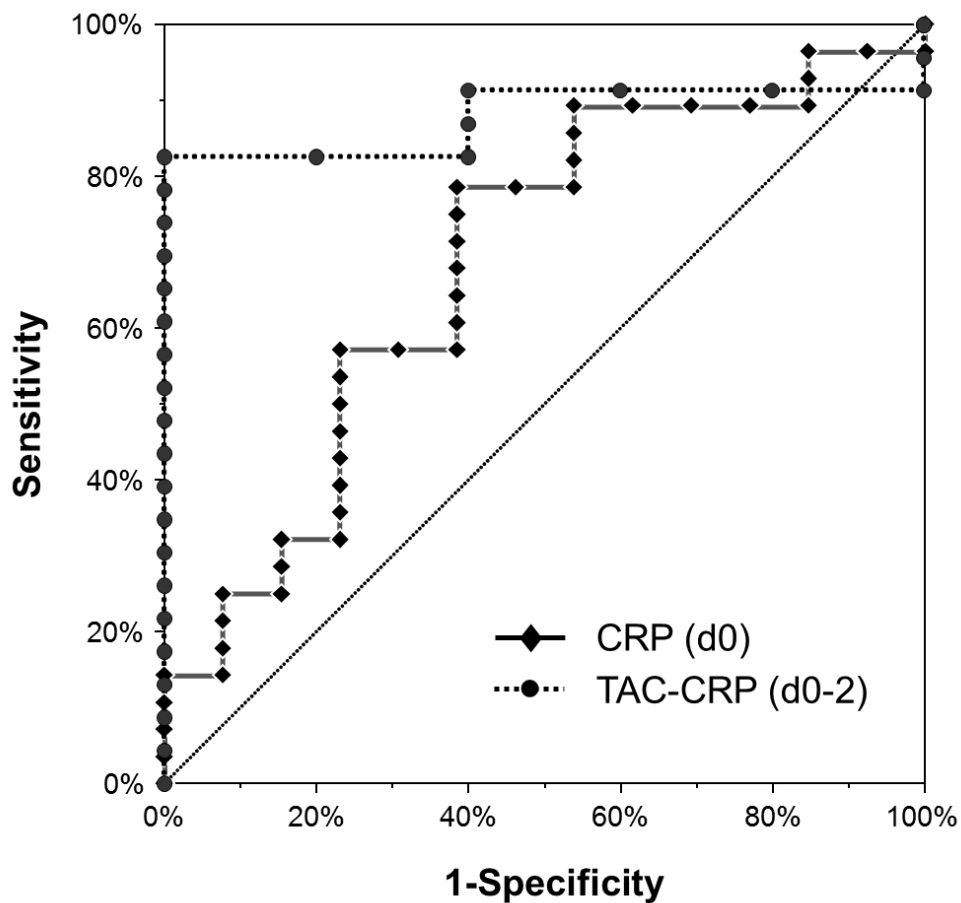


Figure 4A ROC curve representing the predictive value of outcome in dogs with leptospirosis for A) CRP concentration at presentation (d0) and TAC-CRP (d0-2), and B) urea and creatinine concentrations at presentation (d0).

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)

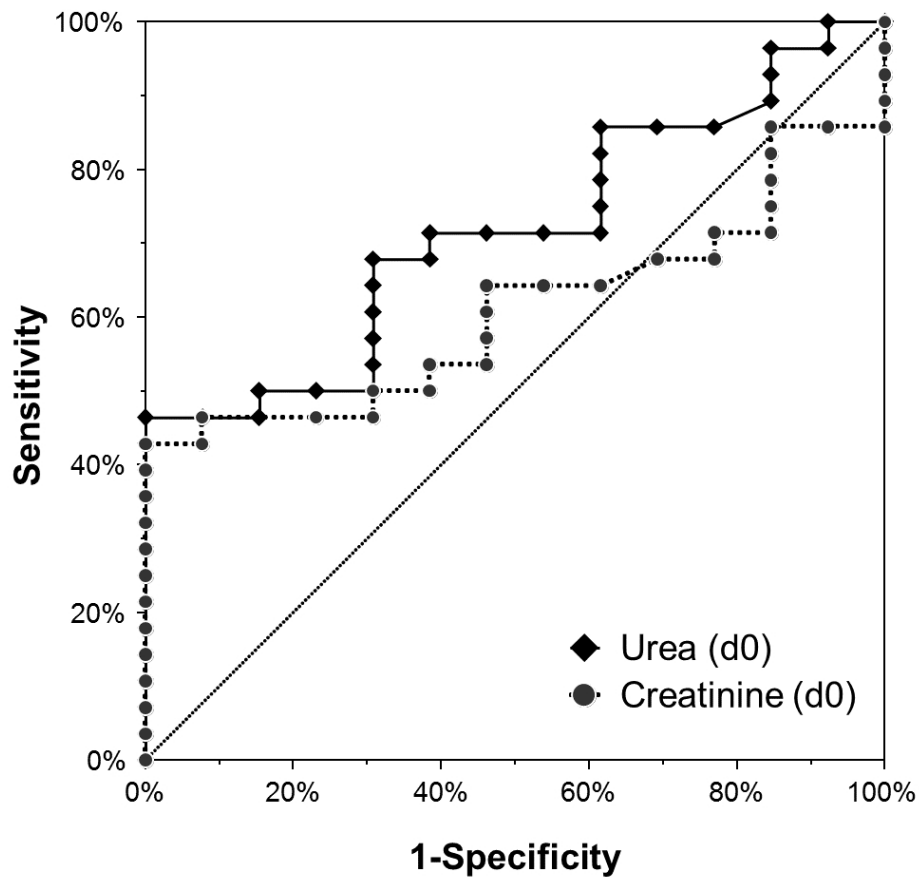


Figure 4B ROC curve representing the predictive value of outcome in dogs with leptospirosis for A) CRP concentration at presentation (d0) and TAC-CRP (d0-2), and B) urea and creatinine concentrations at presentation (d0).

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)

1 Table 1: Clinical and clinicopathological parameters from 41 dogs with leptospirosis and 15 control
 2 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%)

3

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

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

1 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

2

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