

This is the peer-reviewed, manuscript version of an article published in *Veterinary Record*. The final version is available online: <http://dx.doi.org/10.1136/vr.105104>.

The full details of the published version of the article are as follows:

TITLE: Randomised controlled trial of fractionated and unfractionated prednisolone regimens for dogs with immune-mediated haemolytic anaemia

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JOURNAL TITLE: *Veterinary Record*

PUBLISHER: BMJ Publishing Group

PUBLICATION DATE: 2 May 2019 (online)

DOI: 10.1136/vr.105104

1 Randomised controlled trial of fractionated and unfractionated prednisolone
2 regimens for dogs with immune-mediated haemolytic anaemia

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21

22 **WORD COUNT:** 3997 words

23

24 **KEYWORDS:** dog, fractionated, IMHA, prednisolone

25

26 **ABSTRACT**

27 **Methods:** A randomised non-blinded non-inferiority trial was conducted to determine whether treatment
28 with an unfractionated regimen of oral prednisolone was inferior to a fractionated regimen for dogs with
29 primary immune-mediated haemolytic anaemia. Dogs received the same total daily dose of prednisolone
30 as unfractionated (group 1, starting at 4 mg/kg PO once daily) or fractionated (group 2, starting at 2 mg/kg
31 PO twice daily) doses. Questionnaires were administered to owners to assess adverse effects and quality
32 of life (QoL). Endpoints included survival to eight weeks, and changes in QoL and clinicopathological
33 parameters over time.

34 **Results:** Thirty-nine dogs were enrolled in the study, of which 5 were withdrawn and 17 were assigned to
35 each group. The number of cases recruited was insufficient to determine whether unfractionated treatment
36 was inferior to fractionated. Total serum bilirubin decreased more rapidly in dogs in group 2, whereas
37 polydipsia improved more rapidly in group 1. Blood pressure and score for polyuria were higher in dogs
38 in group 2 over time, whereas lymphocyte concentration was lower.

39 **Conclusion:** Administration of the same total daily dose of prednisolone as an unfractionated dose resulted
40 in fewer adverse effects but the effect on survival could not be assessed in this study.

41

42 **INTRODUCTION**

43 Immune-mediated haemolytic anaemia (IMHA) is reported to be the most common autoimmune disease of
44 dogs[1]. Despite its relative frequency, there are few published reports evaluating different forms of
45 immunosuppressive therapy; the majority of these reports have been retrospective analyses, often based on
46 small numbers of dogs and often failing to present essential information regarding case selection and
47 diagnostic criteria[2].

48

49 Glucocorticoids, especially prednisolone or its prodrug prednisone, are widely considered to be the most
50 important component of immunosuppressive therapy for dogs with IMHA. Whereas several previous

51 studies have described the efficacy of glucocorticoids in the treatment of IMHA[3,4], the starting dose, rate
52 of tapering, frequency of administration, and use of additional drugs have varied considerably among
53 reports[2].

54

55 The terminal half-life of orally administered prednisolone is approximately two hours in dogs[5], but the
56 pharmacodynamic effects of the drug are likely to persist for longer because they depend on changes in
57 gene transcription and protein synthesis[6,7]. The clinical effect of a glucocorticoid drug at any particular
58 dose may be described as the product of its potency, chiefly related to its affinity for the glucocorticoid
59 receptor, and its presence at the receptor site, which is dictated by its pharmacokinetic profile[6,8]. This
60 relationship suggests that twice daily administration of prednisolone may increase its efficacy for
61 management of autoimmune diseases by increasing its availability at the receptor site. An uncontrolled
62 observational study of people with glomerulonephritis and after kidney transplants appeared to support this
63 notion because patients receiving twice daily fractionated doses of oral prednisolone had a decreased
64 magnitude of proteinuria and a lesser requirement for additional immunosuppressive drugs compared to
65 once daily dosing[9].

66

67 Conversely, more frequent administration of prednisolone results in greater adrenocortical suppression in
68 dogs[10], and may also increase the risk of typical adverse effects, including polyuria, polydipsia,
69 polyphagia, excessive panting, muscle weakness and muscle wastage[6]. Previous studies have not focused
70 on the impact that these adverse effects could have on the quality of life (QoL) of the patient and their
71 owner, even though these could have a substantial impact on the owner's decision to pursue treatment.
72 Thus, in conceiving this randomised trial, the authors' aim was to compare the survival and QoL of dogs
73 receiving a fractionated or unfractionated regimen of prednisolone. The authors hypothesised that
74 unfractionated administration of prednisolone would not be inferior to fractionated treatment in terms of
75 survival but would result in both a lesser incidence of adverse effects and a more favourable QoL.

76

77 MATERIALS AND METHODS

78 **Trial design:** A randomised controlled non-inferiority trial was conducted to compare the outcome for
79 dogs with primary IMHA treated with prednisolone using two different dose reduction protocols, with an
80 allocation ratio of 1:1. A non-inferiority approach was chosen for evaluation of survival because the authors
81 did not anticipate a significant difference between treatment groups for this parameter. When designing
82 the study, survival to eight weeks after diagnosis was considered to be the primary endpoint, so the sample
83 size calculation was based on this parameter.

84
85 **Sample size calculation:** The authors estimated that at least 28 dogs would be required in each treatment
86 group to demonstrate non-inferiority within the lower margin of -20%, assuming a baseline mortality rate
87 of 10% at eight weeks after diagnosis and with power $(1-\beta)$ 80% and significance value (α) 0.05. The
88 baseline mortality rate was based on calculation of the mortality rate at the same institution among dogs
89 that would have been eligible for this study over the period of two years (2012-2013) before recruitment
90 began. The sample size calculation was completed with an online tool[11]. The lower margin of -20% was
91 selected because previous studies have reported variable mortality rates in different samples of dogs with
92 IMHA treated at tertiary referral institutions, and the authors considered that a margin of at least 20% would
93 be required to prove that a difference between groups was attributable to the treatment allocation rather
94 than the expected variance for this parameter.

95
96 **Participants:** Client-owned dogs were recruited at a single tertiary referral veterinary hospital between
97 April 2014 and November 2015. Dogs were considered eligible for inclusion in this trial if they were
98 anaemic, with a packed cell volume (PCV) of less than 35%, and if they had at least one of the following
99 features suggestive of immune-mediated haemolysis: prominent spherocytosis on examination of a fresh
100 blood smear by a board-certified clinical pathologist or participant in a specialist training programme, a
101 titre of at least 1:16 in a multivalent direct antiglobulin (Coombs') test, or persistent microscopic or
102 macroscopic agglutination of red blood cells after dilution in saline. Dogs were excluded if any underlying

103 cause of IMHA was detected after reviewing results of complete blood count (CBC), serum biochemical
104 profile, serologic tests for endemic arthropod-borne diseases (4DX SNAP test, IDEXX), urinalysis, thoracic
105 radiography or computed tomography (CT) and abdominal ultrasonography or CT.

106

107 After inclusion in the study, dogs were randomised in a sequence generated by a random number
108 calculator[12] to receive the same oral daily dose of prednisolone (Prednidale, Dechra Ltd) either as a single
109 daily dose (group 1) or as fractionated twice daily treatment (group 2), followed by a recommended protocol
110 for reduction of the dose of prednisolone over the following fifteen weeks (shown in Table 1). The first
111 dose reduction was always made as indicated but, after this, the decision to proceed with the recommended
112 reduction was made by the attending clinician based on clinical status and results of follow-up tests. The
113 starting dose of prednisolone and increments for dose reduction were selected based on the clinical
114 experience of the authors and the protocols that were in use at the study institution when the study was
115 designed. Throughout the course of treatment described, dogs in both groups received the same total daily
116 dose of prednisolone but this dose was always administered as a larger number of fractions for those in
117 group 2. The owners, attending veterinary surgeons and trial co-ordinators were not blinded to the
118 allocation of the treatment protocol. If dogs were inappetent, they received daily intravenous injections of
119 dexamethasone sodium phosphate (Dexadreson, MSD Animal Health; at 0.4 mg/kg per day) while
120 hospitalised until they were able to receive oral medications. All dogs also received azathioprine (Imuran,
121 Prometheus Laboratories Inc or Azathioprine Capsules, Nova Laboratories Ltd; median dose 50.4 mg/m²
122 every other day, IQR: 46.7-54.3), omeprazole (Omeprazole, Mylan; median dose 1.1 mg/kg per day, IQR:
123 0.9-1.2), and either aspirin (Soluble Aspirin, Actavis; median dose 0.5 mg/kg per day, inter-quartile range
124 [IQR]: 0.5-0.5) or clopidogrel (Plavix, Bristol-Myers Squibb; median dose 3.8 mg/kg per day, IQR: 3.1-
125 3.8).

126

127 Dogs were considered to have discontinued their allocation if they received an additional
128 immunosuppressive drug during the period of the study or if they deviated from the dose reduction schedule

129 outlined in Table 1 due to a delay of more than one week in making a reduction, due to making a more
 130 rapid reduction than recommended, or if a relapse necessitated an increased dose of prednisolone.

131

132 **Table 1:** Outline of the prednisolone dose reduction schedules utilised in treatment groups 1 and 2.

Time point after diagnosis	Dose of prednisolone	
	Treatment group 1	Treatment group 2
Diagnosis	4 mg/kg PO SID	2 mg/kg PO BID
Week 1	2 mg/kg PO SID	1 mg/kg PO BID
Week 3	3 mg/kg PO every other day	0.75 mg/kg PO BID
Week 5	2 mg/kg PO every other day	0.5 mg/kg PO BID
Week 8	1 mg/kg PO every other day	0.25 mg/kg PO BID
Week 11	0.5 mg/kg PO every other day	0.25 mg/kg PO SID
Week 13	0.5 mg/kg PO q3 days	0.5 mg/kg PO q3 days
Week 15	STOP	STOP

133

134 Following discharge from the hospital, measurement of the PCV was recommended prior to each dose
 135 reduction shown in Table 1, with more complete examinations at three weeks and eight weeks after
 136 diagnosis at the same institution or at the referring veterinary practice. At these visits, procedures were
 137 recommended to monitor progress and detect any adverse effects of treatment, as shown in Table 2, and
 138 questionnaires were administered to owners to assess the QoL of the dog.

139

140 **Table 2:** Outline of the procedures recommended at re-examination visits for dogs in both treatment groups

Time after diagnosis	Procedures recommended
Week 3	CBC ¹ , UA ² , UC ³ , UPC ⁴ , NIBP ⁵ , bodyweight, body condition score, questionnaire 1

Week 8	CBC, BC ⁶ , UA, UC, UPC, NIBP, bodyweight, body condition score, questionnaire 2
--------	---------------------------------------------------------------------------------------------

141

142 1: Complete blood cell count

143 2: Urinalysis, including refractometric urine specific gravity, dipstick and sediment examinations

144 3: Urine culture

145 4: Urine protein: creatinine ratio

146 5: Non-invasive blood pressure measurement

147 6: Serum biochemical profile

148

149 Informed consent was obtained from the owners of dogs for all procedures and for inclusion in this clinical
 150 trial. The trial was approved by the Clinical Research Ethical Review Board at the Royal Veterinary
 151 College, University of London (reference number 2011_1134).

152

153 **Clinicopathological variables:** Complete blood cell counts and serum biochemical profiles were
 154 generated using instruments validated for dogs (ADVIA 2120i, Siemens and ILAB 600, Instrumentation
 155 Laboratory). A fresh blood smear was examined by a board certified clinical pathologist with every CBC.
 156 Systolic blood pressure was measured using a Doppler probe (Model 811-B, Parks Medical Electronics Inc)
 157 after selection of a blood pressure cuff with a width that approximated 40% of the circumference of the
 158 limb of the dog. At least three readings were obtained at every measurement; the arithmetic mean of these
 159 values was used for analysis. All urine samples were obtained by cystocentesis under ultrasonographic
 160 guidance; bacterial culture was performed by applying 2 µl of urine to Columbia agar with 5% sheep blood
 161 and MacConkey agar plates using a sterile hockey stick spreader before incubating aerobically at 37°C for
 162 48 hours.

163

164 **Questionnaires:** A complete copy of the questionnaires used in this trial is available in Supplementary
165 File 1. Briefly, each questionnaire consisted of a number of questions interrogating different aspects of the
166 QoL of the dog (demeanour, activity levels, enthusiasm for exercise, water intake, urination, appetite,
167 panting and muscular strength), together with a question asking the owner to rate the dog's current global
168 QoL, with all results expressed as a single mark on a 100 millimetre visual analogue scale (VAS). For each
169 aspect, owners were also asked to rate the importance of the changes they had observed; these scores were
170 summated to produce a composite score that we described as owner QoL. The questionnaire contained two
171 final questions that asked owners if they felt that their normal activities were restricted as a result of
172 administering medications and if they would treat another dog with IMHA based on their experience with
173 the current patient. An example of the question format is shown in Figure 1. After explanation of the
174 structure of the questionnaire and demonstration of the use of the VAS at the first (week three) re-
175 examination visit, the owner of the dog was allowed to complete this and the subsequent questionnaire
176 without assistance from the attending veterinary surgeon. After completion, the result of the VAS for each
177 question was measured and recorded in an electronic spreadsheet by a single investigator.

178

179 **Outcome measures:** The primary outcome measure in the study was survival to eight weeks after
180 diagnosis; additional outcome measures included changes in quality of life and hematologic and
181 biochemical parameters between diagnosis and the re-examination visits, the proportion of dogs that
182 developed bacteriuria during the first eight weeks of treatment, and the proportion that suffered a relapse
183 during the same period. A relapse was defined as a relative reduction in the packed cell volume of 25% or
184 more compared to the previous visit.

185

186 **Changes to study design after completion of the trial:** Cases were enrolled over a period of 19 months,
187 and the trial was closed eight weeks after the last participant was recruited. An insufficient number of dogs
188 were recruited to power the comparison of mortality between groups, so this outcome measure could not
189 be assessed.

190

191 **Statistical analysis:** All statistical analyses were conducted with commercial software packages (IBM
192 SPSS Statistics for Windows, Version 20.0, IBM Corp and GraphPad Prism version 6.00 for Windows,
193 GraphPad Software). The two treatment groups were compared at baseline to assess whether they were
194 equivalent with respect to parameters that may have prognostic value for dogs with IMHA: age;
195 bodyweight; packed cell volume; platelet, neutrophil and monocyte concentrations; serum albumin,
196 bilirubin, urea and creatinine concentrations; and serum alkaline phosphatase activity[13,14]. Variables
197 were assessed for normality using the Shapiro-Wilks test. Normally distributed variables were then
198 compared using Student's t test, whereas non-normally distributed variables were compared with the Mann-
199 Whitney U test. Categorical proportions were compared with the Chi squared test or Fisher's exact test.

200

201 Changes in parameters over time and differences between treatment groups were assessed with linear mixed
202 effect models according to the intention to treat. Case identity was included as a random factor, whereas
203 time point, treatment group and an interaction term between these two variables were included as fixed
204 categorical factors. If the interaction term was significant in any model, *post hoc* tests were conducted to
205 assess the cause of this interaction. Residuals were assessed visually and using Shapiro-Wilks test: if these
206 were not normally distributed, the dependent variable was logarithmically transformed.

207

208 The manuscript was prepared according to the CONSORT template for reporting of randomised controlled
209 trials[15, 16] (Supplementary File 2) and all results from the trial are available in Supplementary File 3.

210

211 **RESULTS**

212 Thirty-nine dogs were considered eligible for the study and were randomised to treatment group 1 (n=20)
213 or 2 (n=19). Of these dogs, five were withdrawn from subsequent analysis because they did not survive for
214 long enough to receive oral medications (n=2), or because they received a different combination of
215 immunosuppressive medications from the outset of their treatment (n=3). The remaining cases were

216 included in the analysis (n=17 in each group), even if they subsequently deviated from the intended
 217 treatment protocol. Flow of cases in the trial is shown in Figure 2.

218

219 There were no significant differences between groups 1 and 2 at enrolment in terms of age, sex distribution
 220 or selected haematological and biochemical parameters (as shown in Table 3), apart from a difference in
 221 urine specific gravity, which was greater in dogs in group 1 (mean 1.034, SE: 0.003) than in group 2 (1.026,
 222 0.002, $p=0.047$). Nine dogs (52.9%) in group 1 and 8 (47.1%) in group 2 had received immunosuppressive
 223 drugs for up to 3 days prior to referral to the study institution; there was no difference in the proportion of
 224 dogs that had received this treatment (Chi squared, $p=0.598$).

225

226 **Table 3:** Demographic, hematologic and biochemical parameters at baseline in treatment groups 1 and 2.

Parameter		Treatment group 1 (n=17)	Treatment group 2 (n=17)	<i>P</i> value
Age (years)	Median (IQR ¹)	8.0 (4.1-9.0)	7.0 (3.5-8.5)	0.259
Sex (N)				0.600
	Male entire	1	0	
	Male neutered	6	8	
	Female entire	1	2	
	Female neutered	9	7	
Previous immunosuppressive treatment	N (%)	9 (52.9)	8 (47.1)	0.598
Duration of previous immunosuppressive treatment (days)	Median (IQR)	1 (0-1.5)	0 (0-1.0)	0.817

Bodyweight (kg)	Median (IQR)	11.4 (9.3-24.4)	15.6 (7.6-22.0)	0.734
Serum albumin concentration (g/l)	Mean (SD) ²	30.6 (6.5)	31.5 (4.8)	0.643
Serum bilirubin concentration ($\mu\text{mol/l}$)	Median (IQR)	10.5 (1.9-26.1)	13.0 (8.6-30.2)	0.491
Serum urea concentration (mmol/l)	Median (IQR)	7.9 (5.2-10.3)	7.5 (5.3-10.6)	0.809
Serum creatinine concentration ($\mu\text{mol/l}$)	Mean (SD)	56.0 (12.0)	55.6 (11.6)	0.929
Serum alkaline phosphatase activity (U/l)	Median (IQR)	181.0 (112.5-407.0)	203.0 (152.5-222.5)	0.724
Packed cell volume (%)	Median (IQR)	16.0 (11.0-18.0)	13.0 (10.0-17.0)	0.658
Platelet concentration ($\times 10^9/\text{l}$)	Mean (SD)	314 (238)	270 (137)	0.513
Neutrophil concentration ($\times 10^9/\text{l}$)	Median (IQR)	20.4 (12.5-28.6)	16.8 (12.5-19.3)	0.357
Monocyte concentration ($\times 10^9/\text{l}$)	Median (IQR)	1.77 (1.17-2.38)	1.38 (0.85-2.50)	0.474
Lymphocyte concentration ($\times 10^9/\text{l}$)	Median (IQR)	1.19 (0.67-2.17)	0.77 (0.66-1.53)	0.290
Urine specific gravity (kg/l)	Mean (SD)	1.034 (0.011)	1.026 (0.009)	0.047

Urine protein: creatinine ratio	Median (IQR)	0.84 (0.57-5.79)	1.39 (0.30-2.29)	0.792
Systolic blood pressure (mmHg)	Mean (SD)	155 (26.0)	181 (27.3)	0.077

227

228 ¹: Inter-quartile range229 ²: Standard deviation

230

231 No dogs in the study were lost to follow-up within the first eight weeks after diagnosis. One dog in
 232 treatment group 2 suffered a relapse, when the PCV decreased by 50.0% eleven days after the first re-
 233 examination visit; this dog was subsequently euthanised before the second re-examination visit. Overall,
 234 two dogs in treatment group 2 (2/17, 11.7%) were euthanised within eight weeks of diagnosis, whereas all
 235 dogs in treatment group 1 survived.

236

237 Six dogs in treatment group 1 and four in group 2 discontinued their allocated intervention (as shown in
 238 Figure 2). Two dogs began to receive ciclosporin (at 5 mg/kg PO SID and 5 mg/kg PO BID) in addition to
 239 their other drugs due to perceived lack of response to treatment, whereas eight dogs deviated from the
 240 intended dose reduction schedule. In six cases, this was due to a delay of more than one week in completing
 241 a planned dose reduction owing to inadequate control of disease (n=1), delayed presentation for re-
 242 examination (n=2), incorrect instructions given to owners (n=2), or need to repeat a blood sample to assess
 243 clinical progress (n=1). In one further dog, the dose of prednisolone was decreased more rapidly than
 244 recommended because adverse effects were considered to be particularly severe, and the remaining dog
 245 received an increased dose of prednisolone due to relapse (described in the preceding paragraph). There
 246 was no difference between groups in the proportion of dogs deviating from the intended dose reduction
 247 schedule (Chi squared, $p=0.71$). Of the dogs that were treated per protocol, none died in group 1 (n=11)
 248 and 2/13 (15.4%) were euthanised in group 2.

249

250 The median time between diagnosis and the week 3 re-examination visit was 24 days for dogs in group 1
 251 ([inter-quartile range [IQR]: 20-25) and 22 days for group 2 (IQR: 20-24). The median time from diagnosis
 252 until the week 8 visit was 59 days for dogs in group 1 (IQR: 56-66) and 60 days for group 2 (IQR: 56-65).
 253 There was no significant difference between treatment groups for these times (Mann-Whitney U tests,
 254 $p=0.217$ and $p=0.948$, respectively).

255

256 Linear mixed effect models were constructed to evaluate the separate effects of the treatment allocation and
 257 time on clinicopathological parameters and QoL scores (Tables 4 and 5). For QoL scores, data were
 258 available for 11 dogs in group 1 and 7 in group 2. There was a significant interaction between time and
 259 treatment group for serum total bilirubin concentration (TBil) and for the VAS scores for polydipsia and
 260 panting. The score for polydipsia decreased significantly for dogs in treatment group 1 between the first
 261 and second re-examination visits, but not for dogs in group 2 (Figure 3A). Conversely, the TBil decreased
 262 significantly in treatment group 2 but not 1 between the point of diagnosis and the second re-examination
 263 visit (Figure 3B). Despite a significant interaction for panting, *post hoc* tests did not reveal any significant
 264 differences between groups over time (data not shown).

265

266 **Table 4:** Results of linear mixed effect models for clinicopathological variables, expressed as p values for
 267 treatment group, timepoint and their interaction term. Significant results are highlighted in bold.

Parameter	Treatment group	Timepoint	Treatment group*Timepoint
Bodyweight ¹ (kg)	0.581	<0.001	0.822
Packed cell volume ¹ (%)	0.650	<0.001	0.887
Lymphocyte concentration ¹ (x10 ⁹ /l)	0.028	0.100	0.746

Urine specific gravity (l/kg)	0.592	0.053	0.111
Urine protein: creatinine ratio ¹	0.787	0.128	0.975
Serum total bilirubin concentration ¹ (µmol/l)	0.609	<0.001	0.017
Systolic blood pressure (mmHg)	0.019	0.159	0.431

268 1: Variable log-transformed for analysis.

269

270 **Table 5:** Results of linear mixed effect models for visual analogue scores, expressed as *p* values for
 271 treatment group, timepoint and their interaction term. Significant results are highlighted in bold.

Visual analogue score	Treatment group	Timepoint	Treatment group*Timepoint
Lethargy ¹	0.195	0.494	0.242
Activity	0.151	0.228	0.754
Restlessness ¹	0.910	0.571	0.948
Polydipsia	0.354	<0.001	0.045
Polyuria ¹	0.031	0.006	0.160
Polyphagia	0.232	0.025	0.302
Panting	0.951	0.216	0.033
Musculoskeletal strength ¹	0.589	0.054	0.969
Global quality of life ¹	0.885	0.216	0.167
Owner quality of life (summated score)	0.690	0.919	0.576

Owner restriction of activity score	0.110	0.357	0.733
Owner decision to treat another dogs with IMHA	0.194	0.214	0.697

272 1: Variable log-transformed for analysis.

273

274 The mean systolic blood pressure and polyuria score were greater in dogs in treatment group 2 compared
275 to group 1 across time points, whereas the lymphocyte concentration was greater in group 1 (Figure 4A-C).

276 The PCV increased for dogs in both treatment groups over time, whereas the scores for polyuria and
277 polyphagia decreased in both treatment groups (Figures 4B-C and 5A-B). Changes in bodyweight over
278 time were more complex, with an overall decrease from diagnosis to the first re-examination visit, followed
279 by an overall increase between this and the second re-examination visit (Figure 5C).

280

281 There were no associations between treatment group, time, or their interaction term for the three scores
282 used to assess owner QoL. Three dogs in each treatment group were diagnosed with subclinical bacteriuria
283 within the first eight weeks of treatment; there was no difference in prevalence between groups (Fisher's
284 exact test, $p=1.000$).

285

286 **DISCUSSION**

287 This study represents the first report of a clinical trial intended to compare two different protocols for
288 administration of glucocorticoids in dogs with an immune-mediated disease, and the first to provide a
289 detailed and prospective account of the adverse effects experienced by these dogs and their owners during
290 their treatment. Administration of an unfractionated dose of prednisolone resulted in more rapid
291 improvement in the severity of polydipsia, as assessed with an owner questionnaire. Conversely, dogs
292 receiving a more fractionated dose had significantly greater reductions in serum TBil over the course of the

293 study compared to those receiving a less fractionated dose. Across all time points where they were
294 measured, dogs receiving a more fractionated dose had higher systolic blood pressures and had more severe
295 polyuria. It was not possible to assess the effect of the two regimens on survival to eight weeks after
296 diagnosis because an insufficient number of dogs was presented to our institution during the period of time
297 available for the study. This study provides important data on the occurrence of adverse effects associated
298 with the use of glucocorticoids and will act as a pilot study for larger trials that seek to determine the effect
299 of dose fractionation on survival.

300

301 The rate of deviation from the recommended treatment protocols was greater than expected in this study
302 and represents a major source of potential bias. The authors have the impression that these deviations were
303 largely attributable to communication problems among the trial co-ordinators, other attending veterinary
304 surgeons, and the owners of the dogs included in this study. These observations highlight the importance
305 of explicit and intensive communications when conducting prospective studies that involve a relatively
306 large number of stakeholders.

307

308 Beyond the known deviations from the treatment protocol, the authors did not assess whether owners were
309 administering tablets as directed by asking them to keep a medication diary or counting the number of
310 tablets remaining at each visit. This may have been important in a study comparing fractionated and
311 unfractionated regimens because more frequent administration may have been more difficult for some
312 owners, resulting in decreased compliance. Owner compliance with medication has not been studied
313 extensively in veterinary medicine: a previous study of administration of antimicrobials suggested that only
314 27% of owners gave the prescribed number of doses, but the average number of doses administered did not
315 differ between owners of dogs receiving medications two or three times per day[17]. A further study
316 reported similar compliance among owners administering antimicrobials once or twice daily, with a
317 significantly greater number of doses missed if the frequency increased to three times daily[18].

318

319 Neither owners nor veterinary surgeons were blinded to the treatment allocation in this study, which
320 represents a potential source of bias. The decision not to impose blinding was made because dogs with a
321 life-threatening disease were being treated by several different veterinarians, and the authors felt it was
322 important that owners were aware of the medications their dogs were receiving in case of emergency.

323

324 There was a more rapid reduction in the TBil in dogs receiving a more fractionated dose of prednisolone
325 compared to those receiving a less fractionated dose, which may be partly related to the non-significant
326 trend for greater pre-treatment concentrations in the former group. This finding is of interest because TBil
327 has been identified as a negative prognostic factor for dogs with primary IMHA in a number of previous
328 studies[4,19,20]. Serum bilirubin concentration at any moment in time is the product of many different
329 factors, several of which could be perturbed in dogs with IMHA. Conversion of haem to biliverdin in
330 erythrophagocytic macrophages is catalysed by haem oxygenase enzymes; expression of one isoform is
331 induced directly by glucocorticoids[21]. The difference in rate of change of TBil between groups observed
332 in this study could therefore reflect the direct effect of prednisolone on bilirubin production, as also
333 suggested in a trial comparing two different doses of hydrocortisone in people with pituitary
334 insufficiency[22], which reported higher serum concentrations with a larger dose. Conversely, the
335 difference in rate of change could indicate the severity of ongoing abnormal erythrophagocytosis, which
336 may be fully compensated by accelerated erythropoiesis in dogs that have recovered from an acute crisis.

337

338 The serum TBil concentration was measured 8 weeks after starting treatment because the authors sought to
339 achieve a feasible balance among several factors, including the need for monitoring for possible adverse
340 effects associated with treatment, the intention to determine whether clinicopathological factors differed
341 between treatment groups, and the financial cost borne by the owners of enrolled dogs. Ideally, the serum
342 TBil concentration would have been measured more frequently and at an earlier time-point to gain a greater
343 understanding of the kinetics of this variable in both treatment groups.

344

345 Several adverse effects, including polyphagia, polyuria, and polydipsia decreased with time in one or both
346 treatment groups, which was anticipated with the gradual tapering of the dose of prednisolone. There was
347 also a difference in the rate of improvement of polydipsia between treatment groups, suggesting that
348 fractionated administration of prednisolone results in more severe adverse effects in dogs compared to
349 unfractionated dosing. Alternatively, the difference in severity of polydipsia could have been affected by
350 the observed difference in urine specific gravity at the beginning of the trial, which was significantly higher
351 in dogs receiving a less fractionated dose. This may indicate that dogs receiving a more fractionated dose
352 had more severe polyuria and polydipsia from the point of diagnosis, though the authors cannot produce
353 any feasible explanation for this difference because the two groups appeared to be similar with respect to
354 the other parameters compared. No difference was identified in the rate of improvement of polyuria
355 between groups, which could be related to differences in owner observation of drinking and urination.

356

357 The effect of dose fractionation on adverse effects was further supported by evaluation of the lymphocyte
358 concentration, because exposure to glucocorticoids in vitro causes these cells to undergo apoptosis[7] and
359 limits their capacity to proliferate in response to concavalin A[23]. In this study, there was no difference
360 in lymphocyte concentration between groups at diagnosis but the concentration was significantly lower in
361 dogs receiving a more fractionated dose when all timepoints were considered, confirming that greater
362 availability of the drug at the glucocorticoid receptor did produce biological effects.

363

364 This trial had some limitations in addition to those described in the preceding paragraphs. Many of the
365 outcome variables relied on subjective opinions provided by owners, and these may have been biased for
366 several reasons. For example, owners were not asked if they had experience of administering
367 glucocorticoids to dogs before, or whether they usually spent the day at home with their dog, both of which
368 simple factors could have influenced their perception of the severity of adverse effects. Finally, *p* values
369 were not adjusted to account for multiple comparisons between groups due to the limitations inherent to the
370 Bonferroni method[24]. As recommended by Perneger[24], only those tests that were of greatest *a priori*

371 importance were performed, rather than comparing groups with respect to every possible
372 clinicopathological variable. In addition, the results obtained in this study are biologically plausible and
373 can be interpreted easily, which largely abolishes the need for *p* value adjustment.

374

375 In conclusion, an unfractionated regimen of prednisolone produced more rapid amelioration of adverse
376 effects compared to a fractionated regimen, but the effect of treatment allocation on survival, if any, could
377 not be assessed in this study because an insufficient number of dogs was recruited.

378

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442

443


444 **Figure 1: Example of the format of questions included in the questionnaires provided to the owners**
445 **of dogs in the study.** Owners were asked to answer each question by making a single mark on the 100
446 mm scale, as indicated in this example.

Questions on the urinary system:

a. Since discharge, has your dog been drinking more?

Normal	Drinking a lot more	Measurement (mm)
		

Is this issue important for you?

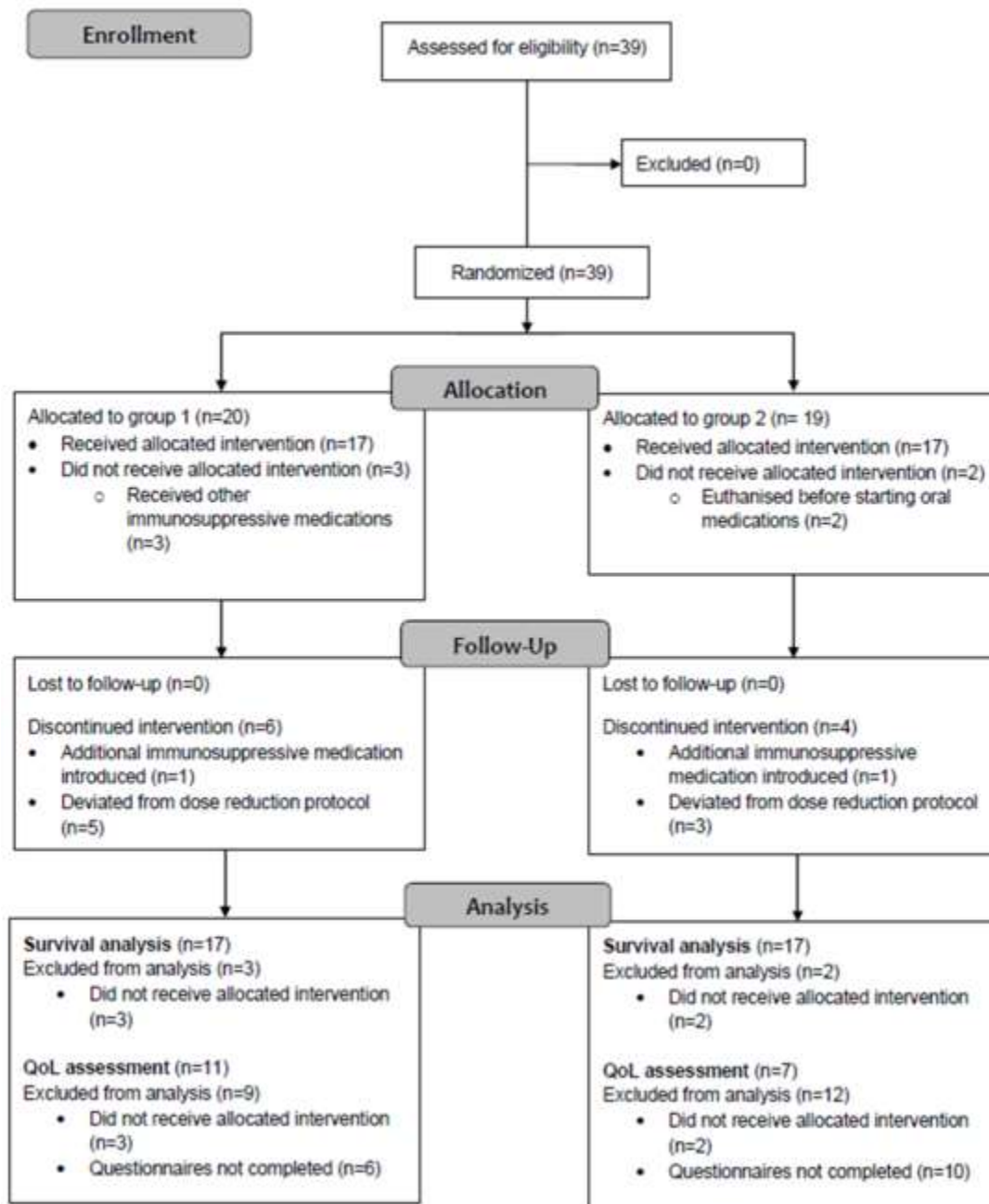
Not important at all	Very important	Measurement (mm)
		

447

448

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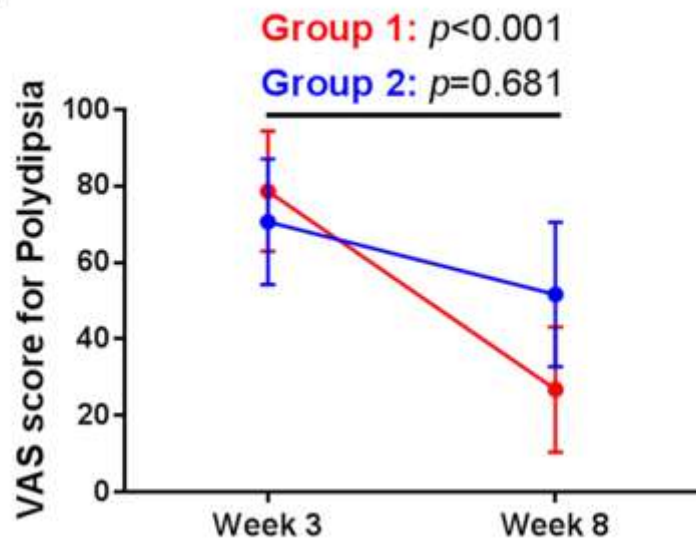
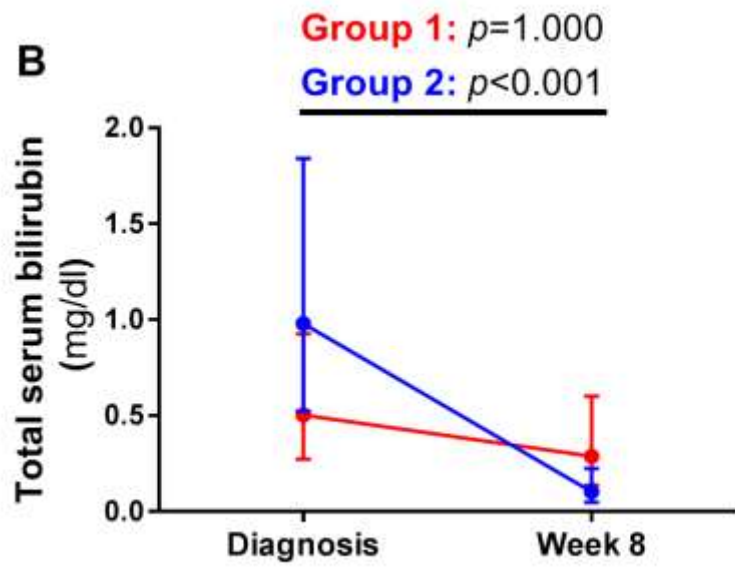
450 **Figure 2: Flow chart of animals included in this trial, formatted according to the CONSORT**
 451 **reporting guidelines.** Please see reference in main text for source of this template.



452

453

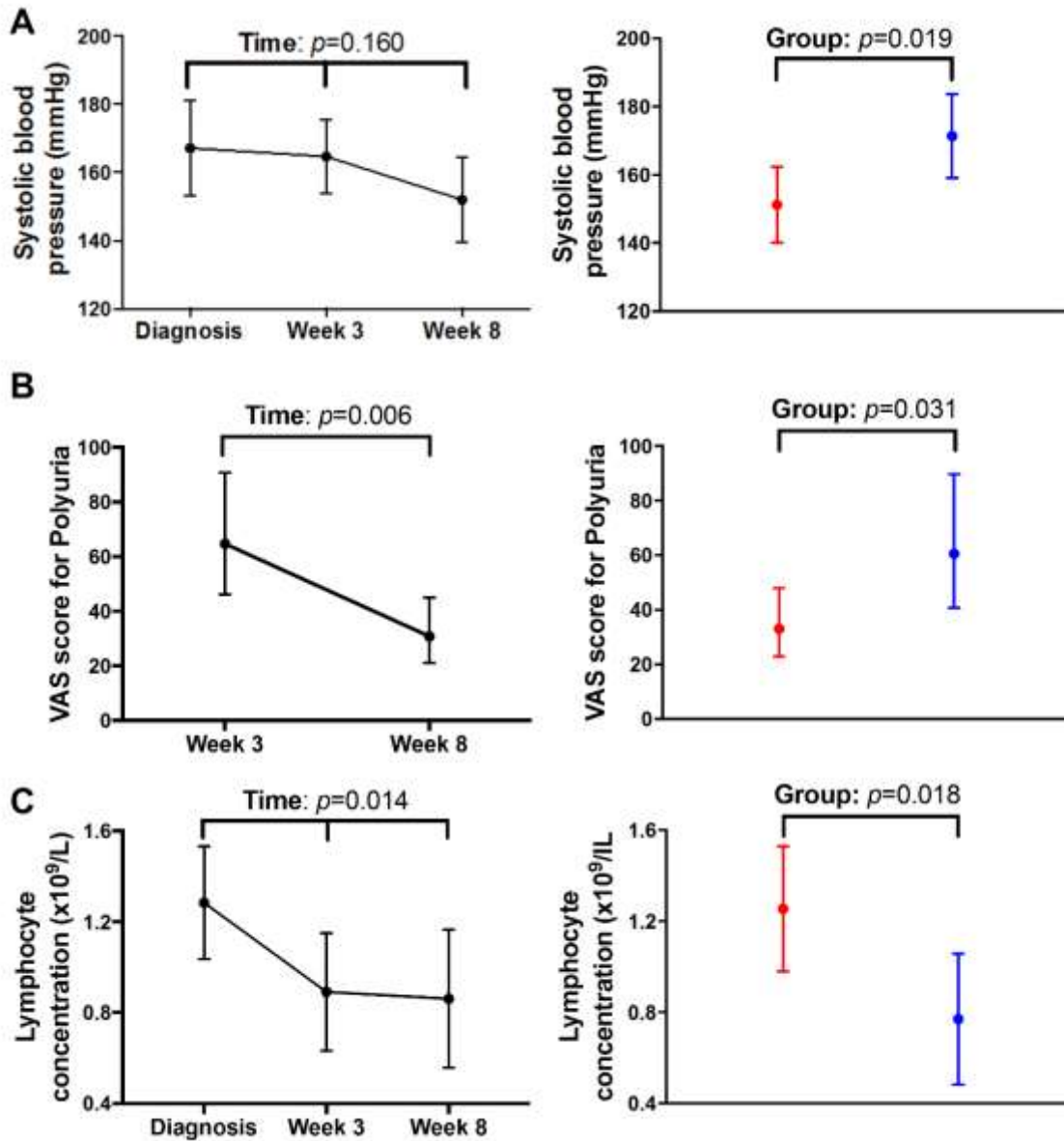
454 **Figure 3: Differences in variables over time and between treatment groups.** All graphs show means
455 with 95% confidence intervals. Red bars: group 1; blue bars: group 2. The visual analogue scale for
456 polydipsia (A) decreased significantly in dogs in treatment group 1 but not 2, whereas the total serum
457 bilirubin concentration decreased significantly in treatment group 2 but not 1 (B). The statistical analysis
458 was performed using \log_{10} values for total bilirubin; the transformation was reversed to produce this
459 figure using model estimates.

A**B**

460

461

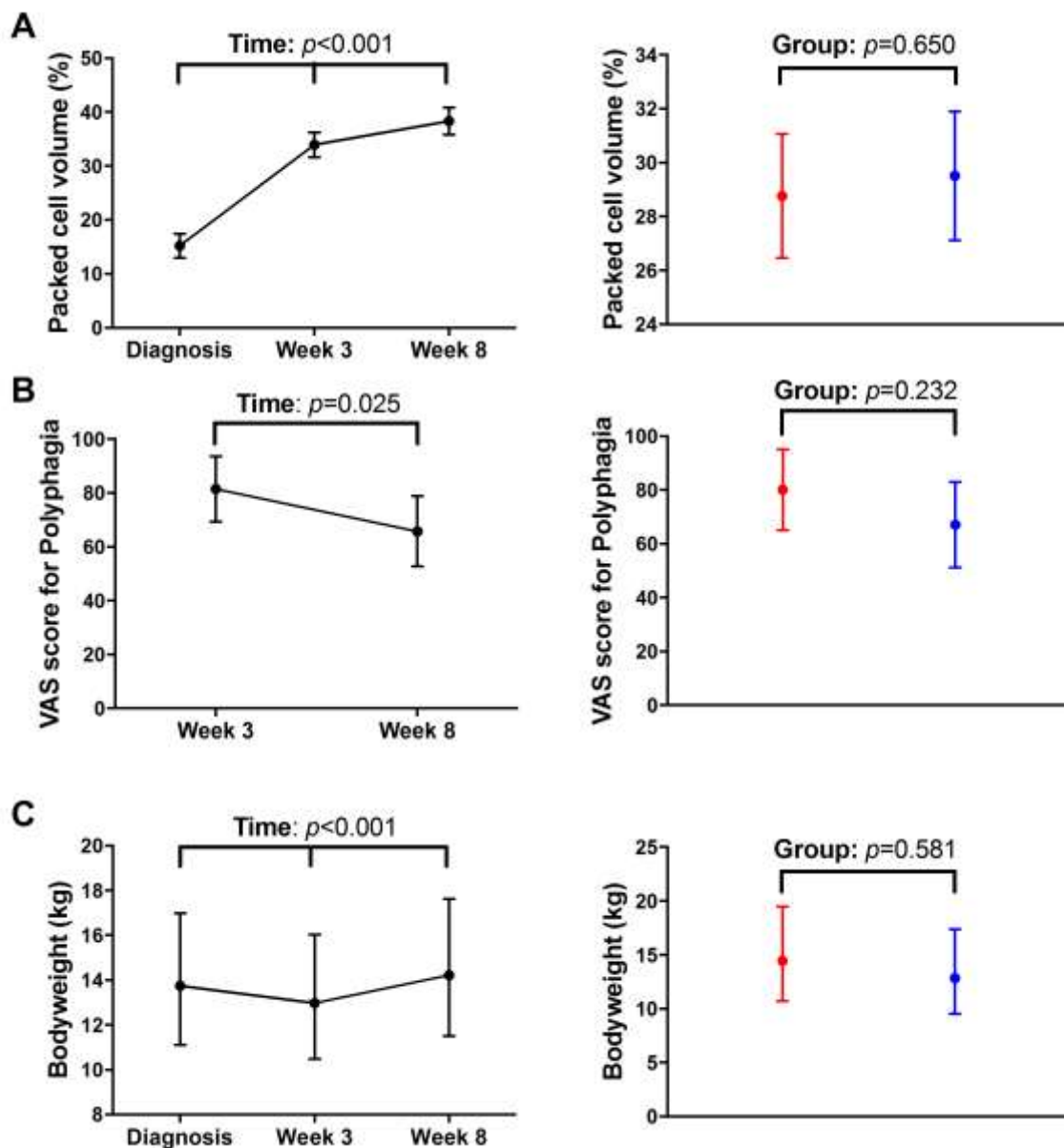
462 **Figure 4: Differences in variables between treatment groups.** All graphs show means with 95%
 463 confidence intervals. Red bars: group 1; blue bars: group 2. The systolic blood pressure (A) and
 464 log(visual analogue score for polyuria) (B) were greater in dogs in group 2 compared to 1 across all time
 465 points, whereas the lymphocyte concentration was greater in group 1 over time (C).



466

467

468 **Figure 5: Differences in variables over time.** All graphs show means with 95% confidence intervals.
 469 Red bars: group 1; blue bars: group 2. The packed cell volume increased significantly over time in dogs
 470 of both treatment groups (A), whereas the visual analogue scale for polyphagia decreased in both groups
 471 over time (B). When the treatment groups were considered together, bodyweight (C) decreased from
 472 diagnosis to week 3 then increased from week 3 to 8. Note that log(visual analogue score for polyuria)
 473 and the lymphocyte concentration also decreased over time in both treatment groups, as shown in Figure
 474 4.



476

477 **SUPPLEMENTARY FILES**

478 **Supplementary File 1: Copy of all questionnaires used in this study.** This document contains a copy
479 of the questionnaires administered to owners of enrolled dogs at week 3 and week 8 after diagnosis, with
480 all questions answered by making a single mark on a 100 millimetre visual analogue scale.

481

482 **Supplementary File 2: CONSORT checklist.** This document contains a checklist of items that are
483 recommended for inclusion in reports of randomised clinical trials, with the location of each item in this
484 document noted.

485

486 **Supplementary File 3: Copy of all data accrued during this trial.** This document contains all clinical
487 and questionnaire data collected for each animal enrolled in the trial.