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

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ABSTRACT

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

Results: Thirty-nine dogs were enrolled in the study, of which 5 were withdrawn and 17 were assigned to each group.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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

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

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

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

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

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

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

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

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

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

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

1: Complete blood cell count

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

3: Urine culture

4: Urine protein: creatinine ratio

5: Non-invasive blood pressure measurement

6: Serum biochemical profile

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

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

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

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

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

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

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

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

RESULTS

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

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

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

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)	*P* 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 (µmol/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 (µmol/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 ($x10^9$/l)	Mean (SD)	314 (238)	270 (137)	0.513
Neutrophil concentration ($x10^9$/l)	Median (IQR)	20.4 (12.5-28.6)	16.8 (12.5-19.3)	0.357
Monocyte concentration ($x10^9$/l)	Median (IQR)	1.77 (1.17-2.38)	1.38 (0.85-2.50)	0.474
Lymphocyte concentration ($x10^9$/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

[1]: Inter-quartile range

[2]: Standard deviation

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

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

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

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

Table 4: Results of linear mixed effect models for clinicopathological variables, expressed as p values for 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

1: Variable log-transformed for analysis.

Table 5: Results of linear mixed effect models for visual analogue scores, expressed as *p* values for 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

1: Variable log-transformed for analysis.

The mean systolic blood pressure and polyuria score were greater in dogs in treatment group 2 compared to group 1 across time points, whereas the lymphocyte concentration was greater in group 1 (Figure 4A-C). The PCV increased for dogs in both treatment groups over time, whereas the scores for polyuria and polyphagia decreased in both treatment groups (Figures 4B-C and 5A-B). Changes in bodyweight over time were more complex, with an overall decrease from diagnosis to the first re-examination visit, followed by an overall increase between this and the second re-examination visit (Figure 5C).

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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

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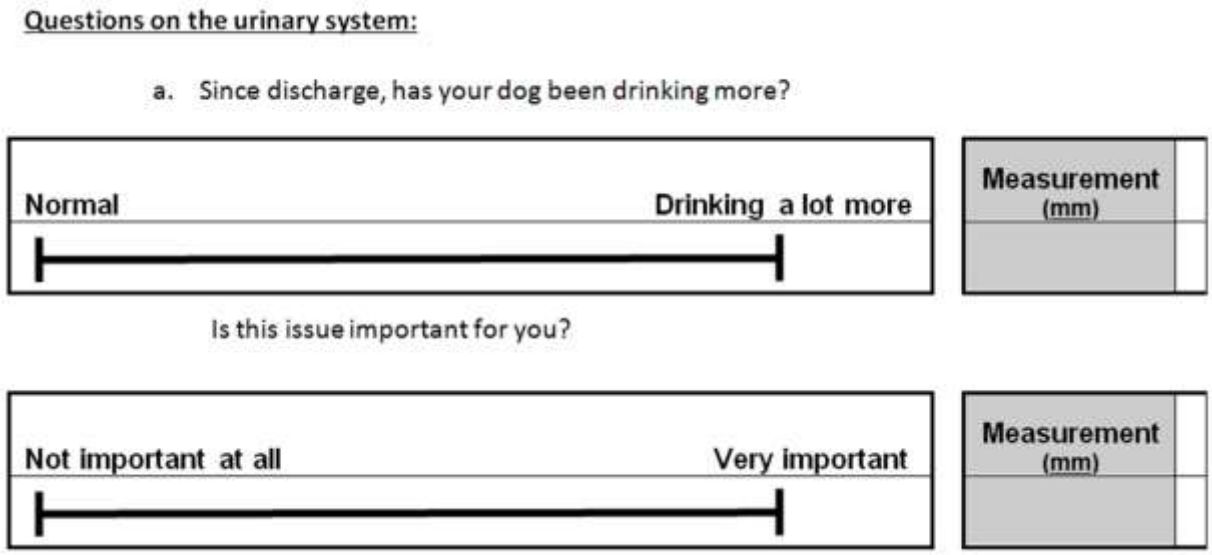
444 **Figure 1: Example of the format of questions included in the questionnaires provided to the owners**
445 **of dogs in the study**. Owners were asked to answer each question by making a single mark on the 100
446 mm scale, as indicated in this example.



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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.

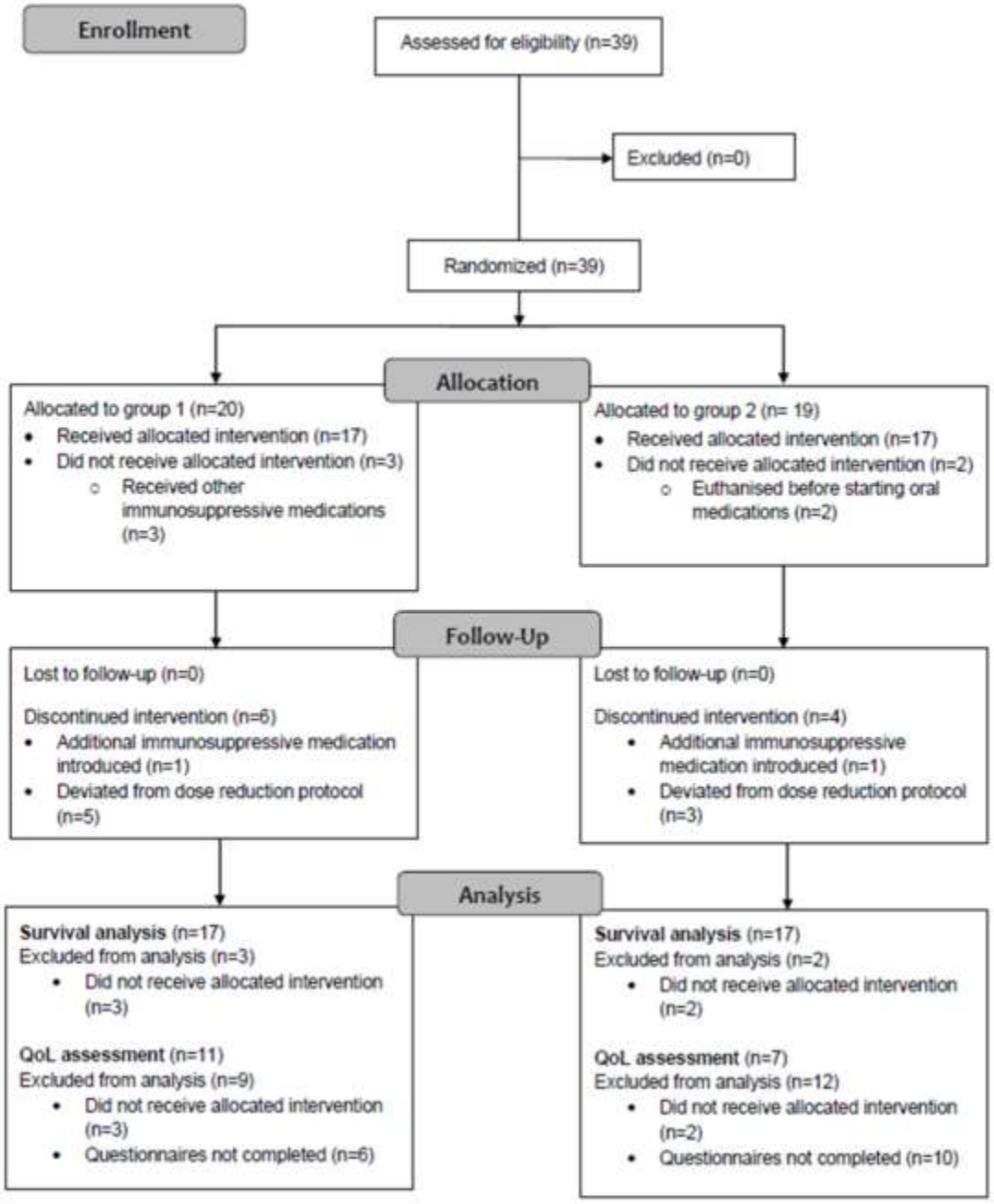

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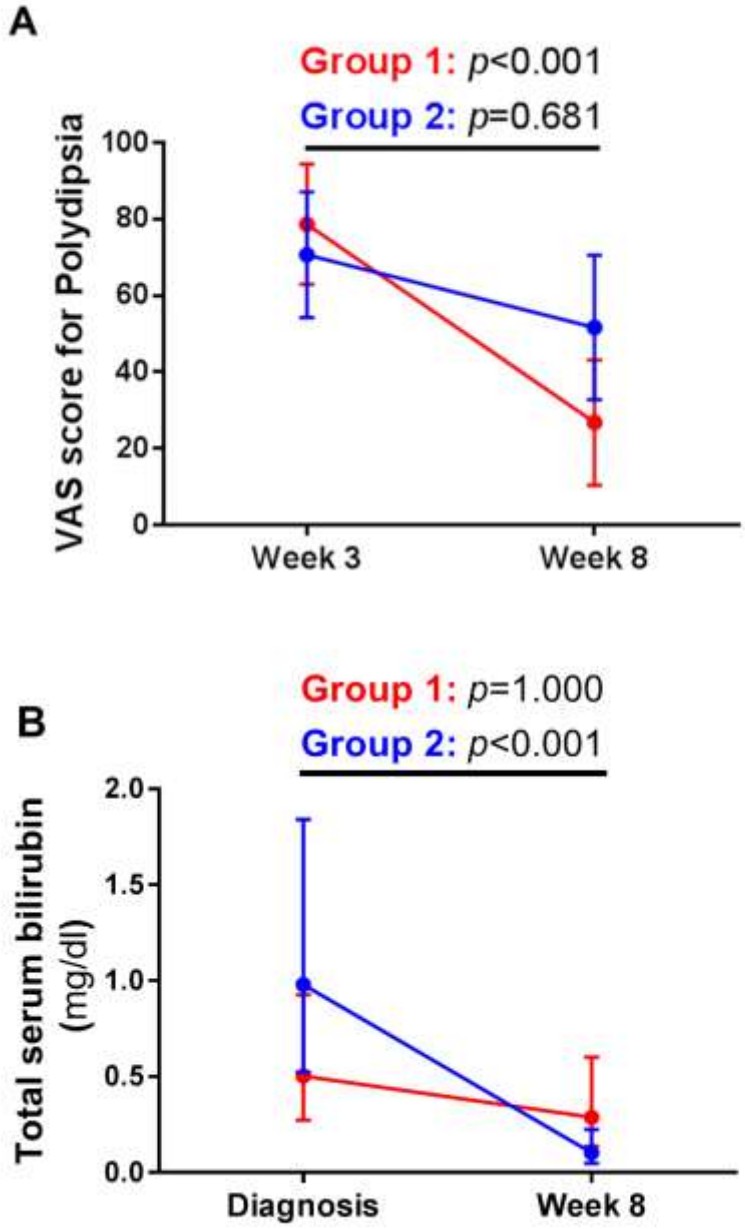
Figure 3: Differences in variables over time and between treatment groups. All graphs show means with 95% confidence intervals. Red bars: group 1; blue bars: group 2. The visual analogue scale for polydipsia (A) decreased significantly in dogs in treatment group 1 but not 2, whereas the total serum bilirubin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


A
Group 1: p<0.001
Group 2: p=0.681
VAS score for Polydipsia
100
80
60
40
20
0
Week 3
Week 8
B
Group 1: p=1.000
Group 2: p<0.001
Total serum bilirubin (mg/dl)
2.0
1.5
1.0
0.5
0.0
Diagnosis
Week 8

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).

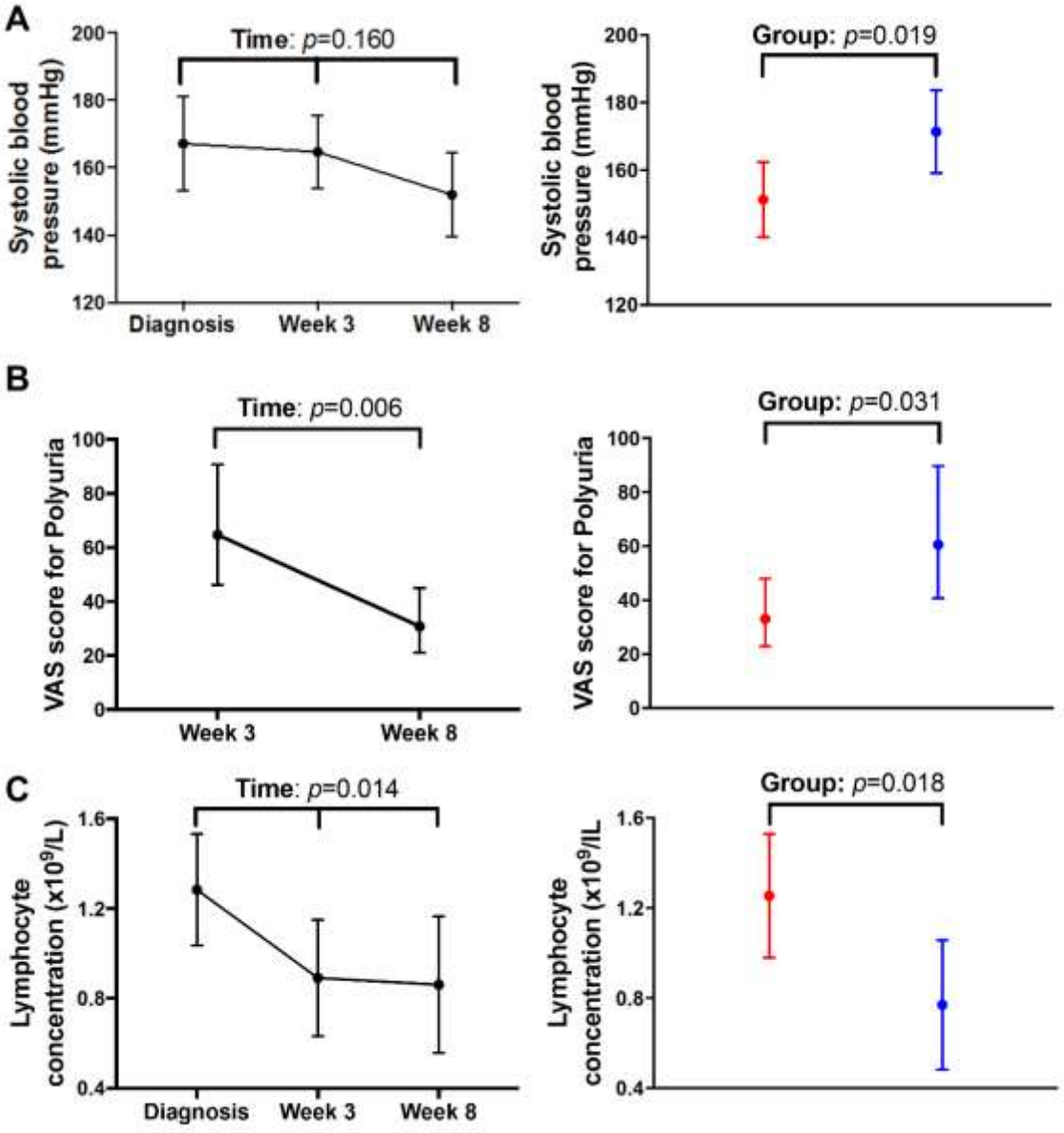


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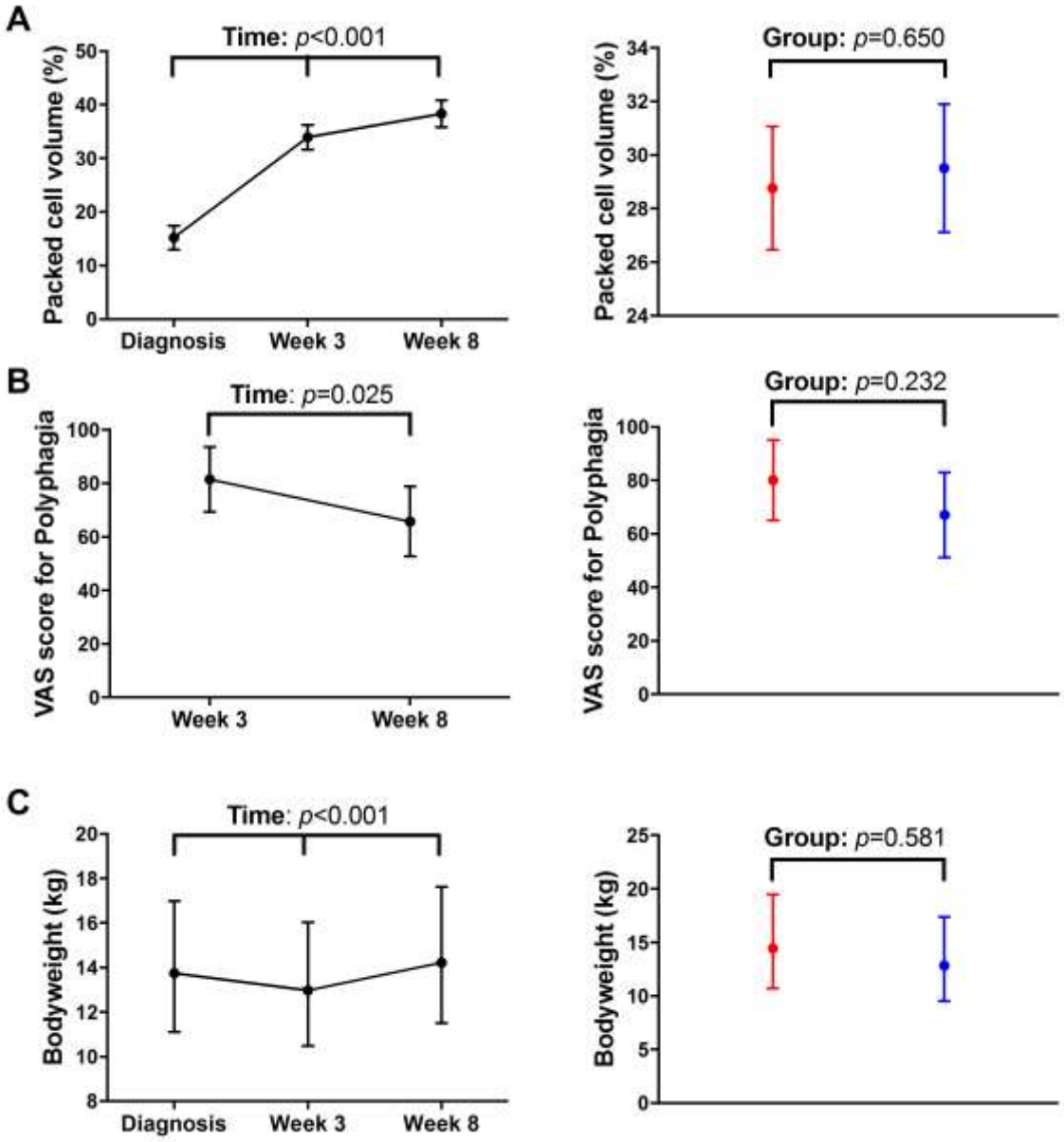
Figure 5: Differences in variables over time. All graphs show means with 95% confidence intervals. Red bars: group 1; blue bars: group 2. The packed cell volume increased significantly over time in dogs of both treatment groups (A), whereas the visual analogue scale for polyphagia decreased in both groups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



SUPPLEMENTARY FILES

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

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

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