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1 **Treatment of Dogs with Compensated Myxomatous Mitral Valve Disease with**
2 **Spironolactone – a Pilot Study**

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13 Short title: Spironolactone in compensated MMVD

14

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16

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19

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21 Bristol, United Kingdom

22

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31 **Treatment of Dogs with Compensated Myxomatous Mitral Valve Disease with**
32 **Spirolactone – a Pilot Study**

33

34 **Abstract**

35 **Objectives:** Spirolactone improves outcome in dogs with advanced myxomatous mitral
36 valve disease (MMVD). Its efficacy in preclinical MMVD is unknown. Hypothesis;
37 administration of spironolactone to dogs with compensated MMVD demonstrating risk
38 factors for poorer prognosis will decrease the rate of disease progression. Aim; to provide
39 pilot data to evaluate preliminary effects and sample size calculation for a definitive clinical
40 trial.

41 **Animals:** Twenty-five client-owned dogs with MMVD with at least one of the following; left
42 atrial-to-aortic ratio (LA:Ao) ≥ 1.5 , normalized left ventricular internal diameter in diastole
43 (LVIDdN) ≥ 1.6 , N-terminal pro-B-type natriuretic peptide (NT-proBNP) >550 pmol/L,
44 cardiac troponin I (cTnI) >0.025 ng/mL.

45 **Methods:** Prospective, single-center, equally randomized, placebo-controlled, double-blinded,
46 parallel grouped pilot study. No dogs were receiving medications for cardiac disease prior to
47 enrolment.

48 **Results:** Twelve dogs received placebo; 13 received spironolactone. One dog in the
49 spironolactone group died suddenly, 1 developed congestive heart failure and 2 received
50 suboptimal spironolactone doses. At enrolment NT-proBNP was significantly higher in the
51 spironolactone group (P=0.005). LA:Ao (P=0.002) and LVIDdN (P=0.005) increased over
52 time in the placebo group, but not the spironolactone group; the change did not differ
53 significantly between groups. The change in biomarker concentrations did not differ
54 significantly between groups; there was a tendency towards an increase in NT-proBNP over

55 time in the placebo group. Enrolment of 76 dogs would be necessary to demonstrate a
 56 difference in the change in LA:Ao over 6 months between groups.

57 **Conclusions:** preliminary results support undertaking a larger clinical trial of treatment of
 58 dogs with preclinical MMVD with spironolactone.

59

60 Keywords: preclinical disease, therapy, canine

61

62 Abbreviations:

ACVIM	American College of Veterinary Internal Medicine
Ao	Aorta
CHF	Congestive heart failure
CKCS	Cavalier King Charles spaniel
cTnI	Cardiac troponin I
ECG	Electrocardiogram
ELISA	Enzyme-linked immunosorbent assay
LA	Left atrium
LA:Ao	Ratio of left atrial to aortic root diameter
LVIDd	Left ventricular internal dimension in diastole
LVIDd/ LFWd	Ratio of left ventricular end-diastolic dimension to left ventricular free wall thickness in diastole

LVIDdN	Left ventricular end-diastolic dimension normalized for body weight
LVIDs	Left ventricular internal dimension in systole
LVIDsN	Left ventricular end-systolic dimension normalized for body weight
LVFWd	Left ventricular free wall thickness in diastole
MMVD	Myxomatous mitral valve disease
NSAID	Non-steroidal anti-inflammatory drug
NT-proBNP	N-terminal pro-B-type natriuretic peptide
PCV	Packed cell volume
UAC	Urinary aldosterone to creatinine ratio

64 Introduction

65 Myxomatous mitral valve disease (MMVD) is the commonest cause of cardiovascular
66 disease in the dog [1, 2]. Valvular degeneration results in regurgitation of blood, leading to
67 progressive volume overload of the left atrium and ventricle, which compensate by eccentric
68 hypertrophy [3]. The American College of Veterinary Internal Medicine (ACVIM)
69 classification system for MMVD describes four disease stages, from A (at risk) to D
70 (decompensated congestive heart failure) [4]. The compensated, preclinical stage of the
71 disease (stage B) is subdivided according to the absence (stage B1) or presence (stage B2) of
72 evidence of compensatory hypertrophy, identified on the basis of chamber enlargement on
73 radiography or echocardiography. The rate of progression of the disease is variable and the
74 development of heart failure is not an inevitable consequence of MMVD [5]. However, even
75 with optimal medical therapy, once dogs with MMVD develop congestive heart failure
76 (CHF), median survival is approximately 270 days [6]. Pimobendan has been shown to delay
77 the onset of congestive heart failure in dogs with ACVIM class B2 MMVD [7]. Further
78 therapeutic strategies, targeting different mechanisms of disease progression, might provide
79 additional clinical benefit in dogs at risk of disease progression.

80 The identification of dogs at greatest risk of progression of MMVD is important in a
81 disease with such variability of outcome. Evidence of cardiac remodelling, such as increased
82 echocardiographic measurements of left atrial [5] and left ventricular size, [8] are associated
83 with decreased survival times in dogs with MMVD. Increases in secondary markers of
84 myocardial stress and injury, specifically, serum N-terminal pro-B-type natriuretic peptide
85 (NT-proBNP) > 524 pmol/L and/ or serum cardiac troponin I (cTnI) > 0.025 ng/mL, are also
86 associated with poorer outcomes [9]. Increased left atrial size is associated with an increased
87 risk of disease progression for dogs in ACVIM class B [10].

88 Activation of the renin-angiotensin-aldosterone system is important in the pathophysiology

89 of cardiac remodelling in canine MMVD [11]. Via its actions on the mineralocorticoid
90 receptor, aldosterone promotes fluid retention, leading to volume-overload and stimulates
91 myocardial fibrosis [12]. Urinary aldosterone to creatinine ratio (UAC) is associated with the
92 rate of change of left ventricular size in dogs with MMVD, suggesting that aldosterone
93 production increases during periods of active remodelling [13]. Spironolactone is a
94 mineralocorticoid receptor antagonist that has been shown to prolong survival times in dogs
95 with advanced MMVD and CHF secondary to MMVD, when given in combination with
96 standard therapy [14]. The use of spironolactone in dogs with compensated MMVD has not
97 been reported, although a study investigating its effects in combination with benazepril is
98 currently ongoing.

99 We hypothesized that chronic oral administration of spironolactone to dogs with
100 compensated MMVD demonstrating risk factors known to be associated with decreased
101 survival times (increased left atrial and ventricular size and increased serum NT-proBNP and
102 cTnI), not receiving any other cardiovascular medications, would result in decreased rates of
103 change of these risk factors over time. The aim of the study was to provide pilot data to
104 evaluate preliminary effects, drug safety and to calculate the number of dogs needed for a
105 definitive clinical trial.

106

107 Animals, Materials and Methods

108 *Study Design*

109 The design of this pilot study was single-centre, prospective, equally randomized, double-
110 blinded, parallel-grouped and placebo-controlled. Each dog participated in the study for a
111 period of six months. The study was conducted in the United Kingdom. The study was
112 approved by the Royal Veterinary College Ethical Committee and specific informed owner

113 consent was obtained (unique reference number 2010 1039).

114

115 *Dogs*

116 Client-owned dogs of a variety of breeds with echocardiographically-confirmed MMVD were
117 prospectively recruited between December 2010 and December 2013 from those already
118 enrolled in a longitudinal study of canine MMVD in first opinion practice [8]. Dogs were
119 referred to the longitudinal study by the veterinarians at 2 London-based first opinion
120 practices after detection of a murmur consistent with mitral regurgitation at any stage in the
121 natural history of the disease. Echocardiography was performed to confirm the diagnosis of
122 MMVD and to exclude the presence of other cardiac diseases. Diagnosis of MMVD was on
123 the basis of characteristic abnormalities of the valve leaflets (thickening, prolapse, or both)
124 and evidence of regurgitant flow across the valve detected by Doppler. Dogs with any other
125 cardiac disease or clinically relevant organ-related or systemic disease were not enrolled in
126 the longitudinal study.

127 To be eligible for inclusion in the present study, a dog had to have echocardiographic
128 evidence of MMVD, defined as above, and demonstrate at least one risk factor for disease
129 progression (evidence of cardiomegaly (defined as left atrial to aortic ratio (LA:Ao) ≥ 1.5
130 and/ or left ventricular end-diastolic dimension, normalized for body weight (LVIDdN)) $>$
131 1.6 [8], serum NT-proBNP > 550 pmol/L [9] and/ or serum cTnI > 0.025 ng/mL [9]. During
132 the screening process, but not during the experimental phase, serum NT-proBNP was
133 measured using the first-generation version of a commercially-available enzyme-linked
134 immunosorbent assay (ELISA).^c

135 Dogs were excluded from the study if they had any of the following: evidence of any
136 congenital or acquired cardiac disease other than MMVD; evidence of kidney disease,

137 hypoadrenocorticism (on the basis of historical, physical examination and routine
138 biochemical findings; ACTH stimulation tests were not performed), hyperkalemia, or
139 hyponatremia; current or previous clinical signs of congestive heart failure or current
140 medical therapy for cardiac disease. The summary of product characteristics for Prilactone
141 recommends that dogs treated concomitantly with spironolactone and non-steroidal anti-
142 inflammatory drugs (NSAIDs) be correctly hydrated,^d and so it was recommended that dogs
143 should not receive NSAIDs, although this was not an absolute exclusion criterion.

144

145 *Randomization*

146 Randomization was by patient (dog). The study was initially designed to recruit 20 dogs.
147 Prior to the enrolment phase, a numbered list of 20 random group assignments (either group
148 A or group B) was compiled by drawing assignments from a hat. Dogs were assigned to
149 groups according to the order in which they were enrolled in the study. Prospectively
150 determined, prognostic factor balance was achieved by minimization to ensure that the
151 number of cavalier King Charles spaniels (CKCS) was equal in each group [15]; briefly, if, in
152 the latter stages of the recruitment phase (from dog number 11 onwards), enrolling a CKCS
153 to the next group assignment according to the randomization list would have resulted in
154 unbalancing of the groups, then the dog was assigned to the alternative treatment group. No
155 other variable was considered prior to enrolment. Data obtained from the first 20 dogs were
156 analysed and reported in abstract form^e and the decision made to recruit an additional 20 dogs
157 to increase the statistical power of the study. An additional numbered list of 20 random
158 assignments (either group C or group D) was compiled in the same way to allow recruitment
159 of additional dogs to the study in a blinded fashion after treatment allocation of groups A and
160 B was revealed at the time of previous data analysis.

161

162 *Blinding*

163 The investigators and owners were blinded to the treatment allocation. Assignment of
164 enrolled dogs to treatment groups was performed by a veterinary nurse to conceal allocation
165 from the investigators responsible for measurement of the variables of interest. Data for the
166 first 20 dogs enrolled (groups A and B) was analyzed prior to recruitment of the additional
167 dogs (groups C and D).^e The blinding codes for the treatment groups were held by the
168 sponsor until the time of each data analysis.

169

170 *Trial medication*

171 Spironolactone verum (Prilactone 10 mg tablets)^f was administered orally at a target dose of 2
172 mg/kg SID, as per registered label instructions, and the dose adjusted to a suitable number of
173 tablets. Placebo was administered PO according to the calculated daily dose for
174 spironolactone verum tablets and adjusted to a suitable number of placebo tablets. The
175 tablets and packaging of the verum and placebo were visually indistinguishable. Dogs
176 received tablets (verum or placebo) orally once daily for 6 months, unless otherwise stated.
177 The dose of the study medication was not adjusted during the study period. Additional
178 appropriate medication was prescribed if clinical signs of cardiac failure developed during the
179 study period. Participation was terminated if clinical signs of another significant medical
180 condition occurred which required additional treatment or warranted euthanasia, or if adverse
181 effects were observed which necessitated cessation of the therapy. A record was kept of other
182 medication used. Compliance was monitored by counting the number of unused pills returned
183 by the owner at the end of the study period. This number was compared with the expected
184 number of pills remaining.

185

186 *Schedule of Events*

187 Prior to enrolment, serum biochemistry and electrolyte measurements were performed.

188 At baseline, enrolled cases underwent a full evaluation, comprising recording of the history,
189 measurement of systolic arterial blood pressure by Doppler sphygmomanometry,^g physical
190 examination, blood sampling, electrocardiography (ECG) and echocardiography, in that
191 order. Electrocardiography was performed in right lateral recumbency and heart rate was
192 measured from a 60-second recording of lead II. Treatment was initiated with either verum or
193 placebo. Re-examinations were scheduled at day 14 and approximately 6 months after
194 inclusion. The tests performed at each study visit are summarized in Figure 1.

195

196 *Clinical Evaluation*

197 At inclusion, demographic characteristics (age, breed, sex and neutering status) were
198 recorded. Body weight and body condition score were recorded at each study visit.

199

200 *Blood sampling and Laboratory Analysis*

201 Blood was collected by jugular venepuncture into serum gel tubes and K3-EDTA-treated
202 tubes. Free-catch urine samples were collected. Samples were chilled at 4°C for up to 6 hours
203 before separation by centrifugation. Packed cell volume (PCV) was measured prior to
204 separation. Serum and urine samples were transported to a commercial laboratory for
205 measurement of routine biochemical parameters and electrolytes.^h The remaining serum and
206 urine were stored at -80°C for batched analysis. Urinary aldosterone concentrations were
207 measured using a previously-validated, commercially-available radioimmunoassay [16]

208 following mild acid hydrolysis and extraction into ethyl acetate, as previously described [13].
209 Serum aliquots were transported to the same commercial laboratory on dry ice. Before
210 analysis, the frozen serum was allowed to thaw slowly at room temperature. Concentrations
211 of cTnI were measured using an ELISAⁱ according to the manufacturer's instructions. The
212 use of this assay has been previously validated for canine samples [17]. Concentrations of
213 NT-proBNP were measured using the second-generation version of a previously-validated
214 canine NT-proBNP ELISA^c according to the manufacturer's instructions [18]. Serum
215 biochemistry and electrolytes, NT-proBNP, cTnI, PCV and UAC were measured at the
216 baseline visit. On day 14, PCV, serum biochemistry and electrolytes and UAC were
217 measured. Full clinical evaluation, plus measurement of PCV, serum biochemistry and
218 electrolytes, NT-proBNP, cTnI and UAC, was repeated at the 6 month time point, unless
219 otherwise stated.

220

221 *Echocardiography*

222 Echocardiography was performed at baseline and at the 6 month visit. Echocardiographic
223 examinations were performed by a single board-certified cardiologist (AB). Dogs were placed
224 in right and then left lateral recumbency on an ultrasound examination table. The
225 echocardiographic examination was performed using an ultra-sound unit^j equipped with 2–4
226 MHz and 3–7 MHz phased array transducers and ECG monitoring. Standard imaging planes
227 were digitally stored. Assessment of mitral valve structures was performed from the right
228 parasternal long-axis view and the left apical 4-chamber view. The LA:Ao was measured
229 from the right parasternal short axis view, as previously described [19]. Left ventricular
230 internal diameters in systole and diastole (LVIDs and LVIDd, respectively) and wall
231 thicknesses were measured from M-mode obtained from the right parasternal short axis view.
232 LVIDs was normalized for body weight (LVIDsN) by the formula: LVIDs/ (body weight

233 [kg])^{0.315}[20]. LVIDd was normalized for body weight (LVIDdN) by the formula: LVIDd/
234 (body weight [kg])^{0.294}[20]. The ratio of LVIDd to left ventricular free wall thickness in
235 diastole (LVFWd) was calculated (LVIDd/ LVFWd) as an indirect estimate of wall stress.
236 Measurements were recorded from at least 3 cardiac cycles and the mean value used in
237 subsequent analyses.

238 The primary outcome comparisons of the study were comparisons of the change in LA:Ao,
239 LVIDdN and serum NT-proBNP and cTnI between groups over a 6 month period. Secondary
240 outcome comparisons were comparisons of the change in other variables (PCV, serum urea,
241 creatinine and electrolyte concentrations, UAC, LVIDsN, LVIDd/ LVFWd ratio, E wave
242 velocity, E/A wave ratio, heart rate and body weight) between groups over the same 6 month
243 period and between-group comparisons at different time points (baseline and 2 and 26 weeks
244 after enrolment). Results are reported according to the Consolidated Standards of Reporting
245 Trials 2010 guidelines for reporting parallel group randomized trials [21].

246

247 *Statistical Analysis*

248 Data were analyzed on an intention to treat basis and data from all dogs that were randomly
249 assigned were included. Statistical analyses were performed using commercially-available
250 software.^k Data were assessed for normality graphically and by use of the Shapiro-Wilk test.
251 Results are reported as mean \pm standard deviations for normally distributed continuous
252 variables or median [range] for non-normally distributed variables. Repeated measures linear
253 mixed models with the random effect of subject (dog) were constructed to compare the
254 change in variables over time, and a compound symmetry (co)variance structure was
255 assumed between residuals of the same subject (dog) in the model. The effects of treatment
256 group (spironolactone vs. placebo), time (treated as a continuous covariate) and interaction

257 between treatment group and time were included in the model. Residuals were assessed
258 graphically for normality. Variables were logarithmically transformed if the residuals were
259 not normally distributed. The assumption of homogeneity of variance was tested by plotting
260 the predicted values against the residual values. Comparisons of continuous variables
261 between groups at different time points were made using independent t-tests or Mann-
262 Whitney U tests, as appropriate. Fisher's exact tests were used to compare proportions
263 between groups at baseline. A value of $P \leq 0.05$ was considered significant. In view of the
264 small sample size and the pilot nature of the study a value of $P < 0.1$ was considered to
265 indicate a tendency towards significance. A sample size calculation was performed on the
266 basis of the data obtained from the present pilot study using commercially-available software,
267 assuming $\alpha = 0.05$ and $\beta = 0.2$ (i.e. power = 0.8).¹

268

269 Results

270 Progress through the phases of the study is summarized in Figure 2. Twenty-five dogs with
271 compensated MMVD diagnosed on the basis of echocardiographic findings were enrolled in
272 the study. Due to the low rate of suitable dogs presenting to the larger longitudinal study, it
273 proved impossible to recruit 40 dogs. Twenty-four dogs had $LVIDdN > 1.6$ and/ or $LA:Ao >$
274 1.5 . Fifteen dogs had a previous measurement of serum cTnI > 0.025 pmol/L. Twenty-one
275 dogs had a previous measurement of serum NT-proBNP > 550 pmol/L. All dogs met the
276 inclusion criteria; 13 dogs demonstrated all three risk factors, 8 dogs demonstrated two of the
277 risk factors and 4 dogs demonstrated one risk factor. Baseline characteristics of the two
278 groups are presented in Table 1. There was no evidence for differences in age, body weight,
279 gender or proportions of CKCS to other breeds between groups. Serum NT-proBNP
280 concentrations were higher in the spironolactone treatment group compared with the placebo
281 group ($P = 0.005$). At baseline there was a tendency for serum potassium ($P = 0.078$) to be

282 higher in the spironolactone treatment group compared with the placebo group. Group-wise
283 comparisons were otherwise unremarkable. No evidence of kidney disease,
284 hypoadrenocorticism, hyperkalemia, or hyponatremia was detected in any dog on serum
285 biochemical and electrolyte analysis.

286 Twelve dogs (5 neutered females, 2 entire males and 5 neutered males) with ages ranging
287 from 6.3 to 13.1 years and body weights ranging from 4.5 to 18.2 kg were assigned to receive
288 placebo. These dogs comprised 7 CKCS, 2 mixed breeds and 1 each of bichon frisé, lurcher
289 and toy poodle. No compliance problems, potential adverse drug reactions or adverse events
290 were reported for dogs in this group. Two dogs had been receiving NSAIDs prior to, but not
291 at the time of, recruitment. NSAID therapy was reinitiated by the primary veterinarian during
292 the trial period in one of these dogs. NSAID therapy was initiated during the trial period in a
293 third dog.

294 Thirteen dogs (2 neutered females and 11 neutered males) with ages ranging from 6.1 to
295 13.4 years and body weights ranging from 1.8 to 23.3 kg were assigned to receive
296 spironolactone. These dogs comprised 8 CKCS, 2 cross-breeds and 1 each of bichon frisé,
297 collie and Chihuahua. One dog in this group received a suboptimal dose of trial medication
298 (1.3 mg/kg once daily) throughout the trial period, due to owner non-compliance. One dog
299 was treated for seasonal allergic dermatitis during the trial period, during which time the trial
300 medication was withdrawn for 10.6 weeks. Trial medication was reinstated following
301 resolution of the dermatitis, which did not subsequently recur.

302 Two dogs in the spironolactone group suffered adverse events during the trial period (one
303 dog developed congestive heart failure requiring medical management and one dog died
304 suddenly). The dog that died suddenly was not known to be in congestive heart failure prior
305 to death. There was no difference in the proportion of dogs experiencing adverse events
306 between groups ($P = 0.480$). One dog had been receiving NSAIDs prior to, but not at the

307 time of, recruitment. NSAID therapy was reinitiated by the primary veterinarian during the
308 trial period; this was the dog that died suddenly, although the death was considered to be
309 unrelated to NSAID therapy.

310 Results of repeated measures linear model analyses for the primary outcome comparisons
311 are summarized in Table 2. Residuals were not normally distributed for serum cTnI.
312 Following logarithmic transformation of this variable the residuals were normally distributed.
313 Because baseline serum NT-proBNP measurements were higher in the group receiving
314 spironolactone, baseline measurements were included in the model as a covariate for this
315 variable. No significant differences were detected for the change over time of any variable
316 between groups. The change in serum NT-proBNP tended to be greater for the placebo group
317 ($P = 0.087$). There was a tendency for serum NT-proBNP concentrations to increase in the
318 placebo group, but not in dogs receiving spironolactone ($P = 0.073$) (Figure 3). Left atrial to
319 aortic ratio ($P = 0.002$) and LVIDdN ($P = 0.005$) increased over time in the placebo group,
320 but not in dogs receiving spironolactone ($P = 0.231$ and $P = 0.194$, respectively, Figures 4
321 and 5); however, in the absence of significant differences in the change over time between
322 groups these findings should be interpreted with caution.

323 Results of repeated measures linear model analyses for the secondary outcome comparisons
324 are summarized in Table 3. Residuals were not normally distributed for UAC, serum
325 creatinine or E wave velocity. Following logarithmic transformation of these variables the
326 residuals were normally distributed. There was a tendency for LVIDd/ LVFWd ratio to
327 increase over time in the placebo group ($P = 0.070$), but not in dogs receiving spironolactone
328 ($P = 0.315$). The change in LVIDd/ LVFWd ratio over time was not different between
329 groups. There was a tendency for body weight to decrease in the dogs receiving
330 spironolactone ($P = 0.066$), but not in the placebo group. The change in body weight was not
331 different between groups.

332 Comparisons between groups at the 2 week time point are summarized in Table 4. No dogs
333 were withdrawn from the study at this time point due to pharmacovigilance concerns. Urinary
334 aldosterone to creatinine ratio was significantly higher in dogs receiving spironolactone than
335 those receiving placebo ($P = 0.006$). There was a tendency for serum potassium to be higher
336 in dogs receiving spironolactone than those receiving placebo ($P = 0.098$). There was no
337 evidence of differences in any other variable between groups at this time point.

338 Comparisons between groups at the 6 month time point are summarized in Table 5. There
339 was a tendency for serum NT-proBNP to be higher in dogs receiving spironolactone than
340 those receiving placebo ($P = 0.051$). There was no evidence of differences in any other
341 variable between groups at this time point.

342 A post-hoc sample size calculation was performed using the mean change in LA:Ao
343 observed from these preliminary data, which suggested that a total sample of 76 dogs (38 per
344 group) would be necessary to demonstrate a significant difference in the change in LA:Ao
345 over 6 months between groups if one existed. However, use of the 95% confidence intervals
346 for the change in LA:Ao for the sample size calculation suggested that the range of total
347 sample size required to demonstrate a difference is 36 to 2936 dogs.

348

349 Discussion

350 The results of the present study suggest that a larger sample size (76 dogs) would be
351 necessary to definitively test the hypothesis that spironolactone slows the rate of progression
352 of preclinical MMVD. Although none of the between groups comparisons reached statistical
353 significance, this pilot study is likely to be underpowered to demonstrate such differences.
354 Four dogs receiving spironolactone failed to adhere to the protocol throughout the entire six
355 month study period; one died suddenly, one developed congestive heart failure necessitating

356 the addition of other therapy, one received a suboptimal dose of medication and
357 administration of trial medication was suspended in one dog for part of the trial period. This
358 is likely to have further reduced the power of the study to detect differences between the
359 groups. A post hoc sample size calculation was performed in order to inform the design of a
360 subsequent, larger study that could be performed to determine whether a real treatment effect
361 exists. Given the 95% confidence interval of the average change in LA:Ao, the number of
362 dogs required could be as few as 36 or as many as 2936. Because of the intrinsic unreliability
363 of such a sample size prediction on the basis of data from a relatively small population it is
364 possible that there is no treatment effect, in which case no difference between groups would
365 be demonstrated even if an infinite number of dogs was studied. No definitive conclusions
366 regarding any effect of spironolactone on disease progression in dogs with MMVD can
367 therefore be drawn on the basis of the results of this pilot study.

368 Echocardiographic measurements of cardiac size (LA:Ao and LVIDdN) increased over
369 time in the placebo group but not in dogs receiving spironolactone, although no difference in
370 the change over time was detected between groups. This suggests that performing a larger,
371 definitive clinical trial is warranted to further investigate whether treatment with
372 spironolactone slows disease progression in dogs with preclinical MMVD. Increasing LA:Ao
373 and LVIDdN are known to be associated with reduced survival times in dogs with MMVD
374 [22]. It is possible that decreasing the rate of increase of cardiac size might delay the onset of
375 congestive heart failure, although large, long-term studies are required to investigate this
376 hypothesis.

377 The radiographic indices of cardiac size in CKCS with compensated MMVD increase in a
378 non-linear fashion with disease progression, reaching their maximal rate of change in the 9
379 months prior to the onset of congestive heart failure [23]. It was expected, therefore, that in
380 this population of dogs with compensated MMVD and risk factors likely to be associated

381 with progression, echocardiographic indices of cardiac size would increase significantly over
382 a six month period. The observation that LA:Ao and LVIDdN increased significantly in the
383 placebo group suggests that this expectation was reasonable. Patients with MMVD that have
384 higher NT-proBNP concentrations and greater heart size tend to have more advanced disease.
385 Thus, since the group of dogs receiving spironolactone had significantly higher NT-proBNP
386 concentrations at baseline it might have been expected that these dogs would have a more
387 rapid rate of change than those in the placebo group. This might have contributed to the
388 failure of the study to demonstrate a difference in the change over time in LA:Ao and
389 LVIDdN. Future studies could use stratification of randomization of recruited cases by
390 echocardiographic indices of heart size and/ or serum NT-proBNP concentrations to ensure
391 that treatment groups are balanced with respect to these variables. In a small pilot study this
392 approach was not feasible.

393 Urinary aldosterone to creatinine ratio was higher in dogs receiving spironolactone
394 compared with placebo at the 2 week time point but not at the 6 month time point.
395 Mineralocorticoid receptor blockade by spironolactone increases aldosterone secretion by
396 stimulation of renin production [24]. It was expected that UAC would remain higher in the
397 group receiving spironolactone throughout the study period. However, data were missing for
398 this variable on 21/ 69 (30.4%) occasions on which it should have been measured due to
399 failure to obtain urine samples. This would have decreased the power of the study to detect
400 significant differences in UAC between treatment groups.

401 There was a tendency for serum potassium concentrations to be higher in dogs treated with
402 spironolactone at baseline and the 2 week time point. Nevertheless, all measurements
403 remained within the range of normal values and so this is not a source of pharmacovigilance
404 concern nor has it been noted as a clinically significant adverse event in the published clinical
405 trials involving spironolactone in dogs [25].

406 There was no evidence that the serum cTnI concentrations changed over time in either
407 treatment group. Rapid increases in serum cTnI concentrations only occur late in the course
408 of the disease, within the last 6 months of life of dogs that die due to MMVD [9]. A rapid
409 increase in serum cTnI would not, therefore, be expected in dogs in the compensated phase of
410 the disease.

411 Fractional shortening is usually increased in the compensated phase of MMVD, as the
412 presence of mitral insufficiency results in altered loading conditions. LVIDsN does not,
413 therefore, increase until the late stages of MMVD [22, 26]. In a similar fashion to serum
414 cTnI, it would not therefore be expected that LVIDsN would rapidly increase over a six
415 month period in this population of dogs.

416 Increased E wave velocities are indicative of increased ventricular filling pressures and the
417 volume of the regurgitant jet [26]. The results of the present study suggest that ventricular
418 filling pressures were not increasing and ventricular diastolic function was not declining in
419 either treatment group, probably reflecting the relatively early stage of the disease in this
420 population. E wave velocities >1.2 m/s have previously been shown to be associated with an
421 increased risk of disease progression in dogs with ACVIM class B MMVD; [10] the majority
422 of dogs in the present study had E wave velocities <1.2 m/s at the baseline visit.

423 This study has a number of limitations. Firstly, the sample size calculation suggests that on
424 the basis of our own data 76 dogs would need to be studied in order to demonstrate a
425 difference in the change in LA:Ao over 6 months between groups if one exists. This pilot
426 study was therefore underpowered to detect such a difference. We have therefore referred to
427 tendencies towards significance in the data with a more lenient P-value of < 0.1 , while
428 recognizing the risks of a type I statistical error inherent in this approach. Secondly, no
429 consensus exists with regard to determining optimal cut-offs for echocardiographic evidence
430 of cardiomegaly in canine MMVD. In the present study, LA:Ao > 1.5 and / or LVIDdN $>$

431 1.6, were used to select dogs at risk of progression. These represent relatively liberal criteria,
432 as the upper 95% confidence interval for LVIDdN is 1.85 [20]. However, in the present
433 study these cut-offs were used only as inclusion criteria, in an attempt to identify dogs with
434 more advanced compensated MMVD that were more likely to experience disease progression
435 over a 6 month period. Previous studies have demonstrated that dogs above cut-off values
436 lower than the value of 1.85 are at increased risk of cardiac related mortality [8]. Thirdly,
437 increases in serum cTnI are not specific for cardiac disease,[27, 28] and so use of this marker
438 as an inclusion criterion has limitations. Nevertheless, no dog was recruited solely on the
439 basis of the cTnI measurement.

440 Fourthly, this study included a high proportion of CKCS. Urinary aldosterone to creatinine
441 ratio has been shown to be higher in CKCS than non-CKCS breeds [13]. It is possible,
442 therefore, that any benefit of mineralocorticoid receptor blockade might be greater in CKCS
443 than other breeds. For this reason, the groups were balanced for numbers of CKCS by the
444 minimization method; the trial was not, therefore, truly randomized. However,
445 mineralocorticoid receptor antagonists have been shown to be beneficial in human patients,
446 regardless of plasma aldosterone concentrations [29]. Large studies that allow for sub-
447 analyses according to breed are necessary to investigate whether breed- specific effects of
448 spironolactone exist.

449 Finally, allocation concealment would ideally have been performed externally rather than
450 by a member of the regular clinic team. Inadequate allocation concealment may lead to
451 selection bias and hence produce spurious results. In the present study, however, every
452 attempt was made to maintain allocation concealment and therefore avoid bias of this nature.

453

454 Conclusions

455 The preliminary findings of this pilot study support undertaking a larger, definitive,
456 prospective, randomized, placebo-controlled, double-blinded clinical trial to further evaluate
457 the effect of spironolactone on disease progression in dogs with preclinical MMVD.

458

459 Footnotes

460 ^c Canine Cardiopet Nt-proBNP, IDEXX Laboratories, Westbrook, ME

461 ^d http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-

462 [_Product_Information/veterinary/000105/WC500063372.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/EPAR_-Product_Information/veterinary/000105/WC500063372.pdf)

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466 ^f CEVA Santé Animale, Libourne, France

467 ^g Park model 811 B, Perimed, Bury St. Edmonds, UK

468 ^h IDEXX Laboratories, Wetherby, UK

469 ⁱ Access Systems AccuTnI Assay, Beckman Coulter Inc., Fullerton, CA

470 ^j Acuson Cypress, Siemens Medical Solutions, Siemens House, Oldbury, Bracknell, UK

471 ^k IBM SPSS version 23, SPSS Inc., Chicago, IL

472 ^l GLIMMPSE version 2.2.4, University of Colorado, Denver, CO

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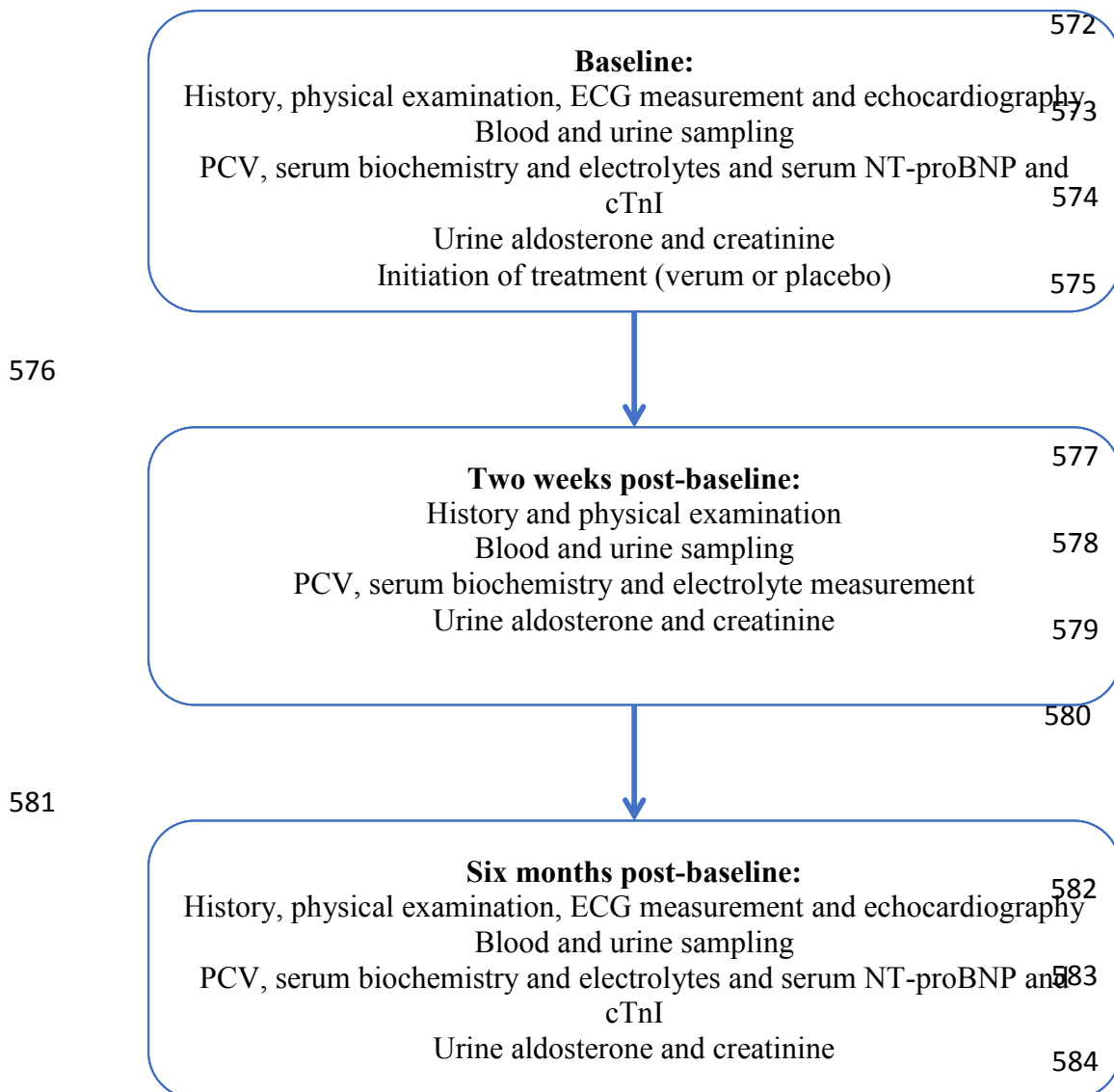
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569

570 Figure 1: Summary flow diagram detailing the tests performed at each visit during the study
 571 period.



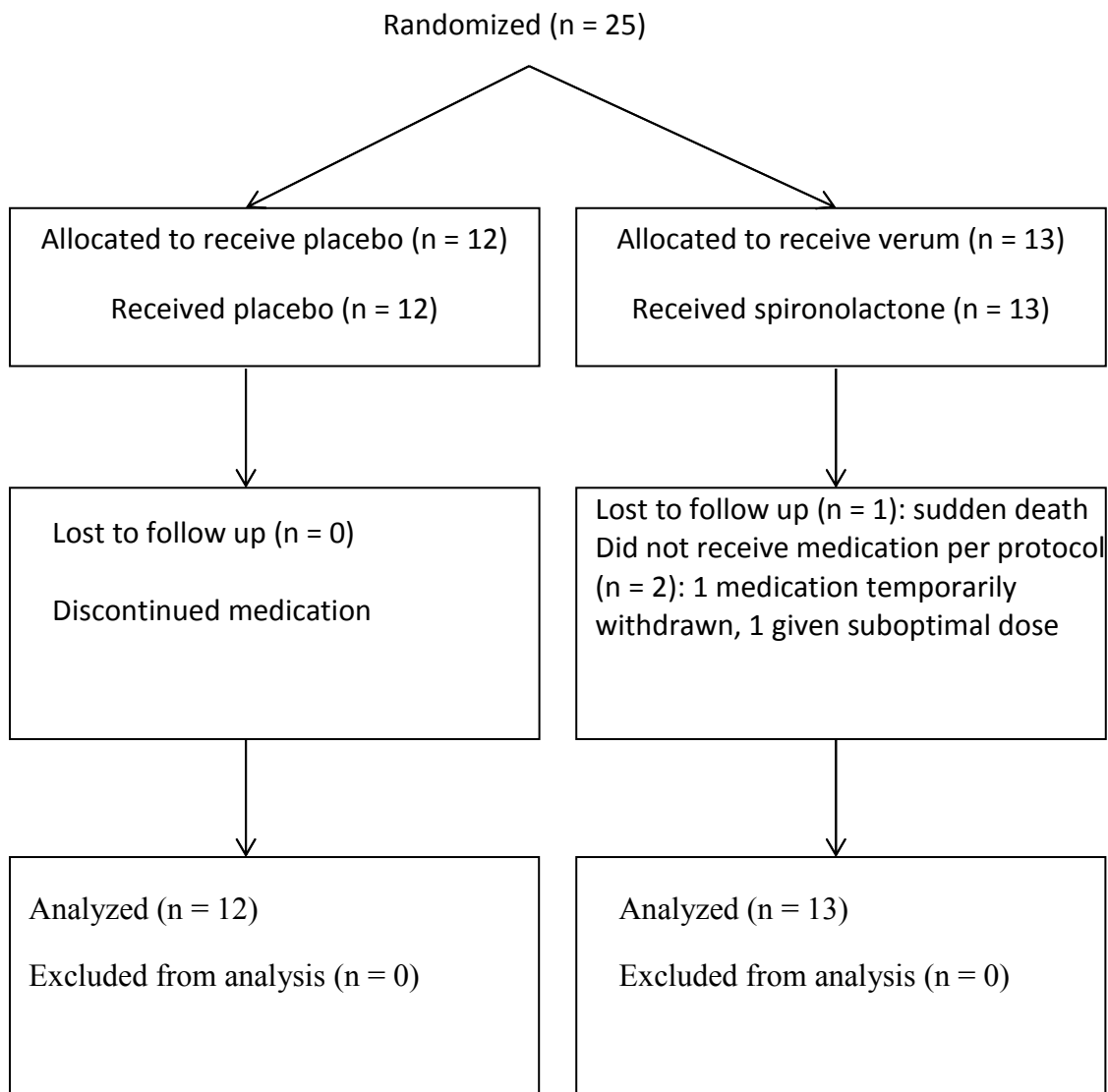
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586 ECG, electrocardiogram; PCV, packed cell volume; NT-proBNP, N-terminal B-type

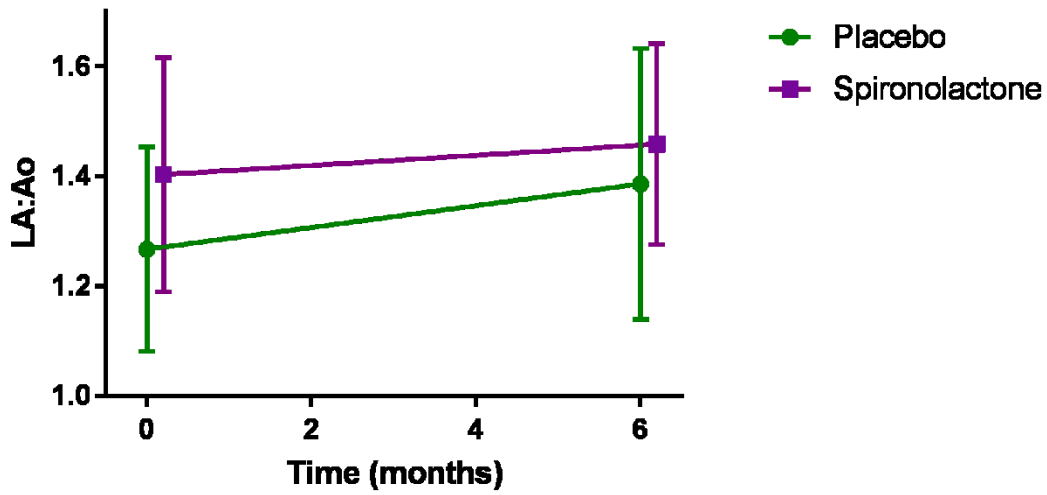
587 natriuretic peptide; cTnI, cardiac troponin I.

588

589 Figure 2: Flow diagram of the progress through the phases of this parallel, randomized trial of
590 two groups. Analysis was on the basis of intention to treat.

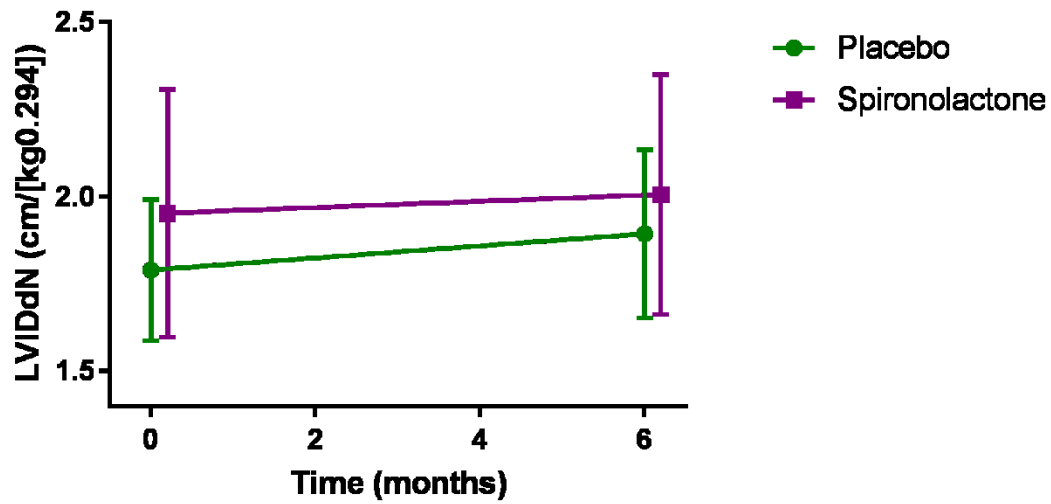


591 Figure 3: LA:Ao in dogs receiving placebo and those receiving spironolactone at baseline (n
592 = 12 and 13, respectively) and at the 6 month time point (n = 12 and 12, respectively). The
593 central tendency represents the mean and the error bars represent the standard deviation.



594

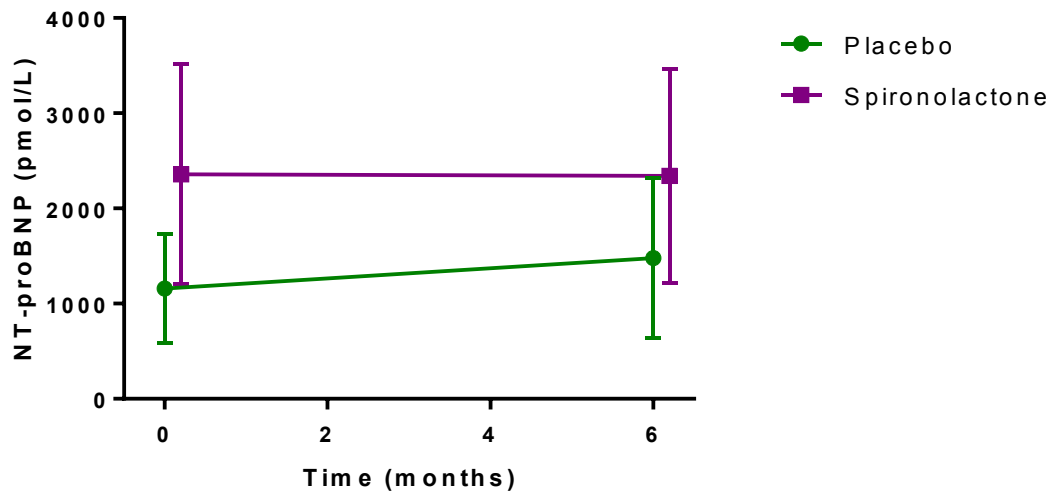
595 Figure 4: LVIDdN ($\text{cm}/[\text{kg}^{0.294}]$) in dogs receiving placebo and those receiving
596 spironolactone at baseline ($n = 12$ and 13 , respectively) and at the 6 month time point ($n = 12$
597 and 12 , respectively). The central tendency represents the mean and the error bars represent
598 the standard deviation.



599

600

601 Figure 5: Serum NT-proBNP concentrations (pmol/L) in dogs receiving placebo and those
602 receiving spironolactone at baseline (n = 12 and 13, respectively) and at the 6 month time
603 point (n = 12 and 11, respectively). The central tendency represents the mean and the error
604 bars represent the standard deviation.



605

606

607 Table 1: Comparisons of clinical, echocardiographic and biomarker data between dogs
 608 receiving placebo and those receiving spironolactone at baseline.

Variable	Group receiving placebo (n = 12)	Group receiving spironolactone (n = 13)	<i>P</i> value
Age (years)	9.4 ±2.0	9.7 ±2.0	0.707
CKCS (yes/ total)	7/ 12	9/ 13	0.688
Sex (male/ total)	7/ 12	11/13	0.202
Body weight (kg)	11.6 ±4.1	11.8 ±6.3	0.940
Systolic blood pressure (mmHg)	156.5 ±29.8	159.4 ±32.0	0.816
Heart rate (ECG) (bpm)	117.3 ±25.0	117.2 ±25.7	0.992
LA:Ao ratio	1.27 ±0.17	1.40 ±0.21	0.105
LVIDdN (cm/[kg ^{0.294}])	1.79 ±0.20	1.95 ±0.35	0.180
LVIDsN (cm/[kg ^{0.315}])	1.05 ±0.13	1.10 ±0.28	0.548
LVIDd/ LVFWd ratio	4.06 ±0.63	4.46 ±0.96	0.229
E wave velocity (m/s)	1.01 ±0.22	0.99 ±0.35	0.883
E/ A wave ratio	1.37 ±0.22	1.40 ±0.50	0.857
Serum NT-proBNP (pmol/L)	1006.0 [541.0, 2108.0]	2434.0 [740.0, 3955.0]	0.005
Serum cTnI (ng/mL)	0.03 [0.01, 0.16]	0.03 [0.01, 0.19]	0.437
UAC (g/mol)	0.079 [0.044, 0.119]	0.088 [0.042, 0.165]	0.744
PCV (%)	42.7 ±6.4	40.9 ±4.6	0.439
Serum urea (mmol/L)	5.3 ±2.2	6.0 ±1.9	0.426

Serum creatinine ($\mu\text{mol/L}$)	68.9 [59.7, 146.3]	70.5 [52.5, 130.6]	0.810
Serum Na^+ (mmol/L)	147.7 \pm 2.5	147.8 \pm 2.0	0.893
Serum K^+ (mmol/L)	4.2 \pm 0.4	4.5 \pm 0.5	0.078
Serum Cl^- (mmol/L)	111.5 \pm 3.6	113.0 \pm 2.5	0.237

609

610 All variables in the group receiving placebo included 12 dogs, except UAC (n = 10), E wave
611 velocity (n = 11) and E/A ratio (n = 11). All variables in the group receiving spironolactone
612 included 13 dogs, except UAC (n = 9). The *P* values for variables for which significant
613 between-group differences were detected ($P < 0.05$) are highlighted in bold text.

614 CKCS, cavalier King Charles spaniel; ECG, electrocardiogram; bpm, beats per minute;
615 LA:Ao ratio, left atrial to aortic root ratio; LVIDdN, left ventricular end-diastolic diameter
616 normalized for body weight; LVIDsN, left ventricular end-systolic diameter normalized for
617 body weight; LVIDd/ LFWd ratio, left ventricular end-diastolic diameter to left ventricular
618 free wall thickness in diastole ratio; NT-proBNP, N-terminal B-type pro-natriuretic peptide;
619 cTnI, cardiac troponin I; UAC, urinary aldosterone to creatinine ratio; PCV, packed cell
620 volume; Na^+ , sodium ions; K^+ , potassium ions; Cl^- , chloride ions.

621 Table 2: Repeated measures linear mixed model analysis of change over 6 months in
 622 variables of primary interest.

Variable	P (between groups)	Unit change in variable per month (B) [95% confidence interval]	SE	P (within group)
Serum NT-proBNP (pmol/L)	0.087			
Placebo group (n = 12)		54.93 [-5.58 to 115.45]	29.22	0.073
Spironolactone group (n = 12)		-18.50 [-78.23 to 41.24]	29.00	0.529
Log (Serum cTnI [ng/mL])	0.708			
Placebo group (n = 12)		-0.010 [-0.035 to 0.015]	0.012	0.420
Spironolactone group (n = 12)		-0.004 [-0.028 to 0.021]	0.012	0.772
LA:Ao ratio	0.110			.
Placebo group (n = 12)		0.020 [0.008 to 0.032]	0.006	0.002
Spironolactone group (n = 12)		0.007 [-0.005 to 0.018]	0.006	0.231
LVIDdN (cm/[kg ^{0.294}])	0.223			
Placebo group (n = 12)		0.017 [0.006 to 0.029]	0.006	0.005
Spironolactone group (n = 12)		0.007 [-0.004 to 0.019]	0.006	0.194

623

624 Repeated measures linear mixed model analysis of change over 6 months in variables of
 625 primary interest (LA:Ao ratio, LVIDdN and serum NT-proBNP and cTnI) for the verum and
 626 placebo groups.

627 P (between groups): probability that the rate of change of the variable is different between the
 628 group receiving placebo and the group receiving spironolactone.

629 Table 3: Repeated measures linear mixed model analysis of change over 6 months in clinical,
 630 echocardiographic and biomarker parameters of secondary interest.

Variable	P (between groups)	Unit change in variable per month (B) [95% confidence interval]	SE	P (within group)
PCV (%)	0.792			
Placebo group (n = 9)		-0.06 [-0.41 to 0.29]	0.17	0.729
Spirolactone group (n = 10)		0.01 [-0.35 to 0.36]	0.17	0.977
Serum urea (mmol/L)	0.406			
Placebo group (n = 12)		0.01 [-0.15 to 0.17]	0.08	0.903
Spirolactone group (n = 12)		0.11 [-0.06 to 0.27]	0.08	0.195
Log (Serum creatinine [μ mol/L])	0.223			.
Placebo group (n = 12)		-0.001 [-0.006 to 0.004]	0.002	0.706
Spirolactone group (n = 12)		0.003 [-0.002 to 0.008]	0.002	0.177
Serum Na ⁺ (mmol/L)	0.407			
Placebo group (n = 12)		-0.071 [-0.254 to 0.113]	0.091	0.441
Spirolactone group (n = 12)		0.036 [-0.144 to 0.216]	0.089	0.690
Serum K ⁺ (mmol/L)	0.328			
Placebo group (n = 12)		0.028 [-0.011 to 0.067]	0.019	0.157
Spirolactone group (n = 12)		0.001 [-0.037 to 0.039]	0.019	0.957
Serum Cl ⁻ (mmol/L)	0.713			
Placebo group (n = 12)		0.023 [-0.258 to 0.306]	0.139	0.871

Spironolactone group (n = 12)		-0.050 [-0.327 to 0.228]	0.138	0.719
Log (UAC[g/mol])	0.223			
Placebo group (n = 9)		0.017 [0.006 to 0.029]	0.006	0.005
Spironolactone group (n = 8)		0.007 [-0.004 to 0.019]	0.006	0.194
Body weight (kg)	0.456			
Placebo group (n = 12)		-0.016 [-0.055 to 0.024]	0.019	0.428
Spironolactone group (n = 12)		-0.036 [-0.074 to 0.002]	0.019	0.066
LVIDsN (cm/[kg ^{0.315}])	0.839			
Placebo group (n = 12)		0.005 [-0.006 to 0.015]	0.005	0.367
Spironolactone group (n = 12)		0.003 [-0.007 to 0.013]	0.005	0.527
LVIDd/ LVFWd ratio	0.531			
Placebo group (n = 12)		0.053 [-0.005 to 0.029]	0.028	0.070
Spironolactone group (n = 12)		0.028 [-0.029 to 0.086]	0.028	0.315
Log (E wave velocity [m/s])	0.734			
Placebo group (n = 11)		0.001 [-0.006 to 0.009]	0.004	0.740
Spironolactone group (n = 12)		-0.001 [-0.008 to 0.007]	0.003	0.887
E/A ratio	0.876			
Placebo group (n = 11)		-0.004 [-0.034 to 0.026]	0.015	0.804
Spironolactone group (n = 12)		-0.007 [-0.035 to 0.022]	0.014	0.625
Heart rate (ECG) (bpm)	0.477			
Placebo group (n = 12)		-0.066 [-1.684 to 1.553]	0.781	0.934
Spironolactone group (n = 12)		-0.858 [-2.449 to 0.733]	0.768	0.276

631

632 Repeated measures linear mixed model analysis of change over 6 months in clinical,

633 echocardiographic and biomarker parameters of secondary interest for the verum and placebo
634 groups.

635 P (between groups): probability that the rate of change of the variable is different between the
636 group receiving placebo and the group receiving spironolactone.

637 P (within group): probability that the rate of change over time within the group = 0.

638 SE, standard error; for definitions of other abbreviations see legend to Table 1.

639 Table 4: Comparisons of selected clinical, echocardiographic and biomarker data between
 640 dogs receiving placebo and those receiving spironolactone at the 2 week time point.

641

Variable	Group receiving placebo (n = 10)	Group receiving spironolactone (n = 10)	P value
UAC (g/mol)	0.063 [0.032, 0.077]	0.236 [0.125, 0.339]	0.006
PCV (%)	46.0 ±7.8	42.9 ±7.4	0.428
Serum urea (mmol/L)	6.3 ±2.2	6.4 ±2.0	0.925
Serum creatinine (µmol/L)	74.7 [63.4, 93.2]	76.4 [67.7, 83.7]	0.940
Serum Na ⁺ (mmol/L)	147.1 ±1.7	147.3 ±1.1	0.780
Serum K ⁺ (mmol/L)	4.5 ±0.2	4.7 ±0.2	0.098
Serum Cl ⁻ (mmol/L)	109.3 ±2.8	111.4 ±3.2	0.144

642 Comparisons of selected clinical, echocardiographic and biomarker data between dogs
 643 receiving placebo and those receiving spironolactone at the 2 week time point. All variables
 644 in the group receiving placebo included 10 dogs, except PCV and UAC, which each included
 645 7 dogs. All variables in the group receiving spironolactone included 10 dogs, except PCV,
 646 which included 9 dogs and UAC, which included 7 dogs. The P values for variables for
 647 which significant between-group differences were detected ($P < 0.05$) are highlighted in bold
 648 text.

649 For definitions of abbreviations see legend to Table 1.

650 Table 5: Comparisons of clinical, echocardiographic and biomarker data between dogs
 651 receiving placebo and those receiving spironolactone at the 6 month time point.

Variable	Group receiving placebo (n = 12)	Group receiving spironolactone (n = 12)	P value
Body weight (kg)	11.5 ±4.2	11.5 ±6.9	0.986
Heart rate (ECG) (bpm)	116.8 ±25.8	111.7 ±20.4	0.592
LA:Ao ratio	1.39 ±0.25	1.46 ±0.18	0.427
LVIDdN (cm/[kg ^{0.294}])	1.89 ±0.24	2.01 ±0.34	0.367
LVIDsN (cm/[kg ^{0.315}])	1.08 ±0.18	1.11 ±0.28	0.747
LVIDd/ LVFWd ratio	4.37 ±0.82	4.59 ±1.12	0.593
E wave velocity (m/s)	1.02 ±0.19	0.96 ±0.28	0.539
E/ A wave ratio	1.33 ±0.13	1.37 ±0.39	0.722
NT-proBNP (pmol/L)	.1188.5 [359.0, 3018.0]	1852.0 [765.0, 4128.0]	0.051
cTnI (ng/mL)	0.025 [0.010, 0.150]	0.030 [0.010, 0.170]	0.378
UAC (g/mol)	0.063 [0.010, 0.410]	0.125 [0.060, 0.370]	0.113
PCV (%)	42.8 ±6.3	42.1 ±5.5	0.792
Serum urea (mmol/ L)	5.7 ±1.7	6.7 ±3.5	0.364
Serum creatinine (µmol/L)	76.1 [51.9, 146.3]	82.4 [54.1, 145.5]	0.514
Serum Na ⁺ (mmol/L)	147.1 ±1.0	147.9 ±1.4	0.127
Serum K ⁺ (mmol/L)	4.5 ±0.3	4.6 ±0.3	0.448

Serum Cl ⁻ (mmol/L)	111.0 ±2.6	112.5 ±3.6	0.258
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652 Comparisons of clinical, echocardiographic and biomarker data between dogs receiving
653 placebo and those receiving spironolactone at the 6 month time point. All variables in the
654 group receiving placebo included 12 dogs, except UAC (n = 9). All variables in the group
655 receiving spironolactone included 12 dogs, except PCV (n = 11), NT-proBNP (n = 11) and
656 UAC (n =6).

657 For definitions of abbreviations see legend to Table 1.