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Glucocorticoid metabolism and the action of 11 beta-hydroxysteroid dehydrogenase 2 in canine congestive heart failure

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23 **Abstract**

24 The enzyme 11-beta-hydroxysteroid dehydrogenase isoenzyme 2 (11BHSD2) is
25 responsible for converting the active glucocorticoid cortisol to inactive cortisone and
26 in the renal medulla protects the mineralocorticoid receptor (MR) from activation by
27 cortisol. Derangements in 11BHSD2 activity can result in reduced conversion of
28 cortisol to cortisone, activation of the MR by cortisol and, consequently, sodium and
29 water retention. The objective of this study was to examine glucocorticoid
30 metabolism in canine congestive heart failure (CHF), specifically to evaluate whether
31 renal 11BHSD2 activity and expression were altered. Dogs were prospectively
32 recruited into one of two phases; the first phase (n = 56) utilized gas
33 chromatography-tandem mass spectrometry to examine steroid hormone
34 metabolites normalised to creatinine in home-caught urine samples. Total serum
35 cortisol was also evaluated. The second phase consisted of dogs (n = 18)
36 euthanased for refractory CHF or behavioural reasons. Tissue was collected from
37 the renal medulla for examination by quantitative reverse transcription polymerase
38 chain reaction, immunohistochemistry and protein immune-blotting. Heart failure did
39 not change urinary cortisol:cortisone ratio ($P = 0.388$), or modify renal expression (P
40 $= 0.303$), translation ($P = 0.427$) or distribution of 11BHSD2 ($P = 0.325$). However,
41 CHF did increase excretion of 5 α -tetrahydrocortisone ($P = 0.004$), α -cortol ($P =$
42 0.002) and α -cortolone ($P = 0.009$). Congestive heart failure modifies glucocorticoid
43 metabolism in dogs by increasing 5 α -reductase and 20 α -hydroxysteroid
44 dehydrogenase activity. Differences between groups in age, sex and underlying
45 disease processes may have influenced these results. However, 11BHSD2 does not
46 appear to be a potential therapeutic target in canine CHF.

47 *Keywords:* Cortisol; Aldosterone; Heart disease; Mineralocorticoid receptor

49 **Introduction**

50 Congestive heart failure (CHF) in dogs is a common sequel to cardiac diseases,
51 such as myxomatous mitral valve disease (MMVD), and the neurohumoral and
52 haemodynamic consequences (Egenvall et al., 2005). Treatment of CHF is
53 multifaceted, but a cornerstone of its management is antagonism of the renin-
54 angiotensin-aldosterone system (RAAS). Activation of the RAAS results in, amongst
55 many other effects, increased cardiac afterload together with sodium and water
56 retention by upregulating angiotensin II and aldosterone expression (Ames et al.,
57 2019). The effects of aldosterone can be antagonised at the level of the distal
58 nephron with mineralocorticoid receptor (MR) antagonists such as spironolactone.
59 These antagonists prevent sodium and potassium exchange within the renal
60 collecting duct, thereby, reducing the excess sodium and water retention that
61 contributes to volume overload. However, despite combining spironolactone with
62 other successful therapeutic agents such as diuretics, pimobendan and angiotensin
63 converting enzyme inhibitors, there is usually progression to end-stage CHF (Group,
64 1999; Haggstrom et al., 2008). Therefore, there is need to understand the
65 mechanisms that contribute to further sodium and water retention, even in the face of
66 RAAS antagonism, so that new treatment strategies targeting these pathways can
67 be developed.

68 The enzyme 11 beta-hydroxysteroid dehydrogenase isoenzyme 2 (11BHSD2) is of
69 interest because it co-localises with the MR in the distal nephron (Krozowski et al.,
70 1995; Kyossev et al., 1996). The MR has a high affinity for cortisol as well as
71 aldosterone; by converting cortisol to inactive cortisone, 11BHSD2 reduces cortisol
72 mediated sodium and water retention (Funder et al., 1988). 11BHSD2 activity can be
73 deduced from the urinary cortisol to cortisone ratio (Best and Walker, 1997). By

74 contrast, the enzyme 11BHS1, which is ubiquitously distributed throughout the
75 body, can increase the availability of cortisol by reactivation of cortisone to cortisol
76 (Jamieson et al., 2000). The overall balance between 11BHS1 and 11BHS2
77 activity can be quantified by determining the ratios of urinary glucocorticoid
78 metabolites that result from hepatic breakdown of cortisol and cortisone by A-ring 5 α -
79 and 5 β -reductases (Boonen et al., 2013; Vantghem et al., 1998) (Fig. 1).

80 In human subjects, congenital or acquired impairment of 11BHS2 activity results in
81 the syndrome of apparent mineralocorticoid excess, expanding vascular volume and
82 causing systemic hypertension, suppressed renin concentration and hypokalaemia
83 (Funder, 2017). Mineralocorticoid receptor antagonists, such as spironolactone or
84 eplerenone, are used to prevent activation of the MR by cortisol in these patients.

85 The aim of this study was to investigate whether CHF in dogs is associated with a
86 reduction in renal 11BHS2 activity. Gas chromatography-tandem mass
87 spectrometry (GC-MS/MS) was used to examine the urinary steroid profile in dogs
88 with and without naturally occurring CHF, and to calculate enzymatic activity within
89 the glucocorticoid metabolic pathway. The effect of CHF on tissue gene and protein
90 expression for 11BHS2 was determined using quantitative reverse transcription
91 polymerase chain reaction (RT-qPCR) and protein immuno-blotting (Western).

92

93 **Materials and Methods**

94 Ethical approval was granted on 14th February 2014 by The University of
95 Edinburgh's Veterinary Ethical Review Committee, VERC 0814.

96 ***Steroid profiling in urine and serum***

97 Dogs were prospectively recruited from the referral and first opinion populations of
98 the Royal (Dick) School of Veterinary Studies between June 2014 and August 2016.
99 All dogs underwent a complete physical examination by a board-certified cardiologist
100 or resident in training under their supervision, and were divided into five groups;
101 healthy control (dogs owned by staff or students that were physically normal and not
102 receiving medication), chronic disease without heart disease (CD), International
103 Small Animal Cardiac Health Council (ISACHC) class I (cardiac disease without
104 clinical signs), ISACHC class II (advanced cardiac disease with mild or controlled
105 CHF) without spironolactone (CHF-S) and ISACHC class II with spironolactone
106 (CHF+S). Dogs not in the control group underwent complete cardiac evaluation
107 including haematology, biochemistry, echocardiography (Vivid-7, GE),
108 electrocardiography (AT-102 Plus, Schiller) and blood pressure measurement
109 (Cardell 9402, Midmark). Additional diagnostic tests, such as thoracic radiography or
110 computed tomography, were performed according to clinical need. Dogs were
111 excluded if they were under six months of age, if they had systemic illnesses that
112 potentially affected cardiovascular function, such as pre-existing renal disease
113 (defined as blood creatinine $>125 \mu\text{mol/L}$ and evidence of renal disease based on
114 imaging, where available), or if they had received glucocorticoids in the previous six
115 months. Dogs with respiratory disease were included in the CD group unless there
116 was evidence of pulmonary hypertension on echocardiography (tricuspid
117 regurgitation $>2.8 \text{ m/s}$ or pulmonic insufficiency of $>2.2 \text{ m/s}$ (Rudski et al., 2010)).
118 Blood samples were obtained by jugular venepuncture in all dogs, apart from
119 controls, shortly after presentation. Samples were centrifuged within an hour of
120 collection at 850g for five minutes and serum separated, and batch stored at $-80 \text{ }^\circ\text{C}$
121 until use (maximum six months). Total serum cortisol concentration was analysed by

122 a chemiluminescent immunoassay (Immulite 1000, Siemens) according to the
123 manufacturer's instructions.

124 Urine samples (free catch, mid-flow, at home, minimum 15mL) were collected by
125 owners on the morning of presentation for all dogs. Urine was stored at -80 °C
126 (maximum six months) before batch analysis of urinary cortisol metabolites and
127 creatinine.

128 ***Urinary GC-MS/MS analysis***

129 The steroid profile of urine samples was determined by GC-MS/MS (Andrews et al.,
130 2002; Best and Walker, 1997) and adapted for tandem mass spectrometry (Homer et
131 al., 2017; Boag et al., 2020). Briefly, steroids were enriched with internal standards
132 and extracted from 15 – 20 mL of urine through Sep-Pak C18 cartridges (Waters,
133 UK), hydrolysed with β -glucuronidase (Sigma), followed by formation of the
134 methoxime-trimethylsilyl derivatives (Sigma) (Best and Walker, 1997). This was
135 followed by gas chromatography (GC) separation and mass analysis on a TSQ
136 Quantum Ultra GC using triple quadrupole mass spectrometer (Thermo Scientific).
137 Steroid extracts were analysed along with calibration standards.

138 The GC-MS/MS method measured cortisol (F), cortisone (E), 5 β -tetrahydrocortisol
139 (5 β THF), 5 α -tetrahydrocortisol (5 α THF), 5 β -tetrahydrocortisone (5 β THE), 5 α -
140 tetrahydrocortisone (5 α THE), α -cortol, β -cortol, α -cortolone and β -cortolone (Homer
141 et al., 2017).

142 Creatinine was used to standardise concentrations of steroid metabolites due to
143 variations in volume and concentration of urine between samples. Creatinine was
144 determined using the creatininase/creatinase specific enzymatic method utilising a
145 commercial kit (Alpha Laboratories Ltd.) adapted for use on a Cobas Fara centrifugal

146 analyser (Roche Diagnostics Ltd) (Borner et al., 1979). Steroid hormone
147 concentration per litre ($\mu\text{g/L}$) was indexed to creatinine (g/L).

148 Ratios of urinary steroid metabolites were derived to investigate activity of various
149 enzymes important to the cortisol/cortisone metabolic pathway. Urinary cortisol to
150 cortisone ratio (F/E) was used to infer renal $11\beta\text{HSD2}$ activity (Best and Walker,
151 1997; Boonen et al., 2013), and whole-body $11\beta\text{HSD}$ balance was determined using
152 the ratio of $(5\alpha\text{THF}+5\beta\text{THF})/(5\beta\text{THE}+5\alpha\text{THE})$, modified from the human urinary
153 steroid method, by the addition of $5\alpha\text{THE}$ to account for the higher levels in dogs
154 compared to humans (Boonen et al., 2013; Vantyghem et al., 1998). Alpha- and
155 beta-reductases irreversibly metabolise cortisol and cortisone, and their relative
156 activities were estimated by the $5\beta\text{THF}/5\alpha\text{THF}$ ratio (Boonen et al., 2013). Total A-
157 ring reduction of cortisol was estimated by the $(5\alpha\text{THF}+5\beta\text{THF})/F$ ratio. Total cortisol
158 metabolite excretion per kg bodyweight was determined by summing $5\alpha\text{THF}$,
159 $5\beta\text{THF}$, $5\beta\text{THE}$, $5\alpha\text{THE}$, cortols and cortolones.

160 ***11BHS2 kidney gene and protein expression***

161 A separate group of dogs was prospectively recruited for this part of the study and
162 included dogs euthanased (intravenous pentobarbitone over-dose (Animalcare, UK))
163 due to refractory CHF (ISACHC stage IIIa/b) and healthy dogs (control), not on
164 medications euthanased for behavioural reasons. The CHF dogs underwent a
165 physical examination, haematology, biochemistry, echocardiography,
166 electrocardiography, thoracic radiography and blood pressure measurement.
167 Controls received only a physical examination. Exclusion criteria were as before.

168 The left kidney was removed within 30 minutes of euthanasia and examined for any
169 gross pathological abnormalities, if any were present the animal was excluded. The

170 renal medulla and cortex were removed by sharp dissection, transversely sectioned
171 and samples of each were then fixed in 10% formalin for a minimum of 48 hours
172 (then paraffin wax embedded for histopathology and immunofluorescence), snap-
173 frozen in dry-ice (then stored at -80 °C for a maximum of six months for Western
174 blots) or stored in RNA later (Ambion, UK; 4 °C for 24 hours then -20 °C for a
175 maximum of six months for quantitative real-time PCR). The left adrenal gland was
176 also removed and prepared, as for the renal medulla and cortex, for
177 immunohistochemistry only.

178 **Total RNA and qPCR**

179 Total RNA was isolated from 50-80 mg samples of renal medulla, cDNA synthesised
180 and real-time qPCR experiments were performed by standard technique, as
181 previously described (Cartwright et al., 2018). Further details can be found in
182 Supplementary material.

183 Melting curve analysis was performed for reference (glyceraldehyde 3-phosphate
184 dehydrogenase (GAPDH) and mitochondrial 28S ribosomal protein subunit 25
185 (MRPS25) and target gene (11BHSD2). Primers were obtained from Eurofins
186 Genomics (Ebersburg) and designed using the Roche primer design software based
187 on *Canis familiaris* sequences from the Ensembl database. The Basic Local
188 Alignment Search Tool (National Center for Biotechnology Information) was utilised
189 to confirm gene specificity. Primer sequences are shown in Table 1. Primer
190 efficiencies were between 92 and 99%. All PCR reactions exhibited one well-defined
191 melting curve peak. Relative expression levels were normalised to the reference
192 genes and calculated using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen, 2001).

193 **Western blots**

194 Samples were thawed on ice and suspended in radioimmunoprecipitation assay
195 buffer (RIPA; Roche). Protein concentrations were determined using the Bio-Rad
196 protein detergent-compatible assay (Bio-Rad). 15 µg of protein was separated using
197 a 10% Bis-Tris gel (Thermo Fisher Scientific), transferred to a nitrocellulose
198 membrane, and probed with goat anti-human 11BHSD2 antibody (1:300, reactivity
199 with *Canis lupus familiaris*, Santa Cruz Biotechnology) and a fluorescent donkey
200 anti-goat secondary antibody (1:800, LI-COR Biosciences). Membranes were
201 stripped and re-probed with a primary β-actin antibody (1:1000, Santa Cruz
202 Biotechnology) used as a loading control followed by a fluorescent donkey anti-rabbit
203 secondary antibody (1:800, LI-COR Biosciences). Bound antibody was detected
204 with a LI-COR Odyssey scanner according to the manufacturer recommendations.
205 Densitometry analysis of protein was performed using LI-COR Image Studio
206 software (Eaton et al., 2013).

207 ***Immunofluorescent staining and imaging***

208 Immunofluorescent staining was performed on renal medulla, renal cortex and
209 adrenal gland (positive tissue control). Detailed methods can be found in
210 Supplementary materials. Polyclonal IgG primary antibodies against 11BHSD2 and
211 nephrin (positive control; Santa Cruz Biotechnology, Germany) were utilised, diluted
212 in 2.5% normal horse serum (Vector Laboratories) (11βHSD2 1:200 dilution, nephrin
213 1:100). The secondary antibody was horse anti-goat IgG (Ready-to-use Vectafluor
214 Dylight-594; Vector Laboratories). Nuclei were counterstained with 4',6-diamidino-2-
215 phenylindole (DAPI; Prolong Gold, Life Technologies). Further details can be found
216 in Supplementary material.

217 Fluorescent images were captured using blue (10 m/s exposure) and green (400 m/s
218 exposure) fluorescent filters (Leica-DMLB; Leica) and examined blindly by a single
219 author (GRM). Five images were taken at x20 magnification per slide. Image
220 analysis software (Photoshop CC, Adobe) was used to examine the relative degree
221 of staining of the inner medulla for 11BHSD2 positive cells in CHF and control
222 animals. The number of stained pixels was identified and compared to the total
223 number of pixels within the image to give a percentage staining.

224

225 ***Statistics***

226 Sigmaplot 13.0 (Systat Software Inc.) was used for all statistical analysis. All data
227 were tested for normality with the Shapiro-Wilk test and were expressed as mean \pm
228 standard deviation (normal) or median with inter-quartile range (IQR; non-normal).
229 Differences in sex between groups was examined using the chi-square test. Multiple
230 comparisons used one-way ANOVA with Tukey *post hoc* tests or Kruskal-Wallis with
231 Dunns pairwise multiple comparison tests according to normality and equality of
232 variance of residuals. A *P*-value <0.05 was considered significant.

233

234 **Results**

235 ***Dog population***

236 A total of 56 dogs (Table 2) were recruited for the steroid profiling study comprising
237 14 control dogs, 14 CD dogs, seven ISACHC-I dogs, 10 CHF-S dogs and nine
238 CHF+S dogs. There was no difference in age ($P=0.79$) or sex ($P=0.66$) between
239 groups. The most frequently identified diseases within each group were as follows;

240 CD, 4/14 pulmonary adenocarcinoma (29%); ISACHC-I, 5/7 MMVD (71%); CHF-S,
241 MMVD 4/10 (40%) and dilated cardiomyopathy (DCM) 4/10 (40%); CHF+S, 5/9
242 MMVD (56%).

243 Eighteen dogs were recruited for the *11BHSD2* expression study, comprising nine
244 CHF and nine control dogs (Table 2). There was no difference in age ($P=0.98$) or sex
245 ($P=0.49$) between groups. The most commonly identified underlying disease in the
246 CHF group was DCM 7/9 (78%).

247 Prescribed treatments are outlined in Table 2 for both studies.

248 ***Total serum cortisol and urinary steroid metabolites***

249 There was no statistically significant difference in total serum cortisol between
250 groups (median (IQR); CD 103.1 $\mu\text{g/g}$ (52.8-176.6); ISACHC-I 54.6 $\mu\text{g/g}$ (46.9-82.5);
251 CHF-S 92.0 $\mu\text{g/g}$ (46.8-121.8); CHF+S 79.5 $\mu\text{g/g}$ (54.9-138.5), $P=0.42$). Alpha-cortol
252 was below the detection limit of the assay in the urine of 3/14 control dogs, 6/14 CD
253 dogs, 2/7 ISACHC-I dogs, 2/10 CHF-S and 4/9 CHF+S, as was urinary cortisol in
254 one CHF+S dog.

255 There was no significant difference in urinary cortisol or cortisone between the
256 groups (Fig. 2a and b). There were significant differences in other urinary steroid
257 metabolites, and the details are illustrated in figure 2. 5α -tetrahydrocortisone was
258 increased in CHF-S ($P=0.021$) and CHF+S ($P=0.032$) compared to dogs with CD
259 (Fig. 2d). Dogs with CHF+S had increased levels of α -cortol (Fig. 2h) compared to
260 control ($P=0.030$) and CD ($P=0.045$). Dogs with CHF-S also had increased levels of
261 α -cortol compared to dogs in the control ($P=0.028$) or CD ($P=0.048$) groups. Alpha-
262 cortolone was different between groups ($P=0.009$), although there was no statistical

263 significance following *post hoc* analysis (Fig. 2g). Total cortisol metabolite excretion
264 was not different between groups (Fig. 2k).

265 ***Activity of steroid metabolising enzymes***

266 There was no difference in renal 11BHS2 activity, as inferred by the F/E ratio (Fig.
267 3a, $P=0.388$), whole body 11BHS2 balance (Fig. 3b, $P=0.644$), total A-ring reduction
268 (Fig. 3c, $P=0.294$) or in relative activity of 5 α - and 5 β -reductases (Fig. 3d, $P=0.199$).

269 ***11BHS2 mRNA and protein levels in control dogs and those with heart failure***

270 *11BHS2* gene expression was identified in the renal medulla in both control and
271 CHF groups, but there was no difference in the level of gene expression between
272 groups ($P=0.303$).

273 The 11BHS2 protein was found in all renal medulla samples, but there was no
274 difference in expression levels between CHF and control (Fig. 4; $P=0.427$).

275 ***Immunofluorescent analysis of 11BHS2 protein expression in the renal*** 276 ***medulla***

277 Positive staining for 11BHS2 was identified in all sections of the renal medulla and
278 cortex (Fig. 5ai and 5aai). In the renal cortex no staining was identified in the
279 glomeruli and proximal convoluted tubule (Fig. 5ai). There was no significant
280 difference in the staining for 11BHS2 in the renal medulla comparing CHF and
281 control groups (Fig. 5b; $P=0.325$).

282

283 **Discussion**

284 This study demonstrates that in dogs with CHF, with or without pharmacological
285 blockade of the MR, there is modified metabolism of cortisol. However, contrary to
286 our hypothesis, there is no reduction in 11BHS2 activity. Instead, there is increased
287 levels of α -cortol and cortolone in CHF, resulting from increased 20- α -hydroxysteroid
288 dehydrogenase activity (Fig 1). The increase in 5 α -THE represents a shift in 5 α
289 reduction over 5 β reduction. This finding suggests that changes to cortisol
290 metabolism are mild in CHF dogs and do not contribute to sodium and water
291 retention via the MR.

292 A previous study had investigated plasma concentrations and urinary excretion of
293 cortisol in dogs with pre-clinical and clinical DCM, and similar to the current study,
294 total serum cortisol levels were not different and could not distinguish between the
295 two groups (Tidholm et al., 2005). Serum cortisol exists in free and bound to cortisol-
296 binding globulin forms (Westphal, 1967). In the current study, free and protein-bound
297 fractions were not measured to determine sensitivity of either to detect CHF. Instead,
298 cortisol and its urinary metabolites were measured by GC-MS/MS, as this provides
299 more information on the exposure of MR to inappropriate activation by cortisol than
300 circulating cortisol (Best and Walker, 1997).

301 While there was a trend towards increased urinary cortisol in dogs in CHF prior to
302 spironolactone therapy, this did not achieve statistical significance, probably due to
303 the wide variation in the data. Markedly increased urinary cortisol has been
304 described in dogs with clinical DCM (Tidholm et al., 2005). While the range of breeds
305 and cardiac diseases in the current study may have been a factor in the variability of
306 the data, it does suggest that in the general dog population urinary cortisol is not an
307 effective biomarker for CHF. Furthermore, unlike the study in DCM dogs, GC-MS/MS
308 was used, as it is the gold standard analytical approach, instead of

309 radioimmunoassays which can cross-react with cortisol metabolites (Lindberg et al.,
310 1982; Tidholm et al., 2005). The current study has quantified the effects of CHF on
311 cortisol (Fig. 2), its metabolites (Fig. 2) and the activity of associated enzymes (Fig.
312 3), and has demonstrated that CHF increases some of these metabolites.

313 The effects of chronic disease states, the stress of which can increase the variation
314 in urinary cortisol concentrations, were separated from the effects of CHF by
315 including two additional groups. While this increased the possibility of a Type II error
316 it did improve the potential clinical utility of the study. Stress effects were also limited
317 by only analysing home-caught urine samples.

318 Our data show that dogs in CHF have increased levels of α -cortol and α -cortolone
319 and so increased 20 α HSD activity (Fig. 1 and 2). The final product of this enzyme,
320 the α -cortols and α -cortolones, are produced in a number of tissues including
321 reproductive organs, the adrenal gland, thymus, brain and kidney (El-Kabbani et al.,
322 2011). It is likely they do not play an important role in the pathogenesis of CHF, but
323 are end-products of modulation of cortisol metabolism by CHF that is not seen in
324 other chronic disease states.

325 The MR is found in non-epithelial tissues such as cardiomyocytes, but these have
326 very low levels of 11BHSD2 (Arriza et al., 1987; Chapman et al., 2013). Therefore, it
327 is necessary to establish whether an increase in 11BHSD2 activity might be linked to
328 increased renal production of 11BHSD2 or a change in its distribution within the
329 kidney. One can speculate that the lack of influence of CHF on expression and
330 translation of the gene encoding *11BHSD2*, and similar distribution in the kidney of
331 healthy controls indicates that any increased activity of 11BHSD2 would be minimal
332 within the range for 11BHSD2 present in the healthy state. Whether or not the same

333 applies to the reductase and HSD enzymes downstream of 11BHSD2 cannot be
334 stated. The clinical usefulness of determining this is questionable since the
335 modulation of cortisol metabolism in CHF does not appear to be maladaptive and, is
336 unlikely to become a therapeutic target. However, it was important to determine
337 whether there was any potentially adverse influence on cortisol metabolism during
338 pharmacological manipulation.

339 Spironolactone is a widely used MR antagonist in CHF cases. Although blockade of
340 MR to reduce sodium and water retention is desirable in CHF, it could lead to
341 accumulations of cortisol, and deleterious clinical consequences if spironolactone
342 was suddenly withdrawn or MR blockade was incomplete. Spironolactone did appear
343 to disrupt the trend towards increased cortisol in CHF, but there was no
344 demonstrable influence on 11BHSD2. There was an increase in 5 α THE production
345 (indicating increased 5 α -reductase activity) when compared to the chronic disease
346 group. However, the absence of any change to 11BHSD balance or significant
347 increase in α -cortolone production suggests spironolactone effect on cortisol
348 metabolism is clinically irrelevant. Similarly, renal disease in human patients treated
349 with spironolactone do not have altered cortisol metabolite excretion or 11BHSD2
350 activity (Hammer et al., 2010). Overall, this supports the conclusion that RAAS
351 activation, rather than decreased renal 11BHSD2 activity, is the major contributor to
352 sodium and water retention via MR in canine CHF.

353 While the preferred method for evaluating urinary metabolites in people involves 24-
354 hour urine collection to adjust for diurnal variation in cortisol secretion, spot urine
355 collection in human studies agree favourably with those from 24-hour sampling
356 (Parikh et al., 2018). Furthermore, diurnal variation does not occur in dogs and the
357 method used in the current study, where urine was collected prior to admission,

358 analysis was performed on a single sample, and glucocorticoid metabolites were
359 normalised to creatinine to account for differing glomerular filtration rates between
360 dogs, was a valid approach (Johnston and Mather, 1978).

361 One confounding limitation of the study was differences in drug administration
362 between groups. This was not controlled for as it was the decision of the attending
363 clinician to prescribe therapy depending on clinical need.

364

365 **Conclusions**

366 In summary, activity of the enzyme 11BHS2 does not appear to be significantly
367 modified in canine CHF. However, other components of the glucocorticoid metabolic
368 pathway are modified by CHF and may contribute to prevention of inappropriate
369 activation of MR by cortisol.

370

371 **Conflict of Interest Statement**

372 The authors have no conflicts of interest to disclose.

373

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386

387 **Appendix: Supplementary material**

388 Supplementary data associated with this article can be found, in the online version,
389 at doi:.....

390

391

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497

498

499 **Tables**

500

501 **Table 1:** Target and reference gene primer sequences. *11BHS2*; 11 beta-
502 hydroxysteroid dehydrogenase, *GAPDH*; glyceraldehyde 3-phosphate
503 dehydrogenase, *MRPS25*; mitochondrial ribosomal protein subunit 25.

Primer		Sequence (5'-3')
<i>11BHS2</i>	Forward	CACTGGAGTTCTCAAAGGCC
	Reverse	TGCCACAGTCACTATACGA
<i>GAPDH</i>	Forward	AATGTATCAGTTGTGGATCTGACC
	Reverse	GCTTCACTACCTTCTTGATGTCG
<i>MRPS25</i>	Forward	TCTTGGGGAAGAACAAGGAA
	Reverse	AGTGGGCGGGTGAGAAAG

504

Table 2: Population characteristics of dogs included in the *in vivo* and *in vitro* phases. ISACHC; International Small Animal Cardiac Health Council, MMVD; myxomatous mitral valve disease, DCM; dilated cardiomyopathy, PDA; patent ductus arteriosus, ME; male entire, MN; male neutered, FE; female entire, FN; female neutered, S; spironolactone, na; not applicable.

	<i>In vivo</i> phase					<i>In vitro</i> phase	
	Control	Chronic disease	ISACHC class I	Heart failure-S	Heart failure+S	Control	Heart failure
n	14	14	7	10	9	9	9
Age	5.7 ± 3.4	6.2 ± 3.5	7.8 ± 3.4	5.9 ± 4.1	6.6 ± 4.3	4.3 ± 1.8 (n=8)	4.2 ± 3.6
Sex	MN n=7, FE n=1, FN n=6	ME n=1, MN n=8, FE n=1, FN n=4	MN n=3, FE n=1, FN n=3	MN n=4, FE n=2, FN n=4	ME n=2, MN n=4, FE n=1, FN n=2	ME n=4, MN n=1, FE n=2, FN n=1, Unknown n=1	ME n=2, MN n=1, FE n=2, FN n=4
Breeds	Cross breed (n=5), Lurcher (n=3), Podenco (n=2), 1 each of Labrador, German shepherd dog, English bulldog, Husky	Labrador (n=2), 1 each of Dobermann, Cocker spaniel, Bearded collie, Standard poodle, Cairn terrier, Leonberger, English springer spaniel, Boxer, Miniature schnauzer, Cross breed, Shih tzu and Husky	Cocker spaniel (n=2), 1 each of Border collie, Cavalier King Charles spaniel, Scottish terrier, Lurcher, Border terrier	Cavalier King Charles spaniel (n=2), Cross breed (n=2), Labrador (n=2), 1 each of Bullmastiff, St Bernard, Hungarian vizsla, German shepherd dog	Cavalier King Charles spaniel (n=2), Labrador (n=2), 1 each of German wire haired pointer, Standard poodle, Cross breed, Cairn terrier, Cocker spaniel	Staffordshire bull terrier (n=5), 1 each of Akita, Pit bull terrier, Bullmastiff, Jack Russell terrier	Flat coat retriever (n=2), Lurcher (n=2), 1 each of Dobermann, Boxer, cross breed, German shepherd dog, Labrador
Disease	na	pulmonary adenocarcinoma (n=4), chronic bronchitis (n=2), other respiratory (n=3), cardiac neoplasia (n=1), chronic diarrhoea (n=1), osteoarthritis (n=1), diagnosis not reached (n=2)	MMVD (n=5), pulmonic stenosis (n=2)	MMVD (n=4), DCM (n=4), PDA (n=1), tricuspid valve dysplasia (n=1)	MMVD (n=5), mitral valve dysplasia (n=3), DCM (n=1)	na	DCM (n=7), mitral valve dysplasia (n=1), tricuspid valve dysplasia (n=1)
Medications	na	pimobendan (n=1), theophylline (n=1), doxycycline (n=1), meloxicam (n=1)	atenolol (n=1),	furosemide (n=10), pimobendan (n=10), benazepril (n=8), diltiazem (n=2)	furosemide (n=9), spironolactone (n=9), benazepril (n=9), pimobendan (n=9), digoxin (n=1), sildenafil (n=1), diltiazem (n=1), hydrochlorothiazide/amiloride (n=1), aspirin (n=1)	na	furosemide (n=9), pimobendan (n=8), benazepril (n=7), spironolactone (n=7), diltiazem (n=1)

509 **Figure legends**

510

511 **Figure 1. A summary of the glucocorticoid metabolic pathway.** The dashed lines
512 indicate that more than one step is involved in this process. 11BHS1; 11 beta-
513 hydroxysteroid dehydrogenase 1, 11BHS2; 11 beta-hydroxysteroid dehydrogenase
514 2.

515

516 **Figure 2. Changes in urinary glucocorticoid metabolite concentrations.** Box
517 and whisker plots showing the differences in normalised glucocorticoid metabolites
518 between groups in ug/g. **a.** Cortisol. **b.** Cortisone. **c.** 5β-tetrahydrocortisone. **d.** 5α-
519 tetrahydrocortisone. **e.** 5α-tetrahydrocortisol. **f.** 5β-tetrahydrocortisol. **g.** α-cortolone.
520 **h.** α-cortol. **i.** β-cortolone. **j.** β-cortol. **k.** Total cortisol metabolite excretion. CD;
521 control, ISACHC; International Small Animal Cardiac Health Council, CHF;
522 congestive heart failure, S; spironolactone, THF; tetrahydrocortisol, THE;
523 tetrahydrocortisone. **P* < 0.05.

524

525 **Figure 3. Changes in the relative activity of glucocorticoid metabolising**
526 **enzymes.** Box and whisker plots showing the differences in the activity of
527 glucocorticoid metabolising enzymes between groups. **a.** Cortisol: cortisone inferring
528 11BHS2 activity. **b.** Whole body balance of 11BHS activity. **c.** Total A-ring
529 reduction. **d.** relative activities of 5α and 5β reductases. CD; control, ISACHC;
530 International Small Animal Cardiac Health Council, CHF; congestive heart failure, S;
531 spironolactone, BHS; beta-hydroxysteroid dehydrogenase.

532 **Figure 4. 11 Beta-hydroxysteroid dehydrogenase 2 protein levels in the renal**
533 **medulla of dogs with congestive heart failure.** Summary data together with
534 representative Western blots for 11BHSD2 and B-actin for heart failure and control
535 animals. Western blots were normalised to B-actin. PC; positive control, CHF;
536 congestive heart failure, 11BHSD2; 11 beta-hydroxysteroid dehydrogenase 2.

537

538 **Figure 5. 11 Beta-hydroxysteroid dehydrogenase 2 protein expression in the**
539 **renal medulla of dogs with congestive heart failure. ai.** Representative cross
540 section of renal cortex from a dog with heart failure. **aii.** Representative cross section
541 of renal medulla from a dog with heart failure. **b.** Box and whisker plot showing no
542 difference in percentage staining between congestive heart failure (CHF) and control
543 groups.

544

545