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

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Abstract

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

Keywords: Cortisol; Aldosterone; Heart disease; Mineralocorticoid receptor

Introduction

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

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

contrast, the enzyme 11BHSD1, which is ubiquitously distributed throughout the body, can increase the availability of cortisol by reactivation of cortisone to cortisol (Jamieson et al., 2000). The overall balance between 11BHSD1 and 11BHSD2 activity can be quantified by determining the ratios of urinary glucocorticoid metabolites that result from hepatic breakdown of cortisol and cortisone by A-ring 5 α - and 5 β -reductases (Boonen et al., 2013; Vantyghem et al., 1998) (Fig. 1).

In human subjects, congenital or acquired impairment of 11BHSD2 activity results in the syndrome of apparent mineralocorticoid excess, expanding vascular volume and causing systemic hypertension, suppressed renin concentration and hypokalaemia (Funder, 2017). Mineralocorticoid receptor antagonists, such as spironolactone or eplerenone, are used to prevent activation of the MR by cortisol in these patients.

The aim of this study was to investigate whether CHF in dogs is associated with a reduction in renal 11BHSD2 activity. Gas chromatography-tandem mass spectrometry (GC-MS/MS) was used to examine the urinary steroid profile in dogs with and without naturally occurring CHF, and to calculate enzymatic activity within the glucocorticoid metabolic pathway. The effect of CHF on tissue gene and protein expression for 11BHSD2 was determined using quantitative reverse transcription polymerase chain reaction (RT-qPCR) and protein immuno-blotting (Western).

Materials and Methods

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

Steroid profiling in urine and serum

Dogs were prospectively recruited from the referral and first opinion populations of the Royal (Dick) School of Veterinary Studies between June 2014 and August 2016. All dogs underwent a complete physical examination by a board-certified cardiologist or resident in training under their supervision, and were divided into five groups; healthy control (dogs owned by staff or students that were physically normal and not receiving medication), chronic disease without heart disease (CD), International Small Animal Cardiac Health Council (ISACHC) class I (cardiac disease without clinical signs), ISACHC class II (advanced cardiac disease with mild or controlled CHF) without spironolactone (CHF-S) and ISACHC class II with spironolactone (CHF+S). Dogs not in the control group underwent complete cardiac evaluation including haematology, biochemistry, echocardiography (Vivid-7, GE), electrocardiography (AT-102 Plus, Schiller) and blood pressure measurement (Cardell 9402, Midmark). Additional diagnostic tests, such as thoracic radiography or computed tomography, were performed according to clinical need. Dogs were excluded if they were under six months of age, if they had systemic illnesses that potentially affected cardiovascular function, such as pre-existing renal disease (defined as blood creatinine >125 µmol/L and evidence of renal disease based on imaging, where available), or if they had received glucocorticoids in the previous six months. Dogs with respiratory disease were included in the CD group unless there was evidence of pulmonary hypertension on echocardiography (tricuspid regurgitation >2.8 m/s or pulmonic insufficiency of >2.2m/s (Rudski et al., 2010)).

Blood samples were obtained by jugular venepuncture in all dogs, apart from controls, shortly after presentation. Samples were centrifuged within an hour of collection at 850g for five minutes and serum separated, and batch stored at -80 °C until use (maximum six months). Total serum cortisol concentration was analysed by

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

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

Urinary GC-MS/MS analysis

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

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

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

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

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

11BHS2 kidney gene and protein expression

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

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

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

Total RNA and qPCR

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

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

Western blots

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

Immunofluorescent staining and imaging

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

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

Statistics

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

Results

Dog population

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

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

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

Prescribed treatments are outlined in Table 2 for both studies.

Total serum cortisol and urinary steroid metabolites

There was no statistically significant difference in total serum cortisol between 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); 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 was below the detection limit of the assay in the urine of 3/14 control dogs, 6/14 CD dogs, 2/7 ISACHC-I dogs, 2/10 CHF-S and 4/9 CHF+S, as was urinary cortisol in one CHF+S dog.

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

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

Activity of steroid metabolising enzymes

There was no difference in renal 11BHSD2 activity, as inferred by the F/E ratio (Fig. 3a, $P=0.388$), whole body 11BHSD balance (Fig. 3b, $P=0.644$), total A-ring reduction (Fig. 3c, $P=0.294$) or in relative activity of 5 α - and 5 β -reductases (Fig. 3d, $P=0.199$).

11BHSD2 mRNA and protein levels in control dogs and those with heart failure

11BHSD2 gene expression was identified in the renal medulla in both control and CHF groups, but there was no difference in the level of gene expression between groups ($P=0.303$).

The 11BHSD2 protein was found in all renal medulla samples, but there was no difference in expression levels between CHF and control (Fig. 4; $P=0.427$).

Immunofluorescent analysis of 11BHSD2 protein expression in the renal medulla

Positive staining for 11BHSD2 was identified in all sections of the renal medulla and cortex (Fig. 5ai and 5aaii). In the renal cortex no staining was identified in the glomeruli and proximal convoluted tubule (Fig. 5ai). There was no significant difference in the staining for 11BHSD2 in the renal medulla comparing CHF and control groups (Fig. 5b; $P=0.325$).

Discussion

This study demonstrates that in dogs with CHF, with or without pharmacological blockade of the MR, there is modified metabolism of cortisol. However, contrary to our hypothesis, there is no reduction in 11BHSD2 activity. Instead, there is increased levels of α -cortol and cortolone in CHF, resulting from increased 20- α -hydroxysteroid dehydrogenase activity (Fig 1). The increase in 5 α -THE represents a shift in 5 α reduction over 5 β reduction. This finding suggests that changes to cortisol metabolism are mild in CHF dogs and do not contribute to sodium and water retention via the MR.

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

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

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

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

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

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

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

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

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

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

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

Conclusions

In summary, activity of the enzyme 11BHSD2 does not appear to be significantly modified in canine CHF. However, other components of the glucocorticoid metabolic pathway are modified by CHF and may contribute to prevention of inappropriate activation of MR by cortisol.

Conflict of Interest Statement

The authors have no conflicts of interest to disclose.

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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:.....

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Tables

Table 1: Target and reference gene primer sequences. *11BHSD2*; 11 beta-hydroxysteroid dehydrogenase, *GAPDH*; glyceraldehyde 3-phosphate dehydrogenase, *MRPS25*; mitochondrial ribosomal protein subunit 25.

Primer		Sequence (5'-3')
<i>11BHSD2</i>	Forward	CACTGGAGTTCTCAAAGGCC
	Reverse	TGCCCACAGTCACTATACGA
<i>GAPDH</i>	Forward	AATGTATCAGTTGTGGATCTGACC
	Reverse	GCTTCACTACCTTCTTGATGTCG
<i>MRPS25</i>	Forward	TCTTGGGGAAGAACAAGGAA
	Reverse	AGTGGGCGGGTGAGAAAG

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)

Figure legends

Figure 1. A summary of the glucocorticoid metabolic pathway. The dashed lines indicate that more than one step is involved in this process. 11BHSD1; 11 beta-hydroxysteroid dehydrogenase 1, 11BHSD2; 11 beta-hydroxysteroid dehydrogenase 2.

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

Figure 3. Changes in the relative activity of glucocorticoid metabolising enzymes. Box and whisker plots showing the differences in the activity of glucocorticoid metabolising enzymes between groups. **a.** Cortisol: cortisone inferring 11BHSD2 activity. **b.** Whole body balance of 11BHSD activity. **c.** Total A-ring reduction. **d.** relative activities of 5 α and 5 β reductases. CD; control, ISACHC; International Small Animal Cardiac Health Council, CHF; congestive heart failure, S; spironolactone, BHSD; beta-hydroxysteroid dehydrogenase.

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

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