Cortisol urinary metabolites in dogs with hypercortisolism, congestive heart failure, and healthy dogs: pilot investigation

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Abstract. Nonadrenal diseases (NAD), including congestive heart failure (CHF), can affect the conversion of cortisone to cortisol favoring the production of cortisol's urinary downstream metabolites 5 α /5 β -tetrahydrocortisol (THF) relative to tetrahydrocortisone (THE). We hypothesized that healthy dogs would have lower urinary levels of cortisol, cortisone, THF, and THE than dogs with hypercortisolism (HC) or CHF, and the latter would have higher urinary levels of THF and lower THE than dogs with HC. Four, 9, and 8 dogs with HC, CHF, and normal health, respectively, were included in a pilot prospective cross-sectional study. A single morning voided urine sample was analyzed for urinary cortisol metabolites by liquid chromatography–mass spectrometry. The percentages of conjugated urinary metabolites were significantly higher in dogs with CHF than in healthy dogs ($p=0.001$), and not different in HC dogs ($p=0.07$). Log-transformed urine cortisol metabolites-to-creatinine ratios in healthy dogs were significantly lower than the 2 other groups ($p<0.001$). The urinary free THE:THF ratio was significantly higher ($p<0.001$) than the urinary total and conjugated THE:THF ratios. Health status did not affect the total, conjugated, and free THE:THF ratios ($p=0.61$). Additional studies are needed to investigate differences in cortisol metabolites between dogs with HC and NAD to accurately discriminate between the groups.

Key words: 11 β -hydroxysteroid dehydrogenase 1; 5 α /5 β -tetrahydrocortisol; canine; Cushing disease; dogs; liquid chromatography–mass spectrometry; tetrahydrocortisone.

Hypercortisolism (HC; Cushing disease) is a common endocrine disease of senior dogs.²⁶ Hypercortisolism may lead to severe complications such as hypertension with end-organ damage, hypercoagulability and thrombosis, and proteinuria.¹⁵ Hypercortisolism also complicates the management of concurrent comorbidities such as diabetes mellitus.¹⁵ An early diagnosis of HC is vital because it can minimize clinical complications and improve the clinical management of concurrent comorbidities. However, HC poses a significant diagnostic challenge, especially in the early stages of the disease, wherein HC appears with ambiguous clinical signs that resemble and overlap with many nonadrenal diseases (NADs).¹

The hypothalamic–pituitary–adrenal axis (HPAA) responses to stress lead to increased cortisol levels.¹⁰ Systemic NAD induces an HPAA response that results in increased cortisol levels. Hormonal tests that are currently employed in the diagnosis of HC are based on the measurement of serum cortisol concentration,^{1,9} hence stimulation of the HPAA by NAD may lead to overlapping results. This becomes especially important when HC is suspected in the background of a preexisting comorbidity (e.g., diabetes mellitus). In those scenarios, the diagnostic dilemma is whether the positive hormonal test results are the result of stress-related stimulation of the HPAA or because of concurrent HC. To date, none of the available tests reliably differentiates between NAD and HC under these conditions.

Cortisol metabolism is complex and involves 10 enzymatic pathways that result in 10 different metabolites.²⁰ However, the profile of cortisol metabolites may vary when NAD directly affects one or more of the cortisol metabolic pathways. Examples include hyperthyroidism and hypothyroidism that affect cortisol metabolism and clearance via thyroid hormones' effects on 11 β -hydroxysteroid dehydrogenase 1 (HSD11B1) and 5 α /5 β -reductases.²⁰ Growth hormone can also alter cortisol metabolism through its effect on insulin growth factor-1 (IGF-1) that increases cortisol clearance by inhibiting hepatic HSD11B1 (conversion of cortisone to cortisol).²⁰ As well, proinflammatory cytokines have been shown to upregulate HSD11B1 and downregulate HSD11B2, favoring the interconversion of cortisone to cortisol.⁴ Therefore,

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Figure 1. The hypothesis working model for evaluation of urinary corticosteroid metabolites. Nonadrenal disease increases the interconversion of cortisone to cortisol through inhibition of 11β -hydroxysteroid dehydrogenase 2 (HSD11B2) and upregulation of 11β -hydroxysteroid dehydrogenase 1 (HSD11B1), subsequently increasing the ratio of the cortisol downstream metabolite, tetrahydrocortisol (THF), to the cortisone downstream metabolite, tetrahydrocortisone (THE). In hypercortisolism compared to NAD, there is a relative increased cortisone downstream metabolism to tetrahydrocortisone, off-setting the THE:THF ratio.

Table 1. Demographic information for dogs included in our study.

Condition	<i>n</i>	Age (y)	Weight (kg)	Sex	Breeds
Hypercortisolism*	4	9.9 ± 1.5	22.8 ± 11.5	2 SF, 1 CM, 1 M	4
Nonadrenal disease†	9	9.2 ± 3.7	23.5 ± 19.3	1 SF, 2 F, 5 CM, 1 M	8
Healthy	8	6.3 ± 2.6	29.5 ± 10.2	6 SF, 1 CM, 1 M	5

CM = castrated male; F = female; M = male; SF = spayed female. Age and weight presented as mean ± SD.

* Dogs were untreated at the time of inclusion.

† Dogs with acute pulmonary edema caused by congestive heart failure.

our goal was to determine if HC and NAD can differentially affect cortisol metabolism, resulting in HC-specific and NAD-specific discriminatory cortisol metabolite profiles.

Based on these differentially stimulated metabolic pathways, we hypothesized that dogs with HC would have lower urinary concentrations of $5\alpha/5\beta$ -tetrahydrocortisol (THF) and higher urinary concentrations of tetrahydrocortisone (THE) compared to dogs with congestive heart failure (CHF), the latter which served as the NAD group in our study (Fig. 1). We also hypothesized that the urinary concentrations of cortisol, cortisone, THF, and THE will be higher in dogs with HC and NAD compared to healthy dogs. The specific aims of our study were: to measure, characterize, and compare the profile of selected urinary cortisol metabolites in healthy dogs, dogs with HC, and dogs with NAD.

Over a period of 18 mo, we prospectively enrolled 21 client-owned dogs into 3 groups: dogs with HC, dogs with NAD, and apparently healthy dogs (Table 1). The first group consisted of 4 dogs with clinical signs; hematologic and biochemical results compatible with HC, confirmed by either an adrenocorticotrophic hormone (ACTH) stimula-

tion test or low-dose dexamethasone suppression test; and imaging studies. The dogs had not received previous treatment for HC. Two of the 4 dogs had a tentative diagnosis of pituitary-dependent hypercortisolism (PDH) based on abdominal ultrasonography. The third dog had a diagnosis of PDH confirmed by the demonstration of a pituitary macroadenoma by computed tomography. The fourth dog had a unilateral adrenocortical tumor diagnosed on abdominal ultrasonography. The second group consisted of 8 dogs with NAD of cardiac etiology. Duration of morbidity was 1 d to 7 mo (mean: 6.5 ± 10.9 wk). All dogs had acute left-sided CHF and required intensive care and stabilization. The outcome for 2 dogs was euthanasia given poor prognosis and deterioration despite treatment. The remainder improved on the therapy implemented and were discharged. We chose to include these dogs to constitute the group of NAD because of previous evidence indicating that structural heart disease leads to activation of the HPAA,²³ and because it is a group that is readily defined in a clinical setting. The third group consisted of 9 apparently healthy dogs with normal history and clinical examination. To the best of our knowledge, none of the dogs received

treatment with topical or systemic glucocorticoids 14 d prior to enrollment into the study.

The University Animal Ethics Committee approved this study (Massey University, protocol 15/07). Informed consent was obtained from all pet owners prior to inclusion in the study. After enrollment, a single voided urine sample was collected from each dog in the morning, after an overnight fast, into sterile containers and stored at -80°C until analysis. At the end of the study (i.e., after 18 mo from its beginning), liquid chromatography–mass spectrometry (LC-MS) was used to measure the concentrations of urinary cortisol, cortisone, THF, and THE, and a kinetic colorimetric assay was conducted to measure the urinary concentration of creatinine. Six assays were run over a 3-wk period in July 2016. Assays took ~ 24 h to run. Quality control and isotopically labeled internal standards accounted for variation during the measuring period.

To detect the total concentration of corticosteroids, a deconjugation enzyme assay was used. Briefly, 100 μL of internal standard (20 ng/mL cortisol-d4 and corticosterone d8; Steraloids, Newport, RI) in water was mixed in a glass tube with 200 μL of urine. For quantitation purposes, a series of standards (0.5–500 ng/mL each steroid; Steraloids) were assayed in parallel with the samples. The solution was then buffered with 200 μL of 0.25 N sodium acetate (pH 5.0) and glucuronidase (255 units)/sulfatase (22.5 units; Crude extract from *Helix pomatia*; Sigma-Aldrich, Auckland, New Zealand), added to the tubes, and then incubated at 37°C in a water bath for 3 h. The reaction was stopped by heating the tubes at 70°C for 5 min. One mL of ethyl acetate (Merck, Darmstadt, Germany) was then added and the solution mixed by vortexing for 30 s. The organic phase was removed to a glass tube and lyophilized by vacuum concentration (Savant SC250EXP; Thermo Scientific, Asheville, NC).

To measure the concentration of free steroids in each, the second set of samples were prepared with an internal standard as above, extracted with ethyl acetate, and the organic layer was dried. The dried extracts were re-dissolved in 70 μL of mobile phase (methanol [Merck]:water, 45:55), vortex-mixed, and transferred to ultra-performance liquid chromatography (UPLC) vials. Twelve μL were injected onto an UPLC mass spectrometer system consisting of an Accela MS pump and autosampler followed by an Ion Max APCI source (in positive ion mode) on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer all controlled by Finnigan Xcalibur software (Thermo Electron, San Jose, CA). The compounds were separated on a Kinetex F5 100 $\times$ 2.1 mm, 2.6- μm column (Phenomenex, Torrance, CA) at 40°C using a methanol:water gradient with a starting composition of 45% methanol. The parent-to-daughter ion transitions followed, and retention times (min) were: cortisol 363.1–121.0 m/z (2.95), cortisol d4 367.1–121.0 (2.95), cortisone 361.1–163.0 (3.0), THF 349.2–301.2 (4.1), THE 347.1–243.1 (4.95), and corticos-

terone d8 355.0–125.2 (4.95). The intra- and inter-assay coefficients of variation (CVs) were 4.2, 3.2, 7.2, and 5.7%, and 6.0, 4.3, 11.1, and 13.3%, respectively, for the total concentration of each steroid.

Creatinine concentrations were measured (COBAS c311 autoanalyzer; Hitachi High Technologies, Tokyo, Japan) by a kinetic colorimetric assay based on the Jaffé method (Roche, Mannheim, Germany).

Conjugated steroid absolute values were calculated by subtracting each of the free steroids from their total concentration. We calculated the ratio between creatinine and the total, free, and conjugated individual urine corticoid and obtained the THE:THF ratio by dividing the ratio of THE:creatinine by THF:creatinine.

All data analyses were performed with SAS University edition (SAS Institute, Cary, NC). The data were examined for normal distribution by inspection of Q-Q plots, histogram, and by the Shapiro–Wilk test. Non-normally distributed data were subjected to transformation (natural log or square root). The descriptive statistics of normally distributed data were described by mean ($\pm$ SD), whereas non-normally distributed data were described by median (min., max.). Analysis of variance was used to test for significant differences between groups with regards to cortisol metabolites and ratios between metabolites. Normally distributed data were analyzed by one-way ANOVA. The Tukey method for post-hoc pairwise comparisons was used to ascertain differences between specific groups. The significance level was set at $p=0.05$.

The canine urinary cortisol metabolites profile was first evaluated. The percentages of conjugated urinary cortisol, cortisone, THF, and THE were calculated by dividing the conjugated urinary cortisol, cortisone, THF, and THE by their respective total urinary concentrations and are presented as median (min., max.; Table 2). In general, all dogs had higher levels of conjugated urinary THE and THF than conjugated cortisol and cortisone. We also found that the percentages of conjugated metabolites in NAD dogs were significantly increased relative to those of healthy dogs ($p=0.001$) but not relative to HC ($p=0.07$); there were no differences between healthy dogs and dogs with HC ($p=0.37$).

The log-transformed urine cortisol metabolite-to-creatinine ratios of the healthy dogs were significantly lower than the 2 other groups ($p<0.05$; Fig. 2, Supplementary Table 1).

In all groups, the urine free THE:THF ratio was significantly higher ($p<0.05$) than the urinary total and conjugated THE:THF ratios (Fig. 3). The total, conjugated, and free THE:THF ratios were not different between groups ($p=0.61$).

To our knowledge, measurement and characterization of THE and THF in canine urine and in various groups (e.g., healthy, CHF, and HC) has not been reported previously. We found that THE and THF in the urine were predominantly conjugated. This implies that their metabolism from cortisol most likely occurs in the liver,²⁰ although the canine kidney

Table 2. Median (min., max.) urinary conjugated cortisol metabolites of healthy dogs and dogs with hypercortisolism and nonadrenal disease. Conjugated cortisol metabolites are expressed as percentages of the total steroid fraction for each metabolite.

Condition/metabolite	Median (%)	Minimum (%)	Maximum (%)	n
Healthy				
Cortisol	9	0	15	8
Cortisone	6	3	11	8
Tetrahydrocortisol	85	42	96	8
Tetrahydrocortisone	58	35	90	8
Hypercortisolism				
Cortisol	12	0	20	4
Cortisone	4	0	10	4
Tetrahydrocortisol	95	95	98	3
Tetrahydrocortisone	72	64	92	4
Nonadrenal disease				
Cortisol	20	14	41	8
Cortisone	5	5	11	9
Tetrahydrocortisol	98	92	99	9
Tetrahydrocortisone	94	34	98	9

n = no. of observations.

Figure 2. Boxplot presentation of the log-transformed cortisol metabolites-to-creatinine ratios in healthy, hypercortisolemic, and congestive heart failure dogs. The horizontal line within each box represents the median; the lower and upper boundaries of each box represent the first and third quartiles; and the whiskers represent the 5th–95th percentile range. C = hypercortisolism dog group; conjugate = urinary conjugated steroid fraction; cL:cr = urinary cortisol:creatinine ratio; cN:cr = urinary cortisone:creatinine ratio; free = urinary free steroid fraction; H = congestive heart failure dog group; N = healthy dog group; the:cr = urinary tetrahydrocortisone:creatinine ratio; thf:cr = urinary tetrahydrocortisol:creatinine ratio; total = urinary total steroid fraction. Letters a and b represent significant differences within each ratio ($p \leq 0.05$).

also has some capacity to perform conjugation reactions.^{6,19} It remains to be determined whether the liver directly secretes the conjugated THE and THF into the blood or THE and THF reach the blood via the enterohepatic circulation. We also found that CHF significantly increased the percentage of conjugated steroids relative to healthy dogs; however, we

are uncertain how significant this finding is from a clinical perspective.

We hypothesized that severe nonadrenal disease would affect cortisol metabolism through upregulation of HSD11B1 and downregulation of HSD11B2, thus favoring the inter-conversion of cortisone to cortisol.⁴ We anticipated an

Figure 3. Boxplot presentation of log-transformed urinary total, conjugated, and free tetrahydrocortisone:tetrahydrocortisol ratios (THE:THF) in healthy, hypercortisolemic, and congestive heart failure dogs. The horizontal line within each box represents the median; the shape within each box represents the mean; the lower and upper boundaries of each box represent the first and third quartiles; and the whiskers represent the 5th–95th percentile range. C = hypercortisolism dog group; H = congestive heart failure dog group; N = healthy dog group; THE = tetrahydrocortisone; THF = tetrahydrocortisol. A significant difference between free THE:THF ratio to the conjugate and total THE:THF ratios was present in all groups (a and b superscripts; $p \leq 0.05$).

increase in the cortisol metabolite THF relative to the cortisone metabolite THE. Indeed, Figure 2 indicates that conjugated and total THF are significantly higher in dogs with HC and CHF than in healthy dogs, whereas THE does not change. This means that downstream cortisol conversion to THF is enhanced in both HC and CHF; however, it did not differ between the 2 groups, possibly given the small sample size. We also used a different approach and analyzed the THE:THF ratio. We found that the health status state (i.e., healthy, HC, and CHF) did not significantly affect the THE:THF ratio ($p=0.61$). It is important to consider that we chose dogs with acute pulmonary edema secondary to structural heart disease to constitute our NAD group. We made that decision because of previous evidence indicating structural heart disease leads to activation of the HPAA,²³ and because it is a group that is easy to define in a clinical setting. Nevertheless, it is also imperative to realize that dogs with advanced heart disease (ACVIM Stage C or D) do not represent the target population of dogs from which HC needs to be distinguished. Dogs with CHF may not have similar metabolic derangements, which could differentially affect cortisol metabolism resulting in the desired NAD-specific versus a HC-specific urine cortisol metabolite profile.

The diagnostic concept of a metabolomic approach implemented in our study has been introduced in human medicine.¹⁴ The concept involves the assessment of a determined set of related biomarkers instead of the analysis of a

single biomarker. Specifically, in Cushing syndrome, the urinary concentrations of steroid hormones have been suggested as novel biomarkers given that the disease results in elevated levels of cortisol, its altered pathway, and, possibly, altered concentrations of its metabolites.¹⁴ Several studies in human patients with Cushing disease have found significantly increased urinary levels of THF and decreased levels of THE,^{11,14} among other alterations in different steroid derivatives. Similarly, a study in human patients with critical illness found a decreased ratio of THE:THF because of a marked reduction of the urinary excretion of THE.² Contrary to the Cushing disease patients in one study¹⁴ and to the findings of our study, the critical patients in another study² had normal levels of urinary THF.^{2,14} These findings suggested that impaired cortisol clearance contributes to the hypercortisolemic state in critical illness.² Hypercortisolemia in critical illness has traditionally been attributed to an augmented increase in cortisol production by activation of the HPAA.^{1,5} However, other mechanisms have been suggested given the paradoxical dissociation between ACTH and cortisol observed in these patients.^{24,25} Possible mechanisms are increased adrenal ACTH-sensitivity, increase in cortisol production by non-ACTH-mediated mechanisms (i.e., cytokines, neuropeptides, catecholamines), and decreased cortisol clearance.^{2,16,24,25}

Previous studies have assessed the accuracy of screening tests for HC in the presence of moderate-to-severe illness.^{3,5,8}

In agreement with these studies, we found that urinary cortisol metabolites do not discriminate patients with NAD from patients with HC. Nevertheless, the cortisol- and cortisone-to-creatinine ratios significantly differentiated healthy dogs from the other groups (Fig. 2). Despite the statistical differences between group averages, the urinary fraction values cannot be used to classify an individual patient into a disease group. Our findings are consistent with previous literature¹ suggesting a high sensitivity and low specificity for this test. Our findings differ from those of a 2014 study that compared the clinical performance of different corticoid immunoassays and gas chromatography–mass spectrometry in healthy dogs and dogs with HC.⁷ In that study, both GC-MS and the immune assays exhibited decreased sensitivity (i.e., sensitivity of 37.5–75%) compared to previous studies that had sensitivity of nearly 100%.^{12,17,21} Possible explanations for the different results between that study and ours are differences in sample size as well as differences in the stages of HC. In our study, we only included dogs in which HC was a new diagnosis and the dogs were naïve to treatment. In the aforementioned study, the dogs were treated with trilostane for various periods of time.

Our study had several limitations. First, group sizes were small, which decreases the statistical power of the study. Second, we may have introduced a selection bias with substantial activation of the HPA axis by selecting animals with severe NAD.²³ In the future, it would be preferable to test the sensitivity and specificity of total, free, and conjugated cortisol metabolites against a control group of dogs with NAD and clinical presentation similar to that of dogs with HC. Third, we compared groups of dogs that were different in age, breed, length of disease, and disease stage. Although this is a limitation, it is also typical of a clinical setting in which a good diagnostic test would be applied to discriminate dogs with hypercortisolism from dogs with NAD. Fourth, we looked at a small number of cortisol metabolites in the urine; additional metabolites and ratios between metabolites might prove to have better discriminatory power. As well, the effect of prolonged storage at -80°C on the stability of urinary glucocorticosteroids and their metabolic derivatives and urine creatinine is largely unknown and was not evaluated in our study. In one study, cortisol plasma levels remained unchanged during a 10-y period when stored at -80°C .¹³ Also, 2 previous studies indicated that urine creatinine remained stable when frozen for a long period of time; at -20°C for up to 3 mo¹⁸ and at -20°C and -80°C for 12 mo.²²

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Supplementary material

Supplementary material for this article is available online.

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