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Circulating renin-angiotensin-aldosterone system activity in cats with systemic hypertension or cardiomyopathy

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Abstract

Background: Activity of the circulating renin-angiotensin-aldosterone system (RAAS) has not been comprehensively characterized in cats with systemic hypertension (SH) or cardiomyopathy (CM), and the effects of furosemide or amlodipine treatment on the RAAS have not been fully evaluated in cats.

Hypothesis/Objectives: To document RAAS activity in cats with SH or CM compared to healthy cats and determine how RAAS profiles change with furosemide or amlodipine treatment.

Animals: Sixty-six client-owned cats: 15 with SH (7 amlodipine-treated, 8 untreated), 17 with advanced CM (7 furosemide-treated, 10 not furosemide-treated), and 34 healthy cats.

Methods: Equilibrium concentrations of RAAS peptides and aldosterone were quantified in serum samples by liquid chromatography-mass spectrometry. Variables were compared between groups using Kruskal-Wallis analysis with post hoc Holms-corrected Dunn's testing.

Results: Compared with healthy cats, cats with CM had higher concentrations of angiotensin I, aldosterone, and plasma renin activity (all $P < .01$), and these differences remained significant ($P < .03$) after considering subgroups of untreated or furosemide-treated cats. Compared with healthy cats, untreated cats with SH showed no differences in RAAS biomarkers, whereas amlodipine-treated cats had higher concentrations of angiotensins I, II, III, IV, and 1-7, aldosterone, and plasma renin activity

Abbreviations: AA2, ratio of aldosterone to angiotensin II (marker of adrenal responsiveness to angiotensin II); ACE, angiotensin converting enzyme; ACEi, angiotensin converting enzyme inhibitor; ACE-S, ratio of angiotensin II to angiotensin I (marker of angiotensin-converting enzyme activity); ACVIM, American College of Veterinary Internal Medicine; ALD, aldosterone; Ang, angiotensin; AT1R, angiotensin type I receptor; AT2R, angiotensin type II receptor; BP, blood pressure; CKD, chronic kidney disease; CM, cardiomyopathy; FS, fractional shortening; LA : Ao, left atrium to aorta ratio; IVSd, interventricular septal thickness in diastole; IRIS, International Renal Interest Society; LVPWd, left ventricular posterior wall thickness in diastole; PRA, plasma renin activity; PRA-S, marker of plasma renin activity; RAAS, renin-angiotensin-aldosterone system; SH, systemic hypertension.

(all $P < .03$). Multivariable analysis determined that furosemide and amlodipine treatments were independent predictors of increased RAAS biomarker concentrations.

Conclusions and Clinical Importance: Cats with CM had increased RAAS activity, whereas cats with untreated SH did not. Furosemide and amlodipine both led to nonspecific activation of both classical and alternative RAAS pathways in cats.

KEYWORDS

amlodipine, angiotensin converting enzyme inhibitor, blood pressure, cardiac, congestive heart failure, feline, furosemide

1 | INTRODUCTION

Excessive activation of the renin-angiotensin-aldosterone system (RAAS) results in pathologic vascular inflammation and remodeling.[1] Studies in animals and humans have shown that angiotensin II (AngII) and aldosterone (ALD) cause proinflammatory effects and cardiovascular remodeling by regulating the expression of cytokines and chemokines in the kidneys, blood vessels, and heart,[2-4] and these pathophysiologic changes ultimately can result in end-organ cardiovascular damage.[5] Decreasing AngII and ALD concentrations thus is considered paramount in the management of cardiomyopathy (CM) and systemic hypertension (SH) in humans.[6-8]

Data regarding the extent and type of RAAS activation in common cardiovascular diseases of cats generally is lacking, including CM and SH. In cats with experimentally-induced left ventricular hypertrophy, increased markers of local tissue RAAS activity have been identified in the myocardium and are associated with myocardial fibrosis.[9] Cats with naturally-occurring asymptomatic CM also have higher plasma ALD concentrations compared to healthy cats.[10] Cats with SH secondary to chronic kidney disease (CKD) typically have higher ALD concentration and suppressed plasma renin activity (PRA) compared to healthy cats,[11,12] whereas in cats with hyperthyroidism, PRA and ALD concentrations do not differ based on presence or absence of concurrent SH.[13]

Treatments used for cardiovascular diseases also can upregulate the RAAS. Furosemide, a loop diuretic and mainstay of treatment for congestive heart failure (CHF), is a potent activator of the RAAS[14,15] by decreasing circulating volume and thus blood flow to the glomerular afferent arterioles, as well as by increasing natriuresis, which activates renin secretion. Treatment-induced RAAS activation also can occur with use of the calcium-channel blocker amlodipine, used for the treatment of SH in Europe[16] and recommended as a first-line treatment for SH in cats.[17,18] It is well-established that amlodipine activates the RAAS in dogs,[19,20] potentially by decreased pressure in the afferent arterioles stimulating renin secretion and gene expression,[21] and recent evidence suggests the same may be true for cats.[12,22]

The RAAS profiles of cats with CM and SH thus are incompletely characterized and are further complicated by concomitant treatments commonly prescribed in these diseases. Furthermore, most existing veterinary research on the RAAS in cats is based primarily on angiotensin-converting enzyme (ACE) activity and urinary aldosterone-to-creatinine ratio, which do not provide an accurate reflection of circulating RAAS activity.[23-29] A more comprehensive understanding of RAAS activation in cats with SH and CM could inform recommendations for RAAS-mitigating treatments at various stages of disease and in conjunction with other treatments.

Our primary objective was to characterize a comprehensive array of circulating RAAS constituents in healthy cats and cats with SH or CM (both treated and untreated), and to compare RAAS activity between disease groups and treatment subgroups. A secondary objective was to explore correlations between circulating RAAS analytes and measures of disease severity (blood pressure [BP], echocardiographic indices) and renal function test results. We hypothesized that profiles of RAAS activity would be higher in cats with CM or SH compared to healthy cats, and that amlodipine- or furosemide-treated cats would have higher concentrations of circulating RAAS activity than untreated cats with similar diseases.

2 | MATERIALS AND METHODS

We utilized surplus serum samples that originally had been obtained for a prior investigation.[30] Procedures for original sampling were approved by the Institutional Animal Care and Use Committees at Iowa State University, and informed owner consent was obtained for each cat.

Client-owned cats presented to the Iowa State University Lloyd Veterinary Medical Center were prospectively recruited into the CM and SH groups between November 2016 and April 2018. Nonhypertensive (systolic blood pressure <160 mm Hg) and euthyroid cats were entered into the CM group based on echocardiographic evidence of CM and at least moderate left atrial enlargement (right parasternal short-axis left atrium-to-aorta ratio [LA : Ao] > 1.7). Cats were entered into the SH group if they had severe hypertension (>180 mm Hg, systolic) deemed not to be situational based either on repeated measurements across different sessions or evidence of target organ damage. Cats with severe systemic disease, including active inflammatory bowel disease, systemic neoplasia, or widespread infectious or inflammatory disease, were excluded. Healthy cats owned by students, staff, and local community residents were recruited during approximately the same time period. Healthy cats were matched to cats in the CM group based on sex and age (within 6 months). Cats

were deemed healthy based on physical examination, blood pressure measurement (<160 mm Hg), echocardiography, and serum creatinine, blood urea nitrogen, and electrolyte concentrations (performed within 6 months or repeated at the time of study sample collection), as well as serum total thyroxine concentrations for cats >8 years of age.

Clinical data collected included signalment, body weight, cardiovascular examination findings, indirect systolic blood pressure measurement (Doppler method from the coccygeal or dorsal pedal artery using a standard protocol[17] by an experienced operator, obtaining an average of at least 3-5 readings), and echocardiography (2-dimensional [2-D], M-mode, color flow, and spectral Doppler). Echocardiography was performed by a board-certified cardiologist or a cardiology resident under the direct supervision of a board-certified cardiologist. Cardiomyopathy phenotype and stage were classified based on current consensus guidelines.[31] Echocardiographic measurements analyzed were: cardiomyopathy phenotype, American College of Veterinary Internal Medicine (ACVIM) disease stage (B2 or C),[31] LA : Ao, interventricular septal thickness in diastole (IVSd), left ventricular posterior wall thickness in diastole (LVPWd), and left ventricular (LV) fractional shortening (FS), all obtained from 2-D right parasternal short-axis views.

Venous blood samples (2 mL) were obtained for analyses pertaining to the primary study. Serum was separated, and surplus serum was frozen and stored at −80°C until RAAS fingerprint analysis. Serum blood urea nitrogen, creatinine, and thyroid concentrations were performed in most cats as part of this clinical visit, and these data were recorded when available. Stage of CKD was assigned based on a modification of the International Renal Interest Society (IRIS) guidelines (categorized as suggested by IRIS guidelines based on single determination of serum creatinine concentration, with no substaging attempted; http://www.iris-kidney.com/pdf/IRIS_Staging_of_CKD_modified_2019.pdf). All cardiovascular medications that cats were receiving at the time of blood sampling were recorded, including dose and duration of medication prescription.

TABLE 1 Demographic, clinical, and treatment variables for 66 cats classified as healthy or diagnosed with cardiomyopathy (CM) or systemic hypertension (SH)

	Data by disease group			*P*-values for group comparisons			
Variable	**Healthy (n = 34)**	**SH (n = 15)**	**CM (n = 17)**	**Overall**	**Healthy vs SH**	**Healthy vs CM**	**CM vs SH**
Male (N, %)	29/34 (85%)	7/15 (47%)	15/17 (88%)	**.006**	**.04**	1	.06
Murmur (N, %)	8/34 (24%)	9/15 (60%)	6/17 (35%)	**.05**	.1	.59	.59
Arrhythmia (N, %)	2/34 (5.9%)	1/15 (6.7%)	7/17 (41%)	**.002**	1.00	**.02**	.13
Age (years)	10.6 (7.9-13.7)	14.6 (12.5-15.9)	9.0 (6.5-11.1)	**<.0001**	**.01**	.29	**.002**
Weight (kg)	5.40 (4.39-5.98)	4.00 (3.71-4.92)	4.72 (3.70-5.45)	**.03**	**.04**	.34	.29
Body condition score (1-9)	6.0 (5.0-7.0)	5.0 (4.0-6.5)	5.0 (4.0-6.0)	.13			
Heart rate (beats/min)	184 (180-200)	210 (220-240)	210 (200-220)	**.01**	**.02**	**.03**	.75
Blood pressure (mm Hg)	143 (131-156)	200 (175-231)	130 (120-150)	**<.0001**	**.0005**	.28	**.0001**
Left atrium to aorta ratio	1.13 (0.99-1.19)	1.18 (1.10-1.36)	2.25 (1.81-2.55)	**<.0001**	.21	**<.0001**	**.0002**
IVSd (cm)	0.46 (0.42-0.49)	0.53 (0.43-0.59)	0.53 (0.47-0.75)	**.01**	.13	**.01**	.45
LVIDd (cm)	1.47 (1.36-1.57)	1.44 (1.18-1.57)	1.56 (1.28-1.71)	.64			
LVPWd (cm)	0.44 (0.42-0.48)	0.49 (0.43-0.56)	0.72 (0.52-0.90)	**<.0001**	.1	**.0002**	.15
LVFS (%)	54.5 (47.8-60.6)	55.6 (52.0-59.7)	43.4 (29.5-54.5)	**<.0001**	.44	**.006**	**.006**
Blood urea nitrogen (mg/dL) (n = 52)	24 (20-30)	27 (23-34)	27 (25-31)	.37			
Creatinine (mg/dL) (n = 52)	1.4 (1.1-2.0)	1.8 (1.5-2.6)	1.7 (1.3-2.0)	.38			
Chronic kidney disease (N, %) (n = 52)	10/23 (43%)	8/14 (57%)	9/15 (60%)	.55			
IRIS stage (n = 52)	1 : 13/23 (57%)	1 : 6/14 (43%)	1 : 6/15 (40%)	.48			
	2 : 8/23 (35%)	2 : 7/14 (50%)	2 : 8/15 (53%)				
	3 : 2/23 (8.7%)	3 : 1/14 (7.1%)	3 : 1/15 (6.7%)				
Any treatment (N, %)	0/34 (0%)	7/15 (47%)	11/17 (65%)	**<.0001**	**.0002**	**<.0001**	.5
ACE inhibitor (N, %)	0/34 (0%)	4/15 (27%)	4/17 (24%)	**.008**	**.03**	**.03**	1
Furosemide (N, %)	0/34 (0%)	0/15 (0%)	7/17 (41%)	**<.0001**	–	**.0006**	**.02**
Amlodipine (N, %)	0/34 (0%)	7/15 (47%)	0/17 (0%)	**<.0001**	**.0002**	–	**.006**

Note: Continuous data are presented as median (interquartile range), while categorical data are presented as number and percentage of cats with each finding. The number of cats with data included is noted in the column header and as denominator values for renal variables with incomplete data sets. *P*-values are shown for overall comparison of disease groups and for post hoc pairwise tests when overall comparison was significant; significant differences ($P < .05$) are indicated in bold.

Abbreviations: IRIS, International Renal Interest Society; IVSd, interventricular septal wall thickness in diastole; LVFS, left ventricular fractional shortening; LVIDd, left ventricular internal dimension in diastole; LVPWd, left ventricular posterior wall thickness in diastole.

TABLE 2 Dose and duration of angiotensin converting enzyme (ACE) inhibitor, furosemide, and amlodipine treatment at time of RAAS biomarker determination in the study sample, including 17 cats with cardiomyopathy (CM) and 15 cats with systemic hypertension (SH)

Class/drug		Number of cats (disease group)	Dose (mg/kg/day)	Duration (days)
ACE inhibitor	Enalapril	2 (CM)	0.43 (0.32-0.54)	1182 (8-2356)
	Benazepril	6 (2 CM, 4 SH)	0.64 (0.34-0.96)	237 (25-513)
Furosemide	Intravenous	4 (CM)	4.0 (2.0-8.0)	0.5 (0–1)
	Oral	3 (CM)	2.4 (2.3-7.1)	21 (18-41)
Amlodipine		7 (SH)	0.36 (0.24-0.81)	269 (5-1182)

Note: Doses are presented as median (range).

TABLE 3 Biomarkers of the renin-angiotensin-aldosterone system in 66 cats classified as healthy or diagnosed with cardiomyopathy (CM) or systemic hypertension (SH)

	Data by disease group			*P*-values for group comparisons			
Variable	Healthy (n = 34)	SH (n = 15)	CM (n = 17)	Overall	Healthy vs SH	Healthy vs CM	CM vs SH
AngI	37.8 (22.5-60.1)	103.2 (28.2-513.5)	178.5 (89.9-409.3)	**<.0001**	**.02**	**<.0001**	.17
AngII	122.6 (76.0-211.6)	188.9 (49.9-322.6)	159.7 (126.7-725.3)	.2			
ALD	148.4 (102.0-182.8)	239.6 (138.1-373.6)	318.6 (240-.3-700.2)	**<.0001**	.1	**.0001**	.06
Ang1-7	19.9 (13.5-37.2)	65.8 (18.5-225.7)	109.6 (48.7-170.6)	**<.0001**	**.01**	**.0001**	.24
Ang1-5	136.1 (54.6-224.3)	119.1 (51.9-260.3)	466.2 (73.4-668.3)	.07			
AngIII	7.48 (2.5-13.4)	10.30 (2.50-25.55)	9.40 (4.40-19.80)	.33			
AngIV	2.95 (2.00-6.15)	6.60 (2.00-12.50)	4.31 (2.80-19.10)	.08			
PRA-S	156.8 (106.2-286.4)	318.4 (76.9-1049.7)	325.2 (220.9-997.1)	**<.0001**	.08	**.004**	.39
ACE-S	3.35 (2.68-3.76)	1.18 (0.62-2.92	1.21 (0.96-1.87)	**<.0001**	**.002**	**<.0001**	.23
AA2	1.11 (0.77-2.03)	1.25 (0.69-2.13)	1.90 (1.71-2.79)	.42			

Note: Data for all cats are presented, including both treated and untreated cats in the CM or SH groups. Units for all biomarker concentrations are pmol/L, and ratios (ACE-S and AA2) are unitless. Continuous data are presented as median (interquartile range). *P*-values are shown for overall comparison of disease groups and for post hoc pairwise tests when overall comparison was significant; significant differences ($P < .05$) are indicated in bold.
Abbreviations: AA2, aldosterone to angiotensin II ratio (marker of adrenal responsiveness to angiotensin II); ACE-S, marker of angiotensin-converting enzyme activity; ALD, aldosterone; Ang, angiotensin; PRA-S, marker of plasma renin concentration.

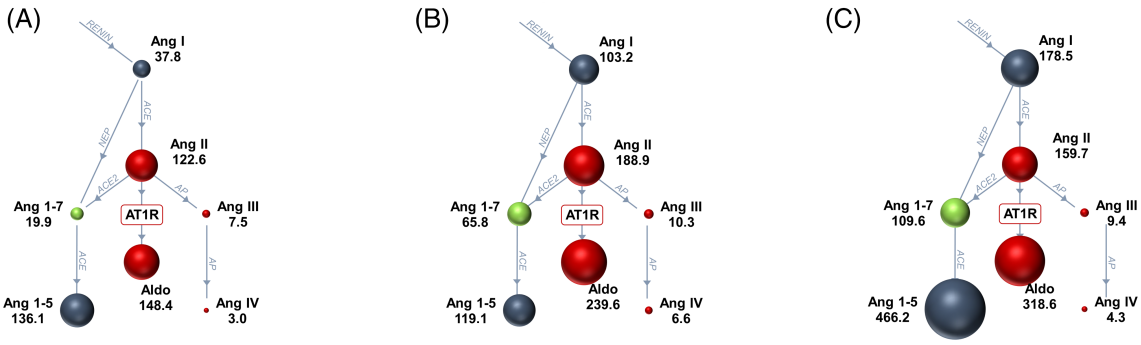

FIGURE 1 Serum equilibrium concentrations and relationships of angiotensin peptides and aldosterone (RAAS fingerprints) in healthy cats (n = 34; A) compared to cats with systemic hypertension (n = 15; B) or cardiomyopathy (n = 17; C). Disease groups include both treated and untreated cats. Sizes of circles are proportional to the median concentration (pmol/L) of each analyte. Ang, angiotensin; Aldo, aldosterone; AT1R, angiotensin type 1 receptor

Equilibrium concentrations of angiotensin I (AngI), AngII, angiotensin III (AngIII), angiotensin IV (AngIV), angiotensin 1-7 (Ang1-7), and angiotensin 1-5 (Ang1-5) as well as ALD were quantified in serum samples by liquid chromatography-mass spectrometry/mass spectroscopy performed at a commercial laboratory (Attoquant Diagnostics, Vienna, Austria), using previously validated and described methods,[32-34] after ex vivo equilibration. Briefly, samples were spiked with a stable isotope-labeled internal standard for

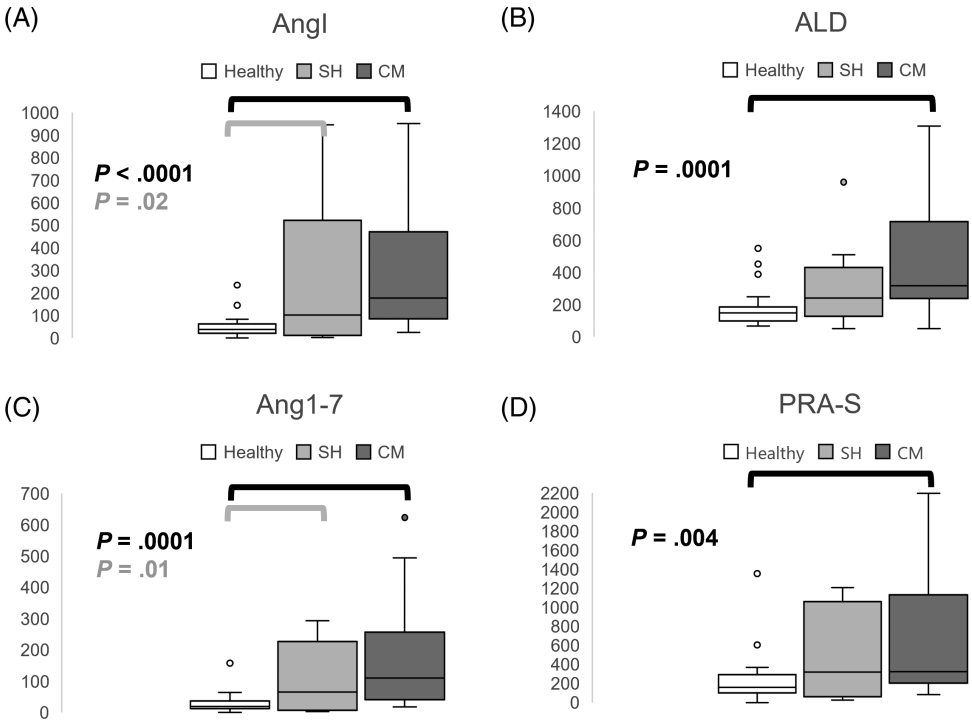

FIGURE 2 Box-and-whisker plots showing serum equilibrium concentrations of selected RAAS peptides by disease group in healthy cats (n = 34) compared to cats with systemic hypertension (n = 15) or cardiomyopathy (n = 17). Disease groups include both treated and untreated cats. Units for all RAAS biomarker concentrations are pmol/L. Boxes represent the interquartile range while the horizontal line in each box represents the group median, whiskers represent minimum and maximum values, and outliers (greater than 1.5 times the interquartile range above the 3rd quartile) are plotted as dots. *P*-values for significant differences between groups are shown. ALD, aldosterone; Ang, angiotensin; CM, cardiomyopathy; PRA-S, marker of plasma renin activity; SH, systemic hypertension

TABLE 4 Biomarkers of the renin-angiotensin-aldosterone system in 17 cats with cardiomyopathy (CM), based on whether cats received furosemide therapy prior to sampling

	Data by disease group and treatment			Adjusted *P*-values		
Variable	CM group no furosemide (n = 10)	CM group furosemide (n = 7)	Healthy cats (n = 34)	CM cats: furosemide vs no furosemide	CM no furosemide vs healthy cats	CM furosemide vs healthy cats
AngI	156 (85.0-233.8)	533.5 (81.2-826.6)	37.8 (22.5-60.1)	.30	**<.0001**	**.003**
AngII	144.1 (78.3-234.4)	725.3 (139.7-900.9)	122.6 (76.0-211.6)	.30	.73	**.03**
ALD	282.7 (201.7-485.1)	687.3 (264.8-1287.3)	148.4 (102.0-182.8)	.30	**.01**	**<.0001**
Ang1-7	83.2 (44.9-131.6)	343.7 (31.2-494.9)	19.9 (13.5-37.2)	.30	**.001**	**.003**
Ang1-5	288.0 (40.6-571.4)	668.3 (76.6-1264.9)	136.1 (54.6-224.3)	.30	.31	**.03**
AngIII	6.1 (4.0-12.4)	19.8 (7.1-41.9)	7.48 (2.5-13.4)	.30	.73	.11
AngIV	3.65 (2.25-6.47)	19.1 (3.4-32.4)	2.95 (2.00-6.15)	.30	.60	**.04**
PRA-S	305.3 (180.9-515.1)	1261.7 (220.9-1727.4)	156.8 (106.2-286.4)	.30	**.02**	**.01**
ACE-S	1.15 (0.59-1.96)	1.31 (1.09-1.72)	3.35 (2.68-3.76)	.67	**<.0001**	**.0004**
AA2	2.21 (1.72-3.02)	1.78 (0.67-2.95)	1.11 (0.77-2.03)	.60	.22	.82

Note: Units for all biomarker concentrations are pmol/L, and ratios (ACE-S and AA2) are unitless. Continuous data are presented as median (interquartile range). *P*-values are shown for treatment comparisons within the CM group, as well as for comparisons of CM treatment subgroups to a group of healthy cats (n = 34). Significant differences (*P* < .05) are indicated in bold.

Abbreviations: AA2, aldosterone to angiotensin II ratio (marker of adrenal responsiveness to angiotensin II); ACE-S, marker of angiotensin-converting enzyme activity; ALD, aldosterone; Ang, angiotensin; PRA-S, marker of plasma renin concentration.

each angiotensin and a deuterated internal standard for ALD (aldosterone D4) after equilibration, and analytes were extracted using C18-based solid-phase extraction. Extracted samples were analyzed using mass spectrometry analysis with a reversed-analytical column (Acquity UPLC C18, Waters) operating in line with a XEVO TQ-S triple quadrupole mass spectrometer (Waters Xevo TQ/S, Milford, MA) in multiple reaction monitoring mode. Internal standards were used to correct for analyte recovery across the sample preparation procedure in each individual sample. Analyte concentrations were calculated from integrated chromatograms considering the corresponding response factors determined in appropriate calibration curves in serum matrix, when integrated

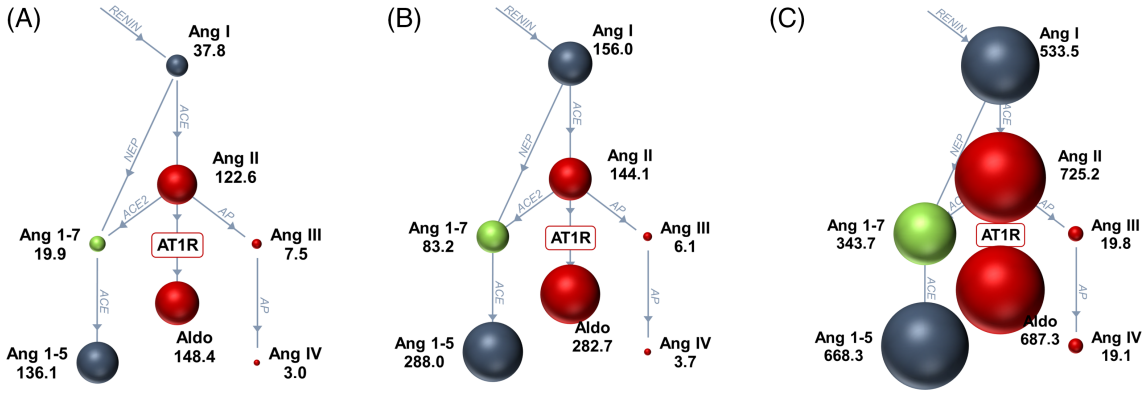

FIGURE 3 Serum equilibrium concentrations and relationships of angiotensin peptides and aldosterone (RAAS fingerprints) in healthy cats (n = 34; A) compared to cats with cardiomyopathy that were either not receiving furosemide treatment (n = 10; B) or furosemide-treated (n = 7; C). Sizes of circles are proportional to the median concentration (pmol/L) of each analyte. Ang, angiotensin; Aldo, aldosterone; AT1R, angiotensin type 1 receptor

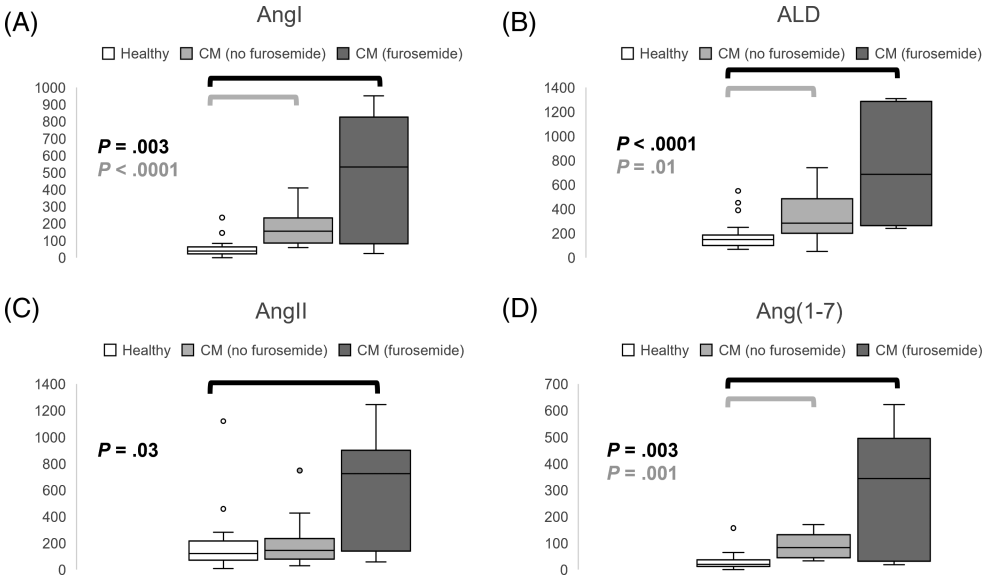

FIGURE 4 Box-and-whisker plots showing serum equilibrium concentrations of selected RAAS peptides by disease group in healthy cats (n = 34) compared to cats with cardiomyopathy not treated with furosemide (n = 10) and cats with cardiomyopathy treated with furosemide (n = 7). Units for all RAAS biomarker concentrations are pmol/L. Boxes represent the interquartile range while the horizontal line in each box represents the group median, minimum and maximum values, and outliers (greater than 1.5 times the interquartile range above the 3rd quartile), and outliers are plotted as dots. *P*-values for significant differences between groups are shown. ALD, aldosterone; Ang, angiotensin; CM, cardiomyopathy; PRA-S, marker of plasma renin activity

signals exceeded a signal-to-noise ratio of 10. The lower limits of quantification for the analytes in feline serum were 3 pmol/L (AngI), 2 pmol/L (AngII), 2.5 pmol/L (AngIII), 2 pmol/L (AngIV), 2.5 pmol/L (Ang1-7), 2 pmol/L (Ang1-5), and 13.9 pmol/L (aldosterone), respectively. Angiotensin-based markers for renin (PRA-S) and angiotensin converting enzyme (ACE-S), were derived from AngII and AngI concentrations by calculating their sum and ratio, respectively,[34,35] whereas the ratio of aldosterone/AngII (AA2-ratio) was calculated to assess adrenal responsiveness after AngII signaling resulting in the release of ALD[36] as described by the analytical laboratory.

2.1 | Statistical analysis

Statistical analyses were performed using commercial software (R software, version 3.5.1, R Foundation for Statistical Computing, Vienna Austria; IBM SPSS Statistics 25.0, IBM Corporation, Armonk, New York). Normality of data was determined using the Shapiro-Wilk test. Quantitative data were summarized as mean ± SD for normally distributed data, and as median (interquartile range) for nonnormally distributed data. Variables were compared between disease and treatment groups using Kruskal-Wallis testing with post hoc Holms-corrected Dunn's test of pairwise comparisons. *P*-values <.05 were

TABLE 5 Biomarkers of the renin-angiotensin-aldosterone system in 15 cats with systemic hypertension (SH), based on whether cats received amlodipine therapy prior to sampling

Variable	Data by disease group and treatment			Adjusted *P*-values		
	SH group No amlodipine (n = 8)	SH group Amlodipine (n = 7)	Healthy cats (n = 34)	SH cats: amlodipine vs no amlodipine	SH no amlodipine vs healthy cats	SH amlodipine vs healthy cats
AngI	46.15 (11.6-97.12)	522 (436.6-2686.6)	37.8 (22.5-60.1)	**.02**	.6	**.0008**
AngII	114.6 (46.25-189.7)	382.3 (220.2-478.9)	122.6 (76.0-211.6)	.28	.58	**.02**
ALD	163.1 (124.2-228.8)	430.2 (261.7-495.2)	148.4 (102.0-182.8)	.45	.65	**.008**
Ang1-7	34.5 (6.525-53.4)	226.6 (220.2-831.5)	19.9 (13.5-37.2)	**.03**	.5	**.001**
Ang1-5	162.9 (53.52-228.43)	94.3 (63.8-266.4)	136.1 (54.6-224.3)	.47	.89	.91
AngIII	6.05 (2.5-10.375)	34.7 (12.1-38.05)	7.48 (2.5-13.4)	.09	.69	**.02**
AngIV	2.75 (2.0-5.85)	14.0 (9.95-22.55)	2.95 (2.00-6.15)	.09	.99	**.003**
PRA-S	162.45 (61.12-294.93)	1059 (870.1-3019.8)	156.8 (106.2-286.4)	**.04**	1	**.002**
ACE-S	2.92 (1.81-4.71)	0.34 (0.185-1.05)	3.35 (2.68-3.76)	**.01**	.71	**<.0001**
AA2	1.525 (1.083-2.658)	1.07 (0.43-1.58)	1.11 (0.77-2.03)	.18	.48	.51

Note: Units for all biomarker concentrations are pmol/L, and ratios (ACE-S and AA2) are unitless. Continuous data are presented as median (interquartile range). *P*-values are shown for treatment comparisons within the SH group, as well as for comparisons of SH treatment subgroups to a group of healthy cats (n = 34). Significant differences ($P < .05$) are indicated in bold.
Abbreviations: AA2, aldosterone to angiotensin II ratio (marker of adrenal responsiveness to angiotensin II); ACE-S, marker of angiotensin-converting enzyme activity; ALD, aldosterone; Ang, angiotensin; PRA-S, marker of plasma renin concentration.

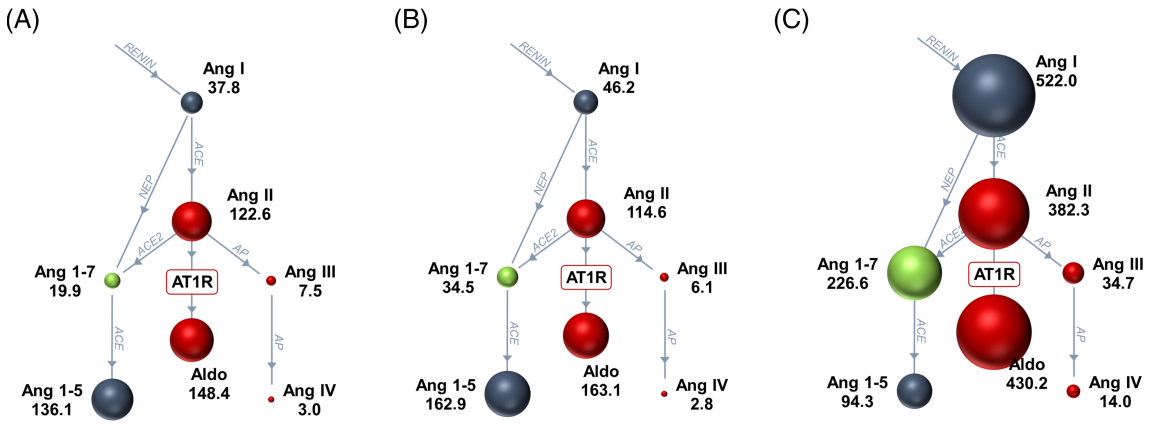

FIGURE 5 Serum equilibrium concentrations and relationships of angiotensin peptides and aldosterone (RAAS fingerprints) in healthy cats (n = 34) compared to cats with systemic hypertension that were either untreated (n = 8) or treated with amlodipine (n = 7). Sizes of circles are proportional to the median concentration (pmol/L) of each analyte. Ang, angiotensin; Aldo, aldosterone; AT1R, angiotensin type 1 receptor

considered significant. Correlations between study variables were assessed using Spearman's correlations (continuous data) or the point biserial method (categorical data). Strength of correlations was classified as negligible (*r*-value of 0-.09), weak (.1-.39), moderate (.4-.69), strong (.7-.89), or very strong (.9-1).[37,38] Logistic regression modeling was performed to determine the influence of disease group and treatment on RAAS metabolites. The following categorical explanatory variables initially were considered in the models: disease group (healthy, SH, CM), furosemide treatment (yes/no), amlodipine treatment (yes/no), and ACEi treatment (yes/no). Biomarkers of the RAAS were considered as continuous outcome variables. Univariable logistic regression analysis initially was performed to obtain *P*-values for each covariate, and variables with *P*-values <.1 were included for consideration in multivariable models. Final modeling was performed with backward selection utilizing the Akaike information criterion.

3 | RESULTS

Serum samples from 66 cats were analyzed, including 15 cats with SH, 17 cats with CM, and 34 healthy cats. The most common breeds were domestic shorthair (43/66, 65%) and domestic longhair (14//66, 21%); other breeds included Maine Coon (n = 2), Persian (n = 2), Siamese (n = 2), and 1 each of Himalayan, Savannah, and Turkish Van.

The SH group comprised 8 cats with a new diagnosis of SH (untreated) and 7 cats receiving antihypertensive treatment

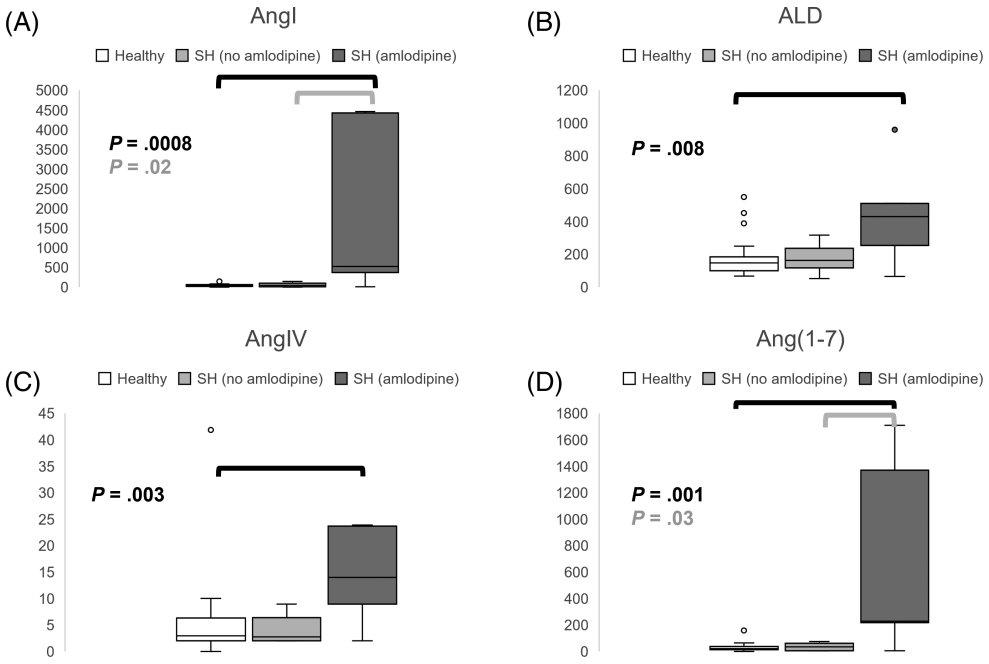

FIGURE 6 Box-and-whisker plots showing serum equilibrium concentrations of selected RAAS peptides by disease group in healthy cats (n = 34) compared to cats with untreated systemic hypertension (n = 8) or systemic hypertension treated with amlodipine (n = 7). Units for all RAAS biomarker concentrations are pmol/L Boxes represent the interquartile range while the horizontal line in each box represents the group median, minimum and maximum values, and outliers (greater than 1.5 times the interquartile range above the 3rd quartile) are plotted as dots. *P*-values for significant differences between groups are shown. ALD, aldosterone; Ang, angiotensin

(3 receiving amlodipine monotherapy, 4 receiving amlodipine plus angiotensin converting enzyme inhibitor [ACEi]). Of the 7 treated cats with SH, BP was adequately controlled (<160 mm Hg) at the time of sampling in 3 cats, whereas 4 cats had uncontrolled BP (>160 mm Hg) at the time of sampling. Underlying systemic diseases associated with SH in this group were CKD (n = 11) and hyperthyroidism (n = 2); SH was presumed to be idiopathic in 2 cats with no obvious predisposing disease.

The CM group comprised 6 cats with preclinical disease (ACVIM stage B2), 8 cats with active CHF (ACVIM stage C decompensated), and 3 cats with previous history of CHF controlled on medications (ACVIM stage C compensated). Of the 11 treated cats with CM, 7 were receiving furosemide and 4 were receiving ACEi; no cat was receiving both furosemide and ACEi at time of sampling. Other medications prescribed before sampling in the CM group included pimobendan (n = 3), clopidogrel (n = 3), apixaban (n = 2), and atenolol (n = 1). No cat was receiving an angiotensin receptor blocker (eg, telmisartan) at the time of sampling.

Basic demographics, clinical and echocardiographic findings, and treatments by disease group are summarized in Table 1. Statistical comparisons identified significant differences between disease groups in many clinical variables, reflective of typical demographics and pathophysiology of CM compared to SH. Renal function test results were available for a subset of 52 cats (23 healthy, 15 CM, and 14 SH) and are presented in Table 1, with no significant differences between groups. Details regarding dose and duration of the most common cardiovascular treatments (ACEi, furosemide, and amlodipine) are provided in Table 2. Other concomitant treatments received by study cats included pimobendan (1.25 mg PO q12h, n = 4), clopidogrel (18.75 mg PO q24h, n = 4), apixaban (0.3125 mg PO q24h, n = 2), methimazole (2.5 mg PO q12h, n = 2), and 1 cat each received atenolol, diltiazem, and aspirin.

Biomarkers of the RAAS by disease group (including both treated and untreated cats), as well as significant differences between disease groups, are shown in Table 3 and Figures 1 and 2. Compared to healthy cats, median concentrations of AngI were 4.7 times higher in CM cats and 2.7 times higher in SH cats. Median concentrations of Ang1-7 were 5.5 times higher in CM compared to healthy cats and 3.3 times higher in SH compared to healthy cats. Cats with CM had median ALD concentrations and PRA-S 2.1 times higher compared to healthy cats. Median ACE-S was 2.8 times lower in CM or SH cats compared to healthy cats.

Differences in RAAS biomarker concentrations within the CM group based on treatment with furosemide are shown in Table 4 and Figures 3 and 4. Compared to healthy cats, cats with CM not treated with furosemide had higher median concentrations of AngI (4.1 times higher), ALD (1.9 times higher), Ang1-7 (4.2 times higher), and PRA-S (1.9 times higher), and lower median ACE-S (2.9 times lower). Compared to healthy cats, furosemide-treated CM cats had higher median concentrations of AngI (14.1 times higher), AngII (5.9 times higher), ALD (4.6 times higher), Ang1-7 (17.3 times higher), Ang1-5 (4.9 times higher), AngIV (6.5 times higher), and PRA-S (8.0 times higher), and lower median ACE-S (2.6 times lower). Within the CM group, no significant differences in RAAS biomarkers were found based on whether or not cats were receiving furosemide treatment.

Differences in RAAS biomarker concentrations in the SH group based on treatment with amlodipine are shown in Table 5 and Figures 5 and 6. Untreated cats with SH showed no differences in RAAS biomarkers compared to heathy cats. Compared to healthy cats, amlodipine-treated cats had significantly higher median concentrations of AngI (13.8 times higher), ALD (2.9 times higher), Ang1-7 (median 11.4 times higher), AngIV (4.8 times higher), and PRA-S (6.8 times higher). Compared to untreated cats with SH, amlodipine-treated cats

TABLE 6 Results of multivariable logistic regression modeling to predict RAAS biomarker concentrations in 66 cats classified as healthy (n = 34) or diagnosed with cardiomyopathy (CM, n = 17) or systemic hypertension (SH, n = 15)

Outcome variable	Significant predictor variables	Parameter estimate	95% confidence interval of parameter estimate	*P*-value
AngI	Furosemide	476.6	12.1-941.1	.04
	Amlodipine	1126.6	603-1650.2	<.0001
	ACEi	821.2	326.6-1315.8	.002
AngII	Furosemide	408.3	225.9-590.7	<.0001
	Amlodipine	184.4	2-366.8	.048
ALD	Furosemide	523.1	356.1-690.1	<.0001
	Amlodipine	219.5	52.4-386.5	.01
Ang1-7	Furosemide	277.4	107.6-448.4	.002
	Amlodipine	404.4	213.1-595.7	<.0001
	ACEi	267.7	87-448.4	.004
Ang1-5	Furosemide	601.6	347.6-855.5	<.0001
AngIII				
AngIV	Furosemide	12.1	5.8-18.3	.0003
	Amlodipine	9.9	3.7-16.2	.002
PRA-S	Furosemide	878.3	335.0-1421.5	.002
	Amlodipine	1353.9	741.5-1966.2	<.0001
	ACEi	734.6	156.3-1313	.01
ACE-S	Amlodipine	−3.32	−5.6 to 1.08	<.0001
	Disease group (CM)	−2.1	−3.7 to −1.1	<.0001
AA2	Amlodipine	13.5	4.7-22.2	.003
	ACEi	−8.4	−16.7 to −0.1	.047

Note: Categorical explanatory variables (disease group and treatment with furosemide, amlodipine, or angiotensin converting enzyme inhibitor [ACEi]) were considered in models to predict each continuous outcome variable (RAAS biomarker concentration). Predictive variables found to be significant in the final model are presented with associated *P*-values and parameter estimates.
Abbreviations: AA2, aldosterone to angiotensin II ratio (marker of adrenal responsiveness to angiotensin II); ACE-S, marker of angiotensin-converting enzyme activity; ALD, aldosterone; Ang, angiotensin; PRA-S, marker of plasma renin concentration.

had higher median concentrations of AngI (11.3 times higher), Ang1-7 (median 6.6 times higher), and PRA-S (6.5 times higher).

Correlation analyses identified only negligible or weak correlations ($|r_s|$ < .4) between RAAS biomarkers and cat sex, age, weight, or renal function test results (see Table S1). Within the SH group, BP was strongly negatively correlated with AngI, Ang1-7, AngIV, and PRA-S, and moderately negatively correlated with AngII, AngIII and ACE-S. Within the CM group, LA : Ao was strongly positively correlated with ALD, and cardiac disease stage was moderately positively correlated with AngI, ALD, Ang1-7, and PRA-S. Interventricular septal thickness in diastole was moderately negatively correlated with AngI, Ang1-7, and PRA-S.

Final multivariable models constructed to predict RAAS biomarkers are shown in Table 6. Furosemide and amlodipine treatment were significant independent predictors of nearly all RAAS biomarkers, whereas ACEi treatment was an additional predictive factor for AngI, Ang1-7, PRA-S, and AA2 ratio. Disease group was not a significant predictor of any RAAS biomarker except for ACE-S.

4 | DISCUSSION

We documented significant increases in circulating RAAS biomarker concentrations in cats with CM or SH compared to healthy cats. Subanalyses by treatment determined that untreated cats with CM had increased circulating RAAS activity compared to healthy cats whereas untreated cats with SH did not. Furthermore, treatment with either furosemide or amlodipine led to significant increases in activity of RAAS metabolites compared to untreated cats with the same disease. These results, as well as multivariable modeling, suggest that cardiovascular treatment was more predictive of RAAS activity than disease group in our study sample.

Untreated cats with CM in our study had evidence of increased RAAS activity compared to healthy cats, including increased concentrations of biomarkers of the classical (eg, AngI and ALD) as well as alternative (eg, Ang1-7) RAAS pathways. This finding is consistent with a previous study of Maine Coon cats with asymptomatic CM in which a majority of cats had increased plasma ALD concentration compared to reference range in normal cats,[10] but differs from a more recent study in which the comprehensive RAAS fingerprint did not differ between healthy cats and untreated cats with asymptomatic CM.[33] This disparity may reflect differences in study populations (research colony vs client-owned cats) or CM disease severity between studies. Previous studies enrolled only asymptomatic cats, whereas 11/17 cats in our study had current or previous CHF. Overall, these findings suggest that advanced CM (LA : Ao > 1.7, with or without CHF) is associated with substantial increases in RAAS activity in cats, suggesting a potential pathophysiologic rationale for RAAS-mitigating treatments in these patients.

Furosemide treatment was associated with further stimulation in RAAS activity in cats with CM compared to healthy cats, again involving increased activity of both the classical (AngI, AngII, ALD) and alternative (Ang1-7, Ang1-5) pathways. This finding is not surprising, given that RAAS activation is a well-established side effect of furosemide treatment in dogs. Indeed, furosemide treatment is utilized as a pharmacologic model of RAAS activation in this species.[39-41] A recent study in healthy dogs characterized comprehensive RAAS fingerprints before and after PO furosemide administration, and identified a pattern of RAAS activity identical to that observed in furosemide-treated cats in our study (increased AngI, AngII, ALD, Ang1-7, Ang1-5, and AngIV).[42] These results suggest that the impact of furosemide on the RAAS is very similar between dogs and cats, and that RAAS-mitigating

treatments might be indicated in cats with advanced CM regardless of the use of furosemide, because cats with advanced CM had significantly increased RAAS activity even without furosemide treatment. The RAAS profiles were not statistically different based on furosemide treatment within the CM group, likely reflecting a combination of increased background RAAS activity in untreated CM cats, small sample size, and wide variability in RAAS biomarker concentrations.

Untreated cats with SH showed no differences in RAAS metabolite concentrations compared to healthy cats. This finding was unexpected given that SH in humans consistently has been linked to activation of both circulating and local renal tissue RAAS.[43,44] However, results of our study are consistent with a previous study of cats that showed no difference in plasma ALD concentration based on presence or absence of SH in cats with hyperthyroidism.[13] Other studies of cats with untreated SH secondary to CKD have shown increased plasma ALD concentration compared to healthy cats, but no differences in AngI or PRA.[11,12] Discrepancies among studies and between species might reflect differences in the underlying disease causing SH (CKD and hyperthyroidism in cats compared to idiopathic SH in humans), variable method of RAAS quantification, local mechanisms of blood pressure regulation in the kidney, or a combination of these factors. Duration and severity of SH also might differ between studies of cats. Although echocardiographic measurements of LV wall thickness and LA : Ao (indicators of target organ damage) were similar to previously published results for cats with SH,[45,46] median LVPWd in our study was lower than that reported in some other studies of SH in cats.[47,48] Because increased RAAS activity often is cited as a potential mechanism for development of SH in people,[43,44] the lack of difference between RAAS biomarker concentrations between SH and healthy cats in our study could reflect failure to identify statistical significance because of small sample size. Alternatively, results of our study might support a role of alternative mechanisms for SH in cats, as previously proposed for cats with hyperthyroidism (increased sensitivity to catecholamines, increased heart rate and cardiac output, or altered baroreceptor setpoints),[49] CKD (abnormal renal sodium retention),[11,12] or idiopathic SH (genetic factors).[50] Although the exact reason remains unclear, results of our study suggest that SH itself was not associated with significantly increased RAAS activity compared to healthy cats in our small sample.

In contrast, amlodipine-treated SH cats showed significant nonspecific increased activity of both classical and alternative RAAS pathways compared to healthy cats (increases in AngI, AngII, ALD, and Ang1-7) and untreated cats with SH (increases in AngI and Ang1-7). These results are generally consistent with previous studies in amlodipine-treated cats with SH. One previous report identified increased PRA (but not ALD) after amlodipine treatment compared to baseline,[12] whereas another study found higher concentrations of circulating RAAS metabolites in amlodipine-treated cats with SH compared to healthy cats.[22] Studies in people receiving nifedipine or amlodipine have found similar significant increases in PRA, likely because of decreased BP causing reflex sympathetic stimulation, whereas effects on ALD have been conflicting.[2,51,52] In dogs, amlodipine upregulates the RAAS, presumably by vasodilation of the glomerular afferent arterioles. Like furosemide, amlodipine is used as a pharmacologic model of RAAS activation in healthy dogs.[19,20] Our study confirms that increased RAAS activity occurs in amlodipine-treated cats, similar to people and dogs, and that this increased activity involves increases in RAAS metabolites from both the classical and alternative RAAS pathways. The clinical relevance of this degree of balanced RAAS activation remains unclear, however, especially because cats with SH showed no baseline RAAS activation before treatment in our study.

Our study found no significant correlations between RAAS analyte concentrations and renal function test results or incidence of CKD, suggesting that decreasing renal function is not associated with increased RAAS activity in cats. This observation is consistent with a previous study that showed no differences in ALD concentrations or PRA in cats based on presence or absence of azotemia.[12] Lack of difference in renal function test results or incidence of CKD among disease groups suggests that the healthy control group was well-matched to diseased cats; approximately 60% of cats in all disease groups had at least IRIS Stage II CKD. Within the SH group, severity of hypertension actually was associated with lower concentrations or results of several RAAS metabolites (AngI, AngII, Ang1-7, AngIII, AngIV, and PRA-S). This seemingly counterintuitive result likely reflects the fact that cats with the highest BP were least likely to be receiving amlodipine at time of sampling, and amlodipine treatment (rather than SH itself) was associated with increased RAAS activity. Within the CM group, certain isolated markers of echocardiographic disease severity were significantly correlated with increases in individual RAAS biomarkers. However, no consistent trends were identified across variables. This result might be explained by relative homogeneity of cardiac disease severity in the CM group, relatively high variability in RAAS biomarkers in this group, or both.

A particular strength of the comprehensive RAAS fingerprint technique is the ability to analyze multiple RAAS metabolites simultaneously, allowing comparisons between classical and alternative RAAS pathways. The classical RAAS pathway refers to the peptide cascade from angiotensinogen to AngI to AngII, mediated by the enzymes renin and ACE, and leading to increased adrenal production of ALD. Physiologic consequences of activation of this classical RAAS cascade include vasoconstriction, sodium and water retention, and myocardial and vascular fibrosis, which are considered maladaptive in the context of chronic increased RAAS activity and represent targets for RAAS-mitigating treatments. It is now well-established that this conventional cascade is not the sole signaling pathway of the RAAS. An alternative RAAS pathway has been characterized that involves breakdown of AngII to Ang1-7 by the enzyme ACE2, with downstream signaling through the Mas receptor leading to vasodilatation, diuresis and natriuresis, and mitigation of vascular inflammation.[53] The alternative RAAS pathway therefore provides an internal counterregulatory mechanism that can partly mitigate the negative effects of AngII and ALD.

This balance between classical and alternative pathway activity is critical to understanding the global effect of a disease or treatment on the RAAS. Nonspecific activation of RAAS, as seen with

both furosemide and amlodipine treatment in our study, increases the circulating pool of AngI and thus leads to increased activity in both classical and alternative cascades; the relative balance of ACE vs ACE2 then determines which enzymatic pathway is prioritized. Furthermore, the ultimate biologic effect of RAAS is determined not by circulating concentrations of biomarkers, but the interaction of biomarkers with their target receptors. For example, although AngII binding to angiotensin type I receptors (AT1R) causes vasoconstriction and sodium retention (biological effects associated with the classical RAAS), binding of AngII to angiotensin type 2 receptors (AT2R) leads to counterregulatory vasodilatory and natriuretic effects.[54] Although classical RAAS activation might be characterized as an adverse effect of furosemide and amlodipine treatment, concurrent increased activity of the alternative RAAS pathway (with resulting vasodilatation and natriuresis) might contribute to the therapeutic benefit of these drugs in CM or SH. This suggests a number of potential therapeutic targets to favorably alter the balance of classical vs alternative RAAS effects, including both traditional treatments (ACEi, angiotensin receptor blockers) and novel treatments (recombinant ACE2 or Ang1-7).[33,55] Our results provide a theoretical rationale for such RAAS-modulating treatments in cats with advanced CM, as well as cats with CM treated with furosemide or cats with SH treated with amlodipine. However, the interplay between the classical and alternative RAAS pathways and complexity of AT1R vs AT2R binding also emphasize that RAAS fingerprinting of biomarker concentrations alone cannot be interpreted as a true surrogate for RAAS activity.

Our study had a number of limitations. The study utilized surplus serum samples harvested from another project. Sample size was relatively small for the CM and SH disease groups, and particularly small for treatment subgroups. Treatments were prescribed at the discretion of the attending clinician and thus were not standardized and had been prescribed for variable amounts of time before sampling. Cats with CM and SH differed in terms of the degree of disease control at the time of sampling. Finally, ACEi treatment was not standardized with respect to other treatments (amlodipine vs furosemide), although ACEi treatment rarely predicted RAAS metabolite concentrations in multivariate modeling.

In conclusion, our study suggests baseline RAAS activation in cats with advanced CM, with furosemide treatment contributing to further activation of the RAAS. Cats with untreated SH showed no difference in RAAS fingerprint compared to healthy cats, whereas amlodipine treatment activated both classical and alternative RAAS pathways in hypertensive cats. Although the net effect of these drugs on the RAAS depends on the balance of ACE vs ACE2 and the relative binding of AngII to AT1R vs AT2R, activation of the alternative pathway could contribute to the clinical benefit of these pharmacotherapeutics in cats with cardiovascular disease.

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CONFLICTS OF INTEREST DECLARATION

Emilie Guillot is the Global Technical Manager (Cardiology, Nephrology, and Hypertension) for the study sponsor, Ceva Sante Animale. Drs. Mochel and Ward have served as consultants for Ceva Sante Animale and have received reimbursement and honoraria for consulting, expert testimony, travel, and service as key opinion leaders (KOLs). Although Ceva Sante Animale provided funding for the project and approved study design, Ceva was not involved in data collection, sample analysis, or statistical analysis of results. No other authors have a conflict of interest.

OFF-LABEL ANTIMICROBIAL USE DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All procedures were approved by the IACUC of Iowa State University. Informed owner consent was obtained for each patient enrolled.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare that human ethics approval was not needed for this study.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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