




# The classical and alternative circulating renin-angiotensin system in normal dogs and dogs with stage B1 and B2 myxomatous mitral valve disease

Hillary H. Hammond<sup>1</sup>  | Marisa K. Ames<sup>1</sup>  | Oliver Domenig<sup>2</sup> |  
 Brian A. Scansen<sup>1</sup>  | Nuen Tsang Yang<sup>3</sup> | Mabelle D. Wilson<sup>3</sup> | Erin Sunshine<sup>1</sup> |  
 Kaitlyn Brunk<sup>1</sup> | Allison Masters<sup>1</sup>

<sup>1</sup>Department of Clinical Sciences, Colorado State University College of Veterinary Medicine and Biomedical Sciences, Fort Collins, Colorado, USA

<sup>2</sup>Clinical and Translational Science Center, Department of Public Health Sciences, Division of Biostatistics, University of California, Sacramento, California, USA

<sup>3</sup>Attoquant Diagnostics GmbH, Vienna, Austria

## Correspondence

Marisa K. Ames, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California, Davis, One Shields Avenue, Davis, CA 95616, USA.  
 Email: [mkames@ucdavis.edu](mailto:mkames@ucdavis.edu)

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## Abstract

**Background:** The behavior of the comprehensive circulating renin-angiotensin system (RAS) in dogs with myxomatous mitral valve disease (MMVD) before to the onset of congestive heart failure remains largely unexplored.

**Hypothesis/Objectives:** The classical and alternative RAS activity and aldosterone concentrations will be significantly higher in dogs with American College of Veterinary Internal Medicine (ACVIM) stage B2 MMVD compared to normal dogs and dogs with ACVIM stage B1 MMVD.

**Animals:** One-hundred seventeen client-owned dogs (normal = 60; B1 = 31; B2 = 26).

**Methods:** Prospective observational study. Angiotensin peptides (AP) and aldosterone concentrations were measured using liquid chromatography and mass spectrometry. Angiotensin converting enzymes 1 and 2 (ACE, ACE2) and renin activity surrogates were calculated from AP concentrations. Equilibrium dialysis (ED) and immediate protease inhibition (PI) methods of AP quantification were compared in 14 healthy dogs.

**Results:** Core RAS activity and aldosterone concentrations did not differ among the 3 groups. However, the balance between the alternative and classical RAS differed, with dogs with stage B2 MMVD having significantly higher ACE2 activity surrogate (ACE2<sub>surr</sub>) when compared to normal dogs (adjusted  $P = .02$ ; ratio of medians for ACE2<sub>surr</sub> [B2:normal], 1.89; 95% confidence interval [CI]: 1.4-2.6). The ED and PI methods of AP quantification were highly correlated (AngI,  $r = .9$ ,  $P < .0001$ ; AngII,  $r = .8$ ,  $P = .001$ ).

**Abbreviations:** AA2<sub>surr</sub>, surrogate of adrenal responsiveness to angiotensin II; ACE2<sub>surr</sub>, surrogate measure of angiotensin-converting enzyme 2 activity; ACEi, angiotensin converting enzyme inhibitor; ACE<sub>surr</sub>, surrogate measure of angiotensin-converting enzyme activity; ACVIM, American College of Veterinary Internal Medicine; ALT<sub>surr</sub>, surrogate measure of alternative renin-angiotensin-system activity; Ang, angiotensin; ANOVA, analysis of variance; AP, angiotensin peptide; CI, confidence interval; CSU-VTH, Colorado State University Veterinary Teaching Hospital; ED, equilibrium dialysis; IQR, interquartile range; LAmAx:Ao, left atrium to aortic ratio obtained from right parasternal long axis 2D-image; LVIDDn, normalized left ventricular internal dimension in diastole; MAS, MAS1 proto-oncogene; MMVD, myxomatous mitral valve disease; PI, protease inhibition; RAAS, renin angiotensin aldosterone system; RAS, renin angiotensin system; Renin<sub>surr</sub>, surrogate measure of renin activity; TR-PG, Tricuspid regurgitation pressure gradient; UAldo:C, urine aldosterone to creatinine ratio; UCD-VMTH, University of California, Davis Veterinary Medical Teaching Hospital; WCI/WRI, width of the confidence interval to width of the reference interval ratio.

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Liquid chromatography and mass spectrometry assays, such as the RAS Fingerprint from Attoquant Diagnostics (Figure 1), are improving our understanding of how the enzymes and angiotensin peptides (AP) of this system change during disease progression and treatment. This assay can be performed by 2 methods: immediate protease inhibition (PI) of the blood sample or simply using untreated serum or plasma. The former method provides an instantaneous picture representing actual concentrations of RAS peptides at the moment the sample was taken. The latter approach uses the concept of equilibrium dialysis (ED) where serum or plasma is incubated *ex vivo* at 37°C for 1 hour. Because angiotensin metabolizing enzymes remain active in the sample and exceed the concentrations of metabolites, an equilibrium of downstream metabolites is reached, where formation and degradation rates of peptides are equal. This situation represents the potential of an individual's RAS and allows for estimation of enzyme activity. The ED approach is more clinically practical and is the approach that has been reported in the veterinary literature thus far.<sup>16-20</sup>

We sought to characterize the comprehensive circulating RAAS (RAS Fingerprint and aldosterone) in normal dogs and to determine the prevalence and magnitude of RAAS activation in dogs with stage B1 and B2 MMVD. Secondary aims were to evaluate week-to-week variation in RAS peptide concentrations in healthy dogs and compare peptide quantification from samples obtained with PI and samples obtained without PI (the latter using ED).

## 2 | MATERIALS AND METHODS

### 2.1 | Healthy dogs

Client-owned apparently healthy dogs were recruited at the Colorado State University Veterinary Teaching Hospital (CSU-VTH). Dogs of all ages, body weight, and neuter status were accepted with an aim to achieve a balance between dogs older than and younger than 6 years of age. There were no restrictions regarding type of diet and feeding schedule. Dogs were excluded if they had a history of preexisting cardiac, endocrine, respiratory, urinary, or renal disease. Additional exclusion criteria included history of polyuria or polydipsia, cough, treatment with pimobendan, ACEi, spironolactone, angiotensin receptor blocker, corticosteroid, sympathomimetic, beta-blocker, or mineralocorticoid. Dogs documented to have a heart murmur or systemic hypertension also were excluded from the healthy dog group.

### 2.2 | Myxomatous mitral valve disease

Client-owned dogs diagnosed with stage B1 and B2 MMVD were prospectively enrolled through the CSU-VTH and University of California Davis Veterinary Medical Teaching Hospital (UCD-VMTH) cardiology services. Additional inclusion criteria included body weight <20 kg and age >5 years. Any neuter status was accepted. There were no restrictions regarding type of diet and feeding schedule. Dogs were

excluded if they had a history of preexisting endocrine, respiratory, urinary, or renal disease. Additional exclusion criteria included history of polyuria or polydipsia, cough, treatment with pimobendan, ACEi, spironolactone, angiotensin receptor blocker, corticosteroid, sympathomimetic, beta-blocker, or mineralocorticoid. Dogs were excluded if diagnostic testing identified concurrent heart disease including severe pulmonary hypertension, congenital defect, other acquired or secondary heart disease or if they had proteinuria, a serum creatinine concentration > 2.0 mg/dL, or systolic blood pressure > 180 mmHg. Dogs with 1+ proteinuria and urine specific gravity <1.040 and all dogs with ≥2+ proteinuria were screened by a urine protein to creatinine ratio and dogs with a ratio > 0.5 were excluded. Dogs with increased tricuspid regurgitation pressure gradients (TR-PG) were excluded if >60 mm Hg.

Physical examination findings, diet, timing of sample collection relative to last meal, and patient demeanor (calm or nervous) were recorded. Dogs were gently restrained for systolic blood pressure measurement in right or left lateral recumbency, a cuff with a width of 30% to 40% of the distal forelimb radius was placed on the forelimb. At least 3 measurements within 10% of each other were averaged. Sedation with butorphanol was allowed after the blood sample and blood pressure measurement were obtained.

### 2.3 | Blood and urine collection

Blood collection was performed with the dog gently restrained in a sitting position or lateral recumbency. Serum samples were allowed to clot at room temperature for 30 minutes before centrifugation at 3000g for 10 minutes at 4°C. Serum was divided with 1 aliquot submitted to the CSU-VTH or the UCD-VMTH Clinical Pathology Laboratory for serum biochemistry analysis and 2 aliquots stored at -80°C and sent out within 4 months for RAS Fingerprint analysis by ED. Blood was collected in an EDTA tube and submitted to the CSU-VTH or the UCD-VMTH Clinical Pathology Laboratory for CBC. A subset of normal dogs returned 7 to 14 days after their initial visit for a second blood collection to measure week-to-week variation and differences between PI and ED analyses. Serum was collected as described above. Additionally, 1.3 mL of whole blood was collected in a lithium heparin tube. Protease inhibitor provided by Attoquant Diagnostics was thawed and re-suspended by 1 minute of vortexing and 60 µL of protease inhibitor was transferred to a microcentrifuge tube suspended in an ice bath. Within 1 minute after collection in the lithium heparin tube, 1.2 mL of blood was transferred to the microcentrifuge tube containing protease inhibitor and the tube was capped and inverted several times and then centrifuged at 3000g for 10 minutes at 4°C. Plasma was transferred to 2 cryovials, stored at -80°C, and sent out within 4 months for RAS Fingerprint analysis. Estimated plasma volume was calculated using Duarte's formula.<sup>21</sup> Urine was collected by voiding or cystocentesis. One aliquot was submitted to the CSU-VTH or UCD-VMTH Clinical Pathology Laboratory for urinalysis and 1 aliquot was centrifuged at 3000g for 10 minutes at 4°C and the aliquot stored at -80°C.

## 2.4 | RAS fingerprint and serum aldosterone analysis (equilibrium and immediately stabilized)

Serum was incubated for 1 hour at 37°C to establish equilibrium concentrations before stabilization with an inhibitor cocktail. The immediately stabilized plasma samples (see above) were thawed on ice. Both the serum and plasma samples were spiked with stable isotope-labeled internal standards for each angiotensin metabolite and aldosterone at a concentration of 200 pg/mL. Angiotensin peptides and aldosterone then were quantified using high-performance liquid chromatography-mass spectrometry. Details of the method are presented in Supporting Information.

Surrogates for enzyme activity, a surrogate for adrenal responsiveness, and a surrogate for the balance of the alternative to classical enzyme activity (ALT<sub>surr</sub>) were determined from the equilibrium concentrations as follows:

- Surrogate measure of ACE activity (ACE<sub>surr</sub>)— AngII/AngI
- Surrogate measure of ACE2 activity (ACE2<sub>surr</sub>)—Ang1,5/AngII
- Surrogate measure of renin activity (renin<sub>surr</sub>)—AngI + AngII
- Surrogate measure of adrenal responsiveness (AA2<sub>surr</sub>)—Aldo/AngII
- Surrogate measure of alternative RAS activity (ALT<sub>surr</sub>)—(Ang1,7 + Ang1,5)/(Ang1 + AngII+Ang1,7 + Ang1,5)

The calculated enzyme surrogates (renin, ACE, and ACE2) have been shown to correlate with directly measured enzyme activity.<sup>22-24</sup> The surrogate measure of adrenal responsiveness (AA2<sub>surr</sub>; the aldosterone to AngII ratio), assesses the responsiveness of the adrenal gland in releasing aldosterone in response to AngII.<sup>25</sup>

## 2.5 | Urine aldosterone to creatinine ratio (UAldo:C)

Two to 5 mL of urine was centrifuged and transferred to a –80°C freezer, within 1 hour of collection and stored for future analysis. A radioimmunoassay was used to measure urine aldosterone concentration and was performed according to the manufacturer's directions (Aldosterone Active Radioimmunoassay, Beckman Coulter Inc., Brea, California) at a veterinary diagnostic laboratory (Michigan State University Veterinary Diagnostic Laboratory, Lansing, MI). This assay measures free aldosterone and the aldosterone 18β-G metabolite (after acid hydrolysis to aldosterone). Urine creatinine concentration was determined using a standard colorimetric assay at the same laboratory.

## 2.6 | Echocardiogram

A complete echocardiogram was performed by a cardiologist or supervised cardiology resident using either a Philips Epiq7 or CVx machine. Standard echocardiographic 2-dimensional, M-mode, and Doppler images were obtained from both right and left parasternal windows.

Echocardiographic variables analyzed and included in the analysis were left atrial-to-aortic ratio (LAmx:Ao; measured on the 2D right parasternal long axis view),<sup>26</sup> normalized left ventricular internal dimension in diastole (LVIDdN; measured on the left ventricular m-mode),<sup>27</sup> and presence of an estimated TR-PG > 35 mm Hg (evaluated as a categorical variable, yes/no). At least 3 measurements for each variable were obtained and averaged. MMVD was confirmed based on thickening and insufficiency of the mitral valve leaflets. Dogs were classified as stage B1 or B2 based on ACVIM guidelines using the left atrial-to-aortic ratio measured at the right parasternal short axis<sup>28</sup> and the LVIDdN as described above.<sup>2</sup>

## 2.7 | Ethics approval

The study was approved by the clinical trials review boards at Colorado State University and the University of California, Davis. Owner permission was obtained by signed consent before enrolling patients.

## 2.8 | Statistical analysis

Power analysis was based on data obtained from a prior study.<sup>16</sup> The difference in serum AngII in normal dogs at baseline and approximately 4 hours after IV furosemide administration mimics mild to moderate RAAS activation and was 23.0 pmol/L with a SD of 38.9 pmol/L. Using these data and an alpha of .05 and power of .80, it was determined that at least 45 dogs per group would be ideal. When creating reference intervals, sample sizes >55 are recommended to increase precision of the upper and lower limits.<sup>29</sup> Keeping budget constraints in mind, enrollment targets for the normal and MMVD groups were set at 60 each. The dogs with MMVD were further divided into 2 groups (B1 and B2) and analyses were performed with 3 groups (normal, B1, and B2). This approach decreased statistical power but was considered to have more potential for clinically relevant hypothesis generation. It was decided a priori to not analyze these data as 2 groups (normal and B1/B2). Normality was assessed using histograms and QQ plots, and non-normally distributed variables were log-transformed before analysis. Normal distribution after transformation was confirmed by inspection of histograms and residual diagnostic plots. Medians and interquartile ranges (IQR) are reported as summary statistics and for group comparisons.

Reference intervals were determined from the 60 normal dogs and represent the central 95% of the study sample's values with the 2.5th and 97.5th quantiles serving as the lower and upper limits, respectively. The 90% confidence intervals (CI) for upper and lower limits of the reference interval and the width of the CI/width of the reference interval (WCI/WRI) were calculated using a robust bootstrap method for obtaining the 5th and 95th quantiles from 1000 ranked bootstrap samples for each of the upper and lower bounds

After log transformation, serum AP and aldosterone concentrations, surrogates, and the UAldo:C were compared among the 3 groups using analysis of variance (ANOVA). Welch's ANOVA p values were

reported in all cases, regardless of variance. Analytes with results below the lower limit of quantification were halved and this result was used in the analysis. Differences in age, time between blood sample and last meal, sex (M, F, SF, MN), and demeanor (calm or nervous) were determined using ANOVA or a Chi-squared test. The Pearson correlation coefficient was used to measure the association between RAAS analytes and these variables. Variables that differed significantly among groups and found to correlate with the RAAS analytes were handled as possible confounders and were used as covariates in a linear regression model.

Echocardiographic variables (LMax: Ao, LVIDdN, pulmonary hypertension [y/n]), serum biochemical variables (serum potassium and creatinine concentrations), and estimated plasma volume were compared among the 3 groups using ANOVA. The association between the RAAS analytes and echocardiographic variables (LMax: Ao, LVIDdN, pulmonary hypertension [y/n]), serum biochemical variables (serum potassium and creatinine concentrations), and estimated plasma volume was evaluated using the Pearson correlation coefficient (continuous variables) or a *t* test (pulmonary hypertension [y/n]) using a pooled or Satterthwaite method depending on equality of variances.

The Pearson correlation coefficient was used to measure the association between PI samples and samples analyzed using the ED (nonprotease inhibited) approach. Strength of correlation was described as very high, high, moderate, low, and negligible for *r* values of .9 to 1.0, .7 to .089, .5 to .69, .3 to .49, and 0 to .29, respectively. Week-to-week variation for each analyte was evaluated by calculating the week-to-week coefficient of variation for each dog. A paired *t* test also was used to compare group differences between the 2 timepoints. The coefficients of variation for several measured analytes (AngI and II, aldosterone, Ang1,5, and Ang1,7) were compared between dogs classified as either normal or nervous using a *t* test and the pooled or Satterthwaite method, depending on equality of variances.

### 3 | RESULTS

#### 3.1 | Dogs

One-hundred thirty-two dogs were enrolled in the study. Fifteen dogs were excluded (11 had proteinuria with UP:C > 0.5 with 2 of these receiving prohibited drugs; 2 were inadvertently enrolled twice, 1 was in heart failure, and 1 normal dog had marked activation of RAAS without any clinically apparent reason and was determined statistically to be an outlier for all APs and aldosterone), leaving 117 dogs included in the analysis cohort. Dogs in the normal group were heavier and younger than dogs in the B1 and B2 groups ( $P < .0001$ ; Table 1). The number of dogs determined to be calm or nervous during blood pressure measurement and venipuncture did not differ between groups. Median (IQR) time between the last meal and blood sampling differed significantly between groups and was 5.2 hours (3.2-14.5), 7.8 hours (3.8-15), and 9.7 hours (6.0-16.6) for the normal, B1, and B2 groups, respectively ( $P = .04$ ).

#### 3.2 | Blood pressure, echocardiography, serum biochemistry, CBC, and urinalysis results

All variables except for LMax: Ao were normally distributed. Abnormalities incidentally detected in the minimum database included 12 dogs with mild (normal = 5, B1 = 1, B2 = 6) and 1 dog (B1 group) with a marked increase in ALP activity and 13 dogs with mild increases in ALT activity (normal = 3, B1 = 4, B2 = 6). Seven dogs had mildly increased BUN concentration (normal = 3, B1 = 3, B2 = 1). Only 1 dog (B1 group) had a mildly increased serum creatinine concentration (1.7 mg/dL). Three dogs were isosthenuric (normal = 2, B2 = 1) and 4 dogs were hyposthenuric (normal = 3, B2 = 1). Serum potassium concentration was significantly higher in the B1 group compared to the normal group (Table 1). Systolic blood pressure was significantly higher in the B1 group compared to the normal group ( $P = .002$ ). Estimated plasma volume was significantly higher in the B2 group compared to the normal group ( $P = .0004$ ) and LMax: Ao and LVIDdN differed significantly among groups as expected ( $P < .0001$ ).

Correlations between analytes (AP and aldosterone concentrations, surrogates, and the UAldo:C) and clinicopathologic and echocardiographic variables were evaluated in all 117 dogs as a single cohort (Table 2). All variables were non-normally distributed. Negligible correlations were found between LMax: Ao and Ang1,5 ( $r = .2$ ,  $P = .03$ ) and Ang1,7 ( $r = .2$ ,  $P = .01$ ). A low, negative correlation was found between LMax: Ao and LVIDdN and ACE<sub>surr</sub> ( $r = -.3$ ,  $P = .004$  and  $r = -.3$ ,  $P = .005$ , respectively). Both LMax and LVIDdN had low or negligible correlation with ACE2<sub>surr</sub> ( $r = .3$ ,  $P = .0004$  and  $r = .2$ ,  $P = .02$ ). The LMax: Ao had low correlation with ALT<sub>surr</sub> ( $r = .3$ ,  $P = .006$ ). Correlation between serum creatinine concentration and ACE<sub>surr</sub> ( $r = .2$ ,  $P = .02$ ) was negligible. Negligible to low, negative correlations were found between serum creatinine concentration and Ang1,5, ACE2<sub>surr</sub>, ALT<sub>surr</sub>, and UAldo:C ( $r = -.2$ ,  $P = .04$ ;  $r = -.3$ ,  $P = .0001$ ;  $r = -.3$ ,  $P = .003$ ;  $r = -.4$ ,  $P < .0001$ , respectively). Negligible correlation was found between serum potassium concentration and ACE<sub>surr</sub> ( $r = .2$ ,  $P = .04$ ). Estimated plasma volume did not have a significant correlation with any of the measured or calculated RAAS variables. Seventeen dogs had estimated TR-PG > 35 mm Hg (B1 = 10 and B2 = 7). Serum aldosterone concentration was significantly lower (median [IQR]: 12 pmol/L [5.0-48.2] vs 44.8 pmol/L [17.9-86.6]) and ALT<sub>surr</sub> significantly higher (0.42 [0.36-0.54] vs 0.37 [0.30-0.46]) in dogs with an increased TR-PG ( $P = .01$  and .03, respectively).

#### 3.3 | RAS fingerprint and serum aldosterone and UAldo: C analysis

Reference intervals were determined for all RAAS analytes measured in the 60 healthy dogs. The WCI/WRI of the lower limit of the reference interval was <0.2 for all RAAS analytes whereas the WCI/WRI of the upper limit was >0.2 for all RAAS analytes. (Table 3)

Results were below the lower limit of quantification in 8% of measurements of directly quantified AP and aldosterone concentrations



**TABLE 1** Median (interquartile range) values for demographic, clinical, and echocardiographic variables for all dogs

Parameter	Normal (N = 60)	B1 (N = 31)	B2 (N = 26)	Reference interval	P value
Age (year)	4.5 (2.7-7.0)	10.6 (8.8-11.7) <sup>a</sup>	11.0 (9.8-12.1) <sup>a</sup>	—	<.0001
Weight (kg)	20.7 (8.4-29.3)	6.2 (4.9-9.0) <sup>a</sup>	8.0 (6.1-9.5) <sup>a</sup>	—	<.0001
Meal to sample time (hours)	5.2 (3.2-14.5)	7.8 (3.8-15.0)	9.7 (6.0-16.6) <sup>a</sup>	—	.02
Blood pressure (mm Hg)	124 (115-138)	146 (128-155) <sup>a</sup>	140 (122-146)	110-160 mmHg	.002
ePV (dL/g)	2.7 (2.3-3.0)	2.9 (2.6-3.4)	3.1 (2.8-3.6) <sup>a</sup>	—	.0004
Potassium (mmol/L)	4.4 (4.3-4.7)	4.7 (4.4-5.0) <sup>a</sup>	4.4 (4.3-4.7)	3.6-4.8 mmol/L	.04
Creatinine (mg/dL)	1.0 (0.8-1.2)	0.9 (0.7-1.1)	0.9 (0.7-1.0)	.8-1.5 mg/dL	.05
LAmx:Ao	2.0 (1.9-2.1)	2.3 (2.2-2.5) <sup>a</sup>	3.0 (2.8-3.3) <sup>a,b</sup>	<2.6	<.0001
LVIDdN	1.4 (1.3-1.5)	1.5 (1.4-1.6) <sup>a</sup>	1.9 (1.8-2.0) <sup>a,b</sup>	<1.7	<.0001

Abbreviations: AePV, estimated plasma volume; LAmx:Ao, left atrium to aortic ratio (left atrium measured at maximal dimension from the two-dimensional right parasternal long axis view); LVIDdN, left ventricular internal dimension in diastole normalized for body weight.

<sup>a</sup>Differs significantly from the normal group.

<sup>b</sup>Differs significantly from the B1 group.

**TABLE 2** Correlation between age, mean to sample time, left atrial to aortic ratio, normalized diastolic left ventricular internal dimension, tricuspid regurgitation pressure gradient and angiotensin peptide and aldosterone concentrations, enzyme surrogates, adrenal function surrogate, and alternative renin-angiotensin system surrogate in 19 normal dogs

Analyte	Person correlation coefficient; P value				t test P value TR-PG >35 mmHg (y/n)
	Age	Meal to sample time (h)	LAmx: Ao	LVIDdN	
AngII (pmol/L)	−0.006; .9	0.1; .2	−0.4; .7	−0.07; .5	.74
Ang1,7 (pmol/L)	<b>0.2; .01</b>	0.1; .2	<b>0.2; .01</b>	0.1; .2	.35
AngI (pmol/L)	0.1; .2	<b>0.2; .03</b>	0.2; .1	0.1; .3	.74
AngIII (pmol/L)	0.09; .3	<b>0.3; .003</b>	0.04; .6	0.9; .3	.49
Ang1,5 (pmol/L)	0.2; .05	0.2; .06	<b>0.2; .03</b>	0.1; .3	.39
AngIV (pmol/L)	0.009; .9	<b>0.2; .04</b>	0.02; .8	0.03; .7	.31
Aldo (pmol/L)	−0.1; .1	0.1; .2	−0.1; .2	0.2; .08	<b>.01</b>
AA2 ([pmol/L]/[pmol/L])	−0.1; .1	0.05; .6	−0.1; .3	0.1; .2	<b>.01</b>
Renin <sub>surr</sub> pmol/L	0.07; .4	0.2; .05	0.09; .4	0.04; .7	.7
ACE <sub>surr</sub> ([pmol/L]/[pmol/L])	−0.1; .07	−0.1; .3	<b>−0.3; .0004</b>	<b>−0.3; .005</b>	.97
ACE2 <sub>surr</sub> ([pmol/L]/[pmol/L])	<b>0.3; .006</b>	0.1; .3	<b>0.3; .0004</b>	<b>0.2; .02</b>	.12
ALT <sub>surr</sub> ([pmol/L]/[pmol/L])	<b>0.3; .005</b>	0.03; .7	<b>0.3; .006</b>	0.1; .2	<b>.03</b>
UAldo:C (μg/g)	0.2; .1	0.08; .5	0.1; .2	0.1; .2	.6

Note: Bold values denote statistical significance at the  $P < 0.05$  level.

Abbreviations: AA2, adrenal function surrogate (Aldo/AngII); ACE<sub>surr</sub>, angiotensin-converting enzyme surrogate (AngII/AngI); ACE2<sub>surr</sub>, surrogate measure of angiotensin-converting enzyme 2 activity (Ang1,5/AngII); Aldo, aldosterone; ALT<sub>surr</sub>, surrogate measure of alternative RAS activity (Ang1,7 + Ang1,5)/(Ang1 + AngII+Ang1,7 + Ang1,5); Ang, angiotensin; Renin<sub>surr</sub>, surrogate measure of renin activity (AngI + AngII); LAmx:Ao, left atrium to aortic ratio (left atrium measured at maximal dimension from the two-dimensional right parasternal long axis view); LVIDdN, left ventricular internal dimension in diastole normalized for body weight; TR-PG: tricuspid regurgitation pressure gradient.

(normal: 29/420, B1: 22/217, and B2 17/182). No significant differences among groups were noted in the classical RAAS peptides (AngI, II, III, IV, Aldo), Ang1,5, and UAldo:C (Figure 2 and Table 4). Angiotensin 1-7 and ACE<sub>surr</sub> were significantly higher in the B2 group when compared to the normal group ( $P = .03$  in both cases; Table 4). The ACE2<sub>surr</sub> was significantly higher in the B2 group when compared to dogs in the normal and B1 groups ( $P = .0005$ ; Tables 4 and S1). The ALT<sub>surr</sub> was significantly higher in the B2 dogs when compared to normal dogs ( $P = .001$ ; Tables 4 and S1). Age and time between last meal and blood sampling were correlated with a few RAAS analytes (Table 2). As such, comparisons of RAAS analytes among groups were repeated after

controlling for age and time between the last meal and blood sampling. After this adjustment, ACE2<sub>surr</sub> remained significantly different between the B2 and normal groups (adjusted  $P = .02$ ; Tables 4 and S1).

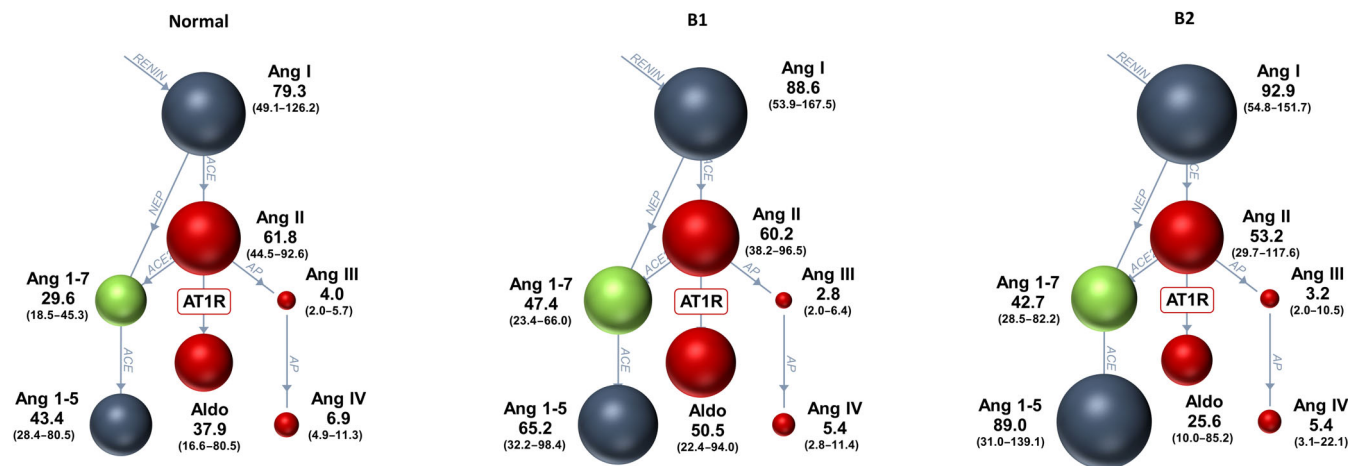
### 3.4 | Week-to-week variation in RAS fingerprint and aldosterone

Nineteen healthy dogs returned 1 week (median, 7 days; range, 4-10 days) after enrollment and initial blood sampling. Median week-

**TABLE 3** Ninety five percent confidence intervals and reference intervals for angiotensin peptide and aldosterone concentrations, enzyme surrogates, adrenal function surrogate, alternative renin-angiotensin system surrogate, and urine aldosterone to creatinine ratio from 60 normal dogs

Analyte	95% CI	Reference interval	90% CI of Lower limit	90% CI of Upper limit	WCI/WRI Lower limit	WCI/WRI Upper limit
AngII (pmol/L)	14.1-226.3	17.2-218.1	9.5-26.7	158.1-264.3	0.08	0.53
Ang1,7 (pmol/L)	6.2-160.2	7.0-160.7	5.4-9.9	97.9-242.7	0.03	0.94
AngI (pmol/L)	27.5-299.6	28.9-284.9	23.0-37.5	179.9-381.2	0.06	0.79
AngIII (pmol/L)	1.0-25.6	1.0-23.7	1.0-1.0	12.7-34.5	0	0.96
Ang1,5 (pmol/L)	11.2-287.0	13.6-278.3	7.9-21.0	162.3-413.5	0.05	0.95
AngIV (pmol/L)	1.0-28.8	1.1-29.9	1.0-2.1	20.8-43.7	0.04	0.80
AA2 ([pmol/L]/[pmol/L])	0.07-4.0	0.1-3.7	0.03-0.1	2.5-4.9	0.03	0.67
Renin <sub>surr</sub> pmol/L	45.3-501.4	49.0-481.3	32.5-67.6	367.0-581.6	0.08	0.50
ACE <sub>surr</sub> ([pmol/L]/[pmol/L])	0.3-1.7	0.3-1.9	0.3-0.4	1.4-3.0	0.07	1.24
ACE2 <sub>surr</sub> ([pmol/L]/[pmol/L])	0.3-2.0	0.3-2.3	0.3-0.4	1.6-4.0	0.09	1.20
ALT <sub>surr</sub> ([pmol/L]/[pmol/L])	0.2-0.5	0.2-0.5	0.2-0.3	0.5-0.6	0.19	0.34
Aldo (pmol/L)	5.0-186.4	5.0-184.4	5.0-5.0	154.8-219	0	0.36
UAldo:C (μg/g)	0.2-1.2	0.2-1.2	0.1-0.2	1.0-1.3	0.04	0.30

Abbreviations: AA2, adrenal function surrogate (Aldo/AngII); ACE<sub>surr</sub>, surrogate measure of angiotensin-converting enzyme activity (AngII/AngI); ACE2<sub>surr</sub>, surrogate measure of angiotensin-converting enzyme 2 activity (Ang1,5/AngII); Aldo, aldosterone; ALT<sub>surr</sub>, surrogate measure of alternative RAS activity (Ang1,7 + Ang1,5)/(Ang1 + AngII+Ang1,7 + Ang1,5); Ang, angiotensin; Renin<sub>surr</sub>, surrogate measure of renin activity (AngI + AngII); WCI/WRI, ratio of the width of the confidence interval to the width of the reference interval.



**FIGURE 2** The renin-angiotensin system (RAS) Fingerprint from 60 normal dogs, 31 dogs with ACVIM stage B1, and 26 dogs with ACVIM stage B2 myxomatous mitral valve disease. Spheres show relative concentrations of angiotensin peptides. Median (interquartile range) concentrations of angiotensin peptides are shown next to the spheres (pmol/L). ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Aldo, aldosterone; Ang, angiotensin; AP, aminopeptidase; AT1R, angiotensin type-1 receptor; DAP, aspartyl aminopeptidase; NEP, neutral endopeptidase (neprilysin).

to-week coefficients of variation for the RAAS analytes and calculated surrogates ranged from 10.4% to 93.5% (Table 5). The ALT<sub>surr</sub> had the lowest coefficient of variation (median, 10.4%; range, 0.9%-27.2%). No analyte differed significantly between weeks 1 and 2. The coefficients of variation for the RAAS analytes did not differ between dogs classified as calm vs those classified as nervous.

### 3.5 | Protease inhibition vs equilibrium dialysis

A second blood sample was immediately protease inhibited in 14 healthy dogs. Two APs (AngI and AngII) were measurable (above the lower limit of quantification) after PI of the blood sample. Correlation between RAS quantification via PI and

**TABLE 4** Median (interquartile range) of angiotensin peptide and aldosterone concentrations, enzyme surrogates, adrenal function surrogate, alternative renin-angiotensin system surrogate, and urine aldosterone to creatinine ratio from all dogs

Analyte	Normal	B1	B2	Unadjusted P value	Adjusted P value
AngII (pmol/L)	61.8 (44.5-92.6)	60.2 (38.2-96.5)	53.2 (29.7-117.6)	.9	.7
Ang1,7 (pmol/L)	29.6 (18.5-45.3)	47.4 (23.4-66.0) <sup>a</sup>	42.7 (28.5-82.2) <sup>a</sup>	<b>.03</b>	.3
AngI (pmol/L)	79.3 (49.1-126.2)	88.5 (53.9-167.5)	92.9 (54.8-151.7)	.5	.8
AngIII (pmol/L)	4.0 (1.2-5.7)	2.8 (1.0-6.4)	3.2 (1.0-10.5)	.3	.1
Ang1,5 (pmol/L)	43.4 (28.4-80.1)	65.2 (32.2-98.4)	89.0 (31.0-139.1)	.1	.3
AngIV (pmol/L)	6.9 (4.9-11.3)	5.4 (2.8-11.4)	5.4 (3.1-22.1)	.5	.5
Aldo (pmol/L)	37.9 (16.6-80.5)	50.5 (22.4-94.0)	25.6 (5.0-86.2)	.2	.5
AA2 ([pmol/L]/[pmol/L])	0.6 (0.3-1.2)	0.72 (0.3-1.5)	0.4 (0.2-1.4)	.3	.3
Renin <sub>surr</sub> pmol/L	147.1 (91.0-220.5)	156.7 (94.9-250.3)	155.8 (99.2-260.2)	.9	.9
ACE <sub>surr</sub> ([pmol/L]/[pmol/L])	0.8 (0.6-1.0)	0.7 (0.5-1.0)	0.6 (0.4-0.8) <sup>a</sup>	<b>.03</b>	.1
ACE2 <sub>surr</sub> ([pmol/L]/[pmol/L])	0.8 (0.5-1.2)	0.9 (0.6-1.3)	1.6 (0.9-2.0) <sup>a,b,c</sup>	<b>.0005</b>	<b>.02</b>
ALT <sub>surr</sub> ([pmol/L]/[pmol/L])	0.34 (0.29-0.42)	0.40 (0.33-0.48)	0.43 (0.38-0.54) <sup>a</sup>	<b>.001</b>	.08
UAldo:C (μg/g)	0.47 (0.34-0.62)	0.49 (0.42-0.91)	0.93 (0.32-1.2)	.07	.5

Note: Bold values denote statistical significance at the  $P < .05$  level.

Abbreviations: ACE<sub>surr</sub>, angiotensin-converting enzyme surrogate (AngII/AngI); ACE2<sub>surr</sub>, surrogate measure of angiotensin-converting enzyme 2 activity (Ang1,5/AngII); AA2, adrenal function surrogate (Aldo/AngII); Aldo, aldosterone; ALT<sub>surr</sub>, surrogate measure of alternative RAS activity (Ang1,7 + Ang1,5)/(Ang1 + AngII+Ang1,7 + Ang1,5); Ang, angiotensin; Renin<sub>surr</sub>, surrogate measure of renin activity (AngI + AngII).

<sup>a</sup>Differs significantly from the normal group.

<sup>b</sup>Differs significantly from the B1 group.

<sup>c</sup>Differs significantly from the normal group after controlling for age and meal to sample time (in multiple regression model).

**TABLE 5** Median (range) of week-to-week coefficient of variation in angiotensin peptide and aldosterone concentrations, enzyme surrogates, adrenal function surrogate, and alternative renin-angiotensin system surrogate in 19 normal dogs

Analyte	Week-to-week CV (%)	Paired t test P value week 1 vs week 2	Paired t test: mean difference; SD of difference
AngII (pmol/L)	37.8 (3.8-89.3)	.6	.08; .7
Ang1,7 (pmol/L)	29.0 (7.6-95.6)	.4	.2; .8
AngI (pmol/L)	28.9 (4.2-101.1)	.8	.05; .7
AngIII (pmol/L)	93.5 (13.9-133.5)	1	.01; 1.6
Ang1,5 (pmol/L)	54.8 (3.1-93.8)	.8	.07; .9
AngIV (pmol/L)	41.5 (4.6-127.9)	.6	.1; 1.0
Aldo (pmol/L)	65.4 (14.0-107.6)	.07	.4; .9
AA2 ([pmol/L]/[pmol/L])	51.8 (6.3-117.3)	.06	.5; 1.0
Renin <sub>surr</sub> pmol/L	29.7 (1.5-95.7)	.8	.03; .7
ACE <sub>surr</sub> ([pmol/L]/[pmol/L])	14.9 (0.1-61.7)	1	.001; .3
ACE2 <sub>surr</sub> ([pmol/L]/[pmol/L])	22.4 (0.1-80.6)	.8	.03; .5
ALT <sub>surr</sub> ([pmol/L]/[pmol/L])	10.4 (0.9-27.2)	.3	.05; .2

Abbreviations: AA2, adrenal function surrogate (Aldo/AngII); ACE<sub>surr</sub>, angiotensin-converting enzyme surrogate (AngII/AngI); ACE2<sub>surr</sub>, surrogate measure of angiotensin-converting enzyme 2 activity (Ang1,5/AngII); Aldo, aldosterone; ALT<sub>surr</sub>, surrogate measure of alternative RAS activity (Ang1,7 + Ang1,5)/(Ang1 + AngII+Ang1,7 + Ang1,5); Ang, angiotensin; Renin<sub>surr</sub>, surrogate measure of renin activity (AngI + AngII).

ED for AngI ( $r = .9$ ,  $P < .0001$ ) and AngII ( $r = .8$ ,  $P = .0006$ ) was very high and high, respectively. The ACE<sub>surr</sub> (AngI + AngII) measured by these 2 methods had high correlation ( $r = .7$ ,  $P = .005$ ).

## 4 | DISCUSSION

We characterized the comprehensive circulating RAS activity and aldosterone concentrations in normal dogs and dogs with stage B1



and B2 MMVD. The circulating classical RAS (AngI, AngII, renin<sub>surr</sub>, ACE<sub>surr</sub>) and aldosterone did not differ among these 3 groups. The balance between the circulating alternative (Ang1,7, Ang1,5, and ACE2) and classical RAS, however, did differ with dogs at stage B2 MMVD having significantly higher estimated ACE2 activity when compared to normal dogs. The week-to-week intra-dog coefficient of variation for measured RAS analytes was high, yet the balance of the alternative to classical RAS peptides (ALT<sub>surr</sub>) remained relatively stable from week to week (median, 10.4%; range, 0.9%-27.2%). We also showed that the less cumbersome ED method of AP quantification was highly correlated with quantification using immediate PI. Ninety-five percent CI for serum concentrations of APs, aldosterone, and enzyme surrogates in normal dogs are presented to facilitate future comparisons and study design.

The circulating, classical RAS activity and aldosterone concentrations did not differ significantly between normal dogs and dogs with stage B1 and B2 MMVD. Before correction for the covariates of age and meal-to-sample time, Ang1,7 was significantly higher in both the B1 and B2 groups as compared to the normal group, the ALT<sub>surr</sub> was significantly higher in the B2 group as compared to the normal group, and the ACE<sub>surr</sub> was significantly lower in the B2 group as compared to the normal group. These differences did not persist after correcting for the 2 covariates. The ACE2<sub>surr</sub> was significantly higher in the B2 group when compared to both B1 and normal, and the difference between the B2 and normal groups persisted after correcting for covariates (Tables 4 and S1). The covariation of age and meal-to-sample time with several analytes may have arisen, in part, from the study design. Healthy dogs were recruited from the Community Practice Service and from students, staff, and faculty at the CSU VMTH and were seen at weekend morning clinics whereas dogs with B1 and B2 MMVD were recruited as they presented to our cardiology clinics. Dogs that fit the clinical inclusion criteria for the healthy group tended to be younger. The correction for these covariates in the statistical plan may be viewed as excessively stringent, because only a few variables covaried. Regardless, this analysis strengthened the finding that increased ACE2 activity accompanied the progression of MMVD in this cohort. The significant changes in the ALT<sub>surr</sub> and Ang1,7 concentrations before the correction is a compelling finding that warrants further study. Whereas the ACE<sub>surr</sub> and AngII concentrations were negatively correlated with Lmax:AO and LVIDDn, the alternative peptides and surrogates (Ang1,7, Ang1,5, ACE2<sub>surr</sub>, and ALT<sub>surr</sub>) were positively correlated with these measures of left heart size (Table 2). This finding supported a shift in the RAS towards increased ACE2 activity and accumulation of alternative RAS peptides as the severity of MMVD increased in this cohort.

Increased circulating ACE2 has been associated with worse severity of structural and functional abnormalities, and is an independent predictor of adverse clinical events in humans with heart failure<sup>30-33</sup> and mortality in patients with acquired aortic stenosis.<sup>34</sup> Angiotensin-converting enzyme 2 catalyzes the conversion of AngI and II to Ang1,9, Ang1,7, and Ang1,5 (Figure 1). Of the resulting metabolites, Ang1,7 is the best studied and has been found to act at the Mas receptor to counteract the vasoconstrictive, prohypertrophic, and profibrotic actions of the classical RAS.<sup>35</sup> Angiotensin-converting enzyme 2 is expressed in the myocardium and is usually tissue bound,

conferring its protective effects locally at the tissue level.<sup>36</sup> Enhanced circulating ACE2 likely implies higher cleavage from tissues by the protease ADAM17 (also called tumor necrosis factor-alpha converting enzyme or TACE) resulting in less tissue ACE2 to mitigate local tissue effects of the classical RAS. Angiotensin II itself induces ADAM17 cleavage of ACE2, creating a vicious cycle of RAS activation and unchecked effects of the classical RAS on tissues.<sup>37</sup> The correlation between increased plasma ACE2 concentration and markers of ADAM17 activation has been demonstrated in people with severe acute respiratory syndrome coronavirus 2 (COVID-19).<sup>38</sup> Although increased unbound ACE2 likely creates a more favorable balance between the alternative and classical RAS in the circulation, it likely reflects a less favorable tissue RAAS environment where the profibrotic and proinflammatory effects of AngII meet little opposition. Additional mechanisms of increased circulating ACE2 concentrations include increased cellular expression and decreased clearance.

Changes in tissue RAS during disease and the impact of treatment on this local system remain largely unexplored in veterinary medicine. Whether the increase in circulating ACE2 in these dogs with stage B2 MMVD reflects higher tissue AngII or ADAM17 activity or both cannot be confirmed from these data, but the hypothesis warrants further investigation. Whether ACE2<sub>surr</sub> or ALT<sub>surr</sub> or both could serve as biomarkers for MMVD disease severity also remains undetermined. The week-to-week intra-dog coefficient of variation for measured individual RAAS analytes was high, which is likely reflective of the many variables that affect renin release. The RAS Fingerprint assay coefficient of variation is <10% within reference intervals and < 15% when close to the lower limit of quantification for all covered analytes. Considering this and given that dogs were screened for systemic and cardiovascular disease, the disparity noted for the individual analytes may reflect variations in stress level, timing of the sample with the most recent meal, hydration, and dietary sodium intake. The ALT<sub>surr</sub>  $([Ang1,7 + Ang1,5]/[Ang1 + AngII + Ang1,7 + Ang1,5])$ , a ratio of alternative to classical RAS peptides, remained relatively stable (median, 10.4%; range, 0.9%-27.2%). This lower variation of the ALT<sub>surr</sub> suggested that the balance of alternative vs classical enzyme activity (i.e., ACE2 vs ACE) remained relatively stable as compared to the AP concentrations. This surrogate may serve as a more reliable indicator of a patient's RAS activity and may have prognostic value.

Neither serum aldosterone concentration nor the UAldo:C differed among groups. The UAldo:C did not change in concert with serum aldosterone concentration and increased with advancing disease whereas serum aldosterone concentration was highest in the B1 group and lowest in the B2 group. The spot blood aldosterone concentrations varied widely in normal dogs, likely reflecting variations in hydration status, dietary sodium intake, and level of excitement. Urine aldosterone concentration represents hours of aldosterone secretion and may be a better indicator of blood aldosterone concentration. However, day-to-day variation in the UAldo:C is high,<sup>39</sup> limiting the utility of this biomarker. Systolic blood pressure mirrored the change in the serum aldosterone concentration and AA2<sub>surr</sub>. This increase in circulating aldosterone concentration is a possible mechanism behind the increased blood pressure in the B1 group.

The RAAS reference intervals calculated from the 60 normal dogs had precise lower limits, but the more clinically relevant upper limits were imprecise (Table 3). It has been recommended that reference interval determination in veterinary species should aim to include at least 120 subjects,<sup>40</sup> yet it is likely this number is higher for many of the RAAS peptides with high WCI/WRI values. The most precise upper limits were noted for ALT<sub>surr</sub> and UAldo:C. Overall RAAS activity in the normal dogs was higher when compared to previously reported results from a cohort of 6 healthy, middle-aged, purpose-bred male Beagles.<sup>16</sup> This difference may be explained by the lack of acclimation in pet dogs as compared to trained research dogs, environmental and dietary differences in owned vs research dogs, breed-related differences in RAS, or small sample size. Regardless of the imprecision of these reference intervals, the 95% CI still should prove useful for future power calculations, study design development, and comparisons.

Quantification of AngI and AngII after immediate PI was highly correlated with the ED approach. Immediate PI of the blood sample provides a snapshot of a patient's circulating AP concentrations at the moment the sample was taken. In these dogs, as is typical in humans,<sup>22</sup> only AngI and II concentrations were high enough to be quantified after PI. The ED approach takes a routinely handled serum or plasma sample and incubates it at body temperature for 1 hour. Because angiotensinogen concentrations markedly exceed those of downstream metabolites, there is constant formation of AngI. Because angiotensin metabolizing enzymes are still active in the sample, stable equilibrium concentrations of downstream metabolites are established, where formation and degradation rates of peptides become equal. The ED approach therefore estimates the capacity of a patient's RAS and allows for assessment of relative enzyme activities. In effect, the ALT<sub>surr</sub> reflects the balance (alternative vs classical) of a patient's RAS.

Our study had strengths and limitations. Although 117 dogs is a relatively large sample size for a veterinary study, division of the MMVD dogs into B1 and B2 groups decreased the statistical power. The planned analysis using 3 groups was decided a priori and thought to have the best potential for generating clinically relevant hypotheses. Age and time between the last meal and blood sampling were correlated with several RAAS analytes. However, a stringent statistical approach was used to correct for this covariation. Ideally, dogs would have been age-matched and the timing between sample collection and the last meal more strictly controlled. Whether this was true covariation or related to study design is not known. Despite using a robust approach to determining reference intervals, the WCI/WRI exceeded 0.2 for all upper limits, indicating that the sample size of 60 normal dogs was inadequate and a larger sample size is needed. Only normal dogs were included in the week-to-week variation evaluation and comparison of PI and ED methods, and additional studies are needed to determine if these findings also apply to diseased dogs. Although systemic health screening was thorough, comorbidities not evident on physical examination, blood tests, urinalysis, or echocardiography may have been missed and may have influenced RAAS activity. Lastly, the exact etiology of an increased TR-PG was not definitively determined and may not have been caused by a postcapillary increase in pulmonary artery pressures in every case.

In conclusion, the comprehensive circulating RAAS was evaluated in a large study sample of healthy dogs and dogs with stage B1 and B2 MMVD. The ACE2<sub>surr</sub> was increased in dogs with stage B2 MMVD compared to normal dogs and may indicate cleavage of ACE2 from the tissues and a less favorable RAS environment in the tissues as this disease progresses. The reference intervals for individual RAAS peptide concentrations were determined, albeit with imprecise upper limits. Although week-to-week variation in RAAS peptide concentrations was high, the ratio of alternative to classical RAAS was relatively stable. High correlation was found between PI samples and samples analyzed using the more clinically practical ED approach. The ALT and ACE2 surrogates, measured by the ED approach, are clinically feasible indicators of RAS dysregulation. Additional studies evaluating whether these biomarkers have an independent association with cardiovascular outcomes, such as heart failure or death, are warranted.

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#### CONFLICT OF INTEREST DECLARATION

Marisa K. Ames has received consulting fees, research support, and honoraria from Ceva Sante Animale/Ceva Animal Health, LLC; Oliver Domenig is a founding member and employee of Attoquant Diagnostics; Brian A. Scansen has received travel support and honoraria from Ceva Animal Health, LLC; Hillary H. Hammond, Nuen Tsang Yang, Mabelle D. Wilson, Erin Sunshine, Kaitlyn Brunk, and Allison Masters do not have a conflict of interest.

#### OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Approvals from Clinical Review Boards from University of California, Davis and Colorado State University.

#### HUMAN ETHICS APPROVAL DECLARATION

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

#### ORCID

Hillary H. Hammond  <https://orcid.org/0000-0003-2828-3113>

Marisa K. Ames  <https://orcid.org/0000-0002-3865-6123>

Brian A. Scansen  <https://orcid.org/0000-0001-7427-2402>

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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