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Characterization of the circulating markers of the renin-angiotensin-aldosterone system in telmisartan- or enalapril-treated dogs with proteinuric chronic kidney disease

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Abstract

Background: Effects of the renin-angiotensin-aldosterone system (RAAS) inhibitors enalapril and telmisartan on circulating RAAS in dogs with proteinuric chronic kidney disease (pCKD) are undescribed.

Objectives: To characterize the RAAS in untreated dogs with pCKD compared to healthy, life-stage- and sex-matched controls, and in dogs with pCKD after 30 days of treatment with enalapril or telmisartan.

Animals: Dogs with pCKD (n = 36) and healthy controls (n = 20).

Methods: Retrospective study of banked samples and previously collected data. Day 0 serum equilibrium concentrations of angiotensin I, II, III, IV, 1-5, and 1-7, and aldosterone, and urinary aldosterone-to-creatinine ratio (UACR) from pCKD dogs were compared to values on day 30 of treatment with enalapril (0.5 mg/kg PO q12) or telmisartan (1 mg/kg PO q24h) and to those of healthy dogs. Data were analyzed using linear mixed models.

Results: Compared with healthy dogs, pCKD dogs had significantly higher Ang I, III, 1-5, and 1-7 concentrations, and UACR. Relative to pretreatment values, day 30 Ang II concentrations were significantly increased and decreased in telmisartan- and enalapril-treated pCKD dogs, respectively (both $P < .001$). Mean (95% confidence interval) percentage change from pretreatment value in serum Ang 1-7 concentration was significantly greater in telmisartan- (753% [489%-1134%]) versus enalapril-treated (149% [69%-268%]) dogs ($P < .001$). Serum aldosterone decreased with

Abbreviations: AA2 ratio, surrogate of adrenal responsiveness to angiotensin II; ABT, aldosterone breakthrough; ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; ACE2-S, surrogate of angiotensin-converting enzyme 2 activity; ACEi, angiotensin-converting enzyme inhibitor; ACE-S, surrogate of angiotensin-converting enzyme activity; Ang, angiotensin; ARB, angiotensin II type I receptor blocker; AT1R, angiotensin II type I receptor; AT2R, angiotensin II type 2 receptor; CKD, chronic kidney disease; pCKD, proteinuric chronic kidney disease; PRA, plasma renin activity; PRA-S, surrogate measure of plasma renin activity; RAAS, renin-angiotensin-aldosterone system; RAASi, renin-angiotensin-aldosterone system inhibitor; SBP, systolic blood pressure; UACR, urinary aldosterone to-creatinine ratio; UPC, urinary protein-to-creatinine ratio.

treatment ($P = .02$ for enalapril, $P < .001$ for telmisartan), with no difference between groups at day 30.

Conclusions and Clinical Importance: Circulating RAAS activity is higher in dogs with pCKD. Compared with enalapril, treatment with telmisartan caused significantly greater increases in the presumed beneficial peptide Ang 1-7.

KEYWORDS
equilibrium analysis, healthy dogs, liquid chromatography-mass spectrometry, renal proteinuria, urinary aldosterone-to-creatinine ratio

1 | INTRODUCTION

Activation of the renin-angiotensin-aldosterone system (RAAS) is associated with accelerated progression of chronic kidney disease (CKD) and the development of proteinuria and systemic arterial hypertension,[1,2] known risk factors for higher renal and all-cause mortality in dogs.[3-5] Treatment with RAAS inhibitors (RAASi) such as angiotensin-converting enzyme inhibitors (ACEi) or angiotensin II type I receptor blockers (ARBs) reduces the magnitude of proteinuria and is considered standard of care for dogs with proteinuric CKD (pCKD).[6-8] In a randomized, double-masked clinical trial of dogs with pCKD, treatment with the ARB telmisartan caused a greater reduction in the magnitude of proteinuria than did treatment with the ACEi enalapril.[9] Preclinical data also shows that telmisartan more completely attenuates the pressor response to exogenous angiotensin (Ang) I than does enalapril or placebo in healthy dogs.[10] These studies suggest telmisartan might more comprehensively inhibit angiotensin II's effects than does enalapril in dogs; however, the relative effects of these drugs on components of the circulating RAAS have not been examined.

Recent research has focused on a counter-regulatory, alternative RAAS pathway involving angiotensin-converting enzyme 2 (ACE2), a homolog of ACE, and alternative Ang peptides, such as Ang 1-7, which can balance the pathologic effects of classical RAAS activation.[11] Methods for extended analysis of the classical and alternative RAAS pathways allow for more thorough assessment of this system and the effect of RAASi, including evaluation of RAAS activity surrogates.[12-16] Although pCKD is common in dogs,[17] extensive characterization of the RAAS has not been performed in those affected by renal proteinuria before or after treatment with an ACEi or ARB. Also, the phenomenon of aldosterone breakthrough (ABT)—defined as an increase in serum aldosterone concentration or urinary aldosterone-to-creatinine ratio (UACR) compared with pretreatment baseline—occurs in 34%-59% of dogs with myxomatous mitral valve disease or pCKD treated with a RAASi,[14,18] but the relative frequency of ABT in ACEi- or ARB-treated dogs has not been systematically studied.

The objectives of this study were 3-fold. (1) We sought to compare markers of the circulating RAAS (ie, serum equilibrium Ang I, II, III, IV, 1-5, and 1-7, and aldosterone concentrations, and UACR) in dogs with pCKD, relative to those of life stage- and sex-matched healthy control dogs. We hypothesized that these markers would be significantly higher in dogs with pCKD. (2) We aimed to evaluate the changes, relative to baseline, in these markers after 30 days of treatment with enalapril or telmisartan. We hypothesized that a subset of dogs with pCKD treated with either enalapril or telmisartan would experience increases in serum aldosterone concentrations and UACR after treatment. (3) We sought to compare these RAAS markers between dogs with pCKD treated with either enalapril or telmisartan for 30 days. We hypothesized that circulating Ang II concentrations would be lower in enalapril- versus telmisartan-treated dogs.

2 | MATERIALS AND METHODS

2.1 | Study design

This was a retrospective study performed on banked serum and urine samples collected from client-owned dogs with naturally occurring pCKD and data from life-stage- and sex-matched healthy control dogs included in an unrelated study. Samples from dogs with pCKD were obtained as part of a prospective, randomized, single-center, double-masked clinical trial designed to investigate the relative antiproteinuric efficacies of enalapril and telmisartan.[9] Clinicopathologic and circulating RAAS data from healthy control dogs were obtained from information collected for an unrelated study comparing RAAS metabolites in healthy dogs and dogs with stage B1 and B2 myxomatous mitral valve disease.[19]

2.2 | Dogs with proteinuric CKD

Thirty-nine dogs with persistent renal proteinuria were recruited at the University of Georgia Veterinary Teaching Hospital. After inclusion, dogs were block-randomized, according to the presence or absence of azotemia and systemic arterial hypertension, to receive enalapril at a dosage of 0.5 mg/kg PO q12h or telmisartan at a dosage of 1 mg/kg PO in the morning and an equal volume of placebo in the evening. Dogs were evaluated at various time points, including pretreatment baseline and after 30 days of treatment with enalapril or telmisartan, at which time whole blood for renal biochemical analyses (including blood urea nitrogen and creatinine concentrations), urine for urinalysis and urinary protein-to-creatinine ratio (UPC), and indirect systolic blood pressure (SBP) readings were obtained for each dog.

Dogs were considered for enrollment in the original study[9] if they had persistent proteinuria with UPC >0.5 if azotemic (ie, blood creatinine ≥1.4 mg/dL, IRIS CKD stages 2-4), or ≥1.0 if nonazotemic (ie, blood creatinine <1.4 mg/dL, IRIS CKD stage 1), documented in ≥2 urine samples collected ≥14 days apart, and ultrasonographic findings consistent with CKD.

On study day 0 (baseline, pretreatment), the following data were collected from each dog: indirect SBP via Doppler ultrasonography (Model 811-B Doppler Ultrasonic Flow Detector, Parks Medical Electronics, Inc, Aloha, Oregon); packed cell volume and total solids; serum biochemical analyses; whole blood renal biochemical analyses (Stat Profile pHOx Ultra, Nova Biomedical Corporation, Waltham, Massachusetts); *Dirofilaria immitis* antigen test (SNAP Heartworm RT Test, IDEXX Laboratories, Westbrook, Maine) or a combined test for *Anaplasma phagocytophilum*, *Anaplasma platys*, *Borrelia burgdorferi*, *Ehrlichia canis*, and *Ehrlichia ewingii* antibodies, and *Dirofilaria immitis* antigen (SNAP 4Dx Plus, IDEXX Laboratories); urinalysis; aerobic bacteriological culture of urine; and abdominal ultrasonography. Dogs were excluded if any of the following were identified at baseline: extrarenal causes of proteinuria or diseases for which treatment might impact the magnitude of proteinuria; average SBP <120 mmHg; moderate hyperkalemia (blood potassium concentration > 6.5 mmol/L); or a history of having received RAASi, corticosteroids, or both, in the 14 days preceding enrollment.

Dogs receiving amlodipine at the time of enrollment were not excluded. In those dogs for which persistent, severe systolic arterial hypertension (ie, SBP ≥ 180 mmHg[20]) was newly documented at screening, amlodipine was started at a dosage of 0.1 mg/kg PO once daily alongside the study medication. At rechecks, the dosage of amlodipine was adjusted at the clinician's discretion, to a maximum dosage of 0.3 mg/kg q12h, targeting SBP between 100 and 180 mmHg. Concurrent treatment with amlodipine was prescribed for 9 ($n = 5$ enalapril- and $n = 4$ telmisartan-treated) dogs for which samples were considered for inclusion in the present study.

After initiation of daily treatment with enalapril or telmisartan, dogs were reevaluated at various time points, including on day 30. Whole blood and urine samples for banking were obtained on each of days 0 and 30 and processed within 1 hour of collection at each time point. Serum and urine were stored at −80°C.

Dogs were included in the present study if serum samples from each of days 0 and 30 were available in sufficient quantity to allow the determination of RAAS components. Availability of banked urine samples was not an exclusion criterion.

2.3 | Healthy dogs

Clinicopathologic and circulating RAAS data from life-stage- and sex-matched healthy dogs were obtained through review of information collected as part of an unrelated prospective study.[19] Briefly, 60 apparently healthy, client-owned dogs were recruited at the Colorado State University Veterinary Teaching Hospital. Dogs underwent physical examination, indirect SBP determination, CBC, serum biochemistry panel, and urinalysis. For dogs with urine dipstick protein greater or equal to 1+ and urine specific gravity <1.040, and all dogs with ≥2+ proteinuria, a UPC was also performed and resulted in exclusion from the study if >0.5. Dogs were also excluded from that study for having a history of cardiac, endocrine, respiratory, urinary, or renal disease or if they had a history of polyuria or polydipsia, or cough. Dogs documented to have a heart murmur, systemic hypertension, or history of treatment with pimobendan, ACEi, spironolactone, ARB, corticosteroid, sympathomimetic, beta-blocker, or mineralocorticoid were also excluded from the healthy dog group.

Data from these dogs were included in the present study if they were matched to an individual within the pCKD group of the same sex and in the same life stage, according to the 2019 AAHA Canine Life Stage Guidelines.[21]

2.4 | Analysis of circulating RAAS components

The circulating RAAS was assessed in banked serum samples from dogs with pCKD by measurement of equilibrium concentrations of Ang I, Ang II, Ang III, Ang IV, Ang 1-5, and Ang 1-7, and aldosterone. Analyses were performed by Attoquant Diagnostics GmbH (Vienna, Austria) using a previously described liquid chromatography-mass spectrometry/mass spectrometry-based technique, RAS-Fingerprint.[13,22,23] The lower limits of quantification for the analytes Ang III, Ang IV, and aldosterone were 2.5 pmol/L, 2.0 pmol/L, and 20 pmol/L, respectively.

Surrogates for renin, angiotensin-converting enzyme (ACE), and ACE2 activity (PRA-S, ACE-S, and ACE2-S, respectively),[12,24] and adrenal responsiveness to Ang II signaling resulting in the release of aldosterone (AA2 ratio)[25] were calculated from the equilibrium concentrations of Ang peptides and aldosterone as follows:

- surrogate measure of ACE activity (ACE-S) = Ang II/Ang I;
- surrogate measure of ACE2 activity (ACE2-S) = Ang 1,5/Ang II;
- surrogate measure of plasma renin activity (PRA-S) = Ang I + Ang II;
- surrogate measure of adrenal responsiveness to Ang II (AA2 ratio) = Aldosterone/Ang II.

Determination of UACR was performed by an external laboratory (Michigan State University Veterinary Diagnostic Laboratory, Lansing, Michigan). Urinary aldosterone was measured using a commercially available radioimmunoassay kit (Active Aldosterone RIA, DSL8600, Beckman Coulter, Fullerton, California). Urinary creatinine was measured using the modified Jaffe method. The UACR was calculated as urinary aldosterone concentration divided by the urinary creatinine concentration and reported in microgram of aldosterone per gram of creatinine.

Banked serum and urine samples from dogs with pCKD were shipped frozen on dry ice in single shipments to the appropriate laboratory. Circulating RAAS data from healthy dogs were generated in this same manner (ie, using RAS-Fingerprint, serum aldosterone

concentration, and UACR) by the same laboratories as part of the original study.[19]

2.5 | Statistical analyses

Analyses were performed using commercially available software (Stata version 16.0, StataCorp LLC, College Station, Texas; GraphPad Prism for MacOS, version 10.2.1, GraphPad Software Inc, La Jolla, California).

Descriptive statistics for demographic, and relevant clinical and clinicopathologic data, including age, sex/neuter status, body weight in kilograms, SBP, blood or serum urea nitrogen, creatinine, albumin, and potassium concentrations, hematocrit, and UPC at baseline (day 0) were calculated for the healthy control group, the pCKD group pair-matched to controls, and for each CKD treatment group (ie, enalapril vs. telmisartan-treated dogs). Distributions of values for clinical variables by study group were examined for normality by visual assessment of histograms and normal quantile plot, and the Shapiro-Wilk test. Normally distributed clinical data are presented as mean ± SD (range).

For the purposes of addressing the main research objectives, mean serum equilibrium concentrations of Ang I, Ang II, Ang III, Ang IV, Ang 1-5, Ang 1-7, and aldosterone, and mean UACR, AA2, PRA-S, ACE-S, and ACE2-S were calculated for the control and CKD groups at pretreatment baseline (study objective 1). For the CKD group, mean concentrations of all evaluated RAAS components, including angiotensin peptide and aldosterone concentrations, UACR, AA2, PRA-S, ACE-S, and ACE2-S were calculated before (day 0) and 30 days after treatment, and mean change in these concentrations for each dog in the study sample and within each treatment group were calculated and compared (study objectives 2 and 3). To explore the possible effects of concurrent calcium channel blockade, age, and cardiac disease on the RAAS, models adjusted for concurrent administration of amlodipine, age, and diagnosis of heart disease, heart murmur, or both were evaluated. No effects of these potential confounding variables were observed, and unadjusted models are reported.

Differences between treatment groups with respect to RAAS markers were assessed using linear mixed models. Mixed Tobit censored regression models were used for variables with values below the limit of quantification. Models comparing dogs with CKD to healthy controls included a random pair effect to account for the dependency in the data that was introduced by the matched study design. Models comparing the effects of enalapril and telmisartan included a random dog effect to account for repeated measurements on the same dogs. Models used restricted maximum likelihood estimation, and degrees of freedom were computed using the Kenward-Roger procedure. Normality of residuals was assessed using normal quantile plots and the Shapiro-Wilk test. Positively skewed variables were log-transformed before analysis to improve the approximation to a normal distribution. Geometric means (GM) and 95% confidence intervals were calculated by back-transforming the means and confidence limits that were estimated on the log-transformed scale. All tests assumed a 2-sided alternative hypothesis, and *P*-values <.05 were considered significant.

3 | RESULTS

3.1 | Animals

For 36 of 39 dogs with pCKD enrolled in the prior study, serum samples from each of days 0 and 30 were available for RAS-Fingerprint and serum aldosterone analyses (Figure 1). Urine samples for UACR were available for 33 of these 36 dogs. For each of 20 dogs with pCKD, a sex- and life-stage pair-matched healthy control dog was identified; of the 20 identified controls, 18 had urine samples available for UACR determination.

3.2 | Circulating RAAS in dogs with pCKD versus healthy controls

Demographic and clinicopathologic characteristics of dogs with pCKD (n = 20) and corresponding sex- and life-stage-matched healthy control dogs (n = 20) are presented in Table 1. Most dogs were of mature adult or senior life stage. Male and female dogs were equally represented. Mean SBP was in the prehypertensive range for the pCKD dogs and the normotensive range for the healthy control dogs, according to the categories set forth by the American College of Veterinary Internal Medicine.[20] Mean blood or serum creatinine and urea nitrogen concentrations were in the nonazotemic range for both groups. Only 4 dogs with pCKD had a blood creatinine concentration ≥1.4 mg/dL (range, 1.6-3.8 mg/dL). Only 1 healthy dog had proteinuria based on urinary dipstick evaluation; therefore, UPC was available for only 1 dog in that group. No dog in the control-matched pCKD group was receiving amlodipine therapy at day 0.

Diet information was available for 33 of 36 dogs with pCKD and all healthy control dogs; 30 dogs with pCKD were fed a prescription commercial clinical renal diet and all but 1 healthy dog was fed a non-renal commercial diet.

Baseline concentrations of RAAS analytes in dogs with pCKD or matched healthy controls are depicted in Figures 2 and 3. There was no significant difference in mean serum equilibrium concentrations of Ang II (GM, 61.8 and 57.8 pmol/L for the control and pCKD groups, respectively; $P = .77$), Ang IV (GM, 7.3 and 8.9 pmol/L for the control and pCKD groups, respectively; $P = .45$), and aldosterone (GM, 37.2 and 44.0 pmol/L for the control and pCKD groups, respectively; $P = .48$). However, relative to healthy dogs, dogs with pCKD had significantly higher mean serum equilibrium concentrations of Ang I (GM, 81.5 and 140.0 pmol/L for the control and pCKD groups, respectively; $P = .04$), Ang III (GM, 4.0 and 7.0 pmol/L for the control and pCKD groups, respectively; $P = .05$), Ang 1-5 (GM, 48.1 and 115.4 pmol/L for the control and pCKD groups, respectively; $P = .01$), and Ang 1-7 (GM, 28.2 and 58.7 pmol/L for the control and pCKD groups,

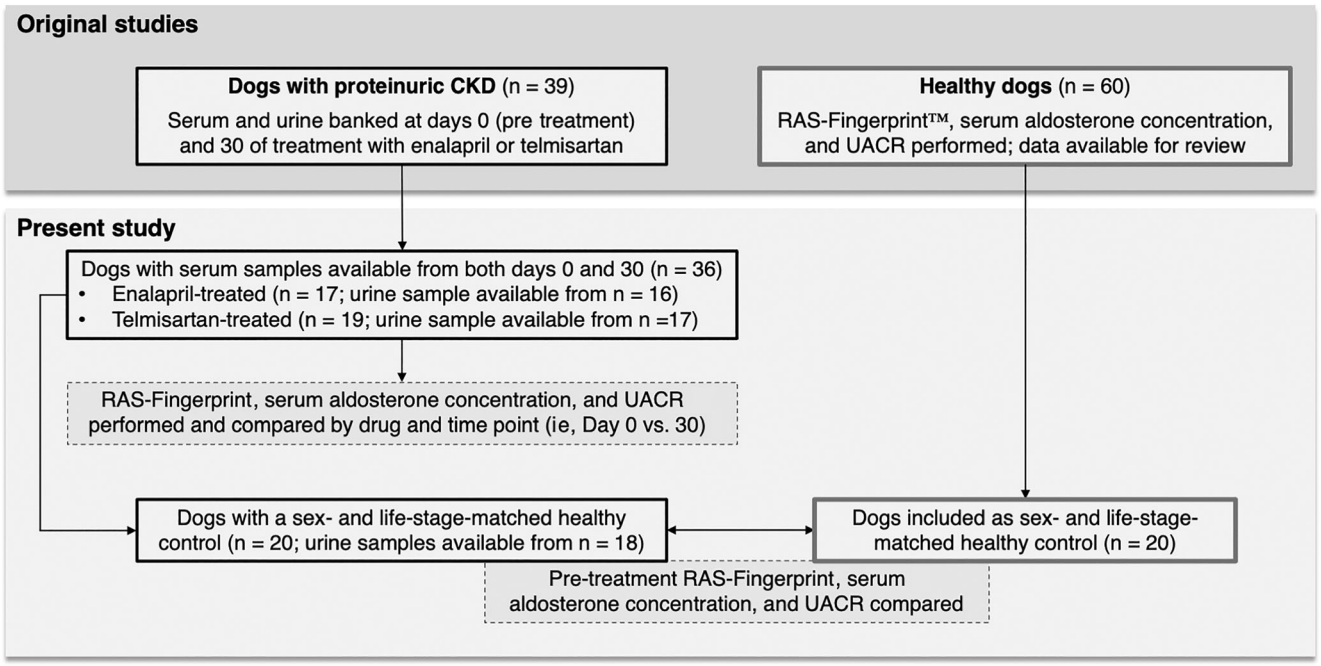

FIGURE 1 Overview of study design. CKD, chronic kidney disease; UACR, urinary aldosterone-to-creatinine ratio.

TABLE 1 Demographic and clinicopathologic data for dogs with proteinuric chronic kidney disease (pCKD; n = 20) and life stage- and sex-pair-matched healthy control dogs (n = 20).

Variable	pCKD dogs	Healthy control dogs
Life-stage (n)		
Young adult	1	1
Mature adult	9	9
Senior	10	10
Age (years)	8.7 ± 2.4 (4.3-14.9)	7.9 ± 2.7 (4-14.8)
Weight (kg)	18.5 ± 12.4 (3.5-42.8)	20.1 ± 11.1 (5-42.6)
Sex (nm/sf)	10/10	10/10
Breeds (n)		
Beagle	3	1
Boston Terrier	2	1
Chihuahua	0	2
Miniature Schnauzer	2	0
Mixed breed dog	2	7
Other	11	9
Systolic arterial blood pressure (mmHg)	149 ± 20 (120-210)	125 ± 16.5 (95-154)
Blood/serum[a] creatinine concentration (mg/dL)	1.2 ± 0.7 (0.6-3.8)	1.0 ± 0.2 (0.7-1.3)
Blood/serum[a] urea nitrogen concentration (mmol/L)	15.1 ± 12.7 (5-64)	18.3 ± 7.3 (10-35)
Blood/serum[a] potassium concentration (mmol/L)	4.3 ± 0.3 (3.9-4.7)	4.4 ± 0.3 (3.9-5.0)
Serum albumin concentration (g/dL)	3.1 ± 0.5 (2.2-3.9)	3.7 ± 0.1 (3.4-3.9)
Hematocrit (%)	44.6 ± 6 (29-52)	51.1 ± 4.4 (45-62)
Urinary protein-to-creatinine ratio	3.9 ± 3.1 (0.8-12.4)	0.24 (n = 1)

Note: Continuous data are presented as mean ± SD (range).
Abbreviations: nm, neutered male; sf, spayed female.
[a]Blood biochemistry was used for pCKD dogs and serum biochemistry was used for healthy dogs.

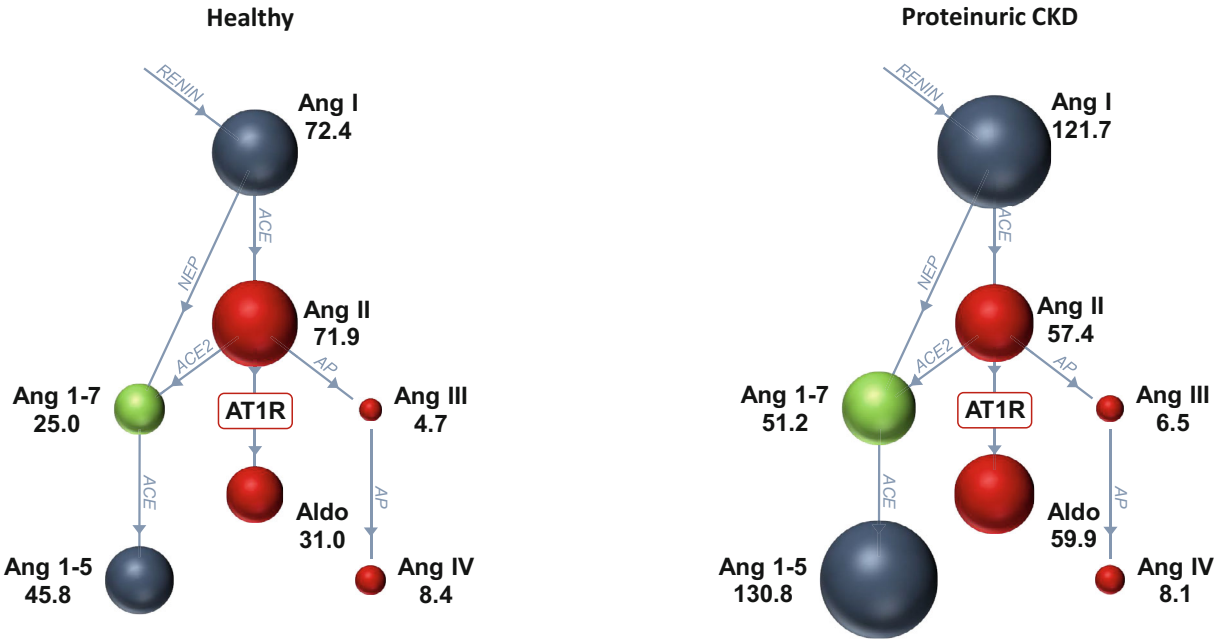

FIGURE 2 Renin-angiotensin-aldosterone system (RAAS) graphs of dogs with proteinuric chronic kidney disease (CKD; n = 20), before treatment with RAAS inhibitors, and healthy life stage- and sex-matched controls (n = 20). The size of each circle is proportional to the median equilibrium concentration (pmol/L) of each peptide or hormone as measured in serum. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Aldo, Aldosterone; Ang 1-5, Angiotensin 1-5; Ang 1-7, Angiotensin 1-7; Ang I, Angiotensin 1; Ang II, Angiotensin II; Ang III, Angiotensin III; Ang IV, Angiotensin IV; AP, aminopeptidase; AT1R, Angiotensin II Receptor Type I; NEP, neprilysin.

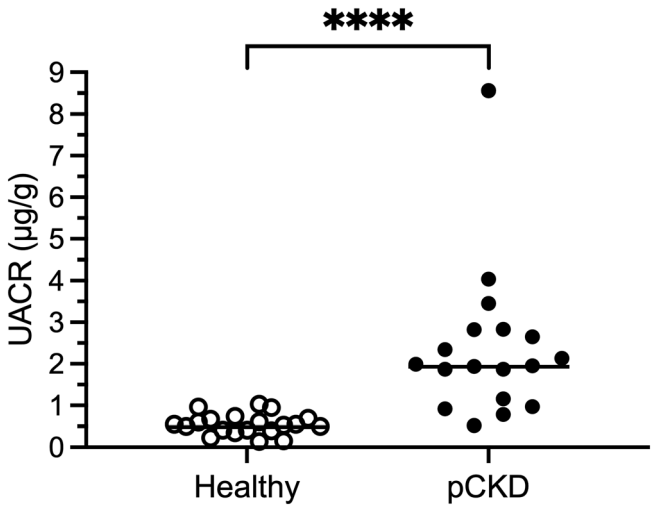

FIGURE 3 Dot plot of urinary aldosterone-to-creatinine ratio (UACR) in dogs with proteinuric chronic kidney disease (pCKD; n = 18) not receiving a renin-angiotensin-aldosterone system inhibitor, and healthy life stage- and sex-matched controls (n = 20). The horizontal bars represent the geometric mean. ****P < .0001.

respectively; P = .02), and UACR (GM, 0.49 and 1.93 µg/g for the control and pCKD groups, respectively; P < .001).

For the calculated values, relative to healthy control dogs, dogs with pCKD had lower mean ACE-S (GM, 0.76 and 0.41 (pmol/L)/(pmol/L) for the control and pCKD groups, respectively; P < .001), and higher mean ACE2-S (GM, 0.78 and 2.0 (pmol/L)/(pmol/L) for the control and pCKD groups, respectively; P < .001). There was no significant difference between groups in the AA2 ratio (GM, 0.62 and 1.00 (pmol/L)/(pmol/L) for the control and pCKD groups, respectively; P = .10) and PRA-S (GM, 147.1 and 201.3 pmol/L for the control and pCKD groups, respectively; P = .19).

3.3 | Circulating RAAS in dogs with pCKD before and after treatment with enalapril or telmisartan

Of the 36 dogs with pCKD included in the present study, 17 (47%) had been randomized to receive enalapril and 19 (53%) had been randomized to receive telmisartan. Urine samples for UACR determination were available from 16 enalapril-treated and 17 telmisartan-treated dogs. Five of 17 enalapril-treated and 4 of 19 telmisartan-treated dogs were receiving concurrent amlodipine therapy. Of these, 5 dogs (n = 3 enalapril- and n = 2 telmisartan-treated) were started on amlodipine on day 0 in keeping with study protocol, and 2 dogs (n = 1 enalapril- and n = 1 telmisartan-treated dogs) had been receiving amlodipine before enrollment.

Baseline demographic and clinicopathologic characteristics of the dogs in each treatment group are presented in Table 2. On average, dogs in both treatment groups were middle-aged and older, with males and females being equally distributed within each group.

TABLE 2 Demographic and pretreatment clinicopathologic characteristics of dogs with proteinuric chronic kidney disease randomized to receive enalapril or telmisartan.

Variable	Enalapril (n = 17)	Telmisartan (n = 19)
Age (years)	10.7 ± 1.5 (8.0-14.5)	9.3 ± 3.0 (4.3-14.9)
Weight (kg)	20.1 ± 12.2 (3.5-41.3)	15.7 ± 11.3 (4.0-42.8)
Sex (nm/sf)	12/5	13/6
Breeds (n)		
Beagle	2	2
Boston Terrier	0	2
Cocker Spaniel	1	1
Fox Terrier	0	2
German Shepherd	1	1
Golden Retriever	2	0
Jack Russell Terrier	3	0
Miniature Schnauzer	0	2
Mixed breed dog	1	1
Soft Coated Wheaten Terrier	1	1
Yorkshire Terrier	2	0
Other	5	7
Systolic arterial blood pressure (mmHg)	157 ± 23.7 (126-210)	157 ± 28.9 (120-220)
Blood creatinine concentration (mg/dL)	1.1 ± 0.6 (0.5-2.3)	1.4 ± 1.1 (0.7-5.0)
Blood urea nitrogen concentration (mmol/L)	14.9 ± 8.1 (8-37)	19.4 ± 16.1 (5-64)
Blood potassium concentration (mmol/L)	4.5 ± 0.5 (3.9-5.5)	4.4 ± 0.3 (3.9-4.9)
Serum albumin concentration (g/dL)	3.3 ± 0.4 (2.6-3.9)	3.1 ± 0.5 (2.2-3.9)
Hematocrit (%)	44.9 ± 4.9 (35-53)	45.6 ± 5.7 (29-53)
Urinary protein-to-creatinine ratio	3.9 ± 4.1 (0.8-15.5)	4.7 ± 3.2 (0.8-13.4)

Note: Continuous data are presented as mean ± SD (range).
Abbreviations: nm, neutered male; sf, spayed female.

Serum equilibrium concentrations of traditional and alternative Ang peptides and aldosterone at day 0 (pretreatment baseline) or on day 30 of treatment with enalapril or telmisartan are presented in Figures 4 and 5. Statistically significant differences in mean values for day 0 RAAS markers between enalapril- and telmisartan-treated dogs were observed only for Ang III and Ang IV concentrations, both of which were significantly higher in the telmisartan group relative to the enalapril group (Table 3). A significant interaction between the effects of drug and day (ie, differing effects of enalapril and telmisartan on RAAS markers) was seen for most analytes, with the exception of serum Ang I and aldosterone concentrations, PRA-S, and UACR. Overall, relative to enalapril-treated dogs, dogs treated with telmisartan for 30 days had significantly higher equilibrium concentrations of the classical Ang peptides Ang I, II, III, and IV, and the alternative Ang peptides Ang 1-5 and 1-7.

The surrogate ACE2-S could not be precisely determined for 2 enalapril-treated dogs whose Ang 1-5 concentrations were below the assay's lower limits of quantification on day 30. At pretreatment baseline, there were no significant differences in ACE-S or ACE2-S between treatment groups (Table 3). However, after 30 days of treatment, enalapril-treated dogs experienced a significant reduction in ACE-S and ACE2-S, whereas telmisartan-treated dogs had no significant changes in these surrogate markers. The surrogate for renin activity, PRA-S, was significantly increased compared with baseline at day 30 in both drug treatment groups. Because serum concentrations of aldosterone were below the assay lower limits of quantification for 11 dogs, the AA2 ratio could only be precisely determined for 11 and 14 enalapril- and telmisartan-treated dogs, respectively. Nonetheless, the AA2 ratio was significantly lower compared with baseline in telmisartan-, but not enalapril-treated dogs. Further, mean AA2 ratio on day 30 was significantly lower in telmisartan- relative to enalapril-treated dogs.

A decrease in mean serum aldosterone concentration was noted after 30 days of treatment in both treatment groups. However, an effect of either enalapril or telmisartan treatment on UACR was not observed. Day 30 serum aldosterone concentration was higher than the corresponding day 0 value for 2 (10.5%) of 19 telmisartan- and 2 (11.7%) of 17 enalapril-treated dogs; and day 30 UACR was higher than the corresponding day 0 value for 8 (47.1%) of 17 telmisartan- and 8 (50.0%) of 16 enalapril-treated dogs for which urine samples were available.

4 | DISCUSSION

The present study demonstrated greater serum equilibrium concentrations of Ang I, Ang III, Ang 1-5, and Ang 1-7, higher ACE2-S, and UACR, and lower ACE-S in dogs with pCKD compared with healthy age- and sex-matched controls. However, no significant differences were noted in the serum equilibrium concentrations of Ang II, Ang III, Ang IV, or aldosterone, or in PRA-S or AA2 ratio between these groups. Consistent with our hypothesis, after 30 days of treatment, Ang II concentrations were significantly lower in dogs with pCKD treated with enalapril (0.5 mg/kg PO q12h) versus those treated with telmisartan (1 mg/kg PO 24 h). Also, ABT, in the form of increased serum aldosterone concentration or UACR relative to baseline, was noted in 11% and 48% of dogs treated with a RAASi, respectively. This phenomenon occurred in similar proportions of dogs of both treatment groups.

Pathologic activation of the RAAS is well-described in CKD.[26,27] Therefore, the lack of significant increases in PRA-S or serum Ang II concentrations in dogs with pCKD compared with healthy controls is noteworthy. This is not an entirely unexpected finding, however, because plasma angiotensin II concentrations and plasma renin activity (PRA) were significantly increased in people with CKD examined in

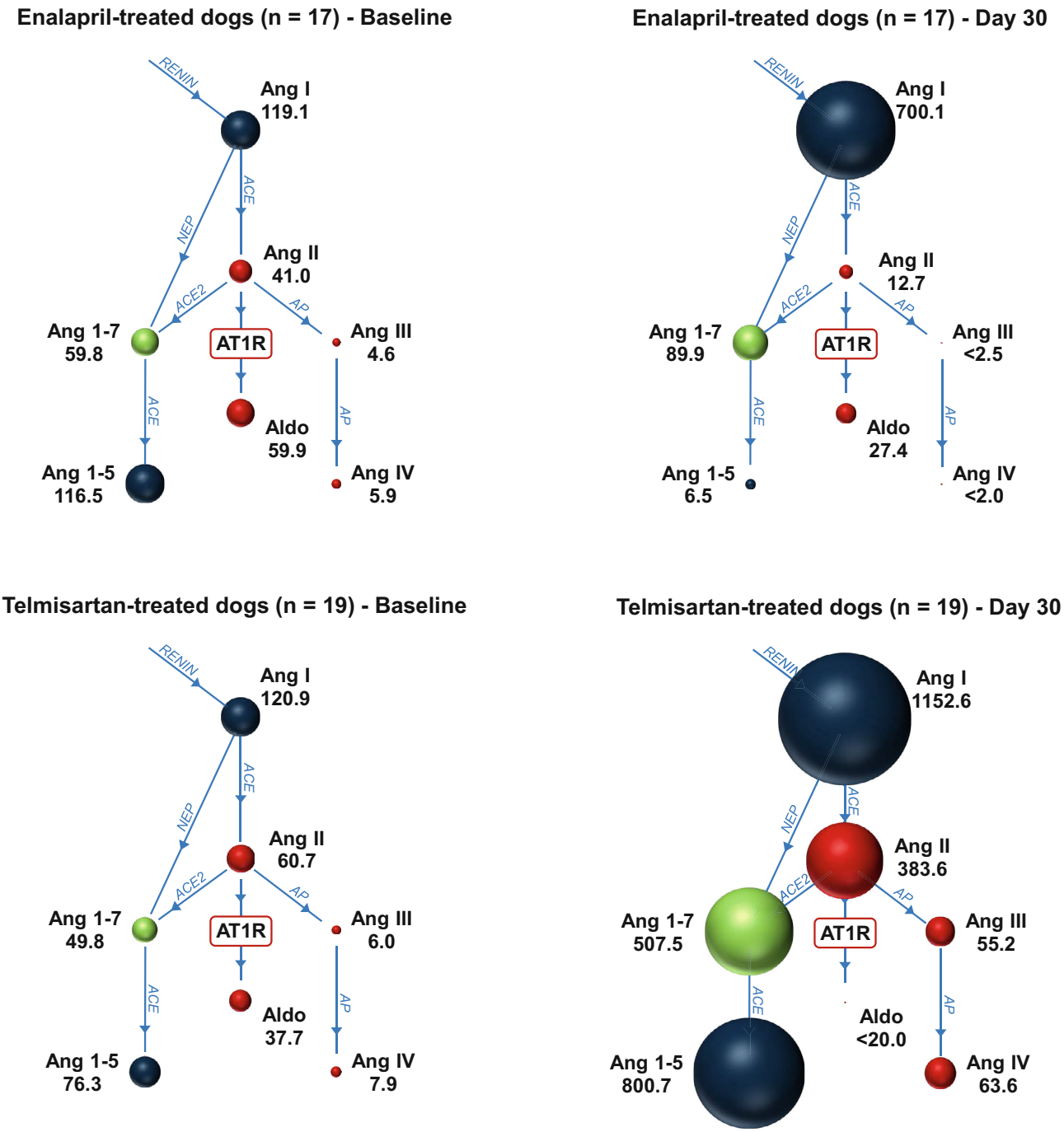

FIGURE 4 Renin-angiotensin-aldosterone system graphs of dogs with proteinuric chronic kidney disease treated with enalapril (0.5 mg/kg PO q12h) or telmisartan (1 mg/kg PO in the morning and placebo in the evening) at day 0 (pretreatment) or day 30 of treatment. The size of each circle is proportional to the median equilibrium concentration (pmol/L) of each peptide or hormone as measured in serum. ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang I, Angiotensin 1; Ang 1-7, Angiotensin 1-7; Ang II, Angiotensin II; Ang III, Angiotensin III; Ang 1-5, Angiotensin 1-5; Ang IV, Angiotensin IV; Aldo, Aldosterone; AP, aminopeptidase; AT1R, Angiotensin II Receptor Type I; NEP, neprilysin.

some[28] but not all studies.[29] In another report, higher PRA and plasma Ang II concentrations were noted in people with naturally occurring hypertensive CKD but not in those with normotensive CKD, compared with healthy controls.[30] It is important to note that while there was not a significant difference in PRA-S or Ang II between pCKD and controls, mean Ang I was significantly higher in pCKD dogs, and there was also a nonstatistically significant trend toward higher PRA-S in that group compared with controls. The lack of significant differences for PRA-S and aldosterone might be because of a type II error, potentially attributable to the relatively small sample size in the present study. We did not perform an a priori power calculation, as our sample size was determined based on the availability of banked samples from

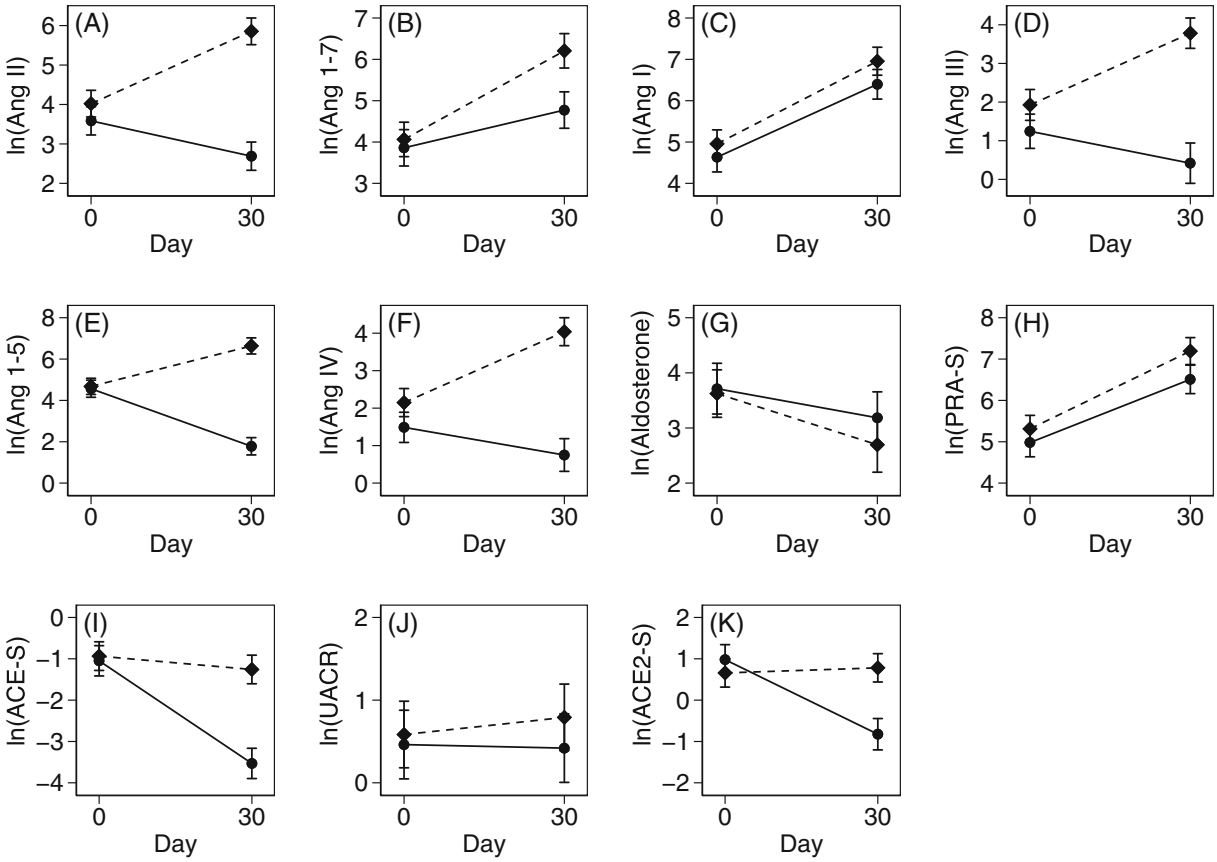

FIGURE 5 Estimated mean (95% confidence intervals) equilibrium concentrations of angiotensin peptides (pmol/L) and aldosterone concentrations (pmol/L), urinary aldosterone-to-creatinine ratio (UACR; μg/g), and calculated markers of renin-angiotensin-system plasma renin activity by drug treatment group (solid line, enalapril; dashed line, telmisartan) and day based on the mixed model analyses. (A) ln(Ang II), natural log of angiotensin II; (B) ln(Ang 1-7), natural log of angiotensin 1-7; (C) Ang I, natural log of angiotensin I; (D) ln(Ang III), natural log of angiotensin III; (E) ln(Ang 1-5), natural log of angiotensin 1-5; (F) ln(Ang IV), natural log of angiotensin IV; (G) ln(Aldosterone), natural log of aldosterone; (H) ln(PRA-S), natural log of plasma renin activity surrogate; (I) ln(ACE-S), natural log of angiotensin-converting enzyme activity surrogate; (J) ln(UACR), natural log of UACR; (K) ln(ACE2-S), natural log of angiotensin-converting enzyme 2 activity surrogate. Mixed Tobit censored regression models were used for Ang III, Ang 1-5, Ang IV, and aldosterone to account for values that were below the limit of quantification. ACE2, angiotensin-converting enzyme 2; ACE2-S, surrogate of angiotensin-converting enzyme 2 activity; ACE-S, surrogate of angiotensin-converting enzyme activity; Ang, angiotensin; PRA-S, surrogate measure of plasma renin activity.

previous studies. Further, only 4/20 (20%) pCKD dogs matched to healthy controls in the present study were systemically hypertensive (ie, SBP ≥160 mmHg) at baseline, and subgroup analyses were not performed. Studies in dogs with experimentally induced renal injury and systemic arterial hypertension have also documented increases in PRA and plasma Ang II relative to preinjury values.[31,32] Therefore, it is reasonable to predict that a similar pattern of RAAS activation might be possible in dogs with naturally occurring disease.

In the original study from which data for the healthy control group of the present report originated,[19] UPC was not assessed unless dogs had proteinuria based on a positive dipstick reading, which was true for only 1 dog. This might have resulted in underestimation of proteinuria in this sample, as proteinuria cannot be definitively ruled out by negative or trace dipstick protein readings.[33] It is possible that some dogs with nonazotemic pCKD might have been misclassified as healthy. Recently, dogs with presumed glomerular disease and UPC >2 were shown to have lower PRA and serum aldosterone concentrations than those with non- or only mildly proteinuric CKD.[34] However, comparison to healthy dogs was not attempted in that study.

The RAAS is a complex system comprising numerous effector molecules and receptors. Therefore, evaluation of single markers such as PRA, Ang II, or aldosterone provides a limited view of this system. The alternative RAAS pathway, which includes peptides and receptors such as Ang 1-7, the angiotensin II type 2 receptor (AT2R) and the Mas receptor, elicits biological functions that oppose those of the classical RAAS and serves as an intrinsic counterbalancing pathway.[11,35] One study of dogs with heart disease found that ACE2 activity and serum equilibrium concentrations of "beneficial" angiotensin peptides, such as Ang 1-7, were significantly higher in dogs with congestive heart failure compared with dogs with preclinical disease, suggesting upregulation of this system.[16] Similarly, the authors of another report found higher concentrations of circulating Ang 1-7 and lower ACE activity in dogs with more advanced heart failure,

despite these dogs being treated with similar dosages of an ACEi.[36] These results mirror the findings of the present study, suggesting the upregulation of beneficial RAAS peptides in the presence of chronic cardiac or kidney disease.

Treatment with RAASi, including ACEi and ARBs, delays the progression of CKD in humans and dogs,[15,37,38] and is the standard of care for dogs with pCKD.[7,8] Since appropriate antagonism of the RAAS is a critical component of the treatment of pCKD, describing the effects of ACEi and ARBs on the extended pathways of Ang production and aldosterone secretion is critical for the development of more effective therapeutic regimens. In the present study, dogs with pCKD treated with the ARB telmisartan or the ACEi enalapril for 30 days experienced changes in the classical Ang peptides and surrogate markers for PRA and ACE activity that are consistent with their predicted effects on the RAAS. Specifically, enalapril therapy resulted in significant decreases in ACE-S and Ang II concentrations. Conversely, Ang II concentrations increased in dogs treated with telmisartan. This is expected; because the angiotensin II type 1 receptor (AT1R) mediates negative feedback inhibition of renin secretion, treatment with telmisartan—a selective blocker of the AT1R—increases the release of renin, the rate-limiting step in the circulating RAAS cascade.[39] However, the potentially deleterious effects of these higher Ang II concentrations are also blocked by telmisartan's antagonism of the AT1R.[40] These changes in circulating RAAS mimic those found in dogs with myxomatous mitral valve disease treated with either an ACEi (enalapril or benazepril) or telmisartan.[16]

Generation of Ang 1-7 can occur through the cleavage of Ang II by ACE2 or cleavage of Ang I by neprilysin.[11] Significant increases in Ang 1-7 were seen in both telmisartan- and enalapril-treated dogs in the present study, which might have been because of increased substrate (ie, increased Ang I for enalapril-treated, and increased Ang I and II for telmisartan-treated dogs) or increased expression of ACE2.[41] However, Ang 1-7 concentrations at day 30 were significantly higher in the telmisartan group, likely because a proportionally greater increase in substrate occurred in that group. The effects of the alternative RAAS are mediated by the AT2R, for which Ang II, Ang III, and Ang 1-7 are ligands,[42] and the Mas receptor, which is activated by Ang 1-7.[43] Proposed renoprotective effects of the AT2R and the Mas receptor include attenuation of inflammation, oxidative stress, fibrosis, and systemic hypertension.[11,44] Therefore, the increases in Ang II, Ang III, and Ang 1-7 observed after 30 days in the telmisartan group represent a theoretical advantage of this drug compared with enalapril.

The phenomenon of ABT has been defined as an increase in serum aldosterone concentration or UACR compared with pretreatment baseline, or an increase in UACR relative to a reference interval derived from healthy dogs.[14,18] In the present study, relatively few pCKD dogs (10.5% and 11.7% of telmisartan- and enalapril-treated dogs, respectively) met the criterion for ABT based on a change in serum aldosterone; however, the proportion increases to approximately one-half of these dogs (47.1% and 50.0% of telmisartan- and enalapril-treated dogs, respectively) when considering change in UACR. The differences in these measures might be attributed to the methodological nuances of each. Serum aldosterone concentration directly reflects aldosterone production, while UACR is additionally influenced by aldosterone excretion dynamics and renal clearance, which are influenced by numerous factors and can vary independently of production rates.[45,46] Therefore, UACR might not be an ideal measure of aldosterone production because of its dependence on renal clearance,[45] which we could not confirm as constant in our study. Therefore, we recommend prioritizing serum aldosterone results as they provide a more direct assessment of aldosterone production. A study evaluating the prevalence of ABT as defined by UACR in dogs with pCKD treated with various dosages of ACEi, ARBs, or both, described the occurrence of this phenomenon in a similar proportion of dogs. However, ABT was not associated with proteinuria outcome in that study,[18] likely reflecting aldosterone's role as one of many players in this extensive system.

The present study did not find a significant effect of concurrent amlodipine therapy on evaluated RAAS components, which is in contrast to prior studies in dogs.[24,47,48] This lack of effect is likely attributable to the fact that only a small number of dogs received amlodipine and these were evenly distributed between the treatment groups.

This study has several limitations that mainly derive from its retrospective nature. First, the relatively small sample size of evaluated groups might have limited our power to detect differences in RAAS markers, which typically show high interindividual variability.[49] Variability in diet also existed; dogs with pCKD were fed a clinical renal, sodium-restricted diet, whereas healthy controls were fed a routine maintenance diet. However, this difference would predictably result in the activation of the classical RAAS and circulating Ang II in dogs with pCKD, which was not appreciated. The time of sample collection and its relationship to feeding or drug administration was not standardized in the original pCKD study. Therefore, diurnal variation of the RAAS or the effects of timing of feeding and dietary sodium intake might have impacted our results.[50,51] Additionally, the small number of azotemic dogs (6 of 36) limits the ability to perform robust correlation analyses between RAAS biomarkers and the degree of GFR decrease. Further studies with larger samples of azotemic animals are necessary to fully understand these relationships. Previous studies have noted effects of sex, age, and breed on RAAS, which introduce variability in the activation of RAAS.[52,53] Although our study attempted to control for the effect of age and sex, it did not control for possible breed differences in ACE gene polymorphism.[14] There are potential discrepancies in the timeline for sample collection and storage. All samples were handled similarly and stored at −80°C, but variations in storage time could influence the results. This limitation should be considered when interpreting our findings. Finally, the evaluation of the RAAS was conducted solely at the circulating level. The activity of the intrarenal renin-angiotensin system, a critical factor in the pathophysiology of kidney diseases,[54] remains to be studied in dogs with pCKD and in response to treatment with ACEi or ARB therapy.

In conclusion, compared with normal controls, dogs with pCKD had alterations in circulating RAAS that were mainly observed in the

TABLE 3 Renin-angiotensin-aldosterone system (RAAS) markers in dogs with proteinuric chronic kidney disease randomized to receive enalapril (0.5 mg/kg PO q12h; *n* = 17) or telmisartan (1 mg/kg PO q24h; *n* = 19) for 30 days.

		Absolute values, geometric mean								
		Day 0			Day 30			Day 0 vs. 30 (*P*-value)		Percent change, mean (95% CI)	
RAAS marker	Interaction between drug and day (*P*-value)	Ena	Tel	Ena vs. Tel (*P*-value)	Ena	Tel	Ena vs. Tel (*P*-value)	Ena (*P*-value)	Tel (*P*-value)	Enalapril	Telmisartan
Ang I (1–10) [pmol/L]	.34	102.8	142.0	.20	599.9	1048.5	0.026	<.001	<.001	483% (309–731%)	638% (428–932%)
Ang II (1–8) [pmol/L]	<.001	36.0	55.7	.083	14.7	348.9	<.001	<.001	<.001	−59% (−71–41%)	525% (347–775%)
Ang III (2–8) [pmol/L]	<.001	3.5	6.9	.025	1.5	43.9	<.001	<.001	<.001	−56% (−72–32%)	540% (366–778%)
Ang IV (3–8) [pmol/L]	<.001	4.4	8.6	.018	2.1	56.8	<.001	<.001	<.001	−52% (−66–32%)	563% (398–784%)
Ang 1–5 [pmol/L]	<.001	96.1	107.7	.69	5.9	762.1	<.001	<.001	<.001	−94% (−96–91%)	608% (381–941%)
Ang 1–7 [pmol/L]	<.001	47.5	58.1	.51	118.3	495.9	<.001	<.001	<.001	149% (69–268%)	752% (489–1134%)
AA2 ratio [(pmol/L)/(pmol/L)]	.021	2.2 (*n* = 11)	1.0 (*n* = 14)	.090	1.8 (*n* = 12)	0.2 (*n* = 7)	<.001	0.63	<.001	−19% (−67–97%)	−83% (−94–55%)
PRA-S [pmol/L]	.15	145.5	202.8	.17	670.7	1330.2	0.005	<.001	<.001	361% (226–551%)	556% (373–810%)
ACE-S [(pmol/L)/(pmol/L)]	<.001	0.35	0.39	.66	0.03 (*n* = 15)	0.28	<.001	<.001	0.13	−92% (−95–87%)	−28% (−52–9%)
ACE2-S [(pmol/L)/(pmol/L)]	<.001	2.67	1.93	.21	0.44	2.18	<.001	<.001	0.48	−84% (−88–76%)	13% (−19–58%)
Aldosterone [pmol/L]	.24	40.9	37.5	.78	24.2	14.8	0.15	0.02	<.001	−41% (−63–7%)	−60% (−75–36%)
UACR [µg/g]	.27	1.59 (*n* = 16)	1.79 (*n* = 17)	.68	1.52 (*n* = 16)	2.21 (*n* = 17)	0.21	0.79	0.19	−4% (−30–32%)	23% (−10–68%)

Note: Day 0 data were collected pretreatment. The number of dogs with available data is presented if it differs from the total number included in each group.
Abbreviations: AA2 ratio, surrogate of adrenal responsiveness to angiotensin II (Aldosterone/Angiotensin II); ACE2-S, surrogate of angiotensin-converting enzyme 2 activity (Ang1-5/AngII); ACE-S, surrogate of angiotensin-converting enzyme activity (AngII/AngI); CI, confidence interval; Ena, enalapril; Tel, telmisartan.

alternative RAAS pathway and the urinary excretion of aldosterone. Treatment with telmisartan, which selectively blocks the AT1R responsible for the classical effects of Ang II, resulted in a significant increase in serum equilibrium concentrations of Ang II and, relative to enalapril, an associated significantly greater increase in the beneficial angiotensin peptide Ang 1-7, creating a shift in the circulating RAAS that favored the alternative counterbalancing RAAS pathway. Treatment with enalapril resulted in a decrease in Ang II and a milder increase in Ang 1-7 compared with pretreatment values. Serum aldosterone concentrations decreased in dogs with pCKD who were treated with either telmisartan or enalapril with no difference between groups, and a subset of dogs in both groups experienced ABT. The clinical importance of the balance between classical and alternative circulating RAAS activation and the phenomenon of ABT in these dogs is undetermined.

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CONFLICT OF INTEREST DECLARATION

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OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

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

Approved by the Clinical Research Committee of the University of Georgia's College of Veterinary Medicine (approval number CR-399).

HUMAN ETHICS APPROVAL DECLARATION

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

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