STANDARD ARTICLE

Effect of angiotensin receptor blockers and angiotensin converting enzyme 2 on plasma equilibrium angiotensin peptide concentrations in dogs with heart disease

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Abstract

Background: The pathophysiology of heart failure involves maladaptive angiotensin peptides (APs) and enzymes, including angiotensin 2 (AT2) and angiotensin converting enzyme (ACE), as well as recently described alternative components, such as angiotensin 1-7 (Ang1-7) and angiotensin converting enzyme 2 (ACE2). The relative effects of different neurohormonal-targeting drugs on balance of APs in dogs with heart disease are unknown.

Hypothesis/Objectives: Plasma AP concentrations differ in dogs receiving angiotensin converting enzyme inhibitors (ACEIs) vs angiotensin receptor blockers (ARBs) and recombinant human ACE2 (rhACE2) will further increase these differences.

Animals: Eight dogs with degenerative mitral valve disease (DMVD).

Methods: Prospective open-label trial. Equilibrium concentrations of APs from plasma during PO ACEI treatment and then after 14 days of PO ARB treatment using telmisartan were measured using liquid chromatography-tandem mass spectroscopy before and after in vitro incubation with rhACE2.

Results: Concentration of Ang1-7 was increased during ARB treatment (Ang1-7: 443 pg/mL; 95% confidence interval [CI] = 247-794 pg/mL) vs ACEI (Ang1-7: 182 pg/mL; 95% CI = 66.2-503 pg/mL; P = .01). Incubation with rhACE2 decreased traditional APs while increasing beneficial alternative APs, and Ang1-7 was significantly higher in the ARB + rhACE2 (880 pg/mL; 95% CI = 560-1383 pg/mL) vs ACEI + rhACE2 (455 pg/mL; 95% CI = 188-1104 pg/mL; P = .03) group. The most favorable theoretical AP profile was achieved in the ARB + rhACE2 group.

Conclusions and Clinical Importance: The AP profile during telmisartan treatment is associated with higher plasma Ang1-7 as compared with during ACEI. This favorable

shift is potentiated in vitro by combination of ARB + rhACE2. These data support potential AP-targeting strategies and drugs in dogs with DMVD.

1 | INTRODUCTION

The angiotensin peptide (AP) system, which is a part of the renin-angiotensin-aldosterone-system (RAAS), plays an important role in the pathophysiology of congestive heart failure (CHF) in the dog.[1-3] Key components are the various APs, such as angiotensin 2 (AT2) that mediate vasoconstriction, sodium retention, and fibrosis.[4] Production of many APs is catalyzed by the dicarboxypeptidase, angiotensin converting enzyme (ACE), which cleaves 2 amino acids from the decapeptide angiotensin 1 (AT1) resulting in the octapeptide, AT2. A component of the newly described alternative AP system is the mono-carboxypeptidase, angiotensin converting enzyme 2 (ACE2), which removes a single amino acid from either AT1 or AT2, resulting in angiotensin 1-9 (Ang1-9) and angiotensin 1-7 (Ang1-7), respectively (Figure 1).[5,6] Despite a difference of only 1 amino acid, Ang1-9, Ang1-7, and other alternative APs, such as angiotensin 1-5 (Ang1-5)

are vasodilatory, natriuretic, and cardioprotective.[7] The best characterized alternative AP is Ang1-7, which binds a G-protein coupled receptor, MasR, and is strongly cardioprotective.[8,9] The discovery of ACE2 offers novel ways to favorably alter the AP system beyond the use of ACE inhibitors (ACEIs).[10]

Angiotensin receptor blockers (ARBs) are drugs that primarily block the type 1 AT2 receptor and are used for treatment of hypertension, proteinuria, and CHF in humans.[11-13] In dogs, ARBs are sometimes used to treat hypertension and proteinuria,[14] but use of ARBs in dogs with heart disease is uncommon. One study[15] of dogs with degenerative mitral valve disease (DMVD) showed that a combination of ARB and neprilysin inhibitor decreased RAAS activity. In another study[16] of purpose-bred dogs fed a low salt diet, the same combination similarly decreased RAAS activity. Unlike ACEIs, ARBs permit unabated AT2 production, but harmful effects are prevented by blockade at the AT2 receptor level.[17] Thus, treatment is characterized by
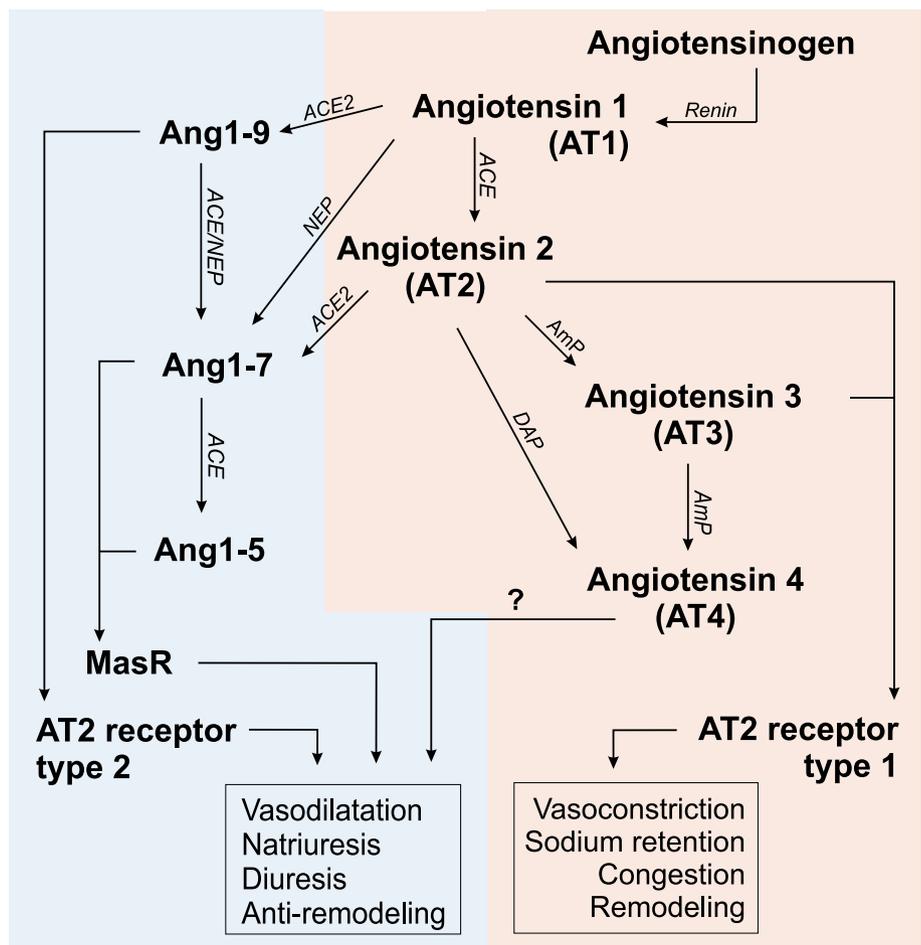
FIGURE 1 The traditional (right hand side, orange background) and alternative (left hand side, blue background) renin-angiotensin-aldosterone system pathway. ACE, angiotensin converting enzyme; ACE2, angiotensin converting enzyme 2; AmP, aminopeptidase; Ang1-5, angiotensin 1-5; Ang1-7, angiotensin 1-7; Ang1-9, angiotensin 1-9; DAP, aspartyl aminopeptidase; NEP, neutral endopeptidase

low AT2 concentrations when using ACEIs and high AT2 concentrations when using ARBs. One potential result of ARB usage is the availability of a large substrate pool of AT2 that could be converted to Ang (1-7) by ACE2 (Figure 1).[18]

The relative concentrations of various traditional and alternative ACE and ACE2-related APs in healthy dogs and dogs with heart disease have been described previously.[19-22] In 1 study,[19] plasma from affected dogs receiving ACEI was incubated with recombinant human ACE2 (rhACE2), resulting in more beneficial APs while simultaneously generating fewer maladaptive APs, but the baseline concentration of AT2 was low because of the presence of ACEI, and the beneficial effect of ACE2 might have been more had dogs been receiving either no ACEI or ARB instead of ACEI. Our hypothesis was that relative plasma concentrations of traditional and alternative APs differ at baseline and in vitro after rhACE2 incubation in dogs receiving ARB as compared with ACEI. Specifically, we hypothesized that concentrations of APs reliant on AT2 as their substrate would be increased in dogs receiving ARBs, and that rhACE2 treatment would further increase these differences in comparison to ACEI. Our objective was to characterize the traditional and alternative APs in a cohort of dogs with DMVD receiving ACEI and then ARB with particular attention to the balance of beneficial APs, such as Ang1-7, Ang1-9, and Ang1-5 compared with maladapative APs, such as AT1 and AT2.

2 | MATERIALS AND METHODS

We designed a prospective open-label study for dogs receiving treatment for DMVD. The study protocol underwent institutional review and approval, and dog owners were required to provide informed consent. Inclusion criteria included a left apical systolic murmur, echocardiographic diagnosis of thickened and prolapsing mitral valve leaflet(s) and mitral regurgitation, as well as clinical history, physical examination, and thoracic radiographs consistent with either stage B2 or C DMVD.[23] Dogs were required to be receiving ACEI at a stable dose for ≥7 days before enrollment. Exclusion criteria included age < 5 years, congenital heart disease, indirect systolic blood pressure > 160 mm Hg or < 100 mm Hg, severe pulmonary hypertension defined as tricuspid regurgitation velocity > 4 m/s, or presence of concurrent systemic disease that was considered severe in the opinion of the investigator. Echocardiographic studies (iE33, Philips Healthcare, Andover, Massachusetts) were performed without sedation. Normalized left ventricular dimension at end diastole (LVIDdN) and end-systole (LVIDsN), left atrial diameter (LADN), aortic root diameter (AoDN), and the left atrial-to-aortic root diameter ratio were obtained from 2-dimensional right parasternal short axis echocardiographic views using previously described formulas and methods.[24,25] At the baseline visit, 5 to 8 mL of venous blood was drawn and split into plain tubes and tubes containing lithium heparin. Owners then were instructed to discontinue ACEI for a 3-day washout period and then to start telmisartan (Mylan Pharmaceuticals, Canonsburg, Pennsylvania) at a dosage of 1 mg/kg PO q24h. Capsules containing the appropriate telmisartan dose

for each dog were prepared by a veterinary compounding pharmacy (Best Pet Rx, New York, New York). After ≥14 days of telmisartan administration, blood pressure measurement and blood sampling were repeated. At the owner's discretion, telmisartan then was either discontinued for 3 days, followed by reinstitution of the dog's previous ACEI treatment, or telmisartan was continued. During the study period, changes to other concurrent cardiac medications were not permitted.

2.1 | Blood assays and surrogate AP system activity

Serum was used to measure blood urea nitrogen, creatinine, sodium, potassium, and chloride concentrations. Plasma was stored at −80°C until batched measurement of equilibrium concentrations of APs, including AT1, AT2, Ang1-9, Ang1-7, Ang1-5, angiotensin 3 (AT3), and angiotensin 4 (AT4), was performed as previously described.[18-20,22,26] Briefly, plasma was spiked with stable isotope-labeled standards for each AP, allowed to reach equilibrium at 37°C, and assayed using liquid chromatography-tandem mass spectrometry (Attoquant Diagnostics, Vienna, Austria). Use of equilibrium assay avoids the need for special collection and handling requirements at the time of blood collection, increases the signal-to-noise ratio of the assay, and results in AP concentrations higher than, but proportional to plasma concentrations using enzyme inhibitors at the time of blood collection.[18,27] To assess the effect of exogenous rhACE2 on the relative concentrations of APs, plasma samples were incubated in vitro with 5 μg/mL of rhACE2 (R&D Systems, Minneapolis, Minnesota) at 37°C, followed by assay of equilibrium concentrations of AT1, AT2, Ang1-9, Ang1-7, and Ang1-5 using previously described methods.[18,19]

2.2 | Statistical analysis

Based on results from a previous study[19] and a goal to demonstrate ≥2-fold difference in Ang1-7 in ACEI plasma vs ARB plasma after incubation with rhACE2, sample size calculation indicated that 8 dogs would be needed to achieve a power of 0.8. Descriptive statistics regarding patient signalment, physical examination findings, and diagnostic results were generated. Descriptive data are presented as mean (SD) or median (range). Comparisons of blood pressure and renal and electrolyte blood test results during ACEI and ARB treatment were performed using paired t tests or Wilcoxon signed rank tests. Concentrations of APs were natural log transformed and assessed for normality using Kolmogorov-Smirnov tests and visual inspection of histograms and QQ plots. Any AP result that was below the lower limit of detection (LLOD) was entered as 0.5 times the LLOD.[28] The central tendencies of various APs are reported as the geometric mean (95% confidence interval). Four different plasma samples were compared, including at baseline during ACEI treatment, after ARB, and after in vitro rhACE2 incubation of ACEI sample plasma (ACEI + ACE2) and ARB sample plasma (ARB + ACE2). These comparisons
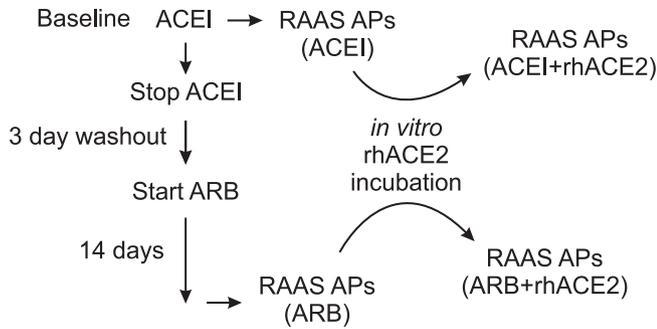
FIGURE 2 Protocol and design of a study investigating the effect of angiotensin converting enzyme inhibitors (ACEIs), the angiotensin receptor blocker (ARB) telmisartan, and in vitro incubation with recombinant human angiotensin converting enzyme 2 (rhACE2) on plasma equilibrium concentrations of angiotensin peptides (APs) in dogs with degenerative mitral valve disease. RAAS, renin-angiotensin-aldosterone system

of AP concentrations were performed using 1-way repeated measures analysis of variance followed by 4 prespecified Holm-Sidak multiple comparison tests of the following group pairs: ACEI vs ARB, ACEI vs ACEI + ACE2, ARB vs ARB + ACE2, and ACEI + ACE2 vs ARB + ACE2. Two unitless surrogates of renin and AP system activity were calculated, including surrogate plasma renin activity (PRA-S) calculated as [AT2] + [AT1] and surrogate ACE activity (ACE-S) calculated as [AT2]/[AT1].[19,29] We mathematically explored the relative activation of alternative APs (ALT) as a ratio of the concentrations of Ang1-9, Ang1-7, and Ang1-5 to the concentrations of AT2 and AT1 (ie, PRA-S; ALT = [Ang1-9] + [Ang1-7] + [Ang1-5])/([AT2] + [AT1]). Correlation among echocardiographic measurements, renal and electrolyte blood test results, and APs while receiving ACEI or ARB were assessed by calculation of either Pearson correlation coefficient or Spearman rank correlation coefficient with Bonferroni correction for multiple comparisons. Figures displaying the various different APs and their relationships were constructed using variably sized circles, the size of which was proportional to the geometric mean equilibrium concentration of each AP. Significance was defined as $P < .05$. Statistical calculations were performed using commercial software (Prism 8, GraphPad Software, San Diego, California). An overview of the study design is shown in Figure 2.

3 | RESULTS

3.1 | Study cohort

Eight dogs presented to the Veterinary Hospital of the University of Pennsylvania were recruited from March to November 2019. Baseline demographic, medication history, and echocardiographic findings are presented in Table 1. Breeds included 2 Chihuahuas, 2 Cavalier King Charles Spaniels, and 1 Beagle, 1 mixed breed dog, 1 Papillion, and 1 Pomeranian. Four dogs were receiving enalapril and 4 dogs were receiving benazepril at baseline. All dogs tolerated ARB and median

TABLE 1 Demographic, medication, and echocardiographic characteristics of the 8 dogs with degenerative mitral valve disease (DMVD) in the study cohort

Age (y)	11.1 (1.6)
Body weight (kg)	6.8 (3.1)
Sex	3 M/5 F
DMVD class (B2/C)	1/7
Furosemide (Y/N)	7/1
Dose (mg/[kg d])	3.4 (2.4-5.5)
Pimobendan (Y/N)	8/0
Dose (mg/[kg d])	0.54 (0.38-0.63)
ACEI (Y/N)	8/0
Enalapril (mg/[kg d])	0.79 (0.63-1.1); n = 4
Benazepril (mg/[kg d])	0.58 (0.38-1.2); n = 4
Days on ACEI	167 (9-791)
Spironolactone (Y/N)	5/3
Spironolactone (mg/[kg d])	1.6 (1.3-2.1)
LVIDdN	1.95 (1.72-2.88)
LVIDsN	1.02 (0.26)
LADN (cm)	1.52 (0.32)
AoDN (cm)	0.74 (0.05)
LAD : AoD	2.08 (0.50)

Note: Data presented as mean (SD) or median (range).
Abbreviations: ACEI, angiotensin converting enzyme inhibitor; AoDN, normalized aortic root diameter; LAD, left atrial diameter; LAD : AoD, left atrial diameter to aortic root diameter ratio; LADN, normalized left atrial diameter; LVIDdN, normalized left ventricular dimension at end diastole; LVIDsN, normalized left ventricular dimension at end systole.

TABLE 2 Hemodynamic and biochemical values while receiving either treatment with angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blocker (ARB)

	ACEI	ARB	P value
Blood pressure (mm Hg)	132 (18.3)	124 (17.4)	.34
BUN (mg/dL)	29 (16)	40 (19)	<.001
Creatinine (mg/dL)	1.0 (0.4)	1.0 (0.3)	.69
Sodium (mEq/L)	143 (3)	144 (4)	.9
Chloride (mEq/L)	110 (105-115)	110 (104-114)	.63
Potassium (mEq/L)	4.5 (0.42)	4.7 (0.4)	.08

Note: Data presented as mean (SD) or median (range).
Abbreviation: BUN, blood urea nitrogen.

duration of dosing was 18 days (range, 15-19 days). Table 2 shows blood pressure and renal and electrolyte blood test results during ACEI and ARB treatment. Blood pressure was not significantly different during ARB and ACEI treatment. Blood urea nitrogen concentration was significantly higher during ARB vs ACEI treatment, but this difference was not associated with clinical signs in any of the dogs.
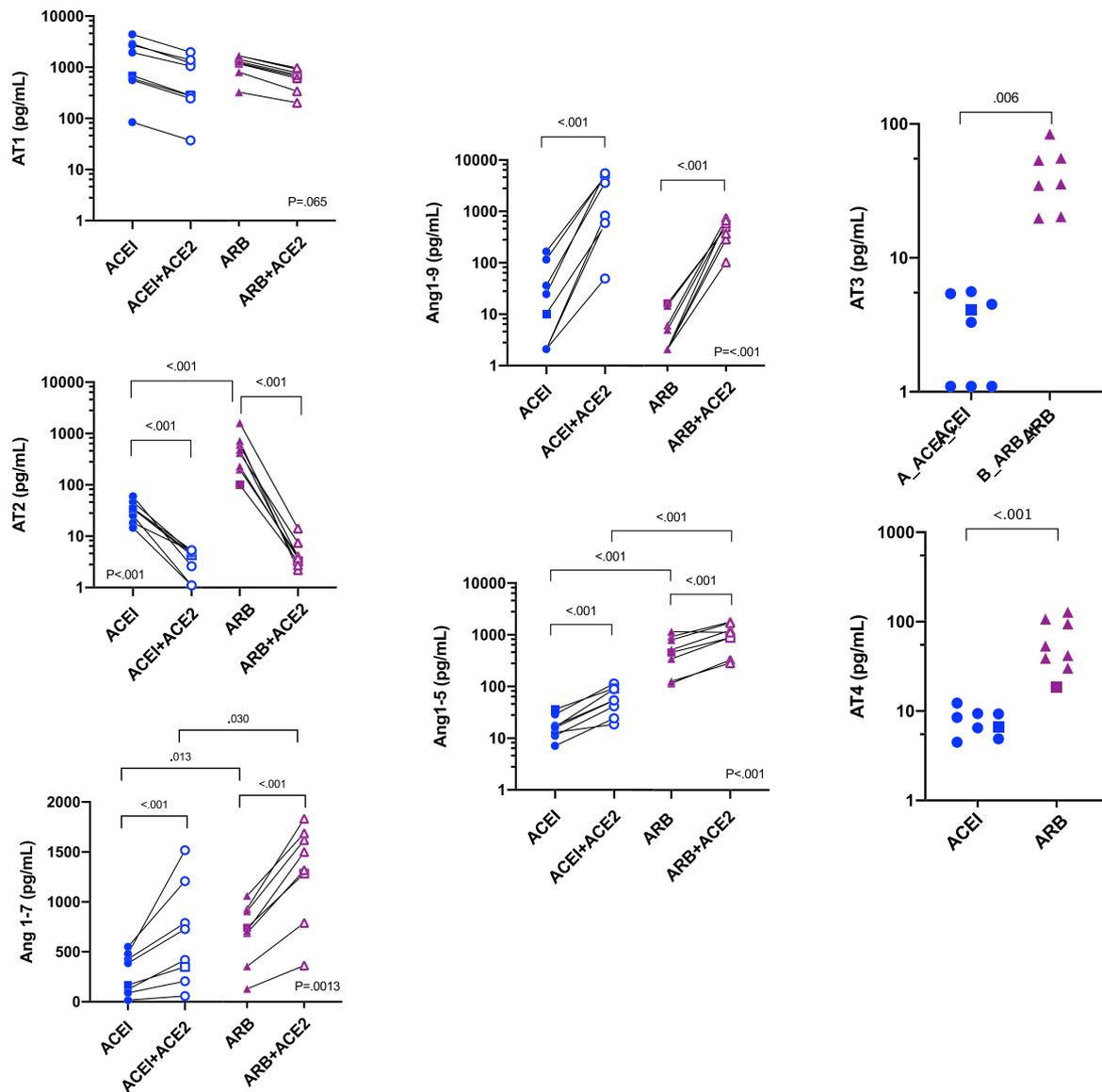
FIGURE 3 Plasma equilibrium concentrations of renin-angiotensin-aldosterone system angiotensin from 8 dogs with degenerative mitral valve disease (DMVD) while receiving angiotensin converting enzyme inhibitor (ACEI) (blue solid circles), angiotensin receptor blocker (ARB) (purple solid triangles) and after in vitro incubation of ACEI plasma with recombinant human angiotensin converting enzyme 2 (ACEI + ACE2) (open blue circles) and ARB plasma with recombinant human angiotensin converting enzyme 2 (ARB + ACE2) (open purple triangles). Data from the 1 dog with stage B2 DMVD are shown as solid and open squares. ACE2, angiotensin converting enzyme 2; ARB, angiotensin receptor blocker; AT1, angiotensin 1; AT2, angiotensin 2; AT3, angiotensin 3; Ang1-5, angiotensin 1-5; Ang1-7, angiotensin 1-7; Ang1-9, angiotensin 1-9
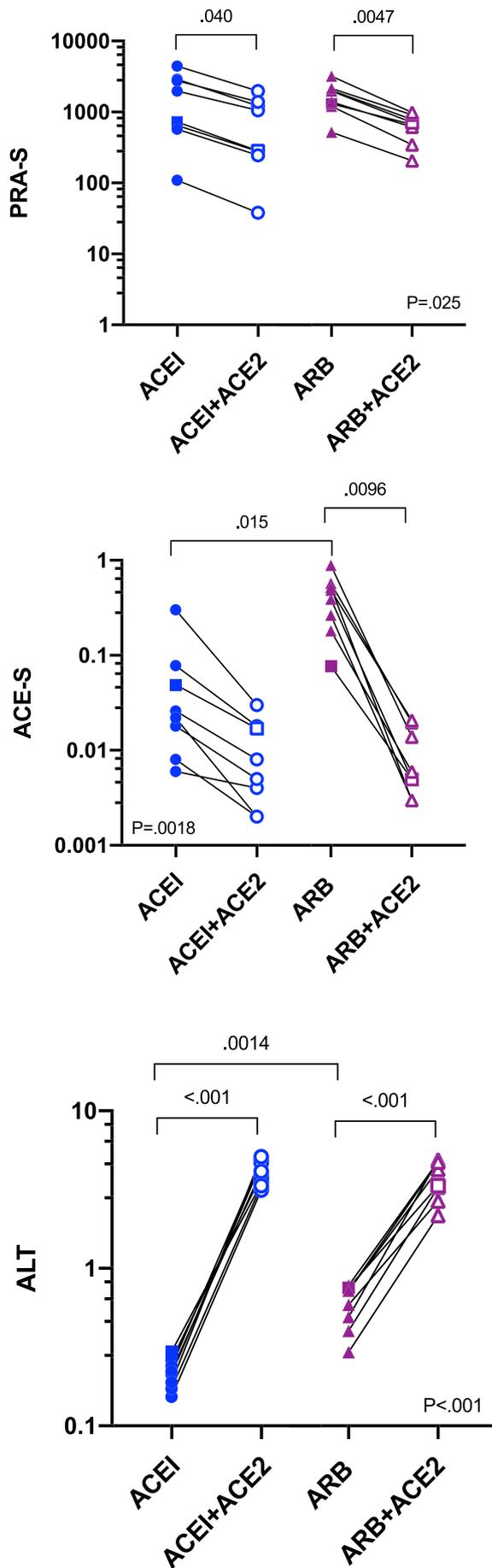
3.2 | Angiotensin peptides during ACEI vs ARB treatment

Equilibrium concentrations of traditional and alternative APs during ACEI and ARB treatment as well as after in vitro incubation with rhACE2 are presented in Figure 3, and surrogate measures of AP system activity are presented in Figure 4. Significantly higher AT2, Ang1-7, Ang1-5, AT3, AT4, ACE-S, and ALT was found in plasma during ARB treatment as compared with plasma during ACEI treatment (Table 3). Specifically, mean concentration of AT2 was 13.1-fold higher and mean concentration of and Ang1-7 was 2.4-fold higher during ARB treatment vs ACEI treatment. Surrogate measure of

angiotensin converting enzyme activity was 6.9-fold higher and ALT was 2.8-fold higher during ARB treatment vs ACEI treatment. Plasma renin activity was similar in the ARB and ACEI groups. No significant correlations were found among echocardiographic and renal and electrolyte blood test results and APs (data not shown).

3.3 | Angiotensin peptides after in vitro incubation with rhACE2

In both the ACEI and ARB groups, AT2 and PRA-S were significantly lower and Ang1-7, Ang1-9, Ang1-5, and ALT were significantly higher

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after in vitro incubation with rhACE2 (Figures 3 and 4 and Table 3). The ACE-S was significantly lower in the ARB + ACE2, but not in the ACEI + ACE2 group. Concentration of Ang1-7 was 1.9-fold higher and concentration of Ang1-5 was 17.2-fold higher in ARB + ACE2 as compared with ACE + ACE2. Concentration of Ang1-9 was 3.0-fold lower in ARB + ACE2 as compared with ACEI + ACE2, but this difference was not statistically significant (P = .08). The relationships and relative amounts of specific APs in the ACEI + ACE2 and ARB + ACE2 groups are presented in Figure 5.

4 | DISCUSSION

Our main finding was that treatment of dogs with DMVD using ACEI or the ARB telmisartan was associated with distinct AP system profiles. The AP profile during ARB treatment was associated with significantly higher concentrations of a variety of APs, including Ang1-7 and AT2, as compared with ACEI treatment. Another finding was that AP profiles could be further manipulated in vitro using rhACE2, which significantly lowered traditional APs, while augmenting alternative APs. This potentially beneficial effect of rhACE2 was greatest in the presence of telmisartan as compared with ACEI. These results increase our understanding of drug effects on the traditional APs, driven by ACE, while simultaneously offering insight into the counterbalancing alternative arm, driven by ACE2. Our study, along with others,[19,22,26] demonstrates that ACEI treatment in dogs with heart disease is associated with relatively low production of APs that are primarily dependent on ACE, particularly AT2, which is produced by cleavage of 2 amino acids from AT1. Our data expand our understanding of ACE production of Ang1-7, which occurs by ACE-mediated cleavage of 2 amino acids from Ang1-9, and also of Ang1-5, which is produced by cleavage of 2 amino acids from Ang1-7. Accordingly, during ACEI treatment, AT2, Ang1-7, and Ang1-5 concentrations were many fold lower than during ARB treatment. In addition to ACE-S being significantly lower, ALT also was significantly lower during ACEI treatment. Inhibition of ACE resulted in a small pool of AT2, which limited the amount of Ang1-7 that could be produced by ACE2. Simultaneously, in the presence of continued AP system input, as evidenced in our study by high PRA-S, higher amounts of AT1 accumulated. In the presence of ACEI, AT1 can be metabolized by ACE2 to Ang1-9, but this conversion appeared inefficient in the absence of exogenous

FIGURE 4 Surrogate measures of plasma renin activity (PRA-S) and angiotensin converting enzyme activity (ACE-S) and the ratio of alternative angiotensin peptides (APs) to angiotensin 1 and angiotensin 2 (ALT), in dogs with degenerative mitral valve disease (DMVD) while receiving angiotensin converting enzyme inhibitor (ACEI) (blue solid circles), angiotensin receptor blocker (ARB) (purple solid triangles) and after in vitro incubation with recombinant human angiotensin converting enzyme 2 using ACEI plasma (ACEI + ACE2) (open blue circles) and ARB plasma (ARB + ACE2) (open purple triangles). Data from the 1 dog with stage B2 DMVD are shown as solid and open squares

TABLE 3 Geometric mean (upper and lower 95% confidence values) of plasma equilibrium concentrations of angiotensin peptides (APs) and mean (SD) of surrogate markers of plasma renin activity (PRA-S) and angiotensin converting enzyme activity (ACE-S), and the ratio of alternative APs to traditional APs (ALT) in 8 dogs with heart disease during angiotensin converting enzyme inhibitor (ACEI) treatment (A), after angiotensin receptor blocker (ARB) treatment (B), after in vitro incubation of ACEI treatment plasma with recombinant human angiotensin converting enzyme-2 (rhACE2) (C), and after in vitro incubation of ARB treatment plasma with rhACE2 (D)

|	ACEI (A)	ARB (B)	ACEI + rhACE2 (C)	ARB ± rhACE2 (D)	P value
AT1 (pg/mL)	1032 (352-3025)	1131 (718-1781)	471 (156-1422)	606 (386-950)	.06
AT2 (pg/mL)	30.5 (20.6-45.1)	399 (194-820)	3.1 (1.8-5.4)	4.3 (2.6-7.1)	<.001 A vs B, $P < .001$ A vs C, $P < .001$ B vs D, $P < .001$
Ang1-9 (pg/mL)	14.1 (3.1-63.6)	4.4 (2.1-9.3)	1255 (317-4968)	415 (243-708)	<.001 A vs C, $P < .001$ B vs D, $P < .001$
Ang1-7 (pg/mL)	182 (66.2-503)	443 (248-793)	455 (188-1104)	880 (560-1383)	.001 A vs B, $P = .01$ A vs C, $P < .001$ B vs D, $P < .001$ C vs D, $P = .03$
Ang1-5 (pg/mL)	16.3 (10.6-25.0)	432 (209-895)	51.6 (30.1-88.6)	886 (495-1583)	<.001 A vs B, $P < .001$ A vs C, $P < .001$ B vs D, $P < .001$ C vs D, $P < .001$
AT3 (pg/mL)	2.6 (1.2-5.2)	38.5 (23.4-63.1)	NA	NA	.006
AT4 (pg/mL)	7.4 (5.5-9.8)	53.1 (30.3-93.1)	NA	NA	<.001
PRA-S	1763 (1507)	1795 (841)	811 (698)	678 (277)	.02 A vs C, $P = .04$ B vs D, $P = .005$
ACE-S	0.063 (0.099)	0.437 (0.265)	0.011 (0.01)	0.01 (0.008)	.002 A vs B, $P = .01$ B vs D, $P = .01$
ALT	0.218 (0.047)	0.6 (0.184)	4.08 (0.75)	3.84 (1.07)	<.001 A vs B, $P = .001$ A vs C, $P < .001$ B vs D, $P < .001$

Abbreviations: Ang1-5, angiotensin 1-5; Ang1-7, angiotensin 1-7; Ang1-9, angiotensin 1-9; AT1, angiotensin 1; AT2, angiotensin 2; AT3, angiotensin 3; AT4, angiotensin 4.

ACE2 activity. With regard to the amount of Ang1-9 that was produced, the presence of ACEI blocked any subsequent ACE-mediated cleavage of Ang1-9 into the more active Ang1-7. Thus, whereas ACEI treatment in dogs with DMVD arrested traditional AP system activity primarily in the upstream part in the pathway, inhibition of ACE also interfered with other more downstream or alternate AP pathways and was not associated with higher alternative AP system activity.

The AP profile during telmisartan treatment was associated with significantly higher ACE-S and ALT as occurred during ACEI. Unlike ACEIs, ARBs do not block conversion of AT1 to AT2. Thus, the finding that telmisartan treatment was associated with significantly higher AT2 concentration and higher ACE-S as compared with ACEI treatment supports this fact. Treatment with telmisartan, in comparison with ACEI, was associated with significantly higher Ang1-7, presumably driven by a large available pool of AT2 together with higher ALT. Other APs, such as AT3 and AT4, which depend on AT2 as their

parent substrate, also were increased. The ACE2 and ACE activity also served to convert AT1 to Ang1-9 and Ang1-9 to Ang1-7, respectively. Thus, production of AT1, by high PRA-S, and AT2, by high ACE2, served to fuel the alternative AP pathway in a way that treatment with ACEI did not. These data should stimulate interest in the potential clinical value of ARBs to leverage ACE and ACE2 to drive production of APs.

Incubation with rhACE2 provided additional insight into AP system pathways. In both ACEI and ARB groups, addition of rhACE2 markedly decreased AT2, PRA-S, and ACE-S, consistent with the counter-regulatory role of ACE2 in the AP system.[30] Despite this, important differences were found among the AP profiles after rhACE2 incubation. The combination of telmisartan and rhACE2 produced significantly higher concentrations of both Ang1-7 and Ang1-5 compared with the combination of ACEI and rhACE2, which presumably was the result of near complete
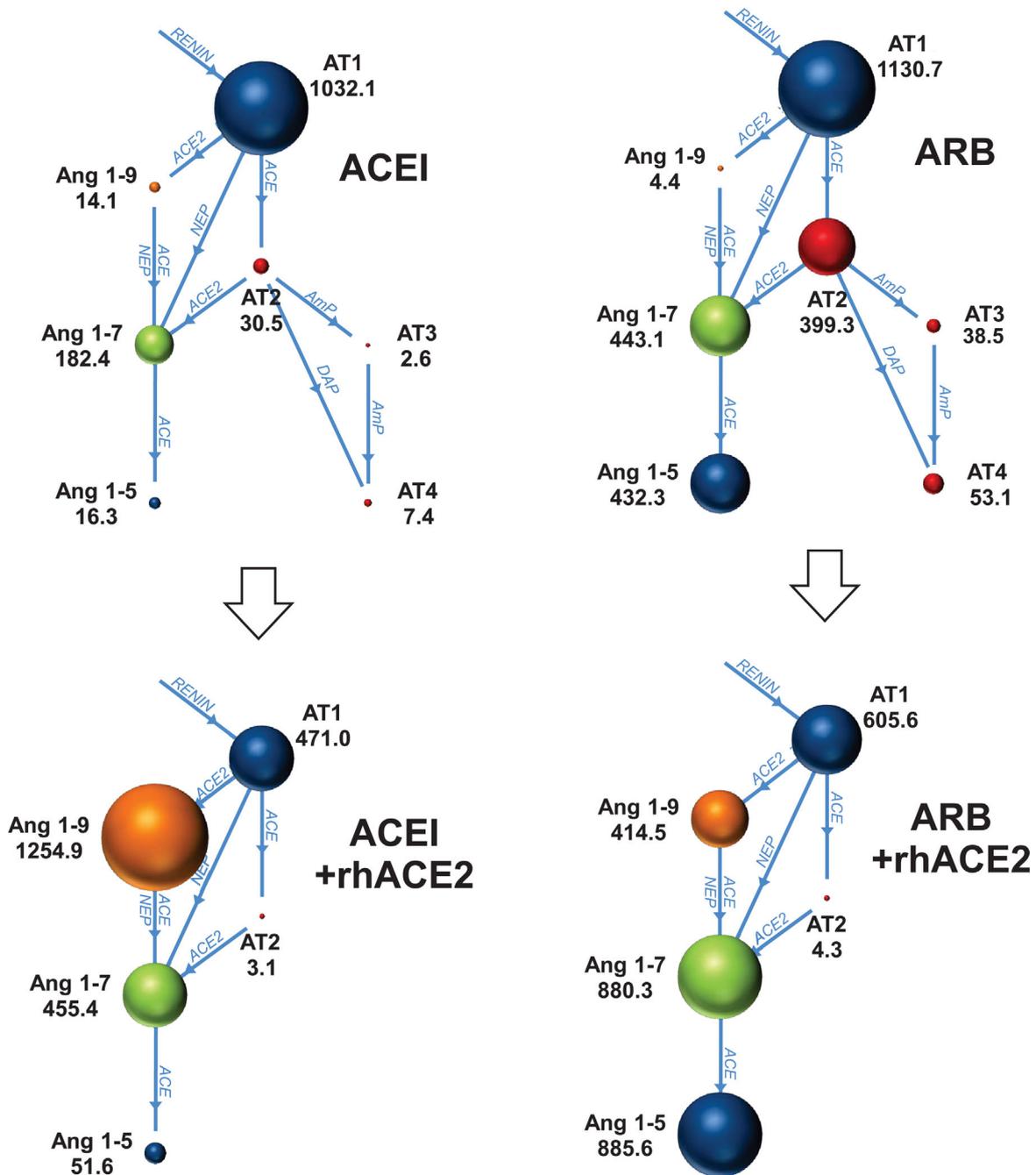
FIGURE 5   Plasma equilibrium concentrations and relationships of angiotensin peptides and angiotensin converting enzyme (ACE) and angiotensin converting enzyme 2 (ACE2) pathways in dogs with degenerative mitral valve disease while receiving treatment with angiotensin converting enzyme inhibitor (ACEI), angiotensin receptor blocker (ARB), and after in vitro incubation of ACEI plasma (ACEI+ACE2) and ARB plasma (ARB + ACE2) with recombinant human ACE2. The sizes of the circles are proportional to the geometric mean concentration (pg/mL) of each angiotensin peptide. AmP, aminopeptidase; Ang1-5, angiotensin 1-5; Ang1-7, angiotensin 1-7; Ang1-9, angiotensin 1-9; AT1, angiotensin 1; AT2, angiotensin 2; AT3, angiotensin 3; AT4, angiotensin 4; DAP, aspartyl aminopeptidase; NEP, neutral endopeptidase

conversion of the large AT2 pool to alternative APs. Another important difference involves Ang1-9. In the presence of ACEI and rhACE2, ACE2 readily converted AT1 to Ang1-9, but further conversion to Ang1-7 was hindered by ACEI. One interpretation of our results is that during ACEI treatment, neutral endopeptidase converts some Ang1-9 to Ang1-7 but less efficiently than does ACE. Thus, the potential augmentation of the alternative APs using

rhACE2 is arrested at the level of Ang1-9 in the presence of ACEI, and beneficial downstream APs, especially Ang1-7, are not produced. In contrast, the upregulation of both ACE and ACE2 pathways during telmisartan, particularly with addition of rhACE2, was associated with the most favorable theoretical AP profile, in which Ang 1-7, Ang1-5, and ALT were highest and AT1, AT2, and PRA-S were lowest.

Results of our study closely mirror findings of a similar study[18] of human plasma, including the relatively low proportion of AT2 to AT1 (ie, low ACE-S) during ACEI treatment and production of high concentrations of Ang1-9 after incubation with rhACE2. Our results also agree with studies of human ARB and ARB + rhACE2 plasma with regard to a high proportion of AT2 to AT1 and production of large amounts of Ang1-5. One difference between studies was that the concentration of Ang1-7 in ARB + rhACE2 plasma from dogs was significantly higher than in ARB plasma, which was not the case in humans. There are several possible explanations for this difference. In our study, plasma samples were paired whereas in the study of humans the cohorts of patients receiving ACEI and ARB consisted of different individuals. In our study, PRA-S was relatively high during both ACEI and telmisartan treatment, which provided a continuous source of AT1 for the alternative AP system during rhACE2 incubation. In the study of humans, PRA-S was substantially lower in the ARB cohort (by 8- to 9-fold) as compared with the human ACEI cohort or our cohort of dogs. In the human ARB + rhACE2 group, without the continual supply of AT1, alternative AP system activity would tend to drive APs to the end of their metabolic pathway (ie, Ang1-5) and deplete APs in the beginning or middle of the pathway (ie, Ang1-9 and Ang1-7).

Our study provides data about less understood APs, such as AT3 and AT4, both of which were significantly higher during telmisartan treatment vs ACEI treatment. The actions of AT3 generally are considered maladaptive because AT3 binds to the type 1 AT2 receptor and promotes hypertension and proliferation and remodeling of vascular smooth muscle,[10,31] which could represent a risk of telmisartan treatment. On the contrary, the actions of AT4 generally are considered beneficial because AT4 binds to its own unique AT4 receptor, as well as the MasR receptor, which is the main receptor for Ang1-7.[32,33] Specific actions of AT4 in the cardiovascular system include protection of the myocardium after ischemia and release of natriuretic peptides.[34,35] Both AT3 and AT4, although produced from AT2, are not formed by ACE or ACE2 (Figure 1), but rather by aminopeptidase and aspartyl aminopeptidase. Understanding of these APs and enzymes in the context of the overall AP system in dogs is incomplete.

Our study had some limitations. Treatment was not blinded or randomized. Experience with ARBs in dogs with DMVD is limited and we felt it was important to provide telmisartan in an open-label fashion. Previous studies[36,37] involving healthy dogs have utilized ACEI washout times from 7 to14 days. We chose 3 days as the longest time we felt comfortable exposing dogs with heart disease to no RAAS-inhibiting treatment. In humans with CHF, even shorter washout duration of 36 hours is used when switching from ACEI to ARBs because of the risk of clinical deterioration when RAAS inhibitors are withdrawn.[12] One convention is to employ a washout period at least 5 times the elimination (excretion) half-life ($t_{1/2}$).[38] The 72-hour washout period exceeded this time for both enalapril (excretion $t_{1/2}$ = 11 hours) and benazepril (excretion $t_{1/2}$ = 3.5 hours).[39] We cannot rule out a carryover effect of ACEI that affected ARB blood samples taken 14 days after the washout period ended, but we note that such an effect would bias results to

the null. All dogs in the study tolerated the switch from ACEI to telmisartan with no clinically apparent adverse effect, but the long-term safety of ARB requires additional study. Our study was not designed to explore the feasibility of in vivo ACE2 treatment nor the effect of different AP profiles on clinical outcomes. Dogs in the study were receiving a variety of different cardiac drugs, which might have affected their baseline AP profile, but these drugs do not preclude our ability to assay APs, which highlighted changes within each dog. In human plasma, diuretics and aldosterone antagonists do not affect circulating ACE2 activity,[40] and previous studies[18] have used methods similar to ours to measure traditional and alternative APs in human patients receiving other RAAS-inhibiting drugs such as spironolactone. The number of dogs was small, and the study likely was underpowered to detect significant differences in all of the different APs, but we still found potentially important differences for many APs and achieved significant results involving the primary AP of interest, Ang1-7. Additional studies of larger populations that specifically account for disease stage, concomitant cardiac medications, and effect on other neurohormonal components of heart failure, such as aldosterone, should be performed to verify and expand our findings.

In conclusion, treatment of dogs with telmisartan was associated with higher activity of both the traditional and alternative AP pathways as compared with that during ACEI. Treatment during telmisartan was associated with higher equilibrium plasma concentrations of a variety of APs, including Ang1-7, Ang1-5, and AT2, as compared with during ACEI treatment. The in vitro combination of telmisartan plasma and rhACE2 was associated with a theoretically optimal AP system profile characterized by high Ang 1-7, Ang1-9, and Ang1-5, and low AT1 and AT2. Treatment with ARBs, such as telmisartan, or combined ARB and ACE2 treatment represents a potential treatment strategy for dogs with DMVD and warrants additional preclinical study.

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