Myxomatous mitral valve disease and associated pulmonary hypertension might increase serum angiopoietin-2 in dogs

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OBJECTIVE
To evaluate the relationships between the severity of myxomatous mitral valve disease (MMVD) and pulmonary hypertension (PH) and serum angiopoietin (Ang)-1 and Ang-2 concentrations in dogs with MMVD.

ANIMALS
74 dogs (control, n = 12; MMVD, n = 62) were included.

METHODS
Serum Ang-1 and Ang-2 concentrations were estimated using the canine-specific ELISA kit. The concentrations were compared between dogs with MMVD and healthy dogs, and they were analyzed according to the severity of MMVD and PH.

RESULTS
The median serum Ang-1 concentration did not differ among the study groups. The median serum Ang-2 concentration was higher in dogs with stage B2 MMVD (P = .041) and acute congestive heart failure (P = .002) than in control dogs. In addition, the median serum Ang-2 concentration was higher in MMVD dogs with PH than in those without PH (P = .031). Serum Ang-2 concentration was correlated with vertebral heart score (r = 0.36, P = .004) and vertebral left atrial score (r = 0.50, P < .001) in dogs with MMVD, and correlated with vertebral heart score (r = 0.63, P = .01), maximum E wave amplitude of the diastolic transmitral flow (r = 0.61, P = .018), ejection fraction (r = -0.77, P < .001), and fractional shortening (r = -0.56, P = .032) in dogs with acute congestive heart failure.

CLINICAL RELEVANCE
Circulating Ang-2 levels increase in dogs with the severity of MMVD and the presence of PH.

Keywords: angiopoietin, biomarker, canine, cardiac disease, pulmonary hypertension

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pulmonary hypertension (PH). In addition, a positive correlation between Ang-2 concentration and heart failure (HF) severity was confirmed, and a significant elevation of Ang-2 levels was observed in patients with acute/chronic HF and nonischemic HF. In contrast, elevated Ang-1 levels are not associated with CVDs. However, information about serum Ang-1 and Ang-2 in dogs with CVDs has not been reported.

Myxomatous mitral valve disease (MMVD) is the most prevalent heart disease in small-breed dogs and progresses slowly, leading to left-sided congestive HF. MMVD can cause PH by chronically increasing the pulmonary arterial wedge pressure. However, the role of the Ang/Tie-2 system in canine CVD has not been reported. We hypothesized that serum Ang-2 concentration may increase in decompensated CHF and PH dogs with MMVD, but Ang-1 concentration may not be associated with the disease severity of MMVD and PH, like in humans. In this study, we evaluated the relationships between the severity of MMVD and serum Ang-1 and Ang-2 concentrations and between PH and serum Ang-1 and Ang-2 concentrations in dogs with MMVD.

Methods

Animals

This cross-sectional study included client-owned dogs with MMVD and healthy dogs who visited our institution between July 2018 and June 2022 and was approved by the local ethics committee [CBNUA-2002-22-01]. Healthy dogs were assigned to the control group based on no clinical signs of vascular, metabolic, inflammatory, or neoplastic disorders or diabetes on physical examination and routine laboratory tests, such as complete blood count and serum biochemistry. The dogs did not have a cardiac murmur on auscultation and had unremarkable findings in the cardiopulmonary system on radiography. The following data of dogs with MMVD were collected on the day of blood sampling: signalment, vital signs, comorbidity, cardiac/pulmonary auscultation, thoracic radiography and echocardiography results, and medical treatment at presentation. Dogs with MMVD were excluded if they had the following comorbidities or abnormalities known to affect circulating Ang concentrations: pneumonia, infection, malignancies, immune-mediated disease, sepsis, hypertension (systolic blood pressure > 160 mm Hg), renal failure (serum creatinine > 1.4 mg/dL), hepatic impairment, obesity (body condition score [9-point scale] ≥ 7), arrhythmia (impulse conduction and formation abnormalities), and a history of immunosuppressive or hormonal therapy within the last 6 months.

Data collection

The following data were collected on the day of blood sampling: signalment, vital signs, comorbidity, cardiac/pulmonary auscultation, thoracic radiography and echocardiography results, and medical treatment at presentation. A commercial digital radiography system (Radiant DICOM Viewer version 2020.2, Medixant Corporation) was used to measure the vertebral heart score (VHS) and vertebral left atrial score (VLAS), which were calculated as previously reported. Echocardiographic examinations were performed by various operators under the supervision of the same experienced operator using the same instrument (Aloka Prosound Alpha 7, from July 2018 to March 2021; Philips EPIQ7, from April 2021 to June 2022). M-mode, Doppler, and 2-D echocardiography were conducted in the left or right recumbency, and the collected echocardiographic parameters included the left atrium to aorta diameter ratio (LA/Ao), the left ventricular end-diastolic internal diameter (LVIDd) normalized for body weight (LVIDDn), end-diastolic volume index, end-systolic volume index, fractional shortening (FS) of the left ventricle (LV), ejection fraction (EF) of LV, maximum E wave amplitude of the transmural transmitral flow (Emax), and the ratio of the transmitral flow E wave and the pulsed wave tissue Doppler E’ wave of the mitral annulus. LA/Ao was obtained using the right parasternal short-axis view at the level of the aortic valve on 2-D echocardiography. The LVIDd and left ventricular end-systolic internal diameter (LVIDs) were evaluated using M-mode echocardiography, taken from the right parasternal short-axis view at the papillary muscle level. LVIDDn was calculated using LVIDd and body weight (LVIDDn = LVIDd [cm]/[body weight (kg)]^0.34). The end-diastolic volume (EDV) and end-systolic volume ( ESV) of LV were calculated using the following formula: EDV = 0.67 X (LVIDd)^3 and ESV = 0.67 X (LVIDs)^3. EF (%) was determined by (EDV - ESV)/EDV X 100 and FS (%) by (LVIDd - LVIDs)/LVIDd X 100. Spectral and tissue Doppler echocardiography allows measurement of early mitral blood flow (E wave), velocity of the mitral annulus (E’ wave), and Emax measured in milliseconds. All parameters were calculated as the average of 2 consecutive measurements. Peak tricuspid regurgitation velocity (TRV) and the number of anatomical sites with echocardiographic signs of PH were also evaluated.

Diagnosis and staging of MMVD and PH

MMVD was diagnosed based on physical examination and echocardiographic findings, such as a left apical systolic murmur and mitral valvular lesions (thickening, prolapse, or both) related to mitral regurgitation. Dogs with MMVD were classified into 3 groups (stage B1, stage B2, and acute CHF) according to MMVD severity based on the American College of Veterinary Internal Medicine consensus. The diagnosis of acute CHF was established based on the following criteria: (1) current clinical signs of left-sided congestive HF (eg, tachypnea or respiratory distress), (2) radiographic evidence of pulmonary edema (eg, pulmonary venous congestion, interstitial, and alveolar patterns), and (3) treatment response with diuretics (reduction in respiratory rate). Dogs with acute CHF were hospitalized and discharged if clinical signs were resolved.
and improvement of pulmonary congestion was confirmed by radiography. The diagnosis of PH was made considering the peak TRV and the number of anatomical changes at 3 sites (the right ventricle, pulmonary artery, and right atrium or caudal vena cava) upon echocardiography. Additionally, dogs with MMVD were divided into 2 subgroups according to TRV (TRV > 3.4 milliseconds and TRV < 3.4 milliseconds) based on the clinical definition of PH proposed by the American College of Veterinary Internal Medicine consensus statement.

**Measurement of serum Ang-1 and Ang-2 concentrations**

Blood samples were collected from the jugular or peripheral veins and placed in serum-separating tubes. Serum was separated by centrifugation (2,000 x g, 10 minutes) at room temperature and stored at -80°C within 2 h of collection until ELISA analysis. The serum Ang-1 concentration was analyzed using a commercial canine-specific ELISA kit (Canine Angiopoietin-1; Immunoassay; Cloud-Clone Corp) according to the manufacturer’s instructions. The intra- and interassay variabilities were < 10% and < 12%, respectively, and the lower limit of detection (LLD) was 1.56 ng/mL according to the manufacturer’s protocol. The tests were run in duplicate by the same person, and the mean value of the wells was used. Eleven samples were below the LLD; thus, the concentration of the samples was set at 1.10 ng/mL for analysis.

Serum Ang-2 concentration was analyzed using a commercial human ELISA test kit (Human Angiopoietin-2; R&D Systems) and validated for use in dogs. The intra- and interassay variabilities were 4.2% and 7.4%, respectively, and the LLD was 46.9 pg/mL. The tests were run in duplicate by the same person, and the mean value of the wells was used. Initially, serum samples were diluted 1:5 with a Calibrator Diluent (Human Angiopoietin-2 Immunoassay; R&D Systems). The samples were further diluted (up to 20 times) if the measured Ang-2 concentration was above the range of the standard curve (46.9 to 3,000 pg/mL), and measurements were repeated.

**Statistical analyses**

All data were analyzed using commercial statistical software (Prism 6; GraphPad Software Inc). The P values were calculated using 2-tailed tests, and the 95% CIs were evaluated to examine the differences between medians. P values < .05 were considered statistically significant. The data are expressed as median (IQR) or mean (SD) if the data had a non-normal or normal distribution, respectively. The D’Agostino-Pearson normality test was used to determine normality. Patient characteristics, including age, weight, body condition score, murmur grade, and systolic blood pressure, were compared among the 4 groups (control, B1, B2, and acute CHF) using a 1-way ANOVA (normally distributed continuous variables), Kruskal-Wallis (non-normally distributed continuous variables), or chi-squared (noncontinuous variables) test. Differences in serum Ang-1 and Ang-2 concentrations and Ang-2:Ang-1 ratio among the control and MMVD stages (control, B1, B2, and acute CHF) were assessed using 1-way ANOVA and Tukey’s multiple comparisons test when normally distributed and Kruskal-Wallis and Dunn’s multiple comparisons tests when non-normally distributed. Differences in serum Ang-1 and Ang-2 concentrations and Ang-2:Ang-1 ratio between dogs with MMVD with TRV > 3.4 milliseconds and those with TRV < 3.4 milliseconds were assessed using the Mann-Whitney U test.

Further analysis was performed for Ang-2, which confirmed significant differences among the study groups. Correlations between serum Ang-2 concentrations and radiographic/echocardiographic variables were assessed using r when normally distributed or r_s otherwise. The receiver operating characteristic curve analysis was used to evaluate the diagnostic value of Ang-2 concentration to differentiate dogs with acute CHF from dogs with stage B1 and B2 MMVD and MMVD dogs with TRV > 3.4 milliseconds from those with TRV < 3.4 milliseconds. The area under the curve (AUC) of the receiver operating characteristic curve, optimum cutoffs, sensitivities, and specificities were measured, and the diagnostic accuracy was categorized as fail (0.5 ≤ AUC < 0.6), poor (0.6 ≤ AUC < 0.7), fair (0.7 ≤ AUC < 0.8), good (0.8 ≤ AUC < 0.9), and excellent (0.9 ≤ AUC < 1.0) based on the AUC value. The optimal cutoff value was selected based on the highest Youden index (sensitivity plus specificity-1). The Wilcoxon signed-rank test was used to evaluate the change in Ang-2 concentration before and after the treatment of acute CHF.

**Results**

**Study population**

Seventy-four client-owned dogs were included in this study, which included 12 healthy control dogs (age, 5.52 [3.5] years; weight, 7.15 [3.27 to 9.75] kg) and 62 dogs with MMVD (age, 10.6 [2.6] years; weight, 3.88 [3.03 to 5.17]) kg. The population characteristics and medication histories are presented (Table 1). The control dogs were significantly younger than the dogs with stage B1 (P = .001), B2 (P < .001), and acute CHF (P < .001). Dogs with stage B2 MMVD and acute CHF had a significantly higher murmur grade than those with stage B1 (both P < .001), and the other characteristics were not different among the groups. The MMVD dogs were of the following breeds: Maltese (n = 28), Pomeranian (n = 9), Shih Tzu (n = 7), Poodle (n = 6), mixed breed (n = 4), Chihuahua (n = 2), Spitz (n = 2), Pekingese (n = 2), Yorkshire Terrier (n = 1), and Bichon Frise (n = 1). Healthy dogs consisted of beagles (n = 3), mixed breeds (n = 3), Maltese (n = 2), and 1 dog each of Poodle, Bichon Frise, Bedlington Terrier, and Dalmatian. Dogs with acute CHF were hospitalized for 1 to several days and discharged if clinical signs were resolved and improvement of pulmonary congestion was confirmed on radiography.
16 dogs in the acute CHF stage, 13 were undergoing CHF for the first time, while 3 had a previous history of CHF at presentation. For a subset of dogs within the acute CHF group, serum samples were obtained at 2 distinct time points: immediately upon presentation (referred to as “before” treatment) and 3 to 14 days after discharge following successful acute CHF management (referred to as “after” treatment). The “after” treatment data were used for comparative assessment to changes induced by CHF treatment.

Serum Ang-1 and Ang-2 concentrations and Ang-2:Ang-1 ratio

The storage duration of the serum samples for the determination of serum Ang-1 and Ang-2 concentrations was 13 (5 to 18) months. Serum Ang-1 concentration did not differ between the control and MMVD stages ($P = .190$) (Figure 1); however, serum Ang-2 concentration was significantly higher in dogs with stage B2 MMVD (8.1 [6.2 to 12.4], $P = .041$) and acute CHF (10.8 [7.6 to 16.8], $P = .002$) compared with that in the control dogs (5.6 [4.0 to 7.1]). The median Ang-2:Ang-1 ratio did not differ between the control and MMVD groups ($P = .618$).

Of the 62 dogs with MMVD, 13 dogs showed TRV > 3.4 milliseconds. Serum Ang-1 concentration was not different between dogs with MMVD with TRV > 3.4 milliseconds (4.4 [2.4 to 10.3], $P = .139$) and those with TRV < 3.4 milliseconds (8.5 [5.4 to 13.5] ng/mL; Figure 2). However, serum Ang-2 concentration was significantly higher in dogs with MMVD with TRV > 3.4 milliseconds compared with dogs with TRV > 3.4 milliseconds (10.8 [8.0 to 11.9] ng/mL, $P = .002$).

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### Table 1—Data for demographic, physical examination, PH, and medication histories at presentation for client-owned dogs with MMVD and healthy dogs (control) who visited between July 2018 and June 2022.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Control</th>
<th>MMVD ($n = 62$)</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B1</td>
<td>B2</td>
</tr>
<tr>
<td>Number</td>
<td>12</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>Age (y)</td>
<td>5.5 (± 3.5)</td>
<td>9.5 (± 2.0)$^a$</td>
<td>10.6 (± 2.7)$^a$</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>4 (33.3%)</td>
<td>9 (52.9%)</td>
<td>16 (55.2%)</td>
</tr>
<tr>
<td>Female</td>
<td>8 (66.7%)</td>
<td>8 (47.1%)</td>
<td>13 (44.8%)</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>7.15 (3.27–9.75)</td>
<td>3.40 (2.87–4.80)</td>
<td>4.20 (3.12–4.99)</td>
</tr>
<tr>
<td>BCS (9-point scale)</td>
<td>4.8 (± 0.9)</td>
<td>4.5 (± 0.8)</td>
<td>4.5 (± 1.1)</td>
</tr>
<tr>
<td>Murmur (6-point scale)</td>
<td>NA</td>
<td>2.9 (± 0.8)</td>
<td>4.0 (± 0.7)$^b$</td>
</tr>
<tr>
<td>SBP (mm Hg)</td>
<td>134.6 (± 19.0)</td>
<td>137.9 (± 17.1)</td>
<td>142.4 (± 17.2)</td>
</tr>
<tr>
<td>PH (TRV &gt; 3.4 m/s)</td>
<td>NA</td>
<td>2 (12%)</td>
<td>6 (21%)</td>
</tr>
<tr>
<td>Medication at presentation</td>
<td>NA</td>
<td>NA</td>
<td>9 (31.0%)</td>
</tr>
<tr>
<td>Pimobendan</td>
<td>NA</td>
<td>NA</td>
<td>8 (7/1) (27.6%)</td>
</tr>
<tr>
<td>ACE inhibitor (enalapril/benazepril)</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Furosemide</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Spironolactone</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Sildenafil</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

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Figure 1—Serum angiopoietin (Ang)-1 and Ang-2 concentrations and the serum Ang-2:Ang-1 ratio were analyzed among client-owned dogs with MMVD and healthy (control) dogs who visited between July 2018 and June 2022. Scattered plot comparing serum Ang-1 (A) and Ang-2 (B) concentrations and serum Ang-2:Ang-1 ratio (C) in the control dogs ($n = 12$) and those with MMVD B1 ($n = 17$), MMVD B2 ($n = 29$), acute CHF ($n = 16$). Serum Ang-1 concentrations and the Ang-2:Ang-1 ratio did not differ among groups. Serum Ang-2 concentration was significantly higher in dogs with MMVD B2 (8.1 [6.2 to 12.4], $P = .041$) and acute CHF (10.8 [7.6 to 16.8], $P = .002$) compared with that in the control dogs (5.6 [4.0 to 7.1]). The horizontal bars show the medians and IQRs from the first to the third quartile. Kruskal-Wallis test with Dunn’s multiple comparison test. $^*P < .05$; $^{**}P < .01$. CHF = Congestive heart failure; MMVD = Myxomatous mitral valve disease.
25.6] ng/mL, \( P = .031 \) compared with MMVD dogs with < 3.4 milliseconds (7.7 [6.2 to 11.2] ng/mL). The serum Ang-2:Ang-1 ratio was significantly higher in MMVD dogs with TRV > 3.4 milliseconds (1.57 [0.94 to 9.3], \( P = .019 \)) compared with MMVD dogs with < 3.4 milliseconds (1.04 [0.54 to 1.85]).

**Correlation between serum Ang-2 concentration and radiographic/echocardiographic variables**

Serum Ang-2 concentrations and radiographic/echocardiographic variables are summarized (Table 2). In dogs with MMVD, serum Ang-2 concentration was positively correlated with VHS (\( r_s = 0.36; 95\% \text{ CI}, 0.11 \text{ to } 0.56; P = .004 \)) and VLAS (\( r = 0.50; 95\% \text{ CI}, 0.28 \text{ to } 0.66; P < .001 \)). However, no correlation was found between LVIDDn, LA/Ao, Emax, the ratio of the transmitral flow E wave and the pulsed wave tissue Doppler E’ wave of the mitral annulus, FS, EF, and serum Ang-2 concentration. In addition, in dogs with acute CHF (n = 16), serum Ang-2 concentration was positively correlated with VHS (\( r = 0.63; 95\% \text{ CI}, 0.19 \text{ to } 0.86; P = .01 \)) and Emax (\( r_s = 0.61; 95\% \text{ CI}, 0.13 \text{ to } 0.86; P = .018 \)) and negatively correlated with EF (\( r_s = -0.77; 95\% \text{ CI}, -0.92 \text{ to } -0.45; P < .001 \)) and FS (\( r_s = -0.56; 95\% \text{ CI}, -0.84 \text{ to } -0.05; P = .032 \)).

**AUC of the serum Ang-2 concentration in MMVD dogs**

The AUC of Ang-2 concentration to distinguish dogs with acute CHF from dogs with stage B1 and B2 was 0.64 (95% CI, 0.44 to 0.81), and the corresponding optimal cutoff of Ang-2 concentration was 10.09 ng/mL, with sensitivity and specificity values of 0.57 (95% CI, 0.29 to 0.82) and 0.77 (95% CI, 0.62 to 0.89), respectively. In addition, the AUC of serum Ang-2 concentration to identify dogs with MMVD with TRV > 3.4 milliseconds was 0.64 (95% CI, 0.44 to 0.81), with sensitivity and specificity values of 0.57 (95% CI, 0.29 to 0.82) and 0.77 (95% CI, 0.62 to 0.89), respectively.

### Table 2—Correlations between serum Ang-2 concentrations and radiographic/echocardiographic variables in the dogs with MMVD described in Table 1.

<table>
<thead>
<tr>
<th>Variables</th>
<th>n</th>
<th>Pearson coefficient (( r ))</th>
<th>( P ) value</th>
<th>N</th>
<th>Spearman coefficient (( r_s ))</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All dogs with MMVD (n = 62)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VHS</td>
<td>62</td>
<td>0.36 (95% CI, 0.11−0.56)</td>
<td>&lt; .001</td>
<td>62</td>
<td>0.36 (95% CI, 0.11−0.56)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>VLAS</td>
<td>62</td>
<td>0.50 (95% CI, 0.28−0.66)</td>
<td>&lt; .001</td>
<td>62</td>
<td>0.22 (95% CI, −0.03 to 0.45)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>LVIDDn</td>
<td>62</td>
<td>0.22 (95% CI, −0.03 to 0.45)</td>
<td>&lt; .001</td>
<td>62</td>
<td>0.20 (95% CI, −0.06 to 0.43)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>LA/Ao</td>
<td>62</td>
<td>0.24 (95% CI, −0.02 to 0.47)</td>
<td>&lt; .001</td>
<td>62</td>
<td>0.01 (95% CI, −0.26 to 0.27)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Emax</td>
<td>60</td>
<td>−0.16 (95% CI, −0.39 to 0.10)</td>
<td>&lt; .001</td>
<td>60</td>
<td>−0.06 (95% CI, −0.31 to 0.19)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>E/E'</td>
<td>58</td>
<td>−0.16 (95% CI, −0.39 to 0.10)</td>
<td>&lt; .001</td>
<td>58</td>
<td>−0.06 (95% CI, −0.31 to 0.19)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>FS</td>
<td>60</td>
<td>−0.16 (95% CI, −0.39 to 0.10)</td>
<td>&lt; .001</td>
<td>60</td>
<td>−0.06 (95% CI, −0.31 to 0.19)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>EF</td>
<td>58</td>
<td>−0.16 (95% CI, −0.39 to 0.10)</td>
<td>&lt; .001</td>
<td>58</td>
<td>−0.06 (95% CI, −0.31 to 0.19)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>EF</td>
<td>62</td>
<td>−0.16 (95% CI, −0.39 to 0.10)</td>
<td>&lt; .001</td>
<td>62</td>
<td>−0.06 (95% CI, −0.31 to 0.19)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>Emax</td>
<td>16</td>
<td>0.63 (95% CI, 0.19−0.86)</td>
<td>&lt; .001</td>
<td>16</td>
<td>0.63 (95% CI, 0.19−0.86)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>FS</td>
<td>15</td>
<td>−0.56 (95% CI, −0.84 to −0.05)</td>
<td>&lt; .001</td>
<td>15</td>
<td>−0.56 (95% CI, −0.84 to −0.05)</td>
<td>&lt; .001</td>
</tr>
<tr>
<td>EF</td>
<td>16</td>
<td>−0.77 (95% CI, −0.92 to −0.45)</td>
<td>&lt; .001</td>
<td>16</td>
<td>−0.77 (95% CI, −0.92 to −0.45)</td>
<td>&lt; .001</td>
</tr>
</tbody>
</table>

Variables were assessed using Pearson’s correlation test when normally distributed or Spearman’s correlation test otherwise. E/E’ = Ratio of the transmitral flow E wave and the pulsed wave tissue Doppler E’ wave of the mitral annulus. EF = Ejection fraction of the LV. Emax = Maximum E wave amplitude of the diastolic transmitral flow. FS = Fractional shortening of the LV. LA/Ao = Left atrium to aorta diameter ratio. LVIDDn = Left ventricular end-diastolic diameter normalized for body weight. VHS = Vertebral heart score. VLAS = Vertebral left atrial score.
> 3.4 milliseconds was 0.71 (95% CI, 0.54 to 0.85), and the corresponding optimal cutoff of serum Ang-2 concentration was 7.75 ng/mL, with sensitivity and specificity values of 0.85 (95% CI, 0.55 to 0.98) and 0.51 (95% CI, 0.36 to 0.67), respectively (Figure 3).

**Change in serum Ang-2 concentrations in response to acute CHF**

After the acute CHF treatment, all 16 dogs survived and were discharged. In 10 of 16 dogs with acute CHF, samples were collected at 2 time points: before (immediately after visiting) and after treatment (3 to 14 days after discharge). The median period of hospitalization was 24 (range, 4 to 72) hours, and the median time point of recollecting samples was 7 (range, 3 to 14) days. At presentation, the dogs were prescribed furosemide (6/16 [38%]), pimobendan (10/16 [63%]), enalapril (8/16 [50%]), benazepril (1/16 [6%]), spironolactone (5/16 [31%]), and sildenafil (4/16 [25%]) (Table 1). Upon discharge, the dogs were administered furosemide (16/16 [100%]), pimobendan (16/16 [100%]), enalapril (12/16 [75%]), benazepril (3/16 [19%]), spironolactone (6/16 [38%]), and sildenafil (5/16 [31%]). There was no difference in the median Ang-2 concentration before (8.61 [5.52–13.6] ng/mL) and after treatment (7.36 [4.08–9.99] ng/mL) (P = .065) (Figure 4).

**Discussion**

In our study, circulating Ang-2 levels were significantly higher in dogs with stage B2 and acute CHF compared with the control dogs. In addition, circulating Ang-2 levels were significantly higher in dogs with MMVD with TRV > 3.4 milliseconds compared with those with TRV < 3.4 milliseconds. These results are consistent with those of previous studies that demonstrated significantly high Ang-2 levels in human patients with CHF and PH. In our study, the Ang-2:Ang-1 ratio was suggested as a marker of endothelial activation and an independent predictor of mortality in humans with acute lung injury. In our study, the Ang-2:Ang-1 ratio was significantly higher in MMVD dogs with PH than in those without PH. In addition, the Ang-2:Ang-1 ratio showed good accuracy in identifying dogs with MMVD with PH (AUC of the Ang-2:Ang-1 ratio, 0.71 [95% CI, 0.55 to 0.87]; these data are not shown). However, the AUC of the Ang-2:Ang-1 ratio was similar to those of Ang-2. Therefore, it is presumed that Ang-2 could solely be used in identifying PH rather than the Ang-2:Ang-1 ratio in dogs with MMVD.

In our study, serum Ang-2 levels were significantly higher in dogs with MMVD B2 and acute CHF compared with control dogs but were not elevated in accordance with the severity of MMVD. This finding...
suggests that Ang-2 reflects the cardiac remodeling progress beyond a certain level. A positive correlation was identified between Ang-2 levels and echocardiographic variables, such as VHS and VLAS, in all dogs with MMVD. Thus, Ang-2 appears to reflect the degree of cardiac remodeling in dogs with MMVD. In addition, circulating Ang-2 levels were significantly correlated with echocardiographic variables (eg, Emax, EF, FS) in dogs with acute CHF. This is consistent with previous reports demonstrating that circulating Ang-2 levels were significantly negatively correlated with EF in human patients with HF.

A high Emax indicates increased LA pressure in the presence of abnormal LV relaxation and increased stiffness. In addition, an increase in regurgitation with worsening MMVD is expected to increase FS. Moreover, the negative correlation between serum Ang-2 and FS was only observed in dogs with acute CHF, reflecting left ventricular systolic dysfunction, although the majority of dogs with MMVD do not have evidence of systolic dysfunction. Therefore, it is possible that serum Ang-2 progressively increases along with worsening MMVD; however, the Ang-2 level reduced due to impaired systolic function, even in some dogs with acute CHF. These suggest that high Ang-2 levels might be due to impaired systolic function and increased LA pressure, which is supported by another study reporting that Ang-2 levels were significantly higher in human patients with HF with severe ventricular dysfunction than in those with normal ventricular function. Thus, circulating Ang-2 levels may reflect hemodynamic impairments in dogs with CHF.

Dogs with CHF had higher levels of the proinflammatory cytokines, including tumor necrosis factor-alpha and interleukin-1, suggesting that CHF may be associated with systemic inflammation. Systemic inflammation and oxidative stress stimulate the secretion of proinflammatory cytokines such as tumor necrosis factor-alpha, which may activate ECs and upregulate Ang-2 expression. Therefore, Ang-2 levels are elevated in dogs with acute CHF by upregulation of Ang-2 expression stimulated by proinflammatory cytokines.

No difference was found in Ang-2 levels before and after acute CHF treatment, although these levels may reflect hemodynamic impairment and myocardial injury. Because of the retrospective nature of this study, there are limitations due to the different time points of sample collection post-treatment and the nonstratified type or dose of medication. As there was no difference before and after treatment, the clinical value of Ang-2 as a therapeutic response or prognostic marker in dogs with CHF is questionable. Serum Ang-2 levels might be elevated in the presence of pulmonary edema or PH. Consequently, we selected “before” samples to determine the baseline Ang-2 values before the initiation of treatment for acute CHF. On the other hand, the “after” values of Ang-2 (post-treatment) might be reduced owing to the resolution of pulmonary edema as a response to the treatment. Therefore, we could not elucidate whether Ang-2 was reduced owing to the effect of CHF treatment or the resolution of CHF. Upon analysis, we observed a seeming decrease in Ang-2 levels after treatment; however, this apparent decrease was not statistically significant. Therefore, we selected “before” values to exclude treatment effect and to focus on the alteration of serum Ang levels according to the progression of MMVD in dogs.

Endothelial dysfunction progressively increases pulmonary vascular resistance and induces PH. Excessive EC proliferation with concomitant neovascularization and inflammation-induced pulmonary vascular remodeling is a common feature of pulmonary arterial hypertension (PAH). Circulating Ang-2 levels are associated with hemodynamic compromise and clinical outcomes and significantly decrease after PAH-targeted therapy in patients with idiopathic PAH, suggesting that circulating Ang-2 might serve as a promising new biomarker of disease severity. Our study showed that MMVD dogs with PH had significantly higher Ang-2 levels than those without PH. Therefore, this finding suggests that Ang-2 may be involved in the pathogenesis of PH and may reflect the severity of PH in dogs. However, the AUC for differentiation between dogs with MMVD with PH and those without PH showed fair diagnostic accuracy, suggesting that Ang-2 cannot be used solely in the diagnosis of PH in dogs with MMVD.

This study has some limitations. First, the study population was relatively small; therefore, we could not stratify our dogs according to age and breed. Specifically, the control dogs were young and did not match the age or breed of the diseased dogs. However, there was no correlation between age and concentration (data not shown, \( P = .338 \)) and Ang-2 concentrations (data not shown, \( P = .490 \)) in the healthy controls; thus, the influence of age was not expected to be significant. In addition, a power calculation to determine an appropriate sample size for the study was not performed initially because no published data regarding serum Ang concentrations in dogs with MMVD existed at the time of study conception. Therefore, the study population was relatively small due to the limitation imposed by study criteria. In addition, the unexpected negative findings of our study, especially no difference in serum Ang-2 concentrations among the MMVD stages, might have been because of a type II error caused by the small sample size. Thus, a larger sample size is required to confirm our findings. Second, we could not confidently exclude the effects of the drug on circulating Ang-2 levels. The use of angiotensin-converting enzyme inhibitors (eg, enalapril, benazepril) and phosphodiesterase-5 inhibitors (eg, sildenafil) may affect circulating Ang-2 levels. The use of angiotensin-converting enzyme inhibitors was administered at the time of presentation in dogs with stage B2 MMVD (data not shown, \( P > .99 \)) and acute CHF (data not shown, \( P = .113 \)). Third, as right heart catheterization is not routinely performed for the diagnosis of PH in clinical veterinary medicine, the correlation between the hemodynamics of PH and circulating Ang-2 concentrations could not be
confirmed in this study. Accordingly, the PH criterion from veterinary medicine was used, and the evaluation of pulmonary arterial pressure through continuous-wave Doppler echocardiography is known to be highly correlated with its evaluation through catheterization.\textsuperscript{21,49} In addition, 2 of 13 dogs whose TRV was > 3.4 milliseconds without other anatomic sites of echocardiographic signs of PH were classified into PH cases, which could have led to some confounding effects because dogs with intermediate to high probability of PH were not completely similar. Finally, it is difficult to determine how much of the performance of the serum Ang-2 in identifying PH was associated with MMVD severity because this study did not include a group of non-MMVD dogs with PH. Additional study including dogs with PH related to diseases other than MMVD is necessary to clarify how the change in serum Ang-2 concentration alters according to the severity of PH related to MMVD. Moreover, the group of MMVD stage C primarily concentrated on acute CHF cases in this study, potentially limiting generalizability to chronic CHF cases. Future investigations that include cases of chronic CHF in MMVD stage C will provide a more comprehensive understanding of Ang-2 dynamics in revealing the nature of MMVD progression.

In conclusion, circulating Ang-2 levels significantly increase in dogs with MMVD with decompensated CHF and PH, suggesting that Ang-2 might play a role in endothelial dysfunction in dogs with advanced heart disease.

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Disclosures

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