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Comparison of N-terminal pro-atrial natriuretic peptide and three cardiac biomarkers for discriminatory ability of clinical stage in dogs with myxomatous mitral valve disease

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ABSTRACT. Plasma N-terminal pro-atrial natriuretic peptide (NT-proANP) concentration increases with progression of myxomatous mitral valve disease (MMVD) in dogs. This multicentre, prospective study compared plasma NT-proANP, N-terminal pro-brain natriuretic peptide (NT-proBNP), ANP, and cardiac troponin I (cTnI) concentrations in dogs with MMVD for their characteristics and discriminatory ability to detect cardiac dilatation and congestive heart failure (CHF). Thirty-six healthy dogs and 69 dogs with MMVD were included. Clinical variables were obtained via physical examination, thoracic radiography, and echocardiography. The discriminatory ability of each cardiac biomarker (CB) to determine the presence or absence of cardiac dilatation (event 1) and CHF (event 2) was evaluated using the receiver operating characteristic curves. Plasma NT-proANP, NT-proBNP, and ANP concentrations showed a significant association with the left atrium/aorta ratio ($P<0.01$). The area under the curve of plasma NT-proANP and NT-proBNP concentrations were 0.72 and 0.75, respectively in event1 and 0.72 and 0.76, respectively in event2. Plasma NT-proANP and NT-proBNP concentrations showed sensitivity 80.0 and 80.0%; specificity 67.6 and 64.7% in event1 (cutoff value; 8,497.81 pg/ml and 1,453.00 pmol/l, respectively) and sensitivity 85.7 and 81.0%; specificity 60.4 and 64.6% in event2 (cutoff value; 8,684.33 pg/ml and 1,772.00 pmol/l, respectively). In dogs with MMVD, plasma NT-proANP, NT-proBNP, and ANP concentrations increase with left atrial enlargement. Particularly, plasma NT-proANP and NT-proBNP concentrations appeared to be equally useful in the discriminatory ability to detect cardiac dilatation and CHF.

KEY WORDS: cardiac biomarker, myxomatous mitral valve disease, N-terminal pro-atrial natriuretic peptide, N-terminal pro-brain natriuretic peptide

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Myxomatous mitral valve disease (MMVD) is the most commonly acquired heart disease in dogs [4, 7]. Its clinical signs include exercise intolerance and dyspnoea due to pulmonary edema [18, 26, 36]. The cardiac biomarker (CB) test is a simple examination for MMVD compared to thoracic radiography and echocardiography. Plasma atrial natriuretic peptide (ANP) concentration is one of the CBs. ANP is released from the atrium in response to atrial wall stretch [23, 28, 44]. Plasma ANP concentration reflects the clinical stages of MMVD and is used as CB in dogs with MMVD [12]. Plasma ANP concentration has a short half-life of 1–4 min and lacks stability, but N-terminal pro-atrial natriuretic peptide (NT-proANP) has a half-life 10 times longer and can be expected to

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be stable in blood [42, 43]. In dogs with recognized clinical signs of MMVD, compared to plasma ANP concentration, plasma NT-proANP concentration showed a higher the area under the curve (AUC) for the detection of congestive heart failure (CHF) [20]. However, no study has evaluated the discriminatory ability of plasma NT-proANP concentration for detection of cardiac dilatation in asymptomatic dogs that treatment for MMVD should be initiated (stage B2 in the most recent consensus statement from the American College of Veterinary Internal Medicine [ACVIM]) [26]. Plasma NT-proANP concentration might be a better marker for assessing cardiac dilatation than plasma ANP concentration in dogs with MMVD.

In dogs with MMVD, CBs, other than plasma ANP and NT-proANP concentrations, have clinical utility include plasma N-terminal pro-brain natriuretic peptide (NT-proBNP) and cardiac troponin I (cTnI) concentrations [14, 16, 33, 34]. NT-proBNP is released into the blood circulation primarily in response to ventricular myocyte stretch, and cTnI is released into the blood with cardiomyocyte injury. Therefore, the mechanism for the increase of these CBs in blood differs from that of the increase in plasma ANP and NT-proANP concentrations [27]. Particularly, plasma NT-proBNP concentration, like plasma NT-proANP concentration, has a long half-life in blood and, unlike plasma cTnI concentration, is elevated in dogs with cardiac disease, regardless of myocyte damage. Plasma NT-proBNP concentration is considered an excellent CB for detecting CHF in dogs with MMVD [38]. ANP can be rapidly secreted at the atrial muscle extension, whereas BNP reportedly increases with long-term stimulation [22, 46]. Thus, plasma NT-proANP and NT-proBNP concentrations may differ in the rate of increase in blood levels in dogs with MMVD. Although there has been a study that plasma NT-proANP and NT-proBNP concentrations were compared for the detection ability of worsening pre-clinical severe mitral regurgitant (MR) [45], these have been no large-scale comparative studies conducted on various dog breeds. In addition, no studies have compared discriminatory ability of cardiac dilatation and CHF of plasma NT-proANP concentration and these three CBs in dogs with MMVD. A comparison of plasma NT-proANP concentration to other CBs that have different secretion mechanisms in dogs with MMVD might be important in selecting a CB for each clinical stage of MMVD in dogs.

This multicentre, prospective study was evaluated the characteristics and discriminatory ability of plasma NT-proANP concentration, plasma NT-proBNP, ANP, and cTnI concentrations to diagnose cardiac dilatation and CHF in dogs with MMVD. We hypothesised that each CB was correlated with different clinical variables, and that plasma NT-proANP and NT-proBNP concentrations have equally better discriminatory abilities than plasma ANP and cTnI concentrations.

MATERIALS AND METHODS

Dogs

This study was a multicentre, prospective study that collected data from a total of 8 centres and was conducted by a total of 17 clinicians. In addition, this study included 105 client-owned dogs. All dogs were examined between June 2018 and November 2019. The blood samples used in this study were collected only from dogs that needed blood collection as part of an examination for health check-up and an examination of complications with treatment of MMVD (e.g., azotemia, electrolyte abnormality). In the control group, these dogs suspected cardiac dilatation on thoracic radiography were performed cardiac examinations as a second opinion. In these cases, no heart murmur was heard, and there were no findings of cardiac dilatation in the imaging examination, so they were judged to have normal hearts. However, some dogs included the control group were performed echocardiography for the present study. All pet owners provided informed consent before inclusion of their dogs in the study. We followed the Guidelines for Institutional Laboratory Animal Care and Use at the Nippon Veterinary and Life Science University (Approval number R2-2).

Healthy dogs constituted the control group (n=36), whereas dogs diagnosed with MMVD constituted the MMVD group (n=69). Dogs in the control group were identified based on medical history, physical examination, serum chemistry, electrocardiography, thoracic radiography, echocardiography, and blood pressure measurement. These evaluations were performed in a quiet room without sedating. Dogs in the MMVD group were classified into stages B1, B2, C, and D according to the most recent consensus statement from ACVIM [26]. Although stage A identifies dogs with a high risk of developing heart disease but with no currently identifiable structural disorder of the heart, dogs in stage A were included in the control group in this study. Among the dogs with MMVD without CHF, dogs with cardiac dilatation were designated as stage B2. The diagnostic criteria for cardiac dilatation are as follows; radiographic vertebral heart score (VHS) of >10.5, echocardiographic left atrium/aorta ratio (LA/Ao) in the right-sided short-axis view in early diastole of ≥1.6, and left ventricular internal diameter in diastole, normalised for body weight (LVIDdN) of ≥1.7 [26]. Dogs with either current or past clinical signs of heart failure caused by MMVD were classified as stage C, and dogs with end-stage MMVD, in which clinical signs of heart failure are refractory to standard treatment, were classified as stage D. In this study, dogs that required more than a total daily dosage of 8 mg/kg of furosemide or the equivalent dosage of torsemide (0.2–0.6 mg/kg), administered concurrently with standard doses of the other medications thought to control the clinical signs of heart failure were diagnosed with stage D. The development of pulmonary edema or pleural effusion confirmed in medical history and physical examination, thoracic radiography, and echocardiography was considered as stage C or D. In this study, dogs with stage B2 or higher and tricuspid regurgitation jet velocity (TR jet) ≥3.0 m/sec and/or pulmonary regurgitation jet velocity (PR jet) ≥2.5 m/sec were diagnosed with postcapillary pulmonary hypertension [41].

Exclusion criteria

Dogs with MMVD were excluded from the study if they met one or more of the following conditions:

• Diagnosis of respiratory disease (e.g., tracheal collapse, bronchial collapse and pneumonia) based on the results of medical history, physical examination, and thoracic radiography.

• Pulmonary hypertension unrelated to the heart (precapillary pulmonary hypertension); precapillary pulmonary hypertension

was diagnosed in cases with mild MMVD (less than ACVIM stage B2) and TR jet ≥3.0 m/sec and/or PR jet ≥2.5 m/sec in this study [41].

• Diagnosis of kidney disease based on the results of medical history, physical examination, and imaging test or serum creatinine levels ≥1.4 (dogs with abnormal kidney structure on ultrasonography, even if creatinine is within the normal range were excluded) [24].

• Diagnosis of arrhythmia based on electrocardiography (e.g., atrial fibrillation, ventricular tachycardia); However, dogs have sinus tachycardia, ventricular or supraventricular premature complex, and sinus arrhythmia were judged not to affect the results and were included in this study.

• Previous treatment for MMVD in dogs with MMVD without cardiac dilatation.

• Disease other than those mentioned above or treatment for condition other than MMVD; However, dogs with benign skin tumors, mild oral disease, and mild patellar dislocation were judged not to affect the results and were included in this study.

Clinical variables

Clinical variables used in the present study were age, body weight, heart rate, respiratory rate (in the hospital environment), murmur intensity, VHS, vertebral LA size (VLAS), MR jet velocity (MR jet), TR jet, PR jet, LA/Ao, LVIDd, LVIDdN, plasma NT-proANP, NT-proBNP, ANP, and cTnI concentrations.

Thoracic radiography

Routine right lateral and dorsoventral radiographic views of the thorax were obtained for each dog using a commercially available digital radiography system. VHS and VLAS were measured as previously described by Buchanan *et al*. and Malcolm *et al*. using right lateral radiographic views [8, 35]. A line was drawn and measured from the ventral border of the carina to the dorsal border of the caudal vena cava where it intersected with the LA. The same line length was drawn beginning at the cranial edge of the fourth thoracic vertebra and expressed in vertebral body units to the nearest 0.1 vertebra as VLAS [35].

Dogs with cough or those with a positive cough test underwent thoracic radiography not only during inspiration but also during exhalation. When >50% narrowing of the tracheal lumen during inspiration or bronchi during expiration was observed, it was determined that there was tracheal or bronchial collapse [37].

Echocardiography

Echocardiography was performed by experienced echocardiographers following the conventional method using an ultrasonographic unit fitted with a 6–12-MHz probe. An electrocardiogram (lead II) was also recorded during echocardiography. Each dog was manually restrained first in right and then in left lateral recumbency. The MR jet was measured from the left apical four chamber view using continuous-wave Doppler echocardiography. The LA and Ao dimensions were obtained from the right parasternal short-axis view in early diastole, and the LA/Ao was calculated [21]. The Ao dimension was measured by placing the first calliper on the midpoint of the convex curvature of the wall of the right coronary aortic sinus, and the second calliper on the point where the aortic wall and non-coronary and left coronary aortic cusps merged. The LA dimension was then measured from this point by extending the Ao line to the blood–tissue interface of the LA wall. The LVIDd was measured from the right parasternal short-axis view at the papillary muscle level using M-mode echocardiography, and the LVIDdN was calculated using the following formula [6, 11]:

$$\text{LVIDdN}=\text{LVIDd [cm]}/\text{body weight [kg]}^{0.294}.$$

CB measurement

Blood was drawn from the external jugular vein of each dog by direct venipuncture. To measure plasma NT-proANP, NT-proBNP, and cTnI concentrations, blood samples were placed in 2-ml vacutainer tubes containing EDTA dipotassium salt (Japan Becton, Dickinson and Co., Fukushima, Japan). To measure the plasma ANP concentration, blood samples were placed in 2-ml vacutainer tubes containing EDTA disodium salt with aprotinin (Japan Becton, Dickinson and Co.). The dispensed blood samples were centrifuged at 1,187 g for 5 min at 4°C. The plasma was stored at −80°C. Each CB was measured in a commercial laboratory: plasma NT-proANP concentration, Kyoritsu Seiyaku, Tokyo, Japan; plasma NT-proBNP concentration, IDEXX Laboratories, Tokyo, Japan; and plasma ANP and cTnI concentrations, FUJIFILM Monolith, Tokyo, Japan.

The plasma NT-proANP concentration was determined using sandwich enzyme-linked immunosorbent assay (ELISA) with two monoclonal antibodies, KS1-6 and biotinylated KS2-2, and streptavidin horseradish peroxidase. These antibodies to canine NT-proANP (31–67) were raised in mice, and these were recognised as different epitopes. The detection limit for NT-proANP assays was 110 pg/ml. The inter-assay used three samples, prepared four concentrations of samples (0, 200, 1,200, and 2,000 pg/ml) and performed three tests. The assay coefficient of variation was 5.0% (1.7–10.7%). In the intra-assay, the measurement date, operator and equipment used were changed, and the same three tests were performed as in those in the inter-assay; the assay coefficient of variation was 10.1% (3.7–18.2%). In addition, the upper limit of measurement of plasma NT-proANP concentration was 2,000 pg/ml. In the case of exceeding this value, plasma was diluted 1/4 to 1/512 with 1% -BSA-PBS solution, and the value obtained by multiplying the measured value (mean value) by the dilution factor of each serial dilution was taken as the plasma concentration of the sample. These were excluded in the serial dilution where the optical density value obtained in the serial dilution exceeds 2.0, and those in which the measured value obtained from the calibration line before multiplying the dilution factor is less than 120 pg/ml, which is the lower limit of quantification. When a total of 14 samples were used for verification between 1/8, 1/16, 1/32,

and 1/64 diluted samples, the median coefficient of variation was 8.0% (5.0−12.3%). Therefore, it was judged that the dilution measurement was justified.

The plasma NT-proBNP concentration was measured using sandwich ELISA, whereas the plasma ANP and cTnI concentrations were measured using chemiluminescent enzyme immunoassay. The upper limits of these CBs were 10,000 pmol/l, 2,000 pg/ml, and 50 ng/ml, respectively. If these upper limits were exceeded, they were measured by serial dilution. Analysis of the dilution linearity was already performed in both cases.

Discriminatory ability of CBs

The discriminatory abilities of cardiac dilation (stage B2 or higher) (event 1) or CHF (event 2) were evaluated in each CB. The development of pulmonary edema or pleural effusion confirmed in the medical history and physical examination, thoracic radiography and echocardiography was considered as event 2. In event 1, plasma concentrations of CBs were comparted between dogs with cardiac dilation (stage B2 or more) (n=35) and those without cardiac dilation (stage B1) (n=34). In event 2, these CBs were compared between dogs with CHF (n=21) and those without CHF (n=47).

Statistical analysis

Statistical analysis was performed using the commercial software SPSS Statistics version 24.0 (IBM Corp., Tokyo, Japan). Data normality was assessed using the Shapiro–Wilk test. The spearman's rank correlation coefficient was used to determine the relationship between CBs and each clinical variable. Then, using the statistically significant clinical variables, a forward–backward stepwise method that uses statistical significance to select the explanatory variables to be used in a multiple regression model was conducted to determine the independent predictor of each CB. In this study, heart murmur intensity was treated as a continuous variable. In addition, residues were confirmed to be normally distributed using the Shapiro–Wilk test, and the assumptions of the model were checked and satisfied. The Kruskal–Wallis test was used to compare the ACVIM stages of clinical variables and each CB; if a significant difference was observed, the Steel–Dwass test was used to compare all pairs of medians. The receiver operating characteristic (ROC) curve analyses were computed and generated to assess the AUC, sensitivity and specificity of CBs in events 1 and 2. The cutoff value was obtained from the point closest to the top left of the figure of the ROC with sensitivity on the vertical axis and 1-specificity on the horizontal axis. Moreover, the AUCs of the four CBs were compared using the Student's *t*-test and Bonferroni correction. A P-value <0.05 was considered statistically significant.

RESULTS

The 36 dogs in the control group had a median age of 6.0 (interquartile range, 1.1–10.0) years and weighed 4.60 (3.50–7.15) kg, whereas the 69 dogs in the MMVD group (ACVIM classification: B1, 34 dogs; B2, 14 dogs; C, 14 dogs; D, 7 dogs) had a median age of 10.0 (8.0–14.0) years and weighed 4.20 (3.30–6.50) kg (Table 1). Table 2 lists the breeds included in the present study. 7 dogs included in stage C or D had ongoing CHF. However, no significant difference was observed in the blood concentrations of each CB depending on whether CHF was ongoing or not in dogs of stage C or D: NT-proANP, P=0.88; NT-proBNP, P=0.44; ANP, P=0.68; and cTnI, P=0.14. In addition, 2 dogs in stage B2 (14.2%), 5 in stage C (35.7%) and 5 in stage D (71.4%) were diagnosed with postcapillary pulmonary hypertension.

The relationships between CBs and clinical variables are shown in Table 3. The results shown in Table 4 are for multiple regression analysis performed using parameters with significant differences in the univariate analysis. In the multiple regression analysis, only LA/Ao was selected as a significant variable associated with the plasma NT-proANP, NT-proBNP, and ANP concentrations, and only VLAS was selected as a significant variable associated with plasma cTnI concentration. Moreover, LA/Ao had a strong correlation with NT-proANP (r=0.666), NT-proBNP (r=0.662), and ANP (r=0.629).

Figure 1 shows the comparison between each stage ACVIM classification in CBs. CBs, other than the plasma cTnI concentration, were significantly different in all ACVIM stages compared to those in the control group (P<0.05). A significant difference was observed in plasma NT-proANP and NT-proBNP concentrations between stages B1 and C and in the plasma ANP concentration between stages B1 and D. Unlike other CBs, the plasma cTnI concentration showed a significant difference only in stages B1 and C compared to that in the control group.

Figure 2 shows the comparison of the ROC curve of each CB for events 1 and 2. In the power analysis with α=0.05, the statistical power of event 1 was 94.9% ± 0.15%, and that of event 2 was 91.6% ± 0.20%. In event 1, plasma NT-proANP and NT-proBNP concentrations were significantly different from plasma cTnI concentration in the AUC (P<0.05). Although there was no significant difference in AUC between CBs, other than plasma cTnI concentration, the sensitivities of plasma NT-proANP and NT-proBNP concentrations were 80.0% (cutoff value; 8,497.81 pg/ml and 1,453.00 pmol/l, 95% confidence interval [CI]; 0.64–0.90 and 0.64–0.90, respectively). In event 2, there was no significant difference in AUC between all CBs. Plasma NT-proANP and NT-proBNP concentrations showed sensitivity of ≥80.0%: NT-proANP, 85.7% (cutoff value; 8,684.33 pg/ml, 95% CI; 0.65–0.95); and NT-proBNP, 81.0% (cutoff value; 1,772.00 pmol/l, 95% CI; 0.60–0.92). In addition, NPV of plasma NT-proANP, NT-proBNP, and ANP concentrations were 90.6%, 88.6%, and 84.2% (95% CI; 0.75–0.96, 0.74–0.95, 0.69–0.92), respectively. The sensitivity, specificity, and NPV of CBs for each event are provided in Tables 5 and 6.

Table 1. Characteristics of dogs in the control and myxomatous mitral valve disease groups

	Control group	MMVD group			
		B1	B2	C	D
Number (head)	36	34	14	14	7
Sex (head)					
Male	8	5	3	0	1
Female	9	2	0	2	0
Neuterd male	13	10	7	5	1
Neuterd female	6	17	4	7	5
Age (year)	6.0 (1.1–10.0)	10.0[a)] (8.5–14.0)	10.0[a)] (8.0–14.0)	10.5 (6.7–14.0)	7.2[b)] (5.6–14.0)
Body weight (kg)	4.60 (3.50–7.15)	4.10 (3.29–6.54)	5.20 (3.72–9.42)	4.05 (2.53–4.91)	4.55 (3.07–6.38)
HR (bpm)	120 (102–150)	130 (120–160)	140 (130–150)	140 (120–171)	138 (116–158)
Respiration rate (bpm)	41 (32–48)	40 (36–57)	42 (36–52)	40 (30–82)	44 (33–58)
Murmur (Levine)	–	3 (2–4)	3 (3–5)	4[b)] (3–4)	5[b, c)] (5–6)
VHS (v)	9.8 (9.5–10.1)	10.3 (9.7–10.6)	11.5[a, b)] (10.9–12.0)	11.2[a, b)] (11.0–12.5)	12.5[a, b)] (11.0–12.8)
VLAS (v)	1.9 (1.7–2.0)	2.2[a)] (2.0–2.4)	2.6[a, b)] (2.2–3.0)	3.0[a, b)] (2.5–3.6)	2.7 (1.8–2.8)
MR jet (m/sec)	–	5.48[a)] (5.04–5.84)	5.95[a, b)] (5.56–6.25)	5.06[c)] (4.75–5.24)	5.08[c)] (4.70–5.43)
TR jet (m/sec)	–	0 (0–1.95)	1.31 (0–2.88)	1.44 (0–3.54)	3.00[b)] (2.16–3.36)
PR jet (m/sec)	–	0 (0–0)	0 (0–0)	0 (0–0)	0 (0–0)
LA/Ao	1.27 (1.13–1.33)	1.43[a, b)] (1.33–1.68)	1.94[a, b)] (1.81–2.27)	2.17[a, b)] (1.92–2.73)	2.36[a, b)] (1.96–2.78)
LVIDd (cm)	2.20 (1.95–2.65)	2.38 (2.05–2.86)	3.28[a, b)] (2.74–3.87)	3.08 (1.16–3.52)	3.31[a, b)] (3.05–3.79)
LVIDdN	1.39 (1.26–1.50)	1.61[a)] (1.39–1.71)	1.90[a, b)] (1.77–2.24)	2.06 (1.92–2.32)	2.21[a, b)] (1.96–2.33)
NT-proANP (pg/ml)	1,694.5 (1,115.7–2,100.2)	2,570.5[a)] (2,064.7–4,444.0)	4,608.5[a)] (2,434.0–5,728.2)	6,302.5[a, b)] (3,678.1–8,226.5)	4,825.0[a)] (3,166.0–8,440.0)
NT-proBNP (pmol/l)	696.5 (449.5–991.5)	1,257.0[a)] (837.2–2,335.2)	1,921.5[a)] (1,390.2–3,752.2)	5,381.0[a, b)] (1,703.5–8,248.2)	3,030.0[a)] (1,772.0–5,934.0)
ANP (pg/ml)	50.45 (38.05–91.65)	126.05[a)] (84.25–197.15)	191.65[a)] (78.55–555.30)	312.40[a)] (96.57–675.92)	433.40[a, b)] (161.10–560.20)
cTnI (ng/ml)	0.032 (0.008–0.062)	0.078[a)] (0.030–0.147)	0.066 (0.028–0.190)	0.127[a)] (0.056–0.372)	0.099 (0.036–0.836)

Data are presented as median (interquartile range). MMVD, myxomatous mitral valve disease; HR, heart rate; VHS, vertebral heart size; VLAS, vertebral left atrial size; MR jet, mitral regurgitant jet velocity; TR jet, tricuspid regurgitant jet velocity; PR jet, pulmonary regurgitant jet velocity; LA/Ao, left atrium/aorta ratio; LVIDd, left ventricular end-diastolic diameter in diastole; LVIDdN, left ventricular end-diastolic diameter normalised for body weight; NT-proANP, N-terminal pro-atrial natriuretic peptide; NT-proBNP, N-terminal pro-brain natriuretic peptide; cTnI, cardiac troponin I; a) significantly different from the control group ($P<0.05$); b) significantly different from stage B1 ($P<0.05$); c) significantly different from stage B2 ($P<0.05$).

DISCUSSION

In this study, the only clinical variable that showed significant differences at all stages of MMVD compared to the control group was LA/Ao, and the CBs selected as factors associated with this clinical variable were plasma NT-proANP, NT-proBNP, and ANP concentrations. Contrary to our hypothesis, these CBs increased with LA expansion, including plasma NT-proBNP concentrations that are released into the blood circulation primarily in response to ventricular myocyte stretching. These results are similar to those reported in previous studies [2, 13, 23]. There is a different regulation of atrial and ventricular BNP synthesis. BNP is secreted from ventricular myocytes quickly after synthesis via a constitutive pathway, but in the atria, it is stored in granules and released by a regulatory pathway [1]. 50–60% of circulating BNP is synthesized in the ventricles in normal individuals [34]. However, a

Table 2. Breed distribution in the control and myxomatous mitral valve disease groups

	Control group	MMVD group
Beagle	6	0
Cavalier King Charles Spaniel	0	10
Chihuahua	5	20
Dachshund	3	6
Miniature Schnauzer	1	5
Papillon	0	4
Poodle (Toy)	10	12
Shiba Inu	2	1
Shih Tzu	2	0
Others	7	11

Table 3. Correlation coefficient of cardiac biomarkers and clinical variables by correlation analysis

	NT-proANP	NT-proBNP	ANP	cTnI
Age (year)	0.258[a)]	0.307[a)]	0.293[a)]	0.409[a)]
Body wight (kg)	−0.308[a)]	−0.330[a)]	−0.313[a)]	−0.178
HR (bpm)	0.097	0.077	0.253[b)]	0.276[a)]
Respiration rate (bpm)	−0.118	−0.105	0.069	0.114
Murmur (Levine)	0.389[a)]	0.211	0.201	0.14
VHS (v)	0.474[a)]	0.420[a)]	0.494[a)]	0.303[a)]
VLAS (v)	0.557[a)]	0.534[a)]	0.550[a)]	0.396[a)]
MR jet (m/sec)	0.494[a)]	0.450[a)]	0.455[a)]	0.312[a)]
TR jet (m/sec)	0.974	0.169	0.117	0.872
PR jet (m/sec)	0.616	0.854	0.808	0.368
LA/Ao	0.666[a)]	0.662[a)]	0.629[a)]	0.413[a)]
LVIDdN	0.324[a)]	0.374[a)]	0.375[a)]	0.127

NT-proANP, N-terminal pro-atrial natriuretic peptide; NT-proBNP, N-terminal pro-brain natriuretic peptide; cTnI, cardiac troponin I; HR, heart rate; VHS, vertebral heart score; VLAS, vertebral left atrial size; MR jet, mitral regurgitant jet velocity; TR jet, tricuspid regurgitant jet velocity; PR jet, pulmonary regurgitant jet velocity; LA/Ao, left atrium/aorta ratio; LVIDd, left ventricular end-diastolic diameter in diastole; LVIDdN, left ventricular end-diastolic diameter normalised for body weight; a), $P<0.01$; b), $P<0.05$.

Table 4. Multiple linear regression analysis of the predictors of cardiac biomarkers

Dependent variable	Independent variable	Partial regression coefficient (95% CI)	Standard partial regression coefficient	*P* value
NT-proANP	LA/Ao	9,172.8 (5,671.3–12,674.2)	0.538	<0.01
NT-proBNP	LA/Ao	3,086.60 (2,286.8–3,886.4)	0.649	<0.01
ANP	LA/Ao	302.9 (232.5–373.3)	0.689	<0.01
cTnI	VLAS	0.85 (0.008–0.161)	0.232	<0.05

NT-proANP, N-terminal pro-atrial natriuretic peptide; NT-proBNP, N-terminal pro-brain natriuretic peptide; cTnI, cardiac troponin I; CI, confidence interval; LA/Ao, left atrium/aorta ratio; VLAS, vertebral left atrial size.

study in human CHF patients indicates that BNP is also secreted from the atrium [32]. Therefore, the correlation between LA/Ao and plasma NT-proBNP concentration in this study may be related to the secretion of BNP from the atrium as MMVD progresses. Moreover, these results suggest that plasma NT-proANP, NT-proBNP, and ANP concentrations increase with LA enlargement even before the diagnostic criteria for stage B2 are met.

In contrast, although VLAS is an indicator of left atrial enlargement this did not correlate with plasma NT-proANP, NT-pro BNP, and ANP concentrations. In addition, VLAS also showed higher values in stage C dogs than in stage D, as with plasma NT-proANP and NT-pro BNP concentrations. The reason may be explained by dilation of the caudal cava. Compared with stage C, dogs with stage D had a higher proportion of dogs with pulmonary hypertension (stage C; 35.7%, stage D; 71.4%). Dogs with pulmonary hypertension dilate the caudal vena cava [31]. VLAS is a line that drawn and measured from the ventral border of the carina to

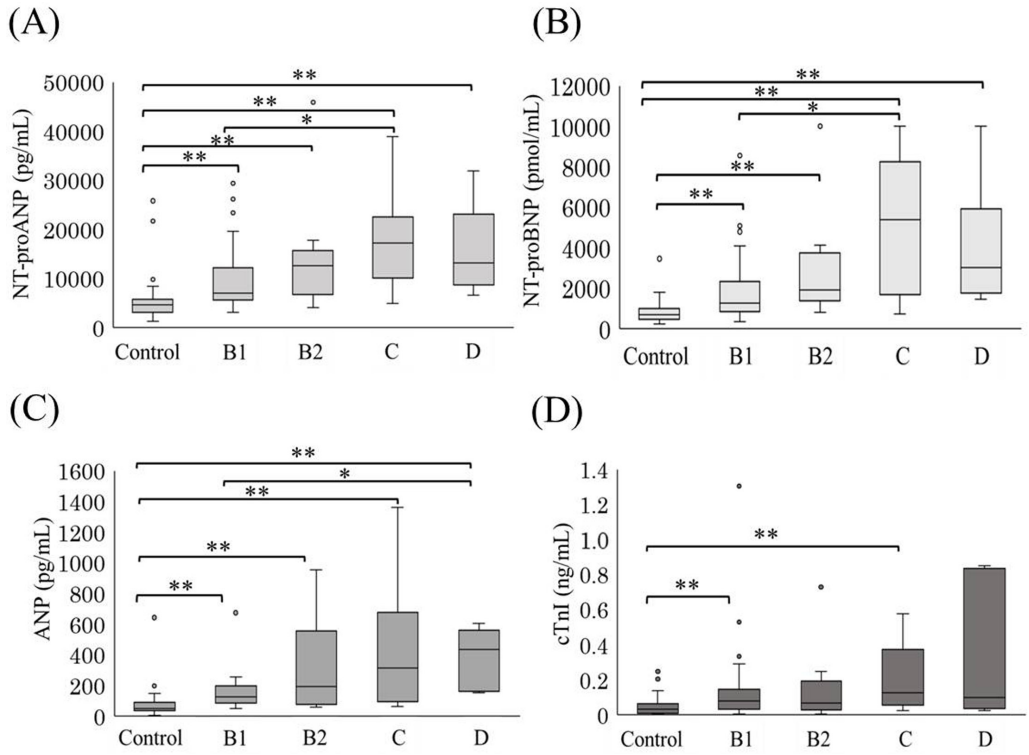

Fig. 1. Comparison between each stage ACVIM classification in plasma NT-proANP (A), NT-proBNP (B), ANP (C), and cTnI (D) concentrations. The box represents the interquartile range (IQR) and the line within, the median. The whiskers reflect the most extreme values that are <1.5 IQR beyond the upper or lower quartiles. ○outlier; **P*<0.05; ***P*<0.01. ACVIM, American College of Veterinary Internal Medicine; CB, cardiac biomarker; IQR, interquartile range; NT-proANP, N-terminal pro-atrial natriuretic peptide; NT-proBNP, N-terminal pro-brain natriuretic peptide; cTnI, cardiac troponin I.

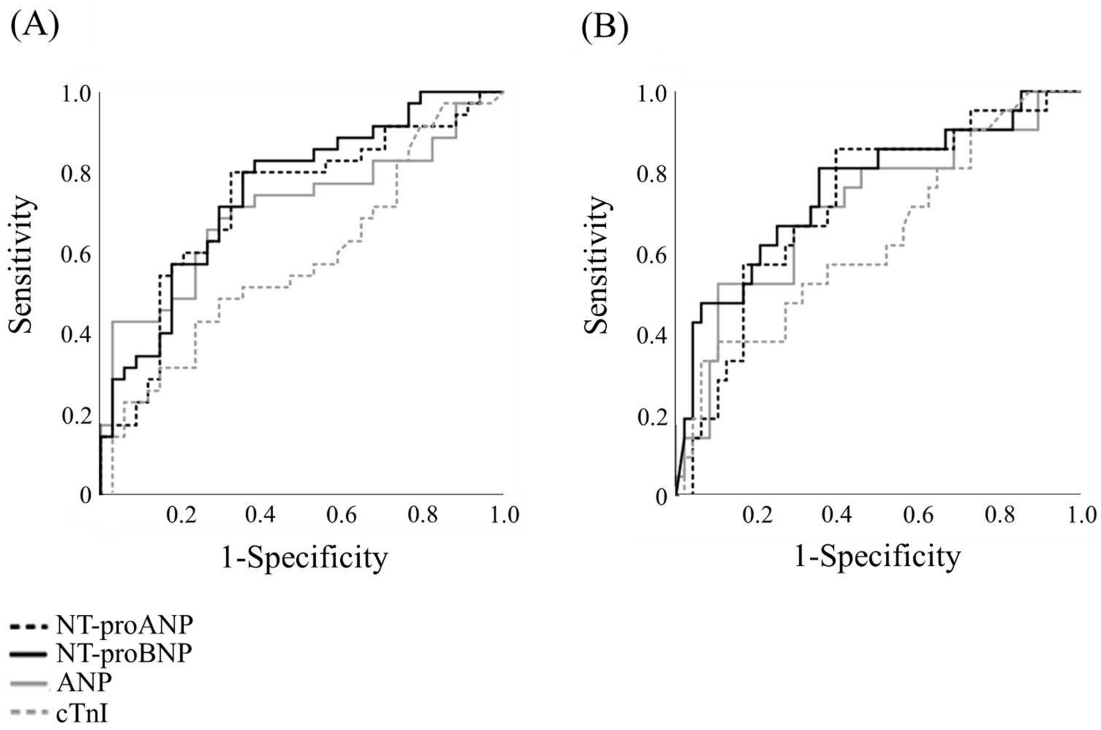

Fig. 2. Comparison of the AUC of CBs for events 1 (A) and 2 (B) using ROC analysis. Event 1: the dogs were determined as stage B2 or more. Event 2: the dogs in the MMVD group had CHF. AUC, area under the curve; CB, cardiac biomarker; ROC, receiver operating characteristic; MMVD, myxomatous mitral valve disease; CHF, congestive heart failure; NT-proANP, N-terminal pro-atrial natriuretic peptide; NT-proBNP, N-terminal pro-brain natriuretic peptide; cTnI, cardiac troponin I.

Table 5. Receiver operating characteristic analysis for detection of dogs determined as stage B2 or more

	NT-proANP	NT-proBNP	ANP	cTnI
Cutoff value	8,497.81 pg/ml	1,453.00 pmol/l	164.20 pg/ml	0.11 ng/ml
AUC	0.72†	0.75†	0.70	0.57
95% CI	0.59–0.84	0.63–0.86	0.58–0.83	0.44–0.71
Sensivity (%)	80.0	80.0	68.6	48.6
95% CI	0.64–0.90	0.64–0.90	0.52–0.81	0.33–0.64
Specificity (%)	67.6	64.7	70.6	70.6
95% CI	0.50–0.80	0.47–0.78	0.53–0.83	0.53–0.83
PPV (%)	71.8	70.0	70.6	63.0
95% CI	0.56–0.83	0.54–0.81	0.53–0.83	0.44–0.78
NPV (%)	76.7	75.9	68.6	57.1
95% CI	0.59–0.88	0.57–0.87	0.52–0.81	0.42–0.70
Likelihood ratio	2.46	2.26	2.33	1.65
95% CI	1.48–4.13	1.39–3.67	1.32–4.11	0.88–3.07

AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; MMVD, myxomatous mitral valve disease; NT-proANP, N-terminal pro-atrial natriuretic peptide; NT-proBNP, N-terminal pro-brain natriuretic peptide; cTnI, cardiac troponin I; †, significantly different from plasma cTnI concentration ($P<0.05$).

Table 6. Receiver operating characteristic analysis for the detection of dogs with congestive heart failure

	NT-proANP	NT-proBNP	ANP	cTnI
Cutoff value	8,684.33 pg/ml	1,772.00 pmol/l	190.40 pg/ml	0.09 ng/ml
AUC	0.72	0.76	0.71	0.63
95% CI	0.59–0.85	0.62–0.89	0.57–0.85	0.48–0.78
Sensivity (%)	85.7	81.0	71.4	57.1
95% CI	0.65–0.95	0.60–0.92	0.50–0.86	0.36–0.75
Specificity (%)	60.4	64.6	66.7	62.5
95% CI	0.46–0.73	0.50–0.76	0.52–0.78	0.48–0.74
PPV (%)	48.6	50.0	48.4	40.0
95% CI	0.33–0.64	0.34–0.65	0.32–0.65	0.24–0.57
NPV (%)	90.6	88.6	84.2	76.9
95% CI	0.75–0.96	0.74–0.95	0.69–0.92	0.61–0.87
Likelihood ratio	2.16	2.28	2.14	1.52
95% CI	1.46–3.20	1.48–3.53	1.32–3.47	0.90–2.56

AUC, area under the curve; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; MMVD, myxomatous mitral valve disease; NT-proANP, N-terminal pro-atrial natriuretic peptide; NT-proBNP, N-terminal pro-brain natriuretic peptide; cTnI, cardiac troponin I.

the dorsal border of the caudal vena cava where it intersected with LA. Therefore, the dorsal border of the caudal vena cava could increase due to the dilation of the caudal vena cava in stage D, and as a result, the VLAS value might be underestimated.

The AUCs between plasma NT-proANP, NT-proBNP, and ANP concentrations were not significantly difference in event 1. Particularly, the CIs for plasma NT-proANP and NT-proBNP concentrations were in close agreement; with stage B2, the discriminatory ability was considered equivalent. Plasma NT-proANP and NT-prBNP concentrations had sensitivity of 80.0%. Thus, these CBs might reflect the diagnostic criteria for stage B2 (diagnostic criteria for cardiac dilatation). This suggests that, in dogs with asymptomatic MMVD, if plasma NT-proANP and NT-proBNP concentrations are below the cutoff values, it is likely that they are not stage B2 or higher.

In event 2, all CBs showed no significant difference in AUC, but plasma NT-proANP and NT-proBNP concentrations showed a specificity ≥80.0% and NPV ≥85.0%. Therefore, the clinical sign (i.e. dyspnea) might be caused by other than CHF if dogs with dyspnea had plasma NT-proANP and NT-proBNP concentrations less than these the cutoff values set in this study.

ANP is stored in atrial muscle secretory granules, and the peptide can be rapidly secreted at the atrial muscle extension [22]. In contrast, BNP is not stored in secretory granules and thus rapid secretion of BNP is impossible [22, 33]. However, the plasma BNP concentration reportedly increases with long-term stimulation [30, 45]. Previous studies have shown that plasma ANP concentration increases with short-term saline loading, but plasma BNP concentration increases with long-term saline loading [9, 29]. However, in the present study, plasma NT-proANP and NT-proBNP concentrations had almost the same discriminatory ability. Unlike bioactive plasma ANP and BNP concentrations, plasma NT-proANP and NT-proBNP concentrations may have equivalent discriminatory abilities in each clinical stage, regardless of the timing of secretion, because these CBs have long half-lives.

Plasma NT-proANP and NT-proBNP concentrations were higher in stage C than in stage D. One of the reasons may be that plasma NT-proANP and NT-proBNP concentrations were depletion. In human studies, both plasma NT-proANP and NT-

proBNP concentrations have been reported to decrease with heart failure and the progression of heart disease due to endogenous mechanisms can no longer contribute adequately to neurohormonal compensation [1, 47]. However, in this study, plasma ANP concentration was higher in stage D than in stage C. In addition, there was a bias in cases in stages C and D, and some dogs in stage D had higher plasma NT-proANP and NT-proBNP concentrations than in stage C cases. Therefore, it was also considered that increasing the number of stage D cases may increase plasma NT-proANP and NT-proBNP concentrations in stage D rather than stage C.

The AUCs of plasma NT-proANP and ANP concentrations were almost identical, and particularly, the almost same AUC values were found for event 2. The difference in AUC, sensitivity, and specificity of plasma NT-proANP and ANP concentrations in event 1 might be due to the probable longer half-life of plasma NT-proANP concentration than that of plasma ANP concentration [42]. Moreover, the same AUC of plasma NT-proANP and ANP concentrations in event 2 might be due to a decrease in blood ANP clearance. A study has reported that a molar ratio of plasma NT-proANP–ANP concentration increases in humans with CHF [3]. A decrease in blood ANP clearance caused by downregulation of ANP receptors is assumed to be further increased the plasma ANP concentration [25].

Plasma cTnI concentration had an overall lower discriminatory ability than other CBs in this study. The increase of plasma troponin I concentration appears to be involved in the myocardial fibrosis as one of the causes. The plasma cTnI concentration is a cardiomyocyte injury marker [17]. In addition, previous studies revealed that high plasma cTnI concentration indicated intramyocardial fibrosis [15]. Plasma cTnI concentration may not have reflected cardiac dilatation and CHF because myocardial fibrosis is not necessarily associated with the clinical stages of MMVD [10]. However, this hypothesis could not be proved by histopathological examination in our study.

This study has a few limitations. First, the AUC, sensitivity, specificity, and likelihood ratios of CBs were lower than those reported in previous studies [39, 40]. This may be due to the effect of age on fluctuations in CBs. Previous studies have reported that the plasma NT-proANP concentration was significantly correlated with age in dogs [3, 14]. In contrast, plasma NT-proBNP concentration was not significantly correlated with age in dogs with MMVD [5]. However, age and body weight were not selected in the multivariate analysis but were significantly correlated in the univariate analysis for plasma NT-proANP, NT-proBNP, and ANP concentrations in the present study. Furthermore, although body weight did not show a significant difference between ACVIM stages, there were significant differences in age between control group and stage B1 or B2 and between stage B1 and D. Improving bias of age may increase the sensitivity, specificity, and likelihood ratio of each CB.

Second, since this our study was a multicentre, prospective study, MR jet, LA/Ao and LVIDd measurements obtained from echocardiography, and VHS and VLAS measurements obtained from thoracic radiography were performed by different individuals in various centres. Moreover, the manufacturers of a radiographic equipment and an ultrasonograph also differed between centres. The intermeasurer variation of each clinical variable could not be verified in the present study, and the measured values could have a measurer bias. In addition, the diagnosis of pulmonary hypertension could not be unified in all centres involved our study. In the present study, the case with ACVIM stage B2 or higher and TR jet ≥3.0 m/sec and/or PR jet ≥2.5 m/sec was diagnosed as postcapillary pulmonary hypertension, but other indicators for pulmonary hypertension could not be used. Therefore, it could not be determined the probability of pulmonary hypertension. In a previous study, the CBs used in the present study reflected the severity of pulmonary hypertension [27]. However, in this our study, TR jet and PR jet showed little significant difference between each ACVIM stage and also did not correlate with CBs. Therefore, it is unlikely that pulmonary hypertension was involved in the results of this study.

Third, a fluoroscopy and bronchoscopy were not used, and respiratory diseases such as bronchial collapse and pneumonia could not be completely differentiated in the present study. Therefore, although dogs with mild MMVD and pulmonary hypertension were diagnosed precapillary pulmonary hypertension, it was not possible to clarify whether the combined postcapillary and precapillary pulmonary hypertension was included in the dogs diagnosed as postcapillary pulmonary hypertension.

Lastly, the present study did not consider the effects of drugs used in these dogs with MMVD. Dogs with stage B2 and higher received treatments for MMVD. All treated dogs received pimobendan (0.25–0.50 mg/kg q12hr), but the use of angiotensin-converting enzyme inhibitors, calcium channel blockers, diuretics, nitroglycerin, and sildenafil varied from individual to individual. To minimise drug effects, dogs with MMVD without cardiac dilatation that have initiated treatment at the time of sample collection were excluded from the present study. Furthermore, LA and LV diameters increased with the progression of ACVIM stages, suggesting that drug treatment had little effect on LA and LV size. However, Häggström *et al*. showed significant reductions in VHS and LVIDd by pimobendan administration in dogs with stage C [19]. Therefore, it cannot be completely ruled out that changes in LA size caused by the drug may have affected the results of the present study. Changes in each CB after drug administration may require further investigation.

In conclusion, plasma NT-proANP, NT-proBNP, and ANP concentrations can increase with LA enlargement with or without clinical signs in dogs with MMVD. Among the four CBs, plasma NT-proANP and NT-proBNP concentrations had comparable discriminatory abilities to detect cardiac dilatation and CHF in dogs with MMVD.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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