



## RESEARCH ARTICLE

### Sex and Neuter Status Affect the Clinical Application of N-Terminal Pro-Brain Natriuretic Peptide as A Survival Biomarker in Dogs with Congestive Heart Failure

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#### ARTICLE HISTORY (18-284)

Received: August 04, 2018  
Revised: March 14, 2019  
Accepted: April 26, 2019  
Published online: May 09, 2019

#### Key words:

Cardiac biomarkers  
Degenerative mitral valve disease  
Relative risk  
Serial measurements

#### ABSTRACT

We aimed to evaluate the utility and sex dependence of serum N-terminal pro-brain natriuretic peptide (NT-proBNP concentration) as a predictor of survival and treatment response in dogs with heart failure. We hypothesized that the serial trend of NT-proBNP levels would be predictive of outcomes and be independent of sex and neuter status in dogs with congestive heart failure secondary to myxomatous mitral valve disease. This prospective study enrolled 18 privately owned dogs with congestive heart failure and no previous diuretic treatment. Serial NT-proBNP measurements were performed at admission (treatment initiation) and during follow-up (on days 7 and 21). The NT-proBNP concentration at admission or at any of the two follow-up examinations did not differ significantly between dogs that survived and those that died of cardiac causes. Intact male dogs with an ascending trend of NT-proBNP levels between admission and the second follow-up died within 200 days, whereas those with a descending trend survived up to 500 days. However, no such relationship was observed for neutered males or spayed females. Our findings suggest that an ascending trend of NT-proBNP levels is indicative of a higher risk of cardiac-related death in intact male dogs with congestive heart failure receiving treatment, warranting careful monitoring. Furthermore, NT-proBNP measurements performed later than two weeks after treatment initiation provide better prognostic capabilities.

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**To Cite This Article:** Chan IP, Hsueh T, Chang CC and Chen WY, 2019. Sex and neuter status affect the clinical application of n-terminal pro-brain natriuretic peptide as a survival biomarker in dogs with congestive heart failure. *Pak Vet J*, 39(3): 347-352. <http://dx.doi.org/10.29261/pakvetj/2019.069>

#### INTRODUCTION

Myxomatous mitral valve disease (MMVD) accounts for 75% of cases of acquired heart disease in small-breed dogs (Borgarelli and Buchanan, 2012). In dogs, congestive heart failure (CHF) commonly occurs secondary to MMVD and is associated with a survival time of 6-24 months (Borgarelli and Haggstrom, 2010). Prognostic indicators of canine CHF secondary to MMVD include parameters related to the progression of cardiomegaly and cardiac dysfunction on diagnostic imaging (Sargent *et al.*, 2015), the choice of loop diuretics and adjunct therapies (Peddle *et al.*, 2012; Haggstrom *et al.*, 2013) and the incidence of complications (Borgarelli *et al.*, 2015; Jung *et al.*, 2016).

N-terminal pro-brain natriuretic peptide (NT-proBNP) is a frequently used cardiac biomarker with clinical

applications for diagnosing heart disease, detecting heart failure, and differentiating cardiogenic respiratory distress in dogs (Fox *et al.*, 2015). Increased serum NT-pro-BNP concentration may reflect CHF deterioration (Oyama *et al.*, 2013). Sex dependency of NT-proBNP levels has been demonstrated in humans and in healthy dogs (Loke *et al.*, 2003; Misbach *et al.*, 2013). Moreover, sex-related differences in prognostic value and survival outcomes predicted based on proBNP levels were found in humans with acute decompensated CHF (Nakada *et al.*, 2016) or acute dyspnea (Christ *et al.*, 2006). However, the effects of sex and neuter status on NT-proBNP concentration and its prognostic value in dogs with CHF secondary to MMVD remain unclear.

Serial monitoring of NT-proBNP levels may be superior to interpreting single time-point NT-proBNP concentrations (Ruau *et al.*, 2015; Winter *et al.*, 2017).

Indeed, serial monitoring with risk categorization based on NT-proBNP thresholds was effective for predicting mortality in 1742 human patients with stable heart failure (Masson *et al.*, 2008). In dogs with symptomatic MMVD, a follow-up NT-proBNP concentration >965 pmol/L and higher severity of heart failure on admission were the only predictors of 12-month mortality (Wolf *et al.*, 2012). The prognostic value of serial NT-proBNP measurements in the field of veterinary medicine remains unclear.

We aimed to evaluate the clinical utility, as well as the sex and neuter status dependence, of serial NT-proBNP measurements as prognostic markers of survival outcomes in dogs with CHF secondary to MMVD.

## MATERIALS AND METHODS

**Patient inclusion and cardiac diagnosis:** This was a prospective, single-blinded clinical study performed between September 2015 and March 2017. Dogs diagnosed with CHF secondary to MMVD and having no history of relevant medication were included. The diagnosis of MMVD was based on characteristic left apical heart murmur, mitral valve lesions (thickened and/or prolapsed leaflets) on two-dimensional echocardiographs, and mitral regurgitation on color and spectral Doppler (Chetboul and Tissier, 2012). The diagnosis of CHF was based on radiographic evidence of cardiogenic pulmonary edema such as pulmonary venous congestion and an interstitial to alveolar pattern of the pulmonary parenchyma combined with an enlarged heart. The exclusion criteria were: congenital or other significant heart disease, hemodynamically significant arrhythmia, acute pulmonary edema secondary to suspected rupture of the chordae tendineae, suspected renal dysfunction (creatinine concentration >1.9 mg/dL), or other significant systemic diseases.

**Radiographic examination:** In most dogs, at least two orthogonal thoracic radiographs were obtained on admission and at the follow-up examinations; in dogs with severe respiratory distress, only dorsoventral radiographs were obtained. The vertebral heart score was calculated based on lateral radiographs.

**Echocardiographic examinations:** All echocardiographic examinations and measurements were performed by a single cardiologist at admission. The left ventricular internal dimension at diastole (LVIDd) was measured and then normalized (LVIDdn) to body weight using the formula  $LVIDd/(body\ weight\ [kg])^{0.294}$ . The end-diastolic volume of the left ventricle (EDV) was obtained using the Teichholz formula and normalized (EDVn) to the body surface area. The diameters of the left atrium and aorta were obtained at the base of the heart using the M-mode in the right parasternal short axis view, and the left atrial-to-aortic diameter ratio (LA/Ao) was calculated. The early diastolic velocity of mitral inflow (E wave) was detected using spectral pulsed-wave Doppler in the left four-chamber long axis view. The velocity of early diastolic motion of the lateral mitral annulus (E' wave) was recorded using color tissue Doppler in the same view and the E/E' ratio was calculated.

**Blood sampling:** Blood was collected from the jugular, cephalic, or saphenous vein. Serum biochemistry analysis, including evaluation of renal function, was performed within 1 hour of blood collection. A validated second-generation proBNP enzyme-linked immunosorbent assay (Cardiopet proBNP test-canine; IDEXX Laboratories Inc., Westbrook, ME, USA) was used for quantification of the serum NT-proBNP concentration in frozen samples sent to a reference laboratory (Cahill *et al.*, 2015).

**Follow-up:** Medical intervention for CHF was initiated at admission. Two follow-up examinations were scheduled, the first at 7 days after treatment (F1), and the second at 14 days after F1 (i.e., 21 days after treatment initiation; F2). Thoracic radiographic examinations and blood sampling including NT-proBNP measurements were performed during each follow-up visit.

**Survival outcomes:** The survival outcomes of the dogs that presented for monthly monitoring of CHF treatment were documented until the end of the study period. If necessary, phone interviews were conducted with the owner or with the referred veterinarians every 6 months after F2.

**Statistical analysis:** Data were analyzed using commercially available statistical software (SAS/STAT software; SAS Institute Inc., Cary, NC, USA). Normally distributed data were expressed as mean  $\pm$  standard deviation, and non-normally distributed data as median (interquartile range). Categorical data were expressed as frequency (percentage). Cox proportional hazard regression and Kaplan-Meier survival analyses were conducted. Statistical significance was set at  $P < 0.05$ .

## RESULTS

**Studied animals:** Of the 83 dogs treated for CHF secondary to MMVD during the study period (September 2015 to March 2017), 37 privately owned animals were prospectively enrolled in the study. Sixteen dogs were excluded from the final analysis because of poor owner compliance (n=12), severe azotemia (n=2), atrial fibrillation (n=1), or acute CHF secondary to rupture of the chordae tendineae (n=1). The final analysis included 21 dogs (7 intact males, 6 neutered males, and 8 neutered females). Five small breeds were represented in this study: Maltese (n=10), Chihuahua (n=3), Miniature Schnauzer (n=2), Pomeranian (n=2), Miniature Poodle (n=2), and non-pedigree dogs (n=2). The following baseline characteristics were noted: age,  $10.3 \pm 1.80$  years; median body weight, 3.40 kg (3.15-4.55 kg); heart rate,  $144 \pm 30.6$  bpm; systolic blood pressure (BP),  $139 \pm 20.0$  mmHg; diastolic BP,  $111 \pm 20.0$  mmHg; blood urea nitrogen (BUN) levels,  $24.6 \pm 7.30$  mg/dL; and median creatinine concentration, 0.90 mg/dL (0.8-1.1 mg/dL).

**Follow-up intervals:** The median time interval between admission and F1 was 7 days (range, 7-12 days), and the median time interval between F1 and F2 was 14 days (range, 14-47 days). The median time between admission and F2 was 25.5 days (range, 17-65 days). The median observation time was 202 days (range, 131-315 days).

**Survival outcomes:** Three types of survival outcomes were defined, namely: (i) survival, (ii) death of cardiac causes, and (iii) death of non-cardiac causes. Dogs that were alive at the end of the study (18 months) were included in the survivor (S) group (11 of 21 dogs, 52.4%). Non-survivors (10 dogs, 47.6%) were categorized according to the cause of death. Dogs with antemortem dyspnea, soft and wet coughing with pink foamy nasal discharge, or sudden death were included in the cardiac-related death (CD) group (n=7). The other dogs were considered to have died of non-cardiac causes (tumor, gastric dilatation-volvulus, severe gastrointestinal disorder). Thus, 18 dogs (S group, n=11; CD group, n=7) were analyzed further.

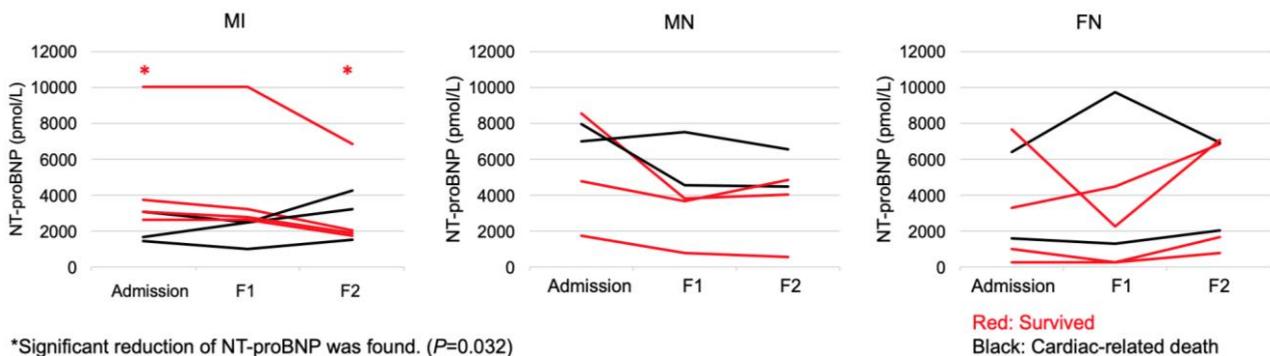
On admission (Table 1), dogs in the CD group had significantly larger LVIDdn ( $P=0.003$ ) and EDVn ( $P=0.02$ ), with no other significant differences between the two groups (t-test or Mann-Whitney U-test). The S and CD groups did not differ regarding NT-proBNP concentrations at admission, F1, or F2 (Table 2).

**Medications:** The medications used included furosemide (18/18, 2-6 mg/kg/day), amlodipine (17/18, 0.2-0.4 mg/kg/day), enalapril (1/18, 0.44 mg/kg/day), spironolactone (1/18, 2 mg/kg/day) and pimobendan (5/18, 0.5 mg/kg/day), with no significant differences between the S and CD groups regarding furosemide or amlodipine dosage (Table 3).

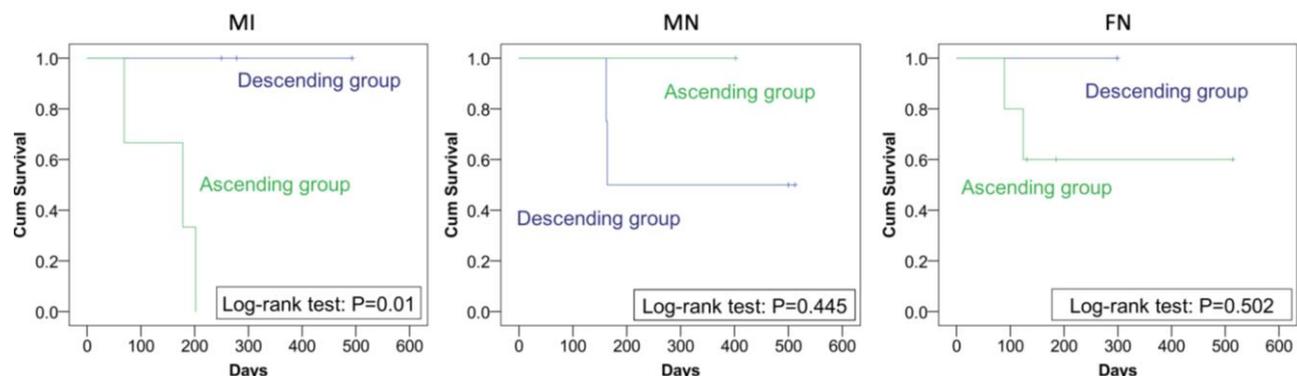
**Classification of NT-proBNP trends:** Dogs were classified into two groups based on the trend of serial NT-

proBNP values. For each case, a line chart was drawn with the time points (admission, F1, F2) on the x-axis and the NT-proBNP concentrations on the y-axis. Dogs with negative slope of the trend line were considered to exhibit a descending trend, whereas those with positive slope of the trend line or NT-proBNP concentration  $>10,000$  pmol/L at two or more evaluations (admission and F1 or admission and F2) were considered to exhibit an ascending trend. The NT-proBNP trends were evaluated for two time periods, namely between admission and F1, and between admission and F2. For each evaluation of NT-proBNP trends, the dogs were stratified based on sex (male, M; female, F) and spay/neuter status (intact, I; neutered/spayed, N). The following subgroups were identified: intact males (MI), neutered males (MN), and neutered females (FN).

**Risk factors:** No statistically significant association with survival free from cardiac death was demonstrated for FN status ( $P=0.497$ ), MI status ( $P=0.452$ ), MN status ( $P=0.237$ ), age ( $P=0.980$ ), body weight ( $P=0.566$ ), systolic BP ( $P=0.771$ ), heart rate ( $P=0.205$ ), BUN levels ( $P=0.595$ ), creatinine levels ( $P=0.302$ ), NT-proBNP levels (admission,  $P=0.685$ ; F1,  $P=0.592$ ; F2,  $P=0.480$ ), vertebral heart score ( $P=0.824$ ), LVIDdn ( $P=0.061$ ), EDVn ( $P=0.057$ ), LA/Ao ( $P=0.726$ ), E wave ( $P=0.641$ ), E/E' ( $P=0.256$ ), furosemide use ( $P=0.581$ ), amlodipine use ( $P=0.646$ ), enalapril use ( $P=0.552$ ), pimobendan use ( $P=0.175$ ), trend of NT-proBNP levels between admission and F1 ( $P=0.433$ ), or trend of NT-proBNP levels between admission and F2 ( $P=0.065$ ).



**Fig. 1:** Serial measurements of NT-proBNP levels in 18 dogs treated for congestive heart failure. The dogs were stratified according to survival (survivors, red; cardiac-related death, black), sex (female, F; male, M), and spay/neuter status (intact, I; neutered/spayed, N). NT-proBNP, N-terminal pro-brain natriuretic peptide.



**Fig. 2:** Cumulative survival among 18 dogs treated for congestive heart failure. The dogs were stratified according to sex (female, F; male, M), spay/neuter status (intact, I; neutered/spayed, N), and serial trend of NT-proBNP levels between admission and the second follow-up visit (ascending, green; descending, blue). NT-proBNP, N-terminal pro-brain natriuretic peptide.

**Table 1:** Admission characteristics of dogs with heart failure

Characteristic	Survived	Cardiac death	P-value
	n=11	n=7	
Age, years	10 (8-10)	11 (9-12)	0.126
Body weight, kg	3.4 (3.0-6.9)	3.4 (3.3-3.8)	0.791
Heart rate, bpm	140±16.3	162±35.7	0.089
Systolic BP, mmHg	142±19.8	138±14.0	0.669
Vertebral heart score	12.4 (11.3-13.4)	12.6 (11.2-13.0)	0.713
BUN, mg/dL	26.9±8.36	23.0±5.80	0.297
Creatinine, mg/dL	0.9 (0.8-1.1)	0.8 (0.8-1.3)	0.596
LVIDdn	1.9±0.2	2.35±0.25	0.003
EDVn, mL/m <sup>2</sup>	125±26.0	204±50.4	0.02
LA/Ao	1.94±0.35	1.96±0.37	0.917
E wave, cm/s	1.50±0.40	1.67±0.38	0.452
E/E'	12.7±4.46	16.8±3.68	0.137

Data are shown as median (interquartile range) or mean ± standard deviation. BP, blood pressure; BUN, blood urea nitrogen; LVIDdn, normalized left ventricular internal dimension at diastole; EDVn, normalized end-diastolic volume; LA/Ao, left atrial-to-aortic diameter ratio.

**Table 2:** Concentration of NT-proBNP at admission and at two time points during follow-up after initiation of treatment (F1, F2)

NT-proBNP (pmol/L)	All (n=18)		P-value
	CD (n=7)	S (n=11)	
Admission	4170±2841	4243±3181	0.961
F1	4170±3313	3113±2700	0.469
F2	4151±2063	3490±2535	0.572
	MI dogs (n=7)		P-value
	CD (n=3)	S (n=4)	
Admission	2070±861.3	4860±3457	0.239
F1	2009±829.9	4674±3561	0.269
F2	3017±1389	3241±2494	0.942
	MN dogs (n=5)		P value
	CD (n=2)	S (n=3)	
Admission	7467±723.4	5012±3408	0.410
F1	6049±2075	2751±1709	0.144
F2	5514±1414	3159±2286	0.295
	FN dogs (n=6)		P-value
	CD (n=2)	S (n=4)	
Admission	4025±3377	3051±3325	0.753
F1	5533±5950	1824±2022	0.536
F2	4489±3447	4088±3313	0.897

The dogs were stratified according to survival (survivors, S; cardiac-related death, CD), sex (female, F; male, M), and spay/neuter status (intact, I; neutered/spayed, N). NT-proBNP, N-terminal pro-brain natriuretic peptide.

**Table 3:** Medication dosage upon admission

Use/Dosage	n	Survived	Cardiac death	P-value
		(n=11)	(n=7)	
		mg/kg/day	mg/kg/day	
Furosemide	11	2 (2-4)	7 2 (2-4)	0.930
Amlodipine	10	0.2 (0.2-0.2)	7 0.2 (0.2-0.2)	0.740
Enalapril	1	0.44	0	NA
Spironolactone	1	2	0	NA
Pimobendan	4	0.5	1 0.5	NA

Dosage is given as median (interquartile range) or actual value. Pimobendan was administered with the same dosage. NA, not applicable.

**Table 4:** Trend of serial NT-proBNP levels between admission and first follow-up (F1, at 7-12 days after treatment initiation)

Serial NT-pro BNP trend (F1)	All (n=18)		Sex and spay/neuter status					
			MI (n=7)		MN (n=5)		FN (n=6)	
	CD	S	CD	S	CD	S	CD	S
Ascending	3	4	1	2	1	0	1	2
Descending	4	7	2	2	1	3	1	2
Fisher's exact test	1.00		1.00		0.4		1.00	
Homogeneity by RR					0.38			

The dogs were stratified according to survival (survivors, S; cardiac-related death, CD), sex (female, F; male, M), spay/neuter status (intact, I; neutered/spayed, N) and trend of serial NT-proBNP values (ascending vs. descending). RR, relative risk; NT-proBNP, N-terminal pro-brain natriuretic peptide.

**Table 5:** Trend of serial NT-proBNP levels between admission and second follow-up (F2, at 17-65 days after treatment initiation)

Serial NT-proBNP trend (F2)	All (n=18)		Sex and spay/neuter status					
			MI (n=7)		MN (n=5)		FN (n=6)	
	CD	S	CD	S	CD	S	CD	S
Ascending	5	4	3	0	0	1	2	3
Descending	2	7	0	4	2	2	0	1
Fisher's exact test	0.33		0.03		1.00		1.00	
Homogeneity by RR					0.04			

The dogs were stratified according to survival (survivors, S; cardiac-related death, CD), sex (female, F; male, M), spay/neuter status (intact, I; neutered/spayed, N), and trend of serial NT-proBNP values (ascending vs. descending). RR, relative risk; NT-proBNP, N-terminal pro-brain natriuretic peptide.

### Survival outcomes and serial NT-proBNP trends:

Homogeneity of relative risk (RR) was tested to evaluate whether the survival outcome of each group (defined based on sex and spay/neuter status) demonstrated the same homogeneity in relation to the trend of the NT-proBNP concentration. The existence of an interaction between the survival outcome and NT-proBNP trend was concluded if the RRs were not homogenous.

The S and CD groups did not differ regarding the serial NT-proBNP trend between admission and F1. Additionally, survival outcomes were homogenous (RR null hypothesis accepted;  $P=0.38$ ) among all subgroups defined based on sex and spay/neuter status (FN,  $P=1.00$ ; MI,  $P=1.00$ ; MN,  $P=0.40$ ) (Table 4).

When considering the entire study sample, the S and CD groups did not differ regarding the serial NT-proBNP trend between admission and F2. However, the survival outcomes were not homogenous (RR null hypothesis rejected;  $P=0.04$ ) across different serial NT-proBNP trends in the MI subgroup (Table 5), where dogs with ascending trend had a significantly higher risk of cardiac death ( $P=0.03$ ) than noted for those with a descending trend.

A significant difference between the S and CD groups regarding the serial NT-proBNP trend between admission and F2 was noted only among MI dogs ( $P=0.032$ ) (Fig. 1). Kaplan-Meier analysis found that, among MI dogs, the survival time was significantly shorter for ascending than for descending NT-proBNP trend ( $P=0.01$ ) (Fig. 2). All MI dogs with descending NT-proBNP trend survived until the end of the study.

## DISCUSSION

We found that the prognostic value of NT-proBNP trends may be affected by sex and spay/neuter status and that, for MI dogs, long-term monitoring (here, 17-65 days after treatment initiation) of NT-proBNP may be more suitable for prognosis of survival outcomes (versus short-term monitoring at 7-12 days after treatment initiation).

In this study, only the echocardiographic parameters of eccentric hypertrophy (LVIDdn and EDVn) differed significantly between the S and CD groups at admission (Table 1). Such echocardiographic parameters are expected to change gradually with MMVD progression, reflecting prognosis (Chetboul and Tissier, 2012; Hezzell *et al.*, 2012). While no such differences were noted for mean NT-proBNP concentration at admission, the standard deviation at each time point was very large (Fig.

1 and Table 2), which indicates a wide range of biologic variability and suggests that serial measurements in individual animals may provide better prognostic power than that of a single measurement. A recent study in dogs with CHF secondary to MMVD reported a normal variability of up to 53.3% (under 95% confidence interval) for NT-proBNP levels, confirming the clinical relevance of evaluating serial changes (Winter *et al.*, 2017).

Unlike previous studies regarding the prognostic value of NT-proBNP in MMVD dogs (Serres *et al.*, 2009; Hezzell *et al.*, 2012), we found no significant association with survival time on Cox proportional hazard regression, and thus could conduct further survival analysis stratified by sex, spay/neuter status, and NT-proBNP trend without concern of interference from other variables. We unexpectedly found that the trend of NT-proBNP concentrations, either until F1 or until F2, did not provide information regarding the survival outcomes in the pooled sample of 18 dogs (Tables 4 and 5). However, after further categorization based on sex and spay/neuter status, MI dogs demonstrated a strong association of survival outcome with the NT-proBNP trend between admission and F2 (Table 5), as all three dogs with ascending NT-proBNP trend died of cardiac causes within 200 days, whereas all four dogs with descending NT-proBNP trend survived up to 500 days (Fig. 2). In some dogs, the trend changed over time (i.e., from admission to F1 and then to F2; Tables 4 and 5), most likely reflecting the wide biological variability of this biomarker. No such relationships were observed for the spay/neuter groups (MN and FN). Unfortunately, we could not clarify the effect of sex hormones in female dogs, as no intact females were included in this study.

Women generally exhibit higher levels of natriuretic peptides, and sex-specific reference ranges have been established (Loke *et al.*, 2003). Estrogen appears to play a role, as women who receive hormonal therapy have higher levels of natriuretic peptides (Redfield *et al.*, 2002). Furthermore, in humans, estrogen has been suggested to have cardioprotective effect by decreasing renin levels and angiotensin-converting enzyme activity, or by decreasing the production of angiotensin receptors and aldosterone. Estrogen also activates natriuretic peptides and other mechanisms that counterbalance the effects of the renin-angiotensin-aldosterone system (de Bold, 1999). On the other hand, it was recently suggested (Chang *et al.*, 2007) that it is the inhibitory effect of free testosterone that lowers the natriuretic peptide concentration in men, not the stimulatory effect of estrogen that increases the concentration in women. Therefore, both sex hormones may play a role in regulating natriuretic peptides. Furthermore, sex hormone-binding proteins may affect the amount of free hormone molecules in the circulation (Chang *et al.*, 2007). It remains unknown whether the effect of sex hormones on natriuretic peptide levels in dogs is similar to that found in humans.

Sterilization is a unique consideration in veterinary medicine. After gonadectomy, male dogs have dramatically decreased testosterone levels and mildly decreased estrogen levels, whereas female dogs have equivalently low testosterone levels and moderately decreased estrogen levels (De Gier *et al.*, 2012). According to a prospective study in humans (Burger *et al.*,

2000), estrogen decreases during menopause, leading to an increase in free testosterone because of the relatively stable concentration of sex hormone-binding proteins. Thus, it can be considered that the changes in hormone levels observed in post-menopausal women resemble those observed in spayed female dogs. Since postmenopausal women have a higher incidence of heart disease and most women included in CHF studies are postmenopausal, we speculate that the levels of sex hormones may also affect CHF prognosis in dogs.

A study involving 26 symptomatic MMVD dogs, predominantly males (n=17) with unknown spay/neuter status, revealed that NT-proBNP concentrations were significantly lower in 12-month survivors than in non-survivors (Wolf *et al.*, 2012). Dogs with a favorable response to therapy, reflected in lower NT-proBNP levels, are thus expected to have better outcomes. In our study, we considered that dogs with lower NT-proBNP levels at 7–30 days after starting CHF treatment with diuretics and vasodilators were having favorable response to medical intervention, with control of volume overload. However, we found no difference in follow-up NT-proBNP levels between dogs who survived and those who died of cardiac causes. Among MI dogs, survival outcomes were better reflected by the longer-term serum NT-proBNP trends (i.e., between admission and 17-65 days after treatment initiation) than by the shorter-term trends (i.e., between admission and 7-12 days after treatment initiation). These findings suggest that, if the NT-proBNP levels of an MI dog remain above the baseline values later than two weeks after initiating therapy, there is a significantly higher risk of CD.

The major limitation of this study was the small sample size, which was due to the strict inclusion criteria. Another limitation was the lack of intact female dogs in the studied population. In addition, most of the CHF dogs admitted to our department had very acute and life-threatening pulmonary edema. These dogs were administered an aggressive furosemide regimen to relieve the respiratory signs prior to blood collection, and thus could not be included in the present study. Another potential limitation is the lack of standardized heart failure therapy among the dogs included in the study. As pimobendan is not readily available in Taiwan, only five of our dogs received this drug. To minimize the bias associated with differences in therapeutic strategy, all cases were handled by the same cardiologist who was unaware of the biomarker levels at the time the treatment plan was established. Finally, this study was conducted at a referral institution, and thus selection bias could not be excluded. Further studies should employ a larger sample size, include intact female dogs, and conduct multiple serial measurements over a longer follow-up.

**Acknowledgements:** We thank Dr. Rita Chang, who facilitates our collaboration with IDEXX, Taiwan.

**Funding:** This study was financially supported by IDEXX, Taiwan.

**Ethics approval:** This study was approved by the Institutional Animal Care and Use Committee of National Chung Hsing University (approval No. 106001).

**Authors contribution:** This study was conceived and designed by IPC and supervised by WYC. TH executed the project and was responsible for organizing and analyzing data. CCC provided advice regarding the statistical analyses. All authors revised the manuscript for important intellectual content and approved the final version to be published.

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