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**Original Article**

# Cytokine expression in peripheral blood mononuclear cells of dogs with mitral valve disease

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**Abstract**

Inflammation plays an important role in the pathogenesis of congestive heart failure (CHF). In humans with CHF, increased production and high plasma concentrations of tumor necrosis factor-α (TNF-α), interleukin (IL)-6, IL-1, IL-8 and transforming growth factor-β (TGF-β) have been associated with disease progression and a negative prognosis. The aim of this study was to investigate whether differences in cytokine mRNA expression exist between clinically healthy dogs and dogs with myxomatous mitral valve disease (MMVD); to determine if the expression is related to the severity of MMVD, and to detect any correlations with echocardiographic parameters of cardiac remodeling. Twenty-three dogs with MMVD of varying severity and six clinically healthy dogs were included in the study. Whole blood samples were obtained for measurement of mRNA expression of IL-1α, IL-1β, IL-6, IL-8, TGF-β1, TNF-α by reverse transcriptase-PCR (RT-PCR).

There were statistically significant differences between clinically healthy dogs and dogs with MMVD for IL-8 and TGF-β1 gene expression. IL-8 expression increased with increasing MMVD severity and TGF-β1 expression was higher in asymptomatic dogs with echocardiographic signs of cardiac remodeling (American College Veterinary Internal Medicine [ACVIM] class B2) than in all other groups. These results could suggest the involvement of these cytokines in different stages of the disease.

*Keywords:* Cardiology; Congestive heart failure; Cytokines; Dog; PCR

## Introduction

Since the first report in 1990 (Levine et al., 1990), numerous studies have shown that inflammation is involved in the pathogenesis of heart failure in human patients. The production of inflammatory mediators, such as cytokines, in association with the activation of various neurohormonal systems, such as the sympathetic nervous system and the renin-angiotensin-aldosterone system, is known to contribute to progression of chronic heart failure in people (Packer, 1992; Chatterjee 2005).

Cytokines are a group of low-molecular weight proteins responsible for autocrine and paracrine signaling influencing the function of neighbouring cells and involved in endocrine signaling throughout the body. Cytokines are produced by cells of the immune system and by all nucleated cell types in the myocardium, including the cardiac myocytes (Parissis et al., 2002; Bozkurt et al. 2010).

In human medicine, increased expression of cytokines has been demonstrated in patients with congestive heart failure (CHF) patients regardless of aetiology (Testa et al., 1996; Aukrust et al., 1999; Hartupee J. et al. 2013) and has been correlated to increased mortality and disease progression (Anker and Von Haehling, 2004; Demyanets et al., 2011).

Over the past few years, there has been increasing interest in the role of pro-inflammatory cytokines in heart failure in veterinary patients. Several studies have focused on myxomatous mitral valve disease (MMVD) (Aupperle et al., 2008; Disartian and Orton, 2009; Obayashi et al., 2011; Zois et al., 2012), as this is the most common cause of CHF in small

breed dogs (Pedersen and Häggström 2000). MMVD is characterized by non-inflammatory, non infectious valvular degeneration that leads to mitral regurgitation (Pedersen and Häggström, 2000; Aupperle and Disartian, 2012). Although the disease appears to be non-inflammatory, upregulation of numerous cytokines has been demonstrated in the mitral valve and myocardium of dogs with MMVD (Oyama and Chittur, 2006; Paslawska et al., 2006; Kiczak et al., 2008). Later studies have measured peripheral cytokine expression and circulating concentrations in dogs with heart disease. Increased blood mRNA expression of interleukin (IL)- 1β and IL-2 was detected in dogs with CHF while other cytokines showed lower (tumor Necrosis Factor [TNF]-α,Transforming Growth Factor (TGF) – β3) or not significantly different (TGF-β1, TGF-β2, IL-8, IL-4, IL-10) expression in dogs with CHF compared to control dogs (Fonfara et al., 2012). Peripheral concentration of several cytokines (IL-6, TNF-α, IL-10) were non-quantifiable (Mavropoulou et al., 2010; Zois et al., 2012) in dogs with different MMVD stages, while others (IL-2, IL-7 and IL-8) decreased with increased disease severity (Zois et al., 2012). These results differ from those reported in human medicine, where increased cytokine levels (particularly IL-6 and TNF- α) have been consistently reported in patients with heart failure and in patients with chronica mitral regurgitatio (Oral et al., 2003; Oikonomou et al. 201)

TGF-β is a cytokine that has gained attention and was implicated in canine MMVD (Aupperle et al., 2008; Obayashi et al., 2011). It belongs to the family of growth factors and three distinct isoforms (TGF-β1, TGF- β2 and TGF- β39 have been identified in mammals (Akhust et al., 1990). TGF- β has an important role in the regulation of cell growth, differentiation and repair in several tissues (Lim and Zhu, 2006), and there is evidence that it may contribute to valve degeneration (Aupperle et al., 2008; Obayashi et al., 2011). Elevated

levels of TGF-β have been demonstrated in valvular heart diseases in humans (Waltenberger et al., 1993; Kim et al., 2008) and also in canine MMVD (Aupperle et al., 2008; Obayashi et al., 2011).

The aim of this study was: (1) to determine levels of cytokine expression in peripheral blood mononuclear cells (PBMC) of dogs with MMVD at different stages and in healthy dogs; and (2) to investigate potential relationships between cytokine expression and echocardiographic indices of MMVD severity, left ventricular (LV) function and remodelling. For this purpose, IL-1, IL-6, IL-8, TGF-β1 e TNF-α PBMC expression was measured by RT-PCR.

## Materials and methods

*Recruitment and examination of dogs*

Cases were recruited prospectively from client-owned dogs presenting for cardiovascular examination to the cardiology service of the Veterinary Teaching Hospital of the University of Parma from January 2009 to July 2010. Informed consent was obtained from all owners and the study protocol was approved by the University of Parma Institutional Animal Care Committee (Protocol number 22/13, 4 February 2013).

Only dogs 5 years or older and with a bodyweight (BW) between 5 and 20 kg were enrolled. Dogs with echocardiographic evidence of MMVD (mitral valve thickening and/or valve prolapse resulting in mitral regurgitation detected with colour Doppler) were included in the MMVD group. The control group was composed of dogs that were presented to the Veterinary Teaching Hospital of the University of Parma for non-therapeutic

ovariohysterectomy/orchiectomy or for preventative cardiac screening. These dogs were enrolled on the basis of a history of good health and no abnormal findings on physical examination, electrocardiography, thoracic radiography, echocardiography, haematology and biochemistry.

Dogs with evidence of cardiac disease (acquired or congenital) other than MMVD, systemic arterial hypertension (systolic blood pressure >160 mmHg), clinical signs and/or haematological/biochemical abnormalities compatible with organ dysfunction, inflammatory, infectious or neoplastic disease and dogs receiving any medication at presentation were excluded from the study.

For all dogs signalment was recorded and clinical history was taken. A complete physical examination, thoracic radiography (right lateral and dorsoventral views), echocardiography, systolic blood pressure measurement (Doppler method), routine haematology and biochemistry were performed. Dogs with MMVD were divided into groups (Stage B1, B2, C), according to the classification system used by the American College of Veterinary Internal Medicine (ACVIM), Specialty of Cardiology (Atkins et al., 2009). Echocardiography was performed without sedation, with dogs positioned in lateral recumbency, using a Megas CPV (Esaote Biomedica) equipped with electronic, phased array transducers of variable frequency (from 2.5 to 7.5 MHz). Standard transthoracic right and left parasternal and subcostal views were obtained for echocardiographic evaluation and measurements (Thomas et al., 1993). Mitral valve morphology was examined, lesions identified and an estimation of regurgitation was performed using the right parasternal long axis and left parasternal apical 2D views. The left

atrium/aortic root ratio (LA/Ao) was obtained from the right parasternal short axis 2D view as previously described (Boon, 2011). The left ventricular internal dimension in diastole (LVIDd) and in systole (LVIDs) were measured from the M-mode echocardiogram, which was obtained from the right parasternal short axis 2D view. The transmitral diastolic flow and mitral regurgitation Doppler recordings were obtained from the left apical 4-chamber view. For the classification of dogs according to the stage of heart failure and for statistical analysis, the following echocardiographic measurements relating to left ventricular function, cardiac remodelling and mitral valve disease severity were recorded: LA/Ao ratio, transmitral inflow E wave velocity to A wave velocity ratio (E/A ratio) and E wave velocity, the LVIDd (Thomas et al., 1993) and LVIDd normalised (nLVIDd) according to allometric scaling ($VDd/BW^{0.294}$), the LVIDs and the LVIDs normalised (nLVIDs) according to the allometric scaling ($VDs/BW^{0.315}$; Cornell et al., 2004). Hearts with LA/Ao ratio > 1.6 and nLVIDd ≥ 1.73 were considered remodelled (Hansson et al, 2002, Borgarelli et al, 2012). Evidence of distended pulmonary veins and pulmonary infiltrate compatible with cardiogenic oedema on thoracic radiography were considered signs of left sided congestive heart failure.

*Blood sampling and processing*

The expression levels of mRNA for relevant cytokines (IL-1α, IL-1β, IL-6, IL-8, TGF-β1 and TNF-α) were determined in canine PBMC. Blood samples (1.5 mL) were obtained via jugular venepuncture and collected in lithium heparin tubes. Immediately after blood collection, PBMC were isolated by density gradient using Histopaque-1077 (Sigma-Aldrich) according to manufacturer's instructions. After isolation, PBMC samples were washed twice in phosphate

buffered saline (PBS), resuspended in RPMI-1640 complete medium supplemented with 40% heat-inactivated (56 °C for 30 min) foetal calf serum (FCS) and 10% dymethylsolfoxide (DMSO) and immediately frozen at −80 °C using a Mr Frosty (Sigma) device gradient and stored in liquid nitrogen the following day. Further processing was performed within 2 weeks of collection. All PBMC samples were thawed and cell viability was evaluated by trypan blue dye exclusion with a result >96%. At that time, $4 \times 10^6$ cells were processed for total cellular RNA extraction using TRI-reagent (Ambion-Life Technologies) according to the manufacturers' instructions. Purity and concentration were assessed by UV-spectrophotometry at 260/280 and 260 nm respectively (GeneQuant Pro, Amersham Pharmacia Biotech-GE Healthcare Life Sciences). RNA integrity and quality were assessed using an Agilent Bioanalyzer 2100 and RNA 6000 Labchip kit (Agilent Technologies). RNA samples were stored at −80 °C until reverse-transcription (RT) phase commenced.

All RNA samples were DNAse-treated (Sigma) prior to cDNA synthesis. Total RNA (1 μg/20 μL) was reverse-transcripted using a high-capacity cDNA RT kit (Applied Biosystems). RT was performed using a PTC-100 Peltier thermal cycler (MJ Research) StepOne according to the manufacturer's instructions under the following thermal conditions: 5 min at 25 °C, 30 min at 42 °C followed by 5 min at 85 °C. All cDNA samples were stored at −20 °C until PCR processing.

The cDNA obtained from each sample was used as a template for PCR performed using a PTC-100 Peltier thermal cycler (MJ Research) and amplified in duplicate. For PCR amplification, 2 μL of cDNA were used in the reaction buffer containing $MgCl_2$ (2 mM), 1 μL

dNTPs (0.2 mM), 0.5 μL DreamTaq Green DNA polymerase (0.05 U/μL; Fermentas Life Science), 5 μL of both forward and reverse primers (2.5 μM; MWG-Biotech) with a final volume of 50 μL. The primers were designed based on published gene sequences (Rottmann et al, 1996, Hegemann et al, 2003) and purchased from Eurofins MWG Operon. Details of each primer set for detection of cytokine gene expression are reported in Table 1. The reaction was run for 3 min at 94 °C followed by 32 cycles (when the reaction was in the middle of the linear range, before reaching the amplification plateau) and a final elongation step at 72 °C for 10 min. For TNF-α, IL-1α, IL1β and IL-8, each cycle consisted of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 1 min. For TGF-β1 each cycle consisted of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min and extension at 72 °C for 1 min. For IL-6 and GAPDH each cycle consisted of denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min 30 s and extension at 72 °C for 2 min.

PCR products were separated by electrophoresis on 2% agarose gel in Sybr Safe (Invitrogen)**,** and visualised under UV light. The average intensity of each band was determined by densitometric analysis with Scion Image (Scion Capture Driver 1.2 for Image-Pro Plus, Scion) in a grey-scale mode. The density of selected band was calculated after background subtraction and values were presented as the ratio of band intensities of each cytokine RT-PCR product over those of the corresponding housekeeping gene GAPDH RT-PCR product. The cytokine/GAPDH ratio was expressed in relative arbitrary units (RAU).

*Statistical analysis*

195 Descriptive statistics were performed and median and interquartile ranges were
196 calculated for echocardiographic parameters and cytokine expression in the different groups
197 (Table 2). Since the homoscedasticity requirements for the parametric ANOVA were not met,
198 the non-parametric Kruskal–Wallis test was used to verify the null hypothesis that the cytokine
199 expression observed in each group came from a population with the same distribution. Post-hoc
200 comparisons were made using the non-parametric Nemenyi–Damico–Wolfe–Dunn procedure
201 (Hollander and Wolfe, 1999). Linear and semi-parametric regression tests were used to
202 investigate the functional shape of the relationship between cytokine expression and
203 echocardiographic parameters. We were not able to reject linearity in the cytokine-
204 echocardiographic measures relationships. $P < 0.05$ was considered significant. Data analysis
205 and graphics were made using the R software (R Core Team, 2013).
206
207 **Results**
208 Twenty nine dogs met the inclusion criteria (19 males and 10 females). Cases had a median
209 age of 12 years and a median BW of 11.3 kg. Breeds included 24 cross-breeds, two Yorkshire
210 terriers and one of each English Cocker spaniel, Miniature poodle, West Highland White terrier.
211 Twenty-three were affected by MMVD and six were determined as healthy and were included in
212 the control group. Of the 23 dogs with MMVD, six had stage B1, eight had stage B2 and nine
213 had stage C disease. Characteristics of each group are presented in Table 2.
214 Significant differences between groups were detected, as expected, for heart rate (**P** = 0.003),
215 LVIDs (**P** = 0.01), LVIDd (**P** = 0.001), nLVIDs (**P** = 0.009), nLVIDd (**P** <0.0001), LA/Ao ratio
216 (**P** <0.001), E/A ratio (**P** = 0.03) and mitral peak E wave velocity (**P** <0.001). BW was not
217 significantly different between groups.

In dogs with mitral valve disease, there were differences in IL-8 and TGF-β1 mRNA concentrations between groups. For IL-8, the pairwise post-hoc comparisons with the control group were significant for the C group (**P** = 0.0013), showing increased IL-8 expression in dogs with more advanced disease (Fig. 1). IL-8 expression was positively associated with nLVIDd, nLVIDs, E/A ratio and peak E wave velocity in a linear fashion (Fig. 2). By contrast, TGF-β1 expression was higher in dogs from group B2 compared to control dogs (**P** = 0.0056; Fig. 3). No relationship was detected between TGF-β1 expression and the echocardiographic parameters considered.

No statistically significant differences were detected between groups for IL-1α (**P** = 0.45), IL-1β (**P** = 0.83), IL-6 (**P** = 0.82) or TNF-α (**P** = 0.50) PBMC expression. No linear or non-linear relationships were detected between the expression of these cytokines and the echocardiographic parameters.

## Discussion

In our study, increased IL-8 and TGF-β1 were detected in dogs affected by MMVD compared with control dogs, and statistically significant differences were identified between groups. In particular, elevated IL-8 mRNA levels were detected in dogs with more advanced MMVD. IL-8 is a cytokine with pro-inflammatory properties. Produced mainly by monocytes and macrophages in response to inflammation, IL-8 is responsible for leukocyte chemoattraction to inflamed tissues (Baggiolini, Clark-Lewis, 1992, Apostolakis

et al, 2009). Increased IL-8 levels have been reported in human patients with CHF (Gullestad et al., 2001) and there is evidence that IL-8 might be involved in myocardial remodelling and failure observed in these patients (Aukrust et al, 2001, Gullestad et al, 2001). In contrast to our results, previous studies failed to show significant differences in circulating levels of IL-8 or whole blood mRNA concentrations of IL-8 in dogs with CHF and control dogs (Fonfara et al, 2012, Zois et al, 2012). The differences observed between the studies could be caused by the different populations and materials used for the investigations. In particular, one study (Fonfara et al., 2012) included dogs with CHF due to various cardiac diseases, not only MMVD. The differences between circulating concentrations of IL-8 and IL-8 mRNA expression by PBMCs might account for the discrepancies in results between our study and those of Zois et al. (2012) in dogs with MMVD. Furthermore, dogs with CHF included by Zois et al. (2012)received heart failure medication, which could have affected cytokine concentrations, as shown by studies in the human literature (Stenvinkel et al, 1999, Ohtsuka et al, 2001). Regression analysis of cytokine expression and echocardiographic parameters of cardiac remodelling, function and loading conditions in this study demonstrated a positive association between IL-8 and nLVIDd, nLVIDs, E/A ratio and peak E velocity of transmitral flow. This is of interest and might suggest an involvement by IL-8 in cardiac remodelling and systolic and diastolic dysfunction in dogs with MMVD.

Our investigation of TGF-β1 mRNA concentrations showed higher expression in the stage B2 group. Increased levels TGF-β1 are known to result in cardiac fibrosis and remodelling in several heart diseases in human patients (Khan, Sheppard, 2006, Lim, Zhu, 2006). In veterinary medicine, increased expression of TGF-β1 and TGF-β3 isoforms was found in mitral valve

tissue from dogs affected by MMVD (Oyama, Chittur, 2006, Aupperle et al, 2008, Obayashi et al, 2011) and some studies (Aupperle et al, 2008, Aupperle, Disartian, 2012, Orton et al, 2012) hypothesised that TGF-β isoforms were involved in the pathogenesis of the disease. The increased TGF-β1 in dogs from the B2 group in the present study could indicate the involvement of this cytokine in MMVD once cardiac remodelling has developed. The recognised fibrinogenic properties of TGF-β could play an important role in cardiac fibrosis in the course of MMVD (Zeisberg et al, 2007, Aupperle, Disartian, 2012, Orton et al, 2012). In contrast to our results, Fonfara et al. (2012) reported lower TGF concentrations in dogs with CHF compared to controls. This might suggest a difference in TGF production depending on cardiac disease and heart failure classification, as the dogs in that study had several cardiac diseases and were presented in heart failure class C.

The present study did not show significant differences in IL-1α, IL-1β, IL-6 and TNF-α mRNA expression between control dogs and those with MMVD or between the heart failure groups. additionally, no significant, linear or non-linear, relationships with the echocardiographic parameters studied were observed for these cytokines.
Increased levels of TNF-α, IL-6 and IL-1 have been consistently described in human patients with CHF (Bozkurt et al, 2010, Hedayat et al, 2010). Previous veterinary reports documented increased IL-1 expression in whole blood from dogs with CHF (Fonfara et al., 2012) and in myocardial cells of dogs with MMVD (Kiczak et al., 2008). Decreased blood TNF-α mRNA expression in dogs with CHF has been reported, and the same study demonstrated that there was no significant difference in IL-6 blood expression between affected dogs and controls (Fonfara et al., 2012). Other reports measured the peripheral IL-6 and TNF-α plasma

protein concentrations in dogs with different degrees of MMVD (Mavropoulou et al, 2010, Zois et al, 2012) and did not demonstrate significant differences between affected dogs and controls, similar to the results of the present study. In the latter studies, however, a large number of dogs had non-quantifiable IL-6 and TNF-α concentrations, making it difficult to draw any conclusions.

Our study had a number of limitations. The use of the densitometric gel analysis of PCR products (semi-quantitative method) is considered less accurate than the quantitative method. Nevertheless, this technique has been previously validated, has demonstrated acceptable performance, and it is an established method for comparisons of cytokine expression (Santos-Gomes et al, 2002, Chamizo et al, 2005, Engel et al, 2005, Panaro et al, 2009). Furthermore, internal quality controls were performed to verify the correct performance of the test and yielded acceptable results, suggesting good test reliability.

Another limitation of our study is the small number of dogs enrolled. However, knowing that pro-inflammatory cytokine levels increase over the course of several inflammatory and non-inflammatory diseases, strict inclusion criteria were used for case enrolment to reduce the possibility of other factors influencing the results. Inclusion criteria from similar human studies were used and we decided to exclude dogs with MMVD that also had concomitant clinical signs and/or haematological or biochemical abnormalities compatible with other organ dysfunction, inflammatory, immune-mediated, infectious or neoplastic diseases (Yndestad et al, 2003, Carrero et al, 2009, Grivennikov, Karin, 2011). Additionally, the number of dogs included in the study was further limited because dogs receiving medications were excluded, as human

medical reports have demonstrated that cardiac therapy can influence cytokine expression (Stenvinkel et al, 1999, Ohtsuka et al, 2001). The small number of dogs involved in this project could also have affected the conclusions of the study. Therefore, the present study should be seen as pilot data and further investigations are required.

## Conclusions

This study demonstrated increased IL-8 and TGF-β1 PBMC expression in dogs affected with MMVD compared to controls, suggesting a potential role of these cytokines in MMVD. Differential expression among groups with various stages of heart failure suggests a role for these cytokines in distinct phases of the disease. The absence of statistically significant differences between groups for IL-6, TNF-α and IL-1 PBMC expression in dogs with MMVD might indicate a variant cytokine activation pattern compared to human patients. Further studies of larger populations of dogs are needed.

## Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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524 **Table 1**

525 Primer sequences and final product size of IL-1α, IL-1β, IL-6, IL-8, TGF-β, TNF-α and
526 glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hegemann et al., 2003).

| Gene | Sequences | Amplicon |
|---|---|---|
| IL-1a | S: 5’-TTGGAAGACCTGAAGAACTGTTAC-3’ | 545 bp |
| | A: 5’-GTTTTTGAGATTCTTAGAGTCAC -3’ | |
| IL1β | S: 5’-CACAGTTCTCTGGTAGATGAGG -3’ | 262 bp |
| | A: 5’-TGGCTTATGTCCTGTAACTTGC -3’ | |
| IL-6 | S: 5’-CTATGAACTCCCTCTCCACAA -3’ | 711 bp |
| | A: 5’-TGCCCAGTGGACAGGTTTCT -3’ | |
| IL-8 | S: 5’-AGGGATCCTGTGTAAACATGACTTCC -3’ | 330 bp |
| | A: 5’-GGAATTCACGGATCTTGTTTCTC -3’ | |
| TGF-β | A: 5’-TTCCTGCTCCTCATGGCCAC -3’ | 393bp |
| | A: 5’-GCAGGAGCGCACGATCATGT -3’ | |
| TNF-a | S: 5’-CTCTTCTGCCTGCTGCAC -3’ | 288 bp |
| | A: 5’-GCCCTTGAAGAGGACCTG -3’ | |
| GAPDH | S: 5’-CCTTCATTGACCTCAACTACAT -3’ | 400 bp |
| | A: 5’-CCAAAGTTGTCATGGATGACC-3’ | |

**Table 2**

Descriptive parameters, echocardiographic measurements and cytokine peripheral blood mononuclear cell expression in dogs with myxomatous mitral valve disease (MMVD) and clinically healthy dogs.

| | All (Range; IQR) | Control (Range; IQR) | B1 stage (Range; IQR) | B2 stage (Range; IQR) | C stage (Range; IQR) |
|---|---|---|---|---|---|
| *n* | 29 | 6 | 6 | 8 | 9 |
| Sex (male:female; *P*=0.405[1]) | 19:10 | 3:3 | 3:3 | 7:1 | 6:3 |
| Age (years; *P*=0.89[2]) | 11 (7-17; 9-12) | 11 (7-14; 10-12.7) | 11 (8-14) [9.2-12) | 11 (8-12; 9-12) | 11 (9-17; 10-12) |
| Bodyweight (kg; *P*=0.41[2]) | 11.3 (5.4-18.4; 8.3-14.2) | 9 (6.5-12.5; 8.1-10.7) | 11.6 (7.2-14.2; 9-12.8) | 13.8 (5.4-18.7; 6.8-17.6) | 13(5.4-17; 10.8-15.2] |
| Heart rate (beats/min; *P*=0.0026[2]) | 120 (90-210; 105-150) | 97 (90-120; 90-116.2) | 112 (90-150; 105-120) | 120 (90-150; 90-127.5) | 170 (120-210; 150-190)[3] |
| LVIDd (cm; *P* <0.001[2]) | 3.85 (2.34-5.71; 3.42-4.68) | 2.79 (2.34-3.60; 2.48-3.37) | 3.62 (2.37-3.95; 3.22-3.79) | 4.44 (3.4-5.16; 3.74-4.79)[3] | 4.68 (3.67-5.71; 4.44-5.12)[3] |

| | | | | | |
|---|---|---|---|---|---|
| LVIDs (cm; *P*=0.01) | 2.30 (1.16-3.40; 1.87-2.53) | 1.82 (1.30-2.30; 1.63-1.88) | 2.19 (1.16-2.34; 1.65-2.33) | 2.42 (1.73-3.15; 1.92-2.64) [3] | 2.53 (1.87-3.40; 2.32-2.80)[3] |
| nLVIDd (*P* < 0.001[2]) | 1.94 (1.20-2.68; 1.69-2.21) | 1.56 (1.20-1.71; 1.34-1.67) | 1.75 (1.32-1.84; 1.67-1.80) | 2.12 (1.88-2.25; 2.0-2.20) | 2.30 (1.82-2.68; 2.06-2.37)[3] |
| nLVIDs (*P*=0.009[2]) | 1.04 (0.62-1.51; 0.93-1.10) | 0.90 (0.72-1.04; 0.79-0.96) | 0.96 (0.62-1.1; 0.81-1.05) | 1.07 (0.98-1.28; 1.02-1.11) | 1.19 (0.88-1.51; 1.04-1.33)[3] |
| LA/Ao ratio (*P* < 0.001[2]) | 1.47 (1.07-2.83; 1.33-1.93) | 1.26 (1.08-1.54; 1.15-1.29) | 1.34 (1.07-1.45; 1.31-1.36) | 1.47 (1.33-2.20; 1.37-1.77) | 2.09 (1.72-2.83; 1.93-2.37)[3] |
| Vmax E wave (m/s; *P* < 0.001 [2]) | 0.85 (0.42-1.86; 0.70-1.22) | 0.62 (0.52-0.85; 0.58-0.72) | 0.75 (0.42-0.86; 0.41-0.80) | 0.91 (0.63-1.33; 0.76-1.06) | 1.36 (1.11-1.86; 1.18-1.61)[3] |
| Vmax A wave (m/s; P= 0.25 [2]) | 0.7 (0.41-1.22; 0.65-0.87) | 0.67 (0.4-0.7; 0.64-0.70) | 0.74 (0.51-0.88; 0.60-0.84) | 0.74 (0.53-1.04; 0.64-0.96) | 0.72 (0.65-1.22; 0.68-1.15) |
| Mitral E/A ratio (*P*=0.03[2]) | 1.12 (0.74-2.24; 0.94-1.45) | 0.93 (0.74-2.05; 0.89-1.03) | 0.99 (0.82-1.44; 0.95-1.04) | 1.15 (0.86-2.10; 0.96-1.33) | 1.57 (1.19-2.24; 1.38-1.78)[3] |
| IL-1α/GAPDH (RAU; *P*=0.45 [2]) | 0.13 (0.0-2.23; 0.0-0.47) | 0.07 (0.0-0.47; 0.01-0.38) | 0.10 (0.0-0.55; 0.0-0.40) | 0.39 (0.0-1.04; 0.14-0.56) | 0.05 (0.0-2.23; 0.0-0.41) |

| | | | | | |
|---|---|---|---|---|---|
| IL-1β/GAPDH (RAU; *P*=0.83 [2]) | 0.74 (0.0-1.46; 0.24-0.95) | 0.57 (0.0-1.11; 0.14-0.77) | 0.76 (0.06-1.15; 0.58-0.91) | 0.80 (0.22-1.05; 0.41-0.92) | 0.62 (0.0-1.46; 0.24-0.99) |
| IL-6/GAPDH (RAU; *P* =0.82 [2]) | 0.0 (0.0-1.04; 0.0-0.05) | 0.02 (0.0-0.18; 0.0-0.05) | 0.0 (0.0-16; 0.0-0.03) | 0.0 (0.0-0.16; 0.0-0.02) | 0.0 (0.0-1.04; 0.0-0.0) |
| IL-8/GAPDH (RAU; *P*=0.005 [2]) | 1.43 (0.25-2.68; 1.13-2.0) | 1.03 (0.26-1.23; 0.78-1.04) | 1.47 (0.70-1.63; 1.24-1.55) | 1.64 (1.18-2.26; 1.28-1.97) | 2.18 (1.08-2.68; 1.36-2.27)[3] |
| TGF-β1/GAPDH (RAU; *P*=0.01 [2]) | 0.56 (0.00-1.35; 0.18-0.61) | 0.22(0.0-0.78) [0.04-0.45] | 0.38(0.0-1.14; 0.21-0.80) | 1.03(0.39-1.35; 0.86-1.14)[3] | 0.56 (0.0-1.01; 0.18-0.61) |
| TNF-α/GAPDH (RAU; *P*=0.50 [2]) | 0.59 (0.0-1.82; 0.45-0.77) | 0.56 (0.48-0.77; 0.50-0.62) | 0.73 (0.0-1.01; 0.62-0.96) | 0.61 (0.20-1.32; 0.41-1.14) | 0.45 (0.0-1.82; 0.30-0.62) |

[1] Fisher's exact test

[2] Kruskal Wallis test Data are shown as median (range; interquartile range).

[3] Significantly different from control group ($P < 0.05$; Nemenyi-Damico-Wolfe-Dunn procedure).

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IQR, interquartile range; LVIDs, Left ventricular internal dimension in systole; LVIDd, Left ventricular internal dimension in diastole; nLVIDs, Left ventricular internal dimension in systole normalized according to allometric scaling; nLVIDd, Left ventricular internal dimension in diastole normalized according to allometric scaling; LA/Ao, Left

atrial to aortic root ratio; Mitral E/A ratio: Mitral inflow E wave velocity to A wave velocity ratio; RAU, relative arbitrary units; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β

## Figure legends

Fig. 1. Interleukin (IL)-8 expression in dogs categorized accordingly the American College Veterinary Internal Medicine (ACVIM) stage. The difference in IL-8/glyceraldehyde-3-phosphate dehydrogenase (GAPDH) distribution across the ACVIM stages are significant at the K-W test ($P = 0.0048$).

Fig. 3. Transforming growth factor-β (TGF-β) expression in dogs categorized according to American College Veterinary Internal Medicine (ACVIM) stage. The difference in TGF-β/ glyceraldehyde-3-phosphate dehydrogenase (GAPDH; relative arbitrary units [RAU]) distribution across the ACVIM stages are significant using the Kruskal-Wallis test ($P = 0.012$).

Fig. 2. Scatterplot of interleukin (IL)-8 and nLVIDs (left ventricular internal dimension in systole normalized according to allometric scaling); nLVIDd (left ventricular internal dimension in diastole normalized according to allometric scaling), E/A ratio (Mitral inflow E wave velocity to A wave velocity ratio), E wave maximum velocity (Vmax). RAU, relative arbitrary units.