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1 **Original Article**

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

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18 **Abstract**

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

30

31 There were statistically significant differences between clinically healthy dogs and dogs
32 with MMVD for IL-8 and TGF- β 1 gene expression. IL-8 expression increased with increasing
33 MMVD severity and TGF- β 1 expression was higher in asymptomatic dogs with
34 echocardiographic signs of cardiac remodeling (American College Veterinary Internal Medicine
35 [ACVIM] class B2) than in all other groups. These results could suggest the involvement of
36 these cytokines in different stages of the disease.

37

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

39 **Introduction**

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

46

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

52

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

57

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

62 breed dogs (Pedersen and Häggström 2000). MMVD is characterized by non-inflammatory,
63 non infectious valvular degeneration that leads to mitral regurgitation (Pedersen and Häggström,
64 2000; Aupperle and Disartian, 2012). Although the disease appears to be non-inflammatory,
65 upregulation of numerous cytokines has been demonstrated in the mitral valve and myocardium
66 of dogs with MMVD (Oyama and Chittur, 2006; Paslawska et al., 2006; Kiczak et al., 2008).
67 Later studies have measured peripheral cytokine expression and circulating concentrations in
68 dogs with heart disease. Increased blood mRNA expression of interleukin (IL)- 1 β and IL-2
69 was detected in dogs with CHF while other cytokines showed lower (tumor Necrosis Factor
70 [TNF]- α , Transforming Growth Factor (TGF) – β 3) or not significantly different (TGF- β 1, TGF-
71 β 2, IL-8, IL-4, IL-10) expression in dogs with CHF compared to control dogs (Fonfara et al.,
72 2012). Peripheral concentration of several cytokines (IL-6, TNF- α , IL-10) were non-quantifiable
73 (Mavropoulou et al., 2010; Zois et al., 2012) in dogs with different MMVD stages, while others
74 (IL-2, IL-7 and IL-8) decreased with increased disease severity (Zois et al., 2012). These results
75 differ from those reported in human medicine, where increased cytokine levels (particularly IL-
76 6 and TNF- α) have been consistently reported in patients with heart failure and in patients with
77 chronica mitral regurgitatio (Oral et al., 2003; Oikonomou et al. 201)

78

79 TGF- β is a cytokine that has gained attention and was implicated in canine MMVD
80 (Aupperle et al., 2008; Obayashi et al., 2011). It belongs to the family of growth factors and
81 three distinct isoforms (TGF- β 1, TGF- β 2 and TGF- β 3) have been identified in mammals
82 (Akhurst et al., 1990). TGF- β has an important role in the regulation of cell growth,
83 differentiation and repair in several tissues (Lim and Zhu, 2006), and there is evidence that it
84 may contribute to valve degeneration (Aupperle et al., 2008; Obayashi et al., 2011). Elevated

85 levels of TGF- β have been demonstrated in valvular heart diseases in humans (Waltenberger et
86 al., 1993; Kim et al., 2008) and also in canine MMVD (Aupperle et al., 2008; Obayashi et al.,
87 2011).

88

89 The aim of this study was: (1) to determine levels of cytokine expression in peripheral
90 blood mononuclear cells (PBMC) of dogs with MMVD at different stages and in healthy dogs;
91 and (2) to investigate potential relationships between cytokine expression and echocardiographic
92 indices of MMVD severity, left ventricular (LV) function and remodelling. For this purpose, IL-
93 1, IL-6, IL-8, TGF- β 1 e TNF- α PBMC expression was measured by RT-PCR.

94

95 **Materials and methods**

96 *Recruitment and examination of dogs*

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

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

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

111

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

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

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

144

145 *Blood sampling and processing*

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

151 buffered saline (PBS), resuspended in RPMI-1640 complete medium supplemented with 40%
152 heat-inactivated (56 °C for 30 min) foetal calf serum (FCS) and 10% dymethylsulfoxide
153 (DMSO) and immediately frozen at –80 °C using a Mr Frosty (Sigma) device gradient and
154 stored in liquid nitrogen the following day. Further processing was performed within 2 weeks of
155 collection. All PBMC samples were thawed and cell viability was evaluated by trypan blue dye
156 exclusion with a result >96%. At that time, 4×10^6 cells were processed for total cellular RNA
157 extraction using TRI-reagent (Ambion-Life Technologies) according to the manufacturers'
158 instructions. Purity and concentration were assessed by UV-spectrophotometry at 260/280 and
159 260 nm respectively (GeneQuant Pro, Amersham Pharmacia Biotech-GE Healthcare Life
160 Sciences). RNA integrity and quality were assessed using an Agilent Bioanalyzer 2100 and
161 RNA 6000 Labchip kit (Agilent Technologies). RNA samples were stored at –80 °C
162 until reverse-transcription (RT) phase commenced.

163

164 All RNA samples were DNase-treated (Sigma) prior to cDNA synthesis. Total RNA
165 (1 µg/20 µL) was reverse-transcribed using a high-capacity cDNA RT kit (Applied
166 Biosystems). RT was performed using a PTC-100 Peltier thermal cycler (MJ Research) StepOne
167 according to the manufacturer's instructions under the following thermal conditions: 5 min at
168 25 °C, 30 min at 42 °C followed by 5 min at 85 °C. All cDNA samples were stored at –20 °C
169 until PCR processing.

170 The cDNA obtained from each sample was used as a template for PCR performed using a
171 PTC-100 Peltier thermal cycler (MJ Research) and amplified in duplicate. For PCR
172 amplification, 2 µL of cDNA were used in the reaction buffer containing MgCl₂ (2 mM), 1 µL

173 dNTPs (0.2 mM), 0.5 μ L DreamTaq Green DNA polymerase (0.05 U/ μ L; Fermentas Life
174 Science), 5 μ L of both forward and reverse primers (2.5 μ M; MWG-Biotech) with a final
175 volume of 50 μ L. The primers were designed based on published gene sequences (Rottmann et
176 al, 1996, Hegemann et al, 2003) and purchased from Eurofins MWG Operon. Details of each
177 primer set for detection of cytokine gene expression are reported in Table 1. The reaction was
178 run for 3 min at 94 °C followed by 32 cycles (when the reaction was in the middle of the linear
179 range, before reaching the amplification plateau) and a final elongation step at 72 °C for 10 min.
180 For TNF- α , IL-1 α , IL1 β and IL-8, each cycle consisted of denaturation at 94 °C for 1 min,
181 annealing at 50 °C for 1 min and extension at 72 °C for 1 min. For TGF- β 1 each cycle consisted
182 of denaturation at 94 °C for 1 min, annealing at 60 °C for 2 min and extension at 72 °C for
183 1 min. For IL-6 and GAPDH each cycle consisted of denaturation at 94 °C for 1 min, annealing
184 at 50 °C for 1 min 30 s and extension at 72 °C for 2 min.

185

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

193

194 *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.

218

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

227

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

232

233 **Discussion**

234

235 In our study, increased IL-8 and TGF- β 1 were detected in dogs affected by MMVD
236 compared with control dogs, and statistically significant differences were identified between
237 groups. In particular, elevated IL-8 mRNA levels were detected in dogs with more advanced
238 MMVD. IL-8 is a cytokine with pro-inflammatory properties. Produced mainly
239 by monocytes and macrophages in response to inflammation, IL-8 is responsible
240 for leukocyte chemoattraction to inflamed tissues (Baggiolini, Clark-Lewis, 1992, Apostolakis

241 et al, 2009). Increased IL-8 levels have been reported in human patients with CHF (Gullestad
242 et al., 2001) and there is evidence that IL-8 might be involved in myocardial remodelling and
243 failure observed in these patients (Aukrust et al, 2001, Gullestad et al, 2001). In contrast to our
244 results, previous studies failed to show significant differences in circulating levels of IL-8 or
245 whole blood mRNA concentrations of IL-8 in dogs with CHF and control dogs (Fonfara et al,
246 2012, Zois et al, 2012). The differences observed between the studies could be caused by the
247 different populations and materials used for the investigations. In particular, one study (Fonfara
248 et al., 2012) included dogs with CHF due to various cardiac diseases, not only MMVD. The
249 differences between circulating concentrations of IL-8 and IL-8 mRNA expression by PBMCs
250 might account for the discrepancies in results between our study and those of Zois et al.
251 (2012) in dogs with MMVD. Furthermore, dogs with CHF included by Zois et al.
252 (2012) received heart failure medication, which could have affected cytokine concentrations, as
253 shown by studies in the human literature (Stenvinkel et al, 1999, Ohtsuka et al, 2001).
254 Regression analysis of cytokine expression and echocardiographic parameters of cardiac
255 remodelling, function and loading conditions in this study demonstrated a positive association
256 between IL-8 and nLVIDd, nLVIDs, E/A ratio and peak E velocity of transmitral flow. This is
257 of interest and might suggest an involvement by IL-8 in cardiac remodelling and systolic and
258 diastolic dysfunction in dogs with MMVD.

259

260 Our investigation of TGF- β 1 mRNA concentrations showed higher expression in the stage
261 B2 group. Increased levels TGF- β 1 are known to result in cardiac fibrosis and remodelling in
262 several heart diseases in human patients (Khan, Sheppard, 2006, Lim, Zhu, 2006). In veterinary
263 medicine, increased expression of TGF- β 1 and TGF- β 3 isoforms was found in mitral valve

264 tissue from dogs affected by MMVD (Oyama, Chittur, 2006, Aupperle et al, 2008, Obayashi et
265 al, 2011) and some studies (Aupperle et al, 2008, Aupperle, Disartian, 2012, Orton et al, 2012)
266 hypothesised that TGF- β isoforms were involved in the pathogenesis of the disease. The
267 increased TGF- β 1 in dogs from the B2 group in the present study could indicate the
268 involvement of this cytokine in MMVD once cardiac remodelling has developed. The
269 recognised fibrinogenic properties of TGF- β could play an important role in cardiac fibrosis in
270 the course of MMVD (Zeisberg et al, 2007, Aupperle, Disartian, 2012, Orton et al, 2012). In
271 contrast to our results, Fonfara et al. (2012) reported lower TGF concentrations in dogs with
272 CHF compared to controls. This might suggest a difference in TGF production depending on
273 cardiac disease and heart failure classification, as the dogs in that study had several cardiac
274 diseases and were presented in heart failure class C.

275

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

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

292

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

300

301 Another limitation of our study is the small number of dogs enrolled. However, knowing
302 that pro-inflammatory cytokine levels increase over the course of several inflammatory and non-
303 inflammatory diseases, strict inclusion criteria were used for case enrolment to reduce the
304 possibility of other factors influencing the results. Inclusion criteria from similar human studies
305 were used and we decided to exclude dogs with MMVD that also had concomitant clinical signs
306 and/or haematological or biochemical abnormalities compatible with other organ dysfunction,
307 inflammatory, immune-mediated, infectious or neoplastic diseases (Yndestad et al,
308 2003, Carrero et al, 2009, Grivennikov, Karin, 2011). Additionally, the number of dogs included
309 in the study was further limited because dogs receiving medications were excluded, as human

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

314

315 **Conclusions**

316

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

324

325 **Conflict of interest statement**

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

328

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332

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524 **Table 1**

525 Primer sequences and final product size of IL-1 α , IL-1 β , 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' A: 5'-GTTTTTGAGATTCTTAGAGTCAC -3'	545 bp
IL1 β	S: 5'-CACAGTTCTCTGGTAGATGAGG -3' A: 5'-TGGCTTATGTCCTGTAACCTGC -3'	262 bp
IL-6	S: 5'-CTATGAACTCCCTCTCCACAA -3' A: 5'-TGCCCAGTGGACAGGTTTCT -3'	711 bp
IL-8	S: 5'-AGGGATCCTGTGTAAACATGACTTCC -3' A: 5'-GGAATTCACGGATCTTGTTTCTC -3'	330 bp
TGF- β	A: 5'-TTCCTGCTCCTCATGGCCAC -3' A: 5'-GCAGGAGCGCACGATCATGT -3'	393bp
TNF-a	S: 5'-CTCTTCTGCCTGCTGCAC -3' A: 5'-GCCCTTGAAGAGGACCTG -3'	288 bp
GAPDH	S: 5'-CCTTCATTGACCTCAACTACAT -3' A: 5'-CCAAAGTTGTCATGGATGACC-3'	400 bp

527 **Table 2**

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

	All	Control	B1 stage	B2 stage	C stage
	(Range; IQR)	(Range; IQR)	(Range; IQR)	(Range; IQR)	(Range; IQR)
<i>n</i>	29	6	6	8	9
Sex (male:female; <i>P</i> =0.405 ¹)	19:10	3:3	3:3	7:1	6:3
Age (years; <i>P</i> =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; <i>P</i> =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; <i>P</i> =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; <i>P</i> <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-1 α /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-1 β /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- β 1/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] ³	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)

530 ¹ Fisher's exact test

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

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

533 GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IQR, interquartile range; LVIDs, Left ventricular internal dimension in systole;

534 LVIDd, Left ventricular internal dimension in diastole; nLVIDs, Left ventricular internal dimension in systole normalized according

535 to allometric scaling; nLVIDd, Left ventricular internal dimension in diastole normalized according to allometric scaling; LA/Ao, Left

536 atrial to aortic root ratio; Mitral E/A ratio: Mitral inflow E wave velocity to A wave velocity ratio; RAU, relative arbitrary units;

537 TNF- α , tumor necrosis factor- α ; TGF- β , transforming growth factor- β

538

539 **Figure legends**

540

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

545

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

550

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

555