

# Inflammation and its association with oxidative stress in dogs with heart failure

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## Research Article

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# Abstract

## Background

Inflammation and oxidative stress can contribute to the development and progression of heart failure. This study aimed to investigate the association between inflammatory and oxidative stress markers in dogs with congestive heart failure (CHF). Associations between the disease severity marker N-terminal pro-B-type natriuretic peptide (NT-proBNP) and markers of inflammation and oxidative stress were also determined.

## Results

Thirty-seven dogs with cardiovascular diseases (dilated cardiomyopathy (16 dogs), myxomatous mitral valve disease (21 dogs)) and ten healthy dogs were included in this prospective study. The patients were further divided into groups with (26) and without CHF (11). We found a significantly higher serum concentration of C-reactive protein ( $P = 0.012$ ), white blood cell ( $P = 0.001$ ), neutrophil ( $P = 0.001$ ) and monocyte counts ( $P = 0.001$ ) in patients with CHF compared to control dogs. The concentration of tumor necrosis factor-alpha (TNF- $\alpha$ ) was significantly higher in patients with CHF compared to patients without CHF ( $P = 0.030$ ). In patients with CHF, TNF- $\alpha$  correlated positively with malondialdehyde ( $P = 0.014$ ,  $r = 0.474$ ) and negatively with glutathione peroxidase (GPX) ( $P = 0.026$ ,  $r = -0.453$ ), and interleukin-6 correlated negatively with GPX ( $P = 0.046$ ,  $r = -0.412$ ). NT-proBNP correlated positively with malondialdehyde ( $P = 0.011$ ,  $r = 0.493$ ). In patients without CHF none of the inflammatory and oxidative stress markers correlated significantly. Furthermore, in the group of all cardiac patients, GPX activity significantly negatively correlated with NT-proBNP ( $P = 0.050$ ,  $r = -0.339$ ) and several markers of inflammation, including TNF- $\alpha$  ( $P = 0.010$ ,  $r = -0.436$ ), interleukin-6 ( $P = 0.026$ ,  $r = -0.382$ ), white blood cell ( $P = 0.032$ ,  $r = -0.369$ ), neutrophil ( $P = 0.027$ ,  $r = -0.379$ ) and monocyte counts ( $P = 0.024$ ,  $r = -0.386$ ).

## Conclusion

Inflammatory and oxidative stress markers are linked in canine CHF patients, but not in patients without CHF. These results suggest complex cross communication between the two biological pathways in advanced stages of CHF.

## Background

Increasing evidence suggests that processes of inflammation are implicated in the pathophysiology and progression of congestive heart failure (CHF) [1, 2]. Inflammation can cause myocardial dysfunction, which leads to endothelial dysfunction and cardiac cachexia. Inflammatory markers are released from the cells of failing myocardium and endothelial cells, blood leukocytes, and platelets, as well as from the liver and lungs [1, 2, 3]. Markers of inflammation, such as C-reactive protein (CRP), cytokines, and their

corresponding soluble receptors, white blood cell count (WBC) and markers of an activated immune system, have been reported to be elevated in human heart failure patients [4, 5, 6, 7]. In canine patients with CHF, WBC, and concentrations of CRP and monocyte chemoattractant protein-1 concentrations have been found to be significantly higher in comparison to control dogs [8, 9, 10, 11, 12, 13, 14, 15]. Besides, interleukin (IL)-2, IL-7, and IL-8 decreased with increasing severity indices of myxomatous mitral valve disease (MMVD) [14]. On the other hand, Rubio and colleagues found no significant differences in a number of cytokines (IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, interferon gamma-induced protein, monocyte chemoattractant protein-1, granulocyte macrophage-colony stimulating factor, tumour necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon gamma- $\gamma$ ) between dogs with different stages of heart failure due to MMVD or dilated cardiomyopathy (DCM) [15].

Inflammation and oxidative stress play an important role in various features of cardiovascular disease, involving endothelial dysfunction, lipid disorders, and myocardial injury [1, 2, 16]. Clinical and experimental studies have provided considerable evidence that oxidative stress, defined as a disturbance in the balance between reactive oxygen species (ROS) and antioxidants, is increased in heart failure and consequently contributes to cardiac remodelling and heart failure [1, 17]. Increased production of ROS and thus increased oxidative stress is implicated in the development of a variety of cardiovascular diseases in human and animal patients [1, 17, 18, 19, 20, 21, 22].

Congestive heart failure, regardless of etiology, is linked with inflammation and enhanced oxidative stress, whereas short-term inotropic support results in the reduction of inflammatory and oxidative stress markers [23]. Biomarkers of oxidative stress and inflammation are promising diagnostic and prognostic markers in heart failure patients [15, 20]. Despite the fact that oxidative stress and inflammation are involved in the progression of heart failure [1, 24] there is still a lack of data on the association between inflammatory and oxidative stress markers in human and canine cardiovascular patients.

Our hypothesis was that inflammatory and oxidative stress markers are associated in canine patients with CHF resulting from MMVD or DCM. To test the hypothesis, we measured selected inflammatory and oxidative stress markers. We correlated them with each other, as well as with the disease severity marker N-terminal pro-B-type natriuretic peptide (NT-proBNP).

## Results

### Dogs

Forty-seven privately owned dogs were included in the study: 37 of them were cardiovascular patients, and ten of them were control dogs. Cardiovascular patients were further divided into two groups; patients with (26) and without (11) CHF. The progression algorithm of dogs during the inclusion into the study is shown in Figure 1. The baseline demographic characteristics of cardiovascular patients with and without CHF and control dogs are presented in Table 1. Control dogs and cardiac patients with and without CHF did not differ significantly in weight; however, patients with and without CHF were significantly ( $P < 0.001$ ) older compared to control dogs.

**Table 1.** Baseline demographic characteristics of canine cardiac patients and control dogs

	Control	All patients	No CHF	CHF
Number	10	37	11	26
Sex Male/Female	3/7	31/6	9/2	22/4
Age (years)	4.4 ± 2.5*	8.8 ± 2.8	9.3 ± 3.6	8.6 ± 2.5
Mean ± SD	1.0–12.5	2.4–14.3	2.4–14.3	4.2–12.5
Min–Max				
Weight Median (IQR)	22.4 (19.2–34.3)	27.8 (13.2–41.9)	31.4 (13.6–61.5)	27.2 (12.8–39.3)
Disease DCM/MMVD	/	16/21	4/7	12/14

\*significant difference when compared to the groups of canine patients with CHF ( $p < 0.001$ ) and without CHF ( $p < 0.001$ ), and the group of all cardiac patients ( $p < 0.001$ )

### Inflammatory and markers of oxidative stress and NT-proBNP concentrations

Concentrations of IL-6 were excluded from the statistical comparison because they were below the lower level of detection.

The results of inflammatory markers and NT-proBNP are presented in Table 2. NT-proBNP concentration was significantly higher in patients with CHF than in patients without CHF ( $P = 0.009$ ) and control dogs ( $P < 0.001$ ); however, NT-proBNP concentration between patients without CHF and control dogs did not differ significantly.

**Table 2** Inflammatory markers (Median, IQR) and NT-proBNP concentrations (median, IQR) in patients and control dogs

	Control (n = 10)	No CHF (n = 11)	CHF (n = 26)
TNF- $\alpha$ (pg/mL)	3.90; 3.90–10.50	3.90; 3.90–3.90	3.90; 6.85–11.90 <sup>a</sup>
IL-6 (pg/mL)	31.3; 31.3–31.3	31.3; 31.3–31.3	31.3; 31.3–31.3
CRP (mg/L)	0.90; 0.78–1.33	1.45; 0.57–7.74	3.53; 1.10–13.18 <sup>b</sup>
WBC ( $\times 10^9/L$ )	6.2; 5.2–8.2	7.7; 6.3–8.9	10.5; 8.1–13.6 <sup>c</sup>
NEUT ( $\times 10^9/L$ )	3.4; 2.8–5.2	5.2; 3.2–6.8	7.2; 5.7–10.3 <sup>b</sup>
Neutrophils (%)	57.3; 51.2–60.7 <sup>d</sup>	66.3; 63.2–75.9	69.5; 61.3–73.5
LYMPH ( $\times 10^9/L$ )	1.83; 1.47–2.35	1.28; 1.18–2.26	1.96; 1.57–2.89 <sup>a</sup>
Lymphocytes (%)	30.7; 24.8–35.0 <sup>d</sup>	17.4; 14.2–26.0	20.0; 14.7–24.6
MONO ( $\times 10^9/L$ )	0.28; 0.21–0.39	0.42; 0.28–0.55	0.60; 0.44–0.84 <sup>c</sup>
Monocytes (%)	4.2; 3.6–6.4	5.5; 4.5–6.2	5.7; 4.3–7.7
NT-proBNP (pmol/L)	822; 507–1201	1207; 927–3086	4773; 2828–8529 <sup>c</sup>

<sup>a</sup>Significant difference ( $P < 0.05$ ) when compared to patients without CHF; <sup>b</sup>Significant difference ( $P < 0.05$ ) when compared to control dogs; <sup>c</sup>Significant difference ( $P < 0.05$ ) when compared to control dogs and patients without CHF; <sup>d</sup>Significant difference ( $P < 0.05$ ) when compared to patients without and with CHF

Serum CRP concentration was significantly higher in patients with CHF than in control dogs ( $P = 0.012$ ). The concentration of TNF- $\alpha$  was significantly ( $P = 0.030$ ) higher in patients with CHF compared to patients without CHF, while no significant difference was found in comparison to control dogs.

White blood cell and monocyte counts were significantly higher in patients with CHF compared to control dogs ( $P = 0.001$ ; and  $P = 0.001$ , respectively) and patients without CHF ( $P = 0.024$  and  $P = 0.049$ , respectively). Neutrophil counts were significantly ( $P = 0.001$ ) higher in patients with CHF than in control dogs. We found significantly ( $P = 0.020$ ) higher lymphocyte counts in patients with CHF than in patients without CHF. Furthermore, we found significantly higher neutrophil percentages and significantly lower lymphocyte percentages in patients without ( $P = 0.002$  and  $P = 0.010$ , respectively) and with CHF ( $P = 0.003$  and  $P = 0.006$ , respectively) in comparison to control dogs.

Regarding oxidative stress parameters (Table 3), we found no significant difference in malondialdehyde (MDA) concentration between groups of patients and control dogs. On the other side, glutathione

peroxidase (GPX) activity was significantly ( $P = 0.042$ ) higher in patients without CHF than in control dogs.

**Table 3** Oxidative stress markers (mean  $\pm$  SD) in patients and control dogs

	Control n = 10	No CHF n = 11	CHF n = 26
MDA ( $\mu\text{mol/L}$ )	1.34 $\pm$ 0.31	1.77 $\pm$ 0.75	1.32 $\pm$ 0.72
GPX (U/g Hgb)	393.7 $\pm$ 43.6	457.6 $\pm$ 43.7*	429.2 $\pm$ 65.6

\* Significant difference ( $P < 0.05$ ) when compared to control dogs

### Correlations

In CHF patients, tumor necrosis alpha (TNF- $\alpha$ ) concentrations correlated significantly positively with MDA concentrations ( $P = 0.014$ ,  $r = 0.474$ ) and negatively with GPX activities ( $P = 0.026$ ,  $r = -0.453$ ). Furthermore, we found a significant negative correlation ( $P = 0.046$ ,  $r = -0.412$ ) between IL-6 concentrations and GPX activities. Percentages of neutrophils and monocyte counts correlated significantly negatively with GPX activities ( $P = 0.024$ ,  $r = -0.460$  and  $P = 0.031$ ,  $r = -0.441$ , respectively). On the other hand, percentage of lymphocytes correlated significantly positively with GPX activities ( $P = 0.006$ ,  $r = 0.542$ ), and lymphocyte counts correlated significantly negatively with MDA concentrations ( $P = 0.050$ ,  $r = -0.388$ ). In this group of patients, we found a significant positive correlation between NT-proBNP and MDA concentrations ( $P = 0.011$ ,  $r = 0.4893$ ).

In patients without CHF, none of the inflammatory and oxidative stress markers correlated significantly.

Additionally, we correlated inflammatory and oxidative stress markers and NT-proBNP in the group of all 37 patients and found several significant correlations. Interestingly, GPX activities correlated significantly negatively with several inflammatory markers, including TNF- $\alpha$  concentrations ( $P = 0.010$ ,  $r = -0.436$ ), IL-6 concentrations ( $P = 0.026$ ,  $r = -0.382$ ), WBC ( $P = 0.032$ ,  $r = -0.369$ ), and neutrophil ( $P = 0.027$ ,  $r = -0.379$ ) and monocyte counts ( $P = 0.024$ ,  $r = -0.386$ ). On the other hand, GPX activities correlated significantly positively with percentages of lymphocytes ( $P = 0.043$ ,  $r = 0.348$ ). Furthermore, we found a significant negative correlation ( $P = 0.009$ ,  $r = -0.423$ ) between MDA concentrations and lymphocyte counts. In the group of all patients, NT-proBNP concentrations correlated significantly negatively with GPX activities ( $P = 0.050$ ,  $r = -0.339$ ) and positively with TNF- $\alpha$  concentrations ( $P = 0.022$ ;  $r = 0.378$ ) and monocyte count ( $P = 0.019$ ,  $r = 0.385$ ).

### Discussion

This paper reports novelties with respect to markers of inflammation and oxidative stress in canine cardiac patients and their relationship as well as their correlation with the disease severity marker NT-

proBNP. Numerous papers reported that heart failure is characterized by local and systemic chronic inflammation [1, 2]. Studies in people have already demonstrated that oxidative stress and chronic inflammation are linked in heart failure patients [1, 2, 16, 20, 25, 26]. Proinflammatory cytokines, especially TNF- $\alpha$ , IL-1, and IL-6, aggravate hemodynamic abnormalities, exert direct toxic effects on the heart and play a role in inflammation, tissue wasting, and weight loss [2]. Canine and human patients with heart failure have elevated levels of a number of inflammatory markers [1, 8, 10, 12, 13, 14, 15, 20]. Our results showed significantly higher TNF- $\alpha$  concentration in patients with CHF in comparison to patients without CHF. In the study of Zois and colleagues [14] TNF- $\alpha$  was not quantifiable in any MMVD dog. Furthermore, no significant difference in TNF- $\alpha$  concentration was observed in dogs with different stages of heart failure due to MMVD or DCM [15]. Due to negative inotropism of TNF- $\alpha$ , this cytokine contributes to interstitial fibrosis, myocyte apoptosis, ventricular remodelling, and systolic dysfunction, [27]. Several papers demonstrated the increased circulatory concentration of proinflammatory cytokines in human heart failure patients [4, 25, 27]. In our study majority of dogs (87%) had non-quantifiable concentrations of IL-6, which is in agreement with the study of Zois and colleagues where more than 75% of dogs had non-quantifiable concentrations of this cytokine [14].

C-reactive protein, WBC, and white blood cell differential counts are common markers of systemic inflammation. In our study serum CRP concentration was significantly higher in CHF patients in comparison to healthy dogs. Increased levels of CRP in dogs with various cardiovascular diseases have also been found in other studies [9, 11, 12, 15, 28]. In our study, significantly higher WBC and monocyte counts and neutrophil percentages and counts compared to control dogs and/or non-CHF dogs and significantly increased neutrophil percentages in patients without CHF compared to control dogs indicate some degree of inflammation. Although the median values of WBC and white blood cell differential counts were within our reference ranges, significant differences in these parameters between our cardiac patients and healthy dogs indicate the development of the inflammatory process. The results are partially in accordance with other studies [8, 10, 13, 15] and our previous study [12]. There have been a few studies investigating WBC and white blood cell differential counts in people [7, 29, 30]. Wall stress present in overloaded heart, causes sterile inflammation that contributes to heart failure. Ischemia in various tissues, including skeletal muscles and gut as a consequence of vasoconstriction and underperfusion in heart failure, also induces inflammation. Underperfused intestinal mucosa leads to increased gut permeability and enhances the translocation of bacteria and their toxins into the blood, thus contributing to systemic inflammation [31].

Increased production of ROS, induced by various triggers, is associated with the severity of inflammation. Decreased pulmonary capillary clearance of ROS positive WBC and platelets in CHF as a consequence of pulmonary congestion increases the production of ROS by mitochondria. A significantly higher number of ROS positive WBC and platelets were found in human patients with CHF in comparison to controls. It has been suggested that circulating WBC, and platelets boost oxidative stress in CHF [32]. Our oxidative stress parameters included MDA, a marker of lipid peroxidation, and GPX, one of the three major intracellular antioxidant enzymes. We found no significant difference in MDA concentrations between groups of cardiac patients and control dogs; however, the activity of GPX was significantly higher in

patients without CHF than in control dogs. Similar results were found in canine cardiovascular patients by Freeman and colleagues [21, 22]. According to our results regarding MDA concentrations, we may assume that oxidative damage to lipids less likely occurred in our cardiac patients; however, increased GPX activity in patients without CHF may indicate a compensatory response to increased production of ROS in the early phase of heart disease. In human CHF patients, increased concentration of MDA [19, 33] and decreased GPX activity have been reported [19, 34].

We found many significant correlations that indicate an association between the inflammatory process and oxidative stress in our CHF patients but not in patients without CHF. The significant positive correlation between TNF- $\alpha$  and MDA found in our study indicates that there is a higher degree of lipid peroxidation with an increased concentration of TNF- $\alpha$ . In human patients with CHF lipid peroxidation markers (MDA, lipid peroxide), and soluble receptors of TNF- $\alpha$  correlated significantly positively [19]. Our results are agreement with the results of Tsutamoto and colleagues [25], who found a relationship between TNF- $\alpha$  and oxidative stress in patients with dilated cardiomyopathy. No significant correlations between TNF- $\alpha$  and total antioxidant capacity and total thiol were found in canine cardiovascular patients with DCM and MMVD [15]. TNF- $\alpha$  induces ROS synthesis via endothelial mitochondria and NAD(P)H and the plasma membrane; furthermore, TNF- $\alpha$  is implicated in the regulation of nitric oxide metabolism [27]. Negative correlations of proinflammatory cytokines TNF- $\alpha$  and IL-6 with GPX suggest that GPX activity decreases with increased inflammation in our CHF patients. This statement is also supported by the negative correlations of neutrophil and monocyte counts with GPX activity. Conversely, percentages of lymphocytes significantly positively correlated with GPX, and lymphocyte count significantly negatively correlated with MDA. The obtained correlations further confirm that inflammation and oxidative stress are associated in canine patients with CHF.

In our study, markers of inflammation and oxidative stress and NT-proBNP correlated significantly in the group of all cardiac patients. Interestingly, GPX activity significantly negatively correlated with several markers of inflammation, such as TNF- $\alpha$ , IL-6, WBC, and neutrophil and monocyte counts. These results suggest the importance of this antioxidant enzyme in cross communication between inflammatory processes and oxidative stress in canine cardiac patients. Furthermore, GPX significantly positively correlated with the percentage of lymphocytes, whereas MDA concentration significantly negatively correlated with lymphocyte count. Additionally, in the group of all patients, we found a significant negative correlation of NT-proBNP with GPX and a significant positive correlation with TNF- $\alpha$  and monocyte count. These results suggest that the severity of the disease is associated with a decrease in GPX activity and increased inflammation. All these correlations further document the cross communication between inflammation and oxidative stress in canine cardiac patients.

Our study has some limitations that should be noted. Sex and age were not matched between our patients and control dogs. Age and/or sex, as well as neuter status, might influence the measured parameters [35, 36].

## Conclusion

In conclusion, most of the measured inflammatory markers were significantly higher in canine patients with CHF than in control dogs. Furthermore, we found that the activity of the antioxidant enzyme, GPX, decreased with increasing concentration of inflammatory markers in the group of all cardiac patients. Significant correlations between markers of inflammation and oxidative stress in patients with CHF but not in patients without CHF suggest complex cross communication between the two biological pathways with the development of CHF.

## Methods

### Dogs

In the present study, 88 privately owned dogs were evaluated. Forty-one dogs were excluded due to allergic diseases, cancer, kidney disease, endocrine disorders, or infectious diseases. Thirty-seven patients with either MMVD or DCM and ten healthy control dogs were prospectively recruited. The same cohort of dogs have been used in our previous study [37]. The ten control dogs were considered healthy according to their history, physical examination, and the routine laboratory results (haematology, biochemical profiles), as well as NT-proBNP levels. Myxomatous mitral valve disease, DCM and heart failure were diagnosed with the help of history, clinical examination, standard electrocardiogram, thoracic radiography, and echocardiography (Vingmed System Five, General Electric Healthcare, Milwaukee, Wisconsin, USA) with one- and two-dimensional modes and colour and spectral Doppler modes. Diseases were diagnosed following ECVIM/ACVIM guidelines [38, 39]. Cardiac therapy included diuretics, angiotensin-converting enzyme (ACE) inhibitors, pimobendan, beta blockers, and digoxin, depending on the needs of individual patients.

Patients were classified according to ACVIM classes into asymptomatic (ACVIM B1 and B2) or heart failure (ACVIM C and D) groups [39].

Signed consent was granted by the owners of the dogs. The Ethical Committee of the Ministry of Agriculture, Forestry and Food, Veterinary Administration of the Republic of Slovenia (Animal Protection Act UL RS 43/2007) approved all procedures.

### Blood sample collection and processing

Blood samples for all laboratory analyses were collected from fasted dogs. Samples for determination of haematological parameters were analysed within one hour after collection. Serum tubes (Vacuette; Greiner Bio-One, Kremsmünster, Austria) stood for 30 minutes at room temperature before centrifugation for 10 minutes at  $1300 \times g$  at room temperature. The serum used for CRP, TNF- $\alpha$ , and IL-6 measurements was frozen at  $-80^{\circ}\text{C}$  and analysed in batch at the end of the study. Serum biochemical parameters were measured on the day of collection. EDTA tubes (Vacuette; Greiner Bio-One, Kremsmunster, Austria) were used for the collection of samples for measurement of plasma NT-proBNP and MDA concentrations. These tubes were centrifuged for 15 minutes at  $1500 \times g$  at  $4^{\circ}\text{C}$  and obtained

plasma samples immediately frozen at -80°C until analysis. Concentrations of NT-proBNP and MDA were measured in batch at the end of the study.

### **Haematological and biochemical analyses**

An automated haematology analyser ADVIA 120 (Siemens, Munich, Germany) was used for haematological measurements. Biochemical parameters (results not reported; urea, creatinine, alkaline phosphatase, alanine aminotransferase;) were measured using automated biochemistry analyser RX Daytona (Randox, Crumlin, United Kingdom). The concentrations of sodium, potassium, and chloride were measured using an electrolyte analyser Ilyte (Instrumentation Laboratory, Lexington, MA, USA).

### **Determination of CRP, TNF- $\alpha$ and IL-6 concentrations**

Serum CRP, TNF- $\alpha$ , and IL-6 concentrations were measured using commercially available ELISA kits (Canine CRP ELISA; Alpco, Salem, NH, USA; Canine TNF- $\alpha$  Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA; Canine IL-6 Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA).

### **Determination of MDA and NT-proBNP concentrations**

The plasma MDA concentration was analysed according to a method described elsewhere [40]. NT-proBNP concentrations were measured in IDEXX Laboratories (Westbrook, USA) according to a method described elsewhere [41].

### **Statistical analysis**

Commercial software (IBM® SPSS® 24, Chicago, IL, USA) was used for data analysis. The normality test (Shapiro-Wilk) was used to assess the distribution of the data. Parameters of interest were compared between groups of dogs (cardiac patients with or without CHF and healthy dogs) using one-way ANOVA and Tukey's HSD test (Gaussian distribution of the data), or Kruskal-Wallis test followed by Bonferroni's method for multiple pairwise comparisons (non-Gaussian distribution of the data). An independent t-test (Gaussian distribution of the data) and the Mann-Whitney U test (non-Gaussian distribution of the data) were used to compare parameters of interest between the groups of patients with and without CHF. The results are reported as means and standard deviations (SD) (Gaussian distribution of the data) and as a median and interquartile range (IQR, 25th to 75th percentile; non-Gaussian distribution of the data). In the groups of patients with and without CHF and the group of all cardiac patients, we performed correlation analyses (non-parametric Spearman's rank correlation) to assess associations between inflammatory and oxidative stress markers and between NT-proBNP and markers of inflammation and oxidative stress. A probability value of less than 0.05 was considered statistically significant.

## **Abbreviations**

CHF: congestive heart failure; CRP:C-reactive protein; DCM:dilated cardiomyopathy; GPX:glutathione peroxidase; IL:interleukin; IQR:interquartile range; LYMPH:lymphocyte count; MDA:malondialdehyde;

MMVD:myxomatous mitral valve disease; MONO:monocyte count; n:number of dogs; NEUT:neutrophil count; NT-proBNP:N-terminal pro-B-type natriuretic peptide; ROS:reactive oxygen species; TNF- $\alpha$ :tumor necrosis alpha; SD:standard deviation; WBC:white blood cell count

## **Declarations**

### **Ethics approval and consent to participate**

The authors confirm that the study was carried out in compliance with the ARRIVE guidelines.

The Ethical Committee of the Ministry of Agriculture, Forestry and Food, Veterinary Administration of the Republic of Slovenia (Animal Protection Act, Official Gazette of the Republic of Slovenia, No. 43/2007) approved all procedures.

All owners signed an informed consent form before enrolling the dogs in the study.

### **Consent for publication**

Not applicable.

### **Availability of data and materials**

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

### **Competing interests**

The authors declare that they have no competing interests.

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### **Authors' contributions**

A.D.P. and A.N.S. formulated the hypothesis, designed the study, carried it out and took part in writing and revising the article. A.D.P. examined and performed the classification of all patients, performed statistical analysis and helped drafting the manuscript. A.N.S. drafted the manuscript, prepared figure and tables, performed the statistical analysis and measurements of glutathione peroxidase activity and interpreted the data. B.V. performed all the necessary collection and preparation of blood samples and helped in evaluating canine cardiac patients. N.Č.K. measured interleukin-6 and tumor necrosis factor-alpha concentrations and interpreted the data. J.S. and V.R. performed measurements of malondialdehyde concentrations and interpreted the data. All authors have read and critically reviewed the manuscript and provided feedback on drafts, as well as approved the final version of the manuscript.

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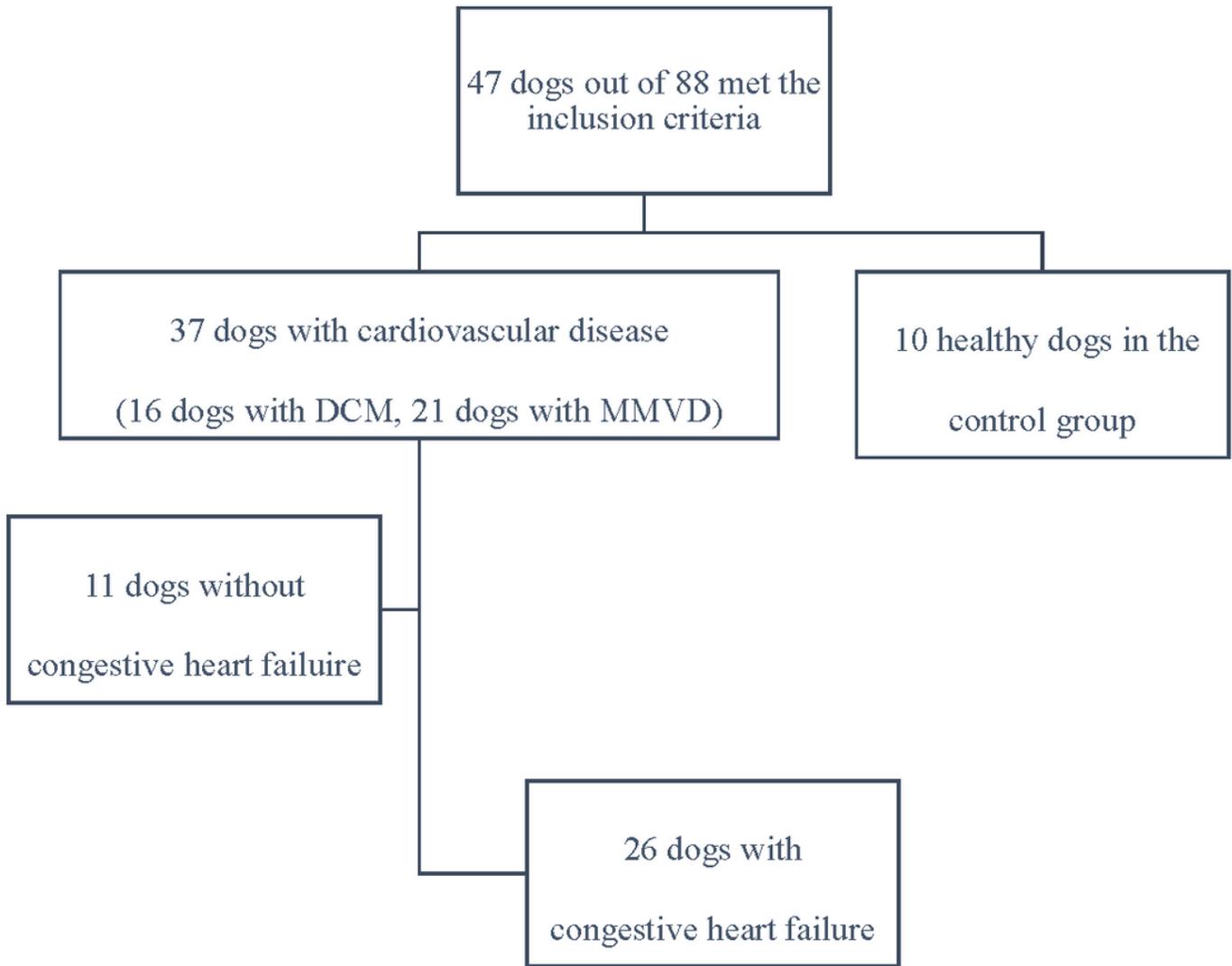
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## Figures



**Figure 1**

The progression algorithm of dogs during the inclusion into the study DCM: dilated cardiomyopathy; MMVD: myxomatous mitral valve disease