Changes of Inflammatory and Oxidative Stress Biomarkers in Dogs with Different Stages of Heart Failure

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Abstract

**Background:** Heart failure (HF) has been associated with changes in inflammatory and oxidative-stress biomarkers. This study aimed to evaluate the changes of a panel of inflammatory and oxidative-stress biomarker in dogs with different stages of HF and its relation with the severity of the disease and the echocardiographic changes. Dogs with HF as a result of myxomatous mitral valve degeneration or dilated cardiomyopathy were included. A total of 29 dogs were classified as stage-A (healthy), B (asymptomatic dogs), C (symptomatic dogs) and D (dogs with end-stage-HF) according to the ACVIM-staging-system. Serum cytokines, and inflammatory and oxidative stress markers were evaluated.

**Results:** KC-like as an inflammatory cytokine was significantly increased in dogs of stage-C (P < 0.01) and -D (P < 0.05) compared with stage-A and -B. Stage-D Dogs showed significantly higher serum CRP and Hp (P < 0.05) but lower serum anti-oxidant capacity (PON1, TEAC, CUPRAC, and thiol) compared to stage-A and -B (P < 0.05). After the treatment, serum levels of CRP, Hp and KC-like decreased but serum anti-oxidant levels increased compared to their pre-treatment values. Left ventricular dimension and LA/Ao ratio correlated positively with CRP, MCP-1, and KC-like but negatively with PON1, GM-CSF, IL-7 and antioxidant biomarkers (P < 0.01).

**Conclusion:** our results indicated that dogs with advanced-stages of HF show increases in positive acute-phase proteins and selected inflammatory cytokines (KC-like), and decreases in antioxidant biomarkers, indicating that inflammation and oxidative-stress act as collaborative partners in the pathogenesis of HF. KC-like may be a more useful parameter to monitor disease progression and treatment efficacy of dogs with severe-HF.

**Background**

Chronic heart failure (CHF) is a progressive clinical syndrome and characterized by exercise intolerance, dyspnea, coughing, lethargy, abdominal distension (ascites), and decrease in the quality and duration of life due to impaired cardiac and pulmonary functions (1). Myxomatous mitral valve degeneration (MMVD) and dilated cardiomyopathy (DCM) are the most common naturally occurring heart disease eventually resulting in CHF in dogs (2, 3) and humans (4).
MMVD is characterized by progressive myxomatous degeneration of mitral valve leaflets leading to mitral regurgitation and left-sided cardiac remodeling with a general preserved systolic function (2). Dilated cardiomyopathy (DCM) has recently emerged as having a genetic basis primarily in large breeds and is characterized by cardiomegaly with predominantly impaired left ventricular systolic function. Ascites is due to right-sided CHF and it is often associated with biventricular failure in giant breeds with DCM (2, 4, 5).

Although both of them are commonly considered non-inflammatory conditions (6, 7), studies have found increased circulating inflammatory cytokines in dogs and humans with CHF due to MMVD (8, 9) and DCM (7, 10). Increased expression and release of inflammatory cytokines such as tumour necrosis factor (TNF-α), as well as serum C-reactive protein (CRP), have been described in humans (11, 12) and dogs with CHF (13). Increased levels of monocyte chemoattractant protein 1 (MCP-1) and decreased levels of interleukins have also been found in dogs with CHF (8), but there is no data available on how CRP or other inflammatory biomarkers can change in severe cases (6). Although increased levels of oxidative stress biomarkers are associated with cardiovascular diseases in dogs (14, 15, 16) and humans (17, 18), there are no studies describing oxidative stress biomarkers such as total antioxidant capacity and thiol, and their relationships with inflammatory biomarkers and echocardiographic variables among the different stages of CHF.

The objectives of this study were to evaluate a panel of serum inflammatory and oxidative stress biomarkers in dogs with different stages of heart failure classified according to the American College of Veterinary Internal Medicine (ACVIM) guidelines (3), and to study the correlation between these biomarkers and echocardiographic variables. Namely, a cytokine panel including 13 cytokines, inflammatory biomarkers such as ferritin, CRP, haptoglobin (Hp), paraoxonase 1 (PON1) and butyrylcholinesterase (BChE) and oxidative stress markers such as total antioxidant capacity (cupric reducing antioxidant capacity - CUPRAC, and trolox equivalent antioxidant capacity - TEAC) and total thiol were evaluated. Furthermore, we sought to evaluate the changes of these biomarkers after treatment in dogs with severe CHF.

Results
Animals
The groups of the study were integrated by different breeds. Stage A group included 3 Labrador, 2 Border Collie, 1 Samoyed, 1 Cavalier King Charles Spaniel, and 1 Anatolian shepherd. Stage B2 group included 2 Golden Retrievers, 1 Cocker Spaniel, 1 Kopay, 1 Jack Russell, and 1 Shih Tzu. Stage C group included 2 Anatolian shepherds, 3 Pekingeses, 1 Shih Tzu, 1 Cavalier King Charles Spaniel, 1 Pincher, and 3 Terriers. Stage D group included 3 Anatolian shepherds, 2 Cocker Spaniels, 1 Pitbull, 1 German shepherd, and 1 mix breed.

Clinical data
There was a statistically significant difference in ages between dogs with stage A and C (P < 0.05) as well as stage A and D (P < 0.01) (Table 1). Heart and respiratory rates increased in parallel according to the severity of the diseases from stages A to D (data not shown) (P < 0.01).

| Parameters                      | Stage A (n = 8) | Stage B2 (n = 6) | Stage C (n = 10) | Stage D (n = 5) |
|---------------------------------|----------------|-----------------|-----------------|----------------|
| Clinical variables              |                |                 |                 |                |
| Age (years)                     | 3.5 ± 0.3 a    | 6.0 ± 1.6 a b   | 8.6 ± 1.6 b *   | 9.1 ± 1.8 b **|
| Body Weight (Kg)                | 23.5 ± 3.8 a   | 19.1 ± 4.7 a    | 16.0 ± 5.6 a    | 34.1 ± 8.5 a   |
| Gender (M/F)                    | 2 / 6          | 2 / 4           | 6 / 4           | 3 / 2          |
| Haematological variables       |                |                 |                 |                |
| WBC (x10^3/mm³)                 | 11.5 ± 1.1 a   | 10.1 ± 1.4 a    | 18.4 ± 2.5 b*** | 18.7 ± 1.5 b***|
| Neu (x10^3/mm³)                 | 7.9 ± 0.8 a    | 7.3 ± 0.9 a     | 14.4 ± 2.2 b**  | 15.7 ± 1.3 b***|
| cTnI ng/mL                      | 0.03 ± 0.04 a  | 0.03 ± 0.03 a   | 1.80 ± 1.39 b***| 5.30 ± 2.01 c***|
| Echocardiographic variables    |                |                 |                 |                |
| RVd (cm)                        | 0.62 ± 0.09 a  | 0.96 ± 0.11 a   | 0.97 ± 0.33 a   | 1.36 ± 0.35 a  |
| IVSd (cm)                       | 0.9 ± 0.1 a    | 1.1 ± 0.1 a     | 0.7 ± 0.0 a     | 1.0 ± 0.1 a    |
| IVSs (cm)                       | 1.1 ± 0.1 a    | 1.3 ± 0.1 a     | 0.9 ± 0.0 a     | 1.2 ± 0.1 a    |
| Lvd (cm)                        | 3.2 ± 0.5 a    | 3.0 ± 0.5 a     | 3.9 ± 0.4 a     | 5.7 ± 2.0 b***|
| LVs (cm)                        | 2.2 ± 0.2 a    | 2.1 ± 0.1 a     | 2.8 ± 0.4 a     | 4.3 ± 0.8 b***\#*
| PWD (cm)                        | 1.0 ± 0.1 a    | 0.9 ± 0.1 a     | 0.6 ± 0.0 b**   | 0.9 ± 0.0 a    |
| PWs (cm)                        | 1.2 ± 0.1 a    | 1.2 ± 0.1 a     | 0.7 ± 0.0 b***  | 1.3 ± 0.1 a    |
| LA/Ao                           | 1.1 ± 0.0 a    | 1.2 ± 0.0 a     | 2.2 ± 0.1 b***  | 2.3 ± 0.1 bc***|
| EPSS (cm)                       | 0.28 ± 0.10 a  | 0.37 ± 0.09 a   | 0.55 ± 0.19 ab  | 1.14 ± 0.30 b* |
| FS (%)                          | 32.2 ± 2.1 a   | 35.0 ± 2.1 a    | 29.5 ± 2.7 a    | 26.7 ± 5.5 a   |
| EF (%)                          | 59.5 ± 3.1 a   | 65.1 ± 2.5 a    | 55.9 ± 4.4 a    | 49.8 ± 8.4 a   |
| MV E/A                          | 1.9 ± 0.21 a   | 2.2 ± 0.2 ab    | 2.2 ± 0.1 ab    | 3.0 ± 0.5 b*   |

Table 1
Selected clinical, hematological and echocardiographic parameters in dogs with different stages (Stages A, B2, C, and D) of heart failure. Mean ± SEM

In the same rows, the difference between different letters was statistically significant, but the difference between all values with the same letter was not significant. * P<0.05; ** P<0.01; *** P<0.001; # compared with Stage B2.

LV- FS and - EF were calculated by Teicholz formula with M-mode echocardiography in the right parasternal long axis view.

**Echocardiographic variables**
Table 1 shows the echocardiographic variables of dogs in this study. M-mode measurements showed that LV dimensions at diastole and systole were increased in stage D compared to those of other stages of HF (P < 0.01). There were statistically increases in LA/Ao ratio (P < 0.01) and EPSS values (P < 0.05) between stage A and B and stage C and D. Mitral valve E/A ratios in stage D were higher than those of other stages of HF (P < 0.05). FS and EF values did not differ statistically between the groups (Table 1).

**Hematologic and serum biochemical results**
Regarding the CBC results, stage C and D showed increases (P < 0.01) in WBC and neutrophil counts, compared to stages A and B (Table-1). RBC and PLT counts did not differ statistically between groups (data not shown). There was a significant increase in cTnI level in stage C (P < 0.01; vs stage A and B) and stage D (P < 0.001; vs stage A, B2 and C) (Table-1). There were no statistically significant differences in biochemical parameters studied between the groups (data not shown).

**Inflammatory biomarkers**
The cytokines results are shown in Fig. 1. Significant increases in KC-like in dogs of stage C (median/interquartile range: 972/689–1188 pg/mL; P < 0.01) and D (median/interquartile range: 917/753–1942 pg/mL; P < 0.05) were found when compared with stage A (median/interquartile range: 377/125–531 pg/mL) and B dogs (median/interquartile range: 222/163–309 pg/mL). No significant differences in IL-2, IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, IP-10, MCP-1, GM-CSF, TNF-α and IFN-γ results were observed between the different groups of dogs (P > 0.05).

A significantly higher serum CRP concentration (Fig. 2) was observed in the stage D of CHF
(median/interquartile range: 37.8/26.9–60.9 µg/mL) compared with stage A (median/interquartile range 2.1/1.5–10.9 µg/mL, P < 0.05) and with stage B2 dogs (median/interquartile range 4.3/1.0–9.7 µg/mL, P < 0.05). Hp concentrations were significantly higher in dogs with stage D when compared with stage A dogs (median/25th–75th percentiles: 2.4/1.6–3.3 versus 4.8/3.9–5.0 g/L, P < 0.05). A significantly lower PON1 activity (Fig. 4) was found in dogs with Stage D of CHF (median/25th–75th percentiles: 1.9/1.4–2.3 IU/L) compared with stage A dogs (median/25th–75th percentiles: 3.9/3.5–4.6 IU/L, P < 0.05) and with dogs with stage B2 (median/25th–75th percentiles: 3.9/3.5–4.3 IU/L, P < 0.05). No significant differences were found in serum ferritin concentrations and BChE activity between stage A dogs and the different groups of dogs with CHF (Fig. 2). Within biomarkers that showed a statistical difference between the groups, the KC-like, CRP (P < 0.01), Hp (P < 0.01) and PON1 (P < 0.05) showed variations after two weeks of the therapy (Fig. 3).

Oxidative stress markers

Variations in the markers of oxidative stress analyzed in the different groups of dogs are shown in Fig. 4. Dogs with stage D heart failure presented significant lower concentrations of serum TEAC (median/25th–75th percentiles: 0.34/0.25–0.36 mmol/L, P < 0.05), CUPRAC (median/25th–75th percentiles: 0.22/0.17–0.25 mmol/L, P < 0.05) and thiol (median/25th–75th percentiles: 0.09/0.06–0.09 mmol/L, P < 0.05) when compared to stage A dogs (median/25th–75th percentiles: 0.49/0.41–0.56 mmol/L [TEAC]; 0.32/0.28–0.40 mmol/L [CUPRAC]; 0.25/0.22–0.36 mmol/L [thiol]) and with stage B2 dogs (median/25th–75th percentiles: 0.46/0.36–0.50 mmol/L, P < 0.01 for TEAC; 0.31/0.27–0.36 mmol/L, P < 0.01 for CUPRAC; 0.21/0.14–0.27 mmol/L, P < 0.01 for thiol). In dogs with stage D of CHF, oxidative stress marker are presented, before and two weeks after the therapy in Fig. 5.

Concentrations of TEAC, CUPRAC, and thiol increased after two weeks of therapy (Fig. 5).

Correlation study

Table – 2 shows all correlations found between echocardiographic parameters and biomarkers studied that showed statistical significance (P < 0.05). The highest correlation (ρ > 0.70) was observed between LA/Ao and serum cTnI (P < 0.001). CRP was positively correlated with LVDd (ρ = 0.40; P = 0.035) and LA/Ao (ρ = 0.60; P = 0.001), in addition KC-like correlated positively with LA/Ao (ρ = 0.52; P
parameters studied KC-like as an inflammatory chemokine seems to be a reliable biomarker to

B2, C and D) and their correlations with echocardiographic findings. Our results show that among

Spearman's rank correlation coefficient between echocardiographic parameters and biomarkers studied of

all dogs included in this study. The correlations included in this table are only those that showed statistical significance (P<0.05).

Discussion

To the best of the authors’ knowledge, this is the first study that reports changes in a panel of inflammatory and oxidative stress biomarkers in dogs with different stages of heart failure (stage A, B2, C and D) and their correlations with echocardiographic findings. Our results show that among parameters studied KC-like as an inflammatory chemokine seems to be a reliable biomarker to
monitor disease severity and treatment efficacy in dogs with CHF. Inflammatory and oxidative stress markers can contribute to cardiac remodeling in the progression of CHF in dogs. In this study, CHF due to MMVD or DCM was diagnosed based on a thorough cardiopulmonary assessment as reported in the previous studies (5, 13), and classified by stage A through D according to the ACVIM consensus statement guidelines (3).

Accumulating evidence indicates that acute-phase proteins (APPs), inflammatory cytokines and oxidative stress may have a role in the pathogenesis of CHF in humans (30) and dogs (8, 31), but it is not known how inflammatory biomarkers changes in the different stages of heart failure. In this study, increased concentrations of CRP and Hp and decreased activity of PON1 in stage D dogs showed that APPs may be associated with end-stage CHF. Similar to our results, significantly higher serum CRP concentration was found in dogs with decompensated CHF compared with compensated dogs with heart disease and healthy dogs (13). Our data are also in agreement with previous studies in which PON1 was described as an enzyme with cardioprotective action in atherosclerosis and related vascular diseases (32), and its activity decreased in humans with CHF (33, 34) and in situations of increased systemic oxidative stress and risk for cardiovascular disease in mouse (35). Therefore, the observed changes in APPs and PON1 activity in this study provide further support for a role of systemic inflammatory activity and oxidative processes in the progression of CHF and a potential anti-oxidant compensatory role of PON1.

Several reports have demonstrated enhanced expression and release of inflammatory cytokines and several chemokines in humans with CHF (36, 37, 38). In this study, a panel of serum cytokines and chemokines was evaluated, and only serum KC-like levels were increased in symptomatic stages of CHF (stage C and D) compared to asymptomatic (stage B2) and healthy dogs (stage A). In a study similar to ours, Zois et al. (8) reported that MCP-1 chemokine was increased in CHF dogs compared to healthy dogs and some ILs decreased with disease severity. Information about KC-like, a major neutrophil chemoattractant, is limited, but it appears to play a role in systemic or generalized inflammation (39). Increased plasma levels of KC-like have been associated with severe cardiac depression in old mice (12). Observed positive correlations between KC-like and other parameters
(CRP, Hp, ferritin, and WBC and neutrophil counts) suggest inflammatory potency of KC-like and its interaction with APPs in the progression of canine CHF. Inflammatory mediators may be released from the failing myocardium itself, and also from circulating WBC, platelets, endothelial cells, and from the liver and lungs and may contribute to myocardial depression and detrimental consequences such as endothelial dysfunction and cardiac myocyte apoptosis (40, 41).

In our study, lower values of all antioxidant biomarkers were found in severe stages of CHF. Similar to our findings, some of the antioxidant biomarkers such as thiol was decreased in humans with CHF (17). Decreased myocardial contractility or pressure/volume overload leads to myocardial ischemia, which in turn induces an increase in xanthine oxidase and decreases in the scavenging activity of superoxide dismutase and glutathione peroxidase (12). It has also been described that oxidative damage to low-density lipoprotein by reactive oxygen species (ROS) can influence the initiation and progression of valve lesions (43). This is in line with the fact that animal models of CHF have suggested that myocardial antioxidant defenses are also impaired (44). Also, an increase in ROS in heart muscle leads to cardiac fibrosis in rats (45). The decrease in antioxidant biomarkers could be connected with the increase in inflammatory biomarkers since the imbalance between antioxidants and oxidants can lead to induction of inflammatory cytokines, besides can cause direct cytotoxicity (44, 46). As changes in markers of inflammation and oxidative stress were detected in stage D dogs, it can be suggested that the degree of inflammatory activity and antioxidant system impairment may be linked to the severity of the disease.

When the individual animals were evaluated before and after the treatment, there was in general, a decrease in the biomarkers of inflammation and an increase in the antioxidant biomarkers, which was associated with the clinical improvement of the dogs. Although the number of animals was low and these findings should be demonstrated in larger and more diverse groups of dogs with severe stages of HF, it could be postulated that biomarkers such as CRP and antioxidant biomarkers could have potential diagnostic and prognostic relevance to monitoring treatment in cases of severe CHF (stage D).

Our findings of a positive correlation between CRP levels and LV diameter and LA/Ao ratio are in
agreement with a previous report made in dogs with CHF (6). This finding and the negative correlation found between these cardiac variables and serum PON1 levels would indicate that APPs have possible role in the pathophysiology of CHF in dogs. On the other hand, the negative correlation between serum CUPRAC, TEAC, thiol levels and LV dimensions may indicate the role of antioxidant system deficit in the development of cardiac remodeling from stage B2 to stage D CHF. Studies have shown that deficit of antioxidant capacity may have a role for myocardial injury and then developing CHF (47) and that CHF after myocardial infarcts are associated with an antioxidant deficit as well as an increase in oxidative stress (48). Based on these findings it could be postulated that antioxidant therapy or supplementation may be beneficial to slow or prevent the progression of CHF in dogs.

The present study is associated with several limitations. First, the sample size was small to obtain results with definitive statistical significance. Dogs were not sub-divided as MMVD and DCM in this study. Despite the fact that they have a different pathogenesis, both of them have resulted in CHF in humans (4) and dogs (2, 3). Second, there were wide-ranging variations in the body weight and various breeds of the included dogs. Both of them can influence several echocardiographic parameters such as LA and LV dimensions and EPSS. The LA/Ao ratio indicates the degree of LA dilation and shows a positive correlation with the severity of heart failure (49, 50). LVIDDN is suggested as a more favourable indicator for evaluation of the degree of LV dilation in dogs (51). Therefore in this study, LA/Ao ratio and LVIDDN were used to describe echocardiographic evidence of cardiac remodeling.

Conclusion
In conclusion, we concluded that CRP and KC-like are increased and antioxidant biomarkers such as TEAC, CUPRAC, and thiol are decreased in more severe stages of CHF, are correlated with some echocardiographic measurements and could be potential biomarkers to monitor disease progression and treatment efficacy of the patients with CHF. Therapeutic strategies for preventing inflammation and oxidative stress may contribute to clinical improvement and slow disease progression.

Methods
This study was performed between July 2018 and May 2019 at the Veterinary Teaching Hospital,
Dogs and groups
This study consisted of a total of 29 client-owned dogs of different breed, age, body weight, and both sexes. The dogs were classified according to the ACVIM staging system (3). Dogs without evidence of cardiopulmonary and other diseases were included as healthy controls (stage A, n = 8). Stage B includes two subgroups (B1 and B2): asymptomatic and presence of heart murmur with (B2) or without cardiomegaly (B1). In this study, only B2 dogs (n = 6) were selected, which was characterized by the presence of heart murmur at mitral valve puncta maxima, and radiographic (vertebral heart score [VHS] > 10.5) and echocardiographic evidence (left atrial to aortic root ratio [LA/Ao] > 1.6 and/or body weight normalized left ventricular internal diameter in diastole [LVIDDN] > 1.7) of left-sided cardiac remodeling due to MMVD. Stage C and D dogs were characterized by the presence of clinical signs associated with CHF. Stage C (n = 10) had a systolic heart murmur (≥ grade 3/6) over the mitral valve area with clinical (coughing, exercise intolerance, etc.), radiological (VHS > 11.0 and pulmonary edema) and echocardiographical evidence of left-sided cardiac remodeling as mentioned above due to MMVD (n = 7) or DCM (n = 3). Stage D dogs (n = 5) had a systolic heart murmur (grade 5–6/6), precordial thrill over the mitral valve area and abdominal distention (ascites), and echocardiographic evidence of left- and right-sided cardiac remodeling due to MMVD (n = 2) or DCM (n = 3), in addition to radiographic evidence of cardiomegaly (VHS > 11.5). Stage D dogs were refractory to standard treatment (pimobendan, furosemide, spironolactone, and enalapril) for CHF. Two weeks later after the initiation of the medical therapy, dogs in stage D were re-examined to collect the data, for two-group comparison: pre- and post-treatment groups.

Case selection
The diagnosis of MMVD was based on the combination of following criteria: the presence of mitral valve prolapse (MVP) and/or thickening of the mitral valve leaflets by 2-D echocardiography on right parasternal long-axis view, and identification of mitral valve regurgitation on left apical 4-chamber view by color Doppler examination (19, 20).

DCM was diagnosed based on the echocardiographic findings such as increased chamber size,
increased E point to septal separation (EPSS) and poor fractional shortening (FS < 25%) along with ECG and thoracic radiographic findings. The diagnosis was confirmed using a scoring system for DCM proposed by the European Society for Veterinary Cardiology (5, 21).

Healthy dogs were recruited from staff and students at the Veterinary Teaching Hospital. All dogs were healthy based on normal physical and cardiovascular examinations and laboratory assessments in which the results of complete blood count (CBC), serum cardiac troponin I (cTnI) and serum biochemistry profile were within the reference ranges suggested for dogs.

Exclusion criteria
According to the results of the analysis, dog with comorbidities such as infectious diseases (pneumonia, urinary tract disease or pyoderma, etc.), non-infectious diseases (renal failure, atopy, inflammatory bowel disease or hepatitis), vector-borne diseases (ehrlichiosis, Lyme, and dirofilariosis, etc.), endocrine diseases (diabetes mellitus, hypothyroidism, hyperthyroidism and Cushing disease among others) and patients with benign or malignant tumors were excluded. If the dogs received any kind of medication (steroids, non-steroids, antibiotics, inotropes or diuretics, etc) prior to admission to the clinic, they did not included to the study, because of the fact that some medication could effects on hematologic and serum biochemistry profile which were analysed here.

Sample Collection And Measurements
Examinations of the cardiopulmonary system
In this study, the cardiopulmonary system was evaluated by a thorough physical examination, electrocardiography (ECG), thoracic radiography and echocardiography in all dogs. Physical examination included body temperature, heart and respiratory rates and cardiac auscultation etc.

Bilateral, ventrodorsal and/or dorsoventral radiographs of each patient were taken, and radiological morphology of the heart, vertebral heart score, lung and thoracic vessels were examined. ECG was recorded without sedation using 3 bipolar standard limb leads. Cardiac rhythm analyses and measurements were performed with a standard calibration (10 mm/mV and 50 mm/sec), as reported in a previous study (22).

A transthoracic echocardiographic examination was performed as reported in 2 previous studies (22, 23). Briefly, cardiac measurements were done using conventional modalities (2-D, M-mode, and color
Doppler) and imaging techniques (right parasternal short and long axis, left apical 4–5 chamber and subcostal views) with phased-array cardiac transducers in all dogs (Caris Plus Esaote, Italy).

**Laboratory analysis**

Venous blood samples were collected via venipuncture from the brachiocephalic veins into EDTA tubes for CBC and serum tubes for biochemistry (inflammatory biomarkers, cytokine panel, and oxidative stress markers) and cardiac troponin I (cTnl) analyses. Serum samples were stored at −80 °C for a maximum of 8 months until analysis.

**Hematological and serum biochemistry analysis**

CBC was measured in the animal hospital lab within one hour after blood collection (HM5, Abaxis), and only white blood cell (WBC) and neutrophil counts were presented in this study. In all dogs, routine serum biochemistry panel including enzyme activities (ALP, ALT, CK, and amylase), total protein, electrolytes (Ca, P), renal damage markers (blood urea nitrogen and creatinine), glucose and total bilirubin was measured (Comprehensive Diagnostic Profile Rotor, VetScan, Abaxis). Serum thyroxine and cholesterol levels were measured using T4/Cholesterol Reagent Rotor (VetScan, Abaxis). Serum cTnl was measured with a portable clinical device (cTnl cartridge, I-Stat, Abaxis).

**Inflammatory biomarkers**

Serum ferritin concentration was measured using a commercial immunoturbidimetric assay (Tina-quant Ferritin, Roche). A commercially available method (Tridelta Ltd., Brey, Ireland) was used for haptoglobin (Hp) concentration measurement. C-reactive protein (CRP) was measured in serum using an immunoturbidimetric assay (CRP OSR6147 Olympus Life and Material Science Europe GmbH, Hamburg, Germany). PON1 and BChE activities were determined following previously validated assays (24, 25). All analysis was performed using the Olympus AU600 (Olympus Diagnostica GmbH) analyzer.

**Serum cytokines measurements**

Milliplex® MAP magnetic bead panel (CCYTO-90K Millipore, Billerica, MA) with an automated analyzer (Luminex 200, Luminex Corporation, Austin, TX) was used to determine concentrations of 13 cytokines (interleukin-2 (IL-2), IL-6, IL-7, IL-8, IL-10, IL-15, IL-18, interferon gamma-induced protein 10 (IP-10), monocyte chemoattractant protein 1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), keratinocyte-derived chemokine (KC)-like, tumour necrosis factor-alpha (TNF-α) and
interferon-gamma (IFN-γ) in blood serum. The assay was performed according to the manufacturer's instructions. Internal quality control material provided by the manufacturer was used to generate a standard curve and calculate concentration for each analyte.

Oxidative stress biomarkers
Trolox equivalent antioxidant capacity (TEAC), based on the enzymatic generation of ABTS radical, and cupric reducing antioxidant capacity (CUPRAC) were determined in serum by using previously validated assays (26, 27). Serum thiol concentrations were measured using the method described by Jocelyn (28) and modified by Costa et al. (29).

Statistical analysis
Data were analyzed using a commercial software tool (GraphPad Prism 6, San Diego, USA). Changes in results between the different groups were assessed by a non-parametric test (Kruskal–Wallis followed by Dunn’s multiple comparison) because of the small sample size. Therefore, they were presented as median and interquartile range. Correlations between variables were determined using the Spearman test. A \( P < 0.05 \) was taken as statistically significant in all cases.

Abbreviations
2-D: two dimensional; ACVIM: American College of Veterinary Internal Medicine; ALP: alkaline phosphatase; ALT: alanine aminotransferase; APPs: acute-phase proteins; BChE: butyrylcholinesterase; CBC: complete blood count; CHF: chronic heart failure; CK: creatinine kinase; CRP: C-reactive protein; cTnI: cardiac troponin I; CUPRAC: cupric reducing antioxidant capacity; DCM: dilated cardiomyopathy; ECG: electrocardiography; EJ: ejection fraction; EPSS: E-point to septal separation; FS: Fractional shortening; GM-CSF: Granulocyte macrophage-colony stimulating factor; Hp: haptoglobin; IFN-γ: interferon gamma; IL: interleukin; IP-10: interferon γ-induced protein 10; KC-like: keratinocyte chemoattractant-like; LA/Ao: left atrium to aorta ratio; LV: left ventricle; LVDd: left ventricular diastole diameter; LVIDDN: normalized left ventricular internal diameter in diastole; MCP-1: monocyte chemoattractant protein 1; MMVD: myxomatous mitral valve degeneration; MVP: mitral valve prolapse; PLT: platelet; PON-1: paraoxonase 1; RBC: red blood cell; ROS: reactive oxygen species; T4: thyroxine; TEAC: trolox equivalent antioxidant capacity; TNF-α: tumour necrosis factor; VHS: vertebral heart score; WBC: white blood cell.
Declarations

Ethics approval and consent to participate

This experiment was approved by the Ethics and Welfare Committee of the Uludag University of Bursa, Turkey (approval No. 2018 – 05 / 02). A signed informed consent was obtained from all owners.

Consent for publication

Not applicable.

Availability of data and materials

All data in this study will be available from the corresponding author upon reasonable previous request and with the permission of the research fund.

Competing interests

The authors declare that they have no competing interests. Zeki YILMAZ (Prof. Dr., corresponding author for this study) is listed as an associate editor in clinical pathology section of this journal (BMC Vet Res). One of the co-authors, Jose J. Ceron is a member of the editorial board (section editor) of this journal.

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Authors’ contributions

CPR: who is listed as a first author and wrote the manuscript draft and carried out laboratory analysis. AS: collected the material and data analysis. MK: conducted the research and interpreted data. RT and JC: interpreted data and corrected the manuscript. JJC: adviser for laboratory analysis, statistic work and manuscript revision. ZY: who is a corresponding author, and supervisor for clinical research, material collection, and finalizing the manuscript. All authors have read and approved the final manuscript.

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Figures
Figure 1

Cytokine results in dogs of different stages of heart failure: Stage A, Stage B2, Stage C and Stage D. IL, interleukin; IP-10, interferon gamma-induced protein 10; MCP-1, monocyte chemoattractant protein 1, GM-CSF, granulocyte-macrophage colony-stimulating factor; KC-like, keratinocyte-derived chemokine; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma.
Values of C-reactive protein (CRP), haptoglobin (Hp) and ferritin in dogs with different stages of heart failure: Stage A, Stage B2, Stage C and Stage D. The plots show median, 25th and 75th percentiles.
Figure 3

Serum levels of keratinocyte chemotactic like (KC-like) (A), C-reactive protein (CRP) (B), haptoglobin (Hp) (C) and paraoxanase-1 (PON-1) (D), before (n=5) and two weeks after the treatments (n=5) in stage D of chronic heart failure. * P<0.05 ** P<0.01
Figure 4

Trolox equivalent antioxidant capacity (TEAC), cupric reducing antioxidant capacity (CUPRAC), and thiol concentrations in serum of dogs with different stages of heart failure: Stage A, Stage B2, Stage C and Stage D. The plots show median, 25th and 75th percentiles.
Figure 5

Serum levels of trolox equivalent antioxidant capacity (TEAC) (A), cupric reducing antioxidant capacity (CUPRAC) (B) and thiol (C), before (n=5) and two weeks after the treatments (n=5) in stage D of chronic heart failure. * P<0.05