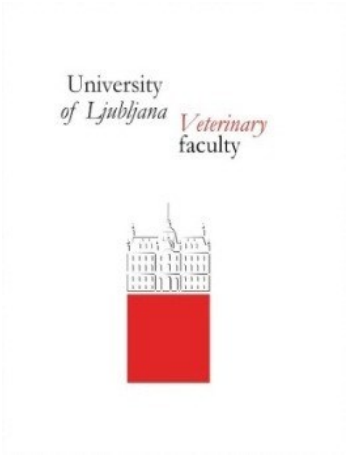

Natalia Druzhaeva

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Doctoral dissertation

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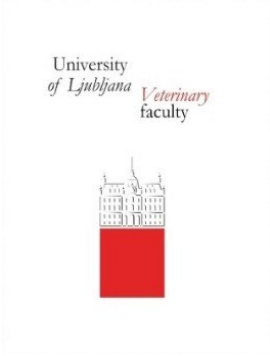

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Natalia Druzhaeva, dr. vet. med.

EFFECT OF COENZYME Q_{10} ON OXIDATIVE STRESS, IMMUNE AND CLINICAL STATUS IN DOGS WITH MYXOMATOUS MITRAL VALVE DEGENERATION

Doctoral Dissertation

VPLIV KOENCIMA Q_{10} NA OKSIDATIVNI STRES TER IMUNSKI IN KLINIČNI STATUS PRI PSIH Z MIKSOMATOZNO DEGENERACIJO MITRALNE ZAKLOPKE

Doktorska disertacija

Ljubljana, 2023

Natalia Druzhaeva

Effect of Coenzyme Q_{10} on oxidative stress, immune and clinical status in dogs with myxomatous mitral valve degeneration

The research work was conducted at the Small Animal Clinic, Veterinary Faculty, University of Ljubljana; Institute of Food Safety, Feed and Environment, University of Ljubljana; Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana.

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I declare that the submitted doctoral dissertation is the result of my own research work, that the results are correctly stated and that I have not violated the copyright and intellectual property of others. I declare that the electronic copy is identical to the printed one.

I dedicate this work to my children Eve and Leon, and my husband, Anatoly. Thank you for your support, encouragement and the sacrifices you all have made along the way.

ABSTRACT

Keywords: dog; myxomatous mitral valve degeneration - disease; congestive heart failure; coenzyme Q_{10}; oxidative stress; inflammation; flow cytometry; lymphocyte subtypes

Coenzyme Q_{10} (CoQ_{10}) is a fat-soluble antioxidant that has long been studied in human patients with cardiovascular disease. A few studies have been performed in dogs, but none assessed the effect of CoQ_{10} supplementation on oxidative stress markers, inflammatory markers and lymphocyte populations in dogs of different breeds with different stages of spontaneous myxomatous mitral valve degeneration (MMVD). In addition, information on lymphocyte subpopulations in dogs in different stages of MMVD is lacking. Furthermore, there are no official recommendations on the dosage of CoQ_{10}, and no dose-ranging studies have been done in dogs with heart disease. To investigate this, we performed a prospective cross-sectional flow cytometry study, a double-blind randomized placebo-controlled CoQ_{10} dose-ranging study, and a double-blind randomized placebo-controlled 3-month CoQ_{10} supplementation study. The results of our research showed that dogs with stable and unstable congestive heart failure (CHF) had a lower percentage of CD4+ T lymphocytes and CD4+/CD8+ ratio and a higher percentage of CD8+ T lymphocytes, and additionally, dogs in unstable CHF had higher CD8+ T lymphocyte concentration, which suggests immune system involvement in the pathogenesis and progression of CHF. A 200 mg daily dose of water-soluble CoQ_{10} was sufficient to achieve at least a 3-fold increase in plasma CoQ_{10} concentration, which enables the biological effects of CoQ_{10}, and can be used in future CoQ_{10} supplementation studies in dogs with MMVD. After a 3-month supplementation with water-soluble CoQ_{10}, a decrease in neutrophil percentage and an increase in lymphocyte count and percentage were noted in dogs with CHF due to MMVD, while there was no effect on other measured biomarkers, lymphocyte subpopulations or echocardiographic and clinical parameters in dogs with or without CHF. The results of our study suggest that CoQ_{10} as a dietary supplement can potentially reduce inflammation in dogs with CHF due to MMVD.

IZVLEČEK

Ključne besede: pes; miksomatozna degeneracija mitralne zaklopke – bolezen; kongestivno srčno popuščanje; koencim Q_{10}; oksidativni stres; vnetje; pretočna citometrija; podvrste limfocitov

Koencim Q_{10} (CoQ_{10}), v maščobah topen antioksidant, ima dolgo zgodovino proučevanja pri bolnikih s srčno-žilnimi boleznimi, pri psih pa je bilo opravljenih zelo malo raziskav. V nobeni od objavljenih raziskav niso proučevali učinek CoQ_{10}, kot dodatka k prehrani, na pokazatelje oksidativnega stresa in vnetja ter podvrste limfocitov pri psih različnih pasem z različnimi stopnjami spontane miksomatozne degeneracije mitralne zaklopke (MDMZ). Poleg tega ni podatkov o koncentraciji in odstotku podvrst limfocitov pri psih v različnih fazah MDMZ ter uradnih navodil o priporočenem odmerku CoQ_{10}. Zasledili nismo niti raziskav za določanje ustreznega odmerka CoQ_{10} pri psih z boleznimi srca. Da bi to raziskali, smo opravili prospektivno presečno raziskavo z uporabo pretočne citometrije, dvojno slepo randomizirano, s placebom nadzorovano raziskavo določanja odmerka CoQ_{10} in dvojno slepo randomizirano, s placebom nadzorovano trimesečno raziskavo dodajanja CoQ_{10}. Izsledki raziskave so pokazali, da so imeli psi s stabilnim in nestabilnim kongestivnim srčnim popuščanjem nižja odstotek CD4+ limfocitov T in razmerje CD4+/CD8+ ter višji odstotek CD8+ limfocitov T, poleg tega so imeli psi v nestabilnem popuščanju srca višjo koncentracijo CD8+ limfocitov T, kar lahko kaže na vpletenost imunskega sistema v patogenezo in napredovanje kongestivnega srčnega popuščanja. 200 mg vodotopnega CoQ_{10} dnevno je zadoščalo za vsaj trikratno povečanje koncentracije CoQ_{10} v plazmi, kar omogoča biološke učinke CoQ_{10}, zato se lahko ta odmerek uporablja v prihodnjih raziskavah suplementacije CoQ_{10} kot dodatka k prehrani psov z MDMZ. Po trimesečnem dodajanju vodotopnega CoQ_{10} se je zmanjšal odstotek nevtrofilnih granulocitov, povečala pa sta se koncentracija in odstotek skupnih limfocitov pri psih s kongestivnim srčnim popuščanjem zaradi MDMZ, medtem ko učinka na druge izmerjene biooznačevalce, subpopulacije limfocitov ali ehokardiografske in klinične parametre pri psih z ali brez srčnega popuščanja nismo ugotovili. Izsledki raziskave kažejo, da lahko CoQ_{10} kot prehranski dodatek potencialno zmanjša sistemsko vnetje pri psih s srčnim popuščanjem zaradi MDMZ.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS:

ACVIM, American College of Veterinary Internal Medicine

CHF, congestive heart failure

CoQ_{10}, coenzyme Q_{10}

CRP, C-reactive protein

cTnI, cardiac troponin I

DAMP, damage-associated molecular pattern

FITC, fluorescein isothiocyanate

GPX, glutathione peroxidase

HF, heart failure

MHC, major histocompatibility complex

MMVD, myxomatous mitral valve degeneration

NF-kB, nuclear factor kappa B

NT-proBNP, N-terminal pro-B type natriuretic peptide

PAMP, pathogen-associated molecular pattern

RAAS, renin-angiotensin-aldosterone system

ROS, reactive oxygen species

RPE, R-phycoerythrin

TNF-α, tumor necrosis factor-α

TNFSR-II, soluble tumor necrosis factor receptor II

1 INTRODUCTION

1.1 SCIENTIFIC PROBLEM IDENTIFICATION

Myxomatous mitral valve degeneration (MMVD) is the most frequent heart disease in dogs and the most frequent cause of heart failure (HF) in this species (Keene et al., 2019). Heart failure is the final syndrome in MMVD and other heart diseases, in which the heart no longer fulfils its role as a pump that supplies the organism with the necessary nutrients and oxygen (Zhang et al., 2017).

Activation of neurohormonal mechanisms, such as the sympathetic nervous system, and the renin-angiotensin-aldosterone system (RAAS), in congestive HF (CHF) is thought to promote immune activation and inflammation, which contribute to the progression of the disease. It has been shown that angiotensin II, which is one of the major end products of the RAAS activity, can promote the synthesis of chemokines, cytokines and adhesion molecules that activate leukocytes and promote their adhesion to endothelium, whereas activation of the sympathetic nervous system and the resulting stimulation of ß-adrenergic receptors on lymphocytes and monocytes may potentially lead to an increase in cyclic adenosine monophosphate and subsequent changes in cytokine production, thereby enhancing local and possibly systemic inflammation (Yndestad et al., 2007; Bennett et al., 2018).

Excessive formation of reactive oxygen species (ROS) can also lead to systemic inflammation, possibly through activation of the transcription factor nuclear factor kappa B (NF-kB) and subsequent increased production of inflammatory cytokines (Yndestad et al., 2007; Fan et al., 2017). On the other hand, it is assumed that cytokines can lead to increased formation of ROS in the cardiovascular system (Yndestad et al., 2007; Szczurek and Szyguła-Jurkiewicz, 2015; Fan et al., 2017).

The immune system consists of innate and adaptive immune mechanisms. Innate immunity is non-specific and responds quickly, while adaptive immunity is slower to respond but generally highly specific. Both components can be involved not only in the immune response to infectious

agents but also to endogenous stimuli, typically associated with cardiovascular diseases (Mann, 2015).

Neutrophils are one of the most important cells of innate immunity playing a principal role in the acute inflammatory response to pathogens, tissue trauma, and necrosis. They are triggered by cytokines, such as interleukin-1, that are produced by macrophages, mast cells and other immune cells after they encounter and recognise pathogen- or damage-associated molecular patterns (PAMPs; DAMPs) using their pattern-recognition receptors (Aiello and Moses, 2016). Neutrophils are phagocytes and their primary function is to phagocytise bacteria and other harmful substances, thus cleaning the site of inflammation. Neutrophils also play a role in chronic diseases characterised by chronic low-grade inflammation (Herrero-Cervera et al., 2022). They are found in the normal heart, but their precise location and the role in healthy heart homeostasis are poorly studied. In heart disease, the role of neutrophils is more evident in marked inflammatory states such as myocardial infarction in which neutrophils are attracted in large numbers to the site of injury (Swirski and Nahrendorf, 2013). In chronic and non-primarily immune-mediated conditions, circulating neutrophils along with other inflammatory cells are also attracted to the heart following the secretion of cytokines and chemokines (Strassheim et al., 2019). Despite being the part of the innate immune response, neutrophils also modulate an adaptive immune response (Strassheim et al., 2019). Circulating neutrophils were previously shown to be increased in dogs with CHF of various non-inflammatory aetiologies (Domanjko Petrič et al., 2018; Hamilton-Elliott et al., 2018; Rubio et al., 2020; Nemec Svete et al., 2021).

Lymphocytes are a subgroup of leukocytes that represent adaptive immunity. There are two major subsets of lymphocytes, T cells and B cells, with T cells presented by cytotoxic T cells (CD8 cells) and secreting helper T cells (CD4 cells) with both CD4 and CD8 cells having regulatory and memory subsets. In a classical immune response, helper T lymphocytes are activated by antigen-presenting cells (dendritic cells and others), which take up the antigen, process it and present it on their surface for T cells (in the major histocompatibility complex [MHC] II), while cytotoxic T lymphocytes are activated by antigens presented in the MHC I complex molecules found on all nucleated cells, including, as was shown in studies in pigs, different types of leukocytes, with few of them expressed also on mammalian cardiomyocytes

(Tizard, 2016). Additionally, a wide array of cytokines plays a role in T lymphocyte activation and proliferation. Once activated, helper T cells, in turn, start secreting cytokines (the type of secreted cytokines depends on the type of the helper T cell), and proliferating, evoking response from other T cells, B cells and affecting multiple other cells. Upon activation, cytotoxic T cells are recruited from lymphoid organs and attracted to the site of infection or injury exerting direct cytotoxic effect. Both helper and cytotoxic T lymphocyte response is strictly limited to the specific antigens (Tizard, 2016; Catchpole and HogenEsch, 2023). Besides, effector T cells do not evoke a response unless the cytokines (such as interferon gamma) from T helper cells are present (Aiello and Moses, 2016). Through these mechanisms, a tight regulation of the immune response is achieved. Lymphocytes are normally found in the heart along with neutrophils and other immune cells (Swirski and Nahrendorf, 2013) and are also recruited in the event of heart disease as cytokines are produced after cardiac injury. T lymphocytes affect the course of heart disease and HF, as lymphocyte activation can lead to faster healing or, on the contrary, faster progression to HF, apoptosis and cardiac remodelling, and activation of B lymphocytes in heart disease brings up deleterious consequences and worsens the disease (Strassheim et al., 2019). The distinct roles of helper and cytotoxic T lymphocytes in the heart have been investigated previously in humans and mice, and it has been shown that in HF both CD4+ and CD8+ lymphocytes infiltrate the myocardium and promote fibrosis and remodeling, with the significant expansion of both subsets in circulation also shown under same pathological conditions (Nevers et al., 2015; Bansal et al., 2017).

Inconsistent changes in the percentages of lymphocyte subtypes have been documented in people with CHF. In one study, an increase in CD4+ T lymphocytes and CD4+/CD8+ ratio was observed in patients with idiopathic and ischemic CHF (Agnoletti et al., 2004), whereas in another study, a decrease in CD4+ T lymphocytes and B lymphocytes and, in elderly CHF patients, also an increase in CD8+ T lymphocytes were observed in people with advanced CHF (Moro-García et al., 2014). Little information is available regarding lymphocyte subtypes in dogs with MMVD and CHF and the findings are contradictory. The early study done in German Shepherds with mitral insufficiency showed a decreased percentage of CD4+ T lymphocytes and decreased CD4/CD8 ratio along with an increased percentage of CD8+ T lymphocytes, but it is not clear if any of these patients were in CHF (Borgarelli et al., 2002). Another study was done in dogs with CHF due to chronic valvular disease and dilated cardiomyopathy and the

decreases in percentages of both CD4+ peripheral blood mononuclear cells and CD8+ T lymphocytes were noted (Farabaugh et al., 2004). In the most recent study, CD4+ T lymphocytes were found to be higher and CD8+ T lymphocytes were found to be lower in CHF dogs compared to healthy old dogs (Piantedosi et al., 2022).

Inflammation is one of the body's most important protective mechanisms to ward off infectious agents, but it also serves to protect against other insults, including non-infectious tissue damage, such as due to hemodynamic overload in heart failure. The acute inflammatory response to an antigen or other stimulus classified as “dangerous” begins with cells (macrophages, mast cells, and others) recognizing PAMPs or DAMPs that bind to pattern recognition receptors (toll-like receptors or others) on these cells and consequently, often via NF-kB, inducing cytokine synthesis and activation of effector inflammatory cells, mainly neutrophils. Increased expression of pattern recognition receptors such as toll-like receptors has been shown in people with advanced HF (Frantz et al., 1999; Birks et al., 2004).

Under normal circumstances, once the goal of inflammation, i.e., elimination of the insult (infectious agent, damaged tissue, or other), is achieved, anti-inflammatory mechanisms are used to rapidly restore homeostasis and terminate inflammation (Mann, 2015; Aiello and Moses, 2016). When the balance between pro-inflammatory and anti-inflammatory cytokines is disturbed, homeostasis cannot be restored, and persistent inflammation, called chronic low-grade inflammation, continues and may contribute to disease progression (Mann, 2015; Bennett et al., 2018). Low-grade inflammation or para-inflammation is the type of inflammation that is limited and does not require major tissue injury (Medzhitov, 2008; Mann, 2015). This type of inflammation is one of the features of human HF with preserved ejection fraction (Mesquita et al., 2021) and is also typical of metabolic diseases associated with cardiovascular pathologies in humans, such as type 2 diabetes, hypertension, as well as obesity and ageing (Hotamisligil, 2006; Van Linthout and Tschöpe, 2017). Low-grade inflammation is also present in canine CHF (Domanjko Petrič et al., 2018).

In addition to neutrophil granulocytes and lymphocytes, many other cells are involved in inflammation, including macrophages, mast cells, dendritic cells, natural killer cells, and others. A wide range of mediators support crosstalk between immune cells and cells of the heart. In

addition, cardiac cells themselves can express cytokines called cardiokines. The main mediators involved in HF are tumor necrosis factor-α (TNF-α) and interleukin-1 (Reina-Couto et al., 2021), and levels of several other inflammatory mediators, including other interleukins and chemokines, have been found to increase in people and dogs with HF (Grosman-Rimon et al., 2019; Rubio et al., 2020; Nemec Svete et al., 2021).

Studies in humans have confirmed that inflammation and thus the immune status, oxidative stress, and the pathogenesis of cardiovascular diseases are closely linked (Yndestad et al., 2007; Szczurek and Szyguła-Jurkiewicz, 2015). This link is of interest to researchers that investigate the effects of exogenous antioxidant supplementation on oxidative stress and clinical status. A lot of attention is paid to the use of the antioxidant coenzyme Q_{10} (CoQ_{10}). Coenzyme Q_{10}, or ubiquinone, is a fat-soluble antioxidant, which is synthesized *de novo* in the organism and is an essential co-factor in the process of oxidative phosphorylation in the mitochondria and an extremely potent endogenous antioxidant (Turunen et al., 2004; Bentinger et al., 2007). Coenzyme Q_{10} is present in the body in both reduced ($CoQ_{10}H_2$ or ubiquinol) and oxidized (CoQ_{10} or ubiquinone) forms, with the reduced form having antioxidant properties. In its reduced form, CoQ_{10} effectively regenerates other antioxidants such as vitamin E and ascorbate (Bentinger et al., 2007). Coenzyme Q_{10} is essential for the functioning of all cells of an animal's body and is present in the membranes of mitochondria and other cell organelles, cell membranes, cytoplasm and blood plasma. It directly or indirectly regulates many bodily functions, including the functioning of the immune system (Crane, 2001; Turunen et al., 2004; Mantle et al., 2021).

The reducing effect of CoQ_{10} supplementation on inflammatory markers has been found in cardiac and other chronic inflammatory diseases in humans and in *in vitro* research (Lee et al., 2012; Lee et al., 2013; Fan et al., 2017; Cirilli et al., 2021; Mantle et al., 2021; Al-Johani et al., 2022; Sue-Ling et al., 2022). It is hypothesized that CoQ_{10} exerts an anti-inflammatory effect by decreasing ROS concentrations and subsequently reducing NF-kB (normally activated by ROS) gene expression (Fan et al., 2017). The anti-inflammatory effect of CoQ_{10} on markers of inflammation has not yet been studied in dogs with CHF.

In people, myocardial CoQ_{10} deficiency was found to be involved in the development and progression of HF (DiNicolantonio et al., 2015). It was also shown that the CoQ_{10} plasma concentration is an independent predictor of mortality in chronic HF patients (Molyneux et al., 2008). In dogs, plasma CoQ_{10} deficiency was not confirmed (Harker-Murray et al., 2000; Svete et al., 2017), but a lower plasma concentration of CoQ_{10} was found to be strongly negatively associated with the severity of the disease (Svete et al., 2017), and recently, lower myocardial CoQ_{10} concentration was found in Cavalier King Charles Spaniels with CHF due to MMVD (Christiansen et al., 2021).

Many studies that have investigated CoQ_{10} as a dietary supplement in addition to standard therapy of HF have reported a positive effect on cardiac contractility, a decrease in plasma levels of inflammatory markers, as well as decrease in disease severity, hospital stays and mortality in people (Belardinelli et al., 2005; Mortensen et al., 2014; Fan et al., 2017; Martelli et al., 2020; Sue-Ling et al., 2022). In dogs, the effect of CoQ_{10} supplementation was investigated in an experimental tachycardia-induced CHF model, and a positive effect of CoQ_{10} supplementation in the dose of 10 mg/kg/day was observed in terms of lower intracardiac pressure in early CHF and less myocardial hypertrophy in severe CHF (Harker-Murray et al., 2000). Later, the study was conducted in dogs of different breeds with spontaneous CHF due to MMVD, which investigated the effect of CoQ_{10} supplementation on echocardiographic parameters including systolic function (ejection fraction) and cardiac troponin I (cTnI; Tachampa et al., 2018). In that study, median cTnI did not change significantly, and shortening fraction and ejection fraction increased. However, these two parameters are not good measures of contractility in this disease due to changing loading conditions. Furthermore, there was no control group included in that study. Another study investigated the effect of oral CoQ_{10} given for 3 weeks on the severity of the disease and owner-perceived quality of life in Cavalier King Charles Spaniels with MMVD with or without CHF, with no positive effects of CoQ_{10} supplementation noted (Christiansen et al., 2020). One more study assessed the effect of supplemental CoQ_{10} on total antioxidant status (measured as ferric reducing antioxidant power) and lipid peroxide concentration (measured as malondialdehyde) in dogs with chronic valvular heart disease (synonym of MMVD; Revathi et al., 2020). The authors confirmed the reducing effect of the supplementation on ferric reducing antioxidant power, but in that study, no placebo or non-treated control MMVD group was included and no plasma CoQ_{10} measurements were

conducted (Revathi et al., 2020). To conclude, no long-term placebo-controlled studies have been performed to date assessing the effects of CoQ_{10} supplementation in dogs of different breeds with spontaneous MMVD. Additionally, out of four CoQ_{10} supplementation studies performed in dogs with heart disease, none assessed CoQ_{10} effects on multiple parameters of oxidative stress and immune status in patients with MMVD with or without CHF (Harker-Murray et al., 2000; Tachampa et al., 2018; Christiansen et al., 2020; Revathi et al., 2020).

Therefore, our aim was to investigate the effect of CoQ_{10} administered in addition to standard cardiac therapy on markers of oxidative stress (F2-isoprostanes concentration, glutathione peroxidase [GPX] activity), inflammatory markers (leukocyte concentration, C-reactive protein [CRP], TNF-α, and soluble TNF-α receptor II [TNFSR-II] concentrations), total lymphocyte percentage and concentration, lymphocyte subtypes (CD21+ B lymphocytes, CD3+ T lymphocytes and their subtypes, CD3+CD4+ T lymphocytes, and CD3+CD8+ T lymphocytes) percentages and concentrations, CD3+CD4+/CD3+CD8+ ratio, selected echocardiographic parameters (left atrium to aorta ratio, normalized left ventricular end-diastolic and end-systolic diameters, early and late transmitral blood flow and their ratio (E/A), tricuspid regurgitation peak gradient, fractional shortening), and clinical status in dogs of different breeds diagnosed with MMVD with or without CHF.

Because there were no data on lymphocyte subtypes in dogs with different stages of MMVD and no recommendation regarding the dosage of supplemental CoQ_{10} in dogs with MMVD, the interim objective was to investigate these before the main study of three months of supplementation with CoQ_{10}.

The research work was comprised of three parts:

1. Flow cytometric study (Research article No 1);

2. Coenzyme Q_{10} dose-ranging study (Research article No 2);

3. Coenzyme Q_{10} 3-month supplementation study (Research article No 3).

1.2 AIM OF THE STUDY AND HYPOTHESES

The hypotheses tested in our research were as follows:

1. Dogs in different stages of MMVD have altered lymphocyte subtype proportions compared to healthy dogs, namely, decreased percentage of B lymphocytes (CD21+) and CD4+ T lymphocytes (T helper cells) as well as decreased CD4+/CD8+ ratio and increased percentage of CD8+ T lymphocytes (cytotoxic T lymphocytes); these alterations are especially evident in unstable heart failure.

2. CoQ_{10} as a dietary supplement improves the clinical status of the affected dog by decreasing oxidative stress (F2-isoprostanes, GPX) and inflammatory markers (leukocytes, CRP, TNF-α and TNFSR-II) and affecting the number of lymphocytes and proportions of lymphocyte subtypes in terms of an increase in total lymphocyte count, B lymphocyte (CD21+) and CD4+ T lymphocyte percentage, and CD4+/CD8+ ratio, and a decrease in CD8+ T lymphocyte percentage and percentage of activated (CD4+CD25+ and CD8+CD25+) lymphocytes.

2 PUBLISHED RESEARCH ARTICLES

2.1 PERIPHERAL BLOOD LYMPHOCYTE SUBTYPES IN DOGS WITH DIFFERENT STAGES OF MYXOMATOUS MITRAL VALVE DISEASE

Podvrste limfocitov v periferni krvi pri psih z različnimi stopnjami miksomatozne degeneracije mitralne zaklopke

Druzhaeva N, Nemec Svete A, Ihan A, Pohar K, Domanjko Petrič A. Peripheral blood lymphocyte subtypes in dogs with different stages of myxomatous mitral valve disease. J Vet Intern Med 2021; 35(5): 2112–22. doi: 10.1111/jvim.16207.

Izvleček v slovenskem jeziku / Abstract in Slovene language

Ozadje: Podatkov o spremembah podvrst limfocitov v periferni krvi pri psih z miksomatozno degeneracijo mitralne zaklopke (MDMZ) je malo.

Cilji: Proučiti podvrste limfocitov v periferni krvi in njihovo korelacijo s parametri vnetja in označevalci napredovanja MDMZ pri psih z različnimi stopnjami bolezni.

Živali: 78 lastniških psov: 65 z MDMZ v stopnjah B2, C in D po klasifikaciji ACVIM (American College of Veterinary Internal Medicine) in 13 zdravih psov v kontrolni skupini.

Metode: Prospektivna presečna raziskava. Opravili smo kardiološki pregled, pretočno citometrijo za določitev posameznih podvrst limfocitov (limfocitov T [CD3+, CD3+CD4+, CD3+CD8+, CD3+CD4+CD8+, CD3+CD4-CD8-] in limfocitov B [CD45+CD21+]) ter merjenje koncentracije N-terminalnega natriuretičnega peptida tipa pro-B, srčnega troponina I in C-reaktivnega proteina.

Rezultati: Odstotek limfocitov CD3+CD4+ je bil značilno nižji pri psih v stabilnem srčnem popuščanju ACVIM C (P = 0,01) in nestabilnem srčnem popuščanju ACVIM C in D (P = 0,003), odstotek limfocitov CD3+CD8+ je bil značilno višji pri psih v stabilnem srčnem popuščanju ACVIM C (P = 0,01) in nestabilnem srčnem popuščanju ACVIM C in D (P = 0,01), koncentracija limfocitov CD3+CD8+ je bila značilno višja pri nestabilnem srčnem popuščanju ACVIM C in D (P = 0,05), razmerje CD3+CD4+/CD3+CD8+ pa je bilo značilno nižje pri stabilnem srčnem popuščanju ACVIM C (P = 0,01) in nestabilnem srčnem popuščanju ACVIM C in D (P = 0,01) v primerjavi z zdravimi kontrolnimi psi.

Zaključki in klinični pomen: Odstotki limfocitov CD3+CD4+ in CD3+CD8+ ter razmerje CD4+/CD8+ so bili spremenjeni pri psih z MDMZ v kongestivnem srčnem popuščanju (ACVIM C, D), ne pa pri psih v stopnji ACVIM B2, kar kaže na vpletenost teh podvrst limfocitov v patogenezo kongestivnega srčnega popuščanja pri psih z MDMZ.

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STANDARD ARTICLE

Journal of Veterinary Internal Medicine Open Access ACVIM American College of Veterinary Internal Medicine

Peripheral blood lymphocyte subtypes in dogs with different stages of myxomatous mitral valve disease

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Abstract

Background: Data on alterations in peripheral blood lymphocyte (PBL) subtypes in dogs with myxomatous mitral valve disease (MMVD) is lacking.

Objectives: To investigate PBL subtypes and their correlation with parameters of inflammation and MMVD progression markers in dogs with different stages of MMVD.

Animals: Seventy-eight client-owned dogs: 65 with MMVD (American College of Veterinary Internal Medicine [ACVIM] classification stages B2, C, and D) and 13 healthy controls.

Methods: Prospective cross-sectional study. Complete cardiac assessment, flow cytometry (T lymphocytes [CD3+], their subtypes [CD3+ CD4+, CD3+ CD8+, CD3+ CD4+ CD8+, CD3+ CD4−CD8−], and B lymphocytes [CD45+ CD21+]) and measurement of N-terminal pro B-type natriuretic peptide, cardiac troponin I, and C-reactive protein concentrations were performed.

Results: The percentage of CD3+ CD4+ lymphocytes was significantly lower in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .003$), the percentage of CD3+ CD8+ lymphocytes was significantly higher in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .01$), CD3+ CD8+ lymphocyte concentration was significantly higher in unstable ACVIM C and D patients ($P = .05$), and the CD3+ CD4+/CD3+ CD8+ ratio was significantly lower in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .01$) compared with healthy controls.

Conclusions and Clinical Importance: The percentages of CD3+ CD4+ and CD3+ CD8+ PBL and CD4+/CD8+ ratio were altered in MMVD dogs with congestive heart failure (ACVIM C, D), but not in ACVIM B2, suggesting involvement of these PBL subtypes in the pathogenesis of congestive heart failure in dogs with MMVD.

Abbreviations: ACVIM, American College of Veterinary Internal Medicine; CHF, congestive heart failure; CRP, C-reactive protein; cTnI, cardiac troponin I; DCM, dilated cardiomyopathy; DN T, double negative T (lymphocytes); DP T, double positive T (lymphocytes); MMVD, myxomatous mitral valve disease; NT-proBNP, N-terminal pro B-type natriuretic peptide; PBL, peripheral blood lymphocyte; WBC, white blood cell.

KEYWORDS
canine, CD21 B lymphocytes, CD3 T lymphocytes, CD4 T helper lymphocytes, CD45, CD8 cytotoxic T lymphocytes, congestive heart failure, degenerative mitral valve disease, flow cytometry

1 | INTRODUCTION

Myxomatous mitral valve disease (MMVD) is common in older dogs,[1] and can progress over time to congestive heart failure (CHF). Several pathophysiological mechanisms are involved in the development and progression of CHF, including neurohumoral activation, immune activation, and inflammation, the latter being under intensive investigation in humans with heart failure.[2-6] Studies have shown that inflammation is also present in dogs with CHF.[7-11] One of the components of the inflammatory response is adaptive immunity, which consists of T lymphocytes (CD3+) including their subtypes, T helper (CD4+) and cytotoxic T lymphocytes (CD8+) and B lymphocytes (CD21+), which are the major subtypes of peripheral blood lymphocytes (PBLs). Lymphocytes are known to orchestrate inflammation by controlling other inflammatory cells and their responses. Their role in the context of cardiovascular disease has been studied extensively in mice and humans, and they are known to promote inflammation of the heart, apoptosis, fibrosis, and remodeling.[12-15] Possibly reflecting this involvement, lymphocytes and their subtype concentrations and percentages have been found to be altered in peripheral blood in people with cardiovascular diseases.[16-19]

In contrast to studies in mice and humans, a single study has investigated changes in PBL subtypes in dogs with cardiac disease.[20] It was found that the percentages of both CD4+ peripheral blood mononuclear cells and CD8+ lymphocytes were decreased in dogs with heart failure caused by dilated cardiomyopathy (DCM) and MMVD compared to healthy dogs.[20] Another study,[21] presented only as an abstract, found a decrease in the percentage of CD4+ and an increase in the percentage of CD8+ lymphocyte subtypes in German shepherds with primary mitral insufficiency.

Our hypothesis was that PBL subtypes in dogs with MMVD (American College of Veterinary Internal Medicine [ACVIM] stages B2, C, D) change with the progression of the disease and differ from those of healthy dogs and that these changes are more pronounced in advanced disease. Therefore, our aim was to investigate changes in the concentrations and percentages of PBL subtypes by multicolor flow cytometry and to correlate PBL subtypes with a biomarker of heart disease severity (N-terminal pro-B type natriuretic peptide, [NT-proBNP]), a biomarker of myocardial damage (cardiac troponin I [cTnI]), the acute phase protein, C-reactive protein (CRP), and with neutrophils and monocytes as cells representing innate immunity, and selected echocardiographic and clinical variables.

2 | MATERIALS AND METHODS

2.1 | Study population

Seventy-eight client-owned dogs were included in this prospective cross-sectional study. Of these, 65 were dogs with various stages of MMVD with or without CHF and 13 were healthy control dogs. Dogs were presented to the cardiology service of the small animal clinic for evaluation (MMVD patients) or health assessment (control dogs) between August 2018 and November 2019.

We included dogs with MMVD of ACVIM classes B2, C, and D.[1] We excluded dogs with concomitant systemic disease (including metabolic, neoplastic, and inflammatory diseases), as well as those with local inflammatory conditions, and those that had received systemic or local immunosuppressive treatment or antibiotics in the past month. We excluded patients as necessary based on history, clinical examination findings, results of hematologic and biochemical analyses, echocardiography, and, if indicated, thoracic radiographs, ECG, abdominal ultrasound findings, and urinalysis. Dogs with asymptomatic MMVD without signs of cardiac remodeling (ACVIM stage B1) were not included. Diagnosis of MMVD and heart failure was made by an experienced veterinarian based on history, clinical examination findings, thoracic radiographs, ECG, and echocardiography using 2-dimensional, M-mode, color, and spectral Doppler modes (GE Vivid E9, General Electric Healthcare). Routine hematologic and biochemical analyses were performed in each dog.

We included healthy small- and medium-sized breed dogs of both sexes older than 5 years of age. They were considered healthy on the basis of clinical examination, echocardiography, and routine hematological and biochemical analyses.

Written informed consent was signed by the owners before enrollment in the study.

2.2 | Groups of MMVD patients in the study

The ACVIM classification was used to divide MMVD dogs into 3 groups. The ACVIM B2 group included asymptomatic treated or untreated dogs with cardiac remodeling and no signs of CHF. The second group included treated dogs in ACVIM stage C, but with stable (compensated) CHF only. The third group included treated dogs in ACVIM stages C and D with advanced decompensated (unstable) CHF, according to the presence of clinical signs such as tachypnea, tachycardia, radiographic signs of pulmonary edema, and echocardiographic signs of increased left atrial pressure.

2.3 | Blood sampling

Blood obtained by jugular or cephalic venipuncture was collected in tubes containing ethylenediaminetetraacetic acid (for CBC with white blood cell [WBC] differential count, flow cytometry, and NT-proBNP) and serum separator tubes (for routine biochemical, cTnI and CRP

analyses). Samples for routine blood analyses (CBC and WBC differential count, biochemistry) were processed by the in-house laboratory within 2 hours. Samples for flow cytometry were stored in the dark at room temperature (approximately 21°C) and analyzed within 24 hours. Samples for NT-proBNP, cTnI, and CRP measurements were centrifuged immediately at 1500*g* at 4°C for 15 minutes (for NT-proBNP) or after complete coagulation at 1300*g* at room temperature for 10 minutes (for cTnI and CRP). Obtained plasma (for NT-proBNP) and serum (for cTnI and CRP) samples were separated and stored at −80°C until analysis.

2.4 | Routine hematological and biochemistry analyses

Hematological analyses were performed using an automated laser-based hematology analyzer (ADVIA 120, Siemens, Munich, Germany). Blood biochemistry was performed using an automated biochemistry analyzer (RX Daytona, Randox, Crumlin, United Kingdom; glucose, urea, creatinine, alanine aminotransferase, alkaline phosphatase, total protein, albumin) and electrolyte analyzer (ILyte, Instrumentation Laboratory, Lexington, Massachusetts; sodium, potassium, chloride).

2.5 | Flow cytometry

Multicolor flow cytometry was used to analyze fresh whole blood samples and determine the percentages of T lymphocytes (CD3+), T helper lymphocytes (CD3+CD4+), cytotoxic T lymphocytes (CD3+CD8+), double positive T (DP T) lymphocytes (CD3+CD4+CD8+), double negative T (DN T) lymphocytes (CD3+CD4−CD8−), and B lymphocytes (CD45+CD21+). Cells were labeled with monoclonal rat and mouse anti-canine antibodies against CD3 (clone CA17.2A12), CD4 (clone YKIX302.9), CD8 (clone YCATE55.9) in a mix (CD3:FITC/CD4:RPE/CD8:Alexa Fluor 647, ref: TC014), CD45 (clone YKIX716.13; Alexa Fluor 488, ref: MCA1042A488), and CD21 (clone CA2.1D6; Alexa Fluor 647, ref: MCA1781A647). All monoclonal antibodies used were manufactured by Bio-Rad Laboratories Inc (Hercules, California). The whole blood lysis method was used according to the manufacturer's protocol.[22] Blood samples were incubated for 30 minutes at 2°C to 8°C with a commercially available triple color cocktail of monoclonal antibodies against CD3, CD4, CD8 in 1 tube and a combination of anti-CD45 and anti-CD21 monoclonal antibodies in another tube. Erythrocytes were lysed using a commercially available red blood cell lysing solution (BD FACS Lysing Solution; BD Biosciences, San Jose, California; ref: 349202) according to the manufacturer's instructions. The samples were centrifuged and the supernatant discarded. Then, 0.1% bovine serum albumin (ref: A9418; Sigma-Aldrich, St. Louis, Missouri)/phosphate-buffered saline (ref: P4417; Sigma-Aldrich) was added and, after centrifugation, the cells were resuspended in phosphate-buffered saline solution and analyzed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, California) with FACSDiva software, version 8.0.1 (BD Biosciences). The flow cytometer was calibrated using BD FACSDiva CS&T Research Beads (BD Biosciences). Compensation controls were performed to correct for fluorescence spillover.

Forward and side scatters were used to gate PBLs based on their size and granularity. For each sample, data for 100 000 events in the lymphocyte gate were acquired for the first tube (CD3, CD4, CD8), and data for 50 000 events were acquired for another tube (CD45, CD21). Forward scatter, side scatter, and fluorescence were used to calculate percentages of lymphocyte subtypes. The absolute concentrations of PBL subtypes were calculated based on CBC and WBC differential count and flow cytometry results.

2.6 | Circulating biomarkers

Plasma NT-proBNP concentrations (pmol/L) were measured using an IDEXX ELISA (IDEXX Laboratories, Leipzig, Germany). Serum cTnI concentrations (μg/L) were measured using a high-sensitivity immunoassay (ADVIA Centaur TnI-Ultra; Siemens). Serum CRP concentrations (ng/mL) were measured using an ELISA (Canine CRP ELISA; Alpco, Salem, New Hampshire).

2.7 | Statistical analysis

Data were analyzed using commercially available software (IBM SPSS 24.0, Chicago, Illinois). The Shapiro-Wilk test was used to determine data distribution. On the basis of the findings, parametric tests or nonparametric tests were used to compare data among groups of dogs. Accordingly, 1-way analysis of variance with post hoc Tukey honestly significant difference (for normally distributed data) or Kruskal-Wallis test followed by pairwise comparisons and Bonferroni adjustments (for non-normally distributed data) were performed to compare variables between groups of patients and healthy dogs. Sex differences were evaluated using Chi-squared analyses. Correlations between variables were performed using the Spearman test (nonparametric data) or Pearson test (parametric data). Results for non-normally distributed data are reported as median and interquartile range and for normally distributed data as mean and SD. For the purpose of correlation analyses, MMVD dogs were divided into 2 groups: ACVIM B2 group and CHF group (all dogs with ACVIM C and D combined). Values of $P < .05$ were considered significant.

3 | RESULTS

One-hundred seventy-three MMVD patients were screened for eligibility, 108 of which were not included in the study because they did not meet the inclusion criteria (n = 99) or the owners did not want to participate in the study (n = 9). At the time of enrollment, 28 healthy dogs were recruited and tested for suitability as control dogs, of which 15 were excluded because they did not meet the inclusion criteria (because of subclinical heart disease [n = 13] or other

pathology [n = 2]). A total of 65 MMVD dogs and 13 healthy control dogs were included in the study. Baseline characteristics of the included dogs are shown in Table 1.

There was a significant age difference between each group of MMVD patients and healthy control dogs, with the healthy dogs being significantly younger than ACVIM B2 ($P = .02$), stable ACVIM C ($P < .001$), and unstable ACVIM C and D dogs ($P = .001$), but no significant age difference was found among the 3 groups of MMVD patients ($P > .05$). It was difficult to include old healthy dogs and many were rejected because of various health problems (mostly asymptomatic MMVD). No significant differences in sex ($P = .12$) and weight ($P > .05$) were found between the cardiac patient and healthy dog groups.

Lymphocyte and their subtype concentrations and percentages are presented in Table 2 and Figures 1 and 2. The percentage of CD3+CD4+ lymphocytes was significantly lower in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .003$) compared with healthy dogs, but no significant differences in CD3+CD4+ lymphocyte concentrations were found between the patient and healthy dog groups. The percentage of CD3+CD8+ lymphocytes was significantly higher in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .01$) compared with healthy controls, and the CD3+CD8+ lymphocyte concentration was significantly higher in unstable ACVIM C and D patients compared with healthy dogs ($P = .05$). The CD3+CD4+/CD3+CD8+ ratio was significantly lower in stable ACVIM C patients ($P = .01$) and unstable ACVIM C and D patients ($P = .01$) compared with healthy dogs. The CD3+CD4+CD8+ and CD3+CD4−CD8− lymphocyte percentages and concentrations were not significantly different between cardiac patient and healthy dog groups. The percentages and concentrations

TABLE 1 Baseline characteristics of 65 dogs with MMVD and 13 healthy dogs

Group	ACVIM B2	ACVIM C stable	ACVIM C and D unstable	Healthy dogs
Number	20	24	21	13
Sex (f/m)	6/14	7/17	11/10	8/5
Spayed/neutered	6/6	2/7	9/2	4/3
Age (years)				
Mean ± SD	10.0 ± 2.1[a]	11.2 ± 1.8[a]	10.7 ± 1.8[a]	7.9 ± 2.2
Range (min-max)	6.0-14.2	7.6-14.3	7.4-13.5	5.0-12.4
Weight (kg)				
Median (IQR)	7.9 (6.1-12.3)	8.3 (5.5-12.5)	7.6 (5.0-10.9)	7.0 (5.1-13.3)
Range (min-max)	3.0-16.8	4.4-33.0	2.4-44.7	2.8-13.5
HR (bpm)				
Mean ± SD	116.0 ± 17.6	128.3 ± 21.2	140.2 ± 20.2	111.5 ± 32.6
Range (min-max)	80.0-150.0	90.0-180.0	100.0-180.0	70.0-200.0
Breeds	4 CKCS, 2 MB, 2 CHI, 2 SHI, 2 TT, 2 WH, 1 PEK, 1 YT, 1 MLT, 1 ECS, 1 STF, 1 CC	8 MB, 4 CKCS, 3 MP, 2 PEK, 1 CHI, 1 YT, 1 TT, 1 MLT, 1 ECS, 1 POM, 1 APBT	7 CKCS, 3 MB, 2 CHI, 1 PEK, 1 YT, 1 MLT, 1 AT, 1 JCH, 1 COTON, 1 IG, 1 DH, 1 GSD	6 MB, 4 SHI, 1 YT, 1 TS, 1 MSCH
Treatment				
Pimobendan	9	24	21	–
ACE inhibitor	6	24	21	–
Furosemide/ torasemide	–	24	21	–
Spironolactone	3	11	9	–
Theophylline	–	2	1	–
Sildenafil	–	1	1	–
Amlodipine	–	1	1	–
Digoxin	–	1	1	–
Potassium chloride	–	1	–	-

Abbreviations: ACE inhibitor, angiotensin-converting enzyme inhibitor; ACVIM, American College of Veterinary Internal Medicine; APBT, American Pit Bull Terrier; AT, Airedale Terrier; bpm, beats per minute; CC, Chinese Crested Dog; CHI, Chihuahua; CKCS, Cavalier King Charles Spaniel; COTON, Coton de Tulear; DH, Dachshund; ECS, English Cocker Spaniel; f, female; GSD, German Shepherd; HR, heart rate; IG, Italian Greyhound; IQR, interquartile range; JCH, Japanese Chin; m, male; MB, mixed breed dog; MLT, Maltese; MMVD, myxomatous mitral valve disease; MP, Miniature Poodle; MSCH, Miniature Schnauzer; PEK, Pekingese; POM, Pomeranian; SHI, Shi Tzu; STF, Staffordshire Terrier; TS, Tibetan Spaniel; TT, Tibetan Terrier; WH, Whippet; YT, Yorkshire Terrier.

[a]Significant difference in comparison to healthy dogs.

TABLE 2 Lymphocytes and their subtype's percentages and concentrations in dogs with MMVD and healthy dogs

Group	ACVIM B2	ACVIM C stable	ACVIM C and D unstable	Healthy dogs
Lymphocytes, total (%)				
Mean ± SD	21.84 ± 6.62	20.19 ± 6.38	21.19 ± 7.09	22.60 ± 4.87
Range (min-max)	11.10-31.70	7.30-32.70	6.80-32.90	15.30-32.40
Lymphocytes, total ($\times 10^9$/L)				
Mean ± SD	1.74 ± 0.64	2.01 ± 0.70	2.11 ± 0.61	1.80 ± 0.60
Range (min-max)	0.80-3.00	0.86-3.87	1.25-3.26	0.98-2.97
CD3 (%)				
Median (IQR)	69.05 (60.38-75.00)	67.25 (51.95-74.00)	68.00 (52.65-74.30)	60.10 (53.50-69.50)
Range (min-max)	38.70-82.70	25.10-83.50	12.60-82.70	41.80-76.00
CD3+ ($\times 10^9$/L)				
Mean ± SD	1.16 ± 0.47	1.24 ± 0.46	1.27 ± 0.53	1.10 ± 0.40
Range (min-max)	0.39-2.23	0.36-2.07	0.35-2.43	0.61-1.81
CD3+CD4+ (%)				
Mean ± SD	47.91 ± 11.66	43.55 ± 8.48[a]	41.78 ± 12.49[a]	55.40 ± 8.91
Range (min-max)	17.00-70.70	31.00-58.50	17.10-65.80	42.80-71.80
CD3+CD4+ ($\times 10^9$/L)				
Mean ± SD	0.56 ± 0.28	0.54 ± 0.22	0.50 ± 0.21	0.60 ± 0.20
Range (min-max)	0.18-1.28	0.14-0.99	0.17-0.93	0.37-1.00
CD3+CD8+ (%)				
Mean ± SD	35.62 ± 12.67	39.66 ± 11.47[a]	40.67 ± 15.70[a]	26.05 ± 8.48
Range (min-max)	16.50-73.20	20.90-61.60	17.70-76.70	13.70-41.50
CD3+CD8+ ($\times 10^9$/L)				
Mean ± SD	0.40 ± 0.19	0.50 ± 0.24	0.55 ± 0.41[a]	0.29 ± 0.15
Range (min-max)	0.12-0.78	0.17-0.93	0.10-1.86	0.08-0.58
CD3+CD4+/CD3+CD8+				
Median (IQR)	1.36 (1.04-2.08)	1.00[a] (0.75-1.71)	1.14[a] (0.60-1.84)	2.28 (1.45-3.20)
CD3+CD4+CD8+ (%)				
Median (IQR)	1.65 (1.10-2.55)	1.35 (0.83-3.15)	1.10 (0.90-1.80)	1.20 (0.90-1.55)
CD3+CD4+CD8+ ($\times 10^9$/L)				
Median (IQR)	0.02 (0.01-0.04)	0.02 (0.01-0.03)	0.01 (0.01-0.02)	0.01 (0.01-0.03)
CD3+CD4−CD8− (%)				
Mean ± SD	14.64 ± 5.52	14.20 ± 5.37	16.20 ± 7.77	17.15 ± 4.66
Range (min-max)	6.20-26.30	4.30-22.50	5.30-36.10	9.20-24.80
CD3+CD4−CD8− ($\times 10^9$/L)				
Median (IQR)	0.14 (0.09-0.26)	0.17 (0.11-0.24)	0.14 (0.11-0.30)	0.15 (0.10-0.32)
CD45+CD21+ (%)				
Mean ± SD	16.70 ± 6.68	18.63 ± 9.08	16.74 ± 7.08	15.08 ± 6.39
Range (min-max)	8.40-36.60	4.80-41.20	3.30-27.90	7.00-28.50
CD45+CD21+ ($\times 10^9$/L)				
Mean ± SD	0.30 ± 0.17	0.39 ± 0.27	0.35 ± 0.19	0.28 ± 0.17
Range (min-max)	0.11-0.67	0.06-1.19	0.09-0.91	0.09-0.68

Abbreviations: ACVIM, American College of Veterinary Internal Medicine; IQR, interquartile range; MMVD, myxomatous mitral valve disease.
[a]Significant difference ($P < .05$) in comparison to healthy dogs.

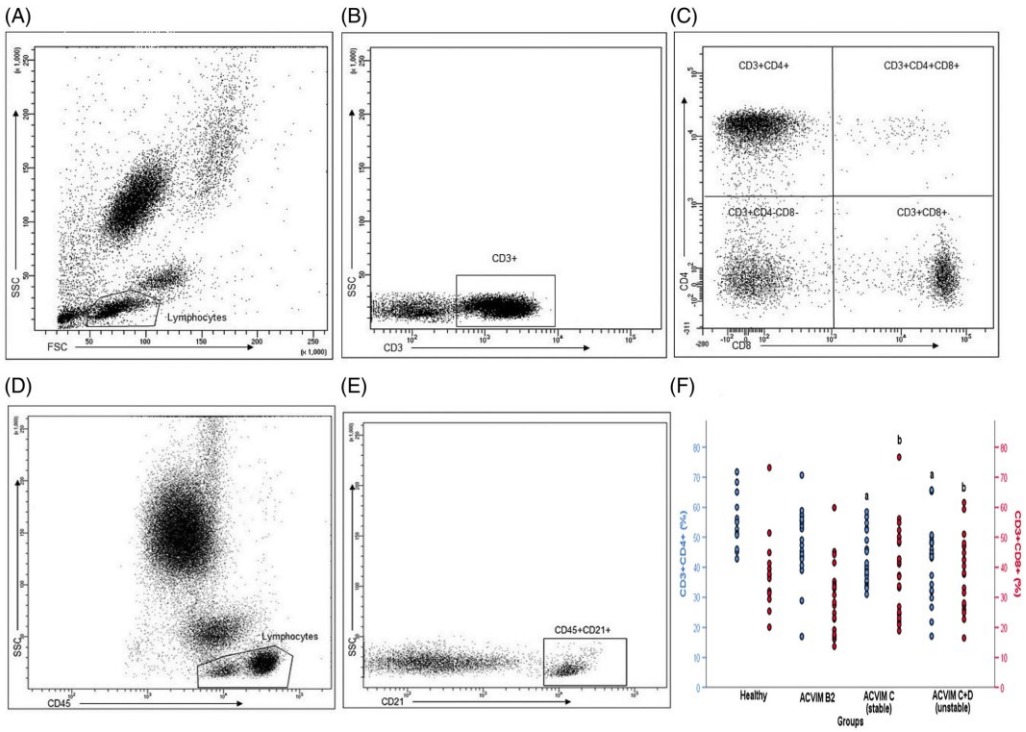

FIGURE 1 Immunophenotyping of canine PBL subtypes (representative sample) with gating strategies for total lymphocytes (SSC/FSC) (A), CD3+ T lymphocytes (B), CD3+CD4+ T helper lymphocytes, CD3+CD8+ cytotoxic T lymphocytes, CD3+CD4+CD8+ DP T lymphocytes, CD3+CD4−CD8− DN T lymphocytes (C), CD45+ lymphocytes (D), CD45+CD21+ B lymphocytes (E) are presented. Scatter plots showing CD3+CD4+ T helper lymphocyte and CD3+CD8+ cytotoxic T lymphocyte percentages in healthy controls and dogs in different stages of MMVD (F). [a]The median values of the stable and unstable CHF patients were significantly lower ($P < .05$) than that of the healthy dogs. [b]The median values of the stable and unstable CHF patients were significantly higher ($P < .05$) than that of the healthy dogs. CD, cluster of differentiation; CHF, congestive heart failure; DN T, double negative T; DP T, double positive T; FSC, forward scatter; MMVD, myxomatous mitral valve disease; PBL, peripheral blood lymphocyte; SSC, side scatter

of CD3+ and CD45+CD21+ lymphocytes and the percentages and concentrations of total lymphocytes did not differ significantly between the patient and healthy dog groups.

The percentages of neutrophils and monocytes (Table 3) did not differ significantly between the patient and healthy dog groups. Neutrophil concentration (Table 3) was significantly higher in unstable ACVIM C and D patients compared with ACVIM B2 patients ($P = .03$), and monocyte concentration (Table 3) was significantly higher in unstable ACVIM C and D patients compared with healthy dogs ($P = .03$) and ACVIM B2 patients ($P = .04$). Total WBC concentration (Table 3) was significantly higher in unstable ACVIM C and D patients compared with healthy dogs ($P = .04$) and ACVIM B2 stage dogs ($P = .02$).

Plasma NT-proBNP concentrations (Table 3) differed significantly between all groups of patients and healthy dogs with higher concentrations in advanced disease, except for ACVIM B2 compared to healthy dogs. Dogs in stable ACVIM C had significantly higher NT-proBNP concentrations than did healthy dogs ($P = .003$) and dogs in ACVIM B2 ($P = .002$). Dogs in the unstable ACVIM C and D groups had higher NT-proBNP concentrations than did healthy dogs ($P < .001$), ACVIM B2 patients ($P < .001$), and stable ACVIM C patients ($P = .04$). Serum cTnI concentrations (Table 3) were significantly higher in unstable ACVIM C and D patients compared with healthy dogs ($P = .01$) and ACVIM B2 patients ($P = .001$). Serum CRP concentrations (Table 3) did not differ significantly between groups of MMVD patients and healthy dogs.

The concentration of CD3+CD4+ lymphocytes correlated significantly and negatively with the percentage of neutrophils in CHF patients (combined stages stable ACVIM C and unstable ACVIM C and D; $r = −0.36$, $P = .01$) and in ACVIM B2 patients ($r = −0.533$, $P = .02$), but not in healthy dogs, and positively with monocyte concentration ($r = 0.318$, $P = .03$) in CHF dogs, but not in ACVIM B2 and

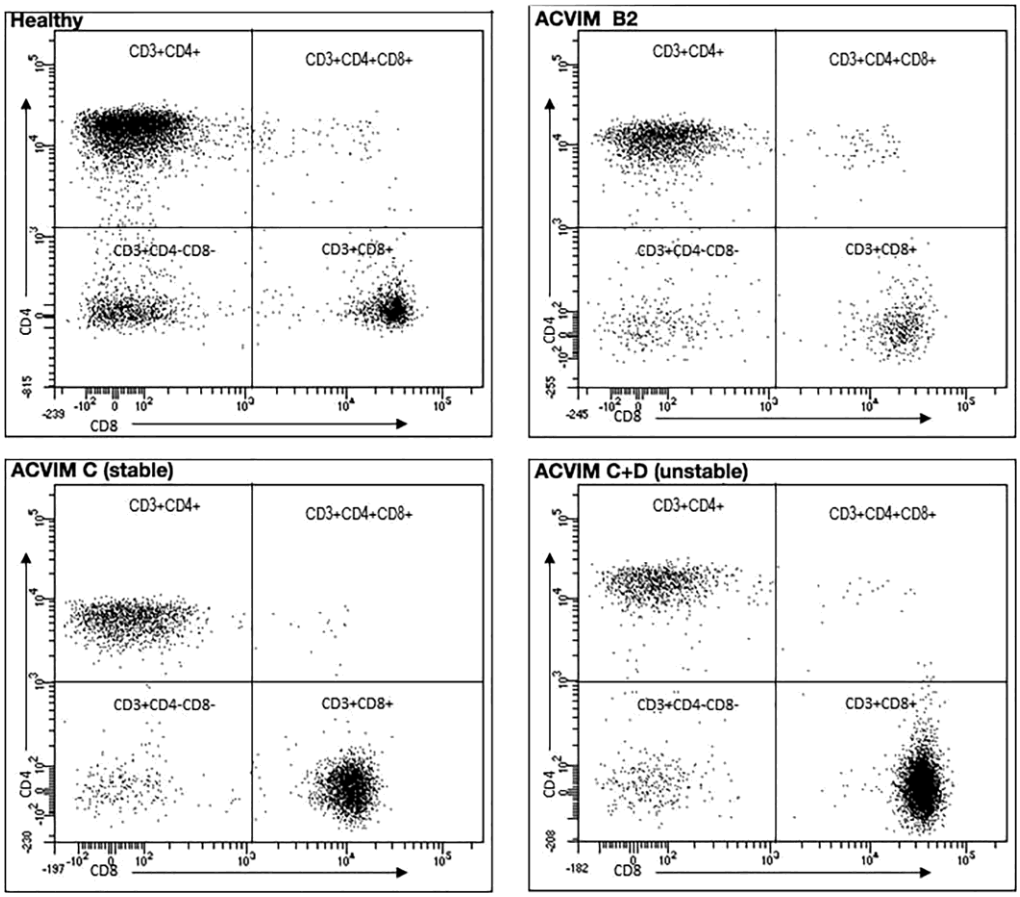

FIGURE 2 Representative flow cytometry plots showing CD3+CD4+ T helper lymphocytes, CD3+CD8+ cytotoxic T lymphocytes, CD3+CD4+CD8+ DP T lymphocytes, and CD3+CD4−CD8− DN T lymphocytes in groups of dogs included in our study—healthy control dogs and dogs with different stages of myxomatous mitral valve disease. DN T, double negative T (lymphocytes); DP T, double positive T (lymphocytes)

healthy controls. The CD3+CD4+CD8+ lymphocyte concentration correlated positively with serum CRP concentration ($r = 0.343$, $P = .02$) in CHF patients, whereas no such correlation was found in ACVIM B2 and healthy controls. The CD3+ lymphocyte concentration correlated negatively with neutrophil percentage in CHF dogs ($r = -0.397$, $P = .01$) and in ACVIM B2 stage ($r = -0.672$, $P = .001$), but not in healthy controls, and positively with monocyte concentration in CHF ($r = 0.328$, $P = .03$), but not in ACVIM B2 or healthy subjects. A significant negative correlation was found between CD45+CD21+ lymphocyte concentration and neutrophil percentage in CHF dogs ($r = -0.406$, $P = .01$) and ACVIM B2 dogs ($r = -0.564$, $P = .01$), but not in healthy dogs. Total lymphocyte percentage correlated negatively with serum CRP concentration in CHF dogs ($r = -0.370$, $P = .01$) and ACVIM B2 dogs ($r = -0.555$, $P = .01$), but not in healthy dogs, and with WBC concentration ($r = -0.563$, $P = .00$) and heart rate ($r = -0.352$, $P = .02$) only in CHF dogs, but not in ACVIM B2 or healthy dogs. Total lymphocyte concentration correlated positively with E wave velocity ($r = 0.316$, $P = .04$) and A wave velocity ($r = 0.330$, $P = .04$) in dogs with CHF, but not in ACVIM B2 or healthy dogs.

In our study, correlations between PBL subtypes and NT-proBNP were not found in any of the MMVD groups or in healthy control dogs. Cardiac troponin I correlated significantly and negatively with the percentage of CD3+CD4−CD8− lymphocytes ($r = -0.451$, $P = .05$) and the concentration of CD45+CD21+ lymphocytes ($r = -0.464$, $P = .04$) in the ACVIM B2 group, but no correlations were found between cTnI and PBL subtypes in dogs with heart failure (combined groups of stable and unstable CHF patients) or in healthy subjects.

TABLE 3 Markers of MMVD progression and inflammation in dogs with MMVD and healthy dogs

Group	ACVIM B2	ACVIM C stable	ACVIM C and D unstable	Healthy dogs
NT-proBNP (pmol/L)				
Median	1324	3122[a,b]	10112[a,b,c]	1428
IQR	880-2107	2284-5599	4888-16586	1031-1742
Cardiac TnI (μg/L)				
Median	0.044	0.082	0.132[a,b]	0.025
IQR	0.032-0.082	0.043-0.130	0.067-0.259	0.012-0.212
CRP (mg/L)				
Median	1.653	2.704	3.559	1.681
IQR	1.022-2.750	1.403-5.485	1.656-6.555	1.164-3.122
WBC ($\times 10^9$/L)				
Mean ± SD	8.11 ± 2.10	10.23 ± 2.56	11.10 ± 4.83[a,b]	8.05 ± 2.26
Range (min-max)	4.01-11.89	5.77-16.20	5.55-23.65	5.14-12.09
Neutrophils (%)				
Mean ± SD	68.09 ± 8.13	70.18 ± 7.63	69.13 ± 7.74	68.55 ± 5.56
Range (min-max)	47.50-79.70	57.50-84.00	57.80-83.90	59.60-77.80
Neutrophils ($\times 10^9$/L)				
Mean ± SD	5.51 ± 1.64	7.27 ± 2.27	7.93 ± 4.30[b]	5.51 ± 1.52
Range (min-max)	2.96-9.32	3.53-11.36	3.31-19.80	3.21-7.86
Monocytes (%)				
Mean ± SD	4.83 ± 1.66	5.16 ± 1.09	5.69 ± 1.36	4.92 ± 2.67
Range (min-max)	2.10-8.80	2.50-6.90	3.10-8.00	2.50-10.60
Monocytes ($\times 10^9$/L)				
Median	0.36	0.48	0.49[a,b]	0.33
IQR	0.23-0.51	0.38-0.69	0.39-0.83	0.25-0.48

Abbreviations: ACVIM, American College of Veterinary Internal Medicine; cardiac TnI, cardiac troponin I; CRP, C-reactive protein; IQR, interquartile range; MMVD, myxomatous mitral valve disease; NT-proBNP, N-terminal pro B-type natriuretic peptide; WBCs, white blood cells.
[a]Significant difference ($P < .05$) in comparison to healthy dogs.
[b]Significant difference in comparison to dogs in ACVIM B2 stage.
[c]Significant difference in comparison to dogs in stable ACVIM C stage.

In addition, serum cTnI concentration correlated positively with plasma NT-proBNP concentration ($r = 0.474$, $P = .001$), serum CRP concentration ($r = 0.641$, $P = .00$), monocyte concentration ($r = 0.358$, $P = .02$), and left atrium to aorta ratio ($r = 0.329$, $P = .03$) in dogs with CHF, but not in ACVIM B2 stage and healthy dogs. Serum CRP concentration correlated positively with WBC concentration ($r = 0.456$, $P = .002$), neutrophil percentage ($r = 0.369$, $P = .01$), neutrophil concentration ($r = 0.477$, $P = .001$), and monocyte concentration ($r = 0.430$, $P = .003$) in CHF patients whereas no such correlations were found in the other groups of dogs in our study.

4 | DISCUSSION

Our study showed that MMVD dogs in CHF (in stable ACVIM C stage and in unstable ACVIM stages C and D) had a significantly lower percentage of CD3+CD4+ (T helper) lymphocytes, a significantly higher percentage of CD3+CD8+ (cytotoxic T) lymphocytes, and consequently a significantly lower CD4/CD8 ratio compared with healthy dogs, while CD3+CD4+CD8+ (DP T), CD3+CD4−CD8− (DN T), and CD21+ (B) lymphocyte percentages or counts were not altered. Besides, dogs in unstable CHF had a significantly higher concentration of CD3+CD8+ (cytotoxic T) lymphocytes, monocytes, and total WBC compared with healthy dogs. These changes were not present in dogs with MMVD without CHF (ACVIM B2 stage). Therefore, we can consider these results indicative of a systemic immune and inflammatory response specifically associated with CHF in dogs with MMVD. In addition, we found several correlations suggesting that systemic inflammation is present in dogs with MMVD when they are in CHF. Our findings facilitate understanding the nature of MMVD and CHF in dogs.

Previous studies have shown that T lymphocytes recruited to the heart can support chronic inflammation and promote cardiomyocyte death, cardiac fibrosis, and adverse cardiac remodeling with subsequent deterioration of cardiac function.[13,14,23-27] The distinct roles of helper (CD4+) and cytotoxic (CD8+) T lymphocytes, which are the 2

major T lymphocyte subsets, have been investigated previously in experimental research, and it has been shown in a mouse model that under pathological conditions both CD4+ and CD8+ lymphocytes infiltrate the heart[14,23] and spread in the circulation.[14] The CD4+ lymphocytes have been shown to promote fibrosis and adverse cardiac remodeling,[12] in both ischemic[14] and nonischemic[13,23] heart failure. The B lymphocytes also play a role in the deterioration of cardiac function because large amounts of autoantibodies associated with increased apoptosis rates and increased circulating components of the complement system have been found in both ischemic and nonischemic heart failure.[15,24,28,29]

Previous studies in human cardiovascular patients showed alterations in CD4+ and CD8+ PBL subtypes. One study of end-stage CHF caused by coronary artery disease and DCM found an increased percentage of CD4+ lymphocytes and CD4+/CD8+ ratio in both groups of patients.[17] Another study found a lower percentage of CD4+ lymphocytes in CHF and an increased percentage of CD8+ lymphocytes in elderly CHF patients, compared to healthy controls.[19] As evidenced by research in humans, the changes in lymphocyte subpopulations are not uniform, which may reflect disease-specific changes, but some investigators suggest these changes may be the result of CHF rather than a specific disease.[17]

Alterations of these lymphocyte subtypes in peripheral blood also have been found in cardiac canine patients. In the first study, performed on 13 German Shepherd dogs with mitral regurgitation (most dogs in this study did not have mitral valve lesions typical of MMVD, as stated by the authors), similar findings to those of our study were seen: a lower percentage of CD4+ lymphocytes, a higher percentage of CD8+ lymphocytes, and a significantly lower CD4+/CD8+ ratio compared to healthy controls.[21] However, it is not clear from the data how many dogs were asymptomatic or in CHF. In contrast to our results, another study found that the percentages of both CD4+ and CD8+ cells were decreased and the CD4+/CB8+ ratio was not changed in dogs in heart failure compared to healthy controls.[20] The difference between these results and our results could be a consequence of the different populations studied, because they included dogs with MMVD and DCM, the latter typically being younger and of different breeds than dogs with MMVD, and the fact that the investigators stated as a limitation that the gated CD4+ cells could not be lymphocytes only, because they did not use antibody against CD3. However, the authors of that study found no differences between DCM and MMVD dogs in any variables and suggested that changes in CD4+ and CD8+ lymphocytes may be a consequence of heart failure and not a specific disease.[20] The mechanisms driving these changes in PBL subtypes have not been studied in canine CHF patients and have been studied in human medicine mainly in coronary artery disease, in which T-cell immunosenescence is a risk factor. The changes in CD4+ and CD8+ lymphocyte subtypes found in our study may be explained by immunosenescence characterized by decreases in some subtypes of T lymphocytes (such as naïve T-cells) and expansion of other T lymphocyte subtypes (such as CD8+ effector memory subsets).[30]

The CD3+ (T) and CD21+ (B) lymphocytes did not differ between the patient and healthy dog groups in our study. A decreased percentage of B lymphocytes was shown in human CHF patients.[19] In dogs with cardiac disease, the percentages of T and B lymphocytes have not been studied extensively, and in the only study[21] in which antibodies against T lymphocytes (CD3) and B lymphocytes (CD21) were used, no changes in these subpopulations were reported. It can therefore be assumed that the disproportions of lymphocyte populations are mainly seen in the T lymphocyte subtypes, whereas the total concentrations and percentages of T and B lymphocytes are not affected.

In contrast to studies in humans that showed either decreased total lymphocyte concentration or decreased lymphocyte percentage and found that lymphopenia correlated with disease severity and mortality in cardiovascular patients,[16-18,31,32] we found no decreased total lymphocyte concentration compared to healthy controls. Previous studies in dogs with CHF did not find lower total blood lymphocytes when comparing CHF patients (all classes combined) and control dogs.[11,20] However, when individual CHF classes were compared with healthy dogs, dogs with severe CHF were found to have significantly lower total lymphocyte counts than healthy dogs and dogs with mild and moderate CHF.[20] The difference between the results of that study and our results could be a result of different populations studied and different methods used.

In our study, we measured concentrations and percentages of CD3+CD4+CD8+ (DP T) and CD3+CD4−CD8− (DN T) lymphocytes in dogs with MMVD. These data obtained in canine cardiac patients have not been reported previously in the veterinary literature. And although these PBL subtypes did not differ between patient groups and control dogs, a significant positive correlation was found between DP T lymphocytes and serum CRP concentration in dogs with heart failure. The DP T lymphocytes have not been studied in cardiovascular disease in humans or dogs and are a cell subset whose role generally is poorly studied.[33] The DP T-cells in dogs have a phenotype, typical of activated T lymphocytes,[34,35] and they have characteristics of both CD4+ (either suppressor or regulatory phenotype) and CD8+ cells (cytotoxic phenotype).[36] They may play a role in autoimmune diseases, viral diseases and cancer,[35] and the correlation found in our study may indicate their role in inflammation in MMVD patients with CHF. Furthermore, it may be of interest that the percentage of DN T lymphocytes found both in cardiac patients and healthy dogs in our study was much higher than in healthy mice and humans, which typically have <5% of DN T-cells.[37,38] Two studies in healthy dogs[39,40] also found a high percentage of DN T lymphocytes in peripheral blood, approximately 10% in 1 study[39] and 15% in another study.[40] The reason for this species-specific difference remains to be investigated. It is known that DN T lymphocytes play a role in host defense, inflammation,[38] autoimmunity,[37] and immune tolerance by suppressing other T lymphocytes and possibly regulating B lymphocytes,[38] although to our knowledge, their role has not been studied in human or canine cardiovascular patients.

We found numerous correlations between various blood variables, including immune cells, and those associated with inflammation and the disease progression. Some of the correlations were only found in dogs with CHF and not in non-CHF (ACVIM B2) or healthy

dogs. Namely, in CHF group, serum CRP concentration correlated positively with DP T lymphocyte concentration and serum cTnI concentration, and total lymphocyte concentration correlated positively with mitral inflow E wave and A wave velocity. These associations may further support the hypothesis of the inflammatory nature and concurrent inflammatory and immunomodulatory changes in CHF.

One of the limitations of our study was the significantly younger age of healthy control dogs compared to the MMVD dogs, but all included healthy dogs were >5 years of age. Because of possible effects of age on the relative and absolute numbers of lymphocytes and their subtypes, with increasing age resulting in lower absolute numbers of all lymphocyte subtypes, lower percentages of CD3+CD4+ and CD21+ lymphocytes, and higher percentages of CD3+CD8+ lymphocytes,[41-44] we aimed to include age-matched control dogs. Initially, 28 age-matched, clinically healthy dogs were recruited for the study, but 15 were excluded after screening because of mitral or tricuspid regurgitation or both (n = 13), arrhythmias (n = 1), or eosinophilia (n = 1). It proved very difficult to find healthy dogs that matched MMVD dogs by age, breed, and weight. It is difficult to determine to what extent the age difference between control and MMVD dogs may have influenced the results of our study. The results of previous studies of PBL changes with age in dogs have been inconsistent regarding which PBL subtypes change with age and which do not. For example, some studies found that the percentage of CD4+ lymphocytes in healthy dogs decreased significantly with age,[41,42] whereas another study found no significant difference.[43] Similarly, the percentage of CD8+ lymphocytes was significantly higher in old dogs compared with young dogs in some studies,[41,44] whereas in other studies this difference was not reported[42] or was reported only for females.[43] Similar discrepancies exist for the absolute concentrations of these PBL subtypes and for the percentages and absolute concentrations of CD3+ (T), CD21+ (B) lymphocytes, and total lymphocytes. Moreover, in some of the studies reporting differences in CD4+ and CD8+ T lymphocytes,[41,42] these differences were significant only when young dogs were compared with old dogs, whereas our control group did not consist of young dogs but of middle-aged and old dogs (all dogs were >5 years of age, and the mean age of the group was 7.9 years).

In addition, unlike dogs with MMVD, our control group consisted of more females than males, although the difference was not significant. Previously, it was found that females of the Labrador Retriever breed had a significantly higher percentage of CD8+ cells than did males of the same breed.[41] However, our healthy dogs had a significantly lower percentage of CD8+ compared to dogs with MMVD, arguing against a possible influence of the sex difference between the dog groups on this variable.

In summary, our study showed that CD4+ (helper) and CD8+ (cytotoxic) T lymphocyte subtypes and CD4+/CD8+ ratio were altered in MMVD dogs with CHF, in contrast to total CD3+ (T), double positive CD3+CD4+CD8+ (T), double negative CD3+CD4−CD8− (T) and CD21+ (B) lymphocyte counts and percentages, and total lymphocyte counts that were not altered. Dogs with CHF had a lower CD4+ percentage, higher CD8+ percentage and concentration (the latter only in unstable CHF), and lower CD4+/CD8+ ratio, supporting the hypothesis that these cell subtypes are involved in the disease processes in dogs with MMVD and CHF. Future studies to elucidate the mechanisms behind the changes found in our study, to explore the prognostic value of these changes and possible therapeutic corrections, and to clarify the role of T helper lymphocytes and cytotoxic T lymphocytes in the pathogenesis and progression of MMVD and CHF in dogs are warranted.

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CONFLICT OF INTEREST DECLARATION

The authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

All procedures complied with the applicable Slovenian governmental regulations (Animal Protection Act, The Official Gazette of the Republic of Slovenia, 43/2007).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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2.2 RANDOMIZED, DOUBLE-BLINDED, CONTROLLED TRIAL OF THE EFFECTS OF COENZYME Q_{10} SUPPLEMENTATION ON PLASMA COENZYME Q_{10} CONCENTRATION IN DOGS WITH MYXOMATOUS MITRAL VALVE DISEASE

Randomizirana dvojno slepa nadzorovana raziskava učinkov dodajanja koencima Q_{10} na plazemsko koncentracijo koencima Q_{10} pri psih z miksomatozno degeneracijo mitralne zaklopke

Druzhaeva N, Petrič AD, Tavčar-Kalcher G, Babič J, Nemec Svete A. Randomized, double-blinded, controlled trial of the effects of coenzyme Q10 supplementation on plasma coenzyme Q10 concentration in dogs with myxomatous mitral valve disease. Am J Vet Res 2021; 82(4): 280–5. doi: 10.2460/ajvr.82.4.280.

Izvleček v slovenskem jeziku / Abstract in Slovene language

Cilj: Določitev odmerka koencima Q_{10} (CoQ_{10}), potrebnega za doseganje vsaj trikratnega povečanja plazemske koncentracije CoQ_{10} pri psih z miksomatozno degeneracijo mitralne zaklopke (MDMZ) in kongestivnim srčnim popuščanjem.

Živali: 18 psov s kongestivnim srčnim popuščanjem zaradi MDMZ in 12 zdravih psov.

Metode: V dvojno slepi randomizirani, s placebom nadzorovani raziskavi so psi z MDMZ dobivali 50 mg ali 100 mg vodotopnega CoQ_{10} (ubikinon; skupni dnevni odmerek 100 mg [n = 5] ali 200 mg [n = 6]) ali placebo (n = 7), *per os*, 14 dni dvakrat na dan, poleg standardnega zdravljenja srčnega popuščanja. Koncentracijo CoQ_{10} v plazmi smo izmerili pri psih z MDMZ pred dodajanjem (izhodišče) in ob različnih časovnih točkah po začetku dodajanja, pri zdravih psih, ki niso dobivali dodatka, pa enkrat. Koncentracije smo primerjali med skupinami in znotraj skupin.

Rezultati: Med zdravimi psi in psi z MDMZ nismo ugotovili značilne razlike v srednji izhodiščni koncentraciji CoQ_{10} v plazmi. Povečanje izhodiščne koncentracije CoQ_{10} v plazmi je bilo od 1,7-kratno do 4,7-kratno oziroma od 3,2-kratno do 6,8-kratno za posamezne pse v skupinah, v katerih so prejemali 100 mg oziroma 200 mg CoQ_{10} dnevno. Sprememba plazemske koncentracije CoQ_{10} po začetku dodajanja je bila značilno višja v primerjavi s placebo skupino po štirih urah ter enem in dveh tednih za pse v skupini z 200 mg ter po enem in dveh tednih za pse v skupini s 100 mg.

Zaključki in klinični pomen: Dnevni odmerek 200 mg CoQ_{10} je zadostoval za doseganje vsaj trikratnega povečanja izhodiščne koncentracije CoQ_{10} v plazmi in se lahko uporablja v raziskavah dodajanja CoQ_{10}, v katere so vključeni psi s kongestivnim srčnim popuščanjem zaradi MDMZ.

Randomized, double-blinded, controlled trial of the effects of coenzyme Q_{10} supplementation on plasma coenzyme Q_{10} concentration in dogs with myxomatous mitral valve disease

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OBJECTIVE
To determine the dose of coenzyme Q_{10} (CoQ_{10}) needed to achieve at least a 3-fold increase in plasma CoQ_{10} concentration in dogs with myxomatous mitral valve disease (MMVD) and congestive heart failure (CHF).

ANIMALS
18 dogs with CHF due to MMVD and 12 healthy dogs.

PROCEDURES
In a randomized, double-blinded, controlled trial, dogs with MMVD were given 50 or 100 mg of water-soluble CoQ_{10} (ubiquinone; total daily dose, 100 mg [n = 5] or 200 mg [6]) or a placebo (7), PO, twice a day for 2 weeks in addition to regular cardiac treatment. Plasma CoQ_{10} concentration was measured in dogs with MMVD before (baseline) and at various time points after supplementation began and in healthy dogs once. Concentrations were compared among and within groups.

RESULTS
No significant difference in median baseline plasma CoQ_{10} concentration was detected between healthy dogs and dogs with MMVD. Fold increases in plasma CoQ_{10} concentrations ranged from 1.7 to 4.7 and 3.2 to 6.8 for individual dogs in the 100-mg and 200-mg groups, respectively. The change in plasma CoQ_{10} concentration after supplementation began was significantly higher than in the placebo group at 4 hours and 1 and 2 weeks for dogs in the 200-mg group and at 1 and 2 weeks for dogs in the 100-mg group.

CONCLUSIONS AND CLINICAL RELEVANCE
A daily CoQ_{10} dose of 200 mg was sufficient to achieve at least a 3-fold increase in plasma CoQ_{10} concentration and may be used in CoQ_{10} supplementation studies involving dogs with CHF due to MMVD. (*Am J Vet Res* 2021;82:280–285)

Coenzyme Q_{10}, also known as ubiquinone, is the only lipid-soluble antioxidant synthesized endogenously in humans and other animals and is an essential electron and proton carrier in the mitochondrial respiratory chain.[1–3] This coenzyme is present in both reduced ($CoQ_{10}H_2$ or ubiquinol) and oxidized (CoQ_{10} or ubiquinone) forms, with the reduced form having antioxidant properties. In its reduced form, CoQ_{10} effectively regenerates other antioxidants such as vitamin E and ascorbate.[1] Coenzyme Q_{10} can also be found in certain foods and may be used as a nutritional supplement for humans with cardiovascular and other diseases (eg, neurodegenerative and neuromuscular disorders, mitochondrial cytopathy, diabetes, cancer, and periodontal disease) as well as for the elderly.[4–6]

ABBREVIATIONS

ACVIM	American College of Veterinary Internal Medicine
CHF	Congestive heart failure
CoQ_{10}	Coenzyme Q_{10}
MMVD	Myxomatous mitral valve disease

In humans, plasma and myocardial CoQ_{10} deficiencies increase with the severity of heart failure.[7] Moreover, a low plasma CoQ_{10} concentration independently predicts death in those with chronic heart failure.[8] Indeed, a meta-analysis[9] of clinical trials revealed that humans with heart failure receiving CoQ_{10} supplementation had a lower mortality rate and greater improvement in exercise capacity than did their placebo-treated counterparts.[9] Numerous studies of the effects of CoQ_{10} supplementation have been conducted in humans with cardiovascular diseases, and several of them show clinical benefits.[6,10–13] Coenzyme Q_{10} supplementation improved left ventricular systolic function in humans with advanced CHF in 1 study,[14] and the investigators suggested that at least a 3-fold increase in baseline plasma CoQ_{10} concentration is necessary for the biological effects of CoQ_{10} supplementation to be appreciated.[14]

In contrast, studies of CoQ_{10} supplementation in dogs with cardiovascular disease are lacking. In the only study[15] we identified of CoQ_{10} supplementation

in dogs with naturally acquired heart disease, a daily dose of 200 mg was used. Research in human medicine suggests that the effectiveness of CoQ_{10} supplementation in the treatment of cardiovascular diseases increases as the dose increases and that the widely used dose of 100 mg/d is suboptimal.[16] To our knowledge, no dose-evaluation studies have been conducted in dogs, and the dose required to achieve a 3-fold increase in plasma CoQ_{10} concentration in dogs is unknown. Therefore, the goal of the study reported here was to determine the dose of CoQ_{10} (ubiquinone) sufficient to achieve a 3-fold increase in plasma CoQ_{10} concentration in dogs with CHF due to MMVD.

Materials and Methods

Animals

Dogs presented for cardiac examination (ie, cardiac patients) or health assessment (control dogs) to the Small Animal Clinic of the University of Ljubljana from November 2018 to April 2019 were recruited for the study by 2 of the authors (ADP and ND). During this recruitment period, 90 dogs with cardiovascular disease and 28 healthy dogs were assessed for eligibility. For all dogs, owners completed a questionnaire regarding their dog's diet, including any nutritional supplements and treats and any medications administered.

Only dogs with stable CHF and MMVD (ACVIM classification stages C and D[17]) that were already receiving long-term treatment for heart failure were eligible for inclusion in the MMVD group. The diagnosis of MMVD was based on results of clinical examination, echocardiography, and thoracic radiography, with all assessments performed by an experienced veterinary cardiologist (ADP). Dogs were excluded from this group if they had ACVIM stage B1 or B2 MMVD, unstable CHF, untreated CHF, or comorbidities (including concomitant systemic disease); had received any nutritional supplements within the past month or any medications not related to CHF treatment; or were very small or had other characteristics that caused difficulty in blood sample collection and potential compromise of the dog's status. Only healthy dogs of small or medium breeds that were > 5 years of age were eligible for inclusion in the control group. Healthy was defined on the basis of results of clinical examination, echocardiography, CBC, WBC differential count, and serum biochemical analysis.

Owner consent was obtained for all included dogs. All procedures complied with applicable Slovenian governmental regulations (Animal Protection Act, the Official Gazette of the Republic of Slovenia, 43/2007).

Study design

In a randomized, double-blinded, controlled trial, dogs with MMVD were randomly assigned to 3 subgroups (2 CoQ_{10} groups and 1 placebo group) by an individual (ANS) uninvolved in patient assessment, diagnostic procedures, treatment, supplement administration, or plasma CoQ_{10} concentration measurements. Dogs assigned to the CoQ_{10} groups received CoQ_{10},[a] PO, in an amount (1.333 or 2.667 g) calculated to provide a total daily dose of 100 or 200 mg of CoQ_{10}, divided into 50 or 100 mg twice a day for 2 weeks in addition to their regular cardiac treatment. Dogs in the placebo group received the placebo, PO, at the same frequency and for the same duration as the CoQ_{10} groups. To ensure blinding, treatments were prepared in syringes (2 syringes/d) containing a water-soluble form of CoQ_{10}[a] (ubiquinone; 7.5% water suspension of CoQ_{10}[b] in the form of an inclusion complex with β-cyclodextrin produced in accordance with a previously filed patent[18]) or color-matched placebo[c] (a solution of methylcellulose [1.5%] and yellow food coloring) and packaged in sequentially numbered sealed bags by the same individual who performed the randomization. Dog owners, veterinarians, and those performing plasma CoQ_{10} concentration measurements were blinded to the study treatment. Owners were instructed to administer the packaged treatments, PO, twice a day (in the morning and in the evening) for 2 weeks.

Jugular or cephalic venous blood samples for plasma CoQ_{10} measurements in dogs with MMVD were collected into tubes containing lithium heparin before (baseline) and at 4 hours and 1 and 2 weeks after study treatment began and 1 week after the last dose was administered. Blood samples were similarly collected once from healthy dogs. Food was withheld from all dogs for 12 hours prior to sample collection. Collected samples were immediately centrifuged at 1,500 × *g* for 15 minutes at 4°C. Plasma was separated and immediately stored at −80°C until analysis.

Determination of plasma CoQ_{10} concentrations

To determine plasma total CoQ_{10} concentration, a modified procedure including oxidation with 1,4-benzoquinone[d] was used.[19,20] Each plasma sample was thawed at room temperature (approx 21°C). A 100-µL portion was transferred to a 1.5-mL microcentrifuge tube, and 100 µL of 1,4-benzoquinone[d] in 1-propanol[d] (0.4 mg/mL) was added. Samples were vortexed and incubated at room temperature for 15 minutes to achieve complete oxidation of CoQ_{10}. Sample components were precipitated with 400 µL of 1-propanol.[d] Tubes were capped, vortexed well, and centrifuged for 5 minutes at 10,000 × *g* and 4°C. The supernatant was transferred to vials.

Measurement of CoQ_{10} concentrations was performed by use of matrix-matched calibration. The concentration range for the calibration curve was 10 to 4,000 ng of CoQ_{10}/mL. This analysis was performed on a liquid chromatography–tandem mass spectrometry system.[e] Chromatographic separation was performed at 40°C on a C18 (1.7 µm; 2.1 × 50 mm) column.[f] The mobile phase consisted of an 80:20:0.1 mixture of acetonitrile[d]:2-propanol[d]:formic

acid[g] in isocratic elution mode. Flow rate and injection volume were 0.4 mL/min and 7.5 µL, respectively. Multiple reaction monitoring transitions at an m/z of 863.8 to 197.1 and of 882.8 to 81.0 were chosen for the determination of CoQ_{10}.

Statistical analysis

Data were analyzed with statistical software.[h,i] To test whether the data were normally distributed, histograms were generated and the Shapiro-Wilk test was performed. On the basis of the findings, parametric tests or nonparametric tests were used to compare data among and within groups of dogs. Accordingly, 1-way ANOVA with the post hoc Tukey honestly significant difference test and independent *t* test were used for comparisons involving body weight and age. The Wilcoxon rank sum test was used to compare baseline plasma CoQ_{10} concentration between all MMVD patients and healthy dogs. The Kruskal-Wallis test followed by pairwise Wilcoxon rank sum tests was used to compare baseline plasma CoQ_{10} concentrations between all 4 groups of dogs and to compare values between the 3 MMVD subgroups at each of the 5 blood sample collection times.

Differences (changes) from baseline in plasma CoQ_{10} concentrations at each time point after supplementation began were calculated for each MMVD group and compared among groups with the Kruskal-Wallis test followed by pairwise Wilcoxon rank sum tests to detect differences between particular group pairs. Within-group analyses included a comparison of plasma CoQ_{10} concentration between baseline and subsequent time points with the Wilcoxon signed rank test. In both sets of analyses, *P* values were adjusted for multiple comparisons by means of the Benjamini-Hochberg correction, except for within-group comparisons for the 200-mg group whereby all *P* values were the same and the Bonferroni correction was used instead. Values of $P < 0.05$ were considered significant.

Results

Of the 90 cardiac patients assessed during the recruitment period, 71 dogs either did not meet the inclusion criteria (n = 66) or had owners who declined participation (5). Of the 28 healthy dogs considered for the control group, 16 were excluded because of subclinical heart disease (n = 13) or other reasons (3). Consequently, 19 client-owned dogs with CHF due to MMVD and 12 client-owned healthy control dogs > 5 years of age were included in the study. Six dogs with MMVD received CoQ_{10} at a total daily dose of 100 mg, 6 received CoQ_{10} at a total daily dose of 200 mg, and 7 received the placebo. One dog in the 100-mg group was excluded from the statistical analysis owing to antimicrobial treatment that began on the fourth day of CoQ_{10} supplementation, leaving 5 dogs in that group.

Healthy dogs included 7 mixed-breed dogs, 3 Shih Tzus, 1 Yorkshire Terrier, and 1 Tibetan Spaniel. Dogs with MMVD included 4 mixed-breed dogs, 3 Pekingese, 2 Shih Tzus, 2 Miniature Poodles, and 1 each of Miniature Pinscher, English Cocker Spaniel, Airedale Terrier, Pomeranian, Coton de Tulear, Cavalier King Charles Spaniel, and Whippet. Sixteen of these 18 dogs had ACVIM stage C MMVD and 2 had stage D MMVD. All dogs with MMVD were clinically stable and not in active CHF. Treatments included furosemide or torasemide, angiotensin-converting enzyme inhibitors, spironolactone, and pimobendan as well as digoxin and diltiazem (n = 1 dog), theophylline and a fluticasone inhalant (1), and potassium chloride (1).

Dogs assigned to the 100-mg group had a higher mean body weight than did those assigned to the 200-mg group, but this difference was not significant **(Table 1)**. Furthermore, no significant differences in mean body weight or age were identified between the 3 MMVD subgroups or in body weight between all dogs with MMVD and healthy dogs. Although all healthy dogs were > 5 years of age, the mean age of those dogs was significantly less than the mean age of all dogs with MMVD.

Median plasma CoQ_{10} concentration for the 12 healthy dogs was 0.095 mg/L (interquartile [25th to 75th percentile] range, 0.070 to 0.143 mg/L). Plasma CoQ_{10} concentrations at the various time points were summarized for dogs with MMVD (**Table 2; Supplementary Figure S1**, available at: avmajournals.avma.org/doi/suppl/10.2460/ajvr.82.4.280). No significant differences in baseline plasma CoQ_{10} concentration were identified among the dog groups. After 2 weeks of CoQ_{10} supplementation, plasma CoQ_{10} concentrations increased significantly from baseline values in the 100-mg and 200-mg groups, whereas they remained unchanged in the placebo group. After 2 weeks of CoQ_{10} supplementation, the fold increase in plasma CoQ_{10} concentration for individual dogs

Table 1—Characteristics of dogs with CHF due to MMVD before receiving water-soluble CoQ_{10} (total daily dose of 100 or 200 mg) or a placebo, PO, for 2 weeks in addition to their regular cardiac treatment and of healthy control dogs.

Characteristic	All dogs with MMVD (n = 18)	100 mg (n = 5)	200 mg (n = 6)	Placebo (n = 7)	Healthy dogs (n = 12)
Sex (female/male)	7/11	2/3	2/4	3/4	6/6
Age (y)	11.6 ± 0.5* (8.6–16.8)	11.7 ± 1.0* (8.7–14.6)	10.9 ± 0.2* (10.7–11.8)	12.2 ± 1.0* (8.6–16.8)	7.9 ± 0.6 (5.0–11.4)
Body weight (kg)	9.4 ± 1.2 (4.4–20.5)	12.1 ± 2.9 (5.6–20.5)	5.8 ± 0.6 (4.4–8.0)	10.4 ± 1.6 (6.7–18.0)	8.3 ± 1.2 (2.8–13.5)

Values for age and body weight represent mean ± SEM (range).
*Mean value differs significantly ($P < 0.05$) from that of healthy dogs.

Table 2—Median (interquartile [25th to 75th percentile] range) plasma CoQ_{10} concentrations (mg/L) for the dogs with MMVD represented in Table 1 before (baseline) and at various time points during and after 2 weeks of daily CoQ_{10} supplementation.

Group	Baseline	4 hours	1 week	2 weeks	3 weeks
100 mg	0.154 (0.062–0.367)	0.165 (0.146–0.340)	0.527* (0.219–1.06)	0.402* (0.219–0.942)	0.190 (0.077–0.373)
200 mg	0.091 (0.042–0.178)	0.185* (0.098–0.292)	0.324* (0.134–0.520)	0.384* (0.165–0.710)	0.148 (0.075–0.211)
Placebo	0.106 (0.100–0.161)	0.107 (0.092–0.189)	0.109 (0.085–0.207)	0.106 (0.094–0.224)	0.106 (0.088–0.193)

*Within a group, the change (ie, difference) in this value from the baseline value differs significantly ($P < 0.05$) from the corresponding change for the placebo group.

ranged from 1.7 to 4.7 and 3.2 to 6.8 in the 100-mg and 200-mg groups, respectively. In the 200-mg group, median plasma CoQ_{10} concentration was significantly greater than at baseline at 4 hours and 1, 2, and 3 weeks after supplementation began without adjustment of *P* values; however, significant differences in plasma CoQ_{10} concentrations were no longer significant after adjustment for multiple comparisons. On the other hand, in the 100-mg group, no significant differences were observed between baseline and any subsequent plasma CoQ_{10} concentrations. In the placebo group, plasma CoQ_{10} concentration remained fairly constant regardless of time point.

Median plasma CoQ_{10} concentration did not differ between the 3 MMVD subgroups at any time point. However, the change in plasma CoQ_{10} concentration after supplementation began was significantly higher than it was in the placebo group at 4 hours and 1 and 2 weeks for dogs in the 200-mg group and at 1 and 2 weeks for dogs in the 100-mg group.

Discussion

In the present study, a total daily CoQ_{10} dose of 200 mg administered PO was found to be sufficient to achieve a 3-fold increase in plasma CoQ_{10} concentration in every dog with CHF due to MMVD after 2 weeks of supplementation.

Coenzyme Q_{10} is widely used as a nutritional supplement for humans with cardiovascular disease,[6,9,12] with reported daily doses ranging from 100 to 300 mg.[6,21] Although CoQ_{10} has also been drawing attention as a nutritional supplement for dogs with heart disease, to the authors' knowledge only 2 reports[15,22] exist of CoQ_{10} supplementation in dogs with heart disease, with 1 report[22] involving dogs with experimentally induced CHF. For dogs with CHF due to MMVD in the present study, the total daily CoQ_{10} doses of 100 and 200 mg were chosen on the basis of data from the veterinary literature[23] and a bioavailability study.[24]

Coenzyme Q_{10} is a lipophilic compound that is practically insoluble in water. Because of the hydrophobicity and large molecular weight of CoQ_{10}, absorption of CoQ_{10} within the gastrointestinal tract when administered PO is slow and limited, resulting in poor bioavailability.[25] Several solubilized formulations of CoQ_{10} have been developed to enable better absorption and, thus, enhanced bioavailability.[10,18,26,27] However, the bioavailability of CoQ_{10} in these formulations varies greatly in humans and dogs.[6,10,24,26–29] The bioavailability of the water-soluble CoQ_{10} formulation used in the present study was shown in healthy Beagles to be better than that of an oil-based form.[24] Similarly, Zaghloul et al[29] demonstrated that soft gelatin capsules containing water-miscible CoQ_{10} formulations (ubiquinone and ubiquinol) are superior to powder-filled formulations with regard to their biopharmaceutical characteristics in healthy dogs. Furthermore, Zmitek et al[27] showed that the same water-soluble formulation of CoQ_{10} used in our study achieved greater bioavailability in healthy men than did soft gelatin capsules containing CoQ_{10} in soybean oil.

Our results indicated that a total daily CoQ_{10} dose of 200 mg resulted in at least a 3-fold increase in plasma CoQ_{10} concentration in every dog in that group after 2 weeks of CoQ_{10} supplementation. Furthermore, median plasma CoQ_{10} concentration was significantly higher than at baseline 1 week after administration ceased. These results suggested that a daily dose of 200 mg would be appropriate for use in supplementation studies involving dogs with CHF due to MMVD. In a previous study[22] involving dogs with experimentally induced CHF, CoQ_{10} administered at 10 mg/kg/d, PO, for 6 weeks resulted in a significant increase in serum CoQ_{10} concentration; however, myocardial CoQ_{10} concentrations were similar to those of healthy dogs. The investigators also noted lower filling pressures and less myocardial hypertrophy in CoQ_{10}-treated dogs.[22] In another study[15] involving dogs with naturally acquired MMVD, twice-daily administration of CoQ_{10} at 100 mg, PO, for 28 days resulted in significantly higher fractional shortening and ejection fraction in dogs weighing < 6 kg; however, these 2 measures of systolic function can be misleading in dogs with MMVD because of increased preload and reduced afterload. As a consequence of these changes in filling pressures, fractional shortening and ejection fraction can be unremarkable to increased in the presence of weakened myocardial systolic function.[30] Plasma CoQ_{10} concentration was not measured, and control dogs were not included in that study.[15] A crossover pharmacokinetics study[31] involving 19 Cavalier King Charles Spaniels with MMVD revealed a significant increase in plasma CoQ_{10} concentration following administration of CoQ_{10} dissolved in vegetable oil, PO, twice daily for 3 weeks. Nevertheless, whether a 3-fold increase in a plasma CoQ_{10} concentration was achieved in all included dogs is unclear.

Plasma and myocardial tissue CoQ_{10} deficiencies have been identified in human cardiac patients.[7,32] In the present study, baseline plasma CoQ_{10} concentrations in most dogs with MMVD were similar to those of healthy dogs, which is in accordance with the results from the previous study[33] of CoQ_{10} concentration in dogs with various cardiovascular diseases. Nevertheless, we are aware of no long-term, placebo-controlled clinical trials of the effects of CoQ_{10} supplementation on the course of cardiovascular disease in dogs. Therefore, further investigation is warranted into the possible effects of CoQ_{10} supplementation in dogs with different cardiovascular diseases.

The present study had some limitations, including the small number of dogs in the MMVD subgroups, which might have affected the precision of our estimates of mean plasma CoQ_{10} concentration in these groups. An additional limitation might be that the doses chosen for CoQ_{10} supplementation were not based on dog body weight as suggested by Tachampa et al.[15] Although no significant difference in mean body weight was found between MMVD subgroups, the low sample sizes may have limited our ability to detect such differences. Regardless of these limitations, we found no significant difference in median plasma CoQ_{10} concentrations between dogs with CHF due to MMVD and healthy dogs, and we found that a daily CoQ_{10} dose of 200 mg was sufficient to achieve at least a 3-fold increase in plasma CoQ_{10} concentration, suggesting this dose may be used in CoQ_{10} supplementation studies involving similar dogs.

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Footnotes

a. Q10Vital liquid, Valens, Šenčur, Slovenia.
b. Ubidecarenone, Xiamen Kingdomway Group Co Xiamen, China.
c. Department of Pharmaceutical Technology, Faculty of Pharmacy, University of Ljubljana, Ljubljana, Slovenia.
d. Sigma-Aldrich Co, St Louis, Mo.
e. Acquity LC-MS-MS system equipped with Xevo TQ detector, Waters, Milford, Mass.
f. UPLC BEH C18 column, Waters, Milford, Mass.
g. Merck & Co, Burlington, Mass.
h. SPSS, version 24.0, IBM Corp, Chicago, Ill.
i. The R Project for Statistical Computing stats and ggplot, R version 4.0.0, The R Foundation, Vienna, Austria.

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2.3 EFFECTS OF COENZYME Q_{10} SUPPLEMENTATION ON OXIDATIVE STRESS MARKERS, INFLAMMATORY MARKERS, LYMPHOCYTE SUBPOPULATIONS, AND CLINICAL STATUS IN DOGS WITH MYXOMATOUS MITRAL VALVE DISEASE

Učinki dodajanja koencima Q_{10} na pokazatelje oksidativnega stresa, vnetja, podvrste limfocitov in klinični status pri psih z miksomatozno degeneracijo mitralne zaklopke

Druzhaeva N, Nemec Svete A, Tavčar-Kalcher G, Babič J, Ihan A, Pohar K, Krapež U, Domanjko Petrič A. Effects of coenzyme Q10 supplementation on oxidative stress markers, inflammatory markers, lymphocyte subpopulations, and clinical status in dogs with myxomatous mitral valve disease. Antioxidants 2022; 11: 1427. doi: 10.3390/antiox11081427.

Izvleček v slovenskem jeziku / Abstract in Slovene language

O učinkih dodajanja koencima Q_{10} (CoQ_{10}) psom z miksomatozno degeneracijo mitralne zaklopke (MDMZ) ni veliko podatkov, zato je bil namen te raziskave raziskati učinke dodajanja CoQ_{10} (kot dodatka k prehrani) na pokazatelje oksidativnega stresa (glutation peroksidazo, F2-izoprostani), vnetja (dejavnik tumorske nekroze-α, topni receptor dejavnika tumorske nekroze-α II, levkocite), podvrste limfocitov (celice T-pomagalke in citotoksični limfociti T, vključno z aktiviranimi limfociti T, in limfociti B) ter ehokardiografske in klinične parametre pri psih z MDMZ. V tej dvojno slepi randomizirani, s placebom nadzorovani longitudinalni raziskavi je 43 psov z MDMZ v stopnjah ACVIM B2 ter ACVIM C in D (kongestivno srčno popuščanje) tri mesece prejemalo vodotopni CoQ_{10} (100 mg dvakrat na dan) ali placebo, 12 zdravih psov, ki niso prejemali nobenega dodatka, je sestavljalo kontrolno skupino. Vsi parametri so bili izmerjeni pred dodajanjem in po trimesečnem dodajanju CoQ_{10} ali placeba pri bolnih psih ter enkrat pri zdravih psih. Dodajanje CoQ_{10} je pozitivno vplivalo na odstotek nevtrofilnih granulocitov ter odstotek in koncentracijo limfocitov pri psih s kongestivnim srčnim popuščanjem (stopnje ACVIM C in D). Zaključimo lahko, da CoQ_{10} kot dodatek k prehrani zmanjša vnetje pri psih z MDMZ in kongestivnim srčnim popuščanjem.

antioxidants

MDPI

Article

Effects of Coenzyme Q_{10} Supplementation on Oxidative Stress Markers, Inflammatory Markers, Lymphocyte Subpopulations, and Clinical Status in Dogs with Myxomatous Mitral Valve Disease

Natalia Druzhaeva [1], Alenka Nemec Svete [1], Gabrijela Tavčar-Kalcher [2], Janja Babič [2], Alojz Ihan [3], Katka Pohar [3], Uroš Krapež [4] and Aleksandra Domanjko Petrič [1,*]

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Abstract: Scarce data exist on the effects of coenzyme Q_{10} (CoQ_{10}) supplementation in dogs with myxomatous mitral valve disease (MMVD). The purpose of this study was to investigate the effect of CoQ_{10} supplementation on oxidative stress markers (glutathione peroxidase, F2-isoprostanes), markers of inflammation (tumor necrosis factor-α, TNF soluble receptor II, leucocytes, and their subtypes), lymphocyte subpopulations (T helper and cytotoxic T lymphocytes, including activated T lymphocytes, and B lymphocytes), and echocardiographic and clinical parameters in dogs with MMVD. In this randomized, controlled, double-blind, longitudinal study, 43 MMVD dogs in stages ACVIM (American College of Veterinary Internal Medicine classification) B2 and ACVIM C and D (congestive heart failure (CHF)) received water-soluble coenzyme Q_{10} (100 mg twice daily) or placebo for 3 months, and 12 non-supplemented healthy dogs served as controls. All parameters were measured before and after supplementation in MMVD dogs and once in healthy dogs. CoQ_{10} supplementation had a positive impact on neutrophil percentage, lymphocyte percentage, and lymphocyte concentration in our cohort of dogs with CHF (ACVIM C and D). Conclusion: CoQ_{10} as an oral supplement may have benefits in terms of decreasing inflammation in dogs with MMVD and CHF.

Keywords: coenzyme Q_{10}; ubiquinone; supplementation; myxomatous mitral valve disease; congestive heart failure; dog; oxidative stress; inflammation; lymphocytes; neutrophils

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1. Introduction

Coenzyme Q_{10} (CoQ_{10}), or ubiquinone, is an endogenously synthesized lipid-soluble compound which is essential for the functioning of all cells of an animal's body. It is present in the membranes of mitochondria and other cell organelles, cell membranes, cytoplasm, and blood plasma. Coenzyme Q_{10} plays a major role in ATP production and antioxidant defense, and it directly or indirectly regulates many bodily functions, including the functioning of the immune system [1–3].

Oxidative stress, defined as "an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage" [4], is present in human cardiovascular diseases (CVD) and heart failure (HF) [5,6]. Low levels of plasma and myocardial CoQ_{10} have been found in human CVD [7–10]. Due to the known fact of CoQ_{10} deficiency in people with CVD, the role of CoQ_{10} in energy

production, and its antioxidant potential, supplemental CoQ_{10} has long been studied as an adjunctive treatment in human HF patients [11–16].

Myxomatous mitral valve disease (MMVD) is a degenerative disease of the mitral and/or tricuspid valve and is the most common cardiac pathology in dogs [17]. As such, it is also the most common cause of canine congestive heart failure (CHF), a debilitating condition which shortens the lifespan of an animal and lowers the quality of life of both the dog and its owner. Treatment of MMVD and CHF is mostly conservative, with surgical mitral valve repair only rarely performed in dogs due to the high cost and relative inaccessibility [17]. The search for new medical agents and dietary supplements with the potential to slow the progression of the disease is ongoing. In accordance with studies, oxidative stress is present in canine cardiac diseases, including MMVD [18–20]. Myocardial CoQ_{10} deficiency has recently been confirmed in Cavalier King Charles Spaniels with CHF due to MMVD [21], and although plasma CoQ_{10} deficiency has not been confirmed in dogs with MMVD or other cardiac pathologies [22,23], CoQ_{10} has drawn interest as a dietary supplement which could potentially positively impact the course of MMVD in dogs due to its antioxidant and other properties. In contrast to human medicine, few CoQ_{10} supplementation studies have been conducted in canine cardiac patients with spontaneous disease [24,25], and no studies have assessed the effect of supplemental CoQ_{10} on oxidative stress parameters.

Inflammation, which is the other hallmark of human [26] and canine [27–30] heart failure (HF), has been shown to be linked to oxidative stress in both people [31,32] and dogs [20,30] with cardiac diseases. The reducing effect of CoQ_{10} supplementation on inflammatory markers has been found in coronary artery disease (CAD) and other chronic inflammatory diseases in people [3,33–35]. It is hypothesized that CoQ_{10} exerts an anti-inflammatory effect by decreasing reactive oxygen species (ROS) concentrations and subsequently reducing nuclear factor kappa B (normally activated by ROS) gene expression [35]. The effect of CoQ_{10} on markers of inflammation has not been studied in dogs with heart diseases.

Besides markers of inflammation, lymphocyte subpopulations have also been reported to be altered in both people with HF [36,37] and dogs with cardiac diseases, including MMVD and CHF [38,39]. In a study of dogs with MMVD conducted by our group [39], a lower percentage of T helper lymphocytes, a higher percentage of cytotoxic T lymphocytes, and a lower T helper/cytotoxic T lymphocyte ratio were documented in dogs with CHF due to MMVD compared to healthy controls, whereas these alterations were not present in the non-CHF group of participating MMVD patients. As far as we know, the effect of CoQ_{10} supplementation on lymphocyte subtypes has not been studied in dogs with heart diseases.

To date, three studies have been conducted to assess the effects of CoQ_{10} supplementation in canine cardiac patients. A few parameters were tested, and some possible benefits were detected in two of these studies [24,25,40]. An earlier study in experimental tachycardia-induced CHF showed that dogs with CHF supplemented with CoQ_{10} for six weeks in total had lower filling pressures in an early stage of CHF and less hypertrophy in severe CHF when compared to non-treated dogs [40]. In the other study, the authors reported, over a period of 28 days of supplementation, an increased shortening fraction and M-mode-derived ejection fraction in a group of dogs of less than 6 kg, which could be just a consequence of a larger end-diastolic diameter [24]. The latest (single-blind, crossover) study [25] to investigate the potential benefits of CoQ_{10} in canine MMVD did not show any positive effects on the severity of the disease and owner-perceived quality of life in Cavalier King Charles Spaniels supplemented with CoQ_{10} for three weeks. The authors of the mentioned study concluded that a long-term placebo-controlled trial is warranted in dogs with MMVD to determine the long-term efficacy of CoQ_{10} on the clinical severity of MMVD.

The aim of the present study was to assess the effect of oral CoQ_{10} supplementation given for three months in addition to standard cardiac therapy on oxidative stress parameters (glutathione peroxidase (GPX), F2-isoprostanes), inflammatory parameters (tumor

necrosis factor-α (TNF-α), soluble TNF receptor II (TNFSR-II), leucocyte populations), lymphocyte subpopulations, cardiac biomarkers (N-terminal pro B-type natriuretic peptide (NT-proBNP) and cardiac troponin I (cTnI)), selected echocardiographic parameters, and clinical signs in dogs of different breeds with spontaneous MMVD with and without CHF.

2. Materials and Methods

2.1. Animals

Dogs with MMVD in stages ACVIM (American College of Veterinary Internal Medicine classification) B2 and ACVIM C and D, as well as healthy control dogs, were recruited for the study. The inclusion process took place between 4 December 2018 and 28 December 2020 at the Small Animal Clinic of the University of Ljubljana. Clinical examination, echocardiography, electrocardiography (if indicated), thoracic radiography (if indicated), routine hematology, and biochemistry analyses were performed at inclusion. All diagnostic procedures were performed by an experienced veterinarian. Diagnosis of MMVD was in all cases confirmed by echocardiography and that of CHF by thoracic radiography and echocardiography. All healthy dogs were subjected to the same diagnostic tests to confirm their eligibility for the healthy control group.

2.1.1. Inclusion Criteria

Dogs were classified as ACVIM B2 if they were asymptomatic (without a history of heart failure) and had advanced mitral regurgitation with subsequent left-sided cardiac remodeling. The criteria for inclusion in this group were as follows: Grade 3/6 cardiac murmur intensity, echocardiographic LA/Ao (left atrial/aortic ratio) $\geq$ 1.6 in early diastole, left ventricular internal end-diastolic diameter normalized to body weight (nLVIDd)$^{1/3}$ $\geq$ 1.7 [17] measured in the right short-axis view, and no evidence of comorbidities at the time of clinical examination and in routine hematology and biochemistry findings. Dogs were included in the cardiac failure group (ACVIM C and D) if they were symptomatic (current or previous symptoms of cardiac failure), had echocardiographically confirmed MMVD, had current or previously diagnosed cardiogenic pulmonary edema on chest radiographs, and had no evidence of comorbidities at the time of clinical examination and in routine hematology and biochemistry findings. Healthy control dogs were included in the control group if they were clinically healthy, had no evidence of cardiac disease on echocardiographic examination, and routine hematology and biochemistry findings showed no evidence of disease.

2.1.2. Exclusion Criteria

Dogs with concomitant diseases (including chronic kidney disease and other metabolic diseases, systemic or local inflammation, and neoplasia) were excluded. Dogs that had not received complete treatment for cardiac disease (pimobendan for B2 dogs or heart failure treatment for ACVIM C and D dogs) at least four weeks before inclusion were not included until they met these criteria. Critically ill dogs were not included until their condition became stable with recommended treatment [17]. Dogs receiving (or having received in the past month) glucocorticoids or other immunosuppressive agents, antibiotics, or dietary supplements were excluded.

2.2. Study Design

In this randomized, double-blinded, placebo-controlled study, we included dogs with MMVD receiving water-soluble CoQ_{10} in a daily dose of 200 mg (100 mg twice a day) or an organoleptically matched placebo for three months. The daily dose of 200 mg was chosen based on the results of our previous research [23]. Dogs were included in the study after complete diagnostic procedures and allocated to either the ACVIM B2 (non-CHF) group or the ACVIM C and D (CHF) group and then further blindly randomized to receive CoQ_{10} or placebo. Randomization was performed by one of the authors (A.N.S.) not involved in the assessment, diagnostics, treatment, following of patients, or any communication with the owners. All diagnostic procedures (including clinical examination, echocardiography, and

blood tests) were performed twice in MMVD dogs, i.e., on the day of inclusion just before the start of supplementation and at the end of the study, approximately 12 h after the final dose of the supplement. Diagnostic procedures, treatment, and all communication with the owners were performed by A.D.P. and N.D., both blinded to the type of supplement the dogs were receiving during the study. At the time of inclusion, all owners completed a questionnaire regarding the dog's diet and the supplements and medications the dogs were receiving. An additional questionnaire regarding the dog's current symptoms was completed at the beginning and at the end of the study. Owners were instructed on the correct administration and the storage of the supplement.

Water-soluble CoQ_{10} (ubiquinone; Q10Vital liquid, Valens, Šenčur, Slovenia) in the form of a 7.5% water suspension (derived from CoQ_{10}, Ubidecarenone (Xiamen Kingdomway Group Co, Xiamen, China) in an inclusion complex with β-cyclodextrin [41]) was used in the study. A single dose comprising 100 mg of CoQ_{10} was equivalent to 1.333 g of the suspension, with the daily dose being equal to 2.667 g. The matched placebo was comprised of cyclodextrin, water, food colorants, and the preservative methyl 4-hydroxybenzoate sodium salt. Both CoQ_{10} suspension and the placebo were packed in identical plastic bottles and put in opaque bags by a person not involved in any diagnostics or treatment before being handed out to veterinarians and subsequently to dog owners. All those performing any status assessment or analyses were blinded to the type of supplement the dogs were receiving.

Healthy dogs underwent clinical and echocardiographic examination only once. They did not receive dietary supplements according to the study protocol. A questionnaire regarding the dog's diet, any treatments, and dietary supplements was completed before enrollment in the study.

2.3. Blood Sampling

Blood was taken by jugular or cephalic venipuncture after 12 h of fasting. The last dose of supplement (CoQ_{10} or placebo) at the end of the supplementation period was given 12 h prior to sampling. Samples were drawn into EDTA-containing tubes (for complete blood count (CBC) with white blood cell (WBC) differential count, flow cytometry, NT-proBNP, F2-isoprostanes (8-iso-prostaglandin F2α (8-isoPGF2α))), serum separator tubes (for routine biochemistry, cTnI, CRP, TNF-α, TNFSR-II), and lithium-heparin tubes (for CoQ_{10}, whole blood GPX activity). Samples for routine hematology and biochemistry were analyzed within 1 h after sampling. Samples for flow cytometry were stored in darkness at room temperature and were analyzed within 24 h. Samples for NT-proBNP, F2-isoprostanes, and CoQ_{10} were immediately centrifuged at 1500 × g at 4 °C for 15 min, and separated plasma was stored at −80 °C until analysis. Samples for cTnI, CRP, TNF-α, and TNFSR-II were centrifuged after complete coagulation at 1300 × g at room temperature for 10 min, and serum was stored at −80 °C until analysis.

2.3.1. Routine Hematology and Biochemistry Analyses

Hematologic analysis was performed with an automated laser-based hematology analyzer (ADVIA 120, Siemens, Munich, Germany). Biochemistry analysis (glucose, urea, creatinine, alanine aminotransferase, alkaline phosphatase, total protein, albumin) was performed with an automated biochemistry analyzer (RX Daytona, Randox, Crumlin, UK). Electrolytes (sodium, potassium, chloride) were measured with an electrolyte analyzer (ILyte, Instrumentation laboratory, Lexington, Massachusetts).

2.3.2. Determination of Oxidative Stress Markers

CoQ_{10} was measured on a liquid chromatography-tandem mass spectrometry system (Acquity UPLC with Xevo TQ detector, Waters, Milford, MA, USA), as described elsewhere [23]. Whole blood GPX activity was measured spectrophotometrically with an automated biochemistry analyzer (RX Daytona, Randox, Crumlin, UK) using a commercial Ransel reagent kit (Ransel, Randox, Crumlin, UK), which is based on the method of Paglia

and Valentine [42]. Concentrations of plasma F2-isoprostanes were measured with a commercially available canine-specific ELISA kit (Canine 8-iso-Prostaglandin F2a (8-isoPGF2α) ELISA kit; MyBioSource.com). The assays were performed according to the original manufacturer's instructions, with all samples assayed in duplicate.

2.3.3. Determination of Inflammatory Markers

Concentrations of serum CRP, TNF-α, and TNFSR-II were measured with commercially available canine-specific ELISA kits (Canine CRP ELISA; Alpco, Salem, NH, USA; Canine TNF-alpha Quantikine ELISA kit; R&D Systems, Minneapolis, MN, USA; Canine Tumor Necrosis Factor Soluble Receptor II (TNFSR-II) ELISA kit; MyBioSource.com). Assays were performed according to the original manufacturer's instructions, with all samples assayed in duplicate.

2.3.4. Flow Cytometry

Flow cytometric analysis of whole blood samples to determine the percentage of lymphocyte subpopulations, including T lymphocytes (CD3+), T helper cells (CD3+CD4+), activated T helper cells (CD3+CD4+CD25+), cytotoxic T lymphocytes (CD3+CD8+), activated cytotoxic T lymphocytes (CD3+CD8+CD25+), double-positive T lymphocytes (DPT) (CD3+CD4+CD8+), double-negative T lymphocytes (DNT) (CD3+CD4-CD8-), and B lymphocytes (CD45+CD21+), was performed as described previously [39]. Briefly, the whole-blood lysis method was used according to the manufacturer's protocol [43]. Blood samples were incubated with monoclonal antibodies at 2 to 8 °C for 30 min. After incubation, the erythrocytes were lysed with a red blood cell lysis solution. The cells were washed with 0.1% bovine serum albumin in phosphate-buffered saline, resuspended in 0.1% bovine serum albumin in phosphate-buffered saline, and analyzed using a FACSCanto II flow cytometer (BD Biosciences, San Jose, California). The flow cytometer was calibrated using BD FACSDiva CS &T Research Beads. Compensation controls were performed to correct for fluorescence spillover. Absolute concentrations of PBL subtypes were calculated based on differential counts of CBC and WBC and flow cytometry results.

2.3.5. Determination of Cardiac Biomarkers

For measurement of plasma NT-proBNP concentrations, IDEXX ELISA (IDEXX Laboratories, Leipzig, Germany) was used. For measurement of serum cTnI concentrations, a high-sensitivity immune-assay (ADVIA Centaur TnI-Ultra; Siemens) was used.

2.4. Echocardiographic and Clinical Assessment

Echocardiography (2-D, M-mode, color, and spectral Doppler (GE Vivid E9, General Electric Healthcare)) was performed before and after the supplementation period. Standardized transthoracic views were used [44]. Dogs were classified as ACVIM B2 (non-CHF) when asymptomatic with cardiac remodeling of the left ventricle and left atrium due to MMVD or ACVIM C and D (CHF due to MMVD), based on the echocardiographic measurements, signs of pulmonary edema on thoracic radiographs, and clinical signs [17]. Due to the large number of measured parameters in this study, only selected echocardiographic measurements were included in the study (LA/Ao, nLVIDd, normalized left ventricular internal systolic diameter (nLVIDs), mitral valve E-wave (MV E) velocity, mitral valve A-wave (MV A) velocity, MV E/A ratio, tricuspid regurgitation pressure gradient (TR PG), and fractional shortening (FS)).

2.5. Statistical Analysis

The data were analyzed using IBM SPSS (version 25, IBM Corp., Armonk, NY, USA). Descriptive statistics were used to obtain basic information about the variables measured. Due to the small number of dogs included in the individual groups, non-parametric tests were used to assess differences in measured parameters. Comparisons of measured

parameters were performed within supplemented groups before and after a 3-month supplementation period with placebo or CoQ_{10} in ACVIM B2 and CHF patients (Wilcoxon signed-rank test), and between supplemented groups—between the placebo and the CoQ_{10} groups before and after a 3-month supplementation period in ACVIM B2 and CHF patient groups (Mann–Whitney test). Furthermore, in each patient group (ACVIM B2 and CHF) supplemented either with placebo or CoQ_{10}, the change in a measured parameter was calculated (delta: the value after supplementation minus the value before supplementation) and the changes (deltas) between placebo and CoQ_{10} groups of ACVIM B2 and CHF patients were compared using the Mann–Whitney test. Additionally, all measured parameters were compared between groups of patients (ACVIM B2 and CHF) and healthy dogs (control group) before supplementation using the Kruskal–Wallis test, followed by pairwise comparisons and Bonferroni adjustments. The results were expressed as medians and interquartile ranges (IQR; 25th to 75th percentiles). A value of $p < 0.05$ was considered significant.

3. Results

Fifty-five client-owned dogs of different breeds (43 dogs with MMVD and 12 healthy control dogs) were included in the study. The inclusion process is shown in Figure 1. The baseline characteristics of all included dogs are shown in Table 1.

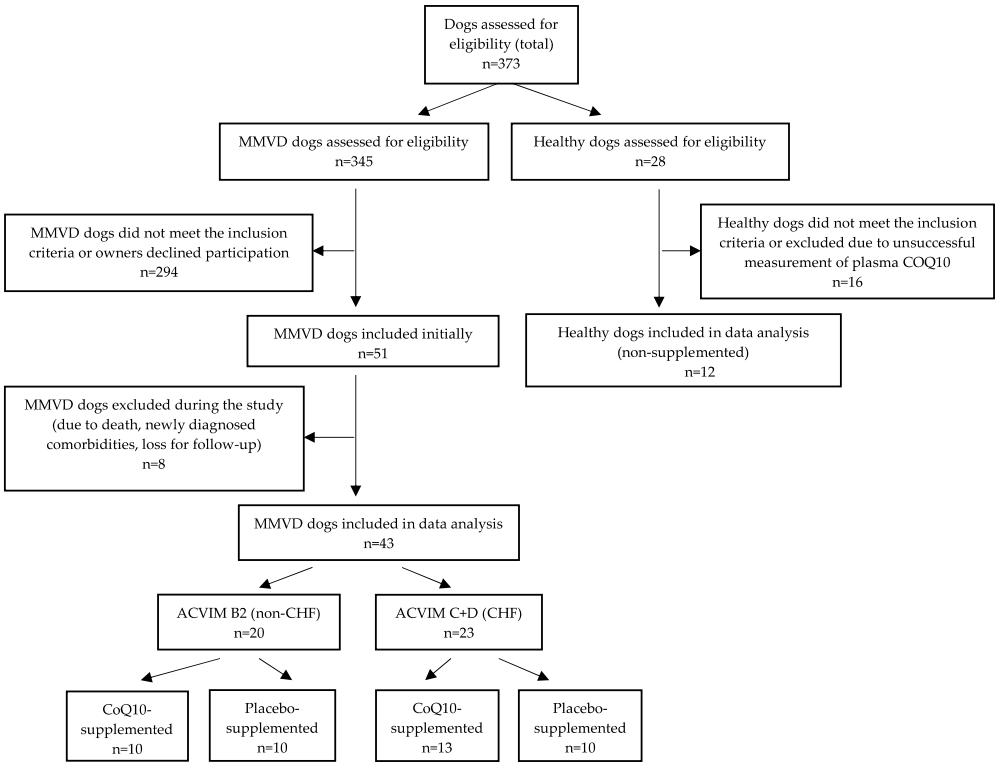

Figure 1. The flow diagram of dogs with MMVD and healthy dogs during the study.

Table 1. Baseline characteristics of dogs with MMVD (stages ACVIM B2 and C, D) and healthy dogs.

	ACVIM B2	ACVIM C, D	Healthy
Number	20	23	12
Sex (f/m)	9/11	9/14	6/6
Spayed/neutered	9/4	8/4	5/4
Age (years) Median (IQR)	11.7 * (9.6–13.5) $p = 0.001$	10.7 * (9.3–11.8)$p = 0.019$	7.9 (6.1–9.5)
Weight (kg) Median (IQR)	8.4 (6.8–11.5)	7.8 (6.0–11.4)	7.2 (4.8–13.4)
CoQ_{10} (mg/L) Median (IQR)	0.176 * (0.125–0.213)$p = 0.019$	0.171 * (0.145–0.213) $p = 0.008$	0.095 (0.070–0.143)
Breeds	5 CKCS, 4 MB, 1 PEK, 1 CHI, 1 MSCH, 1 LA, 1 NT, 1 MLT, 1 TS, 1 ECS, 1 SHI, 1 CC, 1 HAV	11 CKCS, 3 SHI, 2 MB, 2 PEK, 1 MSCH, 1 MLT, 1 ACS, 1 CHI, 1 MP	7 MB, 3 SHI, 1 YT, 1 TS
Treatment			
Pimobendan	20	23	-
ACE inhibitor	11	23	-
Furosemide or torasemide	-	23	-
Spironolactone	-	4	-
Theophylline	6	1	-
Sildenafil	1	-	-
Amlodipine	-	5	-
Potassium chloride	-	3	-

* Significant ($p < 0.05$) difference in comparison to healthy dogs. Abbreviations: ACE, angiotensin-converting enzyme; ACS, American Cocker Spaniel; ACVIM, American College of Veterinary Internal Medicine; CC, Chinese Crested Dog; CHI, Chihuahua; CKCS, Cavalier King Charles Spaniel; CoQ_{10}, coenzyme Q_{10}; ECS, English Cocker Spaniel; f, female; HAV, Havanese Dog; IQR, interquartile range; LA, Lhasa Apso; m, male; MB, mixed breed dog; MLT, Maltese; MMVD, myxomatous mitral valve disease; MP, Miniature Poodle; MSCH, Miniature Schnauzer; NT, Norfolk Terrier; PEK, Pekingese; SHI, Shi Tzu; TS, Tibetan Spaniel; YT, Yorkshire Terrier.

3.1. Within-Group Comparisons of Measured Parameters over a Three-Month Supplementation Period

The results of the within-group comparisons of the measured parameters are shown in Tables 2 and 3 for the ACVIM B2 group and the CHF group, respectively. In both patient groups, three months of CoQ_{10} supplementation resulted in a significant increase in plasma CoQ_{10} concentration compared with baseline. During three months of supplementation, none of the other measured parameters changed significantly in the CoQ_{10}- or placebo-supplemented groups of ACVIM B2 patients (Table 2), whereas in the CHF group (Table 3), supplementation with CoQ_{10} resulted in a significant change in DNT and FS.

The concentration of TNF-α was under the detection limit and as such was not included in the statistical analyses.

3.2. Comparisons of Measured Parameters between the CoQ_{10} and Placebo Groups before and after Three-Month Supplementation

The results of the comparisons of measured parameters between the CoQ_{10}-supplemented group and placebo-supplemented group are shown in Table 2 (for ACVIM B2) and Table 3 (for ACVIM C and D). In ACVIM B2 patients, baseline concentrations of measured parameters did not differ between the CoQ_{10}-supplemented and placebo-supplemented dogs, except for nLVIDd, whereas in the CHF group, none of the baseline concentrations of measured parameters differed significantly between the CoQ_{10}-supplemented and placebo-supplemented groups.

Table 2. Effect of CoQ_{10} (or placebo) supplementation on selected parameters in dogs with MMVD, stage ACVIM B2. Medians and interquartile ranges (IQR) are shown.

Parameter	CoQ_{10} Supplemented				Placebo						
	Baseline	After 3 Months	p *	Delta	Baseline	After 3 Months	p *	Delta	p [a]	p [b]	p [c]
CoQ_{10} (mg/L)	0.190 (0.129–0.271)	1.050 (0.560–1.364)	**0.007**	0.790 (0.439–1.118)	0.158 (0.123–0.199)	0.174 (0.140–0.212)	0.646	−0.009 (−0.024–0.028)	0.364	**<0.001**	**<0.001**
GPX (U/g HGB)	758.0 (692.8–794.8)	765.5 (725.1–793.8)	0.333	11.5 (−13.1–28.5)	701.0 (643.7–806.1)	668.1 (644.7–807.3)	0.508	−0.1 (−79.1–25.7)	0.364	0.070	0.496
F2-isoprostanes (pg/mL)	435.7 (280.0–568.8)	476.4 (417.8–859.6)	0.386	65.2 (−92.7–246.9)	512.8 (335.9–643.6)	639.8 (303.7–858.8)	0.093	82.4 (−10.9–198.9)	0.364	0.762	0.650
TNFSR-II (ng/mL)	0.666 (0.214–2.693)	0.966 6 (0.461–3.721)	0.799	−0.126 (−0.655–0.709)	0.464 (0.348–0.780)	0.662 (0.473–1.225)	0.169	0.220 (−0.097–0.408)	0.597	0.496	0.496
WBC ($\times 10^9$/L)	8.2 (6.1–9.5)	8.6 (7.4–10.5)	**0.037**	0.8 (0.0–2.2)	10.0 (7.1–13.5)	9.1 (7.3–12.4)	0.445	−0.5 (−1.8–1.0)	0.131	0.762	0.070
Neutrophils (%)	70.1 (64.6–72.8)	68.1 (63.7–75.0)	0.959	−0.3 (−4.1–2.9)	68.9 (67.2–77.4)	67.4 (63.8–75.7)	0.092	−2.9 (−4.7–0.7)	0.970	0.821	0.241
Neutrophils ($\times 10^9$/L)	5.7 (4.2–6.6)	5.9 (5.1–7.5)	0.095	0.6 (−0.1–1.2)	6.8 (4.5–9.4)	6.4 (4.7–8.8)	0.285	−0.7 (−1.6–0.7)	0.131	0.597	0.096
Monocytes (%)	4.5 (4.0–5.4)	4.3 (4.0–4.9)	1.000	0.1 (−0.8–0.2)	4.0 (3.3–5.1)	4.2 (3.4–5.0)	0.330	0.2 (−0.3–0.7)	0.345	0.705	0.425
Monocytes ($\times 10^9$/L)	0.32 (0.26–0.50)	0.37 (0.28–0.50)	0.241	0.06 (−0.03–0.13)	0.37 (0.28–0.48)	0.42 (0.31–0.47)	0.799	−0.03 (−0.09–0.11)	0.705	0.880	0.326
NLR	3.2 (2.4–3.9)	3.1 (2.5–4.6)	0.878	0.0 (−0.6–0.5)	3.0 (2.9–5.6)	2.9 (2.3–4.7)	0.139	−0.3 (−1.0–0.1)	1.000	0.821	0.326
Lymphocytes (%)	22.0 (18.8–28.2)	21.4 (16.3–26.7)	0.445	−1.0 (−3.2–2.4)	22.7 (14.1–23.6)	22.9 (16.2–28.1)	0.203	1.2 (−0.6–4.1)	1.000	0.821	0.064
Lymphocytes ($\times 10^9$/L)	2.0 (1.2–2.2)	2.0 (1.4–2.7)	0.093	0.3 (-0.1–0.4)	1.9 (1.5–3.2)	2.2 (1.6–3.1)	0.508	0.0 (-0.1–0.3)	0.406	0.545	0.364
T lymphocytes CD3+ (%)	71.0 (67.1–77.9)	69.1 (53.1–74.4)	0.508	–0.5 (−13.9–3.4)	57.8 (49.2–67.9)	59.4 (45.7–68.5)	0.959	−0.5 (−5.9–6.1)	0.059	0.290	0.597
T lymphocytes CD3+ ($\times 10^9$/L)	1.4 (0.7–1.8)	1.3 (0.9–1.9)	0.241	0.1 (−0.1–0.3)	1.2 (0.7–1.7)	1.4 (0.6–1.7)	0.445	0.1 (−0.2–0.3)	0.705	0.940	0.705
T helper cells CD3+CD4+ (%)	42.0 (28.4–48.5)	40.3 (30.0–45.6)	0.759	−2.0 (−2.5–2.9)	41.5 (35.0–51.2)	43.8 (34.5–49.8)	0.445	−0.2 (−2.7–1.2)	0.880	0.545	0.940
T helper cells CD3+CD4+ ($\times 10^9$/L)	0.41 (0.33–0.63)	0.45 (0.36–0.55)	0.203	0.05 (−0.01–0.08)	0.44 (0.25–0.79)	0.61 (0.18–0.77)	0.646	−0.01 (−0.07–0.08)	1.000	0.496	0.496
Activated T helper cells CD3+CD4+CD25+ (%)	35.8 (21.4–46.1)	30.6 (22.2–44.9)	0.515	−0.8 (−2.5–1.7)	25.7 (16.1–41.1)	23.7 (19.1–35.0)	0.878	2.1 (−5.6–3.7)	0.307	0.290	0.677
Activated T helper cells CD3+CD4+CD25+ ($\times 10^9$/L)	0.16 (0.10–0.22)	0.14 (0.09–0.21)	0.575	0.01 (−0.02–0.03)	0.13 (0.07–0.19)	0.13 (0.05–0.21)	0.959	−0.00 (−0.03–0.03)	0.545	0.650	0.821

Table 2. *Cont.*

Parameter	CoQ_{10} Supplemented				Placebo						
	Baseline	After 3 Months	p *	Delta	Baseline	After 3 Months	p *	Delta	p [a]	p [b]	p [c]
Cytotoxic T lymphocytes CD3+CD8+ (%)	44.2 (33.9–56.7)	44.5 (36.9–58.7)	0.285	2.2 (−2.0–4.6)	36.7 (27.8–44.0)	34.2 (30.3–41.7)	0.721	0.9 (−2.1–2.6)	0.290	0.131	0.408
Cytotoxic T lymphocytes CD3+CD8+ ($\times 10^9$/L)	0.57 (0.26–0.89)	0.57 (0.35–1.02)	0.093	0.07 (−0.02–0.13)	0.39 (0.24–0.64)	0.46 (0.21–0.70)	0.386	0.04 (−0.08–0.12)	0.290	0.406	0.650
Activated cytotoxic T lymphocytes CD3+CD8+CD25+ (%)	8.8 (5.7–15.4)	7.4 (4.3–12.4)	0.241	−1.3 (−2.8–0.9)	9.5 (3.8–14.1)	7.8 (3.1–16.7)	0.646	0.0 (−5.1–1.5)	0.821	0.940	0.733
Activated cytotoxic T lymphocytes CD3+CD8+CD25+ ($\times 10^9$/L)	0.05 (0.03–0.08)	0.04 (0.03–0.06)	0.878	−0.00 (−0.01–0.01)	0.04 (0.01–0.07)	0.04 (0.02–0.06)	0.646	−0.00 (−0.03–0.01)	0.496	0.762	0.940
T helper cells/cytotoxic T lymphocytes ratio (CD4/CD8)	1.0 (0.5–1.4)	0.9 (0.5–1.2)	0.508	−0.0 (−0.1–0.1)	1.1 (0.9–1.6)	1.3 (0.9–1.5)	0.508	−0.0 (−0.2–0.1)	0.450	0.257	1.000
DPT CD3+CD4+CD8+ (%)	0.6 (0.5–1.7)	0.7 (0.5–1.3)	0.766	0.1 (−0.3–0.2)	1.0 (0.6–2.1)	1.0 (0.7–2.7)	0.528	0.1 (−0.3–0.5)	0.206	0.266	0.733
DPT CD3+CD4+CD8+ ($\times 10^9$/L)	0.008 (0.007–0.016)	0.010 (0.007–0.019)	0.386	0.000 (−0.001–0.005)	0.013 (0.009–0.018)	0.015 (0.007–0.027)	0.386	0.001 (−0.002–0.012)	0.326	0.257	0.762
DNT CD3+CD4-CD8- (%)	15.2 (11.9–17.0)	14.6 (10.6–16.2)	0.285	−1.5 (−4.0–0.8)	16.5 (10.8–20.4)	18.2 (10.4–20.8)	0.953	−0.1 (−1.0–1.3)	0.820	0.450	0.199
DNT CD3+CD4-CD8- ($\times 10^9$/L)	0.17 (0.11–0.27)	0.16 (0.13–0.23)	0.799	0.02 (−0.07–0.02)	0.15 (0.10–0.24)	0.16 (0.09–0.27)	0.878	−0.01 (−0.03–0.05)	1.000	0.940	0.880
B lymphocytes CD45+CD21+ (%)	12.3 (8.3–17.2)	13.9 (7.4–16.7)	0.919	−0.1 (−1.4–2.0)	16.9 (10.2–24.6)	13.5 (10.1–22.0)	0.110	−1.5 (−2.3–0.2)	0.130	0.427	0.089
B lymphocytes CD45+CD21+ ($\times 10^9$/L)	0.19 (0.13–0.32)	0.22 (0.14–0.32)	0.285	0.03 (−0.03–0.06)	0.32 (0.13–0.78)	0.29 (0.13–0.68)	0.575	-0.00 (−0.10–0.03)	0.290	0.364	0.364
NT-proBNP (pmol/L)	646.5 (440.3–1031.3)	712.0 (455.0–1044.0)	0.541	0.0 (−143.0–236.3)	974.0 (867.0–1299.0)	953.0 (602.5–1247.5)	0.515	3.0 (−238.0–49.5)	0.102	0.288	0.450
cTnI (µg/L)	0.048 (0.037–0.099)	0.044 (0.031–0.067)	0.959	0.003 (−0.016–0.011)	0.043 (0.030–0.075)	0.039 (0.025–0.066)	0.678	−0.004 (−0.010–0.008)	0.450	0.762	0.970
LA/Ao	2.0 (1.8–2.0)	1.8 (1.5–2.0)	0.333	−0.1 (−0.4–0.1)	1.9 (1.8–2.2)	2.0 (1.7–2.3)	0.721	−0.0 (−0.2–0.2)	0.970	0.272	0.650
nLVIDd	1.7 (1.6–1.8)	1.7 (1.6–1.8)	0.878	−0.0 (−0.1–0.2)	1.9 (1.8–2.0)	1.9 (1.9–2.0)	0.878	0.0 (−0.1–0.1)	**0.034**	0.082	1.000
nLVIDs	0.9 (0.8–1.0)	1.0 (0.8–1.0)	0.333	0.0 (−0.1–0.2)	1.0 (0.8–1.2)	1.0 (0.8–1.1)	0.878	0.0 (−0.1–0.1)	0.290	0.597	0.290
MV E velocity (m/s)	0.96 (0.88–1.09)	0.90 (0.82–1.00)	0.161	−0.10 (-0.21–0.05)	0.87 (0.84–1.14)	1.04 (0.80–1.20)	0.475	0.01 (−0.09–0.17)	0.657	0.328	0.197
MV A velocity (m/s)	0.84 (0.59–0.90)	0.75 (0.65–0.91)	0.889	0.01 (−0.13–0.12)	0.75 (0.60–0.98)	0.74 (0.59–0.82)	0.507	−0.03 (−0.12–0.10)	0.965	0.756	0.503

Table 2. *Cont.*

Parameter	CoQ_{10} Supplemented				Placebo						
	Baseline	After 3 Months	p *	Delta	Baseline	After 3 Months	p *	Delta	p [a]	p [b]	p [c]
MV E/A	1.18 (1.05–1.61)	1.08 (1.00–1.30)	0.575	−0.04 (−0.40–0.27)	1.15 (0.96–1.56)	1.25 (1.05–1.69)	0.114	0.17 (−0.01–0.27)	0.477	0.286	0.286
TR PG (mmHg)	28.0 (20.0–33.0)	29.0 (26.0–51.0)	0.259	6.0 (−6.0–16.5)	28.0 (25.0–50.0)	33.0 (27.0–34.0)	1.000	3.0 (−13.0–7.9)	0.424	0.915	0.367
FS (%)	47.5 (41.8–49.3)	45.0 (38.0–51.0)	0.261	−2.0 (−8.5–2.3)	44.0 (40.5–55.3)	44.0 (41.0–53.5)	0.799	0.0 (−2.3–1.5)	0.791	0.363	0.448
HR (bpm)	130.0 (119.0–150.0)	124.0 (100.0–142.5)	0.321	−2.0 (−12.5–8.5)	135.0 (117.5–140.0)	125.0 (110.0–142.5)	0.713	−10.0 (−10.0–12.5)	0.818	0.676	0.907
Murmur (grade)	4.0 (3.8–4.0)	4.0 (3.0–4.0)	0.157	0.0 (−0.3–0.0)	4.0 (4.0–4.0)	4.0 (4.0–4.0)	0.317	0.0 (0.0–0.0)	0.542	0.194	0.542
Weight (kg)	7.7 (6.0–10.7)	8.2 (5.7–11.2)	0.799	0.0 (−0.2–0.3)	9.2 (7.2–13.8)	9.1 (7.1–14.3)	0.721	−0.0 (−0.3–0.2)	0.257	0.384	0.791
BCS	5.0 (5.0–6.3)	5.0 (5.0–6.3)	1.000	0.0 (0.0–0.0)	5.0 (4.8–5.3)	5.0 (4.0–6.0)	0.655	0.00 (0.00–0.00)	0.170	0.319	0.957

[a] *p*-values resulting from the comparison (Wilcoxon test) of parameters between baseline and the end of the supplementation period in the same group; [a] *p*-values resulting from the comparison (Mann–Whitney test) of parameters between the placebo and the CoQ_{10} groups at baseline; [b] *p*-values resulting from the comparison (Mann–Whitney test) of parameters between the placebo and the CoQ_{10} groups after a 3-month supplementation period; [c] *p*-values resulting from the comparison (Mann–Whitney test) of delta values between the placebo and CoQ_{10} groups. Significant results ($p < 0.05$) are in bold. Abbreviations: A, A-wave (late transmitral blood flow); ACVIM, American College of Veterinary Internal Medicine; BCS, body condition score; cardiac TnI, cardiac troponin I; CD, cluster of differentiation; CoQ_{10}, coenzyme Q_{10}; DNT, double-negative T lymphocytes; DPT, double-positive T lymphocytes; E, E-wave (early transmitral blood flow); FS, fractional shortening; GPX, glutathione peroxidase; HR, heartrate; IQR, interquartile range; LA/Ao, left atrium to aorta ratio; MMVD, myxomatous mitral valve disease; MV, mitral valve; NLR, neutrophil-to-lymphocyte ratio; nLVIDd, normalized end-diastole left ventricular internal dimension; nLVIDs, normalized end-systole left ventricular internal dimension; NT-proBNP, N-terminal pro B-type natriuretic peptide; TNFα, tumor necrosis factor α; TNFSR-II, tumor necrosis factor soluble receptor II; TR PG, tricuspid regurgitation peak gradient; WBC, white blood cells.

Table 3. Effect of CoQ_{10} (or placebo) supplementation on selected parameters in dogs with MMVD and CHF. Medians and interquartile ranges (IQR) are shown.

Parameter	CoQ_{10} Supplemented				Placebo						
	Baseline	After 3 Months	p *	Delta	Baseline	After 3 Months	p *	Delta	p [a]	p [b]	p [c]
CoQ_{10} (mg/L)	0.171 (0.144–0.222)	0.863 (0.598–1.247)	**0.001**	0.705 (0.371–1.071)	0.164 (0.142–0.230)	0.163 (0.138–0.208)	0.575	0.007 (−0.033–0.034)	0.804	**<0.001**	**<0.001**
GPX (U/g HGB)	807.3 (690.8–823.9)	765.9 (711.1–812.3)	0.701	−14.8 (−41.6–32.1)	786.4 (723.3–854.3)	827.8 (729.2–894.1)	0.139	30.8 (7.7–57.7)	0.664	0.137	0.137
F2-isoprostanes (pg/mL)	510.6 (405.7–680.8)	534.1 (467.7–649.8)	0.917	7.4 (−104.2–90.9)	468.2 (338.1–737.8)	658.5 (399.1–813.3)	0.169	85.7 (−35.7–169.5)	0.804	0.756	0.239
TNFSR-II (ng/mL)	2.144 (1.292–3.278)	1.113 (0.802–1.683)	0.071	−0.650 (−2.059–0.270)	0.983 (0.607–4.312)	1.149 (0.713–2.947)	0.515	−0.007 (−1.559–0.322)	0.598	0.843	0.391
WBC ($\times 10^9$/L)	8.8 (6.5–10.0)	9.8 (7.2–11.3)	0.249	0.8 (−0.7–1.3)	9.9 (7.1–12.7)	9.3 (8.0–13.1)	0.799	−0.6 (−1.2–1.4)	0.385	0.577	0.420
Neutrophils (%)	69.3 (63.9–73.9)	67.4 (64.7–72.5)	**0.363**	−2.2 (−4.6–3.0)	66.4 (58.1–71.4)	68.6 (62.7–76.2)	0.241	4.2 (−1.1–10.3)	0.336	0.852	**0.041**
Neutrophils ($\times 10^9$/L)	5.9 (4.4–7.6)	6.0 (4.9–8.1)	0.345	0.5 (−0.6–0.9)	5.9 (4.8–8.6)	6.9 (5.5–7.8)	0.721	0.0 (−1.0–1.4)	0.756	0.577	0.901

Table 3. *Cont.*

Parameter	CoQ_{10} Supplemented				Placebo						
	Baseline	After 3 Months	p *	Delta	Baseline	After 3 Months	p *	Delta	p [a]	p [b]	p [c]
Monocytes (%)	5.1 (4.3–6.2)	5.2 (4.1–6.4)	0.527	−0.2 (−0.5–0.7)	5.1 (4.0–6.3)	4.8 (3.1–5.4)	0.386	−0.6 (−1.5–0.6)	0.664	0.351	0.192
Monocytes ($\times 10^9$/L)	0.46 (0.36–0.61)	0.47 (0.40–0.59)	0.311	0.01 (−0.04–0.09)	0.49 (0.35–0.66)	0.45 (0.37–0.56)	0.445	−0.01 (−0.21–0.13)	0.535	0.664	0.121
NLR	3.3 (2.5–4.6)	3.2 (2.6–3.8)	0.345	−0.3 (−0.8–0.5)	3.0 (2.3–3.8)	3.7 (2.5–4.9)	0.093	0.8 (0.3–1.2)	0.620	0.577	0.055
Lymphocytes (%)	21.5 (16.2–24.4)	22.3 (18.6–25.3)	0.311	1.3 (−2.5–3.9)	22.1 (17.7–28.3)	18.7 (15.7–25.8)	0.139	−3.5 (−9.0– −0.3)	0.598	0.438	**0.044**
Lymphocytes ($\times 10^9$/L)	1.6 (1.4–2.1)	2.0 (1.4–2.6)	0.152	0.0 (−0.2–0.5)	2.0 (1.6–3.2)	1.9 (1.1–3.1)	0.203	−0.3 (−0.7–0.1)	0.107	0.804	**0.041**
T lymphocytes CD3+ (%)	61.6 (50.3–70.7)	63.2 (42.4–74.5)	0.600	1.5 (−5.3–6.6)	58.6 (22.9–64.9)	58.7 (33.5–66.1)	0.169	5.4 (−4.5–15.0)	0.264	0.420	0.264
T lymphocytes CD3+ ($\times 10^9$/L)	1.0 (0.8–1.3)	1.0 (0.8–1.5)	0.152	0.0 (−0.1–0.3)	0.9 (0.4–1.9)	1.2 (0.5–1.8)	0.878	−0.0 (−0.1–0.1)	0.951	0.951	0.352
T helper cells CD3+CD4+ (%)	50.5 (46.5–60.0)	53.1 (45.5–54.7)	0.093	−2.8 (−6.4–1.1)	52.3 (41.7–54.5)	49.3 (44.6–55.7)	0.721	0.5 (−3.6–3.5)	0.385	0.951	0.154
T helper cells CD3+CD4+ ($\times 10^9$/L)	0.47 (0.38–0.72)	0.46 (0.37–0.76)	0.861	−0.00 (−0.09–0.08)	0.45 (0.19–1.00)	0.58 (0.28–0.82)	0.646	−0.01 (−0.09–0.09)	0.804	0.852	1.000
Activated T helper cells CD3+CD4+CD25+ (%)	22.6 (19.2–42.3)	22.7 (17.9–30.6)	0.505	−1.5 (−4.6–2.0)	30.5 (26.8–33.3)	27.2 (25.2–32.6)	**0.037**	−3.5 (−5.6– −0.9)	0.131	0.313	0.402
Activated T helper cells CD3+CD4+CD25+ ($\times 10^9$/L)	0.12 (0.08–0.24)	0.11 (0.09–0.17)	0.650	−0.01 (−0.04–0.03)	0.15 (0.05–0.25)	0.15 (0.09–0.20)	0.445	−0.01 (−0.09–0.03)	1.000	0.535	0.951
Cytotoxic T lymphocytes CD3+CD8+ (%)	29.1 (23.3–33.3)	32.3 (23.0–39.9)	0.075	2.1 (−0.6–8.4)	28.1 (23.8–34.2)	30.3 (25.7–35.9)	0.343	1.3 (−1.7–4.9)	0.877	0.901	0.515
Cytotoxic T lymphocytes CD3+CD8+ ($\times 10^9$/L)	0.28 (0.21–0.39)	0.37 (0.18–0.54)	0.075	0.01 (−0.01–0.19)	0.27 (0.12–0.65)	0.36 (0.14–0.59)	0.386	0.02 (−0.04–0.06)	0.852	0.756	0.420
Activated cytotoxic T lymphocytes CD3+CD8+CD25+ (%)	9.2 (6.0–14.7)	8.3 (5.7–10.6)	0.239	−0.5 (−3.9–1.4)	12.2 (8.7–16.7)	12.4 (5.7–15.7)	0.168	−1.1 (−1.7–0.8)	0.251	0.264	0.901
Activated cytotoxic T lymphocytes CD3+CD8+CD25+ ($\times 10^9$/L)	0.03 (0.02–0.04)	0.03 (0.02–0.04)	0.917	−0.00 (−0.01–0.01)	0.03 (0.01–0.07)	0.03 (0.02–0.06)	0.799	−0.00 (−0.01–0.01)	1.000	0.951	0.710
T helper cells/cytotoxic T lymphocytes ratio (CD4/CD8)	1.6 (1.4–2.6)	1.6 (1.2–2.5)	0.133	–0.3 (–0.5–0.1)	1.9 (1.3–2.1)	1.6 (1.4–2.0)	0.445	−0.0 (−0.5–0.2)	0.710	0.804	0.535

Table 3. *Cont.*

Parameter	CoQ_{10} Supplemented				Placebo						
	Baseline	After 3 Months	p *	Delta	Baseline	After 3 Months	p *	Delta	p [a]	p [b]	p [c]
DPT CD3+CD4+ CD8+ (%)	1.2 (0.8–2.1)	1.0 (0.8–1.9)	0.592	−0.1 (−0.3–0.2)	1.2 (0.8–2.0)	1.2 (1.0–2.2)	0.252	0.1 (−0.1–0.3)	0.975	0.454	0.235
DPT CD3+CD4+CD8+ ($\times 10^9$/L)	0.011 (0.005–0.020)	0.012 (0.005–0.024)	0.972	−0.001 (−0.002–0.004)	0.012 (0.007–0.018)	0.014 (0.007–0.022)	0.575	0.000 (−0.002–0.005)	0.901	0.951	0.852
DNT CD3+CD4-CD8- (%)	17.1 (12.9–24.3)	17.2 (11.2–22.7)	**0.046**	−1.7 (−2.6—0.2)	19.5 (15.0–20.9)	16.9 (13.0–19.4)	**0.028**	−2.1 (−3.0– −0.2)	0.828	0.975	0.576
DNT CD3+CD4-CD8- ($\times 10^9$/L)	0.17 (0.12–0.22)	0.17 (0.11–0.22)	0.972	-0.00 (−0.03–0.03)	0.18 (0.09–0.34)	0.19 (0.10–0.27)	0.241	−0.01 (−0.07–0.01)	0.901	0.804	0.385
B lymphocytes CD45+CD21+ (%)	14.8 (11.8–26.0)	18.5 (13.0–23.2)	0.221	1.0 (−2.0–5.4)	17.3 (15.8–20.1)	14.2 (12.8–21.1)	0.285	−3.3 (−4.6–2.5)	0.535	0.336	0.107
B lymphocytes CD45+CD21+ ($\times 10^9$/L)	0.31 (0.18–0.40)	0.34 (0.25–0.53)	0.152	0.02 (−0.03–0.25)	0.39 (0.27–0.58)	0.26 (0.15–0.67)	0.333	−0.04 (−0.14–0.06)	0.215	0.664	0.121
NT-proBNP (pmol/L)	1600.0 (647.5–2356.5)	1344.0 (767.5–4472.0)	0.382	117.0 (−177.5–1798.0)	2688.0 (1730.0–4271.5)	2647.0 (1215.5–4917.3)	0.799	−35.0 (−867.0–1848.0)	0.137	0.385	0.577
cTnI (µg/L)	0.060 (0.030–0.090)	0.064 (0.039–0.166)	0.249	0.013 (−0.010–0.035)	0.090 (0.062–0.173)	0.135 (0.051–0.263)	0.326	0.004 (−0.023–0.076)	0.117	0.216	0.841
LA/Ao	2.2 (2.1–2.4)	2.2 (2.0–2.4)	0.646	0.0 (−0.2–0.2)	2.2 (2.0–2.3)	2.0 (1.8–2.5)	0.760	−0.1 (−0.2–0.4)	0.344	0.256	0.940
nLVIDd	2.0 (2.0–2.2)	2.1 (1.9–2.3)	0.695	0.0 (−0.1–0.2)	2.1 (1.7–2.4)	1.8 (1.6–2.4)	0.333	−0.1 (−0.4–0.1)	0.895	0.391	0.391
nLVIDs	1.0 (0.7–1.2)	1.1 (0.9–1.4)	0.060	0.2 (0.0–0.3)	1.0 (0.8–1.5)	0.8 (0.7–1.3)	**0.022**	−0.1 (−0.2– −0.0)	0.553	0.391	**0.006**
MV E velocity (m/s)	1.29 (1.12–1.51)	1.18 (1.01–1.68)	0.638	0.05 (−0.14–0.18)	1.19 (1.03–1.53)	1.15 (0.81–1.40)	0.074	−0.13 (−0.22–0.06)	0.368	0.420	0.162
MV A velocity (m/s)	0.79 (0.72–1.02)	0.84 (0.65–0.97)	0.328	-0.04 (−0.15–0.10)	0.85 (0.79–0.96)	0.90 (0.60–1.13)	0.646	−0.05 (−0.15–0.13)	0.620	0.828	0.780
MV E/A	1.49 (1.14–1.85)	1.57 (1.21–1.73)	0.972	0.06 (−0.43–0.33)	1.49 (1.18–1.61)	1.29 (1.06–1.71)	0.285	−0.04 (−0.29–0.12)	0.710	0.239	0.756
TR PG (mmHg)	40.0 (35.8–51.8)	41.0 (35.5–50.0)	0.859	−2.0 (−12.3–10.0)	38.0 (36.5–53.5)	34.0 (26.5–48.5)	0.373	−5.9 (−14.0–7.5)	0.902	0.306	0.567
FS (%)	47.5 (44.0–61.8)	47.0 (36.0–48.8)	**0.049**	−5.5 (−10.3—4)	44.7 (33.8–52.0)	45.5 (38.3–58.5)	0.105	2.5 (−0.3–8.8)	0.321	0.843	**0.007**
HR (bpm)	140.0 (110.0–145.0)	130.0 (120.0–150.0)	0.632	10.0 (−15.0–10.0)	133.0 (120.0–142.5)	130.0 (110.0–142.5)	0.671	−5.0 (−16.5–20.0)	0.850	0.684	0.490
Murmur (grade)	4.00 (4.0–5.0)	4.00 (4.0–4.5)	0.083	0.0 (−0.5–0.0)	4.0 (4.0–5.0)	4.5 (4.0–5.0)	0.564	0.0 (0.0–0.3)	0.442	0.365	0.136
Weight (kg)	8.0 (5.2–11.0)	8.0 (4.7–10.5)	0.328	−0.2 (−0.6–0.1)	7.8 (6.9–11.6)	8.1 (7.1–11.6)	0.285	0.2 (−0.3–0.6)	0.620	0.535	0.163
BCS	5.0 (4.5–6.5)	5.0 (3.5–6.5)	0.206	0.0 (−1.0–0.0)	4.0 (3.75–6.0)	4.5 (3.75–6.25)	0.083	0.0 (0.0–1.0)	0.127	0.775	0.062

* p-values resulting from the comparison (Wilcoxon test) of parameters between baseline and the end of the supplementation period in the same group; [a] p-values resulting from the comparison (Mann–Whitney test) of parameters between the placebo and CoQ_{10} groups at baseline; [b] p-values resulting from the comparison (Mann–Whitney test) of parameters between the placebo and CoQ_{10} groups after a 3-month supplementation period; [c] p-values resulting from the comparison (Mann–Whitney test) of delta values between the placebo and CoQ_{10} groups. Significant results ($p < 0.05$) are in bold. Abbreviations: A, A-wave (late transmitral blood flow); ACVIM, American College of Veterinary Internal Medicine; BCS, body condition score; cardiac TnI, cardiac troponin I; CD, cluster of differentiation; CoQ_{10}, coenzyme Q_{10}; DNT, double-negative T lymphocytes; DPT, double-positive T lymphocytes; E, E-wave (early transmitral blood flow); FS, fractional shortening; GPX, glutathione peroxidase; HR, heartrate; IQR, interquartile range; LA/Ao, left atrium to aorta ratio; MMVD, myxomatous mitral valve disease; MV, mitral valve; NLR, neutrophil-to-lymphocyte ratio; nLVIDd, normalized end-diastole left ventricular internal dimension; nLVIDs, normalized end-systole left ventricular internal dimension; NT-proBNP, N-terminal pro B-type natriuretic peptide; TNFα, tumor necrosis factor α; TNFSR-II, tumor necrosis factor soluble receptor II; TR PG, tricuspid regurgitation peak gradient; WBC, white blood cells.

After the three-month supplementation period, plasma CoQ_{10} concentrations were significantly higher in the CoQ_{10}-supplemented group than in the placebo-supplemented group in ACVIM B2 (Table 2) and CHF dogs (Table 3). No other parameters differed significantly in either patient group.

3.3. Comparisons of Changes (Deltas) in Measured Parameters between CoQ_{10}-Supplemented and Placebo-Supplemented Groups

In both patient groups, ACVIM B2 and CHF, a significantly higher change (increase) in plasma CoQ_{10} concentration was observed in the CoQ_{10}-supplemented groups compared with the change in this parameter in the corresponding placebo groups (Tables 2 and 3). In ACVIM B2 patients (Table 2), no significant differences in the change in other parameters were observed between the placebo and CoQ_{10}-supplemented groups, whereas in CHF patients (Table 3), a significant difference in the change (increase or decrease) in several parameters was observed between the placebo and CoQ_{10}-supplemented groups.

3.4. Comparisons of Measured Parameters between ACVIM B2, CHF, and Healthy Groups

The results of the comparison of measured parameters between ACVIM B2, CHF, and healthy dogs are shown in Tables 1 and 4. Healthy dogs were significantly younger than those in the ACVIM B2 and CHF groups (Table 1). In addition, statistical analysis revealed significant differences in numerous parameters between dog groups, as indicated in Table 4.

3.5. Owner-Perceived Assessment of the Condition of a Dog at the end of the Supplementation Period Compared to That at the Beginning (Subjective Assessment, Not Included in the Statistics)

Out of ten owners of ACVIM B2 dogs supplemented with CoQ_{10} (10 dogs), four assessed the overall condition of the dog as better, four similar, and two worse than it was before the start of supplementation. For ACVIM B2 placebo-supplemented dogs (ten dogs), the results of the overall condition of the dog were as follows: three better, seven similar, and none worse than that prior to supplementation. For CHF CoQ_{10}-supplemented dogs (thirteen dogs), the results of the overall condition of the dog were as follows: seven better, four similar, and two worse than that prior to supplementation. For CHF placebo-supplemented dogs (ten dogs), the results of the overall condition of the dog were as follows: two better, two similar, and six worse than that prior to supplementation.

3.6. Adverse Effects

No significant adverse effects were noticed during the study. Short-lived diarrhea which did not require treatment was noted in 3 out of 23 dogs receiving CoQ_{10} and in 4 out of 20 dogs receiving placebo during the study period.

Table 4. Baseline measurements in MMVD (ACVIM B2 and CHF) and healthy dogs. Medians and interquartile ranges (IQR) are shown.

Parameter	ACVIM B2	CHF	Healthy	*p* [a]	*p* [b]	*p* [c]
CoQ_{10} (mg/L)	0.176 (0.125–0.213)	0.171 (0.145–0.213)	0.095 (0.070–0.143)	**0.006**	**0.019**	**0.008**	1.000
GPX (U/g HGB)	746.5 (675.8–792.1)	792.6 (692.0–827.7)	775.4 (684.1–827.5)	0.317	/	/	/
F2-isoprostanes (pg/mL)	468.9 (323.2–586.1)	510.6 (356.2–727.0)	536.0 (420.2–884.2)	0.373	/	/	/
TNFSR-II (ng/mL)	0.539 (0.247–0.949)	1.716 (0.683–3.426)	0.752 (0.495–0.876)	**0.040**	1.000	**0.048**	**0.006**
WBC ($\times 10^9$/L)	8.4 (6.9–10.7)	9.0 (7.1–10.9)	8.3 (5.9–9.5)	0.463	/	/	/
Neutrophils (%)	69.4 (66.8–74.7)	68.9 (63.3–72.5)	66.9 (62.6–72.9)	0.642	/	/	/
Neutrophils ($\times 10^9$/L)	5.7 (4.9–8.1)	5.9 (4.8–7.8)	5.6 (4.1–6.6)	0.556	/	/	/
Monocytes (%)	4.3 (3.6–5.1)	5.1 (4.1–6.2)	4.1 (3.3–5.7)	0.120	/	/	/
Monocytes ($\times 10^9$/L)	0.35 (0.28–0.46)	0.47 (0.36–0.63)	0.34 (0.27–0.49)	**0.044**	1.000	0.122	0.099
NLR	3.0 (2.9–4.5)	3.2 (2.6–4.3)	2.7 (2.3–4.0)	0.654	/	/	/
Lymphocytes (%)	22.5 (16.9–23.7)	21.5 (16.9–25.4)	24.3 (18.6–27.7)	0.624	/	/	/
Lymphocytes ($\times 10^9$/L)	1.9 (1.3–2.6)	1.8 (1.5–2.5)	1.8 (1.4–2.5)	0.877	/	/	/
T lymphocytes CD3+ (%)	67.1 (53.6–75.9)	61.0 (30.5–67.2)	60.5 (53.9–71.7)	0.180	/	/	/
T lymphocytes CD3+ ($\times 10^9$/L)	1.22 (0.76–1.73)	0.96 (0.62–1.55)	1.04 (0.72–1.66)	0.640	/	/	/
T helper cells CD3+CD4+ (%)	41.7 (31.2–47.9)	52.0 (44.7–56.6)	53.6 (45.2–62.8)	**0.003**	**0.012**	1.000	**0.013**
T helper cells CD3+CD4+ ($\times 10^9$/L)	0.41 (0.31–0.73)	0.47 (0.35–1.55)	0.55 (0.42–0.73)	0.314	/	/	/
Activated T helper cells CD3+CD4+CD25+ (%)	30.2 (19.5–44.7)	26.0 (21.4–32.8)	35.7 (23.0–42.0)	0.497	/	/	/
Activated T helper cells CD3+CD4+CD25+ ($\times 10^9$/L)	0.14 (0.08–0.20)	0.13 (0.07–0.25)	0.19 (0.16–0.24)	0.910	/	/	/
Cytotoxic T lymphocytes CD3+CD8+ (%)	39.1 (31.1–50.1)	29.1 (24.0–33.2)	26.0 (20.3–34.8)	**0.003**	**0.020**	1.000	**0.007**
Cytotoxic T lymphocytes CD3+CD8+ ($\times 10^9$/L)	0.47 (0.26–0.76)	0.28 (0.14–0.40)	0.30 (0.19–0.50)	0.080	/	/	/
Activated cytotoxic T lymphocytes CD3+CD8+CD25+ (%)	9.1 (5.2–14.0)	10.1 (6.2–14.7)	12.5 (10.6–22.6)	0.178	/	/	/
Activated cytotoxic T lymphocytes CD3+CD8+CD25+ ($\times 10^9$/L)	0.05 (0.03–0.07)	0.03 (0.01–0.05)	0.07 (0.03–0.09)	0.456	/	/	/
T helper cells/cytotoxic T lymphocytes ratio (CD4/CD8)	1.05 (0.72–1.40)	1.75 (1.42–2.28)	2.17 (1.31–2.76)	**0.001**	**0.007**	1.000	**0.003**
DPT CD3+CD4+CD8+ (%)	0.7 (0.5–1.8)	1.2 (0.8–2.0)	1.2 (0.9–1.5)	0.295	/	/	/
DPT CD3+CD4+CD8+ ($\times 10^9$/L)	0.011 (0.007–0.015)	0.011 (0.007–0.019)	0.014 (0.008–0.027)	0.719	/	/	/
DNT CD3+CD4-CD8- (%)	15.9 (11.7–18.1)	17.8 (13.5–23.9)	15.8 (12.1–20.5)	0.255	/	/	/
DNT CD3+CD4-CD8- ($\times 10^9$/L)	0.15 (0.11–0.25)	0.17 (0.11–0.26)	0.15 (0.10–0.31)	0.985	/	/	/
B lymphocytes CD45+CD21+ (%)	14.1 (9.9–18.4)	16.9 (12.6–23.1)	13.7 (10.7–16.8)	0.174	/	/	/
B lymphocytes CD45+CD21+ ($\times 10^9$/L)	0.21 (0.14–0.46)	0.32 (0.20–0.49)	0.25 (0.18–0.35)	0.349	/	/	/
NT-proBNP (pmol/L)	950.0 (492.0–1116.0)	1956.0 (1093.0–2924.0)	1452.0 (1110.0–1853.0)	**0.004**	0.341	0.690	**0.001**
Cardiac TnI (µg/L)	0.044 (0.034–0.086)	0.078 (0.037–0.112)	0.022 (0.012–0.189)	0.087	/	/	/

Table 4. *Cont.*

Parameter	ACVIM B2	CHF	Healthy	*p* [a]	*p* [b]	*p* [c]
LA/Ao	1.95 (1.79–2.05)	2.21 (1.98–2.32)	1.36 (1.17–1.60)	**<0.001**	**0.002**	**<0.001**	**0.047**
nLVIDd	1.81 (1.64–1.91)	2.04 (1.85–2.18)	1.36 (1.19–1.66)	**<0.001**	**0.031**	**<0.001**	0.050
nLVIDs	0.90 (0.83–1.03)	1.01 (0.83–1.29)	0.80 (0.66–0.92)	0.066	/	/	/
MV E velocity (m/s)	0.92 (0.85–1.12)	1.28 (1.10–1.53)	0.60 (0.54–0.70)	**<0.001**	**0.006**	**<0.001**	**0.015**
MV A velocity (m/s)	0.79 (0.60–0.91)	0.83 (0.74–0.97)	0.46 (0.39–0.62)	**<0.001**	**0.003**	**<0.001**	0.718
MV E/A	1.17 (1.01–1.56)	1.49 (1.22–1.68)	1.31 (1.04–1.50)	0.175	/	/	/
TR PG (mmHg)[d]	28.0 (21.5–43.3)	39.0 (37.0–49.0)	–	/	/	/	**0.008**
FS (%)	45.5 (41.0–50.8)	44.7 (39.3–58.8)	39.5 (37.0–51.5)	0.556	/	/	/
HR (bpm)	130.0 (120.0–140.0)	136.0 (120.0–140.0)	110.0 (100.0–130.0)	**0.011**	**0.036**	**0.011**	1.000
Murmur (grade)[d]	4.0 (4.0–4.0)	4.0 (4.0–5.0)	–	/	/	/	**<0.001**
BCS	5.0 (5.0–6.0)	5.0 (4.0–6.0)	5.0 (5.0–6.00)	0.205	/	/	/

[a] *p*-values resulting from comparison (Kruskal–Wallis test) of parameters between groups of dogs (ACVIM B2, CHF, healthy); [a] *p*-values resulting from comparison (multiple comparisons with Bonferroni corrections) between ACVIM B2 and healthy dogs; [b] *p*-values resulting from comparison (multiple comparisons with Bonferroni corrections) between CHF and healthy dogs; [c] *p*-values resulting from comparison (multiple comparisons with Bonferroni corrections) between ACVIM B2 and CHF dogs; [d] Mann–Whitney test was used for comparison of these two parameters between ACVIM B2 and CHF dogs. Significant results ($p < 0.05$) are in bold. Abbreviations: A, A-wave (late transmitral blood flow); ACVIM, American College of Veterinary Internal Medicine; BCS, body condition score; cardiac TnI, cardiac troponin I; CD, cluster of differentiation; CHF, congestive heart failure; CoQ_{10}, coenzyme Q_{10}; DNT, double-negative T lymphocytes; DPT, double-positive T lymphocytes; E, E-wave (early transmitral blood flow); FS, fractional shortening; GPX, glutathione peroxidase; HR, heartrate; IQR, interquartile range; LA/Ao, left atrium to aorta ratio; MMVD, myxomatous mitral valve disease; MV, mitral valve; NLR, neutrophil-to-lymphocyte ratio; nLVIDd, normalized end-diastole left ventricular internal dimension; nLVIDs, normalized end-systole left ventricular internal dimension; NT-proBNP, N-terminal pro B-type natriuretic peptide; TNFα, tumor necrosis factor α; TNFSR-II, tumor necrosis factor soluble receptor II; TR PG, tricuspid regurgitation peak gradient; WBC, white blood cells.

4. Discussion

In the present study, we could not confirm the positive effects of CoQ_{10} supplementation on oxidative stress markers, lymphocyte subpopulations, markers of disease severity (circulating cardiac biomarkers and echocardiographic parameters), and TNFSR-II as a marker of inflammation; however, a positive effect was noted on selected inflammatory parameters (neutrophil percentage and lymphocyte percentage and concentration) in dogs with CHF. A daily dose of 200 mg of CoQ_{10} (given as 100 mg twice per day) was well-tolerated and significantly increased plasma CoQ_{10} concentration in both CoQ_{10}-supplemented groups (ACVIM B2 and CHF) in comparison to their basal concentrations and to the concentration of plasma CoQ_{10} measured in corresponding placebo groups.

Out of all significant differences detected during data analysis (Tables 2 and 3), only those found when comparing CoQ_{10}-supplemented groups to placebo-supplemented groups could be contributed to CoQ_{10} supplementation. The most important of them were the significant differences in the median change (delta) of neutrophil percentage and lymphocyte percentage and concentration between the CoQ_{10}-supplemented and placebo-supplemented groups of dogs with CHF. Our results showed that CHF dogs who received CoQ_{10} had a net decrease in their neutrophil percentage during the study period (negative delta), while dogs who received a placebo had a net increase in this parameter (positive delta). CoQ_{10}-supplemented dogs had a net increase in lymphocyte count and percentage (positive delta), while placebo-supplemented dogs had a net decrease in these parameters (negative delta), with all mentioned differences being significant.

An increase in neutrophil count and decrease in lymphocyte count are typical for systemic inflammation, and it is known that low-grade inflammation is present in CHF in people [45,46] and dogs [29,30]. A higher neutrophil percentage has been found in dogs with advanced-stage CHF in comparison to dogs with stable CHF and/or non-CHF dogs or healthy controls [29,30,47]. Low lymphocyte counts are often found in human CVD and were shown to be a predictor of mortality in CVD patients [48–50], but the results of studies in dogs are contradictory, with most of them not detecting decreased lymphocyte count or percentage in canine patients with heart disease [29,30,38,39,47]; however, in one of the mentioned studies, a significantly lower lymphocyte percentage was found in CHF and non-CHF groups of canine cardiac patients compared to that in healthy dogs [30]. In another study, a lower lymphocyte count was shown in dogs with advanced-stage CHF in comparison to that in dogs with stable CHF and healthy controls [38]. In the current study, our CHF group's neutrophil percentage (as well as lymphocyte percentage and concentration) did not differ significantly from ACVIM B2 or healthy dogs at baseline (Table 4). This is likely related to the very selective inclusion process. Pronounced inflammation is most anticipated in untreated, unstable, or critically ill patients [29]; however, only dogs who were receiving proper treatment and were not critically ill were included in our study.

Despite the absence of significant differences in neutrophils and lymphocytes between cardiac patients in CHF and healthy dogs at the baseline (Table 4), during the three-month supplementation period, neutrophil percentage rose in dogs receiving placebo and fell in those receiving CoQ_{10}, and lymphocyte percentage and concentration fell in placebo-supplemented patients and rose in those receiving CoQ_{10}. The positive effect of CoQ_{10} supplementation may be the result of CoQ_{10}'s anti-inflammatory properties. The anti-inflammatory effect of CoQ_{10} has been previously studied in people [3,51]. The association between oxidative stress and inflammation has been shown in dogs with cardiovascular diseases [30], but the anti-inflammatory effect of CoQ_{10} supplementation has not been studied in canine patients. Our study is the first to report the possible benefits of oral CoQ_{10} supplementation in combating inflammation in dogs with CHF. However, in our study, CoQ_{10} did not affect TNFSR-II (despite that this parameter was significantly higher in CHF dogs compared to that in ACVIM B2 and healthy dogs at baseline (Table 4)). No previous studies have assessed the effect of oral CoQ_{10} on serum

TNFSR-II concentration. Instead, the effect on serum TNF-α level was examined. These studies showed that CoQ_{10} supplementation decreased the level of TNF-α in a wide range of diseases, including CVD [51–53]. In our study, we could not assess this as in all dogs but one, TNF-α concentration was under the limit of detection.

Lymphocyte subpopulations were not affected by CoQ_{10} supplementation in our study. The differences in lymphocyte subtypes previously described in dogs with CHF in comparison to healthy dogs [38,39] were also not observed in the present cohort of dogs, possibly because most dogs included in this study were in stable CHF and none were critically ill, whereas many critically ill patients were included in our previous cross-section study [39]. However, we found a significantly lower T helper percentage and CD4/CD8 ratio and a significantly higher percentage of cytotoxic T lymphocytes in ACVIM B2 patients compared with CHF patients and healthy dogs, which we believe may be because the group included the oldest participants or due to advanced cardiac remodeling in this group. However, the dogs in the ACVIM B2 group were significantly older than the healthy dogs but not than those with CHF.

In our study, CoQ_{10} as a supplement did not affect selected oxidative stress markers (plasma F2-isoprostanes concentration and whole blood GPX activity). Being an established lipid peroxidation marker [54,55], F2-isoprostanes were found to be increased in the plasma of human HF patients and are linked both to antioxidant status and heart disease severity [56]. Additionally, urinary 15-F2t-isoprostane concentration was found to be increased in advanced CHF and correlated to the disease severity in non-ischemic CHF in people [57]. In dogs with cardiac diseases, serum 8-F(2alpha)-isoprostanes were found to be significantly higher in CHF in comparison to a healthy control group [18]. In our study, plasma F2-isoprostane concentration did not differ significantly between both groups of MMVD dogs and healthy controls at baseline (Table 4), and no effect of CoQ_{10} supplementation on this parameter was noted. Likewise, whole blood GPX activity, which is another marker of oxidative stress, did not differ between cardiac patients and healthy dogs (similar to another study in dogs [58]) and was not affected by CoQ_{10} supplementation. In people, the results of studies researching the effect of supplementary CoQ_{10} on whole blood GPX activity in different diseases including CVD are not consistent either [34,59,60]. In the present study, orally administered CoQ_{10} did not have an effect on the studied parameters of oxidative stress despite a huge increase (1.7-fold to 11.5-fold) in plasma CoQ_{10} concentration in all but one CoQ_{10}-supplemented dog and a significant increase in all CoQ_{10}-supplemented groups in comparison to their basal plasma CoQ_{10} concentrations and placebo. In future CoQ_{10} supplementation studies in canine cardiac patients, other oxidative stress markers may be chosen for the assessment of possible benefits of supplementation.

No positive effects of CoQ_{10} supplementation on echocardiographic parameters were detected in our study. In our CoQ_{10}-supplemented dogs with CHF, nLVIDs increased, with the positive change being significantly different from the negative change in placebo-supplemented patients. The latest study published on the effects of CoQ_{10} supplementation in MMVD dogs did not find any positive effect on echocardiographic parameters, which is in accordance with our results [25].

Cardiac biomarkers (cTni and NT-proBNP) were also not affected by CoQ_{10} supplementation, a result similar to that in previous studies in dogs with MMVD [24,25]. Owner-perceived quality of life with subjective assessment of changes in the health condition of dogs participating in our study was assessed by a questionnaire, with data not included in the statistics. Subjectively, in CoQ_{10}-supplemented CHF dogs, there were more owners (out of a total number of participants) who noticed improvements in the condition and mood of their dogs in comparison to owners of dogs supplemented with the placebo. In the only study which assessed the quality of life of dogs supplemented with CoQ_{10}, no impact was noted [25]. Since the questionnaire used in the present study was not validated and the data were not statistically analyzed, we cannot draw any conclusions regarding improvement in clinical signs. Results in people have also been inconclusive, with some studies reporting increasing quality of life [61] and others reporting no change [62].

As previously mentioned, for the initial assessment of cardiac patients, the studied parameters were compared between three groups of participating dogs (all ACVIM B2 dogs, all CHF dogs, and healthy dogs) with no regard to the type of later assigned supplement. Data are shown in Table 1 (for age and plasma CoQ_{10} concentration) and Table 4 (for other parameters). Although the main goal of this research was to assess the effects of CoQ_{10} supplementation on selected laboratory and clinical parameters, it is worth mentioning that in both groups of our MMVD patients (ACVIM B2 and CHF), plasma CoQ_{10} concentration was significantly higher in comparison to that in healthy dogs. These results differed from those obtained in our previous research [23], where the basal plasma CoQ_{10} concentration in CHF dogs did not differ significantly from that of healthy dogs (dogs with ACVIM B2 were not included in that study); however, our results are in accordance with another study in which CHF patients receiving cardiac treatment had significantly higher plasma CoQ_{10} concentrations compared to those not receiving cardiac treatment and healthy dogs [22]. To the best of our knowledge, to date, no studies in canine cardiac patients have shown decreased levels of plasma CoQ_{10}. This is in contrast to the results of studies in human cardiovascular patients, where plasma CoQ_{10} levels have been found to be lower than those in healthy subjects and associated with disease severity and mortality [8,63,64]. Despite this discrepancy in results for plasma levels of CoQ_{10} between people and dogs, there is recent evidence that myocardial levels of CoQ_{10} are lower in Cavalier King Charles Spaniels with CHF compared to non-CHF (ACVIM B1 and B2) dogs and healthy controls [21]. This might support the need for future CoQ_{10} supplementation studies in canine CHF patients despite normal or increased plasma CoQ_{10} concentrations.

Our study has some limitations. The most important of these is the relatively low number of dogs included. The inclusion criteria were restrictive as we did not recruit critically ill dogs, those receiving nutritional supplements, or those with comorbidities, all of which made up a large proportion of the dogs assessed for potential participation (Figure 1). We also did not include dogs that were not receiving proper treatment, and dogs that began treatment were included only after at least one month of proper therapy. Some of the dogs included died or were euthanized during the study and are therefore not included in the statistical analysis (Figure 1). Another limitation may be the short duration of the supplementation period. Although our three-month study is the longest study of CoQ_{10} supplementation in dogs with heart diseases, a longer study could potentially show more benefits of oral CoQ_{10} supplementation. On the other hand, the results of a longer study might be difficult to assess due to the number of dogs with heart failure worsening during the supplementation period. Another limitation of our study is that the dose of CoQ_{10} was the same for all dogs and was not based on body weight, as suggested by other authors [24]. MMVD is typical in smaller dog breeds, and all dogs in our study were small or medium-sized (with the heaviest weighing 20.9 kg). The results of our previous dose-ranging study showed that 200 mg per day was sufficient to achieve a 3-fold increase in all supplemented dogs with MMVD regardless of their weight [23], and in the current study, this dose was sufficient to achieve at least a 3-fold increase in most dogs (19 out of 23), including 2 of our cardiac patients who were the heaviest, weighing 17 and 20.9 kg. In addition, for nutritional supplements it might prove impractical to base the dose on the exact weight, although we can suggest that for studies of other cardiac diseases typical of larger breeds, dose adjustment may be used. Additionally, in our study, TNF-α concentration was under the detection limit in all samples but one. The impact of CoQ_{10} supplementation on TNF-α may be assessed in future supplementation studies in canine cardiac patients using different methods, e.g., fluorescent bead-based assays (Luminex Xmap technology) instead of the ELISA method.

5. Conclusions

Our study is the longest of all published trials evaluating the effects of CoQ_{10} supplementation in dogs with heart diseases. Of all parameters assessed, only neutrophil percentage and lymphocyte percentage and concentration were positively affected by

supplementation, which may indicate the anti-inflammatory role of CoQ_{10} in systemic inflammation in dogs with CHF due to MMVD. Studies with a longer supplementation period and a larger number of dogs or studies examining the effect of CoQ_{10} on survival are warranted.

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Informed Consent Statement: Informed consent was obtained from the owners of all dogs involved in the study.

Data Availability Statement: The data from this work are included in this article and may be obtained from the corresponding author.

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3 DISCUSSION

Lymphocyte subtypes, CoQ_{10} dosing and the effects of CoQ_{10} supplementation on oxidative stress, immune and clinical status were investigated in dogs with different stages of MMVD with or without CHF.

3.1 LYMPHOCYTE SUBTYPES IN DOGS WITH MMVD

In the first part of our research, we tested the hypothesis that dogs in different stages of MMVD have altered peripheral blood lymphocyte subtype proportions compared to healthy dogs, namely, decreased B lymphocyte percentage (CD21+) and percentage of CD4+ T lymphocytes (T helper cells) as well as decreased CD4+/CD8+ ratio and increased percentage of CD8+ T lymphocytes (cytotoxic T lymphocytes), and that these alterations are especially evident in unstable heart failure.

In our study, we confirmed in peripheral blood samples that dogs with CHF due to MMVD (in both ACVIM C [stable] and ACVIM C and D [unstable] CHF groups) have a decreased percentage of CD4+ T lymphocytes, decreased CD4+/CD8+ ratio and increased CD8+ T lymphocyte percentage, and that these alterations are more evident in unstable CHF, although they are significant in both CHF groups; also, in terms of absolute count, increase in CD8+ T lymphocytes is significant in ACVIM C and D [unstable] CHF compared to healthy control dogs. None of these changes were noted in dogs with asymptomatic MMVD (ACVIM B2 patients in our study), and we did not confirm a decreased B lymphocyte percentage in dogs in any of the stages of MMVD included in our study.

Previous studies have shown that T lymphocytes (including CD4+ and CD8+ T lymphocytes) may play a role in heart disease progression, promoting apoptosis, fibrosis and cardiac remodelling (Laroumanie et al., 2014; Frieler and Mortensen, 2015; Nevers et al., 2015; Bansal et al., 2017; Sanchez-Trujillo et al., 2017; Blanton et al., 2019; Chiurchiù et al., 2019). Lymphocyte subtypes were found to be altered in peripheral blood in people with cardiovascular diseases. In human patients with idiopathic and ischemic CHF, CD4+ T

lymphocyte proportion was shown to be increased, as well as CD4+/CD8+ ratio, compared to controls (Agnoletti et al., 2004). In contrast, another study that included CHF patients with coronary artery disease, idiopathic dilated cardiomyopathy and other unspecified heart diseases, showed decreased CD4+ lymphocyte percentage in groups of young and elderly CHF patients and increased CD8+ lymphocyte percentage in a group of elderly patients (Moro-García et al., 2014). The discrepancies in results may possibly be explained by the fact that CHF groups in different studies were comprised of patients with CHF of different aetiologies and different age groups. Studies in dogs on peripheral blood lymphocyte subtype alterations in heart disease are lacking. According to the literature search, two studies that included dogs in CHF due to MMVD and dilated cardiomyopathy (Farabaugh et al., 2004) and MMVD dogs with and without CHF (ACVIM B1, B2, C and D; Piantedosi et al., 2022) were published. The latter study was published after completion of the PhD research and publication of the manuscript on the subtypes of peripheral blood lymphocytes in dogs with different stages of MMVD (Druzhaeva et al., 2021).

Findings of the most recent study, conducted by Piantedosi at al. (2022), where lymphocyte subtypes were assessed along with inflammatory markers (TNF-α, interleukin-1ß and interleukin-6) in dogs with MMVD with and without CHF, contradict ours in regards to CD4+ and CD8+ T lymphocyte percentages and CD4/CD8 ratio as CD4+ T lymphocytes and CD4/CD8 ratio were found to be significantly higher and CD8+ T lymphocytes significantly lower in dogs with CHF compared to a group of old healthy control dogs in this study, with no information on age difference between these groups. The reasons for difference between the results of the study by Piantedosi et al. (2022) and our study are not fully clear, considering that MMVD patients and old healthy dogs in their study were of very similar mean age as patients in our study. Additionally, the same anti-canine antibodies (same clones of anti CD3, CD4, CD8 monoclonal antibodies, manufactured by Serotec Ltd. [London, UK], but labelled with different dyes) were used. Besides, group of young control dogs with mean age of 4.1 years was included in Piantedosi et al. (2022) study, but due to overt age difference with MMVD dogs (with mean age of all MMVD dogs being 10.7 years), significant differences between cardiac patients and young healthy control dogs, including lower CD4+ and higher CD8+ T lymphocyte percentages in MMVD dogs, are probably attributed to age difference as the same differences were found when comparing young and old healthy control groups in the same

study. Additionally, Piantedosi et al. (2022) included both untreated and treated patients and those who were treated received non-uniform treatment, while in our study, all CHF dogs had similar treatment regimen and all were treated at the time of inclusion.

The results of the study conducted by Farabaugh and colleagues (2004) also contradict our findings, as the authors found a decrease in both CD4+ peripheral mononuclear blood cells and CD8+ lymphocytes and no changes in the CD4+/CD8+ ratio. The difference between the results of our study and the mentioned study (Farabaugh et al., 2004) could be a result of the different populations studied because they included dogs with MMVD and dilated cardiomyopathy, the latter typically involving younger patients of different breeds than dogs with MMVD, and both age and breed may affect lymphocyte subtype percentages and concentrations. In addition to described earlier effect of age on lymphocyte subpopulations, the longevity of small and large breeds is very different, which likely affects the timeline of changes in the immune system (Holder et al., 2016), with larger shorter-lived breeds experiencing immunosenescence earlier than smaller, longer-lived breeds. Additionally, the investigators stated as a limitation that they could not confirm that gated CD4+ cells were lymphocytes only as they might also be monocytes and neutrophils. This was because they did not use an anti-canine CD3 antibody to exclude CD4+ non-lymphocyte peripheral blood cells. Also, they used different anti-CD4 and anti-CD8 antibodies: rat anti-canine CD4:FITC (fluorescein isothiocyanate) and rat anti-canine CD8:RPE (R-phycoerythrin; Serotec Inc, Raleigh, NC), while in our study, a cocktail of mouse anti-canine antibodies CD3:FITC/CD4:RPE/CD8:Alexa Fluor 647 (Bio-Rad Laboratories Inc; Hercules, CA) was used.

Furthermore, there was another study (presented in a form of research abstract but unpublished) showing similar findings to ours; however, in that study, only dogs of one large breed (German Shepherd) with mitral regurgitation that could be of various aetiologies (not strictly MMVD) were included (Borgarelli et al., 2002).

Myxomatous mitral valve degeneration in dogs is by itself considered a non-inflammatory and non-infectious degenerative pathology with a known genetic component (Madsen et al., 2011; Fox, 2012; Meurs et al., 2018). However, over time, it can progress to CHF, which is known to be accompanied by low-grade inflammation in people (Yndestad et al., 2007) and dogs (Rush

et al., 2006; Polizopoulou et al., 2015; Reimann et al., 2015; Domanjko Petrič et al., 2018; Hamilton-Elliott et al., 2018; Rubio et al., 2020; Nemec Svete et al., 2021; Piantedosi et al., 2022).

The mechanisms behind the lymphocyte subtype alterations found in our and the previous studies have not been studied in canine cardiac patients.

The redistribution of T-cell subtypes with the changes in the ratio of T-lymphocyte subtypes in peripheral blood might be related to inflammation due to myocardial stress and injury and increasing wall stress as heart disease progresses toward HF, RAAS activity increases, and volume and pressure in the ventricles increase. Both wall stress and DAMPs from injured cardiac cells provoke sterile inflammation locally, whereas HF may also induce inflammation in other organs (muscle, intestine) by circulating systemic inflammatory mediators or because of hemodynamic changes. Damage-associated molecular patterns can be secreted due to hypoxia, oxidative damage, and cell death. Sterile inflammation by itself has a similar pathway as inflammation induced by an infectious agent. Damage-associated molecular patterns (various intracellular proteins, mitochondrial DNA) are detected by pattern recognition receptors on cardiomyocytes, macrophages and other cells. This is followed by synthesis of multiple inflammatory mediators (often through activation of NF-kB), including cytokines and chemokines, that directly negatively affect heart function and attract other immune cells including lymphocytes. These attracted immune cells are also a source of cytokines, in addition to cytokines produced by cardiac cells – cardiomyocytes, endothelial cells, fibroblasts, and tissue macrophages (Van Linthout and Tschöpe, 2017). It was shown previously that CD4+ T lymphocytes participate in cardiac fibrosis and remodelling (Laroumanie et al., 2014), and in experimental mice studies it was shown that activated CD4+ T lymphocytes are needed to exert the effects of angiotensin II such as fibrosis and remodelling (Kvakan et al., 2009).

The alterations in T lymphocyte subtypes found in our study may be also attributed to immunosenescence characterized by age-related changes in concentrations and percentages of lymphocytes and their subtypes as well as changes in function. Immunosenescence is linked to human cardiovascular diseases and presents a known risk factor for these diseases (Hoffmann and Spyridopoulos, 2015). Changes in immune cells with increasing age were also studied in

dogs. In study by Fujiwara et al. (2012), that included Beagles of three age groups (0-4, 4-8, >8 years old), negative correlation of age with concentration of total T lymphocytes was noted, as well as a decrease in concentration in naïve CD4+ and CD8+ T lymphocytes with age. Blount and colleagues (2005) included Labrador retrievers from 2 to 10 years old divided to 4 cohorts according to age and found that concentrations of total T lymphocytes, CD3+ and CD8+ T lymphocytes all decreased with increasing age, the percentage of CD4+ T lymphocytes and CD4+/CD8+ ratio decreased, and the percentage of CD8+ T lymphocytes increased with age. In the study by Byrne et al. (2000), Labradors and terriers divided into two groups, young and old, with average age 2 years old and 9 years old, respectively, were included, and authors found lower percentage of CD4+ T lymphocytes in older dogs compared to young, while no difference was noted in the percentages of CD3+ lymphocytes and CD8+ T lymphocytes. In a study by Watabe et al. (2011), dogs from 1 to 17 years old were included, and in them, total lymphocyte concentration, and the concentrations of CD3+, CD4+ T lymphocytes decreased with age, while CD4+ T lymphocyte percentage and CD4+/CD8+ ratio decreased, and CD8+ T lymphocyte percentage increased with age. In another study, conducted by Greeley et al. (2001), Labrador Retrievers of age ranging from 4 to 11 years old were included, and authors found that concentrations of CD3+, CD4+ and CD8+ T lymphocytes all declined with age, while changes in percentages of CD4+ and CD8+ T lymphocytes were not significant, and only in females, the percentage of CD8+ T lymphocytes increased from young to middle age and then did not change significantly with increasing age. In the study by Piantedosi et al. (2022), besides dogs with MMVD, authors included two control groups of healthy dogs – young (mean age of 4.1 years) and old (mean age of 9.6 years), and found significantly higher CD4+ T lymphocyte percentage and significantly lower CD8+ T lymphocyte percentage in young healthy dogs compared to old healthy dogs and MMVD patients that were also old with mean age of 10.7 years.

Therefore, one of the limitations of our study of lymphocyte subtypes in dogs with different stages of MMVD was the significantly younger age of healthy control dogs compared to the cardiac patients, although all included healthy dogs were >5 years of age. Because of possible effects of age on the relative and absolute numbers of lymphocytes and their subtypes discussed above, we aimed to include age-matched control dogs, but most of initially screened dogs were excluded due to occult heart disease or other health problems. It proved very difficult to find

healthy dogs that matched MMVD dogs by age, breed, and weight as majority of older age small breed dogs have mitral and/or tricuspid regurgitation due to age-related myxomatous degeneration, and although clinically insignificant in many of them, it precluded us from inclusion of such dogs in our healthy control group. It is difficult to determine to what extent the age difference between control and MMVD dogs may have influenced the results of our study. The results of previous studies of peripheral blood lymphocyte changes with age in dogs have been inconsistent regarding which lymphocyte subtypes change with age and which do not. For example, some studies found that the percentage of CD4+ T lymphocytes in healthy dogs decreased significantly with age (Byrne et al., 2000; Blount et al., 2005), whereas another study found no significant difference (Greeley et al., 2001). Similarly, the percentage of CD8+ T lymphocytes was significantly higher in old dogs compared with young dogs in some studies (Blount et al., 2005; Watabe et al., 2011), whereas in other studies this difference was not reported (Byrne et al., 2000) or was reported only for females (Greeley et al., 2001). Moreover, in some of the studies reporting differences in CD4+ and CD8+ T lymphocytes (Byrne et al., 2000; Blount et al., 2005) these differences were significant only when young dogs were compared with old dogs, whereas our control group did not consist of young dogs but of middle-aged and old dogs (all dogs were >5 years of age, and the mean age of the group was 7.9 years).

Another limitation was possible impact of standard cardiac treatment on the results of our study. All dogs with MMVD included in our study, were treated with medications for heart disease, and those in both CHF groups were all receiving standard CHF treatment with calcium sensitizer (pimobendane), loop diuretic and angiotensin-converting enzyme inhibitor, while almost half of patients from each CHF group (11 out of 24 in stable CHF group and 9 out of 21 in unstable CHF group) were also receiving mineralocorticoid receptor antagonist (aldosterone). In contrast, healthy control dogs did not receive any treatment. The effect of medications on the results of our study is unknown. Loop diuretics activate RAAS (Lantis et al., 2015), that has pro-inflammatory effects, and angiotensin-converting enzyme inhibitors and mineralocorticoid receptor antagonists both block RAAS, potentially decreasing inflammation related to RAAS activity (Pacurari et al., 2014). Angiotensin-converting enzyme inhibitors were also shown to have immunomodulatory effects on T lymphocytes in cancer (Vallejo Ardila et al., 2020). There is no data on effects of calcium sensitizer pimobendane on lymphocyte subtypes or inflammation. Levosimendan, another calcium sensitizer medication

that is used as inotrope in human HF but rarely used in dogs, showed some anti-inflammatory properties (Adam et al., 2015; Wang et al., 2015), but it is unknown if pimobendan has similar effect.

No research has been done to date to reveal the specific role of CD4+ and CD8+ T lymphocytes in dogs with myxomatous degeneration of the mitral and/or the tricuspid valve. In mice and humans, T lymphocytes play both deleterious and repairing roles in diseased myocardium (Okamoto et al., 2014; Blanton et al., 2019). It is a known fact that lymphocytes infiltrate the heart in people with heart transplants (Bracamonte-Baran et al., 2021), inflammatory heart pathologies such as autoimmune and infectious cardiac disease (Blanton et al., 2019), as seen in myocarditis, which most often has a viral aetiology (Cooper, 2009). However, infiltration of both ventricles with CD4+ and CD8+ T lymphocytes was also shown in mice with induced HF due to pressure overload (Laroumanie et al., 2014), and, in another study, left ventricle tissue infiltration with T lymphocytes was shown in mice with experimental pressure overload HF and in humans with advanced-stage non-ischemic HF (Nevers et al., 2015). These results suggest that in mice and people, myocardial T lymphocyte infiltration is present not only in primary inflammatory infectious or non-infectious diseases but also in HF due to other aetiologies. In the present research, we only studied changes in peripheral blood lymphocyte subtypes while not studying the myocardial tissue itself. And although blood alterations can be a result of lymphocyte activation, proliferation and movement to the specific tissue (including myocardium), the direct evidence of myocardial infiltration in dogs with MMVD is still lacking. There is a possibility that changes we found can be due to other reasons discussed above (age impact and others) or incidental. To prove the direct role of lymphocytes in the failing heart of patients with MMVD, myocardial tissue should be examined in future studies. To conclude, the results of our study demonstrate that there may be an immune response associated with CHF that was not observed in dogs with asymptomatic MMVD, presented by ACVIM B2 patients in our study; therefore, research on the systemic and local immune response and the specific role of T lymphocytes in the heart of dogs with MMVD is warranted.

3.2 COENZYME Q_{10}. A DOSE-RANGING STUDY AND A 3-MONTH SUPPLEMENTATION STUDY

To test the second hypothesis, which questions the effects of dietary supplementation with CoQ_{10} on various parameters of immune system, markers of inflammation, oxidative stress, and the progression of MMVD, we performed a CoQ_{10} dose study to test the ability of doses (100 and 200 mg daily) to increase plasma CoQ_{10} concentration in dogs with CHF and a double-blind placebo-controlled randomized supplementation study to test the effects of CoQ_{10} as a dietary supplement on parameters of oxidative stress, immune and clinical status in dogs with different stages of MMVD.

To test the hypothesis, we first had to determine the dose of water-soluble CoQ_{10} (100 or 200 mg daily) needed to achieve at least a 3-fold increase in plasma CoQ_{10} concentration in dogs with MMVD and CHF, as it was previously shown that such an increase is needed in order to elicit positive effects of supplementation (Belardinelli et al., 2005). In many studies in people with cardiovascular disease, a 2-4-fold increase in plasma CoQ_{10} concentration has been targeted and achieved, and this increase is considered more important than bringing the concentration into line with the range of healthy control subjects or than the dosage used in the studies, because different CoQ_{10} preparations have very different intestinal uptake (Sue-Ling et al., 2022). Even though plasma deficiency has not yet been demonstrated in dogs with MMVD, lower myocardial levels have been found (Christiansen et al., 2021). Additionally, plasma CoQ_{10} status may not always reflect tissue status (Bhagavan and Chopra, 2006) and even with no CoQ_{10} plasma deficiency, supplementation may potentially have a beneficial effect in reducing oxidative stress and inflammation (Martelli et al., 2020; McRae, 2022; Sifuentes-Franco et al., 2022).

In the dose-ranging study, we used water-soluble CoQ_{10}, as it was previously shown to be superior to oil-based CoQ_{10} in dogs (Prosek et al., 2008). Dogs with CHF due to MMVD (ACVIM stages C and D) that were receiving standard cardiac therapy as well as healthy control dogs were included. Cardiac patients were blindly randomized to 3 groups to receive a 100 mg or 200 mg daily dose of CoQ_{10} or placebo for 2 weeks. The CoQ_{10} plasma concentration was measured multiple times during the study – immediately before the first dose of supplement

was given, 4 hours and 1, 2, and 3 weeks after the supplementation began. The results of this study demonstrate that a 200 mg daily dose was needed to achieve a 3-fold increase in plasma and can be used in future CoQ_{10} supplementation studies. Based on these results, a 200 mg daily dose given as 100 mg BID was used in the 3-month CoQ_{10} supplementation study discussed hereafter.

To test the effects of CoQ_{10} supplementation on parameters of oxidative stress, inflammation, lymphocyte subtypes and clinical status in dogs with MMVD with or without CHF, we conducted the 3-month CoQ_{10} supplementation study. The results of our study demonstrate that after supplementation with CoQ_{10}, inflammatory parameters decreased, as neutrophil percentage decreased, and lymphocyte percentage and concentration (total lymphocyte count) increased. These results may potentially suggest possible anti-inflammatory properties of CoQ_{10} in dogs with CHF.

An increase in neutrophil count and a decrease in lymphocyte count are typical for systemic inflammation, and it is known that low-grade inflammation is present in CHF in people (Yndestad et al., 2007; Van Linthout and Tschöpe, 2017) and dogs (Domanjko Petrič et al., 2018; Nemec Svete et al., 2021). A significantly higher neutrophil percentage has been found in previous studies in dogs with advanced-stage CHF, where other possible causes of inflammation were also excluded, in comparison to dogs with stable CHF and/or non-CHF dogs or healthy controls (Domanjko Petrič et al., 2018; Hamilton-Elliott et al., 2018; Nemec Svete et al., 2021). Low lymphocyte counts are often found in human cardiovascular diseases and have been shown to be a predictor of mortality in these patients (Ommen et al., 1998; Acanfora et al., 2001; Charach et al., 2011). However, the results of studies in dogs are contradictory, with most not detecting decreased lymphocyte count or percentage in patients with CHF (Farabaugh et al., 2004; Domanjko Petrič et al., 2018; Hamilton-Elliott et al., 2018; Nemec Svete et al., 2021). Nevertheless, in one of the mentioned studies, a significantly lower lymphocyte percentage was found in CHF and non-CHF groups of canine cardiac patients compared to that in healthy dogs (Nemec Svete et al., 2021). In another study, dogs with advanced-stage CHF showed a lower lymphocyte count in comparison to dogs with stable CHF and healthy controls (Farabaugh et al., 2004).

In the current study, neutrophil percentage as well as lymphocyte percentage and concentration did not differ significantly between the CHF group and ACVIM B2 or healthy dogs at baseline. This is likely related to the very selective inclusion process. Pronounced inflammation is most anticipated in untreated, unstable or critically ill patients (Domanjko Petrič et al., 2018); however, only dogs who were receiving the recommended treatment and were not critically ill were included in our study. Despite the absence of significant differences in neutrophil and lymphocyte concentrations and percentages between cardiac patients in CHF and healthy dogs at baseline, during the three-month supplementation period, neutrophil percentage rose in dogs receiving placebo and fell in those receiving CoQ_{10}, and lymphocyte percentage and concentration fell in placebo-supplemented patients and rose in those receiving CoQ_{10}. These changes seen after CoQ_{10} supplementation may potentially be the result of the anti-inflammatory properties of CoQ_{10}. The mechanisms of the anti-inflammatory effect of CoQ_{10} in cardiovascular disease are still unknown. They are thought to be due to the direct antioxidant effect of the reduced form of CoQ_{10} (ubiquinol), which protects membranes, including those of immune cells, from lipoperoxidation, and is involved in the regeneration of vitamins E and C, as well as by inhibiting the expression of NF-kB and subsequently reducing the production of pro-inflammatory cytokines, and possibly also by increasing the production of adiponectin and thereby reducing the inflammation mediated by TNF-α (Schmelzer at al., 2008; Fan et al., 2017; Zhai et al., 2017; Mantle et al., 2021; Sifuentes-Franco et al., 2022). The anti-inflammatory effect of CoQ_{10} has been previously studied in people (Zhai et al., 2017; Mantle et al., 2021; Sue-Ling et al., 2022), and CoQ_{10} has been shown to decrease some of the inflammatory markers such as CRP, interleukin-6 and TNF-α (Martelli et al., 2020; Sifuentes-Franco et al., 2022).

The association between oxidative stress and inflammation has been shown in dogs with cardiovascular diseases (Nemec Svete et al., 2021), but the anti-inflammatory effect of CoQ_{10} supplementation has not been studied in canine patients. Our study is the first to report the possible benefits of oral CoQ_{10} supplementation in combating inflammation in dogs with CHF.

No effect of CoQ_{10} supplementation on other inflammatory markers (total leukocyte count, CRP, TNFSR-II) or on the parameters of oxidative stress (F2-isoprostanes, GPX), lymphocyte

subtypes, and markers of myocardial damage or disease progression (cTnI and N-terminal pro-B type natriuretic peptide [NT-proBNP]) was noted. The effect on TNF-α was not tested due to failure in the measurement of this parameter in most samples. The effect of CoQ_{10} supplementation on cTnI and NT-proBNP was studied in few recent CoQ_{10} supplementation studies in dogs with MMVD (Tachampa et al., 2018; Christiansen et al., 2020) and no positive effect was noted in those studies that is in accordance with the results of our study, although both studies had flows in design (no control group in one short 28-day study [Tachampa et al., 2018] and too short of a washout period and short duration of CoQ_{10} supplementation [3 weeks] in the crossover study by Christiansen et al. [2020]). The lack of effect of 3-month CoQ_{10} supplementation on the above parameters of inflammation, oxidative stress, and cardiac biomarkers in our study might be partially attributed to the fact that only stable patients were included in our CHF group where inflammation and oxidative stress could be less pronounced, and subsequently, the effect of CoQ_{10} supplementation less obvious or negligible. The inclusion of severely ill patients was restricted due to the owners' acceptance to participate in the study, and due to the fact that such patients had many comorbidities (kidney failure and others) that were contradictory to our inclusion criteria. Additionally, some initially included patients with advanced CHF did not complete the supplementation period due to deterioration and/or death or loss of follow-up. It is possible that in patients with advanced disease presented to cardiac exam or admitted to hospital, as opposed to stable patients participating in our study, the effect of supplemental CoQ_{10} on oxidative stress and inflammatory markers would be more pronounced. Therefore, we believe that CoQ_{10} supplementation should be tested also in severely ill patients in the future, as inflammation and oxidative stress are more pronounced in patients with advanced disease (Domanjko Petrič et al., 2018; Michałek et al., 2020; Rubio et al., 2020; Nemec Svete et al., 2021).

Although the comparisons between ACVIM B2, CHF and healthy dogs before the start of supplementation were not the primary goal of our research, we noted that TNFSR-II was significantly higher in dogs with CHF compared to both ACVIM B2 and healthy dogs. Soluble tumor necrosis factor-α receptor II is a soluble receptor that modulates the effects of TNF-α, the pro-inflammatory cytokine, widely studied and shown to be elevated in HF in humans (Schumacher and Naga Prasad, 2018) and in dogs (Nemec Svete et al., 2021). Tumor necrosis factor-α receptors are found on the surface of many cells, including cardiomyocytes (Kadokami

et al., 2000; Higuchi et al., 2004), and are shed and detected in soluble form in blood and urine although the role of soluble receptors has not yet been well studied in cardiovascular diseases (Schumacher and Naga Prasad, 2018). The potential for TNFSR-II to be the marker of CHF in canine patients could be explored in future studies.

In both our studies involving measurements of basal plasma CoQ_{10} concentrations (Research Articles 2.2 and 2.3), we showed that there is no plasma CoQ_{10} deficiency in dogs with different stages of MMVD. In our 3-month supplementation study, MMVD dogs in the ACVIM B2 and CHF groups had higher plasma CoQ_{10} concentrations than healthy dogs, consistent with previous studies showing no deficiency (Harker-Murray et al., 2000; Svete et al., 2017) and higher levels in treated dogs compared with untreated and healthy subjects; the latter was attributed to the antioxidant properties of the cardiac drugs used (Svete et al., 2017). In people with cardiovascular diseases, low plasma and myocardial CoQ_{10} concentrations have been shown (Folkers et al., 1970; Mortensen et al., 1984; Folkers et al., 1985; Senes et al., 2008). It is believed that plasma concentration is more reflective of tissue concentrations in more severe disease (Folkers et al., 1985), while there is no data regarding correlation between plasma and myocardial levels in healthy subjects. Clinical assessment of CoQ_{10} status is generally based on measurement of plasma CoQ_{10} concentration. However, plasma CoQ_{10} concentrations may not truly reflect tissue CoQ_{10} levels because plasma CoQ_{10} concentrations depend on both dietary intake and lipoprotein concentrations. Nevertheless, plasma CoQ_{10} concentrations are generally used to assess circulating CoQ_{10} levels after supplementation (Mantle et al., 2023). It is currently unclear why dogs with heart disease do not have plasma CoQ_{10} deficiency. Although an earlier study in experimental CHF did not confirm myocardial deficiency in dogs (Harker-Murray et al., 2000), a recent study in Cavalier King Charles Spaniels with MMVD showed lower myocardial CoQ_{10} concentrations compared to healthy control dogs (Christiansen et al., 2021). Plasma CoQ_{10} concentrations were not measured in the dogs participating in that study, and there are no data on simultaneous measurement of myocardial and plasma CoQ_{10} concentrations in dogs. This was done only in pacing-induced heart failure study, in which no serum or myocardial deficiency was observed in CHF dogs compared with the control group (Harker-Murray et al., 2000). Despite the fact that dogs with spontaneous heart disease may have more subtle changes to body CoQ_{10} concentration than people, with just myocardial but not plasma CoQ_{10} deficiency, there may be a rationale for using CoQ_{10} as a

supplement due to its possible anti-inflammatory properties and the potential for increasing myocardial CoQ_{10} levels as well as possibly reducing oxidative stress.

4 CONCLUSIONS

- Dogs with MMVD with stable and unstable CHF, but not asymptomatic MMVD dogs, have altered proportions of T lymphocyte subtypes compared with healthy dogs, possibly reflecting immune system involvement in the pathogenesis and progression of CHF in this cohort of dogs.

- There is no plasma CoQ_{10} deficiency in dogs with MMVD with or without CHF.

- Coenzyme Q_{10} as a dietary supplement is able to decrease the proportion of neutrophils and increase the proportion and concentration of lymphocytes, indicating a possible anti-inflammatory effect of CoQ_{10} supplementation.

- A daily dose of 200 mg of water-soluble CoQ_{10} is sufficient to triple plasma CoQ_{10} concentrations, has been shown to be safe, with no side effects attributable to supplementation, and may be used in future studies of CoQ_{10} supplementation in dogs.

5 SUMMARY

Myxomatous mitral valve degeneration (MMVD) is the most common cardiac disease and the most common cause of congestive heart failure (CHF) in dogs. As such, it is also the most common reason for cardiac-related death in this species. Oxidative stress is present in human and canine CHF. The immune system and inflammation are deeply involved in heart disease progression in people and dogs, and lymphocyte subpopulations may also play an important

role in the immune response related to heart disease. The search for new medications that could decrease oxidative stress and inflammation and slow the progression of the disease and increase the quality of a pet's life is ongoing.

Coenzyme Q_{10} (CoQ_{10}) is a fat-soluble antioxidant that can also be given as a food supplement. Low plasma CoQ_{10} concentration is a constant finding in humans with cardiovascular diseases, and supplemental CoQ_{10} has been extensively studied in human HF patients. Coenzyme Q_{10} has antioxidant properties and may reduce inflammation attributed to CHF.

The goal of our research was to assess the effect of CoQ_{10}, given in addition to standard cardiac therapy, on the parameters of oxidative stress, inflammation, lymphocyte subpopulations, as well as on clinical status, in dogs of different breeds diagnosed with MMVD with or without CHF. As there were no data on lymphocyte subtypes in dogs with different stages of MMVD as well as on the dosing of supplemental CoQ_{10} in dogs with MMVD, the interim goal was to study these prior to the main CoQ_{10} 3-month supplementation study.

Therefore, the research conducted was comprised of three stages:

1. A flow cytometry prospective cross-sectional study to determine the percentages and concentrations of lymphocyte subpopulations in dogs with different stages of MMVD with or without CHF and healthy dogs.

2. A double-blind randomized placebo-controlled study to determine the dose of CoQ_{10} sufficient to achieve at least a 3-fold increase in plasma CoQ_{10} concentration in dogs with CHF due to MMVD.

3. A double-blind randomized placebo-controlled CoQ_{10} supplementation study with the aim of determining the effects of oral CoQ_{10} as an addition to standard therapy of heart failure in dogs on oxidative stress markers, and the immune and clinical status of MMVD dogs with or without CHF.

A decreased percentage of CD4+ T lymphocytes, decreased CD4+/CD8+ ratio and increased CD8+ T lymphocyte percentage in both stable and unstable CHF patients, as well as increased CD8+ T lymphocyte concentration in unstable CHF patients, were found in the first part of our research. These results showed there may be a systemic immune response associated with CHF which is not seen in dogs with asymptomatic MMVD (ACVIM B2 patients in our study).

The mechanisms behind the changes found in our study and those done previously are not completely understood. We assume these changes are due to the activation of autoreactive lymphocytes specific for myocardiocytic elements as antigens.

Myxomatous mitral valve disease in dogs is by itself a non-inflammatory and non-infectious degenerative pathology with a known genetic background. As the disease progresses, it can lead to CHF, which is accompanied by low-grade inflammation. In mice and people, T lymphocytes play both deleterious and reparatory roles in diseased myocardium. It is a known fact that lymphocytes infiltrate myocardium in people with inflammatory heart diseases such as autoimmune and infectious diseases, as seen in myocarditis, which most often has a viral aetiology. However, in mice and people, cardiac T lymphocyte infiltration is present not only in inflammatory infectious or non-infectious diseases but also in heart failure due to other aetiologies. To conclude, the results of our study suggests that there may be a systemic immune response associated with CHF that has not been seen in dogs with asymptomatic MMVD (presented by ACVIM B2 patients). Therefore, it is important to investigate not only systemic but also the local immune response and the specific roles of T lymphocytes in the heart of dogs with MMVD.

In the second part of our research, we found that a 200 mg daily dose of water-soluble CoQ_{10} was sufficient to achieve a 3-fold increase in plasma CoQ_{10} concentration in dogs with CHF due to MMVD. This dose can be used in future CoQ_{10} supplementation studies and was also used in the last part of our research, i.e. the 3-month supplementation study.

Our 3-month supplementation study showed that in dogs with CHF given CoQ_{10} as a dietary supplement neutrophil percentage significantly decreased and lymphocyte percentage and concentration (total lymphocyte count) significantly increased. These results showed possible

anti-inflammatory effect of CoQ_{10}, and to our best knowledge, our study is the first to show such an effect in canine cardiac patients.

Based on our results, we can conclude that lymphocyte subpopulations may be involved in CHF pathogenesis in dogs with MMVD. Alterations were evident in CHF dogs and most pronounced in patients with unstable CHF, as shown in the flow cytometric study. Our dose-ranging study showed that a 200 mg daily dose of water-soluble CoQ_{10} was sufficient to increase plasma CoQ_{10} concentration to the level potentially needed to elicit positive effects of supplementation in dogs with CHF due to MMVD. In the 3-month supplementation study, we found that CoQ_{10} supplementation positively affected neutrophil percentage, as well as lymphocyte percentage and concentration in MMDV dogs with CHF. However, other inflammatory parameters (total leukocyte count, CRP, TNFSR-II), oxidative stress markers (F2-isoprostanes, GPX), lymphocyte subpopulations, and markers of disease progression and myocardial injury (NT-proBNP, cTnI) were not affected. The results of our study suggest the possible anti-inflammatory effect of CoQ_{10} in dogs with CHF due to MMVD.

6 POVZETEK

Miksomatozna degeneracija mitralne zaklopke (MDMZ) je najpogostejša bolezen srca in najpogostejši vzrok kongestivnega srčnega popuščanja pri psih, tako kot tudi najpogostejši vzrok smrti pri tej živalski vrsti. Oksidativni stres je prisoten pri kongestivnem srčnem popuščanju tako pri ljudeh kot pri psih. Imunski sistem in vnetje sta močno vpletena v napredovanje bolezni srca pri ljudeh in psih, zato je možno, da podvrste limfocitov igrajo pomembno vlogo pri imunskem odzivu, vezanem na bolezni srca. Raziskovalci iščejo nova zdravila, ki bi lahko zmanjšala oksidativni stres in vnetje ter upočasnila napredovanje bolezni in izboljšala kakovost življenja psov.

Koencim Q_{10} (CoQ_{10}) je v maščobi topen antioksidant, ki ga lahko uporabljamo tudi kot prehransko dopolnilo. Nizka koncentracija CoQ_{10} v plazmi je povezana s srčno-žilnimi

boleznimi, dodajanje CoQ_{10} pa je bilo obsežno raziskano pri bolnikih s srčnim popuščanjem. Koencim Q_{10} ima antioksidacijske lastnosti in je možno, da zmanjša vnetje, ki je posledica kongestivnega srčnega popuščanja.

Cilj našega raziskovanja je bil oceniti učinke CoQ_{10}, ki so ga pacienti prejemali poleg standardne terapije MDMZ, na parametre oksidativnega stresa in vnetja, limfocitne podvrste ter klinični status pri psih različnih pasem z diagnozo MDMZ s srčnim popuščanjem ali brez njega. Ker ni bilo podatkov o podvrstah limfocitov pri psih z različnimi stopnjami MDMZ, kot tudi ne o priporočenem odmerku CoQ_{10} pri psih z MDMZ, je bil vmesni cilj zbrati te podatke pred izvedbo glavne trimesečne raziskave dodajanja CoQ_{10}.

Naša raziskava je bila sestavljena iz treh delov:

1. Prospektivna presečna raziskava z uporabo pretočne citometrije za določitev odstotkov in koncentracij podvrst limfocitov pri psih z različnimi stopnjami MDMZ in kongestivnim srčnim popuščanjem ali brez njega ter pri zdravih psih.

2. Dvojno slepa randomizirana, s placebom nadzorovana raziskava za določitev odmerka CoQ_{10}, ki bi zadostoval za doseganje vsaj trikratnega povečanja koncentracije CoQ_{10} v plazmi pri psih s kongestivnim srčnim popuščanjem zaradi MDMZ.

3. Dvojno slepa randomizirana, s placebom nadzorovana raziskava dodajanja CoQ_{10} z namenom ugotavljanja vpliva trimesečnega dodajanja peroralnega CoQ_{10} kot podpore standardni terapiji zdravljenja srčnega popuščanja pri psih, na pokazatelje oksidativnega stresa ter imunski in klinični status psov z MDMZ s kongestivnim srčnim popuščanjem ali brez njega.

V prvem delu raziskave smo ugotovili znižan odstotek CD4+ limfocitov T, zmanjšano razmerje CD4+/CD8+ in povečan odstotek CD8+ limfocitov T pri psih s stabilnim in nestabilnim kongestivnim srčnim popuščanjem ter povečano koncentracijo CD8+ limfocitov T pri psih z nestabilnim srčnim popuščanjem. Ti izsledki so pokazali, da je pri kongestivnem srčnem

popuščanju verjetno prisoten sistemski imunski odziv, ki ga pri psih z asimptomatsko MDMZ (ACVIM B2) ne opazimo.

Mehanizmi, ki so odgovorni za spremembe koncentracij podvrst limfocitov, ugotovljene v naši raziskavi in drugih podobnih raziskavah pri psih, niso popolnoma razjasnjeni. Predvidevamo da gre za aktivacijo avtoreaktivnih limfocitov, specifičnih za miokardiocitne elemente kot antigene.

Miksomatozna degeneracija mitralne zaklopke pri psih je sama po sebi nevnetna in nekužna degenerativna bolezen z znanim genetskim ozadjem. Sčasoma lahko napreduje v kongestivno srčno popuščanje, pri katerem je znano, da ga spremlja blago vnetje. Pri miših in ljudeh imajo limfociti T tako škodljivo kot reparacijsko vlogo pri obolelem miokardu. Znano je, da limfociti infiltrirajo srce pri ljudeh z avtoimunskimi in infekcijskimi bolezni srca, npr. pri pacientih z miokarditisom, ki ima najpogosteje virusno etiologijo. Vendar pri miših in ljudeh infiltracija limfocitov T v miokardu ni prisotna le pri hudih vnetnih infekcijskih ali neinfekcijskih boleznih, temveč tudi pri srčnem popuščanju zaradi drugih etiologij. Izsledki naše raziskave so pokazali, da lahko obstaja sistemski imunski odziv, povezan s kongestivnim srčnim popuščanjem, ki pa ga nismo opazili pri psih z asimptomatsko MDMZ (psi v ACVIM B2); potrebne so nadaljnje raziskave tako sistemskega kot tudi lokalnega imunskega odziva in specifične vloge limfocitov T v srcu psov z MDMZ.

V drugem delu raziskave smo ugotovili, da je dnevni odmerek 200 mg vodotopne oblike CoQ_{10} zadostoval za doseganje želenega, vsaj trikratnega povečanja plazemske koncentracije CoQ_{10} v plazmi pri psih s kongestivnim srčnim popuščanjem zaradi MDMZ. Omenjeni odmerek je mogoče uporabiti v prihodnjih raziskavah dodajanja CoQ_{10}, sicer pa smo ga tudi sami uporabili v trimesečni raziskavi dodajanja CoQ_{10}.

Trimesečna raziskava dodajanja CoQ_{10} je pokazala, da se je pri psih s kongestivnim srčnim popuščanjem ki so prejemali CoQ_{10} kot prehranski dodatek značilno zmanjšal odstotek nevtrofilnih granulocitov ter značilno zvišala odstotek in koncentracija skupnih limfocitov. Ti izsledki nakazujejo na protivnetne lastnosti CoQ_{10}. Po našem vedenju je, naša raziskava prva, ki je pokazala možni protivnetni učinek CoQ_{10} pri psih z boleznijo srca.

Na podlagi izsledkov lahko sklepamo, da so podvrste limfocitov verjetno vpletene v patogenezo kongestivnega srčnega popuščanja pri psih z MDMZ, saj so bile spremembe očitne pri psih s kongestivnim srčnim popuščanjem in najizrazitejše pri pacientih v nestabilnem popuščanju, kot je bilo prikazano v prvem delu naše raziskave; dnevni odmerek 200 mg vodotopnega CoQ_{10} zadostuje za najmanj trikratno zvišanje plazemske koncentracije CoQ_{10}, ki je potrebno za biološki učinek CoQ_{10}. Med več parametri, ki so bili testirani v trimesečni raziskavi dodajanja CoQ_{10}, smo ugotovili, da CoQ_{10} znižuje odstotek nevtrofilnih granulocitov ter zvišuje odstotek in koncentracijo limfocitov pri psih z MDMZ in popuščanjem srca, ne vpliva pa na druge vnetne parametre (skupno število levkocitov, C-reaktivni protein, topni receptor dejavnika tumorske nekroze-α II), označevalce oksidativnega stresa (F2-izoprostani, glutation peroksidaza), limfocitne podvrste, označevalce napredovanja bolezni ter poškodbe miokarda (N-terminalni natriuretični peptid tipa pro-B, srčni troponin I). Izsledki naše raziskave kažejo na možen protivnetni učinek CoQ_{10} pri psih s srčnim popuščanjem zaradi MDMZ.

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9 APPENDIX

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Sunday 23 October 2022

> Prof. dr. Alenka Nemec Svete, univ. dipl. inž. kem. inž.
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Natalia Druzhaeva, DVM
Olympus Cove Veterinary Clinic
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Dear Dr Druzhaeva:

First, please allow me to thank you for your commitment to animal health, and to congratulate you on your work.

Thank you for your request, which I have reproduced below:

Our Ph.D. student Natalia Druzhaeva, DVM, is finishing her Ph.D. study. She has published three papers that should be included in her dissertation. One of the published papers is the above one, published in the AJVR.
American Journal of Veterinary Research (AJVR), 2021;82:280. N Druzhaeva, AD Petrič, G Tavčar-Kalcher, J Babič, and Svete A Nemec. Randomized, double-blinded, controlled trial of the effects of coenzyme Q_{10} supplementation on plasma coenzyme Q_{10} concentration in dogs with myxomatous mitral valve disease.

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