Effects of nutritional intervention on dogs diagnosed with early-stage myxomatous mitral valve disease.

by

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B.S., University of North Georgia, 2022

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE

Department of Veterinary Biomedical Sciences
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2026

Approved by:

Major Professor
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Abstract

Myxomatous mitral valve disease (MMVD) is the most prevalent cardiovascular disease in the domestic dog. The disease is characterized by degradation of the mitral valve which results in blood backflowing into the left atrium when the left ventricle contracts, leading to the dilation of the left atrium and left ventricle. Dogs with cardiovascular disease should be fed nutrition to support cardiovascular health, but optimal levels of key nutrients are not well defined for canine MMVD. It was hypothesized that a food formulated to contain increased levels of protein, fat, L-carnitine, taurine, omega-3 fatty acids, and moderate sodium levels (test food) would show evidence of reduced disease progression and support aspects of quality of life compared to a food without elevated levels of key nutrients (control food). Therefore, this study investigated effects of two foods designed to support cardiac health with differing levels of key nutrients in dogs diagnosed with MMVD. The two-by-two factorial study enrolled dogs with MMVD and dogs without MMVD (healthy dogs). The MMVD dogs were stratified and the healthy dogs were randomized to one of two treatment groups, test food (MMVD-TEST group and HEALTHY-TEST group) or control food (MMVD-CONTROL group and HEALTHY-CONTROL group), for 6 months following a 5-week washout period. At the start of the washout period, 10 dogs with MMVD and 10 healthy dogs were enrolled. Of the dogs enrolled, four dogs in the MMVD-TEST group, two dogs in the MMVD-CONTROL groups, four dogs in the HEALTHY-TEST group, and five dogs in the HEALTHY-CONTROL group completed the 6-month feeding study. Caloric intake (kcal/metabolic body weight/day), body weight (BW), body condition score (BCS), muscle condition score (MCS), and blood and urine biomarkers were assessed in dogs with MMVD and healthy dogs. Echocardiographic measurements were assessed in only the MMVD dogs. The MMVD-TEST group maintained ($P > 0.10$) caloric intake, BW, and BCS, but

had increased ($P \leq 0.05$) muscle wasting throughout the study. The MMVD-CONTROL group had reduced ($P \leq 0.05$) caloric intake throughout the study, but maintained ($P > 0.10$) BW, BCS, and MCS. There was no difference ($P > 0.10$) in MCS between the two MMVD treatment groups throughout the study, but the MMVD-CONTROL group had lower caloric intake ($P \leq 0.05$) compared to the MMVD-TEST group at various timepoints. Caloric intake, BW, BCS, and MCS were overall maintained ($P > 0.10$) in the HEALTHY-TEST group and HEALTHY-CONTROL group throughout the study. Both the MMVD-TEST group and MMVD-CONTROL group maintained their MMVD stage throughout the study. Anteroposterior left atrial diameter (LAD), left ventricular internal diameter at end-diastole (normalized for body weight) (LVIDdN), and left ventricular internal diameter at end-systole (LVIDs) decreased by 6% ($P = 0.30$), 12% ($P = 0.03$), and 6% ($P = 0.23$), respectively, in the MMVD-TEST group at 6 months compared to baseline. In the MMVD-CONTROL group, LAD, LVIDdN, and LVIDs increased by 6% ($P = 0.54$), 11% ($P = 0.15$), and 20% ($P < 0.01$), respectively, at 6 months compared to baseline. There was no difference ($P > 0.10$) in left atrial size between the two MMVD treatment groups throughout the study, but LVIDs and LVIDdN was larger ($P \leq 0.05$) in the MMVD-CONTROL group compared to the MMVD-TEST group at the end of the study. Additionally, this research explored the effects of the two study foods on clinical biomarkers and sought to identify exploratory biomarkers associated with canine MMVD. All four treatment groups remained above the lower limit of normal for whole blood taurine (200 nmol/L) and serum 25-hydroxyvitamin D (112 nmol/L) supporting the concept that early-stage MMVD dogs are not at risk for taurine deficiency or low serum vitamin D levels. Although these results should be validated with a longer, larger, and more diverse study since the present study was underpowered

and should be considered a pilot study, this research contributes to the existing data of nutritional intervention in dogs with MMVD.

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Acknowledgements

I would like to express my sincere gratitude to everyone who supported this research. I am deeply grateful to my major professor, Dr. Haley Larson, and my committee members, Dr. Julia Guazzelli Pezzali and Dr. Kathy Gross, for their expert guidance and mentorship.

A special thank you to Dr. Matthew Tanner for his contribution in performing the echocardiograms and for his invaluable mentorship. I also thank Dr. Jen Hafferman, Dr. Luis Montelongo, Selena Tavener, and the Animal Care and Research Technicians for their essential assistance with this study.

I am profoundly grateful to Hill's Pet Nutrition for funding this research. My sincere thanks to Dr. Laura Motsinger and Dr. Leslie Hancock for their mentorship. I also thank Senior Leadership, the Global Clinical Nutrition and Claims team, and my colleagues and peers at Hill's for their unwavering support.

Finally, thank you to my family, friends, and my fiancé, Chad Newton, for their constant encouragement, understanding, and patience throughout this journey. The endless love and support shown during this chapter of my life was vital to my accomplishments and growth.

To everyone, thank you for your belief in my work and for providing the guidance, resources, and encouragement that allowed me to achieve this significant milestone.

Chapter 1 - A review of canine myxomatous mitral valve disease and common pharmaceutical, surgical, and nutritional interventions

Introduction and Review

Myxomatous mitral valve disease (MMVD) is the most prevalent cardiovascular disease in the domestic dog, being most commonly manifested as mitral valve regurgitation. In dogs, MMVD has been reported to account for about 75% of heart disease cases (Keene et al., 2019). The disease is chronic and progressive, worsens with age, and eventually leading to mitral regurgitation as well as congestive heart failure in about 30% of cases (Borgarelli & Haggstrom, 2010; Parker & Kilroy-Glynn, 2012). Predisposition of MMVD in dogs has been linked with various signalment descriptions such as specific breed, body size, age, and sex (O'Brien et al., 2021; Parker & Kilroy-Glynn, 2012). Diagnosis of MMVD is usually associated with the presence of a heart murmur and can be classified into stages depending on disease severity measured using echocardiographic imaging and thoracic radiographs, especially when congestive heart failure develops. Additionally, MMVD can present with complementary clinical signs such as heart rate variability and cachexia, and given that most patients are middle-to-old age, comorbidities such as systemic hypertension, pulmonary hypertension, and arrhythmias are common. As disease severity progresses, cardiac remodeling and congestive heart failure can occur and result in the initiation of disease management through drug therapy, surgery, and nutritional intervention to improve or maintain prognosis and support quality of life in dogs with MMVD. However, disease management can be difficult due to the complexity and variations in treatment plans, impacts of cardiac medications, development of poor quality of life, and owner expenses for care (Beaumier et al., 2018). Nutritional intervention is a common aspect of a multi-modal treatment plan, but the optimal levels of key nutrients for cardiovascular support have not

been well defined. Therefore, further research is needed to better understand optimal levels of these nutrients to support dogs diagnosed with MMVD. This literature review will explain the pathophysiology of MMVD in domestic dogs, predisposed populations, clinical signs and disease staging related to mitral valve regurgitation, various treatment options and nutritional intervention to support dogs with cardiovascular disease.

Myxomatous Mitral Valve Disease in Dogs

The vertebrate heart is a four-chambered organ composed of cardiac muscle that pumps blood to supply oxygen and nutrients to tissues while removing waste products. Each side of the heart contains an atrium and ventricle with one-way valves separating each atrium from their respective ventricle. The right side of the heart is responsible for receiving deoxygenated blood from the body and pumping it through the lungs for oxygenation and removal of carbon dioxide. Deoxygenated blood enters and fills the right atrium through the cranial and caudal vena cava when the ventricle contracts, also known as the systolic phase. When the ventricle begins to relax, the diastolic phase, the right ventricular pressure decreases below the right atrial pressure to create a pressure gradient, which allows for blood to flow across the tricuspid valve, which contains three leaflets, and into the right ventricle (Fishbeck & Sebastiani, 2015; Flores Dueñas et al., 2023). Immediately following ventricular diastole, the right ventricle contracts, and the high ventricular pressure forces the tricuspid valve to shut, preventing backflow of blood into the right atrium (Fishbeck & Sebastiani, 2015). Blood flows out of the right side of the heart, through the pulmonary arteries to the pulmonary capillaries where gas exchange occurs. After gas exchange, oxygenated blood flows to the left atrium via the pulmonary veins. Similar to the right side of the heart, after the oxygenated blood is collected in the left atrium, the bileaflet mitral valve, separating the left atrium and left ventricle, opens and allows blood to flow into the

relaxed left ventricle. Left ventricular contraction generates higher ventricular pressure, which causes the mitral valve to close, and forces blood across the aortic valve and into systemic circulation, delivering oxygen and nutrients to peripheral tissues (Fishbeck & Sebastiani, 2015).

Myxomatous valve disease, one of the most common cardiovascular diseases in the domestic dog, is a pathological condition characterized by abnormally thickened valve leaflets leading to dysfunction of the one-way valve apparatus and is first identified by the onset of a heart murmur. Since the mitral and tricuspid valves function to prevent blood backflow during the contraction of the ventricles, significant dysfunction of the mitral or tricuspid valve apparatus can result in backflow of blood into the left or right atrium, respectively (Coutinho & Antunes, 2021; Neto et al., 2018; O'Brien et al., 2021; Palmisano et al., 2021).

The appearance of MMVD in dogs can vary from small focal thickening to large areas of nodular thickening of the distal part of all leaflets and valve tissue (Borgarelli & Buchanan, 2012). In advanced cases, the myxomatous changes can extend to the chordae tendineae, decreasing their tensile strength, leading to rupture of one or multiple chordae tendineae, which is an indicator of poor prognosis (Ettinger & Buergelt, 1969; Häggström et al., 2005; Kogure, 1980). Since the chordae tendineae span from the valve leaflets to the myocardium and play a crucial role in the one-way valve apparatus, rupture of a major chorda tendinea associated with the mitral valve usually leads to an acute onset of left-sided congestive heart failure (Peddle & Buchanan, 2010). Given that a rupture of a major chorda tendinea is an acute process, the atrial and ventricular cardiac remodeling appreciated with chronic valvular degeneration may be absent.

The typical rate of progression of myxomatous valve disease is slow which allows for the heart to compensate for the increased blood volume by undergoing eccentric hypertrophy of the

associated atrium and ventricle (Keene et al., 2019). Eventually, as cardiac dysfunction becomes more severe, the heart fails to effectively pump blood forward, leading to an increase in central venous pressure or pulmonary venous pressure, and ultimately right-sided or left-sided congestive heart failure, respectively. Common clinical signs associated with congestive heart failure include: restlessness, cough, abdominal distension (associated with right-sided heart failure only), exercise intolerance, episodes of unconsciousness, and respiratory distress (Atkins et al., 2009; Fox, 2012; Keene et al., 2019; Yancy et al., 2013).

Susceptible Canine Populations and Predisposing Factors

Myxomatous mitral valve disease (MMVD) causes mitral regurgitation and accounts for 75% of heart disease cases in dogs (Häggström et al., 2004). Most of the cases are observed in certain breeds, specifically those with a small body size, older dogs, and males, and the disease may be associated with predisposing factors such as closed genetic pools within breeds, a morphological component as a result of a small body size, and overexpression of specific genes (O'Brien et al., 2021; Parker & Kilroy-Glynn, 2012).

Myxomatous mitral valve disease is most commonly reported in small to medium-sized breeds and is usually present with a heart murmur (Svensson et al., 2024). Disease prevalence has been identified in several small breeds, including but not limited to, the Cavalier King Charles Spaniel, Dachshund, Miniature poodle, Chihuahua, Whippet, and Yorkshire terrier (Keene et al., 2019; Meurs et al., 2019; O'Brien et al., 2021). Many breeds represent genetically isolated groups, which results in low heterozygosity within populations leading to the commonality of predisposition to specific conditions (O'Brien et al., 2021). Cavalier King Charles Spaniels and Dachshunds are commonly investigated breeds with MMVD while other predisposed breeds have not been studied to the same extent (Meurs et al., 2019). In addition,

Cavalier King Charles Spaniels display the earliest onset and highest incidence of MMVD (Boswood, 2009; Detweiler & Patterson, 1965b; Madsen et al., 2011; Smith et al., 2015). In an epidemiologic study involving nearly two hundred Dachshunds, factors related to disease severity, such as mitral valve prolapse, jet size (indicative of mitral regurgitation), leaflet thickness, and murmur intensity, were significantly correlated with one another and positively correlated with age (Olsen et al., 1999). One hypothesis for the increased prevalence of MMVD in Cavalier King Charles Spaniels and Dachshunds is an inherited component (Birkegård et al., 2016; Lewis et al., 2011; Olsen et al., 1999; Pedersen, Lorentzen, et al., 1999; Swenson et al., 1996). For example, Madsen et al. (2011) identified two loci as being associated with the development of myxomatous mitral valve disease in Cavalier King Charles Spaniels. It is important to note that purebred dogs have a closed gene pool that contain their own genetic mutation, which expands throughout the population (Meurs et al., 2019; Parker & Kilroy-Glynn, 2012); therefore, predisposed purebred dogs, such as Cavalier King Charles Spaniels, may have a higher risk of developing MMVD.

While MMVD is commonly associated with small breed dogs, valvular disease can also occur in larger dogs. However, this disease appears to be presented differently in large breeds. For example, Borgarelli et al. (2004) recorded that heart murmurs were either undetectable or less intense in German Shepherds with mitral valve prolapse or mitral regurgitation compared to smaller breed dogs with chronic valvular disease. Additionally, Borgarelli et al. (2004) stated that a large portion of the German Shepherd group did not have major mitral valve thickening or prolapse along with significant mitral regurgitation, which may suggest a different cause for the disease. A possible explanation for differences in disease diagnosis and prognosis, in addition to increased prevalence in small breeds, may be related to morphological components or genetics

(Parker & Kilroy-Glynn, 2012). Myxomatous mitral valve disease prevalence is increased in breeds with an average adult body weight of less than 9 kg (Fleming et al., 2011; Serfass et al., 2006; Thrusfield et al., 1985). Cardiovascular conditions were diagnosed in 75% of breeds with an average body weight less than 9 kg compared to only 25% of breeds with an average body weight over 9 kg (Fleming et al., 2011). When observing the morphological differences of small versus large breed dogs, height is hypothesized to be associated with heart disease, especially since an inverse relationship between height and risk of cardiovascular disease is observed in human studies (Gertler et al., 1951; Paajanen et al., 2010; Wannamethee et al., 1998). A similar correlation may be observed in dogs because small breeds are approximately 30% shorter at the shoulder compared to the average breed size; however, additional investigation about height relationships is needed and specific association to valvular disease (Parker & Kilroy-Glynn, 2012). Another hypothesis for the increased prevalence of mitral valve disease in small breeds includes reduced chest diameter or shape of the chest, as abnormal growth of the heart and chest cavity can lead to valve malformation (Raggi et al., 2000; Schutte et al., 1981). Due to the extreme reduction in skeletal size of small breed dogs, investigation of Insulin-like growth factor 1 (IGF-1), organ size, and thorax volume in small versus large breeds may provide insight if there is overcrowding since this hormone affects size variation in dogs (Baker et al., 1993; Sutter et al., 2007; Woods et al., 1996). For example, IGF-1 single-nucleotide polymorphism haplotype is common among all small breeds and missing in giant breeds, suggesting this is a major contributor to the body size of small breed dogs (Sutter et al., 2007). Additionally, IGF-1 is found in cardiac muscle, and therefore, overexpression of Insulin-like growth factor receptor, which is involved in the phosphoinositide 3-kinase-Akt-p70S6K1 pathway in cardiac myocytes, can lead to hypertrophy and may contribute to valve lesions and dysfunction (McMullen et al.,

2004). Finally, Uehara et al. (2019) reported that heart volume and features of large airways are influenced by size of the dog. Specifically, heart volume is inversely related to size, with small breeds having a larger heart volume than large breeds, which may explain clinical differences between breed sizes (Uehara et al., 2019).

In addition to investigating relationships between predisposed breeds and body size, an association between sex and age has been identified in dogs with MMVD. Males are about 1.5 times more likely to be diagnosed with mitral valve disease and detection of a heart murmur compared to females (Häggström et al., 1996; Häggström et al., 1995; Keene et al., 2019; Pedersen, Häggström, et al., 1999). Swenson et al. (1996) observed that heart murmur development and intensity was greater in male Cavalier King Charles Spaniels compared to females. Additionally, Olsen et al. (1999) reported an increase in leaflet thickness and progression of mitral valve prolapse in males compared to females, and the occurrence of congestive heart failure due to mitral regurgitation is approximately 4.5 times greater in male Cocker Spaniels when compared to females (Detweiler & Patterson, 1965b). In addition to increasing prevalence in males, older dogs are associated with increased MMVD development. In Cavalier King Charles Spaniels, 90% develop MMVD over the age of ten years old; although, some have been diagnosed under the age of four (Bagardi et al., 2020; Häggström et al., 1992; Pedersen, Häggström, et al., 1999). Jones and Zook (1965) demonstrated that the percentage of dogs with lesions on the atrioventricular valves, and less commonly, aortic and pulmonary valves was significantly increased with age. Also, Whitney (1974) observed 97% of dogs over the age of nine years old were diagnosed with MMVD compared to 35% of dogs less than five years old during necropsy. In addition to the higher incident of diagnosis, disease stage severity and findings of clinically significant lesions was observed in older dogs (Whitney, 1974). Measures

of disease severity such as mitral valve prolapse, murmur intensity, and leaflet thickness were also demonstrated to be positively correlated with age (Beardow & Buchanan, 1993; Darke, 1987; Detweiler & Patterson, 1965b; Häggström et al., 1992; Olsen et al., 1999; Pedersen, Lorentzen, et al., 1999; Whitney, 1974). Additionally, one study reported that half of a population containing 190 Dachshunds developed heart murmurs at 9.4 years on average (Olsen et al., 1999). The development of MMVD in older dogs has been associated with characteristics in aging.

During the aging process, inflammation increases which promotes inflammatory cytokines that lead to age-related conditions. Senescent cells are cells that stop dividing but do not undergo apoptosis. This is an aging characteristic where accumulation of senescent cells occurs in tissues over time promoting inflammation, tissue dysfunction, and tissue remodeling and, ultimately, leading to age-related cardiac pathology, like MMVD (Grzeczka et al., 2025). Additionally, Transforming Growth Factor-beta (TGF-β) and serotonin (5HT) signaling pathways become dysregulated. These dysregulated pathways drive valvular interstitial cells to become activated into myofibroblasts which show resistance to apoptosis and result in increased extracellular matrix and leaflet thickening of the mitral valve (Grzeczka et al., 2025; Oyama et al., 2020). By understanding the role of TGF-β and 5HT in the development of MMVD in aging dogs, researchers can utilize this knowledge to develop potential therapies to manage MMVD in dogs.

Proper diagnosis and management of MMVD is imperative to ensure the quality of life of dogs affected by the condition, allowing for timely interventions. Thus, having a staging system to assess the severity and progression of the disease is essential, as a well-defined staging system

enables veterinarians to tailor treatment plans based on the specific stage of the disease, providing the most effective care at each point in the course of the disease.

Staging of Myxomatous Mitral Valve Disease

Due to the high prevalence of MMVD in dogs, the American College of Veterinary Internal Medicine Specialty of Cardiology consensus panel revised a staging system that was originally published in 2009 (Atkins et al., 2009; Keene et al., 2019). The 2009 system was adapted from the 2001 American College of Cardiology/American Heart Association classification system for humans, and patients were expected to advance to the next stage unless treatment altered the disease progression (Atkins et al., 2009; Hunt et al., 2001). Prior to the development of the American College of Veterinary Internal Medicine consensus guidelines in 2009, veterinarians were familiar with the New York Heart Association and International Small Animal Cardiac Health Council for functional classification systems (Atkins et al., 2009; Ettinger & Suter, 1970; International Small Animal Cardiac Health Council, 1994). The current 2019 staging system, a revision of the 2009 system, is utilized to help diagnose and manage progressive stages of myxomatous mitral valve disease, including heart failure, by linking morphological changes and clinical signs to four main stages: A, B, C, and D (Keene et al., 2019).

Myxomatous mitral valve disease is usually identified with the presence of a heart murmur (Parker & Kilroy-Glynn, 2012). Approximately 200 years ago, heart murmurs began to be described in humans, and Samuel Levine and A.R. Freeman proposed a 6-level grading scheme to identify murmur intensity and other characteristics to differentiate severity and degrees of pathology (Rishniw, 2018). Levine continued to update the 6-level scheme with additional clarification between the grades and the inclusion of thrills, which are palpable

vibrations that can be felt over the chest in more noticeable murmurs (Rishniw, 2018). Around 1817, Blaine described a similar thrill in dogs that was observed in humans and initiated the commonality of describing heart murmurs in veterinary literature over the next 150 years (Blaine, 1841). In 1965, Detweiler and Patterson published a 5-level grading scheme for heart murmurs in dogs which was followed by the development of an alternative 6-level grading scheme, almost identical to the scheme created by Levine, by Ettinger and Suter in 1970 (Detweiler & Patterson, 1965a; Ettinger & Suter, 1970). The grading scheme created by Ettinger and Suter identifies features of cardiac auscultation and palpation and has been incorporated by veterinarians over the years (Table 1.1) (Côté et al., 2015; Ettinger & Suter, 1970; Rishniw, 2018).

Classification of murmur intensity and auscultation are key components of evaluating MMVD. Over the years, variations of the heart murmur grading scheme have been created (Rishniw, 2018). In one study that graded murmur intensities in small breed dogs with MMVD, there was no clinical difference in disease severity between dogs with grade one and grade two or dogs with grade four and grade five on a 6-level scheme (Ljungvall et al., 2014). Therefore, a 4-level scheme was suggested and each level was renamed to ‘soft’, ‘moderate’, ‘loud’, and ‘thrilling’ (Ljungvall et al., 2014). However, the 4-level grading scheme may not function well in varying breed sizes with MMVD because the different murmur intensities can carry different clinical significance (Rishniw, 2018). The 6-level grading scheme is well established and continues to be taught in both human and veterinary medicine (Rishniw, 2018; Silverman & Wooley, 2008).

Stage A identifies predisposed or high risk dogs that currently do not have structural manipulation of the heart or a heart murmur present, which usually includes small breed dogs

like Cavalier King Charles Spaniels or Dachshunds (Keene et al., 2019). Therefore, breeds predisposed to MMVD should complete yearly auscultations, which includes listening to sounds of the heart, lungs, or other organs, for routine care and screening. Dogs classified as Stage A do not require drug or dietary interventions, but those who are used for breeding are recommended to no longer be bred if evidence of early MMVD development is identified (Birkegård et al., 2016; Swift et al., 2017).

Dogs diagnosed with Stage B are identified with a murmur correlating to mitral valve regurgitation, but do not have clinical signs of congestive heart failure (Keene et al., 2019). Dogs in this classification can be broken down into two substages, B1 and B2, which differ based on evidence of cardiac remodeling observed via echocardiographic imaging, which is the most sensitive and specific diagnostic to assess left atrial and left ventricular dimensions and function that is widely available (Borgarelli et al., 2008). Dogs in Stage B1 do not have evidence of cardiac remodeling secondary to their MMVD and do not meet remodeling criteria to justify initiating drug or dietary interventions. Due to the progressive nature of the disease, reevaluation with an echocardiogram is recommended every 6-12 months (Keene et al., 2019). Stage B2 valve disease is diagnosed when significant cardiac remodeling, specifically left atrial and left ventricular eccentric hypertrophy, is noted. The remodeling is thought to be secondary to more advanced mitral regurgitation leading to decreased cardiac output which, in turn, stimulates mechanisms that increase water retention in the kidneys, systemic vascular resistance, and pathologic cardiac remodeling via the renin-angiotensin-aldosterone system (Hammond et al., 2023). These mechanisms, which are initiated to preserve cardiac output and maintain mean arterial pressure, ultimately increase cardiac workload and contribute to further cardiac

dysfunction. In an attempt to delay the progression, dogs with Stage B2 MMVD warrant the start of intervention options to delay heart failure onset (Keene et al., 2019).

Echocardiographic measurements of the left atrium and left ventricle can predict congestive heart failure risk, medical intervention, and disease prognosis (Atkins et al., 2007; Borgarelli et al., 2012; Borgarelli et al., 2008; Boswood et al., 2016; Häggström et al., 2008; Hezzell et al., 2012; Lord et al., 2010; Moonarmart et al., 2010; Reynolds et al., 2012; Sargent et al., 2015; Strohm et al., 2018). The American College of Veterinary Internal Medicine consensus panel identified various criteria for dogs with Stage B2 MMVD; however, the most reliable way to identify dogs that may benefit from treatment is by observing echocardiographic evidence of left atrium and left ventricle enlargement (Keene et al., 2019). Left ventricle-to-aortic root (LV:Ao), left atrial-to-aortic root (LA:Ao), and left atrial-to-left ventricle (LA:LV) ratios have been shown to be effective for identifying enlargement of the left atrium and left ventricle in dogs with MMVD (Strohm et al., 2018). The LA:Ao ratio is the most commonly used measurement and left atrial enlargement is a strong indicator of disease severity and prognosis (Borgarelli et al., 2008; Sánchez Salguero et al., 2018; Vezzosi et al., 2021). In one study, dogs with a LA:Ao ratio exceeding 1.7 were 2.1 times more at risk of death from cardiac disease than dogs with smaller atria (Borgarelli et al., 2008). Additionally, Ljungvall et al. (2014) showed increasing murmur intensity was associated with left atrial size determined by LA:Ao ratio. Dogs identified as Stage B2 may benefit from drug and dietary interventions (Keene et al., 2019).

Dogs with current or previous clinical signs of congestive heart failure caused by MMVD are identified as Stage C (Keene et al., 2019). Heart failure is defined by an abnormal elevation of central and pulmonary venous pressures at the capillary bed because the heart is unable to move blood forward effectively, and eventually, fluid accumulation occurs in the lungs or body

cavities as a result of further cardiac dysfunction and water retention. The accumulation of fluid in the lungs or body cavities is called congestive heart failure or “backwards heart failure” (Keene et al., 2019). Stage C dogs usually show clinical signs of left-sided congestive heart failure and history of tachypnea or rapid breathing, restlessness, respiratory distress, or coughing (Keene et al., 2019). Although, patients with a history of coughing may not be a result of congestive heart failure because the large population of dogs predisposed to mitral valve disease also experience a high prevalence of chronic tracheobronchial disease, such as tracheobronchial collapse or chronic bronchitis (Keene et al., 2019; Padrid & Amis, 1992). Stage C patients can be managed medically as chronic disease patients and will always be categorized as Stage C regardless of improvement or complete resolution of heart failure signs. However, dogs with successful surgical repair of the mitral valve may be reclassified as Stage B if improvement in cardiac dimensions is observed and the dogs are able to be weaned off of diuretic therapy (Keene et al., 2019). Dogs presenting with acute, decompensated, congestive heart failure symptoms are typically unstable and receive care to optimize preload, afterload, heart rate, contractility, and oxygenation to improve cardiac output, decrease regurgitation, and relieve clinical signs through regulating the blood flow efficiency or hemodynamic status of the patient and tissue oxygen delivery (Keene et al., 2019). Conversely, dogs with chronic, compensated, congestive heart failure, where effective mitral valve repair surgery may not be feasible, are stable, and their treatment focuses on maintaining hemodynamic status and slowing disease progression, maintain body weight, enhance exercise capacity, and improve quality of life to prolong survival (Keene et al., 2019).

The last classification of mitral valve disease is Stage D where dogs are refractory to traditional therapies, and clinical signs of congestive heart failure require specialized

interventions to help keep patients comfortable until end of life (Keene et al., 2019). Mitral valve repair with surgery is possible at this stage but is associated with a higher risk of mortality during the procedure or recovery (Mizuno et al., 2013). Overall, due to the limited number of clinical trials for treatment efficiency and safety and variation in cases of dogs with Stage D, recommendations for treatment strategies are not definitive (Keene et al., 2019).

Myxomatous mitral valve disease can be broken down into four main stages starting with dogs who are predisposed. The initial diagnosis is usually associated with a heart murmur, which is categorized on a 6-level grading scheme. As the disease progresses, dogs are reclassified into new stages depending upon evidence of cardiac remodeling and clinical signs of congestive heart failure. Additionally, dogs with MMVD may also experience complementary clinical signs.

Complementary Clinical Signs Associated with Myxomatous Mitral Valve Disease

Dogs with mitral valve disease may also experience secondary clinical signs associated with MMVD, such as heart rate variability, pulmonary hypertension, and cachexia. These clinical signs may either develop gradually and progress as the disease advances or may only be noticed subtly due to sudden worsening of the disease (Petrič, 2015). These signs are typically associated with advanced stages of MMVD and can help indicate disease progression and severity.

Heart rate variability refers to the variation in the beat-to-beat timing of the heart rate or rhythmic variation between consecutive heartbeats and can be a useful tool for evaluating a cardiovascular event (Billman, 2011). A high heart rate variability represents how an individual responds to various internal and external stimuli to maintain equilibrium, which suggests that individual has a greater cardiovascular fitness (ChuDuc et al., 2013; Young & Benton, 2015). In

veterinary medicine, several factors can influence heart rate variability such as species, breed, age, emotional and physical activity (Haddad et al., 1987; Katayama et al., 2016; Manzo et al., 2009; Rasmussen et al., 2011). Myxomatous mitral valve disease severity has been shown to be inversely related with heart rate variability such that as variability decreases, severity increases. This is observed because as congestive heart failure develops, sympathetic activity increases and heart rate increases to maintain cardiac output. Therefore, when a patient is relaxed, its heart rate does not decrease to the same extent as a healthy dog which decreases heart rate variability (Rasmussen et al., 2012; Rasmussen et al., 2011). Additionally, heart rate increases with increased disease severity (Crosara et al., 2010; Doxey & Boswood, 2004; Häggström et al., 1996; Rasmussen et al., 2011). Heart rate variability impairment can be used as an early detection method of cardiovascular conditions, classify severity of congestive heart failure, monitor and predict progression of MMVD in dogs, and aid in clinical understanding (Chueainta, 2019). In addition to heart rate variability, pulmonary hypertension is another sign commonly associated with mitral valve disease severity.

Pulmonary hypertension is common in dogs with MMVD caused by left-sided heart failure due to elevated pulmonary capillary pressures secondary to increased left-ventricular end-diastolic pressures (Borgarelli et al., 2015; Guazzi & Arena, 2010). At early stages, pulmonary hypertension is reversible; however, it becomes irreversible when pulmonary artery vasoconstriction as well as pulmonary artery and vein remodeling has occurred (Guazzi & Arena, 2010). Pulmonary hypertension has been associated with poor prognosis in humans with left-sided heart failure, including those with MMVD (Barbieri et al., 2010; Ghio et al., 2001). Additionally, studies in dogs have shown an association between pulmonary hypertension and severe MMVD including poor prognosis in dogs with Stage B2 and C (Borgarelli et al., 2015;

Chiavegato et al., 2009; Serres et al., 2006). Overall, moderate and severe pulmonary hypertension is associated with decreased prognosis of dogs with MMVD (Borgarelli et al., 2015). While heart rate and pulmonary hypertension are commonly identified signs associated with MMVD, muscle loss may be more difficult to detect in some patients.

Chronic diseases, such as cardiovascular disease, can increase inflammatory cytokines and reduce the ability to utilize fat in metabolic adaptations resulting in the catabolization of muscle and lean body mass (Lowry & Coyle, 2006; von Haehling et al., 2009). Cachexia is the loss of lean body mass that is commonly associated with chronic diseases such as congestive heart failure, chronic kidney disease, and cancer in companion animals. Cachexia can be defined as mild, moderate or severe muscle loss and/or weight loss of ≥5% in 12 months or less and can stem from increased inflammatory cytokines and decreased appetite (Freeman, 2010; Ineson et al., 2019). The Muscle Condition Score, which assesses muscle loss, and Body Condition Score, which assesses fat composition, are important tools for monitoring dogs with suspected cachexia (World Small Animal Veterinary Association Global Nutrition Committee, 2013a, 2013b). However, cachexia may be masked by the accumulation of fat or water which may prevent early diagnosis in some patients (Freeman, 2012). Cardiac cachexia is well recognized and studied; however, this complementary clinical sign to cardiovascular disease tends to increase morbidity and affect quality of life in many patients (Freeman, 2012). Therefore, it is much easier to prevent than to treat (Freeman, 2009; Freeman, 2012). Lean muscle loss is most apparent in the epaxial, gluteal, scapular, and temporal muscles and is often recognized after the onset of congestive heart failure; although, more advanced muscle loss is observed in dogs with right-sided congestive heart failure (Freeman, 2012; Freeman et al., 1998). Cachexia may be a result of enhanced catabolic processes associated with chronic heart failure (Fonfara et al., 2011).

Inflammatory cytokines are involved in metabolic alterations that contribute to cachexia through decreased appetite, increased energy metabolism, and accelerated loss of lean body mass (Freeman, 2012). Inflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), and interleukin-6 (IL-6) interact with the nuclear factor kappa B (NF-κB) pathway to increase muscle proteolysis, decrease muscle regeneration, and inhibit myoblast differentiation (Freeman, 2012; Hamid et al., 2009; Moresi et al., 2008). Therefore, a decrease in inflammatory cytokines may benefit the patient with their muscle wasting and underlying disease. Consequently, the ubiquitin-proteasome cell signaling pathway influences cachexia because it is activated by the NF-κB pathway, which is stimulated by inflammatory cytokines, such as TNF and IL-1, and reactive oxygen species (Freeman, 2012). Furthermore, TNF impairs the ability of IGF-1 to maintain normal muscle mass and influences the pathogenesis of cachexia (Penna et al., 2010). Another contributor, specifically to cardiac cachexia, is neurohormonal activation which affects myocardial and whole body energy metabolism and protein flux, which is the process of protein turnover for amino acid release and reconversion in metabolic pathways (Freeman, 2012). Muscle loss can be an indicator of poor quality of life leading to the decision of euthanasia and reduce survival time in patients with cardiovascular disease. Many patients who are diagnosed with cachexia also experience anorexia, a decrease or loss in appetite.

Anorexia has been reported to occur in up to 84% of dogs with congestive heart failure and is observed as a sign of decreased quality of life to owners which is a contributing factor to elect euthanasia in 68% of dogs with congestive heart failure (Mallery et al., 1999). Therefore, adequate caloric intake and appetite are important for dogs with cardiovascular disease which can be supported by providing smaller and more frequent meals, offering new food options, adding toppers to increase palatability, and providing appetite stimulants (Freeman, 2012).

Myxomatous mitral valve disease and its clinical appearance can vary with each individual. However, as disease severity increases, quality of life and disease prognosis decrease. Many dogs, depending on disease progression, may benefit from pharmaceutical, surgical, and/or dietary intervention to maintain or improve prognosis.

Treatments and Interventions for Myxomatous Mitral Valve Disease

The American College of Veterinary Internal Medicine consensus panel advises that dogs with Stage B2 MMVD who meet all of the criteria for heart enlargement should start treatment before the onset of congestive heart failure (Keene et al., 2019). In one study of dogs with Stage B2 MMVD, dogs receiving a pharmaceutical drug, pimobendan, which supports blood flow and strengthens cardiac muscular contractions, remained in Stage B2 for an average of 15 months longer before developing congestive heart failure or died as a consequence of MMVD compared to dogs who received a placebo (Boswood et al., 2016). In another study of dogs with MMVD, Li et al. (2019) showed that a food containing key nutrients to support cardiac function was shown to reduce or maintain mitral valve regurgitation compared to dogs fed a control food. Various types of intervention including pharmaceutical, surgical, and nutritional can help support quality of life in dogs with cardiovascular disease.

The pharmaceutical therapy for MMVD and congestive heart failure in dogs has changed over the past fifty years from experimental use of medications to standard treatment (Bagardi et al., 2022). According to the American College of Veterinary Internal Medicine consensus panel, dogs with Stage B2 MMVD should be prescribed pimobendan and angiotensin-converting enzyme inhibitors (Keene et al., 2019). Pimobendan is a drug used to help with vasodilation of peripheral blood vessels through phosphodiesterase 3 inhibition and strengthens muscular contractions of the heart. These two effects work together to allow the heart to pump blood more

effectively (Food and Drug Administration Center for Veterinary Medicine, 2024). Angiotensin-converting enzyme inhibitors play a role in the suppression of the renin-angiotensin-aldosterone-system, decreasing water retention and secondarily decreasing preload while also decreasing afterload by lowering arterial, systolic, and diastolic blood pressure to increase cardiac output without increasing heart rate (Dzau et al., 1980; Gavras et al., 1978; Todd & Heel, 1986; Vidt et al., 1982). In addition to pimobendan and angiotensin-converting enzyme inhibitors, furosemide, dobutamine, and spironolactone are recommended medications for Stage C dogs (Keene et al., 2019). Furosemide is a diuretic to decrease preload by increasing the production of urine and ultimately removing excess fluid and salt for management of respiratory signs associated with excess fluid in cardiac patients. Furosemide can help keep patients in a compensated form of congestive heart failure (Keene et al., 2019). Dobutamine is used to improve left ventricular function by managing blood pressure in patients who are unresponsive to pimobendan and other therapies and in decompensated form of congestive heart failure (Keene et al., 2019). Spironolactone is another medication for management of hypertension and heart failure by competitively blocking aldosterone to reduce sodium reabsorption in the body (Patibandla et al., 2023). Finally, in addition to the medications recommended for Stage B2 and C, Stage D dogs may also be prescribed sildenafil, a drug to manage pulmonary hypertension, other diuretics like torsemide or hydrochlorothiazide to manage respiratory signs, amlodipine or hydralazine to manage hypertension and decrease cardiac afterload, and cough-suppressant medications like hydrocodone (Keene et al., 2019). The appropriate dosing and combination of these medications vary depending on disease severity and patient response (Keene et al., 2019). In addition to drug therapy, surgical repair of the mitral valve is another treatment approach.

Mitral valve surgery is anticipated to substantially reduce the amount of mitral regurgitation and has shown to be successful in dogs (Griffiths et al., 2004; Mizuno et al., 2013; Pennington et al., 2022; Uechi et al., 2012). Successful repair may result in the resolution of clinical signs, reduction in pharmaceutical therapy, extended survival time, and improved quality of life in dogs with MMVD (Pennington et al., 2022; Uechi et al., 2012). Surgical intervention is possible in Stage B2 and C patients with low complication rates (Mizuno et al., 2013; Uechi, 2012; Uechi et al., 2012). Stage D dogs may undergo surgical repair, but an increased mortality rate has been reported (Mizuno et al., 2013). Surgical repair can be an effective treatment option to slow disease progression and improve clinical signs and quality of life; however, the risk of complications associated with surgery increase with disease severity. As part of a multimodal treatment plan, nutritional intervention can play a key role in supporting dogs with MMVD, especially in patients where surgery is not appropriate.

Dietary intervention is also a key component of managing dogs with MMVD. In addition to ensuring dogs with MMVD receive adequate caloric intake, optimal nutrition should be an integral part of any multimodal treatment plan. The food should not only provide the basic nutritional needs of the patient, but also be highly palatable to encourage eating and consider the ideal provision of nutrients that may support cardiovascular health, such as high-quality protein and balanced amino acids, omega-3 fatty acids, sodium, magnesium, potassium, carnitine, and taurine. Furthermore, dietary recommendations may vary depending on disease severity (Keene et al., 2019).

Nutritional Support for Cardiovascular Health

Due to the concern of cachexia in dogs with congestive heart failure, maintaining a healthy body weight and body condition score are imperative for overall health. Therefore,

increased intake of protein, considering the final amino acid balance and protein quality of the food, is crucial. In dogs with severe concurrent renal failure in addition to MMVD, where protein intake should be optimized to meet the amino acid requirement of the animal, selecting high-quality protein sources and ensuring proper amino acid balance becomes even more important, allowing reduction in dietary protein content while still ensuring sufficient intake of indispensable amino acids in their bioavailable form (Bartges, 2012; Brunetto et al., 2021; Keene et al., 2019). While diet alone cannot prevent cachexia, providing a food that contains high-quality protein and adequate calories may support lean muscle maintenance (Fearon, 2008; Rozentryt et al., 2010). Additionally, supplementation of omega-3 fatty acids may support muscle mass and cardiac function.

The three main omega-3 fatty acids are alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). Alpha-linolenic acid is found in plant-based sources, while EPA and DHA are found in marine sources. Additionally, EPA and DHA can be derived from ALA but is very inefficient and therefore, supplementation of EPA and DHA is important. While ALA can provide some anti-inflammatory benefits, these effects are less pronounced compared to EPA and DHA (Baker et al., 2020). Eicosapentaenoic acid and DHA provide benefits by altering the cell membrane ion channels and reducing the formation of pro-inflammatory molecules to help control atrial and ventricular arrhythmias, reduce heart rate, and increase heart rate variability (Endo & Arita, 2016; Sposito et al., 2007). Dogs with cardiac cachexia may benefit from EPA and DHA supplementation, especially EPA, to maintain muscle mass by increasing endogenous protein synthesis (Freeman et al., 1998; Gray & Boit, 2013; Smith et al., 2011). Freeman et al. (1998) showed that supplementation of fish oil (27 mg/kg/day EPA and 18 mg/kg/day DHA) improved cachexia and decreased inflammatory cytokine IL-1 in

28 dogs with heart failure. Cardiac function in dogs can also be supported by EPA and DHA. In one study, dogs with Stage B2 and C MMVD were fed a food supplemented with EPA (0.4% original matter basis) and DHA (0.3% original matter basis) and showed reduced vertebral heart size and normalized left ventricular diameter, contributing to lower volumetric overload (Nasciutti et al., 2021). Additionally, Smith et al. (2007) demonstrated a reduction in arrhythmias with daily supplementation of fish oil (780 mg EPA and 497 mg DHA) compared to daily supplementation of flax oil (1124 mg ALA) for six weeks in Boxers diagnosed with arrhythmogenic right ventricular cardiomyopathy. However, Pasławski et al. (2021) showed no difference in clinical, electrocardiographic, or echocardiographic measurements in dogs diagnosed with B2 and C MMVD when fed a food containing 1.87 g/100 g EPA+DHA, 2.5 g/100 g omega-3 fatty acids, and 1:1 omega-6 to omega-3 ratio (n6:n3) compared to a control food. In addition to the type and amount of omega-3 fatty acids, the n6:n3 should also be considered. A high n6:n3 ratio, or higher content of omega-6 fatty acids, can promote pathogenesis of chronic diseases like cardiovascular disease. On the other hand, a low n6:n3 ratio, or higher content of omega-3 fatty acids, provides suppressive effects of inflammation (Simopoulos & Cleland, 2003). Furthermore, there is limited research to support an ideal n6:n3 for dogs diagnosed with cardiovascular disease. Overall, supplementation of EPA and DHA can help support cardiac function and secondary clinical signs such as cachexia in dogs with MMVD, but the dosage, source, and omega-6 to omega-3 fatty acid ratio of dietary intervention should be considered. Another common nutritional intervention of focus in dogs with cardiovascular disease is reduced dietary sodium but, the optimal level has not been defined.

Sodium is an extracellular electrolyte and acts as an osmotic agent in blood, which increases blood volume (Laflamme, 2022). An increase in blood volume in dogs with

cardiovascular disease can overwork the heart, potentially leading to congestive heart failure. Therefore, in dogs with cardiovascular disease, a restriction in dietary sodium has been recommended, but the restriction level has not been well defined nor is there strong evidence to support the benefits of low sodium intake in dogs specifically (Laflamme, 2022). Rush et al. (2000) showed that a low sodium food (0.04 g/100 kcal) reduced maximum left atrial and standard left atrial size in dogs with chronic valvular disease and dilated cardiomyopathy compared to a moderate sodium food (0.07 g/100 kcal), but serum sodium and chloride concentrations decreased significantly as well when fed the low sodium food. The minimum requirement of sodium set by the National Research Council (NRC) is 300 mg/kg dry matter basis (DMB) and the Association of American Feed Control Officials (AAFCO) recommends 0.08% DMB for adult dogs (Association of American Feed Control Officials, 2025; National Research Council, 2006). Excessive restriction of sodium (0.16 g/kg DMB), which is below the NRC requirement, resulted in clinical signs of sodium deficiency including, but not limited to, restlessness, increased heart rate, and excessive urinary water output in adult beagles (Drochner et al., 1976). While feeding a reduced sodium food might provide benefits to support cardiovascular health, the appropriate level is unknown and maintaining a balance of other electrolytes such as potassium and magnesium should be considered to prevent abnormalities.

Electrolyte abnormalities can contribute to decreased myocardial contractility and heart muscle weakness (Hand et al., 2010). Potassium is a mineral that functions as an electrolyte and interacts with sodium, playing a role in controlling blood pressure. Abbrecht (1972) showed that low dietary potassium intake (0.3 mEq/day) in seven mongrel dogs decreased mean arterial pressure, cardiac output, and left ventricular stroke volume which may be associated with decreased myocardial contractility. Commonly used diuretics in dogs with MMVD, such as

furosemide, excrete sodium and potassium resulting in deficiencies. Although, other common cardiovascular medications, such as spironolactone and angiotensin converting enzyme inhibitors, spare potassium and can lead to hyperkalemia in some patients (Laflamme, 2022). In general, dietary potassium and electrolyte balance is important for cardiovascular health. However, there is limited data to understand what levels of dietary potassium are appropriate for dogs with cardiovascular disease, especially to avoid instances of deficiencies or hyperkalemia when providing pharmaceutical intervention (Laflamme, 2022).

Another important mineral for cardiac function is magnesium, as the latter plays a role in vital functions such as glucose and energy metabolism, protein synthesis, adenosine triphosphate (ATP) synthesis and use, vasodilation to reduce blood pressure, and cardiac function and rhythm (Barbagallo & Dominguez, 2010; Del Gobbo et al., 2013; Fang et al., 2016; Kolte et al., 2014; Mubagwa et al., 2007). Additionally, magnesium can provide anti-inflammatory properties and antioxidant benefits by reducing oxidative stress caused by the production of reactive oxygen species (Barbagallo & Dominguez, 2010; Mak et al., 2013). Hypomagnesemia has been observed in dogs with congestive heart failure (Cobb & Michell, 1992) and, as such, these observations highlight the importance of supplementing dietary magnesium. In one study with 30 Cavalier King Charles Spaniels, 15 without heart murmurs and 15 with heart murmurs, a high prevalence of hypomagnesemia was observed and a majority were fed dietary levels of magnesium between 0.130% and 0.155% (as-fed) prior to enrollment of the study (Pedersen & Mow, 1998). However, no changes were observed in hypomagnesemia when dogs were fed 75 mg of magnesium for four weeks (Pedersen & Mow, 1998). Freeman et al. (2003) showed through collection of food diaries that dogs with chronic valvular disease and dilated cardiomyopathy were consuming higher levels of dietary magnesium per day than recommended by AAFCO (11

mg/100 kcal) in 2002. The current recommendation by AAFCO for magnesium is a minimum of 15 mg/100 kcal for maintenance of adult dogs (Association of American Feed Control Officials, 2025). These past studies further highlight the importance of investigating adequate dietary magnesium levels to support dogs with cardiovascular disease. Potassium and magnesium can support cardiovascular function, but inadequate levels can lead to adverse effects in dogs with cardiovascular disease. Furthermore, the appropriate levels of dietary magnesium and potassium are not clear and may vary by disease condition and treatment plan. In addition to considering adequate dietary provision of EPA, DHA and minerals, supplemented carnitine and taurine intake support cardiac health.

Carnitine is a key compound involved in cardiovascular metabolism and is largely stored in cardiac and skeletal muscles, which accounts up to 98% of the carnitine in the body (Rebouche & Engel, 1983). Carnitine is an amino acid derivative that can be obtained through dietary intake via intact ingredients or in its synthetic form, or it can be metabolic synthesized by dogs from methionine and lysine. To support cardiac metabolism, carnitine must be transported into cardiac muscle through an active membrane transport mechanism (Laflamme, 2022; Sanderson, 2006). Carnitine is involved in the transfer of long-chain fatty acids across the mitochondrial membrane, where they can be oxidized to generate ATP (Hand et al., 2010). As the heart generates about 60% of its total energy from the oxidation of long-chain fatty acids (Neely & Morgan, 1974), carnitine plays a pivotal role in cardiac energy metabolism. Additionally, carnitine can regulate blood volume, without affecting the function of the left ventricle, to protect against congestive heart failure (Elabbassi et al., 2014). Ventricular dysfunction has been associated with fatty acid oxidation within cardiomyocyte cells and a reduction in myocardial carnitine levels (Wang et al., 2018). Li et al. (2019) showed that dietary

intervention of a complete and balanced food, including 2.12% lysine and 1.49% methionine on a DMB as precursors to carnitine and other key nutrients for cardiovascular disease, numerically reduced left atrial size and mitral regurgitation in dogs with either B1 or B2 MMVD. Another study demonstrated that dogs with chronic valvular disease fed a food formulated for cardiovascular support, including 26 mg/100 kcal of L-carnitine, had reductions in echocardiogram measurements compared to dogs with chronic valvular disease fed a placebo diet (Freeman et al., 2006). There is limited research that focused solely on supplemented carnitine or the precursors and, as such, it is unknown what level of carnitine can impact dogs with MMVD. Additionally, it is important to note that L-carnitine should be used as a synthetic source and not D-carnitine, as the latter does not exist naturally in the body and competitively inhibits L-carnitine (Sanderson, 2006).

Taurine is a sulfur containing beta-amino acid involved in myocardial function that can also be obtained from food, through intact ingredients or its synthetic form, or biosynthesized from methionine and cystine in the transsulfuration pathway by dogs (Quilliam et al., 2021; Sanderson, 2006). Similarly to carnitine, taurine is largely found in cardiac muscle and supports energy metabolism through regulation of intracellular calcium to support mitochondrial oxidative phosphorylation to produce ATP and enhance contractility (Griffiths & Rutter, 2009; Jong et al., 2021; Tenaglia & Cody, 1988). As such, a metabolic deficiency in taurine can reduce electron transport chain function and decrease ATP production (Schaffer et al., 2017). Kittleson et al. (1997) investigated the benefits of supplemented carnitine (1 g) and taurine (500 mg) orally three times a day in dogs with dilated cardiomyopathy and showed a significant echocardiographic improvement resulting in the discontinuation of drug therapy and maintenance of a normal quality life compared to dogs who received a placebo. Although, Wesselowski et al. (2022)

demonstrated that plasma or whole blood taurine concentrations did not differ significantly in dogs with different preclinical stages of MMVD or those fed foods that met the World Small Animal Veterinary Association (WSAVA) nutritional guidelines or not. Therefore, additional data is needed to understand what levels of supplemented taurine are appropriate to support cardiovascular function in dogs, especially those diagnosed with MMVD. Additionally, taurine acts as an intracellular antioxidant, providing benefits when inefficient mitochondria in diseased hearts increase production of reactive oxygen species, leading to oxidative stress (Kiyuna et al., 2018). The dietary provision of taurine can provide antioxidant benefits to decrease oxidative stress and support energy production to enhance cardiac function and contractility (Li et al., 2020).

The supplement industry offers a variety of options to support cardiovascular heath in dogs. Supplements are either designed to provide a specific nutrient, such as taurine, or provide a complex of key nutrients in a soft chew, capsule, or tablet. Freeman et al. (2003) found that 31% of dogs with cardiovascular disease were given nutritional supplements, including multivitamins, coenzyme Q10, L-carnitine, taurine, fish oil, and glucosamine. For owners of a dog with MMVD, feeding a food for daily nutrition, giving pharmaceuticals for disease management, and providing a dietary supplement for heart health can be burdensome. By offering a food that is supplemented with key cardiac nutrients, the owner can provide a food that meets the daily nutritional needs of the dog and supports cardiovascular health in one product, which could significantly reduce the load of the owner for disease management in their dog.

Nutritional management can support dogs with MMVD; however, dietary recommendations may vary depending on disease condition, severity, and treatment plans. Dogs diagnosed with Stage B2 MMVD may benefit from a highly palatable and mildly restricted

sodium food with balanced amino acids for maintaining ideal body condition (Freeman et al., 2006; Keene et al., 2019). As dogs progress to Stage C, sodium intake should be restricted modestly while optimizing intake of high-quality protein, unless severe renal failure is present, and ensuring proper amino acid balance (Freeman, 2009; Rush et al., 2000). In efforts to support dogs with cachexia, supplementation of EPA and DHA and maintaining caloric intake, about 60 kcal/kg of body weight, are important (Freeman et al., 2002; Freeman et al., 1998; Keene et al., 2019). Additionally, balancing the dietary provision of minerals, such as potassium and magnesium, in dogs with heart failure is essential and should be monitored (Keene et al., 2019). Ultimately, as MMVD progresses to Stage D, dietary recommendations for Stage C patients should be applied plus the further restriction of sodium without compromising appetite or renal function (Keene et al., 2019). Key nutrients to support cardiovascular health in dogs have been well identified. However, there are some gaps in the current nutrition industry for canine cardiovascular disease, such as optimal levels of these key nutrients, limited definitive efficacy of nutrition on disease progression in dogs with MMVD, and a versatile nutrition for various breed sizes, cardiovascular conditions, and disease severity. First, additional research is needed to better understand optimal dietary recommendations of these nutrients for cardiovascular health in dogs and what levels specifically impact dogs diagnosed with MMVD compared to overall cardiovascular health support or other cardiovascular diseases. Additionally, there are limited studies that support the efficacy of nutrition to reduce disease progression of MMVD in dogs. Furthermore, since each MMVD stage has differences in recommendations for disease management, including nutrition and pharmaceuticals, further research to identify optimal levels of key nutrients for MMVD disease severity is warranted. Finally, since MMVD is primarily

associated with small breed dogs, tailored foods for breed size nutrition for cardiovascular health and palatability preferences should be investigated.

Summary and Conclusion

Myxomatous mitral valve disease is a common cardiovascular disease usually seen in older and small to medium size dogs as well as specific breeds. The disease is chronic and progressive resulting in valvular regurgitation leading to cardiac remodeling and, ultimately, congestive heart failure. A heart murmur is identified and classified on a 6-level scheme proceeding to the staging of MMVD. Dogs classified as Stage B2 should initiate treatment to manage cardiac remodeling and delay the onset of heart failure (Keene et al., 2019). Stage C and D dogs with current or past clinical signs of heart failure should continue treatment to maintain quality of life (Keene et al., 2019). Pharmaceutical, surgical, and nutritional intervention are recommended options and may vary depending on severity and individual cases. Drug therapy can help with blood flow, strengthen cardiac muscles to improve pumping efficiency, decrease cardiac preload, and manage complementary clinical signs such as hypertension and heart rate. Although, some medications may result in mineral imbalances that can negatively affect the patient. Successful surgical repair of the mitral valve can resolve clinical signs and the need for drug therapy as well as improve prognosis and survival time (Pennington et al., 2022; Uechi et al., 2012). However, surgical repair may not be appropriate for dogs with severe disease due to increased risk of complications. Finally, nutritional support can be implemented in early stages and adjusted throughout disease progression. Dogs with cardiovascular disease should be fed a food that is highly palatable and contains adequate calories and high-quality protein with balanced amino acids to support body condition, especially patients with cachexia. Balanced sodium, magnesium, and potassium can support cardiac function and balance electrolyte

concentrations. Supplementation of EPA, DHA, L-carnitine, and taurine can support energy production for the heart and protect against oxidative stress. Further research is needed to better understand optimal levels of these nutrients to support dogs diagnosed with MMVD. Multimodal treatment plans play an important role in the management of MMVD to support overall quality of life and disease progression in dogs.

Table 1.1. Heart murmur grading scheme of cardiac auscultation and palpation features adapted from Ettinger & Suter (1970).

Grade	Features of cardiac auscultation and palpation
1/6	A nearly imperceptible murmur
2/6	A very soft murmur
3/6	A murmur of low intensity
4/6	A murmur of moderate intensity which does not produce a precordial thrill
5/6	A loud murmur heard with a stethoscope barely touching the thorax
6/6	The loudest murmur, heard when the stethoscope is held just away from the thorax

Chapter 2 - Effects of two cardiac foods on food intake and body composition in dogs with myxomatous mitral valve disease and healthy dogs

Madison D. Amundson, Laura A. Motsinger, Leslie Hancock

Introduction

Myxomatous mitral valve disease (MMVD) is the most prevalent cardiovascular disease in the domestic dog, commonly reported in small to medium-sized breeds, and is usually present with a heart murmur (Keene et al., 2019; Svensson et al., 2024). Myxomatous mitral valve disease is caused by the degradation of the mitral valve, which acts as a one-way apparatus between the left atrium and left ventricle. The dysfunction of the mitral valve leads to the backflow of blood into the left atrium from the left ventricle during contraction and increases total blood volume in the left atrium, left ventricle and pulmonary veins. The increase in blood volume triggers the myocardial cells to undergo eccentric hypertrophy, resulting in the dilation of the left atrium and left ventricle. Eventually, MMVD leads to left-sided congestive heart failure (Keene et al., 2019). Like other chronic diseases, MMVD is associated with other clinical signs that can indicate changes in quality of life such as decreases in body weight, lean muscle, and appetite. Owners associate declines in clinical signs as indicators of poor quality of life and often contribute to the election of humane euthanasia in dogs (Freeman, 2012; Mallery et al., 1999).

Cachexia, the involuntary loss of lean body mass, is associated with chronic diseases that increase inflammatory cytokines and reduce the ability to utilize fat and instead catabolize muscle and lean mass (Lowry & Coyle, 2006; von Haehling et al., 2009), which tends to increase

morbidity and affect quality of life in cardiac patients (Freeman, 2012). Cardiac cachexia is well recognized and studied, but it is easier to prevent than treat (Freeman, 2009; Freeman, 2012) and may be a result of enhanced catabolic processes associated with chronic heart failure (Fonfara et al., 2011). Inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1β (IL-1β), and interleukin-6 (IL-6), interact with metabolic pathways like nuclear factor kappa B (NF-κB) resulting in an altered protein flux, such as increased protein catabolism and decreased protein synthesis (Freeman, 2012). Therefore, the reduction of inflammatory cytokines supports patients with muscle wasting and helps manage the underlying disease. Finally, many patients with cachexia also experience anorexia, a decrease or loss in appetite. Anorexia has been recorded to occur in over 80% of dogs with congestive heart failure and was found to be a frequent contributing factor for owners to elect euthanasia in dogs with congestive heart failure (Mallery et al., 1999). As such, offering a highly palatable food designed to provide proper nutrition is critical to support cardiovascular health and overall quality of life in dogs.

Key nutrients such as sodium, potassium, other electrolytes, carnitine, taurine, and omega-3 fatty acids, specifically eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are essential for supporting cardiovascular health in dogs. Additionally, a food with high quality protein and balanced amino acids for muscle synthesis, adequate calories for energy, and increased EPA and DHA to manage cachexia and decrease inflammatory cytokines are needed to support cardiac patients (Freeman et al., 1998). Nonetheless, offering a highly palatable food to entice appetite is essential to ensure cardiac dogs consume enough food to meet their daily nutritional needs and maintain ideal body and muscle condition.

After a dog has been diagnosed with MMVD, a variety of management options are evaluated, including pharmaceutical, surgical, and nutritional options. The disease management

plan of an individual dog can vary depending on the specific MMVD stage and any additional health conditions and/or factors (Keene et al., 2019). Regardless, nutrition is an important aspect of a multimodal management plan. However, ideal nutrient levels for cardiovascular health have not been well-defined for dogs with MMVD. The hypothesis was that dogs with MMVD and healthy dogs fed the test or control food would maintain caloric intake, ideal body weight, body condition score, and muscle condition score. Therefore, the objective of this study was to investigate the effects of feeding two cardiac foods with varying levels of key nutrients on caloric intake, body weight, and body and muscle condition scores in dogs diagnosed with MMVD and compared to healthy dogs.

Methods

Ethics

This study was approved by Hill's Pet Nutrition's Institutional Animal Care and Use Committee and Animal Welfare Committee (CP1098, 08 May 2024).

Study Design and Animals

A two-by-two factorial design was used. A 6-month feeding study included dogs at Hill's Pet Nutrition Center that were diagnosed with a left-sided heart murmur (MMVD dogs) and control dogs without a heart murmur diagnosis (healthy dogs). Healthy dogs were selected to match the sex and age of the MMVD dogs as best as possible. All dogs were fed a washout food for 5 weeks. Prior to the washout period, MMVD dogs were sorted by heart murmur severity only and randomized to either the test food or control food and healthy dogs were completely randomized to either the test food or control food. Dogs with MMVD who were stratified and randomized to the test food and control food are described as the MMVD-TEST group and the MMVD-CONTROL group, respectively. Healthy dogs who were randomized to the test food or

control food are described as the HEALTHY-TEST group and the HEALTHY-CONTROL group, respectively. Throughout the 6-month feeding duration, caloric intake and body weight (BW) were recorded daily and weekly, respectively. Body condition score (BCS; 1 = very thin, 5 = obese), muscle condition score (MCS; 1 = normal, 4 = severe muscle loss) (World Small Animal Veterinary Association Global Nutrition Committee, 2013b), and fecal score (1 = liquid, 5 = firm) were collected monthly. Additionally, fecal score was collected during the transition on to the assigned study food after day 0. One trained evaluator assessed body condition score and muscle condition score in all dogs for the study. Physical exams by a veterinarian were completed on day 0, day 84, and day 168.

Dogs from the Hill's Pet Nutrition Center were enrolled in this study. Dogs were group-housed and were provided regular exercise with access to indoor and outdoor areas. This study did not require any restriction to their normal routines or socialization with other dogs or the caretakers. Dogs were fed solely their assigned study food in individual feeding areas and were fed to maintain ideal body weight. Water was provided ad libitum. After dogs completed the study, they remained at the Hill's Pet Nutrition Center and received continued veterinary care and enrichment.

Dogs were not enrolled if they were in a sodium depletion state, diagnosed with hyperlipidemia, or had a risk of pancreatitis. Dogs could be released from the study if 1) excessive body weight was lost based on veterinarian discretion or 2) the dog became anorexic for 3 or more days. Adverse events were diagnosed, treated, and documented if they occurred and could be cause for dismissal based on veterinarian discretion. Additionally, if medications were needed during the study, medication name and dosage along with any changes throughout the study were documented and dogs could stay enrolled upon veterinarian discretion (Appendix

C). Medications intended to treat symptoms of cardiovascular disease were not a reason for exclusion.

Study Foods

All three foods were offered in a dry form only and formulated for adult maintenance. The washout food was formulated to provide nutritional support aging dogs since MMVD is commonly diagnosed in older dogs. Both the test and control foods were formulated to provide nutritional support to dogs with cardiovascular disease with differing levels in key nutrients for cardiovascular health. The control food was considered a positive control. The test food was formulated with increased levels of protein, fat, L-carnitine, taurine, EPA, DHA, and sodium compared to the control food. On an as-is caloric basis, the analyzed nutritional composition of the washout food for protein, fat, L-carnitine, EPA+DHA and sodium were 46.2 g/1000 kcal ME, 30.2 g/1000 kcal ME, 1.6 mg/1000 kcal ME, <0.04 g/1000 kcal ME, and 0.6 g/1000 kcal ME, respectively. The analyzed nutrition composition for the control food for protein, fat, L-carnitine, EPA+DHA, and sodium were 44.9 g/1000 kcal ME, 48.9 g/1000 kcal ME, 85 mg/1000 kcal ME, <0.04 g/1000 kcal ME, and 0.3 g/1000 kcal ME, respectively. The analyzed nutritional composition of the test food for protein, fat, L-carnitine, EPA+DHA, and sodium were 52.2 g/1000 kcal ME, 51.8 g/1000 kcal ME, 118 mg/1000 kcal ME, 1.7 g/1000 kcal ME, and 0.4 g/1000 kcal ME, respectively. Analyzed dietary taurine was similar across the washout, control, and test foods (0.33 g/1000 kcal ME, 0.32 g/1000 kcal ME, and 0.34 g/1000 kcal ME, respectively). Additional differences in the study foods are provided in Appendix A.

Statistical Analysis

Enrollment BW, age, and heart murmur severity data were analyzed using a linear mixed model. Enrollment data analyses were performed using PROC MIXED in SAS® (version 9.4;

SAS Institute Inc., Cary, NC, USA), and a Tukey adjustment was used in determining significant treatment differences by separation of least square means. Caloric intake, BW, BCS, MCS, and fecal score data were analyzed using a linear mixed model with treatment, day, disease, and the interactions as fixed effects and animal as a random effect. To compare the post baseline time points to baseline, the SLICEDIFF=ADJUST=DUNNETT option for the least square means was used. A $P \leq 0.05$ was considered significant and a $P > 0.05$ and $P \leq 0.10$ was considered a trend. Analyses were performed using PROC GLIMMIX in SAS® (version 9.4; SAS Institute Inc., Cary, NC, USA).

Results

Population

A total of 20 dogs were enrolled into this study, 10 dogs with MMVD and 10 healthy dogs. At enrollment (start of washout period), the mean (SE) age for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 12.4 (1.1) years, 12.0 (1.0) years, 11.2 (0.7) years, and 11.2 (0.7) years, respectively. Mean (SE) BW at enrollment for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 7.7 (0.7) kg, 9.5 (0.7) kg, 8.3 (0.5) kg, and 9.7 (0.5) kg, respectively. In the MMVD dogs, mean (SE) heart murmur severity was 3.8 (0.4) and 3.9 (0.4) on a 6-point scheme for the MMVD-TEST group and MMVD-CONTROL group, respectively, at enrollment. There was no difference ($P > 0.10$) in BW or age between the MMVD-TEST group and MMVD-CONTROL group or HEALTHY-TEST group and HEALTHY-CONTROL group. Finally, there was no difference ($P > 0.10$) in heart murmur severity between the MMVD-TEST group and MMVD-CONTROL group. Each of the four groups contained two males and three females. All dogs enrolled in this study were Beagles,

except two dogs in the MMVD-CONTROL group who were mixed breeds. Signalment information is available in Appendix B.

During the study, there were four MMVD dogs removed from the study, three from the control group and one from the test group. Of the MMVD dogs in the MMVD-CONTROL group, one was removed due to poor quality of life associated with inflammatory bowel disease on day 77, one was removed after day 28 due to weight loss associated with kidney disease, and one was removed after day 54 due to declining mobility issues, weight loss, and decreased appetite, which was deemed unrelated to MMVD. The one MMVD dog in the MMVD-TEST group was removed from the study due to neurological issues due to possible stroke, emboli, or ruptured cardiac valve on day -12 of the washout period. Additionally, one healthy dog was removed from the HEALTHY-TEST group due to renal failure and decreased appetite on day 41. Dogs were included in the statistical analysis up until the point of removal, except for the dog removed during the washout period, who was removed from all analyses except signalment analyses at enrollment.

Caloric Intake and Fecal Score

There was a significant diet by day by disease interaction ($P < 0.01$), a significant day by disease interaction ($P = 0.02$), and there tended to be a diet by disease interaction ($P = 0.06$) for caloric intake (kcal/metabolic body weight (MBW)/day). However, there was no diet by day interaction ($P > 0.10$) for caloric intake (Table 2.1). At day 0, mean (SE) monthly caloric intake for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 112.7 (10.5) kcal/MBW/day, 113.4 (9.4) kcal/MBW/day, 90.5 (9.4) kcal/MBW/day, and 103.0 (9.4) kcal/MBW/day, respectively. The MMVD-TEST group, HEALTHY-TEST group, and HEALTHY-CONTROL group maintained ($P > 0.10$)

caloric intake throughout the 6-month study. However, the MMVD-CONTROL group had decreased ($P < 0.01$) caloric intake on days 56, 84, and 112 compared to day 0. The MMVD-CONTROL group had lower ($P \leq 0.05$) caloric intake at day 56, day 84, and day 112 compared to the MMVD-TEST group, but there was no difference ($P > 0.10$) between the two MMVD treatment groups at day 0, day 28, day 140, or day 168. There was no difference ($P > 0.10$) in caloric intake between the HEALTHY-TEST group and the HEALTHY-CONTROL group throughout the study, except on day 84 where the HEALTHY-CONTROL group tended to have higher ($P = 0.07$) caloric intake compared to the HEALTHY-TEST group. Additionally, there was no difference ($P > 0.10$) in caloric intake between the MMVD dogs and healthy dogs fed the test food throughout the study, except on day 84 where the MMVD-TEST group tended to have higher ($P = 0.08$) caloric intake compared to the HEALTHY-TEST group. The MMVD-CONTROL group had lower ($P \leq 0.05$) caloric intake at day 56, day 84, and day 112 compared to the HEALTHY-CONTROL group, but there was no difference ($P > 0.10$) between the two treatment groups at any other timepoint.

No significant interaction between diet and disease, diet and day, disease and day, or diet, day, and disease ($P > 0.10$) was observed for fecal score (Table 2.1). At day 0, mean (SE) fecal scores for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 4.0 (0.3), 3.8 (0.3), 4.7 (0.3), and 4.8 (0.3), respectively. Dogs in all four groups maintained ($P > 0.10$) a normal, consistent fecal score throughout the transition period onto the study foods and the 6-month feeding period. There was no difference ($P > 0.10$) in fecal score between the two MMVD treatment groups or between the two healthy treatment groups at day 0, day 84, or day 168. Additionally, there was no difference ($P > 0.10$) in fecal score between the MMVD dogs and healthy dogs fed the test food. The MMVD-

CONTROL group had a lower ($P = 0.02$) fecal score at day 0 and tended to have a lower ($P = 0.09$) fecal score at day 3 compared to the HEALTHY-CONTROL group, but there was no difference ($P > 0.10$) between the two groups at any other timepoint.

Body Weight, Body Condition Score, and Muscle Condition Score

There was a significant diet by day by disease interaction ($P = 0.05$) for BW, but no other interactions were significant ($P > 0.10$) (Table 2.1). Mean (SE) BW at the end of the washout period for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 8.3 (0.7) kg, 9.6 (0.6) kg, 8.1 (0.6) kg, and 9.7 (0.6) kg, respectively. Dogs in all four groups maintained ($P > 0.10$) their BW throughout the 6-month feeding period. There was no difference ($P > 0.10$) in BW between the MMVD-TEST group and the MMVD-CONTROL group at any of the study timepoints. The HEALTHY-CONTROL group tended to have a higher BW at day 0 ($P = 0.09$) and day 168 ($P = 0.08$) compared to the HEALTHY-TEST group, but there was no difference ($P > 0.10$) between the two healthy treatment groups at any other timepoint. There was no difference ($P > 0.10$) in BW between the MMVD dogs and healthy dogs fed the test food or between the MMVD dogs and healthy dogs fed the control food at any of the timepoints.

No significant interaction between diet and disease, diet and day, disease and day, or diet, day, and disease ($P > 0.10$) was observed for BCS (Table 2.1). Mean (SE) BCS at the end of the washout period for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 3.0 (0.1), 3.2 (0.1), 2.8 (0.1), and 3.2 (0.1), respectively. Dogs in the MMVD-TEST group, MMVD-CONTROL group, and HEALTHY-CONTROL group maintained ($P > 0.10$) their BCS throughout the 6-month feeding period. The HEALTHY-TEST group also maintained ($P > 0.10$) their BCS, except on day 140 where they

experienced an increase ($P = 0.02$) compared to day 0. There was no difference ($P > 0.10$) in BCS between the MMVD-TEST group and MMVD-CONTROL group throughout the study, except on day 56 where the MMVD-CONTROL group tended to have a higher ($P = 0.08$) BCS compared to the MMVD-TEST group. Additionally, there was no difference ($P > 0.10$) in BCS between the HEALTHY-TEST group and HEALTHY-CONTROL group throughout the study, except on day 0 where the HEALTHY-CONTROL group had a higher ($P < 0.01$) BCS compared to the HEALTHY-TEST group. Furthermore, there was no difference ($P > 0.10$) in BCS between the MMVD dogs and healthy dogs fed the test food or between the MMVD dogs and healthy dogs fed the control food throughout the study, except on day 56 where the MMVD-CONTROL group tended to have a higher ($P = 0.06$) BCS compared to the HEALTHY-CONTROL group.

No significant interaction between diet and disease, diet and day, disease and day, or diet, day, and disease ($P > 0.10$) was observed for MCS (Table 2.1). Mean (SE) MCS at the end of the washout period for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 2.0 (0.4), 2.4 (0.4), 1.8 (0.4), and 1.6 (0.4), respectively. Dogs in the MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group maintained ($P > 0.10$) their MCS throughout the 6-month feeding period. The MMVD-TEST group had an increased ($P \leq 0.05$) MCS on days 84 and 168 and a tendency for an increased ($P = 0.09$) MCS on day 112 compared to day 0. There was no difference ($P > 0.10$) in MCS between the two MMVD treatment groups or between the two healthy treatment groups at any of the timepoints. Additionally, there was no difference ($P > 0.10$) in MCS between the MMVD dogs and healthy dogs fed the control food throughout the study, but the MMVD-TEST group had an increased ($P = 0.04$) MCS at day 82 and tended to have an increased ($P < 0.10$) MCS at day 112 and day 168 compared to the HEALTHY-TEST group.

Discussion

Myxomatous mitral valve disease is the most common cardiovascular disease in dogs and disease management options include pharmaceutical, surgical, and nutritional intervention. Nutrition to support cardiovascular health in dogs is well understood, but optimal levels are ambiguous, especially in dogs diagnosed with MMVD and to support dogs experiencing muscle loss. Therefore, the objective of this pilot study was to investigate the effect of feeding two foods designed to support cardiac health and body and muscle condition with varying levels of key nutrients in dogs diagnosed with MMVD.

In the present study, the MMVD-TEST group maintained caloric intake throughout the 6-month study, which is favorable because owners perceive a reduction of appetite as an indicator of poor quality of life (Freeman, 2012). If dogs consume their recommended amount, they are able to meet their daily nutritional needs, consume enough calories to support their body composition and energy levels, and receive the full benefits of consuming a nutrition designed to support cardiovascular health when offered a complete, balanced, and highly palatable food. The MMVD-TEST group maintained their BW and BCS, but experienced increased muscle wasting at various timepoints throughout the study. On the other hand, the MMVD-CONTROL group had decreased caloric intake at various timepoints throughout the study, but were able to maintain BCS and MCS. Additionally, the MMVD-CONTROL group experienced a decrease in body weight, which could be related to the reduction in calories and nutrients throughout the study, but this was not statically significant.

The association between body composition and survival time has been investigated in humans with cardiovascular disease. Studies have shown that humans with heart failure who had a higher body mass index or weight experienced increased survival rates (Davos et al., 2003;

Fonarow et al., 2007; Horwich et al., 2001; Lavie et al., 2003). Additionally, Rozentryt et al. (2010) showed that humans who received a high-calorie, high-protein nutritional supplement experienced benefits associated with body composition and quality of life. However, research focusing on calorie and protein provision in dogs with cardiovascular disease on body composition and survival is limited. One study, that reviewed medical records of dogs with cardiovascular disease, showed that dogs who gained weight experienced longer survival times compared to dogs who maintained or lost weight (Slupe et al., 2008). While increases in body weight and composition have been associated with improved quality of life in both human and canine cardiac patients, lean body mass is an area of utmost importance. Fat is used as an energy source to conserve lean muscle in healthy animals during times of reduced food intake. However, the opposite is observed in diseased animals where lean body mass becomes the primary energy source (Freeman, 2012). Body condition and muscle condition, which assess primarily fat and muscle, respectively, are not directly correlated (Freeman et al., 2019). Freeman et al. (2019) showed that 59% of overweight dogs also experienced muscle loss, which highlights the importance of evaluating both fat and muscle composition in all patients at every visit.

The analyzed nutritional composition of all three foods exceeded the Association of American Feed Control Officials (AAFCO) minimum recommendation for essential amino acids (Association of American Feed Control Officials, 2025). The increase in muscle wasting observed in the MMVD-TEST group from the present study suggest that amino acid requirements specifically for lean muscle maintenance in dogs with cardiovascular disease should be investigated to understand if they differ from that of healthy dogs or dogs with other chronic conditions. Existing research has shown that the National Research Council (NRC) and AAFCO recommendations for essential amino acids, such as threonine, tryptophan, and

methionine, for example, are too low for adult dogs and can vary based on breed size (Mansilla, Fortener, et al., 2020; Mansilla et al., 2018; Mansilla, Templeman, et al., 2020; Sutherland et al., 2020; Templeman et al., 2019). The results of the present study and evidence in the literature highlights the importance of reevaluating amino acid requirements and focusing on amino acid balance over total protein content, especially in dogs with chronic diseases like MMVD.

Freeman et al. (1998) conducted a study in dogs with stable chronic heart failure to observe the effects of fish oil supplements to reduce cytokines and improve clinical outcomes, such as cachexia. Dogs supplemented with fish oil (27 mg/kg/day EPA and 18 mg/kg/day DHA) experienced decreased interleukin-1β and improved cachexia scores after eight weeks compared to dogs given a placebo (Freeman et al., 1998). In the present study, the MMVD-TEST group consumed an average of 71 mg/kg/day EPA and 46 mg/kg/day DHA (Appendix D), but muscle condition decreased in the MMVD-TEST group throughout the study. In the study by Freeman et al. (1998), cachexia scores were performed by one evaluator on a point scale (0=no cachexia; 4=severe cachexia) using visual examination and palpation of various muscle groups. Similarly, the present study used the muscle condition score (1=normal; 4=severe muscle loss) to assess muscle loss. The muscle condition score is a subjective assessment of lean muscle loss that varies in reproducibility (Freeman et al., 2019), and is performed by visual observation and palpations which can vary depending on the evaluator. Muscle condition score was performed by one, trained evaluator on all dogs throughout the present study. A more objective and accurate technique of lean muscle loss is dual-energy x-ray absorptiometry (DEXA) evaluations. Researchers have used DEXA machines to perform whole-body scans to collect total mass and calculate lean body mass, fat mass, bone mineral content, and bone mineral density of individuals (McGrath et al., 2024). However, DEXA scans were not performed during the

present study to assess lean muscle since companion animals need to be anesthetized for DEXA scans and there is an increased anesthetic risk in older animals and those with health conditions (Freeman et al., 2019; Hughes, 2008). Finally, the increase in muscle wasting may be due to animal-to-animal variation or the limitation of a small sample size per treatment group. The results of the present study suggest that further investigation on the prevention of muscle wasting in dogs with cardiovascular disease is needed with a larger, more diverse population.

The healthy dogs were selected to match the age and sex of the MMVD dogs enrolled. Since these dogs were older and likely to experience some age-related issues, they were considered healthy based on the absence of a heart murmur and cardiac impairment. The inclusion of the healthy cohort provides additional information about the palatability and impact on body composition of the two study foods during long-term feeding. Overall, both the HEALTHY-TEST group and the HEALTHY-CONTROL group maintained caloric intake, BW, BCS, and MCS throughout the study. In a similar study, dogs with MMVD and healthy dogs were enrolled to assess the effects of a cardiac test food versus a control food (Li et al., 2019). At baseline, there was no difference in BW or BCS between the test and control groups for both dogs with MMVD and healthy dogs. Healthy dogs fed the test food had significantly lower BCS at the end of the 6-month study compared to healthy dogs fed the control food. However, the mean BCS of both groups were within the ideal range (Li et al., 2019). The results from the present study and other studies support the inclusion of healthy dogs to provide additional insight about the overall acceptance and support of the study foods. To the authors' knowledge, there is limited data comparing the impact of nutrition on muscle mass in dogs with and without MMVD, which further emphasizes the importance of understanding muscle loss in dogs with MMVD.

There are several limitations of this pilot study. First, the study was underpowered due to the small sample size per treatment group for both the MMVD dogs and healthy dogs. The small sample size was due to the limited availability of dogs diagnosed with a heart murmur. A low number of healthy dogs were also enrolled since the healthy cohort was designed to match the MMVD dogs. Additionally, three MMVD dogs were removed from the MMVD-CONTROL group, one MMVD was removed from the MMVD-TEST group, and one healthy dog was removed from the HEALTHY-TEST group due to concurrent conditions. Following completion of the study, an upper confidence interval was calculated for a clinically meaningful change in MCS to evaluate the appropriate number of dogs needed per treatment group. The residual error from the linear mixed-model was 0.65, resulting in an estimated population standard deviation of 0.81. An increase in MCS < 1.0 was considered to be clinically not significant. Using an alpha = 0.10, a standard deviation of 0.81 and a CI half-width of 1.0, it was determined that seven animals per treatment group is sufficient to detect a change of 1.0. If the upper limit for the CI for change in MCS is less than this amount, then no clinically meaningful change in MCS will be concluded.

Furthermore, the 6-month feeding duration was likely not long enough to understand long-term effects of feeding the study foods, especially with a progressive disease like MMVD. While a study longer than 6 months would provide more insightful information to the effects of the study foods, multiple dogs were removed before the end of the study. This highlights the difficulty of working with senior dogs who experience age-related conditions. Next, a majority of the dogs enrolled in this study were Beagles. Myxomatous mitral valve disease is known to be most prevalent in small breed dogs. Therefore, it would be beneficial to investigate the effects of the study foods on breeds more commonly associated with MMVD, such as Cavalier King

Charles Spaniels as opposed to Beagles. Finally, all of the dogs enrolled in the study were Stage B1 except one dog who was Stage B2, both which are early stages of MMVD, and none of the dogs progressed to the next stage during the study. Dogs experience changes in appetite and body composition as MMVD progresses. Therefore, enrolling a population of dogs with more diverse and progressive stages or a longer study duration to observe disease progression would be impactful to understand the effects of the study food on endpoints associated with patient quality of life.

This pilot study demonstrated that the test food and control food, both designed to support cardiovascular health, resulted in slightly different results of caloric intake and muscle condition score in dogs diagnosed with MMVD. Dogs with MMVD fed the test food maintained caloric intake, body weight, and body condition throughout the study, but did not maintain muscle condition score. However, further research in a larger and more diverse population of dogs with MMVD is needed to understand the impact of the study foods on muscle condition score. A longer study duration with a larger and more diverse population of dogs diagnosed with MMVD is needed to validate the results of the present study. Additionally, next steps of this research include investigating amino acid requirements for dogs with cardiovascular disease.

Funding

This study was funded by Hill's Pet Nutrition.

Disclosures

MDA, LAM, and LH are current employees of Hill's Pet Nutrition.

Table 2.1. Effect of diet, day, disease, and all relevant interactions on caloric intake, body weight, body condition score, muscle condition score, and fecal score of dogs with myxomatous mitral valve disease and healthy dogs after the washout period (day 0) and while fed the test food or control food (day 28, day 56, day 84, day 112, day 140, and day 168). Dogs with myxomatous mitral valve disease fed the test food or control food are labeled as the MMVD-TEST group and MMVD-CONTROL group, respectively. Healthy dogs fed the test food or control food are labeled as the HEALTHY-TEST group and HEALTHY-CONTROL group, respectively.

											P-value						
Endpoint	**Day**	n	MMVD-TEST§	n	MMVD-CONTROL§	n	HEALTHY-TEST§	n	HEALTHY-CONTROL§	**SEM‡**	Diet	Disease	Day	Diet x Disease	Diet x Day	Disease x Day	Diet x Day x Disease
Caloric Intake (kcal / MBW / day)	0	4	112.7	5	113.4	5	90.5	5	103.4	10.5	0.80	1.00	0.64	0.06	0.43	0.02	<0.01
	28	4	111.6	5	102.6	5	102.3	5	105.1	10.5							
	56	4	114.4	3	84.6	4	96.4	5	113.2	10.5							
	84	4	118.6	2	76.2	4	92.4	5	117.9	10.5							
	112	4	113.0	2	81.3	4	96.0	5	115.5	11.5							
	140	4	106.1	2	91.8	4	93.4	5	109.7	11.5							

	168	4	105.8	2	96.4	4	91.0	5	101.8	11.5							
Body Weight (kg)	0	4	8.3	5	9.6	5	8.1	5	9.7	0.7	0.07	0.98	0.88	0.73	0.60	0.70	0.05
	28	4	8.3	5	9.6	5	8.1	5	9.5	0.7							
	56	4	8.3	3	9.7	4	8.3	5	9.6	0.7							
	84	4	8.4	2	9.4	4	8.2	5	9.7	0.7							
	112	4	8.5	2	9.4	4	8.2	5	9.8	0.7							
	140	4	8.5	2	9.3	4	8.3	5	9.8	0.7							
	168	4	8.5	2	9.5	4	8.1	5	9.8	0.7							
Body Condition Score	0	4	3.0	5	3.2	5	2.8	5	3.2	0.1	0.23	0.51	0.90	0.42	0.25	0.73	0.62
	28	4	3.0	5	3.2	5	3.0	5	3.0	0.1							
	56	4	3.0	3	3.2	4	3.0	5	3.0	0.1							

	84	4	3.0	2	3.0	4	3.0	5	3.0	0.2							
	112	4	3.0	2	3.0	4	3.0	5	3.0	0.2							
	140	4	3.0	2	3.0	4	3.3	5	3.0	0.2							
	168	4	3.0	2	3.0	4	3.0	5	3.0	0.2							
Muscle Condition Score°	0	4	2.0	5	2.4	5	1.8	5	1.6	0.4	0.82	0.12	0.35	0.68	0.64	0.25	0.22
	28	4	2.3	5	2.4	5	1.8	5	1.6	0.4							
	56	4	2.3	3	2.4	4	1.5	5	1.6	0.4							
	84	4	2.8	2	2.3	4	1.5	5	1.8	0.4							
	112	4	2.8	2	2.2	4	1.8	5	2.0	0.5							
	140	4	2.5	2	2.2	4	2.0	5	2.2	0.5							
	168	4	3.0	2	2.1	4	2.0	5	2.2	0.5							

Fecal Score¤	0	4	4.0	5	3.8	5	4.7	5	4.8	0.3	0.77	0.12	0.07	0.58	0.97	0.74	0.52
	3	4	4.5	5	3.8	5	4.5	5	4.5	0.3							
	4	4	4.5	5	4.5	5	4.5	5	4.8	0.3							
	5	4	4.5	5	4.2	5	4.8	5	4.8	0.3							
	10	4	4.5	5	4.4	5	4.5	5	4.5	0.3							
	28	4	4.8	5	4.1	5	4.2	5	4.8	0.3							
	56	4	4.8	3	4.6	4	5.0	5	4.6	0.3							
	84	4	4.5	2	4.0	4	4.2	5	4.6	0.5							
	112	4	4.5	2	5.0	4	5.0	5	4.8	0.5							
	140	4	4.8	2	5.0	4	5.0	5	4.6	0.5							

	168	4	4.0	2	4.5	4	4.0	5	4.1	0.5						

§Values are represented as means; ‡Values are represented as pooled standard error of mean; □1=Very thin, 3=Ideal, 5=Obese; °1=Normal muscle mass, 4=Severe muscle loss; ¤1=Liquid, 5=Firm.

Chapter 3 - Echocardiographic measurements of left-sided cardiac eccentric hypertrophy in dogs with myxomatous mitral valve disease

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Introduction

Myxomatous mitral valve disease (MMVD) is the most prevalent cardiovascular disease in the domestic dog, accounting for 75% of heart disease cases (Keene et al., 2019), and leads to mitral regurgitation and congestive heart failure in 30% of cases (Borgarelli & Haggstrom, 2010; Parker & Kilroy-Glynn, 2012). Myxomatous mitral valve disease is usually associated with the presence of a heart murmur and echocardiographic measurements and thoracic imaging are used to determine staging and to monitor progression of the disease.

Myxomatous mitral valve disease is a pathological condition characterized by abnormal thickening of the mitral valve leaflets. The mitral valve separates the left atrium and the left ventricle, acting as a one-way apparatus to allow blood to flow from the atrium to the ventricle as well as preventing backflow of blood into the left atrium when the ventricle contracts. The abnormal thickening of the leaflets leads to dysfunction of the valve apparatus and results in a backwards regurgitant jet of blood, manifesting a heart murmur which can be appreciated on thoracic auscultation (Coutinho & Antunes, 2021; Neto et al., 2018; O'Brien et al., 2021; Palmisano et al., 2021). When the regurgitant volume becomes significant, a drop in cardiac output is appreciated and leads to the upregulation of the renin-angiotensin-aldosterone system that ultimately increases total blood volume in the body (Hammond et al., 2023). The increase in blood volume triggers the myocardial cells to undergo eccentric hypertrophy, resulting in the dilation of the left atrium and left ventricle. The ability of the left side of the heart to dilate prevents intracardiac pressures from rising and prevents the development of congestive heart

failure (Keene et al., 2019). The current 2019 staging system is used to help diagnose and monitor the progression of MMVD and the development of congestive heart failure (Keene et al., 2019). Echocardiographic imaging is the most sensitive diagnostic used to assess left atrial and left ventricular dimensions to determine evidence of eccentric hypertrophy to differentiate dogs from substage B1 and B2 (Borgarelli et al., 2008). Echocardiographic measurements of the left atrium and ventricle predict disease prognosis, congestive heart failure risk, and appropriate medical interventions (Atkins et al., 2007; Borgarelli et al., 2012; Borgarelli et al., 2008; Boswood et al., 2016; Häggström et al., 2008; Hezzell et al., 2012; Lord et al., 2010; Moonarmart et al., 2010; Reynolds et al., 2012; Sargent et al., 2015; Strohm et al., 2018).

Since echocardiography is highly sensitive and specific, it is one of the most reliable ways to monitor disease prognosis (Keene et al., 2019). A complete echocardiogram evaluation utilizes several ultrasound modalities to assess cardiac size and function, but 2-Dimensional (2D) echocardiography provides accurate 2D measurements of chamber size and wall thickness, and it is one of the most important modalities used to estimate chamber volume in dogs (Kuo et al., 2024; Visser et al., 2019). Left atrial-to-aortic root ratio (LA:Ao) and anteroposterior left atrial diameter (LAD) are common measurements to assess eccentric hypertrophy in the left atrium. The LA:Ao is the most frequently used measurement and is a strong indicator of MMVD severity and progression (Borgarelli et al., 2008; Sánchez Salguero et al., 2018; Vezzosi et al., 2021). Due to the significant change in chamber size throughout the cardiac cycle, to properly assess left ventricular size and function, measurements are taken both at the end of the ventricular relaxation (diastole) and ventricular contraction (systole). To assess true ventricular size and to evaluate for left ventricular dilation, end-diastolic measurements, such as left ventricular internal diameter at end-diastole (LVIDd) and left ventricular end-diastolic volume

(LVEDV), are used. In contrast, the function of the left ventricle is assessed at end-systole and is measured as left ventricular internal diameter at end-systole (LVIDs) and left ventricular end-systolic volume (LVESV). Fractional shortening (FS) and left ventricular ejection fraction (LVEF) represent the percentage reduction in diameter and volume, respectively, from end-diastole to end-systole, and provides an estimation in systolic function. These measurements are evaluated together to determine the status of the heart size and function and disease severity.

The American College of Veterinary Internal Medicine (ACVIM) consensus panel advises that Stage B2 dogs who meet all heart enlargement criteria, a) ≥3/6 murmur intensity, b) ≥1.6 echocardiographic LA:Ao, c) ≥1.7 LVIDd normalized for body weight, and d) >10.5 breed-adjusted radiographic vertebral heart score, should begin medical intervention before the onset of congestive heart failure (Keene et al., 2019). The intervention of therapeutic nutrition is a key component of a multimodal plan to manage dogs with MMVD to ensure dogs are receiving optimal nutrition to meet their nutritional needs and support cardiovascular health. Key nutrients, such as L-carnitine, taurine, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), sodium, and other electrolytes, have been identified and studied to understand their impact on cardiovascular health in dogs (Abbrecht, 1972; Freeman et al., 2003; Freeman et al., 2006; Hand, 2010; Kittleson et al., 1997; Laflamme, 2022; Nasciutti et al., 2021; Rush et al., 2000; Smith et al., 2007). However, the optimal levels of these nutrients as a synergistic strategy require further investigation for the management of MMVD in dogs. The hypothesis was that dogs with MMVD fed the test food would show evidence of reduced disease progression as measured by smaller size of the left atrium and left ventricle compared to dogs fed the control food. Therefore, the objective of this study was to investigate a reduction in left-sided eccentric hypertrophy of the

heart if two foods designed to support cardiac health with varying levels of key nutrients were fed to dogs diagnosed with MMVD for 6 months.

Methods

Ethics

This study was approved by Hill's Pet Nutrition's Institutional Animal Care and Use Committee and Animal Welfare Committee (CP1098, 08 May 2024).

Study Design and Animals

A 6-month feeding study enrolled dogs at Hill's Pet Nutrition Center that were diagnosed with a left-sided heart murmur (MMVD dogs). All dogs were fed a washout food for 5 weeks. Prior to the washout period, MMVD dogs were sorted by heart murmur severity only and randomized to either the test food or control food. Dogs with MMVD who were stratified and randomized to the test food and control food are described as the MMVD-TEST group and the MMVD-CONTROL group, respectively. Healthy dogs were not included as the objective was to investigate the effects of the study foods on heart size in dogs with MMVD. Echocardiogram evaluations were performed using a cardiac ultrasound machine (Vivid-q Cardiovascular Ultrasound System, GE Healthcare, Chicago, Illinois, USA) equipped with a 6 MHz transducer on day 0, day 84, and day 168 by a single veterinarian whose practice is limited to cardiology. Dogs were not enrolled if they were in a sodium depletion state, diagnosed with hyperlipidemia, or had a history or risk of pancreatitis. Dogs could be released from the study if 1) excessive body weight was lost based on veterinarian discretion or 2) the dog became anorexic for 3 or more days. Adverse events were diagnosed, treated, and documented if occurred and could be cause for dismissal based on veterinarian discretion. Additionally, if medications were needed during the study, medication name and dosage along with any changes throughout the study were

documented and dogs could stay enrolled upon veterinarian discretion (Appendix C). Medications intended to treat symptoms of cardiovascular disease were not a reason for exclusion.

Dogs from the Hill's Pet Nutrition Center were enrolled in this study. Dogs were group-housed and were provided regular exercise with access to indoor and outdoor areas. This study did not require any restriction to their normal routines or socialization with other dogs or the caretakers. Dogs were fed solely their assigned study food in individual feeding areas and were fed to maintain ideal body weight. Water was provided ad libitum. After dogs completed the study, they remained at the Hill's Pet Nutrition Center and received continued veterinary care and enrichment.

Study Foods

All three foods were offered in a dry form only and formulated for adult maintenance. The washout food was formulated to nutritional support aging dogs since MMVD is commonly diagnosed in older dogs. Both the test and control foods were formulated to provide nutritional support to dogs with cardiovascular disease with differing levels in key nutrients for cardiovascular health. The control food was considered a positive control. The test food was formulated with increased levels of protein, fat, L-carnitine, taurine, EPA, DHA, and sodium compared to the control food. On an as-is caloric basis, the analyzed nutritional composition of the washout food for protein, fat, L-carnitine, EPA+DHA and sodium were 46.2 g/1000 kcal ME, 30.2 g/1000 kcal ME, 1.6 mg/1000 kcal ME, <0.04 g/1000 kcal, ME and 0.6 g/1000 kcal ME, respectively. The analyzed nutrition composition for the control food for protein, fat, L-carnitine, EPA+DHA, and sodium were 44.9 g/1000 kcal ME, 48.9 g/1000 kcal ME, 85 mg/1000 kcal ME, <0.04 g/1000 kcal ME, and 0.3 g/1000 kcal ME, respectively. The analyzed nutritional

composition of the test food for protein, fat, L-carnitine, EPA+DHA, and sodium were 52.2 g/1000 kcal ME, 51.8 g/1000 kcal ME, 118 mg/1000 kcal ME, 1.7 g/1000 kcal ME, and 0.4 g/1000 kcal ME, respectively. Analyzed dietary taurine was similar across the washout, control, and test foods (0.33 g/1000 kcal ME, 0.32 g/1000 kcal ME, and 0.34 g/1000 kcal ME, respectively). Additional differences in the study foods are provided in Appendix A.

Statistical Analysis

Dogs were sorted by heart murmur severity and randomized onto one of the two study foods to minimize differences in starting cardiac function. Enrollment body weight (BW), age, and heart murmur severity data were analyzed using a linear mixed model. Enrollment data analyses were performed using PROC MIXED in SAS® (version 9.4; SAS Institute Inc., Cary, NC, USA), and a Tukey adjustment was used in determining significant treatment differences by separation of least square means. Heart murmur severity, mitral regurgitation, and echocardiogram measurement data were analyzed using a linear mixed model with treatment, day, and the interaction as fixed effects and animal as a random effect. To compare the post baseline time points to baseline, the SLICEDIFF=ADJUST=DUNNETT option for the least square means was used. A $P \leq 0.05$ was considered significant and a $P > 0.05$ and $P \leq 0.10$ was considered a trend. Analyses were performed using PROC GLIMMIX in SAS® (version 9.4; SAS Institute Inc., Cary, NC, USA).

Results

Population

A total of 10 MMVD dogs were enrolled into this study. Mean (SE) age, BW, and heart murmur severity at enrollment (start of washout) were 7.7 (0.7) kg, 12.4 (1.1) years, and 3.8 (0.4) for the MMVD-TEST group and 9.5 (0.7) kg, 12.0 (1.0) years, and 3.9 (0.4) for the MMVD-

CONTROL group. There was no difference ($P > 0.10$) in BW, age, or heart murmur severity at enrollment between the two MMVD treatment groups. Both the MMVD-TEST group and MMVD-CONTROL group contained two males and three females. All dogs enrolled in this study were Beagles, except two dogs in the MMVD-CONTROL group who were mixed breeds. Signalment information is available in Appendix B.

During the study, there were four MMVD dogs removed from the study, three from the MMVD-CONTROL group and one from the MMVD-TEST group. Of the MMVD dogs in the MMVD-CONTROL group, one was removed due to poor quality of life associated with inflammatory bowel disease on day 77, one was removed after day 28 due to weight loss associated with kidney disease, and one was removed after day 54 due to declining mobility issues, weight loss, and decreased appetite, which was deemed unrelated to MMVD. The one MMVD dog in the MMVD-TEST group was removed from the study due to neurological issues due to possible stroke, emboli, or ruptured cardiac valve on day -12 of the washout period. Dogs were included in the statistical analysis up until the point of removal, except for the dog removed during the washout period, who was removed from all analyses except signalment analyses at enrollment.

Some dogs were on medications to support cardiovascular health during the study. Of the dogs in the MMVD-CONTROL group, one dog, who was removed from the study after day 54, started pimobendan (2.5 mg, twice a day) prior to enrollment and another dog, who completed the 6-month study, started pimobendan (2.5 mg, twice a day) prior to enrollment and furosemide (10 mg, once a day) on day 103 of the test period. One dog in the MMVD-TEST group started pimobendan (2.5 mg, twice a day) at day 103 of the test period.

Finally, some dogs required sedation for the echocardiogram evaluations. One dog in the MMVD-TEST group was sedated for all three echocardiogram evaluations. This dog was given 0.3 mg/kg of butorphanol followed by 1.5 mg/kg of propofol intravenously, 0.3 mg/kg of butorphanol followed by 1.9 mg/kg of propofol intravenously, and 0.3 mg/kg of butorphanol followed by 2.9 mg/kg of propofol intravenously at the day 0 echocardiogram, day 84 echocardiogram, and day 168 echocardiogram, respectively. One dog in the MMVD-CONTROL group, who was removed after day 54, was sedated by receiving 0.3 mg/kg of butorphanol intravenously for the day 0 echocardiogram evaluation. Additionally, another dog in the MMVD-TEST group was sedated for the day 168 echocardiogram evaluation, but not the day 0 or day 84 evaluation. This dog was given 0.3 mg/kg of butorphanol intravenously. Sedation was deemed necessary for the safety of this dog and handlers due to progressive resistance during echocardiogram evaluations.

Heart Murmur Severity, Mitral Regurgitation, and MMVD Staging

There was no significant diet by day interaction ($P > 0.10$) observed for heart murmur severity (Table 3.1). Mean (SE) heart murmur severity for dogs in the MMVD-TEST group and MMVD-CONTROL group at day 0 (end of washout) were 4.5 (0.5) and 3.6 (0.4), respectively, on a 6-point scheme (1=Barely audible, 6=Audible with stethoscope off the chest). Heart murmur severity was unchanged at day 168 (0.1, SE = 0.3, $P = 0.84$) in the MMVD-TEST group compared to day 0. In the MMVD-CONTROL group, heart murmur severity tended to increase by day 168 (0.9, SE = 0.4, $P = 0.06$) when compared to day 0 (Figure 3.1A). There was no difference ($P > 0.10$) in heart murmur severity between the two groups at day 0, day 84, or day 168.

There was no significant diet by day interaction ($P > 0.10$) observed for mitral regurgitation severity (Table 3.1). Mean (SE) mitral regurgitation severity in the MMVD-TEST group and MMVD-CONTROL group at day 0 were 1.8 (0.3) and 1.3 (0.3), respectively, on a point scale system (0=None, 3=Severe). Mitral regurgitation severity was unchanged at day 168 (-0.3, SE = 0.2, $P = 0.30$) in the MMVD-TEST group compared to day 0. In the MMVD-CONTROL group, mitral regurgitation severity was unchanged at day 168 ($P = 1.00$) when compared to day 0 (Figure 3.1B). There was no difference ($P > 0$ .10) in mitral regurgitation severity between the two groups at day 0, day 84, or day 168.

At day 0, all MMVD dogs were diagnosed with Stage B1 MMVD, except one dog in the MMVD-TEST group, who completed the 6-month feeding study, was diagnosed with Stage B2. These stages were maintained throughout the 6-month period.

Two-Dimensional (2D) Echocardiographic Measurements

There was no significant diet by day interaction ($P > 0.10$) observed for LA:Ao (Table 3.1). Mean (SE) LA:Ao at day 0 in the MMVD-TEST group and MMVD-CONTROL group were 1.3 (0.1) and 1.3 (0.1), respectively. Compared to day 0, LA:Ao was unchanged by day 168 ($P = 0.88$) in the MMVD-TEST group and in the MMVD-CONTROL group ($P = 1.00$) (Figure 3.2). Additionally, there was no difference ($P > 0.10$) in LA:Ao between the two groups at day 0, day 84, or day 168.

There was no significant diet by day interaction ($P > 0.10$) observed for anteroposterior left atrial diameter along the long axis (LAD) (Table 3.1). Mean (SE) LAD at day 0 for the MMVD-TEST group and MMVD-CONTROL group were 3.3 (0.2) cm and 3.3 (0.2) cm, respectively. Compared to day 0, LAD was unchanged by day 168 in the MMVD-TEST group (-0.2 cm, SE = 0.1, $P = 0.30$) and in the MMVD-CONTROL group (0.2 cm, SE = 0.2, $P = 0.54$)

(Figure 3.3A). There was no difference ($P > 0.10$) in LAD between the two groups at day 0, day 84, or day 168. Additionally, the percent change from baseline over the 6-month study in LAD in the MMVD-TEST group and MMVD-CONTROL group were -6% and 6%, respectively (Figure 3.3B).

There tended to be a diet by day interaction ($P = 0.06$) for left ventricular internal diameter at end-diastole (LVIDd) (Table 3.1). Mean (SE) LVIDd at day 0 for the MMVD-TEST group and MMVD-CONTROL group were 3.1 (0.2) cm and 3.1 (0.2) cm, respectively. Compared to day 0, LVIDd was unchanged by day 168 in the MMVD-TEST group (-0.3 cm, SE = 0.1, $P = 0.12$) and in the MMVD-CONTROL group (0.3 cm, SE = 0.2, $P = 0.18$) compared to day 0 (Figure 3.4A). There was no difference ($P > 0.10$) in LVIDd between the two groups at day 0 or day 84. However, LVIDd was larger ($P = 0.05$) in the MMVD-CONTROL group compared to the MMVD-TEST group at day 168. Additionally, the percent change from baseline over the 6-month study in LVIDd in the MMVD-TEST group and MMVD-CONTROL group were -9% and 11%, respectively (Figure 3.4B).

There was a significant diet by day interaction ($P < 0.01$) and a tendency ($P = 0.08$) for day to have an effect on left ventricular internal diameter at end-systole (LVIDs) (Table 3.1). Mean (SE) LVIDs at day 0 for the MMVD-TEST group and MMVD-CONTROL group were 1.8 (0.1) cm and 1.8 (0.1) cm, respectively. Compared to day 0, LVIDs was unchanged at day 168 (-0.1 cm, SE = 0.1, $P = 0.23$) in the MMVD-TEST group. In the MMVD-CONTROL group, LVIDs increased by day 84 (0.4 cm, SE = 0.1, $P = 0.01$) and day 168 (0.4 cm, SE = 0.1, $P = 0.01$) compared to day 0 (Figure 3.5A). There was no difference ($P > 0.10$) in LVIDs between the two groups at day 0. However, LVIDs was larger at day 84 ($P = 0.04$) and day 168 ($P = 0.04$) in the MMVD-CONTROL group compared to the MMVD-TEST group. Additionally, the

percent change from baseline over the 6-month study in LVIDs in the MMVD-TEST group and MMVD-CONTROL group were -6% and 20%, respectively (Figure 3.5B).

There was no significant diet by day interaction ($P > 0.10$) observed for left ventricular end-diastolic volume (LVEDV) (Table 3.1). Mean (SE) LVEDV for the MMVD-TEST group and MMVD-CONTROL group at day 0 were 28.8 (5.3) mL and 27.2 (4.7) mL, respectively. Left ventricular end-diastolic volume was unchanged at day 168 in the MMVD-TEST group (-3.0 mL, SE = 2.7, $P = 0.47$) and in the MMVD-CONTROL group (-3.4 mL, SE = 3.8, $P = 0.59$) compared to day 0 (Figure 3.6). There was no difference ($P > 0.10$) in LVEDV between the two groups at day 0, day 84, or day 168.

There was no significant diet by day interaction ($P > 0.10$) observed for left ventricular end-systolic volume (LVESV) (Table 3.1). Mean (SE) LVESV for the MMVD-TEST group and MMVD-CONTROL group at day 0 were 9.5 (1.5) mL and 11.9 (1.4) mL, respectively. Left ventricular end-systolic volume (LVESV) was unchanged by day 168 in the MMVD-TEST group (-0.6 mL, SE = 1.3, $P = 0.82$) and in the MMVD-CONTROL group (-1.8 mL, SE = 1.8, $P = 0.54$) compared to day 0 (Figure 3.7). There was no difference ($P > 0.10$) in LVESV between the two groups at day 0, day 84, or day 168.

There was no significant diet by day interaction ($P > 0.10$) observed for left ventricular ejection fraction (LVEF) (Table 3.1). Mean (SE) LVEF for the MMVD-TEST group and MMVD-CONTROL group at day 0 were 63.4% (3.8) and 56.2% (3.4), respectively. Left ventricular ejection fraction (LVEF) was unchanged at day 168 in the MMVD-TEST group (0.5%, SE = 2.4, $P = 0.97$) and in the MMVD-CONTROL group (3.2%, SE = 3.4, $P = 0.56$) compared to day 0 (Figure 3.8). There was no difference ($P > 0.10$) in LVEF between the two groups at day 0, day 84, or day 168.

Motion-mode (M-mode) Echocardiographic Measurements

There was a significant diet by day interaction ($P = 0.03$) for left ventricular internal diameter at end-diastole, normalized for body weight, (LVIDdN) (Table 3.1). At day 0, mean (SE) LVIDdN for the MMVD-TEST group and MMVD-CONTROL group were 1.6 (0.1) and 1.6 (0.1), respectively. Compared to day 0, LVIDdN decreased by day 168 (-0.2, SE = 0.1, $P = 0.03$) in the MMVD-TEST group. In the MMVD-CONTROL group, LVIDdN was unchanged at day 168 (0.1, SE = 0.1, $P = 0.16$) compared to day 0 (Figure 3.9A). There was no difference ($P > 0.10$) in LVIDdN between the two groups at day 0 or day 84. However, LVIDdN was larger ($P = 0.03$) in the MMVD-CONTROL group compared to the MMVD-TEST group at day 168. Additionally, the percent change from baseline over the 6-month study in LVIDdN in the MMVD-TEST group and MMVD-CONTROL group were -12% and 11%, respectively (Figure 3.9B).

Discussion

Myxomatous mitral valve disease is the most common cardiovascular disease in dogs and is usually diagnosed and monitored with echocardiography imaging. Nutrition plays an important role in multimodal treatment plans for dogs with MMVD. However, optimal levels of the key nutrients identified for heart health are ambiguous and vary depending on disease stage. Therefore, the objective of this study was to investigate the effect of feeding two foods designed to support cardiac health with varying levels of key nutrients on echocardiogram measurements in dogs diagnosed with MMVD. In the present study, the MMVD-TEST group showed evidence of smaller left ventricular size through echocardiogram measurements compared to the MMVD-CONTROL group.

The LA:Ao is the most frequently used method to evaluate enlargement of the left atrium by measuring left atrial diameter from the right parasternal short axis view at the beginning of diastole, when the mitral valve begins to open to allow blood flow from the left atrium to the left ventricle, and indexed to the aortic root diameter (Rishniw et al., 2019). This measurement is independent of body size and is a strong indicator of MMVD severity and progression (Borgarelli et al., 2008; Rishniw & Erb, 2000; Sánchez Salguero et al., 2018; Vezzosi et al., 2021). In the ACVIM consensus guidelines for dogs with MMVD, Keene et al. (2019) notes that LA:Ao greater than or equal to 1.6 is one of the Stage B2 criteria for heart enlargement. In the present study, both the MMVD-TEST group and MMVD-CONTROL group remained below the 1.6 threshold at all three timepoints and changes in LA:Ao were not significant. Anteroposterior LAD, which is obtained from the parasternal long-axis of the left atrium, is another measurement used to assess left atrial enlargement in addition to LA:Ao (Marchesotti et al., 2019; Rishniw & Erb, 2000; Strohm et al., 2018). Anteroposterior LAD showed higher intraobserver agreement over time than the LA:Ao ratio (Visser et al., 2019), making it a reliable metric for evaluating progressive left atrial enlargement. Compared to day 0, LAD increased by 6% in the MMVD-CONTROL group and decreased by 6% in the MMVD-TEST group over the 6-month study, but these changes were not statistically significant.

In similar studies, researchers have shown minimal effect on LA:Ao or LAD in MMVD dogs with a nutritional intervention (Li et al., 2019; Nasciutti et al., 2021; Pasławski et al., 2021). In two studies that focused on the effect of omega-3 fatty acids in MMVD dogs, Pasławski et al. (2021) and Nasciutti et al. (2021) observed no statistical difference in LA:Ao between the experimental or control groups or between particular timepoints within each of the groups. In another study that fed an experimental food containing various key nutrients for cardiac health to

dogs diagnosed with B1 and B2 MMVD over 6 months, Li et al. (2019) observed numerical decreases in LA:Ao and LAD in dogs fed the experimental food by the end of the study, but dogs fed the control food experienced a significant increase in LA:Ao and LAD throughout the study. The results of the present study along with existing data suggest that nutrition plays less of a role in reversing cardiac remodeling of the left atrium, but potentially a larger part in maintaining left atrial size. In addition to left atrial measurements, changes in left ventricular dimensions help with assessments of disease progression.

Left ventricular internal diameter can be measured at the end of diastole (LVIDd) and end of systole (LVIDs) using 2-D echocardiography (Visser et al., 2019). In the present study, there was a significant diet by day interaction for LVIDs and there tended to be a diet by day interaction for LVIDd. When compared to day 0, LVIDd and LVIDs increased by 11% and 20%, respectively, in the MMVD-CONTROL group, but decreased by 9% and 6%, respectively, in the MMVD-TEST group over 6 months. In a study that fed a low sodium food or moderate sodium food to dogs with chronic valvular disease, Rush et al. (2000) showed that dogs fed the low sodium food (0.04 g/100kcal) had significant decreases in LVIDd and LVIDs and dogs fed the moderate sodium food (0.07 g/100 kcal) had numerical increases in LVIDd and LVIDs after 28 days of feeding. In the present study, the sodium content of the test food (0.04 g/100 kcal) and the control food (0.03 g/100 kcal) were similar to the low sodium food in the study conducted by Rush et al. (2000). The recommendation for reduced sodium is intended to reduce blood volume and prevent the heart from being overworked, but aggressive sodium restriction can also stimulate water retention, by increasing the renin-angiotensin-aldosterone system activation, which can lead to increased blood volume and, ultimately, cause eccentric hypertrophy of the heart (Ames et al., 2019). Therefore, the amount of sodium restriction recommended varies

based on early-stage and late-stage heart disease (Keene et al., 2019). However, the results of the present study showed that although the control food had lower sodium content than the test food and the MMVD-CONTROL group consumed on average less sodium per kg of body weight per day than the MMVD-TEST group (Appendix D), increases in cardiac size still occurred in the MMVD-CONTROL group. Ultimately, these results contribute to the lack of evidence to support the benefits of feeding a low sodium food to dogs and suggest that other nutritional factors are playing an important role in changes of cardiac size.

Another hypothesis is that synergistic nutrition is the driving factor for favorable changes in cardiac size in dogs with MMVD. In a study that fed a cardiac test food, containing reduced levels of sodium, magnesium, and potassium, but increased levels of EPA, DHA, L-carnitine, and taurine, compared to a placebo food to dogs with chronic valvular disease, Freeman et al. (2006) observed numerical increases in LVIDd and LVIDs in the placebo group, but significant decreases in LVIDd and LVIDs in the test group after 4 weeks of feeding. Nevertheless, long-term feeding is needed to understand the prolonged impact of a nutritional intervention, especially with restricted sodium. The results of the present study and existing data suggest that synergistic nutrition is more impactful on eccentric hypertrophy of the left ventricle compared to the left atrium.

Linear 2D echocardiography is commonly used by veterinary echocardiographers worldwide, especially for left atrial size assessments (Kuo et al., 2024), but researchers have also demonstrated its importance for left ventricular assessments (Strohm et al., 2018; Visser et al., 2019). In addition to 2D echocardiography, M-mode is another common technique especially for left ventricular dimensions (Boon, 2011), but has limitations since it is one-dimensional and relies on geometric assumptions (Smets et al., 2014). However, diagnosis and monitoring of

MMVD relies on measurements obtained from M-mode echocardiography, particularly for LVIDdN (Cerbu et al., 2023). Left ventricular end-diastolic diameter, normalized for body weight, is another aspect of the Stage B2 criteria identified by Keene et al. (2019), such that a threshold greater than or equal to 1.7 is one of the four criteria for advancement to Stage B2. In the present study, there was no difference in LVIDdN between both groups at day 0. In the MMVD-CONTROL group, mean LVIDdN numerically increased, reaching the 1.7 threshold by day 168. On the other hand, LVIDdN significantly decreased by day 168 compared to day 0 in the MMVD-TEST group. At the end of the study, LVIDdN increased by 11% and decreased by 12% in the MMVD-CONTROL group and MMVD-TEST group, respectively, compared to day 0. Furthermore, LVIDdN was significantly larger in the MMVD-CONTROL group compared to the MMVD-TEST group at day 168. Conversely, in the study conducted by Freeman et al. (2006), the authors did not observe significant changes in weight-based left ventricular internal dimension in diastole within or between dogs fed the cardiac and placebo foods for four weeks. Thus, these results highlight the importance of long-term feeding of nutrition designed for cardiovascular health to observe clinical impact.

While dimensional changes provide detailed information about eccentric hypertrophy of the heart, left ventricular function, in conjunction with dimensional measurements, can provide further insight into the current state of the heart (Visser et al., 2019). Left ventricular ejection fraction (LVEF), calculated as the percentage of volume reduction between end-diastole and end-systole, is a common parameter to reflect left ventricular function (Gaudron et al., 1993; McGhie et al., 1991). Unfortunately, correlating LVESV and LVEF with left ventricular systolic function in MMVD patients can be challenging due to significant mitral regurgitation resulting in blood to be ejected into both the left atrium and the aorta, whereas in healthy patients, it is

directed only into the aorta. In both the MMVD-TEST group and MMVD-CONTROL group, LVEDV and LVESV numerically decreased and LVEF numerically increased throughout the study, but these findings were not statistically significant. Volumetric measurements are less commonly reported in nutrition-related studies in dogs with MMVD. However, the results of LVEDV and LVESV align with the dimensional changes observed in the left ventricle in the MMVD-TEST group of the present study. Since increased blood volume is associated with eccentric hypertrophy of MMVD, a decrease in LVEDV and LVESV would be positively associated with a decrease in LVIDd and LVIDs, respectively. This positive association was observed in the MMVD-TEST group of the present study, where LVEDV, LVESV, LVIDd, and LVIDs numerically decreased. However, a negative association was observed in the MMVD-CONTROL group, where LVEDV and LVESV numerically decreased, but LVIDd numerically increased and LVIDs significantly increased. The results of the MMVD-CONTROL group were unexpected, but mitral regurgitation severity was static for all dogs in the control group suggesting that mitral regurgitation did not affect assessment of myocardial function. It is important to note that mitral regurgitation was subjectively assessed in this study, and measuring mitral regurgitant volume objectively could have shown significance. It is hypothesized that since the control food was also designed to support cardiovascular health, the MMVD-CONTROL group experienced a reduction in blood volume, but the reduction in chamber size was not observed due to study length. Another hypothesis is that due to the small sample size and high drop-out rate of the control group, this result may be due to animal-to-animal variation.

Finally, LVEF is an assessment of systolic myocardial function and represents the percent of blood ejected from the left ventricle during contraction (Chetboul & Tissier, 2012). The reference interval for LVEF is 50-65% in normal dogs (Hezzell, 2018). In the present study,

LVEF was unchanged and remained within the 50-65% range for both groups, except on day 84 when LVEF was slightly above the range for the MMVD-TEST group but returned within the range by day 168. Since the dogs in the present study were diagnosed with early-stage MMVD, maintaining within the normal range was expected. In a study by Nasciutti et al. (2021), mean ejection fraction in Stage B2 and C MMVD dogs was 79.4% and 78.9% in the control and test group, respectively, at baseline and numerically decreased by the end of the 12-month study, but these changes were not significant. Various factors influence systolic function and given the slow progressive nature of MMVD and the ability for the heart to compensate for the backflow of blood in early-stages, changes in dimensional measurements could precede noticeable changes in pumping function or clinical signs.

There are several limitations of this pilot study. First, the sample size per treatment group was low due to the limited availability of dogs diagnosed with a heart murmur, and therefore, the study was underpowered. Additionally, three dogs were removed from the MMVD-CONTROL group and one dog was removed from the MMVD-TEST group due to concurrent conditions. Following completion of the study, an upper confidence interval was calculated for a clinically meaningful change in LA:Ao to evaluate the appropriate number of dogs needed per treatment group. The residual error from the linear mixed-model was 0.008, resulting in an estimated population standard deviation of 0.09. Based on the mean LA:Ao at day 0 for both MMVD treatment groups, an increase in LA:Ao < 0.3 cm was considered to be clinically not significant. Using an alpha = 0.10, a standard deviation of 0.09 and a CI half-width of 0.3, it was determined that three animals per treatment group is sufficient to detect a change of 0.3 cm. If the upper limit for the CI for change in LA:Ao is less than this amount, then no clinically meaningful change in MCS will be concluded.

Furthermore, one dog in the test group started pimobendan at day 103 of the study and required increasing levels of propofol for all three echocardiograms and one dog in the test group received butorphanol for the 6-month echocardiogram evaluation only. These two dogs were not excluded from the statistical analysis and could be a limitation to fully understanding the test food's impact on echocardiographic measurements. However, the authors do not believe that butorphanol or the increasing dosage of propofol for the dogs in the MMVD-TEST group would impact echocardiographic measurements of chamber size. If propofol were to impact echocardiographic measurements, the authors hypothesize it would increase blood volume in the chambers, since it is a negative inotrope, and could increase chamber size, in theory. However, there is limited data to understand if propofol impacts echocardiographic chamber size measurements and the opposite trend was observed in the MMVD-TEST group.

Additionally, dogs with MMVD were only stratified by heart murmur severity and not medications at enrollment. There were two dogs in the MMVD-CONTROL group who started pimobendan before enrollment and zero dogs in the MMVD-TEST group who started any medications intended to treat cardiovascular symptoms before enrollment. Furthermore, the 6-month feeding duration may not have been long enough to fully understand the long-term effects of feeding the two study foods, especially with a progressive disease like MMVD. While a study longer than 6 months would provide more impactful results and conclusions, echocardiogram reevaluations are recommended every 6-12 months (Keene et al., 2019), which justified the duration for this initial study. Also, the drop-out rate for this study was high which highlights the difficulty of working with senior dogs who may experience age-related conditions. Some additional limitations include the lack of diversity in breeds, breed sizes, and disease severity. The majority of the dogs enrolled in this study were Beagles, and while MMVD impacts a

variety of breeds, MMVD is known to be more prevalent in small breed dogs. Finally, all of the dogs enrolled in this study were Stage B1 except one dog who was Stage B2 and none of the dogs progressed to the next stage during the study. Therefore, it would be beneficial to investigate the effects of the study foods on breeds more commonly associated with MMVD, in a more diverse population of dogs with more progressive stages of MMVD over a longer study duration.

By offering a complete and balanced food that is highly palatable, dogs are able to meet their daily nutritional needs and receive the full benefits of consuming a nutrition designed to support cardiovascular health. Additionally, this work shows the benefits of synergistic nutrition as part of a multimodal treatment plan. Based on the echocardiographic results of the present study, early-stage MMVD dogs fed the test food showed evidence of smaller cardiac size through left ventricle echocardiogram measurements compared to the MMVD-CONTROL group. The authors hypothesize that the reduction in cardiac size in the test group suggests that the MMVD-TEST group showed evidence of reduced disease progression compared to the MMVD-CONTROL group, but these conclusions need to be validated with a longer and larger study. Although some of the results were not statistically significant, the observed numerical changes in various echocardiogram measurements are still impactful, especially in early-stage MMVD dogs. Additional work should be completed to define the optimal levels of these key nutrients and how they interact with each other to holistically support dogs diagnosed with MMVD. Regardless, this research contributes to the existing data of nutritional intervention in MMVD dogs.

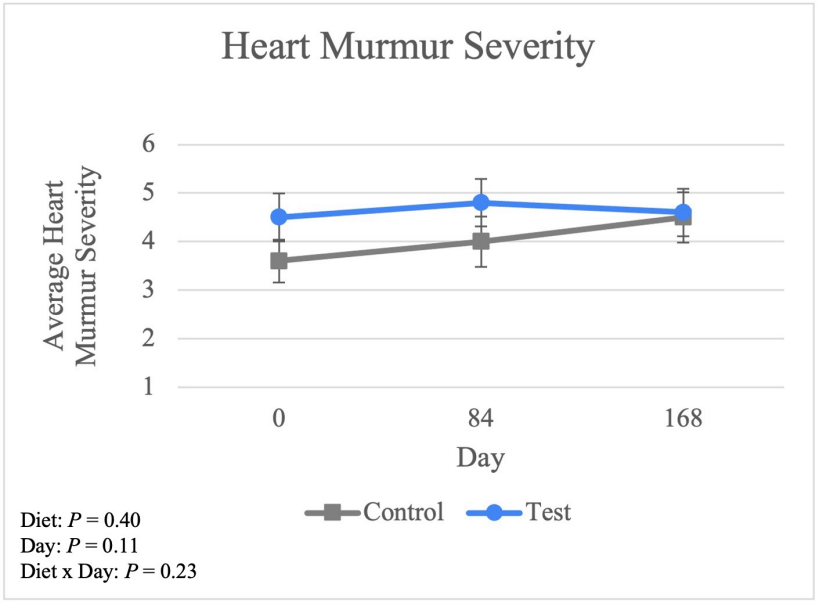
Funding

This study was funded by Hill's Pet Nutrition.

Disclosures

MDA, LAM, and LH are current employees of Hill's Pet Nutrition. MT was an employee of Kansas State University at the time this research was conducted.

A.



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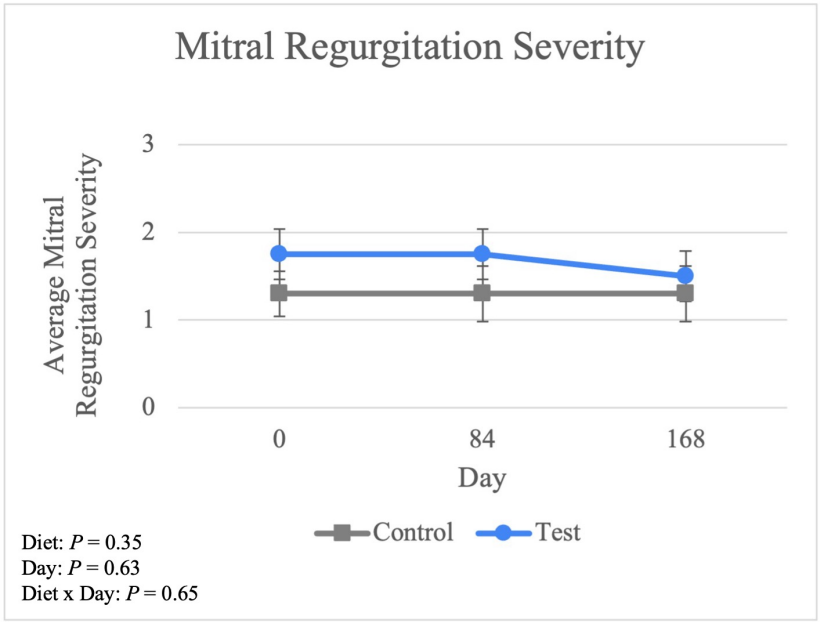


Figure 3.1. Average heart murmur severity (1=Barely audible, 6=Audible with stethoscope off the chest) (A) and average mitral regurgitation severity (0=None, 3=Severe) (B) in dogs with myxomatous mitral valve disease at the end of the washout period (day 0) and while fed either the test or control food (day 84 and day 168). Values are represented as mean ± SE.

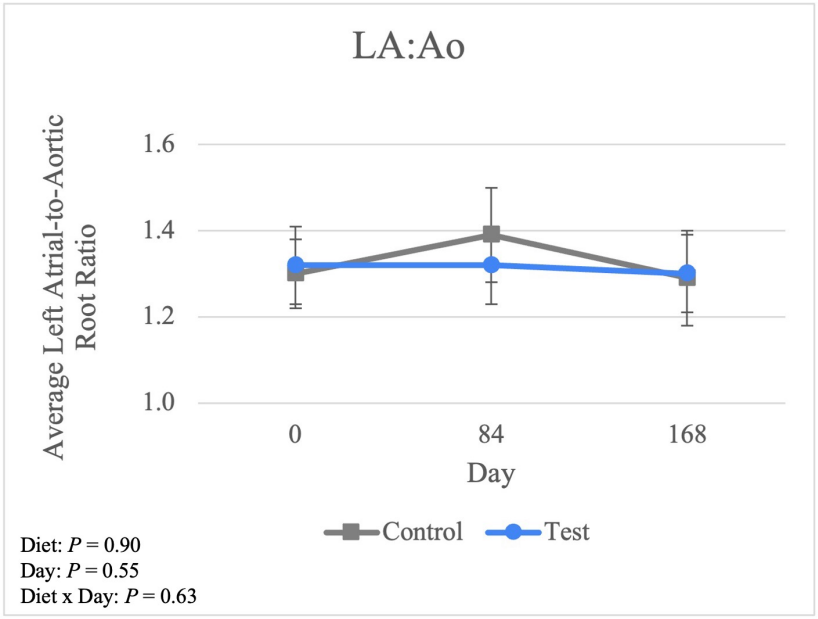
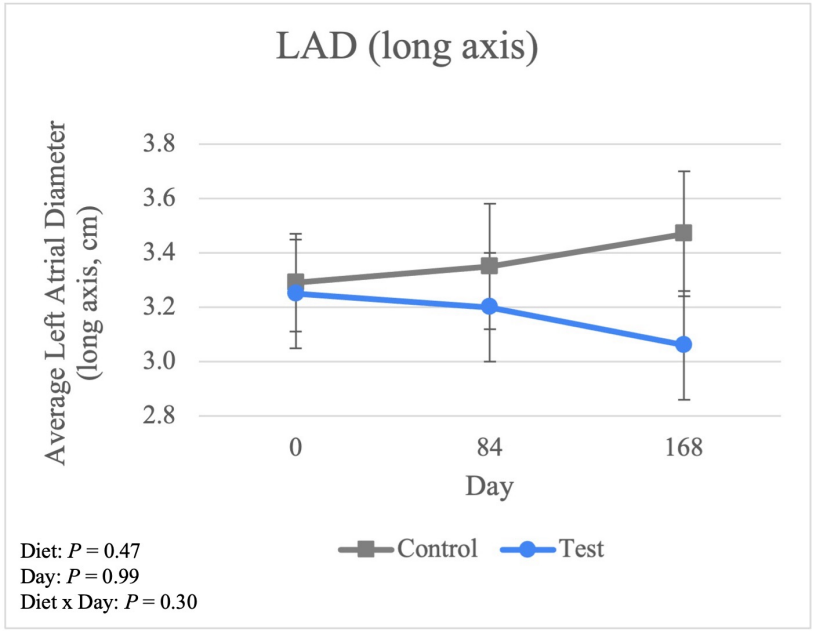


Figure 3.2. Average left atrial-to-aortic root ratio (LA:Ao), assessed by 2-Dimensional echocardiography, in dogs with myxomatous mitral valve disease at the end of the washout period (day 0) and while fed either the test or control food (day 84 and day 168). Values are represented as mean ± SE. A LA:Ao ≥ 1.6 is the threshold for substage B2 dogs (Keene et al., 2019).

A.



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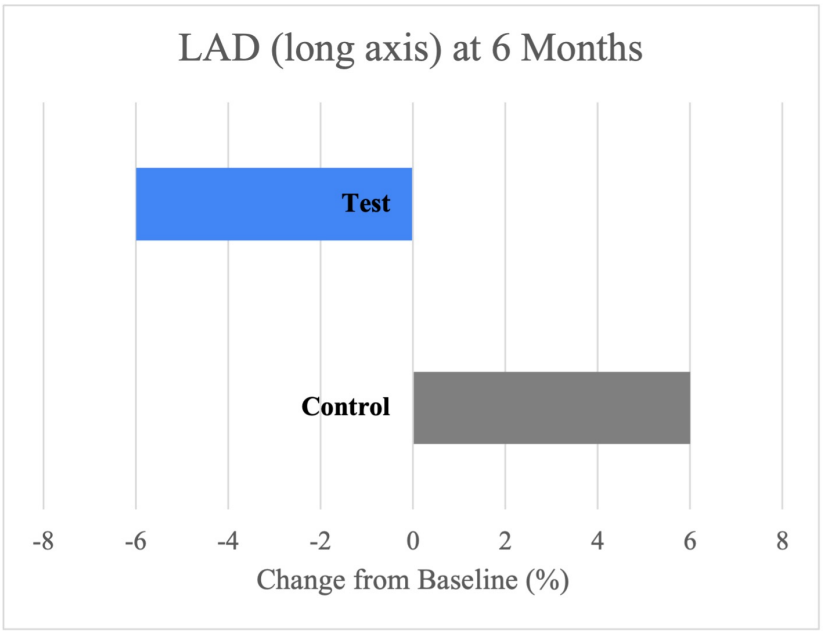
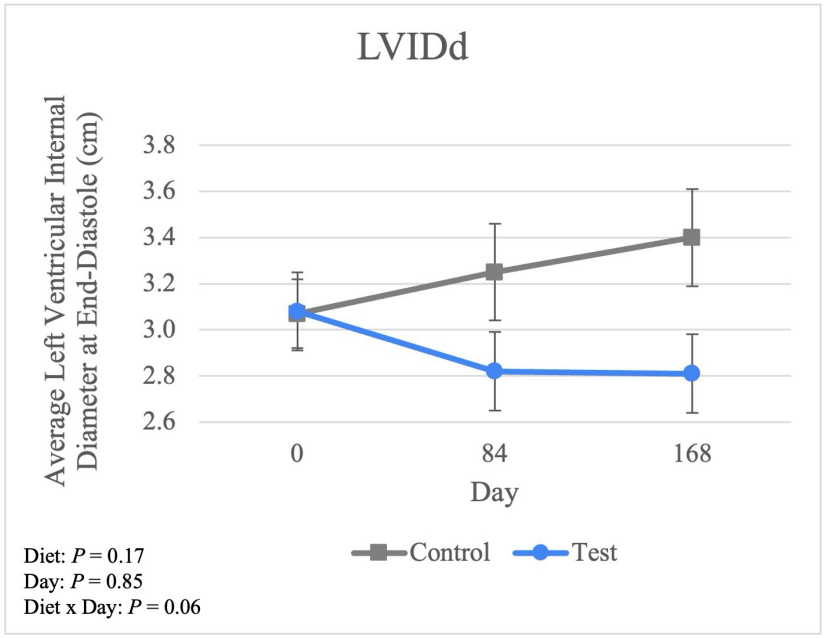


Figure 3.3. Average anteroposterior left atrial diameter (LAD), assessed by 2-Dimensional echocardiography, in dogs with myxomatous mitral valve disease at the end of the washout period (day 0) and while fed either the test or control food (day 84 and day 168) (A). Values are represented as mean ± SE. The percent change in LAD from day 0 to day 168 in dogs with myxomatous mitral valve disease fed either the test or control food (B).

A.



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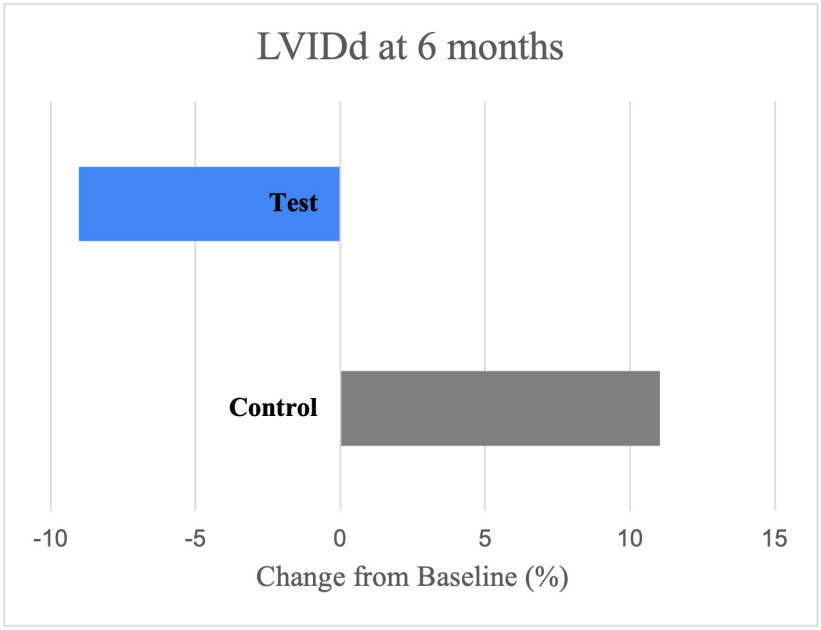
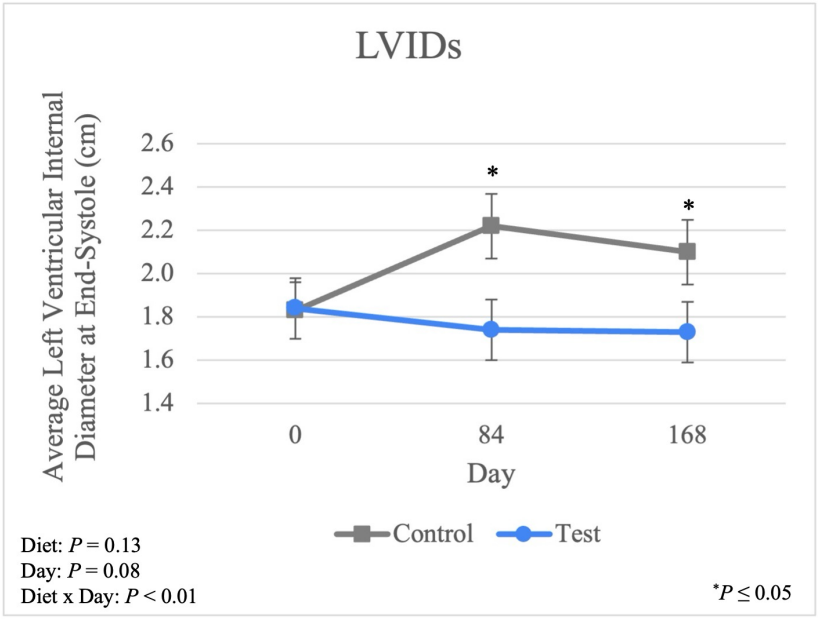


Figure 3.4. Average left ventricular internal diameter at end-diastole (LVIDd), assessed by 2-Dimensional echocardiography, in dogs with myxomatous mitral valve disease at the end of the washout period (day 0) and while fed either the test or control food (day 84 and day 168) (A). Values are represented as mean ± SE. The percent change in LVIDd from day 0 to day 168 in dogs with myxomatous mitral valve disease fed either the test or control food (B). Mean LVIDd in normal, young Beagle dogs has been reported as 2.63 cm (Crippa et al., 1992).

A.



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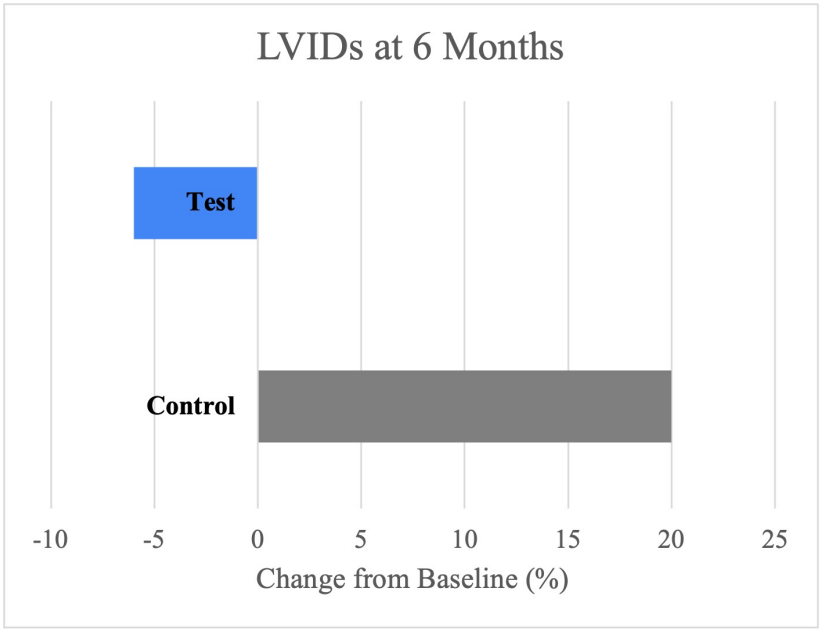


Figure 3.5. Average left ventricular internal diameter at end-systole (LVIDs), assessed by 2-Dimensional echocardiography, in dogs with myxomatous mitral valve disease at the end of the washout period (day 0) and while fed either the test or control food (day 84 and day 168) (A). Values are represented as mean ± SE. The percent change from day 0 to day 168 in LVIDs in dogs with myxomatous mitral valve disease fed either the test or control food (B). Mean LVIDs in normal, young Beagle dogs has been reported as 1.57 cm (Crippa et al., 1992).

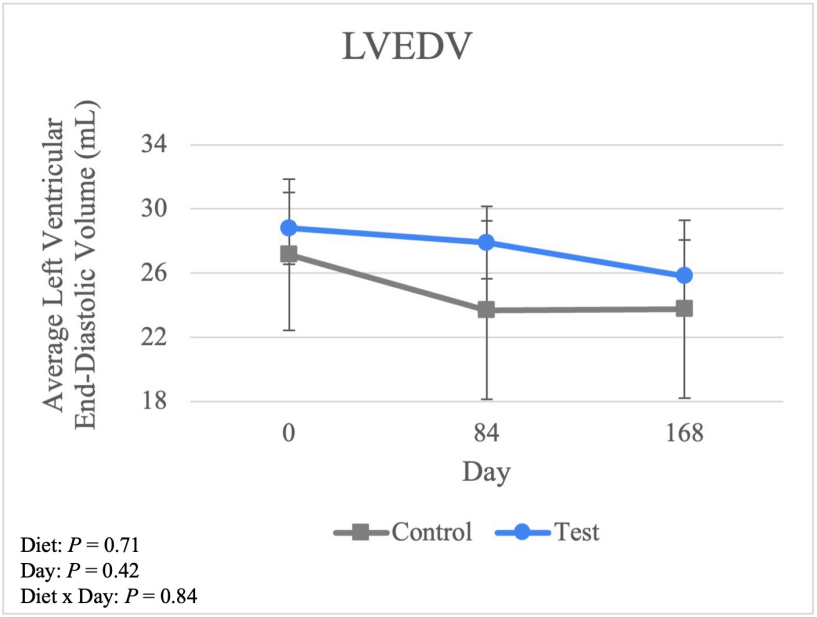


Figure 3.6. Average left ventricular end-diastolic volume (LVEDV), assessed by 2-Dimensional echocardiography, in dogs with myxomatous mitral valve disease at the end of the washout period (day 0) and while fed either the test or control food (day 84 and day 168). Values are represented as mean ± SE.

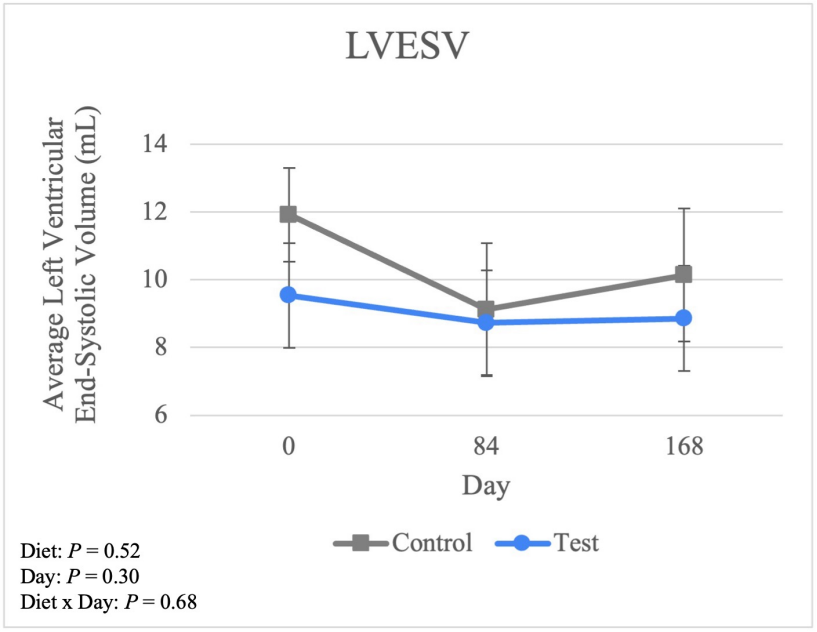


Figure 3.7. Average left ventricular end-systolic volume (LVESV), assessed by 2-Dimensional echocardiography, in dogs with myxomatous mitral valve disease at the end of the washout period (day 0) and while fed either the test or control food (day 84 and day 168). Values are represented as mean ± SE.

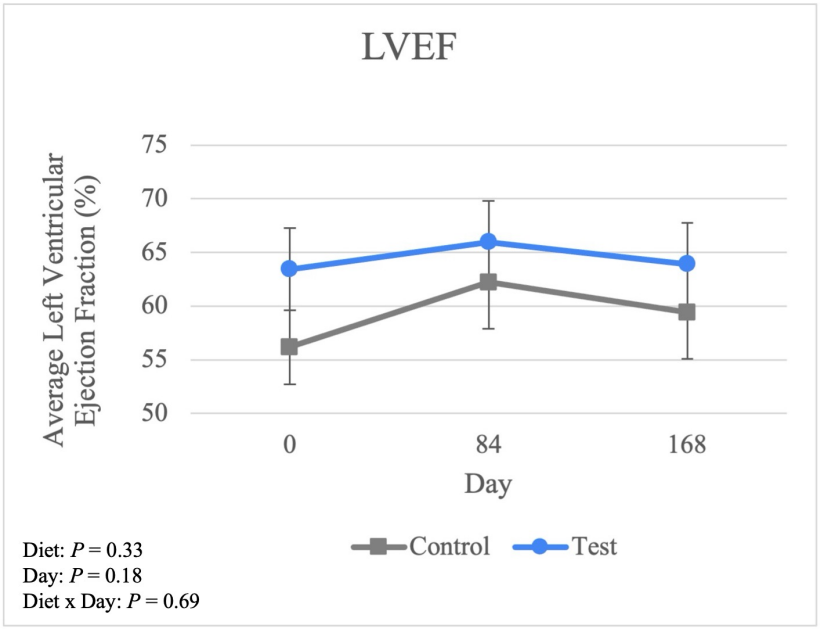
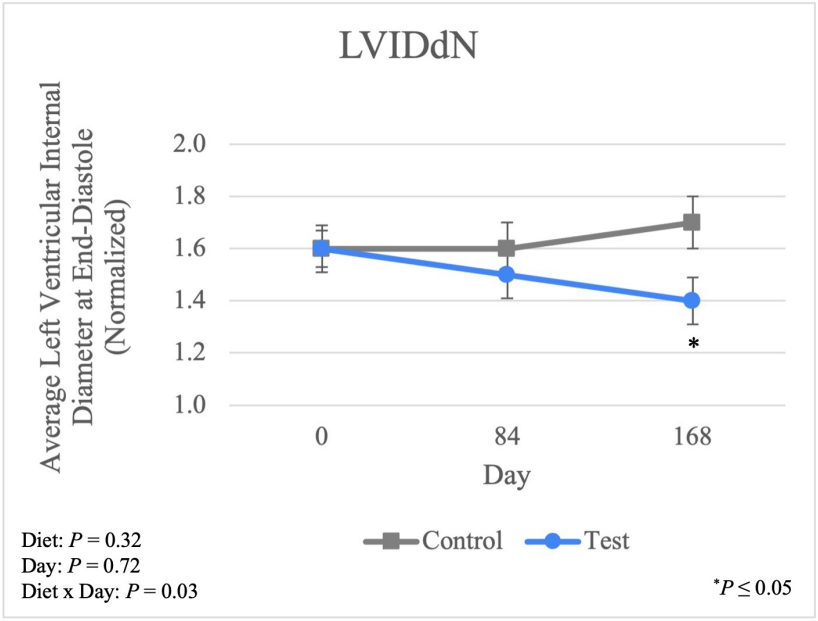


Figure 3.8. Average left ventricular ejection fraction (LVEF), assessed by 2-Dimensional echocardiography, in dogs with myxomatous mitral valve disease at the end of the washout period (day 0) and while fed either the test or control food (day 84 and day 168). Values are represented as mean ± SE. Left ventricular ejection fraction in normal dogs is 50-65% (Hezzell, 2018).

A.



B.

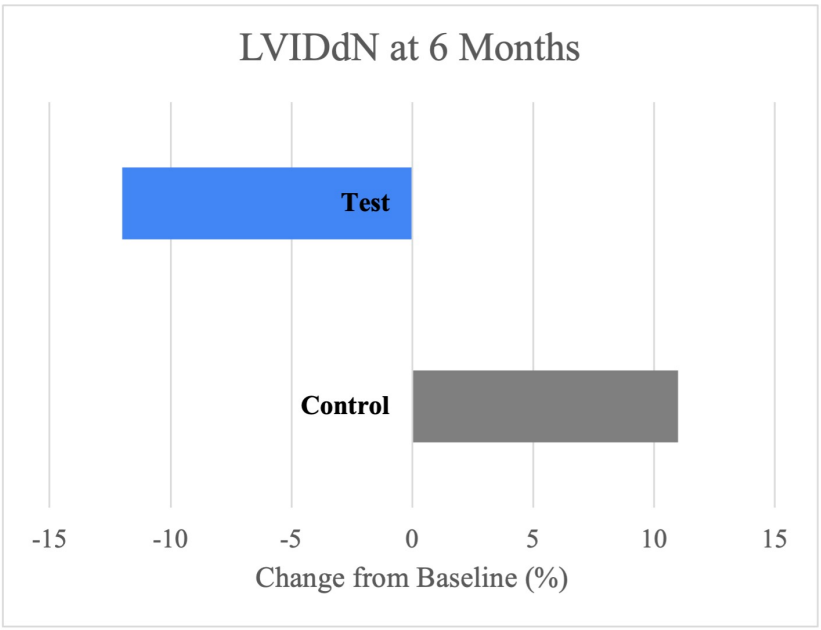


Figure 3.9. Average left ventricular internal diameter at end-diastole, normalized for body weight (LVIDdN), assessed by Motion-mode echocardiography, in dogs with myxomatous mitral valve disease at the end of the washout period (day 0) and while fed either the test or control food (day 84 and day 168) (A). Values are represented as mean ± SE. A LVIDdN ≥ 1.7 is the threshold for substage B2 dogs (Keene et al., 2019). The percent change in LVIDdN from day 0 to day 168 in dogs with myxomatous mitral valve disease fed either the test or control food (B).

Table 3.1. Effect of diet, day, and all relevant interactions on echocardiogram evaluation parameters of dogs with myxomatous mitral valve disease after the washout period (day 0) and while fed the test food or control food (day 84 and day 168). Dogs with myxomatous mitral valve disease fed the test food or control food are labeled as TEST and CONTROL, respectively.

	Day 0[§]		**Day 84**[§]		**Day 168**[§]			**P-value**		
Echocardiogram Evaluation Parameter	**TEST (n=4)**	**CONTROL (n=5)**	**TEST (n=4)**	**CONTROL (n=2)**	**TEST (n=4)**	**CONTROL (n=2)**	**SEM**[‡]	**Diet**	**Day**	**Diet x Day**
2D Echocardiography										
LA:Ao	1.3	1.3	1.3	1.4	1.3	1.3	0.1	0.90	0.55	0.63
LAD, cm	3.3	3.3	3.2	3.4	3.1	3.5	0.2	0.47	0.99	0.30
LVIDd, cm	3.1	3.1	2.8	3.3	2.8	3.4	0.2	0.17	0.85	0.06
LVIDs, cm	1.8	1.8	1.7	2.2	1.7	2.2	0.1	0.13	0.08	<0.01
LVEDV, mL	28.8	27.2	27.9	23.7	25.8	23.8	5.5	0.71	0.42	0.84
LVESV, mL	9.5	11.9	8.7	9.1	8.9	10.1	2.0	0.52	0.30	0.68
LVEF, %	63.4	56.2	66.0	62.2	63.9	59.4	4.3	0.33	0.18	0.69
M-mode Echocardiography										
LVIDdN	1.6	1.6	1.5	1.6	1.4	1.7	0.1	0.32	0.72	0.03
Subjective Assessments										

Heart Murmur Severity□	4.5	3.6	4.8	4.0	4.6	4.5	0.5	0.40	0.11	0.23
Mitral Regurgitation Severity°	1.8	1.3	1.8	1.3	1.5	1.3	0.3	0.35	0.63	0.65

§Values are represented as means; ‡Values are pooled standard error of the mean; □1=Barely audible, 6=Audible with stethoscope off the chest; °0=None, 3=Severe; 2D, 2-Dimensional; M-mode, Motion-mode; LA:Ao, left atrial-to-aortic root ratio; LAD, left atrial diameter; LVIDd, left ventricular internal diameter at diastole; LVIDs, left ventricular internal diameter at systole, LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricle ejection fraction; LVIDdN, left ventricular internal diameter at diastole, normalized

Chapter 4 - Clinical and exploratory biomarkers of canine myxomatous mitral valve disease

Madison D. Amundson, Laura A. Motsinger, Leslie Hancock

Introduction

Myxomatous mitral valve disease (MMVD) is the most common cardiovascular disease in the domestic dog (Keene et al., 2019; Svensson et al., 2024). Canine MMVD is usually present with a heart murmur and is most prevalent in small to medium-sized breeds (Keene et al., 2019). This disease is caused by the degradation of the mitral valve, which acts as a one-way apparatus between the left atrium and left ventricle, leading to the backflow of blood into the left atrium from the left ventricle. Total blood volume increases in the body and the increase in volume triggers the myocardial cells to undergo eccentric hypertrophy, resulting in the dilation of the left atrium and left ventricle. The ability for the heart to dilate prevents intracardiac pressures from rising, but the disease leads to left-sided congestive heart failure, eventually (Keene et al., 2019). Echocardiographic measurements and thoracic imaging are used to diagnose, determine staging, and monitor MMVD. Moreover, biomarker research related to cardiovascular disease led to the development of sensitive methodologies allowing for early detection and monitoring of cardiac conditions (Dhingra & Vasan, 2017).

Carnitine and taurine work synergistically to provide adenosine triphosphate (ATP) to the heart for cardiac function (McCauley et al., 2024). Therefore, low dietary intake of carnitine and/or taurine in the body could result in cardiac dysfunction (Gibbs, 1978; Schaffer et al., 2016; Suga, 1990). Carnitine is an amino acid derivative involved in the transportation of long-chain fatty acids across the mitochondrial membrane, where long-chain fatty acids are oxidized to generate ATP and supply 60% of energy for the heart (Laflamme, 2022; Neely & Morgan,

1974). Carnitine is supplied through dietary intake or can be synthesized by dogs from methionine and lysine. Esterified carnitine is carnitine bound to fatty acids and increased levels of plasma esterified carnitine can indicate a dysfunction in fatty acid oxidation (Scheibe & Grueter, 2021). Similarly to carnitine, taurine is an amino acid that supports energy metabolism by regulating intracellular calcium to support mitochondrial oxidative phosphorylation to produce ATP and enhance contractility (Quilliam et al., 2021; Sanderson, 2006). Additionally, vitamin D plays a role in the pathophysiology of heart disease such as supporting the renin-angiotensin-aldosterone system, regulating intracellular calcium, reducing pro-inflammatory cytokines, and improving blood pressure in humans (Witham, 2011). In veterinary medicine, low serum 25-hydroxyvitamin D was observed in dogs with congestive heart failure secondary to chronic valvular disease or dilated cardiomyopathy compared to normal dogs, which was also associated with poorer outcomes (Kraus et al., 2014).

Commonly used biomarkers in veterinary practice for cardiovascular disease include N-terminal pro-B-type natriuretic peptide (NT-proBNP). N-terminal pro-B-type natriuretic peptide, a biologically inactive form of brain natriuretic peptide (BNP), is used as an indicator of heart failure and cardiac dysfunction because it is released into the bloodstream from myocytes in the left ventricle as a response to left ventricular stretching from pressure or volume overload (Cao et al., 2019; Oyama et al., 2009). Brain natriuretic peptide is a cardiac natriuretic hormone that is produced and synthesized into proBNP by ventricular cardiomyocytes and cleaved into multiple fragments, including NT-proBNP, upon entering the bloodstream (Hall, 2005; Weber & Hamm, 2006). Specifically, NT-proBNP is predominately measured due to having a longer half-life and being more stable during sample collection and handling compared to other fragments (de Lima & Ferreira, 2017). Furthermore, identifying potential novel biomarkers via gene expression could

provide additional options for early detection and monitoring of MMVD in dogs. The nCounter® Gene Expression Assay is a robust and efficient screening method to identify differences in abundance of genes between groups or timepoints. The assay measures abundance with high reproducibility and sensitivity and can profile up to 800 genes in a single reaction (Kulkarni, 2011). While echocardiography is the best way to determine and monitor MMVD, various challenges, including level of echocardiographic expertise, availability of equipment, and cost, can be limiting in veterinary clinics. Therefore, NT-proBNP and other biomarkers can be an option for early detection before echocardiographic and thoracic imaging is needed and provide intermediate monitoring of cardiovascular disease progression. Overall, biomarkers are widely available and provide an informative option when challenges are present (Chanmongkolpanit et al., 2024).

Nutritional intervention is a key part of multimodal management of dogs with cardiovascular disease. Key nutrients for canine cardiovascular health include L-carnitine, taurine, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), sodium, and other electrolytes. However, there is limited data to understand what the optimal levels of these nutrients are and how they interact synergistically to provide nutritional support to dogs with MMVD. The hypothesis was that dogs with MMVD fed a cardiac food with increased levels of taurine, L-carnitine, EPA, and DHA would show increased levels of whole blood and urine taurine and serum 25-hydroxyvitamin D and decreased levels of plasma esterified carnitine and NT-proBNP compared to dogs with MMVD fed the control food. Therefore, the objective of this study was to investigate the effects of feeding two foods designed to support cardiac health with varying levels of key nutrients to dogs with MMVD and on clinical biomarkers. Another objective was to identify potential new biomarkers through nCounter® gene expression.

Methods

Ethics

This study was approved by Hill's Pet Nutrition's Institutional Animal Care and Use Committee and Animal Welfare Committee (CP1098, 08 May 2024).

Study Design and Animals

A two-by-two factorial design was used. A 6-month feeding study included dogs at Hill's Pet Nutrition Center that were diagnosed with a left-sided heart murmur (MMVD dogs) and control dogs without a heart murmur diagnosis (healthy dogs). Healthy dogs were selected to match sex and age of the MMVD dogs as best as possible. All dogs were fed a washout food for 5 weeks. Prior to the washout period, MMVD dogs were sorted by heart murmur severity only and randomized to either the test food or control food and healthy dogs were completely randomized to either the test food or control food. Dogs with MMVD who were stratified and randomized to the test food and control food are described as the MMVD-TEST group and the MMVD-CONTROL group, respectively. Healthy dogs who were randomized to the test food or control food are described as the HEALTHY-TEST group and the HEALTHY-CONTROL group, respectively. Dogs were not enrolled if they were in a sodium depletion state, diagnosed with hyperlipidemia, or had a history or risk of pancreatitis. Dogs could be released from the study if 1) excessive body weight was lost based on veterinarian discretion or 2) the dog became anorexic for 3 or more days. Adverse events were diagnosed, treated, and documented if they occurred and could be cause for dismissal based on veterinarian discretion. Additionally, if medications were needed during the study, medication name and dosage along with any changes throughout the study were documented and dogs could stay enrolled upon veterinarian discretion

(Appendix C). Medications intended to treat symptoms of cardiovascular disease were not a reason for exclusion.

Dogs from the Hill's Pet Nutrition Center were enrolled in this study. Dogs were group-housed and were provided regular exercise with access to indoor and outdoor areas. This study did not require any restriction to their normal routines or socialization with other dogs or the caretakers. Dogs were fed solely their assigned study food in individual feeding areas and were fed to maintain ideal body weight. Water was provided ad libitum. After dogs completed the study, they remained at the Hill's Pet Nutrition Center and received continued veterinary care and enrichment.

Blood and urine samples were collected on day 0, day 84, and day 168 in all groups. Analyses included plasma esterified carnitine, plasma carnitine ratio (esterified carnitine to free carnitine ratio), whole blood taurine, urine taurine, serum 25-hydroxyvitamin D, NT-proBNP, and nCounter® Gene Expression.

Plasma Carnitine Analysis

Whole blood was drawn in a 4 mL sodium heparin tube and centrifuged at 3,000 g for 10 minutes. One mL of plasma was allotted into a 2 mL cryovial, and then stored at -70°C until shipment. All samples were shipped at the end of the study. Plasma carnitine was analyzed by the Biochemical Genetics Laboratory Veterinary Unit at the University of California San Diego and assayed by carnitine acyl transferase, with and without alkaline hydrolysis of esters.

Whole Blood and Urine Taurine Analysis

Whole blood was drawn in 2 mL lithium heparin tubes from each animal. One mL of whole blood was transferred to a 2 mL cryovial, then stored at -70°C until shipment. Urine was collected via cystocentesis from each animal. One mL was allotted to a 2 mL cryovial, then

stored at -70°C until shipment. Both blood and urine samples were shipped immediately following collection and freezing, and analyzed by the Amino Acid Laboratory at the University of California Davis Weill School of Veterinary Medicine using a Biochrom 30 amino acid analyzer (Biochrom, Waterbeach Cambridge, UK).

Serum 25-Hydroxyvitamin D Analysis

Whole blood was drawn in a 4 mL Clot Activator tube, left at room temperature for 30 to 60 minutes to allow for coagulation, and then centrifuged at 3,000 g for 10 minutes for serum extraction. Serum was transferred to a 2 mL cryovial and frozen at -70°C until shipment. All samples were shipped at the end of the study. Serum 25-hydroxyvitamin D was analyzed by the Veterinary Diagnostic Laboratory at Michigan State University.

Serum N-terminal Pro-B-type Natriuretic Peptide Analysis

The Bionote Vcheck® F V2400 fluorescent immunoassay analyzer (Bionote, Big Lake, MN, USA) was used to analyze canine NT-proBNP. Blood samples (4 mL) were collected from each animal in a serum separator tube. For each sample, a pipette was used to draw 100 µL of serum and added to the assay dilution tube. The sample and dilution solution were mixed. Finally, 100 µL of the mixed sample was added to the sample well in the analyzer before starting the analysis. Samples were analyzed promptly following collection.

nCounter® Gene Expression Analysis

Whole blood samples were collected in ribonucleic acid (RNA) PAXgene blood collection tubes and stored at -80°C until analysis after study completion. Total RNA was isolated from each sample using the PAXgene Blood miRNA kit (Qiagen, Germantown, MD, USA). Integrity of total RNA was measured using the Agilent Bioanalyzer (Agilent, Santa Clara, CA, USA). The concentration of total RNA was also measured using the Qubit 3.0 fluorometer

(Thermo Fisher, Waltham, MA, USA). All isolated RNA was stored at -80°C until further analysis.

Following the isolation of total RNA, gene abundance was measured using the nCounter® MAX/FLEX Analysis System (Bruker Spatial Biology, Bothell, WA, USA) following the manufacturer's protocol (formally known as NanoString (Seattle, WA, USA)). The canine immuno-oncology (IO) panel containing 800 genes was used to measure the immune response in dogs diagnosed with MMVD. A Master Mix was created by adding 70 µL of hybridization buffer to the Reporter CodeSet tube. In hybridization tubes, 8 µL of the prepared Master Mix and 5 µL of sample RNA, equaling 200 ng of total RNA input, were added, followed by 2 µL of Capture ProbeSet. The tubes were capped, mixed, spun down, and placed in a 65°C pre-heated thermal cycler, and the hybridization time was set for 18 hours. After the hybridization step, samples were loaded onto the nCounter® MAX/FLEX prep station, followed by the digital analyzer.

Study Foods

All three foods were offered in a dry form only and formulated for adult maintenance. The washout food was formulated to nutritional support aging dogs since MMVD is commonly diagnosed in older dogs. Both the test and control foods were formulated to provide nutritional support to dogs with cardiovascular disease with differing levels in key nutrients for cardiovascular health. The control food was considered a positive control. The test food was formulated with increased levels of protein, fat, L-carnitine, taurine, EPA, DHA, and sodium compared to the control food. On an as-is caloric basis, the analyzed nutritional composition of the washout food for protein, fat, L-carnitine, EPA+DHA and sodium were 46.2 g/1000 kcal ME, 30.2 g/1000 kcal ME, 1.6 mg/1000 kcal ME, <0.04 g/1000 kcal ME, and 0.6 g/1000 kcal

ME, respectively. The analyzed nutrition composition for the control food for protein, fat, L-carnitine, EPA+DHA, and sodium were 44.9 g/1000 kcal ME, 48.9 g/1000 kcal ME, 85 mg/1000 kcal ME, <0.04 g/1000 kcal ME, and 0.3 g/1000 kcal ME, respectively. The analyzed nutritional composition of the test food for protein, fat, L-carnitine, EPA+DHA, and sodium were 52.2 g/1000 kcal ME, 51.8 g/1000 kcal ME, 118 mg/1000 kcal ME, 1.7 g/1000 kcal ME, and 0.4 g/1000 kcal ME, respectively. Analyzed dietary taurine was similar across the washout, control, and test foods (0.33 g/1000 kcal ME, 0.32 g/1000 kcal ME, and 0.34 g/1000 kcal ME, respectively). Additional differences in the study foods are provided in Appendix A.

Statistical Analysis

Enrollment body weight (BW), age, and heart murmur severity data were analyzed using a linear mixed model. Enrollment data analyses were performed using PROC MIXED in SAS® (version 9.4; SAS Institute Inc., Cary, NC, USA), and a Tukey adjustment was used in determining significant treatment differences by separation of least square means. Esterified carnitine, carnitine ratio, whole blood taurine, urine taurine, NT-proBNP, and serum 25-hydroxyvitamin D were examined for normality. The analysis was performed using PROC UNIVARIATE in SAS® (version 9.4; SAS Institute Inc., Cary, NC, USA). Whole blood taurine and urine taurine data were normally distributed. Esterified carnitine, carnitine ratio, NT-proBNP, and 25-hydroxyvitamin D data were determined to be non-normally distributed and a ln-transformation was performed to achieve normality. The transformation was performed using LOGNORMAL in SAS® (version 9.4; SAS Institute Inc., Cary, NC, USA). Esterified carnitine, carnitine ratio, whole blood taurine, urine taurine, NT-proBNP, and serum 25-hydroxyvitamin D were analyzed using a linear mixed model with treatment, day, disease and the interactions as fixed effects and animal as a random effect. To compare the post baseline time points to baseline,

the SLICEDIFF=ADJUST=DUNNETT option for the least square means was used. A $P \leq 0.05$ was considered significant and a $P > 0.05$ and $P \leq 0.10$ was considered a tendency. Analyses were performed using PROC GLIMMIX in SAS® (version 9.4; SAS Institute Inc., Cary, NC, USA). Gene expression data was analyzed with the nSolver software (Bruker Spatial Biology, Bothell, WA, USA). A criterion for significance including an unadjusted P-value of ≤ 0.05 and a fold change ≥ 1.5 or ≤ -1.5 was used.

Results

Population

A total of 20 dogs were enrolled into this study, 10 dogs with MMVD and 10 healthy dogs. At enrollment (start of washout period), the mean (SE) age for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 12.4 (1.1) years, 12.0 (1.0) years, 11.2 (0.7) years, and 11.2 (0.7) years, respectively. Mean (SE) BW at enrollment for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 7.7 (0.7) kg, 9.5 (0.7) kg, 8.3 (0.5) kg, and 9.7 (0.5) kg, respectively. In the MMVD dogs, mean (SE) heart murmur severity was 3.8 (0.4) and 3.9 (0.4) for the MMVD-TEST group and MMVD-CONTROL group, respectively, at enrollment. There was no difference ($P > 0.10$) in BW or age between the MMVD-TEST group and MMVD-CONTROL group or HEALTHY-TEST group and HEALTHY-CONTROL group. Finally, there was no difference ($P > 0.10$) in heart murmur severity between the MMVD-TEST group and MMVD-CONTROL group. Each of the four groups contained two males and three females. All dogs enrolled in this study were Beagles, except two dogs in the MMVD-CONTROL group who were mixed breeds. Signalment information is available in Appendix B.

During the study, there were four MMVD dogs removed from the study, three from the control group and one from the test group. Of the MMVD dogs in the MMVD-CONTROL group, one was removed due to poor quality of life associated with inflammatory bowel disease on day 77, one was removed after day 28 due to weight loss associated with kidney disease, and one was removed after day 54 due to declining mobility issues, weight loss, and decreased appetite, which was deemed unrelated to MMVD. The one MMVD dog in the MMVD-TEST group was removed from the study due to neurological issues due to possible stroke, emboli, or ruptured cardiac valve on day -12 of the washout period. Additionally, one healthy dog was removed from the HEALTHY-TEST group due to renal failure and decreased appetite on day 41. Dogs were included in the statistical analysis up until the point of removal, except for the dog removed during the washout period, who was removed from all analyses except signalment analyses at enrollment.

Plasma Carnitine

No significant interaction between diet and disease, diet and day, disease and day, or diet, day, and disease ($P > 0.10$) was observed for plasma esterified carnitine (Table 4.1). At day 0, mean (SE) esterified carnitine for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 17.5 (2.9) µmol/L, 17.1 (2.6) µmol/L, 14.9 (2.5) µmol/L, and 14.5 (2.0) µmol/L, respectively. Esterified carnitine tended to increase ($P = 0.08$) at day 168 compared to day 0 in the MMVD-CONTROL group and increased at day 84 ($P < 0.01$) and day 168 ($P < 0.01$) when compared to day 0 in the MMVD-TEST group. Compared to day 0, esterified carnitine increased ($P \leq 0.05$) at day 84 and day 168 in both the HEALTHY-TEST group and HEALTHY-CONTROL group. There was no difference ($P > 0.10$) in esterified carnitine between the two MMVD treatment groups or

between the two healthy treatment groups at day 0, day 84, or day 168. Additionally, there was no difference ($P > 0.10$) in esterified carnitine between the MMVD dogs and healthy dogs fed the test food or between the MMVD dogs and healthy dogs fed the control food at any of the timepoints.

There was a significant diet by disease interaction ($P = 0.02$) for plasma carnitine ratio, but no other interactions were significant ($P > 0.10$) (Table 4.1). Mean (SE) carnitine ratio at day 0 for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 0.93 (0.16), 0.80 (0.12), 0.70 (0.12), and 0.81 (0.11), respectively. Compared to day 0, carnitine ratio was unchanged ($P > 0.10$) at day 168 in the MMVD-CONTROL group, but increased ($P = 0.04$) at day 168 in the MMVD-TEST group. Carnitine ratio increased ($P < 0.02$) at day 168 compared to day 0 in both the HEALTHY-TEST group and HEALTHY-CONTROL group. There was no difference ($P > 0.10$) in carnitine ratio between the two MMVD treatment groups or the two healthy treatment groups at day 0, day 84, or day 168, except the HEALTHY-CONTROL group tended to have a higher ($P = 0.06$) carnitine ratio than the HEALTHY-TEST group at day 84. Additionally, there was no difference ($P > 0.10$) in carnitine ratio between the MMVD dogs and healthy dogs fed the test food or between the MMVD dogs and healthy dogs fed the control food at any of the timepoints, except the MMVD-CONTROL group tended to have a lower ($P = 0.08$) carnitine ratio compared to the HEALTHY-CONTROL group at day 168.

Whole Blood and Urine Taurine

No significant interaction between diet and disease, diet and day, disease and day, or diet, day, and disease ($P > 0.10$) was observed for whole blood taurine. Mean (SE) whole blood taurine at day 0 for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST

group, and HEALTHY-CONTROL group were 271.8 (40.3) nmol/mL, 323.0 (36.0) nmol/mL, 225.3 (40.2) nmol/mL, and 270.0 (32.8) nmol/mL, respectively. Whole blood taurine increased ($P < 0.05$) at day 84 and day 168 compared to day 0 in the MMVD-CONTROL group, MMVD-TEST group, HEALTHY-TEST group, and HEALTHY-CONTROL group. There was no difference ($P > 0.10$) in whole blood taurine between the two MMVD treatment groups or between the two healthy treatment groups at day 0, day 84, or day 168. Additionally, there was no difference ($P > 0.10$) in whole blood taurine between the MMVD dogs and healthy dogs fed the test food or between the MMVD dogs and healthy dogs fed the control food at any of the timepoints.

No significant interaction between diet and disease, diet and day, disease and day, or diet, day, and disease ($P > 0.10$) was observed for urine taurine (Table 4.1). At day 0, mean (SE) urine taurine for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 7906.7 (1601.6) nmol/mL, 3272.2 (1240.6) nmol/mL, 4259.8 (1387.0) nmol/mL, and 3584.0 (1132.5) nmol/mL, respectively. Compared to day 0, urine taurine was unchanged ($P > 0.10$) in the MMVD-CONTROL group, MMVD-TEST group, HEALTHY-TEST group, and HEALTHY-CONTROL group at day 168. There was no difference ($P > 0.10$) in urine taurine between the two MMVD treatment groups or between the two healthy treatment groups at day 0, day 84, or day 168, except the MMVD-TEST group had higher ($P = 0.03$) urine taurine compared to the MMVD-CONTROL group at day 0. Additionally, urine taurine was higher ($P = 0.03$) in the MMVD-CONTROL group compared to the HEALTHY-CONTROL group at day 84 and tended to be higher ($P = 0.10$) in the MMVD-TEST group compared to the HEALTHY-TEST group at day 0. Of note, samples for one dog in the MMVD-TEST group were unavailable for all three collection timepoints and samples for one

dog in the HEALTHY-CONTROL group were unavailable for day 84 and day 168 due to resistance during urine collections.

Serum N-terminal Pro-B-type Natriuretic Peptide

No significant interaction between diet and disease, diet and day, disease and day, or diet, day, and disease ($P > 0.10$) was observed for serum NT-proBNP (Table 4.1). At day 0, mean (SE) NT-proBNP for the MMVD-TEST group, MMVD-CONTROL group, HEALTHY-TEST group, and HEALTHY-CONTROL group were 301.7 (70.0) pmol/L, 557.02 (115.7) pmol/L, 311.4 (72.3) pmol/L, and 343.4 (65.1) pmol/L, respectively. Compared to day 0, NT-proBNP was unchanged ($P > 0.10$) at day 168 in the MMVD-CONTROL group and increased ($P = 0.03$) in the MMVD-TEST group at day 168. Additionally, NT-proBNP increased ($P < 0.01$) at day 168 compared to day 0 in the HEALTHY-TEST group and HEALTHY-CONTROL group. N-terminal pro-B-type natriuretic peptide tended to be higher ($P = 0.06$) at day 0 in the MMVD-CONTROL group compared to the MMVD-TEST group, but there was no difference ($P > 0.10$) between the two MMVD treatment groups at day 84 or day 168 or between the two healthy treatment groups at day 0, day 84, or day 168. Additionally, NT-proBNP tended to be higher ($P = 0.10$) in the MMVD-CONTROL group compared to the HEALTHY-CONTROL group at day 0. There was no difference ($P > 0.10$) in NT-proBNP between MMVD dogs and healthy dogs fed the control food or between MMVD dogs and healthy dogs fed the test food at any other timepoint.

Serum 25-Hydroxyvitamin D

There was a tendency for a diet by day interaction ($P = 0.10$) for serum 25-hydroxyvitamin D, but no other interactions were significant ($P > 0.10$) (Table 4.1). Mean (SE) serum 25-hydroxyvitamin D for the MMVD-TEST group, MMVD-CONTROL group,

HEALTHY-TEST group, and HEALTHY-CONTROL group were 271.9 (45.6) nmol/L, 220.6 (33.1) nmol/L, 236.0 (39.7) nmol/L, and 265.5 (36.4) nmol/L, respectively. Compared to day 0, 25-hydroxyvitamin D was unchanged ($P > 0.10$) in the MMVD-CONTROL group, MMVD-TEST group, HEALTHY-TEST group, and HEALTHY-CONTROL group at day 168. There was no difference ($P > 0.10$) in serum 25-hydroxyvitamin D between the two MMVD treatment groups or between the two healthy treatment groups at day 0, day 84, or day 168, except the HEALTHY-CONTROL group tended to have higher ($P = 0.06$) levels of serum 25-hydroxyvitamin D compared to the HEALTHY-TEST group at day 168. Additionally, there was no difference ($P > 0.10$) in serum 25-hydroxyvitamin D between the MMVD dogs and healthy dogs fed the test food or between the MMVD dogs and healthy dogs fed the control food at any of the timepoints.

nCounter® Gene Expression

Samples from four dogs in the MMVD-TEST group and from two dogs in the MMVD-CONTROL group were used in the exploratory gene expression analysis at the end of the study. Seven genes had increased ($P \leq 0.05$ and ≥ 1.5-fold) abundance in the MMVD-TEST group compared to the MMVD-CONTROL group at day 168, including matrix metalloproteinase 1 (MMP1, 4.37-fold), pro-platelet basic protein (PPBP, 2.09-fold), perforin 1 (PRF1, 2.05-fold), granzyme K (GZMK, 1.98-fold), C-X-C motif chemokine receptor 6 (CXCR6, 1.75-fold), C-X3-C motif chemokine receptor 1 (CX3CR1, 1.73-fold), and interleukin 1 alpha (IL-1α, 1.59-fold) (Table 4.2). Additionally, one gene had decreased ($P \leq 0.05$ and ≤ -1.5-fold) abundance in the MMVD-TEST group compared to the MMVD-CONTROL group at day 168, C-X-C motif chemokine ligand 14 (CXCL14; -2.62-fold) (Table 4.3).

Discussion

Myxomatous mitral valve disease is the most prevalent cardiovascular disease in dogs and progresses slowly, eventually leading to congestive heart failure (Keene et al., 2019; Svensson et al., 2024). Nutritional intervention is a key component of multimodal management of cardiovascular disease in dogs. Over time, biomarker research related to cardiovascular disease led to the development of sensitive methodologies allowing for early detection and monitoring of cardiac conditions (Dhingra & Vasan, 2017). Therefore, the objective of this study was to investigate the effects of feeding two foods designed to support cardiac health with varying levels of key nutrients in dogs with MMVD and observe differences in commonly used biomarkers and identify potential new biomarkers.

Carnitine is a key compound involved in cardiovascular metabolism and must be transported into the cardiac muscle (Laflamme, 2022; Rebouche & Engel, 1983). Since the heart is unable to produce carnitine directly, deoxycarnitine is produced and exchanged for carnitine from the bloodstream instead (Li et al., 2020; Siliprandi et al., 1987). In the present study, esterified carnitine increased in the MMVD-CONTROL group and MMVD-TEST group at the end of the study when compared to day 0. Additionally, the carnitine ratio (esterified carnitine to free carnitine ratio) was unchanged throughout the study in the MMVD-CONTROL group, but increased by the end of the study in the MMVD-TEST group. Furthermore, esterified carnitine and carnitine ratio increased in the HEALTHY-TEST group and HEALTHY-CONTROL group by the end of the feeding study.

In a study that evaluated plasma concentrations of carnitine in dogs with congestive heart failure (CHF) secondary to degenerative mitral valve disease (DMVD), dogs with asymptomatic DMVD, and healthy control dogs, Karlin et al. (2019) observed significantly higher total L-

carnitine, carnitine esters, and carnitine ester to free carnitine ratio (E/F carnitine ratio) levels in DMVD dogs with CHF compared to asymptomatic DMVD dogs and healthy control dogs. Mean esterified carnitine and E/F carnitine ratio in healthy dogs were 7.7 μmol/L and 0.29 compared to asymptomatic DMVD dogs (9.8 μmol/L and 0.34) and symptomatic DMVD dogs (23.1 μmol/L and 0.53) (Karlin et al., 2019). In human studies, researchers have observed increased esterified carnitine and E/F carnitine ratio to be associated with heart failure, increased risk of heart failure, and cardiovascular related deaths (Strand et al., 2017; Ueland et al., 2013; Yoshihisa et al., 2017). The increase in esterified carnitine in the bloodstream could be due to impaired fatty acid metabolism by the myocardium in the MMVD-TEST group, resulting in excess esterified carnitine to be released into the bloodstream instead of free carnitine. However, an increase in esterified carnitine was also observed in dogs without a cardiovascular diagnosis. Overall, there is limited data on the relationship between carnitine in the plasma and carnitine in the skeletal and cardiac muscles in dogs (Costa & Labuc, 1994; Keene et al., 1991), but recent studies showed minimal correlation between circulating carnitine and skeletal and cardiac muscle concentrations of carnitine in healthy dogs (McCauley et al., 2024). Therefore, it has been suggested to caution the reliability of blood parameters, like plasma carnitine, to assess cardiac function alone (McCauley et al., 2024).

Taurine is largely found in the heart, supports energy production for the heart, and enhances cardiac contractility (Laflamme, 2022). As such, taurine deficiencies can reduce the function of the electron transport chain and ATP production (Schaffer et al., 2017). Whole blood taurine was analyzed in the present study because whole blood is considered a more accurate representation, as plasma taurine concentrations are more variable and are reported to be poorly correlated to myocardial carnitine concentrations (Streeter et al., n.d.). In the present study,

whole blood taurine was within the normal reference range at day 0 for all four treatment groups, which could be a result of the washout food containing adequate levels of taurine. Since the two MMVD treatment groups were within or above the normal reference range at day 0 and throughout the study, the results suggest that dogs with early-stage MMVD are likely not at risk of being taurine deficient, which aligns with the concept that MMVD is not a result of taurine deficiency. Throughout the present study, whole blood taurine significantly increased in all four treatment groups. Mean whole blood taurine levels were not significantly different between the two MMVD treatment groups or the two healthy treatment groups at any of the timepoints. Furthermore, no differences were found between the MMVD dogs and healthy dogs fed the same food (test or control) at any of the timepoints. Mean values were above the normal reference range for all treatment groups at day 84 and day 168, but dietary taurine on a caloric basis were similar between the washout food, control food, and test food.

In a study of Cavalier King Charles Spaniels with different early stages of MMVD, Wesselowski et al. (2022) showed median whole blood taurine in Stage A dogs, Stage B1 dogs, and Stage B2 dogs were 306 µmol/L, 262 µmol/L, and 280 µmol/L, respectively. Similarly, mean whole blood taurine at day 0 for the MMVD-TEST group and the MMVD-CONTROL group, which contained primarily Stage B1 MMVD dogs, were 271.8 µmol/L and 323.0 µmol/L, respectively. Furthermore, Wesselowski et al. (2022) reported no difference in whole blood or plasma taurine concentrations between Cavalier King Charles Spaniels eating foods that do or do not meet nutritional guidelines set by World Small Animal Veterinary Association (WSAVA). Finally, in the study performed by McCauley et al. (2024), no correlations between plasma, whole blood, skeletal muscle, and cardiac muscle taurine concentrations were observed.

Urine taurine is also used to assess taurine deficiencies, but is a less common analysis. Throughout the study, urine taurine was unchanged within the four treatment groups. Urine taurine was significantly higher in the MMVD-TEST group compared to the MMVD-CONTROL group at day 0. All dogs were fed the washout food for five weeks prior to day 0 and all dogs had early-stage MMVD, so it is unknown why the mean urine taurine was significantly higher in the MMVD-TEST group compared to the MMVD-CONTROL group at day 0. Additionally, urine taurine was higher in the MMVD-CONTROL group and MMVD-TEST group compared to the HEALTHY-CONTROL group and HEALTHY-TEST group at day 84 and day 0, respectively. However, urine taurine was not indexed to urine creatinine, which is important for variability in urine samples, and is a limitation. To the author's knowledge, there is limited data evaluating urine taurine in dogs with MMVD and further research is needed to understand whole blood and urine taurine as biomarkers for canine MMVD.

N-terminal pro-B-type natriuretic peptide is released into the bloodstream by myocardial tissue response to continuous stretching of the atrial and ventricular walls and volume or pressure overload (Oyama et al., 2009). In the present study, mean NT-proBNP in the MMVD-TEST group and MMVD-CONTROL group were within the normal reference range at day 0 and day 84. However, mean NT-proBNP for the MMVD-CONTROL group was above the upper limit of the normal at day 168, and mean NT-proBNP of the MMVD-TEST group significantly increased at day 168 compared to day 0 but remained below the upper limit. Additionally, NT-proBNP increased to the upper limit of normal in the two healthy treatment groups, which was unexpected since these dogs did not have a cardiovascular disease diagnosis. A limitation of this study is the small sample sizes in all four treatment groups and, as such, animal-to-animal variation might have contributed.

Several studies have investigated NT-proBNP levels in dogs with different stages of MMVD and the accuracy and relevance of NT-proBNP as a diagnostic or monitoring biomarker (Chanmongkolpanit et al., 2024; Jang et al., 2023; Khaki et al., 2022; Wolf et al., 2013). In a study that evaluated NT-proBNP values in dogs with different stages of MMVD and healthy control dogs, Wolf et al. (2013) reported NT-proBNP values of all MMVD disease stages were significantly higher than NT-proBNP levels of a control group. Additionally, mean NT-proBNP of stages B2 to Stage D (using the Canine Heart Failure International Expert Forum (CHIEF) staging system) were above 900 pmol/L (Wolf et al., 2013). Another study showed that symptomatic MMVD dogs (2159.9 pmol/L) had significantly higher median NT-proBNP values compared to asymptomatic MMVD dogs (749.0 pmol/L) and control dogs (499.0 pmol/L) (Chanmongkolpanit et al., 2024). Furthermore, asymptomatic MMVD dogs had significantly higher median NT-proBNP values compared to healthy dogs (Chanmongkolpanit et al., 2024). Finally, Jang et al. (2023) reported that systemic hypertensive Stage B MMVD dogs (2345.0 pmol/L) had significantly higher median NT-proBNP values compared to normotensive Stage B MMVD dogs (1083.5 pmol/L), suggesting that NT-proBNP can be evaluated in the presence of systemic hypertension in MMVD dogs. Previous studies show that NT-proBNP in dogs with early-stage MMVD are around the upper limit of the normal reference range. Therefore, NT-proBNP can be a useful biomarker to differentiate between asymptomatic and symptomatic dogs with MMVD and monitor disease progression, especially in clinics that lack echocardiographic imaging capabilities.

Vitamin D plays a role in the pathophysiology of heart disease and vitamin D deficiency increases risk in humans with cardiovascular disease (Osuga et al., 2015). Cardiomyocytes contain vitamin D receptors and a calcitriol-dependent calcium-binding protein, which help

regulate intracellular calcium, heart contractility, and exert anti-hypertrophic effects to regulate cardiac remodeling (Green et al., 2006; Simpson & Weishaar, 1988; Weber et al., 2008). Vitamin D is known to have cardioprotective properties to suppress the renin-angiotensin-aldosterone system, reduce pro-inflammatory cytokines, and improve endothelial dysfunction (Kraus et al., 2014; Patel & Rizvi, 2011). Serum 25-hydroxyvitamin D is not influenced by ultraviolet light exposure because dogs lack the ability to convert ultraviolet light into vitamin D3. Therefore, serum 25-hydroxyvitamin D is representative of exclusively dietary influence (Kraus et al., 2014).

In the present study, serum 25-hydroxyvitamin D remained within the normal reference range for all four treatment groups at all three timepoints and no significant changes were observed. Serum 25-hydroxyvitamin D was higher in the HEALTHY-CONTROL group compared to the HEALTHY-TEST group at day 168, which is likely due to the control food having higher levels of dietary vitamin D compared to the test food. However, a difference in serum vitamin D was not observed between the two MMVD treatment groups at the end of the study. In a previous study, Kraus et al. (2014) observed significantly lower mean serum 25-hydroxyvitamin D in dogs with CHF (100 nmol/L) compared to healthy dogs (123 nmol/L). Additionally, low serum 25-hydroxyvitamin D was associated with poor outcomes in dogs with CHF (Kraus et al., 2014). Furthermore, in a study conducted by Osuga et al. (2015), median serum 25-hydroxyvitamin D for Stage B1, Stage B2, and Stage C/D dogs were 52.5 nmol/L, 33.2 nmol/L, and 13.1 nmol/L, respectively. Serum 25-hydroxyvitamin D was significantly lower in Stage B2 and Stage C/D dogs with chronic valvular heart disease (CVHD) compared to Stage B1 CVHD dogs and was negatively associated with left atrial-to-aortic root ratio and left ventricular end-diastolic diameter normalized for body weight echocardiogram evaluations (Osuga et al.,

2015). There is not a clear understanding of why serum 25-hydroxyvitamin D decreases with cardiovascular disease. The authors hypothesize that the decrease in serum 25-hydroxyvitamin D could be due to the increased metabolism of vitamin D by the heart as MMVD progresses to regulate dysfunction and eccentric hypertrophy. Therefore, the data from the present study and previous studies suggest that vitamin D should be considered an essential nutrient for cardiovascular health and further investigation of appropriate levels of dietary vitamin D for MMVD in dogs should be pursued.

There are a variety of blood biomarkers that are commonly used to monitor MMVD and other cardiovascular diseases in dogs. nCounter® gene expression was used as an exploratory screening technique to generate new and actionable hypotheses to guide further investigation. The canine immuno-oncology (IO) panel used in the present study measures the abundance of 800 genes simultaneously, and genes were considered significant if an unadjusted P-value of $\leq$ 0.05 and a fold change $\geq$ 1.5 or $\leq$ -1.5 were reported. The cutoff was selected based on common use in gene expression research and to avoid losing potential findings. Seven significant genes, matrix metalloproteinase 1 (MMP1), pro-platelet basic protein (PPBP), perforin 1 (PRF1), granzyme K (GZMK), C-X-C motif chemokine receptor 6 (CXCR6), C-X3-C motif chemokine receptor 1 (CX3CR1), and interleukin-1 alpha (IL-1α), were upregulated and one significant gene, C-X-C motif chemokine ligand 14 (CXCL14), was downregulated in the MMVD-TEST group compared to the MMVD-CONTROL group at the end of the 6-month feeding study.

Matrix metalloproteinase 1 (MMP1) is a member of the matrix metalloproteinase family, which hydrolyzes protein and proteoglycan components of the extracellular matrix and is involved with various physiological processes, but can be over expressed in pathological diseases (Pardo & Selman, 2005). Matrix metalloproteinases are activated by other matrix

metalloproteinases, interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and other mediators (Li et al., 2000; Visse & Nagase, 2003). The extracellular matrix of the heart, which can lead to the degradation of the mitral valve in dogs through accumulation of myxomatous substances such as proteoglycans and disrupted collagen and elastic fibers (Grzeczka et al., 2025; Oyama et al., 2020), is regulated by the balance of matrix metalloproteinases and inhibitors of metalloproteinases (Aupperle et al., 2009). Aupperle et al. (2009) investigated the expression of matrix metalloproteinases using polymerase chain reaction (PCR) in normal and diseased canine mitral valves and showed that dogs with chronic valvular disease had significantly increased expression levels of MMP1 compared to dogs with normal valves, who showed low or undetectable levels of MMP1. In the present study, MMP1 was significantly increased in the MMVD-TEST group compared to the MMVD-CONTROL group. Since MMP1 is associated with changes in the extracellular matrix which could be associated to the degradation of the mitral valve in MMVD dogs, the authors hypothesize that an increase in expression of MMP1 would be associated with disease progression, particularly increased regurgitation and cardiac size. However, as seen in Chapter 3 of this thesis, the MMVD-TEST group and MMVD-CONTROL group maintained mitral regurgitation severity, and the MMVD-TEST group showed evidence of smaller cardiac size compared to the MMVD-CONTROL group through echocardiographic measurements of the left atrium and left ventricle. Based on the literature, MMP1 is a potential biomarker for canine MMVD, but should be further investigated and validated.

Pro-platelet basic protein (PPBP), also known as chemokine C-X-C motif ligand 7 (CXCL7), is a pro-inflammatory cytokine and involved in innate immunity, regulating vascular permeability, and has been identified to have a potential connection to cardiovascular disease

(Wang & Ward, 2025). This gene is released from activated platelets in response to vascular injury (Stankiewicz et al., 2014). In two studies that investigated PPBP in Thai men and women, the researchers reported significantly increased PPBP levels in men and women with coronary heart disease compared to healthy men and women (Maneerat et al., 2017; Mansanguan & Maneerat, 2022). In the present study, PPBP was significantly increased in the MMVD-TEST group compared to the MMVD-CONTROL group. To the author's knowledge, there is no data investigating PPBP in dogs diagnosed with MMVD and therefore, PPBP should be investigated and validated to determine if this gene could be a valid biomarker for canine cardiovascular diseases.

In the present study, both perforin 1 (PRF1) and granzyme K (GZMK) were significantly upregulated in the MMVD-TEST group compared to the MMVD-CONTROL group at the end of the study. The Perforin/Granzyme pathway is activated by cytotoxic T lymphocytes to kill normal cardiomyocytes which leads to more damage and inflammation (Santos-Zas et al., 2021; Varda-Bloom et al., 2000). T lymphocytes are part of the adaptive immune system and react to myocardial antigens and initiate the inflammatory process which result in impaired cardiac function, damaged cardiac tissue, and more (Blanton et al., 2019).

Cardiac perforin-positive status is associated with progressive cardiac dysfunction and impaired survival in human patients with inflammatory cardiomyopathy (Escher et al., 2017). Granzymes are important mediators of various pathological conditions and have become an important modulator of tissue remodeling (Zeglinski & Granville, 2020). Circulating levels of granzymes are low or undetectable in healthy individuals, but circulating levels of granzyme A, B, and K are significantly elevated in individuals with conditions associated with chronic inflammation and tissue remodeling (Zeglinski & Granville, 2020). Specifically, granzyme K is

involved in the pro-inflammatory activation of fibroblasts, Protease Activated Receptor (PAR) activation to promote pro-inflammatory responses, and endothelial dysfunction, all of which can lead to cardiovascular tissue remodeling (Cooper et al., 2011; Sharma et al., 2016). To the authors' knowledge, there is limited data that has explored PRF1 and GZMK levels in dogs with MMVD. However, our recent work with nCounter® gene expression showed that dogs with heart murmurs had significantly increased levels of granzyme A, B, and H compared to dogs without heart murmurs (Amundson et al., 2025). Therefore, further investigation and validation of granzymes in dogs with MMVD should be performed as granzymes could act as biomarkers.

C-X-C motif chemokine receptor 6 (CXCR6) is the receptor for C-X-C motif chemokine ligand 16 (CXCL16) which plays an important role in pro-inflammatory responses, extracellular matrix remodeling of the heart, and increases the risk of heart failure (Dahl et al., 2009). In the literature, CXCL16 is more commonly investigated and has been identified as a diagnostic marker and predictor of mortality in humans with cardiovascular disease (Borst et al., 2014). In the present study, CXCR6 levels were significantly increased in the MMVD-TEST group compared to the MMVD-CONTROL group, but CXCL16 levels were not different ($P = 0.77$, 1.1-fold) between the two groups. Overall, there is limited data for CXCR6 in dogs with cardiovascular disease, but the CXCR6/CXCL16 axis should be further investigated in dogs with cardiovascular disease based on the existing data in humans.

C-X3-C motif chemokine receptor 1 (CX3CR1) is the receptor for C-X3-C motif chemokine ligand 1 (CX3CL1) which is involved in the inflammation-driven pathology of cardiovascular disease (Weisheit et al., 2021). Cardiac macrophages play a role in cardiac tissue remodeling and healing (O'Rourke et al., 2019). In situations of pressure overload, CX3CR1 is highly expressed on macrophages and accumulates in the left ventricular tissue (Weisheit et al.,

2014). Weisheit et al. (2021) demonstrated that CX3CR1 is a prerequisite for cardiac hypertrophy and left ventricular dysfunction by showing that mice lacking CX3CR1 had reduced left ventricular hypertrophy and preserved cardiac function. In the present study, the MMVD-TEST group had significantly higher levels of CX3CR1 compared to the MMVD-CONTROL group, but CX3CL1 levels were not different ($P = 0.50$, -1.15-fold) between the two groups. Due to the involvement of CX3CR1 in left ventricular hypertrophy and function, the CX3CR1/CX3CL1 axis should be further investigated and validated in dogs diagnosed with MMVD since degradation of the mitral valve primarily impacts the left atrium and left ventricle.

Cytokines, such as interleukin-1 alpha (IL-1α), are produced by immune system cells and all cell types in the myocardium (Parissis et al., 2002). In dogs with MMVD, the upregulation of various cytokines has been observed in the mitral valve and myocardium (Kiczak et al., 2008; Oyama & Chittur, 2006). In a study that enrolled dogs with different stages of MMVD, mRNA expression was measured from whole blood samples using PCR (Mavropoulou et al., 2016). Mavropoulou et al. (2016) showed no difference in IL-1α between healthy dogs and dogs with different stages of MMVD. The present study demonstrated that IL-1α was significantly higher in the MMVD-TEST group compared to the MMVD-CONTROL group. However, validation and further investigation of IL-1α in dogs diagnosed with MMVD is needed to understand its association with canine MMVD.

Platelets are a major source of chemokines, such as C-X-C motif chemokine ligand 14 (CXCL14), which might play an important role in vascular repair (Witte et al., 2017). Platelet CXCL14 and circulating CXCL14 are inversely related, such that Schories et al. (2023) observed platelet CXCL14 levels were significantly lower and circulating CXCL14 levels were significantly higher in human patients with normal left ventricular ejection fraction compared to

those with impaired left ventricular ejection fraction. Furthermore, Zeng et al. (2019) demonstrated that hypermethylation of Iroquois homeobox 1 (IRX1) decreased CXCL14 which was associated with increased fibrosis and impaired cardiac function in rats. In the present study, CXCL14 was significantly decreased in the MMVD-TEST group compared to the MMVD-CONTROL group. Based on existing data, CXCL14 should be further investigated and validated in dogs with MMVD as a biomarker of disease progression.

There are a few limitations to this study. First, the sample size per treatment group was low due to the limited availability of dogs diagnosed with MMVD, and therefore, the study was underpowered. Additionally, early study removal was high, especially in the MMVD-CONTROL group. Three dogs were removed from the MMVD-CONTROL group, one dog from the MMVD-TEST group, and one dog from the HEALTHY-TEST group due to concurrent conditions. Following completion of the study, an upper confidence interval was calculated for a clinically meaningful change in serum NT-proBNP to evaluate the appropriate number of dogs needed per treatment group. The residual error from the linear mixed-model was 28,973, resulting in an estimated population standard deviation of 170.2. Based on the mean NT-proBNP at day 0 for the MMVD-CONTROL group, a NT-proBNP increase of < 350 pmol/L was considered to be clinically not significant. Using an alpha = 0.10, a standard deviation of 170.2 and a CI half-width of 350, it was determined that four animals per treatment group is sufficient to detect a change of 350 pmol/L. If the upper limit for the CI for change in NT-proBNP is less than this amount, then no clinically meaningful change in NT-proBNP will be concluded.

Additionally, enrolling dogs with other health conditions is a limitation as concurrent conditions could influence biomarker results, especially in exploratory studies using a canine immune response panel. Furthermore, the lack of diversity in breeds, breed sizes, and disease

severity is a limitation. Majority of the dogs were Beagles in this study, but MMVD is predominately observed in various small breed dogs. Finally, all of the dogs enrolled in this study were Stage B1 except one dog who was Stage B2 and none of the dogs progressed to the next stage during the study. Therefore, it would be beneficial to investigate the effects of the study foods on cardiovascular related biomarkers in a diverse population of dogs with more progressive stages of MMVD over a longer study duration.

Nutritional intervention is a key component of managing canine MMVD. However, there is very limited data observing the effects of nutrition on biomarkers in dogs with MMVD, but the results of this study contribute to the existing evidence of biomarkers for canine MMVD. The increase in blood taurine in the two MMVD treatment groups and in the two healthy treatment groups supports the concept that MMVD is not associated with taurine deficiency, particularly early-stage MMVD dogs as seen in the present study. Additionally, the maintained serum 25-hydroxyvitamin D levels in all four treatment groups suggests that early-stage MMVD dogs are not at risk of low serum vitamin D, but this should be further investigated for dogs with more severe stages of MMVD. Finally, the identified significant genes suggest that the MMVD-TEST group was associated with increased disease progression compared to the control group at the end of the 6-month feeding study. However, the identified genes need to be further validated using PCR since the nCounter® technique acts as a screening method and further investigation is warranted to understand if the study foods are affecting protein expression and/or post-translational modifications. Overall, a larger, longer, and more diverse study is needed to validate these results. Furthermore, additional work should be completed to identify reliable biomarkers in canine MMVD and to understand optimal levels of key nutrients and how they interact with each other to holistically support dogs diagnosed with MMVD.

Funding

This study was funded by Hill's Pet Nutrition.

Disclosures

MDA, LAM, and LH are current employees of Hill's Pet Nutrition.

Table 4.1. Effect of diet, day, disease, and all relevant interactions on clinical biomarkers of dogs with myxomatous mitral valve disease and healthy dogs after the washout period (day 0) and while fed the test food or control food (day 84 and day 168). Dogs with myxomatous mitral valve disease fed the test food or control food are labeled as the MMVD-TEST group and MMVD-CONTROL group, respectively. Healthy dogs fed the test food or control food are labeled as the HEALTHY-TEST group and HEALTHY-CONTROL group, respectively.

Biomarker	**Day 0**§		**Day 84**§		**Day 168**§			**P-value**						
	MMVD-TEST (n=4)	MMVD-CONTROL (n=5)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	SEM‡	Diet	Disease	Day	Diet x Disease	Diet x Day	Disease x Day	Diet x Day x Disease
Plasma Esterified Carnitine, µmol/L	17.5	17.1	31.3	21.8	42.3	27.6	7.1	0.41	0.98	<0.01	0.28	0.58	0.18	0.33
	HEALTHY-TEST (n=5)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	SEM‡							
	14.9	14.5	23.6	27.1	40.9	41.0	6.9							
Plasma Esterified to Free Carnitine Ratio	MMVD-TEST (n=4)	MMVD-CONTROL (n=5)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	SEM‡	0.99	0.88	<0.01	0.02	0.82	0.48	0.60
	0.93	0.80	1.09	0.83	1.66	1.08	0.28							
	HEALTHY-TEST (n=5)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	SEM‡							

	0.70	0.81	0.78	1.21	1.37	1.81	0.28							
Whole Blood Taurine, nmol/mL □Normal Reference Range: 200 – 350 nmol/L	MMVD-TEST (n=4)	MMVD-CONTROL (n=5)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	SEM‡	0.67	0.13	<0.01	0.94	0.25	0.77	0.94
	271.8	323.0	545.8	528.9	425.3	382.8	48.9							
	HEALTHY-TEST (n=5)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	SEM‡							
	225.3	270.0	466.5	467.2	374.3	382.8	40.2							
Urine Taurine, nmol/mL	MMVD-TEST (n=3)	MMVD-CONTROL (n=5)	MMVD-TEST (n=3)	MMVD-CONTROL (n=2)	MMVD-TEST (n=3)	MMVD-CONTROL (n=2)	SEM‡	0.40	0.03	0.84	0.81	0.28	0.34	0.11
	7906.7	3272.2	6442.0	7344.9	5502.7	7154.4	1958.2							
	HEALTHY-TEST (n=5)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=4)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=4)	SEM‡							

	4259.8	3584.0	3219.3	1976.3	5010.3	3256.5	1387.0							
Serum NT-proBNP, pmol/L •Normal Reference Range: < 900 pmol/L	MMVD-TEST (n=4)	MMVD-CONTROL (n=5)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	SEM‡	0.06	0.72	<0.01	0.30	0.86	0.41	0.83
	301.7	557.0	250.0	439.9	583.0	1009.0	321.4							
	HEALTHY-TEST (n=5)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	SEM‡							
	311.4	343.4	250.0	355.4	838.5	903.0	194.7							
Serum 25-Hydroxyvitamin D, nmol/L °Normal Reference Range: 112 - 366 nmol/L	MMVD-TEST (n=4)	MMVD-CONTROL (n=5)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	MMVD-TEST (n=4)	MMVD-CONTROL (n=2)	SEM‡	0.43	0.66	0.08	0.31	0.10	0.8	0.32
	271.9	220.6	241.3	281.2	224.0	213.7	54.5							
	HEALTHY-TEST (n=5)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	HEALTHY-TEST (n=4)	HEALTHY-CONTROL (n=5)	SEM‡							
	236.0	265.5	245.0	316.8	193.9	306.1	45.9							

§Values are represented as means; ‡Values are represented as pooled standard error of mean; NT-proBNP, N-terminal pro-B-type natriuretic peptide; □Normal reference range for whole blood taurine provided by the Amino Acid Laboratory at the University of Davis Weill School of Veterinary Medicine, Davis, CA, USA; •Normal reference range for NT-proBNP provided by Bionote, Big Lake, MN, USA; °Normal reference range for serum 25-hydroxyvitamin D provided by the Veterinary Diagnostic Laboratory at Michigan State University, Lansing, MI, USA.

Table 4.2. Average abundance of seven significantly upregulated ($P \leq 0.05$ and ≥ 1.5-fold change) genes in dogs with myxomatous mitral valve disease fed either the test food (MMVD-TEST group) compared to dogs with myxomatous mitral valve disease fed the control food (MMVD-CONTROL group) at the end of the 6-month feeding study (day 168).

Gene	**Abundance in MMVD-TEST Group**[§] (n=4)	**Abundance in MMVD-CONTROL Group**[§] (n=2)	**Fold Change**	**Unadjusted P-value**
MMP1	105.9 (103.2)	24.2 (6.6)	4.37	0.04
PPBP	18874.6 (7224.0)	9019.5 (273.8)	2.09	0.03
PRF1	117.6 (55.9)	57.4 (4.5)	2.05	0.03
GZMK	1333.1 (463.4)	674.5 (120.3)	1.98	0.05
CXCR6	317.8 (76.4)	181.1 (16.5)	1.75	0.01
CX3CR1	844.6 (174.1)	487.6 (51.0)	1.73	0.01
IL-1α	50.8 (11.1)	31.9 (3.8)	1.59	0.03

[§]Values are represented as mean (SE). MMP1, matrix metalloproteinase 1; PPBP, pro-platelet basic protein; PRF1, perforin 1; GZMK, granzyme K; CXCR6, C-X-C motif chemokine receptor 6; CX3CR1, C-X3-C motif chemokine receptor 1; IL-1α, interleukin 1 alpha.

Table 4.3. Average abundance of one significantly downregulated ($P \leq 0.05$ and $\leq$ -1.5-fold change) gene in dogs with myxomatous mitral valve disease fed either the test food (MMVD-TEST group) compared to dogs with myxomatous mitral valve disease fed the control food (MMVD-CONTROL group) at the end of the 6-month feeding study (day 168).

Gene	Abundance in MMVD-TEST Group[§]	Abundance in MMVD-CONTROL Group[§]	Fold Change	Unadjusted P-value
CXCL14	20.1 (0.1)	52.4 (4.2)	-2.62	0.04

[§]Values are represented as mean (SE). CXCL14, C-X-C motif chemokine ligand 14.

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Appendix A - Study Foods

Appendix Table A. Analyzed nutritional composition of the study foods on a caloric basis (unit/1000 kcal ME). The washout food was formulated for canine adult maintenance. The control and test foods were formulated to provide nutritional support for cardiovascular health in dogs with differing levels of protein, fat, L-carnitine, taurine, sodium, EPA and DHA.

Nutrient, unit/1000 kcal	Washout Food	Control Food	Test Food
Protein, g	46.2	44.9	52.2
Fat, g	30.4	48.9	51.8
Nitrogen-free extract, g	165.7	122.0	107.8
Total Dietary Fiber, g	13.9	21.3	20.3
Ash, g	10.7	8.9	9.9
Leucine, g	4.11	4.24	4.18
Lysine, g	2.81	3.11	3.20
Methionine, g	1.00	1.05	1.73
Tryptophan, g	0.53	0.56	0.76
Cystine, g	0.72	0.66	0.78
Taurine, g	0.33	0.32	0.34
L-Carnitine, mg	1.6	85	118
Sodium, g	0.6	0.3	0.4
Potassium, g	2.1	1.5	1.7
Calcium, g	1.9	1.3	1.6
Phosphorus, g	1.2	0.9	1.4

Calcium:Phosphorus	1.6	1.4	1.2
Magnesium, g	0.23	0.30	0.34
EPA, g	0.0	0.0	1.1
DHA, g	0.0	0.0	0.7
EPA+DHA, g	0.0	0.0	1.7
Omega-3 Fatty Acids, g	2.0	0.8	8.0
Omega-6 Fatty Acids, g	8.0	11.6	8.3
Vitamin D, IU	439	470	340

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IU, International Unit; kcal, kilocalorie; kg, kilogram; ppm, parts per million.

[a]Analyzed moisture content of the Washout, Control, and Test foods were 8.2%, 6.6%, and 8.0%, respectively.

[b]Metabolizable energy was calculated as 3596 kcal/kg, 4081 kcal/kg, and 4094 kcal/kg on an as is basis for the Washout, Control, and Test foods, respectively, using the Modified Atwater equation (ME kcal/kg = 10[(3.5 x protein) + (8.5 x fat) + (3.5 x nitrogen-free extract)].

[c]Ingredients for each food were as follows:

Washout: Brewers rice, whole grain corn, whole grain sorghum, whole grain wheat, chicken by-product meal, corn protein meal, egg product, chicken liver flavor, chicken fat, flaxseed, soybean oil, calcium carbonate, lactic acid, pork liver flavor, potassium chloride, L-lysine, choline chloride, sodium chloride, vitamin premix, taurine, and mineral premix.

Control: Whole grain corn, whole grain wheat, chicken fat, chicken by-product meal, cracked pearled barley, corn protein meal, soybean meal, chicken liver flavor, soybean oil, lactic acid, pork liver flavor, calcium carbonate, potassium citrate, L-lysine, choline chloride, L-carnitine, vitamin premix, taurine, DL-methionine, magnesium oxide, mineral premix, and L-tryptophan.

Test: Chicken, brown rice, whole grain wheat, brewers rice, chicken by-product meal, flaxseed, chicken fat, cracked pearled barley, egg product, pork liver flavor, fish oil, corn protein meal, whole grain corn, lactic acid, soybean meal, potassium citrate, calcium carbonate, L-carnitine, vitamin premix, L-lysine, DL-methionine, taurine, mineral premix, L-tryptophan, magnesium oxide.

Appendix B - Signalment Information

Appendix Table B. Signalment information of dogs with myxomatous mitral valve disease and healthy dogs at enrollment/start of washout (day -35). Dogs with myxomatous mitral valve disease assigned the test food or control food are labeled as the MMVD-TEST group and MMVD-CONTROL group, respectively. Healthy dogs assigned the test food or control food are labeled as the HEALTHY-TEST group and HEALTHY-CONTROL group, respectively.

Parameter	**MMVD-TEST Group**	**MMVD-CONTROL Group**	**HEALTHY-TEST Group**	**HEALTHY-CONTROL Group**
Body Weight (kg)§	7.7 (0.7)	9.5 (0.7)	8.3 (0.5)	9.7 (0.5)
Age (years)§	12.4 (1.1)	12.0 (1.0)	11.2 (0.7)	11.2 (0.7)
Heart Murmur Severity (6-point scheme)§	3.8 (0.4)	3.9 (0.4)	-	-
Male, n	2	2	2	2
Female, n	3	3	3	3

§Values are represented as mean (SE).

Appendix C - Medications

Appendix Table C. Medication, dosage, frequency, and duration given to dogs with myxomatous mitral valve disease and healthy dogs during the study. Dogs with myxomatous mitral valve disease fed the test food or control food are labeled as the MMVD-TEST group and MMVD-CONTROL group, respectively. Healthy dogs fed the test food or control food are labeled as the HEALTHY-TEST group and HEALTHY-CONTROL group, respectively.

Dog #	Medication	Dosage	Frequency	Start Day	Duration during study	Study Completion
MMVD-TEST Group						
Dog 1	Lactated ringers	150 mL	Once a day	Prefeed day -2	1 day	Yes
	Gabapentin compounded	100 mg	Once a day	Prior to enrollment	Ongoing	
	Maropitant injectable	0.9 mg/mL	Once a day	Pre-feed day -2	1 day	
	Prednisolone	2.5 mg	Twice a day	Pre-feed day -1	Ongoing	
	Antibacterial and antifungal ointment	1 dollop	Twice a day	Day 113 to day 122	10 days	
	Propofol	6.2 mg/kg	Once a day	Day 125	1 day	
	Compound buprenorphine	0.2 mL	Once a day	Day 125	1 day	
	Maropitant injectable	1.0 mg/mL	Once a day	Day 125	1 day	
	Acepromazine injectable	0.37 mL	Once a day	Day 125	1 day	
Dog 2	None					Yes
Dog 3	Firocoxib	57 mg	Once a day	Prefeed day -12 to day 64	76 days	Yes
	Acepromazine	5 mg	Twice a day	Prior to enrollment to day 14	49 days	
	Propofol	1.5 mg/kg	Once a day	Prefeed day -3	1 day	
	Butorphanol	0.3 mg/kg	Once a day	Prefeed day -3	1 day	
	Propofol	1.9 mg/kg	Once a day	Day 83	1 day	
	Butorphanol	0.3 mg/kg	Once a day	Day 83	1 day	

	Pimobendan compounded	2.5 mg	Twice a day	Day 103	Ongoing	
	Propofol	2.1 mg/kg	Once a day	Day 129	1 day	
	Butorphanol	0.3 mg/kg	Once a day	Day 129	1 day	
	Propofol	2.9 mg/kg	Once a day	Day 165	1 day	
	Butorphanol	0.3 mg/kg	Once a day	Day 165	1 day	
Dog 4	Firocoxib	28.5 mg	Once a day	Prior to enrollment	Ongoing	Yes
	Gabapentin compounded	50 mg	Twice a day	Prior to enrollment	Ongoing	
	Amantadine tablet	25 mg	Once a day	Day 116	Ongoing	
	Maropitant injectable	0.7 mL	Once a day	Day 149	1 day	
	Lactated ringers	150 mL	Once a day	Day 149	1 day	
	Butorphanol	0.3 mg/kg	Once a day	Day 165	1 day	
MMVD-CONTROL Group						
Dog 1	None					Yes
Dog 2	Trilostane	30 mg	Once a day	Prior to enrollment	Ongoing	Yes
	Phenylpropanolamine	12.5 mg	Once a day	Prior to enrollment	Ongoing	
	Pimobendan compounded	2.5 mg	Twice a day	Prior to enrollment	Ongoing	
	Methocarbamol	125 mg	Three times a day	Day 50 to day 61	12 days	
	Gabapentin compounded	100 mg	Three times a day	Day 50 to day 55	6 days	
	Gabapentin compounded	100 mg	Twice a day	Day 56 to day 69	14 days	
	Gabapentin compounded	100 mg	Once a day	Day 70 to day 83	14 days	
	Furosemide	10 mg	Once a day	Day 103	Ongoing	
	Maropitant	24 mg	Once a day	Day 168	3 days	
	Amoxicillin/Clavulanate potassium	125 mg	Twice a day	Day 168	7 days	
Dog 3	Pimobendan compounded	2.5 mg	Twice a day	Prior to enrollment	Ongoing	Removed on day 55
	Alprazolam	1 mg	Twice a day	Prior to enrollment	Ongoing	

	Gabapentin capsule	100 mg	Twice a day	Prefeed day -10 to day 34	45 days	
	Gabapentin capsule	300 mg	Twice a day	Day 35	Ongoing	
	Butorphanol	0.3 mg/kg	Once a day	Prefeed day -3	1 day	
	Firocoxib	57 mg	Once a day	Day 11 to day 17	7 days	
	Firocoxib	57 mg	Once a day	Day 49	Ongoing	
	Maropitant injectable	1.1 mL	Once a day	Day 50	1 day	
	Propofol	2.4 mg/kg	Once a day	Day 50	1 day	
	Compounded buprenorphine	0.2 mL	Once a day	Day 50	1 day	
	Lactated ringers	150 mL	Once a day	Day 50	1 day	
	Amoxicillin/Clavulanate potassium	125 mg	Twice a day	Day 51	Ongoing	
Dog 4	Metronidazole	125 mg	Twice a day	Prefeed day -9 to prefeed day -5	5 days	Removed on day 77
	Prednisolone	2.5 mg	Twice a day	Prior to enrollment to day 48	83 days	
	Prednisolone	5 mg	Twice a day	Day 49	Ongoing	
	Fenbendazole	5 mL	Once a day	Prefeed day -1 to day 4	5 days	
	Lactated ringers	150 mL	Once a day	Prefeed day -1	1 day	
	Gabapentin capsule	100 mg	Twice a day	Prior to enrollment	Ongoing	
	Metronidazole	125 mg	Twice a day	Day 43 to day 52	10 days	
	Mirtazapine	7.5 mg	Once a day	Day 43 to day 52	10 days	
	Mirtazapine	7.5 mg	Once a day	Day 70 to day 74	5 days	
Dog 5	Gabapentin compounded	50 mg	Twice a day	Prior to enrollment	Ongoing	Removed on day 29
	Carprofen	25 mg	Once a day	Prior to enrollment	Ongoing	
HEALTHY-TEST Group						
Dog 1	Gabapentin capsule	100 mg	Twice a day	Prior to enrollment	Ongoing	Yes

	Tobramycine ophthalmic solution without steroid	1 drop	Twice a day	Day 115 to 122	8 days	
	Triple antibiotic ophthalmic liquid with steroid	1 drop	Twice a day	Day 129 to day 136	8 days	
	Cyclosporine ophth	1 dollop	Twice a day	Day 136	Ongoing	
Dog 2	None					Yes
Dog 3	Maropitant	24 mg	Once a day	Day 149 to day 150	2 days	Yes
	Lactated ringers	250 mL	Once a day	Day 150	1 day	
Dog 4	None					Yes
Dog 5	Amoxicillin/Clavulanate potassium	125 mg	Twice a day	Prefeed day -20	Ongoing	Removed on day 41
	Gabapentin capsule	100 mg	Twice a day	Prefeed day -20 to prefeed day -18	3 days	
	Acepromazine injectable	0.2 mL	Once a day	Prefeed day -18	1 day	
	Propofol	5.1 mg/kg	Once a day	Prefeed day -18	1 day	
	Compounded buprenorphine	0.1 mL	Once a day	Prefeed day -18	1 day	
	Maropitant injectable	0.67 mL	Once a day	Prefeed day -18	1 day	
	Atropine ophth ointment	1 dollop	Once a day	Prefeed day -16	1 day	
	Gabapentin capsule	100 mg	Twice a day	Prefeed day -19 to prefeed day -5	15 days	
	Trazadone	50 mg	Once a day	Prefeed day -15	1 day	
	Gentamicin, mometasone, clotrimazole ear ointment	5 drops	Once a day	Prefeed day -14 to prefeed day -10	5 days	
	Firocoxib	28.5 mg	Once a day	Prefeed day -14	Ongoing	
	Trazadone	50 mg	Twice a day	Prefeed day -14 to prefeed day -10	5 days	
	Ofloxacin ophthalmic drops	1 drop	Three times a day	Prefeed day -10 to day 4	14 days	

	Optixcare eye lube	1 dollop	Three times a day	Prefeed day -10 to day 4	14 days	
	Gentamicin, mometasone, clotrimazole ear ointment	5 drops	Once a day	Prefeed day -1 to day 4	5 days	
	Terramycin ophthalmic ointment	1 dollop	Twice a day	Day 4 to day 28	25 days	
	Mirtazapine	7.5 mg	Once a day	Day 39 to day 41	3 days	
HEALTHY-CONTROL Group						
Dog 1	Gentamicin, mometasone, clotrimazole ear ointment	8 drops	Once a day	Day 112 to day 118	7 days	Yes
Dog 2	Propofol	0.5 mg/kg	Once a day	Day 108	1 day	Yes
	Atropine	1 mL	Once a day	Day 108	1 day	
	Meloxicam injectable	0.4 mL	Once a day	Day 108	1 day	
	Acepromazine injectable	0.5 mL	Once a day	Day 108	1 day	
	Meloxicam oral suspension	20 lb	Once a day	Day 109 to day 118	10 days	
Dog 3	Benazepril	2.5 mg	Once a day	Day 41to day 84	44 days	Yes
	Benazepril	2.5 mg	Twice a day	Day 85	Ongoing	
	Amoxicillin/Clavulanate potassium	125 mg	Twice a day	Day 128 to day 132	5 days	
Dog 4	Carprofen	37.5 mg	Once a day	Prior to enrollment	Ongoing	Yes
	Gabapentin capsule	300 mg	Twice a day	Prior to enrollment	Ongoing	
	Bedinvetmab	1 mL	Once a day	Prefeed day -9	1 day	
	Bedinvetmab	1 mL	Once a day	Day 19	1 day	
	Bedinvetmab	1 mL	Once a day	Day 47	1 day	
	Bedinvetmab	1 mL	Once a day	Day 75	1 day	
	Bedinvetmab	1 mL	Once a day	Day 103	1 day	
	Bedinvetmab	1 mL	Once a day	Day 131	1 day	
	Bedinvetmab	1 mL	Once a day	Day 158	1 day	

	Polysulfated glycosaminoglycan	0.35 mL	Once a day every 4 weeks	Day 27	Ongoing	
Dog 5	Trazadone	50 mg	Once a day	Day 111 to day 112	2 days	Yes
	Gabapentin capsule	200 mg	Once a day	Day 112	1 day	
	Cytopoint	1 mL	Once a day	Day 119	1 day	
	Methocarbamol	125 mg	Three times a day	Day 164	Ongoing	
	Gabapentin compounded	50 mg	Three times a day	Day 164	Ongoing	

Prefeed day -32, day 83, and day 165 are the days echocardiograms were performed for baseline (day 0), 3 months (day 84), and 6 months (day 168), respectively. Dogs were given routine heartworm prevention and Bordetella vaccines.

Appendix D - Food and Nutrient Intake

Appendix Table D. Average monthly food intake (grams/day) and average monthly nutrient intake (unit/kg of metabolic body weight (MBW)/day) of dogs with myxomatous mitral valve disease and healthy dogs fed the washout food (day 0) and test food or control food (day 28, day 56, day 84, day 112, day 140, and day 168). Dogs with myxomatous mitral valve disease fed the test food or control food are labeled as the MMVD-TEST group and MMVD-CONTROL group, respectively. Healthy dogs fed the test food or control food are labeled as the HEALTHY-TEST group and HEALTHY-CONTROL group, respectively.

n	Study Day	Metabolic Body Weight (kg)	Food Intake (g/day)	Protein Intake (g/kg of MBW/ day)	Fat Intake (g/kg of MBW/ day)	TDF Intake (g/kg of MBW/ day)	NFE Intake (g/kg of MBW/ day)	Sodium Intake (mg/kg of MBW/ day)	L-carnitine Intake (mg/kg of MBW/ day)	Taurine Intake (mg/kg of MBW/ day)	EPA Intake (mg/kg of MBW/ day)*	DHA Intake (mg/kg of MBW/ day)*
MMVD-TEST Group												
4	0	4.9	158.12	5.38	3.53	1.62	19.28	74.70	0.18	38.80	0.71	2.68
4	28	4.9	137.74	6.02	5.97	2.34	12.43	45.63	13.52	39.43	121.12	78.87
4	56	4.9	141.13	6.17	6.12	2.40	12.74	46.75	13.85	40.41	124.10	80.81
4	84	4.9	147.77	6.40	6.35	2.49	13.22	48.52	14.38	41.93	128.78	83.86
4	112	5.0	142.00	6.10	6.04	2.37	12.59	46.21	13.69	39.93	122.66	79.87
4	140	5.0	133.66	5.74	5.69	2.23	11.85	43.50	12.89	37.59	115.45	75.18
4	168	5.0	132.65	5.70	5.65	2.21	11.76	43.17	12.79	37.31	114.58	74.61
MMVD-CONTROL Group												
5	0	5.5	175.21	5.34	3.51	1.61	19.15	74.21	0.18	38.55	0.71	2.67
5	28	5.5	140.87	4.73	5.16	2.25	12.86	27.38	8.96	33.58	1.06	0.96
3	56	5.5	131.18	4.37	4.77	2.08	11.89	25.30	8.28	31.03	0.98	0.88
2	84	5.4	125.60	4.28	4.67	2.04	11.65	24.80	8.12	30.41	0.96	0.87

2	112	5.4	154.93	5.28	5.76	2.51	14.37	30.59	10.01	37.52	1.18	1.07
2	140	5.3	168.89	5.81	6.33	2.76	15.79	33.62	11.00	41.23	1.30	1.17
2	168	5.4	178.30	6.03	6.58	2.87	16.41	34.93	11.43	42.84	1.35	1.22
HEALTHY-TEST Group												
5	0	4.8	137.50	4.76	3.13	1.43	17.07	66.15	0.16	34.36	0.63	2.38
5	28	4.8	124.75	5.56	5.51	2.16	11.47	42.09	12.47	36.38	111.72	72.75
4	56	4.9	119.29	5.22	5.17	2.02	10.77	39.52	11.71	34.15	104.89	68.30
4	84	4.8	118.61	5.23	5.19	2.03	10.80	39.65	11.75	34.27	105.25	68.54
4	112	4.8	120.03	5.30	5.25	2.06	10.93	40.13	11.89	34.68	106.51	69.36
4	140	4.9	119.28	5.22	5.17	2.02	10.77	39.52	11.71	34.15	104.89	68.30
4	168	4.8	115.67	5.15	5.10	2.00	10.63	39.03	11.56	33.73	103.59	67.46
HEALTHY-CONTROL Group												
5	0	5.5	160.52	4.86	3.19	1.46	17.41	67.46	0.16	35.05	0.64	2.42
5	28	5.4	143.46	4.85	5.29	2.31	13.20	28.10	9.20	34.47	1.09	0.98
5	56	5.5	154.38	5.18	5.65	2.46	14.10	30.00	9.82	36.80	1.16	1.05
5	84	5.5	162.81	5.42	5.92	2.58	14.75	31.40	10.28	38.51	1.21	1.10
5	112	5.5	160.53	5.31	5.79	2.52	14.43	30.72	10.06	37.68	1.19	1.07
5	140	5.5	153.09	5.06	5.52	2.40	13.76	29.30	9.59	35.93	1.13	1.02
5	168	5.5	141.74	4.69	5.11	2.23	12.74	27.13	8.88	33.27	1.05	0.95

DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MBW, metabolic body weight; NFE, nitrogen-free extract, TDF, total dietary fiber; *Typical analysis value used for washout food and control food only since analyzed value was reported as <0.02%.