A pilot study investigating circulating trimethylamine N-oxide and its precursors in dogs with degenerative mitral valve disease with or without congestive heart failure

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Funding Information
Barkley Fund; Cummings School of Veterinary Medicine Companion Animal Health Fund

Background: Pathophysiologic mechanisms for the development and progression of degenerative mitral valve disease (DMVD) remain elusive. Increased concentrations of circulating trimethylamine N-oxide (TMAO) and its precursors choline and L-carnitine are associated with the presence and severity of heart disease in people.

Objectives: To determine if differences exist in plasma concentrations of TMAO, choline, or L-carnitine among dogs with DMVD and congestive heart failure (CHF), dogs with asymptomatic DMVD, and healthy control dogs.

Animals: Thirty client-owned dogs: 10 dogs with CHF secondary to DMVD, 10 dogs with asymptomatic DMVD, and 10 healthy control dogs.

Methods: A pilot cross-sectional study in which echocardiography was performed and fasting plasma concentrations of TMAO, choline, and L-carnitine (total and fractions) were measured.

Results: TMAO (P = .03), total L-carnitine (P = .03), carnitine esters (P = .05), and carnitine esters to free carnitine ratio (E/F ratio; P = .05) were significantly higher in dogs with CHF compared to those with asymptomatic DMVD. TMAO (P = .02), choline (P = .01), total L-carnitine (P = .01), carnitine esters (P = .02), free carnitine (P = .02), and E/F ratio (P = .009) were significantly higher in dogs with CHF compared to healthy controls.

Conclusions and Clinical Importance: Dogs with CHF secondary to DMVD had higher concentrations of TMAO compared to both asymptomatic DMVD dogs and healthy controls. Larger prospective studies are warranted to determine if TMAO plays a role in the development or progression of DMVD or CHF.

KEYWORDS
canine, carnitine, choline, congestive heart failure, heart, intestinal, microbiota

1 | INTRODUCTION

Degenerative mitral valve disease (DMVD) is the most common form of heart disease in the dog.1–3 The disease process has been well studied, but factors contributing to both underlying cause and disease progression remain elusive.3–6 A genetic predisposition exists for the disease (eg, Cavalier King Charles Spaniels, and Dachshunds),7–9 but several other factors have been postulated to play a role, including nutrition, inflammation, and neurohumoral factors, such as serotonin.5,6,10–12

Underlying mechanisms of cardiovascular (CV) disease in people also remain under investigation.13 Recently, the molecule trimethylamine N-oxide (TMAO) has received much attention because of its association with the presence, severity, and outcome of CV disease in people and in rodent models.14–22 TMAO is the oxidized product of trimethylamine, which is produced by gastrointestinal (GI) microbiota from certain dietary nutrients, including choline and L-carnitine (Figure 1).14,15
Increased plasma concentrations of TMAO, choline, and L-carnitine all have been shown to be associated with the presence and severity of CV disease in rodent models and people, and are independent predictors of adverse cardiac events and mortality. A recent meta-analysis found that patients with higher blood concentrations of TMAO had a pooled relative risk of 1.62 (95% confidence interval [CI]: 1.45–1.80) for major adverse CV events and 1.63 (95% CI: 1.36–1.95) for all-cause mortality. In addition, increased blood choline and L-carnitine concentrations both were associated with increased risk for adverse CV events. These associations all were independent of conventional CV risk factors and renal function. Although most studies in humans have focused on TMAO in coronary artery disease, TMAO has been shown to be increased in people with congestive heart failure (CHF) resulting from both ischemic and nonischemic heart disease. Results of 2 recent in vitro studies suggest that TMAO’s nonvascular adverse effects on the heart could be the result of decreased cardiomyocyte contractility and intracellular calcium flux or impaired myocardial energy metabolism. Alternatively, increased TMAO may not be a cause of CV pathology but may be the result of inflammation associated with CV disease.

The role of TMAO and its dietary precursors has not been reported in dogs with CV disease. Therefore, the aim of our pilot study was to determine if differences in plasma concentrations of TMAO, choline, or L-carnitine exist among dogs with DMVD and CHF, dogs with asymptomatic DMVD, and healthy control dogs. We hypothesized that dogs with DMVD would have higher circulating concentrations of TMAO, choline, and L-carnitine compared to healthy control dogs. In addition, we hypothesized that TMAO, choline, and L-carnitine concentrations would be higher in dogs with DMVD and CHF compared to dogs with asymptomatic DMVD.

### 2 MATERIALS AND METHODS

Client-owned dogs with CHF resulting from DMVD (DMVD/CHF; American College of Veterinary Medicine [ACVIM] Consensus Stage C or D) and with asymptomatic DMVD (ACVIM Stage B) were recruited for enrollment in this cross-sectional pilot study. Healthy control dogs (ACVIM Stage A) were recruited from veterinary students and staff and were age-matched to those in the asymptomatic DMVD group. The protocol was approved by the Cummings School of Veterinary Medicine Clinical Studies Review Committee, and written informed consent was obtained from owners before enrollment. The minimum body weight for inclusion was 4 kg. Owners were questioned regarding all medications (including antimicrobials) and supplements administered. Dogs that had received antimicrobial medication within the past 4 weeks were excluded. Dogs currently receiving other medications (except monthly parasite preventative medication or cardiac medications for dogs with DMVD), dietary supplements (except glucosamine, chondroitin, or fish oil), or veterinary diets for noncardiac disease were excluded. Other exclusion criteria included serum creatinine concentration ≥2.1 mg/dL, other known major systemic diseases, such as cancer or diabetes, and other acquired or congenital cardiac structural abnormalities. Healthy dogs that were receiving no medications or dietary supplements (other than those described above) also were enrolled. Owners completed a dietary history form that included the specific flavor and brand of diet, treats, table foods, and foods used to administer medication. The diet that comprised the greatest proportion of calorie intake was named as the “main diet” and used for further analysis. If 2 diets made up equal parts of a dog’s caloric intake, an average of the 2 diets was used.

All dogs were assessed by physical examination, body condition score (on a 1-9 scale), muscle condition score (normal or mild, moderate, or severe muscle loss), and echocardiography (Vivid E9; GE Medical Systems, Milwaukee, Wisconsin). The diagnosis of DMVD was based on signalment, a left apical systolic murmur, typical changes to the mitral valve leaflets on echocardiography, and the presence of mitral regurgitation on color-flow Doppler. To be included in the study, dogs with DMVD had to have at least a grade 3 of 6 systolic murmur and an evidence of left atrial dilatation, defined as a left atrial-to-aortic root ratio of ≤1.6 on 2-dimensional echocardiography. Dogs were classified as having CHF based on a combination of clinical signs, echocardiography, and current or historical radiographic evidence of cardiogenic pulmonary edema. Echocardiography was performed using standard techniques by a board-certified veterinary cardiologist or a supervised cardiologist resident.

Dogs were fasted before their study visit for ≥8 hours. Blood (10 mL total) was collected by venipuncture into ethylenediaminetetraacetic acid tubes (9 mL for CBC, TMAO, choline, and carnitine fractions) and a serum separator tube (1 mL for biochemistry profile). The CBC and biochemistry profile were performed immediately to determine eligibility. All other blood was centrifuged within 30 minutes and separated, and plasma was divided into 4 aliquots and frozen at −80°C until analysis. Blood for TMAO and choline analysis was shipped as a single batch on dry ice to a commercial laboratory (Metabolic Molecular Phenotyping Core-Kannapolis Facilities, Kannapolis, North Carolina). Quantification of TMAO and choline was performed using liquid chromatography-stable isotope dilution-multiple reaction monitoring mass spectrometry as previously published. Blood for analysis of carnitine fractions was shipped as a single batch on dry ice to a commercial laboratory (Metabolic Analysis Labs, Madison, Wisconsin). Total L-carnitine, free carnitine, carnitine esters, and carnitine esters to
free carnitine ratio (E/F ratio) were measured using a radioenzymatic technique according to a previously published protocol.29

The amount of choline in each dog's main diet was obtained from the manufacturers on a mg/1000 kcal basis (or obtained on a percentage basis and converted to mg/1000 kcal using the diet's caloric density). The choline content of other components of the diet (eg, treats, table food, and foods to give medications) was not assessed. The amount of L-carnitine in diets was not readily available, and therefore dietary L-carnitine content was not compared among groups.

2.1 | Statistical analysis

Data were examined graphically and using Shapiro-Wilk tests. Normally distributed data are presented as mean ± SD, and skewed data are presented as median (range). Normally distributed data (eg, dietary choline concentrations) were compared among the 3 groups (ie, DMVD/CHF, asymptomatic DMVD, and healthy controls) using ANOVA with Tukey's post hoc tests, and skewed data (eg, plasma TMAO, choline, and L-carnitine fractions) were compared using Kruskal-Wallis tests with Dwass-Steel-Crithlow-Fligner tests for pairwise comparisons. Categorical variables are compared among groups using chi square tests, and Spearman correlation coefficients were used to compare blood analytes and echocardiographic variables, as well as dietary choline. All statistical analyses were performed using a commercial statistical software (Systat 13.0; Systat Software, Inc., San Jose, California), and P ≤ .05 was considered statistically significant.

3 | RESULTS

3.1 | Animals

Thirty dogs were enrolled, with 10 dogs in each of the 3 groups: DMVD/CHF (ACVIM Stage C or D [n = 10]), asymptomatic DMVD (ACVIM Stage B [n = 10]), and healthy controls (ACVIM Stage A [n = 10]). None of the dogs in the DMVD/CHF group was overtly dyspneic at the time of blood sampling. No significant differences in breed, weight, body condition score, muscle condition score, or sex distribution were found among the groups (Table 1). Age was significantly higher in the DMVD/CHF group compared to the asymptomatic DMVD group, but neither the asymptomatic DMVD and control groups nor the DMVD/CHF and control groups were significantly different from one another. Echocardiographic findings were typical of dogs with DMVD (Table 2). Serum creatinine concentration was not significantly different among groups (Table 3).

Compared to controls, dogs with DMVD/CHF had significantly higher TMAO (P = .02; Figure 2), choline (P = .01; Figure 3), total L-carnitine (P = .01; Figure 4), free carnitine (P = .02), carnitine esters (P = .02), and E/F ratio (P = .009). Compared to dogs with asymptomatic DMVD, dogs with DMVD/CHF had significantly higher TMAO (P = .03; Figure 2), total L-carnitine (P = .03; Figure 4), carnitine esters (P = .05), and E/F ratio (P = .05). Dogs with asymptomatic DMVD were not significantly different from the controls for any of the analytes (Figures 2–4). Age was significantly associated with TMAO (r = 0.532, P = .002), total carnitine (r = 0.468, P = .009), and free carnitine (r = 0.444, P = .01). Serum creatinine concentrations were not associated with any of the analytes.

Only a single dog enrolled in the study was receiving any of the permitted dietary supplements: 1 control dog was receiving fish oil and no dogs were receiving glucosamine or chondroitin. Three dogs each in the asymptomatic DMVD and DMVD/CHF groups were eating a cardiac diet (Royal Canin Veterinary Diet Early Cardiac; Royal Canin USA, Inc, St. Charles, Missouri). Based on the diet history, treats and table food made up only a small percentage of any dog's diet. Choline content of the dogs' main diets was not significantly different among the 3 groups (P = .99). No significant correlations were identified between choline content of the dogs' main diets and blood analytes (all P > .05).

Several significant correlations were found between echocardiographic measurements and blood analytes: left ventricular internal dimension in diastole (LVIDd) was significantly correlated with TMAO (r = 0.383, P = .04), total carnitine (r = 0.577, P = .001), free carnitine (r = 0.560, P = .001), and carnitine esters (r = 0.457, P = .01); normalized LVIDd was significantly correlated with choline (r = 0.434, P = .02), total carnitine (r = 0.446, P = .01), free carnitine (r = 0.385, P = .04), carnitine esters (r = 0.527, P = .003), and E/F ratio (r = 0.461, P = .01); left ventricular internal dimension in systole (LVIDs) was significantly correlated with free carnitine (r = 0.365, P = .05); the ratio of the left atrium to aorta was correlated with carnitine esters (r = 0.486, P = .006) and E/F ratio (r = 0.470, P = .009); and fractional shortening was significantly associated with choline (r = 0.430, P = .02). On multivariate analyses, however, only the groups (ie, DMVD/CHF, asymptomatic DMVD, control) remained significantly associated with these echocardiographic measurements.

4 | DISCUSSION

The finding that dogs with DMVD/CHF have higher TMAO concentrations compared to healthy controls is consistent with studies in human patients with CHF.16,21 In addition, TMAO concentrations were higher in dogs with DMVD/CHF compared to those with asymptomatic DMVD, which also is consistent with studies comparing people with CHF to those with asymptomatic heart disease.16,22 Various explanations have been postulated for the relationship between TMAO and CV disease, including contributions to cardioenal dysfunction, influences on cholesterol metabolism, suppression of bile acid synthesis, effects on platelet reactivity, and changes in Gl permeability resulting from congestion.14–19,21,30 It remains unknown whether increases in blood concentrations of TMAO are a cause or effect of worsening CV disease and CHF, but PO supplementation of TMAO and its precursors has been shown to induce atherosclerotic CV disease in rodents.14,20 Given the findings of our study, in which no significant difference in TMAO concentrations was found between dogs with asymptomatic DMVD and healthy controls, it is possible that increases in TMAO concentrations were a result of CHF, rather than the underlying DVMD. However, ours was a pilot study with a small number of dogs, and additional research is needed to answer this question.

In addition to higher TMAO concentrations, concentrations of TMAO precursors, L-carnitine, and choline were higher in dogs with DMVD/CHF compared to healthy controls, which also is consistent
with studies in people.16,31 Furthermore, total carnitine, carnitine esters, and E/F ratio were significantly higher in dogs with DMVD/CHF compared to those with asymptomatic DMVD. In people, increased carnitine esters and E/F ratio have been shown to be associated with the presence of heart failure, increased risk for worsening heart failure, and CV-related death.31-33 In contrast to studies in humans,16 our results did not show a difference in choline concentrations between dogs with DMVD/CHF and those with asymptomatic DMVD. This finding may be related to the small sample size of our study, to species differences, or to dietary factors.

Dietary habits of humans have been shown to affect not only blood concentrations of choline and L-carnitine but also the capacity to generate TMAO after being subjected to increased PO L-carnitine.34 In our study, a significant correlation was not found between choline content of the dogs’ main diet and plasma choline or TMAO. This finding may be related to our inability to calculate total dietary choline for the dogs, because their diets were comprised of not only a single dog food but sometimes several dog foods, treats, table food, and foods used to administer medications. L-Carnitine content of the diet was even more difficult to obtain from manufacturers because it is not an essential...
nutrient (as is choline) and most manufacturers do not measure it. Interestingly, in people, no clear-cut relationship exists between dietary choline or 1-carnitine and incidence of CV disease or circulating TMAO concentrations,34–37 which indicates a more complex process involving interplay between diet and other variables. Furthermore, even the relationship between plasma choline or 1-carnitine concentrations and CV disease in people appears to be dependent on concurrent increases in plasma TMAO concentrations.14,15

Although not yet studied in dogs, 1 factor that may influence the relationship between diet and CV disease in humans is the GI microbiota.14–22 In vivo TMAO production is regulated not only by dietary intake of nutrients such as choline and 1-carnitine but also by the composition of an individual’s GI bacteria. Several studies have documented an abrupt and significant decrease in TMAO concentrations during antimicrobial administration both in humans and rodents,14,15,21,38 consistent with a role for GI bacteria in TMAO production. Furthermore, modification of diet can rapidly alter the GI microbiome in people.39,40 Therefore, both diet and manipulation of the GI microflora offer potential targets for lowering TMAO concentrations in humans, which in turn could affect the risk of adverse CV events. However, additional research is necessary to determine whether TMAO truly has a pathogenic role in heart disease in humans and if there is any possible relevance for dogs with DMVD.

Our study had a number of limitations that are important to consider. One limitation is small sample size. Our study was designed as a pilot study, and although significant differences were found among the groups in blood analytes, we did not have adequate statistical power to measure associations for blood analytes with potential confounding factors, such as medications or severity of disease. In addition, blood analytes only were measured at a single time point. Changes in TMAO over time could provide valuable information. Larger, prospective studies with multiple time points and long-term follow-up would enable the evaluation of prognostic value and the relationships between concentrations of the various metabolites. In addition, diets were not standardized and we did not measure dietary intake of choline or 1-carnitine and were not able to obtain information on dietary concentrations of 1-carnitine, which also could have affected the results. Only choline concentrations in the main diet were collected, and the choline content of other components of the diet (eg, treats, table food, and foods used for medication administration) was not evaluated. In addition, choline content of the diets relied on manufacturers’ information, rather than on analysis.

### TABLE 2 Two-dimensional echocardiographic variables of dogs with degenerative mitral valve disease and congestive heart failure (DMVD/CHF), asymptomatic DMVD, or healthy controls (Controls)

| Variable | Controls | DMVD | DMVD/CHF | P-value |
|----------|----------|------|----------|---------|
| IVSd     | 0.74 ± 0.12 | 0.72 ± 0.11 | 0.73 ± 0.10 | .85     |
| LVIDd    | 2.73 ± 0.68<sup>b</sup> | 3.41 ± 0.57<sup>ab</sup> | 3.79 ± 1.10<sup>a</sup> | .02     |
| LVWd     | 0.73 ± 0.11 | 0.64 ± 0.10 | 0.72 ± 0.12 | .18     |
| IVSs     | 1.03 ± 0.15 | 1.10 ± 0.18 | 1.18 ± 0.13 | .13     |
| LVIDs    | 1.67 ± 0.54 | 1.82 ± 0.41 | 1.84 ± 0.73 | .76     |
| LVWs     | 1.05 ± 0.20 | 1.21 ± 0.15 | 1.15 ± 0.18 | .14     |
| Left atrium : aorta | 1.60 ± 0.30<sup>b</sup> | 2.41 ± 0.62<sup>a</sup> | 2.53 ± 0.46<sup>b</sup> | <.001   |
| Fractional shortening (%) | 40.6 ± 8.5<sup>b</sup> | 47.0 ± 7.7<sup>ab</sup> | 52.4 ± 8.9<sup>a</sup> | .01     |
| Normalized LVIDd | 1.46 ± 0.18<sup>a</sup> | 1.95 ± 0.24<sup>b</sup> | 2.03 ± 0.34<sup>b</sup> | <.001   |
| Normalized LVIDs | 0.77 ± 0.24 | 0.96 ± 0.18 | 0.93 ± 0.28 | .19     |

Abbreviations: IVSd/s, interventricular septal thickness in diastole/systole; LVIDd/s, left ventricular internal diameter in diastole/systole; LVWd/s, left ventricular free wall thickness in diastole/systole. Normalized left ventricular internal dimension in systole and diastole from M-mode measurements also are listed.41 Data are presented as mean ± SD. All variables are in centimeters unless otherwise noted. The P-value is for comparison among the 3 groups. Variables with different superscript letters within a row are significantly different from one another.

### TABLE 3 Laboratory results for dogs with degenerative mitral valve disease and congestive heart failure (DMVD/CHF), asymptomatic DMVD, or healthy controls (Controls)

| Variable | Controls | DMVD | DMVD/CHF | P-value |
|----------|----------|------|----------|---------|
| Creatinine (mg/dL) | 0.8 ± 0.1 | 0.9 ± 0.3 | 1.1 ± 0.3 | .08     |
| TMAO (μmol/L) | 3.2 (1.3-9.6)<sup>b</sup> | 4.2 (0.8-7.3)<sup>a</sup> | 8.3 (1.6-35.6)<sup>a</sup> | .01     |
| Choline (μmol/L) | 3.7 (2.5-6.1)<sup>b</sup> | 4.4 (3.2-6.7)<sup>ab</sup> | 5.3 (3.5-10.7)<sup>a</sup> | .01     |
| Carotinene | 36.7 (28.3-49.7)<sup>b</sup> | 44.5 (24.6-68.9)<sup>b</sup> | 69.3 (13.7-134.5)<sup>a</sup> | .004    |
| Free (μmol/L) | 29.5 (20.4-42.8)<sup>b</sup> | 33.3 (18.4-49.4)<sup>ab</sup> | 45.2 (9.0-73.8)<sup>a</sup> | .01     |
| Esters (μmol/L) | 7.7 (5.9-10.5)<sup>b</sup> | 9.8 (6.2-19.1)<sup>b</sup> | 23.1 (4.7-60.7)<sup>a</sup> | .006    |
| E/F ratio | 0.29 (0.16-0.40)<sup>b</sup> | 0.34 (0.20-0.52)<sup>b</sup> | 0.53 (0.21-0.82)<sup>a</sup> | .004    |

Abbreviations: E/F ratio, carotinene to free carotinene ratio; TMAO, trimethylamine N-oxide. Data are presented as mean ± SD for creatinine and median (range) for all other variables. The P-value is for comparison among the 3 groups. Variables with different superscript letters within a row are significantly different from one another.
Although no significant difference was found in age between dogs with asymptomatic DMVD and healthy controls or between DMVD/CHF and controls, dogs with DMVD/CHF were significantly older than dogs with asymptomatic DMVD, which is typical for this disease. In studies of humans, age has been found to be correlated with concentrations of TMAO and its precursors, but the relationship of TMAO with CV disease persists after adjustment for age and other traditional CV risk factors.\textsuperscript{14–17,19,21,22,31,33} However, future studies are needed in dogs to identify whether age is a confounding factor in the relationship. Finally, because studies in people consistently show an inverse relationship between renal function and TMAO,\textsuperscript{16–18,22} more specific measures of renal function in these dogs would have been desirable (eg, glomerular filtration rate). However, our study selected dogs without overt renal dysfunction as part of the inclusion criteria, no difference in serum creatinine concentrations was found among groups, and no significant correlation was identified between TMAO concentrations and serum creatinine concentrations.

Despite these limitations, our results showed that circulating concentrations of TMAO and its precursors, choline and \( \text{L-} \)carnitine, are higher in dogs with DMVD/CHF compared to healthy dogs, and concentrations of TMAO and \( \text{L-} \)carnitine are higher in dogs with DMVD/CHF compared to those with asymptomatic DMVD. It remains unknown whether increased concentrations of TMAO, choline, and \( \text{L-} \)carnitine represent a cause or effect of either DMVD or CHF. Therefore, future research on interactions between diet and the GI microbiota is warranted to determine whether TMAO plays a potential role in the development or progression of DMVD or CHF or if it is merely a result of these conditions.
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How to cite this article: Karlin ET, Rush JE, Freeman LM. A pilot study investigating circulating trimethylamine N-oxide and its precursors in dogs with degenerative mitral valve disease with or without congestive heart failure. J Vet Intern Med. 2019;33:46-53. https://doi.org/10.1111/jvim.15347