Original Article

Choline Diet and Its Gut Microbe–Derived Metabolite, Trimethylamine N-Oxide, Exacerbate Pressure Overload–Induced Heart Failure

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Background—Trimethylamine N-oxide (TMAO), a gut microbe–dependent metabolite of dietary choline and other trimethylamine-containing nutrients, is both elevated in the circulation of patients having heart failure and heralds worse overall prognosis. In animal studies, dietary choline or TMAO significantly accelerates atherosclerotic lesion development in ApoE-deficient mice, and reduction in TMAO levels inhibits atherosclerosis development in the low-density lipoprotein receptor knockout mouse.

Methods and Results—C57BL6/J mice were fed either a control diet, a diet containing choline (1.2%) or a diet containing TMAO (0.12%) starting 3 weeks before surgical transverse aortic constriction. Mice were studied for 12 weeks after transverse aortic constriction. Cardiac function and left ventricular structure were monitored at 3-week intervals using echocardiography. Twelve weeks post transverse aortic constriction, myocardial tissues were collected to evaluate cardiac and vascular fibrosis, and blood samples were evaluated for cardiac brain natriuretic peptide, choline, and TMAO levels. Pulmonary edema, cardiac enlargement, and left ventricular ejection fraction were significantly ($P<0.05$, each) worse in mice fed either TMAO- or choline-supplemented diets when compared with the control diet. In addition, myocardial fibrosis was also significantly greater ($P<0.01$, each) in the TMAO and choline groups relative to controls.

Conclusions—Heart failure severity is significantly enhanced in mice fed diets supplemented with either choline or the gut microbe–dependent metabolite TMAO. The present results suggest that additional studies are warranted examining whether gut microbiota and the dietary choline→TMAO pathway contribute to increased heart failure susceptibility. **(*Circ Heart Fail*. 2016;9:e002314. DOI: 10.1161/CIRCHEARTFAILURE.115.002314.)**

Key Words: choline ■ fibrosis ■ metabolomics ■ pulmonary edema ■ ventricular dysfunction, left

Cardiovascular disease (CVD) represents the leading cause of death worldwide. A known environmental risk factor for the development of CVD is a diet rich in lipids and animal products[1]. In the past several years, a role for gut microbiota in the pathogenesis of CVD has been documented.[2–5] The interplay between diet and cardiovascular health has been well studied, establishing that diets high in fat and lipids lead to poor cardiac outcomes.[1] Although historically a causal role for the dietary nutrients cholesterol and fat has been the primary focus, more recent studies have highlighted additional contributory roles of alternative trimethylamine-containing dietary nutrients (eg, choline, phosphatidylcholine, and carnitine) and gut microbial metabolism.[6] Recent studies have shown that there is a mechanistic link between gut microbe–dependent metabolism of dietary choline, phosphatidylcholine, carnitine, and CVD pathogenesis.[2,4,5,7] Specifically, gut microbe metabolism of these dietary nutrients results in production of trimethylamine, which is rapidly converted by host hepatic flavin monooxygenase 3 into trimethylamine N-oxide (TMAO). Involvement of this metaorganismal (gut microbe and host) pathway in both TMAO formation and CVD pathogenesis includes studies that demonstrate the choline-, gut microbiota- and TMAO-dependent acceleration in atherosclerosis in animal models.[2,4,7] Furthermore, reduction in TMAO levels through antisense oligonucleotide mediated inhibition in host hepatic flavin monooxygenase 3 significantly inhibited atherosclerosis development in the low-density lipoprotein receptor null mouse model, and the liver insulin receptor knockout

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mouse model.[8] Numerous mechanistic investigations demonstrate a central role for the choline→TMAO metaorganismal pathway in cholesterol and sterol metabolism, macrophage phenotype, and reverse cholesterol transport.[2,7,8,9] Importantly, recent microbial transplantation studies confirmed that both TMAO production and atherosclerosis susceptibility are transmissible traits.[5]

Clinical studies have substantiated many of the above findings linking gut microbes and TMAO production to CVD. First, the original discovery of the pathway began with an unbiased metabolomics study of plasma from subjects undergoing elective cardiac evaluations, revealing a striking association between plasma levels of choline, TMAO, and betaine with cardiovascular risks among subjects (n=1876).[2] Examination of sequential subjects (n=1020) undergoing elective cardiac diagnostic catheterization shows significantly increased circulating levels of TMAO in patients with more extensive angiographic evidence of coronary artery disease.[2] Furthermore, in a distinct cohort of subjects undergoing diagnostic cardiac catheterization (n=4007), elevated TMAO levels independently predicted increased risk of adverse cardiovascular outcomes, including heart attack, stroke, and death risk.[3] Elevated TMAO levels have also been shown to herald worse overall prognosis among subjects with (and without) impaired kidney function, and to predict enhanced atherosclerotic burden.[10,11] Recently, TMAO levels have been shown to be associated with a 3-fold enhanced risk for prevalent CVD among a community-based multiethnic population (hazard ratio, 3.17; 95% confidence interval, 1.05–9.51).[12] Moreover, elevated TMAO plasma levels were reported in subjects (n=720) with stable heart failure (HF), with elevated levels predicting increased 5-year mortality risks (hazard ratio, 2.2; 95% confidence interval, 1.4–3.4), an association that remained significantly independent of traditional risk factors and cardiorenal indices.[13] Elevated TMAO levels were also reported to predict increased risk of HF among both diabetic subjects (hazard ratio 4.6; 95% confidence interval, 2.0–10.7) and nondiabetic (hazard ratio, 1.9; 95% confidence interval, 1.1–3.4) subjects alike.[14] In further studies, elevated TMAO levels were observed among subjects (n=112) with extensive serial echocardiography, and similarly were associated with more advanced left ventricular (LV) diastolic dysfunction and predicted poorer long-term adverse clinical outcomes independent of cardiac and renal biomarkers.[15] To date, no studies have tested whether dietary choline or TMAO directly promote the development and progression of cardiac dysfunction and HF. In the present study, we investigated the effects of dietary choline or TMAO in the setting of cardiac hypertrophy and HF after transverse aortic constriction (TAC).

Methods

Experimental Animals

C57BL6/J male mice aged 10 to 12 weeks (Jackson Laboratories, Bar Harbor, ME) were utilized for these studies. All animals were housed and maintained in an onsite temperature-controlled animal facility adhering to a 12-hour light/dark cycle and provided with water and rodent chow ad libitum. All animals were humanely cared for in accordance with the Principles of Laboratory Animal Care dictated by the National Society of Medical Research and the Guide for the Care and Use of Laboratory Animals by the National Institutes of Health (Publication No. 85-23, Revised 1996). All animal procedures were approved by Institutional Animal Care and Use Committee of both The Louisiana State University Health Sciences Center and the Cleveland Clinic.

Experimental Diets

Mice were maintained on either a chemically defined control diet (TD.130104), a diet containing 0.12% TMAO added to the standard rodent chow (TD.07865), or a diet containing 1.2% choline added to the standard rodent chow (TD.09041) from Harlan Laboratories, Madison, WI as described previously. These diets were initiated at 3 weeks prior to TAC surgery and maintained for an additional 12 weeks.

TAC Protocol

Cardiac pressure overload and HF were induced using TAC surgery as described previously.[16] The complete experimental protocol for these studies is depicted in Figure 1. Mice were anesthetized using xylazine (2 mg/kg) and ketamine (20 mg/kg) and maintained under anesthesia throughout the procedure by additional administration of xylazine (2 mg/kg) and ketamine (20 mg/kg) as needed. Mice were intubated and mechanically ventilated during the procedure using a Hugo Sachs type 845 minivent. Chest hair was removed from the mice by application of hair removal agent. The area was then cleaned using betadine and alcohol wipes alternating 3×. An incision was made proximally left of the midline of the chest, below the clavicle. The aorta was exposed using blunt dissection. A ligature was placed between the brachiocephalic artery and the left common carotid artery and secured to induce pressure overload. The chest was closed in layers, closing the muscle layer first, then the skin. Mice were removed from ventilation after pedal reflex returned, and it was determined that respiration could be maintained autonomously. Mice were placed in a recovery cage and supplied with 100% O_2 until normal behavior returned.

Echocardiography Assessment

One week before TAC procedure, baseline transthoracic echocardiogram was performed using 30-MHz probe on a Vevo 2100 (Visualsonics) while under light anesthesia with isoflurane (0.25%–0.50%) supplemented with 100% O_2, as described previously.[16] After TAC procedure, echocardiography was performed for 12 weeks at 3-week intervals.

Measurements of Serum Brain Natriuretic Peptide

Brain-type natriuretic peptide (BNP EIA kit; Phoenix Pharmaceuticals, Inc.) serum levels were determined by ELISA at 12 weeks after TAC as described previously.[16]

Histology and Immunochemistry

Hearts were collected at the specified time points and fixed in 10% buffered formalin, embedded in paraffin, and stained with either Masson trichome or Picrosirius red to quantify fibrosis as described previously.[16]

Quantification of TMAO, Choline, Betaine, and Carnitine

Stable isotope dilution liquid chromatography-tandem mass spectrometry (LC/MS/MS) was used for quantification of the total choline, TMA, TMAO, carnitine, and betaine contents of diets, and the concentration of these analytes in plasma, as previously described.[4,17] Briefly, TMAO, choline, betaine, and carnitine were monitored in positive MRM MS mode using characteristic precursor–product ion transitions: *m/z* 76→58, *m/z* 104→60, *m/z* 118→59, and *m/z* 162→60, respectively. The internal standards d9(trimethyl)TMAO (d9-TMAO), d9(trimethyl)choline (d9-choline), d9(trimethyl)betaine, and d3(methyl)carnitine (d3-carnitine) were

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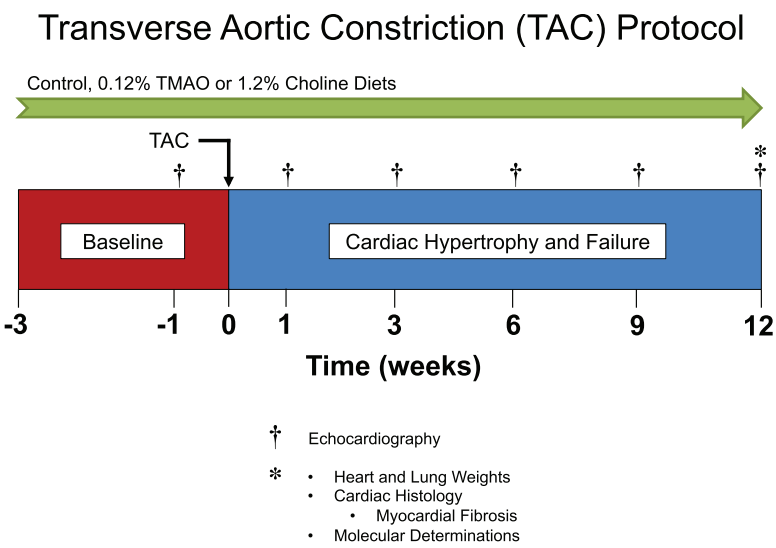

Figure 1. Experimental protocol for transverse aortic constriction (TAC) studies in mice fed either control diet, trimethylamine N-oxide (TMAO), or choline. Mice were fed experimental diets starting at 3 wk before TAC surgery and left ventricular function and structure were evaluated via 2-dimensional echocardiography at 3-wk intervals after baseline echocardiography studies at 1 wk before TAC surgery. On completion of the study, mice were euthanized at 12 wk for heart and lung weights, cardiac histology, and pathology as well as molecular determinations.

added to serum samples before protein precipitation and were similarly monitored in MRM mode at *m/z* 85→68, *m/z* 113→69, *m/z* 127→68, and *m/z* 165→63, respectively. Various concentrations of TMAO, choline, betaine, and carnitine standards and a fixed amount of internal standards were spiked into control serum to prepare the calibration curves for quantification of serum analytes.

Statistical Analysis

All data are presented as median (interquartile range). Kruskal–Wallis or Wilcoxon–Rank sum test was used for between-group comparisons. A linear mixed-effect model was used for repeated measure data to examine the change of all parameters during the 12-week study period. All analyses were performed using R 3.1.2 (Vienna, Austria), and $P<0.05$ were considered statistically significant.

Results

Impact of Dietary TMAO or Choline on Plasma Levels of Metabolites

Analysis of plasma from mice in all groups at 12 weeks post TAC revealed that mice fed either TMAO or choline had significant ($P<0.0001$) increase in the level of circulating TMAO when compared with mice fed a control diet (Table). We also observed significant increases ($P=0.037$) in plasma betaine levels in the mice fed TMAO.

Dietary TMAO Exacerbates Cardiac Dilatation and LV Dysfunction After TAC in Mice

We evaluated the relationship between dietary TMAO and the pathogenesis of HF after TAC in mice. C57BL6/J mice were fed either standard chow diet, diet containing 0.12% TMAO, or diet containing 1.2% choline starting 3 weeks before TAC procedure and maintained on diets for 12 weeks. (Figure 1). Chronic HF was produced by TAC resulting in pressure overload and cardiac hypertrophy. Both groups displayed significant ($P<0.0001$ for LV end-systolic diameter [LVESD]; $P<0.0001$ for LV end-diastolic diameter [LVEDD]; $P=0.0382$ for intraventricular septal wall diastolic diameter; and $P<0.0001$ for LV ejection fraction [LVEF]) LV dilation, hypertrophy, and reduced LVEF when compared with baseline. Mice that consumed 0.12% TMAO exhibited significantly worse cardiac function in all parameters measured after TAC (Figure 2) when compared with mice fed the control diet. An accelerated progression of cardiac dilatation, as evidenced by significantly increased LVESD and LVEDD, was observed in the TMAO group (Figure 2A and 2B). LVESD was significantly greater at 1, 3, and 6 weeks ($P=0.0013$, $P=0.0268$, and $P=0.0268$ versus control, respectively) after TAC. LVEDD was trending toward an increase in the TMAO group versus control group at 6 weeks post TAC ($P=0.0595$). In addition, we also observed worsened adverse ventricular remodeling as determined by interventricular septal dimension ($P=0.0056$ and $P=0.0045$ versus control) at 3 and 6 weeks (Figure 2C). Importantly, mice fed the TMAO diet exhibited significantly decreased LVEF ($P=0.0007$, $P=0.0013$, $P=0.0188$, $P=0.0188$, and $P=0.063$ versus control, respectively) at all time points investigated after TAC surgery (Figure 2D).

Table. Dietary TMAO and Choline 3 Weeks Before TAC Increases Circulating Levels (μmol/L) of Metabolites Indicated in the Pathogenesis of Cardiovascular Disease at 12 Weeks Post TAC

	Choline	Betaine	TMAO	Carnitine
Control (n=10)	22.9 (21.2–26.5)	74.1 (59.5–96.8)	1.7 (1.4–2.3)	25.1 (23.3–27)
TMAO (n=10)	25.1 (19.6–25.8; $P=0.853$)	56.2 (48.7–64.5; $P=0.037$)	25.2 (16.2–41; $P<0.0001$)	26.0 (21.9–26.3; $P=0.65$)
Choline (n=12)	33.4 (25.9–35.5; $P=0.012$)	100.8 (83.6–126.3; $P=0.138$)	26.6 (25.5–30.3; $P<0.0001$)	27.4 (25.6–29.9; $P=0.391$)

Results presented as median and interquartile range, *P* values are comparisons between TMAO or choline vs control using 2-sample Wilcoxon tests. TAC indicates transverse aortic constriction; and TMAO, trimethylamine N-oxide.

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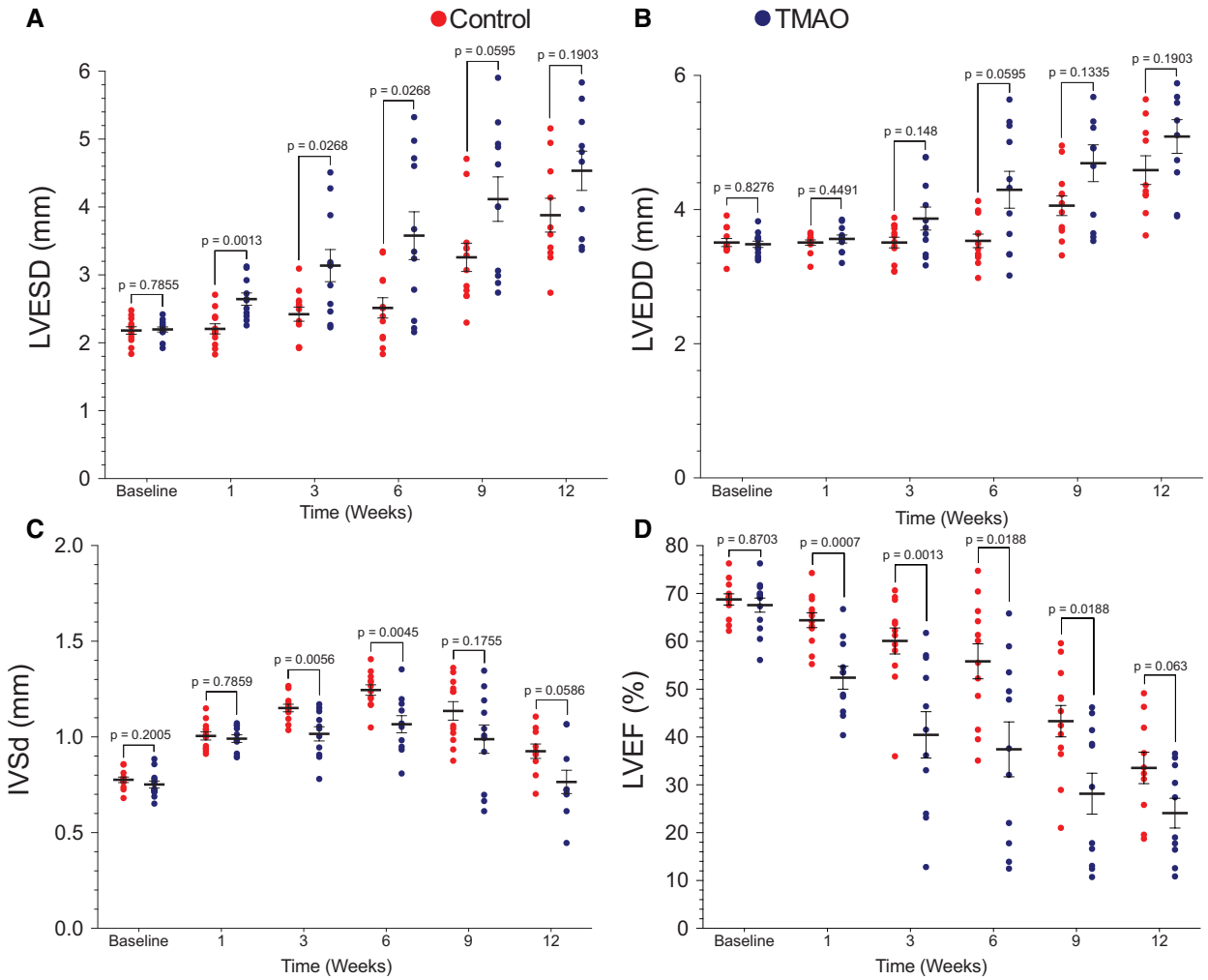

Figure 2. Dietary trimethylamine N-oxide (TMAO) exacerbates cardiac dilation and dysfunction after transverse aortic constriction (TAC) in mice when compared with a control diet. **A**, Left ventricular end-systolic diameter (LVESD; in mm), (**B**) LV end-diastolic diameter (LVEDD; in mm), (**C**) intraventricular septal wall diastolic diameter (IVSd; in mm), and (**D**) LV ejection fraction (LVEF %) from baseline to 12 wk. Results are presented as mean±SEM. A linear mixed-effects model was used to evaluate the change of all parameters during the 12-wk study period when compared with baseline. Two-sample Wilcoxon tests were used to compare between TMAO and control groups at different time points for 10 to 12 animals per group.

Dietary Choline Exacerbates Cardiac Dilatation and LV Dysfunction After TAC in Mice

The effect of TMAO on adverse ventricular remodeling and accelerated HF progression after TAC suggested a potential link between dietary choline and HF severity. To investigate this possibility, we fed mice either control chow or the same diet supplemented with 1.2% choline. Mice that fed the control versus choline-supplemented diets were subjected to the same 12-week TAC experimental protocol. When compared with baseline both groups exhibited LV dilation, hypertrophy, and decremented LVEF (P<0.0001 for LVESD, P<0.0001 for LVEDD, P=0.0007 for intraventricular septal wall diastolic diameter, and P<0.0001 for LVEF). Serial echocardiography analyses revealed that 1.2% dietary choline exerted similar effects on adverse cardiac remodeling as observed in mice that consumed TMAO-supplemented diet (Figure 3). LVESD was significantly (P=0.0454 and P=0.0464, respectively) greater at 1 and 3 weeks after TAC, and LVEDD was trending toward an increase (P=0.0604) at 3 weeks compared with the control diet group (Figures 3A and 3B). LV Interventricular septal thickness was significantly (P=0.0298, P=0.0193, and P=0.0358, respectively) less at 6, 9, and 12 weeks after TAC surgery in the choline group suggesting greater adverse LV remodeling (Figure 3C). Importantly, we also observed significantly worse LVEF (Figure 3D) in mice fed a diet containing 1.2% choline, with significant (P=0.0106, P=0.0108, and P=0.0284, respectively) reduction noted at 1, 3, and 6 weeks after TAC when compared with the control diet group. Collectively, these data clearly demonstrate a detrimental effect of supplemental dietary choline on LV structure and function in the setting of HF in the murine TAC model.

Dietary TMAO and Choline Exacerbate Cardiac Enlargement and Pulmonary Edema After TAC

Body weights and percent survival after HF for mice among the 3 dietary groups revealed no statistically significant

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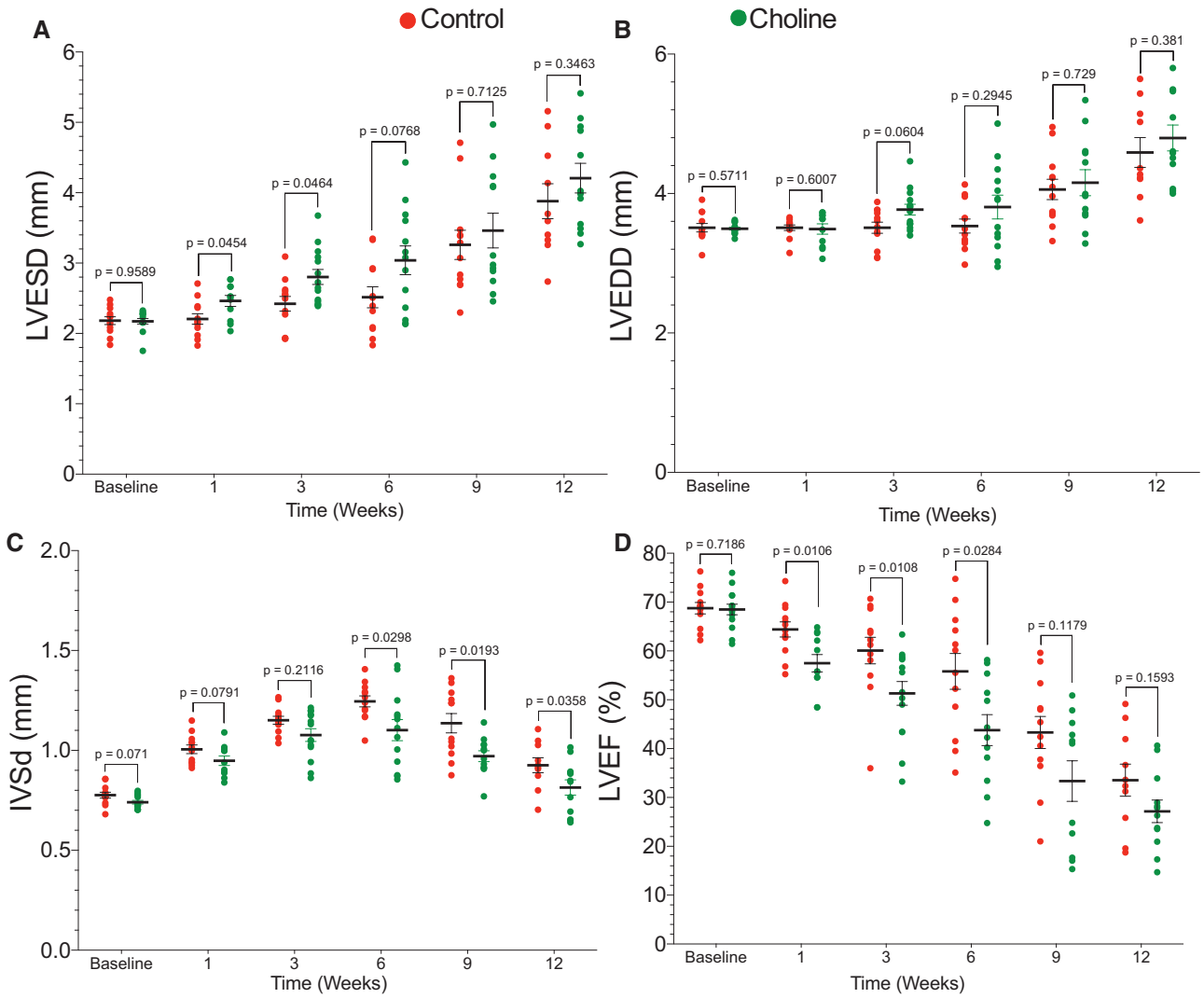

Figure 3. Dietary choline (1.2%) exacerbates cardiac dilation and dysfunction after transverse aortic constriction (TAC) in mice when compared with a control diet. **A**, Left ventricular end-systolic diameter (LVESD; in mm), (**B**) LV end-diastolic diameter (LVEDD; in mm), (**C**) intraventricular septal wall diameter (IVSd; in mm), and (**D**) LV ejection fraction (LVEF %) from baseline to 12 wk. A linear mixed-effects model was used to evaluate the change of all parameters during the 12-wk study period when compared with baseline. Results are presented as mean±SEM. Two-sample Wilcoxon tests were used to compare between choline and control groups at different time points for 10 to 12 animals per group.

differences (Figures I and II in the Data Supplement). Because a major pathological consequence of HF is cardiac enlargement and pulmonary edema, we evaluated myocardial and pulmonary tissue weights as an index of cardiac enlargement and pulmonary edema in mice fed the different diets. First, comparison of control or TMAO diet groups, performed initially, is shown in Figure 4. Comparison of heart weight (normalized to tibia length) at the time of euthanasia 12 weeks after TAC in TMAO fed, and control mice showed a significant (P=0.0005) increase in heart weight in the TMAO group (Figure 4A). Significant (P=0.0005) increases in left atrial weight in the TMAO group when compared with the control group were also noted (Figure 4B). Pulmonary edema, as evidenced by lung weight/tibia length, was also significantly (P=0.0021) increased in mice receiving dietary TMAO (Figure 4C). Parallel changes in cardiac and lung weights were observed in mice fed a diet containing 1.2% choline relative to control diet. Specifically, we observed significant increases in heart weight/tibia length (P=0.0004) and left atrial/tibia length (P=0.0161) in the choline group when compared with control diet (Figure 4D and 4E). However, although a trend toward increase lung weight was observed in the choline fed mice, this difference failed to achieve statistical significance when compared with mice fed the control diet (Figure 4F).

Dietary TMAO and Choline Increase Levels of Circulating BNP in Mice After TAC

We evaluated circulating levels of BNP, a hormone released from myocardial tissues during enhanced stretch and associated with enhanced degree of HF severity, in mice at 12 weeks post TAC (Figure 5D). BNP levels were significantly increased in both the TMAO (P=0.019) and the choline (P=0.017) groups when compared with the mice fed a control diet, with similar BNP results between the TMAO and the choline groups (P=0.792). These data correlate with our echocardiographic data, suggesting that mice in the TMAO

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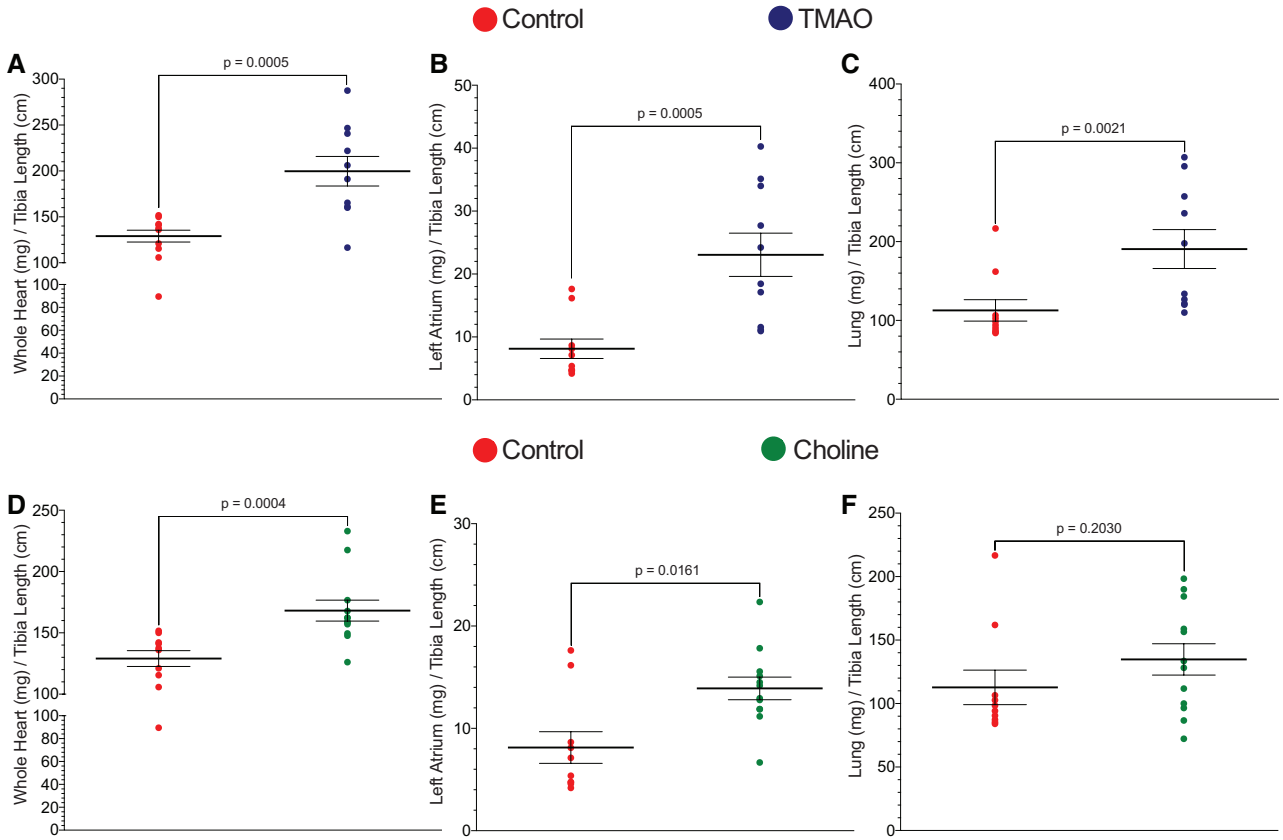

Figure 4. Dietary trimethylamine N-oxide (TMAO) and choline exacerbate cardiac enlargement and hypertrophy after transverse aortic constriction. **A**, Ratio of heart weights:tibia length in TMAO vs control. **B**, ratio of left atrial (LA) weights:tibia length in TMAO vs control. **C**, Ratio of lung weights:tibia length in TMAO vs control. **D**, Ratio of heart weights:tibia length in choline vs control. **E**, Ratio of LA weights:tibia length in choline vs control. **F**, Ratio of lung weights:tibia length in choline vs control. Results are presented as mean±SEM. Two-sample Wilcoxon tests were used to compare between TMAO or choline vs control for 10 to 12 animals per group.

and choline groups experienced more severe LV remodeling and reduction in LV function when compared with mice in the control group. Gross examination of the heart showed significant cardiac enlargement in mice within either the TMAO- or the choline-supplemented dietary groups in comparison with controls (Figure 5A–5C).

Dietary (TMAO) and Choline (TAC) Exacerbate Interstitial and Perivascular Fibrosis After TAC

Myocardial connective tissue responds to cardiac hypertrophy and impaired heart function by the development of fibrosis, which results in LV stiffening, loss of compliance, and further impairments in cardiac contraction and relaxation. To further evaluate cardiac remodeling, we examined cardiac fibrosis formation using 2 histological methods to detect collagen fibers in the heart after TAC (Figure 6). We performed both Masson trichrome and Picrosirius red staining on cardiac tissues at 12 weeks after TAC. With each method of detecting enhanced collagen and fibrosis, we observed significant increases in the hearts of mice fed either a diet supplemented with TMAO or choline relative to normal chow (control) diet. Representative photomicrographs are shown in Figure 6A and 6B for control, choline, and TMAO, and summary data are presented in Figure 6C. Computerized planimetry-based quantification of the intensity of Picrosirius red staining (Figure 6C) affirmed significantly (P=0.0087) greater level of intermuscular fibrosis in the TMAO group when compared with the control group. Using the same method of tissue staining and analysis, we observed similar results in the perivascular sections of heart tissue collected 12 weeks post TAC in control, choline, and TMAO fed mice (Figure 7). Photomicrographs in Figure 7A and 7B show increased levels of perivascular fibrosis in the TMAO group when compared with the control group; this increased significance (P=0.026) is corroborated by quantification of the intensity of the Picrosirius red staining (Figure 7C).

Discussion

The critical importance of diet and overall metabolism in impacting cellular processes is growing in appreciation for its potential to impact the development and severity of CVDs. Moreover, a growing body of data reveals strong associations between gut microbiota formation of the metabolite TMAO and adverse cardiovascular risks. For example, several recent clinical studies have shown that elevated circulating levels of the metabolite TMAO directly correlate with the severity of HF, adverse prognosis in HF subjects, and risk of HF among diabetic and nondiabetic subjects alike.[13,14,18] The present findings extend these human clinical associations, by demonstrating a remarkable effect of dietary supplementation with either choline or its gut microbe–generated metabolite

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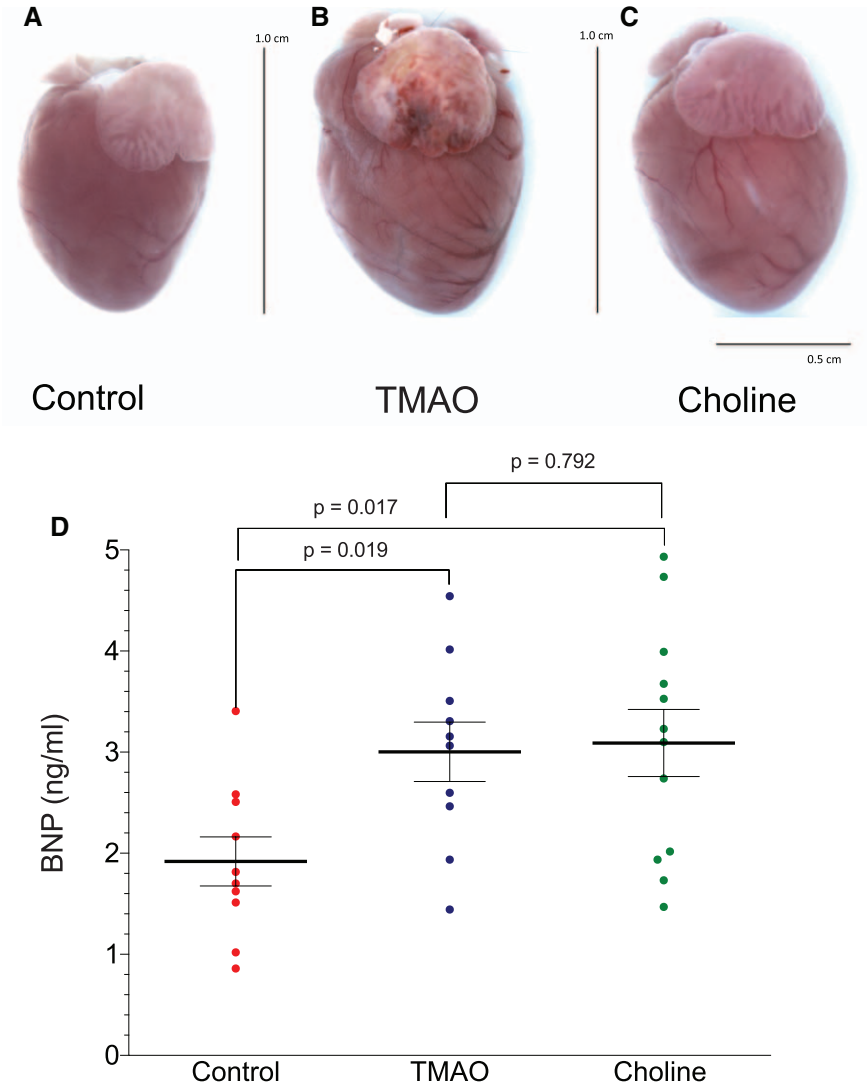

Figure 5. Increased circulating levels of brain natriuretic peptide (BNP) in mice after transverse aortic constriction (TAC) and the development of pressure overload–induced heart failure. **A**–**C**, Representative photomicrographs of control (TAC+control diet), trimethylamine n-oxide treated (TAC+TMAO diet), and choline-treated (TAC+choline diet) hearts at 12 wk after TAC. **D**, Mean circulating BNP levels (ng/mL) in mice 12 wk post TAC. Two-sample Wilcoxon tests were used for 2-group comparison for 10 to 12 animals per study group.

TMAO, and HF progression. Provision of either supplemental choline or TMAO each lead to greater propensity for adverse cardiac remodeling and LV dysfunction in the setting of chronic pressure overload after TAC in naïve mice.

It is now appreciated that there are multiple microbes responsible for the conversion of choline to TMA, as identified in a recently published article.[19] Romano et al[19] show that there are numerous human commensals capable of making TMA from choline and then shown in vivo to produce TMA and TMAO when introduced into gnotobiotic mice. The human commensals examined are also known to inhabit the intestines of mice. Thus, it is not a case of a single species of microbe that forms TMA and TMAO, but numerous microbes that exhibit the biochemical activity needed to convert different TMA containing nutrients into TMA.

Our study revealed that when TMAO levels are elevated in the circulation, susceptibility for development of HF is enhanced as evidenced by pathological LV dilation, reduced LVEF, increased circulating BNP levels, increases in pulmonary edema, and increased myocardial fibrosis. It is interesting to note that recent animal model studies using either choline- or TMAO-supplemented diets similarly revealed enhanced fibrosis in an alternative end organ, the kidney.[20] Specifically, exposure to both diets was noted to provoke tubulointerstitial fibrosis, and enhanced perivascular fibroisis in a manner dose dependently associated with circulating TMAO levels.[20] The enhanced myocardial fibrosis observed in the present study suggests a profibrotic phenotype with high TMAO levels is a common observation and likely plays a contributory role to the worse adverse ventricular remodeling observed with either choline- or TMAO-supplemented diet. Furthermore, the enhanced myocardial fibrosis observed in the animal models herein is consistent with recently reported enhanced indices of diastolic dysfunction and adverse prognosis observed in subjects with HF and higher TMAO levels.[13,14,18]

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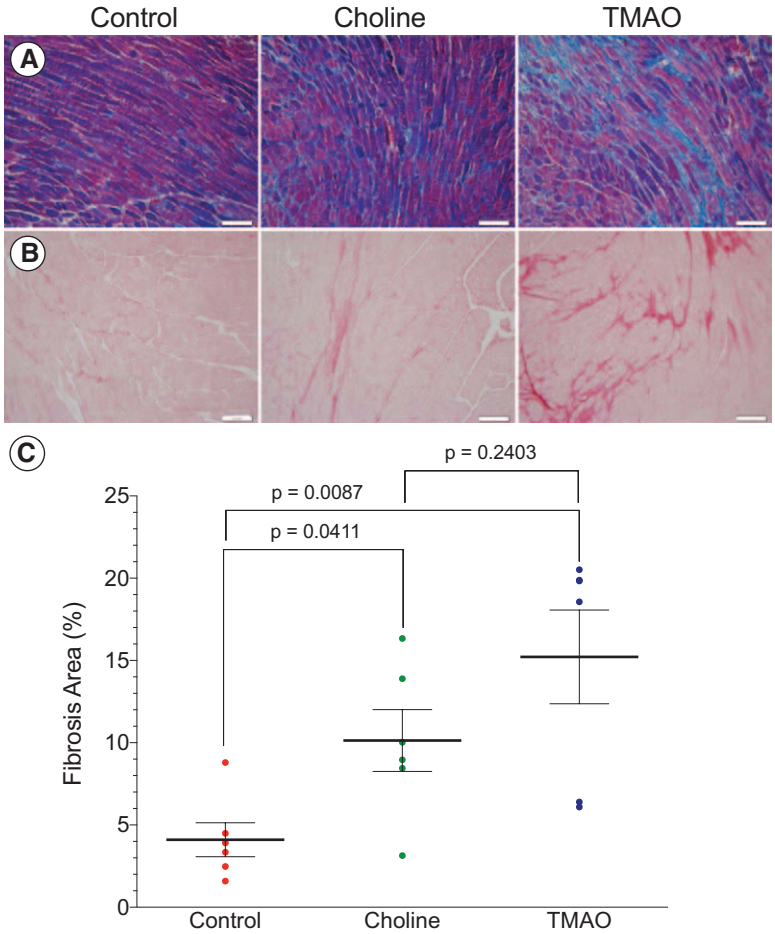

Figure 6. Dietary trimethylamine N-oxide (TMAO) and choline initiated at 3 wk before transverse aortic constriction (TAC) worsens interstitial fibrosis. **A**, Representative photomicrographs of Masson's trichrome–stained heart sections in control, choline, and TMAO groups of mice illustrating intermuscular fibrosis at 12 wk post TAC. **B**, Representative photomicrographs of Picrosirius red–stained heart sections in control, choline, and TMAO mice illustrating intermuscular fibrosis at 12 wk post TAC. **C**, Cardiac fibrosis as a percent (%) of left ventricular area calculated from Picrosirius red–stained heart. Scale bars on photomicrographs represent 500 µm. Results are presented as mean±SEM. Two-sample Wilcoxon tests were used for 2-group comparison for 6 to 7 animals per group.

Elevated levels of betaine were also observed in plasma of mice on the choline diet, but not in the plasma of mice fed a 0.12% TMAO diet. In concordance, recent human studies have shown that elevated betaine levels predict risk for HF[14] and as such we too observed elevated betaine and choline levels predicted poorer prognosis (mortality) in human subjects with HF. However, when TMAO was included in the model (with BNP, traditional CVD risk factors and renal function indices), only TMAO remained a significant predictor of adverse outcome.

Limitations of the present study are worth noting. The murine model is not fully comparable with human physiology because of significant metabolic differences. In our study, we used the TAC protocol to induce HF. Although this is a reliable and consistent model for HF, it does not fully represent human HF and may not accurately predict progression of human disease. Of note, however, it is relevant that TMAO levels observed in the present study in mice (≈25 µmol/L) are well within the elevated levels observed among subjects with either HF or chronic kidney disease and adverse prognosis, where levels in excess of 100 µmol/L can be observed.[10,13] Another limitation of the present study is we do not know at the molecular level how TMAO exerts its adverse effects on cardiac remodeling, fibrosis, and function, and whether there is a specific TMAO receptor or is acting via its known effect on protein conformation and stability.[21,22]

The gut hypothesis in HF has historically focused on the potential involvement of bowel edema with congestion, and gut microbiota generated toxins and lipopolysaccharide leak from reduced barrier function, leading to heightened inflammation and suppressed myocardial function.[19] The present studies, coupled with the recent recognition of elevated plasma TMAO levels as strong independent predictors of HF severity and adverse prognosis,[13,14] suggests the potential importance of both diet and gut microbial pathways to HF susceptibility. Additional studies aimed at manipulating this pathway and monitoring HF risks seem warranted.

Sources of Funding

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Disclosures

Dr Hazen is named as the coinventor on issued and pending patents held by the Cleveland Clinic relating to cardiovascular diagnostics and/or therapeutics; he is also paid as a consultant for the

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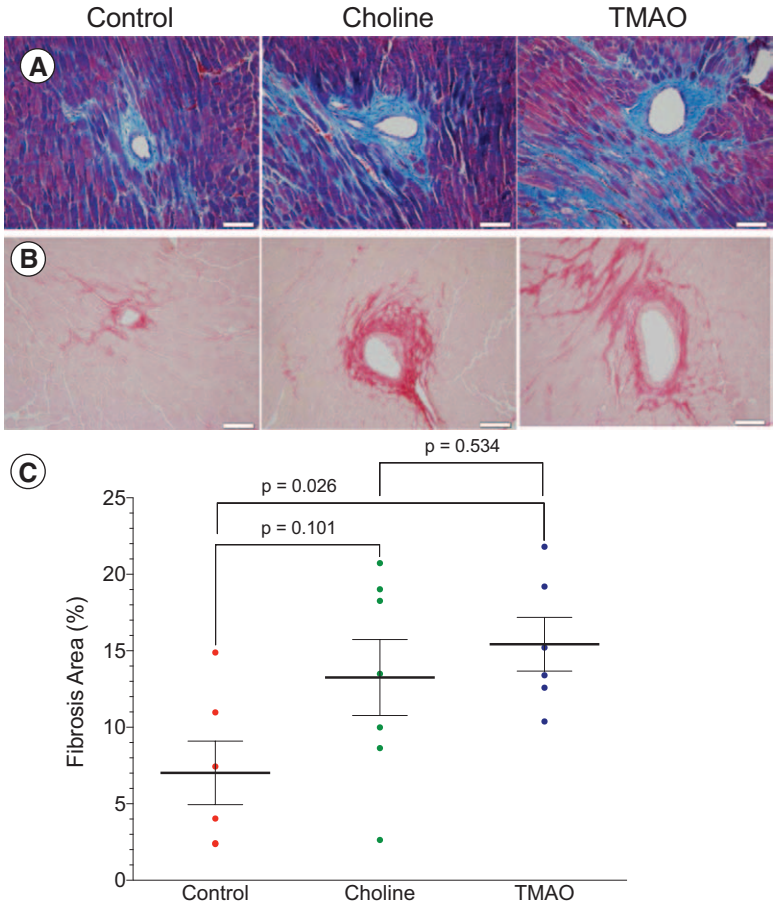

Figure 7. Dietary trimethylamine N-oxide (TMAO) and choline exacerbate perivascular fibrosis in the setting of transverse aortic constriction (TAC). **A**, Representative photomicrographs of Masson trichrome–stained heart sections in control, choline, and TMAO groups of mice illustrating perivascular fibrosis at 12 wk post TAC. **B**, Representative photomicrographs of Picrosirius red–stained heart sections in control, choline, and TMAO mice illustrating perivascular fibrosis at 12 wk post TAC. **C**, Cardiac fibrosis as a percent (%) of left ventricular area calculated from Picrosirius red–stained heart tissue. Scale bars on photomicrographs represent 500 μm. Results are presented as mean±SEM. Two-sample Wilcoxon tests were used for 2-group comparison for 6 to 7 animals per group.

following companies: Cleveland Heart Laboratory, Esperion, and Procter & Gamble; and has received research funds from Astra Zeneca, Pfizer, Procter & Gamble, Roche Diagnostics Inc., and Takeda. He reports having the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics and/or therapeutics from Cleveland Heart Laboratory, Siemens, Esperion, and Frantz Biomarkers, LLC. The other authors report no conflicts.

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CLINICAL PERSPECTIVE

It has been well documented in the literature that diet can affect cardiac outcomes. As the organ with the most direct contact with our environment, the importance of the gut and its microbiome in medicine is gaining appreciation. Recently the role of the gut microbiome in cardiovascular disease has emerged as a significant determinant of overall cardiovascular health and disease. It has been shown that patients with heart failure have significantly increased levels of circulating trimethylamine n-oxide, a metabolite formed by the metabolism of choline via gut microbes. Trimethylamine n-oxide has also been shown to accelerate atherosclerotic plaque formation. In the current study, we examine the effects of dietary trimethylamine n-oxide and choline on the development and progression of heart failure in a mouse model of transverse aortic constriction to better characterize this metabolite and its role in the pathogenesis of heart failure. Our data demonstrate that dietary trimethylamine n-oxide and choline significantly exacerbate cardiac fibrosis and left ventricular adverse remodeling and dysfunction in the setting of cardiac hypertrophy and heart failure.

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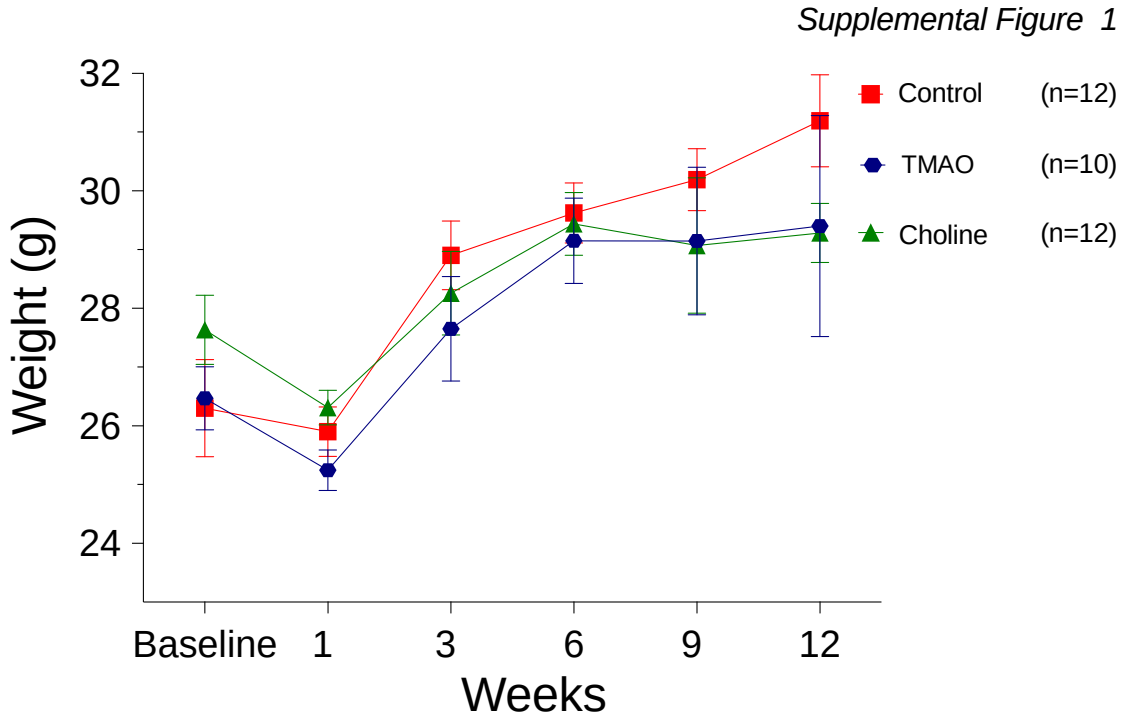
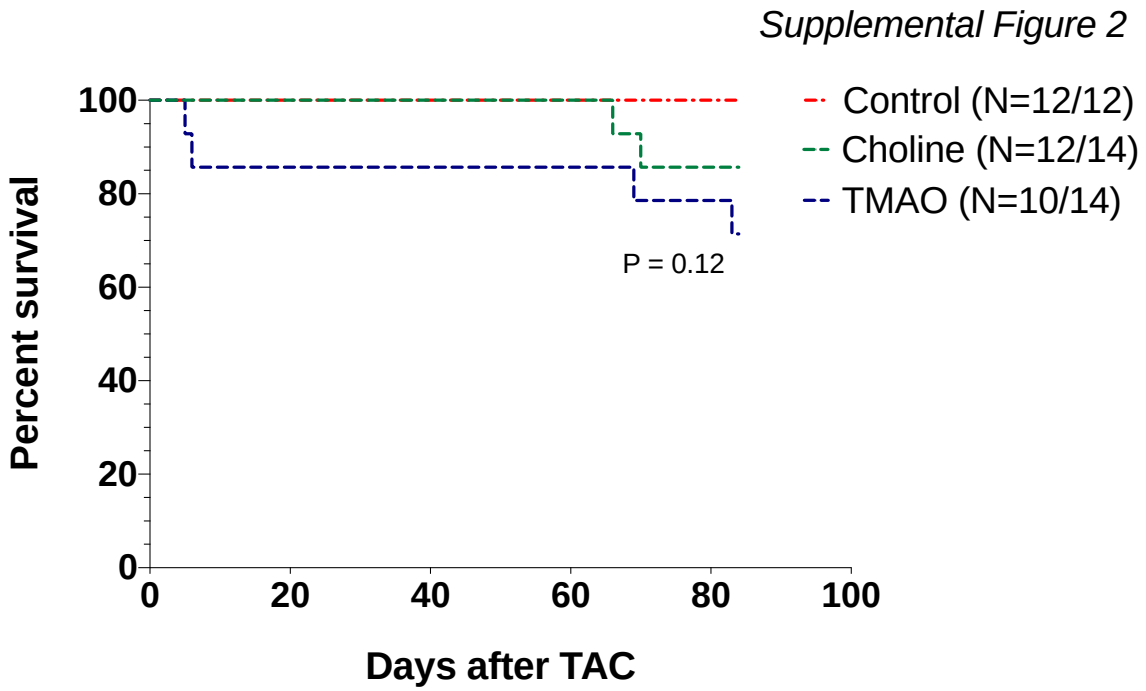
Supplemental Figures

Supplemental Figure 1. Graph of body weight for animals treated with dietary trimethylamine N-oxide and choline starting 3 weeks before transverse aortic constriction (TAC). There was no statistical difference between control (n=12), TMAO (n=12), and choline (n=12).



Supplemental Figure 2. Kaplan-Meier survival curves for 84 days following transverse aortic constriction (TAC). Using a Log-rank test p = 0.12 TMAO vs. control.



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