

THE DIRECT INOTROPIC AND CHRONOTROPIC EFFECTS OF  
TRIMETHYLAMINE-N-OXIDE ON CARDIAC MUSCLE

BY

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## ABSTRACT

**Background:** Cardiovascular disease is endemic among patients with chronic kidney disease (CKD). Growing evidence suggests the gut microbiome plays an obligatory role in cardiovascular pathogenesis. Trimethylamine-N-oxide (TMAO) is a downstream byproduct of intestinal microbe metabolism of phosphatidylcholine and L-carnitine that may directly promote atherosclerosis formation. Since clearance of this uremic metabolite is dependent on urinary excretion, plasma levels of TMAO increase with decrements in kidney function. Association studies have linked plasma levels of TMAO to adverse cardiovascular outcomes in patients with renal dysfunction; however, the direct effect on the heart itself remains largely unexplored.

**Objective:** The objective of this study was to determine if TMAO alone could acutely alter *ex vivo* cardiac contractile function on a beat-to-beat basis.

**Methods:** CD1 adult mouse hearts were extracted, attached to a force transducer, oxygenated, and paced within an organ bath. Changes in contractility were measured after infusing TMAO or vehicle into the organ bath. As a follow-up approach, mouse hearts were reverse perfused through the aorta via a modified Langendorff apparatus to facilitate TMAO delivery into the myocardium. Subsequently, to determine if our findings translated to the human heart, we performed contractility experiments using human atrial appendage biopsy tissue, which was retrieved during cardiopulmonary bypass procedures prior to cannula placement. To investigate whether TMAO alters contractile rate, in a separate series of experiments, the atria and sinoatrial node of isolated mouse hearts were kept intact to allow for spontaneous beating without artificial pacing. Changes in contraction rate (in beats per minute) were measured after treatment with TMAO or vehicle. Additionally, calcium imaging was performed on spontaneously beating embryonic (E18) rat

cardiomyocytes. Changes in intracellular  $\text{Ca}^{2+}$  oscillations were measured, following treatment with 300  $\mu\text{M}$  TMAO or vehicle, using the fluorescent  $\text{Ca}^{2+}$  indicator Fluo-4 AM.

**Results:** Acute exposure to TMAO in the organ bath increased average contraction amplitude 17% and 41% at 300  $\mu\text{M}$  and 3,000  $\mu\text{M}$ , respectively ( $P < 0.05$ ,  $n = 6-7$  animals). Langendorff reverse perfusion of mouse hearts *ex vivo* with 300  $\mu\text{M}$  TMAO generated an even greater response than non-perfusion peripheral exposure and increased isometric force 34% compared to vehicle ( $P < 0.05$ ,  $n = 2-3$ ). Consistent with what we observed in the animal model, incubation of human atrial muscle tissue with TMAO at 3,000  $\mu\text{M}$  increased isometric tension 29% compared to vehicle ( $P < 0.05$ ,  $n = 4-5$ ). Average beating frequency of mouse hearts *ex vivo* increased 27% and 46% compared to vehicle following treatment with TMAO at 300  $\mu\text{M}$  and 3,000  $\mu\text{M}$ , respectively ( $P < 0.05$ ,  $n = 3$ ). Similarly, 300  $\mu\text{M}$  TMAO increased average calcium oscillation frequency within embryonic rat cardiomyocytes by 42% compared to vehicle ( $P < 0.05$ ,  $n = 3-4$ ).

**Conclusions:** TMAO, at pathological concentrations, directly increases the force and rate of cardiac contractility. Initially, these inotropic and chronotropic actions may help maintain cardiac output during CKD; however, chronic increases in isometric tension and beating frequency are known to promote cardiac remodeling, left ventricular hypertrophy, and heart failure. Further *in vivo* studies are needed to determine how chronic exposure to TMAO may contribute to cardiac pathology in CKD and to examine if TMAO represents a therapeutic target for reducing cardiovascular mortality in patients with CKD. Our findings lay the groundwork for future translational research on the intricate relationship between the microbiome, kidneys, and heart.

## ACKNOWLEDGMENTS

To my mentor, **Dr. Michael Wacker**, thank you for your limitless wisdom, patience, and perspective over the years. You have instilled in me a genuine appreciation for basic science research and the pursuit of knowledge. I hope to emulate your passion, energy, and determination in my future career. It has been a true privilege to be your student.

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## INTRODUCTION

The Centers for Disease Control and Prevention estimates that 30 million adults in the United States are living with chronic kidney disease (CKD) as of 2017.<sup>1</sup> CKD is generally defined as progressive, irreversible impairment in renal function and encompasses a spectrum of pathophysiologic processes associated with a decline in glomerular filtration rate (GFR).<sup>2,3</sup> The kidneys have several essential physiologic functions including the filtration of blood and excretion of metabolic and exogenous waste, regulation of volume status and blood pressure, maintenance of acid-base balance and electrolyte homeostasis, and various endocrine functions important for bone metabolism and red blood cell production.<sup>4</sup> Chronic renal insult, most commonly due to diabetes or hypertension, leads to damage and eventual destruction of nephrons, the functional units of the kidney.<sup>5</sup> Initially compensatory hyperfiltration ensues, though in the long term, increased stress on the kidneys propagates overwork injury and pathologic changes such as progressive glomerular distortion and interstitial fibrosis.<sup>6</sup> Therefore as the disease advances, fluid, electrolytes, and metabolic waste normally excreted by the kidneys accumulate within the body leading to widespread homeostatic disturbances and dysfunction including abnormalities in dermatologic, musculoskeletal, neuromuscular, endocrine-metabolic, hematologic, gastrointestinal, and importantly, cardiovascular function.<sup>2</sup>

More than 60% of individuals with CKD also have comorbid cardiovascular disease (CVD),<sup>7</sup> a prevalence that is approximately 9-times higher than that among individuals with normal renal function.<sup>8</sup> It is well established that the presence of CKD is one of the most important risk factors for CVD.<sup>9</sup> However, patients with end-stage renal disease (ESRD) are less prone to receive important cardioprotective interventions and are at increased risk for adverse outcomes and death once cardiac events occur.<sup>10</sup> Thus, CVD is the leading cause of mortality among patients



with CKD — accounting for approximately 50% of deaths in patients with ESRD.<sup>11,12</sup> The management of CKD accounts for an astounding \$48 billion per year in healthcare costs the United States,<sup>13</sup> yet the major expenditure is not in providing renal replacement therapy but the cost of disability from premature CVD.<sup>14</sup>

A complex relationship exists between these two vital organ systems — chronic damage to one organ eventually causes both organs to fail.<sup>15</sup> It was originally suggested that this relationship exists due to coinciding, “traditional” risk factors such as advanced age, hypertension, diabetes mellitus, and dyslipidemia promoting the development and progression of both CVD and CKD.<sup>1,16</sup> However, standard clinical interventions for managing CVD have failed to improve cardiovascular outcomes in patients with CKD.<sup>15,17</sup>

In recent years there has been growing interest in the bacteria living symbiotically within the human intestines. Evolving evidence suggests the gut microbiome is an obligatory contributor in several metabolic pathways linked to chronic cardiometabolic conditions.<sup>18–20</sup> This has spurred a dramatic shift in the understanding and approach to cardiac pathology. There is now a novel appreciation that the intestinal microbiota functions as an endocrine organ capable of producing a myriad of metabolites, such as trimethylamine-N-oxide (TMAO), related to human health and disease.<sup>21</sup>

Formation of TMAO necessitates gut microbial metabolism of dietary choline and L-carnitine.<sup>22–24</sup> Choline, an essential nutrient derived from phosphatidylcholine, and L-carnitine are consumed primarily in the form of fatty, high cholesterol foods such as red meat, liver, and egg yolks.<sup>20</sup> Bacterial metabolism of choline and L-carnitine generates trimethylamine (TMA), an intermediate metabolite, which is absorbed through the intestinal epithelium and carried to the liver in the portal circulation.<sup>25,26</sup> TMA is then oxidized to TMAO by hepatic flavin

monooxygenase 3 (FMO3) enzymatic activity.<sup>27-30</sup> Clearance is dependent almost exclusively on urinary excretion,<sup>31-33</sup> therefore, serum levels climb with decrements in kidney function<sup>34-36</sup> to approximately 100  $\mu$ M in patients with dialysis-dependent CKD.<sup>37</sup>

TMAO has been linked to adverse cardiovascular outcomes, initially identified through a series of untargeted metabolomic studies.<sup>22</sup> Subsequently, elevated levels of TMAO and its precursors were shown to predict major adverse cardiac events such as myocardial infarction, stroke, and death.<sup>23,24</sup> Interestingly, high plasma concentrations of carnitine and choline were only associated with cardiovascular risk in the presence of an intact microbiome, when TMAO was also concomitantly elevated.<sup>24,38</sup> Animal studies have indicated TMAO fosters enhanced macrophage foam cell development and atherosclerotic plaque formation through reductions in reverse cholesterol transport and alterations in expression of macrophage surface receptors.<sup>22,24</sup> These data demonstrating the proatherogenic properties of TMAO,<sup>20,22-25,38</sup> could potentially account for the clinical associations between elevated plasma levels of TMAO and CVD. However, the majority of cardiovascular deaths in patients with ESRD are due to sudden cardiac death, fatal arrhythmia, or heart failure.<sup>39</sup> With relatively fewer deaths attributable to coronary artery disease or myocardial infarction,<sup>39</sup> it is unlikely traditional risk factors account for the excess cardiovascular morbidity and mortality in patients with CKD.<sup>14,15,40</sup> Furthermore, recent evidence indicates elevated TMAO levels correlate with equivalent adverse prognostic value in both ischemic and non-ischemic heart failure,<sup>41</sup> suggesting TMAO may contribute to cardiovascular pathogenesis beyond the development of atherosclerotic complications.

The exact mechanisms through which TMAO promotes CV diseases, including heart failure, have not yet been identified. Studies exploring the direct effects of TMAO on the myocardium are limited. It was recently shown that long-term TMAO administration at a dose of

120 mg/kg in drinking water increased the TMAO concentration in cardiac tissues 22-fold and that this accumulation led to disturbances in both pyruvate and fatty acid oxidation in cardiac mitochondria in mice.<sup>42</sup> Mitochondrial dysfunction and reduced energy metabolism are important mechanisms responsible for the progression of heart failure.<sup>43</sup> It has also been shown in mice fed a Western diet that increased plasma TMAO levels were associated with reduced left ventricular ejection fraction and increased cardiac inflammation and fibrosis — changes that were prevented by treatment of an inhibitor of TMA formation (3,3-Dimethyl-1-butanol).<sup>44</sup> While these initial studies demonstrate that TMAO may have an impact on cardiac function, the objective of our present study was to determine how TMAO alters cardiac function on a beat-to-beat basis. In this study, we test the direct effects of TMAO on isolated mouse and human heart contractile function, as well as the acute effects on spontaneously beating mouse hearts and embryonic cardiomyocytes. These results are critical for understanding the role of TMAO in cardiac function and pathology.

## MATERIALS AND METHODS

### Responsible Conduct of Research

This study was conducted under the approval of the University of Missouri Kansas City Institutional Animal Care and Use Committee (IACUC), Protocol # 1302, and the University of Kansas Medical Center Human Subjects Committee, IRB # 00004440. Animal care and procedures relating to human subjects were performed in accordance with institutional guidelines.

### Chemicals and Reagents

TMAO was purchased from Sigma Chemical (St. Louis, MO). Fluo-4 AM was obtained from Invitrogen (Carlsbad, CA). All remaining reagents were sourced from Sigma (St. Louis, MO).

### Experimental Animals

Twelve-week-old male CD-1 mice (Harlan Laboratories: Madison, WI) were used for experiments with exogenous TMAO. All mice were housed in a temperature-controlled (22 +/- 2°C) room with a 12:12-h light-dark cycle. Animals were fed ad libitum. Mice were anesthetized with 3% isoflurane inhalation prior to tissue harvesting.

### Measurements of Force of Cardiac Contractility

#### **Artificially-paced *ex vivo* mouse hearts.**

The mouse hearts were quickly excised and placed in Ringer's solution [composed of (in mM): 140 NaCl, 2.0 KCl, 2.5 CaCl<sub>2</sub>, 1.0 MgSO<sub>4</sub>, 1.5 K<sub>2</sub>HPO<sub>4</sub>, 10 HEPES, 10 glucose, pH 7.4]. The atria, blood, fat, and excess connective tissues were carefully removed. The hearts were hung vertically and attached to a force transducer between bipolar platinum-stimulating electrodes suspended in 25-ml glass tissue chambers (Radnoti: Monrovia, CA) and bubbled under 100% O<sub>2</sub>. Hearts were stretched to the length of maximum force development and stimulated (SD9

stimulation unit—Grass Technologies: Quincy, MA) with pulses of 1 Hz at 5 ms duration. Hearts were paced for 20 minutes to obtain a stable baseline before treatment with either vehicle or TMAO. Two concentrations of TMAO, 300  $\mu$ M and 3,000  $\mu$ M, were pipetted into the organ bath 30 minutes apart. As a control, equal volumes of vehicle (Ringer's solution) were administered 30 minutes apart. Following treatment with either vehicle or TMAO, norepinephrine (5  $\mu$ M) was administered as a positive control. The contractile data were recorded and analyzed on the LabChart 6 software (AD Instruments: Colorado Springs, CO). Waveform changes were analyzed in the segments corresponding to peak isometric tension (mN). Additional waveform characteristics including maximum slope (mN/s), peak area under the curve (mN x s), and minimum slope (mN/s) were also collected and analyzed. Changes in these parameters could indicate variation in calcium handling mechanisms, such as enhanced calcium release, or modification of the contractile proteins. Experimental data are presented as a fold change from baseline contractile parameters.

**Artificially-paced *ex vivo* mouse hearts with Langendorff perfusion.**

After removal of the heart, the ascending aorta was cannulated to establish access for reverse perfusion of the coronary circulation via a modified Langendorff perfusion setup using a peristaltic pump (Masterflex, 7518-00, Cole-Parmer Instrument Company: Vernon Hills, IL). The cannulated hearts were hung vertically and attached to a force transducer between bipolar platinum-stimulating electrodes suspended in 25-ml glass tissue chambers. Retrograde perfusion of the hearts was initiated with Ringer's solution equilibrated in 100% O<sub>2</sub> to a pH of 7.4. Hearts were stimulated with pulses of 1.0-1.5 Hz at 5 ms duration. Hearts were paced for 20 minutes to obtain a stable baseline before perfusion with either vehicle (Ringer's solution) or TMAO (300  $\mu$ M). Contractility was measured as previously described.

### **Artificially-paced human heart biopsy tissue.**

Atrial appendage cardiac tissue was obtained from the Cardiovascular Research Institute at the University of Kansas Medical Center. Subject qualifying criteria included: 1) being 18 years of age or older, and 2) undergoing an open heart procedure requiring cardiopulmonary bypass (including coronary artery bypass grafting, cardiac valvular repair/replacement, and repair of septal defects). Participation was voluntary and written informed consent was obtained prior to enrollment in the study. Subjects were fully informed of the risks and benefits of participation. There was no reimbursement for patient participation. All adult subjects regardless of age, race or gender were included in this study. Non-English speaking persons were excluded so that there would be no confusion during informed consent. No vulnerable populations were included. Subjects were able to withdraw their consent any time prior to the surgical procedure.

Atrial appendage biopsy tissue was obtained by the cardiac surgeons prior to cannula placement during the cardiopulmonary bypass procedure. Once retrieved, the piece of tissue was placed in a 50-ml sterile tube containing normal saline. This tube was placed on ice to reduce tissue metabolism during transportation to the laboratory.

Upon arrival, the heart tissue was placed in Ringer's solution and carefully cleaned of any connective tissue before being cut into two or three muscle strips depending on the size of the biopsy. Contractility was performed as previously described. REDCap (Research Electronic Data Capture) was utilized to securely store patient data. Once all experimentation was complete, the tissue was disposed of in a biohazard container located in the UMKC School of Medicine laboratory facility.

### **Measurements of Rate of Cardiac Contractility**

#### **Self-paced *ex vivo* mouse hearts.**

The mouse hearts were excised and suspended in 25-ml glass tissue chambers as previously described. However, in these experiments, the atria and sinoatrial node of the isolated hearts were kept intact to allow for spontaneous beating without artificial pacing. Hearts were allowed to self-pace for 20 minutes to obtain a stable baseline rate prior to administration of either TMAO or vehicle. Changes in heart rate (in beats per minute) were measured after treatment with TMAO (300  $\mu$ M or 3,000  $\mu$ M) or vehicle (Ringer's solution) using the LabChart 6 software. Experiments were normalized within each condition to baseline rate of contractility and are presented as a relative change from baseline.

#### **Spontaneously beating rat cardiomyocytes.**

E18 rat cardiomyocytes were plated directly onto 35-mm plastic tissue culture dishes at a density of 10,000 cells/cm<sup>2</sup> in 10% fetal bovine serum (PAA: Piscataway, NJ), 1: 200 penicillin/streptomycin, 50% Ham's F-12 media, and 50% eagle minimum essential media. The myocytes were maintained in a 37°C, 5% CO<sub>2</sub>, and 95% O<sub>2</sub> incubator and allowed 2-5 days of growth, until 100% confluency and spontaneous beating of cell populations occurred. On the day of imaging, media was removed from the dish and the cells were washed in the perfusion system for 10 minutes, which also allowed time for acclimation to the flow of the system. The cells were then loaded on the microscope at 37°C with the Ca<sup>2+</sup> indicator dye, Fluo-4 AM (Invitrogen; 10 $\mu$ M) in calcium imaging buffer (D-MEM/F-12 with 2 mM CaCl<sub>2</sub>) and incubated for 20 minutes. Cells were washed 3 times in calcium imaging buffer and allowed to de-esterify for 10 minutes. Intracellular Ca<sup>2+</sup> oscillations were measured using an inverted microscope with fluorescent imaging capabilities [Olympus IX51 (Olympus: Melville, NY), Hamamatsu Orca-ERGA charge-coupled device camera (Hamamatsu: Bridgewater, NJ), Semrock Bright Line filter set (Semrock: Rochester, NY), EXFO X-cite metal halide light source (EXFO: Mississauga, ON, Canada), and

Slidebook ratiometric software (Intelligent Imaging Innovations)]. TMAO (300  $\mu$ M) or vehicle (calcium imaging buffer) were carefully perfused into the cell culture dishes at a rate of 0.3 ml/min using a VC-8T perfusion system (Warner Instruments: Hamden, CT) and driven by P720/66 high-flow peristaltic pumps (Instech: Plymouth Meeting, PA). The perfusion protocol for acute application of TMAO was as follows: 2 minutes vehicle, 7 minutes TMAO, 6 minutes washout with vehicle. The fluorescent changes from each cell were averaged and used for data analysis. All data were analyzed for average calcium oscillation frequency in the last 30 seconds prior to treatment (baseline) and then in the final 30 seconds after TMAO or vehicle application. This was used to calculate a fold change from baseline, which was then normalized to control (vehicle).

### **Statistical Analysis**

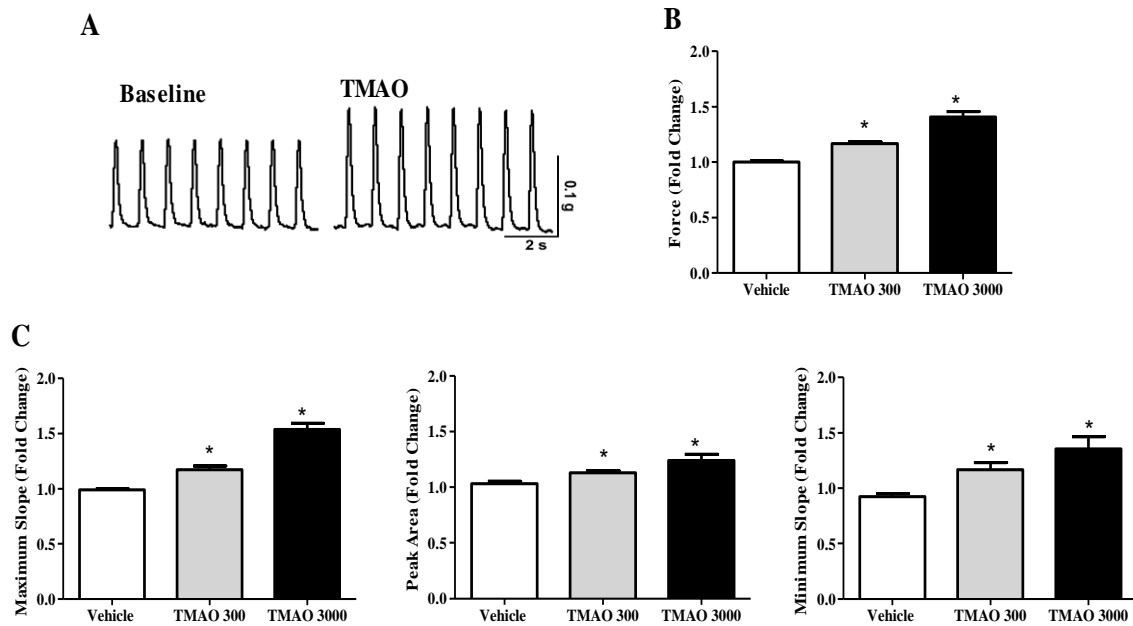
All statistical procedures and graphs were performed with GraphPad Prism 5.0 (La Jolla, CA). Data are presented as the average fold change from baseline  $\pm$  SEM. Data were compared using either an unpaired t-test or a one-way analysis of variance, with the significance set at the  $P \leq 0.05$  level. The one-way ANOVA was followed by Newman-Keuls Multiple Comparison *post hoc* analysis.



## RESULTS

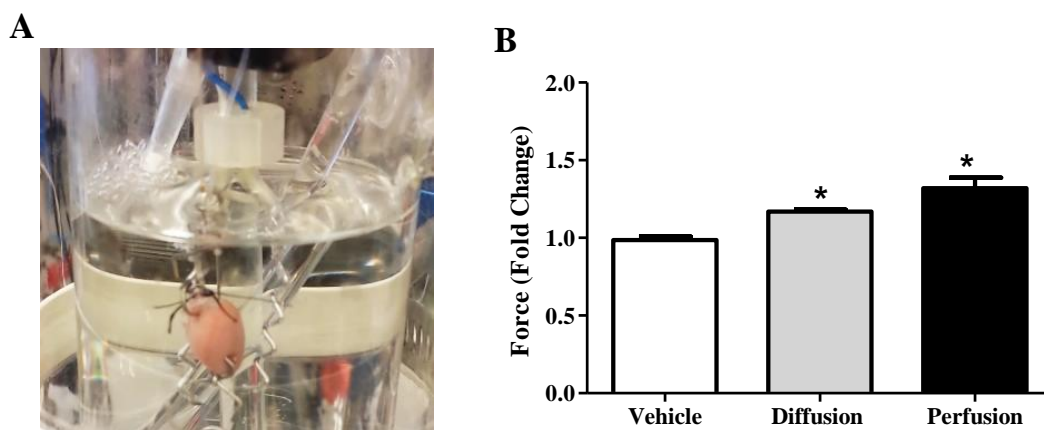
### TMAO-induced Changes in Force of Cardiac Contractility

In order to explore the acute effect of exogenous TMAO on cardiac muscle contractility, we compared the contractile responses elicited by diffusing increasing concentrations of TMAO to vehicle treatment. Peak changes in whole heart contractility were noted between 7 and 18 minutes following addition of TMAO to the organ bath. Figure 1A displays raw tracings of paced hearts following treatment with 3,000  $\mu\text{M}$  TMAO. Acute treatment with TMAO increased average isometric force generation 17% and 41%, at 300  $\mu\text{M}$  and 3,000  $\mu\text{M}$  respectively, compared to vehicle ( $P < 0.05$ ; Fig. 1B). We further analyzed the effect of TMAO on specific characteristics of each contractile waveform. Increasing concentrations of TMAO induced significant increases in the maximum slope (rate of contraction), peak area under the curve, and minimum slope (rate of relaxation) of the waveform when compared with vehicle ( $P < 0.05$ ; Fig. 1C).



**Figure 1. TMAO Increases Cardiac Contractility in *ex vivo* Mouse Hearts** **A:** raw tracings of paced, ventricular contractions at baseline and then following 3,000  $\mu\text{M}$  TMAO. **B:** mean changes in isometric tension (force) normalized to baseline contractions. **C:** mean changes in maximum slope, peak area, and minimum slope of the contractile waveform normalized to baseline contractions ( $n = 6-7$  animals). \*Statistical difference from vehicle ( $P < 0.05$ ). † Statistical difference from 300  $\mu\text{M}$  TMAO ( $P < 0.05$ ).

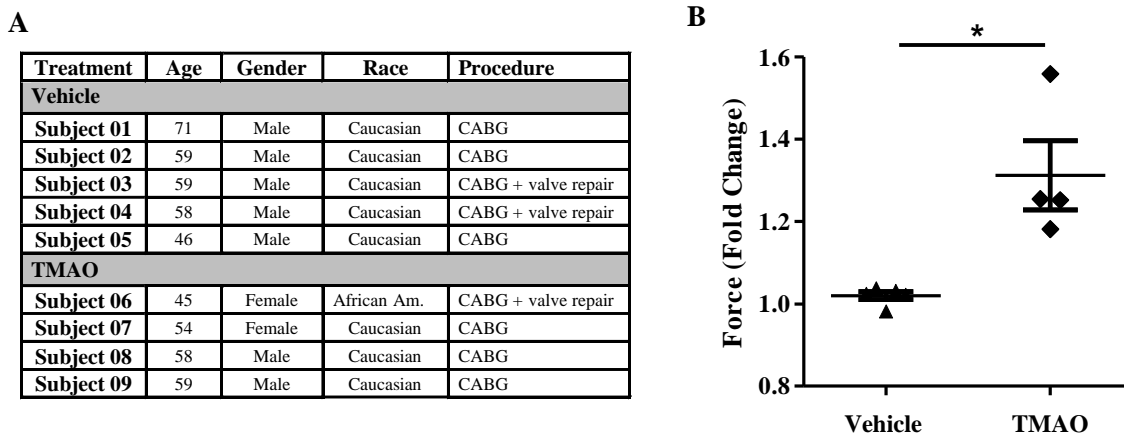
In these initial experiments, TMAO was diluted into the organ bath for diffusion to the heart. In the next series of experiments, we directly perfused TMAO through the coronary arteries. Modified Langendorff perfusion of hearts with 300  $\mu$ M TMAO generated a greater response compared to non-perfusion and increased isometric force 34% compared to vehicle ( $P < 0.05$ ; Fig. 2B). Langendorff perfusion of TMAO also induced statistically significant increases in maximum slope (59%), peak area under the curve (26%), and minimum slope (42%) of the contractile waveform compared to vehicle ( $P < 0.05$ ).



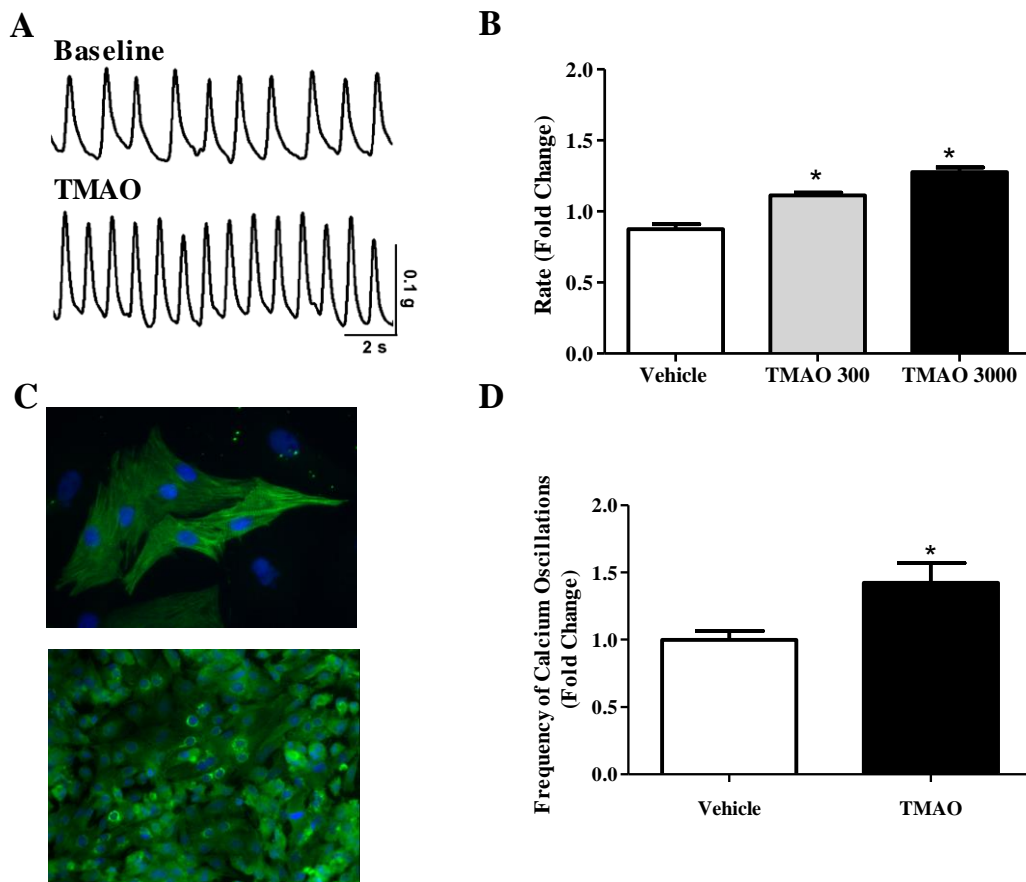
**Figure 2. Langendorff Perfusion of Coronary Arteries with TMAO Generates an Even Greater Response**  
**A:** modified Langendorff perfusion apparatus — a cannula is inserted into the aorta to facilitate reverse perfusion, the heart is connected to the force transducer and hung between bipolar stimulating electrodes. **B:** mean changes in isometric tension induced by 300  $\mu$ M TMAO either infused into the organ bath (Diffusion) or Langendorff perfused through the coronary circulation (Perfusion). All changes have been normalized to baseline contractions and are shown as a fold change ( $n = 2-3$  animals). \*Statistical difference from vehicle ( $P < 0.05$ ). † Statistical difference from diffusion-based delivery of 300  $\mu$ M TMAO ( $P < 0.05$ ).

To determine if our findings translated to human heart tissue, we repeated our studies with human atrial appendage biopsy tissue. Demographic information on the patients from which the samples were obtained is displayed in Figure 3A. Of note, all participants had a documented history of coronary artery disease and hypertension. Other comorbidities included history of myocardial infarction, arrhythmia, diabetes, CKD, hyperlipidemia, and thyroid disease. Two subjects in the TMAO group and three subjects in the control group were taking a beta-blocker at the time of their enrollment in this study. Though these drugs lower blood pressure and reduce

beta-adrenergic receptor activity *in vivo*, all biopsy samples included for analysis generated a greater than 40% increase from baseline in response to the inotropic agent, norepinephrine, used as a positive control. Consistent with what was observed in our animal model, TMAO (3,000  $\mu$ M) increased isometric tension 29% compared to vehicle ( $P < 0.05$ ; Fig. 3B). TMAO also increased maximum slope 29% ( $P < 0.05$ ), minimum slope 32% ( $P < 0.05$ ) and peak area under the curve 12% ( $P > 0.05$ ) compared to vehicle.



We followed these experiments by imaging spontaneously beating isolated embryonic (E18) rat cardiomyocytes. First, to confirm the cardiac phenotype, these cells were stained using fluorescent antibodies to cardiac specific markers — troponin I and atrial natriuretic peptide (ANP) (Fig. 4C). Next, the cells were imaged using the fluorescent  $\text{Ca}^{2+}$  indicator, Fluo-4 AM, to measure changes in intracellular  $\text{Ca}^{2+}$  wave frequency following treatment with TMAO (300  $\mu\text{M}$ ) or vehicle. TMAO increased the calcium oscillation frequency 42% compared to vehicle ( $P < 0.05$ ; Fig. 4D) but did not increase peak intracellular calcium ( $P > 0.05$ ).



**Figure 4. TMAO Increases Rate of Cardiac Contraction** **A:** raw tracings of spontaneously beating isolated mouse hearts at baseline and then following treatment with 3,000  $\mu\text{M}$  TMAO. **B:** mean changes in contraction rate evoked by TMAO or vehicle normalized to baseline rate ( $n = 3$ ;  $P < 0.05$ ). **C:** spontaneously beating embryonic rat cardiac myocytes were stained with two cardiac-specific markers, cardiac troponin I antibody (top image) and atrial natriuretic peptide antibody (bottom image) as well as DAPI to stain the nuclei. **D:** fold change in the average frequency of calcium oscillations normalized to vehicle ( $n = 3-4$ ). \*Statistical difference from vehicle ( $P < 0.05$ ). † Statistical difference from 300  $\mu\text{M}$  TMAO ( $P < 0.05$ ).

## DISCUSSION

There is a strong inverse relationship between kidney function and TMAO levels within the body.<sup>23,25,35,45</sup> In healthy patients the median serum TMAO concentration is approximately 3  $\mu$ M. As CKD advances to ESRD, median serum TMAO concentration climbs roughly 30-fold.<sup>37</sup> Recent studies suggest TMAO could be an independent, nontraditional risk factor for CVD in patients with CKD,<sup>22,46</sup> with elevated concentrations of TMAO having been associated with increased risk for major adverse cardiovascular events (myocardial infarction, stroke, and death),<sup>22,23,38</sup> heart failure,<sup>45,47</sup> and worse overall survival even after adjusting for traditional risk factors.<sup>36,48</sup> Despite these strong clinical associations, the mechanism by which TMAO promotes cardiovascular risk remains poorly understood. To date most studies have focused on vascular function. For example, animal studies have linked increased plasma TMAO to foam cell formation and altered sterol metabolism,<sup>22,24</sup> however, few studies have attempted to uncover the effects of TMAO on the myocardium itself. Therefore, our objective was to determine whether TMAO alone could directly alter cardiac muscle mechanics. The major findings of this study were: 1) acute TMAO exposure increases the force of cardiac contractility in mice 2) these findings translate to human cardiac muscle and 3) exposure to TMAO acutely increases the spontaneous beating rate of mouse hearts as well as that of isolated cardiac myocytes.

### **Inotropic Effects**

We have shown for the first time that TMAO significantly increases isometric tension, maximum slope, peak area under the curve, and minimum slope of the contractile waveform in isolated whole mouse hearts. These increases were significantly greater in Langendorff perfused hearts than when TMAO was pipetted into the organ bath. Increases in *ex vivo* contractility were

also observed in human heart biopsy tissue, confirming that TMAO directly influences human cardiac function.

The cellular mechanism(s) responsible for this improved contractility remain to be elucidated in future studies. However, data from previous studies suggests several possibilities. First, TMAO may augment cytosolic calcium release mechanisms. It has recently been shown that acute TMAO exposure can directly modulate platelet hyperresponsiveness and thrombosis formation through increasing calcium release from intracellular stores.<sup>49</sup> Interestingly, we did not observe a significant increase in peak intracellular calcium when treating isolated rat cardiomyocytes with TMAO. The increase in calcium may have been mitigated by the increase in rate of excitation induced by TMAO. Nevertheless, it may be that the underlying mechanism is largely independent of calcium.

Second, TMAO may be altering the muscle proteins themselves. Growing evidence suggests that TMAO stabilizes the structure of various proteins through preferential hydration.<sup>50–56</sup> Owing to its small size and a combination of both hydrophobic and polar characteristics, TMAO mimics a protein chaperone<sup>57</sup> promoting protein folding and stabilization of secondary structure through formation of hydrogen bonds with surrounding water molecules, which excludes water from the protein's amide backbone.<sup>58–61</sup> At high concentrations (1.0 M) in rabbit skeletal muscle, TMAO has specifically been shown to enhance polymerization of actin<sup>62,63</sup> and stabilization of myosin.<sup>64</sup> In this study the authors showed that at these high concentrations both sarcomere sliding velocity and ATPase activity were decreased, potentially indicating excessive stabilization. Alternatively, TMAO at 50 mM has been shown to increase skeletal muscle power in the rainbow smelt, *Osmerus mordax*, at 10°C. Treatment with TMAO led to greater generation of contractile force and faster relaxation,<sup>65</sup> which the authors attributed to the stabilizing influence of TMAO on

muscle proteins. In our studies we also observed an increase in contractile force and faster relaxation time; therefore, it is possible that similar mechanisms are responsible for altering contractility in cardiac muscle and skeletal muscle, both of which are striated muscle types.

Consistent with this hypothesis, there is robust evidence that TMAO counteracts the effects of urea at the cellular level.<sup>16,18,23–25,44–46</sup> Urea, the predominant nitrogenous product of protein metabolism, is also renally cleared and thus accumulates as the kidneys fail. However, unlike TMAO, urea weakens the hydrogen bonds of water surrounding proteins,<sup>51,58</sup> which results in protein unfolding and loss of function.<sup>66</sup> While studies investigating the direct actions of TMAO on the heart are lacking, the direct effects of urea are better characterized. In a series of similar cardiac contractility experiments, urea depressed myocardial force development.<sup>67,68</sup> It is reasonable, therefore, to postulate both urea and TMAO at pathological concentrations have the capacity to alter the excitation-contraction coupling mechanism in heart muscle and that TMAO may oppose the actions of urea at the myocardium due to its stabilizing properties.

### **Chronotropic Effects**

In the absence of artificial pacing, acute exposure of whole mouse hearts to TMAO significantly increased spontaneous beating rate. TMAO also increased calcium oscillation frequency, which corresponds to cell excitation, in spontaneously beating cardiomyocytes. We utilized these particular cells, primary cardiomyocytes from embryonic rats at day 18 (approximately 2 days before birth), because they are able to autonomously pace<sup>69</sup> and have been shown to change rates in response to known chronotropic agents.<sup>70</sup>

Previous research indicates that more than 85% of the patients with CKD have at least one form of electrocardiogram (EKG) abnormality.<sup>71</sup> Tachycardia and premature contractions are among the most prevalent changes seen in this patient population.<sup>71,72</sup> This may indicate that

TMAO increases pace-making and/or excitatory responses in the heart. Interestingly, in a recent study investigating the role of TMAO in the progression of atrial fibrillation, when locally injected into the four major atrial autonomic ganglionated plexi, TMAO increased susceptibility to atrial fibrillation in canines.<sup>73</sup> These findings in the autonomic nervous system — in light of our data showing a direct, acute effect on the heart — indicate TMAO may facilitate arrhythmia inducibility.

The mechanism responsible for the increase in beating frequency also remains to be elucidated; however, this too may occur through protein stabilization. In a study designed to test the *in vivo* efficacy of TMAO in correcting the Cl<sup>-</sup> transport defect in a mouse model of cystic fibrosis (CF), TMAO partially corrected the Cl-conductance defect in  $\Delta F508$  CF mice.<sup>74</sup> Therefore, TMAO may modify cardiac beating frequency and excitability through stabilization of ion-channels within the muscle membrane, altering ion currents. Further *in vitro* studies are needed to uncover the mechanisms responsible for the changes we observed in spontaneous beating rate.

### **Significance**

There is a growing appreciation for the intricacy of the relationship between the microbiome, kidneys, and cardiac vasculature and its role in chronic disease progression. TMAO has been implicated to play a critical role in this multiorgan cross-talk, yet the importance of the direct impact TMAO has on the heart itself is only now being explored.

At present, it is unclear if TMAO is a harmful byproduct or if it provides some protective benefit.<sup>75</sup> We hypothesize that the acute inotropic and chronotropic actions of TMAO may help to maintain cardiac output during early uremic cardiomyopathy. Among non-diabetic patients with CKD stages 2 to 4, there was no difference in left ventricular ejection fraction compared to healthy controls, in spite of increased diffuse myocardial fibrosis and global cardiac strain.<sup>76</sup> In light of



our findings, TMAO may induce compensatory increases which would allow for preservation of cardiac output and renal perfusion, physiologically advantageous adjustments for patients with renal dysfunction. Yet chronically elevated cardiac contractility and heart rate, while helping to maintain cardiac homeostasis, increases cardiac energy consumption and results in pathologic changes including cardiac remodeling, hypertrophy, fibrosis, and eventual progression to heart failure.<sup>77-83</sup> The prevalence of these maladaptive alterations in cardiac structure and function increases as renal function deteriorates.<sup>84</sup>

In summary, our study is the first to show TMAO alone has direct inotropic and chronotropic effects on the heart. This lays the groundwork for future translational studies investigating the complex multiorgan interplay involved in cardiovascular pathogenesis during CKD. Further studies are needed to illustrate a mechanism of action and to explore whether TMAO represents a therapeutic target for reducing cardiovascular mortality in patients with CKD.

### **Limitations**

A few limitations in our studies should be acknowledged. First, contractility analysis was performed using *ex vivo* mouse hearts. While this allowed us to characterize the direct effect of TMAO on isolated cardiac muscle, contractile mechanics may differ *in vivo*. Further, in the human heart contractility experiments we were able to test atrial appendage tissue but not ventricular muscle. While we obtained similar results between human atrial samples and mouse ventricular tissue, there are species and muscle type differences which are unavoidable. Finally, our *ex vivo* studies were critical for isolating the immediate effects of TMAO on cardiac muscle apart from other endogenous agents released *in vivo*; however, once a heart is removed it remains metabolically active for only a limited time outside of the body, even when oxygenated. Therefore, our setup is less conducive for measuring changes in cardiac function over extended periods.

## **Future Directions**

Due to the disproportionate prevalence of sudden cardiac death among patients with CKD, our group has proposed to further explore the ability for TMAO to induce and promote arrhythmias, such as atrial fibrillation. To do this we would perform EKGs on mouse hearts *ex vivo* in order to characterize alterations in the electrical activity of the heart after TMAO treatment.

We are also interested in further exploring the cellular mechanisms responsible for the inotropic and chronotropic effects we observed. We plan to repeat our contractility experiments treating with both urea and TMAO. If TMAO is able to mitigate the action of urea on the organ level, this would support to the hypothesis that TMAO exerts its effect on the heart through protein stabilization.

Our studies have captured the immediate effects of TMAO on the heart and cardiac cells. Due to the nature of our experimental design, we cannot extrapolate long-term changes in cardiac contractile mechanics. Now that we have described the acute effects of TMAO on the heart, we need to explore how chronically elevated concentrations of TMAO influence cardiac function.

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