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Prognostic Value of Elevated Levels of Intestinal Microbe-Generated Metabolite Trimethylamine-*N*-Oxide in Patients With Heart Failure

Refining the Gut Hypothesis

W.H. Wilson Tang, MD,*† Zeneng Wang, PhD,* Yiying Fan, PhD,‡ Bruce Levison, PhD,* Jennie E. Hazen,* Lillian M. Donahue,* Yuping Wu, PhD,‡ Stanley L. Hazen, MD, PhD*†

ABSTRACT

BACKGROUND Altered intestinal function is prevalent in patients with heart failure (HF), but its role in adverse outcomes is unclear.

OBJECTIVES This study investigated the potential pathophysiological contributions of intestinal microbiota in HF.

METHODS We examined the relationship between fasting plasma trimethylamine-*N*-oxide (TMAO) and all-cause mortality over a 5-year follow-up in 720 patients with stable HF.

RESULTS The median TMAO level was 5.0 μM, which was higher than in subjects without HF (3.5 μM; $p < 0.001$). There was modest but significant correlation between TMAO concentrations and B-type natriuretic peptide (BNP) levels ($r = 0.23$; $p < 0.001$). Higher plasma TMAO levels were associated with a 3.4-fold increased mortality risk. Following adjustments for traditional risk factors and BNP levels, elevated TMAO levels remained predictive of 5-year mortality risk (hazard ratio [HR]: 2.2; 95% CI: 1.42 to 3.43; $p < 0.001$), as well as following the addition of estimated glomerular filtration rate to the model (HR: 1.75; 95% CI: 1.07 to 2.86; $p < 0.001$).

CONCLUSIONS High TMAO levels were observed in patients with HF, and elevated TMAO levels portended higher long-term mortality risk independent of traditional risk factors and cardiorenal indexes. (J Am Coll Cardiol 2014;64:1908–14) © 2014 by the American College of Cardiology Foundation.

From the *Department of Cellular and Molecular Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio; †Department of Cardiovascular Medicine, Heart and Vascular Institute, Cleveland Clinic, Cleveland, Ohio; and the ‡Department of Mathematics, Cleveland State University, Cleveland, Ohio. This research was supported by grants from the National Institutes of Health (NIH) and the Office of Dietary Supplements (R01HL103866, P20HL113452). The GeneBank study was supported by NIH grants P01HL076491, P01HL098055, and R01HL103931, and the Cleveland Clinic Clinical Research Unit of the Case Western Reserve University CTSA (UL1TR 000439). Dr. Wang was partially supported by an American Heart Association Scientist Development Grant (12SDG12050473). Dr. Hazen was partially supported by a gift from the Leonard Krieger endowment and the Foundation LeDucq. Dr. Tang has received investigator-initiated research grant support from Abbott Laboratories, with no personal financial payments. Drs. Wang and Levison are named as co-inventors on pending patents held by the Cleveland Clinic relating to cardiovascular diagnostics and have the right to receive royalty payment for inventions or discoveries related to cardiovascular diagnostics from LipoScience, Inc. Dr. Hazen is named as a co-inventor on pending patents held by the Cleveland Clinic relating to cardiovascular diagnostics; has received consulting fees from Abbott Diagnostics, Cleveland Heart Lab, Esperion, Lilly, LipoScience, Inc., Merck & Co., Procter & Gamble, and Pfizer; has received research funds from Abbott Diagnostics, Cleveland Heart Lab, LipoScience, Inc., Procter & Gamble, Pfizer, and Takeda; and has the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland Heart Lab, Esperion, Frantz Biomarkers, and LipoScience, Inc. All other authors have reported that they have no relationships relevant to the contents of this paper to disclose.

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There is increasing recognition that the gastrointestinal system represents an often overlooked contributor to the pathogenesis of heart failure (HF) syndrome and its adverse complications (1,2). Normally, intestinal barrier function is maintained by a well-balanced intestinal microbial community, intact tight junctions in the mucosa, normal mucosal immunity, and normal sodium and water homeostasis. With HF and associated

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splanchnic circulation congestion, bacterial translocation can occur due to altered intestinal barrier function, bacterial overgrowth, and impaired host defense, leading to endotoxemia that in turn can contribute to systemic inflammation. Several of these features of altered intestinal function have been observed in patients with HF (3,4). Meanwhile, progressive venous congestion in subsets of patients with significant congestive signs and symptoms may lead to unwanted consequences of abdominal congestion, including adverse impact on drug absorption and pharmacokinetics (5,6), and renal glomerular and tubular dysfunction resulting from raised intra-abdominal pressures (7,8).

Intestinal microbiota are implicated in the regulation of multiple host metabolic pathways that contribute to phenotypes such as obesity and insulin resistance (9). Our group has recently described a mechanistic link between intestinal microbe-dependent generation of trimethylamine-*N*-oxide (TMAO) and increased risk for future cardiovascular events (death, myocardial infarction, and stroke) by a pathway involving dietary nutrients such as phosphatidylcholine, choline, and carnitine (10–12). In chronic systolic HF, the relationship between the intestinal microbiota-generated metabolite TMAO and both cardiorenal indexes and long-term clinical prognosis has not been examined. We sought to investigate the relationship between fasting plasma TMAO levels and long-term clinical prognosis in patients with stable HF undergoing cardiac evaluation, particularly in relation with established prognostic markers (B-type natriuretic peptide [BNP] and estimated glomerular filtration rate [eGFR]).

METHODS

STUDY POPULATION. We prospectively enrolled patients with stable cardiac disease seen at the Cleveland Clinic between 2001 and 2007 with a history of HF undergoing elective, nonurgent coronary angiographic evaluation. We excluded those patients who had experienced acute coronary syndrome within the preceding 30 days (cardiac troponin I ≤0.03 ng/ml). All-cause mortality was prospectively tracked for 5 years with the Social Security Death Index and medical chart review and confirmed by follow-up contact. We also performed a cross-sectional comparison of TMAO levels between our cohort of patients with stable systolic HF and an independent set of 300 prospectively recruited, apparently healthy subjects without known cardiac disease from a health-screening program at various locations across Cleveland, Ohio. All subjects for all studies gave written informed consent approved by the Cleveland Clinic Institutional Review Board.

ABBREVIATIONS AND ACRONYMS

BNP = B-type natriuretic peptide

eGFR = estimated glomerular filtration rate

HDL-C = high-density lipoprotein cholesterol

HF = heart failure

hsCRP = high-sensitivity C-reactive protein

TMAO = trimethylamine-*N*-oxide

STUDY DESIGN AND ASSAY MEASUREMENTS. This is a single-center, prospective cohort study approved by the Cleveland Clinic Institutional Review Board. After informed consent was obtained from all patients, fasting plasma blood samples were collected using EDTA tubes, which were then immediately processed and frozen at −80°C until analysis. Fasting plasma TMAO levels were quantified by stable isotope dilution liquid chromatography with online tandem mass spectrometry (LC/MS/MS) on an API 500 triple quadruple mass spectrometer (AB SCIEX, Framingham, Massachusetts), as previously described (10–12). BNP, high-sensitivity C-reactive protein (hsCRP), fasting lipid panel, uric acid, and serum creatinine were measured using the Architect *ci*8200 platform (Abbott Laboratories, Abbott Park, Illinois). Serum arylesterase activity was measured in the same platform as previously described (13).

STATISTICAL ANALYSES. Continuous variables are summarized as mean ± SD if normally distributed and median (interquartile range [IQR]) if not normally distributed. The Student *t* test or Wilcoxon-rank sum test for continuous variables and chi-square test for categorical variables were employed to examine between-group differences. Spearman correlation was used to examine the associations between TMAO and other laboratory measurements. Kaplan-Meier survival plots and Cox proportional hazards analysis were used to determine hazard ratio (HR) and 95% CI for all-cause mortality stratified according to TMAO as a continuous variable (log-transformed per SD increase), as well as according to quartiles. Adjustments were made for individual traditional risk factors, including age, sex, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol (HDL-C), smoking, diabetes mellitus, and BNP, to predict all-cause mortality risks. Net reclassification and area under the

TABLE 1 Baseline Characteristics of Heart Failure Cohort (n = 720)

	Overall	TMAO <5 μM	TMAO ≥5 μM	p Value
Age, yrs	66 ± 10	64 ± 11	68 ± 10	<0.001
Male	59	59	59	1.000
Diabetes mellitus	41	31	51	<0.001
Hypertension	78	76	79	0.316
Ischemic etiology	64	63	65	0.673
LV ejection fraction	35 (25-50)	35 (25-51)	40 (25-50)	0.567
Body mass index, kg/m^2	28.4 (25.1-33.1)	28.7 (25.2-33.3)	28.1 (24.8-32.9)	0.298
Baseline medications				
ACE inhibitors or ARBs	69	71	66	0.206
Beta-blockers	69	73	65	0.031
Loop diuretics	59	53	65	0.001
Statins	61	66	57	0.013
Aspirin	64	66	63	0.505
LDL cholesterol, mg/dl	91 (73-112)	93 (75-113)	88 (71-111)	0.069
HDL cholesterol, mg/dl	32 (26-40)	34 (27-42)	30 (25-38)	<0.001
BNP, pg/ml	294 (114-658)	226 (96-498)	358 (150-907)	<0.001
hsCRP, mg/l	3.9 (1.6-9.0)	2.9 (1.3-8.1)	3.4 (1.3-8.9)	0.585
eGFR, ml/min/1.73 m^2	72 (56-87)	83 (70-93)	60 (44-74)	<0.001
TMAO, mM	5 (3.0-8.5)	3 (2.2-4)	8.5 (6.6-13.6)	<0.001

Values are mean ± SD, %, or median (interquartile range).

ACE = angiotensin-converting enzyme; ARB = angiotensin receptor blocker; BNP = B-type natriuretic peptide; eGFR = estimated glomerular filtration rate; HDL = high-density lipoprotein; hsCRP = high-sensitivity C-reactive protein; LDL = low-density lipoprotein; LV = left ventricular; TMAO = trimethylamine-*N*-oxide.

receiver-operating characteristic curve (AUC) were calculated according to mortality risk estimated using Cox models adjusted for the above-mentioned traditional risk factors with versus without TMAO, as previously described (14,15). All analyses were performed used R 2.15.1 (Vienna, Austria). A p value <0.05 was considered statistically significant.

TABLE 2 Hazard Ratio of Fasting Plasma TMAO Levels for 5-Year All-Cause Mortality

	Quartile 1	Quartile 2	Quartile 3	Quartile 4
Range, μM	<3.03	3.03-5	5-8.51	≥8.51
Unadjusted	1	1.39 (0.87-2.24)	2.19 (1.39-3.43)*	3.42 (2.24-5.23)*
Adjusted model 1	1	1.17 (0.72-1.90)	1.44 (0.90-2.30)	2.20 (1.42-3.43)*
Adjusted model 2	1	1.14 (0.70-1.86)	1.33 (0.83-2.13)	1.75 (1.07-2.86)†
Adjusted model 3	1	1.18 (0.73-1.91)	1.34 (0.84-2.15)	1.85 (1.14-3.00)†

Model 1: adjusted for traditional risk factors, including age, sex, systolic blood pressure, LDL cholesterol, HDL cholesterol, smoking, diabetes mellitus, and BNP (log-transformed). Model 2: adjusted for model 1 plus eGFR (log-transformed). Model 3: adjusted for model 2 plus hsCRP (log-transformed). *p < 0.01. †p < 0.05.

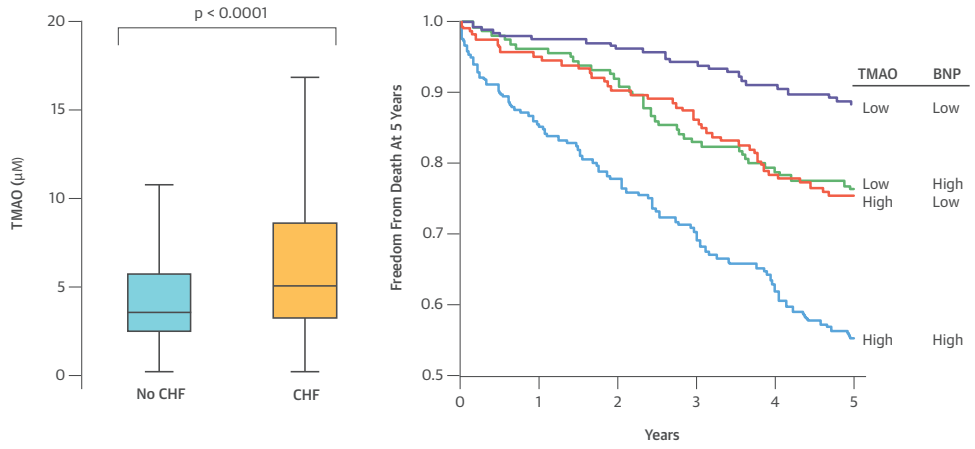
Abbreviations as in Table 1.

RESULTS

BASELINE CHARACTERISTICS. We analyzed 720 patients with a history of HF fulfilling inclusion and exclusion criteria. Baseline characteristics of our study cohort are provided in Table 1. The median TMAO level within the HF cohort was 5.0 μM (IQR: 3.0 to 8.5 μM), which was higher than that in the healthy control cohort (3.5 μM; IQR: 2.3 to 5.7 μM; p < 0.001) (Central Illustration). In our study cohort, patients with elevated TMAO levels tended to be older with a history of diabetes mellitus, renal insufficiency, and lower HDL-C levels. Patients with HF with higher TMAO levels were also more likely to have higher BNP levels and diuretic use and lower beta-blocker use. In contrast, history of hypertension, ischemic etiology, left ventricular ejection fraction, smoking history, body mass index, and sex were similar across TMAO levels. Within the patients with HF cohort, plasma TMAO levels were weakly but significantly inversely correlated with serum arylesterase activity (r = −0.135; p < 0.001), a measure of the antioxidant HDL-associated enzyme paraoxonase 1 (PON1) (12), and positively correlated with serum uric acid levels (r = 0.29; p < 0.001), which are associated with pro-oxidant function (16,17).

FASTING PLASMA TMAO LEVELS AND MORTALITY RISK. Over the 5-year follow-up, 207 deaths occurred in our study cohort. Figure 1 represents the Kaplan-Meier analysis of TMAO stratified by quartiles, which illustrates a graded increased mortality risk that becomes particularly apparent when TMAO levels rise higher than median levels. Importantly, elevated TMAO levels were associated with increased mortality risk within the HF cohort (quartile 4 vs. 1; HR: 3.42; 95% CI: 2.24 to 5.23; p < 0.001) (Table 2). As a continuous variable, an increase in TMAO levels was associated with increased mortality risk at 5 years after adjustment for traditional cardiac risk factors (HR: 1.18; 95% CI: 1.06 to 1.31 per SD; p < 0.01) (Table 2); moreover, mortality risks were similar between ischemic and nonischemic patients with HF, as well as other clinical subgroups (Figure 2). Addition of TMAO to a model of traditional cardiovascular risk factors showed that elevated TMAO levels significantly improved net reclassification (integrated discrimination improvement 16.0%; p < 0.001; net reclassification index 10.9%; p < 0.001) and trended toward improvement in AUC (0.723 to 0.741; p = 0.096).

ASSOCIATION BETWEEN TMAO AND CARDIORENAL INDEXES IN MORTALITY RISK. We found relatively modest but significant correlation between TMAO and BNP levels (r = 0.23; p < 0.001) and stronger



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CENTRAL ILLUSTRATION Comparison of Fasting TMAO Levels Between Patients With Stable Heart Failure and Apparently Healthy Controls

(Left) Trimethylamine-*N*-oxide (TMAO) concentration was higher in patients with stable heart failure than healthy controls and **(Right)** portended poorer survival at higher levels regardless of B-type natriuretic peptide levels. Kaplan-Meier curves for 5-year all-cause mortality with TMAO with TMAO/B-type natriuretic peptide (BNP) stratified at median levels.

inverse correlation between TMAO levels and eGFR ($r = -0.55$; $p < 0.001$) in our study cohort. We further investigated the association between TMAO levels and cardiorenal indexes by constructing a model that included BNP levels and eGFR. In this model, patients in the highest quartile of TMAO (>8.5 μM) remained at a significantly higher mortality risk than those with lower TMAO levels. Specifically, elevated fasting TMAO levels were associated with a 2.2-fold increase in mortality risk after adjustment for traditional risk factors and BNP levels (HR: 2.20; 95% CI: 1.42 to 3.43; $p < 0.01$) and a 1.80-fold increase in mortality risk even after adjustment for traditional risk factors and BNP levels plus eGFR (HR: 1.75; 95% CI: 1.07 to 2.86; $p < 0.05$) and plus hsCRP levels (HR: 1.85; 95% CI: 1.14 to 3.00; $p < 0.05$). Interestingly, within the intermediate BNP range (second tertile 160 to 505 pg/ml), the lower 2 tertiles of TMAO portended a 3.3-fold increase in mortality risk (95% CI: 1.4 to 8; $p < 0.001$), whereas the highest TMAO tertile (>7.2 μM) had a 5.7-fold increased mortality risk (95% CI: 2.5 to 13.2; $p < 0.01$) compared with both low BNP and TMAO levels (Figure 3).

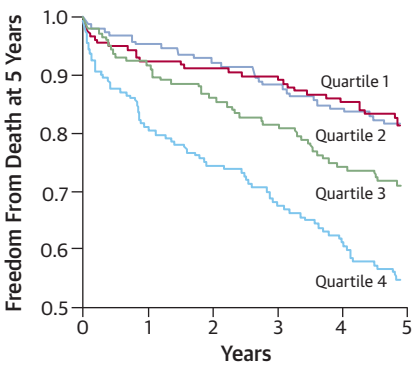

FIGURE 1 Kaplan-Meier Estimates of Risk of All-Cause Mortality According to Quartiles of Plasma Levels of TMAO

Kaplan-Meier curves for 5-year all-cause mortality with trimethylamine-*N*-oxide (TMAO) stratified as quartiles.

DISCUSSION

The key finding to our study is the strong prognostic value of plasma TMAO levels in patients with stable HF incremental to traditional risk factors, cardiorenal indexes (BNP and eGFR), and markers of systemic inflammation (hsCRP). It is intriguing that in the setting of elevated natriuretic peptide levels, which often represent significant myocardial disease progression, a relatively low fasting TMAO level was associated with far lower mortality risk than that seen with elevated levels of both markers. These observations in a large population of HF patients further

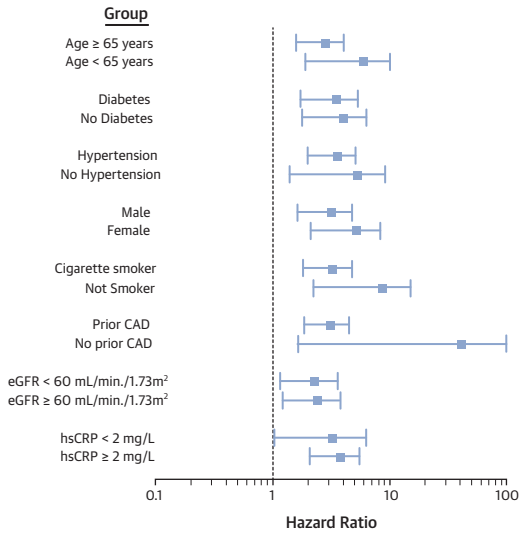

FIGURE 2 Relationship Between Plasma TMAO Concentration and Mortality Risk Stratified According to Baseline Characteristics

Forest plot of the hazard ratio **(squares)** of 5-year all-cause mortality risk comparing first and fourth quartiles of fasting plasma TMAO levels. **Bars** represent 95% CI. The wide CI in some subgroups are in part due to their small sample sizes and event rates. CAD = coronary artery disease; eGFR = estimated glomerular filtration rate; hsCRP = high-sensitivity C-reactive protein; other abbreviation as in Figure 1.

refine the notion of a "gut hypothesis" and suggest a potential association between the gut microbiota pathway of known proatherogenic potential with adverse prognosis in patients with HF.

Although TMAO has been shown to possess proatherogenic properties (10–12), which might partially contribute to the adverse prognosis associated with higher TMAO levels, the equivalent adverse prognostic value of elevated TMAO levels in both ischemic and nonischemic HF suggests the possibility that other potential mechanisms contribute to the increased mortality risks observed in patients with HF with elevated TMAO levels. Our recent identification of the host-microbiome relationship in formation of TMAO (10–12) has generated novel insights into potential contributions of environmental factors within the traditional definition of "self." Although the "gut hypothesis" in HF has prevailed over the years, with evidence implying bacterial translocation and heightened inflammatory responses and oxidative stress, few studies have definitively linked such associations with outcomes or directly demonstrated such processes at the bedside. Our current observations therefore provided direct evidence that accumulation in the systemic circulation of TMAO, an obligatory downstream metabolite of gut microbiota, heralds increased long-term adverse clinical outcomes in patients with HF independently of multiple cardiorenal indexes. It remains to be determined whether elevated TMAO levels are associated with enhanced gut edema and bacterial translocation in HF; we have not identified an alteration in the microbial composition within patients with HF at increased adverse prognosis as the source of elevated TMAO levels among those at increased mortality risk. In the setting of HF, where ischemic or atherogenic processes appear less important in long-term prognosis and progression, the underlying mechanisms for the association between TMAO and adverse outcomes are not entirely understood.

Clearly, other factors such as individual variations in gut microbiota composition and impaired renal clearance may influence systemic TMAO levels. The notion that alterations in microbial composition and gut microbial functional physiology may be part of the HF syndrome is attractive (2). Changes in bacterial composition have been shown to serve as a primary driver of TMAO levels in other studies (10,15). Whether microbial composition changes in HF directly or indirectly contribute to enhanced oxidative stress and inflammation is unclear, but we did note that plasma TMAO levels were modestly yet positively correlated with serum uric acid levels and inversely correlated with PON1 activity as monitored by serum arylesterase activity. Importantly, the association between TMAO levels and eGFR was strong within our study cohort. However, we also observed that TMAO levels conferred mortality risk prediction beyond traditional risk factors, BNP, and eGFR, although the kidneys may be physiologically impaired in eliminating TMAO beyond eGFR.

The present study is the first demonstration of an association between elevated TMAO levels and poor prognosis in patients with HF. Our findings have several important implications that should be discussed. First, by demonstrating the association between TMAO and mortality risks in patients with HF, our findings suggest that gut microbiota composition and functional consequence may contribute to disease progression during HF. With the mechanistic link between TMAO and atherogenesis in animal models (10,12), it is conceivable that progressive vascular remodeling and coronary atherogenesis may occur in the setting of high TMAO levels. Even in those with no known ischemic

heart disease, subclinical concomitant coronary artery disease may occur (18). In fact, previous autopsy findings have shown that progressive coronary vasculopathy is not uncommon in patients with HF nor is the occurrence of sudden cardiac death (19). On the other hand, others have speculated that there exists a potential link between progressive wasting in advanced HF and other chronic disease that can be associated with the gut microbiota axis (20).

Second, the present studies suggest a potential clinical utility for clearance of gut microbiome-generated uremic toxins as a possible adjunct therapy for HF. Of note, recent oral potassium binders have shown some promise in clinical testing in patients with HF and cardiorenal compromise (21,22). The ability of these interventions to eliminate harmful compounds via the alimentary track remains unknown. In the setting of advanced renal diseases, direct binders have been reported to reduce levels of indoxyl sulfate, a tryptophan-based gut microbiome metabolite that has shown antiatherosclerotic effects in animal models (23). Hence, it is conceivable that targeted interventions could directly impact microbiome-host relationships, and further investigations are warranted into direct effects of TMAO and other gut microbiome-derived metabolites on cardiac remodeling.

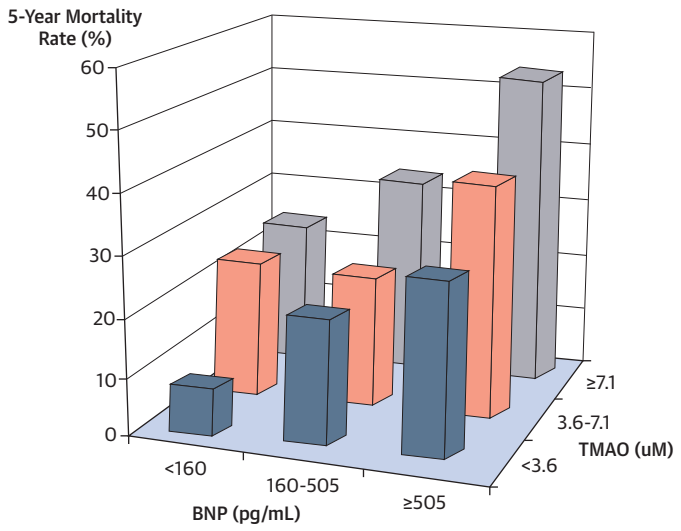

FIGURE 3 Mortality Rates Stratified by Tertiles of BNP and TMAO

Five-year mortality rates according to B-type natriuretic peptide (BNP) tertiles and trimethylamine-*N*-oxide (TMAO) tertiles. All cohorts were significantly different compared with the reference cohort of BNP <160 pg/ml and TMAO <3.6 μM (all $p < 0.01$).

STUDY LIMITATIONS. First, this was a single-center study that recruited patients at the point of cardiac evaluation for coronary angiography; hence, selection bias may have identified a higher proportion of patients with ischemic cardiomyopathy. Also, this was a cohort of patients who underwent elective coronary angiography; thus, right heart catheterization was not performed in a large majority of patients and was not readily available. Also, because of this catchment, the large majority of patients had relatively preserved renal function, and few had advanced cardiorenal diseases or advanced HF (annualized mortality of 5.8%). We also did not have complete information regarding New York Heart Association functional class, presence of cachexia, or atrial fibrillation in our study population, even though we believe that the inclusion of BNP can integrate many of these risk profiles. Another potential limitation is that only a single time-point blood draw after overnight fast was available, and we did not have dietary history to confirm or refute the impact of diet on TMAO levels or their cardiorenal consequences. Specifically, we did not have any information regarding gastrointestinal symptoms and pathology or prior antibiotic use or knowledge of prior supplements other than those taken the day of enrollment. As in most outcome studies, interim events and treatment also may influence the primary outcome of all-cause mortality.

Despite these limitations, our findings point to novel insights that provide mechanistic links between gut microbiota-associated metabolism involved in TMAO formation and cardiorenal pathophysiology. Further investigations to test the hypothesis that targeted interventions are warranted to alter the gut microbiota composition to lower TMAO production or enhance TMAO clearance to determine whether it is possible to alter the natural history of HF disease progression.

CONCLUSIONS

Patients with HF had elevated TMAO levels compared with those without HF. Elevated fasting TMAO levels portended higher long-term mortality risk independently of traditional risk factors, BNP levels, and renal function.

REPRINT REQUESTS AND CORRESPONDENCE: Dr. W.H. Wilson Tang, Cardiovascular Medicine, Cleveland Clinic, 9500 Euclid Avenue, Desk J3-4, Cleveland, Ohio 44195. E-mail: tangw@ccf.org.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The intestinal microbe-dependent metabolite, TMAO is related to the pathogenesis of atherosclerosis and may play a role in the development and progression of heart failure.

TRANSLATIONAL OUTLOOK: Clinical studies should address the effects of dietary modifications and other interventions that alter microbe-generated intestinal TMAO on the development and progression of heart failure.

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