

# Gut Microbiota-Dependent Trimethylamine N-Oxide (TMAO) Pathway Contributes to Both Development of Renal Insufficiency and Mortality Risk in Chronic Kidney Disease

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**Rationale:** Trimethylamine-N-oxide (TMAO), a gut microbial-dependent metabolite of dietary choline, phosphatidylcholine (lecithin), and L-carnitine, is elevated in chronic kidney diseases (CKD) and associated with coronary artery disease pathogenesis.

**Objective:** To both investigate the clinical prognostic value of TMAO in subjects with versus without CKD, and test the hypothesis that TMAO plays a direct contributory role in the development and progression of renal dysfunction.

**Methods and Results:** We first examined the relationship between fasting plasma TMAO and all-cause mortality over 5-year follow-up in 521 stable subjects with CKD (estimated glomerular filtration rate, <60 mL/min per 1.73 m<sup>2</sup>). Median TMAO level among CKD subjects was 7.9 μmol/L (interquartile range, 5.2–12.4 μmol/L), which was markedly higher ( $P<0.001$ ) than in non-CKD subjects ( $n=3166$ ). Within CKD subjects, higher (fourth versus first quartile) plasma TMAO level was associated with a 2.8-fold increased mortality risk. After adjustments for traditional risk factors, high-sensitivity C-reactive protein, estimated glomerular filtration rate, elevated TMAO levels remained predictive of 5-year mortality risk (hazard ratio, 1.93; 95% confidence interval, 1.13–3.29;  $P<0.05$ ). TMAO provided significant incremental prognostic value (net reclassification index, 17.26%;  $P<0.001$  and differences in area under receiver operator characteristic curve, 63.26% versus 65.95%;  $P=0.036$ ). Among non-CKD subjects, elevated TMAO levels portend poorer prognosis within cohorts of high and low cystatin C. In animal models, elevated dietary choline or TMAO directly led to progressive renal tubulointerstitial fibrosis and dysfunction.

**Conclusions:** Plasma TMAO levels are both elevated in patients with CKD and portend poorer long-term survival. Chronic dietary exposures that increase TMAO directly contributes to progressive renal fibrosis and dysfunction in animal models. (*Circ Res.* 2015;116:448-455. DOI: 10.1161/CIRCRESAHA.116.305360.)

**Key Words:** cardiovascular diseases ■ chronic kidney disease ■ fibrosis ■ gut microbiota ■ trimethylamine N-oxide

Patients with chronic kidney disease (CKD) are at increased risk for the development of cardiovascular disease (CVD) beyond traditional risk factors. Yet the mechanism(s) through which CKD is linked to enhanced atherosclerotic heart disease is not fully elucidated. Although a role for uremic toxins in the pathogenesis of cardiovascular and renal disease progression in CKD has been suggested for some time, the precise participants involved, and their mechanisms of action remain unclear. Recent studies have suggested involvement of gut-microbiota in the generation of metabolites that display uremic toxicity.<sup>1</sup> Furthermore, perturbations of the composition of the gut

microbial community in both human and experimental CKD are associated with significant elevations of gut-derived uremic toxins.<sup>2</sup> Such alterations have also been associated with increased systemic inflammatory burden, and thus are suspected to play a role in the pathogenesis of cardiovascular and renal disease progression in subjects with CKD. Bacterial structural components, such as lipopolysaccharide,<sup>3</sup> and metabolites, such as indoxyl sulfate, p-cresyl sulfate, amines, and ammonia,

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### Nonstandard Abbreviations and Acronyms

<b>CI</b>	confidence interval
<b>CKD</b>	chronic kidney disease
<b>CVD</b>	cardiovascular disease
<b>eGFR</b>	estimated glomerular filtration rate
<b>HR</b>	hazard ratio
<b>hsCRP</b>	high-sensitivity C-reactive protein
<b>TMAO</b>	trimethylamine- <i>N</i> -oxide

have been identified as potential microbial by-products capable of initiating proinflammatory cytokine/chemokine cascades seen in the setting of CKD and end-stage renal disease.<sup>4</sup> Thus, it has been hypothesized that gut-microbiota-derived uremic toxins may serve as both therapeutic targets and assessment tools for renal diseases in this vulnerable population.<sup>1,5-7</sup> However, demonstration of a role for specific uremic toxins in CVD pathogenesis has been indirect.

Our group recently identified a novel mechanistic link between gut microbiota metabolism of dietary trimethylamine-containing nutrients and CVD pathogenesis.<sup>8-13</sup> Specifically, gut microbiota-mediated metabolism of phosphatidylcholine, choline, or *L*-carnitine each have been shown to produce trimethylamine-*N*-oxide (TMAO), and in multiple clinical studies, TMAO levels have been shown to be associated with cardiovascular risks.<sup>8-10,13</sup> Furthermore, animal model studies have revealed that TMAO is mechanistically linked to atherosclerosis development through multiple distinct pathways.<sup>8,14</sup> Animal model and human clinical studies demonstrate an obligatory role for gut microbiota in TMAO formation from TMA-containing nutrients, including choline and phosphatidylcholine,<sup>9,10</sup> *L*-carnitine,<sup>8</sup> and more recently,  $\gamma$ -butyrobetaine,<sup>15</sup> and to a lesser degree, the choline oxidation metabolite betaine.<sup>11</sup>

TMAO is cleared by the kidney, and previous studies have reported that TMAO is elevated in subjects with impaired renal function.<sup>16-18</sup> It is thus an attractive hypothesis that TMAO may represent an excellent mechanism-based marker of CVD risk in subjects with impaired renal function. In one large study of subjects with predominantly preserved renal function, we reported the prognostic value of TMAO for predicting 3-year risk for major adverse cardiovascular events (myocardial infarction, stroke, or death) remained significant after adjustments for renal function.<sup>9</sup> However, no studies to date have directly looked at the long-term mortality risk of TMAO among CKD subjects. Interestingly, plasma TMAO levels in apparently healthy donors was identified in a prospective multicenter study aimed at identifying donor factors associated with delayed graft function in renal transplant recipients.<sup>19</sup> A direct role for TMAO in affecting renal functional impairment has not been reported. Herein, we sought to examine both the prognostic value of TMAO among CKD subjects and the potential contribution of dietary-induced, gut microbiota-associated TMAO formation in the development and progression of CKD.

## Methods

### Human Studies

This is a single-center, prospective cohort study approved by the Cleveland Clinic Institutional Review Board. We included adult

subjects (aged,  $\geq 18$  years) who underwent elective diagnostic coronary angiography for cardiac evaluation at our institution from 2001 to 2007 as previously described.<sup>9</sup> We excluded subjects with known acute coronary syndromes or revascularization procedures within 30 days of enrollment or history of congenital heart disease. After informed consent, fasting plasma blood samples were collected using ethylenediaminetetraacetic acid tubes before any drug administration via the arterial sheath, and immediately processed and frozen in  $-80^{\circ}\text{C}$  until analysis. Estimated glomerular filtration rate (eGFR) was calculated according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) creatinine and cystatin C formula,<sup>20</sup> with CKD defined as eGFR  $< 60$  mL/min per  $1.73\text{ m}^2$  (CKD stage 3 or beyond). Ascertainment of all-cause mortality at 5 years was performed by prospective telephone contact and chart review plus interrogation of the Social Security Death Index (up to 2011).

### Plasma Analysis

Trimethylamine-*N*-oxide (TMAO) levels were determined by stable isotope dilution high-performance liquid chromatography with on-line electrospray ionization tandem mass spectrometry on an AB SCIEX 5500 triple quadrupole mass spectrometer (AB SCIEX; Framingham MA) using d9-(trimethyl)-labeled internal standards as previously described.<sup>10,21</sup> High-sensitivity C-reactive protein (hsCRP), fasting lipid panel, cystatin C, and serum creatinine were measured using the Architect ci8200 platform (Abbott Laboratories, Abbott Park, IL).

### Animal Study

To test for a potential contribution of dietary choline or TMAO to promotion of renal dysfunction directly, C57BL6J mice were fed with the following diets for 6 weeks: (1) a chemically defined diet comparable in composition with standard chow diet (Teklad 2018; Harland Laboratories) that contains 0.08% (g/g) total choline; (2) the same diet supplemented with choline (1.0% total); and (3) the same diet supplemented with TMAO (0.12%). A separate study included C57BL6J mice with ApoE<sup>-/-</sup> background fed with the same diet groups for comparing their cystatin C levels at 14 weeks of follow-up using a commercially available mouse ELISA (R&D systems, Minneapolis MN). This study has been approved by the Cleveland Clinic Institutional Animal Care and Use Committee.

### Quantitative Histological Techniques

Mason trichome staining was performed on deparaffinized 5- $\mu\text{m}$  serial kidney sections. The kidney sections were mounted under a Leica DM 2500 microscope and digitized with a QImaging MicroPublisher 5.0 RTV camera for wide field microscopy. Quantitative morphometric analysis was performed on cortical fields ( $\geq 8$  from each animal) lacking major blood vessels, and the collagen volume was determined using automated (for batch analysis) and customized algorithms/scripts (ImageIQ Inc, Cleveland, OH) written for Image Pro Plus 7.0. Briefly, a set of representative images are chosen that demonstrated a wide range of staining intensities and prevalence. In an automated script, these training images were loaded one after another prompting the user to delineate blue pixels representing positive collagen staining using an interactive color picking tool. An iterative color profile or classifier was generated and subsequently applied to all images in a given directory using a fully automated algorithm. Positive pixels, as defined by the color profile, were segmented and summed to provide positive staining area. Total tissue area was determined by extracting the saturation channel, applying a low-pass filter, and thresholding the result. Any area within the general tissue boundary that was empty (ie, white) was removed by converting the original image to grayscale and applying a fixed threshold for nonbackground pixels on adequately white-balanced images. Finally, total tissue area and total stained area were exported to Excel. For postprocessing verification, segmented regions were superimposed onto the original image (green outlines) and saved for each image analyzed.

## Preparation of Tissue Homogenates and Immunoblotting

Equal amounts of protein were prepared using standard biochemical methods and subjected to SDS-PAGE and electrotransfer of proteins from gels to Immobilon-P membranes (Millipore). Membranes were incubated with the following antibodies: SMAD3 and phospho-SMAD-3(Ser<sup>423/425</sup>; Cell Signaling Technology); tubulin (Santa Cruz Biotechnology); kidney injury molecular 1 (Novus Biologicals). Detection of all immunoblots was performed with the SNAP i.d. Protein Detection System (Millipore) and Super Signal Chemiluminescent Substrate Products (Pierce), and band intensity was analyzed by densitometry (ImageQuant; GE Healthcare).

## Statistical Analyses

Continuous variables were summarized as mean±SD if normally distributed and median (interquartile range) if non-normally distributed. The Student *t* test or Wilcoxon rank-sum test for continuous variables and  $\chi^2$  test for categorical variables were used to examine the difference between groups. 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% confidence intervals (95% CI) for all-cause mortality stratified according to TMAO in quartiles. Adjustments were made for individual traditional risk factors, including age, sex, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, diabetes mellitus, and log-transformed hsCRP to predict all-cause mortality risks. Additional adjustment for log-transformed eGFR was also performed. Net reclassification and area under receiver operator characteristic curve were calculated according to mortality risk estimated using Cox models adjusted for above-mentioned traditional risk factors with versus without TMAO as previously described.<sup>22</sup> All analyses performed used R 2.15.1 (Vienna, Austria). *P*<0.05 was considered statistically significant.

## Results

### Elevated TMAO in Patients With Renal Insufficiency Portend Poorer Survival

Baseline clinical and laboratory characteristics of the cohort are reported in Table 1. A total of 3687 subjects were included in this analysis, among which 521 subjects fulfilled criteria for CKD and 3166 subjects for non-CKD. When compared with non-CKD subjects, TMAO levels were elevated in patients with CKD (median TMAO, 7.9 [interquartile range, 5.2–12.4] versus 3.4 [interquartile range 2.3–5.3]  $\mu\text{mol/L}$ ; *P*<0.001; Figure 1A). In the CKD cohort, TMAO modestly correlated with eGFR (*r*=−0.48; *P*<0.001) and cystatin C (*r*=0.46; *P*<0.001), but did not correlate with hsCRP (*r*=0.04; *P*=0.332).

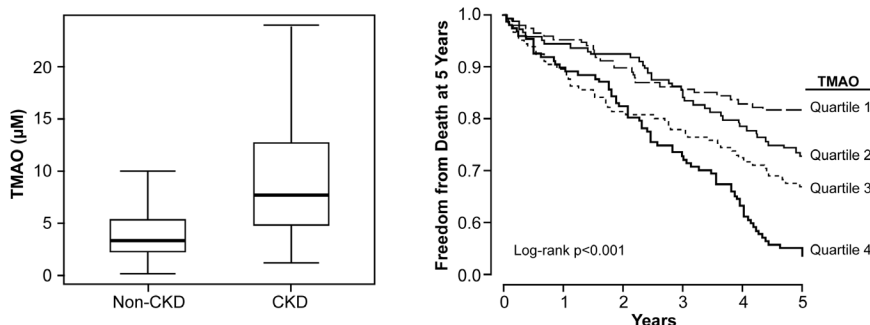
**Table 1. Baseline Characteristics**

Characteristic	Non-CKD Cohort eGFR≥60 (n=3,166)	CKD Cohort eGFR<60 (n=521)	<i>P</i> Value
Age, y	62±11	70±10	<0.001
Men, %	66	48	<0.001
Diabetes mellitus, %	27	53	<0.001
Hypertension, %	69	88	<0.001
Smoking, %	66	61	0.047
History of MI, %	40	53	<0.001
History of stroke, %	5	13	<0.001
History of CABG, %	28	42	<0.001
History of PCI, %	31	30	0.728
LDL, mg/dL	97 (79–118)	93 (72–114)	<0.001
HDL, mg/dL	34 (28–41)	32 (26–40)	<0.001
hsCRP, mg/dL	2.2 (0.9–5.0)	4.1 (1.8–9.6)	<0.001
eGFR, mL/min per 1.73 m <sup>2</sup>	89 (78–101)	49 (38–55)	<0.001
Cystatin C, mg/L	0.9 (0.8–1)	1.5 (1.3–1.8)	<0.001
ACE inhibitor/ARB, %	48	66	<0.001
Statins, %	61	59	0.437
$\beta$ -blockers, %	63	68	0.04
Aspirin, %	75	67	<0.001
TMAO, $\mu\text{mol/L}$	3.4 (2.3–5.3)	7.9 (5.2–12.4)	<0.001

ACE indicates angiotensin-converting enzyme; ARB, angiotensin receptor blocker; CABG, coronary artery bypass grafting; CKD, chronic kidney disease; eGFR, estimated glomerular filtration rate; HDL, high-density lipoprotein; hsCRP, high-sensitivity C-reactive protein; LDL, low-density lipoprotein; MI, myocardial infarction; PCI, percutaneous coronary intervention; and TMAO, trimethylamine-*N*-oxide.

Expressed as % or median (interquartile ranges), except for age as mean±SD.

In the CKD cohort, higher TMAO levels (quartiles 4 versus 1) were associated with a 2.8-fold increase in risk for all-cause mortality at 5 years (unadjusted HR, 2.76; 95% CI, 1.74–4.37; *P*<0.001). After adjusting for traditional CVD risk factors, log-transformed hsCRP, and log-transformed eGFR, higher TMAO levels still were associated with a 1.9-fold poorer 5-year survival (adjusted HR, 1.93; 95% CI, 1.13–3.29; *P*<0.05; Table 2; Kaplan–Meier curve shown in Figure 1B). When stratified according to median levels (7.9  $\mu\text{mol/L}$ ), higher TMAO conferred a 1.7-fold increase in risk for all-cause mortality (HR, 1.70; 95% CI, 1.25–2.30; *P*<0.001) and remained significant after adjusting for traditional risk factors and log-transformed hsCRP



**Figure 1. Prognostic Value of plasma trimethylamine N-oxide (TMAO) levels in the chronic kidney disease (CKD) Cohort.** In a cohort of stable patients undergoing elective diagnostic coronary evaluation, subjects with underlying CKD Stage 3+ (n=521) demonstrated higher levels of fasting plasma TMAO than those with no CKD (n=3,166; *P*<0.01; **A**). Increasing quartiles fasting plasma TMAO levels portend increased risk for all-cause mortality at 5 years in patients with CKD (n=521; **B**).

**Table 2. Cox Proportional Hazards Analysis of Plasma TMAO Levels Stratified by Quartile in Predicting Risk of All-Cause Mortality at 5 Years: CKD and Non-CKD Cohorts**

	TMAO (Range)			
	Quartile 1	Quartile 2	Quartile 3	Quartile 4
CKD cohort (n=521)				
Range, $\mu\text{mol/L}$	<5.2	5.2–7.9	7.9–12.4	$\geq 12.4$
Events	26/129 (20.2%)	42/131 (32.1%)	43/130 (33.1%)	63/131 (48.1%)
Unadjusted HR	1	1.70 (1.04–2.79)*	1.75 (1.07–2.87)*	2.76 (1.74–4.37)†
Adjusted HR	1	1.42 (0.85–2.35)	1.51 (0.90–2.51)	1.93 (1.13–3.29)*
Non-CKD cohort (n=3166)				
Range, mmol/L	<2.3	2.3–3.4	3.4–5.3	$\geq 5.3$
Events	48/787 (6.1%)	59/793 (7.4%)	81/791 (10.2%)	104/795 (13.1%)
Unadjusted HR	1	1.22 (0.83–1.79)	1.70 (1.19–2.43)†	2.21 (1.57–3.12)†
Adjusted HR	1	1.08 (0.74–1.58)	1.23 (0.84–1.78)	1.47 (1.02–2.12)*

Adjusted model: adjusted for traditional risk factors (age, sex, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, smoking, and diabetes mellitus), log(high-sensitivity C-reactive protein), and log(estimated glomerular filtration rate). Events expressed as n (%), HR expressed as HR (95% confidence interval). CKD indicates chronic kidney disease; HR, hazard ratio; and TMAO, trimethylamine-*N*-oxide.

\* $P < 0.05$ .

† $P < 0.001$ .

(adjusted HR, 1.72; 95% CI, 1.16–2.34;  $P < 0.001$ ), as well as with addition of cystatin C to the model (adjusted HR, 1.45; 95% CI, 1.05–2.02;  $P < 0.05$ ). Using median cohort cutoffs with low cystatin C (<1.4 mg/dL) and low TMAO (<7.9  $\mu\text{mol/L}$ ) as reference, those with concomitant high cystatin C and high TMAO had a 3-fold increase in mortality risk (HR, 3.01; 95% CI, 1.97–4.59;  $P < 0.001$ ). These findings are consistent with the notion that elevated TMAO is associated with poor prognosis in patients with established CKD.

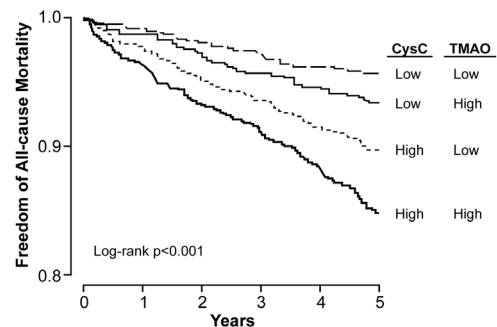
### Increased TMAO Levels in Non-CKD Patients With Elevated Cystatin C

Within the non-CKD cohort (n=3166), the prognostic value of elevated TMAO (quartile 4 versus 1) remained predictive of 5-year mortality risk (HR, 2.21; 95% CI, 1.57–3.12;  $P < 0.001$ ), as well as after adjusting for traditional risk factors, log-transformed hsCRP, and log-transformed eGFR (adjusted HR, 1.47; 95% CI, 1.02–2.12;  $P < 0.05$ ; Table 2). These findings were similar when restricted to subjects with preserved eGFR ( $\geq 60$  mL/min per  $1.73 \text{ m}^2$ ) plus normal cystatin C (<1.4 mg/dL; n=3151). Elevated TMAO levels are associated with higher 5-year mortality risk among subjects with either normal or elevated cystatin C levels (Figure 2). Using median cohort cutoffs with low cystatin C (<0.9 mg/dL) and low TMAO (<3.4  $\mu\text{mol/L}$ ) as reference, those with concomitant high cystatin C and high TMAO had a 3.7-fold increase in mortality risk (HR, 3.67; 95% CI, 2.57–5.23;  $P < 0.001$ ).

### Dietary Choline and Dietary TMAO Both Promote Renal Fibrosis and Dysfunction in Animal Models

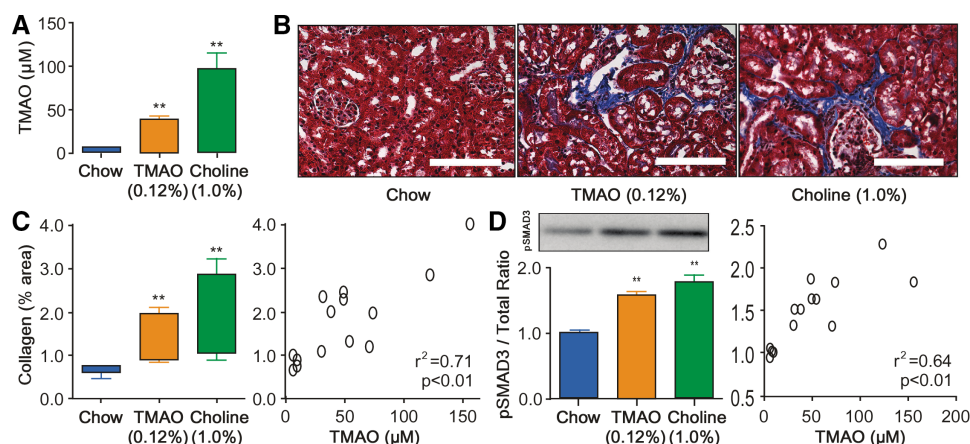
To directly test the hypotheses that either dietary TMAO itself, or dietary nutrients that contribute to gut microbiota-dependent production of TMAO, can affect development and progression of CKD, we performed animal model studies.

Conventionally housed 8-week-old male mice (C57BL/6J background) were fed ad libitum a chemically defined diet comparable with normal chow (0.08% choline) or the same diet supplemented with either choline (1.0% final) or TMAO (0.12%), as described under Methods section of this article. After 6 weeks, we observed significant ( $P < 0.01$ ) increases in TMAO levels in both the TMAO-supplemented and the choline-supplemented groups of mice (Figure 3A), with TMAO levels observed within the range of values detected among CKD subjects studied (97.5 percentile 77.6  $\mu\text{mol/L}$ , 99 percentile 96.3  $\mu\text{mol/L}$ ). Importantly, elevated TMAO levels were associated with corresponding increases in tubulointerstitial fibrosis and collagen deposition (Figure 3B and 3C) and phosphorylation of Smad3, an important regulator of the profibrotic transforming growth factor- $\beta$ /Smad3 signaling pathway during fibrotic kidney disease<sup>23</sup> (Figure 3D). Furthermore, TMAO-fed and choline-fed mice experienced increased kidney injury marker-1 (Figure 4A and 4B). Extending the TMAO/choline feeding to 16 weeks



**Figure 2. Comparative prognostic value of plasma trimethylamine-*N*-oxide (TMAO) and Cystatin C (CysC) in the non-chronic kidney disease (CKD) cohort.** Subjects with elevated CysC ( $\geq 0.9$  mg/dL) and TMAO ( $\geq 3.4$   $\mu\text{mol/L}$ ) had the highest 5-year mortality risk in this non-CKD cohort (n=3188).





**Figure 3. Dietary choline/trimethylamine-*N*-oxide (TMAO) exposure contributes to progressive renal fibrosis in murine model.** Plasma TMAO (**A**) levels are increased after 6 week feeding TMAO (0.12%), or Choline (1.0%) diets vs Chow (0.08% choline) fed mice. Representative Mason trichrome histology (**B**) quantitative morphometry and its relationship with TMAO levels (**C**), and SMAD3 activation by phosphorylation at serine 423/425 (**D**) and its relationship with TMAO levels (**E**) in mouse kidneys after 6 week feeding of Chow (0.08% choline), TMAO (0.12%), and Choline (1.0%) diets. Scale bar, 100 μm. \*\* $P<0.01$  vs Chow,  $n\geq 5$  mice per group.

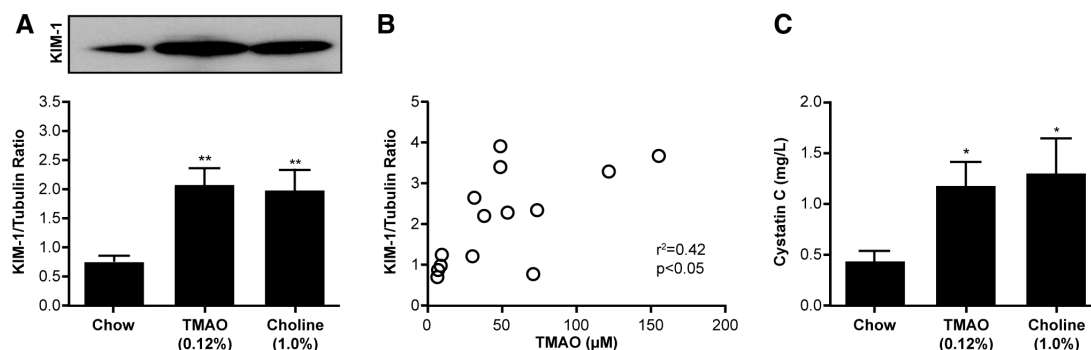
was associated with increased serum cystatin C levels compared with chow-fed mice (Figure 4C). On further examination, striking dose-dependent relationships were noted between plasma TMAO levels and monitored indices of renal histopathologic (Figure 3C and 3D) and functional impairment (Figure 4B).

### Discussion

There are several key findings in this report. First, we observed in subjects with CKD that TMAO levels are not only elevated when compared with non-CKD subjects but also portend poorer overall survival. Second, we observed that within the non-CKD cohort, higher levels of TMAO portend poorer survival both within the cohort of low levels and high levels of cystatin C (stratified at median levels). Interestingly, the prognostic value for the highest TMAO quartile in predicting future mortality risk in this cohort remained robust even after adjustment for traditional risk factors. Third, extending to animal models studies, dietary exposure of either choline or TMAO lead to the development of renal tubulointerstitial fibrosis and early measures of dysfunction (elevated

cystatin C). These studies thus suggest both a causal relationship and clinical relevance of dietary choline-induced, gut microbiota-mediated, TMAO formation in CKD development and progression.

TMAO is a low molecular weight compound that is easily filtered by the kidney and effectively removed by hemodialysis.<sup>16</sup> Considered a nitrogenous waste product whose levels rise with diminished renal function, elevated TMAO levels have been reported in small cohorts ( $n<20$ ) of subjects with either end-stage renal disease or CKD, where levels were shown to correlate with both serum urea and creatinine.<sup>18</sup> Detailed animal and human experiments on the renal clearance of methylamines, such as TMA and TMAO, have been performed and confirm the kidneys as the primary elimination route.<sup>24</sup> Interestingly, the urinary clearances of both TMA and TMAO are higher than the glomerular filtration rate, and TMAO clearance also decreases with increasing dose, which suggests that saturable renal tubular secretion occurs.<sup>25</sup> The majority of a dose of TMA is also excreted in the urine, with varying proportions in the forms of TMA and TMAO being dependent on the dose level.<sup>26</sup> Urinary TMAO levels are



**Figure 4. Dietary choline/trimethylamine-*N*-oxide (TMAO) exposure contributes to progressive renal injury and dysfunction in murine model.** Immunoblot of kidney injury molecular 1 (KIM-1) expression (**A**) and its relationship with TMAO levels (**B**) in mouse kidneys after 6 week feeding of chow (0.08% choline), TMAO (0.12%), and choline (1.0%) diets. Also shown are plasma cystatin C levels (**C**) after 16 week feeding of Chow, TMAO (0.12%), and Choline (1.0%) diets. \*\* $P<0.01$  vs Chow,  $n\geq 5$  mice per group.

reported to rise with episodes of kidney graft dysfunction in renal transplant recipients, suggesting an intrinsic accumulation of TMAO (presumably as an osmolyte like urea) that is released during damage of the renal medullary cells.<sup>27–30</sup> The link between elevated TMAO and adverse prognosis in CKD, and even in the setting of subclinical renal insufficiency (elevated cystatin C in non-CKD patients) observed in our study is consistent with the heightened risk of developing CVD in the CKD population.

Our results from animal studies showed for the first time a direct mechanistic link between dietary choline, or dietary TMAO, and progressive renal dysfunction, even in the C57BL/6J mouse model that is known to be relatively resistant to renal injury.<sup>31</sup> Indeed, exposure to either a high choline diet or a diet supplemented directly with TMAO both led to increased levels of the early kidney injury molecular 1 and enhanced phosphorylation of Smad3, an important regulator of renal fibrosis.<sup>32</sup> A more prolonged exposure to either the high choline diet or the TMAO-supplemented diet both led to increased plasma levels of cystatin C, a sensitive indicator of renal functional impairment. Importantly, the plasma TMAO levels observed within the animal models on either choline or TMAO-supplemented diets were within the range of TMAO levels observed among subjects with CKD. Furthermore, examining TMAO levels as a continuous variable, a dose-dependent relationship between plasma TMAO levels and mortality risk was observed (log[TMAO]: adjusted HR, 1.41; 95% CI, 1.23–1.61;  $P < 0.001$  for CKD cohort; adjusted HR, 1.21; 95% CI, 1.07–1.36;  $P < 0.01$  for non-CKD cohort). Although there is the appearance of a threshold level of TMAO that is associated with increased risk with the predicted outcomes using quartile-based analyses in the current sized study, the biological data collected in the animal model studies (scatter plots comparing TMAO levels versus the CKD-related phenotypes) show a continuous dose-dependent relationship between TMAO levels and renal tubulointerstitial fibrosis (pSMAD3, and various measures of collagen or fibrosis), and renal functional impairment (cystatin C). Interestingly, a recent untargeted metabolomic study from the Framingham Heart Study among subjects with normal renal function identified elevated choline and TMAO levels at baseline were each associated with an increased future risk of developing CKD.<sup>33</sup> Our animal model findings, therefore, provide a potential mechanistic rationale for the Framingham observational data, and collectively, further link elevated systemic TMAO levels with increased susceptibility for the development of CKD.

The prospects that exposure to specific dietary nutrients, such as choline, phosphatidylcholine (lecithin), and *L*-carnitine via gut microbiota may affect susceptibility to the development and progression of both CKD and CVD has important potential public health implications. Randomized nutritional intervention studies in patients with CKD to date have not explored a potential role for choline, phosphatidylcholine, *L*-carnitine, or TMAO (which can be abundant in certain types of fish) in disease progression. Similarly, epidemiological studies are

rather limited on the topic of diet and CKD risks, even though a recommended renal diet is typically low in protein intake. Dietary management of patients with CKD represents a challenge, and much less is known about nutritional factors that might predispose to enhanced risk for development of CKD or its progression. Interestingly, in a substudy ( $n = 3296$ ) among women who had urine microalbumin levels available from the Nurses Health Study,  $\geq 2$  servings of red meat (primary dietary source of *L*-carnitine) per week were directly associated with enhanced risk for development of microalbuminuria (odds ratio, 1.51; 95% CI, 1.01–2.26).<sup>34</sup> Further investigations in dietary predisposition to CKD development and progression are warranted. On the basis of the present studies, we conclude that a diet monitored by following TMAO levels and designed to limit TMAO precursors (low in red meat, meats, liver, egg yolk, and high fat dairy products) and TMAO itself (certain fish) would be an attractive diet to test to see whether it reduces the rate of CKD progression. However, it is important to note that choline is an essential nutrient; therefore, its total elimination from the diet is ill advised and could result in development of a deficiency state.<sup>35</sup>

Collectively, the present data indicate a dietary-induced, intestinal microbiota-dependent mechanism may contribute to both progressive renal fibrosis and dysfunction, and mortality risks, among subjects with CKD. They also build on the recent body of evidence demonstrating a mechanistic link between gut microbiota-associated metabolic dysregulation and cardiovascular risk in humans.<sup>8–12</sup> The discovery of the metaorganismal pathway involved in TMAO generation thus affords a unique opportunity to investigate systematically the potential contributions of discrete participants in the overall diet  $\rightarrow$  microbe  $\rightarrow$  host enzyme pathways for TMAO formation and development and progression of cardiorenal dysfunction. It is interesting that in both animal models and patients with established CKD, pre- and probiotic intervention studies have been performed, with reports of changes in gut microbiota composition and activity. For example, *Lactobacillus acidophilus* or *bifidobacterium* has been reported to reduce inflammatory signaling associated with the microbiota-derived metabolites that accumulate in CKD,<sup>36–39</sup> in addition to improving renal function modestly.<sup>40,41</sup> Similarly, prebiotic compound use to interrupt pathways that lead to gut microbiota-derived uremic toxins, such as indoxyl sulfate and p-cresyl sulfate, has shown some efficacy in both human and animal trials of CKD.<sup>5,42</sup> Additional studies are warranted to see whether dietary interventions, or disruption of the metaorganismal pathway involving TMAO production, may retard the development of CKD, progression of renal functional impairment among subjects with CKD, and adverse CVD event risks in subjects with CKD.

## Conclusions

The gut microbiota-dependent product, TMAO, is associated with both higher risk of progressive renal fibrosis and functional impairment, and poorer long-term survival.

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## Disclosures

Drs. Wang, Levison, and Hazen are named as coinventor on pending patents held by the Cleveland Clinic relating to cardiovascular diagnostics and/or therapeutics. Dr Hazen reports having been paid as a consultant for the following companies: Cleveland Heart Laboratory, Esperion, Liposcience Inc., and P&G. Dr Hazen reports receiving research funds from Cleveland Heart Laboratory, Liposcience Inc, P&G and Takeda. Drs Wang, Levison, and Hazen report having the right to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics and/or therapeutics from Cleveland Heart Laboratory, and Dr Hazen also from the companies shown below: Siemens, Esperion, Frantz Biomarkers, LLC. The other authors report no conflicts.

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## Novelty and Significance

### What Is Known?

- Gut microbe-dependent metabolism of dietary choline, phosphatidylcholine and L-carnitine contributes to the development and progression of atherosclerotic cardiovascular diseases (CVD) via formation of trimethylamine-*N*-oxide (TMAO).
- Patients with chronic kidney disease (CKD) have heightened risk of developing CVD.

### What New Information Does This Article Contribute?

- Plasma TMAO levels are both increased in subjects with CKD, and portend poorer overall survival, independent of traditional risk factors.
- In subjects without CKD, higher levels of TMAO portend poorer survival, both among those with high or low levels of cystatin C. Interestingly, the prognostic value for the highest TMAO quartile in predicting future major adverse cardiovascular events (myocardial infarction, stroke, or death) in this cohort remained robust even after adjustment for traditional risk factors and renal function.

- In animal model studies, dietary exposure to either choline or TMAO each lead to the development of renal tubulointerstitial fibrosis and early measures of dysfunction (elevated cystatin C).

Previous studies have shown that gut microbiota-associated choline metabolism and TMAO generation contribute to the development and progression of atherosclerotic CVD. Hence, we examined the potential contribution of dietary choline and gut microbiota-dependent TMAO production in the development and progression of CKD. These studies indicate both a causal relationship and clinical relevance of dietary choline-induced, gut microbiota-mediated, TMAO formation in CKD development and progression. Our findings support a mechanistic link between TMAO and CVD risk in this vulnerable population. The significance of a dietary-induced CKD susceptibility strongly implies the need to focus preventive efforts on dietary modulation and therapeutic targeting of the gut microbiota-dependent TMAO pathway to retard CKD development, progression, and CVD risks potentially.