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RESEARCH ARTICLE

FMO3–TMAO axis modulates the clinical outcome in chronic heart-failure patients with reduced ejection fraction: evidence from an Asian population

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Abstract **The association among plasma trimethylamine-N-oxide (TMAO), *FMO3* polymorphisms, and chronic heart failure (CHF) remains to be elucidated. TMAO is a microbiota-dependent metabolite from dietary choline and carnitine. A prospective study was performed including 955 consecutively diagnosed CHF patients with reduced ejection fraction, with the longest follow-up of 7 years. The concentrations of plasma TMAO and its precursors, namely, choline and carnitine, were determined by liquid chromatography-mass spectrometry, and the FMO3 E158K polymorphisms (rs2266782) were genotyped. The top tertile of plasma TMAO was associated with a significant increment in hazard ratio (HR) for the composite outcome of cardiovascular death or heart transplantation (HR = 1.47, 95% CI = 1.13–1.91, *P* = 0.004) compared with the lowest tertile. After adjustments of the potential confounders, higher TMAO could still be used to predict the risk of the primary endpoint (adjusted HR = 1.33, 95% CI = 1.01–1.74, *P* = 0.039). This result was also obtained after further adjustment for carnitine (adjusted HR = 1.33, 95% CI = 1.01–1.74, *P* = 0.039). The *FMO3* rs2266782 polymorphism was associated with the plasma TMAO concentrations in our cohort, and lower TMAO levels were found in the AA-genotype. Thus, higher plasma TMAO levels indicated increased risk of the composite outcome of cardiovascular death or heart transplantation independent of potential confounders, and the *FMO3* AA-genotype in rs2266782 was related to lower plasma TMAO levels.**

Keywords chronic heart failure; trimethylamine-N-oxide; flavin monooxygenase 3; single nucleotide polymorphism

Introduction

Heart failure (HF) is a common life-threatening cardiovascular disease particularly in the elderly. This disease is accompanied by high mortality and morbidity rates. In the USA, the incidence of HF approaches 21 per 1000 people over 65 years old, and the lifetime risk for HF is high (20% to 45%) from age 45 to 95 years [1]. In Asia, the prevalence of HF has been estimated to range from 1.26% to 6.70% [2]. Trimethylamine-N-oxide (TMAO) produced from trimethylamine (TMA) is a small circulating organic molecule that has recently emerged as a predictor of cardiovascular disease [3]. The levels of circulating TMAO has a dose-dependent association with the prevalence of cardiovascular disease [4–8]. This marker was used independently to predict the incident risk of major adverse cardiac events, such as myocardial infarction, stroke, or death, after adjustment for traditional

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cardiac risk factors in an American follow-up cohort [4].

Flavin-containing monooxygenase 3 (*FMO3*) plays a predominant role in the oxidation of TMA in the liver. Analysis of the natural genetic variation among inbred strains of mice indicated that *FMO3* and TMAO are significantly correlated, and the TMAO levels explain 11% of the variation in atherosclerosis [9]. Moreover, genetic variations controlling the expression of flavin monooxygenases play an important role in the atherosclerosis burden in hyperlipidemic mice [3]. Knockdown of hepatic *FMO3* by using an antisense oligonucleotide alters the biliary lipid secretion, blunts the intestinal cholesterol absorption, and limits the production of hepatic oxysterols and cholesteryl esters to inhibit atherosclerosis [10,11].

Although *FMO3* activity is well recognized because of its catalysis of TMA to TMAO, the phenotype of the *FMO3* whole genetic knock-out mice indicates the indispensable function of this gene. Functional differences in the *FMO3* activity can occur in humans secondary to variations within the *FMO3* gene. A population study has indicated that the activity of *FMO3* shows extensive functional and molecular genetic variability in human populations [12]. Several studies have also reported associations between these genetic variants and the risk of cardiovascular disease [13]. However, the role of the genetic variations of *FMO3* in metabolites and patients with HF has not been investigated yet. Thus, we conducted a prospective study to explore the association of plasma TMAO-related metabolites (including choline and carnitine) with chronic heart failure (CHF) in a large CHF population with reduced ejection fraction. We also assessed the effect of *FMO3* polymorphisms on the plasma TMAO levels and the risk of major adverse cardiovascular events.

Materials and methods

Cohort studies

The participants enrolled in our study were limited to those of the Chinese Han ethnicity. The Wuhan Cohort ($n = 955$) was a single-center, prospective cohort study approved by the Ethics Committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. Between January 2008 and March 2016, a total of 955 patients diagnosed by cardiologists to have chronic HF with reduced ejection fraction (HFrEF), New York Heart Association (NYHA) function class 2–4, were consecutively enrolled in this study at the Division of Cardiology of the aforementioned hospital. For inclusion, a diagnosis of CHF should be consistent with current guidelines [14], and ejection fraction was less than 40%. Dilated (55%) and ischemic (31%) cardiomyopathies accounted for the majority of the included population. The following exclusion criteria were applied: (1) patients with acute coronary syndromes in one month before the study; (2) patients with significant concomitant diseases, such as severe infection, malignant tumor, or systemic immune disease, and (3) patients who refused to participate in this study. Professional investigators were trained to sort the demographic data, clinical characteristics, and laboratory indices into a standardized format. Patients included were contacted on a regular basis by follow-up investigators through telephone and face-to-face interviews. The median follow-up time was 33 months. Written informed consent was obtained from all individuals at admission.

This study was conducted in accordance with the *Declaration of Helsinki* and the International Conference on Harmonization Guidelines for Good Clinical Practice. The ClinicalTrials.gov Identifier is NCT03461107.

Study endpoints

We defined two study endpoints. The primary outcome was the composite outcome of cardiovascular death, which infers a cardiac origin (such as sudden cardiac death, HF, acute myocardial infarction, cardiovascular procedures, or other cardiovascular causes) unless a noncardiac cause was identified, or heart transplantation. The secondary endpoint comprised the recurrence of HF, first rehospitalization for cardiovascular causes (including angina, arrhythmias, or HF-related symptoms, such as dyspnea or edema), stroke, and all-cause mortality.

Quantification of TMAO and related metabolites by liquid chromatography–mass spectrometry

All blood samples were collected during the fasting state and stored at − 80 °C immediately until analysis. Carnitine, choline, and TMAO were quantified in the Laboratory for Cardiovascular Disease of Peking University. An aliquot (20 μL) of plasma was put into a 1.5 mL tube and mixed with 80 μL of a 10 μmol/L internal standard composed of d9-TMAO, d9-choline, and d9-carnitine in methanol. The protein in the samples was removed by vortexing the samples for 1 min, followed by centrifugation at 20 000× g at 4 °C for 10 min. The supernatant was collected in a mass spectrometry vial with insert previously loaded. Various concentration standards (0–100 μmol/L; 20 μL) were processed using the same procedure to prepare calibration curves. Standard curves were acceptable when the coefficient of determination (R2) reached 0.999.

The supernatant was analyzed by injecting onto a silica column (2.0 mm × 150 mm, Luna 5u Silica 100A; Cat. No. 00F-4274-B0, Phenomenex, Torrance, CA) at a flow rate of 0.5 mL/min by using an LC-20AD Shimadzu pump system, and the SIL-20AXR autosample interfaced with an API 5500Q-TRAP mass spectrometer (AB SCIEX, Framingham, MA). A discontinuous gradient was

generated to resolve the analytes by mixing solvent A (0.1% propanoic acid in water) with solvent B (0.1% acetic acid in methanol) at different ratios from 2% B linearly to 95% B over 5.0 min, held for 1.0 min, and then back to 2% B. The analytes were monitored using electrospray ionization in positive-ion mode with multiple reaction monitoring of precursor and characteristic product-ion transitions of TMAO at m/z 76→58, d9-TMAO at m/z 85→66, choline at m/z 104→59.8, d9-choline at m/z 113.2→68.9, carnitine at m/z 162.1→103, and d9-carnitine at m/z 171.1→102.8. Finally, 40 patients had missing metabolite concentration, and 915 participants were included into our analysis.

Genotyping

The blood samples from the in-patients were put into EDTA anti-coagulated tubes. The genomic DNA (gDNA) was extracted using a commercial DNA extraction kit (Tiangen, Beijing, China) according to the manufacturer's guidelines. The NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) was used to measure the DNA concentration, and the samples were diluted to 5 mg/L. The *FMO3* polymorphism rs2266782 was genotyped by TaqMan single nucleotide polymorphism (SNP) allelic discrimination by using a 7900 HT fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The primers and labeled probes were provided by Applied Biosystems (TaqMan Drug Metabolism Assay, C_2462279_30), and the genotyping analysis was performed by using SDS 2.4. The genotype success rate was 85.8%, and the consensus rate from 45 randomly selected duplicates was 100%.

Statistical analysis

Continuous variable is shown as mean (± SD) or median (interquartile rage) and was analyzed by one-way ANOVA or nonparametric alternatives (Kruskal–Wallis test) among different groups. Categorical variables are presented as number (%) and were compared using the χ^2 test. The relationship between TMAO and other laboratory indices was examined using Spearman's correlation or Pearson's correlation coefficient according to the corresponding data distribution. The Kaplan–Meier method and log-rank test were used separately to depict the survival time of the mentioned metabolites. Cox proportional hazard regression was employed to determine the hazard ratios (HRs) and 95% confidence interval (CI) for 7-year rates of adverse incidences stratified according to TMAO in tertiles. Given that the missing values would increase the I/II type false rate in regression analysis, the missing laboratory variables were imputed with multiple imputation method in mice 3.6.3 of R Package. The missing patterns of variables and goodness of fit were displayed in the supplementary materials (Figs. S1 and S2). The observed variables and imputed variables shared similar distributions, which indicates the excellent performance of data imputation. Individual potential cardiovascular confounders, including sex, age, smoking status, systolic blood pressure (SBP), diabetes mellitus, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), hemoglobin, left ventricular ejection fraction (LVEF), estimated glomerular filtration rate (eGFR), and N-terminal pro B type natriuretic peptide (NT-proBNP), were used to adjust the cardiac outcomes. The subgroups were stratified according to dichotomous population characteristics and risk factors that may affect the mortality risks. The Kaplan–Meier method was used to describe cumulative event rates, and HRs were calculated using univariate and multivariate regression analyses. SPSS version 25.0 (IBM Corp, Armonk, NY) and R (version 3.5.1, Vienna, Austria) were used for statistical analysis. The statistical threshold was 0.05 for two-sided tests.

Results

Baseline characteristics of the participants

The demographic and clinical factors of the 915 included patients are shown in Table 1. In the HFrEF cohort, 69.9% of the patients were men, and the mean age was 57.1 ± 14.1 years. Among these participants, 41.4% were smokers, 77.4% had hypertension, 28.7% had diabetes mellitus, and 29.5% had a history of coronary heart disease. The median plasma TMAO concentration in the overall population was 2.52 μmol/L (interquartile range, 1.20–4.76), which was higher in people over 60 years old (2.84 (1.26–5.26)) than the others (2.30 (1.16–4.32); $P = 0.011$). In addition, patients with NYHA class IV had higher TMAO (NYHA IV, 2.54 (1.04–5.12); NYHA II, 2.32 (1.18–4.06), $P = 0.01$) and choline (NYHA IV, 12.46 (8.96–16.07); NYHA II, 11.05 (8.19–13.99), $P < 0.001$) levels than those with class II. Patients with NYHA class III also had higher TMAO levels than those with class II (NYHA III, 2.66 (1.26–5.12); NYHA II, 2.32 (1.18–4.06), $P = 0.001$), but no difference was found between classes III and IV. Individuals with top tertile plasma TMAO concentrations were more likely to be older and have diabetes, lower estimated glomerular filtration rate (eGFR), and a higher N-terminal pro B type natriuretic peptide (NT-proBNP) level. However, a prior history of hypertension, coronary artery disease and dyslipidemia, fasting glucose, total cholesterol, triglycerides, and LDL cholesterol were not different among the three tertile TMAO level ranges.

Table 1 Baseline characteristics of the HFrEF cohort stratified by TMAO levels ($n = 915$)

	ALL ($n = 915$)	Tertile 1 ⩽1.574 ($n = 306$)	Tertile 2 1.574–3.770 ($n = 304$)	Tertile 3 >3.770 ($n = 305$)	*P* value
Demographic characteristics					
Age (year)	57.1±14.1	55.1±13.9	56.1±13.8	60.2±14.0	<0.001
Male (%)	69.9	67.3	72.4	70.2	0.400
Smoker (%)	41.4	41.2	38.5	44.6	0.310
Drinker (%)	26.8	24.3	29.3	26.9	0.364
SBP (mmHg)	122 (110–138)	120 (107–137)	124 (110–137)	123 (110–140)	0.085
DBP (mmHg)	80 (69–90)	78 (69–88)	88 (73–102)	79 (68–89)	0.483
Heart rate (beat per min)	86 (75–102)	90 (76–104)	88 (73–102)	84 (74–100)	0.079
NYHA class II/III/IV	24.6/44.8/30.6	25.2/40.8/34.0	28.6/46.7/24.7	20.0/46.9/33.1	0.021
Medical history (%)					
Hypertension	77.4	79.7	75.3	77	0.420
Diabetes	28.7	28.1	22	36.1	0.001
Dyslipidemia	16.7	16.7	17.1	16.4	0.970
Coronary artery disease	29.5	29.1	26.3	33.1	0.180
Laboratory measurements					
Fasting glucose (mmol/L)	5.74 (5.02–7.27)	5.77 (5.00–7.36)	5.64 (5.02–6.62)	5.89 (5.07–7.57)	0.518
Total cholesterol (mmol/L)	3.74 (3.13–4.52)	3.76 (3.10–4.55)	3.83 (3.23–4.50)	3.63 (3.02–4.44)	0.287
Triglycerides (mmol/L)	1.08 (0.79–1.55)	1.08 (0.80–1.49)	1.06 (0.84–1.64)	1.08 (0.75–1.55)	0.128
LDL cholesterol (mmol/L)	2.37 (1.87–2.95)	2.37 (1.87–3.02)	2.42 (1.98–2.94)	2.28 (1.79–2.93)	0.440
HDL cholesterol (mmol/L)	0.88 (0.71–1.10)	0.89 (0.68–1.12)	0.91 (0.73–1.11)	0.86 (0.71–1.05)	0.259
NT-proBNP (ng/L)	3804 (1745–8584)	3348 (1566–7904)	3159 (1516–7980)	5038 (2197–9168)	<0.001
hsCRP (mg/L)	5.55 (2.08–15.58)	8.10 (3.20–23.80)	4.20 (1.80–11.00)	4.90 (1.70–21.90)	0.382
eGFR (mL/min/1.73 m^2)	71.1 (54.7–85.5)	72.6 (57.1–88.3)	74.8 (59.5–89.0)	62.8 (45.8–80.0)	<0.001
Hemoglobin (g/L)	134 (119–147)	133 (118–145)	135 (123–148)	133 (117–147)	0.198
Echocardiography					
LVEDD (mm)	64 (58–49)	63 (58–69)	64 (59–70)	64 (58–70)	0.260
LVEF (%)	31 (25–36)	31 (25–36)	31 (26–35)	30 (25–36)	0.641
Medication (%)					
Diuretics	84.9	84	82.2	88.5	0.082
ACEI/ARB	85.5	82	91.4	83	0.001
Beta-blocker	64.2	62.4	65.5	64.6	0.722
Spironolactone	81.5	80.1	80.9	83.6	0.501

Variables are expressed in mean±standard deviation or median (interquartile range). DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; hsCRP, high sensitivity C-reactive protein; HDL, high-density lipoprotein; LDL, low-density lipoprotein; LVEDD, left ventricular end-diastolic dimension; LVEF, left ventricular ejection fraction; NT-proBNP, N-terminal pro B type natriuretic peptide; NYHA, New York Heart Association; SBP, systolic blood pressure; TMAO, trimethylamine N-oxide. Dyslipidemia is defined according to lipid levels or use of anti-dyslipidemia medications during two weeks prior to the experiment.

Clinical and laboratory parameters associated with TMAO

To examine the association between the clinical characteristics and measured metabolites, Spearman's correlation analyses were performed. Spearman's rank correlation coefficients showed that TMAO, carnitine, and choline were all positively related to the NT-proBNP levels ($r = 0.15$, $P < 0.001$; $r = 0.15$, $P < 0.001$; $r = 0.23$, $P < 0.001$, respectively) but negatively related to eGFR (an estimate of renal function) ($r = -0.24$, $P < 0.001$; $r = -0.15$, $P < 0.001$; $r = -0.16$, $P < 0.001$, respectively). Among these metabolites, a positive correlation between TMAO and choline ($r = 0.27$, $P < 0.001$), as well as carnitine ($r = 0.18$, $P < 0.001$), was observed.

Elevated TMAO in patients indicated poorer survival

Over a median follow-up of 33 months, 314 (34.3%) patients died of cardiovascular disease or received a heart transplant. Fig. 1A illustrates the Kaplan–Meier plot of event-free survival time according to the tertile of TMAO, and the plot depicts an increased cardiac mortality with the plasma levels of TMAO. The top tertile was different from

the other tertiles ($P < 0.001$), and the difference was not statistically significant between median and bottom tertiles. Choline and carnitine separately exhibited similar effects (Fig. 1B and 1C). Patients with higher TMAO levels (tertile 3 versus 1) were associated with 1.5-fold risk of primary endpoint, compared with those with lower TMAO levels (unadjusted HR = 1.47, 95% CI = 1.13–1.91, $P = 0.004$). After adjustments for potential confounders (including age, sex, and smoking status), SBP, history of diabetes mellitus, LDL cholesterol, and HDL cholesterol, the elevated TMAO levels were still associated with a 1.3-fold poorer survival rate (adjusted HR = 1.34, 95% CI = 1.02–1.75, $P = 0.034$), even after adjusting for hemoglobin, LVEF, eGFR, and NT-proBNP (adjusted HR = 1.33,

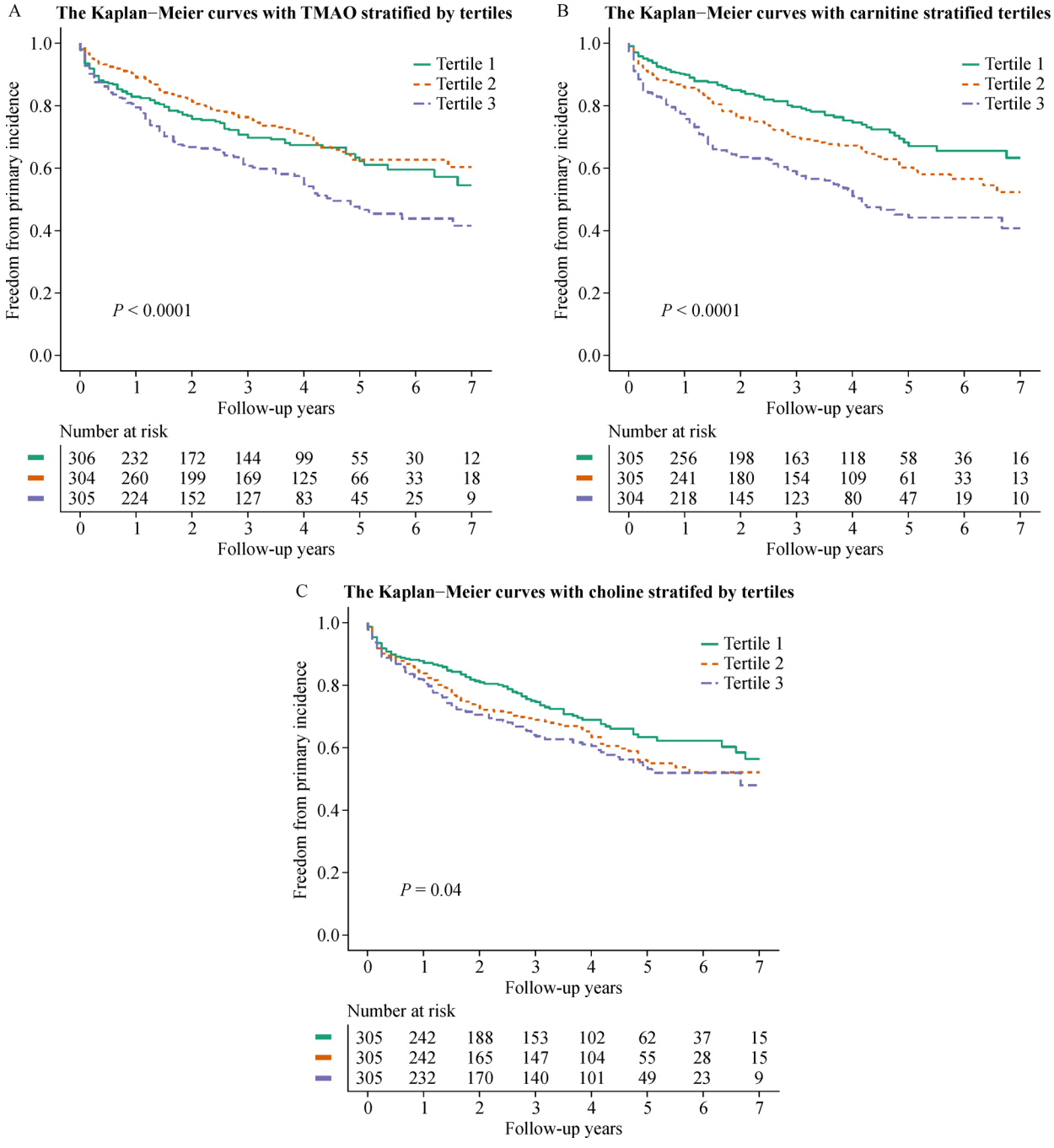

Fig. 1 The Kaplan–Meier curves for primary outcome with metabolites stratified by tertiles. The Kaplan–Meier curves for primary outcome with (A) trimethylamine-N-oxide (TMAO), (B) carnitine, and (C) choline stratified by tertiles. The *P* value was calculated by log-rank test.

Table 2 The hazard ratios (HRs) for the primary outcome according to the tertile of plasma TMAO level

Variables	Tertile 1 ⩽1.574	Tertile 2 1.574–3.770	Tertile 3 >3.770	*P* for Trend
Unadjusted	1	0.84 (0.63–1.12)	1.47 (1.13–1.91)**	<0.001
Adjusted model 1	1	0.83 (0.62–1.10)	1.34 (1.02–1.75)*	0.002
Adjusted model 2	1	0.87 (0.65–1.19)	1.34 (1.02–1.75)*	0.005
Adjusted model 3	1	0.92 (0.68–1.23)	1.33 (1.01–1.74)*	0.018

Model 1, adjusted for sex and age; model 2, adjusted for model 1 plus smoking status, systolic blood pressure (SBP), diabetes mellitus, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL); model 3, adjusted for model 2 plus hemoglobin, left ventricular ejection fraction (LVEF), estimated glomerular filtration rate (eGFR), and N-terminal pro B type natriuretic peptide (NT-proBNP). Data are shown as hazard ratio (95% CI). *P<0.05, **P<0.01.

95% CI = 1.01–1.74, P = 0.039; Table 2).

When the association between TMAO and HFrEF was explored in the secondary outcomes, a high dose-dependent association was found for all-cause mortality ($P < 0.0001$), first rehospitalization for cardiovascular causes (P = 0.002), and recurrence of HF (P = 0.003). At the same time, no statistically significant association was found with stroke (Fig. S3).

Interactions between choline, carnitine, and TMAO predicting cardiac mortality

A positive correlation was found between TMAO and choline (r = 0.27, $P < 0.001$), as well as with carnitine (r = 0.18, $P < 0.001$). Thus, to further explain the relationship between plasma choline, carnitine, and TMAO in predicting cardiovascular mortality, the metabolites were divided into three tertiles, and the 7-year mortality rate referring to each tertile was calculated separately. Another metabolite was combined into the unadjusted model to illustrate the independence of prediction. The analytical results showed that TMAO could still predict the 7-year mortality rate even after adjustment for choline (adjusted HR = 1.35, 95% CI = 1.03–1.78, P = 0.023) or carnitine (adjusted HR = 1.48, 95% CI = 1.13–1.92, P = 0.004) (Table S1). By contrast, the plasma choline could not hold statistical significance under the adjustment for TMAO (P = 0.11). Therefore, the predicting effects of choline may greatly depend on its successor TMAO. From this perspective, TMAO maintained its independent predictive power even under further

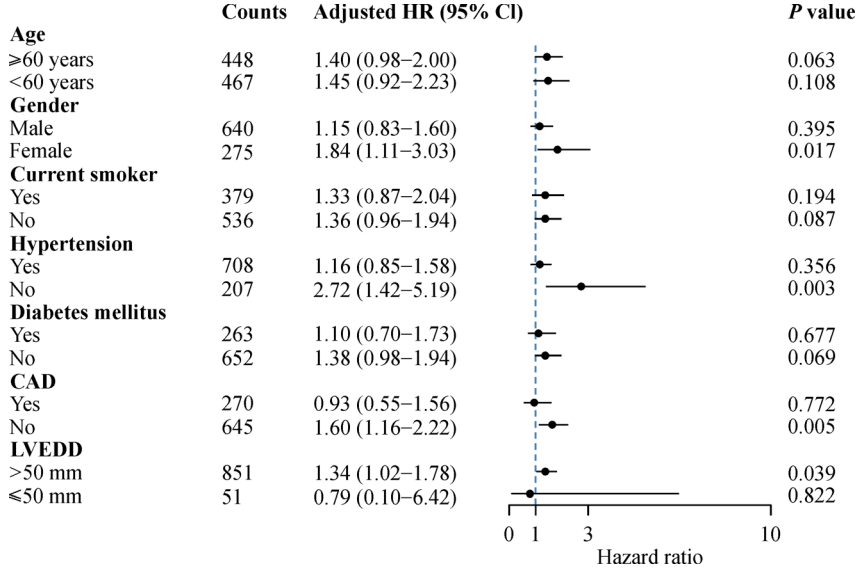

Fig. 2 Hazard ratio (95% CI) of the plasma TMAO levels for the primary outcome among patient subgroups. Comparison of hazard ratios and P values of tertile 3 to tertile 1. Sex, age, smoking status, systolic blood pressure (SBP), diabetes mellitus, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), hemoglobin, left ventricular ejection fraction (LVEF), estimated glomerular filtration rate (eGFR), and N-terminal pro B type natriuretic peptide (NT-proBNP) were adjusted in this model. LVEDD, left ventricular end-diastolic dimension. Thirteen patients have missed LVEDD data.

adjustment for carnitine in the adjusted model 3 (adjusted HR = 1.33, 95% CI = 1.01–1.74, $P = 0.039$).

Subgroup analysis

Fig. 2 represents the HR for the primary endpoint according to the plasma TMAO stratified by subsets of cardiac risk factors. In the adjusted model 3, the risk of primary outcome was stronger in patients without hypertension (adjusted HR = 2.72, 95% CI = 1.42–5.19, $P = 0.003$), females (adjusted HR = 1.84, 95% CI = 1.11–3.03, $P = 0.017$), and those without coronary artery disease (adjusted HR = 1.60, 95% CI = 1.16–2.22, $P = 0.005$). The association between TMAO and mortality was not statistically significant in males and in patients with diabetes or hypertension.

FMO3 gene polymorphism and its association with plasma TMAO levels

FMO3 plays a predominant role in the generation of TMAO in the liver. The effect of *FMO3* polymorphisms on the concentration of plasma TMAO was assessed. The genotypic distribution of rs2266782 was found to be significantly conformed to the Hardy–Weinberg equilibrium by using the χ^2-test. The allele frequency for G was 82% and A was 18% (MAF = 18%). The clinical characteristics of participants across different genotypes are shown in Table S2. Lower TMAO levels were presented in carriers with an AA-genotype of *FMO3* polymorphism than in other genotypes (mean, 3.18 for AA vs. 3.76 for AG and 4.64 for GG), and the AA and GG genotypes showed a statistically significant difference ($P = 0.026$) (Fig. 3).

To uncover the association between TMAO and primary endpoint in the three genotypes, the Kaplan–Meier method was applied to depict the survival curve, and the log-rank test was used to compare the difference between the curves. The associations between TMAO levels and the prognosis of the total HFrEF cohort were consistent in the GG ($P = 0.012$) and GA genotypes ($P = 0.044$). The AA genotype had no statistical difference because of its low incidence (Fig. 4). This result indicated that other factors (such as dietary and gut flora) also influence the generation of TMAO except for *FMO3* SNP.

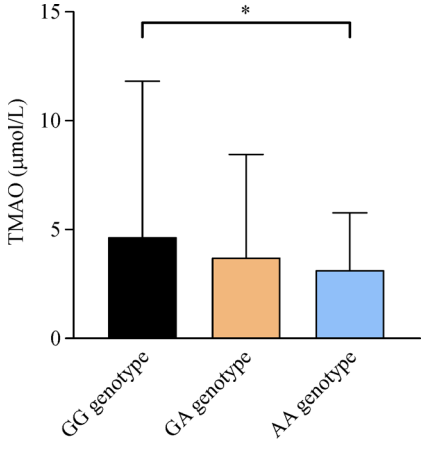

Fig. 3 Association between plasma TMAO and the flavin-containing monooxygenase 3 (FMO3) polymorphisms. The *P* value was calculated by ANOVA. *$P < 0.05$.

Discussion

The function of TMAO and related metabolites were investigated in this HFrEF cohort. The results showed that patients with higher TMAO levels tended to have poorer clinical outcome. TMAO could still be used to predict the risk of the composite outcome of 7-year cardiovascular mortality or transplantation independent of potential confounders and its precursor. The *FMO3* AA-genotype in rs2266782 was found to be associated with reduced plasma TMAO levels. These results provide strong evidence to support TMAO as an independent risk factor for clinical outcomes in HF patients with reduced ejection fraction, and that the polymorphism of *FMO3* gene plays an important role in this process.

TMA, a fish-smelling odorant that is characteristic of degrading seafood, is first produced by the metabolism of TMA-containing substrates, such as phosphatidylcholine (lecithin), choline, betaine, and L-carnitine (found in dietary red meat, fish, and eggs) by gut flora [3,4,15,16]. Then, TMA is absorbed by the intestines and subsequently oxidized by the hepatic flavin monooxygenases to form TMAO [3,9]. The circulating TMAO concentrations are decreased after antibiotic treatment but return to normal upon withdrawal of antibiotics and recolonization of the gut bacteria. TMAO was also undetectable in germ-free mice [3,17,18]. TMAO clearance from the circulation is largely dependent on urinary excretion. Studies have shown a massive elevation of TMAO concentrations (being almost 30-fold higher than that in controls) with advanced chronic kidney disease stage, with median concentrations in dialysis-dependent patients with end-stage renal disease [19]. Moreover, patients display a marked reduction in TMAO concentrations after kidney transplantation. Patients with chronic kidney disease and residual kidney function revealed a strong inverse relationship between eGFR and serum TMAO concentrations [20]. In our study, patients with higher TMAO levels also had reduced eGFR levels, which is consistent with previous studies.

Choline is a constituent of cell and mitochondrial membranes. Higher plasma choline levels have been associated with an increased risk of major adverse cardiac

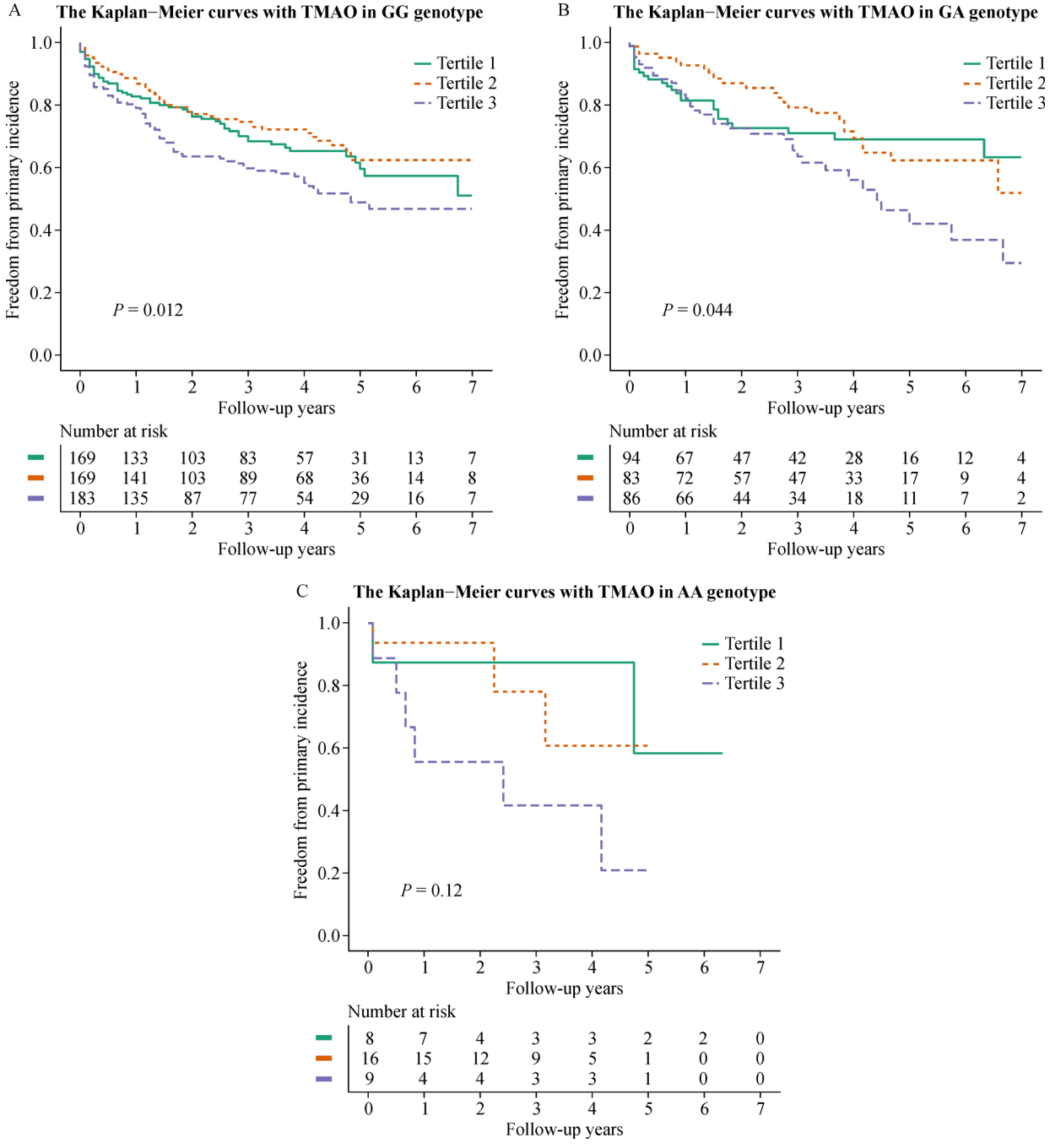

Fig. 4 The Kaplan–Meier curves for the primary outcome with plasma TMAO levels among genotypes (A–C). The *P* value was calculated by log-rank test.

events [21]. Choline has been linked to changes in insulin resistance with increased choline bacterial conversion to TMA and TMAO associated with impaired glucose homeostasis in a nonalcoholic fatty liver animal model [22,23]. The generation of trimethylamine from choline occurs within the intestinal lumen and appears to be exclusively dependent on the presence of intestinal bacteria. This phenomenon is due to the fact that interventions to alter the composition of the gut microbiome have led to marked reductions in circulating TMAO concentrations in both rodents and humans with intact kidney function. Moreover, several studies have suggested a shift in the gut microbiome in patients with HF [24–26]. Thus, increased intestinal absorption and metabolism of

trimethylamine from the precursors or hepatic synthesis of TMAO could have contributed to the elevated TMAO concentrations in patients with HF. In our study, higher TMAO and choline level were found, suggesting a poorer survival including the higher risk of the composite outcome of cardiovascular mortality or transplantation. TMAO remained significant after the adjustment of choline (Table S1), indicating that TMAO has an independent and important impact in HF, which is consistent with a previous report [5]. Another investigation from the same group showed that the prognostic value of choline depended on TMAO, and choline could not be used to predict the MACE risk when TMAO was added to the adjustment model. Similarly, the predictive effects of choline indispensably depended on TMAO in the present study cohort with different ethnic characteristics and endpoints. However, in this study, carnitine also remained significant after adjustment of TMAO, indicating that carnitine may exert function through other pathways.

FMO3 is the liver enzyme that is necessary for oxidizing trimethylamine into TMAO. Global gene expression analyses have suggested that FMO3 has broad effects on lipogenesis and gluconeogenesis mediated through the PPARα and Kruppel-like factor 15 pathways and the beiging of white adipose tissue [11,27]. FMO3 is suppressed by insulin *in vitro* and elevated in obese/insulin-resistant male mice and obese/insulin-resistant humans [28]. Functional differences in the FMO3 activity can occur in humans secondary to variations within the FMO3 gene. Previous studies have reported that the substitution of the A allele for the G allele in the FMO3 polymorphism E158K (rs2266782) would lead to the translation of the amino acid lysine instead of glutamic acid, which is associated with the decreased activity in the main TMAO-generating enzyme FMO3 [29]. In addition to rare genetic mutations in the FMO3 enzyme in trimethylaminuria, SNPs, including E158K (rs2266782) and E308G (rs2266780), are in linkage disequilibrium and have been reported to decrease the metabolic activity of this enzyme (and reduce the conversion of TMA to TMAO under normal physiologic conditions) [30]. Moreover, several studies have reported associations between these genetic variants and disease risk. In patients with hypertension, a greater risk of ischemic stroke was observed among heterozygote *FMO3* E158K and E308G genotype carriers in a Turkish population [13]. The polymorphism E158K in metabolic syndrome remains controversial. One study has shown that *FMO3* SNP E158K might protect children and adolescents from obesity and insulin resistance in an Italian population [31]. By contrast, no significant association was observed between rs2266782 and type 2 diabetes in a Chinese Han population [32]. In the current study, lower TMAO levels were present in carriers with AA-genotype of *FMO3* polymorphism than in other genotypes. Higher plasma TMAO levels were associated with an increased mortality risk in the study cohort. These data collectively suggested that *FMO3* gene SNP rs2266782 could affect its enzyme activity, and, consequently, the concentration of plasma TMAO and the prognosis of HFrEF. Several limitations of this study should be mentioned. First, the possibility of biased diets that may affect the measured data could not be precluded. Second, some values were unavoidably missing, although these values had little impact on the final results. Finally, the cohort was still not large enough to reach conclusions in some stratified analyses, although this study is a rather large-scale study in this field.

Conclusions

Elevated TMAO levels predicted higher risk of the composite outcome of cardiovascular death or transplantation. The prognostic value of TMAO was independent of potential confounders and its precusor. SNP rs2266782 in *FMO3* gene influenced the concentration of the plasma TMAO, and the AA-genotype shared lower TMAO levels.

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Compliance with ethics guidelines

Haoran Wei, Mingming Zhao, Man Huang, Chenze Li, Jianing Gao, Ting Yu, Qi Zhang, Xiaoqing Shen, Liang Ji, Li Ni, Chunxia Zhao, Zeneng Wang, Erdan Dong, Lemin Zheng, and Dao Wen Wang declare that they have no conflict of interest. This study was approved by the ethics committee of Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. This research was conducted in accordance with the *Declaration of Helsinki* and the International Conference on Harmonization Guidelines for Good Clinical Practice. Written informed consent was obtained from all individuals at admission.

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