

Targeted Plasma Metabolomics Reveals Association of Acute Myocardial Infarction Risk with the Dynamic Balance between Trimethylamine-*N*-oxide, Betaine, and Choline

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ABSTRACT: The relationship between trimethylamine-*N*-oxide (TMAO), betaine, and choline with acute myocardial infarction (AMI) end point remains unclear. We analyzed plasma TMAO, betaine, and choline concentrations in AMI cases and non-AMI community-dwelling controls by LC-MS/MS to understand how the balance between these metabolites helps to reduce AMI risk. Results showed that the odds ratio (OR) for the highest versus lowest quartiles of betaine was 0.30 (95% CI, 0.10–0.82) after adjustment for AMI risk factors, and the unadjusted OR for quartile 3 versus quartile 1 of TMAO was 2.47 (95% CI, 1.02–6.17) ($p < 0.05$). The study populations with “high betaine + low TMAO” had a significant protective effect concerning AMI with a multivariable-adjusted OR of 0.20 (95% CI, 0.07–0.55) ($p < 0.01$). Multivariate linear regression showed that the chronological age was correlated with TMAO concentrations among AMI patients (95% CI, 0.05–3.24, $p < 0.01$) but not among the controls. This implies a further potential interplay between age and metabolite combination—AMI risk association.

KEYWORDS: betaine, trimethylamine-*N*-oxide, choline, acute myocardial infarction

INTRODUCTION

Cardiovascular disease is a severe illness endangering human health and is the prime reason for death globally.¹ It is estimated that roughly 17 million people die every year from cardiovascular diseases.² Recent research suggested that gut microbiota is crucial to the development of atherosclerotic cardiovascular disease such as acute myocardial infarction (AMI), especially gut microbial metabolite—trimethylamine-*N*-oxide (TMAO) and related metabolites including betaine and choline.^{3,4}

TMAO is considered the initiator of chronic diseases, such as AMI.⁵ Research has shown that the increase of plasma TMAO concentrations is positively correlated with adverse cardiovascular events.⁶ It was suggested that TMAO disturbed hormone and lipid homeostasis and promoted vascular inflammation, which is a possible link between diet and cardiovascular events, including AMI.^{7,8} Recently, a meta-analysis concluded that higher plasma TMAO was related to a 23% increased risk of cardiovascular events (hazard ratio 1.23, 95% confidence interval 1.07–1.42) and a 55% increase in all-cause mortality.⁹ In fact, studies have pointed out that TMAO is involved in major biological functions of many organisms.⁷ For example, TMAO can be utilized by cells to maintain cell volume under osmotic pressure and hydrostatic stress conditions.⁸ In addition, fish contain high concentrations of trimethylamine (TMA), which will be transformed into TMAO under the action of bacteria in the human intestine.^{5,10} If TMAO causes cardiovascular events, eating more fish will cause an increase of heart disease risk; that is not the case. In fact, a terrific amount of evidence shows that eating fish is linked to cardioprotection.⁵ Therefore, there has been a controversy over whether the increase in the TMAO

concentration is the cause or result of these diseases. TMAO may not be a pathogenic factor or a protective factor but may be a sign of imbalanced homeostasis.^{5,8} Although there have been many studies on the relationship between TMAO and cardiovascular events including AMI risk, no definite conclusion has been reached so far, so further research is necessary.

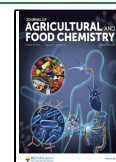
Dietary precursors, for example, choline and betaine, are metabolized to TMA through gut microbiota and oxidized to form TMAO under the function of hepatic flavin monooxygenases (FMOs).^{5,7} TMAO may also be produced by betaine, which is the oxidation product of choline.¹¹ Similar to TMAO, the relationship between plasma betaine and choline concentrations and cardiovascular events, including the end point of AMI end points, remains unclear.^{12,13} The metabolism of betaine links several metabolites that play important roles in humans' and other mammals' health, including choline, homocysteine, and methionine involved in its catabolism.¹⁴ Betaine within the dietary intake range can reduce total homocysteine (tHcy).¹⁵ For example, betaine is a rich component in wheat grains, especially in bran and aleurone,^{16,17} which is considered as a potential contribution to the health value of whole grains.¹⁸ Betaine is important to the remethylation of tHcy,¹⁹ a risk factor for cardiovascular

Received: November 23, 2022

Revised: August 29, 2023

Accepted: September 13, 2023

Published: October 2, 2023



disease, especially stroke.²⁰ It is pointed out that betaine supplementation can reduce the fasting plasma tHcy level of healthy subjects by 15–20%, while after methionine loading, the plasma tHcy level decreases by 20–40%.^{21,22} Betaine and choline can be used as dietary supplements for many purposes, including promoting heart health by reducing homocysteine, aiding digestion, and protecting liver.^{15,22} In addition, other studies have reported that high intake of choline and betaine in diet is related to the reduction of inflammation.²³ However, betaine and choline may have an adverse effect on the concentration of serum lipid, which may increase cardiovascular disease risk to some extent,²⁴ though the increase of cardiovascular risk in patients with elevated choline and/or betaine levels was only observed in patients with concomitantly increased plasma TMAO concentrations.²⁵ However, it is unclear whether the benefits of betaine and choline are greater than the potential adverse effects on serum lipids.²⁶

In conclusion, there is much research about the effects of TMAO, betaine, and choline on cardiovascular diseases, but opinions vary. In addition, most of the existing studies discuss the relationship between a certain metabolite (TMAO, betaine, or choline) and the risk of cardiovascular diseases independently. In fact, these metabolites are closely related to each other, and there is an appropriate dynamic balance between them; we cannot ignore the potential contribution of this balance to the risk of AMI. After all, AMI is a condition of metabolic disturbance.²⁷ Therefore, we hypothesized that the relationship between TMAO and its precursors (betaine and choline), with AMI risk, may depend more on the balance between them. Based on this hypothesis, our present study compared and analyzed the TMAO, betaine, and choline levels in the plasma of patients with AMI and non-AMI community-dwelling controls using liquid chromatography with tandem mass spectrometry (LC-MS/MS). We divided different metabolites into four groups according to the quartiles of metabolite level, further discussed the impact of different metabolite level combinations on AMI risk, and therein presented a “dynamic balance” associated with reduced risk of AMI. Based on this, combined with further analysis and the theoretical basis of previous studies, we will put forward corresponding dietary suggestions and transform this conceptual expression of balance into a statement that is closer to life and has certain practical significance.

MATERIALS AND METHODS

Study Population. This study complied with the relevant laws and institutional guidelines and has been approved by the National Healthcare Group Domain Specific Review Board (ref no. 2013-00248 and 2016-00210). A total of 103 AMI subjects, including 84 STEMI patients and 19 NSTEMI patients, were informed and agreed to participate before the inclusion. AMI plasma samples were obtained from the National University Heart Centre, Tan Tock Seng Hospital, Changi General Hospital, Sarawak General Hospital Heart Centre, and Christchurch District Hospital within 24–48 h after percutaneous coronary intervention. Detailed inclusion and exclusion criteria of the AMI cases may be found in the Supporting Information (Table S1: inclusion and exclusion criteria of AMI cases). Meanwhile, plasma samples from 69 matched non-AMI community-dwelling residents in Singapore were collected as controls. All samples were immediately centrifuged at 4 °C and stored at −80 °C for subsequent analysis.

Chemicals and Standards. Choline chloride, trimethylamine *N*-oxide dihydrate, and glycinebetaine ($C_5H_{11}NO_2$) were obtained from Combi-Blocks Inc. (San Diego, CA, USA), Thermo Fisher Scientific India Pvt. Ltd. (Mumbai, India), and Alfa Aesar (Tewksbury, MA, USA), respectively. Choline chloride (trimethyl-d9) was bought from

Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA). Trimethyl-d9-amine *N*-oxide and *N*-(carboxymethyl)-*N,N,N*-trimethyl-d9-ammonium chloride were purchased from Charslton Technologies Pte Ltd. (Singapore). LC-MS grade acetonitrile and formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA) and Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), respectively.

Metabolomics Analysis. Blood was drawn into labeled 3 × 6 mL K2-EDTA tubes, and the blood was immediately inverted in the tubes 8–10 times and mixed. K2-EDTA blood collected tubes were centrifuged for 10 min at 4000 rpm at 4 °C. The plasma was pooled from the three K2-EDTA blood collected tubes, and the pooled plasma was aliquoted into 2 mL storage tubes.

Based on the method described by Wang et al.,²⁸ plasma TMAO, betaine, and choline were quantified by LC-MS/MS. 80 μ L of cold methanol was added to 20 μ L of plasma sample for protein precipitation. It was centrifuged at 20,000g at 4 °C for 10 min, and then the supernatant was collected and analyzed using an Agilent 1290 UHPLC system equipped with an Agilent 6460 Triple Quadrupole MS (Agilent Technologies, Santa Clara, CA, USA). The chromatographic column used was a HILIC column (2.1 × 100 mm, 1.7 μ m; Waters, Milford, MA, USA). Mobile phase A was water containing 0.1% formic acid, while mobile phase B was acetonitrile containing 0.1% formic acid, with a flow rate of 0.25 mL/min.

Quantification of TMAO, Choline, and Betaine. Stable isotope-labeled compounds were used as internal standards for TMAO, choline, and betaine quantification. TMAO, betaine, and choline were monitored in the positive multiple reaction monitoring (MRM) mode using characteristic precursor-product ion transitions m/z 76 → 58, m/z 118 → 59, and m/z 104 → 60, respectively. The internal standards choline chloride (trimethyl-d9), trimethyl-d9-amine *N*-oxide, and *N*-(carboxymethyl)-*N,N,N*-trimethyl-d9-ammonium chloride were added to the plasma samples before protein precipitation (the concentration of internal standard was 50 ppb) and were similarly monitored in the MRM mode at m/z 85 → 66, m/z 127 → 68, and m/z 113 → 69, respectively. Internal standards were added to TMAO, choline, and betaine standards with different concentrations (0, 10, 50, 200, 500, and 1000 ppb) to prepare calibration curves for quantitative determination of plasma analytes; for plasma samples with concentrations exceeding the concentration range for any of the compounds, they were diluted and reanalyzed. Additionally, quality control (QC) samples were prepared by mixing different samples to calculate the data repeatability. The relevant information about the QC samples and the repeatability are shown in the Supporting Information (Table S2: the relevant information about the QC samples and the repeatability). Other ordinary markers were determined using routine laboratory methods.

Statistical Analysis. All analyses were performed in R 4.0.4 (R Core Team, Vienna, Austria). For the baseline characteristics, differences between groups were examined by the Mann–Whitney test for continuous variables and Fisher’s Exact test for categorical variables. For univariate comparisons of TMAO, betaine, and choline levels, Mann–Whitney test and Kruskal–Wallis test (with Bonferroni correction for pairwise comparisons) were used to assess statistical differences between AMI vs control and STEMI vs NSTEMI vs control groups, respectively. Spearman correlation analysis (with Holm correction for multiple testing) was performed to evaluate relationships among TMAO, betaine, and choline levels and their relationships with the traditional risk factors. The odds ratios (ORs) and 95% confidence intervals (95% CIs) for discriminating the AMI from the control group were obtained by multivariate logistic regression analysis. Traditional risk factors including age, race, gender, smoking status, family history of premature AMI, diabetes, hypertension, dyslipidemia, systolic blood pressure (SBP), diastolic blood pressure (DBP), and the amounts of platelets, creatinine, cholesterol, and high-density lipoprotein C (HDL-C) were adjusted. The relationships between TMAO levels and age were assessed via linear regression with adjustments for gender and SBP and DBP.

RESULTS

Baseline Characteristics and Associations of Betaine, Choline, and TMAO Levels with AMI. The baseline characteristics of AMI cases (including STEMI and NSTEMI) with age-matched non-AMI community-dwelling individuals are shown in the Supporting Information (Table S3: Baseline characteristics of the study populations). There is generally a greater burden of traditional risk factors and predictors of AMI such as high-sensitivity troponin T (hsTnT) in AMI cases. As for the plasma metabolites monitored in this study (Table 1),

Table 1. TMAO, Betaine, and Choline Levels of the Study Populations

metabolites (median [IQR])	AMI (<i>n</i> = 103)	control (<i>N</i> = 69)	<i>p</i> -value ^a
betaine, μM	69.3 (57.9–87.9)	77.9 (66.5–98.7)	9.8×10^{-03}
choline, μM	37.9 (29.5–45.8)	35.1 (30.1–39.9)	1.5×10^{-01}
TMAO, μM	9.4 (6.2–15.2)	8.5 (5.1–17.8)	6.3×10^{-01}

^aAdjusted *p*-values are obtained through comparison between AMI patients and control group. Abbreviations used in the table: TMAO, trimethylamine-*N*-oxide; AMI, acute myocardial infarction; IQR, interquartile range; *n*, number of AMI cases; *N*, number of controls.

betaine demonstrated significant differences in plasma samples between AMI and control groups. Plasma betaine levels in AMI patients were significantly lower than that in control subjects [69.3 (57.9–87.9) versus 77.9 (66.5–98.7) μM ; $p < 0.01$], whereas there was no significant difference in plasma TMAO or choline levels between AMI patients and control. As the numbers of cases and controls in this study were relatively small, we performed a postanalysis power calculation to support our results and facilitate the discussion of any observations. Based on the existing data of the three metabolites (desired fold-differences ~ 1.1) and with a false-discovery rate setting of 0.05, the power was predicted to be 0.61 for a minimum sample group size of 69 (value derived from the control group size in the current work).

When all 103 AMI cases and 69 controls were divided into four equal groups according to quartiles of their plasma metabolite (betaine, choline, or TMAO) levels, statistically significant associations between the metabolites and AMI risk were observed only at the higher quartiles (Table 2). Specifically, for plasma betaine, the ORs for quartile 4 and quartile 3 versus the lowest quartile were 0.33 and 0.40 respectively ($p < 0.05$). As for plasma TMAO, only the OR for quartile 3 versus lowest quartile of TMAO was statistically significant with an OR value of 2.47 ($p < 0.05$). Additional adjusting of AMI risk factors, including age, race, gender, smoking status, family history of premature AMI, diabetes, hypertension, dyslipidaemia, SBP, DBP, and the amounts of platelets, creatinine, cholesterol, and HDL-C, affected the association, and the relationship between TMAO and AMI risk was even attenuated to be nonsignificant.

Effect Evaluation of Different Metabolite Combinations on AMI. Based on previous results, only betaine and TMAO demonstrated significant associations with AMI. Thus, we divided the samples into four combinations based on median cutoffs of betaine and TMAO concentrations (refer to Table 3 for the groupings and concentration ranges). The optimal combination of TMAO and betaine levels with a protective effect for AMI was evaluated based on the ORs

Table 2. ORs of Betaine, Choline, or TMAO Levels in the Respective Quartile Ranges for the Study Populations^a

	quartile 1	quartile 2	quartile 3	quartile 4
betaine				
range (μM)	<60.5	60.6–73.4	73.5–90.6	>90.7
AMI case rate (<i>n</i> / <i>N</i>)	32/11 = 2.90	27/16 = 1.69	23/20 = 1.15	21/22 = 0.95
unadjusted OR (95% CI)	1	0.58 (0.22–1.45)	0.40* (0.14–0.97)	0.33* (0.12–0.80)
adjusted OR (95% CI)	1	0.75 (0.25–2.16)	0.40 (0.09–1.09)	0.30* (0.10–0.82)
choline				
range (μM)	<29.5	29.5–36.0	36.1–44.1	>44.2
AMI case rate (<i>n</i> / <i>N</i>)	26/17 = 1.53	22/21 = 1.05	26/17 = 1.53	29/14 = 2.07
unadjusted OR (95% CI)	1	0.68 (0.29–1.60)	1.00 (0.42–2.38)	1.36 (0.56–3.31)
adjusted OR (95% CI)	1	0.69 (0.26–1.81)	1.08 (0.40–2.90)	1.36 (0.48–3.91)
TMAO				
range (μM)	<5.6	5.6–8.5	8.6–16.0	>16.1
AMI case rate (<i>n</i> / <i>N</i>)	22/21 = 1.05	27/16 = 16.9	31/12 = 2.58	23/20 = 1.15
unadjusted OR (95% CI)	1	1.61 (0.68–3.85)	2.47* (1.02–6.17)	1.10 (0.47–2.57)
adjusted OR (95% CI)	1	2.07 (0.76–5.82)	2.65 (0.93–7.84)	0.93 (0.31–2.83)

^aAdjusted OR is derived from logistic regression models adjusted for traditional risk factors (including age, race, gender, smoking status, family history of premature AMI, diabetes, hypertension, dyslipidaemia, SBP, DBP, and the amounts of platelets, creatinine, cholesterol, and HDL-C). * denotes that there is significant difference with quartile 1 at $p < 0.05$. Abbreviations used in the table: TMAO, trimethylamine-*N*-oxide; AMI, acute myocardial infarction; OR, odds ratio; *n*, number of AMI cases; *N*, number of controls.

obtained (Table 3). In the previous section, logistic regression analysis adjusted for multiple traditional risk factors revealed that higher levels of TMAO and higher levels of betaine are each associated with decreased AMI risk. However, interestingly, when assessed together through the various combinations of TMAO/betaine levels, only those with high betaine + low TMAO levels had a statistically significant protective effect for AMI with an adjusted OR of 0.20 (95% CI, 0.07 to 0.55) ($p < 0.01$). On the other hand, the combination of high TMAO + high betaine did not exhibit statistical significance in association with reduced AMI risk. When the OR is below 1, there is no statistical significance for the low betaine + high TMAO combination as well.

Correlations of Betaine, Choline, and TMAO with Other Cardiovascular Risk Factors/AMI Biomarkers and Age. To identify possible correlations between betaine, choline, and TMAO levels with other cardiovascular risk factors/AMI biomarkers, Spearman's correlation analysis was performed (Figure 1). No significant correlations were found for SBP, DBP, platelets, HDL-C, or NTproBNP levels with the three metabolites. However, significant positive correlations were found between TMAO with age ($p < 0.0001$) and carnitine ($p < 0.01$) and choline with age and carnitine ($p < 0.001$), whereas TMAO was negatively correlated with cholesterol and betaine was negatively correlated with hsTnT ($p < 0.05$).

Table 3. ORs for AMI by Combinations of Betaine and TMAO Level^a

	low betaine + low TMAO	low betaine + high TMAO	high betaine + low TMAO	high betaine + high TMAO
betaine concentration, μM	<73.5	<73.5	>73.5	>73.5
TMAO concentration, μM	<8.6	>8.6	<8.6	>8.6
AMI case rate, n/N	35/14 = 2.50	24/13 = 1.85	14/23 = 0.61	30/19 = 1.58
unadjusted OR (95% CI)	1	0.74 (0.29–1.86)	0.24** (0.09–0.59)	0.63 (0.27–1.46)
adjusted OR (95% CI)	1	0.59 (0.20–1.73)	0.20** (0.07–0.55)	0.46 (0.16–1.25)

^aAdjusted OR is derived from logistic regression models adjusted for traditional risk factors (including age, race, gender, smoking status, family history of premature AMI, diabetes, hypertension, dyslipidaemia, SBP, DBP, and the amounts of platelets, creatinine, cholesterol, and HDL-C). **, $p < 0.01$. Abbreviations used in the table: TMAO, trimethylamine-N-oxide; AMI, acute myocardial infarction; OR, odds ratio; n, number of AMI cases; N, number of controls.

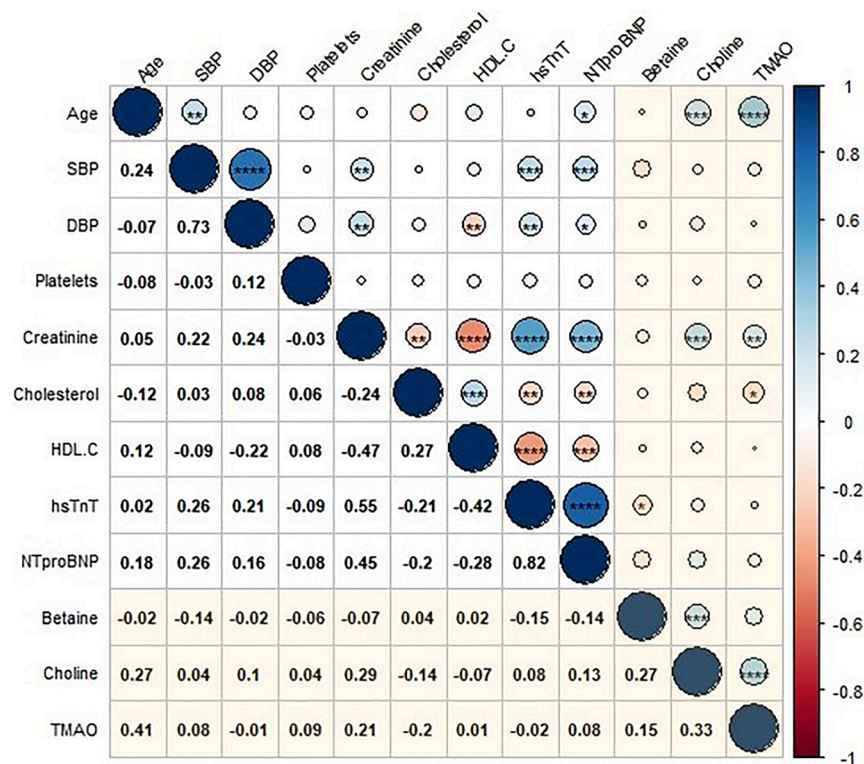


Figure 1. Correlation analysis of betaine, choline, and TMAO with other cardiovascular risk factors/biomarkers. X- and Y-axes are the same set of risk factors. Correlation coefficients (r) of different factors are displayed as values in the lower panel and also indicated on the upper panel according to the color scale given. Red indicates negative correlation and blue indicates positive correlation. The size of the circle indicates the degree of the correlation between factors. Statistical significance is indicated with asterisks: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, with Holm correction for multiple testing. Abbreviations used in the figure: TMAO, trimethylamine-N-oxide; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; hsTnT, high-sensitivity troponin T; NTproBNP, N-terminal-pro hormone brain natriuretic peptides.

Due to the significant correlation between plasma TMAO and age, we further assessed the relationship via a linear regression model, with age as the dependent variable and plasma TMAO as the predictor/independent variable (model adjusted for gender, SBP, and DBP) (Table 4 and Figure 2). The results showed that the plasma TMAO of AMI patients was positively associated with age. Specifically, a $0.18 \mu\text{M}$ (95% CI 0.05 – $3.24 \mu\text{M}$) increase in the mean TMAO concentration for each 1 year increase in age ($p < 0.01$) was observed. Additionally, the coefficients of TMAO in AMI patients was 2.9 times ($0.176/0.06$) higher than that in the control group, suggesting that TMAO is more strongly associated with age in AMI patients with statistical significance ($p < 0.01$) than in controls. Also, for the AMI cases, 31.8% of the variation in chronological age can be explained by this linear model with significant contributions by TMAO ($R^2 = 0.318$, $p < 0.0001$),

while 18.9% of variation in age can be explained by the linear model in the controls as well ($R^2 = 0.189$, $p < 0.01$). The intersection between the two regression lines fitted using AMI versus non-AMI controls was 46.3 years in age.

DISCUSSION

The study on the relationships between TMAO, gut microbiota, Mediterranean diet, and cardiovascular disease is a hotspot recently, where numerous clinical and epidemiological studies have been published.^{28–30} However, the observations regarding TMAO have been heterogeneous and inconclusive, and further research/validation is necessary. The observation and experimental research results indicated that there is a strong positive association between the increase of plasma TMAO concentration and cardiovascular diseases including AMI, and the increase in TMAO levels is associated

Table 4. Regression Coefficients of Age Modeling by TMAO Levels^a

	coefficient (95% CI)	p-value	overall R ²
AMI			
gender (female)	7.41 (2.00–12.82)	7.7×10^{-03}	0.318****
SBP (mm Hg)	0.16 (0.05–0.26)	3.0×10^{-03}	
DBP (mm Hg)	−0.25 (−0.09 to 0.40)	2.4×10^{-03}	
TMAO (μM)	0.18 (0.05–3.24)	1.6×10^{-03}	
control			
gender (male)	1.70 (−2.74 to 6.15)	4.5×10^{-01}	0.189**
SBP (mm Hg)	0.29 (0.12–0.46)	1.3×10^{-03}	
DBP (mm Hg)	−0.36 (−0.11 to 0.61)	5.8×10^{-03}	
TMAO (μM)	0.06 (−0.06 to 0.18)	3.1×10^{-01}	

^aSignificant *p*-values are underlined. **, *p* < 0.01; ****, *p* < 0.0001. Abbreviations used in the table: TMAO, trimethylamine-*N*-oxide; AMI, acute myocardial infarction; OR, odds ratio.

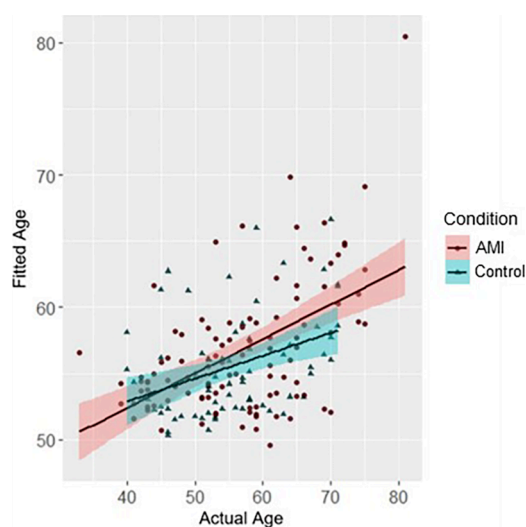


Figure 2. Comparison between actual age and fitted age of AMI patients and healthy subjects respectively, using linear regression of plasma TMAO levels (adjusted for gender, SBP, and DBP). Abbreviations used in the figure: TMAO, trimethylamine-*N*-oxide; AMI, acute myocardial infarction.

with an increase in major adverse cardiac events in patients with AMI.^{8,31,32} Most human observational research suggested a correlation between high plasma TMAO and cardiovascular disease risk.⁵ However, this association does not necessarily mean that TMAO can lead to heart disease. Some studies have shown that TMAO plays important biological functions in various organisms, from bacteria to mammals.⁶ The present study indicates that there is no significant difference in baseline plasma TMAO levels between AMI patients and non-AMI community-dwelling individuals when assessed univariately. In order to magnify the difference, we grouped the plasma metabolite levels of the samples. We divided the study population by half (median), triplets, or quartiles in order to observe the differences. However, we finally decided to use the quartiles because we noticed that if we divided the study population by the median or triplet, meaningful observations would be masked. When all samples were divided into four equal groups based on plasma TMAO values (in ascending order: quartile 1, 2, 3, and 4), the unadjusted OR for quartile 3 versus quartile 1 of TMAO was 2.47 (95% CI, 1.02–6.17) (*p* < 0.05), indicating that TMAO was a significant risk factor for

AMI. This may be because a certain increase of TMAO destroys the internal homeostasis, showing a certain risk effect. Plasma TMAO concentrations are determined by various factors, including diet, intestinal microbiota composition and activity, intestinal blood barrier permeability, liver enzyme activity, and excretion rate.⁸ In addition, interventions and dietary intake before sample collection have also been shown to affect metabolite levels, which were not evaluated in our study. The unification of precollection diet and other related exposures may help to minimize the variability of metabolite measurements and improve comparability between groups.³³

The metabolite TMAO produced by atherogenic intestinal flora is formed by a two-step process triggered by the cleavage of intestinal microbiota-dependent trimethylamine substances (such as choline and betaine) that produce precursor TMA, followed by FMO3 and possibly other FMOs. Studies showed that after adjusting the traditional cardiovascular risk factors, the elevated plasma choline and betaine levels were related to poor prognosis.²⁸ Nevertheless, this poor prognosis was observed only in patients with increased TMAO.^{25,34} Therefore, we speculate that TMAO seems to be the culprit in increasing the risk of cardiovascular disease.²⁵ This phenomenon may depend on the TMAO mechanism. However, since the impact of TMAO on the risk of AMI is not conclusive, it does not rule out that this adverse prognosis is only caused by metabolic disorders or is a marker of disrupted homeostasis in general.⁸ On the other hand, because choline and betaine have different physiological effects, there may be TMAO-independent mechanisms for cardiovascular disease.³⁵ Choline and betaine are metabolically associated with the transmethylation pathway and are considered as methyl donors. Low choline and betaine intake in diet might affect the epigenetic regulation of some genes, thus accelerating the formation of atherosclerosis.³⁶ However, epidemiological investigations have shown an inconsistent association between systemic choline and betaine levels and cardiovascular events.^{12,28,37} For example, some studies indicated that there is no correlation between the plasma choline level and the risk of cardiovascular disease, and our study is consistent with these findings.^{38,39} Some studies indicated that low plasma betaine is related to adverse cardiovascular risk.^{34,40} Moreover, betaine intervention is also considered to be negatively correlated with inflammation.^{23,41} Strong evidence suggested that whole grain foods can help prevent heart disease.^{18,42} Anderson et al.⁴³ pointed out that whole grain intake can significantly reduce the risk of AMI. Although the underlying mechanisms are unclear, the high level of betaine found in wheat aleurone may play a role. Our study is also similar to the results of many studies, indicating that the plasma baseline betaine level in AMI patients is indeed significantly lower than that of non-AMI controls. Moreover, when all samples were divided into four equal groups based on their plasma betaine values, whether adjusting other risk factors or not, the appropriate increase of betaine level will help to reduce the risk of AMI. Nevertheless, considering many inconsistent results of previous studies, we believe that the changes in betaine levels we observed may only indicate nutritional and physiological disorders.⁴⁴ The disruption of betaine homeostasis may lead to complex metabolic consequences, and the interference may be pathogenic.⁴⁵

Guasch-Ferre et al.¹² showed that the ratio of intestinal microbial metabolites had a certain correlation with cardiovascular disease, indicating that the balance between different metabolite concentrations is of great significance for

preventing cardiovascular related diseases. Thus, we compared the effects of different metabolite combinations on the risk of AMI, hoping to find a better indicator or the most conducive metabolite combination among several combinations for reducing the risk in this study. The combination associated with the lowest AMI risk may then reveal the ideal “dynamic balance” between several metabolites. First, according to the results of 3.1 (Table 2), we divided the three substances into two levels by median and obtained a total of eight combinations (high TMAO + high betaine + high choline, high TMAO + high betaine + low choline, high TMAO + low betaine + high choline, high TMAO + low betaine + low choline, low TMAO + high betaine + high choline, low TMAO + high betaine + low choline, low TMAO + low betaine + high choline, and low TMAO + low betaine + low choline). We found that although critical significance can be obtained, the OR has a very large 95% CI, which means that the obtained OR is unreliable and inaccurate and that it may be difficult to accept these values. Considering that the plasma choline level in this study population seems to have no effect on the risk of AMI (Tables 1 and 2), we then analyzed only the effects of four combinations (high betaine + high TMAO, high betaine + low TMAO, low betaine + high TMAO, and low betaine + low TMAO) of betaine and TMAO formation at different levels (high and low) on the risk of AMI (Table 3). The results showed that the effects of different combinations were significantly different, and the best metabolite combination was obtained, that is, high betaine + low TMAO (possibly regardless of choline level). This combination significantly reduced the risk of AMI and can be used as a conceptual “balance” with protective effect in the present study. Our immature opinion on diet and nutrition based on this “dynamic balance” is that within the recommended dietary/nutritional amount, appropriately increasing the ratio of betaine to TMAO, for example, is recommended to supplement more whole grains. Because whole grains are rich in betaine and fiber, this dietary suggestion is helpful in supplementing betaine intake. At the same time, some studies have shown that TMAO-cardiovascular disease is more relevant in individuals with low fiber intake, so whole grain intake can also weaken the correlation between TMAO and cardiovascular disease.^{3,13,43}

Interestingly, this study also found a significant positive inter-relation between plasma TMAO and age in AMI cases. This bears relevance with a previous study, which suggested that the microbial composition of human intestinal tract will change with age, resulting in the increase of TMAO level (intestinal microbial metabolite), which will accelerate endothelial cell aging, vascular aging,⁴⁶ and vascular inflammation⁴⁷ and increase the risk of AMI.^{48,49} We also found that the predicted age of AMI patients is often older than that of the community-dwelling individuals without AMI (especially those over the age of about 45 based on the intersection between the two regression lines fitted using AMI versus non-AMI controls). Based on this, we speculate that after suffering from AMI, it may lead to the aging of the body, which may lead to the increase of the TMAO level by changing the composition of intestinal microorganisms. Physiological aging associated with TMAO or the microbiota could increase the risk of AMI. In this way, a vicious circle may be formed. Further linear regression analysis also showed that after adjusting for gender, SBP, and DBP, the average TMAO concentration increased by 0.18 μM (95% CI 0.05–3.24) ($p <$

0.01) for each 1 year increase in the age of AMI patients. It further exemplifies the strength of association of plasma TMAO with chronological age in AMI patients. On the other hand, the strength of association of TMAO levels with age in non-AMI controls is significantly lower at 0.06. The result means that this malignant closed loop may only exist in people who have been already suffering from AMI. Obviously, if analyzed from this perspective, the above suggestions may need to be further improved. Therefore, in this study, we put forward the following suggestions based on the “dynamic balance” according to the situation of two different patients: (1) for non-AMI people and young (<45 years in age) patients with AMI, they may consider eating more vegetables and less meat and appropriately increase the intake of whole grains; (2) for the middle-aged and elderly (>45 years in age) AMI patients, on the basis of the above dietary suggestions, supplementary food/external interventions that inhibit the “betaine/choline $\rightarrow$ TMAO” pathway, such as probiotics and other symbiotic microorganisms, may be required.⁵⁰

Our research has both important strengths and potential limitations. First, the lack of ethnic diversity (the study population is mainly composed of Southeast Asians) may affect the universality of our research results for other AMI populations. Given that there is merit in studying these metabolites in an underexplored cohort predominantly comprised of Southeast Asians, we hope that our work could be useful in complementing the large-scale studies looking at patients with Western origins and vastly different diets. Second, although many confounding factors were considered and adjusted, there are still some residual factors, especially considering the complexity of the metabolomic analysis. Finally, the number of samples used for the study is very small, and the rigor of the matching of controls is not enough. In this regard, we provide the context that our research scale was severely limited by the recruitment of patients by local hospitals. Therefore, even though we wholeheartedly agree that the controls should have been matched with age, gender, and other possible variables these metabolites are dependent on, we could only match the case and control groups based on the variable (i.e., age) deemed the most pertinent based on literature. The fact that AMI is more common among middle-aged and elderly people leads to problems such as insufficient sample size and a lack of rigor of control matching, which may also lead to an increased risk of statistical errors (such as collinearity). The other variable that would have been highly relevant is gender. However, since AMI patients are usually males, matching by gender based on our limited recruited sample pool proved to be challenging. In any case, any supporting results in this work can be used to verify the conclusions of previous reports, but future research needs to be conducted in a larger population to confirm and expand our findings.

In conclusion, though limited, we found evidence that high betaine + low TMAO may have a protective effect on the risk of AMI. Combined with the important variable of age, reasonable diet/nutrition suggestions are put forward based on the research results. In this way, the conceptualized result (dynamic balance) is translated into statements closer to life, which provide a theoretical basis for reducing/preventing cardiovascular diseases from the perspective of diet/food and has certain practical significance. Of course, our suggestions on diets for different populations are preliminary and should be further confirmed by large-scale prospective studies combined

with mechanistic validation, dietary data, and microbiome data in the future. It should be emphasized that our suggestion is from the perspective of nutrition and dietetics and not from the perspective of medicine. We hope that this study can play a reference and auxiliary role.

■ ASSOCIATED CONTENT

SI Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.2c08241>.

Inclusion and exclusion criteria of AMI cases; relevant information about the QC samples and the repeatability; and baseline characteristics of the study populations (PDF)

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Author Contributions

S.H. wrote the article; S.H. and S.Y.L. devised and designed the research, conducted the experiments, and optimized the analysis method; S.Y.L. performed the statistical analysis; S.H.T. and M.Y.C. obtained, processed, and provided the clinical samples; S.Y.L., S.H.T., M.Y.C., W.N., and S.F.Y.L.

reviewed project administration and funding acquisition. All authors approved the final manuscript. Cofirst author S.H. and S.Y.L. contributed equally to this work.

Funding

This work was supported by the Ministry of Education of Singapore (grant number: R-143-000-B48-114). S.H. would like to thank the China Scholarship Council (CSC, 2020000000). M.Y.C. is supported by grants from the National Medical Research Council, Singapore (CIRG17may014 and MOH-000280).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The authors would like to thank Hui Xin Chong and Poh Geok Per from the NUS Environmental Research Institute for their technical support in the LC-MS/MS analyses and support provided by the Home for Researchers (www.home-for-researchers.com) in cover art.

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