Atherosclerosis 280 (2019) 126–131

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Serum gut microbe-dependent trimethylamine N-oxide improves the prediction of future cardiovascular disease in a community-based general population

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HIGHLIGHTS

- TMAO has a dose-dependent relationship with risk of CVD in the general population.
- Addition of TMAO significantly improved the predictive ability of CVD risk.
- TMAO is a potential novel preventive target for low-risk CVD adults.

ARTICLE INFO

Keywords:
Trimethylamine N-oxide
Cardiovascular disease
Nested case-control study
General population

ABSTRACT

Background and aims: Recent studies have shown that trimethylamine N-oxide (TMAO) is a risk factor for cardiovascular disease (CVD) in different clinical settings, but few studies confirmed the association in a community-based general population.

Methods: This is a nested case-control study from a prospective cohort design. A total of 86 newly diagnosed CVD cases with a median follow-up period of 4.83 years and 86 matched controls were selected for the present analysis.

Results: Using the LC-MS/MS assays, we found that new CVD cases had a higher baseline levels of TMAO than controls [median (inter-quartile): 1.57 (0.79–2.29) μmol/L *v.s* 0.68 (0.23–1.40) μmol/L, $p < 0.001$]. After multivariable adjustment, individuals with TMAO ≥1.89 μmol/L (Q4) and 1.05–1.89 μmol/L (Q3) had odds ratio (OR) for CVD of 2.735 [95% confidence interval (CI): 1.328–5.630] and 2.544 (95% CI: 1.251–5.172) with the lowest quartile (< 0.43 μmol/L) as reference. In addition, comparisons of areas under receiver operator characteristics curves confirmed that a model including TMAO had a better discrimination than one without (0.732 *vs.* 0.664, $p = 0.045$).

Conclusions: In the community-based general population, there was a positive association between TMAO and future risk of CVD. Addition of TMAO improved the prediction of CVD beyond traditional risk factors. We recommend considering TMAO as a potential novel preventive target in the management of low-risk CVD adults.

1. Introduction

Trimethylamine N-oxide (TMAO) is a small metabolite of the dietary lipid phosphatidylcholine through the action of gut flora [1,2]. In the last five years, several studies indicated that elevated serum levels of TMAO is strongly associated with an increased risk for major adverse cardiovascular events (MACE) defined as death, myocardial infarction, or stroke in different clinical settings [3–7]. Wang et al. [6]

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https://doi.org/10.1016/j.atherosclerosis.2018.11.010
Received 13 August 2018; Received in revised form 29 September 2018; Accepted 7 November 2018
Available online 08 November 2018
0021-9150/

firstly revealed the potential role of a complex phosphatidylcholine–choline metabolic pathway involving gut microbiota in contributing to the pathogenesis of atherosclerotic coronary artery disease in animal models. Meanwhile, Tang group [3] demonstrated that increased TMAO levels are positively associated with an increased risk of incident MACE events in 4007 patients undergoing elective diagnostic cardiac catheterization. In patients with chest pain of suspected cardiac origin, Li et al. [7] proved elevated plasma TMAO levels at presentation are independently associated with risk of MACE events over the ensuing 30 day [4th quartile adjusted odds ratio (OR) 6.30; 95% CI: 1.89–21.0, $p < 0.01$].

In addition, the association between increased TMAO levels and future risk of MACE events was replicated in patients with heart failure, chronic kidney disease, and community-acquired pneumonia patients [8–10]. In contrast to these positive results, in a hospital-based case-control study, Yin et al. [11] showed that stroke and transient ischemic attack (TIA) patients had a decreased TMAO levels compared to healthy controls. It is well known that "consistency" is very important to define the causal relationship. When several studies, conducted at different times in different settings, and with different patients, all come to the same conclusion, evidence for a causal relationship is strengthened. However, there is a relatively scarcity of studies evaluating the association in the community-based general population, especially in Chinese adults.

We therefore tested the association between TMAO and risk of future cardiovascular disease (CVD) in a nested case-control study, from a prospective cohort study within a large cohort of the community-based general population followed up for a nearly 5-year period.

2. Patients and methods

2.1. Study population

This is a nested case-control study design from a rural community-based prospective cohort study. From June 2012 to September 2012, 4157 participants aged ≥35 years were subsequently recruited from 19 rural villages (2 towns, Anmin and Helong) of Xifeng County, Liaoning province. All study subjects were invited to return for follow-up from June 2017 to September 2017. From this population, 330 individuals refused or were lost to follow-up and 3827 participants (or their guardians) agreed and completed the follow-up study. For the purposes of this study, individuals with prior stroke (n = 239) and coronary heart disease (CHD) (n = 390), and missing additional blood samples (n = 744) were excluded, leaving 2454 participants free from CVD and with complete blood samples for the present analysis.

During a median follow-up period of 4.83 years, a total of 86 incident cases of newly diagnosed CVD (48 CHD and 38 stroke cases) were identified. Using risk-set sampling [12], we then randomly selected controls who remained free from CVD and matched them to the CVD patients in a 1:1 ratio, according to age (within 3 year), sex (man/woman), duration of the follow-up period (within 1 month), and hypertension (yes/no). On the basis of these criteria, 86 new CVD patients and 86 matched controls free of CVD were selected for the present analysis. China Medical University Research Ethics Committee has approved the research protocol and written informed consent was formally obtained from all the participants or their guardians. Fig. 1 shows the sample size of patients and exclusion criteria.

2.2. Sample processing

Plasma samples (30 μL serum) were aliquoted to a 1.5 mL Eppendorf tube stored in a −80 °C refrigerator and mixed with 90 μL of 10 μmol/L internal standard comprised of d_9-TMAO in methanol. Protein in the samples was precipitated by vortexing for 1 min and then the mixture was centrifuged at 20,000 *g* at 4 °C for 15 min. Supernatant (about 90 μL) was transferred into a 2 mL sealed sample bottle for high-performance liquid chromatography (HPLC) quantitative analysis. According to the method reported by Wang et al. [6], when analyzing TMAO levels, 3 different quality control (QC) samples were run with TMAO concentrations ranging between 0.30 and 30 μmol/L in duplicate, before each sample batch of less than 30 samples. TMAO concentrations from the batch were acceptable when the accuracy of the values determined form each QC sample was within 100 ± 10% of their expected values and the intrabatch CVs for the QC samples were all less than 10%.

2.3. LC-MS/MS

Stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used for quantification of TMAO according to a previously described method [13]. The LC-MS/MS system consisted of a 1260-series LC (Agilent Technologies, USA) coupled to a 6420 triple quadrupole mass spectrometer. Supernatants (1 μL) were analyzed by injection into an Agilent Proshell 120 EC-C18 (50 mm × 2.1 mm, 2.7 μm) with a column temperature of 30 °C. A discontinuous gradient was generated to resolve the analytes by mixing solvent A (0.1% formic acid in water) with solvent B (acetonitrile) at different ratios, starting from 30% B linearly to 50% B over 6 min, then linearly back to 30% B over 4 min. The flow rate was 0.2 mL/min. TMAO was monitored using electrospray ionization in positive-ion mode with multiple reaction monitoring (MRM) of precursor and characteristic production transitions of m/z 76.3 → 58.2. The parameters for the ion monitoring were as follows: spray voltage, 4.5 kV; curtain gas, 15; gas flow (N_2) 16 L/min; nebulizer pressure, 35 psi; collision voltage, 20 eV; dryer temperature, 150 °C; capillary voltage, 3.5 kV.

2.4. CVD definitions

In our study, CVD was defined as incident stroke and/or CHD events during the follow-up period. According to the WHO Multinational Monitoring of Trends and Determinants in Cardiovascular Disease (MONICA) criteria [14], the definition of stroke events included patients presenting with clinical signs and symptoms of subarachnoid hemorrhage, intracerebral hemorrhage, thrombosis and embolism. CHD was defined as myocardial infarction, angina pectoris of which patients received treatment in hospital for ischemic discomfort and diagnosed by coronary angiography, coronary revascularization and sudden death. Coronary revascularization was achieved when a patient underwent percutaneous coronary intervention (for example, angioplasty, stenting, atherectomy and laser ablation) or coronary artery bypass graft [15]. The relevant information was obtained by direct reference to medical records by a single investigator. All materials were independently reviewed by the end-point assessment committee, whose members were all blinded to the study participants' baseline risk factor information.

2.5. Data collection and physical examinations at baseline

The detailed methodology on data collection and physical examinations is similar to our previous studies [16,17]. At baseline examination, all participants were recruited and examined at a single clinic visit by their local doctors in their geographical area of origin. A standard epidemiological questionnaire was used to collect data on demographic variables (age, sex, and ethnicity), smoking status, use of alcohol, information on antihypertensive medications and history of stroke and CHD at baseline. Blood samples were collected for at least fasting 12 h. Serum glucose, uric acid, creatinine, total cholesterol (TC), triglyceride (TG), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C) were measured with automated enzymatic procedure. We used CKD Epidemiology Collaboration (CKD-EPI) equation to calculate the estimated glomerular filtration rate

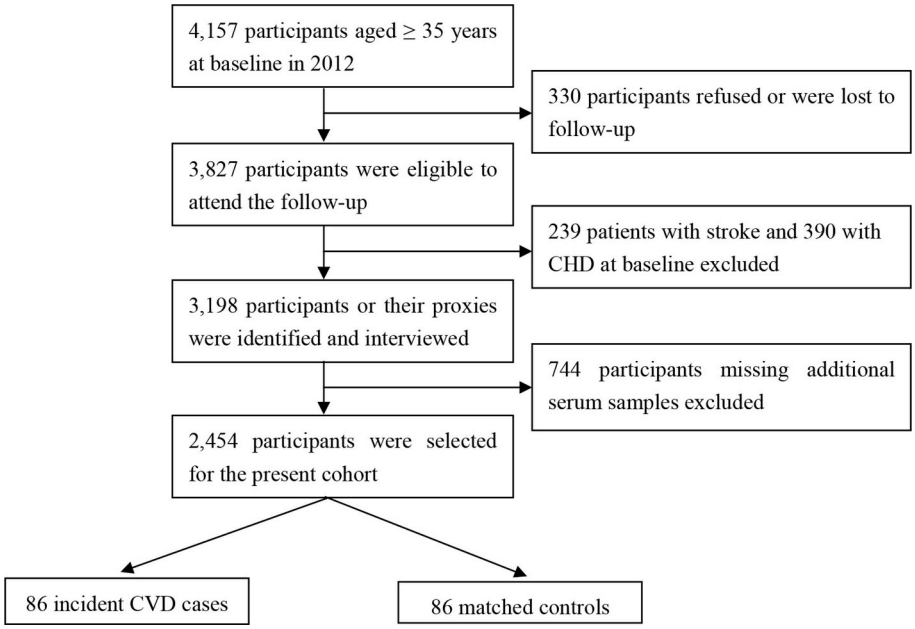

Fig. 1. Flow chart of participant recruitment and derivation of the population used in the final analysis.
CHD, coronary heart disease.

(eGFR) [18]. Blood pressure (BP) was measured using a standardized automatic electronic sphygmomanometer (HEM-907; Omron, Tokyo, Japan).

2.6. Statistical analyses

Continuous variables are presented as mean (standard deviation, SD) or median (inter-quartile) and were compared with Student *t*-test or Mann-Whitney *U* test. Categorical variables are expressed as frequencies and Pearson's χ^2 -tests for independent proportions. Because the incidence-density sampling method was used to match controls to case patients on the basis of the cohort person-time, conditional logistic regression models were used to evaluate the OR and 95% CI for CVD. Before the multivariable conditional logistic regression analysis was conducted, all the preliminary variables were included in an OLS (ordinary least squares) model to test for multicollinearity and the variance inflation factor (VIF) was used to check for the problem of multicollinearity among the independent variables [19].

We constructed 2 models: in model 1, we adjusted for age and ethnicity; and in model 2, we further adjusted for age, ethnicity, and a propensity score. The propensity score was calculated with a linear regression model entering TMAO as the dependent variable and the independent variables included SBP, BMI, antihypertensive medications, current smoking, current drinking, diabetes mellitus, TC, TG, HDL-C, and eGFR. In these multivariable logistic models, TMAO was categorized as quartiles (Q4, ≥1.89 μmol/L; Q3, 1.05–1.89 μmol/L; Q2, 0.43–1.05 μmol/L; Q1, < 0.43 μmol/L) and the lowest quartiles chosen as the reference group. Meanwhile, TMAO were separately entered on a continuous scale (1 μmol/L increment) in these models.

With CVD events as the end point, another two consecutive logistic models of increasing saturation were performed containing (1) systolic blood pressure (SBP), body mass index (BMI), antihypertensive medications, current smoking, current drinking, diabetes mellitus, TC, TG, HDL-C, and eGFR, and (2) TMAO added. To assess the impact on the predictive value of the model of adding in risk factors, the change in −2 log likelihood was calculated for each consecutive model and compared with a χ^2 distribution, with the degrees of freedom reflecting the parameters in each model. In addition, receiver operating characteristic (ROC) curves were constructed, and the areas under the curves (AUC) were calculated to assess the additional discriminant power of each consecutive logistic model.

All analyses were performed with IBM SPSS statistical software version 22.0 (SPSS Inc., Chicago, IL, USA) and SAS statistical software version 9.2 (SAS Institute Inc, Drive Cany, NC, USA). A *p* value less than 0.05 was accepted as indicating statistical significance.

3. Results

Table 1 shows the baseline characteristics of study subjects. Participants who developed new CVD cases during follow-up had a lower levels of eGFR than their counterparts (controls) (92.0 mL/min/1.73 m^2 *vs* 95.9 mL/min/1.73 m^2, $p = 0.042$). There was no significant difference for baseline SBP, BMI, fasting serum glucose, TC, TG, LDL-C, HDL-C, and proportions of ethnicity, diabetes mellitus, antihypertensive

Table 1
Baseline characteristics of CVD patients and controls.

Characteristics	CVD cases	Controls	*p*-value
N	86	86	
Sex, men/women	34/52	34/52	1.000
Age (yrs)	58.8 ± 10.0	57.8 ± 9.8	0.539
Ethnicity, Han/Mongolian/Manchu	46/3/37	51/1/34	0.489
Hypertension, yes/no	40/46	40/46	1.000
SBP (mmHg)	136.6 ± 22.7	134.2 ± 19.9	0.470
BMI (Kg/m^2)	24.3 ± 3.4	23.3 ± 3.5	0.107
TC (mmol/L)	5.12 ± 0.95	4.97 ± 0.98	0.314
TG (mmol/L)[a]	1.35 (0.91–1.83)	1.35 (0.88–2.07)	0.982
HDL-C (mmol/L)	1.26 ± 0.30	1.32 ± 0.35	0.236
LDL-C (mmol/L)	2.93 ± 0.80	2.84 ± 0.75	0.472
Diabetes mellitus, n (%)	11 (12.8)	10 (11.6)	0.816
Glucose (mmol/L)[a]	5.70 (5.34–6.41)	5.88 (5.44–6.30)	0.549
Current smoking, n (%)	30 (34.9)	28 (32.6)	0.747
Current drinking, n (%)	18 (20.9)	15 (17.4)	0.561
Antihypertensive medications, n (%)	9 (10.5)	5 (5.8)	0.265
eGFR (ml/min/1.73 m^2)[a]	92.0 (79.1–104.1)	95.9 (86.0–109.4)	0.042

SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate.

[a] Median and inter-quarter range.

Table 2
Associated factors with TMAO form multivariable linear regression model.

Variables	β coefficient	*p*-value
SBP (mmHg)	−0.005	0.312
BMI (Kg/m^2)	0.038	0.233
TC (mmol/L)	0.037	0.774
TG (mmol/L)	−0.235	0.016
HDL-C (mmol/L)	−0.057	0.873
Diabetes mellitus	0.622	0.066
Current smoking	0.254	0.255
Current drinking	0.235	0.386
Antihypertensive medications	0.851	0.031
eGFR (ml/min/1.73 m^2)	−0.006	0.040

β coefficients and *p* values estimated with linear regression model adjusted for all other characteristics.
TMAO, trimethylamine N-oxide; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; IQR, interquarter range; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate.

medications, current smoking, and current drinking between CVD cases and controls (all $p > 0.05$). As a result of the 1:1 case-control matching criteria, the parameters for sex, age and proportion of hypertension were identical in the 2 groups.

Using the LC-MS/MS assays, we found that participants who developed new CVD cases had a higher baseline levels of TMAO controls [median (inter-quartile): 1.57 (0.79–2.29) μmol/L *vs.* 0.68 (0.23–1.40) μmol/L, $p < 0.001$]. Table 2 shows that TG, eGFR and antihypertensive medications were independently associated with TMAO levels ($p < 0.05$).

Fig. 2 shows the adjusted OR for risk of incident CVD cases as a function of quartiles of TMAO. After adjusting for age and ethnicity, participants with TMAO ≥1.89 μmol/L (Q4) and 1.05–1.89 μmol/L (Q3) *versus* < 0.43 μmol/L (Q1) had ORs for CVD of 2.898 (95% CI: 1.421–5.909) and 2.537 (95% CI: 1.248–5.518), respectively. After further adjustment for other potential confounders, TMAO of ≥1.89 μmol/L (Q4) and 1.05–1.89 μmol/L (Q3) *versus* < 0.43 μmol/L (Q1) had ORs for CVD of 2.735 (95% CI: 1.328–5.630) and 2.544 (95% CI: 1.251–5.172), respectively. TMAO was also considered on a continuous scale in the logistic regression models; an increment of 1 μmol/L was associated with an OR of 1.209 (95% CI: 1.051–1.390) for risk of CVD after adjustment for age and ethnicity and 1.186 (95% CI: 1.021–1.378) after multivariable adjustment.

Fig. 3 shows the ROC curves for risk of incident CVD from multivariable logistic regression models with and without TMAO. With CVD as the end points, the first model incorporating SBP, BMI, antihypertensive medications, current smoking, current drinking, diabetes mellitus, TC, TG, HDL-C, and eGFR had an AUC of 0.664 (95% CI: 0.583–0.746), a change in −2 log likelihood of 224.408. The second model was supplemented by the TMAO and led to a significant increase ($p < 0.001$) in the fit of the model [change in −2 log likelihood of 13.167 with 1 *df*, AUC was 0.732 (95% CI: 0.658–0.806)].

4. Discussion

The association between serum TMAO levels and future risk of CVD has not been well-elucidated in the community-based general population [20–22]. The present study reveals, for the first time, that serum TMAO levels are positively associated with future risk of incident CVD in a prospective cohort sample from community-based rural Chinese adults. There is a dose-dependent relationship between serum TMAO levels and future risk of CVD. Furthermore, TMAO addition significantly increased the predictive ability of the model for incident CVD compared with a model including conventional risk factors alone ($p < 0.05$). These studies thus add a concrete body of data revealing that the gut microbiota-dependent metabolite, TMAO, may serve as a clinically useful and potentially modifiable prognostic marker for incident CVD beyond currently used risk factors and laboratory testing.

In 2011, Wang et al. [6] demonstrated that dietary supplementation of mice with TMAO promoted upregulation of multiple macrophage scavenger receptors linked to atherosclerosis, and supplementation with TMAO promoted atherosclerosis. Next, the association between TMAO and future risk of CVD was widely studied in different cross-sectional and prospective clinical settings [3,5,9,11]. In patients with community-acquired pneumonia, TMAO levels were strongly associated with 6-year all-cause mortality for patients without CAD (adjusted HR, 1.9; 95% CI: 1.2–3.1) [9]. Haghikia et al. [23] indicated that there is a graded relation between TMAO levels and the risk of subsequent cardiovascular events including myocardial infarction, recurrent stroke, and cardiovascular death in patients with recent prior ischemic stroke (fourth quartile versus first quartile; HR, 5.0; 95% CI, 1.7–14.8; $p < 0.01$). However, the positive association between TMAO levels and incident CVD was not confirmed in recent studies [24,25]. In patients receiving hemodialysis, higher serum TMAO levels were not significantly associated with all-cause death (0.84, 95% CI 0.65–1.09)

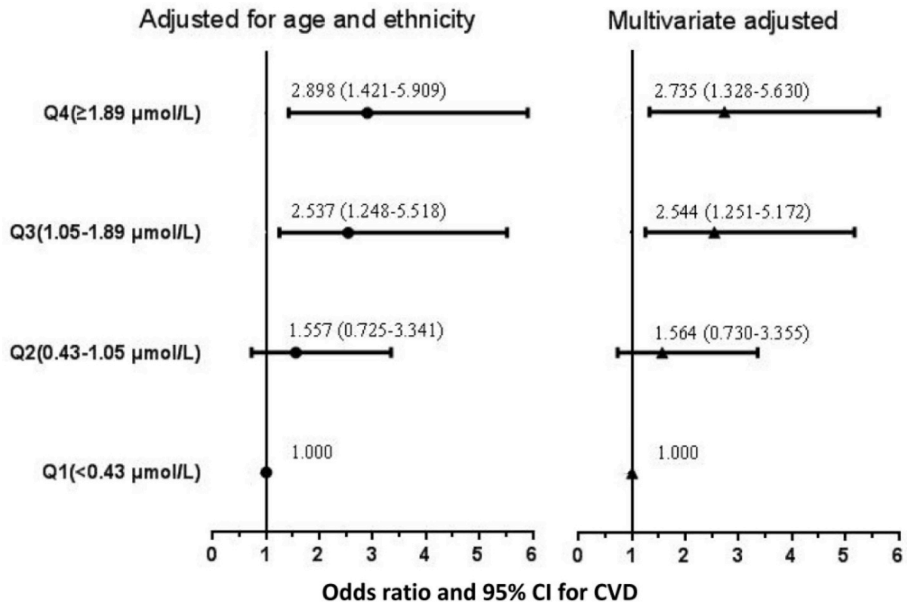

Fig. 2. Adjusted odds ratio and 95% confidence interval for incident CVD risk as a function of baseline trimethylamine N-oxide levels in community-based rural Chinese adults.
Multivariable adjusted for a propensity score (systolic blood pressure, body mass index, antihypertensive medications, current smoking, current drinking, diabetes mellitus, total cholesterol, triglyceride, high-density lipoprotein cholesterol, and estimated glomerular filtration rate).

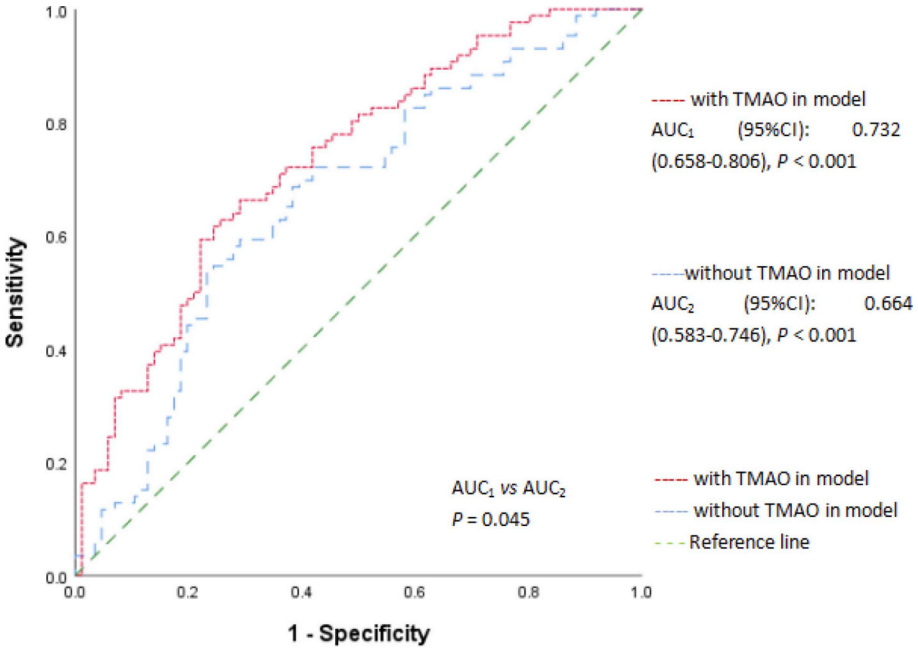

Fig. 3. Receiving operating characteristics curves for incident CVD from multivariable logistic regression models with and without TMAO.
AUC with 95% CI is noted under each curve; probability value is to test diagnostic accuracy against the null hypothesis. Comparison between the AUCs is noted in the bottom panel.

or time to cardiovascular hospitalization or cardiovascular death (0.88, 95% CI 0.57–1.35) [24]. It is noteworthy that the relatively smaller sample size and lower event rates, comparable alternative positive studies, may partly explain the difference.

Recently, several meta-analyses described and validated the dose-dependent relationship between TMAO and incident risks, though these analyses are not community based cohorts [20–22]. Moreover, Schiattarella et al. [21] reported TMAO clinical studies stratified by geographical region, including Asia, and reported that TMAO levels track with incident CVD and mortality risks independently of thr geographic region. However, the studies reviewed in these meta-analyses are not community-based primary prevention subjects, as is reported in the present study. In contrast to these previous studies, we firstly confirmed a dose-independent association between TMAO and increased risk of incident CVD in a community-based general population. An increment of 1 μmol/L was associated with an OR of 1.186 (95% CI: 1.021–1.378) after multivariable adjustment. In addition, in the present study, AUC analysis revealed that the addition of TMAO into a model containing conventional risk factors and other potential confounders resulted in a significant improvement in its predictive ability of future risk of CVD in the general population ($AUC_{\text{with TMAO}}$ *vs.* $AUC_{\text{without TMAO}}$: 0.732 *vs.* 0.663, $p = 0.045$). Our study implied that dynamic assessment of TMAO levels could help improve the prognostic risk of future CVD events in the general population. In addition, we have to point that the median TMAO concentration of the general rural Chinese population is 1.05 μmol/L (interquartile range, 0.43–1.89 μmol/L), which is relatively lower than that of patients with hypertension [26] and CVD [3,6,7]. Whether the lower level of TMAO is because of the effects of the rural Chinese diet or lifestyle in general remains to be further investigated.

TMAO increases the risk of CVD by mainly promoting the pathogenesis of atherosclerosis. Cholesterol is a conventional risk factor for CVD and animal studies showed TMAO affects the level of cholesterol and lipid protein by interfering with the reverse transfer of cholesterol from outside the liver to the liver [27]. In addition, results of animal experiments showed that Cyp7a1, one of the bile acid synthases, which might play a major role in cholesterol elimination, had a lower expression level in the liver of mice treated with TMAO when compared with the untreated group [28]. Meanwhile, TMAO can also cause the accumulation of cholesterol in cells by increasing the expression of the class B scavenger receptor CD36 and scavenger receptor A (SRA) [29]. Studies have also shown that TMAO can induce thrombosis by activating platelet and promoting platelet aggregation [1,30]. TMAO promotes the formation of arterial plaque by up-regulating cell adhesion molecules such as vascular cell adhesion molecule (VCAM-1), intercellular adhesion molecule (ICAM-1), and E-selectin [31]. The above studies provided a pathological mechanism for the association between TMAO and CVD.

The major strength of the present study is the prospective design with a relatively long follow-up period. Thus, all study participants were derived from a socioeconomically homogeneous study population, which can reduce the possibility of bias and confounding factors in our data. Furthermore, in addition to the 1:1 case-control matching criteria for sex, age and prevalent hypertension, a wide variety of other potential confounding cardiovascular factors were controlled for in the present analysis. Thus, the potential for residual confounding factors in our study is at a bare minimum. Finally, this is a nested case-control design from a population-based cohort study, thus giving us more supported evidence for the association between TMAO and incident CVD. There are several limitations for the present study. Firstly, our study cohort consisted of adults selected exclusively from rural areas of China, which may be limited in diversity. We encourage prudent validation in other general populations with greater diversity. Secondly, we did not have more information on dietary history to assess the impact of diet on TMAO levels, which may help us explain the relatively lower TMAO level in these rural residents. Thirdly, the present sample size (86 in each group) in our study seems small, but we will have a > 95% statistical power to detect a 0.6 μmol/L or more difference with at a significance level of 0.05 using a 2-tailed test. In addition, we have to point that there are no enough incident cases for us to exclusively analyze the association between TMAO levels and risk of incident stroke or CHD. Further studies focusing on TMAO and stroke or CHD are encouraged to clarify this issue. Finally, we only quantified baseline TMAO levels one time, and did not test whether dynamic TMAO level is related to CVD.

In conclusion, we provided a direct evidence for the association

between higher TMAO levels and incident CVD in community-based general population, and revealed that the association remains even after adjustment for other potential CVD risk factors. This is an important supplementation evidence for the association between TMAO and incident CVD. Furthermore, TMAO can improve the predictive power for the incident CVD beyond the conventional CVD risk factors. These findings not only broaden the potential clinical utility of TMAO as an independent prognostic marker for CVD in the general population, but also suggest that increased TMAO concentration in the blood is associated with excessive consumption of red meat in the diet [6,24], a potential novel preventive target in the management of low-risk CVD adults in the general population.

Conflicts of interest

The authors declared they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Funding

This research was partly supported by funds from National Nature Science Foundation of China (No. 81773510), Nature Science Foundation of Liaoning Province (No. 20170541048), National Key R& D Program of China (Grant #2017YFC1307600), and Science and Technology Program of Shenyang, China (Grant #17-230-9-06).

CRediT authorship contribution statement

Liqiang Zheng: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Jia Zheng:** Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Yanxia Xie:** Data curation, Investigation. **Zhao Li:** Investigation. **Xiaofan Guo:** Investigation. **Guozhe Sun:** Investigation. **Zhaoqing Sun:** Investigation. **Fuguo Xing:** Conceptualization, Methodology, Writing – review & editing. **Yingxian Sun:** Conceptualization, Funding acquisition, Writing – review & editing.

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