ORIGINAL RESEARCH

Gut Microbiota-Dependent Trimethylamine N-oxide and Cardiovascular Outcomes in Patients With Prior Myocardial Infarction: A Nested Case Control Study From the PEGASUS-TIMI 54 Trial

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BACKGROUND: Trimethylamine N-oxide (TMAO) may have prothrombotic properties. We examined the association of TMAO quartiles with major adverse cardiovascular events (MACE) and the effect of TMAO on the efficacy of ticagrelor.

METHODS AND RESULTS: PEGASUS-TIMI 54 (Prevention of Cardiovascular Events in Patients With Prior Heart Attack Using Ticagrelor Compared to Placebo on a Background of Aspirin - Thrombolysis in Myocardial Infarction 54) randomized patients with prior myocardial infarction to ticagrelor or placebo (median follow-up 33 months). Baseline plasma concentrations of TMAO were measured in a nested case-control study of 597 cases with cardiovascular death, myocardial infarction, or stroke (MACE) and 1206 controls matched for age, sex, and estimated glomerular filtration rate [eGFR]. Odds ratios (OR) were used for the association between TMAO quartiles and MACE, adjusting for baseline clinical characteristics (age, sex, eGFR, region, body mass index, hypertension, hypercholesterolemia, diabetes mellitus, smoking, peripheral artery disease, index event, aspirin dosage and treatment arm), and cardiovascular biomarkers (hs-TnT [high-sensitivity troponin T], hs-CRP [high-sensitivity C-reactive protein], NT-proBNP [N-terminal-pro-B-type natriuretic peptide]). Higher TMAO quartiles were associated with risk of MACE (OR for quartile 4 versus quartile 1, 1.43, 95% CI, 1.06–1.93, P trend=0.015). The association was driven by cardiovascular death (OR 2.25, 95% CI, 1.28–3.96, P trend=0.003) and stroke (OR 2.68, 95% CI, 1.39–5.17, P trend<0.001). After adjustment for clinical factors, the association persisted for cardiovascular death (OR$_{adj}$ 1.89, 95% CI, 1.03–3.45, P trend=0.027) and stroke (OR$_{adj}$ 2.01, 95% CI, 1.01–4.01, P trend=0.022), but was slightly attenuated after adjustment for cardiovascular biomarkers (cardiovascular death: OR$_{adj}$ 1.74, 95% CI, 0.88–3.45, P trend=0.079; and stroke: OR$_{adj}$ 1.82, 95% CI, 0.88–3.78, P trend=0.056). The reduction in MACE with ticagrelor was consistent across TMAO quartiles (P interaction=0.92).

CONCLUSIONS: Among patients with prior myocardial infarction, higher TMAO levels were associated with cardiovascular death and stroke but not with recurrent myocardial infarction. The efficacy of ticagrelor was consistent regardless of TMAO levels.

REGISTRATION: URL: https://www.clinicaltrials.gov; Unique identifiers: PEGASUS-TIMI 54, NCT01225562.

Key Words: antiplatelet therapy ■ cardiovascular death ■ gut microbiota ■ myocardial infarction ■ stroke ■ ticagrelor ■ trimethylamine N-oxide

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CLINICAL PERSPECTIVE

What Is New?

- In patients with prior myocardial infraction, we observed an association between higher trimethylamine N-oxide levels and incident cardiovascular death or stroke events.
- The associations remained significant after adjustment for clinical factors but were slightly attenuated with further adjustment for established biomarkers.

What Are the Clinical Implications?

- Reconciling previous and current findings, trimethylamine N-oxide levels offer insight for risk assessment but, at least among patients with prior myocardial infarction, do not offer additional information for tailoring more intensive antiplatelet therapy.

Nonstandard Abbreviations and Acronyms

ACS	acute coronary syndromes
CVD	cardiovascular disease
eGFR	estimated glomerular filtration rate
hs-CRP	high-sensitivity C-reactive protein
hs-TnT	high-sensitivity troponin T
IQR	interquartile ranges
MACE	major adverse cardiovascular events
MI	myocardial infarction
NT-proBNP	N-terminal-pro-B-type natriuretic peptide hormone
OR	odds ratio
PEGASUS-TIMI 54 trial	Prevention of Cardiovascular Events in Patients With Prior Heart Attack Using Ticagrelor Compared to Placebo on a Background of Aspirin-Thrombolysis in Myocardial Infarction 54
SD	standard deviation
TMAO	trimethylamine N-oxide

Intestinal microbiota have been associated with several pathways (eg, inflammation, obesity, and diabetes mellitus) that affect cardiovascular health.[1,2]

Trimethylamine N-oxide (TMAO) is a circulating metabolite produced by gut microbiota involving nutrient precursors abundant in the western diet (red meat, betaine, choline, carnitine, trimethyllysine),[3–5] and has been associated with incident cardiovascular risk in stable cohorts and patients with acute coronary syndromes.[6–9] TMAO may have prothrombotic properties that are partially reversible with the use of aspirin.[10] In contrast to the setting of an acute coronary syndrome,[11] the prognostic value of TMAO in stable patients with prior myocardial infarction (MI) has not been extensively explored, nor has an interaction with long-term use of more potent antiplatelet therapy. Currently, there is an unmet medical need to identify patients at higher ischemic risk who might derive greater benefit from more intensive preventive strategies after MI.[12,13] This prognostication is especially important to individualize long-term therapies given the variation of residual risk in patients with known cardiovascular disease (CVD).[14]

In the PEGASUS-TIMI 54 trial (Prevention of Cardiovascular Events in Patients With Prior Heart Attack Using Ticagrelor Compared to Placebo on a Background of Aspirin-Thrombolysis in Myocardial Infarction 54), patients with a history of MI during the preceding 1 to 3 years before randomization were assigned to ticagrelor or placebo.[15] Our primary aim was to determine if baseline plasma TMAO levels were independently associated with major adverse cardiovascular events (MACE) in stable patients with prior MI. Our secondary aim was to determine if plasma TMAO levels can identify patients who have a greater risk reduction in MACE with prolonged use of ticagrelor after an MI.

METHODS

We encourage parties interested in collaboration and data sharing to contact the corresponding author directly for further discussions.

Study Population

The PEGASUS-TIMI 54 trial was a randomized double-blind, placebo-controlled trial that tested the cardiovascular benefit of prolonged dual antiplatelet therapy with the addition of ticagrelor (a P2Y$_{12}$ receptor antagonist) to low-dose aspirin among stable patients with a history of MI.[16] The trial tested two doses of ticagrelor, 90 mg twice daily, a well-established dosage regimen in patients with acute coronary syndromes, and 60 mg twice daily versus placebo in a randomly assigned ratio 1:1:1.[16] All patients were on a background therapy of aspirin 75 to 150 mg daily. The protocol, including the biomarker substudy, was approved by the relevant ethics committee at each participating site.

Eligible patients were defined by a history of spontaneous MI 1 to 3 years before the enrollment, were at least 50 years of age, and had one of the following additional high-risk features: age of 65 years or older, diabetes mellitus requiring medication, a second prior spontaneous MI, multivessel coronary artery disease, or chronic renal disease, defined as an estimated glomerular filtration rate (eGFR) <60 mL per minute per 1.73 m^2 body surface area.[15] Briefly, patients were ineligible if they required the use of P2Y$_{12}$ receptor antagonist or anticoagulant therapy during the study period; had a history of bleeding disorder, ischemic stroke, or intracranial bleeding; or had gastrointestinal bleeding within the previous 6 months or a major surgery within the previous 30 days.[15] The study protocol was approved by local institutional review committees and written informed consent was obtained from all participants. Among a total of 21 162 patients who underwent randomization from October 2010 through May 2013, a biomarker substudy was conducted in 8635 participants.[15]

Study Design

We conducted a nested case-control study evaluating the association between TMAO levels and risk of MACE among participants in the PEGASUS-TIMI 54 trial who had a collection of blood sample at the baseline regardless of the randomization arm.[15] Within this study, eligible cases were men or women who experienced the prespecified primary composite end point of cardiovascular death, MI, or stroke. Eligible controls were men or women who remained free from cardiovascular death, MI, or stroke through the end of follow-up. Eligible controls were matched for age (range ±3 years), sex, and eGFR (range ±5 mL/min per 1.73 m^2) with a prespecified ratio of 2 controls for each case. The definitions for cardiovascular death, MI, and stroke have been previously described.[15,16] We initially defined 603 cases and 1206 matched controls (ratio 2:1), but the final sample with measurable TMAO concentrations yielded 597 cases and 1206 controls (see Table S1 for baseline characteristics in the entire PEGASUS-TIMI 54 trial versus the TMAO nested case-control cohort).

Biomarker Analysis

TMAO concentrations in plasma were quantified using stable isotope dilution high-performance liquid chromatography with online tandem electrospray ionization mass spectrometry (LC/MS/MS) using d9-(trimethyl)-labeled internal standard as described previously using a Shimadzu Nexera Ultra High Performance Liquid Chromatograph (UHPLC) system interfaced with Shimadzu 8050 triple quadrupole mass spectrometer.[7,11,17] Exploratory measurements were also done for plasma choline and betaine as potential precursors of TMAO.[4,8,18]

Statistical Analysis

For the baseline characteristics according to TMAO quartiles, continuous variables are presented as medians with interquartile ranges (IQR) and were compared with Kruskal–Wallis test; categorical variables are presented as absolute counts and percentages and were compared with chi-square test. We compared values of TMAO between cases and controls with P values calculated using Wilcoxon Rank Sum tests. We evaluated the correlation between TMAO concentrations and the following continuous variables: age, body mass index (BMI), eGFR, hs-CRP (high-sensitivity C-reactive protein), hs-TnT (high-sensitivity troponin T), and NT-proBNP (N-terminal pro-B-type natriuretic peptide hormone). For our primary aim, we performed conditional logistic regression to explore the association between the exposure (TMAO quartiles) and the binary outcome in 597 cases and 1194 controls matching for age, sex, and eGFR regardless of the randomization arm. We categorized TMAO in quartiles and compared the odds ratio (OR) for the highest quartile versus the lowest quartile and assessed the P value for trend across categories.[11] The trend P value was calculated by testing the coefficient of an ordinal TMAO variable from the logistic regression model. We also used TMAO concentrations as a continuous variable (standardized by subtracting the mean and dividing by the standard deviation) and presented the estimates per increase of one standard deviation (1 SD). To evaluate potential confounding factors, we examined the differences of baseline characteristics across TMAO quartiles. We adjusted our conditional logistic regression model (primary outcome versus not) for baseline CVD clinical risk factors (age, sex, eGFR, diabetes mellitus, BMI, hypertension, hypercholesterolemia, smoking status, peripheral artery disease), region of enrollment, severity of the qualifying index events (type of MI), aspirin dosage, and treatment arm and made further adjustment for the baseline traditional prognostic cardiovascular biomarkers, hs-CRP, hs-TnT, and NT-proBNP. These biomarkers have been previously shown to predict future MACE in patients with coronary artery disease.[13,19,20] Because renal function is a major confounding factor,[21] we added a subgroup analysis comparing baseline eGFR ≥60 mL/min per 1.73 m^2 versus eGFR <60 mL/min per 1.73 m^2. We repeated this same process for each individual end point of cardiovascular death, MI, and stroke using an unmatched logistic regression model, including this time the total population of 597 cases and 1206 controls. For our secondary aim, we

Table 1. Baseline Characteristics Stratified by TMAO Quartile (N=1803)

Variable	1st Quartile N=452 (0.12–3.24 µmol/L)	2nd Quartile N=450 (3.25–4.75 µmol/L)	3rd Quartile N=450 (4.76–7.20 µmol/L)	4th Quartile N=451 (7.22–157.37 µmol/L)	P Value
Matched variables					
Age, median (IQR)	64 (58, 70)	66 (59, 73)	69 (62, 75)	69 (62, 76)	<0.001
Female, n (%)	95 (21.0)	100 (22.2)	115 (25.6)	152 (33.7)	<0.001
eGFR, median (IQR)	75.9 (65.8, 88.0)	71.7 (59.4, 81.8)	64.4 (53.5, 75.6)	55.2 (40.6, 70.6)	<0.001
Other variables					
BMI, median (IQR)	28.3 (25.5, 31.1)	28.2 (25.3, 31.2)	28.4 (25.6, 31.6)	29.9 (26.6, 33.7)	<0.001
White race, n (%)	430 (95.1)	434 (96.4)	440 (97.8)	432 (95.8)	0.185
Region					0.049
Eastern Europe, n (%)	160 (35.4)	151 (33.6)	139 (30.9)	134 (29.7)	
North America, n (%)	143 (31.6)	151 (33.6)	171 (38.0)	189 (41.9)	
Western Europe, n (%)	149 (33.0)	148 (32.9)	140 (31.1)	128 (28.4)	
Smoking status, n (%)					0.002
Never	114 (25.2)	141 (31.3)	156 (34.7)	153 (33.9)	
Former	226 (50.0)	216 (48.0)	212 (47.2)	231 (51.2)	
Current	112 (24.8)	93 (20.7)	81 (18.0)	67 (14.9)	
Hypertension, n (%)	339 (75.0)	357 (79.3)	362 (80.4)	388 (86.0)	0.001
Dyslipidemia, n (%)	356 (78.8)	377 (83.8)	375 (83.3)	372 (82.5)	0.19
Diabetes mellitus, n (%)	100 (22.1)	133 (29.6)	155 (34.4)	224 (49.7)	<0.001
Multivessel CAD, n (%)	306 (67.7)	283 (62.9)	273 (60.7)	292 (64.7)	0.157
>1 prior MI, n (%)	93 (20.6)	81 (18.0)	92 (20.4)	100 (22.2)	0.48
History of PAD, n (%)	33 (7.3)	42 (9.3)	38 (8.4)	57 (12.6)	0.039
Qualifying event					
STEMI, n (%)	229 (50.8)	220 (48.9)	225 (50.0)	172 (38.2)	<0.001
NSTEMI, n (%)	207 (45.9)	220 (48.9)	215 (47.8)	257 (57.1)	0.004
Years from event, median (IQR)	1.7 (1.2, 2.3)	1.6 (1.2, 2.2)	1.7 (1.3, 2.3)	1.6 (1.2, 2.2)	0.132
Medications					
Aspirin, n (%)	450 (99.6)	450 (100)	449 (99.8)	451 (100)	0.30
Aspirin dosage, median (IQR)	81 (80, 100)	81 (80, 100)	81 (80, 100)	81 (80, 100)	0.008
Aspirin ≥100 mg, n (%)	144 (31.9)	138 (30.8)	171 (38.3)	153 (34.0)	0.090
Statin, n (%)	429 (94.9)	424 (94.2)	426 (94.7)	406 (90.0)	0.008
Beta-blocker, n (%)	385 (85.2)	383 (85.1)	384 (85.3)	395 (87.6)	0.67
ACEI or ARB, n (%)	363 (80.3)	359 (79.8)	358 (79.6)	344 (76.3)	0.44
Placebo, n (%)	159 (35.2)	163 (36.2)	152 (33.8)	140 (31.0)	0.26
Ticagrelor 60 mg bid, n (%)	158 (35.0)	130 (28.9)	153 (34.0)	151 (33.5)	
Ticagrelor 90 mg bid, n (%)	135 (29.9)	157 (34.9)	145 (32.2)	160 (35.5)	
Biomarker measurement					
hs-TnT (ng/L) (median, IQR)	6.9 (4.3, 11.1)	8.0 (5.3, 12.8)	9.3 (5.8, 15.6)	12.3 (7.3, 22.5)	<0.001
NT-proBNP (pg/mL) (median, IQR)	131.3 (69.3, 291.2)	163.3 (77.0, 352.3)	181.0 (81.1, 463.0)	276.9 (116.1, 619.1)	<0.001
hs-CRP (mg/dL) (median, IQR)	1.5 (0.7, 3.2)	1.6 (0.7, 3.5)	1.5 (0.8, 3.3)	2.0 (0.9, 4.2)	0.003

Continuous variables are presented as medians with interquartile ranges (IQR) and were compared with Kruskal–Wallis test. Categorical variables are presented as absolute counts and percentages and were compared with chi-square test. ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; hs-CRP, high-sensitivity C-reactive protein; hs-TnT, high-sensitivity troponin T; IQR, interquartile range; MI, myocardial infarction; NSTEMI, non–ST-segment–elevation myocardial infarction; NT-proBNP, N-terminal-proB-type natriuretic peptide; PAD, peripheral artery disease; STEMI, ST-segment–elevation myocardial infarction; and TMAO, trimethylamine N-oxide.

investigated whether the effect of ticagrelor on the primary end point varied by quartiles of TMAO. The interaction P value associated with this test is computed using a likelihood ratio test that compares a full model with the main effect terms for TMAO quartiles, treatment, and an interaction effect of TMAO by treatment, with a reduce model that had main effect terms for TMAO and treatment only. The same

analysis plans were applied for choline and betaine. A *P*<0.05 was considered significant for all analyses.

RESULTS

Baseline Characteristics

Baseline characteristics of the patients, overall and stratified by the quartiles of plasma TMAO quartiles, are shown in Table 1. Patients with higher TMAO quartiles compared to those with lower TMAO quartiles were more likely to be older, female, and had higher BMI, and more frequently had comorbidities including a history of hypertension, diabetes mellitus, impaired renal function, and peripheral artery disease but were less likely to be current smokers (Table 1). TMAO concentrations were moderately negatively correlated with eGFR (r=−0.41, P<0.001) and weakly positively correlated with hs-TNT (r=0.29, P<0.001) and NT-proBNP (r=0.20, P<0.001) (Table S2).

The Association of TMAO Quartiles With Major Adverse Cardiovascular Events

The TMAO concentrations were borderline statistically significantly different between MACE cases and controls, with significant differences between cardiovascular death cases and controls, as well as between stroke cases and controls, whereas no significant differences were found for recurrent MI cases and controls (Figure 1). When analyzed as quartiles, as compared with patients in the first quartile (Q1) of TMAO, the risk for the primary end point of cardiovascular death, MI, or stroke was significantly higher in patients in the highest quartile (Q4) of TMAO using conditional logistic regression (OR for Q4 versus Q1 1.43, 95% CI, 1.06–1.93, P for trend across quartiles=0.015, Table 2). The association was primarily driven by cardiovascular death (Q4 OR 2.25, 95% CI, 1.28–3.96, P trend=0.003) and stroke (Q4 OR 2.68, 95% CI, 1.39–5.17, P trend<0.001). The association with MACE was attenuated after adjustment for clinical factors including age, sex, eGFR, hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, BMI, aspirin dosage, and treatment arm (adjusted OR 1.27, 95% 0.92–1.75, P for trend=0.10) and further adjustment for other biomarkers (hs-TnT, NT-proBNP and hs-CRP) (adjusted OR 1.22, 95% CI, 0.86–1.74, P for trend=0.20). After adjustment for clinical factors, the association remained significant for
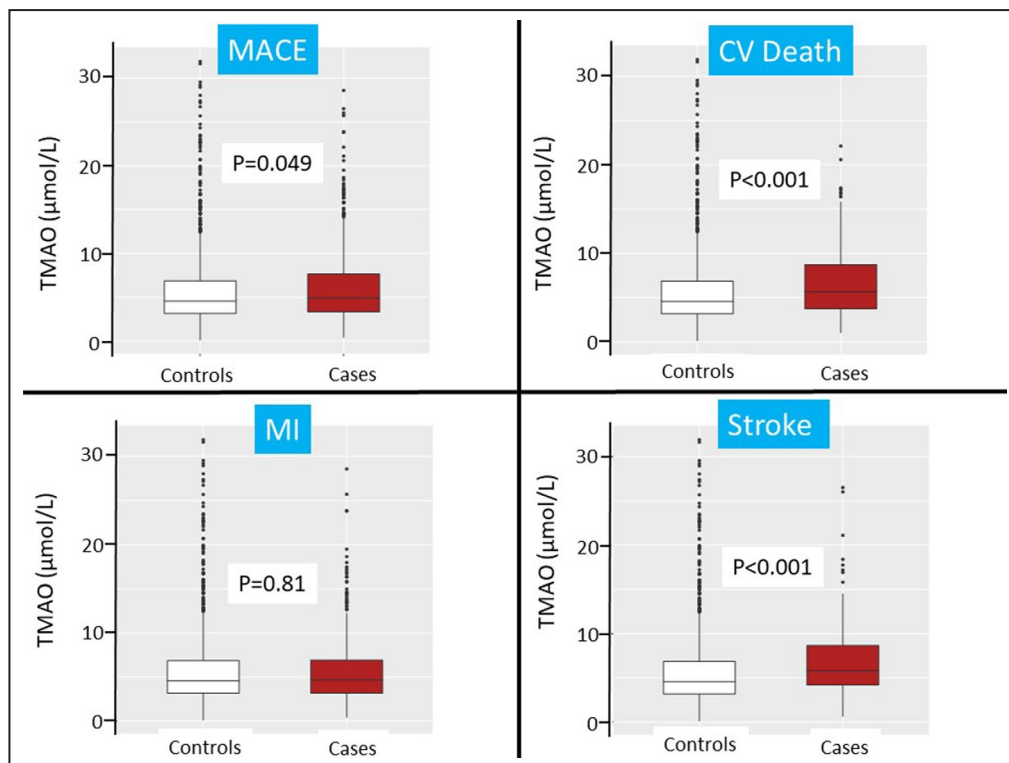

Figure 1. **TMAO concentrations comparing cases vs controls for MACE, cardiovascular death, recurrent MI, and stroke events.**

The *P* values were obtained using Wilcoxon ranking test. Values beyond the 99% percentile are not graphed to avoid an excessive *y* axis. CV indicates cardiovascular; MACE, major adverse cardiovascular events; MI, myocardial infarction; and TMAO, trimethylamine N-oxide.

Table 2. Risk of Cardiovascular Events by TMAO Quartiles

TMAO Quartiles Definition	1st Quartile (N=452) (Range 0.12–3.24 µmol/L)	2nd Quartile (N=450) (Range 3.25–4.75 µmol/L)	3rd Quartile (N=450) (Range 4.76–7.20 µmol/L)	4th Quartile (N=451) (Range 7.22–157.37 µmol/L)	P for Trend
Odds ratios for primary end point (cardiovascular death, MI, and stroke)					
Number of cases (%)	138 (30.5)	141 (31.3)	152 (33.8)	166 (36.8)	
Matched*	Ref	1.03 (0.78–1.37, P=0.81)	1.19 (0.89–1.59, P=0.24)	1.43 (1.06–1.93, P=0.02)	0.015
Odds ratios for cardiovascular death					
Number of cases (%)	23 (5.1)	30 (6.7)	38 (8.4)	48 (10.6)	
Adjustment for matching variable†	Ref	1.32 (0.75–2.34, P=0.34)	1.68 (0.97–2.93, P=0.067)	2.25 (1.28–3.96, P=0.005)	0.003
Odds ratios for recurrent MI					
Number of cases (%)	108 (23.9)	101 (22.4)	101 (22.4)	102 (22.6)	
Adjustment for matching variable†	Ref	0.94 (0.68–1.30, P=0.70)	1.09 (0.79–1.51, P=0.60)	1.10 (0.79–1.55, P=0.57)	0.42
Odds ratios for stroke					
Number of cases (%)	15 (3.3)	19 (4.2)	31 (6.9)	41 (9.1)	
Adjustment for matching variable†	Ref	1.25 (0.62–2.52, P=0.52)	2.05 (1.07–3.93, P=0.031)	2.68 (1.39–5.17, P=0.003)	<0.001

The trend P value was calculated by testing the coefficient of an ordinal TMAO variable from the logistic regression model. eGFR indicates estimated glomerular filtration rate; MI, myocardial infarction; and TMAO, trimethylamine N-oxide.

*Conditional (matched) logistic regression was used for the primary end point with matching variables (age, sex, eGFR).

†Unconditional (unmatched) logistic regression was used for each individual component cardiovascular death, MI, and stroke events adjusting for matching variables (age, sex, eGFR).

cardiovascular death (adjusted OR 1.89, 95% CI, 1.03–3.45, P trend=0.027) and stroke (adjusted OR 2.01, 95% CI<1.01–4.01, P trend=0.022, Figure 2) but was slightly attenuated after further adjustment for biomarkers (adjusted OR fourth versus lowest quartiles for cardiovascular death 1.74, 95% CI, 0.88–3.45, P for trend=0.079; adjusted OR for stroke 1.82, 95% CI, 0.88–3.78, P for trend=0.056). We did not find an association between TMAO quartiles and risk of recurrent MI events, even when modeled as a continuous variable (Table S3). We did not find a significant interaction by baseline renal function, although the associations tended to be stronger for those with eGFR ≥60 mL/min per 1.73 m² compared with those who had an eGFR <60 mL/min per 1.73 m² after adjustment for clinical variables (Table S4). The increased risk association with TMAO quartiles was consistent regardless the randomization arm (pooled ticagrelor versus placebo, Table S5). Neither plasma choline nor betaine was significantly associated with MACE after adjustment for clinical factors (Table S6).

Ticagrelor Efficacy in the Relation to TMAO Quartiles

The efficacy signal of ticagrelor was similar across plasma TMAO quartiles (Q1 to Q4) (adjusted P interaction=0.92, Figure 3), and the effect of ticagrelor was generally consistent for each individual end point (cardiovascular death, MI, or stroke) regardless of baseline TMAO quartiles (Figure S1).

DISCUSSION

In this analysis from the PEGASUS-TIMI 54 trial, patients with prior MI who had higher plasma TMAO were more likely to experience subsequent cardiovascular death or stroke. The associations between TMAO and cardiovascular death or stroke remained significant after adjustment for clinical factors but were slightly attenuated with further adjustment for established biomarkers, with the ORs remaining large but the lower bound of the 95% CIs crossing 1. The associations tended to be stronger for patients with normal renal function compared with those who had an eGFR <60 mL/min per 1.73 m². Nonsignificant association was noted for TMAO quartiles and recurrent MI events. The efficacy of ticagrelor on the primary efficacy end point was consistent regardless of baseline TMAO quartile. We did not observe any statistically significant association between plasma choline or betaine and risks of MACE.[18]

Few studies reported an association between TMAO levels and the mechanisms of stroke events. Among 1244 hypertensive patients in China, a nested-case control study suggested an association between higher TMAO levels and first stroke event, especially with respect to hemorrhagic stroke.[22] In another cohort study of 268 patients who underwent carotid stenting, increased TMAO levels were associated with an increased risk of new ischemic brain lesion on cerebral imaging after stenting.[23] To explore the potential etiology of stroke, two large
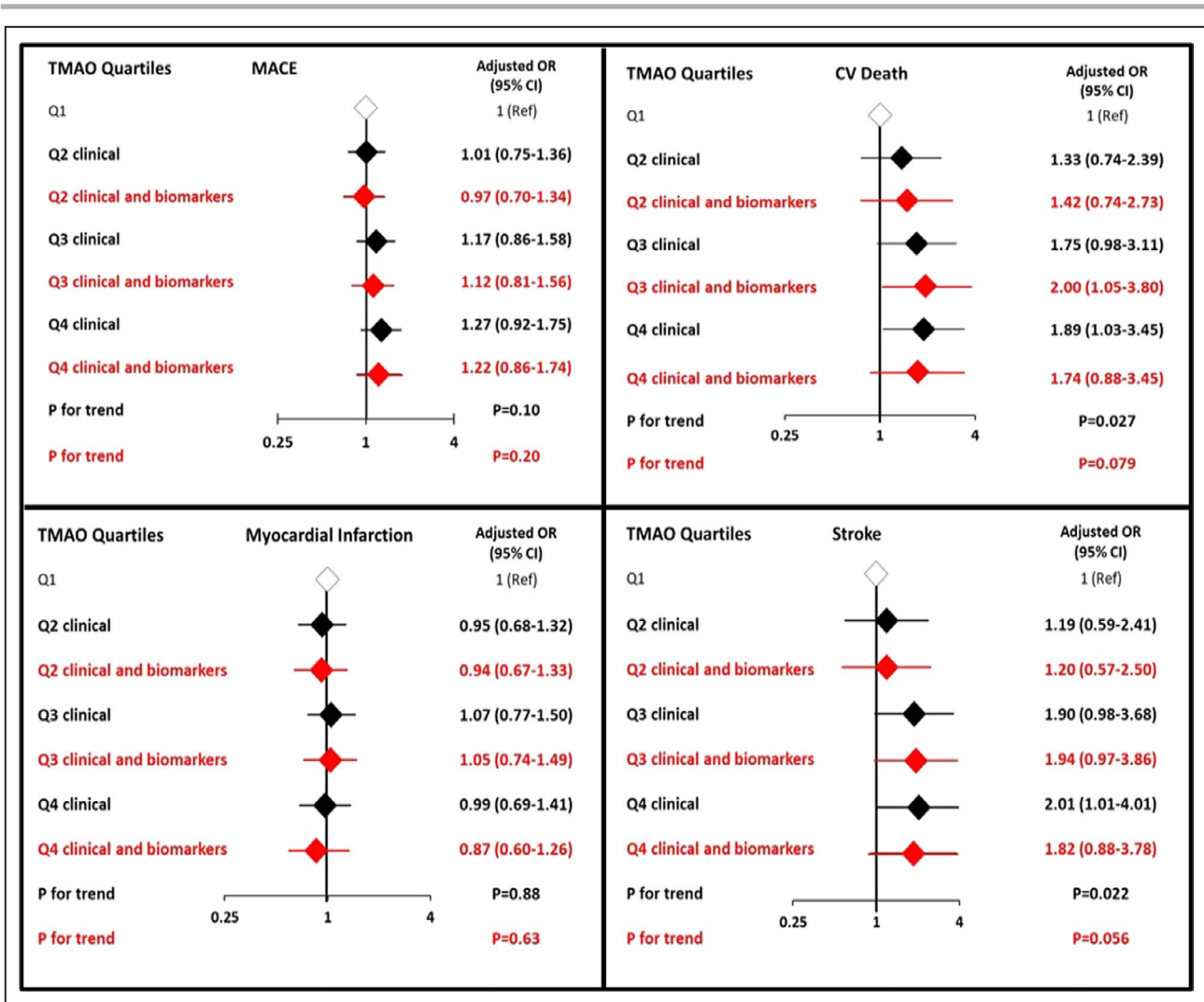
Figure 2. Risks of cardiovascular death, myocardial infarction, and stroke by TMAO quartiles adjusted for clinical variables (black) and biomarkers (red).

MACE was defined as a composite of cardiovascular death, myocardial infarction, and stroke. In the clinical variable model (black), the ORs were adjusted for age, sex, estimated glomerular filtration rate, hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, BMI, aspirin dosage, and treatment arm. In the clinical and biomarkers mode (red), further adjustment was done for hs-CRP (high-sensitive C-reactive protein), hs-TnT (high-sensitivity troponin T); and NT-proBNP (N-terminal-pro hormone BNP). The trend *P* value was calculated by testing the coefficient of an ordinal TMAO variable from the logistic regression model. MACE indicates major adverse cardiovascular events; OR, odds ratio; and TMAO, trimethylamine N-oxide.

observational studies, together including more than 6900 elderly participants, showed an increased risk of incident atrial fibrillation among those with higher TMAO levels even after adjustment for traditional confounding clinical factors.[24] The available findings rather support a possible risk modulation of stroke with TMAO levels, but the evidence is low and more studies are needed to clarify the mechanisms.

As expected, patients with higher TMAO concentrations were more likely to have renal dysfunction. There is existing evidence that TMAO may be involved in the process of chronic kidney disease and that renal dysfunction may lead to higher circulating plasma concentrations of TMAO.[6] In subgroup analyses by baseline eGFR function, we found that the association of TMAO tended to be greater in those with normal renal function.

The prothrombotic properties described with TMAO in previous studies and the possible response to aspirin led us to investigate specifically this microbiota marker in the PEGASUS-TIMI 54 trial.[10,25] Our data show that the clinical efficacy of ticagrelor is preserved regardless of TMAO levels. Reconciling previous and current findings, TMAO levels offer insight for risk assessment but, at least among patients with prior MI, do not offer additional information for tailoring more intensive antiplatelet therapy. The absence of an association with MI in our analysis does not support the role of TMAO as a pure
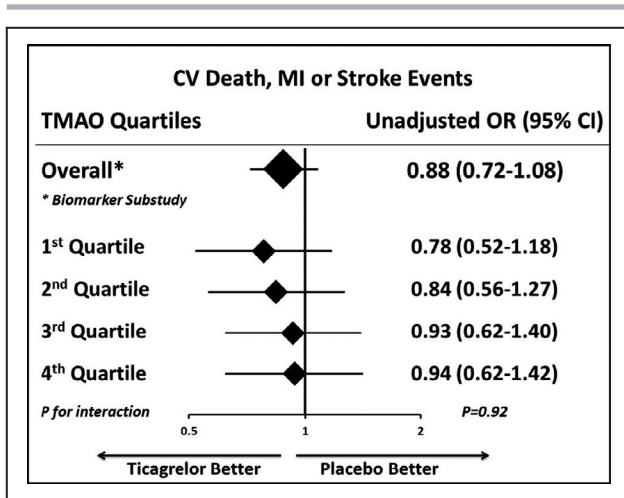
Figure 3. **Treatment effect of ticagrelor vs placebo on the primary end point of cardiovascular death, MI, and stroke by TMAO quartiles.**
CV indicates cardiovascular disease; MI, myocardial infarction; OR, odds ratio; and TMAO, trimethylamine N-oxide.

specific marker of atherothrombotic risks. Some of the prior associations between TMAO and MI might have reflected TMAO's association with multiple cardiovascular comorbidities including age, hypertension, dyslipidemia, diabetes mellitus, and renal dysfunction.

Limitations

Our study findings are applicable just to a secondary prevention population with previous MI and treated with aspirin. However, a previous cohort of patients undergoing elective coronary angiography reported a value of 6.2 µmol/L corresponding approximately to the 75th percentile,[1] which is not dissimilar to our 75th percentile (7.22 µmol/L). Finally, we did not collect data on nutritional habits or use of antibiotics prior to blood sampling (as a potential unmeasured confounding factor associated with TMAO levels).

CONCLUSIONS

Among patients with prior MI, higher systemic TMAO levels were associated with higher risk of cardiovascular death and stroke after adjusting for clinical factors but not with recurrent MI. The efficacy of ticagrelor on cardiovascular outcomes was consistent regardless of baseline TMAO levels.

ARTICLE INFORMATION

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Disclosures

Supplementary Materials

Tables S1–S6
Figure S1

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SUPPLEMENTAL MATERIAL

Table S1. Baseline characteristics of the full PEGASUS-TIMI 54 trial vs. the TMAO Nested Case-Control Cohort.

Variable	Entire Trial (N=21,162)	TMAO Nested Case-Control Cohort (N=1,803)
Matched variables		
Age (median, IQR)	65 (59, 71)	67.9 (59.5, 74)
Female (n, %)	5,050 (23.9)	462 (25.6)
eGFR (median, IQR)	72.4 (61.0, 84.0)	68.2 (55.0, 79.8)
Other variables		
BMI (median, IQR)	27.8 (25.2, 31.2)	28.7 (25.7, 32.0)
White race (n, %)	18,327 (86.6)	1,736 (96.3)
Region		
Eastern Europe (n, %)	6,290 (29.7)	584 (32.4)
North America (n, %)	3,907 (18.5)	654 (36.3)
Western Europe (n, %)	6,138 (29.0)	565 (31.3)
Asia/Pacific	2,369 (11.2)	0 (0)
South America	2,458 (11.6)	0 (0)
Current smoking (n, %)	3,536 (16.7)	353 (19.6)
Hypertension (n, %)	16,407 (77.5)	1,480 (80.2)
Dyslipidemia (n, %)	16,241 (76.8)	1,480 (82.1)
Diabetes mellitus (n, %)	6,806 (32.2)	612 (33.9)
Multivessel CAD (n, %)	12,558 (59.4)	1,154 (64.0)
> 1 Prior MI (n, %)	3,499 (16.5)	366 (20.3)
History of PAD (n, %)	1,143 (5.4)	170 (9.4)
Qualifying event		
STEMI (n, %)	11,329 (53.6)	846 (47.0)
NSTEMI (n, %)	8583 (40.6)	899 (49.9)

Years from event (median, IQR)	1.7 (1.2, 2.3)	1.7 (1.2, 2.3)
Medications		
Aspirin (n, %)	21,132 (99.9)	1800 (99.8)
Statin (n, %)	19,604 (92.6)	1,685 (93.5)
Beta-blocker (n, %)	17,486 (82.6)	1,547 (85.8)
ACEI or ARB (n, %)	17,030 (80.5)	1424 (79.0)

ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; BMI, body mass index; CAD, coronary artery disease; eGFR, estimated glomerular filtration rate; IQR, interquartile range; MI, myocardial infarction; NSTEMI, Non-ST-elevation myocardial infarction; PAD, peripheral artery disease; STEMI, ST-elevation myocardial infarction; TMAO, Trimethylamine N-oxide

Table S2. Spearman Correlation Between TMAO and Cardiovascular Markers.

	TMAO	**Betaine**	**Choline**
TMAO (µM)	1.00	-0.03 (P=0.29)	0.26 (P<0.001)
Betaine (µM)	-0.03 (P=0.29)	1.00	0.35 (P<0.001)
Choline (µM)	0.26 (P<0.001)	0.35 (P<0.001)	1.00
Age (years)	0.21 (P<0.001)	0.07 (P=0.004)	0.19 (P<0.001)
Weight (kgs)	0.05 (P=0.044)	-0.16 (P<0.001)	0.02 (P=0.47)
eGFR(ml/min/1.73m²)	-0.41 (P<0.001)	0.04 (P=0.11)	-0.30 (P<0.001)
BMI (mg/m²)	0.11 (P<0.001)	-0.19 (P<0.001)	0.07 (P=0.002)
Hs-TnT (ng/L)	0.29 (P<0.001)	0.04 (P=0.061)	0.25 (P<0.001)
NT-pro BNP (ng/mL)	0.20 (P<0.001)	0.12 (P<0.001)	0.18 (P<0.001)
Hs-CRP (mg/dL)	0.08 (P<0.001)	-0.06 (P=0.020)	0.10 (P<0.001)

BMI, body mass index; eGFR, estimates glomerular filtration; Hs-CRP, high sensitive C-reactive protein; Hs-TnT, high sensitive Troponin T; MI, myocardial infarction: NT-pro BNP, N-terminal pro B-type natriuretic peptide; TMAO, trimethylamine N-oxide. All biomarkers were log-transformed for this analysis.

Table S3. Risk of Cardiovascular Events by 1-SD Log-Transformed TMAO Increase.

Odds Ratios for Primary Endpoint (CV death, MI and Stroke) by 1-SD Log-Transformed TMAO Increase	
	OR (95% CI)
Matched *	1.09 (0.98-1.21, P=0.12)
Matched with clinical adjustment #	1.05 (0.93-1.18, P=0.43)
Matched with clinical and biomarker adjustment ‖	1.05 (0.92-1.19, P=0.47)
Odds Ratios for CV Death by 1-SD Log-Transformed TMAO Increase	
Matching variable adjustment ¶	1.22 (1.01-1.48, P=0.035)
Multivariate clinical adjustment §	1.13 (0.92-1.39, P=0.24)
Multivariate with clinical and biomarker adjustment †	1.07 (0.85-1.34, P=0.58)
Odds Ratios for MI by 1-SD Log-Transformed TMAO Increase	
Matching variable adjustment ¶	1.04 (0.92-1.18, P=0.51)
Multivariate clinical adjustment §	0.99 (0.87-1.12, P=0.86)
Multivariate with clinical and biomarker adjustment †	0.94 (0.83-1.08, P=0.38)
Odds Ratios for Stroke by 1-SD Log-Transformed TMAO Increase	
Matching variable adjustment ¶	1.25 (1.01-1.53, P=0.038)
Multivariate clinical adjustment §	1.11 (0.88-1.39, P=0.38)
Multivariate with clinical and biomarker adjustment †	1.10 (0.87-1.39, P=0.44)

* Conditional (matched) logistic regression was used for the primary endpoint with matching variables (age, sex, eGFR). # In addition to matching variables (age, sex and eGFR), further adjustment was performed for the following clinical variables: hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, body mass index, aspirin dosage and treatment arm. ‖ In addition to matching and clinical variables, further adjustment was done for log-transformed biomarkers (hs-TnT, NT-proBNP and hs-CRP). ¶ Unconditional (unmatched) logistic regression was used for each individual component CV death, MI and stroke events. § Multivariable adjustment (unmatched) was performed for the following clinical variables: age, sex, eGFR, hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, body mass index aspirin dosage and treatment arm. † In addition to multivariate clinical variables adjustment, further adjustment was done for log-transformed biomarkers (hs-TnT, NT-proBNP and hs-CRP). CV, Cardiovascular; eGFR, estimated glomerular filtration rate; MI, myocardial infarction: TMAO, Trimethylamine N-oxide.

Table S4. Risk of Cardiovascular Events by TMAO Quartiles and eGFR Status (< 60 vs. ≥ 60 ml/min/1.73m²).

	2nd Quartile	3rd Quartile	4th Quartile
Adjusted Odds Ratios for Primary Endpoint (CV death, MI and Stroke) by TMAO Quartiles *			
eGFR ≥ 60	1.77 (0.81-3.84)	2.03(0.96-4.28)	2.14 (1.04-4.40)
eGFR < 60	0.94 (0.68-1.30)	1.07 (0.77-1.50)	0.99 (0.68-1.46)
P for overall interaction = 0.29			
Adjusted Odds Ratios for CV Death by TMAO Quartiles ¶			
eGFR ≥ 60	3.22 (0.67-15.39)	1.83 (0.38-8.81)	3.17 (0.71-14.05)
eGFR < 60	0.99 (0.50-1.96)	1.95 (1.05-3.64)	1.31 (0.63-2.73)
P for overall interaction = 0.097			
Adjusted Odds Ratios for MI by TMAO Quartiles ¶			
eGFR ≥ 60	1.61 (0.66-3.96)	1.93 (0.83-4.52)	1.52 (0.66-3.48)
eGFR < 60	0.87 (0.61-1.25)	0.93 (0.64-1.36)	0.95 (0.63-1.46)
P for overall interaction = 0.44			
Adjusted Odds Ratios for Stroke by TMAO Quartiles ¶			
eGFR ≥ 60	0.97 (0.17-5.57)	2.39 (0.52-11.09)	2.23 (0.50-10.04)
eGFR < 60	1.32 (0.61-2.85)	1.76 (0.82-3.77)	2.22 (1.01-4.87)
P for overall interaction = 0.84			

CV, Cardiovascular disease; eGFR, estimated glomerular filtration rate; MI, myocardial infarction: TMAO, Trimethylamine N-oxide.

* Conditional (matched) logistic regression was used for the primary endpoint with matching variables (age, sex, eGFR). In addition to matching variables (age, sex and eGFR), further adjustment was performed for the following clinical variables: hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, body mass index, aspirin dosage and treatment arm.

¶Multivariable adjustment (unmatched) was performed for the following clinical variables: age, sex, eGFR, hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, body mass index, aspirin dosage and treatment arm.

Table S5. Risk of Cardiovascular Events by TMAO Quartiles and Antiplatelet Regimen.			
Odds Ratios for Primary Endpoint (CV death, MI and Stroke) by TMAO Quartiles			
	2nd Quartile	3rd Quartile	4th Quartile
Placebo	1.05 (0.47-2.37)	1.23 (0.49-3.09)	1.56 (0.60-4.02)
Pooled Ticagrelor	0.92 (0.59-1.44)	1.09 (0.71-1.68)	1.18 (0.75-1.87)
P for overall interaction = 0.952			
Odds Ratios for CV Death by TMAO Quartiles §			
Placebo	1.06 (0.41-2.72)	1.22(0.49-3.02)	1.31 (0.51-3.36)
Pooled Ticagrelor	1.55 (0.73-3.28)	2.17 (1.04-453)	2.34 (1.12-4.89)
P for overall interaction = 0.742			
Odds Ratios for MI by TMAO Quartiles §			
Placebo	1.06 (0.62-1.79)	0.83 (0.48-1.45)	0.99 (0.56-1.74)
Pooled Ticagrelor	0.88 (0.58-1.34)	1.23 (0.82-1.86)	0.99 (0.65-1.52)
P for overall interaction = 0.399			
Odds Ratios for Stroke by TMAO Quartiles §			
Placebo	1.12 (0.32-3.85)	2.36 (0.79-7.04)	2.28 (0.74-7.00)
Pooled Ticagrelor	1.24 (0.538-2.93)	1.65 (0.72--3.74)	1.91 (0.85-4.32)
P for overall interaction = 0.895			

In addition to matching variables (age, sex and eGFR), further adjustment was performed for the following clinical variables: hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, body mass index, aspirin dosage and treatment arm.
§ Multivariable adjustment (unmatched) was performed for the following clinical variables: age, sex, eGFR, hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, body mass index, aspirin dosage and treatment arm.
CV, Cardiovascular disease; MI, myocardial infarction: TMAO, Trimethylamine N-oxide.

Table S6. Risk of Cardiovascular Events by Choline and Betaine Quartiles.

Odds Ratios for Primary Endpoint (CV death, MI and Stroke) #				
	2nd Quartile	3rd Quartile	4th Quartile	P for trend
Choline	1.03 (0.77-1.39)	1.28 (0.95-1.72)	1.06 (0.78-1.44)	P=0.43
Betaine	0.78 (0.58-1.04)	0.75 (0.56-1.01)	0.81 (0.60-1.09)	P=0.16
Odds Ratios for CV Death §				P for trend
Choline	0.81 (0.47-1.39)	1.04 (0.61-1.76)	0.64 (0.37-1.13)	P=0.24
Betaine	0.78 (0.47-1.31)	0.76 (0.44-1.32)	1.24 (0.75-2.06)	P=0.41
Odds Ratios for MI §				P for trend
Choline	1.07 (0.77-1.49)	1.33 (0.95-1.85)	1.15 (0.81-1.62)	P=0.25
Betaine	0.82 (0.60-1.13)	0.80 (0.58-1.10)	0.77 (0.55-1.08)	P=0.13
Odds Ratios for Stroke §				P for trend
Choline	0.98 (0.53-1.81)	1.22 (0.67-2.20)	0.82 (0.44-1.56)	P=0.71
Betaine	0.71 (0.40-1.26)	0.83 (0.47-1.47)	0.89 (0.50-1.59)	P=0.79

In addition to matching variables (age, sex and eGFR), further adjustment was performed for the following clinical variables: hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, body mass index, aspirin dosage and treatment arm.

§ Multivariable adjustment (unmatched) was performed for the following clinical variables: age, sex, eGFR, hypertension, hypercholesterolemia, diabetes mellitus, peripheral artery disease, qualifying index event, smoking, region, body mass index, aspirin dosage and treatment arm.

CV, Cardiovascular disease; MI, myocardial infarction.

Figure S1. Treatment Effect of Ticagrelor Versus Placebo on the Composite and Individual Endpoints of CV Death, MI and Stroke By TMAO Quartiles.
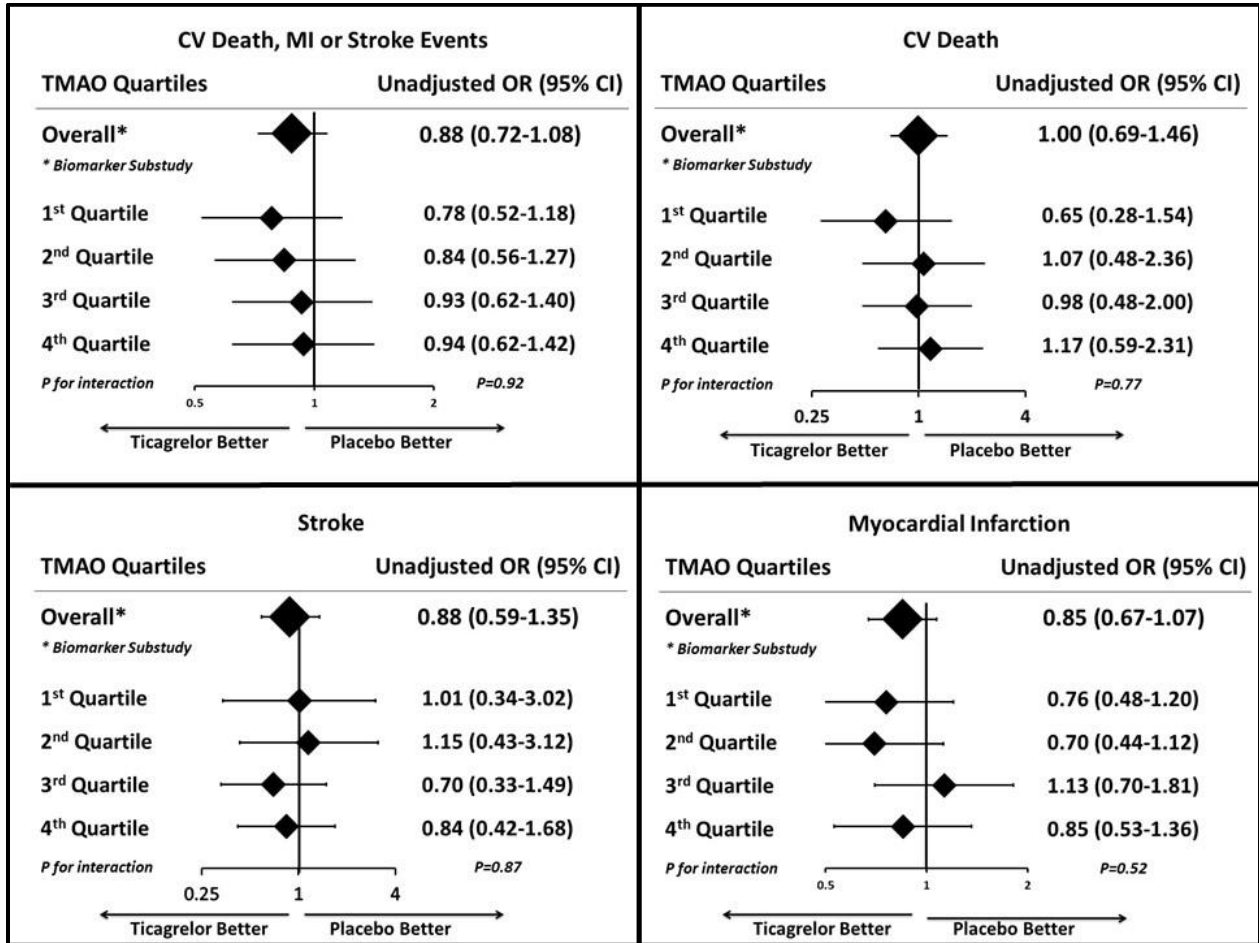
CI, confidence intervals; CV, Cardiovascular disease; MI, myocardial infarction; OR, odds ratio; TMAO, Trimethylamine N-oxide.