6-7-2016

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Plasma Trimethylamine *N*-Oxide, a Gut Microbe–Generated Phosphatidylcholine Metabolite, Is Associated With Atherosclerotic Burden

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The incidence of atherosclerotic coronary artery disease (CAD), a common cardiovascular disease (CVD), has been increasing and remains a leading cause of death around the world (1,2). Despite the considerable attention to traditional risk factors (including age, sex, hypertension, dyslipidemia, smoking, and diabetes) and use of modern pharmacotherapies, including high potency statin therapy, at least a 50% residual risk remains (2–4). Therefore, we are interested in identifying novel cardiovascular risk factors to improve both our understanding of the processes that contribute to CVD pathogenesis that have not been explained by traditional or known risk factors, and the prevention and treatment of CVD (5).

There is growing appreciation that gut microbes participate in the overall metabolism of their host, and contribute to and are associated with cardiometabolic disease phenotypes in both animal models of disease and in humans (6–9). In particular, a role for gut microbes as participants in the development of atherosclerosis and CVD has recently become acknowledged (10–12). Specifically, a gut microbial metabolite, trimethylamine *N*-oxide (TMAO), has been shown to be atherogenic (12,13), and recent studies that examined microbial transplantation in recipients confirmed a direct causal role for gut microbes in transmitting atherosclerosis susceptibility and overall TMAO production (10). TMAO arises from gut microbiota metabolism following ingestion of diets rich in phosphatidylcholine (or lecithin), the major dietary source of choline, and carnitine, an abundant nutrient in red meat (11,12,14,15). Moreover, elevated TMAO levels have been shown to predict a future risk of major adverse cardiac events (MACE), an increased prevalence of CVD, and have shown a relationship with the number of diseased coronary vessels (11,12,14). However, a relationship between plasma TMAO levels and detailed characterization and quantification of atherosclerosis burden has not been investigated.

The SYNTAX (Synergy Between Percutaneous Coronary Intervention with Taxus and Cardiac Surgery) trial produced the SYNTAX score, which is an angiographic scoring system to determine the complexity and burden of atherosclerotic CAD (16,17). The anatomical SYNTAX score has been shown to predict MACE and long-term prognosis risks among stable patients with CAD who have undergone coronary revascularization (18,19). However, due to some limitations of this score, including lack of clinical variables and a purely anatomical focus, the SYNTAX score II was recently developed, with a presumed improved prognostic value. It consists of a combination of 2 anatomical (SYNTAX score and unprotected left main CAD) and 6 clinical variables (age, creatinine clearance, left ventricular ejection fraction, sex, chronic obstructive pulmonary disease, and peripheral artery disease [PAD]). The SYNTAX score II has shown better long-term (4-year) mortality prediction between coronary artery bypass graft (CABG) surgery and percutaneous coronary intervention (PCI) than the original SYNTAX score (20). Patients with diffuse lesions present a higher risk for an adverse outcome after coronary revascularization due to a higher incidence of restenosis, poor run-off in the target CABG, and an increased risk of adverse cardiovascular outcomes (21–23).

In this study, we aimed to examine the relationships between plasma TMAO levels and coronary artery atherosclerotic lesion complexity and burden quantified by SYNTAX scores and lesion characteristics.

METHODS

This is a single-center prospective cohort study approved by the Cleveland Clinic Institutional Review Board. All subjects provided written informed consent.

From the total of 989 patients who underwent elective diagnostic cardiac catheterization at the Cleveland Clinic between 2012 and 2014 who did not have evidence of acute coronary syndrome (cardiac troponin T level: <0.03 μg/l), we specifically excluded patients with a history of cardiac transplantation, PCI, CABG, and who had undergone a noncoronary artery procedure (structural heart procedure or for PAD), and patients who had no evidence of significant CAD (total exclusion: 636 patients) (**Figure 1**). Therefore, 353 consecutive patients with evidence of significant CAD, defined by a diameter stenosis of ≥50% in vessels ≥1.5 mm, were included in this study.

ANGIOGRAPHIC ANALYSIS. Images of coronary angiograms were obtained with Syngo Dynamics cardiovascular imaging software (Siemens Medical Solutions USA, Inc., Malvern, Pennsylvania). The SYNTAX score was calculated for each patient using a computer program that consisted of sequential and interactive self-guided questions according to the SYNTAX score calculator version 2.11, and divided into tertiles according to the SYNTAX trial, which

ABBREVIATIONS AND ACRONYMS

AUC = area under the receiver-operating curve

BMI = body mass index

CABG = coronary artery bypass graft

CAD = coronary artery disease

CI = confidence interval

CVD = cardiovascular disease

eGFR = estimated glomerular filtration rate

hs-CRP = high-sensitivity C-reactive protein

hs-cTnT = high-sensitivity cardiac troponin T

MACE = major adverse cardiac events

NRI = net reclassification index

OR = odds ratio

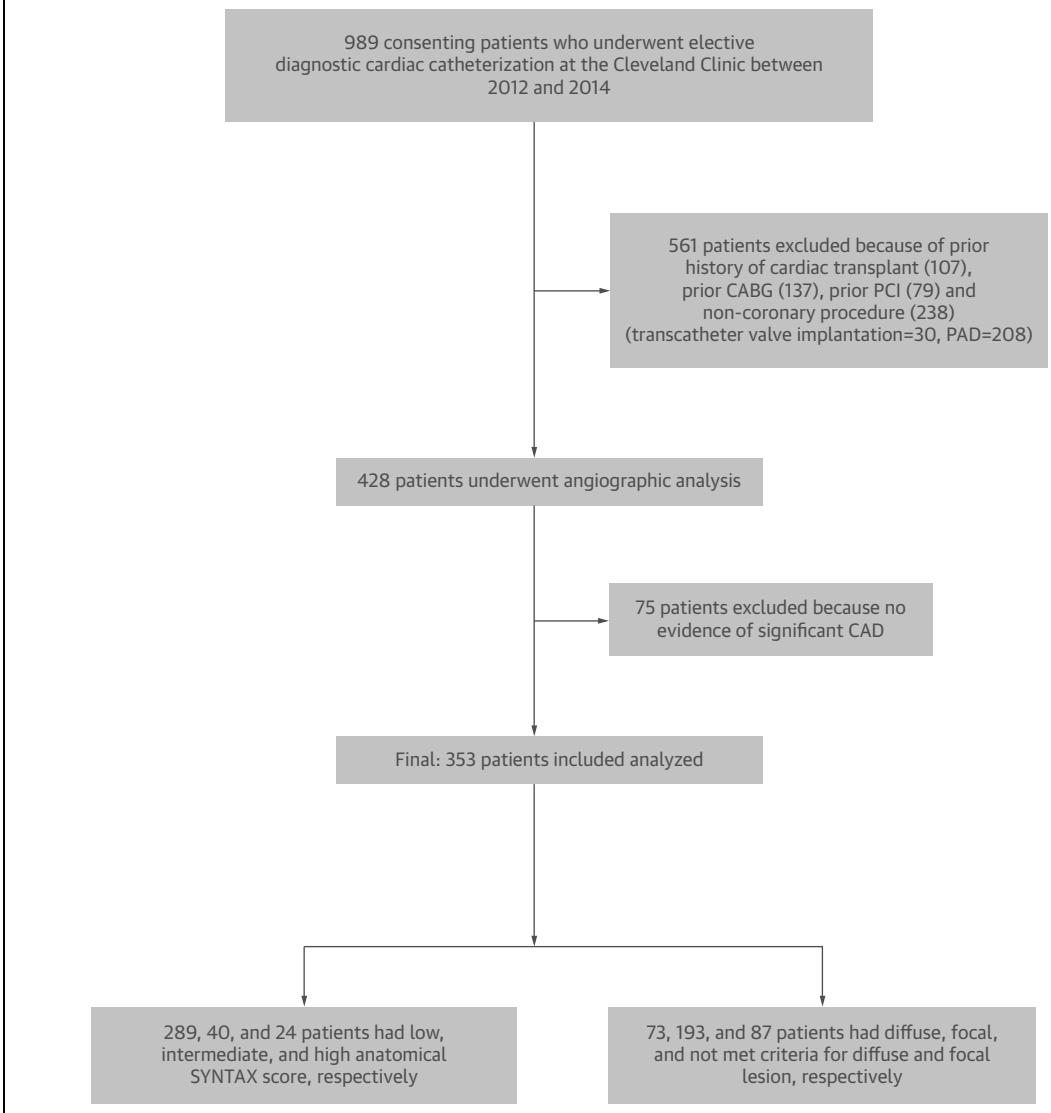
PAD = peripheral arterial disease

PCI = percutaneous coronary intervention

SYNTAX score = Synergy Between Percutaneous Coronary Intervention with Taxus and Cardiac Surgery score

TMAO = trimethylamine *N*-oxide

FIGURE 1 CONSORT (Consolidated Standards of Reporting Trials) Diagram



The flow diagram showing the process used to define the study population is depicted. CABG coronary artery bypass surgery; CAD coronary artery disease; PAD peripheral artery disease; PCI percutaneous coronary intervention; SYNTAX Synergy Between Percutaneous Coronary Intervention with Taxus and Cardiac Surgery.

were defined as low (0 to 22), intermediate (23 to 32), and high (≥33) SYNTAX scores. The SYNTAX score II was calculated for each patient using a nomogram and stratified according to tertiles of SYNTAX score II for PCI as previously described (20,24). A diffuse lesion was defined as de novo CAD of >75% of the length of any segment(s) proximal to the lesion, at the site of the lesion, or distal to the lesion that had a vessel diameter of <2.0 mm; it was considered non-diffuse if the arteries contained no such lesion (25). A focal lesion was defined as de novo CAD of >50% reduction in luminal diameter and a lesion length of <20.0 mm. We indicated diffuse lesions if at least 1 diffuse lesion characteristic was present, focal lesions if pure focal lesions were present, and nondiffuse and nonfocal lesions if they did not meet the criteria for those lesions. All angiograms were reviewed by an interventional cardiologist who was blinded to TMAO level and clinical variable data.

LABORATORY TESTING. After informed consent was obtained from all patients, fasting blood samples were collected using ethylenediaminetetraacetic acid

tubes at the time of cardiac catheterization, which were then immediately processed and frozen at 80°C until analysis. TMAO levels in plasma were determined using stable isotope dilution high-performance liquid chromatography with online tandem mass spectrometry on an API 5000 triple quadrupole mass spectrometer (AB SCIEX, Framing-ham, Massachusetts), as previously described (12,26). The assay shows inter- and intraday reproducibility (all coefficient of variations <7%) and accuracy (>98.5% across low, mid, and high values). Routine laboratory tests and high-sensitivity C-reactive pro-tein (hsCRP) were measured using the Architect *ci*8200 platform (Abbott Laboratories, Abbott Park, Illinois), and high-sensitivity cardiac troponin T (hs-cTnT) was measured by a high-sensitivity (fifth generation) assay on a Roche Cobas e411 platform (Roche Diagnostics, Indianapolis, Indiana). Estimated glomerular filtration rate (eGFR) was esti-mated using the Modification of Diet in Renal Disease equation.

STATISTICAL ANALYSIS. Continuous data are presented as mean ± SD or median (interquartile range [IQR]) and compared with the Student *t* test or nonparametric test when appropriate. Categorical variables are presented as number (percent) and compared between groups with chi-square tests. Spearman correlation analysis was used to examine the associations between TMAO and all clinical and laboratory variables. Comparisons among ≥3 groups were evaluated by 1-way analysis of variance or the Kruskal-Wallis test according to whether or not the distribution was normal. Ordinal logistic regression analysis, adjusted for traditional Framingham risk factors (including age, sex, hypertension, diabetes mellitus, smoking, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglycerides), lesion characteristics, hsCRP, eGFR, body mass index (BMI), and medications (angiotensin-converting enzyme inhibitor/angiotensin receptor blocker, beta-blockers, statins, and aspirin) were used to examine the association of TMAO with higher SYNTAX score, SYNTAX score II, and hs-cTnT tertiles. Univariate and multivariate logistic regression analyses were used to determine independent predictors of diffuse or focal lesions; the variables were en-tered into the model, including the previously mentioned traditional risk factors and hs-cTnT (log-transformed). Category-free net reclassification indexes (NRI) and area under the receiver-operating characteristic curves (AUC) were calculated to eval-uate the incremental predictive ability of TMAO for predicting intermediate or high SYNTAX score (>22) and SYNTAX score II (>21) and adjusted for the same covariates from the regression model with traditional risk factors, lesion characteristic, hsCRP, eGFR, BMI, and medications (27). All analyses were performed using JMP Pro version 10 (SAS Institute, Cary, North Carolina) and R (version 3.1.2, Vienna, Austria). A p value <0.05 was considered statistically significant.

RESULTS

PATIENT CHARACTERISTICS. Baseline characteristics of the 353 patients included in our study are shown in Table 1. The mean age was 65 years of age, 79% were men, 80% had hypertension, and 30% had diabetes. The median TMAO was 5.48 μM (IQR: 3.41 to 9.76 μM), the median SYNTAX score was 11.0 (IQR: 4.0 to 18.5), and 289 (82%), 40 (11.3%), and 24 (6.8%) patients had low, intermediate, and high SYNTAX scores, respectively. The baseline characteristics according to SYNTAX score tertiles showed that a patient with a higher SYNTAX score was more likely to be older and have hypertension and diabetes. In contrast, sex, a history of smoking, and BMI were similar across SYNTAX score tertiles (Table 1). TMAO levels were significantly higher with an increasing SYNTAX score, SYNTAX score II, and hs-cTnT tertiles (Central Illustration).

CORRELATIONS WITH PLASMA TMAO LEVELS. Plasma TMAO levels were strongly correlated with both SYNTAX score and SYNTAX score II (Spearman's correlation: r 0.61 and 0.62, respectively; both $p < 0.0001$). Plasma TMAO was also correlated with hs-cTnT, a measure of subclinical myonecrosis, because all hs-cTnT levels were below the diagnostic cutoff for myocardial infarction (r 0.29; $p < 0.0001$). Following ordinal logistic regression analysis adjusting for traditional risk factors (including age, sex, hypertension, diabetes mellitus, smoking, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride) and lesion characteristics, elevated TMAO levels remained independently associated with a higher tertile of the SYNTAX score (adjusted odds ratio [OR]: 4.68; 95% confidence interval [CI]: 2.35 to 9.29; $p < 0.0001$), SYNTAX score II (adjusted OR: 2.02; 95% CI: 1.72 to 3.01; p 0.0001), and hs-cTnT (adjusted OR: 1.34; 95% CI: 1.02 to 1.78; p 0.037). However, when further adjusted for hsCRP, BMI, eGFR, and medications, the association between TMAO and hs-cTnT was no longer significant, whereas SYNTAX score and SYNTAX score II remained significant (Table 2).

TABLE 1 Baseline Characteristics

	Total (N 353)	SYNTAX Score			p Value
		Low (0–22) (n 289)	Intermediate (23–32) (n 40)	High (≥33) (n 24)	
Age, yrs	65.0 ± 11.0	64.0 ± 11.0	67.0 ± 9.0	68.0 ± 10.0	0.02
Male	79.0	78.0	88.0	75.0	0.35
BMI, kg/m^2	29.4 (26.4 33.4)	29.0 (29.3 30.6)	30.2 (28.9 32.9)	32.7 (29.2 34.7)	0.22
Systolic BP, mm Hg	128.8 ± 19.4	128.6 ± 18.8	125.1 ± 21.9	137.4 ± 21.9	0.06
Diabetes mellitus	30.3	26.0	47.5	54.2	0.001
Hypertension	80.5	77.5	92.5	95.8	0.012
Current smoker	60.2	59.9	60.0	65.2	0.40
Unprotected left main CAD	5.7	1.7	22.5	25	<0.001
LDL cholesterol, mg/dl	81 (63 104)	82.0 (64.0 105.0)	70.0 (51.0 85.0)	78.0 (62.0 103.0)	0.03
HDL cholesterol, mg/dl	45.0 (36 55)	45.0 (37.0 55.0)	43.0 (30.0 59.0)	42.0 (35.0 49.0)	0.26
Triglyceride, mg/dl	123.0 (84 159)	119.0 (84.0 154.0)	146 (92.0 215.0)	142.0 (88.0 178.0)	0.09
eGFR, ml/min/1.73 m^2	95.33 (72.5 116.1)	97.44 (75.4 115.6)	85.68 (54.4 123.1)	79.78 (59.9 114.3)	0.004
Medications					
Aspirin	74.0	64.0	75.0	75.0	0.98
ACEI or ARB	36.0	44.0	68.0	42.0	<0.001
Statin	66.0	70.0	70.0	50.0	0.203
Beta blocker	58.0	68.0	62.0	58.0	0.79
TMAO, μM	5.48 (3.41 9.76)	4.84 (3.1 7.5)	12.02 (7.66 15.34)	19.69 (16.1 26.28)	<0.001
hs cTnT, ng/l	10.41 (7.03 18.04)	9.73 (6.86 15.83)	16.82 (9.74 27.65)	12.39 (8.49 28.78)	0.003
Diffuse lesion characteristics	20.7	17.0	32.5	45.8	<0.001

Values are mean ± SD, %, or median (interquartile range).

ACEI angiotensin-converting enzyme inhibitors; ARB angiotensin receptor blocker; BMI body mass index; BP blood pressure; CAD coronary artery disease; eGFR estimated glomerular filtration rate; HDL high-density lipoprotein; hs-cTnT high-sensitivity cardiac troponin T; LDL low-density lipoprotein; SYNTAX Synergy Between Percutaneous Coronary Intervention with Taxus and Cardiac Surgery; TMAO trimethylamine *N*-oxide.

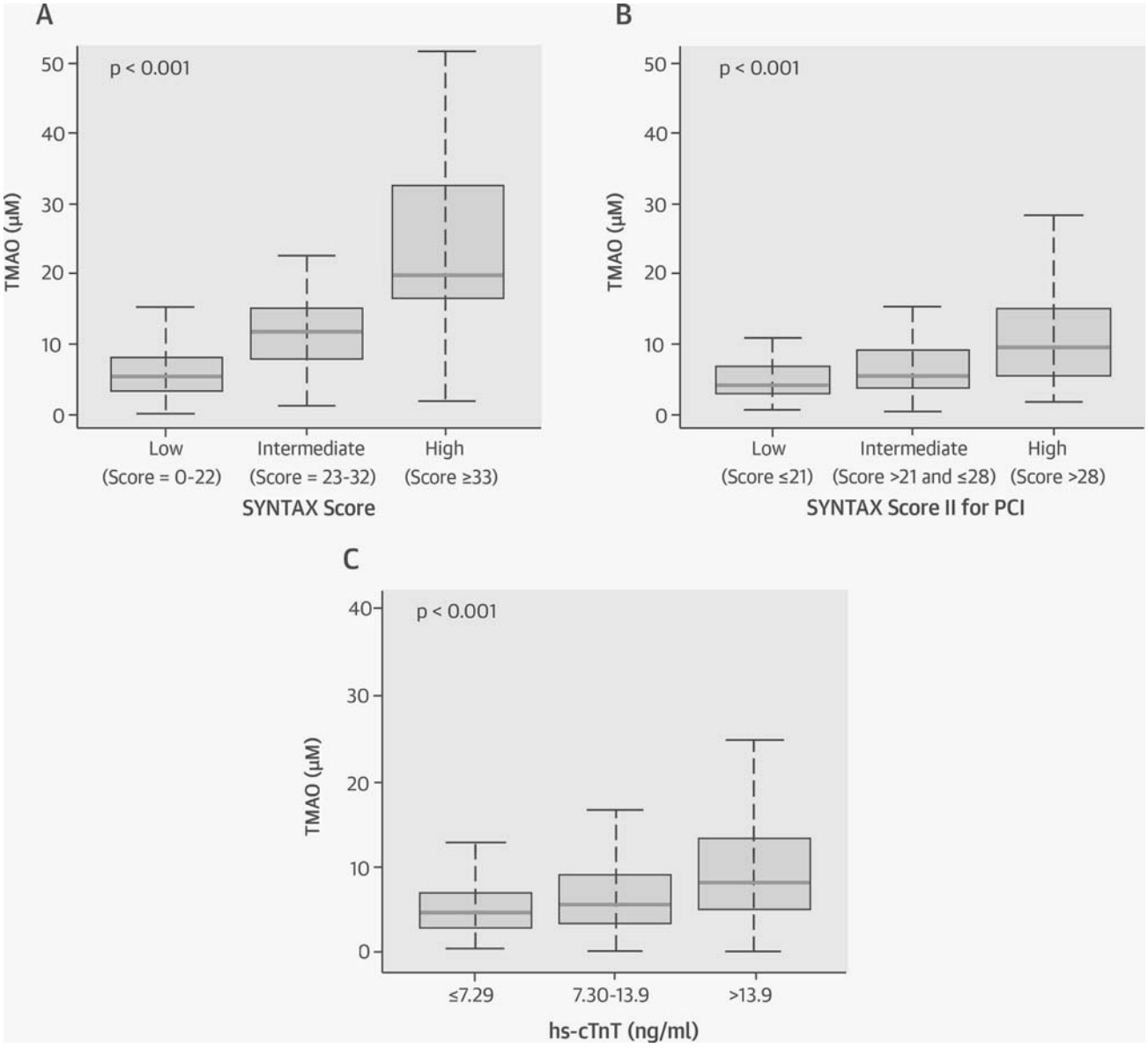
Plasma TMAO levels were significantly higher in patients with diffuse lesions than in patients with focal lesions (8.4 μM [IQR: 5.7 to 14.0 μM] vs. 4.4 μM [IQR: 5.2 to 13.5 μM]; $p < 0.0001$) (Figure 2C). Furthermore, plasma TMAO was significantly higher in patients with nonfocal and nondiffuse lesions than in patients with focal lesions (8.3 μM [IQR: 4.5 to 13.4 μM] vs. 4.4 μM [IQR: 5.2 to 13.5 μM]; $p < 0.0001$) (Figure 2C). Interestingly, the frequency of diffuse lesions was significantly increased with increasing SYNTAX score and SYNTAX score II tertiles (17% in low SYNTAX score vs. 45.8% in high SYNTAX score and 11.4% in low SYNTAX score II vs. 20.7% in high SYNTAX score II; $p < 0.0001$ for all) (Figures 2A and 2B). In contrast, the frequency of focal lesions was significantly lower with increasing SYNTAX score and SYNTAX score II tertiles ($p < 0.0001$) (Figures 2A and 2B). Following multivariate adjustments, elevated TMAO levels remained associated with an increased likelihood of having diffuse lesions (adjusted OR: 2.05; 95% CI: 1.45 to 2.90; p 0.0001) and a decreased likelihood of having focal lesions (adjusted OR: 0.46; 95% CI: 0.31 to 0.68; p 0.0001).

PREDICTION OF HIGH ATHEROSCLEROTIC BURDEN. High atherosclerosis burden was identified by the presence of intermediate or high SYNTAX score and SYNTAX score II. We tested whether TMAO levels could help predict enhanced coronary atherosclerotic burden, and whether addition of TMAO to models that included traditional risk factors, lesion characteristic, hsCRP, eGFR, BMI, and medications helped to predict enhanced CAD atherosclerotic burden. Addition of TMAO to the fully adjusted model showed that elevated TMAO levels significantly improved NRI and trended toward improvement in AUC for predicting high atherosclerosis burden, albeit not at a statistically significant level (SYNTAX score: NRI 0.87; $p < 0.001$; AUC: from 0.83 to 0.88; p 0.07; and SYNTAX score II: NRI 0.58; $p < 0.001$; AUC: from 0.92 to 0.93; p 0.07).

DISCUSSION

The main finding of our study is the strongly significant association between fasting plasma TMAO levels in an independent and contemporary cohort of stable patients with CAD and quantitative indexes of coronary atherosclerosis burden (quantified by the SYNTAX score and SYNTAX score II). An additional major finding is the observed correlation between TMAO levels and evidence of subclinical myonecrosis (quantified by hs-cTnT) in patients

CENTRAL ILLUSTRATION **Relationship Between TMAO and Measures of CAD Burden and Subclinical Myonecrosis**



Senthong, V. et al. J Am Coll Cardiol. 2016;67(22):2620 8.

Trimethylamine *N* oxide (TMAO) has links to coronary artery disease (CAD) pathogenesis and is associated with adverse outcomes. One way to measure CAD complexity and burden is via the SYNTAX score that came from the SYNTAX (Synergy Between Percutaneous Coronary Intervention with Taxus and Cardiac Surgery) trial; the more recent SYNTAX score II has been shown to have improved prognostic ability. In 353 stable patients with evidence of atherosclerotic CAD, concentrations of TMAO were significantly higher with **(A)** increasing SYNTAX score, **(B)** SYNTAX score II, and **(C)** subclinical myonecrosis (quantified by high sensitivity cardiac troponin T [hs cTnT]) tertiles. PCI percutaneous coronary intervention.

with stable CAD who underwent elective coronary angiography (28), although renal insufficiency could be a confounder. Furthermore, elevated TMAO levels were found to serve as an independent predictor of higher SYNTAX score, higher SYNTAX score II, and elevated hs-cTnT, even after adjustments for traditional risk factors and lesion characteristics on coronary artery angiography. A higher plasma TMAO level was also shown to serve as an independent predictor for the presence of

TABLE 2 Association Between TMAO and SYNTAX Scores and hs-cTnT

	SYNTAX Score		SYNTAX Score II		hs-cTnT*	
	OR (95% CI)	p Value	OR (95% CI)	p Value	OR (95% CI)	p Value
Unadjusted	6.57 (3.37 12.80)	<0.0001	2.28 (1.72 3.01)	<0.0001	1.56 (1.20 2.02)	0.0008
Adjusted model†	4.82 (2.43 9.57)	<0.0001	1.88 (1.36 2.60)	0.0001	1.14 (0.88 1.47)	0.3147

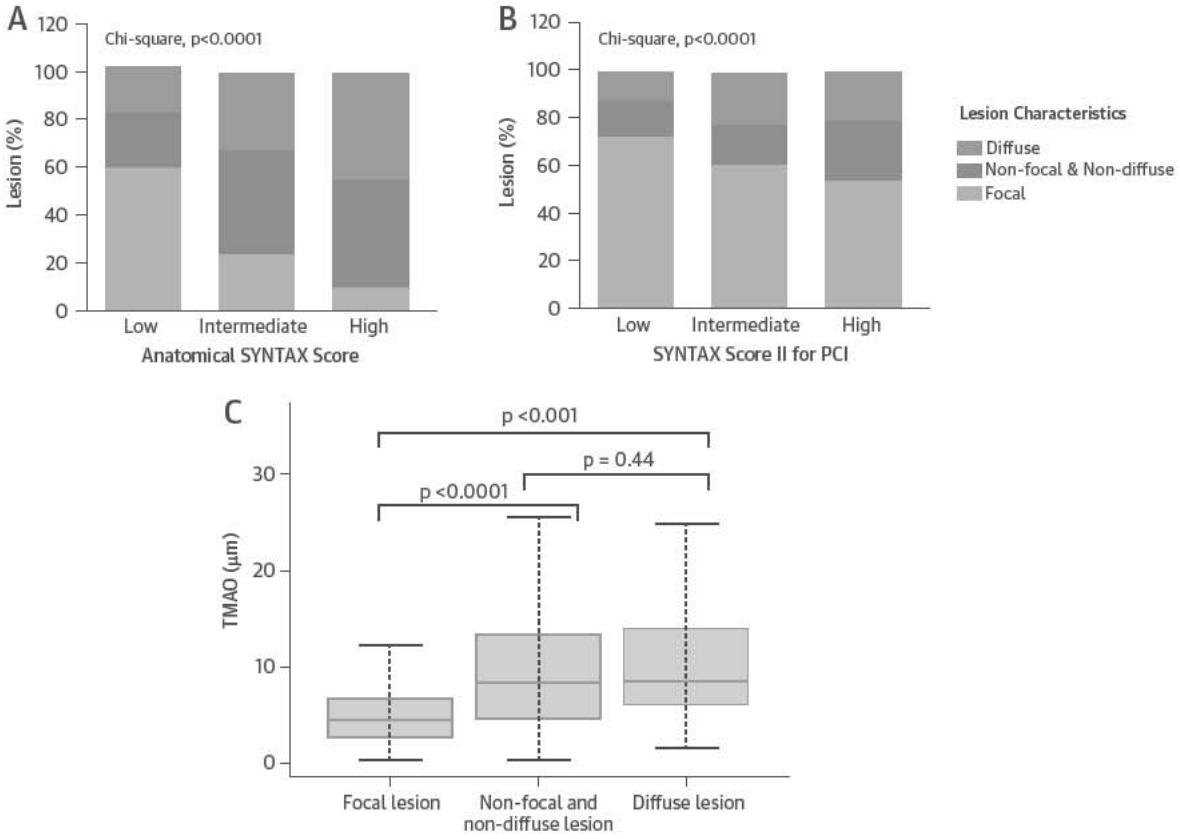
*Subclinical myonecrosis hs-cTnT >0.01 ng/ml. †Included age, sex, triglyceride, HDL cholesterol, LDL cholesterol, diabetes mellitus, hypertension, smoking, diffuse lesion, focal lesion, log high-sensitivity C-reactive protein, BMI, log eGFR, statins, ACEI or ARB, aspirin, and beta-blocker.

CI confidence interval; OR odds ratio; other abbreviations as in Table 1.

diffuse coronary lesion characteristics, which often represent high severity and the atherosclerotic burden of CAD. Moreover, the addition of TMAO resulted in a significant increase in the NRI for prediction of high atherosclerotic burden over traditional risk factors, lesion characteristics, hsCRP, eGFR, BMI, and medications. Taken together, our findings further demonstrate that TMAO levels are associated with a greater coronary atherosclerotic burden.

Gut microbes play an important role in global host metabolism, including the production of vitamins and other essential nutrients, and regulation of many aspects of host immunity (6-8). Changes in gut microbial composition has been linked to diseases such as obesity, insulin resistance, chronic kidney disease, inflammatory bowel disease, and CVD (12,29-34). TMAO has previously been shown to directly promote pro-atherosclerotic effects in vivo using animal model studies. The present study extends these observations

FIGURE 2 TMAO, Atherosclerotic Burden, and Lesion Characteristics



Diffuse, nonfocal and nondiffuse, and focal lesion frequency was compared according to **(A)** SYNTAX score and **(B)** SYNTAX score II for PCI tertiles. **(C)** Fasting trimethylamine *N* oxide (TMAO) levels were compared according to lesion characteristics. Abbreviation as Figure 1.

by showing striking and independent associations between TMAO and enhanced coronary artery atherosclerotic burden. Thus, the results are consistent with the gut microbial TMAO pathway being mechanistically linked with the development of CAD and the pathogenesis of CVD (12–14,35).

Previous studies showed that the SYNTAX score is a useful tool to risk stratify outcomes in stable patients with complex CAD (with or without unprotected left main CAD) who have undergone revascularization by PCI or CABG. These studies independently predicted MACE and all-cause mortality, which increased in the higher SYNTAX score tertiles (18,19). A high SYNTAX score is also a marker of systemic atherosclerotic burden, which likely correlates with a poor prognosis (16,17).

In our study, plasma TMAO levels were correlated with both the SYNTAX score (anatomical factors) and the SYNTAX score II (both anatomical and clinical factors). We also found elevated plasma TMAO to be an independent predictor for the presence of diffuse lesion characteristics, which represent markers of higher atherosclerotic burden and are associated with adverse outcomes in CAD (22,36). In contrast, elevated plasma TMAO levels showed an inverse and independent association with the presence of focal lesions, which indicates a lower atherosclerotic burden. Importantly, despite the present study cohort including stable subjects without evidence of acute coronary syndrome (cardiac troponin T level, <0.03 μg/l) at presentation, elevated plasma TMAO was associated with evidence of subclinical myonecrosis, as indicated by a higher level of hs-cTnT. It is thus conceivable that subclinical myo-necrosis or high severity and an atherosclerotic burden of CAD may occur in the setting of elevated plasma TMAO levels. Taken together, our current findings provided the additional support for a pro-atherogenic effect of elevated systemic levels of TMAO. The mechanism of the relation between plasma TMAO and atherosclerotic burden might be explained by our previous studies that showed that higher plasma TMAO was correlated with greater atherosclerotic plaque size in both the arterial wall and aortic root in mice on diets supplemented with either TMAO or choline (12). TMAO may have direct biological activity that facilitates the development or propagation of atherosclerosis plaque and suppres-sion of reverse cholesterol transport in in vivo mouse models (12,13). Moreover, recent studies suggest that flavin monooxygenase 3, the major host enzyme responsible for forming TMAO from gut microbe-generated trimethylamine, is a master regulator of tissue cholesterol and sterol metabolism (37). Furthermore, results from recent studies in mice fed a high-fat diet also suggest that dietary TMAO may exacerbate impaired glucose tolerance, obstruct hepatic insulin signaling, and promote adipose tissue inflammation, which have been related to the complexity and degree of atherosclerotic burden of CAD (38). It is also of interest that recent studies have further implicated flavin monooxygenase 3 in atherosclerosis development in rodent models of diabetes (39).

STUDY LIMITATIONS. This was a single, tertiary referral center that recruited patients at the point of cardiac evaluation; therefore, we could not exclude selection bias for patients who underwent diagnostic cardiac catheterization (especially with relatively preserved renal function). Because the patient population was a contemporary cohort (the last patient was enrolled in 2014), we did not have long-term outcome data. Despite all subjects being recruited at the time of coronary angiography while fasting for at least 10 h in anticipation of coronary angiography, we could not exclude the potential for dietary intake of choline or TMAO (e.g., large consumption of some fish species) within 24 h before blood sampling. The relatively low number of the patients in the intermediate and high SYNTAX score tertiles was a also limitation of our study. Furthermore, improvement in AUC for a model is often very small in magnitude, yet the category-free NRI may tend to overstate the incremental value of a biomarker.

CONCLUSIONS

Fasting plasma TMAO level is an independent predictor of high atherosclerotic burden in patients with CAD. Higher TMAO levels can predict the presence of increased atherosclerotic burden and complexity, as evidenced by higher SYNTAX scores and diffuse lesion characteristics. Despite the associative nature in this cross-sectional study, these findings are consistent with numerous previous mechanistic demonstrations that have linked TMAO to the pathogenesis of atherosclerosis, and the multiple reported findings that have demonstrated associations between TMAO and cardiovascular risks. Further investigations into the mechanisms of elevated TMAO leading to atherosclerotic burden and myonecrosis are warranted.

PERSPECTIVES

COMPETENCY IN MEDICAL KNOWLEDGE: The intestinal microbe-generated phosphatidylcholine metabolite TMAO is related to the pathogenesis of atherosclerotic CAD.

TRANSLATIONAL OUTLOOK: Further research is needed to determine whether dietary or pharmacological interventions that reduce plasma levels of TMAO can prevent or retard the progression of coronary atherosclerosis.

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