THE ASSOCIATION OF PLASMA TRIMETHYLAMINE N-OXIDE IN INDIAN PATIENTS WITH CORONARY ARTERY DISEASE WITH OR WITHOUT TYPE 2 DIABETES MELLITUS: A PROSPECTIVE COHORT STUDY

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Abstract

Background: The role of trimethylamine-N-oxide (TMAO) in the development of coronary artery disease (CAD) remains controversial, and prospective data are few. We aimed to investigate the association between plasma TMAO in CAD with or without diabetes mellitus (DM).

Methods: This study was based on the community-based prospective cohort study in India. A total of 50 CAD, 50 CAD with DM patients and 50 healthy individuals aged 18-75 years were included from 2020 to 2022. CAD and DM was ascertained during follow-up visits. After getting written informed consent, the demographic characteristics, clinical profile, detailed nutrition history, laboratory investigations were collected from the patients. The blood samples of4 ml was collected from study participants and the samples were processed to measure plasma concentration of TMAO using Triple Quadrupole LC/MS/MS with online electrospray ionization tandem mass spectrometry.

Results: CAD patients had significantly higher plasma levels of TMAO than control controls (1.10±0.83 µM vs. 2.04±0.61 µM). Moreover, there was no statistically significant difference in the plasma levels of TMAO between CAD-T2DM patients and control subjects (1.35±1.81 µM vs. 1.10±0.83 µM). Similarly, chronic consumption of red meat, fish, egg and dairy products raised plasma TMAO levels in individuals with CAD and CAD-DM in comparison with individuals who do not eat red meat (CAD: 2.10±0.60 vs. 1.68±0.65 µM vs. 1.09±0.88 vs. 0.48±0.10 µM).

Conclusion: This is the first prospective study to evaluate the plasma levels of TMAO in CAD patients in the Indian population. TMAO was found to be substantially higher in patients with CAD and red meat consumers when compared to controls and no red meat consuming participants. However, there was no significant impact of diabetes on the concentration of TMAO.

Keywords: trimethylamine, trimethylamine N-oxide, CAD, T2DM, hepatic flavin monooxygenases

Introduction

One of the trading causes of mortality and disability in adults with diabetes is coronary

artery disease (CAD) [1]. Patients with type 2 diabetes mellitus (T2DM) are more likely than those without T2DM to develop CAD [2]. Additionally, cardiovascular diseases like CAD account for 75% of T2DM patients' deaths. The majority of CAD cases in T2DM patients are characterized by small, diffuse, calcified, multivessel involvement (multivessel disease, or MVD), which makes the condition more complex [3]. It often requires coronary revascularization in addition to effective medical treatment to control angina. Until now, not much is known about the onset and progress of CAD in people with diabetes [4]. The clinical significance of assessing CAD-T2DM risk is that it can help determine disease treatment and preventative methods [5]. To reduce disease morbidity and mortality when both CAD and T2DM occur, comprehensive cardiovascular risk factor prevention should be implemented as practice [6]. Rather than merely reducing blood glucose or blood pressure, a comprehensive therapy establish should involve treating abnormalities in blood lipids, glucose, and pressure, as well as adequate activity, weight control, and smoking cessation [7]. Many research have found that consuming pattern plays an essential influence in CAD-T2DM risk [8].

Trimethylamine-N-oxide (TMAO), a major intestinal microbial metabolite, is derived from dietary choline, betaine, and L-carnitine. The gut bacteria metabolizes it to trimethylamine (TMA), which is then rapidly oxidized into TMAO by the hepatic flavin monooxygenases (FMOs) (Scheme 1) [9]. A small organic substance called TMAO is contained in certain foods, including dairy, red meat, fish, and poultry [10]. Recent research using metabolomics and animal models have investigated the relationship between TMAO and atherosclerosis [11]. Furthermore, a number of studies have discovered an association between blood TMAO levels and the risk of CAD [12-17]. These studies found that, independent of traditional risk factors, elevated plasma TMAO levels were associated with an increased risk of major adverse cardiac events. Moreover, some of these studies observed that plasma TMAO levels independently predicted a high atherosclerotic burden in patients with CAD and that high TMAO levels predicted worse long-term clinical outcomes in patients with heart failure. Moreover, another study found that hyperglycemia was associated to higher TMAO plasma levels. Nevertheless, research in India has not yet examined the connection between TMAO levels and the risk of CAD in T2DM patients. In individuals with T2DM, circulating levels of TMAO may therefore predict and increase incident risks for CAD, offering a unique idea and approach for the prevention of CAD-T2DM. The purpose of this study was to investigate the association between plasma TMAO in CAD with or without T2DM.

Scheme 1: Oxidation of trimethylamine to trimethylamine N-Oxide catalyzed by the enzyme hepatic FMO3.

$$TMA + NADPH + O_2 + H^+ \xrightarrow[FMO3]{\text{Hepatic}} TMAO + NADP^+ + H_2O$$

Materials and Methods

Participants

This study consists of 50 CAD patients and 50 patients with CAD-T2DM visited the Cardiology Dept at SRM Medical College Hospital and Research Centre (Chennai, India) between Jan 2021 and June 2023. Three subject groups made up the study's participants: 50 controls, 50 CAD patients, and 50 CAD patients with T2DM. Table 1 provides a summary of the study population's demographic and clinical characteristics.

Study approval for ethics and informed consent

The study protocols and procedures for handling of human blood samples were approved by the SRM Medical College and Research Centre ethics committee (EC No: 1899). Written informed consent including consent to store plasma and perform further analysis on blood samples was obtained from all participants after oral and written information.

Collection, storage and processing of human blood samples

Each research participant provided a single whole-blood sample (5 ml), which was collected in with and without EDTA-containing vacutainers and processed to obtain plasma and serum. At -80°C, samples were frozen for further analyses.

Laboratory methods of biochemical parameters

HbAlC was determined by HPLC method, renal function tests such as urea and creatinine was measured by urease-GLDH and Jaffe's kinetic method respectively. Lipid profile including total cholesterol, triglycerides and HDL was determined by CHOO-POD, enzymatic GPO-POD, direct antibody inhibition methods respectively. CPK-MB was measured by UV kinetic G6P method. Other parameters such as BUN, LDL and VLDL were calculated. Calculations of reference intervals and 90% confidence intervals were performed.

Determination of TMAO by LC-MS/MS

To determine TMAO by LC-MS/MS all chemicals standards, and reagents used were either analytical or high-performance liquid chromatography (HPLC)-grade reagents. The water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA) before use. The quantification of T1v1AO serum levels was performed according to the high-performance liquid chromatography coupled with MS/MS (HPLC-MS/MS) (Agilent LC-MS/MS). Briefly, 200 µL of plasma sample was taken and madeup with 1 ml of milli-Q water. Vortex and centrifuge for 6000 rpm, filtered the solution using 0.22 µm Syringe filter and injected into LC-MS/MS Instrument. The Poroshell 120, EC-C18 column was

used (4.6 x 50mm, 2.7µm particle size) at temperature 40°C. The mobile phase consist of 10 Mm Ammonium formate and Acetonitrile. MS was performed on an G6430A Triple Quadrupole mass s pectrometer (Agilent) with electrospray ionization in the positive mode. Ion transitions used for quantitation were *mlz* 76⟶58 for TMAO and *mlz* 85⟶66 for the internal standard.

Statistical analysis

The results are presented as mean± standard deviation (SD). All data were subjected to analysis of variance using the SPSS software (version 16. 0 software). Differences between the means were tested by one-way ANOVA and all detected significant differences were further evaluated by student's t-test. To evaluate the levels of TMAO to predict CAD patients, the area under the curves (AUC) was calculated after the receiver operating characteristic (ROC) curves were constructed. The level of significance chosen was $P < 0.05$.

Results

Clinical features of the Study Population

The study consisted of three subject groups: 50 CAD patients 50 CAD patients with T2DM patients, and 50 controls. The clinical and demographic characteristics of the study participants are shown in Table 1. In all the subject groups, there was no significant difference in the age, cholesterol level and sex distribution, most of CAD-DM patients were hypertensive (46%) compared to control and CAD patients (8% and 18%). Smoking and alcohol consumption was more in CAD group when compared to other groups.

Levels of biochemical parameters and TMAO in Patients and controls

The highest number of MIs occurred in diabetics with mean HbAlc levels of 9.6% compared to controls and CAD patients (5.2% and 5.6%). There was a significant association between increasing HbAlc levels and the incidence of MI. There was no difference in the mean concentration of urea, BUN, creatinine, cholesterol, LDL and VLDL between patients and controls. The level of triglycerides and CPK-MB was increased in patient group when compared to controls and the HDL was higher in controls compared to patient groups. LC-MS/MS analysis was used to determine the concentration of TMAO in plasma. TMAO demonstrated differences in plasma samples between control, CAD, and CAD-T2DM (Fig. 1). The levels of circulating TMAO were higher in CAD patients than in control subjects (2.04±0.61 µM vs. 1.10±0.83 µM). In addition, plasma levels of TMAO were increased in CAD patients with T2DM

compared with control (l.35±1.8lμM vs. 1.10±0.83 μM) but not statistically significant (Table 2). The ROC curve for predicting CAD using TMAO was plotted and the AUC was 0.63(p=0.08). (Fig. 2)

TMAO concentration in consumers of red meat

The concentration of TMAO is lightly higher in red meat consuming CAD patients when compared to patients who are not consuming red meat (2.10±0.60 μM vs. 1.68±0.65 μM). Similarly the level of TMAO is higher in red meat consuming CAD-DM patients when compared to CAD-T2DM patients who are not consuming red meat (1.09±0.88 μM vs. 0.48±0.10 μM). The level of biochemical parameters and TAMO between the red meat consuming patients and the patient's not consuming red meat were shown in Table 3.

Level of TMAO in patients with different age group

The TMAO level is higher in CAD patients with age group of 31-60 years when compared to patients with 61-75 years (1.88±0.94 μM vs. l.69±0.66 μM). Unlikely the TMAO level is slightly higher in CAD-DM patients with age group of 61-75 years when compared to patients with 31-60 years (1.35±1.0 μM vs. 1.33±2.19 μM). The level of biochemical parameters and TAMO between the CAD and CAD-DM with different age groups were shown in Table 4.

Discussion

This study is the first to report an association in the Indian population between plasma TMAO and CAD and CAD-T2DM patients. It was revealed that TMAO was a substantial risk factor for cardiovascular diseases, and that it was an independent predictor of a high atherosclerotic impact and heart failure in individuals. Despite the fact that these prior studies included a significant number of patients, there was no investigation that focused on the relationship between TMAO and Indian CAD/CAD-DM patients with controls. In this cross-sectional study the TMAO concentration was increased in CAD and CAD-DM patients compared to controls but not statistically significant. We found that the TMAO, in addition to the traditional prognostic indicators, was not an independent predictor of CAD in CAD patients.

It has been found that only γBB and L-carnitine levels are elevated in patients with carotid atherosclerosis, however TMAO or TML levels are unaltered, despite the fact that various studies have demonstrated that TMAO plays a causal role in atherogenesis[18]. The result was consistent with that of the previous study. In 2016 Meyer et al., performed a clinical study, in that study they tested the prospective associations between TMAO and coronary artery calcium (CAC) and carotid intima-media thickness (cIMT). TMAO

did not correlate with CAC incidence, CAC development, or cIMT, three indicators of atherosclerosis, in that population-based investigation [19]. These findings suggest that in healthy early-middle-aged adults, TMAO may not have a substantial impact on increasing the risk of early atherosclerosis disease. However, TMAO increased the risk of CVD in patients who had particular histories, such as those undergoing dialysis or infected with HIV. As a result, the involvement of TMAO in atherogenesis remains controversial, in 2019 Sinha et al., reported that the development of atherosclerotic plaque was associated with betaine and L-carnitine rather than basal levels of TMAO and choline [20]. Moreover, after adjusting for confounding variables, there was no significant increase in the odds ratio (OR) for myocardial infarction among subjects with high carnitine levels who were also HIV-positive, but there was an OR increase for those with high levels of betaine, TMAO, or choline. Our findings confirm these previous findings and show that TMAO plays no substantial role in atherogenesis.

Some studies show that TMAO may be most predictive of CVD among individuals with known comorbidities, including diabetes mellitus, heart failure, or chronic kidney disease, though such results have been inconsistently reported [21-23]. Elevated levels of TMAO indicate a higher consumption of TMAO precursors, some of which are also thought to increase the risk of CVD. Statistical correction for dietary precursors such as red meat, eggs, and fish had no significant impact on effect estimates in this study. The significance of TMAO dietary precursors in CVD risk is unclear with red meat being proven to raise CVD risk while fish is thought to be cardioprotective. Furthermore, recent study suggests that taking the TMAO precursor L-carnitine orally may help to reduce plasma lipoprotein(a) [24].

Studies in the general population have shown that TMAO largely mediates the effects of betaine and carnitine on CVD [25]. Despite this well-established relationship, we did not find a significant association between TMAO and CAD in our cohorts. This finding is supported by several previous studies that have shown an inconsistent relationship between TMAO and CVD among People living with HIV. A recent study by Shan et al found an association between incident carotid artery plaque and TMAO in people living with HIV; however, they did not evaluate carnitine or cardiovascular events [26]. Alterations to gut mucosa and microbiota secondary to HIV infection likely alter these metabolites, which could help explain the lack of association seen between TMAO and CVD in people living with HIV compared with the general population. Adding to the growing evidence for the role of gut microbiota in HIV-

associated CVD, Kehrmann et al found signature divergences in the gut microbiota of individuals with and without coronary artery disease in a cohort of PLWH [27].

The specific processes by which TMAO and/or its precursor metabolites worsen atherosclerosis remain unclear. L-carnitine may have an impact on mitochondrial function since it is essential for the movement of fatty acids across the mitochondrial membrane. While L-carnitine may have pro-inflammatory functions, Sinha et al. found no association between L-carnitine levels and high-sensitive CRP, d-dimer, or interleukin-6. In contrast, low-grade inflammation in a German population has been favorably correlated with plasma TMAO level. To address this issue, comprehensive biochemical and/or cell biology research are required.

Conclusion

In this cross-sectional study, we found that red meat and fish were the main dietary sources of TMAO and that plasma TMAO level was not a risk factor for CAD in an aged Indian population. We assume that Indian consuming red meat and fish in diet is not as much as like western countries, and will reduce the impacts of TMAO on atherosclerosis and CAD. Further research is required to validate our findings in other populations and to use a longitudinal study design to demonstrate the causal association between TMAO metabolite and CAD.

Limitation of the study

The main limitation of our study is the small sample size and MACE, which has an effect on the statistical findings. Larger sample sizes are required to conclude this issue. Furthermore, we also did not assess the occurrence of MACE in the control group.

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Conflict of Interest Statement

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

Table 1. Baseline Characteristics of the patients and controls

Characteristics	Control (*n* = 50)	CAD (*n* = 50)	CAD-DM (*n* = 50)
Age	49.2±7.1	52±11.3	55.3±9.7
Hypertensive (%)	4 (8)	9 (18)	23 (46)
Smoking (%)	5 (10)	15 (30)	12 (24)
Alcohol consumption	5 (10)	24 (48)	11 (22)
Hypercholesterolemia	-	5 (10)	4 (8)

Table 2. Biochemical parameters and TMAO level in subjects

Characteristics	Control (*n* = 50)	CAD (*n* = 50)	CAD-DM (*n* = 50)
HbA1c	5.2±0.4	5.6±0.3	9.6±2.1
Urea (mg/dL)	21.5±2.0	26.9±10.9	29.3±14.0
BUN	11.0±1.9	11.8±5.5	14.5±7.0
Creatinine	0.8±0.2	0.9±0.4	1.3±1.5
Cholesterol (mg/dL)	141±19.5	161.5±51.0	164.8±32.5
Triglycerides (mg/dL)	93.7±17.2	181.7±153.7	224.3±204.4
LDL (mg/dL)	95.7±16.3	152.5±119.3	121.3±40.6
VLDL (mg/dL)	19.5±3.6	30.6±16.2	21.7±7.9
HDL (mg/dL)	80.5±8.2	45.3±18.9	45±13.8
CPK-MB (U/L)	19.6±2.9	55.5±71.7	56.2±34.4
TMAO (μM)	1.10±0.83	2.04±0.61	1.35±1.81

Table 3. Biochemical parameters and TMAO level in patients with red meat consumer and not consuming red meat.

Characteristics	CAD		CAD-DM	
	Meat	No Meat	Meat	No Meat
HbA1c	6.10±0.8	5.8±0.8	9.65±2.60	9.9±1.57
Urea (mg/dL)	28.7±8.6	31.3±15.2	30.1±11.4	28.3±2.5

BUN	11.7±4.3	14.3±7.09	15.8±5.6	16.6±2.51
Creatinine	0.97±0.49	1.26±0.81	1.11±0.68	0.97±0.11
Cholesterol (mg/dL)	178.4±55.1	114±1.41	177.3±17.6	140.3±.38.3
Triglycerides (mg/dL)	148.6±65.1	128.5±75.6	223.0±48.6	230.3±63.0
LDL (mg/dL)	138.8±123.4	141.5±0.70	97.8±27.9	87.6±9.50
VLDL (mg/dL)	27.1±11.2	48.0±28.2	21.0±5.2	25.3±3.0
HDL (mg/dL)	48.7±23.8	32.0±5.6	51.7±16.7	42.3±4.9
CPK-MB (U/L)	86.5±42.7	90.0±41.3	60.6±48.4	80.3±21.7
TMAO (µM)	2.10±0.60	1.68±0.65	1.09±0.88	0.48±0.10

Table 4. Biochemical parameters and TMAO levels in patients with different age groups.

Characteristics	**CAD**			**CAD-DM**		
Age	**18-30**	**31-60**	**61-75**	**18-30**	**31-60**	**61-75**
HbA1c	-	5.5±0.36	5.9±0.14	-	10.02±2.3	8.83±0.89
Urea (mg/dL)	40.5±18.5	25.5±9.7	29.2±9.63	-	30.0±16.38	29.2±10.0
BUN	19±9	10.9±4.77	13.45±4.5	-	14.52±8.06	14.68±5.46
Creatinine	0.8±0.1	0.97±0.42	0.88±0.31	-	1.06±0.72	1.02±0.51
Cholesterol (mg/dL)	-	176±54.0	137±15.8	-	178.25±12.5	143±32
Triglycerides (mg/dL)	-	260.8±170.0	102.6±33.0	-	302±154	-
LDL (mg/dL)	-	173±128.9	86±19.1	-	118.6±19.4	124±48.6
VLDL (mg/dL)	-	40.1±14.7	19.2±5.19	-	24.3±6.0	-
HDL (mg/dL)	-	42.2±7.4	49.6±26.1	-	44.5±13.8	45.6±11.3
CPK-MB (U/L)	24.5±2.5	58.9±37.8	66.6±10.2	-	65±85.7	43.4±36.3
TMAO (µM)	-	1.88±0.94	1.69±0.66	-	1.33±2.19	1.35±1.0

Fig. 1. The plasma levels of TMAO in CAD, CAD-T2DM and controls

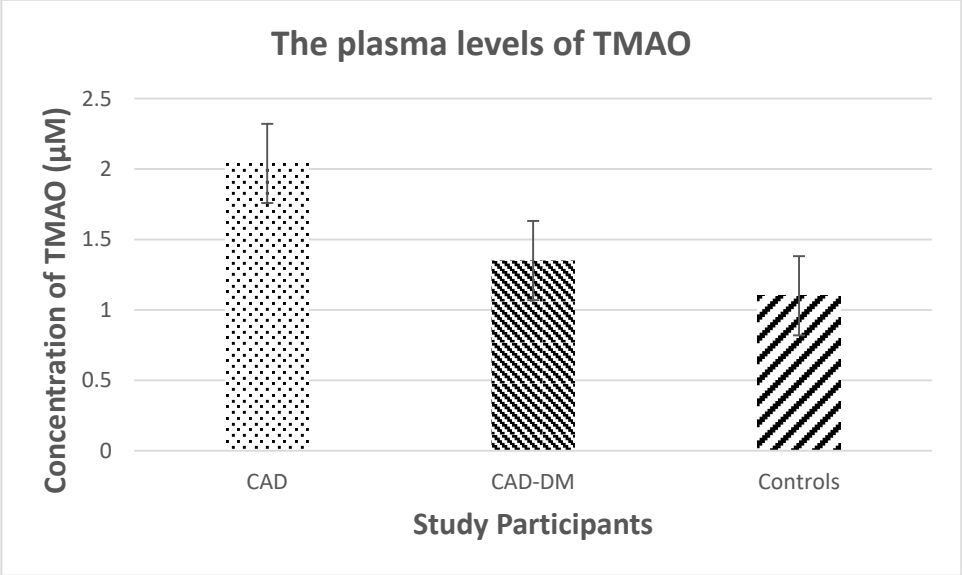
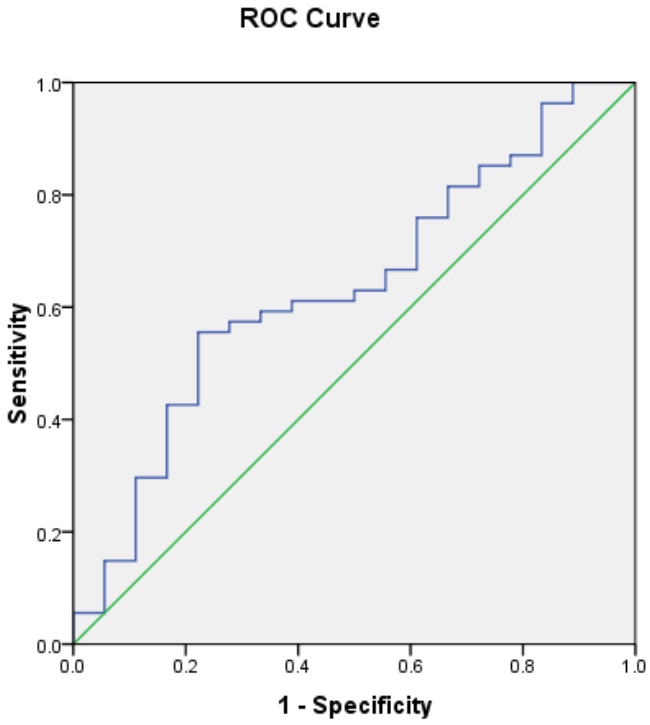


Fig. 2. Receiver operator characteristic analysis of TMAO for predicting CAD.



Area Under the Curve

Test Result Variable(s):CAD TMAO

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
.635	.075	.088	.489	.781

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

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