

ORIGINAL ARTICLE

Is Trimethylamine N-oxide (TMAO) Associated with NF- κ B mRNA Expression in Patients with Coronary Artery Disease?

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Abstract

Background: Trimethylamine N-oxide (TMAO), a gut microbiota metabolite, is associated with cardiovascular disease (CVD) development. TMAO can trigger an inflammatory response by inducing the nuclear factor-kappa B (NF- κ B) signaling cascade and increasing the expression of pro-inflammatory cytokines, contributing to the worsening of CVD. This study aimed to evaluate the association between TMAO plasma levels and inflammation in patients with coronary artery disease (CAD).

Methods: A cross-sectional study was carried out including 29 patients with CAD. Peripheral blood mononuclear cells (PBMC) were isolated from fasting blood samples, and NF- κ B and vascular cell adhesion protein 1 (VCAM1) mRNA expression were estimated using real-time quantitative PCR. We determined TMAO plasma levels by LC-MS/MS and TNF- α by ELISA. Routine biochemical parameters were evaluated using an automatic biochemical analyzer. Correlations were estimated by Spearman or Pearson test. Statistical significance was set at the level of $p < 0.05$.

Results: All patients presented TMAO levels within the normal range according to EUTox (normal range: 2.83 ± 1.53 mg/L; CAD patients: $0.2 [0.1 \text{ to } 0.2]$ ng/ μ L). TMAO plasma levels were positively correlated with NF- κ B mRNA expression (0.555 ; $p = 0.002$).

Conclusion: TMAO plasma levels may be associated with NF- κ B mRNA expression in patients with CAD and may contribute to the pathogenesis of this disease.

Keywords: Coronary Artery Disease; Inflammation; Oxidative Stress; NF-kappa B.

Introduction

Metabolites originating from an imbalanced gut microbiota have been reported to play an essential role in the development of cardiovascular diseases (CVD).¹⁻⁴ Distinct gut microbiota species contribute to several metabolites that can impact human health,^{1,5} such as the generation of trimethylamine (TMA) by *Clostridia*, *Shigella*, *Proteus*, and *Aerobacter*. TMA is transformed into trimethylamine N-oxide (TMAO) via hepatic flavin monooxygenase 3.^{2,6,7}

TMAO has been linked to the development and progression of chronic diseases, such as atherosclerosis

and cardiometabolic diseases.^{2,5} In recent years, a growing body of evidence has described the relationship between gut microbiota, the immunomodulatory effects of TMAO, and atherosclerosis plaque formation.⁸⁻¹⁰ Indeed, circulating TMAO levels can be used as an independent predictor of cardiovascular risk.^{11,12}

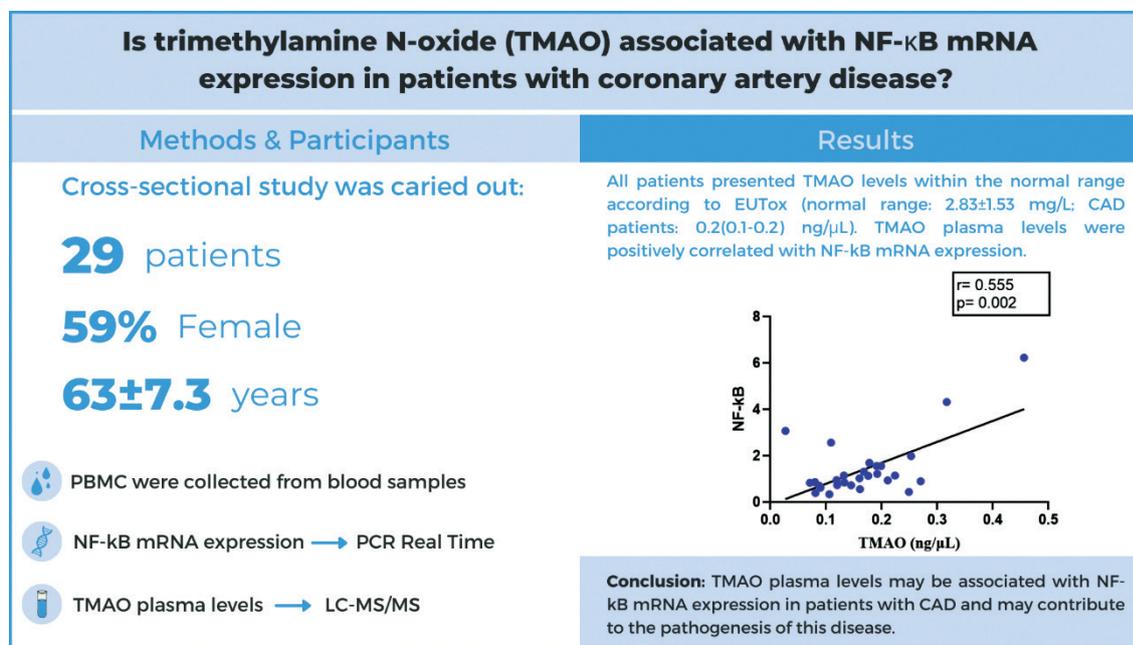
Also, elevated plasma TMAO levels induce a nuclear factor- κ B (NF- κ B) signaling cascade and increase the expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), adhesion molecules, and chemokines, intensifying the inflammatory response within endothelial and smooth muscle cells.¹³

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Central Illustration: Is Trimethylamine N-oxide (TMAO) Associated with NF-κB mRNA Expression in Patients with Coronary Artery Disease?



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PCR Real Time ⊕ Real-time PCR. EUTox (normal range: 2.83±1.53 mg/L; CAD patients: 0.2(0.1-0.2) ng/μL). ⊕ EUTox (normal range: 2.83 ± 1.53 mg/L; CAD patients: 0.2 [0.1 to 0.2] ng/μL). NF-κB ⊕ NF-κB. CAD: coronary artery disease; NF-κB: nuclear factor-kappa B; PBMC: peripheral blood mononuclear cells; TMAO: trimethylamine N-oxide; LC-MS/MS: Liquid Chromatography-Mass Spectrometry.

Furthermore, TMAO can induce atherosclerosis through several mechanisms; it has been correlated with some biomarkers of inflammation and endothelial dysfunction such as TNFα, interleukin-6 (IL-6), and endothelin-1 (ET-1),^{14,15} and it could be used as a biomarker to predict CVD prevalence and elevated incidence of main adverse cardiovascular events, such as stroke and myocardial infarction.^{11,12,14,16} Thus, understanding atherosclerotic immunopathology induced by changes in host microbiota can lead to identifying new therapeutic targets in CVD treatment.^{10,17} The objective of this study was to evaluate the association of circulating TMAO levels with inflammatory markers in patients with coronary artery disease (CAD).

Methods

Study sample

This study included 29 patients over 18 years of age with a previous diagnosis of CAD or abnormal findings on myocardial perfusion scintigraphy through a convenience sample. Data about the presence of

comorbidities, such as diabetes mellitus, hypertension, and dyslipidemia, were collected from medical records. This is a secondary investigation of a randomized controlled clinical trial that analyzed the effects of a Brazil nut-enriched diet on oxidative stress and transcription factor mRNA expression involved with inflammation in patients with CAD.¹⁸ Patients with autoimmune and infectious diseases, HIV, chronic kidney disease, and cancer and women who were pregnant or lactating were not included in this study.

All data presented in the current study were collected at the baseline of the previous clinical trial.¹⁸ The written consent form was obtained from all participants at the beginning of the study.

Myocardial perfusion scintigraphy

Myocardial perfusion scintigraphy is a non-invasive procedure that uses physical or pharmacological stress to identify the presence of ischemia. The main application of myocardial perfusion scintigraphy is in the diagnosis and prognostication of CVD. Subjects with ischemia on myocardial perfusion scintigraphy

present a higher risk for adverse outcomes than those with a normal test.^{19,20}

Anthropometric assessment

Anthropometric assessments were conducted by a trained dietitian using standard techniques. Body mass index (BMI) was estimated by the ratio of weight in kilograms to height in meters squared.²¹

Blood sample collection and isolation of peripheral blood mononuclear cells (PBMC)

Fasting blood samples were taken by venous puncture in a tube containing EDTA (1.0 mg/ml) as an anticoagulant. Plasma was separated by centrifugation (15 min, 3500 rpm, 4 °C) and stored in tubes at -80 °C until analysis. Blood samples with EDTA were diluted in PBS, and cells were separated into 5 ml Histopaque (Sigma-Aldrich) by centrifugation (1700 rpm, 30 min, 18 °C). PBMC were isolated and washed twice with cold PBS and re-suspended and stored (-80 °C) with 1 ml of recovery cell culture freezing medium (Invitrogen) for RNA isolation.

Biochemical analysis

Glucose, ultra-sensitive C-reactive protein (CRP), total cholesterol, high-density lipoprotein (HDL), triglycerides, urea, creatinine, alanine aminotransferase, and aspartate aminotransferase plasma levels were determined using Bioclin® kits and an automatic biochemical analyzer (Bioclin BS-120 chemistry analyzer). Low-density lipoprotein (LDL) was calculated by the Friedwald equation:²²

$$\text{LDL} = (\text{total cholesterol}) - (\text{HDL} - \text{triglycerides}/5)$$

Castelli's risk index was estimated to evaluate atherogenic indices. Castelli risk index I refers to the ratio of total cholesterol to HDL, and Castelli risk index II to the ratio of LDL to HDL. Castelli index I < 4.3 and Castelli index II < 2.9 were considered reference values.²³

TNF plasma levels were measured by Peprotech® ELISA kits according to manufacturer protocol. Plasma levels of TMAO were determined by LC-MS/MS, according to Missailidis et al.²⁴ Briefly, aliquots obtained from plasma were coded with internal standards consisting of TMAO-D9 in methanol and water as a recovery standard. TMAO, choline, and betaine were

then detected with an Agilent 6490 triple quadrupole mass spectrophotometer.

Gene expression

NF- κ B and *VCAM-1* mRNA expressions in PBMCs were estimated using real-time quantitative PCR, according to Cardozo et al.²⁵ To detect *NF- κ B* (Hs00765730_m1), *VCAM-1* (Hs01003372_m1), and the control gene *GAPDH* (Hs02758991_g1), TaqMan Gene Expression Assays (Applied Biosystems) were used. PCR amplification was executed using the ABI Prism 7500 Sequence Detection System (Applied Biosystems) and standard cycling conditions. *NF- κ B* and *VCAM-1* mRNA expressions were normalized against *GAPDH*, and the expression levels were calculated using the delta-delta threshold cycle method.

Statistical analysis

Data distribution was evaluated by the Shapiro-Wilk test and is presented as the mean \pm standard deviation or median and interquartile range (p25-p75), according to data normality. The correlations between variables were estimated by Spearman or Pearson correlation tests. Linear regression analysis was used to verify the impacts of age, sex, and BMI on TMAO correlations; the necessary assumptions for linear regression were met. Statistical significance was set at the level of $p < 0.05$. Categorical variables were tested using the chi-square test and were presented as frequencies (%). The statistical analyses were executed by SPSS 20 software (Chicago, IL, USA) and GraphPad Prism version 8 (San Diego, CA, USA).

Results

Table 1 shows the general characteristics and anthropometric parameters of the patients. According to BMI, 69% of patients were overweight or obese. No patients reported using food or dietary supplements. Only 3 participants were smokers, and 9 reported drinking alcohol regularly.

As shown in Table 2, all patients had normal TMAO according to the European Uremic Solutes Database (EUTox-dB).^{26,27}

Positive correlations were found between TMAO and NF- κ B mRNA expression ($r = 0.555$; $p = 0.002$) (Figure 1), independently of age, sex, and BMI. No correlation was found between TMAO and other inflammatory markers, such as TNF- α , CRP, and VCAM-1 mRNA expression.

Table 1 – General characteristics and anthropometric parameters of patients with CAD

Parameters	CAD patients (n=29)
Age (years)	63±7.3
Male/Female	12/17
Hypertension (%)	100
Diabetes mellitus (%)	41
Dyslipidemia (%)	79
BMI (kg/m ²)	28.9±4.9

Data are expressed as mean ± standard deviation or median (p25-p75). CAD: coronary artery disease; BMI: body mass index.

Table 2 – Biochemical parameters and gene expression of patients with CAD

Parameters	CAD patients	Reference values
Glucose (mg/dL)	93.0(81.0-118.0)	<99
CRP (mg/dL)	1.8(0.8-6.5)	<0.9
Total cholesterol (mg/dL)	147.0(132.0-185.5)	<200
HDL (mg/dL)	45.3±10.5	>60
LDL (mg/dL)	81.1±30.8	<100
Triglycerides (mg/dL)	101.0(75.2-173.5)	<150
Castelli index I (mg/dL)	3.4(2.8-3.9)	<4.3
Castelli index II (mg/dL)	1.8±0.7	<2.9
Urea (mg/dL)	25.6±5.4	6 to 24
Creatinine (mg/dL)	0.6±0.2	Men: 0.74 to 1.35 Women: 0.59 to 1.04
Aspartate aminotransferase (U/L)	19.0±8.3	8 to 33
Alanine aminotransferase (U/L)	8.5(5.0-11.7)	4 to 36
TNF-α (pg/mL)	124.3(98.6-154.3)	13.3±3.0
TMAO (ng/μL)	0.2(0.1-0.2)	2.83±1.53
NF-κB mRNA expression (a.u.)	1.0(0.7-1.6)	-
VCAM-1 mRNA expression (a.u.)	0.8(0.6-2.1)	-

Data are expressed as mean ± standard deviation, and non-parametric data are expressed as median (p25-p75). CAD: coronary artery disease; TMAO: trimethylamine N-oxide; TNF: tumor necrosis factor; CRP: C-reactive protein; HDL: high-density lipoprotein; LDL: low-density lipoprotein.

Discussion

This short report showed a positive correlation between TMAO plasma levels and *NF-κB* mRNA expression in patients with CAD. To our knowledge, no clinical study has reported a correlation between TMAO and inflammatory markers in patients with CAD.

TMAO can promote atherosclerosis by changing cholesterol metabolism, inhibiting bile acid synthesis, stimulating foam cell formation by activating CD36 scavenger receptor, and stimulating platelet aggregation, thus contributing to acute coronary syndrome.^{4,9,28,29} Indeed, TMAO has been associated with aortic plaque lesion area and the risk of developing CAD, peripheral artery disease, and stroke.³⁰ Many clinical studies have demonstrated that high TMAO levels are related to several adverse cardiac events, including death in patients with heart failure, left ventricular diastolic dysfunction, chronic kidney disease, and atherosclerotic CAD.^{24,31-34}

Furthermore, increased TMAO levels induce vascular inflammation and oxidative stress.¹³ Accordingly, elevated TMAO levels are related to a greater expression of pro-inflammatory cytokines, such as TNF, and direct activation of the *NF-κB* signaling pathway.^{13,35} The results of this study are supported by experimental studies that demonstrated an association between TMAO levels and inflammatory biomarkers.^{13,28,35-37} Geng et al.²⁸ showed *in vitro* that TMAO promotes inflammation induction via upregulated TNF, IL-6, and ICAM1 gene transcription in atherosclerotic plaque lesions. Moreover,

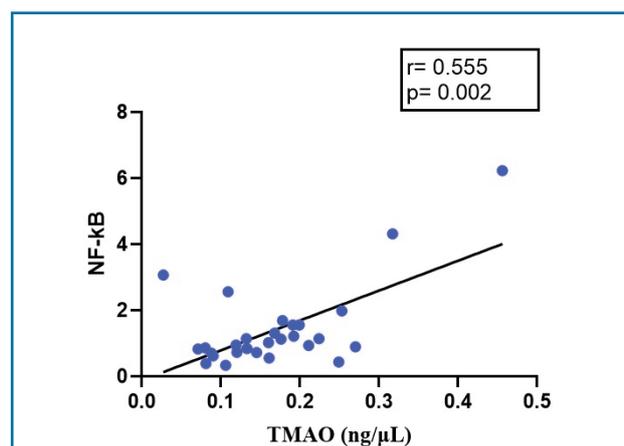


Figure 1 – Positive correlation between baseline plasma TMAO (ng/μL) and NF-κB mRNA expression (r = 0.555; p = 0.002) in patients with CAD (n = 29).
NF-κB: nuclear factor-kappa B; TMAO: trimethylamine N-oxide

Seldin et al.¹³ have demonstrated that treatment with TMAO in endothelial and smooth muscle cells increased inflammatory markers and promoted the increase of NF- κ B mRNA expression. Chen et al.³⁵ showed that obese mice presented high TMAO levels and inflammatory cytokines, such as TNF and IL-1 β . A study showed a positive correlation between TMAO concentrations and low-grade inflammation, where adult patients with high TMAO plasma levels had higher TNF- α plasmatic levels.³⁶ Also, in apparently healthy individuals, high TMAO plasma levels were associated with CAD development.³⁸ In patients with angina pectoris, TMAO levels were associated with inflammatory markers, including IL-1 β and CRP.³⁷ High levels of the inflammatory biomarker CRP are well associated with atherosclerosis complications and CVD risk. Moreover, an *in vitro* study carried out in endothelial progenitor cells demonstrated that TMAO induced inflammation and elevated oxidative stress.^{37,39}

Several pathways by which TMAO can activate inflammation have been suggested. First, TMAO can interfere with the expression of pro-inflammatory cytokines from NF- κ B, leading to the phosphorylation of NF- κ B, thereby promoting the production of cyclooxygenase 2, IL-6, E-selectin, and ICAM1.¹³ Second, TMAO stimulates oxidative stress, an essential activator of the inflammasome, and consequently promotes the activation and expression of the TXNIP-NLRP3 pathway, leading to the production of inflammatory cytokines (1 β and IL-18).⁴⁰ Finally, TMAO induces inflammation through NLRP3 by inhibiting the SIRT3-SOD2-mtROS signaling pathway.⁴¹ Sirtuin 3 (SIRT3) is an enzyme that modulates mitochondrial reactive oxygen species (mtROS) production, leading to the expression of mitochondrial antioxidant enzymes as manganese superoxide dismutase 2 (SOD2).^{42,43} Thus, TMAO inhibits SOD2 and SIRT3 expression and activity, promoting oxidative stress and activation of NLRP3 and pro-inflammatory cytokines.⁴¹

Although we report no association of TMAO levels with VCAM-1 expression, it should be highlighted that TMAO stimulates the VCAM-1 expression in human umbilical vein endothelial cells through the activation of the NF- κ B pathway.⁴⁴ Thus, the increased expression of these molecules can contribute to inflammation, endothelial dysfunction, foam cell production, and atherosclerosis plaque formation.⁴⁴⁻⁴⁶

Due to the strong association between TMAO, inflammation, and atherosclerosis, gut microbiota modulation has been suggested to be a novel therapeutic

target for CVD.^{17,47,48} The main strategies include dietary intervention, fecal microbiota transplantation, antimicrobials, bacterial enzyme inhibitors, prebiotics, or probiotics.¹⁰ Among these, nutritional interventions have been highlighted for improving the gut microbiota composition and inhibiting TMA-producing bacteria.⁷ Indeed, choline, betaine, and L-carnitine obtained from dietary sources like red meat, milk, eggs, and fish enter the gastrointestinal tract where they can undergo metabolism by bacteria, including *Clostridia*, *Shigella*, *Proteus*, and *Aerobacter*. This metabolic process leads to the generation of TMA, which subsequently gets absorbed and, upon reaching the liver, undergoes further transformation into TMAO through the action of the enzyme flavin monooxygenase 3.^{2,6,7} Thus, "food as medicine" can be considered a promising strategy to reduce TMAO levels and disease.⁴⁹

This short report should be interpreted with some limitations, the main one being the small sample size. In addition, the small sample size did not allow a sample stratification to analyze other associations as TMAO levels and NF- κ B expression in participants with diabetes. Furthermore, no other inflammatory markers were analyzed. Thus, further studies with a larger sample size are necessary to elucidate the correlation between TMAO and inflammatory pathways and whether this association translates into clinical effects.

Conclusions

In conclusion, the association between TMAO and NF- κ B indicates that this gut metabolite may be a relevant target to regulate a persistent inflammatory phenotype in patients with CAD. Further understanding of these intriguing links may provide a staggering opportunity for better treatment of CVD.

Acknowledgments

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Author Contributions

Conception and design of the research: Da Cruz B, Coutinho-Wolino KS, Cardozo L, Stockler-Pinto MB;

acquisition of data: Da Cruz B, Mesquita CT; analysis and interpretation of the data: Da Cruz B, Coutinho-Wolino KS, Cardozo L, Mafra D, Stockler-Pinto MB; statistical analysis: Da Cruz B, Coutinho-Wolino KS, Pereira AD; obtaining financing: Stockler-Pinto MB; writing of the manuscript: Da Cruz B, Coutinho-Wolino KS; critical revision of the manuscript for intellectual content: Cardozo L, Pereira AD, Mesquita CT, Stenvinkel P, Bergman P, Mafra D, Stockler-Pinto MB.

Potential Conflict of Interest

No potential conflict of interest relevant to this article was reported.

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Study Association

This article is part of the research project of associate professor submitted by Ludmila Cardozo, from Universidade Federal Fluminense.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Faculty of Medicine of the Universidade Federal Fluminense under the protocol number 826.041. All the procedures in this study were in accordance with the 1975 Helsinki Declaration, updated in 2013. Informed consent was obtained from all participants included in the study.

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