# Gut microbiota–derived metabolites and risk of coronary artery disease: a prospective study among US men and women

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## ABSTRACT

**Background:** Accumulating evidence has suggested that human gut microbiota metabolize certain dietary compounds and subsequently produce bioactive metabolites that may exert beneficial or harmful effects on coronary artery disease (CAD) risk.

**Objectives:** This study examined the joint association of 2 gut microbiota metabolites, enterolactone and trimethylamine N-oxide (TMAO), that originate from intake of plant-based foods and animal products, respectively, in relation to CAD risk.

**Methods:** A prospective nested case—control study of CAD was conducted among participants who were free of diabetes, cardiovascular disease, and cancer in the Nurses' Health Study II and the Health Professionals Follow-up Study. Plasma concentrations of enterolactone and TMAO, as well as choline and L-carnitine, were assayed among 608 CAD case-control pairs.

**Results:** A high enterolactone and low TMAO profile was associated with better diet quality, especially higher intake of whole grains and fiber and lower intake of red meats, as well as lower concentrations of plasma triglycerides and C-reactive protein. Participants with a high enterolactone/low TMAO profile had a significantly lower risk of CAD: the multivariate-adjusted OR was 0.58 (95% CI: 0.38, 0.90), compared with participants with a low enterolactone/high TMAO profile. No significant interaction between enterolactone and TMAO on CAD risk was observed. Neither TMAO nor enterolactone alone were associated with CAD risk in pooled analyses. In women, a higher enterolactone concentration was significantly associated with a 54% lower CAD risk ($P$ trend = 0.03), although the interaction by sex was not significant.

**Conclusions:** Our results show that a profile characterized by high enterolactone and low TMAO concentrations in plasma is linked to a healthful dietary pattern and significantly associated with a lower risk of CAD. Overall, these data suggest that, compared with individual markers, multiple microbiota-derived metabolites may facilitate better differentiation of CAD risk and characterization of the relations between diet, microbiota, and CAD risk. *Am J Clin Nutr* 2021;114:238–247.

**Keywords:** coronary artery disease, gut microbiota, metabolites, epidemiology, prospective study

## Introduction

Healthful dietary patterns, such as the Alternative Healthy Eating Index (AHEI), healthful plant-based diet index (hPDI), Dietary Approaches to Stop Hypertension (DASH), and Mediterranean diet, share some key common components, including higher intake of whole grains, fruits, and vegetables and lower intake of animal products, especially red meats, processed meats, and their constituents (1–4). These healthful diets are associated with a lower risk of developing coronary artery

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Abbreviations used: AHEI, Alternative Healthy Eating Index; CAD, coronary artery disease; CRP, C-reactive protein; CVD, cardiovascular disease; hPDI, healthful plant-based diet index; HPFS, Health Professionals Follow-Up Study; MET, metabolic equivalents; MI, myocardial infarction; NHSII, Nurses' Health Study II; TMAO, trimethylamine N-oxide.

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disease (CAD) (1–3). Accumulating evidence suggests that metabolites exclusively produced by microbiota from foods that characterize healthful dietary patterns may modulate CAD risk and thus constitute an important pathway through which diet influences CAD risk (5–7). Enterolignans and trimethylamine N-oxide (TMAO) represent 2 groups of gut microbiota–derived metabolites that come from 2 distinct groups of foods and may have opposite cardiovascular effects (8–11).

Enterolignans, primarily enterolactone and enterodiol, are metabolites exclusively produced by gut microbiota from plant lignans that are commonly present in flax seeds, other seeds, whole grains, legumes, coffee, fruits, and vegetables (8, 9). TMAO is a gut microbiota metabolite of choline and L-carnitine, which are abundant in red meats and other animal products (10, 12, 13). Both animal experiments and human studies have suggested that enterolignans might confer health benefits related to cardiometabolic diseases (8, 14), whereas TMAO may promote cardiovascular disease (CVD) (15, 16). Despite the accumulating evidence from animal and human experiments that demonstrates the contrasting cardiovascular effects of these 2 groups of metabolites, epidemiological evidence regarding these compounds and CAD risk is somewhat mixed. For example, some but not all prospective human studies have shown an inverse association between enterolignan concentrations and CAD risk (17–20). Likewise, abundant evidence has suggested potential adverse effects of TMAO on atherosclerosis and CVD, especially among high-risk individuals, such as patients undergoing elective diagnostic cardiac catheterization or patients with prevalent kidney disease (10, 11, 21, 22). However, some recent epidemiological studies in more generalized, healthy populations found no association for TMAO (23–25). These previous studies are subject to a few common limitations, such as small sample size, inclusion of heterogeneous CVD outcomes, confounding by existing chronic conditions, and insufficient adjustment of covariates (e.g., physical activity and dietary factors). In addition, given the close relations among diet, gut microbiota, and related metabolites and that a dietary pattern often exerts stronger health effects than its individual components (26), it is biologically plausible that a combination of the gut microbiota metabolites may also help better differentiate CAD risk, although this has not been examined.

To fill these knowledge gaps, we aimed to prospectively investigate the joint associations of enterolactone and TMAO with CAD risk in 2 well-characterized cohorts, Nurses' Health Study II (NHSII) and Health Professionals Follow-Up Study (HPFS) participants. We hypothesized that a high enterolactone and low TMAO profile is significantly associated with a lower CAD risk. We further examined the relation of these 2 biomarkers with dietary factors and CVD risk markers in this analysis.

## Methods

### Study population

NHSII is an ongoing prospective cohort study of 116,430 US female registered nurses, aged 25 to 42 y, who were enrolled in 1989. HPFS consists of 51,529 US male health professionals, aged 40 to 75 y, who responded to a baseline questionnaire in 1986. Information on demographics, lifestyle, and medical history was assessed at baseline and updated every 2 y through self-administered questionnaires in both cohorts. A total of 18,159 men provided blood samples in 1993–1995 in HPFS, and 29,611 NHSII women provided blood samples in 1995–2000. The blood samples were shipped to a central biorepository via overnight courier, and then immediately processed, divided into aliquots, and dispensed into cryotubes as plasma, buffy coat, and red blood cells, which were stored in the vapor phase of liquid nitrogen freezers at $\leq$-130°C (9).

### Nested case-control study design

Among pariticipants who provided blood samples and were free of CVD and cancer at sample collection, we identified 608 participants with incident CAD (187 women in NHSII and 421 men in HPFS) between blood draw and June 2017. Using the risk-set sampling approach, controls were selected randomly among participants who were free of CVD at the time the index CAD case was diagnosed and matched in a 1:1 ratio to cases for age at blood sample collection, sex, month of sample collection, and smoking status (**Supplementary Figure 1**). Participants from the 2 cohorts were pooled to maximize statistical power in the absence of heterogeneity in results between the 2 cohorts. The current study was approved by the institutional review board of the Brigham and Women's Hospital and the Human Subjects Committee Review Board of Harvard TH Chan School of Public Health, and the return of the questionnaires was considered implied consent.

### Ascertainment of CAD

Incident CAD included nonfatal myocardial infarction (MI) and fatal CAD. We requested permission to review medical records when participants reported having a nonfatal MI on any biennial questionnaires. Study physicians who were blinded to the participant questionnaire reports reviewed all medical records. MI was ascertained using the World Health Organization criteria, including typical symptoms, elevated cardiac enzyme concentrations, and characteristic electrocardiographic findings (27). Deaths were identified from the National Death Index, or reports by next of kin or postal authorities. Fatal CAD was ascertained if CAD was listed as the cause of death on the death certificate and the history of CAD was evident through reviewing hospital records or autopsy reports.

### Laboratory measurements

In the current study, samples from each case-control pair were shipped together, handled identically, and analyzed in the same run by the same technicians in a random sequence. Plasma concentrations of TMAO and its precursors, including L-carnitine and choline, were measured using an established stable isotope dilution HPLC with ESI-MS/MS. Plasma concentrations of enterolactone were measured by using ESI orbitrap LC-MS (28). In the current analysis, we measured enterolactone only, which accounts for the vast majority of total enterolignan levels in the circulation. The average intra-assay CV was 3.0% for enterolactone, 1.5% for TMAO, 3.1% for L-carnitine, and 2.8% for choline in NHSII; or 5.9% for enterolactone, 10.5%

for TMAO, 5.9% for L-carnitine, and 7.7% for choline in HPFS.

In addition, triacylglycerol , total cholesterol, HDL cholesterol, C-reactive protein (CRP), and glycated hemoglobin (HbA1c) were also measured for the current analysis. A Cobas MiraPlus chemistry autoanalyzer (Roche) was used to measure total and HDL cholesterol (Pointe Scientific, kits H7510 and H7545), and triglycerides (Pointe Scientific, kit T7532). LDL cholesterol was derived using the Friedewald equation, which is based on amounts of triglycerides, total and HDL cholesterol for a valid range of triglyceride concentrations (29). The assays for these biomarkers have been documented elsewhere (30, 31).

### Assessment of diet and other covariates

Diet was assessed using a validated semiquantitative FFQ that inquired about intake of 130 food items every 4 y in NHSII (since 1991) and HPFS (since 1986) (32). The FFQs were designed to estimate the usual diet consumed over the past year. The validity and reproducibility of the assessments of individual food items, including fruits, vegetables, red meats, beverages, and whole grains, have been demonstrated in previous studies (33, 34). Nutrient intake was computed by multiplying the frequency of consumption of each relevant food item by its content of the nutrient and then summing intake of the nutrient across all food items. AHEI was derived to evaluate the overall quality of diet. A higher score indicated a better diet quality. In addition, hPDI was created based on nutrient and culinary similarities within the broad categories of healthy plant foods, less healthy plant foods, and animal foods (1). A higher index reflected higher intake of healthy plant foods and lower intake of animal foods. The details of the 2 diet indices have been described previously (1, 35).

Information on demographics, physical activity, smoking status, alcohol consumption, menopausal status, and use of postmenopausal hormones (women only), family history of MI or cancer, medical history, and presence of hypertension, hypercholesterolemia, CVD, cancer, or other diseases was updated via biennial questionnaires. BMI was calculated as self-reported weight in kilograms divided by the square of height in meters (kg/m$^2$). Physical activity was estimated as metabolic equivalents (METs)/wk based on the average hours spent on various activities (32).

### Statistical analysis

Partial Spearman correlation coefficients ($r_s$) were calculated among controls to examine the correlations between enterolactone, TMAO, L-carnitine, choline, dietary factors, and other CVD risk factors, with multivariate adjustment for age, sex, month of sample collection, smoking status, alcohol consumption, BMI, physical activity, family history of MI, aspirin use, and fish intake. Covariates were primarily derived from the questionnaires administered in 1994 in HPFS and 1995 in NHSII when blood samples were collected.

Participants were grouped into tertiles according to the distribution of metabolites among controls. We also categorized the study population into 4 groups according to a combination of enterolactone and TMAO concentrations. Low or high TMAO and enterolactone concentrations were sex based and assigned according to the median levels among the controls. Conditional logistic regression was applied to examine the associations between these metabolites and CAD risk. In addition to matching factors (i.e., age, sex, month of sample collection, fasting status at time of collection, and smoking status), we also adjusted for alcohol intake (0, 0.1–4.9, 5.0–9.9, ≥10.0 g/d), physical activity (METs-h/wk), BMI (≤24.9, 25.0–29.9, ≥30.0 kg/m$^2$), family history of MI (yes or no), aspirin use (yes, no), fish intake (servings/d), AHEI score, and presence of diabetes, hypertension, or hypercholesterolemia (yes, no). Missing data (<0.5%) on the covariates were replaced with valid values assessed in the previous cycle (i.e., questionnaires administered in 1992 in HPFS and 1993 in NHSII). $P$ values for linear trend were calculated by modeling the median value of each tertile as a continuous variable. A Wald test was used to calculate $P$ value for an interaction term between TMAO and enterolactone (as continuous variables). Restricted cubic spline regressions with 3 knots were applied to test the dose–response relation of these metabolites with CAD risk after excluding participants in the lowest 5% and highest 5% of these metabolites to minimize potential impact of outliers (36). Tests for nonlinearity were based on the likelihood ratio test, comparing the model with linear term only to the model with the linear plus cubic spline terms. In addition, we examined the extent to which the associations of AHEI and hPDI with CAD risk could be explained by high enterolactone/low TMAO profile, using an SAS macro %MEDIATE based on the work by Lin et al. (37). Likewise, we estimated the extent to which the association of high enterolactone/low TMAO profile with CAD risk could be explained by lipids, inflammation, and HbA1c.

All statistical analyses were performed with SAS software, version 9.4 (SAS Institute Inc.). Two-sided $P < 0.05$ was considered statistically significant.

## Results

The characteristics of the CAD cases and controls at blood collection in NHSII and HPFS are shown in **Table 1**. CAD cases and controls had similar distributions of the matching factors. Otherwise, CAD cases had a high-risk profile in both cohorts, compared with controls. For example, CAD cases had a higher BMI, were more likely to have a family history of MI, and a history of hypercholesterolemia, hypertension, and diabetes, and had higher levels of blood lipids, CRP, and HbA1c than controls. The characteristics of CAD cases and controls in pooled population of the 2 cohorts are shown in **Supplementary Table 1**.

**Table 2** presents the baseline characteristics according to joint distributions of enterolactone and TMAO in the pooled population. Compared with participants with the low enterolactone and high TMAO profile, individuals with a high enterolactone and low TMAO profile had a higher level of physical activity, drank more alcohol, and had lower levels of lipids and CRP. **Table 3** demonstrates the least-square mean of dietary intake according to joint distributions of enterolactone and TMAO in the pooled population. Comparing with participants with the low enterolactone and high TMAO profile, individuals with high enterolactone and low TMAO profile had a significantly higher

**TABLE 1** Baseline characteristics of CAD cases and controls in NHS II and HPFS ($n = 1216$)[1]

| Characteristics | NHS II | | | HPFS | | |
|---|---|---|---|---|---|---|
| | Cases (n = 187) | Controls (n = 187) | *P* value | Cases (n = 421) | Controls (n = 421) | *P* value |
| Demographics and lifestyle factors | | | | | | |
| Age at blood draw, y | 45.7 (4.1) | 45.7 (4.2) | 0.97 | 63.7 (8.7) | 63.6 (8.7) | 0.92 |
| BMI, kg/m$^2$ | 27.9 (6.9) | 25.4 (5.0) | <0.001 | 26.1 (3.2) | 25.6 (3.2) | 0.03 |
| Physical activity, METs-h/wk | 9.6 (4.2, 20.9) | 11.7 (4.0, 22.9) | 0.23 | 25.6 (11.3, 44.7) | 28.0 (13.1, 53.8) | 0.06 |
| Smoking status, % | | | 0.54 | | | 0.65 |
| Current smoker | 23.0 | 19.3 | | 8.1 | 6.4 | |
| Former smoker | 19.3 | 23.0 | | 48.6 | 49.3 | |
| Never smoker | 57.7 | 57.7 | | 43.3 | 44.3 | |
| Menopause status, yes, % | 34.8 | 36.9 | 0.67 | — | — | — |
| Family history of MI, % | 33.2 | 20.9 | 0.007 | 42.8 | 31.1 | <0.001 |
| Hypercholesterolemia, % | 38.0 | 26.7 | 0.02 | 50.4 | 40.4 | 0.004 |
| Hypertension, % | 23.0 | 11.2 | 0.003 | 38.2 | 29.7 | 0.009 |
| Diabetes mellitus, % | 6.4 | 0.5 | 0.002 | 8.6 | 4.8 | 0.03 |
| Aspirin use, % | 29.4 | 24.6 | 0.29 | 25.9 | 22.8 | 0.30 |
| Dietary factors | | | | | | |
| Total energy, kcal/d | 1859.6 (522.2) | 1748.7 (451.9) | 0.03 | 2004.7 (529.6) | 2012.2 (522.2) | 0.84 |
| Alcohol, g/d | 0.6 (0.0, 2.8) | 1.0 (0.0, 4.9) | 0.13 | 4.6 (1.0, 13.7) | 8.4 (1.9, 19.0) | <0.001 |
| Fruits, serving/d | 1.8 (1.2) | 1.7 (1.2) | 0.72 | 2.6 (1.4) | 2.6 (1.2) | 0.94 |
| Vegetable, serving/d | 2.7 (1.4) | 2.5 (1.2) | 0.34 | 3.3 (1.6) | 3.4 (1.5) | 0.30 |
| Red meats, serving/d | 0.9 (0.6) | 0.7 (0.4) | <0.001 | 1.2 (0.7) | 1.1 (0. 7) | 0.24 |
| Fish, serving/d | 0.3 (0.2) | 0.2 (0.1) | 0.02 | 0.3 (0.2) | 0.3 (0.2) | 0.65 |
| Egg, serving/d | 0.2 (0.2) | 0.1 (0.1) | 0.02 | 0.3 (0.2) | 0.3 (0.3) | 0.20 |
| Whole grains, g/d | 19.9 (11.8) | 21.5 (16.1) | 0.26 | 26.9 (17.3) | 26.9 (15.7) | 0.99 |
| Alternate healthy eating index | 47.8 (9.6) | 49.3 (9.8) | 0.14 | 41.3 (8.9) | 42.0 (8.6) | 0.24 |
| Healthful plant-based diet index | 53.6 (7.6) | 54.9 (7.3) | 0.07 | 55.0 (7.3) | 55.4 (7.1) | 0.43 |
| Cardiovascular risk markers* | | | | | | |
| Total cholesterol, mg/dL | 210.1 (41.3) | 202.2 (33.5) | 0.04 | 210.2 (39.9) | 201.4 (34.4) | 0.001 |
| LDL cholesterol, mg/dL | 151.0 (37.9) | 144.7 (30.4) | 0.08 | 136.0 (34.4) | 127.7 (30.7) | <0.001 |
| HDL cholesterol, mg/dL | 28.6 (11.4) | 33.3 (10.1) | <0.001 | 42.6 (11.3) | 47.4 (13.6) | <0.001 |
| Triglyceride, mg/dL | 152.3 (99.9) | 120.5 (68.7) | <0.001 | 158.3 (89.7) | 133.3 (75.0) | <0.001 |
| hsCRP, mg/L | 2.7 (1.0, 5.8) | 1.6 (0.5, 3.8) | 0.002 | 1.4 (0.6, 2.7) | 1.0 (0.5, 2.1) | 0.002 |
| HbA1c, % | 5.4 (1.1) | 5.1 (0.3) | <0.001 | 5.8 (1.0) | 5.6 (0.7) | 0.001 |
| Gut microbiota–related metabolites | | | | | | |
| TMAO, $\mu$M | 3.8 (2.6, 6.4) | 3.7 (2.7, 5.9) | 0.55 | 4.0 (2.6, 6.3) | 3.5 (2.4, 5.6) | 0.05 |
| L-carnitine, $\mu$M | 42.6 (10.0) | 41.5 (8.8) | 0.24 | 42.6 (12.6) | 42.6 (17.5) | 0.94 |
| Choline, $\mu$M | 16.0 (4.3) | 15.7 (5.9) | 0.54 | 22.5 (7.3) | 22.2 (8.1) | 0.67 |
| Enterolactone, nM | 2.9 (1.0, 6.8) | 4.7 (2.1, 10.2) | 0.003 | 9.2 (3.6, 18.9) | 9.9 (4.5, 19.2) | 0.69 |

[1]Controls were matched in a 1:1 ratio to cases for age at blood sample collection, sex, month of sample collection, and smoking status. Data are means (SDs), medians (IQRs), or percentage (%). *In the controls, 2 participants had missing values of total cholesterol, LDL and HDL cholesterol, triglyceride, and CRP, and 10 participants had missing value of HbA1c. Abbreviations: CAD, coronary artery disease; CRP, C-reactive protein; HbA1c, glycated hemoglobin; HPFS, Health Professionals Follow-Up Study; hsCRP, high-sensitivity C-reactive protein; MET, metabolic equivalents; MI, myocardial infarction; NHSII, Nurses' Health Study II; TMAO, trimethylamine-N-oxide.

consumption of whole grains and fiber, as well as a higher hPDI, and lower intake of red meats.

**Supplementary Table 2** shows the partial Spearman correlation coefficients of enterolactone, TMAO, L-carnitine, and choline with diet, lifestyle and CVD risk markers among controls in the pooled population. After multivariate adjustment, weak-to-modest correlations were observed among enterolactone, TMAO, L-carnitine, and choline ($r_s$ ranged from 0.17 to 0.37, all $P < 0.001$). Higher enterolactone was significantly associated with higher intake of fruit, whole grains, and fiber, higher hPDI and physical activity, and lower BMI and CRP ($r_s$ ranged from −0.15 to 0.20, all $P < 0.01$), while TMAO was not significantly associated with any dietary and lifestyle factors.

Higher enterolactone concentrations were significantly associated with a lower risk of CAD in women (NHSII), but not in men (HPFS) (**Table 4**), although no significant interaction by sex was observed. After multivariate adjustment including matching factors, physical activity, BMI, menopause status (for women only), family history of MI, and dietary factors, women in the highest tertile had an OR: 0.46 (95% CI: 0.22, 0.93) compared to those in the lowest tertile ($P$ for trend = 0.03). When further adjusting for LDL-C, HDL-C, triglyceride, and CRP, the results were largely unchanged. Although TMAO tertiles were not significantly associated with CAD risk in the pooled population [comparing extreme tertiles, OR: 1.23 (95% CI: 0.89, 1.70), per 1 unit increment in log-transformed TMAO was marginally significantly associated with a 52% increased

**TABLE 2** Baseline characteristics according to concentrations of enterolactone and TMAO in the pooled populations from NHS II and HPFS ($n = 1216$)[1]

| | Low enterolactone high TMAO[2] | Low enterolactone low TMAO | High enterolactone High TMAO | High enterolactone low TMAO |
|---|---|---|---|---|
| Demographics and lifestyle factors | | | | |
| Number | 310 | 339 | 331 | 236 |
| Age at blood draw, y draw, years | 55.5 (11.4) | 55.3 (10.6) | 62.8 (10.8) | 59.1 (10.3) |
| BMI, kg/m$^3$ | 27.1 (5.5) | 26.0 (4.2) | 25.7 (3.7) | 25.4 (3.4) |
| Physical activity, METs-h/wk | 15.8 (4.9, 35.2) | 19.1 (6.3, 37.5) | 25.3 (10.0, 44.1) | 26.1 (12.5, 43.2) |
| Smoking status, % | | | | |
| Current smoker | 18.9 | 12.2 | 6.5 | 8.4 |
| Former smoker | 35.8 | 36.7 | 43.6 | 45.8 |
| Never smoker | 45.3 | 51.1 | 49.9 | 45.8 |
| Family history of MI, % | 32.9 | 28.9 | 35.3 | 40.2 |
| Hypercholesterolemia, % | 41.0 | 40.4 | 41.4 | 43.2 |
| Hypertension, % | 28.7 | 27.1 | 33.8 | 24.1 |
| Diabetes mellitus, % | 5.8 | 4.4 | 7.8 | 4.2 |
| Aspirin use, % | 24.8 | 22.7 | 30.5 | 21.6 |
| Total energy, kcal/d | 1895.5 (537.1) | 1971.1 (520.2) | 1952.9 (549.4) | 1964.0 (469.2) |
| Alcohol, g/d | 1.6 (0, 7.9) | 3.3 (0.3, 11.0) | 5.2 (1.0, 14.5) | 5.9 (1.4, 14.6) |
| Cardiovascular risk markers[3] | | | | |
| Total cholesterol, mg/dL | 208.0 (40.6) | 203.7 (36.8) | 206.3 (37.3) | 205.4 (34.7) |
| LDLcholesterol, mg/dL | 139.0 (37.0) | 136.3 (33.7) | 136.1 (34.3) | 135.6 (30.7) |
| HDL cholesterol,, mg/dL | 37.3 (14.9) | 39.5 (13.6) | 43.4 (12.9) | 42.9 (12.9) |
| Triglyceride, mg/dL | 161.0 (102.9) | 141.0 (79.4) | 135.5 (78.7) | 132.4 (69.5) |
| hsCRP, mg/dL | 1.9 (0.9, 4.5) | 1.2 (0.6, 3.3) | 1.2 (0.5, 2.6) | 1.1 (0.6, 2.3) |
| HbA1c, % | 5.6 (1.0) | 5.5 (0.7) | 5.7 (0.8) | 5.6 (1.0) |
| Gut microbiota–related metabolites | | | | |
| TMAO, $\mu$M | 5.4 (4.3, 7.5) | 2.4 (1.8, 3.0) | 6.0 (4.5, 9.2) | 2.7 (2.1, 3.1) |
| L-carnitine, $\mu$M | 44.9 (12.2) | 39.6 (10.3) | 45.3 (18.1) | 39.1 (10.8) |
| Choline, $\mu$M | 20.1 (7.6) | 18.6 (5.7) | 22.9 (9.5) | 19.6 (6.2) |
| Enterolactone, nM | 2.6 (1.0, 5.2) | 3.3 (1.2, 5.5) | 17.3 (11.8, 27.2) | 15.5 (11.0, 24.6) |

[1]Data are means (SDs), medians (IQRs), or percentage (%). Abbreviations: CRP, C-reactive protein; HbA1c, glycated hemoglobin; hsCRP, high-sensitivity C-reactive protein; HPFS, Health Professionals Follow-Up Study; MET, metabolic equivalents; MI, myocardial infarction; NHSII, Nurses' Health Study II; TMAO, trimethylamine N-oxide.

[2]Low enterolactone or high TMAO was based on median levels among the controls.

[3]In the controls, 2 participants had missing values of total cholesterol, LDL and HDL cholesterol, triglyceride, and CRP, and 10 participants had missing value of HbA1c.

risk of CAD [OR: 1.52 (95% CI: 1.00, 2.32); $P = 0.05$]. In addition, higher concentrations of choline, but not L-carnitine, were associated with an increased risk of CAD in the pooled population.

**Table 5** shows the joint associations of enterolactone and TMAO concentrations with CAD risk in pooled population. Comparing with participants with low enterolactone/high TMAO, individuals with high enterolactone/low TMAO concentrations had a multivariate-adjusted OR (95% CI) of 0.61 (0.40, 0.92). After further adjustment of LDL-C, HDL-C, triglyceride, CRP, and HbA1c, the association attenuated slightly (OR [95% CI]: 0.58 [0.38, 0.90]). However, we did not detect significant interaction between enterolactone and TMAO with respect to CAD risk ($P$ interaction = 0.76). High enterolactone/low TMAO profile was estimated to account for 28.2% (95% CI: 1.6%, 90.3%; $P = 0.02$) of the association between hPDI and CAD risk, or 4.2% (95% CI: 0.2%, 43.3%; $P = 0.22$) of associations of AHEI with CAD risk. In addition, 16.8% (95% CI: 4.1%, 48.5%; $P = 0.04$) of the association between high enterolactone/low TMAO profile and CAD risk could be explained by lipids, CRP, and HbA1c.

In a sensitivity analysis, similar findings were observed when using a random effects model to pool the results of the 2 cohorts.

**Supplementary Figure 2** presents the dose–response relations between enterolactone, TMAO, and CAD risk in the pooled population. No significant linear relations were detected.

## Discussion

In this prospective study among 2 cohorts of US men and women, a high enterolactone/low TMAO profile was correlated with better diet quality as reflected by higher hPDI scores, as well as individual components, including higher intake of whole grains, fiber, and fruits, and lower intake of red meats. In addition, this profile was correlated with a more favorable distribution of CVD risk markers, in comparison with a low enterolactone/high TMAO profile. Lastly, men and women with this profile also had lower risk of developing CAD, after adjustment of established and potential CVD risk factors. This association was robust in sensitivity analyses.

Few prior studies have examined joint associations of enterolignans and TMAO with risk of CAD, and existing evidence of associations between individual markers and CAD risk is somewhat mixed. A few prospective studies addressed associations between enterolactone concentrations and CAD risk primarily among men (17–20). For example, in Finnish men,

**TABLE 3** Least-square mean of dietary factors according to concentrations of enterolactone and TMAO in pooled populations from NHS II and HPFS ($n = 1216$)[1]

| | Low Enterolactone High TMAO[2] | Low Enterolactone Low TMAO | High Enterolactone High TMAO | High Enterolactone Low TMAO |
|---|---|---|---|---|
| Dietary factors | | | | |
| Fruits, serving/d | 2.3 (2.2, 2.4) | 2.2 (2.1, 2.3) | 2.5 (2.4, 2.6)* | 2.3 (2.1, 2.4) |
| Vegetable, serving/d | 3.1 (3.0, 3.2) | 3.1 (3.0, 3.2) | 3.1 (3.0, 3.3) | 3.1 (2.9, 3.2) |
| Red meats, serving/d | 1.09 (1.03, 1.14) | 1.01 (0.96, 1.06)* | 1.06 (1.01, 1.12) | 0.97 (0.91, 1.03)** |
| Fish, serving/d | 0.28 (0.26, 0.31) | 0.28 (0.26, 0.30) | 0.27 (0.25, 0.30) | 0.27 (0.25, 0.30) |
| Egg, serving/d | 0.24 (0.21, 0.26) | 0.23 (0.20, 0.25) | 0.26 (0.23, 0.28) | 0.24 (0.22, 0.27) |
| Whole grains, g/d | 23.7 (22.2, 25.2) | 24.1 (22.7, 25.5) | 26.4 (24.9, 27.9)* | 26.0 (24.3, 27.7)* |
| Fiber, g/d | 21.3 (20.8, 21.9) | 21.6 (21.1, 22.1) | 21.9 (21.3, 22.4) | 22.4 (21.8, 23.0)** |
| Coffee, cup/d | 1.81 (1.64, 1.99) | 1.72 (1.56, 1.89) | 2.29 (2.12, 2.46)** | 2.02 (1.83, 2.23) |
| Nut, serving/d | 0.53 (0.47, 0.59) | 0.47 (0.41, 0.53) | 0.49 (0.44, 0.55) | 0.50 (0.43, 0.57) |
| P/S ratio | 0.50 (0.48, 0.51) | 0.48 (0.47, 0.50) | 0.47 (0.46, 0.49) | 0.49 (0.47, 0.51) |
| Alternate healthy eating index | 43.8 (42.7, 44.8) | 43.7 (42.7, 44.7) | 43.0 (42.0, 44.1) | 45.0 (43.8, 46.2) |
| Healthful plant-based diet index | 54.1 (53.3, 54.8) | 54.2 (53.5, 54.9) | 55.7 (55.0, 56.5)** | 56.0 (55.1, 56.8)** |

[1]Adjustment for matching factors, including age (years), sex (male, female), month of sample collection, fasting status at time of collection, and smoking status (never, former, or current), and alcohol intake (0, 0.1–4.9, 5.0–9.9, ≥10.0 g/d), physical activity (in tertiles), BMI (≤24.9, 25.0–29.9, ≥30.0 kg/m$^2$), family history of MI (yes, or no), aspirin use (yes, no), presence of diabetes, hypertension, or hypercholesterolemia (yes, no), and other dietary factors (individual foods were mutually adjusted). *$P < 0.05$ and **$P < 0.01$, compared with the first group. Data are least-square means (95% CIs). Abbreviations: HPFS, Health Professionals Follow-Up Study; NHSII, Nurses' Health Study II; P/S ratio: ratio of polyunsaturated to saturated fat; TMAO, trimethylamine N-oxide.

[2]Low enterolactone or high TMAO was based on median levels among the controls.

serum enterolactone levels were significantly associated with a lower risk of developing acute coronary events or CAD-related mortality (17, 18), while a nonsignificant association was observed in another study among an independent cohort of male Finnish smokers (19). In a Dutch population primarily consisting of men, neither enterolactone nor enterodiol was associated with nonfatal MI, and data for fatal CAD were not available (20). In addition, several important covariates, such as physical activity, diet quality, and family history of MI, were not taken into account in the previous studies. In our study, we found an interesting inverse association between enterolactone and CAD risk in women, but not men.

In contrast to the relatively sparse data for enterolignans, a significant number of studies have been conducted to examine prospective associations between TMAO concentrations and CAD risk. Among patients with existing conditions, TMAO concentrations were consistently associated with a higher CVD (10, 21, 22, 38). For example, studies found that higher TMAO concentrations were associated with increased risk of multiple adverse cardiovascular events, such as MI, stroke, or death, among patients undergoing elective coronary angiography, or patients with prevalent kidney disease or chronic systolic heart failure (21, 22, 39, 40). In contrast, among largely healthy individuals, the association between TMAO concentrations and CAD risk has been less consistently demonstrated (23–25). For instance, in 2 separate groups of postmenopausal women (n = 1571), Paynter et al. found that TMAO was not associated with CAD risk (25). The reasons underlying the contrasting findings in individuals with existing chronic diseases compared with largely healthy populations are unclear, although differences in TMAO assessments, short half-life of TMAO in circulation, and influences of pathophysiology of the chronic conditions on TMAO metabolism may partially account for the inconsistency. Nonetheless, the associations observed in the current analysis partially mirror the prevailing evidence of lack of associations of TMAO in healthy men and women.

Another important fact to consider when interpreting TMAO associations with CAD is that there are potentially heterogeneous sources of TMAO in human circulations. In contrast to enterolignans that are exclusively produced by human gut microbiota through processing plant lignans (8), microbial production of TMA may not be the sole source of TMAO in the circulation. Accumulating evidence has demonstrated that TMAO functions as an osmolyte for marine organism to counteract the adverse effects of pressure (41, 42). As such, TMAO can be absorbed directly into human circulation without the assistance of gut microbiota to produce the intermittent TMA in the gut. While TMAO can be a sensitive marker for red meat intake, as demonstrated in feeding trials showing that red meat, but not white meat or nonmeat protein, increased TMAO concentrations in plasma and urine (43), whether TMAO is a specific marker for red meat intake is less clear. In free living individuals, the correlation between habitual red meat intake and circulating TMAO levels is not well established, and in our population of men, fish intake as assessed by 2 wk of 7-d diet records was more strongly, positively correlated with plasma TMAO than red meat (44). Our study also observed no significant correlation between TMAO and intake of red meats or other animal products. It is possible that the diet-TMAO and TMAO-CAD associations might vary depending on participants' habitual diets and short-term intake of animal foods containing choline and carnitine, as well as the gut microbiota (45, 46).

The rationale for considering enterolignans and TMAO in the current analysis is based on the consideration that enterolignans and TMAO are both constitutive by-products of microbiota (especially after we controlled for fish intake), could be simultaneously produced on an omnivore diet, and, more importantly, converge on specific metabolic pathways involving

**TABLE 4** Associations of enterolactone and TMAO with CAD risk ($n = 1216$)[1]

| | T 1 | T 2 | T 3 | $P_{trend}$ |
|---|---|---|---|---|
| Enterolactone | | | | |
| NHS II | | | | |
| Median (range)[2] | 1.06 (0.35, 2.95) | 4.70 (2.99, 8.25) | 13.3 (8.30, 77.1) | |
| Case/total | 94/157 | 56/118 | 37/99 | |
| Model 1[3] | 1.00 | 0.61 (0.37, 0.98) | 0.37 (0.21, 0.65) | <0.001 |
| Model 2[4] | 1.00 | 0.76 (0.42, 1.37) | 0.42 (0.21, 0.83) | 0.01 |
| Model 3[5] | 1.00 | 0.78 (0.41, 1.46) | 0.46 (0.22, 0.93) | 0.03 |
| HPFS | | | | |
| Median (range)[2] | 2.44 (0.35, 6.02) | 9.88 (6.03, 15.0) | 25.2 (15.1,211.4) | |
| Case/total | 151/291 | 136/277 | 134/274 | |
| Model 1[3] | 1.00 | 0.89 (0.64, 1.24) | 0.88 (0.62, 1.24) | 0.51 |
| Model 2[4] | 1.00 | 0.96 (0.67, 1.36) | 0.96 (0.66, 1.40) | 0.84 |
| Model 3[5] | 1.00 | 0.92 (0.64, 1.33) | 0.91 (0.61, 1.35) | 0.66 |
| Pooled | | | | |
| Median (range)[2] | 2.05 (0.35, 4.66) | 8.32 (4.68, 12.3) | 22.1 (12.4,211.4) | |
| Case/total | 238/441 | 177/380 | 193/395 | |
| Model 1[3] | 1.00 | 0.73 (0.55, 0.97) | 0.79 (0.59, 1.06) | 0.21 |
| Model 2[4] | 1.00 | 0.82 (0.60, 1.12) | 0.93 (0.67, 1.29) | 0.84 |
| Model 3[5] | 1.00 | 0.82 (0.60, 1.13) | 0.94 (0.67, 1.32) | 0.91 |
| TMAO | | | | |
| NHS II | | | | |
| Median (range)[2] | 2.30 (0.90, 3.00) | 3.70 (3.10, 4.80) | 7.00 (4.90, 42.1) | |
| Case/total | 60/125 | 63/123 | 64/126 | |
| Model 1[3] | 1.00 | 1.13 (0.70, 1.84) | 1.12 (0.68, 1.86) | 0.72 |
| Model 2[4] | 1.00 | 1.17 (0.66, 2.08) | 1.11 (0.62, 1.99) | 0.82 |
| Model 3[5] | 1.00 | 1.12 (0.61, 2.05) | 1.05 (0.57, 1.94) | 0.93 |
| HPFS | | | | |
| Median (range)[2] | 2.08 (0.07, 2.79) | 3.52 (2.80, 4.63) | 6.71 (4.65, 98.5) | |
| Case/total | 128/268 | 136/277 | 157/297 | |
| Model 1[3] | 1.00 | 1.07 (0.75, 1.53) | 1.26 (0.88, 1.79) | 0.19 |
| Model 2[4] | 1.00 | 0.99 (0.67, 1.46) | 1.22 (0.83, 1.79) | 0.25 |
| Model 3[5] | 1.00 | 0.95 (0.64, 1.42) | 1.19 (0.80, 1.77) | 0.29 |
| Pooled | | | | |
| Median (range)[2] | 2.18 (0.07, 2.89) | 3.59 (2.90, 4.65) | 6.82 (4.67, 98.5) | |
| Case/total | 185/388 | 199/401 | 224/427 | |
| Model 1[3] | 1.00 | 1.09 (0.82, 1.45) | 1.24 (0.92, 1.65) | 0.16 |
| Model 2[4] | 1.00 | 1.07 (0.79, 1.46) | 1.25 (0.91, 1.71) | 0.16 |
| Model 3[5] | 1.00 | 1.05 (0.77, 1.43) | 1.23 (0.89, 1.70) | 0.19 |

[1]ORs (95% CIs) were calculated by conditional logistic regression analysis. Abbreviations: CAD, coronary artery disease; HPFS, Health Professionals Follow-Up Study; MI, myocardial infarction; NHSII, Nurses' Health Study II; TMAO, trimethylamine-N-oxide.

[2]The median levels among the controls.

[3]Model 1, adjusted for matching factors, including age at blood sample collection (years), sex (male, female, for pooled analysis only), month of sample collection, fasting status at time of collection, and smoking status (never, former, or current).

[4]Model 2, further adjusted for alcohol intake (0, 0.1–4.9, 5.0–9.9, ≥10.0 g/d), physical activity (in tertiles), BMI (≤24.9, 25.0–29.9, ≥30.0 kg/m$^2$), menopause status (yes, no; for women only), family history of MI (yes, or no), aspirin use (yes, no), and presence of diabetes, hypertension, or hypercholesterolemia (yes, no).

[5]Model 3, further adjusted for intake of fruits, vegetables, red meats, fish, egg, whole grains, fiber, nut, coffee, and ratio of polyunsaturated to saturated fat (all as continuous).

glucose metabolism and lipid metabolism with contrasting effects. In both in vivo and in vitro studies, enterolignans demonstrate clear antioxidant properties: they inhibit lipid peroxidation (47), reduce oxygen species production (48), induce gene expression of antioxidant enzymes (49), and reduce vitamin E catabolism (50). Enterolignans are phytoestrogens that bind preferentially to estrogen receptor-$\alpha$ (ER$\alpha$) over ER-$\beta$ and lead to subsequent ER-mediated gene transcription (51–53), which might explain the inverse association between enterolactone and CAD in our NHSII cohort. Interestingly, enterolignans also increase the levels of sex-hormone-binding protein (54), which leads to reduced free estradiol, improved insulin resistance, and a lower diabetes risk (55, 56). Enterolignans may also improve insulin resistance by decreasing inflammation (57) and inducing adiponectin expression (58). In contrast, multiple lines of research demonstrate the potentially detrimental effects of TMAO on CVD health. Supplementation of TMAO or its precursors to atherosclerosis-prone mice led to enhanced

**TABLE 5** Joint associations of enterolactone and TMAO concentrations with CAD risk in pooled population ($n = 1216$)[1]

| | Low enterolactone high TMAO | Low enterolactone low TMAO | High enterolactone high TMAO | High enterolactone low TMAO |
|---|---|---|---|---|
| Case/total | 169/310 | 176/339 | 168/331 | 95/236 |
| Model 1[2] | 1.00 | 0.87 (0.64, 1.19) | 0.81 (0.58, 1.13) | 0.54 (0.38, 0.77)* |
| Model 2[3] | 1.00 | 0.93 (0.67, 1.30) | 0.90 (0.62, 1.29) | 0.60 (0.40, 0.90)* |
| Model 3[4] | 1.00 | 0.96 (0.68, 1.36) | 0.92 (0.63, 1.33) | 0.61 (0.40, 0.92)* |
| Model 4[5] | 1.00 | 0.99 (0.69, 1.42) | 0.91 (0.61, 1.35) | 0.58 (0.38, 0.90)* |

[1]High TMAO or enterolactone was defined as the concentrations above the median levels among the controls. ORs (95% CIs) were calculated by conditional logistic regression analysis. *$P < 0.05$, compared with the first group. Abbreviations: CAD, coronary artery disease; CRP, C-reactive protein; HbA1c, glycated hemoglobin; HPFS, Health Professionals Follow-Up Study; MET, metabolic equivalents; MI, myocardial infarction; mycNHSII, Nurses' Health Study II; TMAO, trimethylamine-N-oxide.

[2]Model 1, conditioned on matching factors, including age at blood sample collection (years), sex (male, female), month of sample collection, fasting status at time of collection, and smoking status (never, former, or current).

[3]Model 2, further adjusted for alcohol intake (0, 0.1–4.9, 5.0–14.9, ≥15.0 g/d), physical activity (METs-h/week), BMI (≤24.9, 25.0–29.9, ≥30.0 kg/m$^2$), family history of MI (yes, or no), aspirin use (yes, no), and presence of diabetes, hypertension, or hypercholesterolemia (yes, no).

[4]Model 3, further adjusted for intake of fruits, vegetables, red meats, fish, egg, whole grains, fiber, nut, coffee, and ratio of polyunsaturated to saturated fat (all as continuous).

[5]Model 4, further adjusted for LDL and HDL cholesterol, triglyceride, CRP, and HbA1C (all as continuous).

cholesterol accumulation in macrophages and atherosclerotic plaque development (13, 59), through inhibiting reverse cholesterol transport (10). Moreover, TMAO supplementation promoted the production of inflammatory cytokines and induced glucose intolerance through obstructing hepatic insulin signaling pathway and modulating inflammation-related gene expression in adipose tissue (60).

The strength of the current study included a prospective study design, high follow-up rate, detailed information on lifestyles and diet with good quality, rigorous quality control of laboratory procedures, careful adjustment for a wide array of covariates, and consideration of both enterolignans and TMAO. Several limitations should be discussed as well. First, we only had a single measurement of enterolactone and TMAO, which was more likely to reflect relatively short-term concentrations. Given that enterolactone and TMAO are products of dynamic interactions between diet and gut microbiota, repeated measurements of these metabolites over time would be a more desirable approach to better reflect long-term exposure levels. Second, only 2 gut microbiota metabolites, i.e., enterolactone and TMAO, were included in the current study, although our findings provided preliminary evidence to support the notion that multiple microbiota-derived metabolites might indeed better discriminate CAD risk than individual markers. Future research is warranted to consider a more comprehensive list of gut microbiota-derived metabolites. Third, our study participants were all health professionals, and most were Caucasians, which limits generalizability of our findings to other ethnic groups, who may exhibit distinctive dietary patterns. Fourth, although our analysis was based on an a priori hypothesis, the role of multiple testing or chance cannot be excluded. Last, unmeasured confounding or residual confounding cannot be entirely ruled out, either.

In conclusion, in this prospective investigation among 2 cohorts of US women and men, a profile characterized by high enterolactone and low TMAO concentrations was linked to a healthful dietary pattern and significantly associated with a lower risk of CAD. More prospective studies are warranted to confirm these findings and to extend this research by including other gut microbiota metabolites that potentially mediate diet-disease associations.

The authors' responsibilities were as follows—QS, EBR: designed the research; GL conducted analyses; GL: wrote the first draft of the paper; all authors: contributed to the interpretation of the results and critical revision of the manuscript for important intellectual content; and all authors: read and approved the final version of the manuscript. The authors report no conflicts of interest.

## Data Availability

Data described in the manuscript, code book, and analytic code will be made available upon request pending application and approval from the corresponding author.

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