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Dietary meat, trimethylamine N-oxide-related metabolites, and incident cardiovascular disease among older adults: the Cardiovascular Health Study

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Supplemental Materials
Expanded Materials & Methods
Online Figures S1–S3
Online Tables S1–S6
References 7, 25, 44, 45, 33, 34, 36, 55, 56

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Abstract

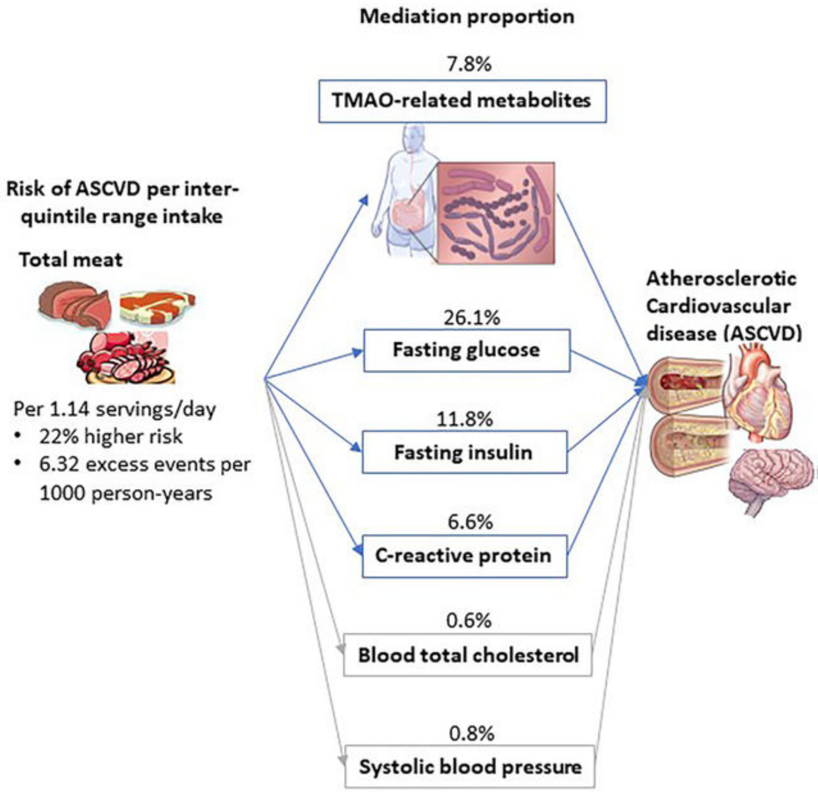
Background: Effects of animal source foods (ASF) on atherosclerotic cardiovascular disease (ASCVD) and underlying mechanisms remain controversial. We investigated prospective associations of different ASF with incident ASCVD and potential mediation by gut microbiota-generated trimethylamine N-oxide (TMAO), its L-carnitine-derived intermediates γ-butyrobetaine and crotonobetaine, and traditional ASCVD risk pathways.

Methods: Among 3,931 participants from a community-based U.S. cohort aged 65+ years, ASF intakes and TMAO-related metabolites were measured serially over time. Incident ASCVD (myocardial infarction, fatal coronary heart disease, stroke, other atherosclerotic death) was adjudicated over 12.5 years median follow-up. Cox proportional hazards with time-varying exposures and covariates examined ASF-ASCVD associations; and additive hazard models, mediation proportions by different risk pathways.

Results: After multivariable-adjustment, higher intakes of unprocessed red meat, total meat, and total ASF associated with higher ASCVD risk, with hazard ratios (95%CI) per interquintile range of 1.15 (1.01–1.30), 1.22 (1.07–1.39), and 1.18 (1.03–1.34), respectively. TMAO-related metabolites together significantly mediated these associations, with mediation proportions (95%CI) of 10.6% (1.0–114.5), 7.8% (1.0–32.7), and 9.2% (2.2–44.5), respectively. Processed meat intake associated with a nonsignificant trend toward higher ASCVD (1.11; 0.98–1.25); intakes of fish, poultry, and eggs were not significantly associated. Among other risk pathways, blood glucose, insulin, and C-reactive protein, but not blood pressure or blood cholesterol, each significantly mediated the total meat-ASCVD association.

Conclusions: In this large, community-based cohort, higher meat intake associated with incident ASCVD, partly mediated by microbiota-derived metabolites of L-carnitine, abundant in red meat. These novel findings support biochemical links between dietary meat, gut microbiome pathways, and ASCVD.

Graphical Abstract



Keywords

trimethylamine-N-oxide; atherosclerotic cardiovascular disease; animal source food; red meat; fish; mediation

Keywords

Diet and Nutrition; Biomarkers; Risk factors; Cardiovascular Disease; Mechanisms; Epidemiology

Introduction

Animal source foods (ASF), including unprocessed red meat, processed meat, fish, poultry, and eggs, are major components of many diets. The impact of these different foods on atherosclerotic cardiovascular disease (ASCVD) have been widely studied but remain controversial. Evidence is particularly sparse among older adults, the age group at the highest risk for ASCVD and in whom adequate intakes of high-quality protein, which is rich in ASF, appears important to offset aging-related losses of muscle mass and strength.[1–3]

The resulting controversies are exacerbated by poorly understood potential mechanisms underlying these associations. A historical focus on saturated fat, for example, has been tempered by evidence that its health effects vary according to the food source,[4] suggesting relevance of other compounds in ASF. Growing evidence highlights newly discovered, gut microbiota-generated metabolites of ASF.[5–7] These include trimethylamine N-oxide

(TMAO), generated by microbial metabolism of dietary L-carnitine (abundant almost exclusively in red meat) and choline (present in a variety of ASF). Gut microbial metabolism of L-carnitine also generates two intermediates γ-butyrobetaine and crotonobetaine, each of which can then be further converted to TMAO (Figure 1). In experiments, TMAO promotes macrophage foam cell formation,[8] vascular inflammation and inflammasome activation,[9–12] endothelial dysfunction,[13] platelet hyperreactivity and thrombosis,[14, 15] and decreases reverse cholesterol transport.[16] In large clinical samples of patients with prevalent diseases, although not other small studies with less robust designs,[17, 18] higher plasma levels of TMAO were associated with higher risk of ASCVD and total mortality.[8, 16, 19–23] It has been hypothesized that TMAO and its gut microbiota- generated intermediates may partly mediate the effects of consumption of ASF on ASCVD.[24] However, no research has assessed this hypothesis. Investigation of such mediation would help advance understanding of potential mechanisms linking these ASF to ASCVD, as well as reasons for heterogeneous associations with ASCVD of different ASF.

To address these important research gaps, we investigated the associations of different ASF with incidence of ASCVD in a prospective, community-based cohort of older adults. We further evaluated the extent to which plasma levels of TMAO, γ-butyrobetaine, and crotonobetaine (referred to hereafter as TMAO-related metabolites) jointly mediated the identified associations.

Materials and Methods

Because of the sensitive nature of the data collected for this study, requests to access the dataset from qualified researchers trained in human subject confidentiality protocols may be sent to the Cardiovascular Health Study Collaborative Health Studies Coordinating Center at CHSdata@uw.edu.

Study population

The Cardiovascular Health Study (CHS) is a multi-center, community-based, prospective cohort study designed to investigate risk factors for coronary heart disease and stroke in older adults. The study design and participant recruitment have been described.[25, 26] Briefly, in 1989–90, 5,201 non-institutionalized adults aged ≥ 65 years were recruited from random samples of Medicare eligibility lists in 4 US communities. To enrich minority recruitment, an additional 687 African American participants were recruited in 1992–93 using similar methods, resulting in 5,888 total participants. Trained personnel assessed participants' demographic characteristics, lifestyle, medical history, and other health related phenotypes during annual in-clinic exams with intervening phone interviews every 6 months through 1999. Thereafter, participants were contacted every 6 months by phone for follow-up through June 2015. Follow-up for vital status was nearly 100% complete. The study was approved by the institutional review board of each participating university. All participants provided written informed consent.

After excluding participants without joint assessments of diet and TMAO, with extreme reported energy intake (<500 or >5000 kcal/day), and with prevalent CVD (myocardial infarction [MI], stroke, angina, coronary revascularization) at the time of their first joint assessment of diet and TMAO, a total of 3,931 participants were included in this investigation (Supplemental Figure S1). Compared to included individuals, excluded individuals were slightly more likely to be male, non-White, older, less educated, and less healthy by self-report (Supplemental Table S1).

Assessment of dietary habits (exposures)

We focused on foods with significant associations with ASCVD in prior meta-analyses of generally middle-aged populations, including processed meat, unprocessed red meat, and fish.[27–32] We hypothesized that processed meat and unprocessed red meat consumption would be positively associated with incidence of ASCVD, and that these associations would be partly mediated by plasma levels of TMAO-related metabolites; and that fish consumption would be inversely associated with ASCVD, and that its association would become stronger (i.e., more protective) after accounting for plasma levels of TMAO-related metabolites. In exploratory analyses, we examined the associations of secondary dietary exposures including total meat (i.e., unprocessed red meat plus processed meat), poultry, eggs, and total of these ASF with ASCVD. Dairy foods were not included given these are not appreciable dietary sources of TMAO precursors.

Usual dietary habits over the past year were assessed in 1989–90 using a validated 99-item picture-sort food-frequency questionnaire (FFQ) adapted from the National Cancer Institute,[33, 34] and again in 1995–96 using a validated Willett semi-quantitative FFQ.[35–37] The Pearson correlation coefficients between the Willett FFQ and two 1-week dietary records ranged from 0.56 to 0.83 for individual food groups of meats, eggs, and fish.[36] For each FFQ, participants were asked to indicate how often, on average, they had eaten given amounts of various foods during the past year. The picture-sort FFQ used a 5-category frequency of intake ranging from “never” to “almost every day or at least five times per week”, based on medium portion sizes. The Willett FFQ used a 10-category frequency of intake ranging from “never or less than once per month” to “6+ per day”, with defined standard portion sizes. Frequencies of intake were converted to servings/day using the midpoint of the relevant response category.[38, 39] Food intakes were adjusted for total energy using the residual method.[40]

Assessment of plasma TMAO-related metabolites (mediators)

We primarily focused on the joint mediation by TMAO and its two intermediate gut microbiota-dependent metabolites derived from dietary L-carnitine, γ-butyrobetaine and crotonobetaine. We also explored path-specific mediated associations by each TMAO-related metabolite and mediation by plasma levels of each nutrient precursor (i.e., choline, betaine, carnitine). Measurements were performed using stored frozen (−80 °C) fasting blood samples collected at enrollment (1989–90 or 1992–93) and again in 1996–97. Each biomarker was quantified using its deuterium-isotopologue as internal standard via a stable-isotope dilution assay coupled with high-performance liquid chromatography, with online electrospray ionization tandem mass spectrometry on a Shimadzu 8050 mass spectrometer. All laboratory measurements were performed at the Cleveland Clinic Lerner Research Institute, with laboratory CVs < 10% for each metabolite, as previously described.[7]

Assessment of traditional ASCVD risk factors and other covariates

At each in-clinic exam, information on sociodemographics, lifestyle, anthropometrics, medical history, medications (including antibiotic use in the past 2 weeks), and other risk factors were assessed by trained personnel using standardized questionnaires and physical examination.[25] Physical activity (excluding chores, kcal/week) was assessed by a modified Minnesota Leisure Time Activities Questionnaire.[41, 42] Information was collected on alcohol intake, including usual frequency and types of alcoholic beverages (wine, beer, liquor), smoking status (never, former, or current; lifetime pack-years), and self-perceived general health (excellent, very good, good, fair, poor). Anthropometrics were directly measured, as were two seated resting blood pressure measurements. Fasting glucose, total cholesterol, HDL cholesterol, and triglyceride levels were measured from collected blood samples using standardized methods; and LDL cholesterol level calculated using the Friedewald formula excluding patients with hypertriglyceridemia. C-reactive protein (CRP) was measured using a high-sensitivity enzyme-linked immunosorbent assay.[43] Cystatin-C and creatinine were measured and used to calculate estimated glomerular filtration rate (eGFR) using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation.[44–46] Diabetes was defined by treatment with oral hypoglycemic agents or insulin, fasting plasma glucose ≥ 126 mg/dl, or 2-hour post-oral glucose challenge ≥ 200 mg/dl.[47]

Assessment of ASCVD (outcome)

The primary outcome was incident ASCVD, defined as a composite of first definite or probable MI, fatal coronary heart disease (CHD), stroke (excluding transient ischemic attack), or other atherosclerotic death. Potential ASCVD events were identified during annual examinations and interim telephone interviews.[48] All ASCVD events were adjudicated continuously from baseline through June 2015 by centralized committees based on information from interviews, medical records, physician questionnaires, death certificates, medical examiner forms, Health Care Financing Administration hospitalizations and available brain imaging.[48, 49] The detailed methods for follow-up and classification of events have been published.[48, 49] Briefly, MI was classified based on chest pain, cardiac enzymes and electrocardiogram findings. Fatal events with suspected coronary cause not meeting criteria for MI were classified as fatal CHD if occurring within 72 hours of chest pain or with an antecedent history of CHD.[39] Stroke was defined as neurological deficit of rapid onset lasting longer than 24 hours unless death supervened or as a subarachnoid hemorrhage.

Statistical Analysis

Cox proportional hazards models with time-varying exposures and covariates assessed the association (hazard ratio, HR) between each dietary exposure and incidence of ASCVD (i.e., main dietary association). Time at risk was calculated from the first joint availability of diet and TMAO measures (i.e., time zero) to the occurrence of ASCVD, death due to non-ASCVD reasons, or last study contact, whichever occurred first (Supplemental Figure S2). The proportional hazards assumption was examined using a test based on Schoenfeld residuals.[50] Given that two covariates (sex, self-perceived health status) violated the assumption, we used risk-set stratified Cox models for these two covariates in all multivariable-adjusted analyses.

To leverage serial dietary measures, reduce exposure misclassification, and obtain estimates of long-term dietary intake, we assessed time-varying cumulative averages of dietary consumption (i.e. cumulative updating):[40] dietary measures at or before the first TMAO measure in 1989–90 were related to ASCVD risk until the timepoint of the second TMAO measure in 1996–97, and the average of serial dietary measurements at or before 1996–97 was related to subsequent risk until 2015. For participants with only one dietary measure, that dietary measure was carried forward. Dietary exposures were analyzed as linear terms measured in units of the difference between the midpoints of the first and fifth quintiles (inter-quintile range, IQR). In sensitivity analyses, we used restricted cubic splines to explore potential non-linear associations and used the most recent intake (i.e., simple updating) instead of cumulative updating for time-varying dietary exposures.

We adjusted for pre-specified covariates including age, sex, race, study site, education, and household income, and time-varying smoking status, alcohol intake, physical activity, self-perceived health status, antibiotic use, and dietary habits including intakes of total energy, fruits, vegetables, dietary fiber, total dairy, and mutual adjustment for the other ASF. In sensitivity analyses, we further adjusted for traditional CVD risk factors that may be intermediate outcomes on the causal pathway between diet and ASCVD, including body mass index (BMI), waist circumference, diabetes, systolic blood pressure, diastolic blood pressure, LDL cholesterol, HDL cholesterol, triglycerides, CRP, anti-hypertensive medication use, lipid lowering medication use. In sensitivity analysis, we further adjusted for eGFR which could be both a confounder and mediator (i.e., on the causal pathway) of TMAO's effects on ASCVD.[51] TMAO is renally cleared,[7] which could make eGFR a confounder; and TMAO also experimentally causes renal fibrosis and dysfunction,[52, 53] which could make eGFR a mediator of the association with ASCVD. In this context, including eGFR in the primary model would be subject to overadjustment. All time-varying covariates were updated at the time of TMAO updating using the most recent measure. Covariates with missing values were imputed using single imputation via best-subset regression; previous studies in CHS have documented minimal differences in results using this approach compared to multiple imputation.[54]

We used additive hazard models to perform causal mediation analyses.[55–57] The three TMAO-related metabolites (TMAO, γ-butyrobetaine, crotonobetaine) were analyzed as time-varying linear variables. Simple updating was used for these mediators to ensure that mediators were measured no earlier than the measurement of dietary exposures (Supplemental Figure S2). The associations between each dietary exposure and ASCVD (measured by rate difference) were decomposed into those independent of and mediated via the three TMAO-related metabolites based on the conceptual diagram shown in Figure 2. Mediation proportions were defined as the mediated association/ | (independent association + mediated association) |. A detailed description of calculations of independent and mediated associations was included in supplemental materials. Supplemental Figure S3 (study design flowchart) summarizes all main analyses performed in the study. Given that animal feeding studies have established the causal interconversions between these metabolites but not the precise order of the pathways,[6, 58] we evaluated alternative conceptual diagrams changing the sequence of the three mediators, and findings were not appreciably changed (data not shown). We also explored and compared mediation proportions for traditional ASCVD risk factors.

We explored effect modification by baseline renal function (eGFR <60 vs. ≥ 60 mL/min/1.73m^2) for main dietary associations, based on recent findings suggesting renal function could be an effect modifier of the TMAO-ASCVD association,[51] using multiplicative interaction terms between each dietary exposure and eGFR. In post-hoc exploratory analyses, we similarly explored effect modification by age ((≥ vs. < median, 72 years), sex, race/ethnicity (White vs. non-White), education level (<high school, high school, some college, or college graduate), and smoking status (never smoked, former smoker, or current smoker); with Bonferroni correction for testing of these exploratory interactions (5 interaction variables × 7 dietary exposures=35 comparisons; corrected threshold of significance: 0.05/35=0. 0014). Analyses were performed using Stata version 14.2 (StataCorp) and R version 4.0.3 (The R Foundation). Statistical significance for main dietary associations was assessed using a two-sided alpha=0.05. Statistical significance of mediation was assessed by the 95% confidence intervals.

Results

Participant characteristics

Among participants at baseline, mean (SD) age was 72.9 (5.5) years, most were female (63.5%), and 12.0% were non-White (Table 1). Educational attainment ranged from < high school (25.9%) to college graduates (21.4%). About 20% of participants had diabetes, 40% were on anti-hypertensive mediations, and 3% had taken antibiotics in the previous 2 weeks. Participants with higher unprocessed red meat intake were more likely to be male, current smokers, less educated, physically inactive, and have prevalent diabetes; and have lower intakes of fruits, vegetables, and dietary fiber. Patterns of participant characteristics across quintiles of processed meat intake were similar (Supplemental Table S2). Opposing patterns were observed for fish intake, with participants having higher intake being more likely female, never or former smokers, more educated, and to have higher intakes of fruits, vegetables, and dietary fiber (Supplemental Table S3).

Correlations between dietary exposures and TMAO-related biomarkers

At baseline, small (Spearman rho=0.05 to 0.07) but statistically significant positive correlations were seen between plasma TMAO levels and self-reported intakes of unprocessed red meat, total meat, fish, and total ASF, but not processed meat, poultry, or eggs (Table 2). The L-carnitine metabolites γ-butyrobetaine and crotonobetaine positively correlated with intakes of unprocessed red meat, processed meat, total meat, and eggs (rho= 0.03 to 0.15), and inversely correlated with intakes of fish and poultry (rho= −0.03 to −0.08). Correlations for nutrient precursors of TMAO (choline, betaine, carnitine) are also shown. Similar modest diet-biomarker associations have been reported previously for TMAO,[59–61] which could relate to imperfect measurement of self-reported diet, the temporal difference between assessment of usual dietary habits (1 year) vs. shorter term dietary variations that alter TMAO levels (weeks), and/or inter-individual biologic variation in microbial conversion of precursors to TMAO.

Associations of dietary exposures with the risk of ASCVD (main dietary associations)

The median follow-up was 12.5 years (range:0.01 to 26.0). Numbers of events among participants included in the analysis of each dietary exposure are shown in Table 3. After adjusting for sociodemographic factors, lifestyle, dietary factors, and antibiotics use, higher intake of unprocessed red meat was associated with 15% higher incidence of ASCVD per IQR (HR=1.15, 95%CI: 1.01–1.30, P=0.031) (Table 3). Processed meat intake was associated with a similar but nonsignificant trend toward higher ASCVD risk (HR=1.11 [0.98–1.25], P=0.089). Total meat intake (unprocessed red meat + processed meat) was associated with 22% higher incidence of ASCVD (HR=1.22 [1.07–1.39], P=0.004).

Intakes of fish, poultry, and eggs were not significantly associated with incident ASCVD. Total ASF intake was associated with 18% higher risk (HR=1.18 [1.03–1.34], P=0.016) per IQR. Dietary associations estimated by additive hazard models (used for mediation analyses) showed similar findings on the scale of rate difference (Table 3), although the association for unprocessed red meat did not reach statistical significance (rate difference: 4.0 events per 1000 person-years per IQR intake, 95%CI: −0.1, 8.0, P=0.059).

Analyses of dose-response relationships between the extent of intakes of the various ASF and ASCVD (assessed using restricted cubic splines) showed key significant associations (Figure 3). In particular, increasing intakes of unprocessed red meat and total meat were dose-dependently significantly associated with increased risk of ASCVD. Processed meat ingestion trended toward both an overall and a threshold association, but neither achieved statistical significance. A non-linear relationship was suggested for poultry (P-nonlinearity<0.001), with lower ASCVD risk up to a nadir of about 0.4 servings/day, and then diminished benefits thereafter; this nonlinear association was no longer statistically significant (P-nonlinearity=0.083) in sensitivity analyses removing observations with extreme exposures (i.e., the top and bottom 1% of the exposure distribution).

Gut microbiota-generated metabolites of L-carnitine significantly mediate ASF-associated ASCVD risk

In mediation analyses, the three gut microbiota-generated metabolites of dietary L-carnitine (TMAO, γ-butyrobetaine, and crotonobetaine) appeared to jointly mediate part of the association between unprocessed red meat intake and incident ASCVD. Among the total 3.92 (0.42 +3.50) excess ASCVD events per 1000 person-years associated with each IQR higher intake, 0.42 events (95%CI: 0.04, 0.85) or 10.6% (95%CI: 1.0, 114.5) appeared attributable to plasma levels of these metabolites (Table 3). The three microbial metabolites also significantly mediated part of the associations of total meat and total ASF with ASCVD, accounting for 7.8% (95%CI: 1.0, 32.7) and 9.2% (95%CI: 2.2, 44.5) of the observed excess risk, respectively. In exploratory analyses examining path-specific mediated associations, the four paths via crotonobetaine alone (γ-butyrobetaine→crotonobetaine→ASCVD, γ-butyrobetaine→ crotonobetaine→TMAO→ASCVD, crotonobetaine→ASCVD, and crotonobetaine→TMAO→ASCVD; see Figure 2) also significantly mediated the associations of unprocessed red meat, processed meat, total meat, and total ASF with ASCVD risk.

Interestingly, fish intake was not associated with ASCVD risk overall, but had an estimated adverse impact mediated through plasma levels of these gut microbial metabolites (0.45 excess ASCVD events per 1000 person-years [0.07, 0.88] per IQR), mostly related to TMAO. No significant mediated associations were observed for any foods for the paths via γ-butyrobetaine or TMAO alone. Nor did we observe significant mediated associations by any of the nutrient precursors (i.e., carnitine, choline, and betaine) (Supplemental Table S4).

Mediation of ASF-associated ASCVD risk by traditional risk factors

Evaluating traditional ASCVD risk factors as mediators, neither blood cholesterol levels nor blood pressure levels significantly mediated the associations of unprocessed red meat, processed meat, or total meat with ASCVD (Table 4). In contrast, fasting blood glucose and insulin were each significant mediators of these associations; for example, mediating 26.1% (12.7, 82.7) and 11.8% (4.3, 43.2) of the total meat-ASCVD association, respectively. CRP also significantly mediated the associations of intakes of processed meat (13.9% [2.8, 192.7]) and total meat (6.6% [0.4, 27.5]), but not unprocessed red meat (0.9% [−18.6, 21.2]), with ASCVD.

Sensitivity analyses

Results for the main dietary associations and mediation by the TMAO-related metabolites were not appreciably changed when using simple updating of dietary intakes in place of cumulative updating (Supplemental Table S5). Results were also similar after further adjustments for additional CVD risk factors (Supplemental Table S6). Spearman correlations between eGFR and TMAO, γ-butyrobetaine, and crotonobetaine were −0.31, −0.34, and −0.38, respectively ($P<0.001$ each). After additional adjustment for eGFR which could be both a confounder and mediator for the metabolites-ASCVD associations, although the magnitude of main dietary associations remained similar, mediation proportions were attenuated and no longer statistically significant. Examination of the modeling results revealed that this was related to attenuation of the mediator-outcome association (i.e., associations of these metabolites with ASCVD), rather than the exposure-mediator association. Renal function did not significantly modify the associations between any of the ASF and the risk of ASCVD (P-interaction>0.10 for each). Exploratory analyses identified no significant interactions of the ASF-ASCVD relationships by age, sex, race/ethnicity, education level, or smoking status (P-interaction>0.0014 each).

Discussion

In this large, community-based prospective cohort of older adults, higher intakes of unprocessed red meat, total meat, and total ASF were each associated with higher risk of ASCVD, with processed meats trending toward higher risk. These associations were partly (~8–11%) mediated by plasma levels of three dietary L-carnitine-derived gut microbiota-generated metabolites: TMAO, γ-butyrobetaine, and crotonobetaine. Path-specific analyses suggested that plasma crotonobetaine accounted for the largest proportion of the observed mediation. Intakes of fish, poultry, and eggs were not statistically significantly associated with ASCVD. To our knowledge, this is the first study to investigate the association of ASF with ASCVD and potential mediation by gut microbiota-generated TMAO-related metabolites.

Prior studies of ASF and CVD have primarily included middle-aged participants. In these studies, processed meat intake most consistently associates with higher risk, while associations for unprocessed red meat have been smaller and less consistent.[28, 29, 62–72] A recent meta-analysis suggests similar overall magnitudes of associations as in our investigation, with HRs (95% CIs) for CVD of 1.18 (1.10 – 1.30) for processed meat and 1.08 (1.03 – 1.16) for unprocessed red meat (scaled to the same servings as in our study).[29] Associations for fish intake have generally been specific to coronary events, especially sudden death,[27, 72–74] but less consistently with stroke or total ASCVD [29] – for example, a recent meta-analysis identified a pooled HR for CVD per 2 weekly fish servings of 1.00 (95%CI: 0.98 – 1.02).[29] Eggs and poultry have also generally had minimal or neutral associations with ASCVD in prior analyses. [29, 71, 72, 75–78] Our findings for ASF and ASCVD in this population of older US adults, average age 73 years at baseline and followed for an average of 13 years, were generally consistent with these previous studies. We demonstrated a linear dose-response relationship between higher unprocessed red meat and total meat intake and higher incidence of ASCVD later in life. Processed meat was associated with a similar magnitude of increased risk, although the association did not achieve statistical significance, perhaps related to the relatively low intake in CHS of processed meat (median: 0.2 servings/day) vs. unprocessed red meat (median: 0.4 servings/day).

Several ingredients and mechanisms have been proposed to explain potential harmful effects of meat intake on ASCVD. These include contents of saturated fat, cholesterol, and heme iron in red meats, as well as sodium, nitrites, and high temperature cooking of processed meats.[66] However, true mechanisms are surprisingly poorly understood. Mounting evidence indicates heterogeneous health effects of saturated fat on blood cholesterol levels and ASCVD depending on the type of saturated fat as well as the food source.[4, 79] A consensus has also emerged that dietary cholesterol has little meaningful effects on blood cholesterol levels or ASCVD risk at amounts commonly consumed.[80, 81] Consistent with this, in our analysis, neither blood cholesterol levels nor blood pressure levels significantly mediated the associations between unprocessed red meat, processed meat, or total meat and incidence of ASCVD. In contrast, blood glucose levels and insulin sensitivity (measured by fasting insulin) mediated a significant proportion of the meat-ASCVD associations. Red meat is the major source of dietary heme iron, which is implicated as a causal factor in development of type 2 diabetes [82–85] and associated with increased CVD.[86] In a previous mediation analysis, increased cardiovascular mortality associated with processed red meat intake was mediated by both heme iron (24.1%) and nitrite (72.0%) intake; and with unprocessed red meat intake, mediated by heme iron (20.8%) with a large portion of the remaining excess risk unexplained.[70] Our findings support mechanisms related to glucose-insulin homeostasis, and therefore potentially heme content, as one important pathway whereby meat consumption may influence ASCVD. TMAO-related metabolites may play a role here, since TMAO has been mechanistically linked to hyperglycemia and insulin resistance.[87 88] Systematic inflammation as assessed by CRP also mediated a significant proportion of the association between processed meat, but not unprocessed red meat, and ASCVD.

Our novel findings further suggest that L-carnitine derived microbiome metabolites play a larger mediating role in meat-ASCVD associations than blood pressure or blood cholesterol levels. This result is consistent with, and may partly help explain, the neutral associations of saturated fat consumption with CVD;[4, 89] and suggest that attention to other meat constituents and risk pathways is needed. The interplay between diet, the gut microbiota, and microbial-generated metabolites increasingly appears to be a novel pathway linking ASF, especially red meat, to cardiovascular health.[5, 90] Dietary L-carnitine, a nutrient abundant almost exclusively in red meat, can be metabolized to γ-butyrobetaine and crotonobetaine, and ultimately TMAO through the action of gut microbiota and hepatic flavin monooxygenases (Figure 1);[16, 58] and habitual red meat ingestion increases plasma TMAO more than other animal or plant-based protein sources.[7] Our findings suggest that TMAO, γ-butyrobetaine, and crotonobetaine together explain about 8–11% of observed excess ASCVD risk associated with intakes of unprocessed red meat and total meat. Exploratory analyses suggested that γ-butyrobetaine and especially crotonobetaine may be at least as important as TMAO in such mediation, important given that these specific metabolites are derived from the carnitine-pathway, rather than the alternative choline-pathway, for TMAO production.

In experimental studies, TMAO promotes cholesterol accumulation in macrophages by upregulating cell surface expression of the proatherogenic scavenger receptors CD36 and SR-A1;[8] inhibits reverse cholesterol transport and alters sterol metabolism;[16] enhances vascular inflammation through activation of mitogen-activated protein kinase and nuclear Factor-κB signaling[9] and inflammasome activation;[91] impairs endothelial function by increasing superoxide-associated oxidative stress;[13] and promotes platelet hyperresponsiveness and thrombosis potential by enhancing stimulus-dependent Ca^{2+} release from intracellular stores.[14] TMAO also induces hyperglycemia by binding to endoplasmic reticulum stress kinase PERK (EIF2AK3).[88] Possible pro-atherogenic effects of γ-butyrobetaine and crotonobetaine have not been reported. Our findings highlight the need to investigate whether these metabolites have independent physiologic effects, or simply provide an additional or even superior measure of overall tissue exposure to TMAO.

The estimated mediation proportions were attenuated by about half following adjustment for eGFR. Because these metabolites are renally cleared, impaired renal function could confound their associations with ASCVD. On the other hand, mechanistic studies demonstrate that TMAO directly causes renal tubulointerstitial fibrosis, reduced renal filtration, and elevated cystatin-C levels.[52] In addition, suppression of TMAO generation prevents renal impairment in animal models.[53] Thus, impaired renal function may also be an intermediate outcome (i.e., mediator) on the causal pathway between these TMAO-related metabolites and ASCVD. Future experimental studies are needed to further investigate the potential interplay and extent of confounding vs. mediation between intakes of ASF, these gut microbial metabolites, renal function, and ASCVD.

Prior work by our group and others has demonstrated that cardiovascular benefits of fish consumption or omega-3 supplementation may depend on outcomes examined, with stronger associations for CHD, especially fatal CHD or arrhythmic cardiac death, than stroke or total ASCVD;[29, 92, 93] and on fish preparation methods, with protective associations for tuna fish or broiled/baked fish but not fried fish or fish sandwiches.[93] The specificity for coronary events is consistent with the experimental impact of omega-3 fatty acids on stabilization of partially depolarized, acutely ischemic myocytes, reducing susceptibility to acute ventricular arrhythmias.[94] Our focus in the present analysis was on total fish consumption and total ASCVD, and the absence of significant association with this compositive endpoint is consistent with extant literature.[29, 92] Nonetheless, while there was no evidence of overall association (HR=1.00), our mediation analyses suggest the relationship between fish intake and these TMAO-related plasma metabolites was associated with some excess risk. Other beneficial compounds in fish could offset this estimated harm, so the overall association of fish with total ASCVD was neutral. In contrast to meats, the largest mediated association for fish was via TMAO, consistent with fish being a rich source of choline (a precursor of TMAO) but not L-carnitine (a precursor of γ-butyrobetaine, crotonobetaine, and TMAO).

The association of poultry consumption with CVD has not been well-studied,[29] especially in older populations. Our exploratory analyses suggested a possible non-linear dose-response, with lowest risk at about 0.4 servings/day. Adequate intakes of protein can help prevent aging-related loss of muscle mass, improving physical functioning and long-term health outcomes later in life.[1–3] Our findings suggest that poultry might be a healthy source of protein for older adults when consumed moderately. However, the non-linear analyses were exploratory, so conclusions based on them may be due to chance, and should be interpreted with caution until confirmed in other investigations.

Our study has several strengths. The relationship between ASF, plasma levels of microbiome-derived TMAO-related metabolites, and ASCVD events has not been fully established and warrants careful investigation, especially in community-based prospective cohorts such as the CHS with well-measured metabolite biomarkers, ASCVD risk factors (including detailed sociodemographics, traditional CVD risk factors, and lifestyle habits), and a sufficient number of outcomes. Such findings are less subject to bias, residual confounding, and reverse causation; have greater statistical power; and have greater generalizability than many of the prior studies. We evaluated multiple ASF in relation to risk of ASCVD in older adults, the age group at the highest risk; and further investigated potential mediation by a novel set of microbiome-derived metabolites, providing an important new piece of evidence in the puzzle of diet, the microbiome, and ASCVD.

Potential limitations should be considered. The observational design cannot exclude residual confounding. However, we adjusted for a broad range of well-measured risk factors for ASCVD, and results were robust except for additional adjustment for eGFR, which could be both a mediator and confounder for the metabolites-ASCVD associations. Factors known to influence TMAO's generation such as host hepatic flavin-containing monooxygenase (FMOs) and choline TMA-lyase carried by some bacterial species in the gut were not measured; yet, there is no established evidence that these enzymes alter dietary intake or affect CVD risk via paths independent of TMAO. Thus, the potential for major confounding that could fully account for the observed associations, conditional upon all other covariates in the model, is not high. Although we applied statistical methods for causal mediation analysis, the study findings are observational and cannot prove causality. Dietary habits were self-reported, which could cause non-differential measurement errors with respective to ASCVD and metabolite measurements. Because dietary data in CHS were validated against nutrients rather than foods, we could not perform correction for such measurement errors, although we took advantage of serial measures from two validated FFQs. Analyses for secondary dietary exposures should be interpreted with caution, although the total meat-ASCVD association (P=0.004) would remain significant after adjusting for multiple comparisons (adjusted alpha=0.05/4=0.0125). Our findings may not be generalizable to young populations, different races, or other nations.

Conclusions

In this large, community-based cohort of older US adults, higher intakes of unprocessed red meat, total meat, and total ASF were associated with higher incidence of ASCVD, partly explained by plasma levels of γ-butyrobetaine, crotonobetaine, and TMAO. The higher risk of ASCVD associated with meats further appeared partly mediated by glucose-insulin homeostasis and systematic inflammation, but not blood pressure or blood cholesterol levels. These novel findings support a biochemical link between dietary meat intake, carnitine-related gut microbiome pathways, and ASCVD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation:

ASF	animal source food
TMAO	trimethylamine N-oxide
ASCVD	atherosclerotic cardiovascular disease
CRP	C-reactive protein

CHS	Cardiovascular Health Study
FFQ	food frequency questionnaire
eGFR	estimated glomerular filtration rate
CHD	coronary heart disease
MI	myocardial infarction
HR	hazard ratio
IQR	inter-quintile range

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Highlights

- In a community-based cohort of older US adults aged ≥ 65 years, higher intakes of unprocessed red meat, total meat (unprocessed red meat plus processed meat), and total animal source foods were prospectively associated with a higher incidence of atherosclerotic cardiovascular disease (ASCVD) during a median follow-up of 12.5 years.
- These associations were partly mediated (8–11% of excess risk) by plasma levels of gut microbiota-generated metabolites, including trimethylamine N-oxide (TMAO) and its two intermediates derived from L-carnitine, abundant in red meat.
- The higher risk of ASCVD associated with meat intake was also partly mediated by glucose-insulin homeostasis and systematic inflammation, but not blood pressure or blood cholesterol levels.
- Intakes of fish, poultry, and eggs were not significantly associated with ASCVD.

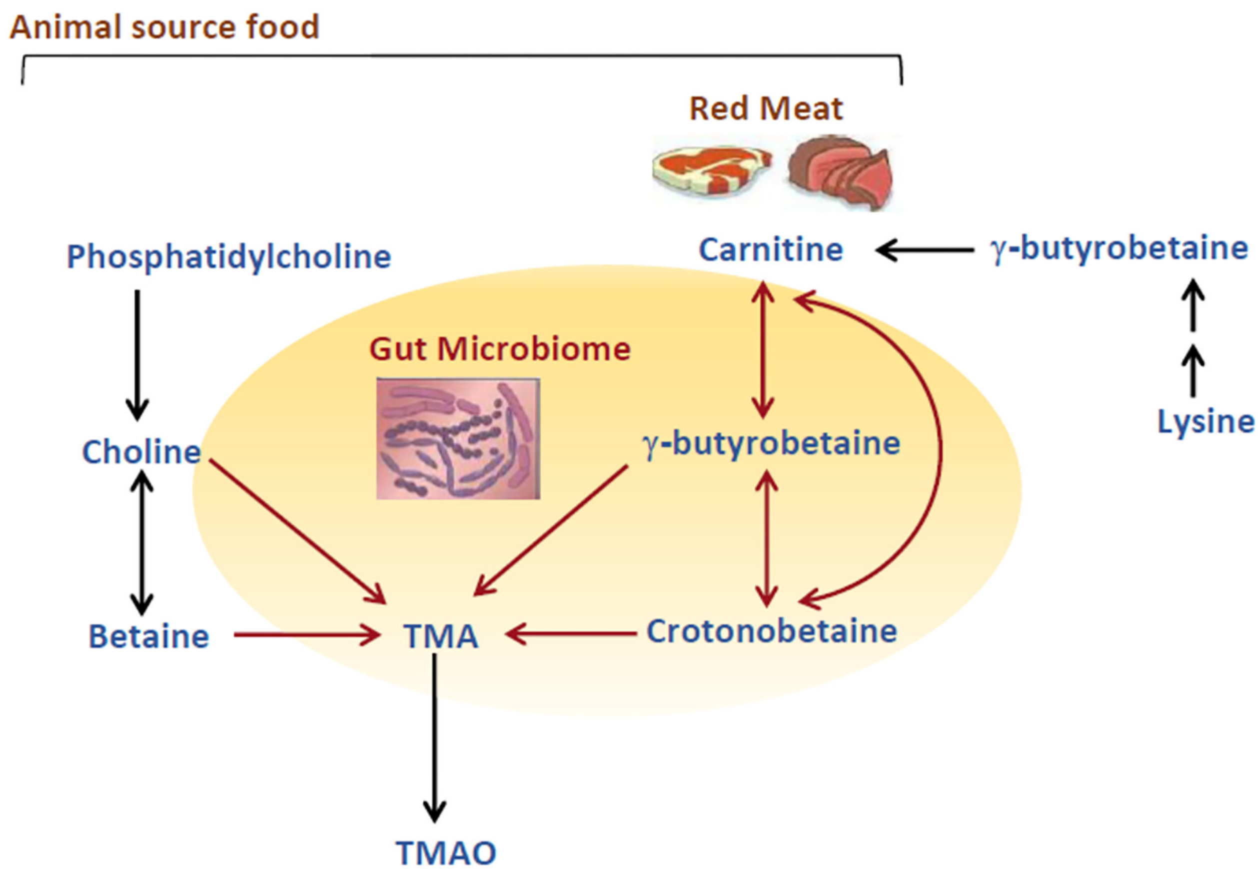

Figure 1. Pathways for generation of trimethylamine N-oxide (TMAO) and its intermediates.

Arrows in black represent transformations performed by the host, and arrows in red represent transformations performed by gut microbes. The endogenous biosynthesis of carnitine involves multiple steps from lysine to γ-butyrobetaine, indicated by a chain of arrows. In healthy subjects, γ-butyrobetaine is also endogenously synthesized from lysine, independent of gut-microbiota (6,58). In contrast, production of TMAO and crotonobetaine are profoundly suppressed by antibiotic administration (58), supporting a dominant role of gut microbial metabolism in their generation.

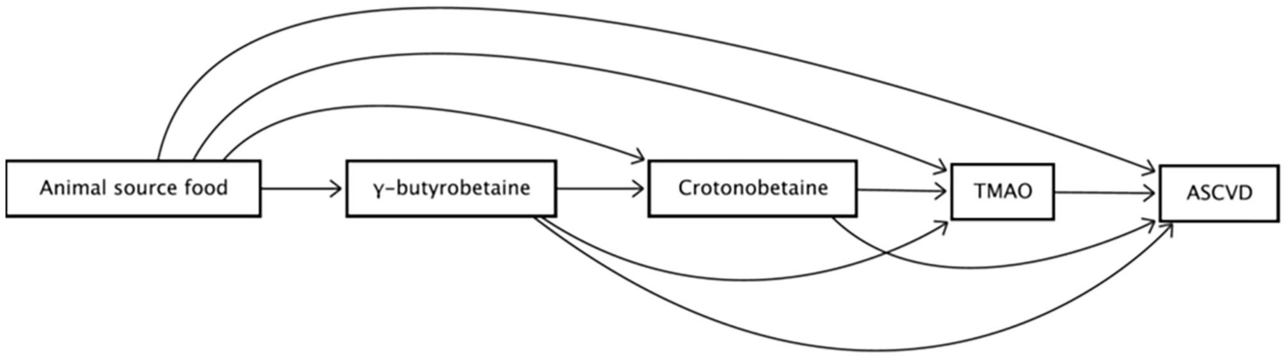

Figure 2. Conceptual diagram of dietary exposures, gut microbiota-generated TMAO-related metabolites (mediators), and ASCVD.

For mediation modeling, eight potential causal pathways were jointly assessed: 1) ASF (animal source food)→ASCVD through other pathways; and ASF to ASCVD via: 2) **γ**-butyrobetaine→ASCVD; 3) **γ**-butyrobetaine→ crotonobetaine→ASCVD; 4) **γ**-butyrobetaine→ crotonobetaine→TMAO→ASCVD; 5) **γ**-butyrobetaine→TMAO→ASCVD; 6) crotonobetaine→ASCVD; 7) crotonobetaine→TMAO→ASCVD; 8) TMAO→ASCVD. Confounders are not shown in the graph to focus on the main causal pathways and for better visualization.

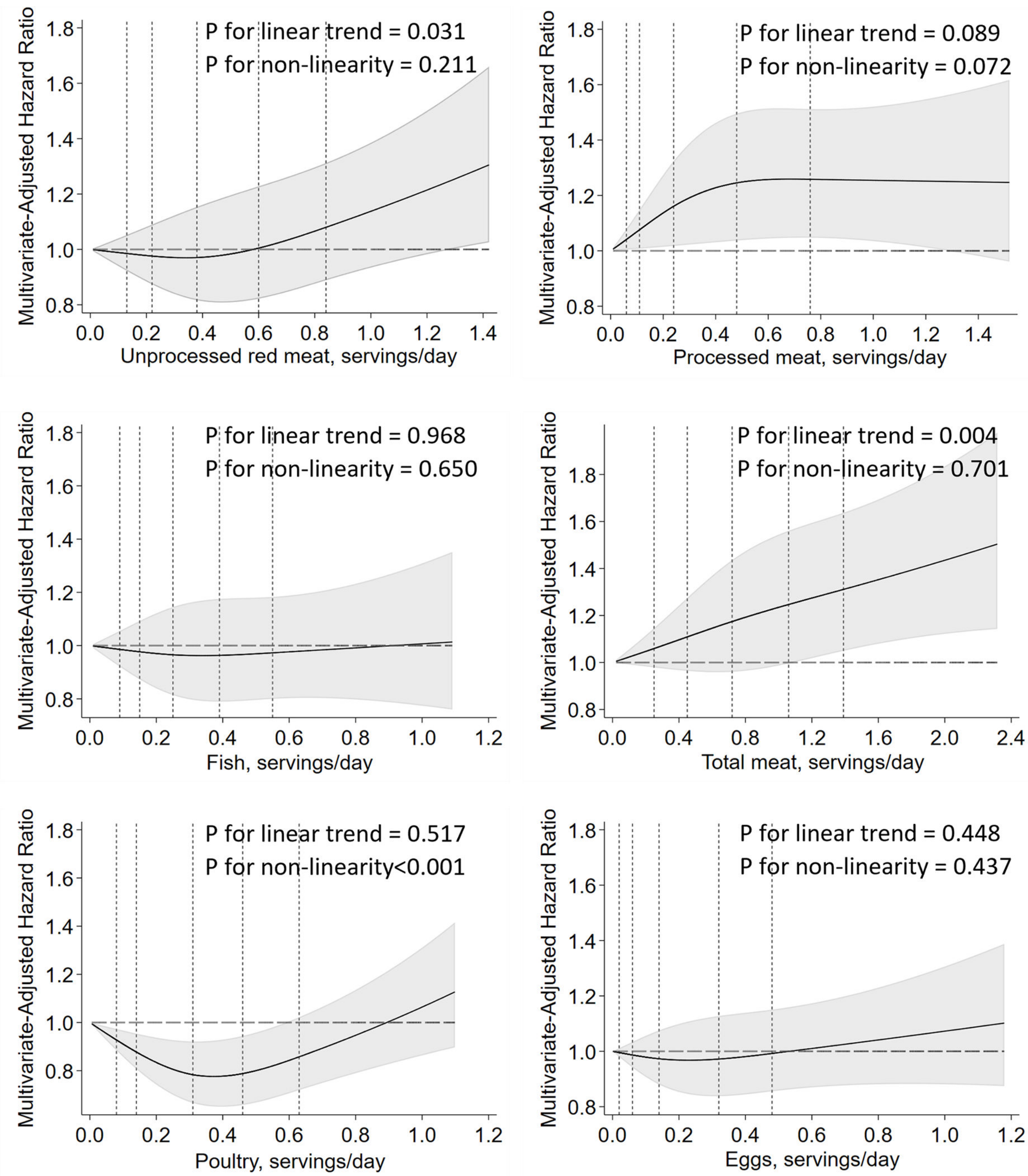

Figure 3. Multivariable adjusted relationships between intakes of each ASF and the risk of ASCVD, evaluated using restricted cubic splines.

Knots were evaluated at the 10th, 50th, and 90th percentiles. Dotted vertical lines represent, from left to right, the 10th, 25th, 50th, 75th, and 90th percentiles of dietary intake. Covariates are specified in Table 3. The top 1% of the exposure distribution was not shown for better visualization.

Table 1.

Population characteristics of older U.S. men and women in the Cardiovascular Health Study, by quintiles of unprocessed red meat intake at baseline

	Q1	Q2	Q3	Q4	Q5	Total
N	778	778	778	778	777	3889 *
Range, servings/day	(0.01–0.18)	(0.18–0.30)	(0.30 – 0.47)	(0.47 –0.69)	(0.69 – 4.49)	(0.01– 4.49)
Sociodemographic factors						
Age, years	72.9 ± 5.5	72.7 ± 5.4	73.2 ± 6.0	73 ± 5.7	72.5 ± 5.4	72.9 ± 5.6
Male	233 (29.9)	260 (33.4)	296 (38.0)	336 (43.2)	294 (37.8)	1419 (36.5)
Race						
White	673 (86.5)	672 (86.4)	684 (87.9)	692 (88.9)	700 (90.1)	3421 (88.0)
Non-white	105 (13.5)	106 (13.6)	94 (12.1)	86 (11.1)	77 (9.9)	468 (12.0)
Income						
<$11,999	172 (22.1)	179 (23.0)	168 (21.6)	163 (21.0)	188 (24.2)	870 (22.4)
$12,000 to 24,999	259 (33.3)	264 (33.9)	312 (40.1)	299 (38.4)	286 (36.8)	1420 (36.5)
$25,000 to $49,999	218 (28.0)	231 (29.7)	200 (25.7)	223 (28.7)	195 (25.1)	1067 (27.4)
>$50,000	129 (16.6)	104 (13.4)	98 (12.6)	93 (12.0)	108 (13.9)	532 (13.7)
Education						
<High school	180 (23.1)	181 (23.3)	206 (26.5)	211 (27.1)	230 (29.6)	1008 (25.9)
High school	226 (29.0)	236 (30.3)	229 (29.4)	221 (28.4)	223 (28.7)	1135 (29.2)
Some college	180 (23.1)	186 (23.9)	183 (23.5)	193 (24.8)	173 (22.3)	915 (23.5)
College graduate	192 (24.7)	175 (22.5)	160 (20.6)	153 (19.7)	151 (19.4)	831 (21.4)
Lifestyle factors						
Smoking						
Never	386 (49.6)	359 (46.1)	390 (50.1)	359 (46.1)	357 (45.9)	1851 (47.6)
Former	324 (41.6)	330 (42.4)	288 (37.0)	321 (41.3)	312 (40.2)	1575 (40.5)
Current	68 (8.7)	89 (11.4)	100 (12.9)	98 (12.6)	108 (13.9)	463 (11.9)
Alcohol, drinks/week	0.02 (0–1.25)	0.04 (0–1.50)	0.02 (0–1.25)	0.02 (0–1.50)	0 (0–1.00)	0.02 (0–1.25)
Physical activity, kcal/week	699 (158–1643)	690 (205–1530)	600 (140–1650)	623 (158–1470)	496 (0–1328)	622 (135–1530)
General Health						
Excellent	125 (16.1)	114 (14.7)	122 (15.7)	102 (13.1)	112 (14.4)	575 (14.8)
Very good	225 (28.9)	213 (27.4)	216 (27.8)	223 (28.7)	202 (26.0)	1079 (27.7)

	Q1	Q2	Q3	Q4	Q5	Total
Good	296 (38.0)	298 (38.3)	308 (39.6)	314 (40.4)	291 (37.5)	1507 (38.8)
Fair	119 (15.3)	140 (18.0)	120 (15.4)	120 (15.4)	153 (19.7)	652 (16.8)
Poor	13 (1.7)	13 (1.7)	12 (1.5)	19 (2.4)	19 (2.4)	76 (2.0)
Body mass index (kg/m^2)	26.0 ± 4.5	26.2 ± 4.6	26.9 ± 4.6	27.0 ± 4.7	27.1 ± 4.9	26.6 ± 4.7
Medical history						
Anti-hypertensive medication use	302 (38.8)	321 (41.3)	313 (40.2)	316 (40.6)	319 (41.1)	1571 (40.4)
Lipid-lowering medication use	42 (5.4)	48 (6.2)	38 (4.9)	32 (4.1)	25 (3.2)	185 (4.8)
Oral hypoglycemic agents or insulin	37 (4.8)	59 (7.6)	38 (4.9)	60 (7.7)	57 (7.3)	251 (6.5)
Antibiotic use (prior 2 weeks)	28 (3.6)	18 (2.3)	19 (2.4)	24 (3.1)	23 (3.0)	112 (2.9)
Diabetes	134 (17.2)	153 (19.7)	148 (19.0)	171 (22.0)	183 (23.6)	789 (20.3)
Diet						
Fruits, servings/day	2.5 ± 1.1	2.3 ± 1.0	2.2 ± 1.1	2.1 ± 1.0	1.9 ± 1.0	2.2 ± 1.1
Vegetables, servings/day	2.9 ± 1.4	2.6 ± 1.2	2.6 ± 1.2	2.5 ± 1.2	2.5 ± 1.2	2.6 ± 1.3
Dietary fiber, g/day	33.6 ± 10.9	31.0 ± 10.0	29.5 ± 9.5	28.7 ± 9.0	26.6 ± 9.2	29.9 ± 10.0
Dairy products, servings/day	1.4 ± 0.7	1.4 ± 0.6	1.3 ± 0.6	1.2 ± 0.6	1.1 ± 0.6	1.3 ± 0.6
Total ASF, servings/day	1.3 ± 0.6	1.5 ± 0.6	1.6 ± 0.6	1.8 ± 0.5	2.2 ± 0.7	1.7 ± 0.7
Processed meats, servings/day	0.3 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	0.4 ± 0.3	0.4 ± 0.3
Fish, servings/day	0.4 ± 0.3	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2
Poultry, servings/day	0.4 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	0.3 ± 0.2	0.4 ± 0.2	0.3 ± 0.3
Eggs, servings/day	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.3	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
Total energy, kcal/day	1783.4 ± 527.1	1687.0 ± 542.7	1858.0 ± 752.6	2016.7 ± 685.8	1735.6 ± 603.8	1816.1 ± 638.4
TMAO-related plasma biomarkers,						
TMAO, μM/L	4.6 (3.1–6.9)	4.3 (3.1–7.3)	4.6 (3.1–7.5)	5.1 (3.5–8.1)	5.0 (3.4–8.2)	4.7 (3.2–7.7)
Choline, μM/L	9.4 (8.0–10.9)	9.6 (8.1–11.1)	9.5 (8.0–11.1)	9.7 (8.4–11.4)	9.4 (8.0–11.3)	9.5 (8.1–11.2)
Betaine, μM/L	38.0 ± 13.9	37.3 ± 12.8	36.8 ± 12.4	37.0 ± 13.2	36.0 ± 12.5	37.0 ± 13.0
Carnitine, μM/L	35.9 ± 8.5	36.8 ± 7.7	37.1 ± 8.1	37.7 ± 8.3	38.0 ± 8.3	37.1 ± 8.2
γ -butyrobetaine, μM/L	0.96 ± 0.29	0.99 ± 0.31	1.03 ± 0.34	1.05 ± 0.34	1.07 ± 0.37	1.02 ± 0.34
Crotonobetaine, μM/L	0.021 (0.010–0.027)	0.022 (0.010–0.028)	0.023 (0.010–0.030)	0.023 (0.010–0.029)	0.024 (0.010–0.031)	0.023 (0.010–0.029)

Values are N (%), mean ± SD, or median (interquartile range) at analysis baseline (1989–1990 for 3335 participants and 1996–97 for 554 participants).

Food intakes were energy-adjusted.

*Number of participants with joint availability of unprocessed red meat and TMAO measures was 3889.

TMAO: trimethylamine N-oxide

Table 2.

Spearman correlations between intakes of ASF (servings/day) and TMAO-related plasma biomarkers at baseline

	Unprocessed red meat	Processed meat	Total meat†	Fish	Poultry	Eggs	Total ASF‡
No. of participants	3889	3891	3871	3891	3898	3908	3843
TMAO	0.060**	0.008	0.047**	0.069**	0.000	0.017	0.072**
γ -butyrobetaine	0.110**	0.123**	0.135**	−0.064**	−0.043**	0.032*	0.073**
Crotonobetaine	0.096**	0.126**	0.147**	−0.043**	−0.033*	0.078**	0.114**
Choline	0.021	0.101**	0.077**	−0.048**	−0.040*	0.088**	0.046**
Betaine	−0.050**	0.038*	−0.005	−0.039*	−0.050**	0.060**	−0.011
Carnitine	0.090**	0.088**	0.111**	−0.017	−0.028	0.015	0.066**

Values are spearman correlation coefficients at analysis baseline. Red color represents positive values, with darker red representing larger positive correlations. Green color represents negative values, with darker green representing larger negative correlations.

Dietary intakes were energy-adjusted using the residual method.

*P<0.05

**P<0.01

†Total meat: Unprocessed red meat plus processed meat

‡Total ASF (animal source food): Unprocessed red meat, processed meat, fish, poultry, and eggs.

TMAO: trimethylamine N-oxide.

Table 3.

Risk of incident ASCVD associated with time-varying intakes of each ASF (per IQR), and joint mediation by time-varying TMAO, γ-butyrobetaine, and crotonobetaine

	Unprocessed red meat	Processed meat	Total meat *	Fish	Poultry	Eggs	Total ASF †
Inter-quintile range (10th, 90th), serving/day	0.71 (0.13, 0.84)	0.71 (0.06, 0.76)	1.14 (0.25, 1.39)	0.46 (0.09, 0.55)	0.55 (0.08, 0.63)	0.47 (0.02, 0.48)	1.50 (0.93, 2.43)
No. of cases/total N ‡	1655/3889	1653/3891	1644/3871	1658/3891	1660/3898	1664/3908	1634/3843
Person-years	50894	50962	50589	50979	51066	51243	50142
Main dietary association §							
Hazard ratios (95%CI) ¶	1.15 (1.01, 1.30)	1.11 (0.98, 1.25)	1.22 (1.07, 1.39)	1.00 (0.89, 1.13)	1.04 (0.92, 1.18)	1.04 (0.94, 1.14)	1.18 (1.03, 1.34)
No. of excess events per 1000 persons per year (95%CI) #	3.95 (−0.15, 8.05)	3.56 (−0.46, 7.58)	6.32 (1.77, 10.87)	0.49 (−3.27, 4.25)	1.77 (−2.13, 5.67)	1.43 (−2.20, 5.06)	5.79 (1.36, 10.22)
Mediation analyses **							
No. of excess events per 1000 persons per year (95%CI)							
Dietary association independent of metabolites	3.50 (−0.64, 7.64)	3.33 (−0.69, 7.35)	5.77 (1.20, 10.34)	0.06 (−3.76, 3.88)	1.80 (−2.12, 5.72)	1.36 (−2.27, 4.99)	5.22 (0.75, 9.69)
Dietary association mediated via metabolites	0.42 (0.04, 0.85)	0.18 (−0.05, 0.45)	0.49 (0.06, 0.98)	0.45 (0.07, 0.88)	−0.04 (−0.26, 0.16)	0.06 (−0.17, 0.29)	0.53 (0.14, 0.98)
Via γ-butyrobetaine ††	0.20 (−0.08, 0.52)	0.04 (−0.05, 0.19)	0.21 (−0.06, 0.54)	0.00 (−0.10, 0.11)	0.00 (−0.11, 0.10)	−0.08 (−0.25, 0.03)	0.08 (−0.03, 0.28)
Via crotonobetaine ††	0.31 (0.04, 0.62)	0.17 (0.01, 0.41)	0.39 (0.03, 0.79)	0.17 (0.01, 0.39)	−0.06 (−0.24, 0.06)	0.09 (−0.01, 0.26)	0.36 (0.02, 0.75)
Via TMAO ††	0.11 (−0.01, 0.30)	0.02 (−0.09, 0.16)	0.10 (−0.02, 0.30)	0.31 (−0.03, 0.69)	0.01 (−0.10, 0.13)	0.01 (−0.09, 0.11)	0.20 (−0.02, 0.48)
Mediation proportions (%) ‡‡	10.6 (1.0, 114.5)	5.1 (−1.7, 63.2)	7.8 (1.0, 32.7)	NA §§	−2.4 (−71.3, 20.6)	4.2 (−30.5, 92.7)	9.2 (2.2, 44.5)

Models were adjusted for age (years), sex, race (white vs. non-white), study site (4 categories), education (<high school, high school, some college, or college graduate), income (<$11,999, $12,000 to 24,999, $25,000 to $49,999, or >$50,000), and time-varying self-reported health status (excellent, very good, good, fair, or poor), smoking status (never smoked, former smoker, or current smoker), alcohol intake (drinks/week), physical activity (kcal/week, log transformed for additive hazard model), antibiotic use (yes vs. no), and intakes of total energy (kcal/day, log-transformed for additive hazard models), fruits (servings/day), vegetables (servings/day), dietary fiber (g/day), total dairy products (servings/day), and the other animal source foods mutually adjusted (servings/day). Imputed values were used when animal source foods were adjusted covariates.

* Total meat: unprocessed red meat plus processed meat.

† Total ASF (animal source food): sum of unprocessed red meat, processed meat, fish, poultry, and eggs

[‡]Given that data availability varied by dietary exposures, the number of participants included in the analyses of each exposure was different.

[§]Dietary associations were estimated from models without the three metabolites.

[¶]Hazard ratios were estimated from Cox models

[#]No. of excess events were estimated from additive hazard models.

[**]Mediation analyses were performed using additive hazard models. Confidence intervals excluding zero indicate statistically significant mediated association or mediation proportion. TMAO and crotonobetaine were log transformed.

[††]Refers to any paths passing through the specified metabolite. The three paths via γ-butyrobetaine, crotonobetaine, and TMAO were not mutually exclusive. See Figure 2 for details.

[‡‡]Mediation proportion was defined as mediated association/|(independent association + mediated association)|.

[§§]Given that the dietary association for fish was close to null, mediation proportions for fish would not be meaningful and were not calculated.

TMAO: trimethylamine N-oxide. IQR: interquintile range, comparing the midpoints of the first and fifth quintiles.

Table 4.

Risk of incident ASCVD associated with time-varying intakes of meats (per IQR) and mediation by time-varying traditional CVD risk factors

	Unprocessed red meat	Processed meat	Total meat *
Mediator: Total cholesterol			
No. of excess events per 1000 persons per year (95%CI)			
Dietary association independent of cholesterol	3.9 (−0.2, 8.0)	3.5 (−0.5, 7.6)	6.3 (1.8, 10.8)
Dietary association mediated via cholesterol	0.0 (−0.1, 0.1)	0.0 (−0.1, 0.2)	0.0 (−0.1, 0.2)
Mediation proportions (%)	0.5 (−3.2, 8.4)	0.8 (−2.8, 12.9)	0.6 (−1.1, 4.4)
Mediator: Fasting triglycerides			
No. of excess events per 1000 persons per year (95%CI)			
Dietary association independent of triglycerides	4.0 (−0.1, 8.1)	3.3 (−0.7, 7.4)	6.2 (1.6, 10.7)
Dietary association mediated via triglycerides	0.0 (−0.2, 0.2)	0.1 (−0.1, 0.3)	0.1 (−0.1, 0.3)
Mediation proportions (%)	0.3 (−10.4, 11.3)	1.6 (−8.5, 30.1)	1.6 (−2.4, 8.8)
Mediator: LDL cholesterol			
No. of excess events per 1000 persons per year (95%CI)			
Dietary association independent of LDL	3.9 (−0.2, 8.0)	3.6 (−0.5, 7.6)	6.3 (1.8, 10.8)
Dietary association mediated via LDL	0.0 (−0.1, 0.1)	0.0 (−0.1, 0.1)	0.0 (−0.1, 0.1)
Mediation proportions (%)	0.2 (−3.6, 6.0)	0.1 (−5.3, 6.4)	0.2 (−1.6, 2.7)
Mediator: HDL cholesterol			
No. of excess events per 1000 persons per year (95%CI)			
Dietary association independent of HDL	3.9 (−0.2, 8.0)	3.6 (−0.5, 7.6)	6.3 (1.7, 10.8)
Dietary association mediated via HDL	0.1 (0.0, 0.2)	0.0 (−0.1, 0.1)	0.1 (−0.1, 0.2)
Mediation proportions (%)	2.0 (−1.1, 20.6)	−0.3 (−12.1, 8.1)	0.9 (−1.6, 5.7)
Mediator: Systolic blood pressure			
No. of excess events per 1000 persons per year (95%CI)			
Dietary association independent of SBP	3.8 (−0.3, 7.9)	3.7 (−0.3, 7.7)	6.3 (1.8, 10.9)
Dietary association mediated via SBP	0.1 (−0.3, 0.5)	−0.1 (−0.5, 0.4)	0.1 (−0.4, 0.5)
Mediation proportions (%)	2.1 (−21.7, 33.7)	−1.6 (−48.3, 18.9)	0.8 (−10.8, 11.2)
Mediator: Diastolic blood pressure			
No. of excess events per 1000 persons per year (95%CI)			
Dietary association independent of DBP	3.9 (−0.2, 8.0)	3.7 (−0.3, 7.6)	6.4 (1.8, 10.9)
Dietary association mediated via DBP	0.1 (−0.1, 0.2)	0.0 (−0.2, 0.2)	0.1 (−0.1, 0.3)
Mediation proportions (%)	1.3 (−5.6, 17.1)	1.0 (−8.3, 18.0)	1.0 (−2.7, 6.4)
Mediator: Fasting glucose			
No. of excess events per 1000 persons per year (95%CI)			
Dietary association independent of glucose	3.4 (−0.7, 7.5)	2.4 (−1.5, 6.4)	4.9 (0.4, 9.4)
Dietary association mediated via glucose	0.9 (0.4, 1.5)	1.2 (0.7, 1.9)	1.7 (1.0, 2.6)
Mediation proportions (%)	21.5 (8.5, 175.8)	34.0 (13.7, 366.1)	26.1 (12.7, 82.7)
Mediator: C-reactive protein			
No. of excess events per 1000 persons per year (95%CI)			

	Unprocessed red meat	Processed meat	Total meat *
Dietary association independent of glucose	4.0 (−0.1, 8.1)	2.9 (−1.1, 6.9)	5.8 (1.3, 10.4)
Dietary association mediated via glucose	0.0 (−0.3, 0.4)	0.5 (0.1, 0.9)	0.4 (0.0, 0.8)
Mediation proportions (%)	0.9 (−18.6, 21.2)	13.9 (2.8, 192.7)	6.6 (0.4, 27.5)
Mediator: fasting insulin			
No. of excess events per 1000 persons per year (95%CI)			
Dietary association independent of glucose	3.7 (−0.4, 7.8)	3.1 (−0.9, 7.1)	5.7 (1.2, 10.3)
Dietary association mediated via glucose	0.4 (0.1, 0.7)	0.6 (0.2, 1.0)	0.8 (0.3, 1.3)
Mediation proportions (%)	9.6 (2.7, 90.6)	15.7 (4.8, 186.6)	11.8 (4.3, 43.2)

Additive hazard models were adjusted for age (years), sex, race (white vs. non-white), study site (4 categories), education (<high school, high school, some college, or college graduate), income (<$11,999, $12,000 to 24,999, $25,000 to $49,999, or >$50,000), and time-varying self-reported health status (excellent, very good, good, fair, or poor), smoking status (never smoked, former smoker, or current smoker), alcohol intake (drinks/week), physical activity (kcal/week, log transformed for additive hazard model), antibiotic use (yes vs. no), and intakes of total energy (kcal/day, log-transformed for additive hazard models), fruits (servings/day), vegetables (servings/day), dietary fiber (g/day), total dairy products (servings/day), and the other animal source foods mutually adjusted (servings/day). Imputed values were used when animal source foods were adjusted covariates.

Confidence intervals excluding zero indicate statistically significant association or mediation proportion. Triglycerides, CRP, and fasting insulin were log-transformed.

*Total meat: unprocessed red meat plus processed meat.

IQR: interquintile range, comparing the midpoints of the first and fifth quintiles.