SUPPLEMENTAL MATERIALS

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

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Methods

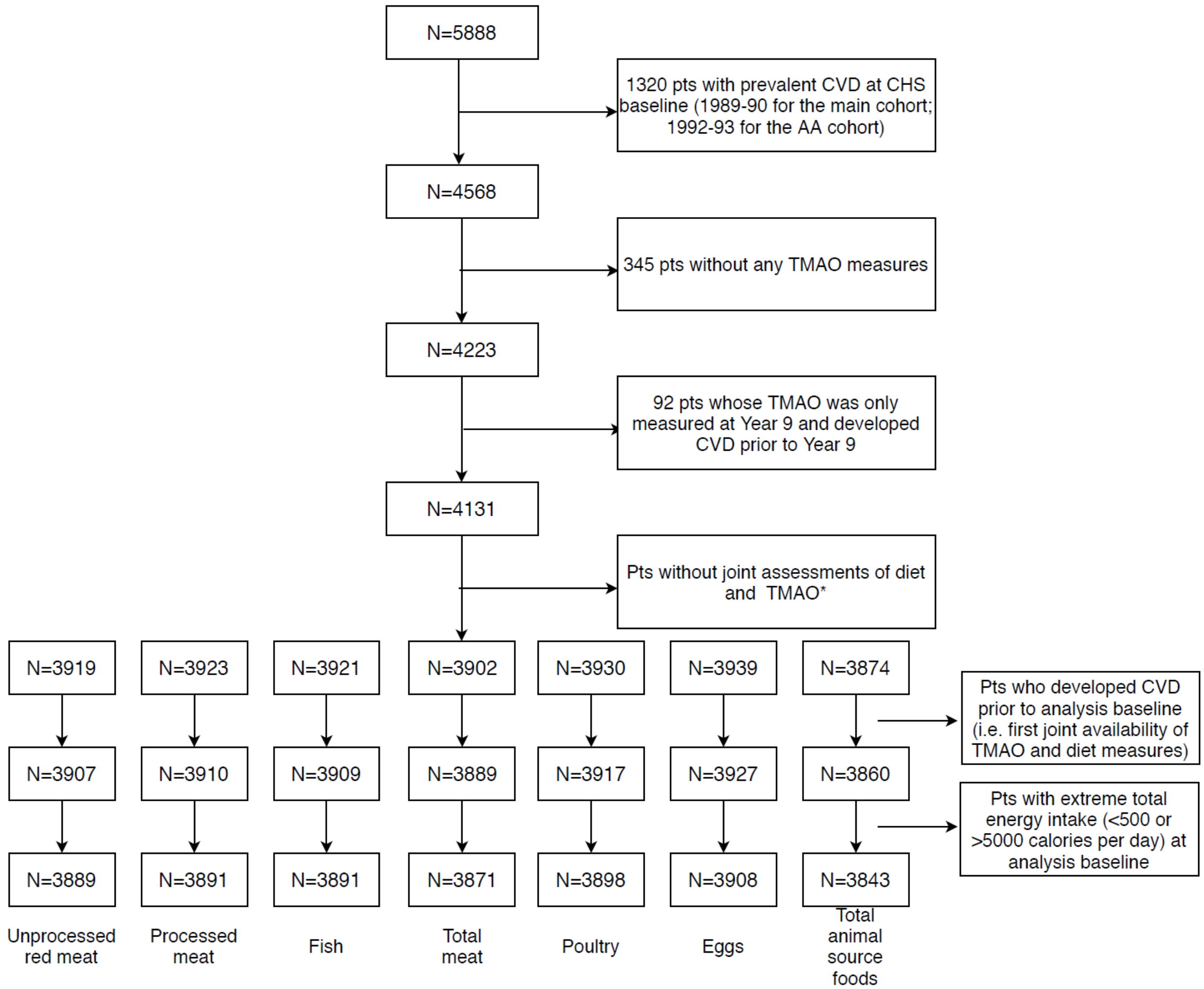

Figure S1. Flowchart of participants included in the analyses of each dietary exposure. Data availability varied by dietary exposures. A total of 3931 participants had available data on at least one exposure. Abbreviation: AA, African-American; CHS, Cardiovascular Health Study; CVD, cardiovascular disease; Pts, participants; TMAO, trimethylamine N-oxide.

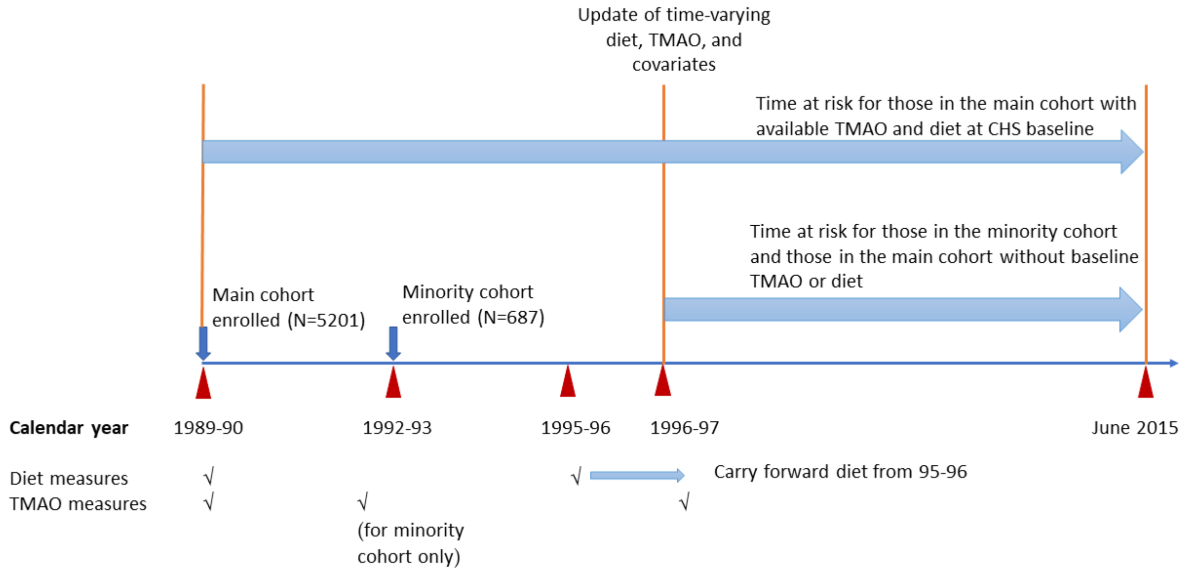

Figure S2. Study design and timing of TMAO and diet assessment. Plasma TMAO-related biomarkers measured at 1989-90 were evaluated as mediators for the association between usual dietary intake at 1989-90 and ASCVD risk until 1996-97, and biomarkers measured at 1996-97 were evaluated as mediators for the association between the average of dietary intakes at 1989-90 and 1996-97 and subsequent risk. Time - varying covariates were updated whenever TMAO-related biomarkers were updated. We used simple updating for non-dietary covariates and cumulative updating for dietary covariates. Abbreviation: CHS, Cardiovascular Health Study; TMAO, trimethylamine N-oxide. Follow-up for ASCVD events started at the baseline TMAO measure and continued through June 2015.

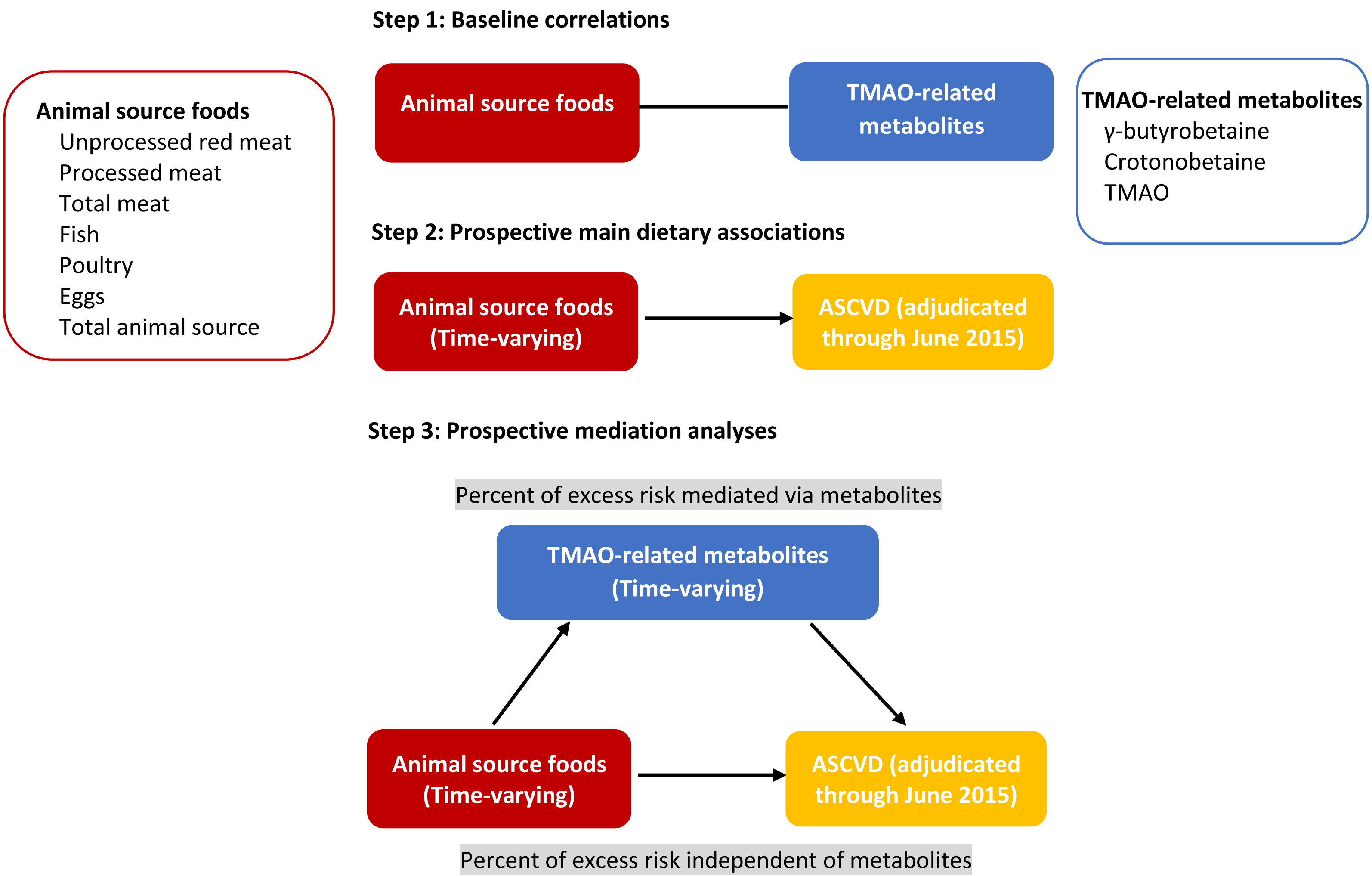

Figure S3. Study flowchart. TMAO: trimethylamine N-oxide; ASCVD: atherosclerotic cardiovascular disease

Systematic and random measurement errors

Systematic measurement errors may be present when a variable is not measured using the "gold standard" method. However, many of the main covariates used in CHS were measured using a "gold standard" approach for epidemiologic studies. For example, TMAO-related metabolites were measured using a targeted, mass spectrometry-based approach, with low lab CVs.[7] Anthropometrics and blood pressure were directly measured by trained personnel, and the average of 2 blood pressure readings taken after a 5-minute rest was used.[25] Laboratory measures such as lipids, CRP, glucose, creatinine, and cystatin C were all directly measured from fasting blood samples using standardized methods[25, 44]. Creatine, measured using a colorimetric assay, was further calibrated to isotope dilution mass spectrometry measures from the Cleveland Clinic Research Laboratory.[45] Therefore, the magnitude of systematic measurement errors in each of these factors should not be large.

Habitual diet was measured using food frequency questionnaires (FFQ) validated against multiple 24-hour dietary recalls or week diet records.[33-34,36] Nevertheless, self-reported FFQs can have measurement errors compared to the more objective approach of diet records, although diet records have the separate limitation of assessing a very short period of time (e.g., 24 hours or 1 week), rather than true habitual intake. Thus, there is no perfect measure of true long-term dietary intake.

Given the above points and the unavailability of data (i.e., regression coefficients from the model regressing a gold standard measure such as dietary record or recall on FFQ measures and all covariates) needed for performing dietary measurement correction, we did not perform corrections for systematic measurement errors.

Cohort studies that only evaluate a single measure of exposures and covariates at baseline are subject to random measurement errors due to possible within-subject variations in these measures over time. We have in part addressed this type of error by using time-varying exposures, mediators, and covariates with updated values over time.

Statistical analysis

The causal mediation analysis method of Huang and Yang [55] under the Aalen additive hazard model was used to assess the evidence for mediation. For each dietary exposure, to decompose the association with ASCVD into those independent of and mediated via the three TMAO-related metabolites, we first fit three linear mixed models for γ-butyrobetaine (M1), crotonobetaine (M2), and TMAO (M3), respectively. The model for γ-butyrobetaine included the dietary exposure (A) and all covariates (X); the model for crotonobetaine included the dietary exposure, γ-butyrobetaine, and all covariates; the model for TMAO included the dietary exposure, γ-butyrobetaine, crotonobetaine, and all covariates. These three models are shown below:

$$M_{1it} = \alpha_0 + \alpha_A A_{it} + \alpha_X X_{it} + \epsilon_{M1it}$$
$$M_{2it} = \beta_0 + \beta_A A_{it} + \beta_{M1} M_{1it} + \beta_X X_{it} + \epsilon_{M2it}$$
$$M_{3it} = \gamma_0 + \gamma_A A_{it} + \gamma_{M1} M_{1it} + \gamma_{M2} M_{2it} + \gamma_X X_{it} + \epsilon_{M3it},$$

where the error term ϵ_{M1it}, ϵ_{M2it}, and ϵ_{M3it} are independent and normally distributed with mean zero and model-specific variances.

At the second step, we fit an Aalen additive hazard model for the outcome (time to ASCVD) that included the dietary exposure, γ-butyrobetaine, crotonobetaine, TMAO, and all covariates:

$$\lambda(t|A_{it}, M_{1it,} M_{2it}, M_{3it}, X_{it}) = \lambda_i(t) = \lambda_0(t) + \lambda_A A_{it} + \lambda_{M1} M_{1it} + \lambda_{M2} M_{2it} + \lambda_{M3} M_{3it} + \lambda_X X_{it},$$

where $\lambda_i(t)$ is the hazard of developing ASCVD for participant i at $time$ t ; $\lambda_0(t)$ is the baseline hazard, and λ_A, λ_{M1}, λ_{M2}, λ_{M3}, and λ_X are regression coefficients for the dietary exposure, γ-butyrobetaine, crotonobetaine, TMAO, and covariates X, respectively. The assumptions of constant hazard difference were examined, and no variables violated this assumption.

As in Huang and Yang [55], the natural direct effect measuring the association between a dietary exposure and ASCVD that is not mediated by any of the three mediators,

$$\lambda(t|A_{1t}, M_{1it}(A_{2t}), M_{2it}(A_{3t}, M_{1it}(A_{2t})), M_{3it}(A_{4t}, M_{1it}(A_{2t}), M_{2it}(A_{3t}, M_{1it}(A_{2t}))), X_{it}) -$$
$$\lambda(t|A_{0t}, M_{1it}(A_{2t}), M_{2it}(A_{3t}, M_{1it}(A_{2t})), M_{3it}(A_{4t}, M_{1it}(A_{2t}), M_{2it}(A_{3t}, M_{1it}(A_{2t}))), X_{it}),$$

was assumed not to depend on the value of $A_{2t},\ A_{3t}, or\ A_{4t}$. It was estimated by $\lambda_A(A_1 - A_0)$, where $A_1 - A_0$ is the dietary exposure inter-quintile range.

Similarly, the natural indirect effects measuring the associations between a dietary exposure and ASCVD mediated via the three mediators (i.e., mediated association) were also assumed not to depend on the exposure levels leading to adjustment values of the mediators absent from the pathway. We estimated these natural indirect effects by the following 7 path-specific associations:

(1) A->γ-butyrobetaine->ASCVD, estimated by $\alpha_A\lambda_{M1}$;

(2) A->γ-butyrobetaine-> crotonobetaine->ASCVD, estimated by $\alpha_A\beta_{M1}\lambda_{M2}$;

(3) A->γ-butyrobetaine-> crotonobetaine->TMAO->ASCVD, estimated by $\alpha_A\beta_{M1}\gamma_{M2}\lambda_{M3}$;

(4) A->γ-butyrobetaine->TMAO->ASCVD, estimated by $\alpha_A\gamma_{M1}\lambda_{M3}$;

(5) A->crotonobetaine->ASCVD, estimated by $\beta_A\lambda_{M2}$;

(6) A->crotonobetaine->TMAO->ASCVD, estimated by $\beta_A\gamma_{M2}\lambda_{M3}$;

(7) A->TMAO->ASCVD, estimated by $\gamma_A\lambda_{M3}$,

and the overall natural indirect effect measuring the association between a dietary exposure and ASCVD mediated by one or more of the three mediators was estimated by the sum of these 7 path-specific associations.

Confidence intervals of the path-specific associations and overall mediated association were computed using a resampling method taking 10,000 random draws from multivariate normal distribution of estimates for $(\alpha_A, \beta_A, \gamma_A, \beta_{M1}, \gamma_{M1}, \gamma_{M2}, \lambda_A, \lambda_{M1}, \lambda_{M2}, \lambda_{M3})$. Assumptions of independence, positivity, consistency, and normality of mediators similar to those made by Huang and Yang[55] and by Lange and Hansen[56] need to be made with extensions to the scenario of three mediators.

Results

Table S1. Comparison of baseline characteristics between participants excluded and included in the study

	Excluded	Included
N	637	3931
Age, years	75.2 ± 6.5	72.1 ± 5.2
Male	344 (54.0)	1436 (36.5)
Race		
White	386 (60.6)	3454 (87.9)
Non-white	251 (39.4)	477 (12.1)
Income		
<$11,999	249 (39.1)	878 (22.3)
$12,000 to 24,999	213 (33.4)	1438 (36.6)
$25,000 to $49,999	127 (19.9)	1079 (27.4)
>$50,000	48 (7.5)	536 (13.6)
Education		
<High school	257 (40.3)	1026 (26.1)
High school	138 (21.7)	1142 (29.1)
Some college	123 (19.3)	926 (23.6)
College graduate	119 (18.7)	837 (21.3)
Smoke		
Never Smoked	293 (46.0)	1884 (47.9)
Former Smoker	255 (40.0)	1572 (40.0)
Current Smoker	89 (14.0)	475 (12.1)
Alcohol, drinks/week	0.0 (0.0 - 1.0)	0.0 (0.0 - 1.3)
Physical activity, kcal/week	518 (0 - 1370)	634 (158 - 1590)
General Health		
Excellent	72 (11.3)	648 (16.5)
Very good	129 (20.3)	1101 (28.0)
Good	231 (36.3)	1463 (37.2)
Fair	171 (26.8)	638 (16.2)
Poor	34 (5.3)	81 (2.1)
Body mass index (kg/m^2)	26.9 ± 5.1	26.6 ± 4.7
Medical history		
Anti-hypertensive drugs	288 (45.2)	1534 (39.0)
Lipid-lowering drugs	26 (4.1)	175 (4.5)
Oral hypoglycemic agents or insulin	82 (12.9)	234 (6.0)
Antibiotics	18 (2.8)	108 (2.7)
Prevalence of diabetes	179 (28.1)	760 (19.3)

Comparison was performed among 4568 subjects without prevent cardiovascular disease at baseline. A participant was considered as “included” if he/she was included in at least one analysis.

Table S2. Population characteristics of older U.S. men and women in the Cardiovascular Health Study, by quintiles of processed meat intake

	Q1	Q2	Q3	Q4	Q5	Total
N	779	778	778	778	778	3891
Range, servings/day	(0.01 – 0.09)	(0.09 – 0.18)	(0.18 – 0.34)	(0.34 – 0.60)	(0.60 – 2.82)	(0.01 – 2.82)
Sociodemographic						
Age, years	72.7 ± 5.4	72.4 ± 5.2	72.8 ± 5.8	73.2 ± 6.1	73.1 ± 5.7	72.9 ± 5.6
Male	202 (25.9)	211 (27.1)	266 (34.2)	356 (45.8)	385 (49.5)	1420 (36.5)
Race						
White	706 (90.6)	705 (90.6)	696 (89.5)	678 (87.1)	632 (81.2)	3417 (87.8)
Non-white	73 (9.4)	73 (9.4)	82 (10.5)	100 (12.9)	146 (18.8)	474 (12.2)
Income						
<$11,999	143 (18.4)	154 (19.8)	152 (19.5)	189 (24.3)	229 (29.4)	867 (22.3)
$12,000 to 24,999	271 (34.8)	274 (35.2)	287 (36.9)	292 (37.5)	297 (38.2)	1421 (36.5)
$25,000 to $49,999	218 (28.0)	233 (29.9)	233 (29.9)	197 (25.3)	188 (24.2)	1069 (27.5)
>$50,000	147 (18.9)	117 (15.0)	106 (13.6)	100 (12.9)	64 (8.2)	534 (13.7)
Education						
<High school	125 (16.0)	168 (21.6)	187 (24.0)	246 (31.6)	281 (36.1)	1007 (25.9)
High school	231 (29.7)	220 (28.3)	228 (29.3)	222 (28.5)	235 (30.2)	1136 (29.2)
Some college	192 (24.6)	205 (26.3)	193 (24.8)	178 (22.9)	147 (18.9)	915 (23.5)
College graduate	231 (29.7)	185 (23.8)	170 (21.9)	132 (17.0)	115 (14.8)	833 (21.4)
Lifestyle						
Smoking						
Never Smoked	410 (52.6)	389 (50.0)	377 (48.5)	355 (45.6)	318 (40.9)	1849 (47.5)
Former Smoker	305 (39.2)	305 (39.2)	316 (40.6)	334 (42.9)	318 (40.9)	1578 (40.6)
Current Smoker	64 (8.2)	84 (10.8)	85 (10.9)	89 (11.4)	142 (18.3)	464 (11.9)
Alcohol, drinks/week	0.0 (0.0 - 1.3)	0.0 (0.0 - 1.3)	0.0 (0.0 - 2.0)	0.0 (0.0 - 2.0)	0.0 (0.0 - 1.0)	0.0 (0.0 - 1.3)
Physical activity, kcal/week	680 (158 - 1645)	602 (131 - 1443)	653 (198 - 1480)	601 (131 - 1610)	540 (90 - 1473)	620 (135 - 1530)
General Health						
Excellent	156 (20.0)	106 (13.6)	120 (15.4)	109 (14.0)	84 (10.8)	575 (14.8)
Very good	217 (27.9)	244 (31.4)	216 (27.8)	208 (26.7)	196 (25.2)	1081 (27.8)
Good	288 (37.0)	295 (37.9)	325 (41.8)	281 (36.1)	321 (41.3)	1510 (38.8)
Fair	103 (13.2)	125 (16.1)	108 (13.9)	160 (20.6)	154 (19.8)	650 (16.7)
Poor	15 (1.9)	8 (1.0)	9 (1.2)	20 (2.6)	23 (3.0)	75 (1.9)
Body mass index (kg/m2)	25.9 ± 4.6	26.3 ± 4.7	26.6 ± 4.5	27.2 ± 4.7	27.1 ± 4.8	26.6 ± 4.7

Medical history						
Anti-hypertensive drugs	292 (37.5)	301 (38.7)	341 (43.8)	321 (41.3)	325 (41.8)	1580 (40.6)
Lipid-lowering drugs	55 (7.1)	38 (4.9)	46 (5.9)	22 (2.8)	27 (3.5)	188 (4.8)
Oral antihyperglycemic drugs or insulin	32 (4.1)	40 (5.1)	43 (5.5)	54 (6.9)	81 (10.4)	250 (6.4)
Antibiotics	23 (3.0)	20 (2.6)	17 (2.2)	22 (2.8)	33 (4.2)	115 (3.0)
Prevalent of diabetes	113 (14.5)	146 (18.8)	161 (20.7)	164 (21.1)	204 (26.2)	788 (20.3)
Diet						
Fruits, servings/day	2.7 ± 1.1	2.3 ± 1.0	2.2 ± 1.0	2.0 ± 0.9	1.8 ± 0.9	2.2 ± 1.1
Vegetables, servings/day	3.2 ± 1.4	2.8 ± 1.2	2.6 ± 1.2	2.4 ± 1.1	2.2 ± 1.2	2.6 ± 1.3
Dietary fiber, g/day	34.7 ± 10.7	32.3 ± 9.9	29.8 ± 9.2	27.6 ± 8.8	25.1 ± 8.3	29.9 ± 10.0
Dairy products, servings/day	1.4 ± 0.7	1.3 ± 0.6	1.3 ± 0.6	1.2 ± 0.6	1.1 ± 0.6	1.3 ± 0.6
Total animal source foods, servings/day	1.3 ± 0.6	1.4 ± 0.5	1.6 ± 0.5	1.8 ± 0.5	2.3 ± 0.7	1.7 ± 0.7
Unprocessed meat, servings/day	0.3 ± 0.3	0.4 ± 0.3	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3	0.5 ± 0.3
Fish, servings/day	0.4 ± 0.3	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.2 ± 0.2	0.3 ± 0.2
Poultry, servings/day	0.4 ± 0.3	0.4 ± 0.3	0.3 ± 0.3	0.3 ± 0.2	0.3 ± 0.3	0.3 ± 0.3
Eggs, servings/day	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.3	0.2 ± 0.2
Total energy intake, calories/day	1792.5 ± 557.2	1741.4 ± 560.0	1767.0 ± 669.7	1957.1 ± 724.6	1837.1 ± 654.0	1819.0 ± 640.6
TMAO-related biomarkers						
TMAO, µM	4.7 (3.1 - 8.2)	4.7 (3.2 - 7.8)	4.7 (3.1 - 7.4)	4.6 (3.2 - 7.7)	4.8 (3.4 - 7.6)	4.7 (3.2 - 7.7)
Choline, µM	9.3 (7.9 - 10.7)	9.3 (8.0 - 11.2)	9.4 (8.0 - 11.1)	9.8 (8.4 - 11.4)	9.8 (8.4 - 11.7)	9.5 (8.1 - 11.2)
Betaine, µM	36.5 ± 12.9	36.6 ± 13.7	36.8 ± 13.4	37.6 ± 12.3	37.6 ± 12.6	37.0 ± 13.0
Carnitine, µM	35.7 ± 7.8	37.0 ± 8.6	37.5 ± 7.8	37.5 ± 8.3	37.9 ± 8.4	37.1 ± 8.2
γ -Butyrobetaine, µM	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.3	1.1 ± 0.3	1.1 ± 0.4	1.0 ± 0.3
Crotonobetaine, µM	0.021 (0.010 - 0.027)	0.022 (0.010 - 0.028)	0.022 (0.010 - 0.029)	0.023 (0.010 - 0.029)	0.025 (0.020 - 0.031)	0.023 (0.010 - 0.029)

Values are N (%), mean ± SD, or median (IQR) at analysis baseline (1989-1990 for 3334 subjects and 1996-97 for 557 subjects).
Food intakes were energy-adjusted.
TMAO: trimethylamine N-oxide

Table S3. Population characteristics of older U.S. men and women in the Cardiovascular Health Study, by quintiles of fish intake

	Q1	Q2	Q3	Q4	Q5	Total
N (range, servings/day)	779 (0.01 – 0.12)	778 (0.12 – 0.21)	778 (0.21 – 0.31)	778 (0.31 – 0.44)	778 (0.44 – 2.09)	3891 (0.01 – 2.09)
Sociodemographic						
Age, years	73.9 ± 6.2	73.2 ± 5.7	73.1 ± 5.6	72.2 ± 5.4	71.9 ± 5.0	72.9 ± 5.6
Male	343 (44.0)	300 (38.6)	315 (40.5)	259 (33.3)	201 (25.8)	1418 (36.4)
Race						
White	695 (89.2)	681 (87.5)	672 (86.4)	702 (90.2)	668 (85.9)	3418 (87.8)
Non-white	84 (10.8)	97 (12.5)	106 (13.6)	76 (9.8)	110 (14.1)	473 (12.2)
Income						
<$11,999	209 (26.8)	168 (21.6)	164 (21.1)	158 (20.3)	168 (21.6)	867 (22.3)
$12,000 to 24,999	326 (41.8)	305 (39.2)	275 (35.3)	277 (35.6)	241 (31.0)	1424 (36.6)
$25,000 to $49,999	174 (22.3)	219 (28.1)	232 (29.8)	225 (28.9)	216 (27.8)	1066 (27.4)
>$50,000	70 (9.0)	86 (11.1)	107 (13.8)	118 (15.2)	153 (19.7)	534 (13.7)
Education						
<High school	263 (33.8)	197 (25.3)	204 (26.2)	175 (22.5)	169 (21.7)	1008 (25.9)
High school	218 (28.0)	235 (30.2)	230 (29.6)	222 (28.5)	232 (29.8)	1137 (29.2)
Some college	179 (23.0)	184 (23.7)	180 (23.1)	194 (24.9)	178 (22.9)	915 (23.5)
College graduate	119 (15.3)	162 (20.8)	164 (21.1)	187 (24.0)	199 (25.6)	831 (21.4)
Lifestyle						
Smoke						
Never Smoked	366 (47.0)	387 (49.7)	363 (46.7)	373 (47.9)	362 (46.5)	1851 (47.6)
Former Smoker	293 (37.6)	300 (38.6)	316 (40.6)	328 (42.2)	340 (43.7)	1577 (40.5)
Current Smoker	120 (15.4)	91 (11.7)	99 (12.7)	77 (9.9)	76 (9.8)	463 (11.9)
Alcohol, drinks/week	0.0 (0.0 - 1.0)	0.0 (0.0 - 1.5)	0.0 (0.0 - 1.5)	0.0 (0.0 - 1.3)	0.0 (0.0 - 1.3)	0.0 (0.0 - 1.3)
Physical activity, kcal/week	490 (79 - 1365)	663 (140 - 1605)	630 (158 - 1470)	716 (184 - 1653)	603 (135 - 1553)	613 (135 - 1530)
General Health						
Excellent	107 (13.7)	117 (15.0)	108 (13.9)	125 (16.1)	118 (15.2)	575 (14.8)
Very good	204 (26.2)	218 (28.0)	217 (27.9)	222 (28.5)	221 (28.4)	1082 (27.8)
Good	286 (36.7)	294 (37.8)	320 (41.1)	303 (38.9)	304 (39.1)	1507 (38.7)
Fair	160 (20.5)	131 (16.8)	121 (15.6)	114 (14.7)	125 (16.1)	651 (16.7)
Poor	22 (2.8)	18 (2.3)	12 (1.5)	14 (1.8)	10 (1.3)	76 (2.0)
Body mass index (kg/m2)	26.1 ± 4.7	26.5 ± 4.6	26.7 ± 4.6	26.8 ± 4.7	27.0 ± 4.8	26.6 ± 4.7

Medical history						
Anti-hypertensive drugs	310 (39.8)	320 (41.1)	306 (39.3)	310 (39.8)	331 (42.5)	1577 (40.5)
Lipid-lowering drugs	22 (2.8)	34 (4.4)	37 (4.8)	39 (5.0)	56 (7.2)	188 (4.8)
Oral antihyperglycemic drugs or insulin	63 (8.1)	48 (6.2)	50 (6.4)	46 (5.9)	44 (5.7)	251 (6.5)
Antibiotics	24 (3.1)	19 (2.4)	26 (3.3)	18 (2.3)	23 (3.0)	110 (2.8)
Prevalent of diabetes	171 (22.0)	142 (18.3)	172 (22.1)	164 (21.1)	140 (18.0)	789 (20.3)
Diet						
Fruits, servings/day	2.0 ± 1.1	2.1 ± 1.0	2.2 ± 1.1	2.3 ± 1.1	2.3 ± 1.0	2.2 ± 1.1
Vegetables, servings/day	2.2 ± 1.3	2.4 ± 1.2	2.6 ± 1.1	2.8 ± 1.2	3.0 ± 1.3	2.6 ± 1.3
Dietary fiber, g/day	28.3 ± 10.7	29.2 ± 10.3	29.8 ± 9.1	31.2 ± 9.8	31.2 ± 9.8	29.9 ± 10.0
Dairy products, servings/day	1.2 ± 0.6	1.3 ± 0.6	1.3 ± 0.6	1.3 ± 0.6	1.3 ± 0.7	1.3 ± 0.6
Total animal source foods, servings/day	1.6 ± 0.7	1.6 ± 0.6	1.6 ± 0.6	1.7 ± 0.6	2.0 ± 0.7	1.7 ± 0.7
Unprocessed meat, servings/day	0.5 ± 0.4	0.5 ± 0.3	0.5 ± 0.3	0.4 ± 0.3	0.4 ± 0.4	0.5 ± 0.3
Processed meat, servings/day	0.5 ± 0.4	0.4 ± 0.3	0.4 ± 0.3	0.3 ± 0.3	0.3 ± 0.3	0.4 ± 0.3
Poultry, servings/day	0.3 ± 0.2	0.3 ± 0.2	0.3 ± 0.2	0.4 ± 0.3	0.4 ± 0.3	0.3 ± 0.3
Eggs, servings/day	0.2 ± 0.3	0.2 ± 0.3	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
Total energy intake, calories/day	1789.7 ± 624.9	1761.1 ± 611.9	1895.0 ± 674.1	1884.3 ± 615.4	1761.3 ± 658.1	1818.2 ± 639.8
TMAO-related biomarkers						
TMAO, µM	4.6 (3.1 - 6.9)	4.6 (3.2 - 7.4)	4.7 (3.2 - 7.5)	5.0 (3.2 - 8.5)	4.9 (3.4 - 8.3)	4.7 (3.2 - 7.7)
Choline, µM	9.7 (8.2 - 11.4)	9.6 (8.1 - 11.4)	9.5 (8.2 - 11.2)	9.6 (8.2 - 11.2)	9.2 (8.0 - 10.8)	9.5 (8.1 - 11.2)
Betaine, µM	37.6 ± 13.5	37.5 ± 13.5	37.6 ± 12.8	36.3 ± 12.8	35.8 ± 12.2	37.0 ± 13.0
Carnitine, µM	37.4 ± 8.2	37.1 ± 8.0	37.3 ± 8.7	36.7 ± 8.3	37.0 ± 7.9	37.1 ± 8.2
γ -Butyrobetaine, µM	1.1 ± 0.4	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.3
Crotonobetaine, µM	0.023 (0.010 - 0.030)	0.023 (0.010 - 0.029)	0.022 (0.010 - 0.030)	0.023 (0.010 - 0.028)	0.022 (0.010 - 0.028)	0.023 (0.010 - 0.029)

Values are N (%), mean ± SD, or median (IQR) at analysis baseline (1989-1990 for 3339 subjects and 1996-97 for 552 subjects).
Food intakes were energy-adjusted.
TMAO: trimethylamine N-oxide

Table S4. Risk of ASCVD associated with intakes of each animal source food (per IQR) and mediation by non-microbiota derived precursors

	Unprocessed red meat	Processed meat	Total meat [*]	Fish	Poultry	Eggs	Total animal source foods[†]
Mediator: Choline							
No. of excess events per 1000 persons per year (95%CI)							
Dietary association independent of choline	4.0 (-0.1, 8.1)	3.6 (-0.4, 7.6)	6.3 (1.8, 10.9)	0.5 (-3.3, 4.3)	1.9 (-2.1, 5.8)	1.4 (-2.3, 5.0)	5.8 (1.3, 10.2)
Dietary association mediated via choline	-0.1 (-0.3, 0.0)	0.0 (-0.1, 0.1)	-0.1 (-0.2, 0.1)	0.0 (-0.2, 0.1)	-0.1 (-0.3, 0.0)	0.1 (0.0, 0.2)	0.0 (-0.2, 0.1)
Mediation proportions (%)	-2.3 (-28.5, 0.7)	0.2 (-7.8, 9.8)	-0.9 (-5.9, 1.1)	NA[‡]	-6.4 (-108.9, 0.6)	4.4 (-2.5, 83.7)	-0.9 (-6.3, 1.4)
Mediator: Betaine							
No. of excess events per 1000 persons per year (95%CI)							
Dietary association independent of betaine	4.1 (0.0, 8.1)	3.6 (-0.4, 7.7)	6.5 (1.9, 11.0)	0.5 (-3.3, 4.3)	1.8 (-2.1, 5.7)	1.4 (-2.3, 5.0)	5.9 (1.4, 10.3)
Dietary association mediated via betaine	-0.1 (-0.3, 0.1)	0.0 (-0.2, 0.0)	-0.1 (-0.4, 0.1)	0.0 (-0.1, 0.1)	0.0 (-0.1, 0.1)	0.1 (-0.1, 0.2)	0.0 (-0.2, 0.0)
Mediation proportions (%)	-2.5 (-31.5, 2.6)	-1.3 (-17.1, 1.9)	-2.0 (-11.2, 1.4)	NA[‡]	-1.1 (-26.6, 7.0)	4.0 (-7.1, 84.5)	-0.8 (-5.8, 1.0)
Mediator: Carnitine							
No. of excess events per 1000 persons per year (95%CI)							
Dietary association independent of carnitine	3.9 (-0.2, 8.1)	3.6 (-0.5, 7.6)	6.3 (1.7, 10.9)	0.5 (-3.3, 4.3)	1.8 (-2.2, 5.7)	1.4 (-2.2, 5.1)	5.8 (1.3, 10.2)
Dietary association mediated via carnitine	0.0 (-0.3, 0.3)	0.0 (-0.1, 0.1)	0.0 (-0.3, 0.4)	0.0 (-0.1, 0.1)	0.0 (-0.1, 0.1)	0.0 (-0.1, 0.1)	0.0 (-0.2, 0.3)
Mediation proportions (%)	0.3 (-12.5, 20.4)	0.1 (-8.1, 10.9)	0.5 (-6.1, 9.3)	NA[‡]	-0.1 (-10.9, 10.4)	0.1 (-16.2, 17.4)	0.6 (-4.1, 8.0)

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), antibiotic use (yes vs. no), and intakes of total energy (kcal/day, log-transformed), 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.

Choline was log transformed.

[*]Total meat: unprocessed red meat plus processed meat.

[†]Total animal source foods: sum of unprocessed red meat, processed meat, fish, poultry, and eggs.

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

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

Table S5. Risk of ASCVD associated with intakes of each animal source food (per IQR), and joint mediation by TMAO, γ-butyrobetaine, and crotonobetaine: sensitivity analyses using the most recent intake (simple update)

	Unprocessed red meat	Processed meat	Total meat [*]	Fish	Poultry	Eggs	Total animal source foods[†]
Main dietary association[‡]							
Hazard ratios (95%CI) [§]	1.14 (1.01, 1.27)	1.07 (0.96, 1.18)	1.17 (1.04, 1.32)	1.00 (0.90, 1.12)	1.05 (0.94, 1.18)	1.04 (0.97, 1.12)	1.13 (1.01, 1.27)
No. of excess events per 1000 persons per year (95%CI) [¶]	3.9 (0.1, 7.7)	2.6 (-1.0, 6.1)	5.5 (1.3, 9.6)	0.6 (-2.8, 4.0)	2.3 (-1.5, 6.2)	1.5 (-1.6, 4.7)	5.0 (1.0, 9.1)
Mediation analyses[#]							
No. of excess events per 1000 persons per year (95%CI)							
Dietary association independent of metabolites	3.5 (-0.3, 7.3)	2.4 (-1.2, 6.0)	5.0 (0.8, 9.2)	0.2 (-3.3, 3.6)	2.4 (-1.5, 6.2)	1.5 (-1.7, 4.6)	4.5 (0.5, 8.6)
Dietary association mediated via metabolites	0.4 (0.1, 0.8)	0.1 (-0.1, 0.3)	0.4 (0.1, 0.8)	0.4 (0.1, 0.8)	-0.1 (-0.3, 0.1)	0.0 (-0.1, 0.2)	0.4 (0.1, 0.8)
Mediation proportions (%)	10.8 (1.7, 97.2)	3.5 (-7.6, 65.5)	7.8 (1.1, 37.5)	NA[**]	-3.2 (-70.2, 7.1)	3.2 (-22.8, 73.5)	8.8 (2.2, 49.4)

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 animal source foods: sum of unprocessed red meat, processed meat, fish, poultry, and eggs

[‡]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. TMAO and crotonobetaine were log transformed.

[**]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 S6. Risk of ASCVD associated with intakes of each animal source food (per IQR), and joint mediation by TMAO, γ-butyrobetaine, and crotonobetaine: sensitivity analyses with additional adjustments

	Unprocessed red meat	Processed meat	Total meat [*]	Fish	Poultry	Eggs	Total animal source foods[†]
Adjust for traditional CVD risk factors[‡]							
Main dietary association[§]							
Hazard ratios (95%CI) [¶]	1.15 (1.01, 1.30)	1.09 (0.97, 1.23)	1.21 (1.06, 1.38)	1.01 (0.89, 1.14)	1.03 (0.91, 1.17)	1.05 (0.95, 1.15)	1.17 (1.03, 1.34)
No. of excess events per 1000 persons per year (95%CI) [#]	4.0 (-0.1, 8.1)	2.8 (-1.2, 6.8)	5.8 (1.2, 10.3)	0.7 (-3.1, 4.5)	1.6 (-2.3, 5.5)	1.7 (-1.9, 5.4)	5.6 (1.1, 10.1)
Mediation analyses[**]							
No. of excess events per 1000 persons per year (95%CI)							
Dietary association independent of metabolites	3.5 (-0.6, 7.6)	2.7 (-1.3, 6.7)	5.2 (0.7, 9.8)	0.4 (-3.4, 4.2)	1.7 (-2.2, 5.6)	1.8 (-1.9, 5.5)	5.2 (0.7, 9.7)
Dietary association mediated via metabolites	0.5 (0.1, 0.9)	0.2 (-0.1, 0.4)	0.5 (0.1, 1.0)	0.3 (-0.1, 0.7)	0.0 (-0.3, 0.2)	0.0 (-0.3, 0.2)	0.4 (0.0, 0.9)
Mediation proportions (%)	11.6 (1.9, 127.9)	5.6 (-3.8, 96.9)	8.9 (1.2, 44.3)	NA[††]	-2.0 (-73.3, 25.0)	-2.1 (-75.9, 29.7)	7.4 (0.6, 39.0)
Adjust for traditional CVD risk factors[‡] plus continuous eGFR							
Main dietary association[§]							
Hazard ratios (95%CI) [¶]	1.15 (1.01, 1.30)	1.09 (0.97, 1.23)	1.20 (1.05, 1.38)	1.00 (0.89, 1.13)	1.04 (0.92, 1.18)	1.06 (0.96, 1.16)	1.18 (1.03, 1.35)
No. of excess events per 1000 persons per year (95%CI) [#]	4.0 (-0.1, 8.1)	2.9 (-1.1, 6.9)	5.8 (1.3, 10.3)	0.6 (-3.1, 4.4)	2.0 (-1.9, 5.9)	2.0 (-1.7, 5.7)	6.0 (1.5, 10.5)
Mediation analyses[**]							
No. of excess events per 1000 persons per year (95%CI)							
Dietary association independent of metabolites	3.8 (-0.4, 7.9)	2.8 (-1.2, 6.8)	5.6 (1.0, 10.2)	0.5 (-3.3, 4.3)	2.0 (-2.0, 5.9)	2.0 (-1.8, 5.7)	5.8 (1.2, 10.3)
Dietary association mediated via metabolites	0.2 (-0.2, 0.6)	0.1 (-0.2, 0.3)	0.2 (-0.3, 0.7)	0.2 (-0.2, 0.6)	0.0 (-0.1, 0.2)	0.0 (-0.2, 0.2)	0.2 (-0.3, 0.7)
Mediation proportions (%)	4.9 (-8.5, 61.3)	2.3 (-13.0, 47.3)	3.7 (-5.8, 25.4)	NA[††]	2.0 (-10.6, 43.8)	0.8 (-30.8, 49.3)	3.8 (-5.3, 24.4)

Both 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 animal source foods: sum of unprocessed red meat, processed meat, fish, poultry, and eggs

[‡]Traditional CVD risk factors included BMI (kg/m^2), waist circumference (cm), SBP (mmHg), DBP (mmHg), HDL cholesterol (mg/dL), LDL cholesterol(mg/dL), triglycerides (mg/dL), diabetes status (Yes/No), CRP (mg/L), anti-hypertensive drugs(Yes/No), and lipid lowering drugs (Yes/No).

[§]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. TMAO and crotonobetaine were log transformed.
††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.

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Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent - Male					
Parent - Female					

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)	Persistent ID / URL

DNA/cDNA Clones

Clone Name	Sequence	Source / Repository	Persistent ID / URL

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
Data	The Cardiovascular Health Study	https://chs-nhlbi.org/Internal/CHSData (request should be sent to CHS CC)
Code	Tufts University	Not publicly available

Other

Description	Source / Repository	Persistent ID / URL

DOI [to be added]