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Choline Metabolites and 15-Year Risk of Incident Diabetes in a Prospective Cohort of Adults: Coronary Artery Risk Development in Young Adults (CARDIA) Study.

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Authors

Sprinkles, Jessica

Lulla, Anju

Hullings, Autumn

et al.

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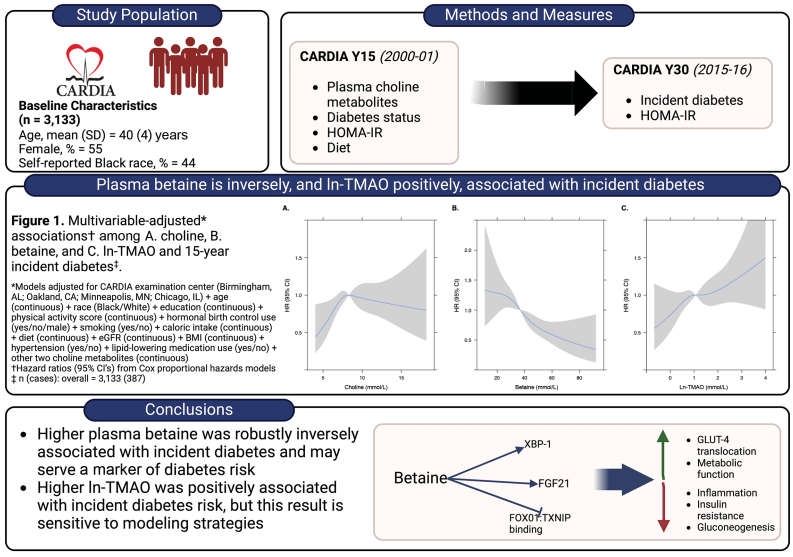

eGFR, estimated glomerular filtration rate; FGF21, fibroblast growth factor 21; HOMA-IR, HOMA of insulin resistance; HR, hazard ratio; ln, natural log; TMAO, trimethylamine N-oxide; TXNIP, thioredoxin-interacting protein; Y, year.

ARTICLE HIGHLIGHTS

- **Why did we undertake this study?**
  Previous reports support a beneficial effect of betaine in diabetes risk, but results have been inconsistent for trimethylamine N-oxide and choline. There has been a lack of research among people of early middle age, when preventive activities may be most relevant.
- **What is the specific question we wanted to answer?**
  Are choline metabolites associated with 15-year incident diabetes in a population-based cohort of early middle-aged adults?
- **What did we find?**
  Betaine was inversely associated with 15-year risk of incident diabetes (hazard ratio 0.76 [95% CI 0.67, 0.88] per 1-SD unit). This finding was robust across analytic approaches. Trimethylamine N-oxide was positively associated with incident diabetes in some, but not all, analyses.
- **What are the implications of our findings?**
  Robust associations for betaine suggest betaine's potential value as a biomarker for diabetes risk.

Choline Metabolites and 15-Year Risk of Incident Diabetes in a Prospective Cohort of Adults: Coronary Artery Risk Development in Young Adults (CARDIA) Study

Jessica K. Sprinkles,[1,2] Anju Lulla,[1] Autumn G. Hullings,[2,3] Isis Trujillo-Gonzalez,[1,2] Kevin C. Klatt,[4] David R. Jacobs Jr.,[5] Ravi V. Shah,[6] Venkatesh L. Murthy,[7] Annie Green Howard,[8] Penny Gordon-Larsen,[2,3] and Katie A. Meyer[1,2]

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ORIGINAL ARTICLE

OBJECTIVE

The potential for choline metabolism to influence the development of diabetes has received increased attention. Previous studies on circulating choline metabolites and incident diabetes have been conducted in samples of older adults, often with a high prevalence of risk factors.

RESEARCH DESIGN AND METHODS

Participants were from year 15 of follow-up (2000-2001) in the Coronary Artery Risk Development in Young Adults (CARDIA) Study (*n* = 3,133, aged 33–45 years) with plasma choline metabolite (choline, betaine, and trimethylamine N-oxide [TMAO]) data. We quantified associations between choline metabolites and 15-year risk of incident diabetes (*n* = 387) among participants free of diabetes at baseline using Cox proportional hazards regression models adjusted for sociodemographics, health behaviors, and clinical variables.

RESULTS

Betaine was inversely associated with 15-year risk of incident diabetes (hazard ratio 0.76 [95% CI 0.67, 0.88] per 1-SD unit betaine), and TMAO was positively associated with 15-year risk of incident diabetes (1.11 [1.01, 1.22] per 1-SD unit). Choline was not significantly associated with 15-year risk of incident diabetes (1.05 [0.94, 1.16] per 1-SD).

CONCLUSIONS

Our findings are consistent with other published literature supporting a role for choline metabolism in diabetes. Our study extends the current literature by analyzing a racially diverse population-based cohort of early middle-aged individuals in whom preventive activities may be most relevant.

Dietary choline is an essential micronutrient with several metabolic fates, including metabolites betaine and trimethylamine N-oxide (TMAO) (1). Choline can be oxidized to betaine, predominantly in the liver and kidney. In the gut, choline is metabolized by the gut microbiota to form trimethylamine, which is further oxidized

[1]Nutrition Research Institute, University of North Carolina at Chapel Hill, Kannapolis, NC
[2]Department of Nutrition, University of North Carolina at Chapel Hill, Chapel Hill, NC
[3]Carolina Population Center, University of North Carolina at Chapel Hill, Chapel Hill, NC
[4]Department of Nutrition Sciences and Toxicology, University of California, Berkeley, Berkeley, CA
[5]Division of Epidemiology and Community Health, School of Public Health, University of Minnesota-Twin Cities, Minneapolis, MN
[6]Department of Cardiovascular Medicine, Vanderbilt University Medical Center, Nashville, TN
[7]University of Michigan, Ann Arbor, MI
[8]Department of Biostatistics, University of North Carolina at Chapel Hill, Chapel Hill, NC

Corresponding author: Katie A. Meyer, ktmeyer@unc.edu

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in the liver by flavin monooxygenase 3, to generate TMAO (2,3). Freely circulating choline, betaine, and TMAO can be influenced by dietary intake, endogenous synthesis, and clearance dynamics. Various nutritional epidemiological investigations have observed that concentrations of circulating choline, betaine, and TMAO appear altered in chronic disease states, leading to increased interest in whether altered choline metabolism is a risk factor for disease development (2,4–10). Several mechanisms have been proposed through which choline, betaine, and TMAO may influence diabetes risk, but to date, observational studies have varied in design and population characteristics.

In cross-sectional analyses, choline and TMAO have been positively associated, and betaine inversely associated, with prevalent diabetes, fasting glucose, HbA_{1c}, and HOMA of insulin resistance (HOMA-IR) (2,4–6,11,12). In prospective studies, betaine has remained inversely associated with incident diabetes, while choline and TMAO associations are often attenuated (8,10,11,13,14). Most prospective studies have been conducted in high-risk cohorts (i.e., older age, prevalent cardiometabolic disease) in which the association may differ from younger cohorts due to potential modifying factors, such as liver and kidney function (15). This is important, as early middle age is a period of increasing metabolic risk. In addition, most previous studies have had limited racial diversity, which may fail to capture diversity in dietary intakes and other risk factors that modify choline metabolism. Studies of diverse populations with representative levels of risk factors and with available dietary intake and thorough covariate data are necessary to increase the generalizability of findings, as well as to draw implications in clinical disease prevention.

The Coronary Artery Risk Development in Young Adults (CARDIA) Study provides a unique opportunity to address important gaps in the literature by examining these associations in a population-based U.S. cohort of self-reported Black and White individuals of early middle age. We hypothesized that choline and TMAO are positively associated, and betaine negatively associated, with 15-year risk of incident diabetes and 15-year changes in HOMA-IR. The results of this study will contribute new data on the associations among dietary intake, choline metabolites, and glycemic outcomes through analyzing a population-based and sociodemographically diverse U.S. cohort at early middle age, when preventive efforts are most valuable.

RESEARCH DESIGN AND METHODS

Study Sample

The prospective CARDIA Study began in 1985–1986 with the recruitment of 5,115 self-identified Black and White men and women aged 18–30 years from four U.S. urban centers: Birmingham, Alabama; Chicago, Illinois; Minneapolis, Minnesota; and Oakland, California (16). A majority of the surviving cohort have attended nine follow-up examinations. Study participants provided informed consent at each examination, and the institutional review board at each CARDIA site approved examination protocols.

The current study used metabolite data generated from year 15 (2000–2001) plasma samples, with follow-up through year 30 (2015–2016). Of the surviving cohort, 72% (n = 3,671) attended the year 15 examination, and 3,492 participants had available stored plasma samples. Women who reported being pregnant at the year 15 examination (n = 16) or who had prevalent diabetes at year 15 (n = 154) were excluded from all analyses. Other exclusions were based on missing year 15 covariate data in multivariable adjusted regression analysis (n = 189), including missing data on diabetes status (n = 5), diet (n = 158), physical activity (n = 10), smoking (n = 5), BMI (n = 8), estimated glomerular filtration rate (eGFR) (n = 4), hypertension (n = 6), and lipid-lowering medication use (n = 2) (Supplementary Fig. 1). After exclusions due to missing data, the final analytic sample for all analyses of incident diabetes consisted of 3,133 participants. For analysis of prospective changes in HOMA-IR, participants missing data on year 15 HOMA-IR (n = 29) and year 30 HOMA-IR (n = 669) were excluded, leaving a sample of 2,530 participants.

Blood Sample Collection and Targeted Metabolomics

Participants were asked to fast for at least 12 h and to avoid smoking and physical activity for 2 h prior to the examination. Blood was drawn by venipuncture, separated through centrifugation, aliquoted, flash frozen, and stored at −70°C.

Plasma choline, betaine, and TMAO were quantified by The University of North Carolina at Chapel Hill Nutrition Obesity Research Center Human Phenotyping Core using stable isotope dilution liquid chromatography-multiple reaction monitoring mass spectrometry (17). An Atlantic HILIC Silica 3 μm 4.6 × 50 mm column (Waters Corporation) was used for chromatography, along with an ACQUITY UPLC system (Waters Corporation). For the mass spectrometric analysis, an ACUITY TQ detector (Waters Corporation) equipped with an electrospray ionization probe set to positive ion mode was used. The internal standards TMAO-d9, choline-d9, and betaine-d9 were monitored at ion transitions of $85 > 66$, $113 > 45$, and $127 > 68$ mass-to-charge ratios, respectively. Calibration curves constructed from the peak area ratios of the analyte to its internal standard allowed for quantification. The assay has a wide dynamic range (choline 0.122–250 μmol/L, betaine 0.488–1,000 μmol/L, TMAO 0.061–62.5 μmol/L), high intra- and interday precision (coefficient of variation $<$6%), and high accuracy ($<$15% error).

Assessment of Diabetes, HbA_{1c}, Fasting Glucose, and Insulin

Plasma glucose and insulin were quantified from fasted samples using the hexokinase method and a radioimmunoassay technique, respectively. HbA_{1c} was quantified from whole blood by ion exchange high-performance liquid chromatography (Tosoh G7). Diabetes and HOMA-IR were assessed at years 15, 20, 25, and 30. Diabetes was defined as a fasting glucose ≥7.0 mmol/L, 2-h oral glucose tolerance test ≥11.1 mmol/L, HbA_{1c} ≥6.5%, or reported use of glucose-lowering medication. For sensitivity analysis, prediabetes was defined as fasting glucose of 5.6 to $<$7.0 mmol/L. HOMA-IR values were generated from fasting glucose and insulin concentrations using a standard equation (18).

Covariates

At each examination, CARDIA used interviewer-administered standard questionnaires to assess participant sociodemographic variables (age, self-reported race, sex, education), pregnancy status (women), smoking history, and medication use, including birth control, lipid-lowering, antihypertensive, and diabetes-related drugs. Physical activity was assessed using the validated interviewer-administered CARDIA Physical Activity History, which queried

engagement in 13 activities over the past year, from which a total activity score was calculated by multiplying the frequency and intensity of each activity and summing over all activities (19).

At years 0, 7, and 20, participants were asked to complete an interviewer-administered diet history, which assessed usual dietary intake over the past month (20). The University of Minnesota's Nutrition Coordinating Center coded dietary intake variables into 166 food groups, which were further grouped into 46 food groups. The mean intake of food groups at years 7 and 20 was used for analysis. Food groups were included if they were dietary precursors for choline metabolites or were significantly associated with at least one choline metabolite. Food groups included eggs, processed red meat, lean red meat, regular red meat, chicken, fish, refined grains, whole grains, seeds and nuts, green vegetables, yellow vegetables, other vegetables, fruit, and fried foods. In addition, we adjusted for total caloric intake and the CARDIA a priori diet quality score, as described previously (21).

Trained field center staff used standardized and validated protocols for clinical measures. Weight and height were measured in the clinic using a calibrated scale. A random zero sphygmomanometer was used to measure blood pressure three times while the participant was in a resting state, and the mean of the second and third measures was calculated for analysis. Hypertension was defined as a systolic pressure $\geq$140 mmHg or diastolic pressure of $\geq$90 mmHg or use of hypertensive medications. The Northwest Lipid Research Laboratory at the University of Washington performed lipid analyses on fasting blood samples. Enzymatic procedures were used to quantify concentrations of total cholesterol, HDL cholesterol (HDL-C), and triglycerides. The Friedewald equation was used to estimate LDL cholesterol. Hexokinase coupled to glucose-6-phosphate dehydrogenase was used to measure serum glucose and radioimmunoassay for insulin. Serum creatinine was measured using the Jaffe method. The 2009 Chronic Kidney Disease Epidemiology Collaboration equation was used to calculate the eGFR (mL/min/1.73 m^2) based on serum creatinine, age, race, and sex (22).

Statistical Analysis

We generated descriptive statistics for participants across quartiles of plasma choline, betaine, and TMAO. Differences across quartiles were tested using χ^2 test for categorical variables and Kruskal-Wallis test for continuous variables. Correlations among metabolite concentrations were assessed using Pearson correlation coefficients. HOMA-IR and TMAO were natural log (ln) transformed for analysis to improve normality. Choline metabolites were standardized (mean 0, SD 1) for analysis and modeled using restricted cubic spline, continuous, and quartile specifications. We used Cox proportional hazards regression to quantify associations between baseline metabolite concentration and 15-year risk of incident diabetes and linear regression to estimate prospective associations between metabolites and 15-year changes in ln-HOMA-IR, with results presented as geometric mean ratios (GMRs). To account for potential nonlinearity in associations, we included three model specifications for metabolite exposures, including continuous, metabolite quartiles, and cubic spline regression.

Multivariable adjusted regression models were adjusted for covariates measured at year 15 to align with the timing of metabolite quantification. Model 1 CARDIA examination center (Birmingham, AL; Chicago, IL; Minneapolis, MN; Oakland, CA), age (continuous), sex (male, female), self-reported race (Black, White), highest education attained (continuous years), physical activity (continuous), current smoking (yes, no), diet (continuous), hormonal birth control use (yes, no [women only]), eGFR (continuous), BMI (continuous), hypertension (yes, no), and lipid-lowering medication use (yes, no). Model 2 additionally adjusted for all three metabolites (continuous). In a prospective analysis of HOMA-IR, we adjusted for HOMA-IR at baseline.

We considered potential effect modification by sex, race, and eGFR by including cross-product interaction terms in Cox proportional hazards models. In sensitivity analysis, we *1*) excluded participants with prediabetes at baseline, *2*) excluded participants who developed diabetes within the first 5 years of follow-up, and *3*) added time-varying covariates to the Cox proportional hazards model. Statistical analyses were conducted using Stata (version 18.0) and *R* (version 4.2.3) software. Statistical significance was defined as $\alpha < 0.05$.

Data and Resource Availability

Information on accessing CARDIA data can be found at https://www.cardia.dopm.uab.edu/.

RESULTS

Choline Metabolites and Participant Characteristics

Table 1 shows participant characteristics overall and according to quartiles of plasma TMAO, choline, and betaine. Among the 3,133 participants included in this study, 55% were female, 44% self-reported as Black race, and mean (SD) age was 40 (4) years. Overall mean (SD) metabolite concentrations were 8.51 (2.22) and 38.19 (13.32) μmol/L for choline and betaine, respectively. Overall median (interquartile range [IQR]) TMAO concentration was 2.9 (2.0–4.6) μmol/L. Females had lower concentrations of all three metabolites compared with males; Black individuals had lower concentrations of choline and TMAO compared with White individuals. Participants with higher concentrations of choline and TMAO tended to have a more adverse clinical risk profile, with higher triglycerides and lower HDL-C and eGFR. In contrast, betaine was inversely associated with BMI, triglycerides, and total cholesterol but positively associated with current smoking. Pearson correlation coefficients for choline metabolites were ln-TMAO and choline r = 0.13, ln-TMAO and betaine r = 0.07, and choline and betaine r = 0.39. Over the 15-year follow-up period, 387 participants developed diabetes.

Metabolites were also associated with dietary variables (Supplementary Table 1). All three metabolites were positively associated with the intake of eggs, regular red meat, and total red meat. In addition, betaine was positively associated with seeds and nuts, choline and betaine were positively associated with processed red meat and refined grains, choline and TMAO were positively associated with lean red meat, and TMAO and betaine were positively associated with whole grains. Given the relevance of diet on choline metabolites, we more fully examined associations between metabolites and dietary components with multivariable adjusted linear regression (Supplementary Fig. 2). After adjusting for sociodemographic variables,

Table 1—Participant characteristics according to quartiles of ln-TMAO, choline, and betaine: CARDIA, 2000–2001

	Overall	Q1	Q2	Q3	Q4	*P*†
Choline						
n	3,133	784	783	783	783	
TMAO, median (IQR), μmol/L	2.90 (2.00–4.60)	2.60 (1.80–4.10)	2.80 (1.84–4.50)	2.90 (2.10–4.70)	3.30 (2.30–5.20)	<0.001
Choline, μmol/L	8.51 (2.22)	6.05 (0.68)	7.65 (0.39)	8.94 (0.40)	11.41 (1.88)	<0.001
Betaine, μmol/L	38.19 (13.32)	31.37 (10.31)	35.98 (11.10)	39.84 (12.39)	45.58 (14.78)	<0.001
Self-identified Black/African American race, %	44	49	42	44	41	0.01
Female, %	55	70	59	51	42	<0.001
Age, years	40.22 (3.60)	39.75 (3.62)	40.14 (3.60)	40.24 (3.69)	40.77 (3.44)	<0.001
Education, years	15.86 (2.99)	15.87 (2.55)	15.93 (2.62)	15.97 (3.94)	15.66 (2.63)	0.14
Current smoking, %	21	21	20	20	22	0.5
Birth control use, % (women only)	16	17	15	18	14	0.4
Physical activity intensity score, median (IQR)	286.0 (144.0–496.0)	271.0 (122.8–459.0)	267.0 (134.5–477.5)	293.0 (144.0–508.0)	318.0 (158.5–537.5)	<0.001
BMI, kg/m^2	28.49 (6.72)	27.13 (6.06)	27.85 (6.27)	28.96 (6.92)	30.01 (7.21)	<0.001
Triglycerides, mg/dL, median (IQR)	81.0 (58.0–120.0)	72.0 (53.0–105.0)	78.0 (56.0–109.0)	86.0 (62.0–123.0)	94.0 (64.5–141.0)	<0.001
Cholesterol, mg/dL	184.35 (34.34)	179.70 (34.49)	183.26 (31.87)	186.54 (33.93)	187.90 (36.41)	<0.001
HDL-C, mg/dL	50.88 (14.41)	53.16 (15.32)	51.13 (14.34)	49.88 (13.70)	49.33 (13.98)	<0.001
Hypertension, %	18	12	17	18	24	<0.001
Developed incident diabetes, *n* (%)	387 (12)	77 (10)	93 (12)	110 (14)	107 (14)	0.04
Prediabetes, %	4	2.7	3.2	4.9	5.0	0.038
HOMA-IR	2.28 (2.00)	2.14 (1.42)	2.16 (1.73)	2.32 (1.82)	2.52 (2.76)	<0.001
eGFR, mL/min/1.73 m^2	103.56 (16.61)	106.12 (16.04)	103.43 (15.81)	103.58 (16.55)	101.11 (17.64)	<0.001
Betaine						
n	3,133	784	783	783	783	
TMAO, median (IQR), μmol/L	2.9 (2.0–4.6)	2.7 (1.9–4.3)	2.8 (1.9–4.7)	3.1 (2.1–4.8)	3.0 (2.1–4.7)	<0.001
Choline, μmol/L	8.51 (2.22)	7.47 (1.95)	8.04 (1.84)	8.73 (2.01)	9.81 (2.33)	<0.001
Betaine, μmol/L	38.19 (13.32)	23.27 (4.75)	33.30 (2.18)	40.78 (2.35)	55.44 (11.22)	<0.001
Self-identified Black/African American race, %	44	40	46	43	46	0.073
Female, %	55	83	60	44	35	<0.001
Age, years	40.22 (3.60)	40.39 (3.53)	40.19 (3.58)	40.17 (3.68)	40.14 (3.62)	0.6
Education, years	15.86 (2.99)	15.97 (2.60)	15.90 (2.57)	15.83 (3.95)	15.72 (2.62)	0.2
Current smoking, %	21	18	21	21	23	0.076
Birth control use, % (women only)	16	34	5.6	2.6	2.3	<0.001
Physical activity intensity score, median (IQR)	286.0 (144.0–496.0)	236.0 (103.5–404.5)	274.0 (138.5–472.5)	300.0 (159.0–512.0)	345.0 (170.5–572.0)	<0.001
BMI, kg/m^2	28.49 (6.72)	28.99 (7.31)	28.92 (6.71)	28.34 (6.62)	27.69 (6.13)	<0.001
Triglycerides, mg/dL, median (IQR)	81.0 (58.0–120.0)	88.0 (66.8–130.0)	79.5 (58.0–119.0)	78.0 (56.0–115.0)	78.0 (56.0–113.0)	<0.001
Cholesterol, mg/dL	184.35 (34.34)	186.81 (34.91)	184.47 (32.80)	184.31 (34.81)	181.80 (34.70)	0.065
HDL-C, mg/dL	50.88 (14.41)	53.17 (15.42)	49.90 (14.05)	50.76 (14.14)	49.67 (13.75)	<0.001
Hypertension, %	18	17	18	17	18	>0.9
Developed incident diabetes, *n* (%)	387 (12)	124 (16)	102 (13)	86 (11)	75 (9.6)	0.001
Prediabetes, %	4.0	5.2	4.5	4.4	1.8	0.004
HOMA-IR	2.28 (2.00)	2.45 (1.88)	2.37 (1.63)	2.29 (2.19)	2.03 (2.22)	<0.001
eGFR, mL/min/1.73 m^2	103.56 (16.61)	103.19 (17.20)	103.81 (15.96)	102.54 (16.48)	104.71 (16.74)	0.074
Ln-TMAO						

Continued on p. 1989

Table 1—Continued

	Overall	Q1	Q2	Q3	Q4	P†
n	3,133	784	783	783	783	
TMAO, median (IQR), μmol/L	2.90 (2.00–4.60)	1.50 (1.20–1.80)	2.40 (2.20–2.60)	3.50 (3.20–4.10)	6.90 (5.50–10.10)	<0.001
Choline, μmol/L	8.51 (2.22)	8.07 (2.00)	8.39 (2.03)	8.73 (2.17)	8.87 (2.54)	<0.001
Betaine, μmol/L	38.19 (13.32)	36.56 (12.93)	37.63 (12.97)	39.78 (14.10)	38.79 (13.06)	<0.001
Self-identified Black/African American race, %	56	48	58	61	58	<0.001
Female, %	55	66	57	51	48	<0.001
Age, years	40.22 (3.60)	39.97 (3.58)	40.08 (3.60)	40.31 (3.55)	40.53 (3.67)	0.007
Education, years	15.86 (2.99)	15.76 (2.59)	15.93 (3.90)	15.97 (2.62)	15.77 (2.66)	0.2
Current smoking, %	21	19	21	22	21	0.5
Birth control use, % (women only)	16	17	16	18	12	0.2
Physical activity intensity score, median (IQR)	286.00 (144.00–496.00)	279.00 (124.00–468.50)	277.00 (145.50–473.50)	318.00 (152.00–545.00)	278.00 (144.00–493.00)	0.013
BMI, kg/m^2	28.49 (6.72)	28.36 (7.05)	28.34 (6.52)	28.49 (6.69)	28.75 (6.60)	0.14
Triglycerides, mg/dL, median (IQR)	81.00 (58.00–120.00)	77.00 (56.00–110.00)	82.00 (59.00–119.50)	84.00 (58.50–126.00)	83.00 (60.00–124.00)	0.006
Cholesterol, mg/dL	184.35 (34.34)	184.17 (32.78)	183.59 (34.06)	184.76 (33.92)	184.86 (36.56)	0.9
HDL-C, mg/dL	50.88 (14.41)	52.90 (14.30)	50.73 (14.23)	50.37 (14.32)	49.50 (14.61)	<0.001
Hypertension, %	18	18	15	18	19	0.2
Developed incident diabetes, *n* (%)	387 (12)	89 (11)	99 (13)	95 (12)	104 (13)	0.7
Prediabetes, %	4.0	2.4	4.0	4.2	5.1	0.05
HOMA-IR	2.28 (2.00)	2.13 (1.52)	2.26 (2.04)	2.37 (2.50)	2.37 (1.81)	0.022
eGFR, mL/min/1.73 m^2	103.56 (16.61)	107.63 (15.88)	103.17 (15.94)	102.24 (15.71)	101.20 (18.11)	<0.001

Data are mean (SD) unless otherwise indicated. †Kruskal-Wallis rank sum test for continuous variables and Pearson χ^2 test for categorical variables.

health behaviors, and total energy intake, choline was inversely associated with the diet quality score and positively associated with eggs, processed red meat, and refined grains; betaine was positively associated with the diet quality score, green vegetables, and whole grains and inversely associated with lean red meat, regular red meat, and total red meat. TMAO was not associated with the diet quality score or any of the food groups examined.

Choline Metabolites and Incident Diabetes

We tested associations between metabolites and incident diabetes in multivariable adjusted regression analysis. In analysis of metabolites modeled continuous, choline was not associated (HR 1.05 [95% CI 0.94, 1.16]), while betaine was inversely associated (0.76 [0.67, 0.88]) and ln-TMAO was positively associated (1.11 [1.01, 1.22]) with 15-year risk of incident diabetes (Table 2). To address possible nonlinearity in associations, we additionally specified metabolite exposures as quartiles. Compared with quartile 1 (Q1), all quartiles of betaine were inversely associated with 15-year risk of diabetes (Q2: HR 0.69 [95% CI 0.53, 0.91], Q3: 0.61 [0.45, 0.83], Q4: 0.50 [0.35, 0.70]). Q2–Q4 of TMAO were positively associated with the 15-year risk of incident diabetes compared with Q1, but this association was not statistically significant (Q4 vs. Q1: HR 1.33 [95% CI 0.99, 1.78]).

We used spline regression to address apparent nonlinearity in associations, particularly for choline and TMAO (Fig. 1). CIs were broad but generally supported results from prior specifications. HRs were nonmonotonic across concentrations of choline (Fig. 1*A*) and TMAO (Fig. 1*C*), while betaine displayed a relatively monotonic inverse association with 15-year incident diabetes (Fig. 1*B*). Low concentrations of choline appeared inversely associated with diabetes, while confidence bounds for TMAO did not clearly exclude the null value.

We next tested potential effect modification of metabolite-diabetes associations by sex and race. For choline and TMAO, these analyses did not support clear differences in associations across sex (Supplementary Table 2 and Supplementary Fig. 3) or racial (Supplementary Table 3 and Supplementary Fig. 4) groups in continuous, quartile, or spline specifications. The inverse association

Table 2—Multivariable adjusted hazard ratios (95% CI) for choline metabolites and 15-year incident diabetes, *n* (cases) = 3,133 (387)

	HR (95% CI) across quartiles of metabolites†				HR (95% CI) per 1-SD metabolite‡
	Q1 (reference)	Q2	Q3	Q4	
Choline§					
n (cases)	784 (77)	783 (93)	783 (110)	783 (107)	
Model 1	1.00	1.18 (0.87, 1.60)	1.11 (0.82, 1.50)	0.99 (0.73, 1.35)	0.96 (0.87, 1.06)
Model 2	1.00	1.29 (0.95, 1.76)	1.32 (0.97, 1.81)	1.27 (0.91, 1.76)	1.05 (0.94, 1.16)
Betaine					
n (cases)	784 (124)	783 (102)	783 (86)	783 (75)	
Model 1	1.00	0.71 (0.54, 0.93)	0.63 (0.47, 0.85)	0.52 (0.38, 0.72)	0.78 (0.69, 0.89)
Model 2	1.00	0.69 (0.53, 0.91)	0.61 (0.45, 0.83)	0.50 (0.35, 0.70)	0.76 (0.67, 0.88)
TMAO					
n (cases)	784 (89)	783 (99)	783 (95)	783 (104)	
Model 1	1.00	1.31 (0.98, 1.75)	1.22 (0.90, 1.64)	1.34 (1.00, 1.79)	1.11 (1.01, 1.22)
Model 2	1.00	1.29 (0.96, 1.73)	1.24 (0.92, 1.67)	1.33 (0.99, 1.78)	1.11 (1.01, 1.22)

Model 1 included CARDIA examination center (Birmingham, AL; Oakland, CA; Minneapolis, MN; Chicago, IL), age (continuous), race (Black, White), education (continuous), physical activity score (continuous), hormonal birth control use (yes, no, male), dietary intake (continuous food groups, diet quality, caloric intake), eGFR (continuous), BMI (continuous), hypertension (yes, no), and lipid-lowering medication use (yes, no). Model 2 additionally adjusted model 1 for the other two choline metabolites (continuous). †From Cox proportional hazards models. ‡TMAO was ln transformed for continuous specification. §Metabolite mean (SD): choline 8.51 (2.22), betaine 38.19 (13.32), ln-TMAO 1.47 (0.57).

between betaine and 15-year risk of incident diabetes appeared stronger among males compared with females. In some, but not all, specifications, the inverse association between betaine and incident diabetes appeared stronger among White compared with Black participants. However, we note that our sample size may not have been sufficient to robustly test these associations, and caution is warranted in interpretation.

We next tested whether associations between choline metabolites and incident diabetes may be modified by kidney function given prior studies of cardiometabolic outcomes (23). In this analysis, the inverse association between betaine, modeled continuous, and incident diabetes was stronger among participants with lower eGFR (HR 0.64 [95% CI 0.45, 0.91], 0.78 [0.66, 0.91], and 0.86 [0.64, 1.15] for eGFR $<$90, 90–120, and $>$120 mL/min/1.73 m^2, respectively) (Supplementary Table 4). Results were materially similar when metabolites were modeled as quartiles, but this analysis was limited by sample size (Supplementary Table 4).

We conducted several sensitivity analyses of metabolite-diabetes associations. First, we excluded participants with prediabetes at baseline (*n* = 427). In this analysis, incident diabetes associations were attenuated for both betaine and TMAO. Betaine remained inversely associated with incident diabetes (HR 0.80 [95% CI 0.68, 0.95]), while the positive association between TMAO and incident diabetes was no longer statistically significant (1.05 [0.93, 1.19]) (Supplementary Table 5). Next, we excluded participants who developed

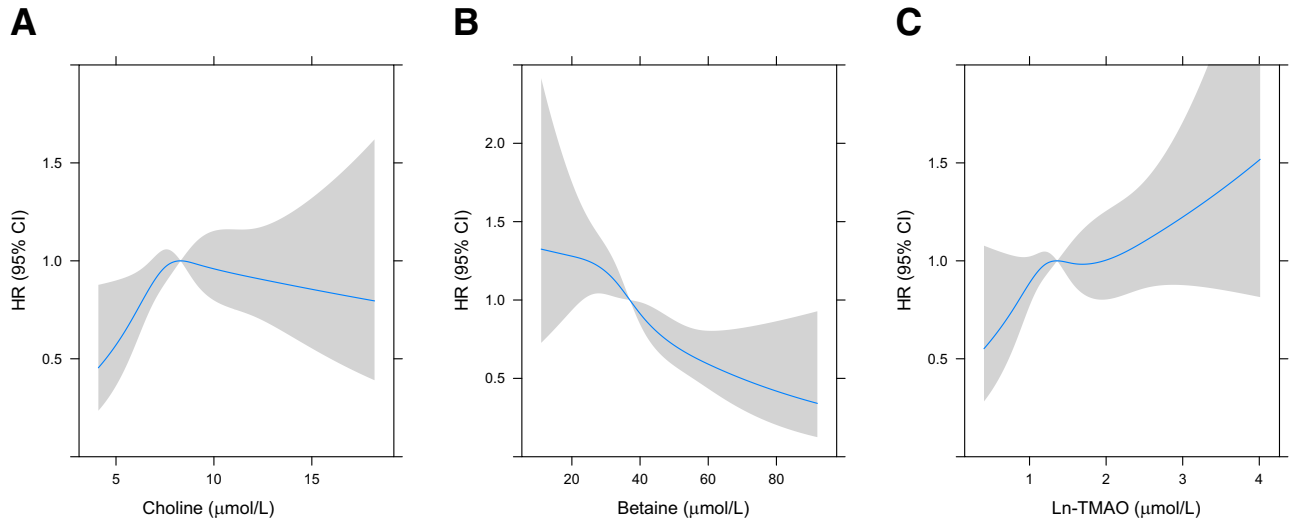

Figure 1—Multivariable adjusted associations among choline (*A*), betaine (*B*), and ln-TMAO (*C*) and 15-year incident diabetes. Models were adjusted for CARDIA examination center (Birmingham, AL; Oakland, CA; Minneapolis, MN; Chicago, IL), age (continuous), race (Black, White), education (continuous), physical activity score (continuous), hormonal birth control use (yes, no, male), smoking (yes, no), caloric intake (continuous), diet (continuous), eGFR (continuous), BMI (continuous), hypertension (yes, no), lipid-lowering medication use (yes, no), and the other two choline metabolites (continuous). *n* (cases) overall = 3,133 (387).

diabetes within the first 5 years of follow-up. As in the analysis excluding participants with prediabetes, associations were attenuated for both betaine and TMAO. Betaine remained significantly inversely associated with incident diabetes, while TMAO was no longer significantly associated with incident diabetes (Supplementary Table 6). The association between choline and incident diabetes was not affected by either exclusion. Finally, we ran Cox proportional hazards models with time-varying covariates, including physical activity score, eGFR, BMI, hypertension, and lipid-lowering medication use. After accounting for time-varying covariates, the positive association between choline and incident diabetes strengthened (1.11 [1.03, 1.21]). Betaine and TMAO associations were unchanged (Supplementary Table 7). We interpret the change in choline results with caution, as we did not have repeated metabolite measures and it is possible that clinical covariates measured after the study baseline may have been impacted by baseline choline.

Choline Metabolites and Insulin Resistance

In addition to incident diabetes, we studied 15-year changes in insulin resistance based on the HOMA-IR measure. Betaine, modeled continuous, was inversely associated with 15-year changes in ln-HOMA-IR (GMR 0.95 [95% CI 0.93, 0.97]) (Table 3). Similarly, betaine quartiles, compared with Q1, were inversely associated with 15-year changes in ln-HOMA-IR (Q2: GMR 0.95 [95% CI 0.90, 1.01]; Q3: 0.90 [0.85, 0.95]; Q4: 0.86 [0.80, 0.91]). Choline and TMAO were not associated with HOMA-IR in either continuous or quartile specifications (Table 3). Given the lack of significant results and the less variable results across quartiles, we did not conduct spline regression for associations between metabolites and HOMA-IR.

CONCLUSIONS

In a U.S. prospective cohort study of early to late middle-aged Black and White participants, we quantified associations between plasma choline metabolites and 15-year risk of incident diabetes, as well as 15-year changes in HOMA-IR. Betaine was robustly inversely associated with both outcomes, across all levels of covariate adjustment, and in all sensitivity analyses, including those testing for potential reverse causality. In contrast, positive associations between TMAO and study outcomes were sensitive to model specification, and choline was generally not associated with outcomes. Interaction models suggested that inverse association between betaine and incident diabetes may be stronger among males compared with females and among participants with decreased kidney function.

Our results contribute to the growing body of literature on choline metabolism and glycemic outcomes and are supported by mechanistic work showing that betaine and TMAO may influence glucose and insulin metabolism. Animal models have identified pathways through which TMAO and betaine can impact insulin signaling, hyperglycemia, metabolic dysfunction, gluconeogenesis, and glycogen synthesis (24–28). One specific pathway that has been described is through FoxO1, which is a target for both TMAO and betaine but in opposing ways (24). TMAO activates FoxO1 to promote hyperglycemia and metabolic dysfunction, while betaine inhibits insulin-induced FoxO1 binding to the thioredoxin-interacting protein to modulate hepatic inflammation and improve insulin resistance (24). Further mechanistic studies to unravel the opposing effects of betaine and TMAO on FoxO1 may contribute to understanding the role of choline metabolism in cardiometabolic disease. Betaine also decreases gluconeogenesis through XBP-1, increases glucose transport through improvements in GLUT-4 translocation, and increases fibroblast growth factor 21 levels to improve metabolic function (25,29). Betaine serves as a methyl donor and may influence diabetes risk through one-carbon metabolism. Low availability of one-carbon metabolism nutrients, including betaine, reduces availability of methyl group supply

Table 3—Prospective associations between choline metabolites and 15-year changes in ln-HOMA-IR (n = 2,530)

	GMR (95% CI)† for HOMA-IR across quartiles of metabolites				GMR (95% CI) per 1-SD metabolites‡
	Q1 (reference)	Q2	Q3	Q4	
Choline§					
n (cases)	784 (77)	783 (93)	783 (110)	783 (107)	
Model 1	1.00	1.01 (0.96, 1.06)	1.01 (0.96, 1.06)	0.99 (0.94, 1.05)	0.99 (0.97, 1.01)
Model 2	1.00	1.03 (0.98, 1.08)	1.05 (1.00, 1.11)	1.05 (1.00, 1.12)	1.01 (0.99, 1.03)
Betaine					
n (cases)	784 (124)	783 (102)	783 (86)	783 (75)	
Model 1	1.00	0.96 (0.91, 1.01)	0.91 (0.87, 0.97)	0.88 (0.83, 0.93)	0.95 (0.94, 0.97)
Model 2	1.00	0.95 (0.90, 1.01)	0.90 (0.85, 0.95)	0.86 (80, 0.91)	0.95 (0.93, 0.97)
TMAO					
n (cases)	784 (89)	783 (99)	783 (95)	783 (104)	
Model 1	1.00	1.01 (0.96, 1.07)	1.00 (0.95, 1.06)	1.00 (0.95, 1.05)	1.00 (0.99, 1.02)
Model 2	1.00	1.01 (0.96, 1.06)	1.01 (0.96, 1.06)	1.00 (0.95, 1.05)	1.00 (0.99, 1.02)

Sequential multivariable adjusted linear regression models were used. Model 1 included CARDIA examination center (Birmingham, AL; Oakland, CA; Minneapolis, MN; Chicago, IL), age (continuous), race (Black, White), education (continuous), year 15 ln-HOMA-IR (continuous), physical activity score (continuous), hormonal birth control use (yes, no, male), dietary intake (continuous food groups, diet quality, caloric intake), eGFR (continuous), BMI (continuous), hypertension (yes, no), and lipid-lowering medication use (yes, no). Model 2 additionally adjusted model 1 for the other two choline metabolites (continuous). †Determined through exponentiating the β-coefficients from linear regression models. ‡TMAO was ln transformed in continuous models. §Metabolite mean (SD): choline 8.51 (2.22), betaine 38.19 (13.32), ln-TMAO 1.47 (0.57).

for DNA methylation of genes in glucose and lipid metabolism, contributing to β-cell dysfunction, insulin resistance, and glucose intolerance (30).

Our study addresses important gaps in the literature through analysis of a U.S. population-based cohort of participants who were aged 33–45 years at the time of metabolite measurement. Previous studies have been largely limited to adults who are older (8,31,32), potentially missing an important window for preventive activities, or among participants with a higher prevalence of cardiometabolic risk factors than the general population (7,10,33), which may confound or modify metabolite associations. Such differences in participant characteristics may contribute to inconsistent findings across studies. Our findings that plasma choline was not associated and plasma betaine was inversely associated with incident diabetes is consistent with previous studies, except for the Cardiovascular Health Study, which found no association between plasma betaine and incident diabetes in older adults (7,8,10,31–33).

In contrast, published findings for TMAO have been inconsistent, with TMAO inversely associated (7), positively associated (32), and not associated (8) with incident diabetes. In our analysis, TMAO associations with incident diabetes were sensitive to model specification. For example, the positive association between TMAO and incident diabetes observed in our primary analysis was attenuated and no longer statistically significant after excluding participants with prediabetes at baseline or participants who developed diabetes within the first 5 years of follow-up. These results suggest that inconsistencies across studies could, to some extent, reflect residual confounding or modification by subclinical glycemic status. The sample's TMAO distribution may be another important source of variability across studies. In CARDIA, we observed an overall median of 2.9 (IQR 2.0–4.6) μmol/L, while other studies have reported lower (median 1.54 [IQR 0.86–2.91] μmol/L) [32]) and higher (5.6 [3.6–9.3] μmol/L [10]) concentrations. Higher TMAO concentrations have been documented in individuals with diabetes, metabolic syndrome, and decreased renal function (15), and it is possible that increased cardiometabolic risk may be limited to higher TMAO concentrations.

We found that the inverse association between betaine and incident diabetes was stronger among, or limited to, men. This result is consistent with findings from the Prevention of Renal and Vascular End-Stage Disease (PREVEND) study (33). However, it is possible that our study was not sufficiently powered for robust analysis of interactions. In addition, as in the PREVEND study, we noted that circulating betaine concentrations were lower among women compared with men, and it is possible that betaine concentrations may not have been sufficiently high to impart benefit among female CARDIA participants. Many sex-specific differences in one-carbon metabolism have been well acknowledged in the literature (34). We also found that the association between betaine and incident diabetes was modified by eGFR, with stronger inverse associations at lower eGFR levels. A primary function of betaine is as an osmoprotectant, and high urinary betaine excretion has been observed in people with diabetes and/or kidney disease (26).

Choline metabolites are influenced by diet (39), but whether circulating measures can serve as dietary biomarkers in U.S. adults meeting the adequate intake for choline is unclear (35,36). Few studies have assessed associations between food group intake and choline metabolites. In CARDIA, circulating choline was inversely, while betaine was positively, associated with a diet quality score. Plasma choline was positively associated with choline-rich foods, including eggs and red meat, and refined grains, which likely reflects the pattern of food consumption. Similarly, betaine was associated with foods that contribute dietary betaine, such as green vegetables and whole grains and inversely associated with chicken and red meat. TMAO was not associated with dietary intake.

Observational data cannot establish causality. In a randomized controlled trial of betaine supplementation among 27 participants with obesity and prediabetes, effects on metabolic outcomes were mixed. After 12 weeks, the betaine arm had significantly decreased fasting glucose and, following oral glucose, insulin concentrations, but other measures were not impacted, including glucose area under the curve, C-peptide, and insulin sensitivity (assessed by clamp) (37). The Mendelian randomization approach may hold promise for future studies investigating the role of betaine and other choline metabolites in identifying betaine as a genetically altered metabolite associated with diabetes risk (38).

This study has several strengths and limitations. The U.S.-based multicenter CARDIA cohort comprises Black and White men and women with socioeconomic and geographic diversity, which may improve the generalizability of the results. A broad range of covariates are measured at CARDIA examinations using standardized and validated protocols, though the potential for residual confounding remains. In addition, repeated-measures data allowed us to control for changes in BMI and kidney function over follow-up, though we lacked repeated measures of plasma metabolites. Metabolite concentrations were measured from a single fasting sample. It is possible that within-person variability in metabolite concentrations may have contributed extraneous variability, though reliability studies have indicated that choline and betaine are relatively stable among healthy adults (34). CARDIA lacks data on islet autoantibody tests or other clinical measures to distinguish type 2 from type 1 diabetes. We excluded all prevalent diabetes at baseline, when participants were aged 33–45 years, but we cannot discount the potential for adult-onset type 1 diabetes among our incident cases (39). We recognize that our analysis was conducted in a relatively small study sample, particularly for stratum-specific estimates, which limited precision in analysis of effect modification.

Given the existing literature and the results of this population-based study in early middle-aged adults, further studies are needed to determine population characteristics that may influence the association between plasma betaine and diabetes risk. For example, our finding that betaine was inversely associated with incident diabetes in men but not women requires replication in larger samples. In addition, work is needed to improve the assessment of causality, such as Mendelian randomization in observational cohorts and larger, more representative trials than previously conducted (37). The extent to which plasma betaine can be modified by diet to reduce diabetes risk is not known. The Diabetes Prevention Program observed that in high-risk participants, betaine concentrations increased in response to intensive lifestyle interventions, which were the most effective in reducing diabetes risk (40). These results suggest the possibility that

increased circulating betaine may contribute to decreased diabetes risk through recommended healthy lifestyle changes.

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This article has been reviewed by CARDIA for scientific content.

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