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Prospective Associations of Systemic and Urinary Choline Metabolites with Incident Type 2 Diabetes

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BACKGROUND: Several compounds in the choline oxidation pathway are associated with insulin resistance and prevalent diabetes; however, prospective data are scarce.

We explored the relationships between systemic and urinary choline-related metabolites and incident type 2 diabetes in an observational prospective study among Norwegian patients.

METHODS: We explored risk associations by logistic regression among 3621 nondiabetic individuals with suspected stable angina pectoris, of whom 3242 provided urine samples. Reclassification of patients was investigated according to continuous net reclassification improvement (NRI >0).

RESULTS: After median (25th to 75th percentile) follow-up of 7.5 (6.4–8.7) years, 233 patients (6.4%) were registered with incident type 2 diabetes. In models adjusted for age, sex, and fasting status, plasma betaine was inversely related to new-onset disease [odds ratio (OR) per 1 SD, 0.72; 95% CI, 0.62–0.83; $P < 0.00001$], whereas positive associations were observed for urine betaine (1.25; 1.09–1.43; $P = 0.001$), dimethylglycine (1.22; 1.06–1.40; $P = 0.007$), and sarcosine (1.30; 1.13–1.49; $P < 0.001$). The associations were maintained in a multivariable model adjusting for body mass index, hemoglobin A_{1c}, urine albumin-to-creatinine ratio, estimated glomerular filtration rate, C-reactive protein, HDL cholesterol, and medications. Plasma betaine and urine sarcosine, the indices most strongly related to incident type 2 diabetes, improved reclassification [NRI >0 (95% CI) 0.33 (0.19–0.47) and 0.16 (0.01–0.31), respectively] and showed good within-person reproducibility.

CONCLUSIONS: Systemic and urinary concentrations of several choline metabolites were associated with risk of incident type 2 diabetes, and relevant biomarkers may improve risk prediction.

The choline oxidation pathway comprises the sequential metabolism of choline to betaine, dimethylglycine, and sarcosine (Fig. 1). This pathway yields 1-carbon units for the production of the universal methyl donor, *S*-adenosylmethionine, concentrations of which may be low among patients with type 2 diabetes (T2D)[9] *(1)*. Both choline and betaine are involved in mobilizing lipids from the liver *(2)*, and betaine supplementation may alleviate hepatic lipid accumulation *(3)* that is commonly related to insulin resistance (IR) and T2D. In addition, several steps in the choline oxidation pathway take place within the mitochondrion and are tightly connected to the mitochondrial respiratory chain *(4)*, linking choline metabolism to adequate mitochondrial function.

Lower circulating betaine concentrations have been reported among patients with T2D than among those without established T2D *(5, 6)*, and a recent report proposed a relationship between low plasma dimethylglycine and incident diabetes *(7)*. Moreover, the intestinal microflora take part in turning dietary choline into trimethylamine *N*-oxide (TMAO) *(8)*, and higher plasma TMAO concentrations have been observed among patients with T2D than among those without T2D *(9)*. Also, several studies have found very high urinary betaine concentrations among patients with diabetes *(10, 11)*. We previously observed strong positive correlations between urine betaine, dimethylglycine, and sarcosine among patients with coronary heart disease and found

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[9] Nonstandard abbreviations: T2D, type 2 diabetes; IR, insulin resistance; TMAO, trimethylamine *N*-oxide; WENBIT, Western Norway B-Vitamin Intervention Trial; Hb A_{1c}, glycated hemoglobin; HOMA2, updated homeostatic model assessment; BMI, body mass index; eGFR, estimated glomerular filtration rate; CRP, C-reactive protein; HDL-C, HDL cholesterol; NRI, net reclassification improvement; ICC, intraclass correlation coefficient; OR, odds ratio; BHMT, betaine-homocysteine *S*-methyl transferase.

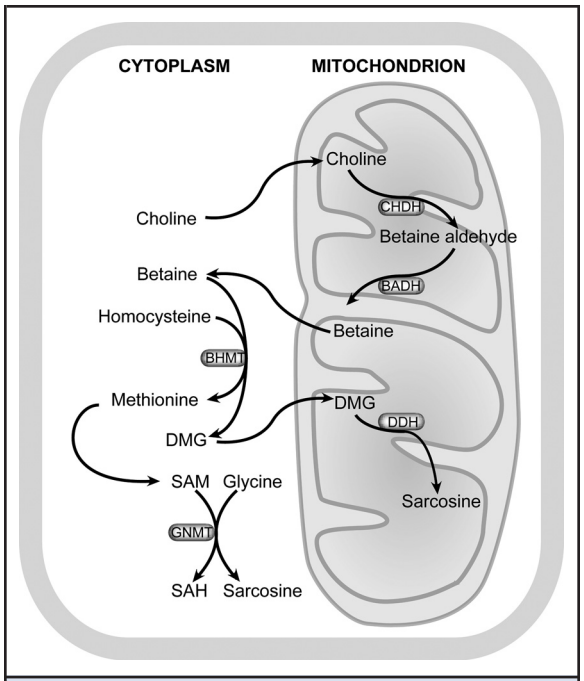

Fig. 1. Choline metabolism and its ramifications to homocysteine and methyl group metabolism.

BADH, betaine-aldehyde dehydrogenase; CHDH, choline dehydrogenase; DDH, dimethylglycine dehydrogenase; DMG, dimethylglycine; GNMT, glycine-*N*-methyltransferase; SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; SDH, sarcosine dehydrogenase.

that high urine betaine was associated with new-onset diabetes during follow-up for approximately 3 years *(11)*.

These findings suggest that IR and diabetes are associated with altered downstream choline metabolism, and potentially also dietary choline intake. Moreover, patients with diabetes seem to have increased excretion of several choline metabolites in the urine. However, it is not known whether compounds related to the choline oxidation pathway other than plasma dimethylglycine and urine betaine are associated with incident T2D in long-term prospective studies; hence, we now report on these issues in a large prospective observational cohort study with long-term follow-up.

Materials and Methods

STUDY DESIGN

The source population has been described elsewhere *(12)*. In short, 4164 patients were evaluated for suspected stable angina pectoris at 2 Norwegian university hospitals from 2000 to 2004. About two-thirds were included in the Western Norway B-Vitamin Intervention Trial (WENBIT) and randomized to receive folic acid + vitamin B_{12} + vitamin B_6, folic acid + vitamin B_{12}, vitamin B_6 alone, or placebo *(13)*.

For baseline analyses, we excluded 94 patients with type 1 diabetes or without data on glycated hemoglobin (Hb A_{1c}) or plasma glucose, choline, betaine, and dimethylglycine, leaving 4070 patients (see Supplementary Fig. 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol62/issue5). When carrying out analyses on end points and repeated measurements, we further excluded 449 patients with established T2D. This left 3621 patients for follow-up, of whom 3242 had provided urine samples.

CLINICAL AND BIOCHEMICAL DATA

The collection of anamnestic, clinical, and routine biochemical information has been described *(11, 12)*. We measured plasma TMAO, choline, betaine, and dimethylglycine; serum sarcosine; and urine choline, betaine, dimethylglycine, and sarcosine by LC-MS/MS *(14)* or GC-MS/MS *(15)*. Within-day CVs for the assays were as follows: choline, 5.4%–5.9%; betaine, 5.5%–7.2%; dimethylglycine, 6.7%–11.7%; *(14)*; sarcosine 5%; and TMAO 2.1%–3.1% *(16)*. Concentrations of compounds in the urine were given per mole creatinine to correct for dilution.

We estimated daily total intake of energy, as well as choline and betaine according to the USDA Database for the Choline Content of Common Foods *(17)*, among 1939 patients who provided information on average dietary habits during the last year from food frequency questionnaires, as described elsewhere *(18)*. We also calculated β-cell function, insulin sensitivity, and IR among 877 fasting patients without established type 2 diabetes at baseline with the computer-based updated homeostatic model assessment (HOMA2), as previously reported *(19)*.

All patients provided written informed consent, and the study was carried out according to the Declaration of Helsinki.

STUDY END POINTS

Patients were classified as having incident T2D when diagnosed according to the *International Classification of Diseases, Revision 10* (codes E11–E14) at their discharge summary from a stay in a Norwegian public hospital. Data were obtained from the Cardiovascular Disease in Norway project (http://www.cvdnor.no) *(20)*, and follow-up ended on December 31, 2009. For the subset included in WENBIT and during in-trial follow-up, cases were additionally identified as incident self-reported T2D or newly diagnosed T2D according to fasting or nonfasting plasma glucose ≥126 and ≥200 mg/dL (≥7.0 and ≥11.1 mmol/L), respectively *(21)*.

STATISTICAL ANALYSES

Continuous and categorical variables are given as medians (25th to 75th percentiles) and counts (%), respectively. Between-group differences were tested by use of age, sex, and fasting status (fasting defined as ≥8 h since last meal) adjusted mixed linear regression models for continuous and logistic regression models for categorical dependent variables. We explored associations between choline metabolites and indices of IR and glucose homeostasis with partial Spearman rank correlation, adjusted for age, sex, and fasting status. We investigated changes in choline metabolites from baseline to the 1-year WENBIT study visit according to study treatment allocation, with mixed linear modeling.

All metabolites had right-tailed distributions and were log-transformed and standardized before entry into logistic regression models when investigating their relationships with incident T2D. Estimates are reported as per 1 SD, and obtained unadjusted; adjusted for age, sex, and fasting status; and additionally adjusted for several established risk factors of T2D and potential confounders: body mass index (BMI), Hb A_{1c}, urine albumin-to-creatinine ratio, estimated glomerular filtration rate (eGFR), C-reactive protein (CRP), HDL cholesterol (HDL-C), and the use of loop diuretics, thiazides, β-blockers, statins, ACE inhibitors, and angiotensin receptor blockers at discharge from the baseline hospital visit. Potential nonlinear relationships between choline-related metabolites (as continuous, nontransformed variables) and incident type 2 diabetes were investigated by generalized additive modeling of the logistic regression models adjusted for age, sex, and fasting and potential break-points as tested by the Davies test for segmented regression. To identify the choline metabolites that were most strongly associated with incident T2D, we simultaneously included choline metabolites that were independently associated with the end point in the univariate analyses into a stepwise backward elimination logistic regression model otherwise containing the variables in the multivariate model. The selection was determined by improvement in the Akaike information criterion. For the choline metabolites still left in the model, we explored their individually added improvement in model discrimination by calculating the *c*-statistic and the integrated discrimination index and assessed reclassification by determining the continuous (category-free) net reclassification improvement (NRI >0). Within-individual reproducibility for the same variables was explored by calculating their intraclass correlation coefficients (ICCs), with values of 0.40 to 0.75 and ≥0.75 suggesting fair to good and excellent reproducibility, respectively *(22)*.

The software packages IBM SPSS Statistics for Windows, version 22.0 (IBM Corp.) and R version 3.0.2 (R Foundation for Statistical Computing; packages *nlme*, *ppcor*, *mgcv*, *segmented*, *Hmisc*, *ROCR*, *PredictABEL*, and *ICC*) were used for statistical analyses, and the significance level was 0.05 for all models.

Results

BASELINE CHARACTERISTICS

Among 4070 patients at baseline, 71.9% were men, 26.6% were fasting, and overall median (25th to 75th percentile) age, BMI, and Hb A_{1c} were 62 (55–70) years, 26.3 (24.2–29.0) kg/m^2, and 6.1% (5.4%–6.8%), respectively. Compared with nondiabetic patients, those with T2D had higher serum CRP and triglycerides and lower HDL-C. Patients with T2D also had higher plasma TMAO and choline but lower plasma betaine; however, the daily intake of neither choline nor betaine differed according to diabetic status. Urinary concentrations of all choline metabolites were approximately 2- to 3-fold higher among patients with T2D than among those without T2D, and as expected, patients with T2D had higher urine albumin-to-creatinine ratios (Table 1). Because the diagnosis of diabetes requires 2 independent measurements in otherwise symptom-free individuals *(21)*, we did similar calculations after excluding 1108 patients having Hb A_{1c} ≥6.5%, fasting plasma glucose ≥126 mg/dL (7.0 mmol/L), or random plasma glucose ≥200 mg/dL (11.1 mmol/L), but without a diagnosis of T2D at baseline, indicating possible but yet not verified diabetes. Similar trends were observed (see online Supplementary Table 1).

As depicted in online Supplementary Figs. 2 and 3, higher plasma choline and lower plasma betaine concentrations were related to a generally more adverse diabetes risk profile. Plasma dimethylglycine had positive associations with HOMA2-IR and CRP. Serum sarcosine was associated with a favorable risk profile, especially among patients with T2D. Plasma TMAO correlated positively with HOMA2-IR, as well as with plasma choline, dimethylglycine, and serum sarcosine. In urine, most choline metabolites were positively related to an adverse risk profile, and particularly strong associations were observed with plasma glucose and Hb A_{1c} among patients with established T2D.

All choline oxidation pathway metabolites in plasma and serum showed moderately strong positive intercorrelations. Even stronger associations were seen among the various choline metabolites in urine, and in particular between betaine, dimethylglycine, and sarcosine (partial Spearman $\rho \geq 0.69$, $P < 0.001$). However, systemic and urinary concentrations of each metabolite were only moderately positively correlated, and among patients with T2D, we observed a negative correlation between plasma and urine betaine.

Table 1. Baseline characteristics according to type 2 diabetes at baseline.[a]

Characteristic	Type 2 diabetes at baseline: No n[b]	No: Median (25th–75th percentile) or n (%)	Yes n[b]	Yes: Median (25th–75th percentile) or n (%)	*P*
Age, years	3621	62 (55–69)	449	65 (58–72)	<0.000001
Male sex	3621	2603 (71.9)	449	325 (72.4)	0.48
Prior cardiovascular disease.	3621	2044 (56.4)	449	298 (66.4)	0.001
Current smoking	3621	1175 (32.4)	449	113 (25.2)	0.09
Estimated total daily intake	1735		204		
Energy, kcal		2052 (1663–2503)		1912 (1530–2384)	0.12
Choline, mg		242 (193–302)		240 (182–299)	0.83
Betaine, mg		136 (105–169)		133 (104–168)	0.20
BMI, kg/m^2	3618	26.1 (24.1–28.7)	449	28.1 (25.4–31.4)	<0.000001
Hb A_{1c}, %	3621	6.0 (5.3–6.6)	449	7.7 (6.7–8.9)	<0.000001
Plasma glucose, mg/dL	3621	99 (90–112)	449	180 (139–225)	<0.000001
HOMA-2[c]	877				
β-cell function, %		53 (43–80)		–	–
Insulin sensitivity, %		238 (88–265)		–	–
Insulin resistance		0.40 (0.40–1.10)		–	–
eGFR, mL · min^{-1} · (1.73 m^2)$^{-1}$	3621	91 (79–99)	448	90 (74–99)	<0.001
Plasma/serum					
CRP, mg/L	3620	1.74 (0.85–3.51)	449	2.15 (1.09–4.81)	0.009
HDL-C, mg/dL	3620	49 (39–58)	449	43 (35–50)	<0.000001
Triglycerides, mg/dL	3617	130.2 (94–185)	449	160 (114–233)	<0.000001
Alanine aminotransferase, IU/L	3025	28 (20–38)	378	30 (22–42)	0.005
Total homocysteine, μmol/L	3621	10.4 (8.7–12.5)	449	10.7 (8.6–12.9)	0.30
Methionine, μmol/L	3621	25.6 (22.5–31.9)	449	26.7 (22.5–33.1)	0.31
Choline metabolites, μmol/L					
TMAO	3610	5.6 (3.6–9.3)	446	7.2 (4.3–12.3)	0.0004
Choline	3621	9.6 (8.2–11.4)	449	10.1 (8.4–12.2)	0.10
Betaine	3621	39.4 (32.5–48.1)	449	35.6 (28.3–45.0)	<0.000001
Dimethylglycine	3621	4.1 (3.4–5.1)	449	4.2 (3.2–5.2)	0.88
Sarcosine	3345	1.5 (1.2–1.8)	422	1.4 (1.1–1.8)	0.92
B-vitamins					
Riboflavin, nmol/L	3605	11.0 (7.4–18.0)	442	12.9 (8.6–21.2)	<0.001
Folate, nmol/L	3619	10.0 (7.3–14.6)	449	10.8 (7.9–15.6)	0.008
Cobalamin, pmol/L	3181	362 (275–466)	400	358 (270–464)	0.53
5′-pyridoxal phosphate, nmol/L	3605	41.5 (29.6–59.8)	442	39.0 (27.4–59.4)	0.21
Urine					
Choline metabolites, mmol/mol creatinine					
Choline	3242	1.98 (1.47–2.69)	399	2.81 (1.85–4.28)	<0.000001
Betaine	3242	6.96 (4.67–42.25)	399	22.27 (10.21–45.25)	<0.000001
Dimethylgycine	3242	3.0 (1.98–4.64)	399	5.61 (3.50–8.09)	<0.000001
Sarcosine	3242	0.13 (0.09–0.20)	398	0.25 (0.15–0.42)	<0.000001
Albumin, g/mmol creatinine	2952	0.52 (0.38–0.86)	375	1.01 (0.55–3.06)	<0.000001

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Table 1. Baseline characteristics according to type 2 diabetes at baseline.[a] *(Continued from page 758)*

Characteristic	Type 2 diabetes at baseline: No n[b]	No: Median (25th–75th percentile) or n (%)	Yes n[b]	Yes: Median (25th–75th percentile) or n (%)	*P*
Medications and supplements, n (%)					
Before baseline					
Statin	3621	2601 (71.8)	449	349 (77.9)	0.009
Folic acid	3407	313 (9.2)	408	42 (10.3)	0.39
Multivitamins	3407	520 (15.3)	408	46 (11.3)	0.05
At discharge from hospital					
Statin	3621	2875 (79.4)	–	–	–
Folic acid[d]	3407	226 (6.6)	–	–	–
Multivitamin[d]	3407	203 (6.0)	–	–	–

[a] *P* values were adjusted for age, sex, and fasting status. To convert plasma glucose from mg/dL to mmol, multiply by 0.05556; HDL-C from mg/dL to mmol, multiply by 0.02586; and triglycerides from mg/dL to mmol, multiply by 0.01129.
[b] Patients with valid measurements.
[c] Fasting patients without established diabetes.
[d] Patients in WENBIT were instructed not to use any additional vitamin supplements.

CHANGES IN CHOLINE METABOLITES OVER 1 YEAR

Among patients allocated to placebo treatment in WENBIT, we observed a slight increase in the systemic concentrations of all 4 choline metabolites, as well as for urine choline, dimethylglycine, and sarcosine (see online Supplementary Table 2). Relative to placebo, treatment with folic acid + vitamin B_{12} augmented the increase in plasma choline and betaine but lowered plasma and urine dimethylglycine and urine sarcosine. Compared with placebo, vitamin B_6 treatment was associated with an even larger increase in plasma dimethylglycine but less prominent increments in serum sarcosine and urine choline concentrations. No significant differences in temporal plasma TMAO were found, nor were differences found according to WENBIT study treatment.

ASSOCIATIONS BETWEEN CHOLINE METABOLITES AND INCIDENT T2D

By the end of 2009, and during a total follow-up time of median (25th to 75th percentile) 7.5 (6.4, 8.7) years, 88.2% of 3621 patients without known T2D at baseline had been admitted to any Norwegian public hospital at least once, among whom we identified 191 cases of incident T2D according to hospital discharge diagnoses. Additionally, 42 cases of new-onset T2D were reported during WENBIT follow-up study visits throughout 2006, bringing the number of end points to 233 (6.4% overall incidence rate).

As expected, patients who later received a diagnosis of new T2D had higher baseline BMI, Hb A_{1c}, plasma glucose, and HOMA2-IR than those who did not develop T2D (Table 2). Patients who developed T2D also had lower plasma betaine but higher urine betaine and sarcosine concentrations, whereas we observed no differences in plasma TMAO or reported total intake of choline and betaine.

In logistic regression analyses adjusted for age, sex, and fasting status, incident T2D was strongly associated with lower baseline plasma betaine [odds ratio (OR) per 1 SD, 0.72; 95% CI, 0.62–0.83; $P < 0.00001$] and higher urine betaine (1.25; 1.09–1.43; $P = 0.001$), dimethylglycine (1.22; 1.06–1.40; $P = 0.007$), and sarcosine (1.30; 1.13–1.49; $P < 0.001$). No statistically significant associations were found for plasma or urinary choline, plasma TMAO, plasma dimethylglycine, or serum sarcosine (Table 3), nor was there any relationship between urine creatinine and incident T2D (data not shown).

The generalized additive modeling plots in Fig. 2 show age-, sex-, and fasting-adjusted dose–response relationships between metabolites of the choline oxidation pathway and incident T2D. The inverse relationship observed for plasma betaine leveled off at higher plasma betaine concentrations ($P = 0.03$). No statistically significant nonlinear relationships were observed between any of the other metabolites and incident T2D ($P \geq 0.08$).

The risk associations were not statistically significantly different when excluding the 1108 patients with indices of possible T2D at baseline (see online Supple-

Table 2. Baseline characteristics according to incident type 2 diabetes during follow-up.[a]

Characteristic	Type 2 diabetes during follow-up: No n[b]	No: Median (25th–75th percentile) or n (%)	Yes n[b]	Yes: Median (25th–75th percentile) or n (%)	*P*
Age, years	3388	62 (54–69)	233	62 (56–70)	0.06
Male sex	3388	2434 (71.8)	233	169 (72.5)	0.61
Prior cardiovascular disease	3388	1897 (56.0)	233	147 (63.1)	0.06
Current smoking	3388	1103 (32.6)	233	72 (30.9)	0.96
Estimated total daily intake	1605		130		
Energy, kcal		2053 (1672–2509)		1997 (1596–2418)	0.77
Choline, mg		242 (193–302)		242 (182–301)	0.71
Betaine, mg		136 (105–169)		135 (105–169)	0.59
BMI, kg/m^2	3385	26.0 (23.9–28.4)	233	28.9 (26.3–31.4)	<0.00001
Hb A_{1c}, %	3388	5.9 (5.3–6.6)	233	6.2 (5.5–7.0)	<0.00001
Plasma glucose, mg/dL	3388	99 (90–110)	233	119 (104–150)	<0.00001
HOMA-2[c]	835		42		
β-cell function, %		53 (43–80)		67 (46–106)	0.19
Insulin sensitivity, %		247 (90–266)		101 (39–219)	<0.00001
Insulin resistance		0.40 (0.40–1.10)		1.00 (0.48–2.55)	<0.00001
eGFR, mL · min^{-1} · (1.73 m^2)$^{-1}$	3388	91 (79–99)	233	91 (80–98)	0.13
Plasma/serum					
CRP, mg/L	3387	1.71 (0.84–3.43)	233	2.34 (1.17–4.32)	0.09
HDL-C, mg/dL	3387	50 (42–58)	233	43 (35–50)	<0.00001
Triglycerides, mg/dL	3384	127 (93–182)	233	174 (120–238)	<0.00001
Alanine aminotransferase, IU/L	2835	27 (20–38)	190	32 (24–48)	0.008
Total homocysteine, μmol/L	3388	10.4 (8.7–12.5)	233	10.5 (8.6–12.9)	0.79
Methionine, μmol/L	3388	26.5 (22.5–31.8)	233	26.9 (22.4–33.9)	0.08
Choline metabolites, μmol/L					
TMAO	3378	5.5 (3.6–9.3)	232	5.9 (4.2–9.4)	0.77
Choline	3388	9.6 (8.2–11.4)	233	10.0 (8.2–11.4)	0.73
Betaine	3388	39.5 (32.8–48.2)	233	36.9 (28.8–44.6)	0.0009
Dimethylglycine	3388	4.1 (3.4–5.1)	233	4.2 (3.4–5.2)	0.56
Sarcosine	3127	1.5 (1.2–1.8)	218	1.5 (1.2–1.8)	0.84
B-vitamins					
Riboflavin, nmol/L	3372	11.0 (7.3–17.8)	233	12.2 (7.7–20.2)	0.42
Folate, nmol/L	3386	10.0 (7.3–14.6)	233	10.2 (7.8–15.6)	0.78
Cobalamin, pmol/L	2969	363 (275–467)	212	350 (275–445)	0.27
5′-pyridoxal phosphate, nmol/L	3372	41.5 (29.7–59.9)	233	41.8 (28.4–59.8)	0.21
Urine					
Choline metabolites, mmol/mol creatinine					
Choline	3037	1.97 (1.47–2.69)	205	2.03 (1.45–2.93)	1.00
Betaine	3037	6.91 (4.67–10.86)	205	8.18 (4.97–13.60)	<0.00001
Dimethylgycine	3037	3.02 (1.96–4.56)	205	3.32 (2.15–5.43)	0.61
Sarcosine	3037	0.13 (0.08–0.20)	205	0.16 (0.09–0.24)	<0.00001
Albumin, g/mmol creatinine	2778	0.51 (0.37–0.84)	192	0.67 (0.46–1.33)	0.89

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Table 2. Baseline characteristics according to incident type 2 diabetes during follow-up.[a] *(Continued from page 760)*

	Type 2 diabetes during follow-up				
	No		Yes		
Characteristic	n[b]	Median (25th–75th percentile) or n (%)	n[b]	Median (25th–75th percentile) or n (%)	*P*
Medications and supplements, n (%)					
Before baseline					
Statin	3388	2432 (71.8)	233	169 (72.5)	0.76
Folic acid	3184	294 (9.2)	223	19 (8.5)	0.74
Multivitamins	3184	488 (15.3)	223	32 (14.3)	0.73
At discharge from hospital					
Statin	3388	2678 (79.0)	233	197 (84.5)	0.05
Folic acid[d]	3184	215 (6.8)	223	11 (4.9)	0.71
Multivitamin[d]	3184	191 (6.0)	223	12 (5.4)	0.76

[a] *P* values were adjusted for age, sex, and fasting status. To convert plasma glucose from mg/dL to mmol, multiply by 0.05556; HDL-C from mg/dL to mmol, multiply by 0.02586; and triglycerides from mg/dL to mmol, multiply by 0.01129.
[b] Patients with valid measurements.
[c] Fasting patients without established diabetes.
[d] Patients in WENBIT were instructed not to use any additional vitamin supplements.

mentary Table 3; *P* for interaction ≥0.17), of whom 99 (8.9%) received a later diagnosis of T2D. The risk estimates were similar also when considering only end points registered during the in-trial follow-up period among participants in WENBIT (see online Supplementary Table 4).

In the multivariate model (n = 2949, 191 events), the risk associations were essentially unaltered (Table 3), also when further adjusting for estimated daily total intake of either choline or betaine in the subgroup of patients with dietary data (data not shown). B-vitamin status is closely metabolically linked to the choline oxidation pathway, but adjusting for serum folate, serum cobalamin, plasma riboflavin, plasma 5′-pyridoxal phosphate, or WENBIT study treatment did not influence overall risk estimates (data not shown).

Table 3. Associations of systemic and urine choline metabolites with incident type 2 diabetes.[a]

Metabolite	Univariate model	*P*	Adjusted for age, sex, and fasting status	*P*	Multivariate model[b]	*P*
Plasma/serum						
TMAO	1.05 (0.92–1.20)	0.46	1.02 (0.88–1.17)	0.81	1.08 (0.91–1.27)	0.39
Choline	1.06 (0.93–1.22)	0.37	1.01 (0.87–1.16)	0.94	0.89 (0.75–1.06)	0.19
Betaine	0.78 (0.69–0.89)	<0.001	0.72 (0.62–0.83)	<0.00001	0.74 (0.63–0.88)	<0.001
Dimethylglycine	0.99 (0.86–1.13)	0.83	0.94 (0.82–1.09)	0.42	0.92 (0.77–1.09)	0.33
Sarcosine	0.97 (0.84–1.11)	0.65	0.93 (0.81–1.08)	0.34	1.07 (0.91–1.26)	0.43
Urine[c]						
Choline	1.09 (0.96–1.25)	0.20	1.07 (0.92–1.23)	0.39	1.09 (0.93–1.29)	0.30
Betaine	1.27 (1.11–1.45)	0.001	1.25 (1.09–1.43)	0.001	1.23 (1.06–1.43)	0.006
Dimethylglycine	1.23 (1.07–1.42)	0.003	1.22 (1.06–1.40)	0.007	1.19 (1.01–1.39)	0.03
Sarcosine	1.31 (1.14–1.50)	<0.001	1.30 (1.13–1.49)	<0.001	1.25 (1.07–1.46)	0.004

[a] Data are OR (95% CI) per 1 SD of logarithmically transformed variable.
[b] Includes age, sex, fasting status, BMI, Hb A_{1c}, eGFR, CRP, HDL-C, urine albumin-to-creatinine ratio, and the use of loop diuretics, thiazides, β-blockers, statins, ACE inhibitors, and angiotensin receptor blockers.
[c] Corrected for urine creatinine.

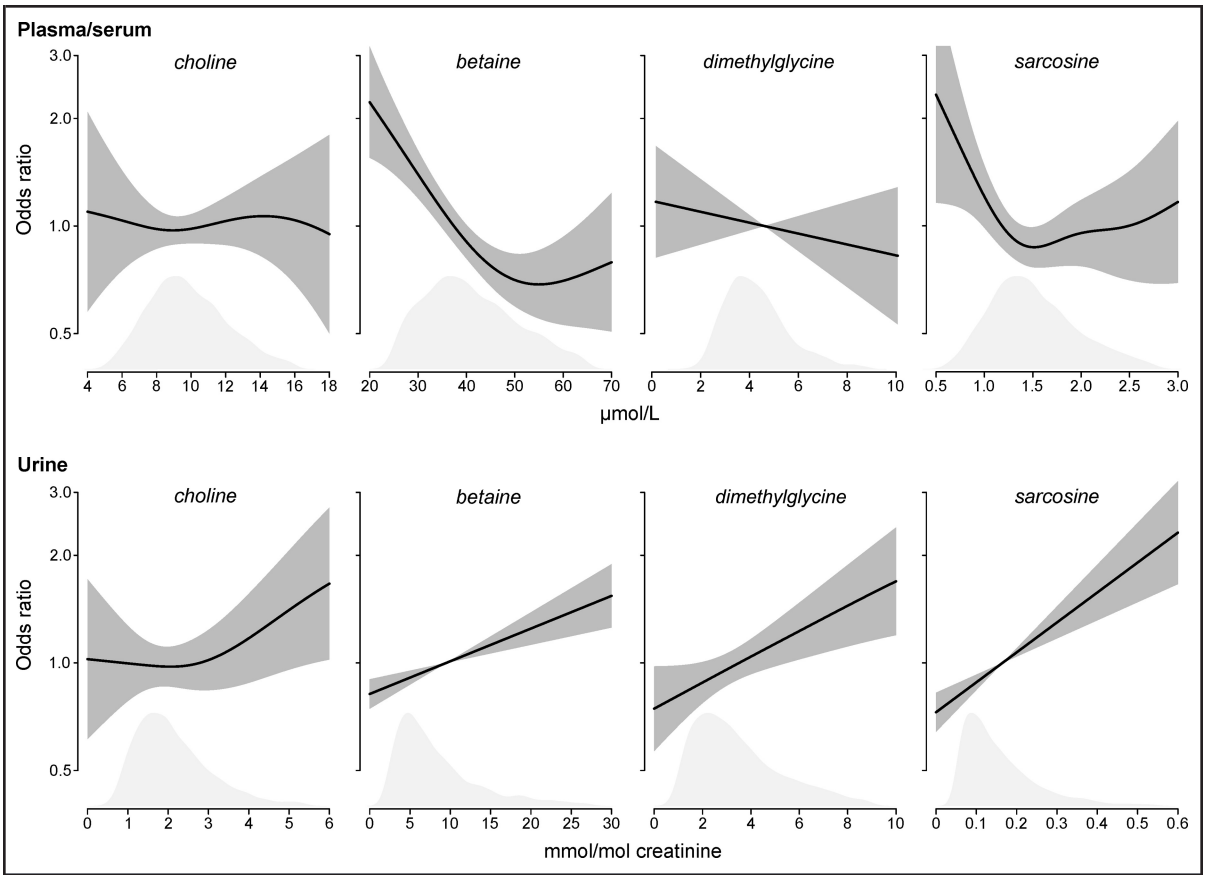

Fig. 2. Relationships between systemic and urinary choline-related metabolites and incident type 2 diabetes.
Solid lines depict the smoothed spline of the generalized additive logistic regressions model, adjusted for age, sex, and fasting status. The shaded areas depict 95% CIs. Density plots are aligned along the *x* axes.

When stepwise backwards elimination was used for the multivariate model also including plasma betaine as well as urine betaine, dimethylglycine, and sarcosine, only plasma betaine and urine sarcosine remained of the choline metabolites in the final model.

MODEL DISCRIMINATION AND RECLASSIFICATION

As shown in Table 4, adding plasma betaine or urine sarcosine to the multivariate logistic regression model improved the reclassification of patients at risk, as well as the integrated discrimination index, although the increments in *c*-statistics did not reach statistical significance.

TEST-RETEST RELIABILITY OF PLASMA BETAINE AND URINE SARCOSINE

On the basis of 560 paired measurements among patients allocated to placebo treatment, the ICC was 0.62 (95% CI,

Table 4. Model discrimination and reclassification.[a]

	c-statistic (95% CI)	*P*	NRI >0 (95% CI)	*P*	IDI (95% CI)	*P*
Basic model[a]	0.750 (0.714–0.786)		–	–	–	–
+ plasma betaine	0.751 (0.715–0.787)	0.79	0.33 (0.19–0.47)	<0.000001	0.0084 (0.0038–0.0130)	<0.001
+ urine sarcosine	0.757 (0.721–0.793)	0.19	0.16 (0.01–0.31)	0.03	0.0048 (0.0002–0.0094)	0.04

[a] The basic model included age, sex, fasting status, BMI, Hb A_{1c}, eGFR, CRP, HDL-C, urine albumin-to-creatinine ratio, and the use of loop diuretics, thiazides, β-blockers, statins, ACE inhibitors, and angiotensin receptor blockers.

0.56–0.66) for plasma betaine and 0.69 (95% CI, 0.64–0.74) for urine sarcosine. Similar results were obtained among patients receiving vitamin B_6 alone, whereas lower ICCs were found among those allocated to folic acid + vitamin B_{12} (see online Supplementary Table 5).

Discussion

In the current prospective cohort study, we found that lower plasma concentrations of betaine and higher urinary concentrations of several metabolites downstream in the choline oxidation pathway were associated with incident T2D among patients evaluated for coronary heart disease and followed for an average of >7 years. The relationships were robust in sensitivity analyses and when adjusting for several traditional diabetes risk factors and potential confounders. Moreover, plasma betaine and urine sarcosine improved risk prediction of incident T2D and showed good within-person reproducibility.

Patients with T2D had slightly higher baseline plasma choline than nondiabetic patients, a finding supported by positive associations between plasma choline and components of the metabolic syndrome in a Norwegian general population sample *(23)*. Increased plasma TMAO among patients with T2D is consistent with findings from a small cross-sectional study among patients with heart failure *(9)*. However, the prospective associations between choline, TMAO, and diabetes are somewhat inconsistent, as lower plasma TMAO and higher urine choline have been associated with development of gestational diabetes *(24)*, whereas we found no associations between either choline or TMAO and incident T2D in the current investigation.

The present study also shows for the first time that low plasma betaine concentrations strongly predicted new-onset T2D, and that patients who later developed T2D had baseline plasma betaine concentrations comparable to those with existing disease. As opposed to the findings from a recent Swedish Mendelian randomization study *(7)*, we did not observe significant associations between plasma dimethylglycine and incident T2D. Also, there was no overall relationship between serum sarcosine and incident T2D; however, lower serum sarcosine was observed among patients with established T2D both in the current study and in a survey among US men *(25)*.

Higher urine betaine concentrations have previously been observed among patients with T2D or the metabolic syndrome *(10, 11)*, and increased urinary betaine and sarcosine concentrations were found among patients with T2D vs patients with maturity-onset diabetes of the young *(26)*, indicating a role of IR. Accordingly, the current study showed that the highly correlated urine betaine, dimethylglycine, and sarcosine concentrations were all positively associated with HOMA2-IR and incident T2D, and that urine sarcosine was the urinary choline metabolite most strongly associated with new-onset disease.

Betaine is obtained either directly from the diet or via the irreversible conversion of choline inside the mitochondrion. In the present study, the dietary intake of neither choline nor betaine differed according to established or incident T2D, and adjusting for intake did not influence the risk estimates. It is therefore likely that the current associations between downstream choline metabolites and new-onset T2D reflect metabolic traits rather than dietary habits.

In line with a study in the general population *(23)*, plasma betaine and choline demonstrated divergent associations with several indices of IR. This suggests impaired oxidation of choline to betaine in patients at higher risk of developing T2D, potentially linking the current findings with mitochondrial dysfunction, a hallmark of IR *(27)*. Low plasma betaine concentrations may also reflect altered flux over the cytosolic enzyme betaine-homocysteine *S*-methyl transferase (BHMT). BHMT is abundant in the liver and the kidneys *(28)* and catalyzes the betaine-dependent remethylation of homocysteine, forming methionine and dimethylglycine. The hepatic BHMT pathway has wide metabolic ramifications, including methylation status and lipid handling *(29)*, as well as insulin and energy homeostasis *(30)*. A potential interplay between betaine status, BHMT activity and transcription, and expression of the nuclear transcription factor peroxisome proliferator-activated receptor α might also affect pancreatic β-cells *(31–33)*. Altered BHMT flux in the kidneys—which, in addition to the liver, harbor most of the betaine in the body *(10)*—may partly explain higher urine dimethylglycine and sarcosine concentrations among patients at higher risk of T2D. Treatment with folic acid + vitamin B_{12} seemed to increase betaine and decrease dimethylglycine and sarcosine in both plasma/serum and urine during follow-up. This indicates reduced flux over BHMT, although folic acid may also lower sarcosine production via reduced remethylation of glycine by glycine-*N*-methyltransferase in the cell cytosol *(34)*. However, adjusting for study treatment did not alter the associations between choline metabolites and incident T2D among WENBIT participants, nor did a recent Mendelian randomization study find any association between BHMT polymorphisms and T2D risk *(35)*. Our observational data on steady-state concentrations does not allow conclusions regarding flux through complex metabolic pathways. Nevertheless, these results point to a direction of causality in which IR or the diabetic states affect the BHMT reaction, rather than the other way around.

Possible mechanisms behind increased urinary betaine, dimethylglycine, and sarcosine include increased renal choline uptake, metabolism, and excretion. Betaine is freely filtered in the glomeruli but has a low fractional renal clearance, probably because of extensive reuptake in the proximal tubular system *(36)*. Sarcosine undergoes similar renal handling *(37)*, whereas the renal processing of dimethylglycine

has not been described. However, all 3 metabolites were strongly correlated in the urine, suggesting common handling by the kidneys. Individuals at increased risk of diabetes, but without overt renal insufficiency, have signs of renal tubular dysfunction *(38)*. Thus, it is plausible that impaired tubular reuptake may have influenced the positive associations between incident T2D and urinary concentrations of betaine, dimethylglycine, and sarcosine in our population, who had overall eGFRs within reference intervals and no signs of albuminuria at baseline.

Adding either plasma betaine or urinary sarcosine to other established predictors of incident T2D improved model performance and risk prediction. Among patients randomized to placebo or treatment with vitamin B_6 alone, these metabolites also showed good within-person reproducibility. For plasma betaine, this confirms recent results obtained from data in the Nurses' Health Study *(39)*, and high reproducibility is imperative when considering novel clinical biomarkers, as biomarker status can be obtained from a single measurement.

The strengths of this study are the size of the well-characterized cohort, including data on all choline metabolites in both blood and urine, as well as the long follow-up time. In addition, the results were robust in multivariate models and sensitivity analyses; however, residual confounding is an inherent limitation of observational data. Our results also need to be validated in populations with different characteristics, especially patients who are younger and without coronary heart disease, the latter condition being connected to IR *(40)*. The proportion of patients who developed T2D was comparable to similar cohorts *(41)*; however, since the diagnosis of T2D partly relied on hospital discharge reports, we cannot rule out misclassification of cases. Nevertheless, the relationships between choline metabolites and incident T2D were similar when only including WENBIT patients with end points occurring during close in-trial follow-up, thereby strengthening the reliability of our findings.

Baseline plasma glucose and Hb A_{1c} were high among 1108 patients with possible, yet not verified, T2D at baseline. If not diabetic, these patients were probably prediabetic, with a high risk of subsequently developing T2D *(42)*. On the other hand, the reproducibility of prediabetes has been questioned *(43)*, and only approximately 1 of 10 patients with possible T2D was reported with incident disease during follow-up in the current study. This suggests that it may not be appropriate to classify all patients with possible T2D as having T2D at baseline. Although no significant interaction was observed when excluding these patients, most risk estimates became statistically nonsignificant, probably owing to loss of statistical power. The concentrations of choline metabolites were similar when comparing those without T2D and those with possible T2D. In addition, the majority of end points (57.5%) were registered among those without any signs of T2D at baseline; hence, including patients with possible T2D in the prospective analyses was unlikely to introduce any serious bias, although some influence by reverse causation cannot be excluded. There is, however, a need for validation of our results among patients who are more rigorously classified according to diabetes status both at baseline and during follow-up.

We applied the widely used NRI > 0 for reclassification analyses, although we do acknowledge the limitations of the method *(44)*. The potential clinical utility for plasma betaine and urine sarcosine should therefore be assessed in future studies by also exploring alternative reclassification measures *(45)*.

In summary, this large-scale, prospective, observational cohort study of patients with suspected stable angina pectoris showed that lower plasma betaine and higher urine betaine, dimethylglycine, and sarcosine predicted incident T2D, and that the relationships were not explained by traditional risk factors or potential confounders. Firm conclusions about pathophysiologic mechanisms cannot be drawn from epidemiological studies alone; however, the current findings may reflect impaired renal tubular uptake, alterations in BHMT flux, and activation of key elements in energy and insulin homeostasis, with potential ramifications also to mitochondrial function and methyl group metabolism. Information on plasma betaine and urine sarcosine status also improved the reclassification of patients at risk of T2D, and high within-person reproducibility for plasma betaine and urine sarcosine allows the assessment of biomarker status by a single measurement.

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