Research Article

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# Serum Trimethylamine N-oxide, Carnitine, Choline, and Betaine in Relation to Colorectal Cancer Risk in the Alpha Tocopherol, Beta Carotene Cancer Prevention Study

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## Abstract

**Background:** Trimethylamine N-oxide (TMAO), a choline-derived metabolite produced by gut microbiota, and its biomarker precursors have not been adequately evaluated in relation to colorectal cancer risk.

**Methods:** We investigated the relationship between serum concentrations of TMAO and its biomarker precursors (choline, carnitine, and betaine) and incident colorectal cancer risk in a nested case–control study of male smokers in the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study. We measured biomarker concentrations in baseline fasting serum samples from 644 incident colorectal cancer cases and 644 controls using LC/MS-MS. Logistic regression models estimated the ORs and 95% confidence interval (CI) for colorectal cancer by quartile (Q) of serum TMAO, choline, carnitine, and betaine concentrations.

**Results:** Men with higher serum choline at ATBC baseline had approximately 3-fold greater risk of developing colorectal cancer over the ensuing (median ± IQR) 14 ± 10 years (in fully adjusted models, Q4 vs. Q1, OR, 3.22; 95% CI, 2.24–4.61; $P_{trend} < 0.0001$). The prognostic value of serum choline for prediction of incident colorectal cancer was similarly robust for proximal, distal, and rectal colon cancers (all $P < 0.0001$). The association between serum TMAO, carnitine, or betaine and colorectal cancer risk was not statistically significant ($P = 0.25$, 0.71, and 0.61, respectively).

**Conclusions:** Higher serum choline concentration (but not TMAO, carnitine, or betaine) was associated with increased risk of colorectal cancer.

**Impact:** Serum choline levels showed strong prognostic value for prediction of incident colorectal cancer risk across all anatomical subsites, suggesting a role of altered choline metabolism in colorectal cancer pathogenesis. *Cancer Epidemiol Biomarkers Prev; 26(6); 945–52. ©2017 AACR.*

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## Introduction

Recent studies provide convincing evidence that individuals with higher serum trimethylamine N-oxide (TMAO) have greater risk of several detrimental outcomes, including atherosclerosis, cardiovascular disease (CVD), and adverse thrombotic events (1–4). TMAO is a metabolite formed by host hepatic metabolism of intestinal bacteria–derived trimethylamine (TMA), which is in turn derived from several nutrients that can be obtained through the diet—choline, carnitine, or (to a lesser extent) betaine (1, 2, 5, 6). Despite the potential relevance of TMAO to the gut, there is limited evidence evaluating the association between TMAO and its biomarker precursors in relation to the risk of colorectal cancer, the third leading cause of cancer-related deaths in the United States (7).

Only one study, to the best of our knowledge, has investigated the association between baseline circulating TMAO concentrations and incident risk of colorectal cancer (8). In 835 matched case–control female pairs from the Women's Health Initiative (WHI) Observational Study, higher plasma TMAO concentration was associated with a 3-fold greater risk of rectal cancer (8). Moreover, in that study, plasma choline concentration was positively associated with rectal cancer risk, whereas plasma betaine concentration was inversely associated with colorectal cancer (8). The association between TMAO and colorectal cancer risk among men has not yet been examined. Further epidemiologic evidence is needed to gain a better understanding of the relationship between serum TMAO, its precursor biomarkers, and incident colorectal cancer risks, particularly among men.

Several mechanistic links between TMAO, its biomarker precursors, and colorectal cancer risk are plausible. One potential link

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**Note:** Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

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between TMAO and colorectal cancer risk is its involvement in inflammatory pathway upregulation (9). Choline and betaine may be involved in carcinogenesis through their roles as methyl donors in one-carbon metabolism (10, 11). There are also several lines of evidence linking diet in general and choline specifically, to both colorectal cancer and TMAO. Red and processed meats are a shared risk factor for both cardiovascular disease (CVD; refs. 12, 13) and colorectal cancer (7, 14–19) and these foods are also dietary sources of TMAO precursors (carnitine and choline); in humans, higher meat intake is associated with higher circulating and urinary TMAO levels (2, 20).

Collectively, these studies suggest the need for further examination of TMAO and related metabolites in relation to colorectal cancer risk. Herein we investigated the association between serum levels of TMAO and its nutrient precursors, choline, carnitine, and betaine, and prospective colorectal cancer risk in a nested case–control study of men.

## Materials and Methods

### Study population

We conducted a nested case–control study within the Alpha Tocopherol, Beta Carotene Cancer Prevention (ATBC) Study, described in detail elsewhere (21). Briefly, the ATBC Study was a large randomized, double-blind, placebo-controlled, primary prevention trial of vitamin E (50 mg/day DL-α-tocopheryl acetate) and beta-carotene (20 mg/day β-carotene) among 29,133 male Finnish smokers ages 50–69 years at baseline; the primary endpoint in the ATBC Study was lung cancer occurrence. ATBC excluded men with a prior cancer or serious illness and men who reported current use of high levels of vitamin E, A, or beta-carotene. Study supplementation occurred from enrollment (1985–1988) until death or the end of the trial (April 30, 1993) and follow-up is continuing through the Finnish Cancer Registry and the Register of Causes of Death (22). Our study includes follow-up through December 31, 2011. The ATBC Study obtained written informed consent from all participants and was approved by the institutional review boards at the US National Cancer Institute and the Finnish National Public Health Institute.

***Selection of cases and controls.*** We included all identified colorectal cancer cases ($n = 644$; International Classification of Diseases 9, codes 153–154) and an equivalent number of controls. Incident colorectal cancer cases in ATBC were identified by the Finnish Cancer Registry (22), which provides nearly complete ascertainment of cases. Outcomes of interest included total and site-specific colorectal cancers (proximal colon ICD-9 153.1, 153.4–153.6; distal colon ICD-9 153.2, 153.3, 153.7; rectum ICD-9 154.0–154.1).

ATBC participants were eligible for this nested case–control study if they had an available baseline serum specimen of adequate volume with $\leq$1 prior freeze–thaw cycle and no prior rare cancer (whose specimens were reserved for other studies). A total of 20,846 participants met these criteria, including 644 cases and 20,199 potential controls. Incidence density matching was used to select one control alive and free of colorectal cancer from each case's risk set without replacement. Within the risk sets of cases, controls were matched 1:1 on age at randomization (±5 years) and within the pool of eligible controls, we selected the specimen that minimized the difference in thaw count and serum draw date between the chosen control and the case. Two colorectal cancer cases were diagnosed with cancer at both the proximal colon and rectum and thus are counted once in the overall analyses for colorectal cancer but also contribute to each of the site-specific case numbers.

### Laboratory analysis

ATBC collected overnight fasting serum samples at the pre-randomization baseline study visit. Samples were stored at −70°C and the median time from blood collection until colorectal cancer diagnosis was 14 years (range 1 month–26 years). Biospecimens were shipped overnight on dry ice to the Cleveland Clinic laboratory that measured serum TMAO, choline, carnitine, and betaine concentrations. Metabolites were analyzed by stable isotope dilution LC/MS-MS using established methods (1, 2) on a Shimadzu LCMS-8050 CL Triple Quadrupole Liquid Chromatograph Mass Spectrometer with Nexera LC-30AD CL UHPLC interface. Investigators performing LC/MS-MS were blinded to sample identity (other than barcode label) and to case–control status. Specimens were divided into 27 batches and case–control pairs were included in the same batch. Blinded quality control specimens were randomly inserted into each batch; these samples comprised approximately 10% of all specimens assayed and assay values from these specimens were used to calculate coefficients of variation. The average inter-batch coefficients of variation for the blind duplicate control specimens across all analyses were between 3%–5% as follows: carnitine 3%, choline 4%, TMAO 5%, and betaine 5%.

### Covariate assessment

At baseline, ATBC administered a questionnaire that collected data on demographics, medical history, physical activity and smoking, and height and weight were measured. Total energy intake was estimated by a 276-item food frequency questionnaire (FFQ) that participants completed at baseline; usual intake of specific foods in grams per day (g/day) over the past 12 months was calculated by linkage to a food-composition database of the National Public Health Institute in Finland.

### Statistical analysis

We compared baseline characteristics of colorectal cancer cases and controls using *t* tests. We used Spearman correlations to describe the association between the serum biomarkers (TMAO, choline, carnitine, betaine). We used unconditional logistic regression models to estimate the ORs and 95% confidence interval (CI) for colorectal cancer for each quartile of the serum biomarkers (based on the distribution of controls). *P* values for trend (denoted as $P_{trend}$) were calculated by testing whether the regression coefficient for a continuous exposure, which was defined as the median value within each quartile, differed from zero. All models were adjusted for batch (categorical) and age (continuous). The fully adjusted model included age, batch, years smoked, cigarettes per day, education, body mass index (BMI), physical activity, and total energy intake. We also evaluated models adjusted for alcohol consumption and aspirin use and considered models excluding cases that occurred within the first two years and, separately, the first 5 years of follow-up. We explored potential interactions between the biomarker concentration quartile and years smoked, number of cigarettes per day, quartile of alcohol intake and BMI (<median, ≥median). In supplemental analyses, we investigated whether there were

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differences in serum biomarker concentrations by cancer stage at diagnosis or by the ATBC Study randomization arm. Statistical significance was defined as $P < 0.05$ and tests of significance were two-sided. Analyses were conducted in SAS 9.3 (SAS Institute).

## Results

At baseline, the mean age of study participants was 57 years and most (>80%) men were married (Table 1). On average, these smokers initiated smoking at age 19, smoked about one pack of cigarettes per day and had regularly smoked for 36 years. Most baseline characteristics, including demographics, smoking and dietary intake, were comparable between incident colorectal cancer cases and controls. There was a small difference between cases and controls in body weight [mean 80.4 vs. 78.9 kg among cases and controls ($P = 0.03$), respectively] but the difference in BMI was not statistically significant ($P = 0.06$). Nominal but nonstatistically significant differences between cases and controls were observed for aspirin use ($P = 0.09$) and alcohol consumption ($P = 0.05$); there was no difference in reported intake of folate ($P = 0.94$). Comparing self-reported dietary intake of choline- and carnitine-containing foods between cases and controls (Supplementary Table S1), there were no significant differences for red meat ($P = 0.79$), processed meat ($P = 0.30$), fish ($P = 0.07$), or eggs ($P = 0.13$).

Serum TMAO, choline, carnitine, and betaine concentrations were moderately intercorrelated (Supplementary Table S2). Among controls, the Spearman correlation coefficients ($P$ value) for these biomarkers were as follows: TMAO versus carnitine 0.22 ($P < 0.0001$), TMAO versus choline 0.23 ($P < 0.001$), carnitine versus choline 0.36 ($P < 0.0001$), and betaine versus choline 0.40 ($P < 0.0001$). The magnitude and significance of these associations were similar among cases.

In this study, no statistically significant association was observed between serum levels of TMAO and risk of total or site-specific colorectal cancer (Table 2). In the fully adjusted model, the estimated risk of colorectal cancer in the highest quartile of serum TMAO was not significantly different ($P = 0.25$) compared with the lowest quartile (OR 1.20; 95% CI, 0.86–1.68). In investigations by anatomical subsite, we observed similarly elevated (but nonsignificant) point estimates among those in the highest quartile of serum TMAO for cancer of the proximal colon; there was no association between TMAO and rectal cancer. OR estimates from models that were further adjusted for alcohol intake and aspirin use were largely unchanged (Supplementary Table S3).

**Table 1.** Baseline characteristics of colorectal cancer cases and controls in a nested case–control study within the ATBC Study

| | Colorectal cancer cases ($N = 644$) | | Controls ($N = 644$) | | |
|---|---|---|---|---|---|
| | ***N*** | **Value** | ***N*** | **Value** | ***P*** |
| Age at baseline (years) | 644 | 57 (5) | 644 | 57 (5) | 0.39 |
| Married, No. (% yes) | 644 | 530 (82) | 644 | 539 (84) | 0.46 |
| Education, No. (% yes) | 644 | | 644 | | 0.19 |
| <HS | | 186 (29) | | 216 (34) | |
| Some college or technical school | | 421 (65) | | 396 (61) | |
| College graduate | | 37 (6) | | 32 (5) | |
| Height (cm) | 643 | 174.0 (6.0) | 644 | 173.7 (6.1) | 0.39 |
| Weight (kg) | 643 | 80.4 (12.6) | 644 | 78.9 (11.4) | **0.03** |
| Body mass index (kg/m$^2$) | 643 | 26.5 (3.8) | 644 | 26.1 (3.4) | 0.06 |
| Heavy physical activity, No. (% yes) | | | | | |
| Leisure time | 644 | 39 (6) | 643 | 45 (7) | 0.49 |
| Occupational | 644 | 51 (8) | 644 | 64 (10) | 0.21 |
| Physical activity ≥3 times/week[a], No. (% yes) | 644 | 118 (18) | 644 | 120 (19) | 0.89 |
| Family history of colorectal cancer, No. (% yes) | 436 | 25 (6) | 485 | 22 (5) | 0.41 |
| Cigarettes per day | 644 | 19.4 (8.4) | 644 | 20 (9) | 0.93 |
| Years smoked | 644 | 35.9 (8.3) | 644 | 35.9 (7.9) | 0.95 |
| Pack-years | 644 | 35.2 (17.5) | 644 | 35.4 (18.0) | 0.90 |
| Age at smoking initiation | 644 | 19.6 (4.9) | 644 | 19 (4) | 0.11 |
| Aspirin use, No. (% yes) | 489 | 70 (14) | 536 | 98 (18) | 0.09 |
| Supplement use, No. (% yes) | 643 | 126 (20) | 643 | 125 (19) | 0.94 |
| Calcium | 637 | 64 (10) | 638 | 70 (11) | 0.59 |
| Vitamin D | 637 | 39 (6) | 638 | 52 (8) | 0.16 |
| Dietary Intake (per day) | | | | | |
| Energy (kcal) | 614 | 2691.5 (708.6) | 616 | 2668.3 (708.2) | 0.57 |
| Folate including supplements (μg) | 614 | 339.8 (98.7) | 616 | 340.2 (102.2) | 0.94 |
| Alcohol (g) | 614 | 18.8 (20.9) | 616 | 16.5 (20.3) | 0.05 |
| Serum biomarkers (μmol/L) | | | | | |
| Trimethylamine N-oxide (TMAO) | 644 | 4.8 (3.9) | 644 | 4.7 (3.9) | 0.51 |
| Median (25th–75th percentile) | | 3.73 (2.61–5.46) | | 3.6 (2.5–5.2) | |
| Choline | 644 | 15.2 (15.2) | 644 | 10.4 (9.9) | **<0.01**[b] |
| Median (25th–75th percentile) | | 10.24 (7.90–15.68) | | 8.7 (7.0–10.5) | |
| Carnitine | 644 | 34.2 (7.4) | 644 | 33.9 (7.2) | 0.40 |
| Median (25th–75th percentile) | | 33.79 (29.91–38.23) | | 33.5 (29.8–38.3) | |
| Betaine | 644 | 33.08 (13.72) | 644 | 32.4 (11.2) | 0.33 |
| Median (25th–75th percentile) | | 30.72 (25.05–38.54) | | 30.6 (24.8–37.6) | |

NOTE: Two participants had diagnoses of both colon and rectal cancers on the same date and only contribute once to these data. Values are mean (SD) unless otherwise noted. *P* values represent a *t* test for difference in unadjusted means. Bold *P*-values denote $P < 0.05$.

[a]Leisure-time physical activity.

[b]$P < 0.0001$.

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**Table 2.** ORs (95% CIs) of colorectal cancer ranked by quartile of serum TMAO

| | | OR (95% CI) by quartile of serum TMAO (μmol/L) | | | | |
|---|---|---|---|---|---|---|
| | ***N*** | **1 (≤2.5)** | **2 (>2.5–3.6)** | **3 (>3.6–5.4)** | **4 (>5.4)** | $P_{trend}$ |
| Colorectal cancer | | | | | | |
| No. cases/No. controls | 644/644 | 154/167 | 159/166 | 163/156 | 168/155 | |
| Age and batch adjusted | 644/644 | 1.00 (Ref.) | 1.04 (0.76–1.42) | 1.14 (0.83–1.57) | 1.20 (0.86–1.68) | 0.26 |
| Fully adjusted[a] | 642/643 | 1.00 (Ref.) | 1.04 (0.76–1.42) | 1.15 (0.83–1.58) | 1.20 (0.86–1.68) | 0.25 |
| Cancer of the proximal colon | | | | | | |
| No. cases/No. controls | 169/169 | 43/53 | 38/47 | 51/37 | 37/32 | |
| Age and batch adjusted | 169/169 | 1.00 (Ref.) | 0.99 (0.54–1.84) | 1.83 (0.99–3.41) | 1.60 (0.80–3.22) | 0.12 |
| Fully adjusted[a] | 169/169 | 1.00 (Ref.) | 1.00 (0.54–1.86) | 1.84 (0.99–3.43) | 1.60 (0.80–3.22) | 0.12 |
| Cancer of the distal colon | | | | | | |
| No. cases/No. controls | 153/153 | 30/35 | 50/36 | 34/45 | 39/37 | |
| Age and batch adjusted | 153/153 | 1.00 (Ref.) | 1.63 (0.82–3.24) | 0.85 (0.41–1.78) | 1.22 (0.58–2.56) | 0.95 |
| Fully adjusted[a] | 152/153 | 1.00 (Ref.) | 1.73 (0.86–3.46) | 0.81 (0.38–1.70) | 1.21 (0.57–2.56) | 0.89 |
| Rectal cancer | | | | | | |
| No. cases/No. controls | 282/282 | 76/68 | 62/71 | 63/67 | 81/76 | |
| Age and batch adjusted | 282/282 | 1.00 (Ref.) | 0.77 (0.48–1.25) | 0.83 (0.50–1.36) | 0.97 (0.59–1.60) | 0.87 |
| Fully adjusted[a] | 281/281 | 1.00 (Ref.) | 0.76 (0.47–1.24) | 0.83 (0.50–1.36) | 0.94 (0.57–1.57) | 0.92 |

NOTE: Logistic regression models adjusted for batch (categorical, 27 batches) and age (continuous). Quartiles of serum TMAO were based on the distribution among controls. Medians for each quartile (based on controls) were used in trend test; medians for TMAO quartiles 1 through 4 were 2.0, 3.1, 4.4, and 7.7, respectively. Two participants had cancer of the proximal colon and rectum and are counted in both disease-stratified analyses.

[a]Model additionally adjusted for the number of years smoked regularly (continuous), number of cigarettes smoked per day (continuous), education (college graduate, some college or technical school, versus ≤ high school), body mass index (continuous, kg/m$^2$), heavy physical activity in leisure (yes/no), heavy occupational physical activity (yes/no), physical activity frequency (≥3 times per week, versus <3), total energy intake (continuous, kcal/day).

In addition to serum TMAO concentrations, we also examined serum metabolites that are precursors of TMAO—namely, choline, carnitine, and betaine. Serum choline was strongly and statistically significantly associated with incident colorectal cancer risk (Table 3); participants with higher serum choline had greater risk of developing colorectal cancer over the ensuing follow-up period ($P_{trend} < 0.0001$). Compared with those in the lowest quartile of choline, the ORs (95% CIs) for colorectal cancer development in the fully adjusted model among increasing choline quartiles 2–4 were 1.05 (0.73–1.51), 1.26 (0.86–1.84), and 3.38 (2.37–4.80). The direction and significance of the association between serum choline levels and colorectal cancer risk was consistent for cancers of the proximal colon, distal colon, and rectum. Furthermore, the risk associated with choline persisted after eliminating cases that occurred early during follow-up (first 2 years, and separately, first 5 years; Supplementary Table S4). Further adjustment for alcohol intake and aspirin use did not appreciably alter estimates (Supplementary Table S3). In fully adjusted models estimating colorectal cancer risk, there were no significant interactions between serum choline and years smoked ($P = 0.47$), number of cigarettes smoked per day ($P = 0.74$), quartile of alcohol intake ($P = 0.97$), or BMI ($P = 0.54$).

There was no significant association between serum carnitine and risk of total or site-specific colorectal cancer (Table 4).

**Table 3.** ORs (95% CIs) of colorectal cancer ranked by quartile of serum choline

| | | OR (95% CI) by quartile of serum choline (μmol/L) | | | | |
|---|---|---|---|---|---|---|
| | ***N*** | **1 (≤7.0)** | **2 (>7.0–8.7)** | **3 (>8.7–10.5)** | **4 (>10.5)** | $P_{trend}$ |
| Colorectal cancer | | | | | | |
| No. cases/No. controls | 644/644 | 113/161 | 108/161 | 116/160 | 307/162 | |
| Age and batch adjusted | 644/644 | 1.00 (Ref.) | 1.04 (0.73–1.500) | 1.25 (0.86–1.83) | 3.37 (2.37–4.79) | **<0.01**[a] |
| Fully adjusted[b] | 642/643 | 1.00 (Ref.) | 1.05 (0.73–1.51) | 1.26 (0.86–1.84) | 3.38 (2.37–4.80) | **<0.01**[a] |
| Cancer of the proximal colon | | | | | | |
| No. cases/No. controls | 169/169 | 33/44 | 28/39 | 31/46 | 77/40 | |
| Age and batch adjusted | 169/169 | 1.00 (Ref.) | 1.02 (0.50–2.06) | 1.10 (0.52–2.31) | 3.35 (1.66–6.76) | **<0.01**[a] |
| Fully adjusted[b] | 169/169 | 1.00 (Ref.) | 1.02 (0.50–02.06) | 1.08 (0.51–2.29) | 3.37 (1.67–6.81) | **<0.01**[a] |
| Cancer of the distal colon | | | | | | |
| No. cases/No. controls | 153/153 | 24/39 | 22/40 | 30/31 | 77/43 | |
| Age and batch adjusted | 153/153 | 1.00 (Ref.) | 1.01 (0.46–2.22) | 2.36 (1.02–5.51) | 4.24 (1.98–9.05) | **<0.01**[a] |
| Fully adjusted[b] | 152/153 | 1.00 (Ref.) | 0.99 (0.45–2.18) | 2.27 (0.97–5.31) | 4.07 (1.89–8.75) | **<0.01**[a] |
| Rectal cancer | | | | | | |
| No. cases/No. controls | 282/282 | 76/68 | 62/71 | 63/67 | 81/76 | |
| Age and batch adjusted | 282/282 | 1.00 (Ref.) | 1.19 (0.68–2.07) | 1.09 (0.61–1.95) | 4.06 (2.34–7.04) | **<0.01**[a] |
| Fully adjusted[b] | 281/281 | 1.00 (Ref.) | 1.22 (0.70–2.14) | 1.09 (0.61–1.96) | 4.09 (2.35–7.12) | **<0.01**[a] |

NOTE: Logistic regression models adjusted for batch (categorical, 27 batches) and age (continuous). Quartiles of serum choline were based on the distribution among controls. Medians for each quartile (based on controls) were used in trend test; medians for choline quartiles 1 through 4 were 6.0, 7.8, 9.4, and 12.9, respectively. Bold *P*-values denote $P<0.05$

[a]$P < 0.0001$.

[b]Model additionally adjusted for the number of years smoked regularly (continuous), number of cigarettes smoked per day (continuous), education (college graduate, some college or technical school, versus ≤ high school), body mass index (continuous, kg/m$^2$), heavy physical activity in leisure (yes/no), heavy occupational physical activity (yes/no), physical activity frequency (≥3 times per week, versus <3), total energy intake (continuous, kcal/day).

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**Table 4.** ORs (95% CIs) of colorectal cancer ranked by quartile of serum carnitine and betaine

| | | OR (95% CI) by quartile of serum biomarker (μmol/L) | | | | |
|---|---|---|---|---|---|---|
| **Carnitine** | ***N*** | **1 (≤29.8)** | **2 (>29.8–33.5)** | **3 (>33.5–38.3)** | **4 (>38.3)** | $P_{trend}$ |
| Colorectal cancer | | | | | | |
| No. cases/No. controls | 644/644 | 157/161 | 151/161 | 178/161 | 158/161 | |
| Age and batch adjusted | 644/644 | 1.00 (Ref.) | 0.97 (0.70–1.35) | 1.15 (0.83–1.59) | 1.02 (0.73–1.43) | 0.74 |
| Fully adjusted[a] | 642/643 | 1.00 (Ref.) | 0.97 (0.70–1.35) | 1.15 (0.83–1.59) | 1.03 (0.73–1.44) | 0.71 |
| Cancer of the proximal colon | | | | | | |
| No. cases/No. controls | 169/169 | 38/50 | 42/46 | 50/34 | 39/39 | |
| Age and batch adjusted | 169/169 | 1.00 (Ref.) | 1.34 (0.70–2.59) | 2.22 (1.13–4.36) | 1.44 (0.72–2.87) | 0.16 |
| Fully adjusted[a] | 169/169 | 1.00 (Ref.) | 1.33 (0.69–2.58) | 2.21 (1.12–4.34) | 1.42 (0.71–2.84) | 0.17 |
| Cancer of the distal colon | | | | | | |
| No. cases/No. controls | 153/153 | 42/36 | 25/42 | 46/41 | 40/34 | |
| Age and batch adjusted | 153/153 | 1.00 (Ref.) | 0.49 (0.24–1.00) | 0.96 (0.49–1.89) | 1.05 (0.50–2.20) | 0.60 |
| Fully adjusted[a] | 152/153 | 1.00 (Ref.) | 0.49 (0.24–1.02) | 0.97 (0.49–1.90) | 1.06 (0.51–2.22) | 0.60 |
| Cancer of the rectum | | | | | | |
| No. cases/No. controls | 282/282 | 69/66 | 73/62 | 70/77 | 70/77 | |
| Age and batch adjusted | 282/282 | 1.00 (Ref.) | 1.12 (0.67–1.85) | 0.85 (0.51–1.41) | 0.84 (0.51–1.40) | 0.37 |
| Fully adjusted[a] | 281/281 | 1.00 (Ref.) | 1.15 (0.69–1.91) | 0.84 (0.51–1.41) | 0.85 (0.51–1.41) | 0.37 |
| **Betaine** | ***N*** | **1 (≤24.75)** | **2 (>24.75–30.62)** | **3 (>30.62–37.56)** | **4 (>37.56)** | $P_{trend}$ |
| Colorectal cancer | | | | | | |
| No. cases/No. controls | 644/644 | 152/161 | 169/161 | 154/161 | 169/161 | |
| Age and batch adjusted | 644/644 | 1.00 (Ref.) | 1.12 (0.82–1.54) | 1.02 (0.74–1.41) | 1.13 (0.82–1.56) | 0.58 |
| Fully adjusted[a] | 642/643 | 1.00 (Ref.) | 1.12 (0.82–1.53) | 1.02 (0.74–1.40) | 1.12 (0.81–1.55) | 0.61 |
| Cancer of the proximal colon | | | | | | |
| No. cases/No. controls | 169/169 | 40/49 | 41/31 | 43/45 | 45/44 | |
| Age and batch adjusted | 169/169 | 1.00 (Ref.) | 1.68 (0.87–3.23) | 1.20 (0.64–2.25) | 1.33 (0.69–2.53) | 0.62 |
| Fully adjusted[a] | 169/169 | 1.00 (Ref.) | 1.71 (0.88–3.30) | 1.24 (0.66–2.33) | 1.34 (0.70–2.57) | 0.60 |
| Cancer of the distal colon | | | | | | |
| No. cases/No. controls | 153/153 | 38/37 | 41/40 | 34/40 | 40/36 | |
| Age and batch adjusted | 153/153 | 1.00 (Ref.) | 1.00 (0.52–1.92) | 0.81 (0.41–1.58) | 1.11 (0.57–2.18) | 0.83 |
| Fully adjusted[a] | 152/153 | 1.00 (Ref.) | 1.00 (0.52–1.93) | 0.79 (0.40–1.57) | 1.17 (0.59–2.31) | 0.73 |
| Rectal cancer | | | | | | |
| No. cases/No. controls | 282/282 | 62/60 | 81/78 | 68/67 | 71/71 | |
| Age and batch adjusted | 282/282 | 1.00 (Ref.) | 1.12 (0.69–1.82) | 1.09 (0.66–1.81) | 1.08 (0.65–1.80) | 0.86 |
| Fully adjusted[a] | 281/281 | 1.00 (Ref.) | 1.10 (0.68–1.78) | 1.05 (0.63–1.75) | 1.02 (0.61–1.71) | 0.98 |

NOTE: Logistic regression models adjusted for batch (categorical, 27 batches) and age (continuous). Quartiles of serum carnitine were based on the distribution among controls. Medians for each quartile (based on controls) were used in trend test; medians for carnitine quartiles 1 through 4 were 26.4, 31.9, 35.5, and 41.1, respectively; medians for betaine quartiles 1 through 4 were 21.4, 27.9, 33.6, and 44.1, respectively.

[a]Model additionally adjusted for the number of years smoked regularly (continuous), number of cigarettes smoked per day (continuous), education (college graduate, some college or technical school, versus ≤ high school), body mass index (continuous, kg/m$^2$), heavy physical activity in leisure (yes/no), heavy occupational physical activity (yes/no), physical activity frequency (≥3 times per week, versus <3), total energy intake (continuous, kcal/day).

Comparing the highest quartile to the lowest quartile of carnitine, the OR (95% CI) for colorectal cancer was 1.03 (0.73–1.44) in the fully adjusted model ($P_{trend} = 0.71$). The $P_{trend}$ for total colorectal cancer and cancers of the proximal colon, distal colon and rectum were not statistically significant. Similarly, no statistically significant association was observed between serum betaine and colorectal cancer risk (Table 4) in fully adjusted models; comparing the highest quartile to the lowest quartile of betaine the OR (95% CI) for colorectal cancer was 1.12 (0.81–1.55).

Several additional analyses were undertaken in efforts to fully describe the aforementioned biomarker–cancer associations. We investigated whether the biomarker concentrations differed by ATBC intervention arm, as our case–control study population is derived from a large randomized trial and thus some (74%) participants were randomized to one of the three active intervention arms (α-tocopherol supplements, β-carotene supplements, or both) with the remaining 26% in the placebo arm. However, as expected there was no evidence that these serum biomarker concentrations differed by intervention arm; in fully adjusted logistic regression models, for example, ATBC intervention arm was not predicted by serum choline ($P = 0.93$) or, separately, by serum TMAO ($P = 0.10$). We found no evidence of variation in serum biomarker concentrations according to stage of cancer diagnosis; in fully adjusted logistic regression models neither serum choline ($P = 0.65$) nor TMAO ($P = 0.40$) predicted stage of cancer at diagnosis ($P = 0.65$). There was no interaction between BMI and quartile of serum TMAO (type III $P = 0.09$) or choline (type III $P = 0.54$). To facilitate comparisons to other studies, we also present the associations between each biomarker and risk of colon cancer, defined as cancer diagnoses of either the proximal or distal colon, in Supplementary Table S5; risk estimates for colon cancer are similar to overall colorectal cancer findings in that serum choline was positively associated with risk.

## Discussion

We identified a strong association between serum choline [the presumed major dietary source of TMA (23), from which TMAO is derived] and the risk of colorectal cancer, whereby men in the

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highest quartile of serum choline demonstrated a significantly increased 3-fold risk of developing colorectal cancer compared with men in the lowest quartile. This association was consistent across all three examined anatomical subsites of colorectal cancer including cancers of the proximal colon, distal colon, and rectum. In this first prospective study of TMAO and colorectal cancer risk among men, we did not observe a significant association. There was also no association noted between serum levels of either carnitine or betaine, alternative dietary precursors of TMAO, and colorectal cancer development.

To our knowledge, only one prospective study has previously investigated the association between serum TMAO and colorectal cancer risk (8). In contrast to our null TMAO-colorectal cancer findings for total and site-specific colorectal cancer, the WHI observed that women with higher plasma TMAO had an increased risk of rectal cancer and, among women with low plasma B12, greater risk of overall colorectal cancer. While statistically significant, the WHI point estimate for rectal cancer risk in the highest quartile of TMAO had a very wide CI; in addition, despite the positive finding for rectal cancer, TMAO was not significantly associated with risk of overall colorectal cancer or cancers of the proximal or distal colon in the WHI (8). Whether sex explains the different TMAO findings in ATBC and the WHI with respect to colorectal cancer risk is unknown. However, it should be noted that prior epidemiologic studies examining predictors of TMAO did not observe an influence of sex on TMAO concentrations, although females included in these studies have predominantly been of post-menopausal age (1, 24). Several other differences between the ATBC and WHI populations, including differences in the underlying distribution of biomarker concentrations, may have contributed to the divergent findings. Serum choline concentrations (μmol/L), for example, were more variable and slightly higher, on average, among controls in this study (mean 10.4 SD 9.9) compared with controls in the WHI (mean 9.4; SD 2.2) (8). Serum TMAO concentrations, in contrast, were comparable between controls in ATBC and WHI, with median (25th–75th percentile) concentrations of 3.6 (2.5–5.2) and 3.8 (2.6–5.7), respectively (8); it is thus unlikely that the lack of association between TMAO and colorectal cancer in ATBC, in contrast to the positive association reported by WHI, is due to differences in the distribution of serum TMAO concentrations. Although ATBC and WHI utilized different specimen types (serum and plasma, respectively), this is unlikely to explain the divergent TMAO findings given that studies comparing side-by-side plasma versus serum levels of TMAO recovered from subjects at the same time show no differences in TMAO levels from the two matrices (25). Further epidemiologic studies are needed to fully evaluate the association between serum TMAO and colorectal cancer in both sexes.

In addition to TMAO and choline, we investigated serum carnitine and betaine in relation to colorectal cancer risk. As expected, we found a modest direct correlation between serum concentrations of TMAO and carnitine; however, there was no association between serum carnitine concentration and colorectal cancer risk. We did not detect an association between serum betaine and colorectal cancer risk; this is in contrast to two previously reported inverse associations between betaine and both colorectal cancer (8) colorectal adenoma (26). While betaine was reported to have an inverse correlation with colorectal cancer risk in the WHI study (8), we observed no association between betaine concentration and incident colorectal cancer development in this study of men.

A link between choline and colorectal cancer risk has been previously reported (8, 27). The gut microbiota converts dietary choline, typically in the form of phosphatidylcholine, to TMA (1), which is the precursor for TMAO. We observed a strong increased risk of colorectal cancer with higher serum choline. This observation is consistent with the modest positive association detected by the nested case–control study in the WHI (8), although our risk estimates are substantially higher [OR (95% CI); 3.38 (2.37–4.80) compared with 1.22 (0.88–1.70) for colorectal cancer; 4.09 (2.35–7.12) compared with 2.44 (0.93–6.40) for rectal cancer]. In contrast, a nested case–control study within the European Prospective Investigation into Cancer and Nutrition (EPIC) detected an inverse association between serum choline and colorectal cancer risk among women and a null association among men (28). Null associations were also reported for serum choline and colorectal adenoma in a cross-sectional Norwegian study (26). The mixed findings for serum choline and colorectal cancer risk reported by observational epidemiologic studies may be due to a variety of factors including differences in study populations. The ATBC study population was confined exclusively to male smokers. Whether tobacco use impacts the relationship between choline and colorectal cancer risk is unknown. However, sex may contribute to differences in the association between choline and colorectal cancer risk; estrogens are known to increase the activity of the Phosphatidylethanolamine N-methyltransferase (PEMT) pathway by which phosphatidylcholine is synthesized (29). in addition, there may be differences in the distribution of serum choline concentration between different study populations. As previously mentioned, controls in this study had slightly higher mean serum choline concentration compared with controls in WHI (8); this may partly explain the higher ORs observed in this study compared with WHI. Furthermore, there is evidence that the magnitude of the upper end of range of serum choline concentrations (95th percentiles) was higher in colorectal cancer cases in this study (37.4 μmol/L) compared with EPIC cases (14.2 μmol/L; ref. 28).

The potential relation of choline to cancer is complex (29). Currently, there is a limited understanding of choline's role in cancer etiology, although a prior study demonstrated that choline kinase is overexpressed in human colorectal cancer cells (27); this enzyme initiates the first and rate-limiting step of converting choline to phosphatidylcholine. Choline kinase is a potential new target for cancer treatments, as associations have been reported for choline kinase-α expression/activity and both malignancy and increased cellular proliferation (30). Increased total choline-containing compounds, referred to as the "colonic phenotype," is a recently identified metabolic hallmark of malignant transformations (31). Differential uptake of choline, which can be measured by positron emission tomography (32), has been noted in several cancers. There is other evidence that activated choline metabolism may result from the malignancy itself, rather than as a result of enhanced proliferation (33). In our study, the elevated risk of colorectal cancer with higher serum choline persisted even after excluding cases that occurred early during follow-up (the first two years and, separately, the first 5 years); thus, it is unlikely, but not impossible, that our risk estimates reflect the promotion of growth of precancerous lesions by choline. A similar case exists for folate, a nutrient with a central role in one-carbon metabolism, where folate deficiency promotes carcinogenesis but folate supplementation is thought to promote tumor growth and progression (34). Studies in mice have shown that diets deficient in

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methyl donors (choline, folic acid, methionine, and vitamin B12) and supplemented with homocysteine can change the intestinal epithelium and result in prolonged protection against colorectal tumor development (35).

Strengths of this study include the prospective design, large sample size, the ability to stratify by tumor site and state-of-the-art assay methods. The consistency in the direction of the significant choline risk estimates in this study and the WHI lends support to the validity of our choline findings and a connection between choline metabolism and colorectal cancer development. Limitations of this study include the use of a single blood specimen to measure biomarkers, which may not reflect long-term concentrations. In addition, as this sample is comprised of male Finnish smokers, the results herein may not be generalizable to other populations; the epidemiologic data to date raise the need for additional studies that evaluate both sexes. Finally, choline status can be modulated by several factors including folate nutritional status (36, 37) and the composition of the intestinal gut microbiome (38); however, neither factor was measured in this study. Folate nutritional status may differ between the ATBC and WHI study populations given that Finland does not require mandatory folic acid fortification of staple foods, in contrast to the United States (39); however, WHI analyses did not detect differences in the association between plasma metabolites and colorectal cancer risk by fortification period (8) and thus folate fortification (or lack thereof) is unlikely to have a substatntial impact on our findings. Although no significant association between TMAO and colorectal cancer risk was observed in the current study, whether or not alternative choline and gut microbial processes or pathways contribute to colorectal cancer development remain to be examined.

In this study of male Finnish smokers, we did not detect an association between serum TMAO and colorectal cancer risk. Men with high serum choline had a statistically significant 3-fold increase in colorectal cancer risk compared with men with low serum choline. Future studies should investigate serum choline and colorectal cancer risk in more diverse study populations.

## Disclosure of Potential Conflicts of Interest

Z. Wang has ownership interest (including patents) in Cleveland Heart Lab. S.L. Hazen reports receiving a commercial research grant from AstraZeneca, Proctor and Gamble, Pfizer Inc., and Takeda, has ownership interest (including patents) in Cleveland Heart Lab, and is a consultant/advisory board member for Esperion and Proctor and Gamble. No potential conflicts of interest were disclosed by the other authors.

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**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** K.A. Guertin, X.S. Li, B.I. Graubard, Z. Wang, R. Sinha
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