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Associations of Serum Trimethylamine N-oxide and its precursors with colorectal cancer risk in the Prostate, Lung, Colorectal, Ovarian Cancer Screening Trial Cohort

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Abstract

Background: Dietary intake influences gut microbiome composition, which in turn may be associated with colorectal cancer (CRC). Associations of the gut microbiome with colorectal carcinogenesis may be mediated through bacterially regulated metabolically active metabolites, including trimethylamine N-oxide (TMAO) and its precursors, choline, *l*-carnitine, and betaine.

Methods: We investigated prospective associations of circulating TMAO and its precursors with CRC risk. We measured TMAO, choline, betaine, and *L*-carnitine in baseline serum samples from 761 incident CRC cases and 1:1 individually matched controls in the prospective Prostate, Lung, Colorectal, Ovarian Cancer Screening Trial Cohort (PLCO) using targeted fully quantitative LC-MS/MS panels. We estimated prospective associations of the metabolites with CRC risk using multivariable conditional logistic regression. We also investigated associations of *a priori*-selected dietary exposures with the four metabolites.

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Conflicts of interest: Dr. Hazen reports being named as co-inventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics, being a paid consultant formerly for Procter & Gamble in the past, and currently with Zehna Therapeutics. He also reports having received research funds from Procter & Gamble, and Zehna Therapeutics, and being eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Procter & Gamble, Zehna Therapeutics, and Cleveland HeartLab, a wholly owned subsidiary of Quest Diagnostics. Dr. Wang reports being named co-inventor on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics and being eligible to receive royalty payments for inventions or discoveries related to cardiovascular diagnostics or therapeutics from Cleveland HeartLab, a wholly owned subsidiary of Quest Diagnostics, Procter & Gamble. There are no other conflicts of interest to report.

Results: TMAO and its precursors were not associated with CRC risk overall, but TMAO and choline were positively associated with higher risk for distal CRC (Continuous $OR_{Q90\ Vs.\ Q10}$=1.90(95% confidence interval [CI]: 1.24, 2.92; p=0.003) and 1.26(95% CI: 1.17, 1.36; p<0.0001), respectively. Conversely, choline was inversely associated with rectal cancer ($OR_{Q90\ vs.\ Q10}$(95% CI)=0.77[0.76, 0.79; P <0.001]). Red meat, which was previously associated with CRC risk in PLCO, was positively associated with TMAO (Spearman rho = 0.10; p = 0.0003).

Conclusions: Serum TMAO and choline may be associated with higher risk of distal CRC and red meat may be positively associated with serum TMAO. Our findings provide insight into a potential microbially mediated mechanism underlying CRC etiology.

Precis:

In a prospective study among men and women in the United States, we measured circulating TMAO, choline, betaine, and *L*-carnitine and found that TMAO and choline were positively associated with distal colon cancers. Our findings support future studies into underlying mechanisms and potential TMAO-targeted interventions to mitigate CRC risk.

Keywords

metabolomics; colorectal cancer; choline; Trimethylamine N-oxide; diet

INTRODUCTION

In the United States, colorectal cancer (CRC) is the second leading cause of cancer deaths among men and women combined (1). Epidemiologic studies have supported the importance of environmental exposures, such as diet and lifestyle, in CRC risk (2). Many CRC-relevant environmental exposures largely shape the human colon which hosts trillions of bacteria comprising the gut microbiome (3). In turn, there is growing interest in the potential roles of metabolites that the gut microbiome produces and regulates in initiation and progression of the adenoma-carcinoma sequence (4).

Choline, *L*-alpha glycerylphosphorylcholine, phosphatidylcholine, *L*-carnitine, and betaine are diet- and endogenously-derived (5,6) and contain trimethylamine substrates that are cleaved by gut bacteria to form trimethylamine (TMA). TMA is absorbed, travels via portal circulation to the liver and is oxidized by flavin monooxygenases as trimethylamine oxide (TMAO) (7). TMAO and its precursors have most strongly been implicated in cardiovascular disease (8–10). Recently, studies have implicated these metabolites in colorectal carcinogenesis. The exact mechanisms underlying the role of these metabolites in promoting CRC remain to be fully elucidated but may involve similar mechanisms underlying their role in CVD including roles in inflammation, oxidative stress, and bi-directional relationships with the gut microbiome (7). Overall, however, epidemiologic evidence is conflicting thus far. Using stable isotope dilution (targeted, quantitative) LC/MS-MS platforms, choline was strongly, positively associated with CRC risk in prospective nested case-control studies conducted in plasma in the Women's Health Initiative (WHI) (11) and in serum in the Alpha Tocopherol Beta Carotene (ATBC) study (12), but plasma choline was inversely associated with CRC risk in the EPIC cohort (13). Thus, further

investigation of associations of choline, other TMAO precursors (betaine and *L*-carnitine), and TMAO measured in blood, with CRC risk is warranted.

We investigated prospective associations of serum TMAO, choline, betaine, and *L*-carnitine with incident CRC in a population of US men and women, and further considered these associations in the context of CRC dietary risk factors.

METHODS

Study population

This study was nested in the intervention arm of the Prostate, Lung, Colorectal and Ovarian (PLCO) Cancer Screening Trial (N=77,443), a large, multicenter, randomized controlled trial in the United States that enrolled almost 155,000 men and women aged 55–74 years from 1993 to 2001 to evaluate the effectiveness of screening regimens for prostate, lung, colorectal, and ovarian cancers as described previously (14). Participants in the screening arm underwent flexible sigmoidoscopy screening for CRC at baseline and again during follow-up 3 or 5 years later. The study was approved by the human subjects review boards at the National Cancer Institute and at the 10 study centers, and all participants provided written informed consent.

Among those who completed a baseline risk factor questionnaire, provided consent for biospecimens to be used in etiologic studies, did not have CRC at study entry according to questionnaire data and screening sigmoidoscopy, did not have a history of self-reported cancer, Crohn's disease, ulcerative colitis, familial polyposis, Gardner's syndrome or colorectal polyps at baseline, did not have a rare cancer during follow-up, and had at least 6-months of follow-up and available baseline serum, we identified 761 first primary incident CRC cases (ICD-0-3 codes: C180, C182–189, C199, C209, C260, excluding ICD morphologies in the range of 8240–8249, which are carcinoid/neuroendocrine tumors and have different etiology). Cases were individually matched to controls on age at randomization (+/−5-year age group), sex, race, year of randomization, thaw count, and year/season of blood draw. Eligible controls met the same inclusion/exclusion criteria and were alive and CRC/rare cancer-free at date of CRC diagnosis. All colorectal cancer cases were histologically confirmed through medical record review and/or via linkage to cancer registries for cancer diagnoses up to 2014.

Dietary and covariate information

Participants completed a risk-factor questionnaire and a 137-item food-frequency questionnaire (FFQ), the PLCO Dietary Questionnaire (DQx), assessing dietary intake over the past year (15). The DQx is similar in format to two validated FFQs (16,17). A standard portion size and 10 possible frequency-of-consumption values were given ranging from 'never' to '2+ times/day', with typical portion sizes assessed for 60 of the 137 items. The values of foods, beverages, and nutrients in grams were sex-specifically derived based on the participants' responses and their reported serving size.

Quantitation of serum concentrations of TMAO and its precursors by LC/MS/MS

We measured TMAO and its precursors (choline, betaine, and *L*-carnitine) by liquid chromatography-tandem mass spectrometry (LC/MS-MS), as described previously (12,18). Briefly, biospecimens were shipped overnight on dry ice to the Cleveland Clinic laboratory that previously measured serum TMAO, choline, *L*-carnitine, and betaine concentrations in the ATBC study (12). Metabolites were analyzed by stable isotope dilution LC/MS-MS using established methods on a Shimadzu LCMS-8060 CL Triple Quadrupole Liquid Chromatography Mass Spectrometer with Nexera LC-30AD CL UHPLC interface (10,18).

Investigators performing LC/MS-MS were blinded to sample identity (other than barcode label) and to case–control status. Case–control pairs were included adjacently in the same batch with pairs in random order. To assess intra- and inter-batch reproducibility, we used three types of pooled, replicate blinded quality control (QC) specimens that were randomly inserted into each batch. Technical reproducibility, as estimated by coefficients of variation (CVs) from blinded replicates, was excellent except for choline in one QC. All average inter-batch CVs were <8% for TMAO, *L*-carnitine, and betaine. For choline, they were 7.42%, 6.89%, and 33.15% for QCs 1, 2, and 3, respectively (Supplemental Table 1). The reasons underlying the low CV for choline are likely due to thawing as QC 3 had a range of 0, 1, and 2 thaw counts; whereas QCs 1 and 2 all had two thaw counts. To address potential thawing issues that could have led to the low CVs for choline, we conducted analyses stratified by number of thaws and our results were largely similar.

Statistical analysis

We summarized population characteristics by case-control status using Chi square tests for categorical variables, ANOVA for normally distributed variables, and Kruskal-Wallis tests for non-normally distributed variables. We calculated Pearson correlations between all metabolites. We used multivariable conditional logistic regression to estimate associations of TMAO and its precursors with CRC. We used polytomous logistic regression to investigate associations by anatomic CRC site. We categorized the metabolites into quantiles based on the distribution among the controls. We also assessed associations of metabolite ratios with CRC given the interdependence of the metabolites (e.g., via the choline/betaine/*L*-carnitine to trimethylamine to TMAO pathway) and given the strength of associations observed in prior studies of these metabolites and CRC (11).

Consideration for inclusion of covariates in the above-described multivariable logistic regression models were specified based on biological plausibility, previous literature, and magnitude of the change in the estimated association upon addition/removal from the multivariate model (summarized in table footnotes). We also ran diet-adjusted models among the subset of the cohort that completed a DQx, specifically adjusting for red and processed meat intake, but the estimates of association were generally similar.

To investigate potential effect modification, separate analyses were conducted for each exposure within categories of selected participant characteristics, such as dichotomized age, sex, and BMI. We tested whether TMAO- and TMAO precursor-CRC associations were modified by intake of dietary folate, red meat, and fish. We tested for multiplicative

interactions by including the cross-product term of each metabolite and exposure of interest in separate logistic regression models. As a sensitivity analysis to address reverse causation, we also excluded those diagnosed within the first 5 years after randomization.

To estimate associations of diet with TMAO/TMAO precursors, we estimated partial Spearman correlations of *a priori* selected dietary components that are established or possible CRC risk/protective factors that are relevant to TMAO (i.e., red and processed meat, fish, white meat, dairy, whole and refined grains, and eggs).

RESULTS

The median number of years from randomization to CRC diagnosis was 9.4 years. The characteristics of the matched cases and controls are shown in Table 1. On average, cases were more likely to have a comorbidity, and to consume higher total energy and red meat. Spearman correlations among TMAO and its precursors are shown in Supplemental Table 2. TMAO was most strongly correlated with *L*-carnitine and choline (Rs=0.17 and 0.18, respectively).

As shown in Table 2, serum TMAO and precursor metabolites were not associated with overall CRC. In contrast, as shown in Table 3, multiple metabolites were associated with distal colon and rectal cancers. Comparing those in the 90th versus 10th percentile, choline was inversely associated with rectal cancer [$OR_{Q90\ vs.\ Q10}$ (95% CI)= 0.77 (0.76, 0.79; P <0.001)]; whereas TMAO and choline were positively associated with distal colon cancer [$OR_{Q90\ vs.\ Q10}$ (95% CI)=1.90 (1.24, 2.92; P=0.003) and 1.26 (1.17, 1.36; P<0.001), respectively]. *L*-carnitine was moderately positively associated with rectal cancer [$OR_{Q90\ vs.\ Q10}$ (95% CI)= 1.08 (1.06, 1.11); p<0.001]. There was statistically significant heterogeneity by anatomic site for distal compared to proximal TMAO associations (P=0.001) and choline associations (P=0.02). The associations of the various ratios of metabolites with overall CRC (Supplemental Table 3), were generally weak and/or close to null. When we stratified our analyses by various participant characteristics, associations of the metabolites with overall CRC did not substantially differ across the characteristics (Supplemental Table 4). Findings were also similar after excluding those diagnosed within the first five years of follow-up (Supplemental Table 5).

Partial Spearman correlations for the associations of selected dietary exposures with the metabolites are shown in Table 4. We found that red meat intake [g/day] was positively correlated with circulating TMAO (Rs=0.10, *P*=0.0003). No other diet-metabolite associations were statistically significant after Bonferroni correction, though dairy and whole grains were slightly inversely associated with *L*-carnitine concentrations (Rs=−0.08 for both and P's=0.004 and 0.003, respectively). The associations were generally similar among CRC cases (by subsite and combined) and controls (data not shown).

DISCUSSION

The goal of this study was to investigate associations of diet- and gut microbiome-related metabolites – TMAO, choline, betaine, and *L*-carnitine – with incident CRC in a large, prospective cohort of US men and women. We found that the metabolites were not

associated with CRC considered as a single endpoint but were associated with distal colon and rectal cancer risk. Notably, there was almost a 2-fold higher risk of distal colon cancer comparing those in the 90th relative to 10th percentile of circulating TMAO. Conversely, choline was inversely associated with rectal cancer. In PLCO, unprocessed red meat intake was previously associated with a 47% higher risk of CRC for every 50 g/1,000 kcals of intake per day (19). These findings were consistently replicated in other epidemiological studies (20). In our study, red meat was correspondingly positively associated with circulating TMAO. Below we discuss potential implications and support for our findings pertaining the interrelationships of diet, the gut microbiome and its metabolites, and CRC risk.

TMAO has most strongly, consistently been associated with the development of cardiovascular disease but the mechanisms underlying TMAO's role in colorectal carcinogenesis specifically are still unclear. This molecule does exhibit pro-inflammatory and pro-oxidative properties (7) that may influence colorectal carcinogenesis. In a recent study, TMAO increased CRC cell proliferation and angiogenesis *in vitro* and *in vivo* increased tumor burden in mice (21). Choline may also have pro- or anti-carcinogenic effects, perhaps depending on whether it is destined to the likely anti-carcinogenic one-carbon metabolism pathway versus to the likely pro-carcinogenic gut microbiome-TMAO pathway (13). Further, abnormal choline metabolism may be implicated in tumorigenesis (22). In a meta-analysis of eight shotgun sequencing CRC case-control studies, bacterial choline trimethylamine-lyase genes were more abundant among individuals with CRC (23). Making connections between these metabolites and the trillions of microbes residing in the gut may provide potential insight into carcinogenic mechanisms and downstream interventions to suppress TMAO/TMAO-precursor production. However, bacteria in the stool may not strongly, directly reflect TMAO-related metabolites in circulation as supported thus far (24,25).

Interestingly, we found that the associations of TMAO and its precursors with CRC were generally strong and positively associated with distal colon cancers, generally had null associations for proximal colon cancers, and choline was inversely associated with rectal cancers. In addition to differences in embryologic origin and physiologic functions, from the colon to the rectum, as the fecal stream passes, the composition of the gut microbiome and its metabolically active metabolites may be altered (26). Choline has pleotropic roles in carcinogenesis. Choline may have anticarcinogenic functions via one carbon metabolism and contributing to normal DNA methylation (13). Conversely, it can also be converted to TMAO during the potentially pro-carcinogenic process of gut microbial conversion via bacterial genes like *CutD* (21, 23). The conflicting evidence for choline-CRC associations in the literature and in the study described herein may reflect a highly nuanced and complex interrelationship reflecting both anatomical differences and choline functionality. We also found that red meat was positively associated with TMAO, similar to some (25,27) but not all (28) recent cross-sectional studies. Our findings of TMAO by CRC anatomic site followed a somewhat similar pattern of associations observed in PLCO and two other large, prospective cohorts of red meat with CRC. In a meta-analysis of these three studies, the positive associations of red meat with CRC were stronger for distal colon cancers (19).

Overall, our observed anatomic site-specific TMAO associations are biological plausible and mirror associations of another established CRC risk factor.

Three previous prospective studies investigated the associations of circulating concentrations of choline, *L*-carnitine, betaine, and TMAO with CRC risk. In a cohort of Finnish male smokers (N=644 CRC cases and N=644 controls), there was a 3.4-fold higher CRC risk for those in the highest relative to lowest quartile of choline (12), in contrast to our null choline-overall CRC findings. In a cohort of postmenopausal women, those in the highest relative to lowest quartile of TMAO had a 65% higher risk for CRC (11). Somewhat like our findings, the associations in WHI were stronger for rectal cancers and those in the highest relative lowest quartile of TMAO and choline concentrations had a 3.4-fold and 2.9-fold higher rectal cancer risk, respectively. In contrast to our null findings, these investigators found that those in the highest relative to lowest quartile of betaine and the betaine/choline ratio had a 32% and 44% lower CRC risk, respectively. Finally, in the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort, choline was inversely associated with overall CRC risk (29). These studies were conducted among Europeans, male smokers, and/or post-menopausal women, thus it is plausible the population differences (e.g., in the gut microbiome) may have influenced the differences in findings. Differences may also be attributable to differences in sample collection/type (e.g., plasma [WHI and EPIC] versus serum [PLCO, ATBC]). Further, WHI and ATBC had fasted blood; whereas fasting was not a requirement for PLCO participants providing blood. Despite these differences, the quantitative serum concentrations as measured in ATBC and PLCO were relatively similar among CRC cases.

A study limitation was the potential freeze-thaw effects for choline (comparing QCs with higher versus lower thaws), which we were able to identify with rigorous QC analyses; however, we found that the choline-CRC associations did not differ by the number of thaws and we matched cases and controls by thaw counts. In addition, circulating TMAO and its precursors may have temporal variability, potentially attenuating our findings. In an untargeted metabolomic analysis (UHPLC-MS/MS) among 100, non-overlapping participants in PLCO of serum samples collected at baseline and either year-1 or between years 3–5, the intraclass coefficients (ICCs; higher is more stable) for relative concentrations of choline, *L*-carnitine, TMAO, and betaine between baseline and year 1 were 61.12%, 76.53%, 17.50%, and 72.69%, respectively. Between baseline and years 3–5, the ICCs were slightly lower for all metabolites except TMAO which was slightly higher (26.42%). Such temporal variability likely attenuated our findings – meaning that the true TMAO-CRC associations are likely stronger than what we observed. We estimated the diet-metabolite associations based on a population that was enriched with CRC cases. However, the individuals were disease-free at the time of questionnaire completion and the associations were similar when restricted among the controls. Further, dietary questionnaires have known limitations including recall error, which would have likely attenuated our observed associations. Despite these limitations, in PLCO, a number of established pro-carcinogenic dietary patterns and foods have been associated with CRC (19). Future studies should collect serial samples. Overall, our study was strengthened by our prospective analysis of serum metabolites in relation to CRC and a somewhat larger study population that enabled site-specific analyses.

Taken together, we found that TMAO and its precursors were associated with distal colon and rectal, but not proximal colon, cancers among men and women in the PLCO study. Future studies should include serial blood samples collected in large, diverse study populations, characterize microbial biochemical pathways contributing to TMAO production, investigate underlying mechanisms whereby TMAO and its precursors may promote colorectal carcinogenesis, and include interventions to investigate how to modulate TMAO concentrations, such as via dietary intervention, to reduce CRC risk or related biomarkers in preclinical and clinical studies. In the future, public health interventions may be developed to inform dietary recommendations for limiting inputs to the TMAO pathway across population groups, especially high-risk groups, to mitigate the risk of CRC in the population.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments:

Cancer incidence data have been provided by the Alabama Statewide Cancer Registry, Arizona Cancer Registry, Colorado Central Cancer Registry, District of Columbia Cancer Registry, Georgia Cancer Registry, Hawaii Cancer Registry, Cancer Data Registry of Idaho, Maryland Cancer Registry, Michigan Cancer Surveillance Program, Minnesota Cancer Surveillance System, Missouri Cancer Registry, Nevada Central Cancer Registry, Ohio Cancer Incidence Surveillance System, Pennsylvania Cancer Registry, Texas Cancer Registry, Utah Cancer Registry, Virginia Cancer Registry, and Wisconsin Cancer Reporting System. All are supported in part by funds from the Center for Disease Control and Prevention, National Program for Central Registries, local states or by the National Cancer Institute, Surveillance, Epidemiology, and End Results program. The results reported here and the conclusions derived are the sole responsibility of the authors.

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Data availability:

Data described in the manuscript, code book, and analytic code will be made available upon request pending proposal approval and completion of a Data Transfer Agreement (see https://cdas.cancer.gov/learn/plco/instructions/?type=data)

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Table 1.

Selected characteristics of participants in Prostate, Lung, Colorectal and Ovarian Screening Cohort (N = 761 cases and N=761 controls)

	Cases		Controls	
	N (%)	Mean (SD)	N (%)	Mean (SD)
Demographics				
Age		63.94 (5.21)		63.79 (5.24)
Male	383 (50.33)		383 (50.33)	
White	665 (87.39)		665 (87.39)	
Married or partnered	555 (73.03)		608 (79.89)	
College graduate or higher	233 (30.66)		269 (35.39)	
Medical History				
Aspirin use > once/week	364 (47.83)		355 (46.65)	
Ibuprofen use > once/week	207 (27.20)		211 (27.73)	
Current or former HRT use	239 (31.41)		257 (33.77)	
Family history of CRC	102 (13.88)		83 (11.35)	
History of colon polyps	71 (9.33)		37 (4.87)	
Has comorbidity[b]	209 (27.54)		163 (21.42)	
Has diabetes	69 (9.07)		45 (5.91)	
Has gallbladder disease	103 (13.53)		77 (10.12)	
Has cardiovascular disease	77 (10.12)		65 (8.54)	
Lifestyle behaviors				
BMI (kg/m^2)		28.10 (5.14)		26.93 (4.45)
Alcohol intake (g/day)		0.92 (2.12)		0.82 (1.80)
Smokes > 20 cigarettes per day	139 (18.39)		131 (17.21)	
Exercises < 1 hour per week	216 (31.44)		221 (31.04)	
Dietary Intake				
Total energy intake (kcal/day)		2,090 (938.79)		2,056 (851.56)
Calcium (g/1000 kcal/day)		618.84 (316.64)		645.92 (323.98)
Fiber (g/1000 kcal/day)		11.80 (3.52)		12.15 (3.44)
Folate (g/1000 kcal/day)		349.45 (205.86)		367.20 (205.15)

	Cases		Controls	
	N (%)	**Mean (SD)**	**N (%)**	**Mean (SD)**
Vitamin B12 (g/1000 kcal/day)		6.93 (9.02)		7.28 (9.49)
Fish (g/day)		20.84 (26.58)		20.65 (25.91)
Red meat (g/day)		80.20 (74.01)		73.42 (62.88)
Fruit and vegetable (g/day)		664.46 (329.97)		658.89 (305.93)
Metabolite Values				
Betaine (μM)		36.43 (14.55)		36.57 (15.66)
Choline (μM)		13.61 (6.70)		14.44 (16.43)
L-Carnitine (μM)		39.27 (8.71)		39.03 (9.13)
TMAO (μM)		5.08 (5.25)		5.03 (7.46)

[a] P-values were estimated using Chi square tests for categorical variables, ANOVA for normally distributed variables, and Kruskal-Wallis tests for non-normally distributed variables. Values represent either mean (SD) or N (%); abbreviations: BMI, body mass index; CRC, colorectal cancer; HRT, hormonal replacement therapy; SD, standard deviation.

[b] Comorbidities include the presence of at least one of the following: diabetes, gallbladder disease, or heart disease.

Table 2.

Associations[a] of circulating TMAO and its precursors with incident colorectal cancer in the Prostate, Lung, Colorectal and Ovarian Screening Cohort (N = 761 cases and N=761 controls)

	Overall			Males			Females		
	N	**OR (95% CI)**	**P[b]**	**N males**	**OR (95% CI)**	**P**	**N females**	**OR (95% CI)**	**P**
Continuous concentrations [range] (ng/mL)									
TMAO [0.231; 163.00]	761	1.12 (0.84, 1.48)	0.44	383	1.08 (0.73, 1.60)	0.71	378	1.11 (0.73, 1.69)	0.63
Betaine [0.028; 209.00]	761	0.95 (0.67, 1.34)	0.77	383	0.89 (0.57, 1.39)	0.62	378	0.93 (0.58, 1.48)	0.76
Choline [5.26; 316.00]	761	1.03 (0.75, 1.40)	0.87	383	0.94 (0.61, 1.46)	0.79	378	1.36 (0.86, 2.14)	0.19
L-carnitine [0.027; 83.70]	761	0.94 (0.68, 1.30)	0.69	383	0.80 (0.50, 1.26)	0.33	378	1.31 (0.83, 2.06)	0.24
TMAO									
Quartile 1 [0.231; 2.65]	177	1.00		82	1.00		95	1.00	
Quartile 2 [2.65; 3.80]	183	1.05 (0.78, 1.40)		101	1.28 (0.83, 1.96)		82	0.85 (0.56, 1.28)	
Quartile 3 [3.80; 5.57]	180	0.93 (0.68, 1.26)		96	1.11 (0.71, 1.73)		84	0.75 (0.49, 1.16)	
Quartile 4 [5.58; 163.00]	221	1.14 (0.85, 1.52)	0.44	104	1.21 (0.79, 1.84)	0.71	117	1.04 (0.68, 1.60)	0.63
Betaine									
Quartile 1 [0.028; 26.70]	187	1.00		99			88	1.00	
Quartile 2 [26.80; 35.30]	203	1.10 (0.81, 1.49)		104	0.96 (0.63, 1.45)		99	1.18 (0.75, 1.87)	
Quartile 3 [35.30; 44.30]	179	0.90 (0.66, 1.22)		89	0.85 (0.55, 1.32)		90	0.88 (0.55, 1.41)	
Quartile 4 [44.30; 209.00]	192	1.01 (0.73, 1.39)	0.77	91	0.87 (0.55, 1.37)	0.62	101	1.01 (0.62, 1.63)	0.76
Choline									
Quartile 1 [5.26; 11.00]	182	1.00		103	1.00		79	1.00	
Quartile 2 [11.00; 12.80]	175	0.96 (0.71, 1.30)		94	0.85 (0.55, 1.31)		81	1.08 (0.69, 1.69)	
Quartile 3 [12.80; 15.00]	196	1.10 (0.81, 1.50)		83	0.77 (0.49, 1.20)		113	1.54 (0.98, 2.42)	
Quartile 4 [15.00; 316.00]	208	1.10 (0.80, 1.52)	0.87	103	0.98 (0.62, 1.55)	0.79	105	1.27 (0.79, 2.04)	0.19
Carnitine									
Quartile 1 [0.027; 33.20]	199	1.00		105	1.00		94	1.00	
Quartile 2 [33.20; 38.60]	155	0.73 (0.54, 0.99)		88	0.76 (0.49, 1.19)		67	0.67 (0.43, 1.04)	
Quartile 3 [38.60; 44.50]	190	0.90 (0.67, 1.22)		93	0.77 (0.50, 1.20)		97	0.96 (0.63, 1.48)	
Quartile 4 [44.50; 83.70]	217	0.99 (0.72, 1.36)	0.69	97	0.78 (0.49, 1.24)	0.33	120	1.16 (0.74, 1.83)	0.24

[a]Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using conditional logistic regression. Odds ratios represent risk at the 90th percentile as compared with the 10th percentile of log metabolite intensity (OR = eβ(X90-X10) where β is the coefficient for the metabolite modeled continuously and X90 and X10 are metabolite values at the 90th and 10th percentiles based on the control distribution); all the models above were adjusted for sex, age, race, season and calendar year of blood draw, total years of cigarettes smoked, number of cigarettes per day, education, body mass index, regular non-steroidal anti-inflammatory drug (NSAID)/aspirin use, marital status, family history of CRC, hormone therapy use, and comorbidities including diabetes, gallbladder and cardiovascular diseases.

Table 3.

Associations[a] of circulating TMAO and its precursors with incident colorectal cancer by anatomical site in the Prostate, Lung, Colorectal and Ovarian Screening Cohort (N = 761 cases and N=761 controls)

	N	OR (95% CI)	P-value	P-heterogeneity
TMAO				
Proximal	533	0.95 (0.71, 1.25)	0.70	ref
Distal	127	1.90 (1.24, 2.92)	0.003	0.001
Rectal	92	1.05 (0.63, 1.75)	0.86	0.75
Betaine				
Proximal	533	0.98 (0.77, 1.26)	0.90	ref
Distal	127	0.79 (0.61, 1.03)	0.08	0.26
Rectal	92	1.14 (0.91, 1.42)	0.24	0.51
Choline				
Proximal	533	0.90 (0.73, 1.11)	0.31	ref
Distal	127	1.26 (1.17, 1.36)	<0.001	0.02
Rectal	92	0.77 (0.76, 0.79)	<0.001	0.63
L-Carnitine				
Proximal	533	1.12 (0.91, 1.38)	0.29	ref
Distal	127	1.03 (0.97, 1.10)	0.30	0.49
Rectal	92	1.08 (1.06, 1.11)	<0.001	0.88

[a]Odds ratios (ORs) and 95% confidence intervals (CIs) were estimated using polytomous logistic regression. Odds ratios represent risk at the 90th percentile as compared with the 10th percentile of log metabolite intensity (OR = $e\beta(X90-X10)$ where β is the coefficient for the metabolite modeled continuously and X90 and X10 are metabolite values at the 90th and 10th percentiles based on the control distribution); all the models above were adjusted for sex, age, race, season and calendar year of blood draw, total years of cigarettes smoked, number of cigarettes per day, education, body mass index, regular non-steroidal anti-inflammatory drug (NSAID)/aspirin use, marital status, family history of CRC, hormone therapy use, and comorbidities including diabetes, gallbladder and cardiovascular diseases.

Table 4.

Partial Spearman correlations[a] for associations of dietary exposures with circulating TMAO and its precursors in the Prostate, Lung, Colorectal and Ovarian Screening Cohort (N = 653 cases and N= 683 controls)

	Fish		Red meat		Processed meat		White meat		Dairy		Whole grain		Refined grains		Eggs	
	Rs	***P***	**Rs**	***P***	**Rs**	***P***	**Rs**	***P***	**Rs**	***P***	**Rs**	***P***	**Rs**	***P***	**Rs**	***P***
Betaine	0.01	0.65	0.00	0.86	−0.03	0.21	0.02	0.46	−0.07	0.02	0.04	0.14	0.04	0.16	0.04	0.20
L-carnitine	0.02	0.43	0.05	0.05	−0.02	0.58	0.03	0.34	−0.08	0.004	−0.08	0.003	0.01	0.62	0.02	0.51
Choline	0.02	0.41	0.03	0.28	−0.07	0.02	−0.01	0.65	−0.03	0.30	−0.05	0.09	0.05	0.08	0.05	0.06
TMAO	0.03	0.35	0.10	0.0003	−0.06	0.04	0.00	0.92	−0.05	0.10	−0.03	0.31	−0.03	0.31	0.00	0.96

[a]All the models above were adjusted for CRC status, sex, age, race, season and calendar year of blood draw, total years of cigarettes smoked, number of cigarettes per day, education, body mass index, regular non-steroidal anti-inflammatory drug (NSAID)/aspirin use, marital status, family history of CRC, comorbidities including diabetes, gallbladder and cardiovascular diseases, energy intake, hormone therapy use, folate intake, vitamin B12, fruits and vegetables, calcium intake, fiber intake, fish intake, red meat intake (the latter three covariates were only included in pertinent models, and all nutrients were energy adjusted using the nutrient density method).