Gut Microbiome–Dependent Metabolic Pathways and Risk of Lethal Prostate Cancer: Prospective Analysis of a PLCO Cancer Screening Trial Cohort

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ABSTRACT

Background: Diet and the gut microbiome have a complex interaction that generates metabolites with an unclear effect on lethal prostate cancer risk. Identification of modifiable risk factors for lethal prostate cancer is challenging given the long natural history of this disease and difficulty of prospectively identifying lethal cancers.

Methods: Mass spectrometry was performed on baseline serum samples collected from 173 lethal prostate cancer cases and 519 controls enrolled in the Prostate, Lung, Colorectal, and Ovarian Cancer Screening trial. Baseline serum levels of choline, carnitine, betaine, γ-butyrobetaine, crotonobetaine, phenylacetylglutamine, hippuric acid, and p-cresol sulfate were quantified and analyzed by quartile. Conditional multivariable logistic regression analysis associated analyte levels with lethal prostate cancer incidence after adjusting for body mass index and PSA. The Cochran–Armitage test evaluated analyte level trends across quartiles.

Results: Relative to those in the first quartile, cases with the highest baseline levels of choline (Q4 OR: 2.19; 95% CI, 1.23–3.90; *P*-trend: 0.005) and betaine (Q4 OR: 1.86; 95% CI, 1.05–3.30; *P*-trend: 0.11) exhibited increased odds of developing lethal prostate cancer. Higher baseline serum levels of phenylacetylglutamine (Q4 OR: 2.55; 95% CI, 1.40–4.64; *P*-trend: 0.003), a gut microbiome metabolite of phenylalanine with adrenergic activity, were also associated with lethal prostate cancer.

Conclusions: Baseline serum levels of one-carbon methyl donors and adrenergic compounds resulting from human and gut microbiota–mediated metabolism are associated with increased lethal prostate cancer risk.

Impact: Dietary composition, circulating metabolite levels, and downstream signaling pathways may represent modifiable risk factors associated with incident lethal prostate cancer. Beta-adrenergic blockade represents an additional target for oncologic risk reduction.

Introduction

Epidemiologic research has linked Western diets (rich in energy, red meat, high-fat dairy, and processed foods) with an increased risk of advanced prostate cancer (1, 2). This finding is exemplified by ecological studies demonstrating increased risk among immigrant populations that adopt Western dietary patterns following relocation (3–5). Other large-scale studies have also demonstrated that egg, poultry, and red meat consumption enhances the risk of developing advanced or lethal prostate cancer (6, 7). Notably, these animal products are enriched in choline, which has been independently associated with increased prostate cancer incidence and mortality (8, 9).

The gut microbiome plays a critical role in generating compounds that circulate within their human hosts (**Fig. 1**; refs. 10, 11). One such pathway centers on the generation of trimethylamine (TMA) via the gut-microbiota-mediated metabolism of betaine compounds (including γ-butyrobetaine and crotonobetaine), choline, and carnitine. TMA is subsequently oxidized in the liver to trimethylamine N-oxide (TMAO), a physiologically active compound known to activate inflammatory pathways and alter cholesterol metabolism (12, 13). Higher serum levels of TMAO have been linked to colorectal and hepatocellular carcinoma, as well as cardiovascular disease (CVD) risk (14–17). Although higher levels of TMAO were associated with an aggressive prostate cancer diagnosis in the alpha-tocopherol beta-carotene (ATBC) cancer prevention trial, it is unknown how TMAO levels affect prostate cancer mortality (18).

Amino acid metabolites that result from a combination of host and gut microbiome metabolism have also been associated with human disease. For example, hippuric acid, p-cresol sulfate, and phenylacetylglutamine (PAGln) have each been linked with CVD (19–21). Interestingly, PAGln was found to activate various α- and β-adrenergic receptors, providing a novel mechanism through which these metabolites exert their physiologic effects (21). These metabolites have not been studied clinically with regard to prostate cancer outcomes.

Given that TMAO precursors have been associated with prostate cancer risk, we sought to investigate how alterations in the gut microbiota–dependent metabolism of choline, carnitine, and other TMA precursors affect lethal prostate cancer risk. We also aimed to

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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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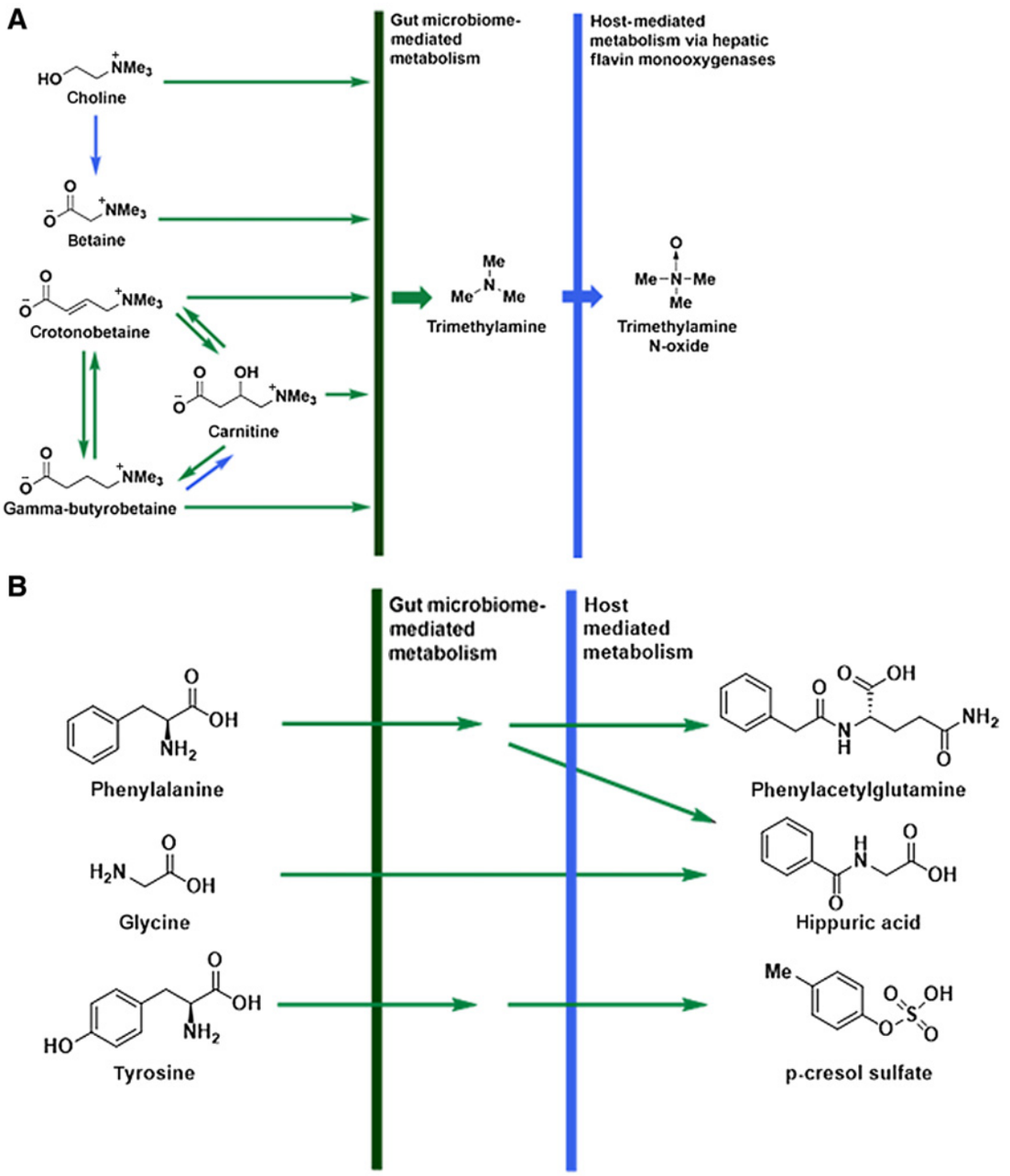

Figure 1.

Gut microbiome–mediated metabolism of TMA precursors and select amino acids. **A,** Precursors to TMA—including choline, carnitine, betaine, γ-butyrobetaine, and crotonobetaine—are consumed through the diet. These nutrients may be converted to other TMA precursors by the host (shown via blue arrows) or by gut microbiota (shown via green arrows). A minority of these precursor nutrients reach the large intestine and are metabolized by gut microbiota to TMA, which is ultimately absorbed by the host. Via the portal circulation, TMA reaches the liver, where it is oxidized to trimethylamine N-oxide by flavin-containing monooxygenases (FMOs). TMAO then enters the systemic circulation, where it may exert its physiologic effects on its human host. **B,** Amino acids—including phenylalanine, glycine, and tyrosine—are ingested through dietary protein. In the stomach and small intestine, these amino acids are liberated by proteases. A minority of these amino acids reach gut microbiota in the large intestine, where they are converted to phenylacetylglutamine (PAG), hippuric acid, and p-cresol sulfate, respectively. These gut microbiota–derived amino acid derivatives are absorbed by the host and ultimately enter the systemic circulation where they may alter physiologic and metabolic processes in the host.

study whether amino acid derivatives previously associated with CVD influence the risk of prostate cancer mortality. Therefore, we conducted a nested case–control study in which baseline levels of these nutrients and metabolites were measured in prediagnostic serum samples obtained through the Prostate, Lung, Colorectal, and Ovarian (PLCO) cancer screening trial. We hypothesized that higher baseline levels of choline, carnitine, TMAO, and PAGln are associated with an increased risk of incident lethal prostate cancer.

Materials and Methods

PLCO cohort

The PLCO cancer screening trial was a large, population-based, randomized controlled trial designed to evaluate effects of cancer screening in men and women between the ages of 55 and 74 (22). Between November 1993 and July 2001, ten screening centers in the United States enrolled 76,685 men, who were randomized to an intervention arm or a control arm. Men in the intervention arm received screening for prostate cancer via PSA and digital rectal exam for the first six years of the trial and were followed for at least seven years thereafter. Trial data were collected through December 2009 and included baseline blood sample collection (prior to cancer diagnosis), demographic information, and screening results. The primary endpoint of this trial was cancer-specific mortality, though multiple secondary endpoints related to cancer screening and morbidity were examined. Through 2015, data were collected on participant deaths via administration of the Annual Study Update questionnaire, by physician or family report, or by performing National Death Index Plus searches. A Death Review Process was subsequently performed to evaluate participant deaths and determine if a PLCO cancer was responsible (23).

Nested case–control study design

We used a nested case–control design. Baseline serum specimens from 173 lethal prostate cancer cases and 519 controls without lethal prostate cancer were analyzed. Samples were collected from men assigned to the intervention arm of the PLCO trial not previously diagnosed with prostate cancer. The 173 cases represent all prostate cancer deaths in the intervention arm for which prediagnostic samples were available. The controls include men never diagnosed with prostate cancer and those diagnosed with prostate cancer who did not expire within the observation period of the trial. Metastatic events were not considered to be lethal events, unless the patient was determined to have died from a prostate cancer–specific cause during the observation period of the study. Cases and controls were defined in this fashion to identify risk factors that predispose patients to aggressive prostate cancer that may require earlier and more definitive intervention.

At the time of death, cases were matched at a 1:3 ratio with randomly selected controls in the corresponding risk set on the basis of race, age (within 2 years), time of baseline blood draw (within 6 months), and enrollment date (within 1 year). This study was designed to identify potential drivers of lethal disease, hence the focus on lethal prostate cancer using strict risk-set sampling.

Serum levels of choline, carnitine, betaine, γ-butyrobetaine, crotonobetaine, TMAO, PAGln, hippuric acid, and p-cresol sulfate levels were quantified from a single baseline measurement of analyte levels from apparently healthy subjects who were then prospectively followed for incident development of prostate cancer. Study approval was obtained from the NCI and Cleveland Clinic Institutional Review Board following a competitive application and peer review process.

Reagents for metabolomic analysis

d_9-[N,N,N-trimethyl]choline (d_9-choline), L-carnitine:HCL (methyl-D3, d_3-carnitine), *d_9*-trimethylamine *N*-oxide (d_9-TMAO), hippuric acid (benzoyl-D5, d_5-hippuric acid), and p-cresol sulfate potassium salt (D7, d_7-p-cresol sulfate) were purchased from Cambridge Isotope Laboratories. p-Cresol sulfate (potassium salt) was purchased from Cayman Chemical. *d_9*-[N,N,N-trimethyl]-betaine (d_9-betaine) and Nα-(Phenyl-d5-acetyl)-L-glutamine (d_5-phenylacetylglutamine) were purchased from C/D/N Isotopes. d_9-[N,N,N-trimethyl-γ-butyrobetaine (d_9-γ-butyrobetaine) and d_9-[N,N,N-trimethyl]-crotonobetaine (d_9-crotonobetaine) were synthesized as previously described (24–26). All other reagents were purchased as HPLC grade from either Sigma-Aldrich or Fisher Scientific Chemicals.

Metabolomics analysis

Twenty microliters of serum was mixed with 80 μL of cold methanol containing an isotope-labeled internal standard mix composed of d_9-choline, d_9-TMAO, d_9-betaine, d_3-carnitine, d_9-γ-butyrobetaine, d_9-crotonobetaine, d_7-p-cresol sulfate, d_5-phenylacetylglutamine and d_5-hippuric acid (each 5 μmol/L). The mixture was vortexed and centrifuged at 20,000 × *g* at 4°C for 10 minutes. 0.2 μL of supernatant was injected onto silica column (150 × 2 mm, 00F-4274-B0, Phenomenex) at a flow rate of 0.25 mL/min using a Vanquish high-performance liquid chromatography system interfaced with a Thermo Quantiva mass spectrometer (Thermo Scientific) and a TSQ Quantiva Triple Quadrupole mass spectrometer (Thermo Scientific). A liquid chromatography gradient generated from two solvents (A: 0.1% propionic acid in water, B: 0.1% acetic acid in methanol) at a flow rate of 0.25 mL/min was used to resolve analytes. Samples were run sequentially and continuously on the LC/MS/MS system, with laboratory personnel blinded to the case status of the samples.

Targeted metabolites and isotope-labeled internal standards were monitored using electrospray ionization in positive-ion mode (except for p-cresol sulfate, which was monitored using negative-ion mode) with multiple reaction monitoring (MRM) as previously reported (27). Transitions of m/z 104 → 60 for choline, 162 → 60 for carnitine, 118 → 58 for betaine, 146 → 87 for γ-butyrobetaine, 144 → 59 for crotonobetaine, 76 → 59 for TMAO, 265 → 130 for PAGln, 180 → 105 for hippuric acid, and 187 → 107 for p-cresol sulfate were utilized to quantify these analytes as shown in Supplementary Fig. S1. Internal standards were similarly monitored using ESI with MRM of ion transitions of m/z 113 → 69 for d_9-choline, 165 → 63 for d_3-carnitine, 127 → 66 for d_9-betaine, 155 → 87 for d_9-γ-butyrobetaine, 153 → 66 for d_9-crotonobetaine, 85 → 66 for d_9-TMAO, 270 → 130 for d_5-PAGln, 185 → 110 for d5-hippuric acid, and 194 → 114 for d_7-p-cresol sulfate. Parameters for the ion monitoring were optimized for individual metabolites and internal standards and dwell time was set at 20 ms. Argon was used as the CID gas and nitrogen (99.95% purity) was used otherwise.

Various concentrations of standards were mixed with fixed concentrations of an isotope-labeled internal standard mix to prepare calibration curves. The standard curves for quantitation of choline, carnitine, betaine, γ-butyrobetaine, crotonobetaine, PAGln, hippuric acid, and p-cresol sulfate are shown in Supplementary Fig. S2. Squared correlation coefficients were greater than 0.99 for each metabolite, demonstrating the reliability of this analytic approach.

Statistical analysis

Serum measurements were analyzed by quartile, with the distribution of analyte levels among the controls used to determine quartile (Q) thresholds. Multivariable conditional logistic regression analysis was used to assess the association of analyte levels with lethal prostate cancer after conditioning on case status and adjusting for PSA and BMI. The odds ratio (OR) and 95% confidence interval (95% CI) of developing lethal prostate cancer were reported for each quartile of nutrient and metabolite levels, with the first quartile serving as the reference. Trend of increasing ORs was also assessed based on quartile medians using the Cochran–Armitage test. For other comparisons between cases and controls, median and interquartile ranges or counts

Table 1. Baseline clinical and pathologic characteristics.[a]

Full cohort	Controls $n = 519$	Cases $n = 173$	P value[b]
Age, years, median (IQR)	66 (61–69)	66 (61–69)	Matching factor[c]
Race			Matching factor[c]
White	477 (91.9)	159 (91.9)	
Black	33 (6.4)	11 (6.4)	
Other	9 (1.7)	3 (1.7)	
BMI, kg/m^2, median (IQR)	26.6 (24.7–29.0)	26.9 (25.1–29.6)	**0.025**
PSA nearest diagnosis, ng/mL			
Median (IQR)	5.8 (4.5–8.2)	7.3 (4.9–16.3)	0.055
<10	71 (83.5)	113 (65.3)	**0.038**
10–20	11 (12.9)	22 (12.7)	0.939
>20	3 (3.5)	38 (22.0)	**0.011**
Subset with prostate cancer diagnosis	$n = 85$ (16.4%)	$n = 173$ (100%)	
Clinical T stage			
T1	53 (62.4)	55 (31.8)	**0.007**
T2	30 (35.3)	94 (54.3)	0.160
T3	0 (0)	10 (5.8)	0.998
T4	0 (0)	6 (3.5)	0.998
Unknown or not evaluated[d]	2 (2.4)	8 (4.6)	
Clinical N stage			
N0	63 (74.1)	112 (64.7)	0.487
N1	0 (0)	6 (3.5)	0.998
Nx	20 (23.5)	55 (31.8)	0.665
Unknown or not evaluated[d]	2 (2.4)	0 (0)	
Clinical M stage			
M0	69 (81.2)	113 (65.3)	**0.029**
M1	1 (1.2)	38 (22.0)	**0.003**
Mx	13 (15.3)	0 (0)	0.429
Unknown or not evaluated[d]	2 (2.4)	22 (12.7)	
Gleason score on biopsy			
6	39 (45.9)	34 (19.7)	**0.002**
7	16 (18.8)	43 (24.9)	0.948
8	10 (11.8)	31 (17.9)	0.233
9	5 (5.9)	36 (20.8)	**0.017**
10	0 (0)	7 (4.0)	0.998
Unknown or not evaluated[d]	15 (17.6)	22 (12.7)	

Note: Bold values denote P values < 0.05.
Abbreviations: BMI, body mass index; IQR, interquartile range; PSA, prostate-specific antigen.
[a]Data are presented as number (percentage) of cases or controls unless otherwise specified.
[b]Significance testing was performed using univariate conditional logistic regression based on available observations among those who received a prostate cancer diagnosis.
[c]Patient factors used to match subjects at the time of case mortality as part of strict risk-set sampling.
[d]Number reported represents cases or controls who underwent prostate cancer staging evaluation with missing clinical information.

and percentages were reported, though significance was based on univariate conditional logistic regression where P values are provided. All analyses were conducted using R (version 3.6.3) using a significance threshold of 0.05.

Results

One hundred seventy-three lethal prostate cancer cases and 519 controls, well matched by age and race, were analyzed (**Table 1**). A minority of controls were diagnosed with nonlethal prostate cancer [16.4% (85/519)] by the end of the observation period. Median PSA levels nearest prostate cancer diagnosis were similar between cases and controls (7.3 vs. 5.8 ng/dL, $P = 0.06$). A majority of lethal prostate cancers were diagnosed at clinical stage T2, compared with a minority of the nonlethal cancers [54.3% (94/173) vs. 35.3% (30/85)]. Prostate cancer deaths occurred a median of 11.69 years following baseline blood draw (range, 0.62–20.29 years; SD: 3.96 years) with 50% of prostate-specific cancer deaths occurring between 8.48 and 14.09 years after sample provisioning.

Using conditional univariate logistic regression modeling, median baseline levels of TMA-associated nutrients were compared (**Table 2**). Choline (13.9 vs. 13.1 μmol/L, $P = 0.003$) and carnitine (42.3 vs. 41.0 μmol/L, $P = 0.03$) were higher in subjects who developed incident lethal prostate cancer compared with those who did not. No statistically significant differences were observed among cases and controls with respect to baseline circulating levels of betaine (46.1 vs. 42.8 μmol/L, $P = 0.46$), γ-butyrobetaine (0.67 vs. 0.63 μmol/L, $P = 0.29$), crotonobetaine (0.07 vs. 0.07 μmol/L, $P = 0.34$), or TMAO (4.00 vs. 3.63 μmol/L, $P = 0.07$). Interestingly, baseline levels of the gut microbiota–derived metabolites PAGln (2.13 μmol/L vs. 1.75 μmol/L, $P = 0.002$) and p-cresol sulfate (19.7 vs. 15.0 μmol/L, $P = 0.01$) were higher in men who subsequently developed lethal prostate cancer, whereas baseline levels of hippuric acid (2.66 vs. 2.38 μmol/L, $P = 0.99$) were similar between cases and controls.

Table 2. Distribution of serum analyte levels among cases and controls.[a]

Metabolite, μmol/L	Controls ($n = 519$)	Cases ($n = 173$)	*P* value[b]
Choline	13.1 (11.0–16.0)	13.9 (12.1–16.9)	**0.003**
Carnitine	41.0 (34.0–48.3)	42.3 (36.2–50.5)	**0.03**
Betaine	42.8 (35.6–54.0)	46.1 (37.3–54.7)	0.46
γ-Butyrobetaine	0.63 (0.53–0.79)	0.67 (0.56–0.82)	0.29
Crotonobetaine	0.07 (0.06–0.09)	0.07 (0.06–0.10)	0.34
TMAO	3.63 (2.46–5.72)	4.00 (2.92–6.21)	0.07
PAGln	1.75 (0.96–3.03)	2.13 (1.27–3.40)	**0.002**
Hippuric acid	2.38 (1.07–4.99)	2.66 (1.21–5.00)	0.99
P-cresol sulfate	15.0 (7.5–27.6)	19.7 (10.6–31.7)	**0.01**

Note: Bold values denote *P* values < 0.05.
Abbreviations: IQR, interquartile range; PAGln, phenylacetylglutamine; TMAO, trimethylamine N-oxide.
[a]Serum analyte concentrations are presented as median (IQR).
[b]Calculated using univariate logistic regression modeling conditioned on case status.

The association between nutrient precursors of the gut microbiota–dependent metabolite TMAO and lethal prostate cancer risk was evaluated across analyte quartiles (**Fig. 2**). Notably, participants with higher baseline serum levels of choline (Q3 OR: 2.37; 95% CI, 1.35–4.17; Q4 OR: 2.19; 95% CI, 1.23–3.90) or betaine (Q3 OR: 2.13; 95% CI, 1.22–3.70; Q4 OR: 1.86; 95% CI, 1.05–3.30) exhibited increased odds of developing lethal prostate cancer (relative to Q1) in the conditional multivariable logistic regression model adjusted for PSA and BMI. Lethal prostate cancer risk increased across quartiles of choline in a dose-dependent fashion (*P*-trend: 0.005), a trend not observed with increasing quartiles of betaine (*P*-trend: 0.11). Higher baseline serum levels of carnitine were inconsistently associated with lethal prostate cancer in this model, with only Q2 (OR: 1.95; 95% CI, 1.12–3.39) and Q4 (OR: 2.00; 95% CI, 1.13–3.56) achieving statistical significance (*P*-trend: 0.08). None of the other TMA-associated analytes monitored showed significant associations with incident lethal prostate cancer, including γ-butyrobetaine (Q4 OR: 1.35; 95% CI, 0.76–2.37; *P*-trend: 0.11), crotonobetaine (Q4 OR: 0.99; 95% CI, 0.59–1.66; *P*-trend: 0.73), and TMAO (Q4 OR: 1.46; 95% CI, 0.86–2.50; *P*-trend: 0.16).

Gut microbiota–dependent metabolites of aromatic amino acid catabolism were also targeted for analysis (**Fig. 3**). Notably, participants with higher serum levels of baseline PAGln (Q3 OR: 2.54; 95% CI, 1.41–4.58; Q4 OR: 2.55; 95% CI, 1.40–4.64) exhibited increased odds (relative to Q1) of developing incident lethal prostate cancer in the conditional regression model adjusted for BMI and PSA. The risk of prostate cancer–specific mortality generally increased across quartiles of PAGln, suggesting a dose-dependent relationship (*P*-trend: 0.003). Higher levels of hippuric acid (Q3 OR: 1.79; 95% CI, 1.04–3.09; Q4 OR: 1.49; 95% CI, 0.84–2.62; *P*-trend: 0.51) and p-cresol sulfate (Q3 OR: 1.70; 95% CI, 1.00–2.90; Q4 OR: 1.56; 95% CI, 0.92–2.64 *P*-trend: 0.01) were inconsistently linked with an increased risk of lethal prostate cancer in the aforementioned model.

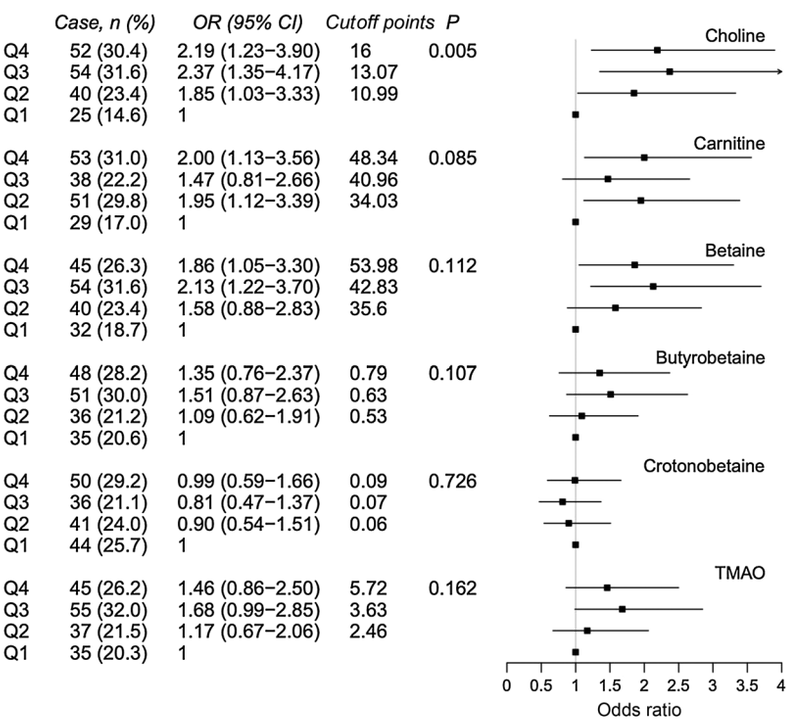

Figure 2.
Forest plot displaying odds associated with lethal prostate cancer by quartile of TMA-associated analytes. The forest plot above displays the OR and 95% CI associated with developing incident lethal prostate cancer by quartile (Q) of baseline serum levels of TMA-associated nutrients or metabolite, relative to the odds calculated for those with baseline serum levels in the first quartile (Q1). The number and percentage of lethal prostate cancer cases with baseline serum levels in each quartile are also displayed, as well as the cutoff points (in micromolar) that define the range of each quartile. The *P*-trend value (*P*), calculated using the Cochran–Armitage test, is also reported and represents the probability that the change in odds ratios across quartiles is due to chance alone. For example, the forest plot shows that participants with baseline serum choline levels in the second, third, and fourth quartiles have increased odds of developing incident lethal prostate cancer (Q2 OR: 1.85, Q3 OR: 2.37, Q4 OR: 2.19) and that the trend of increasing risk with higher quartile designation is statistically significant ($P = 0.005$).

	Case, n (%)	OR (95% CI)	Cutoff points	P
Q4	52 (30.4)	2.55 (1.40–4.64)	3.03	0.002
Q3	58 (33.9)	2.54 (1.41–4.58)	1.75	
Q2	35 (20.5)	1.57 (0.85–2.89)	0.96	
Q1	26 (15.2)	1		
Q4	43 (25.0)	1.49 (0.84–2.62)	4.99	0.513
Q3	49 (28.5)	1.79 (1.04–3.09)	2.38	
Q2	42 (24.4)	1.42 (0.82–2.46)	1.07	
Q1	38 (22.1)	1		
Q4	53 (30.8)	1.56 (0.92–2.64)	27.63	0.014
Q3	56 (32.6)	1.70 (1.00–2.90)	15.02	
Q2	29 (16.9)	0.88 (0.49–1.58)	7.48	
Q1	34 (19.8)	1		

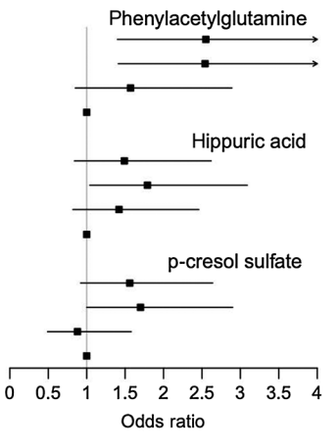

Figure 3.

Forest plot displaying odds associated with lethal prostate cancer by quartile of gut microbiome–derived amino acid metabolites. The forest plot above displays the OR and 95% CI associated with developing incident lethal prostate cancer by quartile (Q) of baseline serum levels of gut microbiome–derived amino acids, relative to the odds calculated for those with baseline serum levels in the first quartile (Q1). The distribution of lethal cases across quartiles, quartile cutoff points, and *P*-trend statistics are also shown. The forest plot shows that participants with baseline serum phenylacetylglutamine levels in the third and fourth quartiles have increased odds of developing incident lethal prostate cancer (Q3 OR: 2.54, Q4 OR: 2.55), and that the trend of increasing risk with higher quartile designation is statistically significant ($P = 0.002$).

Discussion

The impact of diet, the gut microbiome, and host metabolism on human disease has become increasingly evident (28). In this nested case–control study, we show that men with higher levels of choline, betaine, or PAGln have approximately double the odds (relative to Q1) of being later diagnosed with incident lethal prostate cancer. Although previous studies have associated choline, TMAO, or both with oncologic risk, this study is the first to associate an increased risk of lethal prostate cancer with metabolites with gut microbiome–related metabolites (e.g., PAGln) and their precursors (e.g., choline and betaine; refs. 14, 15, 18). Interestingly, our study differed from the results of the ATBC cancer prevention trial, which associated higher levels of TMAO with a diagnosis of aggressive prostate cancer (18).

The significant association of high serum concentrations of PAGln with lethal prostate cancer is intriguing. PAGln is a gut microbiota–dependent metabolite of dietary phenylalanine, an amino acid consumed via dietary protein (29). Phenylalanine is released by digestive proteases and may be converted to PAGln by anaerobic microorganisms in the large intestine (30). Nemet and colleagues used both genetic and pharmacologic studies to demonstrate that this gut microbiota–derived metabolite signals in hosts through multiple adrenergic receptors (α2A, α2B, and β2 adrenergic receptors) (21). In both cellular systems and animal models, numerous cardiovascular-associated effects promoted by PAGln were shown to be attenuated by beta blockers. It is therefore highly relevant that sympathetic signaling, specifically through the β2 adrenergic receptor, has been shown to increase prostate cancer aggressiveness by dysregulating apoptosis, enhancing cell migration, augmenting metastatic potential, and accelerating the epithelial–mesenchymal transition observed in some tumors (31). Moreover, a recent meta-analysis examining 16,825 patients associated beta-blocker use with lower prostate cancer–specific mortality (HR: 0.85; 95% CI, 0.77–0.95; ref. 32). It is tempting to speculate that inhibition of PAGln-driven sympathetic signaling among subjects with higher PAGln levels may account for this interesting clinical observation. Additional investigation is necessary to understand how PAGln elevations modulate lethal prostate cancer risk and whether beta-blocker therapy attenuates PAGln-driven adrenergic hyperstimulation.

One of the more striking findings of the present study is that baseline levels of choline and betaine predicted heightened incident risk of lethal prostate cancer, suggesting that this elevation in oncologic risk is independent of gut microbiome–related metabolism. A prior report associated increased choline intake with greater lethal prostate cancer risk, whereas a previous nested case–control study linked higher levels of serum choline with increased odds of any (lethal or nonlethal) prostate cancer diagnosis (8, 9). Choline may be implicated in oncogenesis given that it serves as a structural component of various phospholipids, making this nutrient essential for maintenance of the cell membrane and cancer growth (33). Specifically, phosphatidylcholine may be metabolized to signaling molecules, including phosphocholine and diacylglycerol, that transduce mitogenic commands for cell growth (34). Choline metabolism has also been observed to be dysregulated in prostate cancer, particularly when choline kinase, an enzyme that facilitates the rate-limiting step in the phosphatidylcholine biosynthesis, is overexpressed (35). These alterations in choline metabolism are particularly relevant, given the clinical utility of using ^{11}C-choline and ^{18}F-choline PET imaging to restage prostate cancer with heightened malignant potential (36).

Serum elevations in the choline oxidation product, betaine, were also associated with lethal prostate cancer. Betaine serves as a methyl donor in pathways that yield *S*-adenosylmethionine, a substrate that mediates DNA and histone methylation (37). Though our study is the first to positively associate elevated baseline betaine levels with incident lethal prostate cancer, other research evaluating dietary intake concluded that greater betaine consumption offers protection from lethal prostate cancer (38). Interestingly, meta-organismal (involving both bacteria and host) betaine and choline metabolism has been shown to alter global DNA methylation patterns, induce epigenetic changes, and even influence behavior in offspring in rodent models (39). Though it is presently unknown whether betaine and choline metabolism induces oncogenic changes in host prostate tissue that result in lethal prostate cancer development, this possibility should be considered.

Further investigation is necessary to assess how choline, betaine, and other one-carbon methyl donors affect gene regulation in lethal prostate cancer carcinogenesis.

The discovery that choline, betaine, and PAGln are independently associated with increased risk of lethal prostate cancer has potential implications for prostate cancer risk modification and screening. If confirmed in independent clinical studies, men with elevated levels of these analytes may consider dietary or pharmacologic risk reduction strategies that modulate meta-organismal choline, betaine, or phenylalanine metabolism. It would also follow that prospective studies might investigate whether assessment of choline, betaine, and PAGln levels help identify patients for whom earlier initiation of PSA screening might be warranted. A large portion of patients with lethal prostate cancer in this study had GS 7 disease, suggesting levels of these analytes could be considered when weighing eligibility for active surveillance in intermediate-risk patients.

This study had several limitations. As an association-based clinical study, this work cannot demonstrate a causal connection between analytes and lethal prostate cancer risk. Additionally, these findings should be considered to be hypothesis-generating until they can be validated by an additional set of baseline serum samples prospectively collected from patients followed closely for the development of lethal prostate cancer. The Prostate Cancer Biorepository Network and NCI Early Detection Research Network could potentially be accessed for validation purposes. Our reliance on a single baseline measurement of targeted nutrients and metabolites could be considered another weakness, given that dietary patterns, lifestyle, and composition of the gut microbiome may not remain static over time. However, similarly designed studies have also associated prediagnostic levels of TMA pathway metabolites with overtly recognizable diseases such as colorectal cancer and CVD (14, 17). Evaluating baseline blood levels of metabolites before incident development of lethal prostate cancer was a strength, as was the nested case–control design that permitted robust control of time-dependent exposures through strict risk-set sampling.

Conclusion

Baseline serum elevations in choline and betaine are associated with incident lethal prostate cancer independent of other nutrients and metabolites in the TMAO pathway when adjusted for PSA and BMI. This association was also shown with higher circulating levels PAGln, a phenylalanine metabolite resulting from gut microbiota metabolism that signals via adrenergic receptors. These findings collectively suggest that diet and the meta-organismal metabolism of choline, betaine, and phenylalanine warrant further study in relation to lethal prostate cancer development.

Authors' Disclosures

Z. Wang reports being named as 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 fully owned subsidiary of Quest Diagnostics, and Procter & Gamble. M.J. Stampfer reports grants from NIH during the conduct of the study. S.L. Hazen reports being named as 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 fully owned subsidiary of Quest Diagnostics, and Procter & Gamble. S.L. Hazen also reports being a paid consultant for Procter & Gamble, and having received research funds from Procter & Gamble and Roche Diagnostics. No disclosures were reported by the other authors.

Authors' Contributions

C.A. Reichard: Conceptualization, formal analysis, investigation, writing–review and editing. **B.D. Naelitz:** Formal analysis, investigation, writing–original draft. **Z. Wang:** Formal analysis, investigation. **X. Jia:** Investigation, methodology, writing–review and editing. **J. Li:** Formal analysis, investigation, writing–review and editing. **M.J. Stampfer:** Conceptualization. **E.A. Klein:** Conceptualization, resources, supervision, writing–review and editing. **S.L. Hazen:** Conceptualization, resources, supervision, funding acquisition, investigation, methodology, writing–review and editing. **N. Sharifi:** Conceptualization, resources, supervision, funding acquisition, investigation, methodology, writing–original draft.

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References

1. Ambrosini GL, Fritschi L, de Klerk NH, Mackerras D, Leavy J. Dietary patterns identified using factor analysis and prostate cancer risk: a case control study in Western Australia. Ann Epidemiol 2008;18:364–70.
2. Chan JM, Gann PH, Giovannucci EL. Role of diet in prostate cancer development and progression. J Clin Oncol 2005;23:8152–60.
3. Kolonel LN, Hankin JH, Nomura AM. Multiethnic studies of diet, nutrition, and cancer in Hawaii. Princess Takamatsu Symp 1985;16:29–40.
4. Hemminki K, Forsti A, Khyatti M, Anwar WA, Mousavi M. Cancer in immigrants as a pointer to the causes of cancer. Eur J Public Health 2014;24:64–71.
5. Gann PH. Diet and prostate cancer risk: the embarrassment of riches. Cancer Causes Control 1998;9:541–3.
6. Richman EL, Kenfield SA, Stampfer MJ, Giovannucci EL, Chan JM. Egg, red meat, and poultry intake and risk of lethal prostate cancer in the prostate-specific antigen-era: incidence and survival. Cancer Prev Res 2011;4:2110–21.
7. Sinha R, Park Y, Graubard BI, Leitzmann MF, Hollenbeck A, Schatzkin A, et al. Meat and meat-related compounds and risk of prostate cancer in a large prospective cohort study in the United States. Am J Epidemiol 2009;170:1165–77.
8. Richman EL, Kenfield SA, Stampfer MJ, Giovannucci EL, Zeisel SH, Willett WC, et al. Choline intake and risk of lethal prostate cancer: incidence and survival. Am J Clin Nutr 2012;96:855–63.
9. Johansson M, Van Guelpen B, Vollset SE, Hultdin J, Bergh A, Key T, et al. One-carbon metabolism and prostate cancer risk: prospective investigation of seven circulating B vitamins and metabolites. Cancer Epidemiol Biomarkers Prev 2009;18:1538–43.
10. Simo C, Garcia-Canas V. Dietary bioactive ingredients to modulate the gut microbiota-derived metabolite TMAO. New opportunities for functional food development. Food Funct 2020;11:6745–76.
11. Oliphant K, Allen-Vercoe E. Macronutrient metabolism by the human gut microbiome: major fermentation by-products and their impact on host health. Microbiome 2019;7:91.
12. Wang Z, Klipfell E, Bennett BJ, Koeth R, Levison BS, Dugar B, et al. Gut flora metabolism of phosphatidylcholine promotes cardiovascular disease. Nature 2011;472:57–63.
13. Tremaroli V, Backhed F. Functional interactions between the gut microbiota and host metabolism. Nature 2012;489:242–9.

14. Bae S, Ulrich CM, Neuhouser ML, Malysheva O, Bailey LB, Xiao L, et al. Plasma choline metabolites and colorectal cancer risk in the Women's Health Initiative Observational Study. Cancer Res 2014;74:7442–52.
15. Guertin KA, Li XS, Graubard BI, Albanes D, Weinstein SJ, Goedert JJ, et al. Serum trimethylamine N-oxide, carnitine, choline, and betaine in relation to colorectal cancer risk in the alpha tocopherol, beta carotene cancer prevention study. Cancer Epidemiol Biomarkers Prev 2017; 26:945–52.
16. Liu ZY, Tan XY, Li QJ, Liao GC, Fang AP, Zhang DM, et al. Trimethylamine N-oxide, a gut microbiota-dependent metabolite of choline, is positively associated with the risk of primary liver cancer: a case-control study. Nutr Metab 2018;15:81.
17. Tang WH, Wang Z, Levison BS, Koeth RA, Britt EB, Fu X, et al. Intestinal microbial metabolism of phosphatidylcholine and cardiovascular risk. N Engl J Med 2013;368:1575–84.
18. Mondul AM, Moore SC, Weinstein SJ, Karoly ED, Sampson JN, Albanes D. Metabolomic analysis of prostate cancer risk in a prospective cohort: the alpha-tocolpherol, beta-carotene cancer prevention (ATBC) study. Int J Cancer 2015; 137:2124–32.
19. Shafi T, Meyer TW, Hostetter TH, Melamed ML, Parekh RS, Hwang S, et al. Free levels of selected organic solutes and cardiovascular morbidity and mortality in hemodialysis patients: results from the Retained Organic Solutes and Clinical Outcomes (ROSCO) Investigators. PLoS One 2015;10:e0126048.
20. Cosola C, Rocchetti MT, Cupisti A, Gesualdo L. Microbiota metabolites: pivotal players of cardiovascular damage in chronic kidney disease. Pharmacol Res 2018; 130:132–42.
21. Nemet I, Saha PP, Gupta N, Zhu W, Romano KA, Skye SM, et al. A cardiovascular disease-linked gut microbial metabolite acts via adrenergic receptors. Cell 2020;180:862–77.
22. Andriole GL, Crawford ED, Grubb RL 3rd, Buys SS, Chia D, Church TR, et al. Mortality results from a randomized prostate-cancer screening trial. N Engl J Med 2009;360:1310–9.
23. Miller AB, Feld R, Fontana R, Gohagan JK, Jatoi I, Lawrence W Jr, et al. Changes in and impact of the death review process in the prostate, lung, colorectal and ovarian (PLCO) Cancer Screening Trial. Rev Recent Clin Trials 2015;10:206–11.
24. Koeth RA, Levison BS, Culley MK, Buffa JA, Wang Z, Gregory JC, et al. gamma-Butyrobetaine is a proatherogenic intermediate in gut microbial metabolism of L-carnitine to TMAO. Cell Metab 2014;20:799–812.
25. Morano C, Zhang X, Fricker LD. Multiple isotopic labels for quantitative mass spectrometry. Anal Chem 2008;80:9298–309.
26. Löster H, Seim H. Synthesis of [methyl-14C]crotonobetaine from DL-[methyl-14C]carnitine. J Labelled Compd Radiopharm 1996;38:179–86.
27. Wang Z, Bergeron N, Levison BS, Li XS, Chiu S, Jia X, et al. Impact of chronic dietary red meat, white meat, or non-meat protein on trimethylamine N-oxide metabolism and renal excretion in healthy men and women. Eur Heart J 2019;40: 583–94.
28. Koh A, Backhed F. From association to causality: the role of the gut microbiota and its functional products on host metabolism. Mol Cell 2020;78:584–96.
29. Poesen R, Claes K, Evenepoel P, de Loor H, Augustijns P, Kuypers D, et al. Microbiota-derived phenylacetylglutamine associates with overall mortality and cardiovascular disease in patients with CKD. J Am Soc Nephrol 2016;27: 3479–87.
30. Seakins JW. The determination of urinary phenylacetylglutamine as phenylacetic acid. Studies on its origin in normal subjects and children with cystic fibrosis. Clin Chim Acta 1971;35:121–31.
31. Braadland PR, Ramberg H, Grytli HH, Tasken KA. beta-Adrenergic receptor signaling in prostate cancer. Front Oncol 2014;4:375.
32. Lu H, Liu X, Guo F, Tan S, Wang G, Liu H, et al. Impact of beta-blockers on prostate cancer mortality: a meta-analysis of 16,825 patients. Onco Targets Ther 2015;8:985–90.
33. Glunde K, Bhujwalla ZM, Ronen SM. Choline metabolism in malignant transformation. Nat Rev Cancer 2011;11:835–48.
34. Zeisel SH, da Costa KA. Choline: an essential nutrient for public health. Nutr Rev 2009;67:615–23.
35. Challapalli A, Trousil S, Hazell S, Kozlowski K, Gudi M, Aboagye EO, et al. Exploiting altered patterns of choline kinase-alpha expression on human prostate tissue to prognosticate prostate cancer. J Clin Pathol 2015;68:703–9.
36. Ackerstaff E, Pflug BR, Nelson JB, Bhujwalla ZM. Detection of increased choline compounds with proton nuclear magnetic resonance spectroscopy subsequent to malignant transformation of human prostatic epithelial cells. Cancer Res 2001;61:3599–603.
37. Li Z, Vance DE. Phosphatidylcholine and choline homeostasis. J Lipid Res 2008; 49:1187–94.
38. Han P, Bidulescu A, Barber JR, Zeisel SH, Joshu CE, Prizment AE, et al. Dietary choline and betaine intakes and risk of total and lethal prostate cancer in the Atherosclerosis Risk in Communities (ARIC) Study. Cancer Causes Control 2019;30:343–54.
39. Romano KA, Martinez-Del Campo A, Kasahara K, Chittim CL, Vivas EI, Amador-Noguez D, et al. Metabolic, epigenetic, and transgenerational effects of gut bacterial choline consumption. Cell Host Microbe 2017;22:279–90.