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INTRODUCTION: **We recently described the sulfur microbial diet, a pattern of intake associated with increased gut sulfur-metabolizing bacteria and incidence of distal colorectal cancer (CRC). We assessed whether this risk differed by CRC molecular subtypes or presence of intratumoral microbes involved in CRC pathogenesis (*Fusobacterium nucleatum* and *Bifidobacterium* spp.).**

METHODS: **We performed Cox proportional hazards modeling to examine the association between the sulfur microbial diet and incidence of overall and distal CRC by molecular and microbial subtype in the Health Professionals Follow-Up Study (1986–2012).**

RESULTS: **We documented 1,264 incident CRC cases among 48,246 men, approximately 40% of whom had available tissue data. After accounting for multiple hypothesis testing, the relationship between the sulfur microbial diet and CRC incidence did not differ by subtype. However, there was a suggestion of an association by prostaglandin synthase 2 (PTGS2) status with a multivariable adjusted hazard ratio for highest vs lowest tertile of sulfur microbial diet scores of 1.31 (95% confidence interval: 0.99–1.74, $P_{trend} = 0.07$, $P_{heterogeneity} = 0.04$) for PTGS2-high CRC. The association of the sulfur microbial diet with distal CRC seemed to differ by the presence of intratumoral *Bifidobacterium* spp. with an adjusted hazard ratio for highest vs lowest tertile of sulfur microbial diet scores of 1.65 (95% confidence interval: 1.14–2.39, $P_{trend} = 0.01$, $P_{heterogeneity} = 0.03$) for *Bifidobacterium*-negative distal CRC. We observed no apparent heterogeneity by other tested molecular markers.**

DISCUSSION: **Greater long-term adherence to the sulfur microbial diet could be associated with PTGS2-high and *Bifidobacterium*-negative distal CRC in men. Additional studies are needed to further characterize the role of gut microbial sulfur metabolism and CRC.**

SUPPLEMENTARY MATERIAL accompanies this paper at http://links.lww.com/CTG/A652.

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INTRODUCTION

Colorectal cancer (CRC) is the third most common and second most lethal cancer globally (1). A modifiable lifestyle is a major risk factor in the development of sporadic CRC (2–5). The link between dietary intake and CRC risk has been well-documented (6–8). However, the degree to which gut microbial communities mediate the relationship between diet and carcinogenesis is less well-understood (9,10). Specifically, sulfur-metabolizing bacteria have been implicated in CRC etiopathogenesis (11–16). This phylogenetically diverse group of microbes can convert dietary sulfur into hydrogen sulfide (H_2S) gas (17). Data from *in vitro* and animal studies have linked H_2S in the colorectum to several carcinogenic processes, including direct DNA damage (18–20), promotion of a proinflammatory phenotype (17,21), disruption of the protective colonic mucus bilayer (22), and inappropriate cell cycle progression (11). We recently identified a gut microbiome–derived dietary pattern—the sulfur microbial diet—associated with a greater abundance of sulfur-metabolizing bacteria in healthy adults. This dietary pattern was characterized by a higher intake of processed meats and low-calorie drinks and decreased intake of vegetables and legumes, foods previously linked to risk of CRC (23). Long-term adherence to this pattern of intake was subsequently associated with an increased incidence of distal colon and rectal cancers (23).

Separately, several studies have previously described molecular and intratumoral microbial heterogeneity of CRC by both anatomic subsite (24–26) and dietary intake (27–33)—i.e., different dietary risk factors may give rise to heterogenous tumors with respect to molecular subtypes, the intratumoral microbes they harbor, and even where in the colorectum these neoplastic lesions tend to occur. In particular, several studies have shown that the association between dietary risk factors and CRC may differ by Kirsten rat sarcoma (*KRAS*), v-raf murine sarcoma viral oncogene homolog B1 (*BRAF*), phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (*PIK3CA*), long-interspersed nucleotide element-1 (LINE-1) methylation, microsatellite instability (MSI), CpG island methylator phenotype (CIMP), prostaglandin synthase 2 (PTGS2) (cyclooxygenase-2), and CTNNB1 (beta-catenin) status (27,28,31,34–40). Similarly, previous work suggests that diet can alter the abundance of 2 important microbes implicated in CRC pathogenesis, *Fusobacterium nucleatum* and *Bifidobacterium* spp. (41–43).

We hypothesized that the association between a diet associated with a greater abundance of sulfur-metabolizing bacteria and distal CRC is driven by particular molecular CRC subtypes or relative enrichment or depletion of intratumoral CRC-associated microbes (44,45). The study of these molecular and microbial CRC subtypes may offer a more complete and precise mechanistic understanding of the relationship between dietary intake, the gut microbiome, and colorectal tumorigenesis. Thus, in a large US prospective cohort, we examined the relationship between the sulfur microbial diet and incidence of CRC according to several molecular tumor markers and intratumoral microbes previously linked to CRC.

METHODS

Study population

We enrolled participants from the Health Professionals Follow-up Study (HPFS). The HPFS is an ongoing prospective cohort study of 51,529 US male podiatrists, dentists, osteopathic physicians, veterinarians, pharmacists, and optometrists. Participants were aged 40–75 years at enrollment in 1986 and followed with biennial questionnaires on medical, lifestyle, and other health-related information. Dietary intake was assessed every 4 years through validated semiquantitative food frequency questionnaires. Follow-up among eligible subjects exceeds 90% (46). The cohort has previously been described in detail (46). The study protocol was approved by the Institutional Review Boards of the Brigham and Women's Hospital, the Harvard T.H. Chan School of Public Health, and those of participating registries as required.

Assessment of the sulfur microbial diet

Self-reported dietary intake was assessed through semiquantitative food frequency questionnaires administered every 4 years from 1986 to 2010. These questionnaires have been validated and described in detail (47). The food frequency questionnaire includes 131 food items with specified serving sizes, of which participants indicated their average frequency of consumption over the past year. Intake frequency ranged from never or less than once per month to 6+ times per day and was converted to servings/d. Total caloric intake was calculated by summing energy intake across all food groups.

Using a previously described method, we linked dietary intake of food and the log-transformed relative abundance of 43 putative sulfur-metabolizing species (see Table 1, Supplementary Digital Content 1, http://links.lww.com/CTG/A652) (23). Briefly, the sulfur microbial diet was derived using long-term dietary intake and longitudinal stool metagenomes from 307 men in the Men's Lifestyle Validation Study, a nested developmental cohort within the HPFS (~0.6% of the original HPFS population). Reduced rank regression and stepwise linear regression analyses were used to identify food groups associated with increased or decreased relative abundance of 43 sulfur-metabolizing bacterial species by summing the intake of foods retained from the final stepwise linear regression analyses weighted by their regression coefficients. The component food groups were processed meat, liquor, and low-calorie drinks (each positively associated with the abundance of sulfur-metabolizing bacteria), as well as beer, fruit juice, legumes, mixed (other) vegetables, and sweets/desserts (each negatively associated). We found that the sulfur microbial diet explained 2% of variation in Bray-Curtis distances (R^2), comparable in magnitude with recent antibiotic use (1.5%).

To represent long-term usual dietary habits (48), sulfur microbial diet scores were updated at each follow-up cycle using the cumulative average method with each score averaged across all assessments before the current questionnaire. Dietary scores were then categorized into tertiles. The food-based sulfur microbial diet score represents a data-driven prediction for how much sulfur-metabolizing bacteria an individual may harbor over the long term. Notably, although the sulfur microbial diet shared some foods with dietary patterns previously linked to CRC (e.g., the Western diet) (27,49–51), sulfur microbial diet scores were not associated with Western dietary scores, which suggests that the sulfur microbial diet may capture a novel signal in the established diet-CRC relationship.

Assessment of CRC cases and subtype

Cases of incident CRC were reported by participants on biennial questionnaires or were identified by next of kin, postal authorities, the National Death Index, or death certificates. Study physicians blinded to risk factor status reviewed relevant records to

confirm cases and extract data on anatomic site, histology, and stage of the tumor. Distal colorectal tumors were defined as tumors located from the splenic flexure to the rectum. For CRC cases with available tumor tissue, we retrieved formalin-fixed paraffin-embedded tissue blocks from hospitals throughout the United States, as previously described (52). Adjacent normal tissue and tumor sections from all CRC cases were reviewed by a pathologist (S.O).

The presence of intratumoral *F. nucleatum* (53) and *Bifidobacterium* spp. (54) was assessed by real-time polymerase chain reaction (PCR). Positivity was defined as a detectable level of bacterial DNA, and negativity was defined as an undetectable level as previously described. PCR and pyrosequencing were performed to detect mutations in *KRAS* (codons 12, 13, 61, and 146) (55), *BRAF* (codon 600) (56), and *PIK3CA* (exons 9 and 20) (57). LINE-1 methylation status was measured by PCR on bisulfite-treated DNA and pyrosequencing and categorized as high if ≥ 60% of sites were methylated and low if <60% were methylated (58). MSI status was determined using 10 microsatellite markers (D17S250, D18S55, D18S56, D18S67, D18S487, D2S123, D5S346, BAT25, BAT26, and BAT40) (59). Tumors were classified as MSI-high if 30% or more of the markers demonstrated instability. We quantified DNA methylation using polymerase chain reaction in 8 CIMP-specific promoters (*MLH1*, *NEUROG1*, *RUNX3*, *CACNA1G*, *CDKN2A* [p16], *CRABP1*, *IGF2*, and *SOCS1*) (60). We classified tumors as CIMP-high if 6 or more promoters were methylated and as CIMP-low/negative if 0–5 promoters were methylated (61). PTGS2 (cyclooxygenase-2) (62) and nuclear CTNNB1 (beta-catenin) (52) expression was measured by immunohistochemistry as described previously and graded as negative/low, intermediate, and high.

Assessments of covariates

Height and weight were reported at study inception, and weight was updated biennially. Body mass index (BMI) was calculated as weight in kilograms/height in meters squared. Physical activity was self-reported using validated questionnaires every 2–4 years (63). We also assessed and updated the age they started or stopped smoking, number of cigarettes smoked daily, family history of CRC among first-degree relatives, regular use of aspirin, previous health care engagement (assessed as a visit to a care provider in the past 2 years), and history of lower gastrointestinal endoscopy.

Statistical analyses

We excluded participants with a history of CRC or inflammatory bowel disease before study baseline or during follow-up. We also excluded individuals with missing information on dietary intake and those who reported implausible energy intake (<800 or >4,200 kcal/d) at baseline. Follow-up time accrued from study enrollment until the date of CRC diagnosis, death from any cause, or the end of follow-up (January 31, 2012), whichever occurred first.

To examine whether the association between the sulfur microbial diet and CRC differed by subtype, we used Cox proportional hazards models with a duplication method for competing risks and computed age and multivariable-adjusted hazard ratios (HRs) and their 95% confidence intervals (CIs). We tested for heterogeneity by using a meta-regression method with a subtype-specific random effect term (64). We separately evaluated heterogeneity by subtype among distal colon and rectal tumors to evaluate subsite-specific effects, given the previously observed association between the sulfur microbial diet and tumors arising from the distal colorectum (23).

To test for trend, participants were assigned the median value of their dietary pattern tertile. Multivariate analyses were adjusted for family history of CRC in a first-degree relative (yes/no), BMI (categories, <21, 21–24.9, 25–29.9, 30–31.9, and ≥32 kg/m^2), physical activity (metabolic equivalent task hr/wk, quintiles), smoking (categories: never, past, and current: 1–14, 15–24, and ≥25 cigarettes/d), regular aspirin use (yes/no), total caloric intake (continuous), previous lower endoscopy within the past 2 years (yes/no), and physical examination in the past 2 years (yes/no). All analyses were additionally stratified by age (continuous) and calendar year. Covariates were chosen *a priori* and prospectively updated. For missing data, nonmissing data from 1 previous data cycle were carried forward. Two-sided P values <0.005 were considered statistically significant as recommended (65). SAS version 9.4 (Cary, NC) was used for all statistical analysis.

RESULTS

Among 48,246 eligible participants with 1,077,517 person-years of follow-up, the mean age of participants at study baseline was 54.2 ± 9.8 years. Subjects in the highest tertile of the sulfur microbial diet generally had poorer health-related indices. They tended to have higher BMIs, consume more alcohol, and were more likely to smoke and take aspirin regularly (Table 1).

We documented 1,264 incident cases of CRC. Among CRC cases with information on anatomic site, we identified 637 distal colon and rectal cancers. Tumor tissue data were available for approximately 40% of total CRCs and 50% of distal colon and rectal cancers. The baseline characteristics of participants with CRC whose tumors we analyzed were overall similar to those of participants whose tumors we did not analyze (data not shown).

We examined whether the association between the sulfur microbial diet and total CRC differed by relevant molecular tumor subtypes (Table 2). After accounting for multiple hypothesis testing, there was no conclusive evidence of heterogeneity. However, the association of the sulfur microbial diet with CRC incidence seemed to differ by PTGS2 status ($P_{heterogeneity} = 0.04$) when accounting for multiple testing with an α level of 0.005. Multivariable HRs for the highest tertile of sulfur microbial diet scores (vs the lowest) were 1.31 (95% CI: 0.99–1.74; $P_{trend} = 0.07$) for PTGS2-high subtype, 0.99 (95% CI: 0.61–1.59; $P_{trend} = 0.95$) for PTGS2-intermediate subtype, and 0.65 (95% CI, 0.36–1.15; $P_{trend} = 0.17$) for PTGS2-negative/low subtype. There was no evidence for heterogeneity by *KRAS*, *BRAF*, *PIK3CA*, LINE-1, CIMP, MSI, or CTNNB1 status. Similarly, the relationship between the sulfur microbial diet and total CRC did not differ by the presence of intratumoral microbes *F. nucleatum* or *Bifidobacterium* spp. (Table 3).

Given our previous findings linking the sulfur microbial diet and cancers of the distal colorectum, we subsequently focused on CRC that had arisen in the distal colon or rectum. We found trends for PTGS2-high (HR 1.58 [95% CI: 1.09–2.28], $P_{trend} = 0.02$), CTNNB1-high (HR 2.10 [95% CI: 1.04–4.21], $P_{trend} = 0.04$), and *KRAS*-wildtype tumors (HR 1.52 [95% CI: 1.06–2.17], $P_{trend} = 0.02$), but we did not observe statistically significant heterogeneity ($P_{heterogeneity} = 0.08$, $P_{heterogeneity} = 0.25$, and $P_{heterogeneity} = 0.06$, respectively) (Table 4). There was no apparent heterogeneity by *BRAF*, *PIK3CA*, LINE-1, CIMP, or MSI status among distal colon and rectal cancers (Table 4). We observed that the association of the sulfur microbial diet with distal colon and

Table 1. Baseline age-standardized characteristics by the sulfur microbial diet score, HPFS (1986)

	Sulfur microbial diet score		
	Tertile 1 (n = 16,123)	Tertile 2 (n = 16,195)	Tertile 3 (n = 15,928)
Age, yr	54.2 (10.0)	54.2 (9.8)	54.1 (9.6)
BMI, kg/m^2	25.0 (3.1)	25.4 (3.2)	26.2 (3.4)
Physical activity, METS-hr/wk	21.0 (27.3)	18.3 (26.3)	16.9 (24.4)
Former smokers, %	39	41	46
Current smokers, %	7.8	8.4	13
White race, %	96	95	96
Family history of CRC, %	15	15	15
Regular aspirin use, %	28	29	31
Screening lower endoscopy within the past 2 yr, %	27	27	27
Physical examination within the past 2 yr, %	52	51	50
Alcohol intake (g/d)	11.9 (16.1)	8.1 (10.8)	14.3 (18.0)
Total energy intake, kcal/d	2,268 (623)	1863 (552)	1829 (582)
Dietary intake (servings/wk)			
Processed meats	1.9 (2.1)	2.2 (2.2)	3.7 (3.9)
Liquor	1.1 (2.6)	1.5 (3.0)	4.8 (7.6)
Low-calorie drinks	1.4 (2.8)	2.0 (3.4)	7.1 (9.6)
Beer	3.6 (6.7)	1.2 (2.3)	1.0 (1.9)
Fruit juice	8.0 (8.0)	5.0 (4.3)	3.7 (3.9)
Legumes	4.5 (3.2)	2.7 (1.7)	2.2 (1.6)
Other vegetables	5.1 (4.0)	3.3 (2.3)	2.7 (2.1)
Sweets and desserts	10.9 (11.2)	6.1 (5.5)	4.8 (5.1)

All values other than age have been directly standardized to the age distribution (in 5-year age groups) of the entire study population. Mean (SD) is presented for continuous variables.
BMI, body mass index; CRC, colorectal cancer; HPFS, Health Professionals Follow-Up Study; kcal, kilocalories; METS, metabolic equivalent of tasks; m, meters.

rectal cancers seemed to differ by the presence of intratumoral *Bifidobacterium* spp., although the difference was not statistically significant ($P_{heterogeneity} = 0.03$) when accounting for adjustment for multiple testing with an α level of 0.005 (Table 5). The multivariable HR for the highest tertile of sulfur microbial diet scores (vs the lowest) was 1.65 (95% CI: 1.14–2.39; $P_{trend} = 0.01$) for *Bifidobacterium*-negative distal CRC, but these associations were not observed in *Bifidobacterium*-negative total CRC ($P_{trend} = 0.27$). Similar to the result with overall CRC, there was no observed heterogeneity in the association of diet and incidence of distal colon and rectal cancers by *F. nucleatum* status (Table 5).

DISCUSSION

In a large US prospective cohort of men, we found that the association between the sulfur microbial diet and CRC incidence may differ by molecular and microbial subtypes. We found that a previously described sulfur microbial dietary pattern associated with an increased abundance of cancer-associated sulfur-metabolizing bacteria may be more strongly associated with PTGS2-high tumors. Finally, we observed a relative depletion of intratumoral *Bifidobacterium* spp., a taxon associated with beneficial health and anticancer effects, associated with higher sulfur microbial diet scores. These results provide early evidence that may link lifestyle and the development of specific CRC subtypes through dietary modulation of gut microbial communities.

Diet has played a long-recognized role in altering CRC risk (8,50,66). Modulation of the gut microbiome may be one mechanism through which certain dietary exposures contribute to or protect against carcinogenesis along the colorectum (10,67,68). Among intestinal microbial communities, sulfur-metabolizing bacteria are an important class of organisms implicated in CRC tumorigenesis. Through the metabolism of dietary sulfur, these organisms may release H_2S, a compound whose gut luminal concentration is critically determined by the abundance of sulfur-metabolizing bacteria, as opposed to the sulfur content of foods alone (69,70). Interestingly, beer intake contributed negatively to sulfur microbial diet scores. Although some data have shown an increased risk of CRC associated with heavy beer consumption (71), our results may suggest that modest beer consumption, in combination with other food groups, could be protective against CRC through modulation of the sulfur-metabolizing bacteria. Both H_2S and sulfur-metabolizing bacteria have been implicated in carcinogenesis in the colorectum, possibly through mechanisms related to free radical formation (18–20), immunomodulation (21), disruption of the protective colonic mucus bilayer (22), and impaired cell cycle arrest and apoptosis (11).

Table 2. Association between sulfur microbial diet scores and incidence of CRC by molecular subtype

Molecular subtype	T1	T2	T3	P_{trend}	$P_{heterogeneity}$[a]
KRAS					
KRAS-wildtype CRC					
No. of cases (n = 339), No.	101	123	115		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.23 (0.94–1.61)	1.23 (0.93–1.62)	0.14	
Multivariable HR (95% CI)[c]	1 (referent)	1.19 (0.91–1.56)	1.15 (0.87–1.52)	0.34	
KRAS-mutant CRC					0.24
No. of cases (n = 223), No.	79	76	68		
Age-adjusted HR (95% CI)[b]	1 (referent)	0.97 (0.70–1.33)	0.95 (0.68–1.32)	0.73	
Multivariable HR (95% CI)[c]	1 (referent)	0.94 (0.68–1.29)	0.89 (0.63–1.24)	0.47	
BRAF					
BRAF-wildtype CRC					
No. of cases (n = 517), No.	161	181	175		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.13 (0.91–1.41)	1.18 (0.94–1.48)	0.15	
Multivariable HR (95% CI)[c]	1 (referent)	1.10 (0.88–1.37)	1.11 (0.88–1.39)	0.40	
BRAF-mutant CRC					0.22
No. of cases (n = 45), No.	17	18	10		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.07 (0.55–2.07)	0.64 (0.29–1.41)	0.29	
Multivariable HR (95% CI)[c]	1 (referent)	1.04 (0.53–2.02)	0.60 (0.27–1.32)	0.22	
PIK3CA					
PIK3CA-wildtype CRC					
No. of cases (n = 434), No.	135	157	142		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.17 (0.92–1.48)	1.13 (0.89–1.44)	0.33	
Multivariable HR (95% CI)[c]	1 (referent)	1.14 (0.9–1.45)	1.07 (0.84–1.38)	0.59	
PIK3CA-mutant CRC					0.68
No. of cases (n = 94), No.	33	31	30		
Age-adjusted HR (95% CI)[b]	1 (referent)	0.93 (0.57–1.53)	1.01 (0.61–1.67)	0.98	
Multivariable HR (95% CI)[c]	1 (referent)	0.90 (0.55–1.48)	0.96 (0.58–1.58)	0.85	
LINE-1					
LINE-1 methylation high CRC					
No. of cases (n = 364), No.	115	131	118		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.15 (0.89–1.49)	1.13 (0.86–1.47)	0.39	
Multivariable HR (95% CI)[c]	1 (referent)	1.12 (0.86–1.44)	1.05 (0.80–1.37)	0.74	
LINE-1 methylation low CRC					0.89
No. of cases (n = 200), No.	64	71	65		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.12 (0.79–1.57)	1.10 (0.77–1.56)	0.61	
Multivariable HR (95% CI)[c]	1 (referent)	1.08 (0.77–1.52)	1.02 (0.71–1.45)	0.93	
CIMP					
CIMP-low/negative CRC					
No. of cases (n = 454), No.	144	154	156		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.08 (0.85–1.36)	1.17 (0.93–1.49)	0.19	
Multivariable HR (95% CI)[c]	1 (referent)	1.05 (0.83–1.33)	1.10 (0.87–1.40)	0.44	
CIMP-high CRC					0.15
No. of cases (n = 63), No.	24	26	13		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.10 (0.63–1.92)	0.60 (0.31–1.19)	0.18	

Table 2. (*continued*)

Molecular subtype	T1	T2	T3	P_{trend}	$P_{heterogeneity}$[a]
Multivariable HR (95% CI)[c]	1 (referent)	1.07 (0.61–1.87)	0.56 (0.29–1.12)	0.12	
MSI					
Non–MSI-high CRC					
No. of cases (n = 489), No.	157	166	166		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.07 (0.85–1.34)	1.16 (0.92–1.45)	0.22	
Multivariable HR (95% CI)[c]	1 (referent)	1.04 (0.83–1.30)	1.08 (0.86–1.37)	0.51	
MSI-high CRC					0.97
No. of cases (n = 64), No.	17	29	18		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.74 (0.95–3.17)	1.16 (0.59–2.25)	0.67	
Multivariable HR (95% CI)[c]	1 (referent)	1.68 (0.92–3.07)	1.08 (0.55–2.11)	0.84	
PTGS2					
PTGS2-negative/low CRC					
No. of cases (n = 89), No.	31	39	19		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.29 (0.80–2.08)	0.69 (0.39–1.23)	0.26	
Multivariable HR (95% CI)[c]	1 (referent)	1.25 (0.78–2.02)	0.65 (0.36–1.15)	0.17	
PTGS2-intermediate CRC					0.04
No. of cases (n = 106), No.	36	36	34		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.03 (0.65–1.64)	1.05 (0.65–1.69)	0.86	
Multivariable HR (95% CI)[c]	1 (referent)	1.01 (0.63–1.60)	0.99 (0.61–1.59)	0.95	
PTGS2-high CRC					
No. of cases (n = 328), No.	93	118	117		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.30 (0.98–1.71)	1.39 (1.05–1.84)	0.03	
Multivariable HR (95% CI)[c]	1 (referent)	1.26 (0.95–1.67)	1.31 (0.99–1.74)	0.07	
CTNNB1 (Nuclear)					
Nuclear CTNNB1-negative/low CRC					
No. of cases (n = 239), No.	73	96	70		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.36 (1.00–1.86)	1.08 (0.77–1.51)	0.64	
Multivariable HR (95% CI)[c]	1 (referent)	1.33 (0.98–1.81)	1.03 (0.73–1.44)	0.88	
Nuclear CTNNB1-intermediate CRC					0.12
No. of cases (n = 170), No.	54	58	58		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.09 (0.75–1.59)	1.18 (0.81–1.72)	0.40	
Multivariable HR (95% CI)[c]	1 (referent)	1.07 (0.73–1.55)	1.12 (0.77–1.64)	0.56	
Nuclear CTNNB1-high CRC					
No. of cases (n = 87), No.	24	28	35		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.21 (0.70–2.10)	1.65 (0.98–2.79)	0.06	
Multivariable HR (95% CI)[c]	1 (referent)	1.18 (0.68–2.05)	1.56 (0.92–2.65)	0.09	

BRAF, v-raf murine sarcoma viral oncogene homolog B1; CI, confidence interval; CIMP, CpG island methylator phenotype; CRC, colorectal cancer; CTNNB1, catenin beta-1; HR, hazard ratio; KRAS, Kirsten rat sarcoma; LINE-1, long-interspersed nucleotide element-1; MSI, microsatellite instable; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PTGS2, prostaglandin synthase 2; T, tertile.

[a]Heterogeneity was tested using a meta-regression method with a subtype-specific random effect term among multivariable models.

[b]Models stratified by age and calendar year and adjusted for total caloric intake (kcals/d).

[c]Models stratified as above and additionally adjusted for family history of CRC in any first-degree relative, BMI (categories), physical activity (METS-hr/wk), smoking (never vs past vs current), recent aspirin use, history of previous lower gastrointestinal endoscopy, and history of physical examination.

Our finding of possible diet-induced heterogeneity of risk builds on a well-established body of research on the molecular heterogeneity of CRC. Several previous studies have linked certain dietary exposures with an increased risk of specific CRC molecular subtypes (27–32). In addition, there are data supporting distinct gut microbial communities with different CRC

Table 3. Association between sulfur microbial diet scores and incidence of CRC by intratumoral microbes of interest

Molecular subtype	T1	T2	T3	P_{trend}	$P_{heterogeneity}$[a]
Fusobacterium nucleatum					
F. nucleatum–negative CRC					
No. of cases (n = 462), No.	145	170	147		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.19 (0.94–1.49)	1.11 (0.88–1.41)	0.38	
Multivariable HR (95% CI)[c]	1 (referent)	1.15 (0.92–1.45)	1.05 (0.82–1.33)	0.72	
F. nucleatum–positive CRC					0.35
No. of cases (n = 60), No.	21	12	27		
Age-adjusted HR (95% CI)[b]	1 (referent)	0.57 (0.28–1.16)	1.37 (0.77–2.44)	0.25	
Multivariable HR (95% CI)[c]	1 (referent)	0.56 (0.27–1.14)	1.30 (0.73–2.32)	0.33	
Bifidobacterium spp.					
Bifidobacterium spp.–negative CRC					
No. of cases (n = 378), No.	115	132	131		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.15 (0.89–1.48)	1.23 (0.95–1.60)	0.11	
Multivariable HR (95% CI)[c]	1 (referent)	1.12 (0.87–1.45)	1.16 (0.89–1.51)	0.27	
Bifidobacterium spp.–positive CRC					0.33
No. of cases (n = 148), No.	49	55	44		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.14 (0.77–1.68)	0.98 (0.65–1.48)	0.93	
Multivariable HR (95% CI)[c]	1 (referent)	1.10 (0.74–1.62)	0.91 (0.60–1.39)	0.68	

CI, confidence interval; CRC, colorectal cancer; HR, hazard ratio; spp, species; T, tertile.
[a]Heterogeneity was tested using a meta-regression method with a subtype-specific random effect term among multivariable models.
[b]Models stratified by age and calendar year and adjusted for total caloric intake (kcals/d).
[c]Models stratified as above and additionally adjusted for family history of CRC in any first-degree relative, BMI (categories), physical activity (METS-hr/wk), smoking (never vs past vs current), recent aspirin use, history of previous lower gastrointestinal endoscopy, and history of physical examination.

subtypes (72,73). Our results extend these findings and provide new insights in carcinogenic processes induced by the complex interaction between dietary intake and microbial ecology.

We expand on the link between dietary exposures, gut microbial ecology, and carcinogenesis. The genus *Bifidobacterium* seems to inhibit colorectal carcinogenesis (45,74), modulate gut mucosal barrier function (75), and enhance antitumor immunity (76). Our finding that higher sulfur microbial diet scores may be associated with a higher incidence of *Bifidobacterium*-negative distal CRC may suggest that this relationship could be mediated by alterations in a distinct class of microbes capable of metabolizing sulfur. Although it is unclear why this association was observed only among distal tumors, it may be a result of heterogeneity in microbial biogeography (77,78). By contrast, we did not identify heterogeneity by *F. nucleatum* status. A species capable of dietary sulfur metabolism, *F. nucleatum* is relatively rare in healthy populations and was not included in the derivation of the sulfur microbial diet. The observed absence of an effect by *F. nucleatum* status may reflect distinct regional ecology in the distal colorectum, where *F. nucleatum*–positive tumors are less common (79,80).

Although the precise mechanism by which diet and microbial communities could increase the risk of PTGS2-high cancers is unknown, our findings may implicate carcinogenesis by proinflammatory mechanisms and abnormalities in bile acid metabolism (81,82). PTGS2 is involved in the conversion of free arachidonic acid into prostaglandins, which are potent proinflammatory mediators in colorectal carcinogenesis (83,84). PTGS2-mediated inflammation secondary to cell membrane damage (82), activation of tumor-associated fibroblasts (85), and epidermal growth factor receptor signaling (86) can be stimulated in response to deoxycholic acid, a secondary bile acid. Deoxycholic acid is a potential carcinogen that is produced in high levels in individuals who consume diets high in animal protein, a primary component of the sulfur microbial diet. Animal protein contains high levels of sulfur-containing amino acids such as taurine and cysteine, the former of which can be transformed to deoxycholic acid in the presence of taurine-respiring bacteria such as *Bilophila wadsworthia* (82), a microbe whose relative enrichment was associated with higher sulfur microbial diet scores (23). Taken together, these studies offer possible mechanistic explanations for the elevated risk associated with a key component of the sulfur microbial diet, directly implicating aberrant signaling along PTGS2 pathways.

There are several strengths to this study. We leveraged a large, prospective cohort with over 20 years of follow-up with validated dietary instruments. Consequently, we were able to assess the link between long-term dietary trends and development of cancer. At the same time, the prospective nature of data collection limited the potential for recall and ascertainment bias. The detailed data across a wide range of lifetime exposures allowed us to adjust for multiple potential confounders. Finally, the sulfur microbial diet was previously identified through incorporating metagenomic taxonomic assignment and metatranscriptomic functional activity.

Table 4. Association between sulfur microbial diet scores and incidence of distal colon and rectal cancers by molecular subtype

Molecular subtype	T1	T2	T3	P_{trend}	$P_{heterogeneity}$[a]
KRAS					
KRAS-wildtype distal CRC					
No. of cases (n = 208), No.	53	72	83		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.35 (0.94–1.94)	1.66 (1.16–2.36)	0.005	
Multivariable HR (95% CI)[c]	1 (referent)	1.30 (0.91–1.88)	1.52 (1.06–2.17)	0.02	
KRAS-mutant distal CRC					0.06
No. of cases (n = 109), No.	36	39	34		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.09 (0.69–1.72)	1.02 (0.63–1.64)	0.95	
Multivariable HR (95% CI)[c]	1 (referent)	1.03 (0.65–1.63)	0.93 (0.57–1.50)	0.76	
BRAF					
BRAF-wildtype distal CRC					
No. of cases (n = 310), No.	85	108	117		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.27 (0.94–1.70)	1.46 (1.09–1.96)	0.01	
Multivariable HR (95% CI)[c]	1 (referent)	1.22 (0.91–1.63)	1.34 (1.00–1.81)		0.05
BRAF-mutant distal CRC					0.43
No. of cases (n = 8), No.	3	3	2		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.01 (0.20–5.00)	0.68 (0.11–4.07)	0.68	
Multivariable HR (95% CI)[c]	1 (referent)	0.98 (0.20–4.85)	0.62 (0.10–3.75)	0.61	
PIK3CA					
PIK3CA-wildtype distal CRC					
No. of cases (n = 257), No.	69	91	97		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.32 (0.96–1.82)	1.49 (1.08–2.05)	0.02	
Multivariable HR (95% CI)[c]	1 (referent)	1.27 (0.92–1.76)	1.37 (0.99–1.90)	0.06	
PIK3CA-mutant distal CRC					0.53
No. of cases (n = 39), No.	12	14	13		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.15 (0.53–2.49)	1.17 (0.53–2.59)	0.70	
Multivariable HR (95% CI)[c]	1 (referent)	1.10 (0.51–2.38)	1.08 (0.49–2.39)	0.86	
LINE-1					
LINE-1 methylation high distal CRC					
No. of cases (n = 186), No.	55	62	69		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.14 (0.79–1.65)	1.36 (0.94–1.95)	0.10	
Multivariable HR (95% CI)[c]	1 (referent)	1.09 (0.75–1.59)	1.24 (0.86–1.79)	0.26	
LINE-1 methylation low distal CRC					0.61
No. of cases (n = 134), No.	34	50	50		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.48 (0.95–2.30)	1.57 (1.01–2.45)	0.05	
Multivariable HR (95% CI)[c]	1 (referent)	1.42 (0.91–2.20)	1.42 (0.91–2.23)	0.13	
CIMP					
CIMP-low/negative distal CRC					
No. of cases (n = 285), No.	81	98	106		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.21 (0.89–1.64)	1.39 (1.03–1.88)	0.03	
Multivariable HR (95% CI)[c]	1 (referent)	1.17 (0.86–1.59)	1.28 (0.94–1.74)	0.12	
CIMP-high distal CRC					0.30
No. of cases (n = 10), No.	4	5	1		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.26 (0.34–4.72)	0.29 (0.03–2.63)	0.34	

Table 4. (*continued*)

Molecular subtype	T1	T2	T3	P_{trend}	$P_{heterogeneity}$[a]
Multivariable HR (95% CI)[c]	1 (referent)	1.22 (0.32–4.54)	0.27 (0.03–2.42)	0.29	
MSI					
Non–MSI-high distal CRC					
No. of cases (n = 299), No.	83	104	112		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.27 (0.94–1.70)	1.45 (1.08–1.95)	0.01	
Multivariable HR (95% CI)[c]	1 (referent)	1.22 (0.90–1.64)	1.34 (0.99–1.81)	0.06	
MSI-high distal CRC					0.06
No. of cases (n = 12), No.	2	4	6		
Age-adjusted HR (95% CI)[b]	1 (referent)	2.04 (0.37–11.14)	3.30 (0.66–16.44)	0.13	
Multivariable HR (95% CI)[c]	1 (referent)	1.96 (0.36–10.72)	3.04 (0.61–15.15)	0.16	
PTGS2					
PTGS2-negative/low distal CRC					
No. of cases (n = 40), No.	13	16	11		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.28 (0.61–2.66)	0.95 (0.42–2.13)	0.91	
Multivariable HR (95% CI)[c]	1 (referent)	1.22 (0.58–2.55)	0.87 (0.38–1.95)	0.74	
PTGS2-intermediate distal CRC					0.08
No. of cases (n = 49), No.	16	16	17		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.03 (0.51–2.07)	1.15 (0.58–2.29)	0.71	
Multivariable HR (95% CI)[c]	1 (referent)	1.00 (0.50–2.00)	1.05 (0.52–2.10)	0.90	
PTGS2-high distal CRC					
No. of cases (n = 206), No.	51	75	80		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.49 (1.04–2.15)	1.71 (1.19–2.45)	0.004	
Multivariable HR (95% CI)[c]	1 (referent)	1.44 (1.00–2.08)	1.58 (1.09–2.28)	0.02	
CTNNB1 (Nuclear)					
Nuclear CTNNB1-negative/low distal CRC					
No. of cases (n = 110), No.	27	45	38		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.70 (1.05–2.76)	1.53 (0.93–2.53)	0.11	
Multivariable HR (95% CI)[c]	1 (referent)	1.64 (1.01–2.67)	1.42 (0.85–2.35)	0.20	
Nuclear CTNNB1-intermediate distal CRC					0.25
No. of cases (n = 114), No.	37	32	45		
Age-adjusted HR (95% CI)[b]	1 (referent)	0.87 (0.54–1.40)	1.31 (0.84–2.04)	0.23	
Multivariable HR (95% CI)[c]	1 (referent)	0.84 (0.52–1.36)	1.22 (0.78–1.91)	0.37	
Nuclear CTNNB1-high distal CRC					
No. of cases (n = 60), No.	12	23	25		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.94 (0.96–3.92)	2.27 (1.13–4.55)	0.02	
Multivariable HR (95% CI)[c]	1 (referent)	1.88 (0.93–3.79)	2.10 (1.04–4.21)	0.04	

BRAF, v-raf murine sarcoma viral oncogene homolog B1; CI, confidence interval; CIMP, CpG island methylator phenotype; CRC, colorectal cancer; CTNNB1, catenin beta-1; HR, hazard ratio; KRAS, Kirsten rat sarcoma; MSI, microsatellite instable; LINE-1, long-interspersed nucleotide element-1; PIK3CA, phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha; PTGS2, prostaglandin synthase 2; T, tertile.

[a]Heterogeneity was tested using a meta-regression method with a subtype-specific random effect term among multivariable models.

[b]Models stratified by age and calendar year and adjusted for total caloric intake (kcals/d).

[c]Models stratified as above and additionally adjusted for family history of CRC in any first-degree relative, BMI (categories), physical activity (METS-hr/wk), smoking (never vs past vs current), recent aspirin use, history of previous lower gastrointestinal endoscopy, and history of physical examination.

We also acknowledge several limitations. Despite the large size of the cohort, there was limited power to detect heterogeneity in the association of dietary exposure and subtypes of CRC. The sulfur microbial diet was derived among bacterial species with sufficient prevalence and abundance in the healthy human gut. As a result, our dietary exposure may not necessarily capture specific

Table 5. Association between sulfur microbial diet scores and incidence of distal colon and rectal cancers by intratumoral microbes of interest

Molecular subtype	T1	T2	T3	P_{trend}	$P_{heterogeneity}$[a]
F. nucleatum					
F. nucleatum–negative distal CRC					
No. of cases (n = 256), No.	72	92	92		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.28 (0.93–1.75)	1.37 (0.99–1.89)	0.05	
Multivariable HR (95% CI)[c]	1 (referent)	1.23 (0.9–1.69)	1.27 (0.91–1.76)	0.16	
F. nucleatum–positive distal CRC					0.17
No. of cases (n = 32), No.	10	5	17		
Age-adjusted HR (95% CI)[b]	1 (referent)	0.5 (0.17–1.46)	1.83 (0.83–4.02)	0.10	
Multivariable HR (95% CI)[c]	1 (referent)	0.48 (0.16–1.41)	1.70 (0.77–3.74)	0.14	
Bifidobacterium spp.					
Bifidobacterium spp.–negative distal CRC					
No. of cases (n = 212), No.	49	80	83		
Age-adjusted HR (95% CI)[b]	1 (referent)	1.61 (1.12–2.32)	1.79 (1.24–2.57)	0.002	
Multivariable HR (95% CI)[c]	1 (referent)	1.55 (1.08–2.24)	1.65 (1.14–2.39)	0.01	
Bifidobacterium spp.–positive distal CRC					0.03
No. of cases (n = 84), No.	31	25	28		
Age-adjusted HR (95% CI)[b]	1 (referent)	0.81 (0.47–1.37)	0.96 (0.57–1.62)	0.86	
Multivariable HR (95% CI)[c]	1 (referent)	0.77 (0.45–1.32)	0.88 (0.52–1.49)	0.62	

CI, confidence interval; CRC, colorectal cancer; HR, hazard ratio; spp, species; T, tertile.
[a]Heterogeneity was tested using a meta-regression method with a subtype-specific random effect term among multivariable models.
[b]Models stratified by age and calendar year and adjusted for total caloric intake (kcals/d).
[c]Models stratified as above and additionally adjusted for family history of CRC in any first-degree relative, BMI (categories), physical activity (METS-hr/wk), smoking (never vs past vs current), recent aspirin use, history of previous lower gastrointestinal endoscopy, and history of physical examination.

pathogenic bacterial strains or less prevalent but highly active taxa in the CRC microbiome (e.g., *F. nucleatum*) (44,87,88). Our study was conducted among male, mostly white health professionals, potentially limiting the generalizability of our results. It is possible that stronger associations between diet and CRC incidence would have been observed in different cohorts with a wider range of dietary quality.

In conclusion, we demonstrate that long-term adherence to a dietary pattern associated with a greater abundance of sulfur-metabolizing bacteria may be associated with a greater incidence of PTGS2-high CRC and *Bifidobacterium*-negative distal CRC among adult men. Our data suggest that dietary modulation of the gut microbiome may be an attractive population-level preventive strategy in high-risk individuals. Additional studies are needed to further characterize the relationship between dietary sulfur metabolism, gut microbial ecology, and CRC among large and diverse study populations.

CONFLICTS OF INTEREST

Guarantors of the article: Daniel R. Sikavi, MD, Long H. Nguyen, MD, MS, Koichiro Haruki, MD, PhD, Tomotaka Ugai, MD, PhD, Shuji Ogino, MS, MD, PhD, Curtis Huttenhower, PhD, and Andrew T. Chan, MD, MPH.

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Use of standardized official symbols: We use Human Genome Organization (HUGO)-approved official symbols (or root symbols) for genes and gene products, including BRAF, CACNA1G, CDKN2A, CRABP1, CTNNB1, IGF2, KRAS, MLH1, NEUROG1, PIK3CA, PTGS2, RUNX3, SOCS1, and WNT, all of which are described at www.genenames.org. Gene symbols are italicized, whereas symbols for gene products are not italicized.

Study Highlights

WHAT IS KNOWN

- ✓ Sulfur-metabolizing bacteria have been implicated in colorectal cancer (CRC).
- ✓ We previously showed that a diet that enriches for sulfur-metabolizing bacteria was associated with an increased risk of distal CRC in adult men.
- ✓ It is unclear whether this relationship is driven by associations with particular molecular or microbially-enriched tumor subtypes.

WHAT IS NEW HERE

- ✓ The sulfur microbial diet may be associated with PTGS2-high CRC in adult men.
- ✓ The sulfur microbial diet may be associated with distal colon and rectal cancers depleted of intratumoral *Bifidobacteria* in adult men.

TRANSLATIONAL IMPACT

- ✓ Dietary modulation of the gut microbiome may contribute to colorectal carcinogenesis.
- ✓ Dietary manipulation of the gut microbiome may reduce CRC risk.

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