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Dietary Patterns and Risk of Colorectal Cancer Subtypes Classified by *Fusobacterium nucleatum* in Tumor Tissue

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Abstract

Importance—*Fusobacterium nucleatum* appears to play a role in colorectal carcinogenesis through suppression of host immune response to tumor. Evidence also suggests that diet influences intestinal *F. nucleatum.* However, the role of *F. nucleatum* in mediating the relationship between diet and the risk of colorectal cancer is unknown.

Objective—To test the hypothesis that the associations of prudent diets (rich in whole grains and dietary fiber) and Western diets (rich in red and processed meat, refined grains, and desserts) with colorectal cancer risk may differ according to the presence of *F. nucleatum* in tumor tissue.

Design—Prospective cohort study.

Setting—The Nurses' Health Study (1980–2012) and the Health Professionals Follow-up Study (1986–2012).

Participants—121,700 US female nurses and 51,529 US male health professionals aged 30 to 55 years and 40 to 75 years, respectively, at enrollment.

Exposures—Prudent and Western dietary patterns.

Main Outcomes and Measures—Incidence of colorectal carcinoma subclassified by *F. nucleatum* status in tumor tissue, determined by quantitative polymerase chain reaction.

Results—We documented 1,019 incident colon and rectal cancer cases with available *F. nucleatum* data among predominantly white 137,217 individuals over 26–32 years of follow-up encompassing 3,643,562 person-years. The association of prudent diet with colorectal cancer significantly differed by tissue *F. nucleatum* status ($P_{heterogeneity}$ = .01). Prudent diet score was associated with a lower risk of *F. nucleatum*-positive cancers [P_{trend} = .003; multivariable hazard ratio of 0.43 (95% confidence interval 0.25–0.72) for the highest vs. the lowest prudent score quartile], but not with *F. nucleatum*-negative cancers (P_{trend} = .47). Dietary component analyses suggested possible differential associations for the cancer subgroups according to intakes of dietary fiber ($P_{heterogeneity}$ = .02). There was no significant heterogeneity between the subgroups according to Western dietary pattern scores ($P_{heterogeneity}$ = .23).

Conclusions and Relevance—Prudent diets rich in whole grains and dietary fiber are associated with a lower risk for *F. nucleatum*-positive colorectal cancer but not *F. nucleatum*-negative cancer, supporting a potential role for intestinal microbiota in mediating the association between diet and colorectal neoplasms.

INTRODUCTION

Accumulating evidence suggests that the human gut microbiome is linked to colorectal cancer development.[1–4] *Fusobacterium nucleatum* has been found to be enriched in colorectal cancer tissue relative to normal adjacent colonic tissue, and is detected at higher levels in stool among colorectal cancer cases compared to cancer-free controls.[1,5–10] Recent experimental data suggest that *F. nucleatum* may contribute to colorectal carcinogenesis through modulation of host immunity and activation of pathways associated with cellular proliferation.[9,11,12] Furthermore, a higher amount of *F. nucleatum* in colorectal cancer tissue has been linked to shorter survival, proximal tumor location, and specific tumor molecular features such as high-level CpG island methylator phenotype and microsatellite instability.[13–15]

Prudent dietary patterns – rich in fruits, vegetables, and whole grains – have been associated with a lower risk of colorectal cancer and adenoma, as reviewed in a recent systematic meta-analysis.[16–22] In contrast, Western dietary patterns – dominated by red and processed meats – have been linked with colorectal carcinogenesis.[16,18] Although mechanisms underlying these diet-cancer associations remain unclear, it is postulated that the gut microbiota may play a mediating role.[23] Recently, in a dietary intervention study, stool *F. nucleatum* levels markedly increased after participants were switched from a prudent-style, high-fiber, low-fat diet to a low-fiber, high-fat diet.[24] In addition, accumulating data suggest that low fiber consumption and high meat intake may be associated with altered bacterial and metagenomic profiles as well as an inflammatory phenotype determined by serum levels of metabolites.[25–28]

Based on these findings, we hypothesized that the inverse association between prudent diets and risk of colorectal cancer might be more evident for a cancer subgroup enriched with tissue *F. nucleatum* than for that without detectable tissue *F. nucleatum*. To test this hypothesis, we utilized two U.S.-nationwide prospective cohort studies, the Nurses' Health Study and the Health Professional Follow-up Study. These two studies offered a unique opportunity to integrate prospectively collected, regularly updated dietary intake data with tissue microbial features in incident colorectal cancers that occurred over long-term follow-up.

METHODS

Study population

We used data drawn from two ongoing prospective cohort studies, the Nurses' Health Study (NHS) and the Health Professionals Follow-up Study (HPFS). The NHS began in 1976 among 121,700 U.S. female nurses aged 30 to 55 years at enrollment. The HPFS began in 1986 among 51,529 U.S. male health professionals aged 40 to 75 years at enrollment. In both cohorts, participants have returned questionnaires every two years with follow-up rates exceeding 90% to provide information about lifestyle and dietary factors, medication use, and diagnoses of colorectal cancer and other diseases.

A total of 137,217 individuals (47,449 men and 89,768 women) were included in this study. We excluded participants with implausibly high or low caloric intakes (i.e., <600 or >3,500 kcal/day for women and <800 or >4,200 kcal/day for men), missing dietary pattern data, or with a history of ulcerative colitis or cancer (except for non-melanoma skin cancer) prior to baseline (1980 for the NHS and 1986 for the HPFS) (see eMethods). The Institutional Review Board at the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health approved this study.

Assessment of diet

Participants reported average food intake over the preceding year (of each questionnaire return) through semi-quantitative food frequency questionnaires (FFQ), which have been previously validated and described.[29] Total nutrient intake was calculated by summing intakes from all foods, and adjusted for total energy intake by the residual method. As previously described, total dietary fiber was calculated according to methods from the Association of Official Analytic Chemists.[30] For this analysis, we used information from FFQs administered in following years: 1980, 1984, 1986, 1990, 1994, 1998, 2002, 2006, and 2010 for the NHS, and 1986, 1990, 1994, 1998, 2002, 2006, and 2010 for the HPFS.

Assessment of colorectal cancer cases

In both cohorts, incident cases of colorectal cancer were reported by participants through 2012 follow-up for the HPFS and NHS. We identified and confirmed lethal colorectal cancer cases through information from various sources including next-of-kin, the National Death Index, death certificates, and medical records. A study physician, blinded to exposure information, reviewed records and extracted data on histological type, anatomic location, and stage. We attempted to collect formalin-fixed paraffin-embedded (FFPE) tissue specimens from hospitals throughout the U.S. as previously detailed.[9] Cases with available tissue data (n = 1,019) for the current study were similar to those without tissue data (n = 2,241) with regard to patient and clinical characteristics (see eMethods).

Fusobacterium nucleatum analysis

DNA was extracted from colorectal cancer tissue obtained from sections of FFPE tumor blocks using QIAamp DNA FFPE tissue kits (Qiagen). We performed a real-time polymerase chain reaction (PCR) assay using custom TaqMan primer/probe sets (Applied Biosystems) for the *nusG* gene of *F. nucleatum*.[9] The interassay coefficient of variation of cycle threshold (Ct) values from each of five selected specimens in five different batches was <1% for all targets in the validation study.[14] *F. nucleatum* positivity was defined as a detectable level of *F. nucleatum* DNA within 45 PCR cycles, and *F. nucleatum* negativity as an undetectable level with a proper amplification of human reference gene *SLCO2A1*.

Statistical analyses

We used SAS software version 9.3 (SAS Institute Inc.) for all statistical analyses, and all statistical tests are two-sided. To account for multiple testing for the two primary hypotheses (related to Prudent and western dietary scores) associated with the two tumor subtype

variables, we adjusted the two-sided α level to .01 (≈ .05/4) by simple Bonferroni correction in our primary and secondary analysis.

Two maximally uncorrelated dietary patterns – one named "prudent" and another named "Western" – were derived by principal component analysis (PCA), as previously described and validated with good reproducibility.[16,31] Factor loadings were derived based on the correlations between food groups and the two derived factors. Each participant was assigned a factor score, determined by adding the reported frequencies of food group intakes, weighted by the factor loadings. These factor scores were then standardized to have a mean of 0 and standard deviation of 1. To capture long-term habitual consumption, we calculated the cumulative average of the prudent (or Western) dietary pattern scores from preceding FFQs up to each questionnaire cycle. Then, the cumulative average score was categorized into sex-specific quartiles, and used as the primary exposure variable.

Using Cox proportional hazards models, we computed hazard ratios (HR) to examine the association of the prudent (or Western) dietary score with incidence of colorectal cancer. To test for trend, participants were assigned to the median score of their sex-specific dietary pattern quartile and then this variable was entered into the models as a continuous term. The covariates included in the multivariable models are described in Table 1 and the supplementary methods.

To examine whether the association between dietary patterns and incidence of colorectal cancer subgroups differed according to tissue *F. nucleatum* status, we used Cox proportional hazards regression models with a duplication method for competing risks data. As our primary hypothesis testing, we tested for heterogeneity by using a likelihood ratio test, comparing a model that allows for separate associations of dietary patterns and risk of cancer subgroups according to *F. nucleatum* status with a model that assumes a common association.[32] In secondary analyses, we examined heterogeneity of the associations with cancer subgroups in relation to dominant factor loadings for the prudent dietary pattern using cumulative average intakes of fruits, vegetables, legumes, and whole grains as well as energy-adjusted intakes of fat, fiber, and protein, all of which were categorized into quartiles.

RESULTS

Two major, uncorrelated dietary patterns were identified by factor analysis. The prudent dietary pattern was characterized by high intake of vegetables, fruits, whole grains, and legumes, while the Western dietary pattern was characterized by red and processed meats, refined grains, and desserts (eTable 1). Consistent with prior analyses,[16] participants with high prudent scores in the HPFS and NHS tended to smoke less, exercise more, and have greater rates of lower gastrointestinal endoscopy whereas Western pattern scores were associated with behaviors typically considered unhealthy (eTable 2).

After 26 years (in HPFS) and 32 years (in NHS) of follow-up encompassing 3,643,562 person-years, we documented 1,019 incident colorectal cancers with available data on tissue *F. nucleatum* status. Among these cancer cases, there were 125 (12%) *F. nucleatum*-positive

tumors and 894 (88%) *F. nucleatum*-negative tumors. We examined the association of prudent and Western dietary pattern scores with incidence of overall colorectal cancer. Western dietary pattern scores showed a trend towards associations with overall risk of colorectal cancer in the HPFS (eTable 3), and the combined cohort (Table 1); however, statistical significance was not reached with the adjusted α level of.01. We did not observe significant heterogeneity in the associations of the dietary scores with colorectal cancer risk between the two cohorts ($P \geq .21$). To maximize statistical power, we used the combined cohort for further analyses.

We then tested our primary hypothesis that the association of prudent and Western diets with colorectal cancer incidence might differ according to the presence of *F. nucleatum* in tumor tissue. Notably, the association between prudent dietary pattern and risk of colorectal cancer significantly differed by tumor *F. nucleatum* status ($P_{\text{heterogeneity}} = .01$) (Table 1). We found a significant inverse association of prudent dietary scores with *F. nucleatum*-positive cancer risk ($P_{\text{trend}} = .003$), but not with *F. nucleatum*-negative cancer risk ($P_{\text{trend}} = .47$). Comparing participants in the highest prudent dietary score quartile to those in the lowest quartile, the multivariable hazard ratio (HR) for *F. nucleatum*-positive tumors was 0.43 [95% confidence interval (CI), 0.25–0.72]; in contrast, the corresponding HR for *F. nucleatum*-negative tumors was 0.95 (95% CI, 0.77–1.17). We found similar differential associations by *F. nucleatum* status in men (HPFS) and women (NHS) though statistical power was limited (eTable 4). In addition, though statistical power was limited, we found similar results when levels of *F nucleatum* were categorized as low or high on the basis of the median cut point among *F. nucleatum*-positive cases, as performed in our previous analyses (eTable 5).[9] As we observed that the fraction of colorectal cancers enriched with *F. nucleatum* gradually decreased from cecum to rectum,[33] we conducted exploratory analyses stratified by tumor location (eTable 6). The differential association of prudent diet score with colorectal cancer by tissue *F. nucleatum* status appeared to be consistent in both proximal and distal cancer strata.

When we examined the association of the Western dietary pattern with colorectal cancer subgroups according to tumor *F. nucleatum* status, although Western dietary pattern scores appeared more strongly associated with *F. nucleatum*-positive cancer risk, there was no significant heterogeneity between the subgroups ($P_{\text{heterogeneity}} = 0.23$) (Table 1).

In a secondary analysis, we sought to determine if specific food groups might explain the observed differential associations between prudent dietary patterns and risk of colorectal cancer according to *F. nucleatum* status. We examined the top four dominantly contributing food groups to the prudent diet pattern (vegetables, fruits, legumes, and whole grains) in relation to the risk of colorectal cancer according to *F. nucleatum* status (eTable 7). We observed no significant heterogeneity (with the adjusted α of.01).

Finally, to further determine whether any specific macronutrient components of the prudent dietary pattern might explain the observed differential associations according to *F. nucleatum* status, we explored associations of fiber, fat, and protein intake with colorectal cancer subgroups (eTable 8). There appeared to be heterogeneity in the differential association of fiber intake with cancer subgroups classified by *F. nucleatum* status

($P_{heterogeneity}$ =.02), similar to the findings for prudent dietary pattern scores. Comparing participants in the highest quartile of fiber intake (>26 g per day for men and >19 g per day for women) to those in the lowest quartile (<18 per day for men and <13 g per day for women), the multivariable hazard ratio (HR) for *F. nucleatum*-positive tumors was 0.54 [95% confidence interval (CI), 0.32–0.92]; in contrast, the corresponding HR for *F. nucleatum*-negative tumors was 1.13 (95% CI, 0.92–1.40). In further exploratory analyses, we found that intakes of cereal-derived fiber might be differentially associated with colorectal cancer according to *F. nucleatum* status ($P_{heterogeneity}$ =.01) (eTable 9). We did not observe such heterogeneity for fat or protein.

DISCUSSION

In the two U.S.-nationwide prospective cohorts, we found that participants with higher long-term prudent dietary pattern scores were associated with a lower risk of *F. nucleatum*-positive colorectal cancers but not *F. nucleatum*-negative cancers. Our data also suggest that higher intakes of dietary fiber, one of the components of the prudent diet, may be associated with a lower risk of *F. nucleatum*-positive colorectal cancer but not *F. nucleatum*-negative cancer. These findings support the hypothesis that the possible cancer-preventative effects of prudent diets rich in dietary fiber may be mediated by modulation of specific species in the gut microbiota, and subsequent alteration of the amount of *F. nucleatum* in local colonic tissue. To our knowledge, our study represents the first to examine the intersection of diet and incidence of colorectal cancer subgroups according to microbial status in human tumor tissue.

The potential role of diet in modulating the risk of a variety of diseases including colorectal cancer has been widely recognized[23,34] According to the World Cancer Research Fund and American Institute for Cancer Research, foods with fiber including whole grains are one of the strongest factors linked to decreasing the risk of colorectal cancer.[35] Importantly, however, there has been considerable heterogeneity in the epidemiological data associating prudent dietary patterns and its major components with colorectal cancer.[36] Our results here suggest that the inconsistency in the association of prudent dietary patterns (and its components) with lower colorectal cancer risk may be in part due to differential associations with cancer subgroups according to *F. nucleatum* in tumor tissue. In addition, given our recent findings between increasing amounts of *F. nucleatum* DNA in colorectal cancer tissue and worsened survival,[14] our data lends additional support to the promotion of healthy diets to reduce mortality from colorectal cancer.

The precise mechanism by which prudent diets rich in dietary fiber may lower *F. nucleatum*-enriched cancer incidence remains unclear. Accumulating evidence suggests that long-term dietary fiber intake has a profound impact on the gut microbiome, specifically through promotion of microbial diversity and by lowering levels of inflammatory metabolites.[25,37–40] Of note, a recent study showed that a two-week feeding intervention switching rural-dwelling South Africans from a high-fiber, low-fat diet to a low-fiber, high-fat diet was associated with an increase in *F. nucleatum* measured by PCR in the stool.[24] In addition, some have hypothesized that the variation observed in *F. nucleatum* levels in colorectal cancers collected from Spain, Vietnam, Japan, and the U.S. may be due to differences in

dietary practices in these countries.[5,41] Furthermore, in a cross sectional study, participants with advanced adenoma were associated with lower dietary fiber intakes as well as distinct fecal microbiome communities when compared to healthy controls.[42] It is plausible that abundance of microbiota-accessible carbohydrates from prudent diets may influence bacterial fermentation of dietary fiber resulting in altered levels of short-chain fatty acids. These changes may alter pH, increase transit time of gut contents, or lead to differences in local immune surveillance, which are less hospitable for non-native species such as *F. nucleatum* to establish themselves in the colonic niche and potentiate colorectal carcinogenesis.[24,25,43,44] Taken together, these data provide evidence for substantial influences of diet on the gut microbiome, which may in turn influence tumorigenesis.

There are several strengths in this study. First, our dietary data were prospectively collected and have been well-validated.[29] Second, our data were detailed and updated such that we could examine long-term effects of overall dietary patterns, specific food groups, and macronutrients in relation to colorectal cancer risk. Third, we collected detailed data on multiple potential confounders, although residual confounding cannot be excluded. Finally, our molecular pathological epidemiology (MPE) research[45] provides refined risk estimates for specific cancer subgroups such as *F. nucleatum*-positive cancer, and thereby offers insights into pathogenesis and causality. Notably, molecular subtyping in the MPE approach can gather pathogenetically similar cases, and thus can enhance statistical inference (even with a relatively small number of cases).[46] The current study represents emerging unique microbial MPE research,[47] where the microbial feature in tumor tissue can serve as a pathogenic signature.

We acknowledge limitations of this study. First, this study is observational, and residual confounding may be an issue. Nevertheless, adjustment for a variety of known risk factors for colorectal cancer showed no substantial impact on our results. Second, our diet data were derived from food frequency questionnaires (FFQ), and subject to measurement errors. Nonetheless, studies have shown that FFQ can better capture long-term dietary intakes than detailed diet diaries in a limited period.[48] Third, with the use of FFPE tissue specimens, routine histopathology procedures might have influenced performance characteristics of our PCR assay to detect *F. nucleatum*. Nonetheless, we conducted a rigorous validation study, which showed high precision of our PCR assay to detect *F. nucleatum*.[9] Moreover, our assay has previously been shown to have high specificity for *F. nucleatum*.[6] Fourth, we could not collect FFPE blocks from all colorectal cancer cases in the cohorts; nonetheless, cases with available tissue were generally similar to those without tissue with regard to patient characteristics. Fifth, because our participants were all health professionals and mostly white, generalizability of our findings to other populations need to be examined in future studies.

In summary, we have shown that prudent diet is associated with a lower risk of *F. nucleatum*-positive colorectal cancer but not *F. nucleatum*-negative cancer. Our data generate new hypotheses about how the intestinal microbiota may mediate the association between diet and colorectal neoplasms. Further studies are needed to confirm these findings and determine the potential utility of characterization of *F. nucleatum* in colonic mucosa, tumor, or stool as a biomarker for personalized nutritional, probiotic or antibiotic interventions. In

addition, our findings underscore the importance of future large-scale prospective studies that examine the gut microbiota to understand the complex intersection of diet, the gut microbiome, and carcinogenesis.[49]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CI	confidence interval
Ct	cycle threshold
FFPE	formalin-fixed paraffin-embedded
FFQ	food frequency questionnaire
HPFS	Health Professionals Follow-up Study
HR	hazard ratio
NHS	Nurses' Health Study
PCA	principal component analysis
PCR	polymerase chain reaction
SD	standard deviation

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Table 1

Hazard ratios (HRs) of incident colorectal cancer, overall and by *F. nucleatum* status, according to prudent or Western dietary score quartiles in the combined cohort of the Health Professionals Follow-up Study (1986–2012) and the Nurses' Health Study (1980–2012).

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}[3]	$P_{heterogeneity}$[4]
Prudent dietary pattern						
Overall colorectal cancer						
Person-years	913,569	907,676	912,395	909,922		
No. of cases (n=1,019)	250	248	268	253		
Age-adjusted HR (95% CI)[1]	1 (referent)	0.93 (0.77–1.11)	0.90 (0.75–1.08)	0.79 (0.65–0.95)	.01	
Multivariable HR (95% CI)[2]	1 (referent)	0.95 (0.80–1.14)	0.95 (0.79–1.14)	0.85 (0.69–1.03)	.08	
F. nucleatum (+) colorectal cancer						
No. of cases (n=125)	43	26	34	22		
Age-adjusted HR (95% CI)[1]	1 (referent)	0.54 (0.33–0.89)	0.67 (0.42–1.05)	0.40 (0.24–0.67)	.0001	
Multivariable HR (95% CI)[2]	1 (referent)	0.56 (0.34–0.92)	0.70 (0.44–1.10)	0.43 (0.25–0.72)	.003	
F. nucleatum (−) colorectal cancer						.01
No. of cases (n=894)	207	222	234	231		
Age-adjusted HR (95% CI)[1]	1 (referent)	1.01 (0.83–1.22)	0.96 (0.79–1.16)	0.88 (0.72–1.08)	.15	
Multivariable HR (95% CI)[2]	1 (referent)	1.04 (0.86–1.26)	1.00 (0.83–1.22)	0.95 (0.77–1.17)	.47	
Western dietary pattern						
Overall colorectal cancer						
Person-years	910,656	910,525	910,465	911,916		
No. of cases (n=1,019)	244	275	243	257		
Age-adjusted HR (95% CI)[1]	1 (referent)	1.24 (1.04–1.48)	1.21 (1.00–1.46)	1.46 (1.18–1.82)	.001	
Multivariable HR (95% CI)[2]	1 (referent)	1.19 (1.00–1.43)	1.12 (0.92–1.36)	1.29 (1.03–1.62)	.05	
F. nucleatum (+) colorectal cancer						
No. of cases (n=125)	25	33	33	34		
Age-adjusted HR (95% CI)[1]	1 (referent)	1.42 (0.84–2.40)	1.59 (0.94–2.69)	1.92 (1.12–3.29)	.01	
Multivariable HR (95% CI)[2]	1 (referent)	1.37 (0.81–2.31)	1.49 (0.88–2.53)	1.69 (0.98–2.90)	.05	

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}[3]	$P_{heterogeneity}$[4]
F. nucleatum (−) colorectal cancer						.23
No. of cases (n=894)	219	242	210	223		
Age-adjusted HR (95% CI)[1]	1 (referent)	1.25 (1.03–1.50)	1.16 (0.95–1.42)	1.42 (1.13–1.78)	.006	
Multivariable HR (95% CI)[2]	1 (referent)	1.20 (0.99–1.44)	1.08 (0.88–1.33)	1.25 (0.99–1.58)	.12	

[1] Stratified by age, calendar year, and gender and adjusted for total caloric intake (kcal/day)

[2] As above, and additionally adjusted for family history of colorectal cancer in any first-degree relative, history of previous endoscopy, pack-years of smoking (never, 0–4, 5–19, 20–39, or ≥40), body mass index (kg/m^2), physical activity (MET-hours/week), regular aspirin or NSAID use (≥2 tablets/week).

[3] Tests for trend were conducted using the median value of each quartile category as a continuous variable.

[4] We tested for heterogeneity by using a likelihood ratio test, comparing a model that allows separate associations for the two colorectal cancer subgroups (i.e., *F. nucleatum*-positive and negative subgroups) with a model that assumes a common association.

Abbreviations: CI, confidence interval; HR, hazard ratio; MET, metabolic equivalent task; NSAID, non-steroidal anti-inflammatory drug.