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Associations Between Intake of Folate and Related Micronutrients with Molecularly Defined Colorectal Cancer Risks in the Iowa Women's Health Study

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Abstract

Folate and related micronutrients may affect colorectal cancer (CRC) risk, but the molecular mechanism(s) remain incompletely defined. We analyzed associations between dietary folate, vitamin B6, vitamin B12 and methionine with incident CRC, overall and by microsatellite instability (MSS/MSI-L or MSI-H), CpG island methylator phenotype (CIMP-negative or CIMP-positive), *BRAF* mutation (negative or positive), and *KRAS* mutation (negative or positive) status in the prospective, population-based Iowa Women's Health Study (IWHS; 55–69 years at baseline; n = 41,836). Intake estimates were obtained from baseline, self-reported food frequency questionnaires. Molecular marker data were obtained for 514 incident CRC cases. Folate intake was inversely associated with overall CRC risk in age-adjusted Cox regression models, while methionine intake was inversely associated with overall CRC risk in multivariable-adjusted models (RR = 0.81; 95% CI = 0.69–0.95; p trend = 0.001 and RR = 0.72; 95% CI = 0.54–0.96; p trend = 0.03 for highest versus lowest quartiles, respectively). None of the dietary exposures were associated with MSI, CIMP, *BRAF*, or *KRAS* defined CRC subtypes. These data provide minimal support for major effects from the examined micronutrients on overall or molecularly defined CRC risks in the IWHS cohort.

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Keywords

Colorectal Cancer; Alcohol; Older Women; Cohort Study

INTRODUCTION

Colorectal cancer (CRC) is the third most common malignancy among women in the United States, with an estimated 69,360 new cases occurring in 2011 (1). Dietary factors are thought to have a major influence on CRC risk, although the effects of specific micro- and macronutrients remain controversial (2, 3). Folic acid, a water soluble B-complex vitamin (B9), has been widely investigated as a candidate CRC chemoprevention agent, given its pivotal involvement in key physiologic processes such as one-carbon metabolism, nucleic acid synthesis, and epigenetic modification (4–7). Yet, the relationship between folate intake and colorectal carcinogenesis appears to be quite complex (8, 9).

Several molecular mechanisms have been proposed through which relative folate availability could modulate CRC risk (10). For example, folate deficiency can lead to misincorporation of uracil during DNA replication, with consequent changes in gene transcription and chromosomal stability (11, 12). Decreased folate availability may also reduce *S*-adenosylmethionine (SAM) production, resulting in aberrant DNA methylation and altered protein expression patterns (13, 14). Conversely, folate sufficiency might facilitate increased cellular proliferation by making DNA precursors more readily available (15).

A number of previous studies have examined folate-associated CRC risks, using a variety of study designs in diverse subject populations, with mixed results. To estimate the risk association based on prospective observational studies only, Kim et al. conducted a pooled analysis of 13 cohort studies, involving a total of 725,134 participants. Higher versus lower folate intake was found to reduce CRC risk by approximately 15% overall (RR = 0.85; 95% confidence interval [CI] = 0.77–0.95) (16). However, summary risk estimates based on existing clinical trial data have been less convincing of a protective effect. In a meta-analysis of 6 randomized controlled trials, Carroll, et al. identified no statistically significant benefits associated with folic acid supplementation (at doses of 500 µg/d to 20 mg/d over intervention periods of 2 to 7 years) with respect to either recurrent adenoma among 2,143 post-polypectomy subjects (n = 3 trials) or incident CRC among 11,180 average-risk subjects (n = 3 trials).

Because colorectal carcinogenesis is thought to involve at least 3 major pathways (17), it seems conceivable that etiologic heterogeneity (currently defined by molecular markers) could be contributing to the inconsistent and/or inconclusive associations between folate intake and CRC risk described across prior reports. To date, relatively few studies have investigated folate-associated CRC risks by molecularly defined subtypes, which may yield further insights regarding the anti-carcinogenic (or pro-carcinogenic) potential of this micronutrient (18–21). In the present study, we used data and tissue resources from the population-based Iowa Women's Health Study (IWHS), a prospective cohort study of cancer risks in older women, to evaluate associations between folate and related micronutrients (vitamins B6, B12 and methionine) with incident CRC overall, as well as by subtypes defined by microsatellite instability (MSI), CpG island methylator phenotype (CIMP), *BRAF* mutation, or *KRAS* mutation status. These data update and extend a previous study of these dietary factors and CRC risk among IWHS participants (22) by including the molecularly defined risk estimates, as well as additional cohort follow-up time.

MATERIALS AND METHODS

This study was reviewed and approved by the institutional review boards for human research of the University of Iowa, University of Minnesota, and Mayo Clinic.

Subjects

A detailed description of the methods used for the IWHS subject recruitment and enrollment has been published elsewhere (23). In brief, a 16-page questionnaire was mailed out at baseline (January 1986) to 99,826 randomly selected women, ages 55–69 years, who resided in Iowa and held a valid driver's license. Baseline questionnaires were returned by 41,836 women (42%), who comprise the full IWHS cohort. As previously reported (24), demographic characteristics and CRC incidence rates were comparable between baseline survey responders and non-responders. Vital status and state of residence were determined by mailed follow-up surveys in 1987, 1989, 1992, 1997, and 2004, as well as through linkage to Iowa death certificate records. Non-responders to the follow-up surveys were also cross-matched with the National Death Index to identify decedents. Migration out of the IWHS cohort has been estimated at approximately 1% per year (25). Exclusion criteria for the present molecular epidemiology study were defined as (not mutually exclusive): follow-up < 1 day (n=10), history of malignancy other than non-melanoma skin cancer (n = 3,830), and incomplete or implausible dietary intake data (n = 3,096), leaving 35,216 women in the resulting analytic cohort.

Exposure Assessment

Self-reported demographic, dietary, lifestyle, medication, and other CRC risk factor data were collected during the baseline IWHS evaluation. Dietary habits were assessed using a semi-quantitative food frequency questionnaire adapted from the 126-item instrument developed by Willett and colleagues (26). Folate, B6, B12, and methionine intakes were computed by multiplying the frequency response by the nutrient content of the specified portion sizes, with additional intake from supplement use included when reported.

Case Ascertainment

Incident CRC cases were identified through annual linkage with the Iowa Cancer Registry, which is a member of the National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) program (27). Each year, a computer-generated list of IWHS subjects was matched to SEER registry data using a combination of first, last, and maiden name; zip code; birth date; and social security number. CRC cases were identified using International Classification for Diseases in Oncology (ICD-O) codes of 18.0, 18.2–18.9, 19.9, and 20.9, with tumors located in the cecum, ascending colon, hepatic flexure, transverse colon and splenic flexure defined as proximal colon cancers and tumors located in the descending colon, sigmoid colon, rectosigmoid junction, and rectum defined as distal colorectal cancers.

Tissue Collection and Processing

Archived, paraffin-embedded tissue specimens were requested for molecular epidemiology research from incident CRC cases diagnosed among IWHS subjects through 12/31/2002. Tissue specimens were retrieved from 732/1255 (58%) cases, as previously reported (28). Baseline demographics and general tumor characteristics (size and stage) for incident CRC cases with retrieved versus non-retrieved tissue specimens were not significantly different. Paraffin blocks were serially cut into 5- or 10-micron thick sections, stained with hematoxylin and eosin, and marked for areas of neoplastic (>50%) and normal tissue. Tumor and normal tissues were scraped from unstained slides and placed into separate tubes for DNA extraction using the QIA-amp Tissue Kit (QIAGEN, Valencia, CA), according to the

manufacturer's instructions. A total of 169 retrieved CRC cases were subsequently excluded from the present study due to inadequate/unusable tissue from the first primary CRC or multiple primary CRCs at the time of initial diagnosis. Coupled with other study exclusions outlined above, 514 incident CRC cases were available for the molecular marker assays.

Molecular Markers

The laboratory methods used for assessing MSI, CIMP and *BRAF* mutation status in CRC cases among IWHS participants have been previously reported (28, 29). In brief, MSI testing was performed on paired tumor and normal DNA samples for each CRC case subject, using an established panel of 4 mononucleotide repeats (BAT25, BAT26, BAT40 and BAT34C4), 5 dinucleotide repeats (ACTC, D5S346, D18S55, D17S250 and D10197) and 1 complex marker (MYCL) (30, 31). MSI status was classified as microsatellite instability-high (MSI-H) or microsatellite instability-low/microsatellite stable (MSS/MSI-L) if $\geq 30\%$ or $< 30\%$ of the markers, respectively, demonstrated instability (30, 31). For the CIMP analyses, tumor DNA was treated with sodium bisulfite and analyzed using automated realtime PCR-based MethyLight to amplify methylated CpG sites in the promoter regions of an established gene panel (*CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3* and *SOC31*) (32). CIMP status was classified as CIMP-positive or CIMP-negative if hypermethylation was observed in ≥ 3 or 0–2 markers, respectively (32). To identify *BRAF* mutations, tumor DNA was analyzed using fluorescent-allele specific polymerase chain reaction to detect alterations in exon 15 (V600E point mutation). *BRAF* mutation status was classified as *BRAF* mutation-positive or *BRAF* mutation-negative. *KRAS* mutation status was determined by PCR amplification of tumor DNA, using primers for exon 2 (codons 12 and 13). Thermocycler conditions were 95° C for 10 minutes, followed by 35 cycles of 95° C for 30 seconds, 60° C for 30 seconds, and 72° C for 30 seconds, with a final extension at 72° C for 10 minutes. The PCR product was cleaned using Shrimp Alkaline Phosphatase and Exonuclease I, followed by sequencing with the Applied Biosystems PRISM BigDye Terminator v1.1 cycle sequencing kit on an ABI PRISM 3730 DNA analyzer (ABI Carlsbad, CA). Data analysis was performed using Mutation Surveyor software (Softgenetics, State College, PA). *KRAS* mutation status was classified as *KRAS* mutation-positive or *KRAS* mutation-negative.

Among the 514 evaluable CRC cases, molecular marker status was successfully characterized for MSI in 500 cases (97%), CIMP in 486 (95%) cases, *BRAF* mutation in 497 (97%) cases, and *KRAS* mutation in 470 (91%) cases.

Statistical Analyses

Data were descriptively summarized using frequencies and proportions for categorical variables and means and standard deviations for continuous variables. Among the incident CRC cases, we assessed pair-wise agreement between the various molecular marker values using kappa coefficients, as previously reported from a similar IWHS study (33, 34). Follow-up time was calculated as the difference between the date of completing the baseline questionnaire and age at first CRC diagnosis, date of move from Iowa, or date of death; if none of these events occurred, the subject was assumed to be alive, cancer-free and living in Iowa through 12/31/2002. Cox proportional hazards regression models were fit to estimate relative risks (RRs) and 95% confidence intervals (CIs) for associations between folate intake and incident CRC, overall and by anatomically and molecularly defined subsets. All eligible IWHS subjects were included in these initial regression analyses, regardless of eventual cancer status. CRC incidence was modeled as a function of age, as age is a better predictor of cancer risk in our cohort than follow-up time (35). Baseline folate intake was defined *a priori* as our primary dietary exposure of interest, and was analyzed based on the quartile distribution among all IWHS subjects, as well as in relation to the U. S.

recommended daily allowance (RDA) in 1989 of 180 µg/d for adults > 18 years of age (36); this value was chosen because it was the closest RDA to the IWH baseline evaluation and also defined folate intake using the same methods applied for estimating exposure from the baseline FFQ data. Associations between baseline vitamin B6, vitamin B12, and methionine intakes with incident CRC were modeled as quartile distributions in secondary analyses. Tests for trend for the quartile-based variables were carried out by ordering the categorized micronutrient intake values from lowest to highest and including the resulting variable as a one degree-of-freedom linear term in a Cox proportional hazards model.

We initially assessed associations between folate, B6, B12, and methionine intakes with incident CRC overall. Two sets of Cox regression models were fit: one adjusting for age and one adjusting for age and other potential CRC risk factors, including body mass index (BMI; quartiles), waist to hip ratio (WHR; quartiles), smoking status (ever, never), exogenous estrogen use (ever, never), physical activity level (low, moderate, high), self-reported diabetes mellitus (DM; yes, no), and daily intake (quartiles) of total energy (kcal/d), total fat (g/d), sucrose (g/d), red meat (g/d), calcium (mg/d), vitamin E (mg/d) and alcohol (none, 3.4 g/d, > 3.4g/d). As family history of CRC and non-steroidal anti-inflammatory drugs were not routinely recorded at baseline, these variables were not included in the current data analyses. Interactions between folate and the other micronutrients of interest were tested by including a cross-product term in the relevant proportional hazards models. Subsequent analyses were conducted to examine CRC risks defined by tumor subtypes according to anatomic location (proximal colon or distal colorectum), microsatellite instability phenotype (MSI-H or MSS/MSI-L), CIMP status (CIMP-positive or CIMP-negative), *BRAF* status (*BRAF* mutation-positive or mutation-negative), and *KRAS* status (*KRAS* mutation-positive or mutation-negative). For the molecularly-defined subtype analyses, the outcome variable was defined as incident CRC with the phenotype of interest; all other incident CRCs (including those with the missing or unknown values for the specific marker) were considered censored observations at the date of diagnosis. Low event rates within strata of molecularly-defined CRC subtypes did not allow for meaningful analyses of potential interactions between the micronutrient exposures of interest. We also examined associations between folate intake (as our exposure of primary interest) and CRC risk based on case subsets defined by tissue availability (available versus not available), using the same multi-outcome analytic approach as described above, to determine if incomplete tissue access introduced any association biases. All statistical tests were two-sided, and all analyses were carried out using SAS (SAS Institute, Inc., Cary, NC) and R (R foundation for statistical computing, Vienna, Austria) software systems.

RESULTS

Baseline demographic characteristics for the 35,216 women included in the final analytic cohort are shown in Table 1 by quartiles of folate intake. Daily folate intake ranged from 43.5 to 2555.2 µg/d, with a median value of 350.7 µg/d. Quartile boundaries were set at 251.0 µg/d (quartile 1; reference), 251.1 – 350.7 µg/d (quartile 2), 350.8 – 573.4 µg/d (quartile 3), and 573.5 µg/d (quartile 4). Higher folate intake was associated with increased baseline age, exogenous estrogen use, physical activity level, self-reported DM, and intake of total energy, total fat, sucrose, red meat, calcium and vitamin E, as well as lower BMI and smoking prevalence. Relatively strong correlations were observed between folate intake and vitamin B6 (0.80), vitamin B12 (0.58), and methionine (0.37) intake. For CRC cases with available molecular marker data, the distributions by subtype were: 365 (73%) MSS/MSI-L and 135 (27%) MSI-H; 338 (70%) CIMP-negative and 148 (30%) CIMP-positive; 359 (72%) *BRAF* mutation-negative and 138 (28%) *BRAF* mutation-positive; and 316 (67%) *KRAS* mutation-negative and 154 (33%) *KRAS* mutation-positive tumors.

In the age-adjusted risk models, quartile distributions of folate intake were inversely associated with incident CRC overall (p trend = 0.001). Compared to quartile 1, women with folate intake levels in quartiles 2, 3, and 4 had risk estimates of $RR = 1.00$ (95% CI = 0.86–1.16), $RR = 0.83$ (95% CI = 0.71–0.97) and $RR = 0.81$ (95% CI = 0.69–0.95), respectively. Age-adjusted analysis of folate intake equal to or above the RDA (180 μ g/d) also suggested lower CRC risk compared to folate intake below the RDA ($RR = 0.86$; 95% CI = 0.72–1.03; p value = 0.11). However, after adjusting for other potential confounding factors, the folate-related risk estimates for CRC overall were attenuated and were no longer statistically significant (Table 2). Multivariable adjusted risk models based on folate intake from dietary versus supplemental sources yielded similar associations ($RR = 0.86$; 95% CI = 0.70–1.06 and $RR = 1.01$; 95% CI = 0.84–1.22, respectively, for comparisons of extreme quartiles). A series of sensitivity analyses adjusting only for age and one additional covariate from the multivariate model, for each covariate in turn, identified calcium intake as the confounding variable primarily responsible for attenuation of the folate effect (age- and calcium-adjusted association of folate with CRC $p = 0.18$), followed by vitamin E intake ($p = 0.06$). No other variables substantively attenuated the folate association (folate $p < 0.02$ when considering each of the other covariates). Null associations were also observed between folate intake and anatomic subsite-specific CRC risks in the multivariate models (p trend > 0.05 for each comparison). When incident CRC was restricted to the subset of cases for whom complete molecular marker data were available, the multivariable-adjusted risk estimates for folate intake based on quartile distribution ($RR = 1.02$; 95% CI = 0.72–1.46; p trend = 0.74 for comparison of extreme quartiles) or RDA level ($RR = 1.08$; 95% CI = 0.76–1.53; p value = 0.68 for comparison of $\geq$ versus < 180 μ g/d) were not appreciably different from the risk associations observed for incident CRC overall, indicating that tissue availability status did not introduce a major selection bias.

Multivariate-adjusted risk estimates for quartile distributions of vitamin B6 and vitamin B12 were not statistically significant for CRC overall or by anatomic subsite (Table 2). For methionine intake, an inverse association was observed with incident CRC overall ($RR = 0.72$; 95% CI = 0.54–0.96; p trend = 0.03 for comparison of extreme quartiles) that appeared to be driven by a greater reduction in distal than proximal CRC risk.

Risk associations for individual micronutrients and molecularly defined CRC subtypes are shown in Table 3. In general, folate intake did not appear to differentially influence CRC risk based on MSI, CIMP, *BRAF*, or *KRAS* status. More specifically, folate intake in the highest versus lowest quartiles yielded risk estimates for MSS/MSI-L and MSI-H tumors of $RR = 1.05$ (95% CI = 0.69–1.61) and $RR = 1.01$ (95% CI = 0.51–2.02); for CIMP-negative and CIMP-positive tumors of $RR = 0.93$ (95% CI = 0.60–1.44) and $RR = 1.34$ (95% CI = 0.69–2.60); for *BRAF* mutation-negative and *BRAF* mutation-positive tumors of $RR = 1.04$ (95% CI = 0.68–1.59) and $RR = 0.85$ (95% CI = 0.43–1.70); and for *KRAS* mutation-negative and *KRAS* mutation-positive tumors of $RR = 0.98$ (95% CI = 0.62–1.54) and $RR = 1.17$ (95% CI = 0.60–2.28). Null associations were also observed for each of the molecularly defined CRC subtypes and folate intake above or equal to versus below the RDA threshold, as well as quartile distributions of vitamin B6, vitamin B12, and methionine intake (Table 3).

DISCUSSION

Data from this prospective, population-based cohort study provide limited support for an etiologic association between folate intake, including both dietary and supplement sources, and incident CRC among older women. Further evaluation by CRC subsite, as well as by molecularly defined subtypes, did not meaningfully influence the observed risk associations. Although we did find that folate intake was inversely associated with CRC risk overall in the

age-adjusted models, the potential protective effect was no longer apparent after adjusting for additional co-factors. Based on additional sensitivity analyses, the inclusion of calcium intake, which was correlated with folate intake in this cohort ($r = 0.40$) and has been shown to reduce CRC risk in previous clinical trials (37, 38), appeared to impose the greatest level of risk attenuation. Thus, much of the perceived benefit associated with increased folate intake in the minimally adjusted risk models from our study was likely due to confounding. Additional analyses of vitamin B6, vitamin B12, and methionine intake were generally unremarkable, although methionine was found to be associated with lower risk for distal, but not proximal, CRC. In aggregate, these data provide minimal support for major effects from the examined micronutrients on overall or molecularly defined CRC risks in the IWHS cohort.

Previous studies from our group have shown that relatively common exposures, such as cigarette smoking and postmenopausal hormone use, exhibit different risk patterns for molecularly distinct CRC subsets in the IWHS cohort (28, 33). However, based on limited available data, the relationship between folate intake and MSI-, CIMP-, *BRAF*- or *KRAS*-defined CRC subtypes remains inconclusive. In a case-cohort study participants enrolled in the Netherlands Cohort Study ($n = 648$ CRC case subjects and 4,059 subcohort members), de Vogel, et al. initially found no statistically significant association between dietary folate and incident CRC overall or with respect to *MLH1* hypermethylation, *MLH1* protein expression, MSI or *BRAF* status (39). Further analyses based on a smaller NCS subcohort ($n = 609$ CRC case subjects and 1,663 subcohort members) demonstrated null associations between folate intake and both CIMP-positive and CIMP-negative cases (40). Genetic variation in select methyl-metabolizing genes (*MTHFR*, *MTR*, *MTRR*) and other epigenetic regulators (*DNMT3B*, *EHMT1*, *EHMT2*, *PRDM2*) also had no apparent influence on the folate-associated CRC risks. A recent report based on 185 CRC case subjects from the EPIC-Norfolk study also failed to detect a statistically significant association between folate consumption and mismatch repair deficient tumors (41). Using combined resources from the Nurses' Health Study and the Health Professionals Follow-up Study ($n = 88,971$ women and 47,371 men; 669 incident colon cancer case subjects), Schernhammer, et al. reported that subjects with folate intake ≥ 400 $\mu\text{g}/\text{d}$ were 25% less likely to develop incident colon cancer than subjects with folate intake < 200 $\mu\text{g}/\text{d}$ (42). However, the inverse association did not differ by MSI or *KRAS* status. In a subsequent study restricted to Nurses' Health Study participants, these investigators found no statistically significant associations between folate intake and CIMP- or *BRAF*-defined colon cancer subtypes (based on $n = 375$ and 386 molecularly characterized cases, respectively), based on multivariate risk models (20). Similarly, a series of reports from separate case-control studies of colon cancer ($n = 1,154$ case subjects and 2,410 control subjects) and rectal cancer ($n = 750$ case subjects and 1,205 control subjects) conducted by Slattery and colleagues have not convincingly demonstrated a unique role for dietary folate in MSI-, CIMP-, *BRAF*-, or *KRAS*-defined colon cancer risks, although higher vs. lower folate intake was associated with increased risk for CIMP-positive rectal cancers among men (OR = 3.2; 95% CI = 1.5–6.7), but not women (21, 43–45). Data from the current prospective, population-based study of older women are consistent with the absence of a risk or protective association between folate intake and molecularly-defined CRC subtypes.

Cereal products were fortified with folic acid in America and Canada in 1998, Chile in 2000, and other countries soon after as a public health initiative to combat the incidence of neural-tube-related birth defects. Since this implementation, there have been documented increases in plasma and RBC folate concentrations at the population level (46). Some reports have also suggested that folate fortification has contributed to increased CRC risk (47, 48). However, data from the IWHS cohort do not suggest that higher folate intake is positively

associated with incident CRC risk, overall or by anatomically or molecularly defined subtypes.

Major strengths of our study include the large, prospective, population-based design; prolonged follow-up; ability to adjust for multiple confounding factors; comprehensive CRC case ascertainment; extensive tissue availability with high assay success rates for multiple molecular markers; and absence of appreciable selection bias associated with the tissue collection, processing or analysis methods. With respect to potential design limitations, we acknowledge that our data are derived from a homogenous subject population (older, primarily Caucasian women) and the applicability of these findings to more diverse groups requires further investigation. Also, as folate exposure was quantified based on a single, self-reported dietary survey rather than repeated assessment and/or direct biologic measurement (such as plasma folate level), some degree of misclassification may have been introduced over the prolonged follow-up period. However, we are not aware of a plausible rationale to suspect that such misclassification, if present, would be differently distributed between cases and non-cases, or across subtype-specific CRC case groups. Therefore, the possibility that factors related to the exposure measurement had a major influence on our observed risk associations seems remote. Lastly, given that tissue samples were available only from CRC case subjects, we were unable to evaluate potential effect modification from folate metabolizing gene polymorphisms on our reported risk estimates. Nonetheless, as noted above, no differences in folate-associated CRC risks were found by polymorphism status in the Netherlands Cohort Study (40), providing some reassurance that unmeasured genetic variation likely had minimal, if any, effect on our observations.

In summary, despite a seemingly strong mechanistic rationale, our study did not indicate any consistent patterns of differential effects from folate, vitamin B6, vitamin B12, or methionine intake on subtype-specific CRC risks among older women. These findings are in keeping with the preponderance of existing observational data. Further evaluation of associations between the examined micronutrients (particularly methionine) and additional molecular markers and/or integrated carcinogenic pathways (17) may help to clarify or refute the functional significance of these dietary exposures in colorectal carcinogenesis.

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Table 1

Baseline Subject Characteristics, by Folate Intake

Characteristic	FOLATE INTAKE ^a			
	Quartile 1 (< 251.0 µg/d)	Quartile 2 (251.1–350.7 µg/d)	Quartile 3 (350.8–573.4 µg/d)	Quartile 4 (> 573.5 µg/d)
Subjects, N	8,804	8,804	8,804	8,804
Age, years	61.8 (4.2)	62.0 (4.2)	62.1 (4.2)	62.1 (4.2)
Body Mass Index, kg/m ²	27.1 (5.1)	27.2 (5.2)	27.0 (5.1)	26.6 (5.0)
Waist-to-Hip Ratio	0.84 (0.09)	0.84 (0.09)	0.84 (0.09)	0.83 (0.08)
Smoking Status, N (%)				
never	5,306 (61.1)	5,815 (67.0)	5,949 (68.5)	5,745 (66.1)
ever	3,374 (38.9)	2,869 (33.0)	2,737 (31.5)	2,950 (33.9)
Exogenous Estrogen Use, N (%)				
never	5,617 (64.3)	5,499 (63.0)	5,437 (62.3)	4,926 (56.5)
ever	3,113 (35.7)	3,231 (37.0)	3,288 (37.7)	3,786 (43.5)
Physical Activity, N (%)				
low	4,919 (56.8)	4,221 (48.7)	3,813 (43.9)	3,511 (40.5)
moderate	2,133 (24.6)	2,436 (28.1)	2,520 (29.0)	2,461 (28.4)
high	1,607 (18.6)	2,013 (23.2)	2,357 (27.1)	2,695 (31.1)
Self-reported diabetes mellitus, N (%)	446 (5.1)	546 (6.2)	558 (6.4)	522 (6.0)
Total Energy, kcal/d	1,388.5 (400.2)	1,796.4 (446.5)	2,063.3 (633.6)	1,945 (672.2)
Total Fat, g/d	55.8 (21.0)	69.0 (23.7)	77.3 (30.1)	71.7 (30.1)
Sucrose, gm/d	31.6 (17.3)	41.3 (19.5)	48.0 (23.3)	45.1 (23.6)
Red Meat, g/day	76.9 (54.6)	92.6 (58.6)	101.1 (68.1)	89.4 (66.5)
Calcium, mg/d ^a	776.6 (447.0)	1,014 (464.3)	1,213.1 (505.4)	1,378.6 (598.2)
Methionine, g/d	1.4 (0.5)	1.8 (0.6)	2.1 (0.8)	2 (0.8)
Vitamin E, mg/d ^a	35.4 (112.1)	40.8 (117.0)	58.9 (139.0)	131.9 (195.2)
Alcohol consumption, g/d	3.6 (8.6)	3.9 (9.0)	3.8 (8.8)	3.8 (9.0)
Folate supplement use, N (%)	145 (1.6)	391 (4.4)	2,141 (24.3)	7,981 (90.7)

Results presented as mean (standard deviation) unless otherwise indicated.

^aIncluding supplements.

Table 2

Associations Between Folate, Vitamin B6, Vitamin B12, and Methionine Intake Levels with Incident Colorectal Cancer (CRC), Overall and by Anatomic Subsite

Micronutrient Intake ^a	Person-Years	ANY CRC (N = 1,298)		PROXIMAL CRC (N = 673)		DISTAL CRC (N = 597)	
		Events, N	RR (95% CI) ^b	Events, N	RR (95% CI) ^b	Events, N	RR (95% CI) ^b
Folate							
Quartile Distribution, µg/d							
Q1 (250.9)	142,477	352	1.00 (Ref)	184	1.00 (Ref)	163	1.00 (Ref)
Q2 (251.0 – 350.7)	143,152	358	1.04 (0.88–1.24)	191	1.05 (0.83–1.32)	162	1.04 (0.81–1.33)
Q3 (350.8–573.4)	142,999	300	0.91 (0.74–1.10)	161	0.91 (0.69–1.19)	130	0.86 (0.64–1.15)
Q4 (573.5)	141,705	288	0.95 (0.76–1.20)	137	0.81 (0.59–1.11)	142	1.08 (0.77–1.49)
<i>p trend</i>			0.46		0.16		0.95
RDA Threshold, µg/d^c							
< 180	50,211	128	1.00 (Ref)	62	1.00 (Ref)	63	1.00 (Ref)
180	520,122	1,170	0.95 (0.76–1.17)	611	1.03 (0.76–1.39)	534	0.87 (0.64–1.18)
<i>p trend</i>			0.60		0.85		0.37
Vitamin B6							
Quartile Distribution, mg/d							
Q1 (1.72)	143,227	344	1.00 (Ref)	171	1.00 (Ref)	167	1.00 (Ref)
Q2 (1.73–2.42)	144,623	352	1.05 (0.88–1.25)	207	1.21 (0.96–1.54)	139	0.88 (0.68–1.14)
Q3 (2.43–3.88)	141,667	315	1.05 (0.86–1.30)	154	1.01 (0.76–1.35)	152	1.09 (0.80–1.47)
Q4 (3.89)	140,816	287	1.08 (0.86–1.35)	141	0.96 (0.69–1.32)	139	1.23 (0.88–1.70)
<i>p trend</i>			0.55		0.58		0.14
Vitamin B12							
Quartile Distribution, µg/d							
Q1 (250.9)	142,373	336	1.00 (Ref)	177	1.00 (Ref)	153	1.00 (Ref)
Q2 (251.0 – 350.7)	143,988	320	1.01 (0.85–1.20)	177	1.02 (0.80–1.29)	136	0.98 (0.76–1.27)
Q3 (350.8–573.4)	142,561	342	1.07 (0.91–1.27)	174	0.99 (0.79–1.26)	163	1.18 (0.92–1.50)
Q4 (573.5)	141,412	300	1.05 (0.86–1.27)	145	0.91 (0.69–1.19)	145	1.19 (0.90–1.58)

		ANY CRC (N = 1,298)		PROXIMAL CRC (N = 673)		DISTAL CRC (N = 597)	
	Person-Years	Events, N	RR (95% CI) ^b	Events, N	RR (95% CI) ^b	Events, N	RR (95% CI) ^b
Micronutrient Intake ^a							
<i>p trend</i>			0.46		0.46		0.08
Methionine							
Quartile Distribution, g/d							
Q1 (250.9)	141,785	358	1.00 (Ref.)	174	1.00 (Ref)	175	1.00 (Ref)
Q2 (251.0 – 350.7)	142,129	339	0.96 (0.80–1.15)	181	0.93 (0.73–1.18)	154	0.85 (0.65–1.11)
Q3 (350.8–573.4)	142,651	322	0.88 (0.70–1.11)	168	0.89 (0.67–1.18)	149	0.78 (0.56–1.09)
Q4 (573.5)	143,769	279	0.72 (0.54–0.96)	150	0.80 (0.59–1.10)	119	0.54 (0.35–0.83)
<i>p trend</i>			0.03		0.17		0.008

Definitions for proximal and distal CRC subsites provided in the Methods section.

^aAs reported during the IWHS baseline evaluation (1986);

^badjusted for age, BMI, WHR, smoking status, exogenous estrogen use, physical activity level, history of DM, and daily intakes of total energy, total fat, sucrose, red meat, calcium, methionine, vitamin E, and alcohol.

^cRecommended daily allowance (RDA) for U.S. adults > 18 years of age in 1989 (36).

Table 3

Associations Between Folate Intake Level and Incident Colorectal Cancer (CRC), by Molecularly Defined Subtypes

MICRONUTRIENT INTAKE ^a	PERSON-YEARS	EVENTS, N	RR (95% CI) ^b	EVENTS, N	RR (95% CI) ^b
			MICROSATELLITE INSTABILITY (MSI)		
			MSS/MSI-L (n = 365)		
Folate					
Quartile Distribution, µg/d					
Q1 (250.9)	142,477	89	1.00 (Ref.)	36	1.00 (Ref.)
Q2 (251.0 – 350.7)	143,152	109	1.32 (0.96–1.82)	38	0.93 (0.56–1.56)
Q3 (350.8–573.4)	142,999	85	0.94 (0.64–1.38)	28	0.77 (0.42–1.43)
Q4 (573.5)	141,705	82	1.05 (0.69–1.61)	33	1.01 (0.51–2.02)
<i>p trend</i>			0.81		0.86
RDA Threshold, µg/d ^c					
180	50,211	31	1.00 (Ref.)	10	1.00 (Ref.)
180	520,122	334	1.09 (0.72–1.65)	125	1.12 (0.54–2.30)
<i>p trend</i>			0.69		0.76
Vitamin B6					
Quartile Distribution, mg/d					
Q1 (1.72)	143,227	88	1.00 (Ref.)	31	1.00 (Ref.)
Q2 (1.73–2.42)	144,623	106	1.19 (0.85–1.67)	37	1.21 (0.70–2.09)
Q3 (2.43–3.88)	141,667	86	1.07 (0.72–1.59)	36	1.40 (0.74–2.63)
Q4 (3.89)	140,816	85	1.20 (0.78–1.85)	31	1.12 (0.54–2.29)
<i>p trend</i>			0.52		0.71
Vitamin B12					
Quartile Distribution, µg/d					
Q1 (250.9)	143,227	83	1.00 (Ref.)	34	1.00 (Ref.)
Q2 (251.0 – 350.7)	144,623	106	1.42 (1.02–1.98)	32	1.02 (0.61–1.71)
Q3 (350.8–573.4)	141,667	91	1.21 (0.86–1.69)	37	0.92 (0.51–1.66)
Q4 (573.5)	140,816	85	1.28 (0.88–1.85)	32	0.99

MICRONUTRIENT INTAKE ^a	PERSON-YEARS	EVENTS, N	RR (95% CI) ^b	EVENTS, N	RR (95% CI) ^b
		MICROSATELLITE INSTABILITY (MSI)			
		MSS/MSI-L (n = 365)		MSI-H (n = 135)	
<i>p trend</i>			0.51		
Methionine					
Quartile Distribution, g/d					
Q1 (250.9)	143,227	90	1.00 (Ref.)	29	1.00 (Ref.)
Q2 (251.0 – 350.7)	144,623	108	1.44 (1.01–2.05)	34	1.17 (0.64–2.15)
Q3 (350.8–573.4)	141,667	87	1.05 (0.66–1.66)	41	1.48 (0.72–3.03)
Q4 (573.5)	140,816	80	0.89 (0.51–1.54)	31	1.06 (0.43–2.59)
<i>p trend</i>			0.44		0.79
		CpG ISLAND METHYLATOR PHENOTYPE (CIMP)			
		CIMP-negative (n = 338)		CIMP-positive (n = 148)	
Folate					
Quartile Distribution, µg/d					
Q1 (250.9)	142,477	80	1.00 (Ref)	39	1.00 (Ref)
Q2 (251.0 – 350.7)	143,152	107	1.38 (0.99–1.91)	37	0.89 (0.53–1.49)
Q3 (350.8–573.4)	142,999	79	0.87 (0.59–1.30)	31	0.95 (0.52–1.72)
Q4 (573.5)	141,705	72	0.93 (0.60–1.44)	41	1.34 (0.69–2.60)
<i>p trend</i>			0.34		0.44
RDA Threshold, µg/d ^c					
180	50,211	25	1.00 (Ref.)	13	1.00 (Ref.)
180	520,122	313	1.23 (0.78–1.95)	135	0.99 (0.52–1.89)
<i>p trend</i>			0.37		0.98
Vitamin B6					
Quartile Distribution, mg/d					
Q1 (1.72)	143,227	80	1.00 (Ref.)	30	1.00 (Ref.)
Q2 (1.73–2.42)	144,623	100	1.18 (0.83–1.67)	41	1.53 (0.89–2.63)
Q3 (2.43–3.88)	141,667	84	1.02 (0.68–1.54)	37	1.94 (1.04–3.64)
Q4 (3.89)	140,816	74	1.05 (0.67–1.64)	40	1.87 (0.94–3.70)

MICRONUTRIENT INTAKE ^a	PERSON-YEARS	EVENTS, N	RR (95% CI) ^b	EVENTS, N	RR (95% CI) ^b
		MICROSATELLITE INSTABILITY (MSI)			
		MSS/MSI-L (n = 365)		MSI-H (n = 135)	
<i>p trend</i>			0.99		0.08
Vitamin B12					
Quartile Distribution, µg/d					
Q1 (250.9)	143,227	80	1.00 (Ref.)	35	1.00 (Ref.)
Q2 (251.0 – 350.7)	144,623	102	1.30 (0.93–1.82)	31	0.92 (0.54–1.58)
Q3 (350.8–573.4)	141,667	79	1.02 (0.72–1.45)	43	1.26 (0.77–2.07)
Q4 (573.5)	140,816	77	1.11 (0.76–1.64)	39	1.18 (0.67–2.08)
<i>p trend</i>			0.89		0.31
Methionine					
Quartile Distribution, g/d					
Q1 (250.9)	143,227	82	1.00 (Ref.)	34	1.00 (Ref.)
Q2 (251.0 – 350.7)	144,623	97	1.30 (0.90–1.88)	38	1.40 (0.79–2.49)
Q3 (350.8–573.4)	141,667	82	0.93 (0.58–1.50)	45	1.95 (0.98–3.88)
Q4 (573.5)	140,816	77	0.79 (0.45–1.41)	31	1.42 (0.60–3.37)
<i>p trend</i>			0.26		0.31
		BRAF MUTATION STATUS			
		BRAF mutation-negative (n = 359)		BRAF mutation-positive (n = 138)	
Folate					
Quartile Distribution, µg/d					
Q1 (250.9)	142,477	85	1.00 (Ref)	38	1.00 (Ref)
Q2 (251.0 – 350.7)	143,152	109	1.31 (0.95–1.82)	38	0.96 (0.57–1.59)
Q3 (350.8–573.4)	142,999	84	0.90 (0.61–1.33)	31	0.92 (0.50–1.67)
Q4 (573.5)	141,705	81	1.04 (0.68–1.59)	31	0.85 (0.43–1.70)
<i>p trend</i>			0.69		0.65
RDA Threshold, µg/d ^c					
180	50,211	26	1.00 (Ref.)	14	1.00 (Ref.)
180	520,122	333	1.23 (0.78–1.92)	124	0.85 (0.45–1.61)

MICRONUTRIENT INTAKE ^a	PERSON-YEARS	EVENTS, N	RR (95% CI) ^b	EVENTS, N	RR (95% CI) ^b
		MICROSATELLITE INSTABILITY (MSI)			
		MSS/MSI-L (n = 365)		MSI-H (n = 135)	
<i>p trend</i>			0.37		0.62
Vitamin B6					
Quartile Distribution, mg/d					
Q1 (1.72)	143,227	86	1.00 (Ref.)	31	1.00 (Ref.)
Q2 (1.73–2.42)	144,623	104	1.12 (0.80–1.57)	39	1.40 (0.81–2.40)
Q3 (2.43–3.88)	141,667	87	1.00 (0.67–1.49)	38	1.70 (0.90–3.20)
Q4 (3.89)	140,816	82	1.11 (0.72–1.71)	30	1.07 (0.52–2.23)
<i>p trend</i>			0.77		0.78
Vitamin B12					
Quartile Distribution, µg/d					
Q1 (250.9)	143,227	82	1.00 (Ref.)	33	1.00 (Ref.)
Q2 (251.0 – 350.7)	144,623	103	1.33 (0.95–1.85)	35	1.04 (0.61–1.77)
Q3 (350.8–573.4)	141,667	88	1.14 (0.81–1.59)	40	1.16 (0.70–1.94)
Q4 (573.5)	140,816	86	1.26 (0.87–1.83)	30	0.87 (0.48–1.60)
<i>p trend</i>			0.49		0.80
Methionine					
Quartile Distribution, g/d					
Q1 (250.9)	143,227	89	1.00 (Ref.)	27	1.00 (Ref.)
Q2 (251.0 – 350.7)	144,623	101	1.30 (0.91–1.86)	38	1.58 (0.86–2.89)
Q3 (350.8–573.4)	141,667	85	0.99 (0.63–1.57)	46	2.00 (0.97–4.10)
Q4 (573.5)	140,816	84	0.94 (0.54–1.64)	27	1.03 (0.41–2.56)
<i>p trend</i>			0.59		0.84
		KRAS MUTATION STATUS			
		KRAS mutation-negative (n=316)		KRAS mutation-positive (n=154)	
Folate					
Quartile Distribution, µg/d					
Q1 (250.9)	142,477	78	1.00 (Ref)	32	1.00 (Ref)

MICRONUTRIENT INTAKE ^a	PERSON-YEARS	EVENTS, N	RR (95% CI) ^b	EVENTS, N	RR (95% CI) ^b
		MICROSATELLITE INSTABILITY (MSI)			
		MSS/MSI-L (n = 365)		MSI-H (n = 135)	
Q2 (251.0 – 350.7)	143,152	91	1.09 (0.77–1.54)	49	1.82 (1.09–3.05)
Q3 (350.8–573.4)	142,999	74	0.90 (0.60–1.35)	38	1.15 (0.62–2.13)
Q4 (573.5)	141,705	73	0.98 (0.62–1.54)	35	1.17 (0.60–2.28)
<i>p trend</i>			0.74		0.92
RDA Threshold, µg/dc		27	1.00 (Ref.)	7	1.00 (Ref.)
180	50,211	289	0.98 (0.63–1.54)	147	2.00 (0.89–4.53)
180	520,122		0.93		0.10
<i>p trend</i>					
Vitamin B6					
Quartile Distribution, mg/d					
Q1 (1.72)	143,227	68	1.00 (Ref.)	35	1.00 (Ref.)
Q2 (1.73–2.42)	144,623	93	1.37 (0.95–1.96)	45	1.22 (0.72–2.08)
Q3 (2.43–3.88)	141,667	80	1.34 (0.88–2.05)	39	1.10 (0.60–2.04)
Q4 (3.89)	140,816	75	1.32 (0.83–2.10)	35	1.07 (0.55–2.10)
<i>p trend</i>			0.32		0.96
Vitamin B12					
Quartile Distribution, µg/d					
Q1 (250.9)	143,227	75	1.00 (Ref.)	31	1.00 (Ref.)
Q2 (251.0 – 350.7)	144,623	73	1.16 (0.81–1.64)	49	1.75 (1.03–2.96)
Q3 (350.8–573.4)	141,667	84	1.12 (0.79–1.59)	39	1.36 (0.79–2.34)
Q4 (573.5)	140,816	81	1.18 (0.80–1.75)	35	1.38 (0.76–2.50)
<i>p trend</i>			0.50		0.72
Methionine					
Quartile Distribution, g/d					
Q1 (250.9)	143,227	74	1.00 (Ref.)	37	1.00 (Ref.)
Q2 (251.0 – 350.7)	144,623	87	1.29 (0.88–1.89)	45	1.35 (0.78–2.36)
Q3 (350.8–573.4)	141,667	89	1.26 (0.78–2.02)	35	0.94 (0.46–1.93)

MICRONUTRIENT INTAKE ^a	PERSON-YEARS	EVENTS, N	RR (95% CI) ^b	EVENTS, N	RR (95% CI) ^b
		MICROSATELLITE INSTABILITY (MSI)			
		MSS/MSI-L (n = 365)		MSI-H (n = 135)	
Q4 (573.5)	140,816	66	0.88 (0.49–1.60)	37	0.88 (0.37–2.10)
<i>p trend</i>			0.68		0.59

^a as reported during the IWH baseline evaluation (1986);

^b adjusted for age, BMI, WHR, smoking status, exogenous estrogen use, physical activity level, history of DM, and daily intakes of total energy, total fat, sucrose, red meat, calcium, methionine, vitamin E, and alcohol.

^c Recommended daily allowance (RDA) for U.S. adults 18 years of age in 1989 (36).