

# Methylenetetrahydrofolate Reductase, Diet, and Risk of Colon Cancer<sup>1</sup>

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## Abstract

**Individuals with different forms of the 5,10-methylenetetrahydrofolate reductase (*MTHFR*) gene, carriers of the C677T mutation versus wild type, show differences in enzyme levels; these differences have been hypothesized to be related to DNA methylation and, perhaps, to the nucleotide pool size. Using data from an incident case-control study, we evaluated the combined effect of dietary intake of folate, methionine, vitamin B<sub>6</sub>, vitamin B<sub>12</sub>, and alcohol and various forms of the *MTHFR* gene on risk of colon cancer. Individuals homozygous for the variant form of the *MTHFR* gene (*TT*) had a slightly lower risk of colon cancer than did individuals who were wild type [*CC*, odds ratio (*OR*) = 0.8, 95% confidence interval (*CI*) = 0.6–1.1 for men; and *OR* = 0.9, 95% *CI* = 0.6–1.2 for women]. High levels of intake of folate, vitamin B<sub>6</sub>, and vitamin B<sub>12</sub> were associated with a 30–40% reduction in risk of colon cancer among those with the *TT* relative to those with low levels of intake who were *CC* genotype. Associations were stronger for proximal tumors, in which high levels of intake of these nutrients were associated with a halving of risk among those with the *TT* genotype. The inverse association with high levels of these nutrients in those with the *TT* genotype was stronger among those diagnosed at an older age. Although imprecise, the inverse association with the low-risk diet that was high in folate and methionine and without alcohol was observed for both the *TT* genotype (*OR* = 0.4, 95% *CI* = 0.1–0.9) and the *CC/CT* genotype (*OR* = 0.6, 95% *CI* = 0.4–1.0), but this association was not seen with the high-risk diet for either the *TT* or *CC/CT* genotype. Although associations were generally weak, these findings suggest that those with differing *MTHFR* genotypes may have**

**different susceptibilities to colon cancer, based on dietary consumption of folate, vitamin B<sub>6</sub>, and vitamin B<sub>12</sub>.**

## Introduction

Dietary folate, methionine, vitamin B<sub>6</sub>, vitamin B<sub>12</sub>, and alcohol have been associated with colon cancer in some but not all epidemiological studies (1–4). The underlying mechanisms whereby these dietary factors are associated with colon cancer are unknown. One hypothesized unifying mechanism is that these factors act together through their involvement in DNA methylation processes (1, 2). Methylation of DNA plays an important role in gene regulation (5, 6). In colonic neoplasms, generalized hypomethylation along with hypermethylation of some unmethylated cytosine-rich areas can be found frequently (7–9). This imbalance in methylation of DNA is thought to result in abnormal expression of oncogenes and tumor suppressor genes (10). The methylation process involves several steps and several dietary factors such as folate, methionine, vitamin B<sub>12</sub>, and vitamin B<sub>6</sub> are potentially involved in these processes. The folate pathway is also important in determining the availability of nucleotides for DNA synthesis.

*MTHFR*<sup>3</sup> catalyzes the reduction of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major circulatory form of folate and carbon donor for the remethylation of homocysteine to methionine. Folate, in the form of methyltetrahydrofolate and vitamin B<sub>12</sub> transmethylase, is involved in these pathways (11, 12), as is vitamin B<sub>6</sub>, the cofactor for serine-hydroxymethyltransferase, which could have an impact on the availability of 5,10-methylenetetrahydrofolate. Methionine is a precursor for *S*-adenosylmethionine, the methyl donor for most biological transmethylation reactions in the body, including that of DNA (11, 12). Alterations in plasma homocysteine can result from genetic or nutrient-related disturbances in the transsulfuration or remethylation pathways of homocysteine metabolism (13). Thus, *MTHFR* is involved in regulating plasma homocysteine concentration and maintaining an adequate methionine pool.

A variant of the human *MTHFR* gene that results in an alanine to valine substitution has been described at bp 677. This mutation codes for a thermolabile enzyme with reduced *MTHFR* activity, resulting in elevated plasma homocysteine levels. Individuals who are homozygous for this variant (*TT*) have been reported as having 30% of normal enzyme activity, and heterozygotes (*CT*) have been reported as having 65% of normal enzyme activity (13). How dietary factors interact with variations in *MTHFR* in relation to colon cancer is generally unknown. Using data from a large case-control study of incident colon cancer, we explored these associations.

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<sup>3</sup> The abbreviations used are: *MTHFR*, 5,10-methylenetetrahydrofolate reductase; KPMCP, Kaiser Permanente Medical Care Program; *OR*, odds ratio; *CI*, confidence interval; *BMI*, body mass index.

## Materials and Methods

**Study Population.** Study participants were from the KPMCP of Northern California, an eight-county area in Utah (Davis, Salt Lake, Utah, Weber, Wasatch, Tooele, Morgan, and Summit Counties), and the metropolitan Twin Cities area (Anoka, Carver, Dakota, Hennepin, Ramsey, Scott, and Washington Counties) of Minnesota. The racial composition of the study population was 4.2% black (not Hispanic), 4.4% Hispanic, and 91.4% white (not Hispanic). Eligibility criteria for cases included: diagnosis with first primary colon cancer (International Classification of Diseases-Oncology, Ed. 2, codes 18.0 and 18.2–18.9) between October 1, 1991, and September 30, 1994; age between 30 and 79 years at the time of diagnosis; and mental competence to complete the interview. Cases with tumors in the rectosigmoid junction or rectum (defined up to 15 cm from the anal opening) and cases with pathology report designated familial adenomatous polyposis, Crohn's disease, or ulcerative colitis were not eligible. A rapid-reporting system was used to identify all incident cases of colon cancer resulting in the majority of cases being interviewed within 4 months of diagnosis. Of those whom we were able to ask to participate in the study, 76% cooperated; this represents 64.5% of the eligible cases. The main reasons for lack of response were: refusal of patient's physician (4.6%), patient illness or death (10.6%), patient had moved or we were unable to locate the patient (3.3%), and patient refusal (17%).

Methods used to ascertain controls have been reported (14). Control subjects from the KPMCP were randomly selected from membership lists. In Utah, control subjects under 65 years of age were identified from random-digit dialing and lists of individuals with a current Utah driver's license or state identification; control subjects 65 years of age and older were randomly identified from Health Care Financing Administration (social security) lists. In Minnesota, control subjects were identified from lists of individuals with a Minnesota driver's license or state identification. Controls were matched to cases by 5-year age groups and sex. Of all controls asked to participate, 64% cooperated. Reasons for nonparticipation have been described (15). The primary reason for nonparticipation was that we were unable to locate the subjects (752 selected controls). Of these, 663 were controls selected from Minnesota, where new driver's license holders were added on a regular basis, but people who died or moved were not removed. This resulted in a 10% overall difference in response rate, depending on whether these 663 controls were kept in the denominator or left out during calculations.

**Data Collection.** Data were collected from study participants by trained and certified interviewers using laptop computers. The referent period for the study was the year 2 years prior to the date of selection (the date of diagnosis for cases or date of selection for controls). The interview took ~2 h to complete. Study quality control methods have been described (16, 17).

**Dietary Intake.** Dietary intake data were ascertained using an adaptation of the CARDIA diet history (17–19). With this questionnaire, participants were asked to recall foods eaten, the frequency with which they were eaten, foods eaten as additions to other foods, and use of fats during food preparation. Nasco food models were used to help participants estimate their usual serving size; cue cards were used to help participants report foods in a consistent manner. Foods eaten away from home that were prepared with fat were assigned the fat that is most commonly used at medium-priced restaurants. For certain food items, many foods within a category could be eaten, *e.g.*, cereal; in these cases, participants were asked to report the three most common types. Seasonal consumption was obtained for fresh and canned fruits and for four vegetables (corn on the cob,

tomatoes, and summer and winter squash). Nutrient values for specific foods were calculated using the Nutrition Coordinating Center Nutrient Database version 19 (20). Nutrient values were calculated from foods only and do not include values from supplements. Data on alcohol (average grams consumed per day and standard servings of specific types of alcohol) were obtained as part of the diet history questionnaire. Type of alcohol consumed during the referent period was obtained for usual weekdays (Monday through Thursday) and weekend days (Friday, Saturday, and Sunday).

**Vitamin and Mineral Supplements.** Study participants were asked if they took multivitamins on a regular basis, defined as at least three times a week for 1 month during the referent year. The frequency that multivitamins were taken was obtained, although long-term duration of use was not. Multivitamin use was reported by 27.5% of male cases and 29.4% of male controls and 36.5% of female cases and 37.5% of female controls. Participants were also asked whether they took other vitamin or mineral supplements. Fewer than 1% of the participants reported taking individual supplements of folate, vitamin B<sub>12</sub>, or vitamin B<sub>6</sub>; ~1% reported taking a B complex supplement. For this study, multivitamin use was categorized as follows: using supplements, if they reported taking any multivitamins, folate, vitamin B<sub>12</sub>, vitamin B<sub>6</sub>, or B complex supplements; or not using supplements, if they did not report taking these supplements during the referent period.

**MTHFR.** Of the 4403 cases and controls with valid study data, 3680 (84%) had blood collected. Genomic DNA for the University of Minnesota and KPMCP samples was extracted at the University of Utah using a PureGene kit (Gentra Systems, Inc., Minneapolis, MN). University of Utah samples were obtained from immortalized cells. These cell lines were grown, and DNA was extracted using phenol chloroform. Of the 3680 individuals with DNA, 3283 had *MTHFR* genotype data. This represents ~75% of those who were interviewed. *MTHFR* data were not available for 397 individuals because either insufficient DNA was available to perform the test or the quality of the DNA was such that it was difficult to clearly determine individual genotype. Quality control methods used included running three control samples per 96-well tray.

Genomic DNA was amplified using PCR. The variant allele is created by a C to T bp change at nucleotide 677 that creates a *HinfI* restriction site. The identification of *MTHFR* genotype test was detected by PCR, followed by a *HinfI* enzyme digest. This region of genomic DNA was amplified using PCR, as described previously (13). The uncut PCR product comprised 198 bases. Digestion of DNA with *HinfI* for the alanine to valine polymorphism removed 23 bases from this product, leading to a 175-bp product. DNA from individuals homozygous for this polymorphism, therefore, showed only this 175-bp band; heterozygotes showed both the 175- and 198-bp bands; and homozygous wild type individuals showed only the 198-bp band. Cases and controls were classified as *TT* if they were homozygous for the C677T allele, *TC* if they were heterozygous for the C677T allele, and *CC* if they were homozygous for the C677 (wild-type) allele.

**Statistical Methods.** Initial analyses were done using spline regression models. This was done to determine visually whether differences in associations with dietary factors existed by genotype. Logistic regression models were run two ways to estimate associations. (a) We assessed interaction by using as a common referent point those at the highest risk: *CC* genotype and low intakes of nutrients. Conducting analyses in this manner allowed us to determine whether there was a difference in association across combined categories of dietary factor and genotype. (b) We stratified the data by genotype. In these

Table 1 Associations between MTHFR and colon cancer

MTHFR genotype	Men			Women			All subjects		
	Cases (n)	Controls (n)	OR (95% CI) <sup>a</sup>	Cases (n)	Controls (n)	OR (95% CI)	Cases (n)	Controls (n)	OR (95% CI)
CC	372	435	1.0	301	392	1.0	673	827	1.0
CT	378	423	1.0 (0.9–1.3)	277	359	1.0 (0.8–1.3)	655	787	1.0 (0.9–1.2)
TT	74	109	0.8 (0.6–1.1)	65	98	0.9 (0.6–1.2)	139	207	0.9 (0.7–1.1)
Distal tumors									
CC	175	435	1.0	141	392	1.0	316	827	1.0
CT	189	423	1.1 (0.9–1.5)	130	359	1.0 (0.8–1.3)	329	787	1.1 (0.9–1.3)
TT	41	109	1.0 (0.7–1.5)	32	98	0.9 (0.6–1.5)	73	207	1.0 (0.7–1.3)
Proximal tumors									
CC	185	435	1.0	154	392	1.0	339	837	1.0
CT	177	423	1.0 (0.8–1.3)	142	359	1.0 (0.8–1.4)	319	787	1.0 (0.8–1.2)
TT	33	109	0.7 (0.5–1.1)	31	98	0.8 (0.5–1.3)	64	207	0.8 (0.6–1.1)

<sup>a</sup> Adjusted for age, BMI, long-term vigorous physical activity, energy intake, dietary fiber, and usual number of cigarettes smoked.

analyses, we addressed the question, what is the risk of colon cancer, given a certain genotype, at specific levels of nutrient intake?

ORs and 95% CIs were calculated from these unconditional logistic regression models. In these models, age at selection, BMI [weight/(height)<sup>2</sup> for men; weight/(height)<sup>1.5</sup> for women] reported for the referent period, long-term vigorous leisure-time physical activity, as determined using an adaptation of the CARDIA physical activity history (21, 22), total energy intake, dietary fiber, and usual number of cigarettes smoked per day (persons who never smoked were the referent group) on a regular basis were included as covariates. Categories of exposure for nutrients were based upon the distribution in the control population for men and women separately. Because few differences in risk were noted for the middle three quintiles and more stable estimates of risk were obtained, we made three categories of approximately the lowest quintile for men and women, the middle quintiles, and approximately the upper quintile of intake. Nutrients were expressed per 1000 kcal.

Analyses were performed separately for men and women to determine whether differences existed by sex and because most of the literature has focused on either men or women. Age-specific analyses were performed using age 60 as the cutoff point. This was done to allow comparisons with studies focusing on adenomas. Tumor site within the colon was defined as proximal (cecum through transverse colon) or distal (splenic flexure, descending, and sigmoid colon), as classified by the tumor registries. Site-specific analyses were done because much of the literature suggests that stronger associations between folate and alcohol are seen for distal colon tumors. Surveillance Epidemiology and End Results summary stage data (local, regional, and distant) were used to evaluate associations by stage at time of diagnosis.

## Results

Of all cases, 45.9% had the CC genotype, 44.7% had the CT genotype, and 9.5% had the TT genotype (Table 1); the distribution among controls was 45.4, 43.2, and 11.4%, respectively. We observed no differences in colon cancer risk associated with MTHFR genotype by sex or subsite. Results are reported with the CC genotype as the referent group; however, if the CC and CT genotypes are combined and used as the referent group, the OR for TT genotype is the same, at 0.8, although the estimate is more precise (95% CI = 0.7–1.1 compared to 0.5–1.3).

High intakes of folate, vitamin B<sub>6</sub>, and vitamin B<sub>12</sub> were associated with a 30–40% reduction in risk of colon cancer among those with the TT genotype relative to those with low

Table 2 Associations between nutrients and MTHFR status in men and women

MTHFR genotype	OR (95% CI) <sup>a</sup>		
	Low	Intermediate	High
Folate <sup>b</sup>			
CC	1.0	0.8 (0.7–1.1)	0.8 (0.6–1.2)
CT	1.0 (0.8–1.3)	0.9 (0.7–1.2)	0.8 (0.6–1.1)
TT	0.8 (0.5–1.3)	0.8 (0.6–1.2)	0.6 (0.4–1.0)
Vitamin B <sub>6</sub>			
CC	1.0	0.8 (0.6–1.0)	0.9 (0.7–1.3)
CT	0.9 (0.7–1.2)	0.9 (0.7–1.2)	0.9 (0.6–1.2)
TT	0.8 (0.5–1.3)	0.8 (0.6–1.2)	0.6 (0.4–1.0)
Vitamin B <sub>12</sub>			
CC	1.0	1.1 (0.8–1.4)	1.0 (0.8–1.4)
CT	1.1 (0.7–1.9)	1.1 (0.9–1.4)	0.9 (0.6–1.2)
TT	1.2 (0.7–1.9)	0.9 (0.6–1.2)	0.7 (0.5–1.2)
Methionine			
CC	1.0	0.9 (0.7–1.2)	0.8 (0.6–1.0)
CT	1.0 (0.7–1.3)	0.9 (0.7–1.2)	0.9 (0.6–1.2)
TT	0.9 (0.5–1.4)	0.7 (0.5–1.0)	0.8 (0.5–1.3)
Alcohol			
CC	1.0	0.9 (0.7–1.2)	0.8 (0.6–1.1)
CT	0.9 (0.7–1.1)	1.0 (0.8–1.3)	1.2 (0.9–1.6)
TT	1.0 (0.7–1.4)	0.5 (0.3–0.8)	1.0 (0.6–1.6)
Multivitamin supplement <sup>c</sup>			
CC	1.0		1.1 (0.9–1.3)
CT	1.0 (0.8–1.2)		1.1 (0.9–1.4)
TT	0.9 (0.7–1.2)		0.9 (0.6–1.3)

<sup>a</sup> Nutrients were evaluated as nutrients per 1000 kcal and adjusted for sex, age, BMI, long-term vigorous physical activity, usual number of cigarettes smoked per day, total energy intake, and dietary fiber.

<sup>b</sup> Cut-off points for men were: folate, 126/197; vitamin B<sub>6</sub>, 0.8/1.2; vitamin B<sub>12</sub>, 1.9/3.31; methionine, 0.74/1.0; alcohol, 1/20. Cut-off points for women were: folate, 141/214; vitamin B<sub>6</sub>, 0.85/1.21; vitamin B<sub>12</sub>, 1.88/3.31; methionine, 0.75/1.0; alcohol, 1/20.

<sup>c</sup> Includes any reported use of multivitamin supplements or any supplement that contains folate or B vitamins during the referent year. Low, used no multivitamin supplement; High, used multivitamin supplement.

levels of intake who were CC genotype (Table 2). Associations for methionine, alcohol, and multivitamin supplement users did not vary appreciably by MTHFR genotype. Statistically significant differences in association were not observed by sex, although the strength of some associations varied by sex (data not shown).

The combined associations for MTHFR genotype with dietary intake of folate, vitamin B<sub>6</sub>, and vitamin B<sub>12</sub> were most

Table 3 Associations between nutrients and MTHFR status by tumor site

MTHFR genotype	Proximal, OR (95% CI) <sup>a</sup>			Distal, OR (95% CI)		
	Low	Intermediate	High	Low	Intermediate	High
Folate						
CC	1.0	0.8 (0.6–1.1)	0.9 (0.6–1.4)	1.0	0.9 (0.6–1.2)	0.8 (0.5–1.2)
CT	0.9 (0.7–1.3)	0.9 (0.6–1.2)	0.8 (0.6–1.3)	1.1 (0.8–1.6)	1.0 (0.7–1.3)	0.7 (0.5–1.2)
TT	0.7 (0.4–1.3)	0.8 (0.5–1.4)	0.5 (0.2–0.9)	0.9 (0.5–1.5)	0.9 (0.5–1.4)	0.8 (0.5–1.5)
Vitamin B <sub>6</sub>						
CC	1.0	0.8 (0.6–1.1)	1.1 (0.7–1.6)	1.0	0.7 (0.5–1.0)	0.8 (0.5–1.1)
CT	1.0 (0.7–1.4)	0.8 (0.6–1.2)	1.0 (0.6–1.4)	0.8 (0.6–1.2)	0.9 (0.7–1.3)	0.8 (0.5–1.2)
TT	0.8 (0.4–1.5)	0.8 (0.5–1.3)	0.5 (0.3–0.9)	0.8 (0.4–1.4)	0.8 (0.5–1.3)	0.7 (0.4–1.3)
Vitamin B <sub>12</sub>						
CC	1.0	1.3 (0.9–1.8)	1.2 (0.8–1.8)	1.0	0.8 (0.6–1.2)	0.9 (0.6–1.3)
CT	1.3 (0.9–1.8)	1.3 (0.9–1.8)	1.0 (0.7–1.5)	1.0 (0.7–1.5)	1.0 (0.7–1.4)	0.8 (0.6–1.2)
TT	1.1 (0.6–2.2)	1.0 (0.6–1.6)	0.6 (0.3–1.3)	1.1 (0.6–2.1)	0.8 (0.5–1.2)	0.9 (0.5–1.6)

<sup>a</sup> Nutrients were evaluated as nutrients per 1000 kcal and adjusted for sex, age, BMI, long-term vigorous physical activity, usual number of cigarettes smoked per day, total energy intake, and dietary fiber.

Table 4 Associations between nutrients and MTHFR status by age at time of diagnosis

MTHFR genotype	≤60 years, OR (95% CI) <sup>a</sup>			>60 years, OR (95% CI)		
	Low	Intermediate	High	Low	Intermediate	High
Folate						
CC	1.0	0.8 (0.5–1.3)	0.7 (0.3–1.3)	1.0	0.9 (0.6–1.2)	0.9 (0.6–1.4)
CT	1.0 (0.6–1.6)	0.7 (0.4–1.1)	0.5 (0.3–1.1)	1.0 (0.7–1.4)	1.0 (0.8–1.4)	0.9 (0.6–1.3)
TT	1.1 (0.5–2.7)	1.0 (0.4–1.9)	0.6 (0.2–1.7)	0.7 (0.4–1.2)	0.8 (0.5–1.3)	0.6 (0.4–1.1)
Vitamin B <sub>6</sub>						
CC	1.0	0.7 (0.5–1.2)	0.6 (0.3–1.2)	1.0	0.8 (0.6–1.1)	1.0 (0.7–1.5)
CT	0.7 (0.5–1.2)	0.7 (0.4–1.1)	0.8 (0.4–1.7)	1.0 (0.7–1.4)	1.0 (0.7–1.4)	0.9 (0.6–1.3)
TT	0.6 (0.2–1.3)	1.0 (0.5–2.0)	1.1 (0.4–2.9)	0.9 (0.5–1.7)	0.8 (0.5–1.2)	0.5 (0.3–0.9)
Vitamin B <sub>12</sub>						
CC	1.0	0.9 (0.5–1.5)	0.9 (0.5–1.5)	1.0	1.1 (0.8–1.5)	1.1 (0.7–1.5)
CT	1.0 (0.6–1.8)	0.8 (0.5–1.3)	0.7 (0.4–1.3)	1.2 (0.8–1.7)	1.3 (0.9–1.7)	0.9 (0.6–1.3)
TT	1.6 (0.6–4.2)	0.7 (0.4–1.6)	1.0 (0.4–2.6)	1.0 (0.5–1.8)	0.9 (0.6–1.3)	0.6 (0.4–1.2)

<sup>a</sup> Nutrients evaluated as nutrients per 1000 kcal and adjusted for sex, age, BMI, long-term vigorous physical activity, usual number of cigarettes smoked per day, total energy intake, and dietary fiber.

marked for those with proximal tumors (Table 3). In this subset of the population, high intakes of nutrients were associated with a halving of risk for those with the *TT* genotype relative to *CC* genotype and low levels of intake. This combination did not appear to have the same strength of association with distal tumors as we observed for proximal tumors. Neither alcohol nor methionine showed these site-specific associations when men and women were assessed together. Associations with folate, vitamin B<sub>6</sub>, and vitamin B<sub>12</sub> were observed for both men and women separately, although the inverse association with folate was stronger among men (data not shown) and high levels of intake of methionine were associated with a 2-fold increase in risk among women (data not shown).

The inverse associations noted with high vitamin B<sub>6</sub> and vitamin B<sub>12</sub> and the *TT* *MTHFR* genotype were stronger among those who were diagnosed when they were over 60 years of age (Table 4). Although these trends in association were different by age at time of diagnosis, interaction terms including age, nutrient, and *MTHFR* genotype did not significantly improve the fit of the regression models at the  $P = 0.05$  level.

Evaluation of colon cancer risk by examination of the levels of folate and methionine (Table 5) in conjunction with *MTHFR* genotype showed those at lowest risk of colon cancer were participants who had the *TT* genotype and high intakes of both folate and methionine. Although risk estimates were very imprecise, a protective effect for these combined factors appeared to exist for both nondrinkers and drinkers of alcohol.

Evaluation of the combined effect of these variables by grouping them into categories of low, intermediate, and high risk (Table 6) indicated that the *TT* genotype was inversely associated with risk in the presence of a low-risk dietary pattern. In the presence of a high-risk diet, the inverse association with the *TT* genotype disappeared for the total population and older individuals and was not statistically significant, although the values were very imprecise among younger individuals.

Evaluation of associations by stage at diagnosis was undertaken to allow a better comparison with data from studies of adenomas. We did not observe differences in associations for the nutrients examined in conjunction with *MTHFR* status by stage at time of diagnosis (data not shown).

## Discussion

*MTHFR* converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the major circulatory form of folate in the body and the primary methyl donor for the methylation of homocysteine to methionine. This pathway is key in the methylation of DNA, and abnormalities in this process can result in altered expression of oncogenes and tumor suppressor genes (10). Although variants of the *MTHFR* gene have been shown to result in different levels of enzyme activity, it is generally not known how diet and other environmental factors influence the carcinogenic process, given inherent variation in enzyme levels.

Table 5 Combined effects of folate, methionine, and alcohol on risk of colon cancer

Folate <sup>a</sup>	Methionine	All subjects		Non-alcohol drinkers		Alcohol drinkers	
		CC/CT, OR (95% CI) <sup>b</sup>	TT, OR (95% CI)	CC/CT, OR (95% CI)	TT, OR (95% CI)	CC/CT, OR (95% CI)	TT, OR (95% CI)
Low	Low	1.0	1.0 (0.5–2.3)	1.0	0.9 (0.2–4.0)	1.0	1.2 (0.4–3.1)
	Intermediate	0.9 (0.7–1.3)	0.7 (0.4–1.2)	1.0 (0.6–1.6)	1.1 (0.5–2.4)	0.9 (0.6–1.3)	0.4 (0.1–0.9)
	High	1.0 (0.7–1.6)	0.9 (0.2–2.8)	1.1 (0.6–2.1)	0.5 (0.1–2.7)	0.9 (0.5–1.8)	— <sup>c</sup>
High	Low	0.9 (0.6–1.4)	0.7 (0.3–1.7)	1.0 (0.5–1.8)	0.7 (0.2–2.3)	0.8 (0.4–1.5)	0.5 (0.1–2.5)
	Intermediate	0.6 (0.4–0.9)	0.6 (0.3–1.0)	0.6 (0.4–1.1)	0.8 (0.3–1.8)	0.6 (0.4–1.1)	0.4 (0.2–1.0)
	High	0.8 (0.5–1.2)	0.3 (0.1–0.9)	0.7 (0.4–1.2)	0.4 (0.1–1.1)	0.9 (0.5–1.7)	— <sup>c</sup>

<sup>a</sup> High folate was defined as >205 µg per 1000 calories; high methionine was defined as >1.0 g per 1000 calories; low folate was defined as ≤135 µg per 1000 calories; low methionine was defined as <0.75 g per 1000 calories; intermediate levels fell between these values.

<sup>b</sup> Nutrients were evaluated as nutrients per 1000 kcal and adjusted for sex, age, BMI, long-term vigorous physical activity, usual number of cigarettes smoked per day, total energy intake, and dietary fiber.

<sup>c</sup> Too few numbers in cell to estimate associations.

Table 6 Associations between MTHFR and high- and low-risk diets as determined by combined levels of folate, methionine, and alcohol<sup>a</sup>

MTHFR genotype	OR (95% CI) <sup>b</sup>		
	High risk	Intermediate risk	Low risk
CC/TT cases/controls	109/96	1180/1449	39/64
TT cases/controls	11/10	121/179	7/18
All subjects			
CC/CT	1.0	0.8 (0.6–1.0)	0.6 (0.4–1.0)
TT	1.0 (0.4–2.4)	0.7 (0.5–1.0)	0.4 (0.1–0.9)
Subjects ≤60 years			
CC/CT	1.0	0.7 (0.4–1.2)	0.6 (0.2–1.9)
TT	0.6 (0.1–2.6)	0.8 (0.4–1.7)	0.2 (0.02–1.8)
Subjects >60 years			
CC/CT	1.0	0.8 (0.5–1.0)	0.6 (0.3–1.0)
TT	1.3 (0.4–4.5)	0.6 (0.4–0.9)	0.4 (0.2–1.2)

<sup>a</sup> Low risk was defined as folate ≥195 µg per 1000 calories, methionine, ≥1.0 g per 1000 calories and non-alcoholic beverage drinker. High risk was defined as folate ≤140 µg per 1000 calories, methionine ≤0.80 g per 1000 calories, and alcohol >10 g per day. Values between those for low and high are considered intermediate.

<sup>b</sup> Nutrients were evaluated as nutrients per 1000 kcal and adjusted for sex and age.

Although there is a biologically plausible mechanism that supports studying *MTHFR* variants in conjunction with dietary factors and colon cancer, few studies have evaluated these associations. Those that have evaluated these associations have generally had a small sample size, resulting in imprecise estimates of association (23, 24); have included tumors located in both the colon and rectum (23); and have, in some instances, looked at colonic adenomas rather than cancers (25). The frequencies of the variant *TT* genotype reported here, 9.5% of cases and 11.4% of controls, are similar to the prevalence of this genotype reported in other studies, where the prevalence has ranged from 9.3% in controls and 11.7% in cases (25) to 15% in controls and 9% in cases (24). As in the study by Chen *et al.* (23) and Ma *et al.* (24), we observed that the variant form of *MTHFR* was associated with lower risk of colon cancer than the *CC* or wild-type genotype. Although the magnitude of the association was less than that reported previously, our point estimates of 0.8 for men (95% CI = 0.6–1.1) and 0.9 for women (95% CI = 0.6–1.2) are within the CIs reported by Chen *et al.* (23) in their study of men (95% CI = 0.3–1.1) and by Ma *et al.* (24) in their study of men (95% CI = 0.2–0.9). In contrast to these findings, in a study of adenomas, the *TT* variant genotype was reportedly associated with an estimated 40% increase in the risk of colonic adenomas (25). Our own

study of adenomas<sup>4</sup> is consistent with these findings, showing a marginally reduced risk with the *TT* genotype.

Our findings on the associations with dietary factors and *MTHFR* genotype vary slightly from those presented by Chen *et al.* (23); however, in almost all instances, our point estimate is within their reported 95% CIs. Results were similar for folate, in that high intakes of folate were associated with reduced risk of colon cancer for those with the *TT* genotype. Thus, if the associations are regarded as causal, people with reduced enzyme activity obtain the most protection from high levels of folate, vitamin B<sub>6</sub>, and vitamin B<sub>12</sub> intakes. Assessment of vitamin supplements showed association trends that were similar to but weaker than those observed for other nutrients. However, supplement information was obtained for the referent period and other studies have suggested that folate supplements are protective only if they are taken for over 15 years (26). Our study on adenomas shows that the risk among individuals with the *TT* genotype and adequate or high intakes of folate and so on differs little from that among those with the *TT* genotype alone; however, those with a low intake and the *TT* genotype are at elevated risk of adenoma.<sup>4</sup>

Associations have not been previously examined by age at diagnosis or tumor site within the colon. However, most studies have focused on younger men (2), or on younger populations in studies of adenomas (24). We observed stronger associations with a low intake of nutrients independent of genotype for those diagnosed at a younger age, whereas among those diagnosed at older ages high levels of folate, vitamin B<sub>6</sub>, and vitamin B<sub>12</sub> were more associated with reduced risk in individuals with the *TT* genotype. Plasma homocysteine concentrations have been shown to increase with age, independent of B vitamin status (27). This is thought to result from an age-related decline in cystathionine β-synthase and possibly other enzymes involved in homocysteine metabolism (27). In our study, associations appear to be strongest for those with proximal tumors. The reason for this site-specific association is not clear. Other genes involved in methylation processes, such as methionine synthase (28), may be involved in DNA methylation processes and may be influenced by dietary factors. Incorporation of data on other genotypic variations may alter estimates of associations with colon cancer risk.

In studying colon cancer, we are evaluating associations with later events in the carcinogenic process than those considered in studies of adenomas. On the basis of the epidemiologic

<sup>4</sup> C. M. Ulrich, E. Kampman, J. Bigler, S. M. Schwartz, C. Chen, R. Bostick, L. Fosdick, S. A. Beresford, Y. Yasui, and J. D. Potter. Colorectal adenomas and the C677T *MTHFR* polymorphism: evidence for gene-environment interaction?, submitted for publication.

logical literature, there are reasons to believe that dietary factors such as folate, methionine, and alcohol are related to earlier rather than later events (17). Likewise, there are data that suggest that DNA methylation abnormalities are early events in the neoplastic process (5–12). This could influence our results in two ways. (a) Associations may be weaker for colon cancer than for adenomas because late rather than early events in the disease pathway are being examined. Only some adenomas advance to cancers. Studying characteristics of those adenomas most likely to advance to tumors may provide insight into these differences. (b) Using the referent period of the year 2 years prior to diagnosis may have limitations in capturing the most relevant time period. These differences may account for some of the differences observed from studies of adenomas and from studies in which cases were identified from cohort studies in which exposure information has generally been collected at an earlier time. However, results from the Nurses' Health Study, which evaluated the same dietary and genetic factors reported here, did not observe an inverse association with the *TT* variant of the *MTHFR* gene, and differences in associations with diet based on *MTHFR* genotype were not observed (25).

Not all individuals included in the original study had DNA available for analyses. Therefore, it is possible that differences in exposures of interest between those with and without DNA may exist. Assessment of potential differences in these two groups showed that there were no differences in levels of BMI, physical activity, sex, tumor site, total energy intake, folate, vitamin B<sub>6</sub>, vitamin B<sub>12</sub>, or methionine. However, those with genotype data were more likely to report drinking alcohol (14.6 g per day for drinkers) than those without genotype data (alcohol intake, 12.13 g per day;  $P = <0.01$ ). It is unlikely that differences in alcohol intake influence our data because we did not detect an association between colon cancer and alcohol in the larger group of cases (4), nor did we observe differences in association by level of reported alcohol.

In summary, the variant form (*TT*) of the *MTHFR* gene appears to be slightly inversely associated with colon cancer; the heterozygote (*CT*) and wild-type (*CC*) genotypes appear to have similar associations with colon cancer. Although there were some suggestions of differences in effect from combined dietary intake and genotype profiles, associations were generally weak. Inverse associations generally were stronger for tumors in the proximal tumor and for older age groups; however, low intakes of folate, vitamin B<sub>6</sub>, and vitamin B<sub>12</sub> were associated with slight increases in risk for all genotypes among those who were diagnosed at a younger age. These age-specific differences in association could contribute to differences observed between studies in the literature.

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### References

- Freudenheim, J. L., Graham, S., Marshall, J. R., Haughey, B. P., Cholewinski, S., and Wilkinson, G. Folate intake and carcinogenesis of the colon and rectum. *Int. J. Epidemiol.*, 20: 368–374, 1991.
- Giovannucci, E., Rimm, E. B., Ascherio, A., Stampfer, M. J., Colditz, G. A., and Willett, W. C. Alcohol, low-methionine-low-folate diets and risk of colon cancer in men. *J. Natl. Cancer Inst. (Bethesda)*, 87: 265–273, 1995.
- Giovannucci, E., Stampfer, M. J., Colditz, G. A., Rimm, E. B., Trichopoulos, D., Rosner, B. A., Speizer, F. E., and Willett, W. C. Folate, methionine, and alcohol intake and risk of colorectal adenoma. *J. Natl. Cancer Inst. (Bethesda)*, 85: 875–884, 1993.
- Slattery, M. L., Schaeffer, D., Edwards, S. L., Ma, K. N., and Potter, D. J. Are dietary factors involved in DNA methylation associated with colon cancer? *Nutr. Cancer*, 28: 52–62, 1997.

- Laird, P. W., and Jaenisch, R. DNA methylation and cancer. *Hum. Mol. Genet.*, 3: 1487–1495, 1994.
- Cedar, H. DNA methylation and gene activity. *Cell*, 53: 3–4, 1988.
- Issa, J. P., Vertino, P. M., Wui, J., Sazawal, S., Celano, P., Nelkin, B. D., Hamilton, S. R., and Baylin, S. B. Increased cytosine DNA-methyltransferase activity during colon cancer progression. *J. Natl. Cancer Inst. (Bethesda)*, 85: 1235–1240, 1993.
- Goetz, S. E., Vogelstein, B., Hamilton, S. R., and Feinberg, A. P. Hypomethylation of DNA from benign and malignant human colon neoplasms. *Science (Washington DC)*, 228: 187–200, 1985.
- Makos, M., Nelkin, B. D., Lerman, M. I., Latif, F., Abar, B., and Baylin, S. B. Distinct hypermethylation patterns occur at altered chromosome loci in human lung and colon cancer. *Proc. Natl. Acad. Sci. USA*, 89: 1929–1933, 1992.
- Baylin, S. B., Makos, M., We, J., Chiu Yen, R. W., de Bustros, A., Vertino, P., and Nelkin, B. D. Abnormal patterns of DNA methylation in human neoplasia: potential consequences for tumor progression. *Cancer Cells*, 3: 382–390, 1991.
- Selhub, J., and Miller, J. W. The pathogenesis of homocysteinemia: interruption of the coordination regulation by S-adenosylmethionine of the remethylation and transsulfuration of homocysteine. *Am. J. Clin. Nutr.*, 55: 131–138, 1992.
- Hoffman, R. M. Altered methionine metabolism, DNA methylation and oncogene expression in carcinogenesis. A review and synthesis. *Biochim. Biophys. Acta*, 738: 49–87, 1984.
- Frost, P., Blom, H. J., Milos, R., Goyette, P., Sheppard, C. A., Matthews, R. G., Boers, G. J., den Heijer, M., Kluijtmans, L. A., and vanden Heuvel, L. P. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat. Genet.*, 10: 111–113, 1995.
- Slattery, M. L., Potter, J. D., Caan, B. J., Edwards, S., Coates, A., Ma, K. N., and Berry, T. D. Energy balance and colon cancer: beyond physical activity. *Cancer Res.*, 57: 75–80, 1997.
- Slattery, M. L., Edwards, S. L., Caan, B. J., Kerber, R. A., and Potter, J. D. Response rates among control subjects in case-control studies. *Ann. Epidemiol.*, 5: 245–249, 1995.
- Edwards, S., Slattery, M. L., Mori, M., Berry, T. D., Caan, B. J., Palmer, P., and Potter, J. D. Objective system for interviewer performance evaluation for use in epidemiologic studies. *Am. J. Epidemiol.*, 140: 1020–1028, 1994.
- Slattery, M. L., Caan, B. J., Duncan, D., Berry, T. D., Coates, A., and Kerber, R. A computerized diet history questionnaire for epidemiologic studies. *J. Am. Diet. Assoc.*, 94: 761–766, 1994.
- McDonald, A., Van Horn, L., Slattery, M. L., Hilner, J., Bragg, C., Caan, B., Jacobs, D., and Lin, K. The CARDIA dietary history: development and implementation. *J. Am. Diet. Assoc.*, 9: 1104–1112, 1991.
- Liu, K., Slattery, M. L., Jacobs, D. R., Jr., Cutter, G., McDonald, A., Van Horn, L., Hilner, J., Caan, B., Bragg, C., Dyer, A., and Havlik, R. A study of the reliability and comparative validity of the CARDIA dietary history. *Ethn. Dis.*, 4: 15–27, 1994.
- Dennis, B., Ernst, N., Hjortland, M., Tillotson, J., and Grambsch, V. The NHLBI nutrition system. *J. Am. Diet. Assoc.*, 77: 641–647, 1980.
- Jacobs, D. R., Jr., Hahn, L. P., Haskell, W. L., Pirie, P., and Sidney, S. Validity and reliability of a short physical activity history: CARDIA and the Minnesota Heart Health Program. *J. Cardiopulm. Rehabil.*, 9: 448–459, 1989.
- Slattery, M. L., and Jacobs, D. R., Jr. Assessment of ability to recall physical activity of several years ago. *Ann. Epidemiol.*, 5: 292–296, 1995.
- Chen, J., Giovannucci, E., Kelsey, K., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Spiegelman, D., Willett, W. C., and Hunter, D. J. A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. *Cancer Res.*, 56: 4862–4864, 1996.
- Ma, J., Stampfer, M. J., Giovannucci, E., Artigas, C., Hunter, D. J., Fuchs, C., Willett, W. C., Selhub, J., Hennekens, C. H., and Rozen, R. Methylenetetrahydrofolate reductase polymorphism, dietary interactions, and risk of colorectal cancer. *Cancer Res.*, 57: 1098–1102, 1997.
- Chen, J., Giovannucci, E., Hankinson, S. E., Ma, J., Willett, W. C., Spiegelman, D., Kelsey, K. T., and Hunter, D. J. A prospective study of methylenetetrahydrofolate reductase and methionine synthase gene polymorphisms, and risk of colorectal adenoma. *Carcinogenesis (Lond.)*, 19: 2129–2132, 1998.
- Giovannucci, E., Stampfer, M. J., Colditz, G. A., Hunter, D. J., Fuchs, C., Rosner, B. A., Speizer, F. E., and Willett, W. C. Multivitamin use, folate, and colon cancer in women in the Nurses' Health Study. *Ann. Intern. Med.*, 129: 517–524, 1998.
- Selhub, J., Jacques, P. F., Wilson, P. W. F., Rush, D., and Rosenberg, I. H. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *J. Am. Med. Assoc.*, 270: 2693–2698, 1993.
- Chen, L. H., Liu, M.-L., Hwang, H.-Y., Chen, L.-S., Korenberg, J., and Shane, B. Human methionine synthase. cDNA cloning, gene localization and expression. *J. Biol. Chem.*, 272: 3628–3634, 1997.