

Vitamins B₂, B₆, and B₁₂ and Risk of New Colorectal Adenomas in a Randomized Trial of Aspirin Use and Folic Acid Supplementation

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Abstract

Background: Folate, other vitamin B cofactors, and genes involved in folate-mediated one-carbon metabolism all may play important roles in colorectal neoplasia. In this study, we examined the associations between dietary and circulating plasma levels of vitamins B₂, B₆, and B₁₂ and risk colorectal adenomas. **Methods:** The Aspirin/Folate Polyp Prevention Study is a randomized clinical trial of folic acid supplementation and incidence of new colorectal adenomas in individuals with a history of adenomas (*n* = 1,084). Diet and supplement use were ascertained through a food frequency questionnaire administered at baseline. Blood collected at baseline was used to determine plasma B-vitamin levels. We used generalized linear regression to estimate risk ratios (RR) and 95% confidence intervals (95% CI) as measures of association. **Results:** We found a borderline significant inverse association with plasma B₆ [pyridoxal 5'-phosphate

(PLP)] and adenoma risk (adjusted RR Q4 versus Q1, 0.78; 95% CI, 0.61-1.00; *P*_{trend} = 0.08). This association was not modified by folic acid supplementation or plasma folate. However, the protective association of PLP with adenoma risk was observed only among subjects who did not drink alcohol (*P*_{interaction} = 0.03). Plasma B₂ (riboflavin) was inversely associated with risk of advanced lesions (adjusted RR Q4 versus Q1, 0.51; 95% CI, 0.26-0.99; *P*_{trend} = 0.12). No significant associations were observed between adenoma risk and plasma vitamin B₁₂ or dietary intake of vitamin B₂ and B₆. When we examined specific gene-B-vitamin interactions, we observed a possible interaction between methylenetetrahydrofolate reductase -C677T and plasma B₂ on risk of all adenomas.

Conclusion: Our results suggest that high levels of PLP and B₂ may protect against colorectal adenomas. (Cancer Epidemiol Biomarkers Prev 2008;17(8):2136-45)

Introduction

Folates play potentially important roles in carcinogenesis because of their role as major carriers of the one-carbon groups needed for intracellular methylation reactions and nucleotide synthesis (1, 2). Folate-associated one-carbon metabolism (FOCM) depends on B-vitamin cofactors for many of the key reactions involved. Thus, methylenetetrahydrofolate reductase (MTHFR) requires vitamin B₂ (riboflavin); the reactions catalyzed by methionine synthase (MTR) reductase (MTRR) and MTR require vitamin B₁₂ (cyanocobalamin) and cystathionine-β-synthase (CBS) requires vitamin B₆ (pyridoxine). However, understanding of the relationships

between B-vitamins and risk of incident colorectal adenomas is limited.

The role of vitamins B₂, B₆, and B₁₂, particularly dietary intake, has been investigated in several studies of colorectal adenomas and cancer. In the majority of studies, intake of vitamin B₂ (3-7) and vitamin B₁₂ (3, 5, 6, 8-12) has been unassociated with colorectal adenoma or cancer risk. However, there is stronger evidence to support an inverse association between dietary vitamin B₆ and risk of colorectal adenomas or cancer (3, 4, 12-17). In addition, levels of the main active form of circulating B₆, pyridoxal 5'-phosphate (PLP), were found to be inversely associated with risk of colon cancer in the Nurses' Health Study (14).

Other studies have investigated whether selected polymorphisms in FOCM genes coding for enzymes that require B-vitamins as cofactors modify the relationship between dietary intake of the vitamins and risk of colorectal adenomas or cancer, but results have been inconsistent (5, 6, 18-25). A few studies have also reported on the role of polymorphic FOCM genes on risk of adenomas in individuals with a previous history of adenomas (26, 27) but have not yet explored the

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interrelationships between these polymorphisms and intake or blood levels of B-vitamins.

In this randomized clinical trial of aspirin use and folic acid supplementation, we examined the associations of baseline plasma levels and dietary intake of vitamins B₂, B₆, and B₁₂ with risk of colorectal adenoma occurrence. We also assessed whether any B-vitamin association with adenoma risk was modified by folic acid supplementation, baseline plasma folate, multivitamin use, alcohol use, smoking, age, or polymorphisms in key FOCM genes, MTHFR, MTRR, MTR and CBS.

Materials and Methods

Study Design. The Aspirin/Folate Polyp Prevention Study is a randomized, double-blind, placebo-controlled trial of the efficacy of oral aspirin, folic acid, or both to prevent colorectal adenomas in patients with a history of adenomas (28). Recruitment began in July 6, 1994 and ended in March 20, 1998. The study was originally designed to investigate the chemopreventive potential of aspirin. Shortly after enrollment began (after 100 subjects had been randomized), the study was extended to incorporate folic acid supplementation in a 3 × 2 factorial design, with 1 mg folic acid or placebo incorporated into each aspirin treatment arm. The study protocol was approved by the institutional review board at all nine clinical centers and written informed consent was obtained from all study participants. The findings regarding aspirin and folic acid have been reported (28, 29).

Study Population: Randomization, Interventions, and Follow-up. Eligible individuals had at least one of the following: one or more histologically confirmed adenomas removed within 3 months before recruitment, one or more histologically confirmed adenomas removed within 16 months before recruitment and a lifetime history of two or more confirmed adenomas, or a histologically confirmed adenoma at least 1 cm in diameter removed within 16 months before recruitment. All participants were required to have had a complete colonoscopy documenting an absence of lesions remaining in the large bowel within 3 months of study entry and anticipated colonoscopy follow-up 3 years later. After completion of a 3-month aspirin run-in period, compliant individuals who wished to continue participating were randomized in a 1:1 ratio to 1 mg/d folic acid or placebo within strata defined by clinical center, sex, and age (≤60 versus >60 years). Initially, 3 years of treatment and follow-up was planned. However, because of concern that a longer exposure to folic acid might be required to observe an antineoplastic effect (30), participants were asked to continue on the study treatment for a subsequent (second) colonoscopic surveillance cycle (usually 3 or 5 years). The present analysis includes 1,084 individuals who completed the first follow-up colonoscopy at least 1 year after randomization. When a surveillance colonoscopy was not done at the end of the first follow-up interval, we used the last examination at least 1 year after randomization; on or before September 28, 2001 was used to demarcate the end of the follow-up period.

Data Collection

Questionnaires. All participants completed a questionnaire regarding personal characteristics, medical history,

and lifestyle habits. Dietary information was collected using the Block food frequency questionnaire administered to participants at baseline. The validity and reliability of the food frequency questionnaire has been described previously (31). Questions assessed the average consumption of a food item during the past year. Daily dietary nutrient intakes were calculated by multiplying frequency responses by the nutrient contents of the specified portion sizes using a comprehensive database. Brand and type of multivitamin supplement use were collected. Individuals who regularly consumed multivitamins (at least once per week) were considered to be "multivitamin users." Total daily alcohol intake was calculated as the sum of alcohol content from beer, wine, and liquor.

Measurement of Baseline B-Vitamin Levels. Vitamin B assays were conducted at University of Bergen. Blood samples were collected from nonfasting participants into 7 mL Vacutainer brand tubes containing EDTA at baseline. After collection, specimens were immediately put on ice and then centrifuged at 1,100 × g for 10 min. Whole blood, plasma, and buffy coat fractions were stored at -20°C and then transferred to Dartmouth Medical School where they were stored at -80°C until analysis.

Vitamin B₂ (riboflavin) and B₆ (PLP, the main active form of vitamin B₆ and pyridoxal) were determined in plasma by liquid chromatography-tandem mass spectrometry and vitamin B₁₂ by microbiological assay using published methods (32, 33).

Genotyping Assays. The following polymorphisms were considered in this study: CBS-1080C>T, A360A (rs1801181) and CBS-699C>T, Y233Y (rs234706), MTHFR-677C>T, V222A (rs1801133) and MTHFR-1298C>T, A429E (rs1801131), and MTR-2756A>G, D919G (rs1805087), and MTRR-66A>G, I22M (rs1801394). These polymorphisms, selected based on previous studies suggesting possible biological functions, were genotyped using the 5-nuclease TaqMan allelic discrimination assay on the ABI 7900HT (Applied Biosystems). All oligo primers and dual-labeled allele discrimination probes were designed and developed either using Assay-by-Design (Applied Biosystems) or the Primer Express software 2.0 (Applied Biosystems). Each assay contained quality-control DNA of the homozygous wild-type, heterozygous, and homozygous variant alleles for the respective polymorphisms in addition to the no-target controls. Laboratory staff was blinded to outcome status. Call rates for each polymorphism were >98%. Specific experimental details are available upon request from the authors.

Study Outcomes. Adenoma occurrence was determined by colonoscopy and pathology review. All important medical events reported by participants were verified with medical record review. Records for all large bowel procedures (endoscopy or surgery) were obtained, and slides for all tissue removed from the bowel were retrieved and sent to a single-study pathologist for uniform review. Lesions were classified as neoplastic (adenomatous, including sessile serrated adenomas) or nonneoplastic.

The primary study outcome was the occurrence of one or more colorectal adenomas detected at least 1 year

following randomization up to the end of the first follow-up period. A secondary outcome was the occurrence of one or more advanced lesions, defined as invasive carcinoma or adenomas with at least 25% villous component, high-grade dysplasia, or estimated size of ≥ 1 cm.

Statistical Methods. Fisher's exact and Student's *t* tests were used to compare individuals who had an adenoma occurrence and those that did not with regard to categorical and continuous variables, respectively. Spearman's rank correlation coefficient was used to calculate the correlation between plasma and dietary vitamin B levels. Baseline plasma quartile levels of vitamins B₂, B₆, and B₁₂ and dietary quartiles levels for vitamins B₂ and B₆ were the main exposures of interest. We did not examine dietary vitamin B₁₂ use because it was not estimated by the food frequency questionnaire software. Overdispersed generalized linear models for the Poisson family as an approximation to the binomial family were used to compute crude and adjusted risk ratios to assess the risk of at least one new adenoma. Covariates included in the models were age, sex, center, duration of follow-up, aspirin treatment group, and baseline multivitamin use. We included multivitamin use in our final models as an indirect adjustment for other nutrients/vitamins and unknown lifestyle factors, although exclusion of this variable from adjusted models did not substantially change any of the results. We also report a fully adjusted model including plasma levels or dietary intake of other B-vitamins (including folate). Further adjustment for body mass index, smoking, alcohol use, family history of colorectal cancer, 25-OH vitamin D levels, dietary calcium intake, dietary methionine, and red meat consumption did not substantially change the estimated relative risk, and we report here the more parsimonious models. For plasma measures, we also adjusted for time from blood draw to measurement (years) to account for possible sample degradation, but this variable was not statistically significant in any of the regression models nor did the estimates of risk change substantially.

The possibility that baseline factors modified the B-vitamin effect was assessed in these models with the use of interaction terms and Wald tests (with or without adjustment for other variables). The following baseline factors were considered: folic acid supplement group (yes versus no), plasma folate (above versus below median), alcohol use (0 versus ≥ 1 drink per day), smoking (never versus ever), age (above versus below median), and multivitamin use (none versus regular use of at least once per week). We conducted stratified analysis to obtain stratum specific estimates of risk and confidence interval (95% CI). We assessed the potential effect modification of these variables only for the risk of any adenoma occurrence because of small numbers of advanced lesions.

We also tested whether there were statistically significant interactions between polymorphisms in folate genes and B-vitamin plasma/dietary levels on adenoma risk. We considered six polymorphisms in four key genes coding for enzymes that require vitamins B₂, B₆, or B₁₂ as cofactors: CBS-1080C>T and CBS-699C>T, MTHFR-677C>T and MTHFR-1298C>T, MTR-2756A>G, and MTRR-66A>G. We assessed Hardy-Weinberg equilibrium

by using a contingency table χ^2 test to compare observed genotype frequencies to those expected under Hardy-Weinberg equilibrium. For the majority of polymorphisms, we grouped the heterozygotes with the common homozygotes, in agreement with findings from previous studies, which suggest a recessive model for polymorphisms in MTHFR (34, 35), MTRR (36), and CBS (37). For MTR-2756A/G, we grouped the heterozygotes with the less common homozygote variants to accommodate limited numbers. For dietary intake of vitamins B₂ and B₆, we used the median to define subgroups when examining gene-vitamin B interactions.

All analyses of study folate treatment were conducted according to the principle of intention to treat. Two-sided *P* values < 0.05 were considered statistically significant. We did not adjust for multiple testing as per convention in epidemiologic studies testing *a priori* defined hypotheses. Stata (version 9.2) was used for all analyses.

Results

Characteristics of Study Participants. Table 1 summarizes the characteristics of the study participants that completed a follow-up colonoscopy at least 1 year after randomization. Most were male ($n = 690$, 64%) and the overall mean age was 57.4 years (SD = 9.6). Of the 1,084 subjects with endpoint data, 471 (43.5%) were found to have one or more colorectal adenoma at least 1 year after randomization. Subjects with new adenomas did not differ significantly from those without in terms of baseline dietary and total folate but were more likely to have lower baseline plasma folate levels ($P = 0.025$) and consume more alcohol ($P = 0.006$). The mean (SD) time from randomization to completion of the first follow-up interval was 32.7 (3.6) months.

Correlation between Plasma B-Vitamins. Plasma B-vitamins were significantly correlated with each other. Plasma B₂ was modestly correlated with PLP ($\rho = 0.44$), pyridoxal ($\rho = 0.50$), B₁₂ ($\rho = 0.30$), and folate ($\rho = 0.45$). Plasma PLP and pyridoxal were strongly correlated ($\rho = 0.82$) and both were modestly correlated with plasma vitamin B₁₂ ($\rho = 0.38$ and 0.34 , respectively) and folate ($\rho = 0.48$ and 0.53 , respectively). Similarly, plasma B₁₂ and folate were modestly correlated ($\rho = 0.34$).

Association of Baseline B-Vitamin Plasma and Dietary Levels and Adenoma Risk. Baseline PLP concentration was borderline inversely associated with risk of any adenoma (RR for Q4 versus Q1, 0.78; 95% CI, 0.61-1.00; $P_{\text{trend}} = 0.08$; Table 2). However, there was no association between PLP levels and risk of advanced lesions (RR for Q4 versus Q1, 0.89; 95% CI, 0.46-1.73). Baseline pyridoxal levels were also inversely associated with all adenoma risk (RR for Q4 versus Q1, 0.78; 95% CI, 0.62-0.99), an association that was attenuated after further adjustment for plasma B₂, B₁₂, and folate (RR for Q4 versus Q1, 0.86; 95% CI, 0.66-1.12). For advanced lesions, the pyridoxal RR for Q4 versus Q1 was 0.65 (95% CI, 0.32-1.32 in a fully adjusted model). Baseline dietary vitamin B₆ intake was modestly correlated with plasma PLP and pyridoxal ($\rho = 0.17$ and 0.15 , respectively; $P < 0.001$) but did not show any association with risk of any adenoma (RR for Q4 versus Q1, 1.05; 95% CI,

0.78-1.41) or advanced lesions (RR for Q4 versus Q1, 0.99; 95% CI, 0.46-2.12; Table 3). We were also able to examine total B₆ intake (diet plus supplements) and observed nonsignificant associations similar to those for dietary B₆ (data not shown).

Baseline plasma vitamin B₂ was not significantly associated with adenoma risk (adjusted RR for Q4 versus Q1, 0.95; 95% CI, 0.75-1.20), although there was a statistically significant inverse association for advanced lesions (RR for Q4 versus Q1, 0.51; 95% CI, 0.26-0.99; $P_{\text{trend}} = 0.12$; Table 2). Baseline dietary vitamin B₂ was modestly correlated with plasma levels ($\rho = 0.1$; $P < 0.001$) and showed a borderline nonsignificant inverse association with risk of all adenomas (RR for Q4 versus Q1, 0.83; 95% CI, 0.64-1.08; Table 3) but not with advanced lesions (RR for Q4 versus Q1, 0.78; 95% CI, 0.39-1.57; Table 3).

There was no significant association between baseline plasma B₁₂ and risk of any adenoma (RR for Q4 versus Q1, 0.91; 95% CI, 0.73-1.15) or advanced lesions (RR for Q4 versus Q1, 0.94; 95% CI, 0.52-1.69; Table 2).

When we examined multivitamin use with adjustment for plasma vitamins B₂, B₆, and B₁₂ and folate, we observed no association with any adenoma (RR for Q4 versus Q1, 1.02; 95% CI, 0.85-1.23) or advanced lesions (RR for Q4 versus Q1, 1.07; 95% CI, 0.66-1.73).

Effect Modification of Folic Acid Treatment Group, Baseline Plasma Folate, Age, Smoking, Multivitamin, and Alcohol Use at Baseline. Table 4 provides estimates of risk for any adenoma according to baseline plasma B₂, B₆, and B₁₂ stratified by folic acid treatment group, multivitamin use, and alcohol use. There was no evidence that folic acid supplementation modified the association between any plasma B-vitamin and adenoma risk (Table 4). In addition, baseline plasma folate levels did not modify the associations of plasma vitamin B₂ ($P_{\text{interaction}} = 0.75$), PLP ($P_{\text{interaction}} = 0.69$), pyridoxal ($P_{\text{interaction}} = 0.68$), or vitamin B₁₂ ($P_{\text{interaction}} = 0.19$) with adenoma risk (data not shown). Similarly, multivitamin use at baseline did not significantly modify the association between plasma B₂, B₆ (PLP and pyridoxal), or B₁₂ and adenoma risk. However, alcohol use did appear to interact significantly with the association of PLP and risk of all adenomas: the inverse association was evident only in nondrinkers ($P_{\text{interaction}} = 0.03$). No other significant interactions with pyridoxal or dietary B₂ or B₆ were observed. Smoking status and age did not significantly modify any of the associations with plasma or dietary intake of B₂, B₆, or B₁₂ (all $P_{\text{interaction}}$ values > 0.05).

Selected Gene-Nutrient Interactions and Adenoma Risk. There was no evidence of deviation from

Table 1. Characteristics of individuals in the Polyp Prevention Trial of Aspirin and Folate who completed first year follow-up

Characteristics	Adenoma occurrence	No adenoma occurrence	<i>P</i> *
No. participants	471	613	
Age at baseline, mean \pm SD (y)	59.0 \pm 9.3	56.2 \pm 9.6	<0.001 [†]
Male sex, <i>n</i> (%)	321 (68.2)	369 (60.2)	0.007
Body mass index >30 kg/m ² , <i>n</i> (%)	117 (24.9)	125 (20.4)	0.080
Current cigarette smoker, <i>n</i> (%)	86 (18.5)	72 (11.8)	0.002
Colorectal cancer in first-degree relative, <i>n</i> (%)	143 (37.1)	189 (37.4)	0.949
Self-identified as White, <i>n</i> (%)	412 (87.5)	516 (84.2)	0.125
Aspirin use at baseline, <i>n</i> (%)	291 (61.8)	358 (58.5)	0.274
Aspirin treatment group, <i>n</i> (%)	300 (63.7)	421 (68.7)	0.085
Folate treatment group, <i>n</i> (%)	221 (51.8)	280 (50.0)	0.584
Baseline plasma folate, mean \pm SD (nmol/L)	21.9 \pm 15.3	24.4 \pm 19.1	0.025 [†]
Baseline plasma B ₂ , mean \pm SD	30.6 \pm 56.8	27.7 \pm 43.6	0.374 [†]
Baseline plasma B ₆ , mean \pm SD	76.3 \pm 89.2	81.3 \pm 86.8	0.378 [†]
Baseline plasma B ₁₂ , mean \pm SD	317.1 \pm 141.3	333.7 \pm 177.1	0.112 [†]
Multivitamin use, <i>n</i> (%)	153 (32.6)	226 (37.1)	0.125
Dietary intake, mean \pm SD			
Dietary folate intake (mg/d)	317.9 \pm 150.4	320.7 \pm 162.4	0.772 [†]
Total folate [§] (mg/d)	460.2 \pm 309.5	461.9 \pm 284.7	0.927 [†]
Dietary vitamin B ₂ (mg/d)	30.6 \pm 56.8	27.7 \pm 43.6	0.729 [†]
Total vitamin B ₆ [§] (mg/d)	2.6 \pm 2.2	2.5 \pm 2.0	0.718 [†]
Total vitamin B ₁₂ [¶] (mg/d)	2.6 \pm 5.8	2.5 \pm 4.6	0.779 [†]
Alcohol (drinks/d), <i>n</i> (%)			
0	121 (26.8)	206 (35.4)	0.006
≤ 1	223 (49.4)	271 (46.6)	
≥ 2	107 (23.7)	105 (18.0)	
Adenoma characteristics (at baseline)**			
<i>n</i> (mean \pm SD)	1.7 \pm 1.1	1.5 \pm 0.9	<0.001 [†]
Large adenomas (>1 cm), <i>n</i> (%)	107 (22.7)	140 (22.8)	0.962
Villous histology, <i>n</i> (%)	60 (12.7)	93 (15.2)	0.254
Proximal location, <i>n</i> (%)	248 (52.7)	244 (39.8)	<0.001

* χ^2 test.

[†]Two-sample *t* test.

[‡]81 and 325 mg/d aspirin treatment groups combined.

[§]Total folate and total B₆ = dietary + supplements.

^{||}Total B₂ from dietary sources only.

[¶]Total B₁₂ from supplements only.

**Using standard definitions by Polyp Prevention Study Group (28, 29).

Table 2. Association of vitamin B₂, B₆, and B₁₂ baseline plasma levels and risk of colorectal adenomas

	Baseline plasma vitamin B level				P*
	Q1	Q2	Q3	Q4	
Vitamin B ₂ (riboflavin), nmol/L	2.1-8.9	9.0-15.0	15.1-27.5	27.6-479.0	
All adenomas					
<i>n</i> recurrences/total	109/247	111/249	104/244	106/246	
Adjusted RR (95% CI) [†]	1.00	1.00 (0.81-1.22)	0.89 (0.72-1.10)	0.88 (0.71-1.10)	0.18
Adjusted RR (95% CI) [‡]	1.00	1.01 (0.82-1.24)	0.93 (0.75-1.15)	0.95 (0.75-1.20)	0.51
Advanced lesions					
<i>n</i> recurrences/total	29/247	22/249	30/244	18/246	
Adjusted RR (95% CI) [†]	1.00	0.76 (0.45-1.30)	0.96 (0.58-1.59)	0.55 (0.30-1.01)	0.13
Adjusted RR (95% CI) [‡]	1.00	0.76 (0.44-1.31)	0.95 (0.57-1.59)	0.51 (0.26-0.99)	0.12
Vitamin B ₆ (PLP), nmol/L	4.7-35.6	35.7-53.3	53.4-84.3	84.7-857.0	
All adenomas					
<i>n</i> recurrences/total	116/248	113/245	111/248	90/245	
Adjusted RR (95% CI) [†]	1.00	0.97 (0.79-1.18)	0.91 (0.74-1.12)	0.75 (0.59-0.94)	0.02
Adjusted RR (95% CI) [‡]	1.00	1.00 (0.82-1.22)	0.95 (0.77-1.17)	0.78 (0.61-1.00)	0.08
Advanced lesions					
<i>n</i> recurrences/total	23/248	19/245	36/248	21/245	
Adjusted RR (95% CI) [†]	1.00	0.77 (0.43-1.37)	1.44 (0.86-2.42)	0.86 (0.46-1.60)	0.73
Adjusted RR (95% CI) [‡]	1.00	0.77 (0.43-1.39)	1.48 (0.87-2.52)	0.89 (0.46-1.73)	0.61
Vitamin B ₁₂ , pmol/L	21.4-235.9	236.4-300.3	300.5-388.8	389.2-2,479	
All adenomas					
<i>n</i> recurrences/total	115/249	119/246	98/248	99/247	
Adjusted RR (95% CI) [†]	1.00	1.02 (0.84-1.25)	0.85 (0.69-1.05)	0.88 (0.71-1.11)	0.12
Adjusted RR (95% CI) [‡]	1.00	1.02 (0.84-1.25)	0.86 (0.69-1.06)	0.91 (0.73-1.15)	0.22
Advanced lesions					
<i>n</i> recurrences/total	27/249	28/246	19/248	25/247	
Adjusted RR (95% CI) [†]	1.00	1.01 (0.61-1.70)	0.69 (0.39-1.24)	0.95 (0.53-1.68)	0.57
Adjusted RR (95% CI) [‡]	1.00	1.02 (0.61-1.71)	0.69 (0.38-1.24)	0.94 (0.52-1.69)	0.55

*P_{trend}.

† Adjusted for age, sex, center, duration of follow-up, aspirin treatment group, and multivitamin use.

‡ Adjusted for age, sex, center, duration of follow-up, aspirin treatment group, multivitamin use, and plasma B₂, B₆, B₁₂, and folate as appropriate.

Hardy-Weinberg equilibrium for any of the polymorphisms investigated. As reported previously by us, MTHFR-C677T and MTHFR-C1298T were not significantly associated with adenoma risk in adjusted models (38). The relative risk was 0.99 (95% CI, 0.85-1.15) for

MTR-2756A/G or G/G versus A/A, 0.97 (95% CI, 0.83-1.14) for MTRR-66G/G versus A/A or A/G, and 0.80 (95% CI, 0.62-1.03) for CBS-699T/T versus C/C or C/T. We observed a significantly increased risk for any adenoma recurrence among individuals with the

Table 3. Association of baseline intakes of vitamins B₂ and B₆ with risk of colorectal adenomas

	Baseline dietary vitamin B intake*				P [†]
	Q1	Q2	Q3	Q4	
Vitamin B ₂ , mg	0.39-1.19	1.20-1.61	1.62-2.13	2.14-6.56	
All adenomas					
<i>n</i> recurrences/total	117/260	115/261	120/260	100/255	
Adjusted RR (95% CI) [†]	1.00	0.94 (0.77-1.15)	0.96 (0.79-1.17)	0.84 (0.69-1.03)	0.14
Adjusted RR (95% CI) [§]	1.00	0.94 (0.77-1.16)	0.95 (0.77-1.18)	0.83 (0.64-1.08)	0.23
Advanced lesions					
<i>n</i> recurrences/total	28/260	30/261	31/260	19/255	
Adjusted RR (95% CI) [†]	1.00	1.05 (0.64-1.73)	1.09 (0.67-1.79)	0.70 (0.40-1.22)	0.28
Adjusted RR (95% CI) [§]	1.00	1.10 (0.66-1.84)	1.16 (0.67-2.00)	0.78 (0.39-1.57)	0.66
Vitamin B ₆ , mg	0.33-1.12	1.13-1.54	1.55-2.01	2.02-6.20	
All adenomas					
<i>n</i> recurrences/total	108/259	122/258	112/261	110/258	
Adjusted RR (95% CI) [†]	1.00	1.08 (0.88-1.32)	0.98 (0.80-1.20)	0.95 (0.77-1.16)	0.42
Adjusted RR (95% CI) [§]	1.00	1.12 (0.90-1.39)	1.04 (0.82-1.32)	1.05 (0.78-1.41)	0.91
Advanced lesions					
<i>n</i> recurrences/total	25/259	31/258	29/261	23/258	
Adjusted RR (95% CI) [†]	1.00	1.24 (0.75-2.06)	1.08 (0.65-1.80)	0.85 (0.49-1.46)	0.46
Adjusted RR (95% CI) [§]	1.00	1.30 (0.76-2.25)	1.18 (0.65-2.15)	0.99 (0.46-2.12)	0.92

*Ranges are based on 2,000 calories/d.

† P_{trend}.

‡ Adjusted for age, sex, center, duration of follow-up, aspirin treatment group, and multivitamin use.

§ Adjusted for age, sex, center, duration of follow-up, aspirin treatment group, multivitamin use, and intake of B₂, B₆, and folate as appropriate.

Table 4. Association of vitamin B₂, B₆ (PLP) and B₁₂ baseline plasma levels and the risk of colorectal adenomas by folate treatment group, multivitamin and alcohol use at baseline

	Baseline plasma vitamin B levels								<i>P</i> [†]	<i>P</i> [‡]
	Q1		Q2		Q3		Q4			
	Recurrences/ total	RR (95% CI)*	Recurrences/ total	RR (95% CI)*	Recurrences/ total	RR (95% CI)*	Recurrences/ total	RR (95% CI)*		
Folic acid treatment group										
Vitamin B ₂ [§]	56/118	1.00	50/112	0.97 (0.72-1.30)	51/112	0.97 (0.71-1.31)	47/115	0.85 (0.62-1.18)	0.36	0.66
Vitamin B ₆	57/115	1.00	49/112	0.90 (0.67-1.20)	61/126	0.99 (0.74-1.33)	37/104	0.75 (0.53-1.07)	0.23	0.58
Vitamin B ₁₂ [¶]	60/125	1.00	56/118	0.96 (0.72-1.27)	46/109	0.89 (0.65-1.20)	43/107	0.86 (0.63-1.19)	0.30	0.58
Placebo group										
Vitamin B ₂ [§]	42/109	1.00	50/114	1.12 (0.81-1.53)	46/112	1.00 (0.72-1.39)	49/111	1.10 (0.78-1.55)	0.75	
Vitamin B ₆	49/105	1.00	51/114	0.95 (0.70-1.28)	39/102	0.79 (0.57-1.10)	48/125	0.76 (0.54-1.06)	0.07	
Vitamin B ₁₂ [¶]	40/97	1.00	50/103	1.14 (0.83-1.58)	44/122	0.88 (0.63-1.23)	53/125	1.10 (0.79-1.54)	0.97	
No multivitamin use at baseline**										
Vitamin B ₂ [§]	90/203	1.00	80/180	0.97 (0.77-1.22)	72/148	0.99 (0.78-1.27)	47/103	0.93 (0.71-1.24)	0.71	0.43
Vitamin B ₆	97/205	1.00	95/203	0.99 (0.80-1.23)	69/155	0.93 (0.73-1.19)	28/71	0.84 (0.61-1.17)	0.31	0.89
Vitamin B ₁₂ [¶]	93/206	1.00	89/177	1.05 (0.84-1.32)	61/142	0.92 (0.72-1.18)	47/111	0.95 (0.72-1.26)	0.52	0.68
Multivitamin use at baseline**										
Vitamin B ₂ [§]	18/41	1.00	31/68	1.12 (0.72-1.76)	32/96	0.79 (0.50-1.25)	59/143	0.94 (0.60-1.45)	0.47	
Vitamin B ₆	18/39	1.00	18/42	1.04 (0.63-1.72)	42/93	0.97 (0.63-1.48)	62/174	0.74 (0.49-1.13)	0.08	
Vitamin B ₁₂ [¶]	21/40	1.00	30/69	0.87 (0.57-1.33)	37/106	0.69 (0.45-1.04)	52/135	0.77 (0.52-1.15)	0.17	
No alcohol use at baseline										
Vitamin B ₂ [§]	22/59	1.00	25/72	0.86 (0.56-1.34)	30/77	0.89 (0.58-1.37)	35/88	0.93 (0.61-1.42)	0.86	0.91
Vitamin B ₆	33/78	1.00	33/71	1.13 (0.78-1.63)	29/74	0.85 (0.58-1.26)	17/73	0.53 (0.33-0.84)	0.01	0.03
Vitamin B ₁₂ [¶]	22/58	1.00	32/68	1.17 (0.77-1.76)	29/81	0.87 (0.57-1.34)	29/91	0.83 (0.54-1.29)	0.19	0.50
Alcohol use (≥1 drink/d) at baseline										
Vitamin B ₂ [§]	83/176	1.00	78/163	1.03 (0.81-1.30)	73/159	0.96 (0.75-1.23)	67/147	0.96 (0.73-1.26)	0.67	
Vitamin B ₆	74/155	1.00	74/161	0.96 (0.75-1.23)	81/167	1.04 (0.81-1.33)	72/162	0.93 (0.70-1.23)	0.86	
Vitamin B ₁₂ [¶]	86/174	1.00	82/168	0.95 (0.75-1.21)	66/158	0.86 (0.67-1.11)	68/147	0.97 (0.75-1.27)	0.63	

*Adjusted for age, sex, center, duration of follow-up, aspirin treatment group, multivitamin use, and plasma B₂, B₆, B₁₂, and folate as appropriate.

[†] *P*_{trend}.

[‡] *P*_{interaction}.

[§]Vitamin B₂ (riboflavin): Q1, 2.1 to 8.9 nmol/L; Q2, 9.0 to 15.0 nmol/L; Q3, 15.1 to 27.5 nmol/L; and Q4, 27.6 to 479.0 nmol/L.

^{||}Vitamin B₆ (PLP): Q1, 4.7 to 35.6 nmol/L; Q2, 35.7 to 53.3 nmol/L; Q3, 53.4 to 84.3 nmol/L; and Q4, 84.7 to 857.0 nmol/L.

[¶]Vitamin B₁₂: Q1, 21.4 to 235.9 pmol/L; Q2, 236.4 to 300.3 pmol/L; Q3, 300.5 to 388.8 pmol/L; and Q4, 389.2 to 2,479 pmol/L.

**Adjusted for age, sex, center, duration of follow-up, aspirin treatment group, and plasma B₂, B₆, B₁₂, and folate as appropriate.

Table 5. Selected gene-vitamin B interactions and risk of colorectal adenomas

Genotype	<i>n</i> recurrences/total		Adjusted RR (95% CI)*	<i>P</i> _{interaction}
	Vitamin B level below the median (reference)	Vitamin B level above the median		
Baseline plasma vitamin B ₂ (riboflavin), nmol/L	2.1-15.0	15.1-479.0		
MTHFR-677CC + C/T	195/413	179/416	0.87 (0.73-1.03)	
MTHFR-677T/T	18/52	19/44	1.40 (0.85-2.30)	0.07
MTHFR-1298A/A + A/C	197/431	178/416	0.90 (0.76-1.07)	
MTHFR-1298C/C	16/35	21/45	0.92 (0.56-1.54)	0.94
Baseline plasma vitamin B ₆ (PLP), pmol/L	21.4-300.3	300.5-2,479		
CBS-1080 C/C+C/T	182/403	159/402	0.90 (0.75-1.08)	
CBS-1080 T/T	37/56	32/62	0.72 (0.49-1.03)	0.25
CBS-699 C/C+C/T	195/404	179/422	0.89 (0.75-1.06)	
CBS-699 T/T	25/57	13/44	0.67 (0.40-1.13)	0.30
MTHFR-677CC + C/T	196/410	178/419	0.90 (0.76-1.07)	
MTHFR-677T/T	24/51	13/45	0.66 (0.39-1.10)	0.25
Baseline plasma vitamin B ₁₂ , nmol/L	4.7-53.3	53.4-857.0		
MTR-2756A/A	72/155	63/151	0.92 (0.71-1.20)	
MTR-2756A/G + G/G	151/310	127/314	0.87 (0.72-1.05)	0.70
MTRR-66A/A + A/G	163/335	139/342	0.86 (0.72-1.03)	
MTRR-66G/G	59/129	51/123	0.96 (0.72-1.29)	0.50
MTHFR-677CC + C/T	198/411	177/421	0.90 (0.77-1.07)	
MTHFR-677T/T	24/52	13/45	0.69 (0.41-1.17)	0.33

*Adjusted for age, sex, center, duration of follow-up, aspirin treatment group, multivitamin use and plasma B₂, B₆, B₁₂, and folate as appropriate.

CBS-1080T/T genotype compared with those with at least one C allele (RR, 1.29; 95% CI, 1.06-1.56).

There was a suggestive, but nonsignificant, interaction between the MTHFR-C677T polymorphism and baseline plasma vitamin B₂ on risk of all adenomas (Table 5). Among subjects who were homozygotes for the (variant) T allele, there was a nonsignificantly increased risk of adenomas associated with higher plasma vitamin B₂ (adjusted RR, 1.40; 95% CI, 0.85-2.30), whereas those who were heterozygotes or homozygotes for the common 677C allele showed a reduced risk (adjusted RR, 0.87; 95% CI, 0.73-1.03; *P*_{interaction} = 0.07). The association between baseline median dietary vitamin B₂ levels and adenoma risk did not significantly differ by MTHFR-677C/T or MTHFR-1298C/T genotypes (data not shown).

There was no indication that the risk of adenomas associated with baseline plasma vitamin B₁₂ differed by MTHFR-677C/T, MTR-2756A/G, or MTRR-66A/G genotypes or that the association between baseline plasma vitamin B₆ [PLP or pyridoxal (data not shown)] and adenoma risk differed by MTHFR-677C/T, CBS-1080C/T, or CBS-699C/T genotypes. Baseline dietary levels of vitamin B₆ also showed no evidence of effect modification by these genotypes (data not shown).

Discussion

In this randomized clinical trial of aspirin use and folic acid supplementation, we found a borderline significant inverse association between baseline plasma vitamin B₆ (PLP, the active form of B₆) levels and risk of all adenomas but not advanced lesions. The inverse association was not modified by folic acid supplementation or baseline plasma folate, although we did observe a significant interaction between plasma PLP levels and alcohol use. Dietary and total intake of B₆ as well as plasma B₁₂ were not associated with risk of adenomas or

advanced lesions. Circulating levels of B₂ were inversely associated with risk of advanced lesions, whereas dietary intake showed a nonsignificant inverse trend for all adenomas. There were no significant interactions between polymorphic FOCM genes and their respective B-vitamin cofactors, except possibly for MTHFR-C677T and plasma B₂.

In FOCM, vitamin B₆ acts as a cofactor for serine hydroxymethyltransferase (SHMT), which catalyzes the formation of glycine and 5,10-methylenetetrahydrofolate, a key carrier of one-carbon groups in nucleotide synthesis and DNA repair. In addition, vitamin B₆ is a cofactor for CBS, which catalyzes the irreversible conversion of homocysteine to cystathionine, reactions that may affect intracellular S-adenosylhomocysteine levels *in vivo* (39). Vitamin B₆ is also involved in ~100 enzymatic reactions (40) and has been shown to reduce oxidative stress as well as cell proliferation and angiogenesis (41, 42), all effects that could result in reduced risks of neoplasia. Vitamin B₆ is found in poultry, fish, liver, whole grains, nuts, and legumes, and deficiency has been associated with impaired FOCM (43) and chemically induced colon tumor formation in animal models (41).

In contrast to our results for plasma levels, we found no statistically significant association of baseline dietary vitamin B₆ with adenoma occurrence. Other observational studies have reported an inverse relationship between dietary vitamin B₆ intake and risk of colorectal adenomas (3, 4) and a more consistent association with cancer (7, 12-17). Our negative results could have been due to measurement error in the estimation of intake, which would tend to exert a conservative bias (44). We are aware of only one other study that examined plasma vitamin B₆ (PLP) levels and colorectal adenoma and cancer risk. Wei et al. showed a significant inverse association for PLP and risk of colon cancer and a borderline significant risk of incident advanced distal colorectal adenomas among women (14).

Previous studies have investigated the possible interaction between polymorphisms in MTHFR and dietary intake of vitamin B₆ on risk of colorectal cancer and adenomas. A stronger protective effect of vitamin B₆ on adenoma risk has been observed among individuals with the MTHFR-677T/T genotype compared with those with at least one C allele (6, 20, 21), although this interaction was statistically significant in only one study (6). There has been no previous investigation regarding interactions between plasma or dietary vitamin B₆ and two functional polymorphisms in the B₆-dependent CBS enzyme (CBS-699C/T and CBS-1080C/T; ref. 45) on risk of adenomas.

Vitamin B₂ (found in dairy products, poultry, fish, grains, and enriched food products) is the precursor for flavin mononucleotide and flavin adenine dinucleotide, key cofactors in multiple oxidation/reduction reactions including the reduction of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, catalyzed by MTHFR (46). Our data did not suggest a significant association between plasma riboflavin levels and risk of any adenoma, although we observed a borderline significant inverse association with risk of advanced lesions and suggestive associations with dietary intake. Other studies have also reported nonsignificant associations of dietary vitamin B₂ with risk of colorectal adenomas (3-7). The only study to observe a significant protective effect of dietary B₂ was a case-control study of colorectal adenomas in The Netherlands (8). Contrary to The Netherlands, foods in the United States have been enriched with vitamin B₂ since 1940s (47). Thus, our study population may have relatively high B₂ intake (48), leaving us with insufficient variation in our population to observe significant associations.

We also investigated whether the MTHFR-677C/T polymorphism modified the relationship between plasma levels of vitamin B₂ and adenoma risk. The MTHFR-677T allele codes for a valine at position 222 in the flavin adenine dinucleotide binding domain of the enzyme, and the variant enzyme has relatively lower affinity for the flavin adenine dinucleotide cofactor (49, 50), suggesting that a high level of the flavin adenine dinucleotide cofactor may stabilize the variant enzyme just as folate does (51, 52). However, we observed a nonsignificant increase in adenoma risk for those with the MTHFR-677T/T genotype and plasma B₂ levels above the median compared with those with that genotype and lower plasma B₂ levels. There was a borderline significant association between plasma B₂ and any adenoma occurrence among those with at least one C allele. These findings contrast with a report that dietary intake of B₂ is inversely associated with adenoma risk in those with the MTHFR-677T/T genotype (8). Other studies found no evidence of interaction between dietary vitamin B₂ and MTHFR-677C>T on risk of colorectal adenomas (3, 8) or cancer (5). When we examined dietary vitamin B₂ levels and adenoma risk, there was no suggestion of effect modification by the MTHFR-677C/T genotype.

Vitamin B₁₂ is a cofactor of MTR, which, paired with MTRR, catalyzes transfer of methyl groups from 5-methylenetetrahydrofolate to homocysteine via vitamin B₁₂. Vitamin B₁₂ is found exclusively animal products, and deficiencies of either enzyme or the B₁₂ cofactor essentially trap 5-methylenetetrahydrofolate molecules at the B₁₂ transfer step (53). In agreement with our findings, the majority of studies for both

adenoma (3, 8, 11) and cancer risk (5, 6, 10, 12) have found no evidence of an association with dietary vitamin B₁₂. There has been considerable study of the role of polymorphisms in MTR-2756A>G and MTRR-66A>G, including assessment of their interaction with vitamin B₁₂ in colorectal neoplasia (6, 22, 26, 54-57). The MTRR-A66G variant allele has been associated with a lower affinity for MTR and possibly less efficient reducing capacity (58), whereas the MTR-A2756G polymorphism has been inconsistently reported to affect plasma homocysteine and folate levels (59, 60). Data regarding MTRR-G66 have been inconsistent in this regard (5, 6, 26, 61, 62). As in our study, the MTR-2756A>G polymorphism does not appear to play a role in colorectal carcinogenesis (22, 54, 57). Additionally, our data are consistent with other studies (6, 20) that have found no evidence of interaction between MTHFR-677C>T and B₁₂.

In agreement with our findings, other investigations have not found that folate status modified the association of plasma or dietary vitamin B intakes with risk of colorectal neoplasia. The Nurses' Health Study did not find a statistically significant stronger protective effect of high PLP levels among individuals with low plasma folate, nonusers of multivitamins or B-vitamin supplements on colorectal cancer or adenoma risk (14). At least one other study also failed to observe a significant interaction between intakes of vitamins B₂, B₆, and B₁₂ and dietary folate on risk of colorectal adenomas (3). These data may reflect limited statistical power to detect interaction but may also suggest that PLP may protect against colorectal neoplasia through biological pathways other than FOCM. We observed a potential interaction between PLP and alcohol. Alcohol is a known folate "antagonist" that affects dietary methyl supply. In addition to effects on folate availability, alcohol is metabolized into acetaldehyde, which has been shown to be carcinogenic in the colorectum possibly through inhibition of DNA methylation and interactions with retinoid metabolism (63). Findings from two other studies (12, 13) observed a protective effect of vitamin B₆ among heavy drinkers, and further study is needed to better understand these relationships.

This study has several limitations. The generalizability of our results may be limited as all participants in this clinical trial were volunteers who had a previous history of at least one colorectal adenoma and so represent a restricted part of the colon cancer risk spectrum. We had a limited sample size to investigate risk of advanced lesions, a clinically important endpoint. We used a validated semiquantitative food frequency questionnaire, but these are still subject to measurement error, which may introduce substantial biases, generally conservative (44). In addition, we did not consider all polymorphisms in CBS, MTR, MTRR, and MTHFR, and we do not have any information on other key polymorphic FOCM genes. For example, SHMT is a vitamin B₆-dependent enzyme involved in purine and pyrimidine nucleotide synthesis, and previous investigation has suggested a potential interaction between the SHMT-1420C>T polymorphism and dietary levels of vitamin B₆ in colorectal adenoma risk (19).

Strengths of this study include the measurement of baseline plasma vitamin B levels (although in nonfasting bloods), genotypes in key FOCM genes, and the large

sample size. Because of the prospective design, recall or selection biases are unlikely to explain our findings in this study. In addition, inclusion only of individuals with a clean colonoscopy in this prospective clinical trial allowed us to assess the effect of B-vitamins on incident rather than prevalent adenomas and thereby to make clear the temporal relationships between intake and adenoma occurrence. Furthermore, the high follow-up rates in this study (29) minimize the concern that differential rates of follow-up affected our results.

In summary, our results suggest that high baseline levels of PLP and B₂ may protect against new colorectal adenomas among individuals with a previous history of adenomas.

Disclosure of Potential Conflicts of Interest

J. Baron: Wyeth supplied folate and placebo tablets for the trial; P. Ueland: Speakers bureau/honoraria, ownership interest. The other authors disclosed no potential conflicts of interest.

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