Responses of Biomarkers of Folate and Riboflavin Status to Folate and Riboflavin Supplementation in Healthy and Colorectal Polyp Patients (The FAB2 Study)

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Abstract

Epidemiologic data suggest that increasing folate intake may protect against colorectal cancer. Riboflavin may interact with folate to modulate the effect. A double-blind randomized placebo-controlled intervention study (the FAB2 Study) was carried out in healthy controls and patients with colorectal polyps (adenomatous and hyperplastic) to examine effects of folic acid and riboflavin supplements on biomarkers of nutrient status and on putative biomarkers of colorectal cancer risk (DNA methylation and DNA damage; to be reported elsewhere). Ninety-eight healthy controls and 106 patients with colorectal polyps were stratified for the thermolabile variant of methylene tetrahydrofolate reductase, *MTHFR* C677T, and were randomized to receive 400 μg of folic acid, 1,200 μg of folic acid, or 400 μg of folic acid plus 5 mg of riboflavin or placebo for 6 to 8 weeks. Blood samples and colon biopsy samples were collected for the measurement of biomarkers of folate and riboflavin status. Supplementation with folic acid elicited a significant increase in mucosal 5-methyl tetrahydrofolate, and a marked increase in RBC and plasma, with a dose-response. Measures of riboflavin status improved in response to riboflavin supplementation. Riboflavin supplement enhanced the response to low-dose folate in people carrying at least one T allele and having polyps. The magnitude of the response in mucosal folate was positively related to the increase in plasma 5-methyl tetrahydrofolate but was not different between the healthy group and polyp patients. Colorectal mucosal folate concentration responds to folic acid supplementation to an extent comparable to that seen in plasma, but with a suggestion of an upper limit. (Cancer Epidemiol Biomarkers Prev 2007;16(10):2128–35)

Introduction

Diets low in folate may be associated with an increased risk of colorectal cancer or of colorectal adenomas. Case-control studies have suggested that people with high folate intakes have a lower risk of colorectal cancer and precursor adenomatous polyps than those with low intakes (1, 2). Results from prospective cohort studies are inconsistent. Larsson et al. (3) reported a strong protective effect of folate against colon cancer in women, whereas Su and Arab (4) reported a significant protection in men. Other cohort studies (5, 6) or nested case-control studies (7) suggest a weak protective effect against colorectal cancer or colorectal adenoma, whereas yet others reported no evidence of an independent protective effect of folate (8). Similarly, there was little evidence for a protective effect of dietary folate in the Health Professional's Follow-up Study (9). Most folic acid intervention trials in humans have been conducted in patient groups with an increased risk of colorectal cancer, in which various intermediate end points were used, with some encouraging results (10, 11). In contrast, some studies in animals have suggested that high doses of folic acid might enhance colorectal tumorigenesis, depending on the dose and whether this is given after neoplastic foci are established (12-14). Furthermore, results of a folic acid and aspirin intervention trial (15) suggest that there are circumstances in which there might also be adverse effects of folic acid supplementation in humans.

People carrying the TT variant for the C677T single nucleotide polymorphism in the gene expressing methylene tetrahydrofolate reductase (*MTHFR*), may be at reduced risk of colorectal cancer (16), although reports are not consistent and interactions with other dietary and lifestyle factors modulate the folate-genotype interaction (17, 18). Other reports suggest that risk of colorectal adenoma recurrence may be higher in 677TT variants, in conjunction with low folate status (19). Elevated plasma total homocysteine, a biomarker of poor folate

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status, is associated with increased risk of adenoma recurrence (20, 21). Other B vitamins that act as cofactors in homocysteine metabolism (vitamins B_2, B_6, and B_{12}) might also contribute to overall risk of colorectal neoplasia (8, 20).

There is interest in the value of putative biomarkers of colorectal cancer risk as surrogate end points in intervention trials, especially in their responsiveness to exposure to dietary components. We have conducted a study (The FAB2 Study) to evaluate the determinants of mucosal folate status in three groups of volunteers at differential colorectal cancer risk and the responsiveness of various measures of DNA stability and colonic mucosal folate to supplemental folic acid or folic acid with riboflavin in healthy controls and those with colorectal polyps. This article will focus on the biochemical responses to intervention in these latter two groups. Details regarding the molecular biology and the dietary analysis will be described elsewhere.

Materials and Methods

All chemicals were purchased from Sigma unless otherwise stated.

Study Protocol. Patients over the age of 40 years referred for flexible sigmoidoscopy or colonoscopy at North Tyneside General Hospital were provided with written information about the study prior to clinic attendance. Patients who showed an interest in the study and who satisfied the entry requirements, met research personnel and provided informed written consent together with a baseline blood sample and a completed food frequency questionnaire prior to attending the clinic. Ethical approval was obtained from the joint ethics committee of Newcastle and North Tyneside Health Authority, University of Newcastle upon Tyne and University of Northumbria (Ref: 2002/376). Respondents were excluded if they (*a*) reported current supplemental vitamin use or had taken vitamin supplements within the preceding 3 months, (*b*) were pregnant or breast-feeding, (*c*) were alcoholics, (*d*) had hereditary nonpolyposis colorectal cancer, familial adenomatous polyposis, or inflammatory bowel disease, or (*e*) consumed any medicines known to interact with folate.

At sigmoidoscopy or colonoscopy, baseline biopsies of normal rectal mucosa were taken 15 cm from the anal margin, snap-frozen in liquid nitrogen, and stored at −80°C; any lesion thought to be a polyp was biopsied. Patients were classified into three groups: colorectal cancer, colorectal polyp (adenomatous and hyperplastic polyps), and no evidence of neoplasia. The latter two groups were randomized to treatment groups, with stratification according to *MTHFR* C677T genotype. Volunteers were randomized to receive one of four treatments, i.e., placebo capsule, 400 μg of folic acid, 1,200 μg of folic acid, or 400 μg of folic acid with 5 mg of riboflavin, daily. Blood samples and repeat rectal mucosal biopsies were collected 6 to 8 weeks later.

Blood Handling. All venous blood samples were taken following an overnight fast. Blood samples (40 mL) were collected at baseline and following 6 to 8 weeks of intervention. Whole blood was stored at −80°C for the measurement of S-adenosyl methionine and S-adenosyl homocysteine, both intermediates in the conversion of methionine to homocysteine. An aliquot of whole blood was stored in lysate reagent (folate kit; Abbott Laboratories) at −80°C for the measurement of whole blood total folate. The hematocrit was measured on fresh whole blood for the calculation of RBC folate. A further aliquot of whole blood was stored for the analysis of the C677T *MTHFR* polymorphism. Plasma was stored at −80°C for the measurement of plasma 5-methyl tetrahydrofolate (5MeTHF; in 10% ascorbic acid), total homocysteine, pyridoxal phosphate, pyridoxic acid, and flavin concentrations. Washed and packed RBC were stored at −80°C for the measurement of riboflavin status.

Biochemical Analyses

Folate Status Variables. Plasma 5MeTHF was measured by reversed-phase high-performance liquid chromatography with fluorescence detection, using a modification of the method described by Loehrer et al. (22). Intrabatch coefficient of variation (CV) was 2.5%, interbatch CV was 6.9%. For the measurement of colonic 5MeTHF, biopsy samples were thawed, weighed, and 400 μL of 0.5% ascorbic acid added prior to homogenization with an Ultraturrax T-8 microhomogenizer (IKA). Homogenates were centrifuged at 10,000 × *g* at 4°C for 5 min and the supernatant analysed as for plasma folate. The CV for mucosal folate in eight biopsy samples collected from the same region of the bowel was 6.5%.

Riboflavin Status Variables. Plasma flavins were measured using reversed-phase high-performance liquid chromatography using a modification (16) of the method by Capo-chichi et al. (23). Intrabatch CVs were 2.4%, 7.7%, and 1.4% whereas interbatch CVs were 9.1%, 0.8%, and 10.0% for flavin adenine dinucleotide, flavin mononucleotide, and riboflavin, respectively. Erythrocyte glutathione reductase activation coefficient (EGRAC) was determined using a spectrophotometric technique automated for the Cobas Bioautoanalyser (24). A threshold of 1.40 was used to indicate biochemical riboflavin deficiency.

Other B Vitamins. Plasma pyridoxal phosphate and pyridoxic acid were measured by high-performance liquid chromatography using a Chromsystems kit. Intrabatch CVs were 2.5% and 2.2% whereas interbatch CVs were 1.0% and 1.3% for pyridoxal phosphate and pyridoxic acid, respectively. Plasma vitamin B_{12} concentration was measured by a chemiluminescent microparticle immunoassay using a kit from Abbott Laboratories Diagnostics. Quality assurance was provided by participation in the WEQAS scheme. CV (between- and within-run) was <10%.

Methylation Cycle Intermediates. S-Adenosyl methionine and S-adenosyl homocysteine were measured in whole blood using reversed-phase high-performance liquid chromatography with UV detection, using a modification of the method described by Loehrer et al. (22). The intrabatch CVs were 4.4% and 13.1% whereas interbatch CVs were 10.8% and 38.1% for S-adenosyl methionine and S-adenosyl homocysteine, respectively. Plasma total homocysteine was measured by immunoassay using an Abbott IMX Analyser (25). Interbatch CV was 6.4%.

MTHFR C677T Genotyping. MTHFR genotyping was carried out on DNA extracted from whole blood (26).

Statistical Methods. Baseline data were log-transformed before analysis where appropriate. Baseline data were examined according to *MTHFR* C677T genotype, histology, and treatment group using ANOVA followed by the Scheffe test where post hoc analysis was warranted. Associations between variables were examined using Spearman's rank-order coefficient of correlation. An initial analysis was conducted to reveal which of a number of chosen factors should be included as covariates in the analysis of the effects of the intervention. Thus, ANCOVA was carried out for each variable, using histology, treatment, gender, smoking, age, alcohol consumption, and baseline value as covariates. Levene's equality of variance was carried out and the residuals were analyzed. Results are expressed as mean and SE except where log transformation of data was undertaken, in which case results were expressed as geometric mean and confidence intervals (CI).

Results

The average period of supplementation was 45 days (SD 7.0). Ninety-five subjects with no evidence of colorectal polyps or cancer completed the intervention. One hundred and two subjects with colorectal polyps completed the intervention, 12 of whom had hyperplastic polyps, and the remainder had adenomatous polyps. One person was diagnosed with cancer after being randomized to treatment and was removed from the study. Of the 197 subjects who completed the study, a further 18 were removed from the analysis because information regarding tobacco and alcohol consumption were not available. The analysis was therefore carried out on 179 subjects, ages 40 to 87 years, of whom 88 were males and 91 were female. Fifty-five men and 38 women had colorectal polyps, of which 9.5% of the sample was homozygous for the C677T *MTHFR* polymorphism.

Table 1 shows baseline biochemical data for the entire cohort and according to *MTHFR* C677T genotype. Analysis for each variable was restricted to subjects for whom there were data at baseline and postintervention for that variable. Plasma homocysteine concentration was higher than the reported median for men or women in the most recent National Diet and Nutrition Surveys of adults and the elderly in the U.K. (27, 28) but plasma B_{12} values and EGRAC values (vitamin B_2) were comparable. Mean plasma concentration of 5MeTHF suggested good overall folate status. Whole blood

Table 1. Baseline biochemistry according to *MTHFR* C677T genotype

Variable		*MTHFR* C677T genotype			
		CC	CT	TT	All
Plasma 5MeTHF (nmol/L)	*N*	72	77	16	165
	Mean	39.86	43.73	36.70	41.40
	SD	21.26	25.65	16.70	23.50
Mucosal 5MeTHF* (nmol/g)	*N*	72	71	15	158
	Mean	0.680	0.649	0.543	0.652
	CI	0.557-0.831	0.543-0.778	0.375-0.785	0.576-0.739
Plasma tHcy* (μmol/L)	*N*	70	78	16	134
	Mean	12.71	13.26	13.4	13.00
	CI	6.38-27.98	12.34-14.25	11.35-15.81	12.14-13.28
EGRAC*	*N*	70	77	17	164
	Mean	1.37	1.37	1.38	1.37
	CI	1.33-1.43	1.34-1.41	1.30-1.46	1.35-1.39
Plasma riboflavin* (nmol/L)	*N*	71	76	17	164
	Mean	10.44	11.39	11.39	10.87
	CI	8.80-12.40	9.59-13.54	8.14-13.47	9.92-12.21
Plasma FAD (nmol/L)	*N*	71	75	17	163
	Mean	47.60	48.77	53.77	48.80
	SD	10.58	11.88	14.68	11.72
Plasma FMN* (nmol/L)	*N*	71	75	17	163
	Mean	2.90	3.07	2.77	2.86
	CI	2.45-3.42	2.53-3.73	1.86-4.15	2.68-3.35
Plasma flavins (nmol/L)	*N*	71	76	17	164
	Mean	65.15	67.82	69.10	66.80
	SD	19.74	21.32	13.72	19.94
Plasma B12 (pmol/L)	*N*	78	81	17	75
	Mean	323	341	300	329
	SD	226	199	83	289
Whole blood SAM (μmol/L)	*N*	35	34	6	75
	Mean	1.72	1.89	2.79	1.88
	SD	2.68	3.83	4.34	3.35
Plasma pyridoxic acid (nmol/L)	*N*	27	20	9	56
	Mean	29.24	32.78	31.43	30.86
	SD	14.89	15.18	6.11	13.88
Plasma pyridoxal phosphate (nmol/L)	*N*	27	20	9	56
	Mean	46.96	52.84	64.69	51.92
	SD	24.61	58.24	35.46	40.99

Abbreviations: tHcy, total homocysteine; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; SAM, S-adenosyl methionine.
*Analysis on log-transformed data; geometric mean and 95% CI are reported.

S-adenosyl homocysteine concentrations are not reported because the plasma concentration decreased to below the limits of sensitivity of the assay employed.

Table 2 shows baseline biochemistry according to bowel histology. There were no significant differences for any variables between people with normal bowel histology or those with colorectal polyps. Although plasma homocysteine concentrations were higher in the polyp group, this failed to reach significance ($P = 0.067$).

Baseline data were analyzed according to treatment group. ANOVA suggested group differences in plasma 5MeTHF ($P < 0.05$) but post hoc analysis (Scheffe test) revealed no significant differences between any two treatment groups. Associations between blood biomarkers of vitamin status tended to reflect common food sources of B vitamins or a common functional role in maintaining homocysteine homeostasis. Of interest was a highly significant ($P = 0.001$) positive association between colon mucosal folate and plasma 5MeTHF (Fig. 1) and a weaker but significant ($P = 0.009$) negative association between colon folate and plasma homocysteine.

Table 2. Baseline biochemistry according to histology

Variable		Histology	
		Normal	Polyp
Plasma 5MeTHF (nmol/L)	*N*	76	89
	Mean	41.47	41.26
	SD	23.96	22.4
Mucosal 5MeTHF* (nmol/g)	*N*	71	87
	Mean	0.570	0.732
	CI	0.46-0.70	0.634-0.844
Plasma tHcy* (μmol/L)	*N*	76	88
	Mean	12.53	13.49 †
	CI	11.63-13.49	12.67-14.36
EGRAC*	*N*	77	87
	Mean	1.387	1.362
	CI	1.35-1.43	1.33-1.39
Plasma riboflavin* (nmol/L)	*N*	76	88
	Mean	11.23	10.58
	CI	9.34-13.50	9.25-12.10
Plasma FAD (nmol/L)	*N*	75	88
	Mean	51.58	46.40
	SD	13.8	9.00
Plasma FMN* (nmol/L)	*N*	75	88
	Mean	3.066	3.011
	CI	2.36-5.62	2.62-3.46
Plasma flavins* (nmol/L)	*N*	76	88
	Mean	70.56	63.56
	CI	21.2-150.4	37.2-163.1
Plasma B12 (pmol/L)	*N*	85	91
	Mean	311.3	345.9
	SD	190.1	214.4
Whole blood SAM (μmol/L)	*N*	24	51
	Mean	2.34	1.66
	SD	3.73	3.17
Plasma pyridoxic acid (nmol/L)	*N*	34	22
	Mean	30.35	31.64
	SD	13.26	15.07
Plasma pyridoxal phosphate (nmol/L)	*N*	34	22
	Mean	48.36	57.4
	SD	25.15	57.92

Abbreviations: tHcy, total homocysteine; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; SAM, S-adenosyl methionine.
*Analysis on log transformed data; geometric mean and 95% CI are reported.
† Compared with control group ($P = 0.067$).

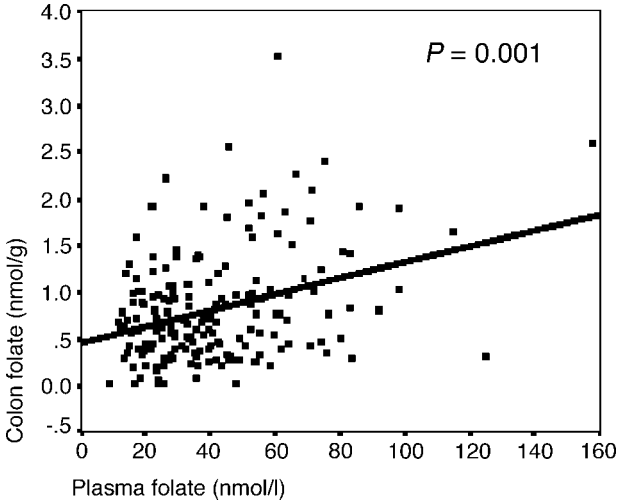

Figure 1. Associations between colon mucosal folate and plasma folate concentrations at baseline ($r = 0.259$, $P = 0.001$).

Effects of Intervention. Effects of intervention were analyzed using ANOVA, using appropriate covariates. In practice, this meant that baseline values were used as covariates for all variables; additional covariates were included as detailed in the Results. Table 3 shows postintervention mean values for each variable, adjusted to a standardized baseline, with SEs or CIs, as appropriate. The standardized covariates are included as a footnote. Plasma 5MeTHF, colon mucosal folate, homocysteine, plasma riboflavin, plasma total flavins, and EGRAC all showed significant responses to intervention.

Plasma 5MeTHF. All treatments elicited a significant improvement in plasma 5MeTHF compared with placebo ($P < 0.001$). When considering all the data, a clear dose-response effect of folic acid supplement on plasma 5MeTHF was observed (Fig. 2A). Closer inspection of the data revealed a three-way interaction between supplement, genotype, and histology ($P < 0.001$; Table 4). Although low-dose folic acid led to an increase in 5MeTHF irrespective of genotype and histology ($P < 0.001$), a further increase in plasma 5MeTHF in response to high-dose folic acid was significant only in those subjects carrying one or more T alleles and having colorectal polyps ($P < 0.0001$) and in subjects carrying two C alleles and with normal histology ($P = 0.008$). There was also evidence of an interaction between riboflavin, genotype, and histology in determining the plasma response to folic acid supplements, such that riboflavin enhanced the effect of low folate dose in those subjects carrying at least one T allele and having polyps ($P = 0.018$).

Colon Mucosal Folate. Colon mucosal folate concentration also increased in response to the folic acid supplement. The low-dose folic acid increased colon folate by a factor of 1.64 ($P = 0.003$), and in the presence of riboflavin, by a factor of 2.0 ($P < 0.0001$), suggesting an enhancing effect of riboflavin with the low folic acid dose. There was little evidence of a dose-response effect of folic acid (Fig. 2B).

Riboflavin Status. Plasma riboflavin ($P < 0.01$) and plasma total flavins ($P < 0.05$) showed a significant increase in response to the supplement containing riboflavin (Table 3). A significant genotype ($P < 0.05$), supplement, and histology interaction was seen for both variables. Thus, the greatest response in plasma riboflavin was seen in those subjects with wild-type *MTHFR* C677T and with normal histology (postintervention mean, 37.1 nmol/L; CI, 27.7-49.8). The biggest response in plasma total flavins was seen in subjects with wild-type *MTHFR* C677T and having normal histology (postintervention mean, 96.6 nmol/L; CI, 86.9-106.3). EGRAC data were log-transformed for analysis, and in addition to baseline values, smoking status was included as a covariate. EGRAC showed the anticipated reduction in response to a riboflavin supplement ($P < 0.001$). Folic acid elicited a significant reduction in plasma homocysteine ($P < 0.001$), but there was no dose-response effect.

Associations between Changes in Biochemical Variables. Data were examined for associations between the magnitude of the response to intervention for selected variables, using data from all intervention groups. Of particular interest was the significant correlation between changes in colon mucosal folate and plasma folate ($P < 0.001$) and between colon mucosal folate and plasma homocysteine ($P < 0.01$).

Discussion

Supplementation with folic acid and riboflavin for 45 days resulted in the anticipated improvements in conventional measures of folate and riboflavin status, with some effects of genotype. In addition, the study showed that mucosal folate concentration increases in response to supplemental folic acid, with evidence of an upper limit in response. The effect of riboflavin (5 mg/d) given in addition to the low folic acid dose (400 μg/d), was to elicit a moderately enhancing effect on circulating and mucosal folate, but with some interactions with genotype and histology.

This is the first study to examine the response of colon mucosal folate to short-term low folic acid supplement and shows that folic acid supplements in the physiologic range (400 μg) can elicit a significant increase in mucosal folate concentration. Although there was a dose-response effect for plasma folate, this was not evident for colon mucosal folate. Thus, unlike plasma folate, colon mucosal folate seems to exhibit an upper threshold in response to a moderate folic acid supplement, which may reflect regulation over the uptake of folate by colonocytes or the transport of folate from the cell. We have measured folate in the form of 5MeTHF monoglutamate. It is understood that once inside a cell, folate is acted on by folylpolyglutamyl synthase, and that the resulting polyglutamate forms of folate are retained in the cell (29). The activity of γ-glutamate hydrolase reforms the monoglutamate, which is thought to pass readily across the cell membrane and is not retained in the cell. However, there have been few reports of folate concentrations in colonic mucosa. Kim et al. (30, 31) used a microbiological assay to measure total folate in colonic mucosa of 20 people with adenomatous polyps and reported a mean value of ~0.70 nmol/g (SE, 0.085) prior to supplementation, similar to the baseline value of 0.65 nmol/g (CI, 0.58-0.74) for colonic mucosal 5MeTHF reported here. This suggests that the majority of colon mucosal folate is present in the monoglutamate 5MeTHF form. Meenan et al. (32) reported a lower concentration of total folate in neoplastic colonic epithelial cells than

Table 3. Effects of a folate and riboflavin supplement on blood and colon biochemical variables

Variable		Treatment group			
		Placebo	Low folate*	Low folate + riboflavin†	High folate‡
Plasma 5MeTHF (nmol/L)	Mean	44.3	81.7§	82.1§	114.4§
	SE	5.3	4.7	4.9	4.6
Mucosal 5MeTHF‖ (nmol/g)	Mean	0.69	1.12§	1.4§	1.3§
	Lower CI	0.56	0.94	1.16	1.08
	Upper CI	0.84	1.34	1.69	1.55
Plasma tHcy‖ (μmol/L)	Mean	13.2	11.3§	10.9§	11.7§
	Lower CI	12.3	10.6	10.1	10.9
	Upper CI	14.3	12.1	11.7	12.5
EGRAC‖	Mean	1.39	1.37	1.23§	1.41
	Lower CI	1.35	1.33	1.2	1.37
	Upper CI	1.44	1.41	1.26	1.45
Plasma riboflavin (nmol/L)	Mean	11.5	10.1	23.8§	10.4
	Lower CI	9.6	8.6	20.3	8.8
	Upper CI	13.7	11.9	28.0	12.2
Plasma flavins‖ (nmol/L)	Mean	67.5	67.3	84.1§	62.1
	Lower CI	61.6	62.1	78.8	56.7
	Upper CI	73.4	72.6	89.4	67.4

NOTE: Covariates appearing in the model were evaluated on the basis of the following values: baseline plasma folate, 41.2 nmol/L; age, 60.7 years; baseline colon folate, 0.65 nmol/g; baseline plasma tHcy, 13.03 μmol/L; baseline EGRAC, 1.37; baseline plasma riboflavin, 10.89 nmol/L; total plasma flavins, 66.5 nmol/L.
Abbreviation: tHcy, total homocysteine.
*400 μg of folic acid daily.
† 400 μg of folic acid plus 5 mg of riboflavin.
‡ 1,200 μg of folic acid daily.
§Significantly different from placebo effect ($P < 0.05$), $P < 0.01$.
‖Analysis on log-transformed data; geometric mean and 95% CI are reported.

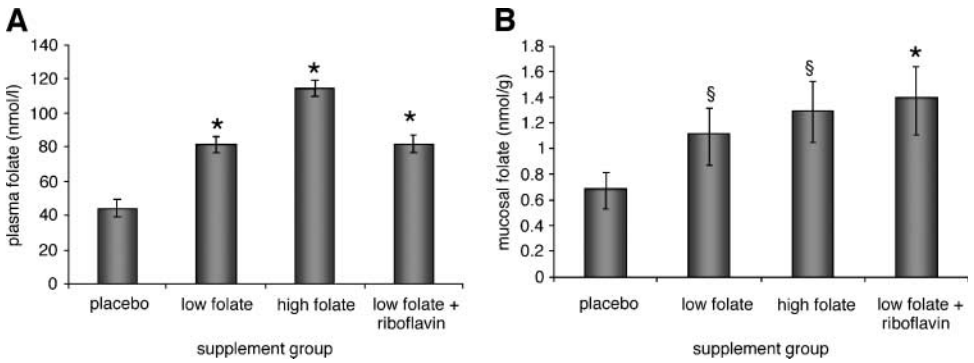

Figure 2. Response of plasma (**A**) and colon mucosal (**B**) folate to supplements of folic acid and riboflavin. Postintervention values corrected to a baseline of 41.2 nmol/L of plasma folate (*columns,* mean; *bars,* SE) and 0.65 nmol/g of mucosal folate (geometric mean, 95% CI). Low folate, 400 μg of folic acid/d; high folate, 1,200 μg of folic acid/d; low folate + riboflavin, 400 μg of folic acid + 5 mg riboflavin/d. *, $P < 0.001$, significantly different from placebo; §, $P < 0.01$, significantly different from placebo.

in healthy adjacent epithelium but this may reflect an increased rate of utilization of folate in rapidly proliferating cells rather than a specific role of localized folate deficiency in carcinogenesis.

We showed a significant correlation between colonic mucosal 5MeTHF and plasma 5MeTHF in the entire cohort, as well as when the polyp group and normal subjects were examined separately. We also observed a significant positive relationship between the magnitude of the change in colonic mucosal 5MeTHF and plasma folate following intervention. Meenan et al. (32) found no relationship between folate in the circulation and folate in isolated colonocytes, in contrast with Kim et al. (30), who reported a relationship between colonic mucosal folate and circulating folate concentration. This group also showed a 2.5-fold increase in colonic mucosal folate concentration following 6 months of high-dose (5 mg/d) folic acid. They also reported a further increase in colon folate following a further 6-month high-dose supplementation but no such effect in plasma or RBC folate (31). The same authors reported that colon mucosal folate concentrations correlated with plasma folate concentrations only in unsupplemented individuals and suggested that this reflected an upper limit to the ability of colonic mucosa to retain folate. Although this is a plausible explanation for an upper limit of colonic mucosal folate concentrations, their data do not show such an upper limit convincingly. Kim's data suggest that the lack of a correlation between plasma folate and colon folate in the supplemented group is due to an upper threshold for serum folate at the folic acid dose given and not to an upper threshold for colon folate. Thus, although we support the notion of an upper limit for colon mucosal folate, the data by Kim et al. (30, 31) do not provide evidence for this. Our data show that 1,200 μg of folic acid/d for 45 days does not elicit any additional significant increase in colon folate over 400 μg of folic acid/d, which does indeed suggest an upper limit to folate concentration in this tissue. An upper limit to colon mucosal folate concentrations has previously been observed in rats (33). It remains to be seen whether the explanation lies in the activity of cellular folate–metabolizing enzymes, or to the limited ability of mucosal colonocytes to take up folate from the gut lumen. The relevance of this phenomenon to the susceptibility of the colon to malignant transformations is also not understood.

The strong inverse relationship between colon folate and plasma total homocysteine seen only in the polyp patients is suggestive of a stronger regulatory control of the methylation cycle in mucosal cells from patients with polyps. This is interesting in view of the fact that folylpolyglutamate synthases are widely expressed in tumor cells (34), and that colon mucosal folate

Table 4. Interaction between *MTHFR* C677T genotype, histology, and intervention to determine plasma 5MeTHF

Intervention*	CT and TT †						CC †					
	Normal			Polyp			Normal			Polyp		
	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*
Placebo	48.9	9.7	11	37.7	9.4	11	33.8	12.2	7	57.0	11.1	9
Low folate ‡	79.2	9.9	10	65.5	7.8	16	88.4	10.0	10	93.6	9.5	11
Low folate + riboflavin§	75.4	8.7	13	87.6‖	7.2	19	87.9	9.2	13	77.7	13.0	6
High folate¶	89.9	9.6	11	127.6**	8.8	14	130.0**	10.0	13	110.1	8.8	14

*Plasma 5MeTHF increased in the low folate groups, irrespective of genotype or histology ($P < 0.001$).
† CC, CT, and TT are genotypes for the *MTHFR* C677T polymorphism.
‡ 400 μg of folic acid daily.
§ 400 μg of folic acid plus 5 mg of riboflavin.
‖ This group showed a significant increase in plasma 5MeTHF compared with the low folic acid, no riboflavin group ($P = 0.018$).
¶ 1,200 μg of folic acid daily.
** These groups showed a significant increase in plasma 5MeTHF compared with the low-dose folic acid group ($P < 0.01$).

concentration was higher, although not significantly so, in biopsies collected from polyp patients. This supports the idea of a ''field effect'' in people with enhanced risk of colorectal cancer (35).

The observed inverse association between colon mucosal folate and plasma homocysteine reflects the importance of cellular folate metabolism to extracellular homocysteine concentrations (36), and confirms that modest folic acid supplements can elicit significant homocysteine-lowering (37). The study has revealed interactions between genotype and histology in determining response to folate and riboflavin supplementation. In patients with normal colon histology, the increase in plasma 5MeTHF in response to high-dose folic acid was diminished in people carrying at least one T allele, which is consistent with reports of a poorer response to folate supplements in people homozygous for *MTHFR* C677T in the general population (38, 39). This may be explained by the reduced activity of *MTHFR* and therefore a reduced rate of conversion of 5,10-methylene THF to 5MeTHF in these subjects. In contrast, in patients with polyps, a significant dose-response effect was seen only in people carrying the T allele. We have no explanation for this observation. In addition, riboflavin enhanced the effect of low-dose folic acid on plasma folate in polyp patients carrying a T allele, suggesting that riboflavin can partly compensate for the reduction in MTHFR activity in heterozygotes and homozygotes for the C677T mutation, through its cofactor role for this enzyme. The effect seems to be related to the poorer response to low folic acid supplementation in the polyp patients. Others have recently reported an interaction between riboflavin and genotype that may influence the risk of colorectal cancer. Le Marchand et al. (18) reported a case-control study in which the lowest risk for colorectal cancer was in those people with the TT *MTHFR* C677T variant and in the highest tertile for riboflavin intake.

Animal studies have highlighted the importance of the timing of doses of folic acid in determining the nature of the effect on colorectal carcinogenesis (13, 14). Clearly, important questions have yet to be addressed with respect to the putative protective effect of dietary folate or folic acid supplements in reducing the risk of colorectal cancer, including a safe and effective range of intakes.

Conclusion

Colorectal mucosal folate concentration shows a response to folate supplementation that is comparable with that seen for plasma or RBC folate, but with evidence that there may be an upper limit. Increasing folate intake has been shown to elicit various responses that may be beneficial in reducing the risk of colorectal cancer and these effects may be mediated through increased concentrations of colonic mucosal folate.

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Responses of Biomarkers of Folate and Riboflavin Status to Folate and Riboflavin Supplementation in Healthy and Colorectal Polyp Patients (The FAB2 Study)

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