

Uracil misincorporation into DNA and folic acid supplementation^{1–3}

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ABSTRACT

Background: Folate deficiency decreases thymidylate synthesis from deoxyuridylate, which results in an imbalance of deoxyribonucleotide that may lead to excessive uracil misincorporation (UrMis) into DNA during replication and repair.

Objective: We evaluated the relation between UrMis in different tissues and the effect of folate supplementation on UrMis.

Design: We analyzed UrMis concentrations in rectal mucosa ($n = 92$) and white blood cells (WBCs; $n = 60$) among individuals randomly assigned to receive supplementation with 1 mg folate/d or placebo, who were then evaluated for colorectal adenoma recurrence.

Results: As expected, total homocysteine was significantly lower among the study participants who received active folate treatment (Wilcoxon's $P = 0.003$) than among those in the placebo group. The median UrMis concentration in rectal mucosa and WBCs among individuals treated with folate was not significantly lower than that in those who received placebo (Wilcoxon's $P = 0.17$). UrMis concentrations in both rectal mucosa and WBCs did not correlate significantly with folate measured in plasma and red blood cells. UrMis in rectal mucosa was marginally associated with an increased risk of adenoma recurrence (odds ratio per SD: 1.43; 95% CI: 0.91, 2.25).

Conclusions: UrMis measurements in WBCs are not a robust surrogate for UrMis measurements in the rectal mucosa (Spearman correlation coefficient = 0.23, $P = 0.08$). Furthermore, folate supplementation in an already replete population (half treated with folic acid supplements and all exposed to folic acid fortification of the food supply) was not significantly associated with reduced UrMis in rectal mucosa cells or WBCs. Large-scale studies are needed to evaluate whether excessive UrMis concentrations are an important risk factor for colorectal neoplasia. This trial was registered at clinicaltrials.gov as NCT00272324. *Am J Clin Nutr* 2010;91:160–5.

INTRODUCTION

Folate, a water-soluble B vitamin, is an important cofactor for DNA methylation reactions and nucleotide synthesis (1). In observational studies, there is an inverse association between folate intake (2–13) or circulating folate concentrations (3, 14, 15) and the risk of colorectal cancer and adenomas. However, in the Aspirin/Folate Polyp Prevention Study, supplementation with 1 mg folic acid/d did not reduce colorectal adenoma risk (16). In secondary analyses, there was evidence of increased risk, with higher rates of advanced adenomas (unadjusted relative risk: 1.67; 95% CI: 0.93, 1.37) and multiple adenomas (unadjusted relative risk: 1.20; 95% CI: 1.23, 4.35). Folate plays a dual role in animal carcinogenesis (17, 18). Thus, folate deficiency may promote carcinogenesis, whereas high folate status may stimulate

the growth of existing preneoplastic and malignant lesions (9, 19).

Deficient cellular folate decreases thymidylate synthesis, producing deoxyribonucleotide pool imbalances. This results in uracil misincorporation (UrMis) into DNA during replication and repair (20–22). In addition, UrMis can also occur from spontaneous hydrolytic deamination of cytosine. Repair of uracil residues produces double-strand breaks, deletions, chromosomal breaks, micronucleus formation, and loss of heterozygosity (20–23). The resultant DNA damage may contribute to the increased risk of cancer (24) associated with folate inadequacy (25).

It is not known to what extent folate supplementation in a population with folic acid fortification of the food supply affects UrMis or whether there are differences between white blood cells (WBCs) and rectal mucosa tissue in the extent of UrMis or in the response to folate supplementation. To clarify these issues, we evaluated the relation between UrMis and the risk of colorectal adenomas among 98 individuals in the Aspirin/Folate Polyp Prevention Study who were randomly assigned to receive folate treatment or placebo and then evaluated for adenoma recurrence.

SUBJECTS AND METHODS

Study population

The participants were enrolled in the Aspirin/Folate Polyp Prevention Study, a double-blinded, placebo-controlled clinical trial of aspirin and folate as chemopreventive agents against the occurrence of new colorectal adenomas. Recruitment at 9 North

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American clinical centers, began on 6 July 1994 and ended on 20 March 1998. An independent data and safety monitoring committee reviewed the study semiannually. Human subjects committees at the clinical centers approved the study protocol and the materials distributed to the participants. All participants provided written informed consent (16, 26).

Using a 3×2 factorial design, the trial compared 81 and 325 mg aspirin/d with placebo and 1 mg folic acid/d with placebo. Initially, the trial was designed to investigate only aspirin, but shortly after enrollment began, it was expanded to examine folic acid (100 individuals were randomly assigned before the folic acid component was initiated). All participants had a recent history of a histologically confirmed colorectal adenoma and a complete colonoscopy within 3 mo before enrollment (qualifying examination), with no known polyps left in the bowel. A total of 1021 subjects were randomly assigned to the folic acid intervention between 25 September 1994 and 15 July 1998, at recruitment sites associated with 9 clinical centers in North America. The principal findings of the Aspirin/Folate Polyp Prevention Study were previously reported (16, 27, 28).

"Year 3" in this analysis is defined as the time of the surveillance colonoscopy ≈ 3 y after the qualifying examination (36–40 mo after the baseline colonoscopy). The principal outcome of the study was the occurrence of one or more adenomas determined by colonoscopy detected in the period starting 1 y after randomization to the end of the year 3 surveillance follow-up examination. Also evaluated was the occurrence of advanced lesions, including tubulovillous or villous adenomas, large adenomas (≥ 1 cm in diameter), those with severe dysplasia, and invasive cancer. The folic acid intervention was initially designed for a 3-y treatment period. However, because a longer exposure to folic acid may be required to observe an inverse association with colorectal neoplasia, participants completing their first follow-up colonoscopy, at year 3, were invited to continue their blinded randomized treatment (folic acid or placebo; 16) for an additional colonoscopic surveillance cycle (typically 3 or 5 y). This second surveillance cycle and follow-up interval is defined as the interval from the time after the year 3 colonoscopy through the subsequent second colonoscopy or 1 October 2004, whichever came first. Of the 1021 participants initially randomly assigned to receive folate or placebo, 729 (71.4%) continued with their randomized treatment, and 607 (59.5%) completed their second follow-up examination with a mean (\pm SD) follow-up time of 41.8 ± 11.8 mo. Participants still on randomized treatment during the second surveillance cycle were recruited to undergo a mucosal biopsy for the UrMis. Plasma folate and plasma homocysteine data presented here are year 3 measurements. Forty-four participants were approached in the context of a regularly scheduled surveillance colonoscopy, during which standard mucosal biopsy samples were collected at the beginning of the procedure ≈ 15 cm from the anal verge; 48 subjects had a "no prep" procedure, and the biopsy samples were collected through a sigmoidoscope.

Biochemical methods

The uracil content of DNA was measured by gas chromatography–mass spectrometry, as previously described by Mashiyama et al (29) and Blount and Ames (30). Briefly, uracil was obtained from 5 μ g DNA from WBCs and rectal mucosa

biopsy samples by incubation with 4 U uracil DNA glycosylase (New England Biolabs, Ipswich, MA) in TE buffer for 3 h at 37°C. 4,5- $^{13}\text{C}_2$ Uracil (200 pg; Cambridge Isotope Laboratories, Andover, MA) was added as an internal standard after glycosylase digestion, and the samples were dried in a speed vacuum concentrator. We used a larger volume of commercially available enzyme and corrected for background noise by subtracting a blank sample. The residue was resuspended in a mixture of 50 μ L acetonitrile, 10 μ L triethylamine, and 1 μ L 3,5-bis(trifluoromethyl)benzyl bromide and shaken to derivatize for 25 min at 37°C, followed by the addition of 50 μ L water (HPLC grade) to improve extractability. *N1,N3*-(3,5-bis[trifluoromethyl]benzyl)uracil, a derivatized form, was extracted into 100 μ L isooctane. The sample (5 μ L) was injected onto a Series 6890 Gas Chromatograph equipped with a 7683 automated liquid sampler (Agilent Technologies, Santa Clara, CA) in the splitless mode. Adequate separation of *N1,N3*-(3,5-bis[trifluoromethyl]benzyl)uracil from interfering compounds was achieved on a capillary column (HP-5MS; Hewlett-Packard, Palo Alto, CA). A 5973N mass selective detector (Agilent Technologies) using negative chemical ionization MS was used for analysis with selected ion monitoring at *m/z* 337 and 339.

Plasma folate concentrations were measured close to year 3, according to previously reported methods (31). Red blood cell (RBC) folate was measured with the ACS:180 folate chemiluminescence assay (Bayer Corporation, Tarrytown, NY) (32). However, the hematocrit at baseline was used because it was not available at year 3. Total homocysteine (tHcy) in plasma was measured according to the principles described by Araki and Sako (33).

Statistical analysis

We evaluated differences between the randomized placebo and folate treatment groups using contingency table chi-square testing for categorical variables and *t* tests for continuous variables. Correlations between variables were evaluated by using Spearman's correlation coefficient. In this study, UrMis values are presented as the average of 2 duplicate laboratory measurements repeated from the same sample. Intraclass correlation coefficients for these measurements were calculated as between-person variance divided by the total variance: 0.69 for log-transformed UrMis concentrations in rectal mucosa samples and 0.73 for log-transformed UrMis concentrations in WBC samples. To assess associations with adenoma occurrence in the interval up to and including the year 3 examination, we used unconditional logistic regression for the analyses to compute odds ratios (ORs) and 95% CIs. All statistical tests were 2-sided. Statistical analyses were performed with SAS (version 9.1; SAS Institute, Cary, NC).

RESULTS

A total of 94 subjects were evaluated in this study. UrMis was successfully measured in 60 WBC samples (30 placebo samples and 30 folic acid supplementation samples) and 92 rectal mucosa samples during the second surveillance cycle. The mean baseline age of study participants included in this analysis was 57 y (Table 1). There were no statistically significant differences between treatment groups in baseline characteristics. Overall, most of the

TABLE 1
Characteristics of the study population, by treatment group¹

	Placebo (n = 42)	Folate (n = 52)	P value
Sex [n (%)]			0.29 ²
Male	28 (66.7)	29 (55.8)	
Female	14 (33.3)	23 (44.2)	
Age (y) ³			0.83 ⁴
Mean ± SD	57.8 ± 7.8	57.4 ± 8.8	
Race [n (%)]			1.00 ²
White	40 (95.2)	50 (96.2)	
Other	2 (4.8)	2 (3.8)	
Baseline alcohol intake [n (%)]			0.47 ²
0 drinks/d	13 (31.7)	17 (33.3)	
<0.5 drinks/d (≈<7 g/d)	9 (22.0)	15 (29.4)	
≥0.5 drinks/d (>7 g/d)	19 (46.4)	19 (37.3)	
Baseline smoking status [n (%)]			0.92 ²
Never	22 (52.38)	25 (48.08)	
Former	17 (40.48)	23 (44.23)	
Current	3 (7.14)	4 (7.69)	
Lifetime number of adenomas at baseline [n (%)]			0.51 ²
1–2	26 (61.9)	36 (69.2)	
≥3	16 (38.1)	16 (30.8)	
Uracil misincorporation, mucosa ^{5,6}			
Median (interquartile range)	4.31 (1.17)	4.21 (1.31)	0.17 ⁷
Year 3 plasma folate (ng/mL) ⁸			
Mean ± SD	9.81 ± 3.95	29.43 ± 15.73	<0.0001 ⁴
Median (interquartile range)	8.85 (5.10)	24.80 (26.25)	<0.0001 ⁷
Year 3 RBC folate (ng/mL) ⁸			
Mean ± SD	680.77 ± 223.67	1023.72 ± 207.43	<0.0001 ⁴
Median (interquartile range)	648.90 (275.37)	1014.06 (229.95)	<0.0001 ⁷
Year 3 tHcy (μmol/L) ⁸			
Mean ± SD	9.69 ± 2.39	8.64 ± 2.45	0.04 ⁴
Median (interquartile range)	9.21 (1.51)	8.09 (2.36)	0.003 ⁷

¹ tHcy, total homocysteine; RBC, red blood cell.

² Fisher's exact test.

³ Age at randomization.

⁴ *t* test.

⁵ Uracil measurements in mucosa from rectal biopsy samples.

⁶ Total sample size = 92 for rectal mucosa uracil measurements; total sample size = 60 for white blood cell uracil measurements.

⁷ Wilcoxon's rank-sum by folate treatment status.

⁸ Year 3 of this study was defined as the time of the surveillance colonoscopy ≈3 y after the qualifying examination.

participants were white (95% of placebo subjects and 96% of those in the folic acid treatment group), alcohol intake was moderate, and <8% of all participants were current smokers. Sixty-seven percent of participants in the placebo group were men, and 56% of those who were randomly assigned to folic acid. In the placebo group, 38% of the participants had >3 lifetime adenomas at baseline compared with 30% in the folate group ($P = 0.51$).

In this folate-replete population (defined in this study as half treated with folic acid supplements and all exposed to folic acid fortification of the food supply), the median prerandomization tHcy was 9.22 μmol/L (interquartile range: 3.03 μmol/L). Postrandomization differences between treatment groups in plasma and RBC folate were in the expected direction (Table 1). Postrandomization median tHcy concentrations (μmol/L) were significantly lower in the folate treatment group (8.09; interquartile range: 2.36; mean ± SD: 8.64 ± 2.45) than in the placebo group (9.21; interquartile range: 1.51; Wilcoxon's $P =$

0.003). Year 3 plasma folate and RBC folate were highly correlated (Spearman correlation = 0.71, $P > 0.0001$; **Table 2**).

The overall median UrMis (pg/μg DNA) was 2.17 (interquartile range: 1.97) in WBCs and 4.29 (interquartile range: 1.28) in rectal mucosa. We observed weak, nonsignificant, inverse relations between mucosal UrMis and year 3 RBC folate (Spearman correlation = −0.18, $P = 0.09$) and plasma folate (Spearman correlation = −0.15, $P = 0.14$). There were no associations between UrMis in WBC and plasma folate (Spearman correlation = 0.006, $P = 0.97$) or RBC folate (Spearman correlation = 0.003, $P = 0.97$). Individuals receiving folate supplementation had nonsignificantly lower UrMis in the rectal mucosa than did those receiving placebo: the median mucosal UrMis (pg/μg DNA) in folate-treated subjects was 4.21 (interquartile range: 1.31) compared with 4.31 (interquartile range: 1.17) among placebo subjects (Wilcoxon's $P = 0.17$; Table 1). There was also no effect of folic acid supplementation on WBC UrMis (Wilcoxon's $P = 0.88$; Table 1).

TABLE 2Spearman correlations between folate and uracil misincorporation (UrMis; in pg/ μ g DNA) measurements¹

	Year 3 plasma folate ² (n = 94)	Year 3 RBC folate ² (n = 94)
Year 3 plasma folate ² (n = 94)	—	0.71 (<i>P</i> < 0.0001)
Year 3 ³ UrMis in mucosa ^{2,4} (n = 92)	-0.15 (<i>P</i> = 0.14)	-0.18 (<i>P</i> = 0.09)
Year 3 ³ UrMis in WBC ^{2,4} (n = 60)	0.006 (<i>P</i> = 0.97)	0.003 (<i>P</i> = 0.97)

¹ Analysis includes participants who received placebo or folic acid supplementation. RBC, red blood cell; WBC, white blood cell count.² All measurements as continuous variables.³ Year 3 of this study was defined as the time of the surveillance colonoscopy \approx 3 y after the qualifying examination.⁴ Total sample size = 92 for rectal mucosa uracil measurements; total sample size = 60 for WBC uracil measurements.

There was a modest association between UrMis in WBCs and measurements in mucosal rectal biopsy samples, but it was only of borderline statistical significance (Spearman correlation = 0.23, *P* = 0.08). Although not statistically significant, the mean UrMis in rectal biopsy samples was higher in individuals with adenoma recurrence (median: 4.35; interquartile range: 1.08) than in those without (median: 4.24; interquartile range: 1.09; Wilcoxon's *P* = 0.18; **Table 3**). There were no statistically significant differences by adenoma recurrence. The OR for adenoma recurrence was 1.43 (95% CI: 0.91, 2.25) per SD of mucosa UrMis; for WBC UrMis, the OR per SD was 0.66 (95% CI: 0.33, 1.32).

DISCUSSION

We analyzed UrMis concentrations in rectal mucosa (*n* = 92) and WBC samples (*n* = 60) among individuals randomly assigned to supplementation with 1 mg folic acid/d or placebo and who were then evaluated for adenoma recurrence. We found that active folic acid treatment was not significantly associated with reduced UrMis in either tissue. Furthermore, UrMis concentrations in mucosa were only weakly correlated with concentrations measured in WBCs. Although sample size is a limitation, these data suggest that UrMis concentrations in WBC are not suitable surrogates for UrMis measurements in rectal mucosa.

Folic supplementation had the expected effect on serum folate and tHcy as evidenced by higher folate and lower tHcy at follow-up in the subjects supplemented with folic acid. However, the median UrMis measured in rectal mucosa biopsy

samples and WBCs was not significantly lower among individuals treated with folic acid than among those randomly assigned to receive placebo. Nonetheless, there was a suggestive, not statistically significant, association of UrMis concentrations in rectal biopsy samples with an increased risk of adenoma recurrence.

Population-based studies have reported a conflicting relation between folate status and UrMis. A few studies did not observe a significant association between UrMis and folate status (34, 35). In more recent epidemiologic studies, UrMis in peripheral blood lymphocytes decreased significantly after folate supplementation in healthy volunteers (36). However, in another study, high-dose supplementation with 5 mg folic acid/d and 1.1 mg vitamin B-12/d for 6 mo was associated with increased UrMis in rectal biopsy samples, especially among participants with the *MTHFR* 677 TT genotype (37). In a recent study among 431 participants of the Boston Puerto Rican Health Study, SNPs in the uracil processing genes *DUT*, *UNG*, and *SMUG1* were significantly associated with UrMis concentrations, but plasma folate, vitamin B-6, vitamin B-12, and riboflavin were not significantly associated (38). Data from a folate-replete population suggest that UrMis in leukocytes may depend on the amounts of other B vitamins such as plasma B-12 concentrations (39).

Dietary and supplemental folate has been shown to be inversely associated with the risk of colorectal neoplasia (6, 8, 9, 14, 15, 40–43), although findings from recent trials suggest that supplementation in replete populations does not reduce risk (16). Folate deficiency, possibly by provoking altered DNA global (genomic) methylation levels (44), imbalanced deoxyribonucleotide pools,

TABLE 3Association of uracil misincorporation (UrMis; in pg/ μ g DNA) and risk of adenoma recurrence¹

	Adenoma	No adenoma	OR (95% CI) ²	OR (95% CI) ³
UrMis in mucosa ⁴			1.46 (0.94, 2.28)	1.43 (0.91, 2.25)
<i>n</i>	36	56		
Mean \pm SD	4.45 \pm 0.79	4.12 \pm 0.92		
Median (interquartile range)	4.35 (1.08)	4.24 (1.49)		
UrMis in WBC ⁴			0.64 (0.32, 1.28)	0.66 (0.33, 1.32)
<i>n</i>	30	30		
Mean \pm SD	2.33 \pm 1.05	2.93 \pm 2.32		
Median (interquartile range)	2.11 (1.57)	2.23 (2.23)		

¹ Analysis included participants who received placebo or folic acid supplementation and were then evaluated for adenoma recurrence within the first 3 y. OR, odds ratio; WBC, white blood cell count. Wilcoxon's *P* values for adenoma recurrence status: mucosa (*P* = 0.18), WBC (*P* = 0.58).² OR for adenoma recurrence adjusted for age by using logistic regression.³ OR for adenoma recurrence adjusted for age and folate randomization status by using logistic regression.⁴ UrMis as a continuous variable (calculated per SD of UrMis).

chromosomal breakage (20), and consequent DNA instability may have implications for cancer risk (45).

Strengths of the study were the careful laboratory measurements in paired rectal mucosa and WBC specimens from participants randomly assigned to folate supplementation. Our UrMis measures, with a robust intraclass correlation coefficients, fell well within the published range measurements from population-based studies (29, 39, 46–48). A limitation of our methodology was that the cloned enzyme used in our study may not have removed all of the uracil present in the DNA. A newer method for measuring UrMis (49), published after our study samples were analyzed, incorporated technical changes that resulted in a reduction in uracil concentrations at baseline. We achieved a similar result by subtracting a blank sample, but recognize that the concentration of uracil in healthy WBCs may have been lower than previously reported. However, we were limited by a small sample size and were unable to assess potential effect modification by genetic polymorphisms such as the *MTHFR C677T* and *MTHFR A1298C* variants (35, 38). The blood samples for plasma and RBC folate measurements were drawn up to several years before the time when the samples for UrMis were obtained; variation over time in these measurements might weaken any associations among them. Furthermore, the relatively high plasma folate and low tHcy values in the study population limit the generalizability of the findings to folate-replete populations.

In conclusion, we found that the effect of high-dose folic acid supplementation on UrMis concentrations in both blood WBCs and colorectal mucosa may be minimal in a folate-replete population. The effect of folate on UrMis may only be detectable in a population with a significant number of individuals with lower B vitamin status. Additional large-scale studies are needed to evaluate whether UrMis may be a marker of risk of colorectal neoplasia.

The authors' responsibilities were as follows—JAB: study design; JAB, JS, W-HC, and PMU: data acquisition; and JAB, AH, and DJH: analysis and interpretation of the data and drafting of the manuscript. All authors contributed to critical revision of the manuscript. Wyeth, which markets folic acid supplements, provided the study agents for the clinical trial on which this research is based. There were no other potential conflicts of interests.

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