

## Lower Risk of Cervical Intraepithelial Neoplasia in Women with High Plasma Folate and Sufficient Vitamin B12 in the Post-Folic Acid Fortification Era

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### Abstract

The purpose of this study was to determine the influence of plasma folate and vitamin B12 concentrations on cervical cancer risk in the U.S. after the folic acid fortification era. The study included 376 premenopausal women of childbearing age who tested positive for infections with high-risk (HR) human papillomaviruses (HPVs) and were diagnosed with cervical intraepithelial neoplasia (CIN) grade 2 or higher (CIN 2+, cases) or  $\leq$ CIN 1 (noncases). CIN 2+ (yes/no) was the dependent variable in logistic regression models that specified plasma folate concentrations combined with plasma B12 concentrations as the independent predictors of primary interest, adjusting for age, race, education, smoking, parity, number of lifetime male sexual partners, use of contraceptives, waist circumference, physical activity, healthy eating index, and circulating concentrations of vitamins A, C, tocopherol, and total carotene. Women with supraphysiologic concentrations of plasma folate ( $>19.8$  ng/mL) who also had sufficient plasma vitamin B12 ( $\geq 200.6$  pg/mL) had 70% lower odds of being diagnosed with CIN 2+ ( $P = 0.04$ ) when compared with women with plasma folate of  $\leq 19.8$  ng/mL and plasma vitamin B12 of  $< 200.6$  pg/mL. Our results do not corroborate the concern that supraphysiologic plasma folate concentrations seen in the post-U.S. folic acid fortification era increase the risk of CIN in premenopausal women of childbearing age. In fact, higher folate is associated with significantly lower risk of CIN, especially when vitamin B12 is sufficient, demonstrating the importance of vitamin B12 in the high-folate environment created by the folic acid fortification program.

In 1998, the Food and Drug Administration mandated the addition of folic acid (synthetic form of folate, a water-soluble B vitamin) to grain products to reduce the risk of neural tube defects in the United States. This has induced a population-wide increase in folate intake, which in some subgroups may have exceeded the body's physiologic needs. Serum or plasma folate concentrations of  $>45$  nmol/L (19.8 ng/mL) in fasting blood samples are often considered supraphysiologic. According to the National Health and Nutrition Examination Survey, by 1999 to 2000,  $\sim 25\%$  of the U.S. population had supraphysiologic serum folate concentrations (1), although this may be an overestimate due to inclusion of nonfasting blood samples in the analyses that produced these findings. Supraphysiologic folate concentrations are associated with the pres-

ence of unmetabolized folic acid (2), which may interfere with the diagnosis of pernicious anemia due to vitamin B12 deficiency (3), and may also have different effects on folate binding proteins or transporters (4), potentially interfering with normal folate metabolism. Also, there is some evidence to suggest that adverse effects of higher folate may only occur when vitamin B12 status is lower as shown for cognitive impairment and anemia (5) and for insulin resistance (6).

The scientific literature has discussed possible adverse effects of higher folate intake on several health conditions (7), including cancer (8). The evidence available on specific adverse health outcomes, however, is inadequate (9, 10). Reviews of the safety and toxicity of folate that were published before initiation of the folic acid fortification in the United States concluded that folic acid is safe under most circumstances even at supraphysiologic amounts (11). In addition, it is important to consider that fortification not only raises the concentration of unmetabolized folic acid but also the concentrations of total folate in the body, reducing the risk of diseases associated with low-folate status (12). Finally,  $\sim 25\%$  of the adults (13) and most pregnant women in the United States have consumed multivitamin preparations containing folic acid for many decades (14) and most likely had unmetabolized folic acid in their circulation as a result (15). Observations of these groups have produced no credible evidence of any adverse health effects. Thus, whether high systemic folate levels

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induced by the fortification program are harmful to humans is still largely a matter of speculation.

The evaluation of potential effects of high folate levels is further complicated by measurement problems. First, because of lack of consensus on what circulating concentrations of folate might cause harm for a given disease, studies have mostly used cut points based on their study populations (e.g., median, tertiles) to categorize individuals. This approach may be efficient in the presence of linear effects, but may obscure associations if there are disease-specific thresholds, and does not allow clear comparisons across studies. Use of different assays (e.g., microbiological versus protein binding based, most commonly used) to measure folate adds another layer of complexity. Protein binding–based assays seem to be less sensitive and reliable than microbiological assays, and consistently give lower folate values. It is widely accepted that the microbiological assay based on growth of *L. casei*, a well-validated method is the gold standard for measuring total folates (16, 17).

Our previous research has shown that circulating folate status significantly influences the natural history of infections with carcinogenic or high-risk (HR) human papillomaviruses (HPV), the major risk factor for cervical intraepithelial neoplasia (CIN) grades higher than 2 (CIN 2+), precursor lesions for invasive cervical cancer. Our results showed that higher circulating concentrations of folate were associated with a lower likelihood of becoming HR-HPV positive and of having a persistent HR-HPV infection, and when infected, a greater likelihood of clearing HR-HPV (18) and lower likelihood of developing HR-HPV–associated CIN 2+ (19). Folate supplementation is simple and inexpensive, and may be effective as an approach to cervical cancer prevention, or possibly as an adjuvant to HPV vaccines. However, the possibility of a paradoxical increase in the risk of CIN 2+ due to supraphysiologic folate concentrations in blood, and the influence of folate-vitamin B12 interactions on CIN risk have not been evaluated in the folic acid fortification era. The purpose of this study was to determine the joint influence of supraphysiologic plasma folate and vitamin B12 concentrations on CIN risk in the folic acid fortification era, using the microbiological assay for assessing total folate status.

## Materials and Methods

### Study participants

The present analysis is based on a subset of women enrolled after the initiation of the folic acid fortification program in United States (2004–2006) for an ongoing prospective follow-up study funded by the National Cancer Institute (R01 CA105448, Prognostic Significance of DNA & Histone Methylation). The main purpose of the parent study is to investigate whether alterations in folate-related biomarkers such as DNA and histone methylation and DNA damage in cervical cells can be useful in identifying underlying precancerous lesions and whether these markers can be used to identify women who are at risk of developing those lesions because of their exposure to HR-HPVs. All women enrolled in this study were diagnosed with abnormal cervical cells (atypical squamous cells of undetermined significance, low-grade, or high-grade squamous intraepithelial lesion) by the Health Department in Birmingham, Alabama, and were referred to a colposcopy clinic at the University of Alabama at Birmingham for further examination by colposcopically directed biopsies. All women met specific study eligibility criteria: 19 y or older (none above age 50 y), no previous history of cervical or other lower genital cancer, no previous hysterectomy, or known destructive therapy of the cervix; currently

not pregnant and not actively trying to become pregnant within the next 2 y; no current use of antifolate medications such as methotrexate, sulfasalazine, or phenytoin; and ability to provide informed consent with a reasonable likelihood of follow-up, i.e., no obvious plans to leave the area during the course of the study. At the enrollment visit, ~20% of women were diagnosed with CIN 2+ and were treated using the loop electrosurgical excisional procedure. Approximately 80% of these women diagnosed with  $\leq$ CIN 1 lesions and were considered to be free of true preneoplastic lesions and eligible to enter the 24-mo follow-up phase of the study.

Seven hundred and three women were enrolled in the main study. For the present analysis, we excluded women of ethnicities other than Caucasian American or African American ( $n = 9$ ), who were missing a baseline histologic diagnosis ( $n = 54$ ) or had a diagnosis of invasive cancer ( $n = 1$ ), who did not have HPV results ( $n = 19$ ) or tested negative for all of the 13 types of HR-HPV ( $n = 150$ ). To allow additional analyses of dietary intake data in the same subset in future analyses, it was also necessary to exclude an additional 67 HR-HPV–positive women whose dietary questionnaire data were missing, had serious errors, or showed abnormal caloric intake. Because of the importance of fasting blood samples for the measurement of plasma folate, 27 women were excluded because they had given nonfasting blood samples. Thus, 376 women are included in this report. Of these, 103 women were diagnosed with CIN 2+ [cases, including CIN 2 ( $n = 62$ ), CIN 3 ( $n = 38$ ) or carcinoma *in situ* ( $n = 3$ )] and 273 women were diagnosed with  $\leq$ CIN 1 [noncases, including normal cervical epithelium ( $n = 13$ ), HPV cytopathic effect ( $n = 31$ ), reactive nuclear enlargement ( $n = 48$ ) or CIN 1 ( $n = 181$ )].

All H&E-stained tissue sections were reviewed by the study pathologist (WCB). When there was disagreement with the diagnosis stated in the pathology report generated by the University of Alabama at Birmingham Surgical Pathology, a third pathologist was consulted. In all such cases there was agreement between the study pathologist and the third reviewer, and amendments were submitted by the PI to modify the original diagnosis reports.

Data from a detailed risk factor questionnaire and from a CDC physical activity questionnaire were available for all women included in this analysis, as well as height, weight, and waist circumference (WC) measurements obtained using standard protocols. Pelvic examinations and collection of cervical cells and biopsies were carried out following clinical protocols established for the colposcopy clinic. Fasting blood samples were collected from all women. All protocols were approved by the Institutional Review Board at the University of Alabama at Birmingham.

### Methods

Age at study entry, race, level of education, cigarette smoking frequency, parity, use of hormonal/oral contraceptives, and lifetime number of male sexual partners were ascertained from the risk factor questionnaire administered by the study personnel. The healthy eating index (HEI) (Block scale of 0–100) was obtained from the Block questionnaire data. CDC physical activity questionnaire data were summarized as two categories according to the number of minutes of moderate physical activity/week ( $<150$  or  $\geq 150$ ). WC data were summarized as two categories,  $>88$  cm or  $\leq 88$  cm. Exfoliated cervical cells collected with a cervical brush and immediately rinsed in 10 mL of PBS and fasting blood samples collected in EDTA containing blood collection tubes, which were kept cold after collected were transported to the laboratory on ice within 2 h of collection. In the laboratory, cervical cell suspensions were centrifuged and the resulting pellets were resuspended in fresh PBS. Aliquots used for HPV genotyping were stored in PreservCyt Solution at  $-20^{\circ}\text{C}$ .

DNA was extracted from cervical cells using the QIAamp MiniElute Media kit (Qiagen, Inc.) by following manufacturer's instruction for HPV genotyping test. HPV genotyping test (Linear array; Roche Diagnostics) was done according to the manufacturer's instructions by a research associate trained by personnel from Roche Diagnostics. Briefly,

**Table 1.** Risk factor and micronutrient profiles of the study population

Risk factors*	Cases n (%) or median	Noncases n (%) or median	P
No. women	103 (27)	273 (73)	
Median age at enrollment (y)	23	23	0.875
Education: less than high school	26 (25.2)	54 (19.8)	0.248
Completed high school or greater	77 (74.8)	219 (80.2)	
Race: African American	59 (57.3)	187 (68.5)	0.041
Caucasian American	44 (42.7)	86 (31.5)	
WC: >88 cm	54 (52.9)	168 (61.5)	0.132
≤88 cm	48 (47.1)	105 (38.5)	
Healthy eating index (Block scale of 0-100)	53	54	0.330
Moderate activity/wk: ≥150 min	19 (18.4)	61 (22.3)	0.410
<150 min	84 (81.6)	212 (77.7)	
Current smoker: Yes	53 (51.5)	92 (33.7)	0.002
No	50 (48.5)	181 (66.3)	
Parity: 2+ live births	45 (43.7)	78 (28.6)	0.010
1 live birth	33 (32.0)	93 (34.1)	
0 live births	25 (24.3)	102 (37.4)	
Life time sexual partners: ≥2	100 (98.0)	257 (94.5)	0.172 <sup>†</sup>
<2	2 (2.0)	15 (5.5)	
Hormonal/oral contraceptives: ever used	50 (49.0)	124 (46.1)	0.615
Never used	52 (51.0)	145 (53.9)	
Micronutrient concentrations by cut point			
Folate (ng/mL): >19.8	9 (8.8)	39 (14.5)	0.146
Folate (ng/mL): ≤19.8	93 (91.2)	230 (85.5)	
Vitamin B12 (pg/mL): ≥200.6	84 (81.6)	245 (89.7)	0.032
Vitamin B12 (pg/mL): <200.6	19 (18.4)	28 (10.3)	
Median micronutrient concentrations			
Folate (ng/mL)	12.55	11.30	0.417
Vitamin B12 (pg/mL)	373.88	402.54	0.361
Vitamin C (μg/mL)	15.13	14.46	0.531
α-Tocopherol (mg%)	0.77	0.80	0.737
γ-Tocopherol (mg%)	1.95	2.06	0.139
Vitamin A (μg%)	32.05	32.46	0.998
Total carotene (μg%)	84.55	87.93	0.707

NOTE: P values for  $>\chi^2$  from Kruskal-Wallis test for median values. P values from a  $\chi^2$  test are shown for frequency values.

\*Nonreference category is listed first.

<sup>†</sup>Fisher's exact test two-sided  $Pr \leq P$ .

target DNA amplified by PCR uses the PGM09/11 L1 consensus primer system and includes coamplification of a human cellular target,  $\beta$ -globin, as an internal control. Detection and HPV genotyping are achieved using a reverse line-blot method, and this test includes probes to genotype for 37 anogenital HPV types [6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, 52, 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and CP6108]. HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68 are considered to be HR-HPV types and all other types are considered to be low risk in this analysis.

Circulating concentrations of micronutrients were determined using protocols previously established and validated in the Nutrition Sciences Laboratory at the University of Alabama at Birmingham (20). Briefly, plasma folate was measured using the *L. casei* microbiological assay, plasma vitamin B12 using a competitive radio-binding assay (SimulTRAC-SNB; MP Biomedicals), vitamins A, E, and C by high performance liquid chromatography, and total carotene by spectrophotometry. All plasma samples were stored at  $-70^\circ\text{C}$  and tested within 2 to 3 mo of collection. We monitor the reproducibility of our

micronutrient assays by including two pooled samples (low and high) that have been assayed at least 30 times to determine the mean and SD. This serves as the basis for the quality control for the assays as determined by Westgard "multirule" procedure.

### Statistical analysis

We used descriptive statistics to characterize the 103 cases and 273 noncases. The Pearson  $\chi^2$  statistic was used to assess differences between observed and expected frequencies. Proportions were compared using a two-sided  $\chi^2$  test. Differences in median micronutrient concentrations and other continuous variables were evaluated using a two-sided Kruskal-Wallis test.

Unconditional logistic regression models specified a binary indicator of CIN 2+ diagnosis (yes/no) as the dependent variable. We examined all circulating concentrations of micronutrients (folate, vitamins B12, A, C,  $\alpha$  and  $\gamma$  tocopherol, and total carotene) as potential independent risk factors, adjusting for age as a continuous variable and race, education level, smoking status, parity, lifetime number of sexual partners, degree of physical activity, HEI, and WC as

**Table 2.** Relationship between micronutrients and other factors with the diagnosis of CIN 2+ at baseline

Risk factors	OR adjusted for demographic and nutritional factors (95% CI)	P
Age at enrollment (y)	0.9 (0.9-0.995)	0.034
Less than high school education	0.6 (0.3-1.1)	0.108
African American	0.6 (0.4-1.1)	0.117
WC: >88 cm	0.8 (0.5-1.4)	0.523
Healthy eating index tertiles: $\geq 59$ , $\geq 50$	0.8 (0.6-1.2)	0.270
$\geq 150$ min moderate activity/wk	0.6 (0.3-1.1)	0.095
Current smoker	1.95 (1.1-3.4)	0.017
Parity, 2+, 1 or 0	1.9 (1.3-2.7)	<0.001
Lifetime sex partners $\geq 2$	2.5 (0.5-12.0)	0.243
Ever used hormonal/oral contraceptives	1.0 (0.6-1.7)	0.940
Folate (ng/mL): >19.8	0.7 (0.3, 1.6)	0.366
Vitamin B12 (pg/mL): $\geq 200.6$	0.5 (0.2-0.98)	0.044
Vitamin C tertiles: $\geq 18.23$ , $\geq 12.47$ $\mu\text{g/mL}$	1.1 (0.8-1.5)	0.610
$\gamma$ -Tocopherol: $\geq 2.36$ , $\geq 1.72$ mg%	0.8 (0.6-1.1)	0.131
Vitamin A tertiles: $\geq 38.4$ , $\geq 26.66$ $\mu\text{g}\%$	0.96 (0.7, 1.3)	0.780
Total carotene tertiles: $\geq 100.45$ , $\geq 72.61$ $\mu\text{g}\%$	1.2 (0.8-1.6)	0.365

categorical variables. We categorized plasma folate levels into supra-physiologic ( $>45$  nmol/L or  $>19.8$  ng/mL) and low-physiologic ( $\leq 45$  nmol/L), and vitamin B12 into sufficient ( $\geq 148$  pmol/L or  $\geq 200.6$  pg/mL), and insufficient ( $<148$  pmol/L or  $<200.6$  pg/mL; ref. 21). We categorized all other micronutrient concentrations according to the tertile values of cases and noncases combined, and compared the highest tertile and the middle tertile with the lowest tertile used as the common reference category.

In a variation of the model described above, we specified combined categories of plasma folate and vitamin B12 as the independent predictors of primary interest. We evaluated the strength of each association by estimating the odds ratio (OR) and its 95% confidence interval (CI), and its statistical significance using Wald's  $\chi^2$  statistic of the null hypothesis that the OR of 1. All statistical analyses were conducted using SAS Version 9.1.3 (SAS Institute).

## Results

All women were of premenopausal age. All except three women were  $<45$  years of age, and therefore, 99% of the population was of childbearing age (Table 1). As compared with the 273 noncases, the 103 cases were significantly less likely to be African American ( $P = 0.04$ ), and more likely to be current smokers ( $P = 0.002$ ). Parity was significantly higher in cases than in noncases ( $P = 0.01$ ): cases had two or more births more frequently than noncases; and noncases had zero births more frequently than cases. More noncases had supra-physiologic plasma folate concentrations, although the difference was not statistically significant. Sufficient plasma B12 ( $\geq 200.6$  pg/mL) was significantly more frequent among noncases (89.7%) than

**Table 3.** Relationship of plasma folate and plasma vitamin B12 interaction and other factors with diagnosis CIN 2+

Risk factors	OR adjusted for demographic and nutritional factors (95% CI)	P
Age at enrollment (y)	0.9 (0.9-0.995)	0.033
Less than high school education	0.6 (0.3-1.1)	0.083
African American	0.6 (0.4-1.1)	0.115
WC: >88 cm	0.8 (0.5, 1.4)	0.462
Healthy eating index tertiles: $\geq 59$ , $\geq 50$	0.8 (0.6, 1.2)	0.278
$\geq 150$ min moderate activity/wk	0.6 (0.3, 1.2)	0.122
Current smoker	1.9 (1.1-3.4)	0.017
Parity: 2+, 1 or 0	1.9 (1.3-2.8)	<0.001
Lifetime sex partners: $\geq 2$	2.5 (0.5-12.1)	0.241
Ever used hormonal/oral contraceptives	1.0 (0.6-1.7)	0.954
Folate: >19.8 ng/mL; vitamin B12: $\geq 200.6$ pg/mL; $n = 45$	0.3 (0.1, 0.9)	0.039
Folate: >19.8 ng/mL; vitamin B12: $\geq 200.6$ pg/mL; $n = 279$	0.5 (0.2-0.97)	0.042
Folate: >19.8 ng/mL; vitamin B12: $<200.6$ pg/mL; $n = 44$		1.000 (reference)
Vitamin C tertiles: $\geq 18.23$ , $\geq 12.47$ $\mu\text{g/mL}$	1.1 (0.8-1.5)	0.641
$\gamma$ -Tocopherol: $\geq 2.36$ , $\geq 1.72$ mg%	0.8 (0.6-1.1)	0.151
Vitamin A tertiles: $\geq 38.4$ , $\geq 26.66$ $\mu\text{g}\%$	1.0 (0.7-1.3)	0.875
Total carotene tertiles: $\geq 100.45$ , $\geq 72.61$ $\mu\text{g}\%$	1.2 (0.8-1.6)	0.367

among cases (81.6%;  $P = 0.03$ ). The two groups were similar with regard to other variables, including potential risk factors for CIN (e.g., lifetime number of sex partners, HEI, degree of physical activity or degree of obesity assessed by the WC, and concentrations of other micronutrients).

In logistic regression analyses, the odds of CIN 2+ diagnosis were 30% lower among women with supraphysiologic plasma folate (OR, 0.7; 95% CI, 0.3-1.6), indicating that the data are compatible with a strong protective effect as well as with a modest increase in risk, but are statistically incompatible with a large excess risk of CIN 2+ among women with supraphysiologic folate concentrations (Table 2). Having sufficient vitamin B12 ( $\geq 200.6$  pg/mL) was significantly associated with 50% lower odds of CIN 2+ diagnosis ( $P = 0.044$ ). Being a current smoker and having more live births (2+ versus 1 versus 0) also were significantly associated with elevated odds of CIN 2+ ( $P = 0.02$  and  $<0.001$ , respectively).

In the logistic regression analysis of combined folate and vitamin B12 categories, the reference group was women with low-physiologic plasma folate ( $\leq 19.8$  ng/mL) and insufficient vitamin B12 ( $<200.6$  pg/mL;  $n = 44$ ). Forty-five women had supraphysiologic plasma folate and sufficient vitamin B12, and 279 women had low-physiologic plasma folate and sufficient vitamin B12. Only three women had supraphysiologic plasma folate and insufficient B12, and this group was excluded from the analysis. Five women were not included in this analysis because of missing folate or vitamin B12 data. Women with supraphysiologic plasma folate and sufficient plasma B12 had 70% reduced odds of CIN 2+ (OR, 0.3; 95% CI, 0.1-0.9;  $P = 0.039$ ) when compared with those with low-physiologic plasma folate and insufficient plasma B12 (Table 3). Women with low-physiologic plasma folate and sufficient vitamin B12 had 50% reduced odds for the presence of CIN 2+ (OR, 0.5; 95% CI, 0.2-0.97;  $P = 0.042$ ) when compared with the same reference group. In this model, higher parity and being a current smoker were associated with higher risk for CIN 2+, whereas older age was associated with lower risk of CIN 2+. Other micronutrients were not significantly associated with CIN 2+.

## Discussion

A report by Mason et al. (22) that documented a temporal trend of colorectal cancer incidence in the United States and Canada after fortification suggested that higher folate intake associated with fortification was associated with an increase in the risk of colorectal cancer. Because of the limitations inherent in these data, the observations cannot prove a causal link (23). By contrast, in two large prospective cohorts (Nurses' Health Study and The Health Professionals Follow-Up Study) higher prediagnostic concentrations of plasma folate not only were not associated with an increased risk of death from colorectal cancer, but predicted lower mortality rates overall (24). Another investigation has shown that the incidence of some childhood cancers has declined because folic acid fortification began in the United States (25). A randomized trial (polyp prevention study) for the prevention of recurrent colorectal adenomas in men and women with a recent history of the disease was conducted in the US post-folic acid fortification era. The findings of this study, in which participants assigned to the treatment arm received 1 mg folic acid per day, showed that

folic acid did not reduce colorectal adenoma risk, and suggested increased risk for advanced and multiple lesions in the folic acid treatment arm (26). Because of the different folate assays used (microbiological versus radio-binding) and because the folate cut points used for categorizing study participants into higher or lower folate status vary greatly between observational studies, it is not easy to draw conclusions on the folate-colorectal cancer link based on these studies. Given the important public health implications of the folic acid fortification program, it is necessary to conduct further studies to assess the effects of folic acid fortification on cancer.

Our recent and ongoing studies implement standard procedures that ensure high quality data, including consistency in the collection of blood samples (i.e., known fasting/nonfasting status) and in the assessment of folate (microbiological assays carried out within a short storage time period after blood collection), enabling us to address important questions about the effects of folic acid fortification on cervical cancer risk. The present analysis focused on prevalent CIN at the baseline, and analysis of follow-up data will allow studying both CIN incidence and recurrence of CIN after treatment of CIN lesions detected at study enrollment.

Randomized trials conducted in the pre-folic acid fortification era with 10 or 5 mg of folic acid per day showed that these doses of folic acid had no significant effects on the regression of established cervical precancer lesions (27, 28), but the results did not suggest enhancement of the progression to higher grade lesions. These studies do not provide evidence against the hypothesis that higher folate status (higher than the amount provided by the current folic acid fortification program or supraphysiologic blood concentrations) may prevent the development of cervical precancer or cervical cancer in HR-HPV-infected women who are free of preneoplastic lesions. Randomized trials conducted in individuals who are at risk for cancer but are free of preneoplastic lesions at the time of enrollment, and who are well-characterized with respect to folate intake, systemic concentrations of folate, and folate-related biomarkers will provide more definitive evidence on the effects of folic acid on carcinogenesis. A study of this nature that will address the role of folate for cervical carcinogenesis is under way, and completion is expected by 2011 (R01CA102489, PI: Piyathilake). The current report was based on women who are similar to the women screened for enrollment into the ongoing trial. Our results show that supraphysiologic concentrations of plasma folate are not associated with higher odds of CIN 2+ diagnosis at baseline, especially in the presence of sufficient vitamin B12. About 12% of the women in this study had supraphysiologic concentrations of plasma folate and sufficient vitamin B12 at entry. Women in this group were 70% less likely to be diagnosed with CIN 2+. Women with low-physiologic plasma folate concentrations and sufficient vitamin B12 were also 50% less likely to be diagnosed with CIN 2+. This may be because none of the women included in this analysis are folate deficient according to normal plasma folate range established for our laboratory in the pre-folic acid fortification era (3-10 ng/mL). In fact, 48% of women who were categorized as having low-physiologic plasma folate had concentrations between 10 and 19.8 ng/mL, and 39% had concentrations between 3 and 10 ng/mL. These results suggest that considerable protection against CIN might be afforded by higher intakes of folate or folic acid leading to

supraphysiologic concentrations of plasma folate, but that even lesser amounts would provide some protection, provided that these women have sufficient vitamin B12. Because we did not find adequate numbers of women who had supraphysiologic concentrations of plasma folate and insufficient vitamin B12, we were unable to evaluate the possibility that CIN risk may be higher in women with such a combination. The rarity of this micronutrient profile in this group of women at high risk of cervical cancer suggests that this concern may be of little public health relevance. To our knowledge, this is the first report to suggest a joint effect of folate and vitamin B12 on cervical cancer risk in the folate fortification era.

To maintain a plasma-tissue concentration gradient, monoglutamate forms of folate present in plasma (predominantly 5-methyl tetrahydrofolate) need to be converted to polyglutamates within tissues. Because 5-methyl tetrahydrofolate is a poor substrate for the synthesis of folate polyglutamates, it must be demethylated to tetrahydrofolate before being polyglutamated (29) through a vitamin B12-dependent reaction. Vitamin B12 deficiency is associated with inefficient polyglutamation of folates (30), suggesting that in the presence of sufficient vitamin B12, high folate levels may lead to efficient polyglutamation of folates. Efficient formation of biologically active forms of polyglutamates may influence many important functions of the folate pathway, which includes the methylation of DNA and histones, or DNA stability mediated by alterations in DNA damage, synthesis, or repair. Biologically active forms of polyglutamates are also critical for the removal of homocysteine (Hcy). National Health and Nutrition Examination Survey data obtained after folic acid fortification began in the United States indicate that in subjects with adequate vitamin B12, concentrations of Hcy decrease significantly as serum folate increases, but that in subjects with low serum vitamin B12, Hcy concentrations increase as serum folate increases starting at ~9 ng/mL (31). Thus, the effect of folate on Hcy is highly dependent on vitamin B12 levels.

Hcy precursor S-adenosyl-Hcy is a powerful competitive inhibitor of S-adenosylmethionine-dependent methyltransferases (32). Studies suggest that the adverse effects of hyperhomocysteinemia on the epigenetic control of gene expression can be reverted by treatment with folic acid in patients with renal disease (33). Increased plasma Hcy is associated with increased S-adenosyl-Hcy and lymphocyte DNA hypomethylation in healthy individuals (34). Individ-

uals with hyperhomocysteinemia have increased genetic damage (35), which could be due to DNA instability associated with hyperhomocysteinemia (36). Hyperhomocysteinemia has been proposed as a risk factor for cancer and as a potential tumor marker (37) because of the associated effects on DNA damage. Supplementation with folic acid and vitamin B12, more efficiently, the combination of folic acid and vitamin B12, has been shown to reduce DNA damage as indicated by lower frequency of micronuclei in peripheral leukocytes (38). An *in vitro* study documented both pro-oxidant and antioxidant effects of folic acid on lipid peroxidation, whereas vitamin B12 showed only antioxidant effects (39). These observations suggest that antioxidative properties of vitamin B12 might enhance the beneficial effects of folate on DNA. Cohort studies have shown an association between the frequency of cells with DNA damage (as indicated by the presence of micronuclei) and cancer risk (40). Thus, the reduced odds of CIN observed in our study among women with supraphysiologic concentrations of plasma folate and sufficient vitamin B12 may be due to enhanced methylation of DNA or reduced DNA damage.

In summary, our results do not corroborate the concern that the higher concentrations of circulating folate seen in the post-U.S. folic acid fortification era may increase the risk of CIN in women of childbearing age. Instead, the combination of higher folate and sufficient vitamin B12 is associated with significantly reduced odds of preneoplastic lesions of the cervix. Thus, the evidence provided by this study corroborates the hypothesis that high levels of folate and vitamin B12 in the folate fortification era exert a protective effect against cervical carcinogenesis. Confirmation of these findings in a prospective follow-up study will increase the scientific credibility of the observed associations.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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