

Genomic DNA methylation decreases in response to moderate folate depletion in elderly women¹⁻⁴

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See corresponding editorial on page 903.

ABSTRACT

Background: Methylation of genomic DNA is dependent on an adequate supply of folate coenzymes. Previous data support the hypothesis that abnormal DNA methylation plays an integral role in carcinogenesis. To date, no studies assessing the effect of inadequate folate status on DNA methylation in older women (aged >63 y) have been reported.

Objective: The effect of moderate folate depletion followed by folate repletion on leukocyte genomic DNA methylation was investigated in elderly women (aged 60–85 y) to evaluate whether DNA methylation could be used as a functional indicator of folate status.

Design: Healthy, postmenopausal women ($n = 33$) consumed a moderately folate-depleted diet (118 μg folate/d) for 7 wk, followed by 7 wk of folate repletion with 200 or 415 $\mu\text{g}/\text{d}$, each provided as 2 different dietary treatments for a total of 4 treatment groups ($n = 30$). Leukocyte DNA methylation was determined on the basis of the ability of DNA to incorporate [³H]methyl groups from labeled *S*-adenosylmethionine in an in vitro assay.

Results: Incorporation of [³H]methyl groups increased significantly ($P = 0.0025$) in response to folate depletion, suggesting undermethylation of DNA. No significant changes were detected in [³H]methyl incorporation in any group over the 7-wk repletion period compared with postdepletion values.

Conclusions: DNA methylation status may be used as a functional indicator of moderately depleted folate status. The slow response to the repletion diets observed suggests that normalization of DNA methylation after moderate folate depletion may be delayed in older women. *Am J Clin Nutr* 2000;72:998–1003.

KEY WORDS DNA methylation, genomic DNA, folate, homocysteine, humans, elderly, women

INTRODUCTION

The advent of the new dietary reference intakes (DRIs) has redefined the role of recommended micronutrient intakes and shifted the focus from prevention of nutrient deficiency to reduction of disease risk. A key component of this new approach is establishing reliable functional indicators of nutrient status that may predict disease risk before a severe nutrient deficiency ensues. The identification and use of functional indicators is also

important in the determination of nutrient intakes adequate to support key metabolic functions (1).

Folate in the form of *N*⁵-methyltetrahydrofolate is required for methylation of homocysteine to form methionine, which is modified to *S*-adenosylmethionine, a universal methyl donor in a variety of biochemical reactions, including methylation of DNA. Several studies have provided evidence that abnormal DNA methylation plays an integral role in carcinogenesis, as reviewed recently (2). Methylation of DNA plays a role in genome stability and gene expression (3–5) and may affect expression of tumor suppressor genes (6–9). Therefore, the role of folate in maintenance of normal DNA methylation has been hypothesized as one mechanism by which cancer risk may be altered (2, 10). The potential for changes in folate status to negatively influence genome integrity (11–13), including DNA methylation (14), provides an impetus for determining whether DNA methylation could be an adequate and reliable functional indicator of folate status.

Previous studies in which DNA methylation was monitored in response to folate intake or supplementation were conducted in younger individuals (aged ≤ 63 y) (14–16). Hypomethylation of human lymphocyte DNA, observed in postmenopausal women aged ≤ 63 y consuming a low-folate diet, was reversed with subsequent folate supplementation (14). Because the DRIs include further stratification of older age groups (ie, 51–70 and >70 y of age), it is important to investigate the effects of folate status on DNA methylation in older age categories. In establishing the 1989 recommended dietary allowance (RDA; 17) of folate for older women (180 $\mu\text{g}/\text{d}$), there were no age-specific data available for persons >50 y and the value was established by extrapolating data

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from younger groups. Data were insufficient to provide a basis for estimating folate DRIs (18), which include the RDA stated as 400 μg dietary folate equivalents/d, in age categories >51 y because the only controlled metabolic study conducted in women aged >51 y did not include subjects aged >63 y (14).

The purposes of this study were to investigate the effects of controlled folate intake on leukocyte genomic DNA methylation in women aged 60–85 y and to compare the relative effectiveness of either 200 [1989 RDA (17)] or 400 [1980 RDA (19)] μg folate/d in restoring genomic DNA methylation after consumption of a moderately folate-depleted diet. This study is the first investigation in subjects aged >63 y in which changes in DNA methylation were evaluated in response to controlled folate intakes and it provides new information regarding the role of marginal folate intake in reduced DNA methylation.

SUBJECTS AND METHODS

Protocol and subjects

This metabolic study was conducted by using a depletion-repletion protocol approved by the Institutional Review Board of the University of Florida. Informed consent was obtained from each subject. Thirty-three healthy, postmenopausal women (aged 60–85 y) completed the depletion phase and 30 subjects completed the entire study. Subjects were recruited from the community of Gainesville, FL, and were initially screened by phone. Subsequent screening included a personal interview, physical exam, and blood chemistry profile. Exclusion criteria included being aged <60 or >85 y, use of tobacco products, chronic use of alcohol, use of hormone replacement therapy or antifolate medications, vegetarianism, having a body weight $>120\%$ of ideal, having an abnormal blood chemistry profile, and having a chronic disease condition such as cardiovascular disease, cancer, diabetes, or hypertension. Serum folate, red blood cell (RBC) folate, and vitamin B-6 and B-12 concentrations were within normal limits for subjects at baseline (ie, ≥ 7 nmol/L, ≥ 317 nmol/L, ≥ 20 nmol/L, and ≥ 130 pmol/L, respectively), as were plasma total homocysteine (tHcy) concentrations (ie, ≤ 16 $\mu\text{mol/L}$).

For the 98-d (14-wk) study period, subjects consumed breakfast and dinner in the General Clinical Research Center located at Shands Hospital at the University of Florida. Lunch and snacks were provided for each subject to consume at home. Over-the-counter medication use was limited to ibuprofen and stool softeners. The research team was in daily contact with study subjects to ensure adherence to the diet.

Study design and diet

This investigation was conducted as 4 separate 14-wk feeding trials. Each trial period consisted of a 7-wk folate-depletion period followed by a 7-wk folate-repletion period. During the depletion phase, subjects consumed a folate-deplete diet providing an average of 118 ± 25 μg folate/d. During the repletion phase, subjects were randomly assigned to 1 of 4 dietary treatments that consisted of consumption of the depletion diet plus supplemental folate. The treatment groupings were designed to compare the effects of 2 different folate repletion intakes [ie, ≈ 200 and 400 μg folate/d] on normalization of folate status after depletion. Subjects in groups A and C consumed an average of 200 μg folate/d, whereas subjects in groups B and D consumed 415 μg folate/d. Subjects in groups

A and B received folic acid and a percentage of their total folate as orange juice (36% and 38%, respectively), whereas groups C and D received folic acid as their sole source of additional folate. Supplemental folic acid was provided in 40 mL apple juice consumed daily. The average folate content of the orange juice was 73.7 ± 4.5 μg per 240 g juice (225 mL).

The folate-depleted diet included a 5-d menu cycle comprising foods typical of a mixed diet and similar to that reported previously (20). The mean daily folate content of the 5-d menus ranged from 100 to 134 $\mu\text{g/d}$. The same diet was used throughout the study, with folate supplementation by folic acid or orange juice during the repletion phase. Except for folate, the nutrient content of the diet was estimated by using the Minnesota Nutrient Data System (version 2.7; Nutrition Coordinating Center at the University of Minnesota, Minneapolis). The diet provided an average energy intake of 7929 kJ (1895 kcal)/d (13% protein, 65% carbohydrate, and 22% fat). To provide adequate and equivalent amounts of nutrients other than folate, subjects were provided with a daily custom-formulated multivitamin supplement (Tishcon Corporation, Westbury, NY) and a separate iron supplement (providing 18 mg Fe/d; General Nutrition Center, Pittsburgh) to ensure that the intake of all other vitamins and minerals met the 1989 RDA. Subjects were weighed weekly and monitored for weight loss or gain. Subjects with a weight change that varied by $>5\%$ from baseline during any given week had their daily energy intake adjusted accordingly through the use of regular and fat- or sugar-free foods. Caffeine intake was limited to the equivalent of ≈ 180 mL (6 oz) caffeinated coffee and 355 mL (12 oz) caffeinated soda per day.

To minimize the amount of naturally occurring folate in the diet, low-folate food items, such as canned fruit and vegetables, were served. In addition, food items such as rice, chicken, potatoes, green beans, and carrots were boiled 3 times and the cooking water discarded after each boiling to help leach endogenous folate from the food (21, 22). The folate concentration of the diet was determined by using a modification of the trienzyme extraction method of Martin et al (23) and analysis by the microplate adaptation of the *Lactobacillus casei* microbiological assay (24, 25).

The folic acid supplement was prepared fresh just before the start of each 14-wk supplementation period. Folic acid (product number F-7876; Sigma Chemical Co, St Louis) was prepared in a sodium phosphate buffer solution (catalog no. 3817-01; JT Baker, Phillipsburg, NJ) at pH 7.0. The volume of folic acid solution necessary to achieve the desired supplementation was added to 40 mL apple juice contained in a 50-mL conical tube. Each supplement tube was immediately wrapped in aluminum foil and frozen at -10°C until the night before use in the study, when the apple juice supplements were thawed under refrigeration. The concentrations of the folic acid supplements were verified by using ultraviolet spectrophotometry (Beckman DU 640; Beckman Instruments, St Louis) with absorbance at 282 nm and an extinction coefficient of 27 600.

Specimen collections and analytic methods

Leukocytes were obtained at baseline and weeks 7 and 14 by using whole blood collected in tubes with EDTA (Vacutainer; Becton Dickinson, Rutherford, NJ) and centrifuged at $2000 \times g$ for 30 min at 4°C (Astel model 4237R centrifuge; ALC, Milan, Italy). At least 500 μL leukocytes was removed and mixed well with 50 μL dimethyl sulfoxide. The samples were frozen (-30°C) before further processing. DNA was extracted from



leukocyte samples by using the modified method of Sambrook et al (26). Isolated cell protein was treated with RNase for 10 min at 37°C followed by treatment with proteinase K for 1 h at 50°C. DNA was extracted in successive treatments with tris buffer-saturated phenol, phenol:chloroform:isoamyl alcohol (25:24:1, by vol), and chloroform:isoamyl alcohol (24:1 by vol). DNA was precipitated with ethanol and resuspended at 37°C in tris-EDTA, pH 7.4. The DNA was stored at -35°C before use in the methylation assay. DNA concentration was determined by ultraviolet spectrophotometry (Beckman DU 640; Beckman Instruments) with absorbance at 260 and 280 nm. All DNA samples had 260-to-280 absorbance ratios ≥ 1.7 . DNA size was estimated by using gel electrophoresis and all samples processed were found to have most of their DNA >40 kilobases in size.

DNA methylation was determined by using the modified method of Balaghi and Wagner (27), in which DNA is incubated with [³H]methyl *S*-adenosylmethionine in the presence of the *Sss*I prokaryotic methylase enzyme. The ability of DNA to incorporate [³H]methyl groups in vitro is inversely related to endogenous DNA methylation. The following reaction mixture was prepared in a 1.5-mL microtube on ice: 0.5 μ g DNA, 3 U (2 μ L) *Sss*I methylase enzyme (product no. 226L; New England Biolabs, Beverly, MA), 3 μ L [³H]methyl *S*-adenosylmethionine (399.6 GBq/mmol, 20.35 GBq/L, catalog number NET155; New England Nuclear, Boston), 3 μ L 10 \times NEB buffer (New England Biolabs), and sterile-filtered water to a total reaction volume of 30 μ L. The mixture was incubated at 30°C for 1 h and placed on ice for 5 min. Fifteen microliters was loaded onto a 2.5-cm, round, DE81 ion-exchange paper filter (catalog no. 3658325; Whatman, Maidstone, United Kingdom) and fitted on a 25-mm, 200-mL filter funnel unit (product no. 4203; Gelman Sciences, Ann Arbor, MI) attached to a vacuum source. The filter was washed successively 3 times with 15 mL each 0.5 mol phosphate buffer/L (pH 8.0), once with 2 mL 70% ethanol, and once with 2 mL 100% ethanol. The filter was dried at room temperature and placed in a liquid scintillation vial with 10 mL scintillation fluid (ScintiSafe 30%, product SX23-5; Fisher Scientific, Fair Lawn, NJ). Samples were analyzed by using a Beckman LS 6000SC (Beckman Instruments, Fullerton, CA) liquid scintillation counter. Each DNA sample was processed in duplicate and each processing run included samples for background (reaction mixture with all components except DNA), a positive control [deoxyguanine-deoxycytosine (dG-dC)-dC-dG, product no. P-9389; Sigma Chemical Co], and a negative control (hyper-methylated DNA from a pooled DNA sample). DNA extracted from a pooled whole-blood sample was used to determine the interassay and intraassay CVs (9.0% and 3.9%, respectively).

Statistical methods

Descriptive statistics in the text and tables are given as the means \pm SDs. One-way analysis of variance (ANOVA) was used to test for differences in age, weight, vitamin status, hematocrit, [³H]methyl incorporation, serum folate, and plasma tHcy concentrations between groups at baseline. To account for subject variability on entry into the study, analysis of covariance (ANCOVA) was used to evaluate group differences in [³H]methyl incorporation and serum folate and plasma tHcy concentrations at weeks 7 and 14 with adjustment for either baseline or week 7 values, respectively. Least-squares means were used to describe the magnitude of the differences between each group and were evaluated at the average covariate value (baseline for the week 7 analysis

and week 7 for the week 14 analysis). If the overall test for dietary treatment group differences was significant at $P < 0.05$, multiple pairwise comparisons within each ANCOVA were carried out by using Bonferroni-corrected P values. Correlations between [³H]methyl incorporation and other variables were examined by using Pearson correlation coefficients. A sign test for proportion of trends analysis (28) was used to compare the expected and observed combination of trends for [³H]methyl incorporation and plasma tHcy, serum folate, and RBC folate concentrations over the depletion and repletion phases.

Specifically, regression analysis was used to determine the slope of each individual's response over the specified time period. The signs of the regression slope values (positive or negative) were tallied and the observed proportion tested against the proportion expected by chance alone (ie, there are 4 possible trends: +/+, +/-, -/+, and -/-, such that by chance alone the proportion of any possible combination is 25% or 0.25). Differences were considered significant at $P < 0.05$. All statistics were computed by using SAS version 6.12 (SAS Institute, Cary, NC).

RESULTS

No significant difference was detected in mean age, weight, or hematocrit between dietary treatment groups. Significant differences in baseline serum folate, RBC folate, plasma tHcy, and vitamin B-6 and B-12 concentrations were not detected between the dietary treatment groups, and hematocrit values did not change significantly over the 14-wk study period. Detailed data and analyses related to folate and homocysteine status response (29) and the effect of methylenetetrahydrofolate reductase genotype are reported elsewhere (30).

[³H]Methyl incorporation into leukocyte DNA for all subjects by folate intake (ie, 200 or 415 μ g/d) group at baseline, week 7, and week 14 are included in **Table 1**. No significant differences were detected in baseline [³H]methyl incorporation between the dietary treatment groups. Over the depletion phase, there was a significant increase in mean [³H]methyl incorporation for all subjects (**Figure 1**) and in [³H]methyl incorporation for both the 200 and 415 μ g-folate/d groups (**Figure 2**). At week 7, no significant differences were detected in mean [³H]methyl incorporation between the folate intake groups or the dietary treatment groups (data not shown). Over the repletion phase (weeks 7-14), significant decreases in [³H]methyl incorporation were not detected overall for all subjects or for the 200- or 415- μ g folate/d groups. At week 14, no significant differences were detected in mean [³H]methyl incorporation between the folate intake groups or the dietary treatment groups (data not shown).

Over the depletion and repletion phases of the study, an inverse relation between serum folate or RBC folate concentrations and [³H]methyl incorporation was expected (eg, it was expected that serum folate concentrations would decrease and [³H]methyl incorporation would increase with folate depletion). Conversely, a direct relation between plasma tHcy concentration and [³H]methyl incorporation was expected in response to folate depletion and repletion. Over the depletion phase, an inverse relation between serum folate or RBC folate concentrations and [³H]methyl incorporation was observed in 27 of 33 subjects ($P < 0.001$, sign test for trend). A direct relation was observed between plasma tHcy concentrations and [³H]methyl incorporation in 24 of 33 subjects ($P < 0.001$, sign test for trend). In response to folate repletion, the inverse relation between serum



TABLE 1
[³H]Methyl incorporation into leukocyte DNA¹

	Baseline	Week 7	Week 14
		<i>dpm</i>	
All subjects	30392 ± 3855 [33]	33132 ± 3882 [33] ²	32532 ± 4477 [30]
Range	23829–39758	26127–42661	22976–47382
By folate intake			
200 µg/d			
Unadjusted	31132 ± 4355 [17]	34419 ± 3895 [17] ³	33873 ± 5115 [14]
Range	24327–39758	26127–42661	26574–47382
Least-squares mean	NA	34052	33505
415 µg/d			
Unadjusted	29606 ± 3190 [16]	31765 ± 3477 [16] ⁴	31360 ± 3598 [16]
Range	23829–35445	26895–37661	22976–38444
Least-squares mean	NA	32156	31681

¹Unadjusted values are $\bar{x} \pm \text{SD}$; *n* in brackets. Least-squares mean, average covariate value adjusting for baseline at week 7 and week 7 at week 14; NA, not applicable.

^{2–4}Significantly greater than baseline (ANCOVA with baseline as the covariate); ²*P* = 0.0025, ³*P* = 0.0012, ⁴*P* < 0.001.

folate and [³H]methyl incorporation was observed in 37% of subjects (*P* = 0.10, sign test for trend) whereas the direct relation between plasma tHcy concentration and [³H]methyl incorporation was observed in only 30% of subjects (*P* = 0.28, sign test for trend). For a significant proportion of subjects (53%; *P* = 0.0024, sign test for trend), an increase in both [³H]methyl incorporation and serum folate concentration was observed during the repletion phase. A significant positive correlation (*r* = 0.35, *P* = 0.043) was detected between [³H]methyl incorporation and plasma tHcy concentrations at baseline. However, no other significant correlations were observed between overall [³H]methyl incorporation and serum folate, RBC folate, or plasma tHcy concentrations at any time during the study.

DISCUSSION

Moderate folate depletion in elderly women resulted in a significant increase in overall mean [³H]methyl incorporation into leukocyte DNA, with a total of 27 of 33 subjects showing an increase at week 7 compared with baseline. This increase in [³H]methyl incorporation suggests reduced rates of DNA methylation on the basis of the inverse relation between in vitro and endogenous methylation. The decreased rate of DNA methylation observed at week 7 was coupled with serum folate concentrations indicative of inadequate folate status and significantly greater plasma tHcy concentrations relative to baseline (Table 2) (29). At week 7, 60% of subjects had a serum folate concentration considered marginally deficient (7–14 nmol/L, or 3–6 ng/mL) or deficient (<7 nmol/L, or 3 ng/mL) (29). These results are consistent with other published data (14) indicating a significant increase in [³H]methyl incorporation into the lymphocytes of postmenopausal women after a 9-wk folate-restricted diet (56 µg/d for 36 d followed by 111 µg/d for 28 d).

In response to folate repletion, significant differences in [³H]methyl incorporation were not detected at week 14 compared with week 7, suggesting that 7 wk of repletion with 200 or 415 µg folate/d did not significantly increase endogenous DNA methylation. In fact, a significant proportion of subjects continued to respond during repletion with increasing [³H]methyl incorporation even though significant changes were detected in blood folate indexes (Table 2) (29). These findings suggest that

leukocyte DNA methylation may be slow to respond to folate repletion relative to increases in blood folate concentrations. The leukocyte cell population consists primarily of neutrophils (60–70%) and lymphocytes (20–25%) and is expected to have an average turnover time of ≈14 d (31). Given this relatively short time period of cellular turnover, leukocyte DNA methylation rates may be expected to increase significantly, with a corresponding decline in [³H]methyl incorporation, after 7 wk of folate repletion. However, this was not observed in either folate intake group and suggests a delayed DNA methylation response to folate repletion. These findings suggest that a longer repletion period (ie, >7 wk) or repletion with higher amounts of folate (ie, >415 µg/d) may have been required to observe significant increases in the methylation status of hypomethylated DNA in elderly women. This is consistent with kinetic estimates of the very slow in vivo turnover of whole-body folate pools (32).

The limitations of assessing the adequacy of folate intake on the basis of changes in blood folate concentrations alone are well recognized and efforts have been focused on identifying status indicators that reflect abnormalities in function. For example,

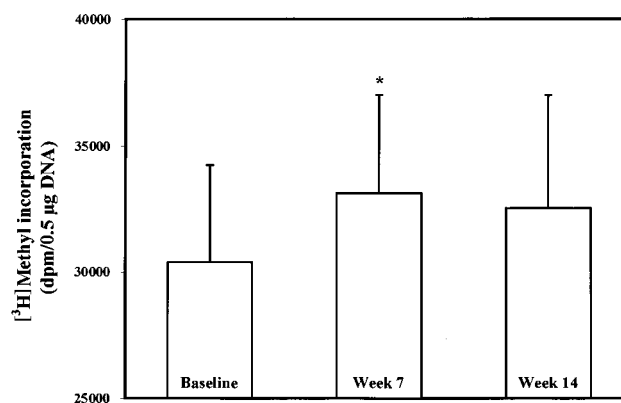


FIGURE 1. Mean (\pm SD) [³H]methyl incorporation into leukocyte DNA of all subjects at baseline, week 7 (postdepletion), and week 14 (postrepletion). *Least-squares mean [³H]methyl incorporation at week 7 significantly greater than at baseline, *P* = 0.0025 (ANCOVA).

TABLE 2
Serum folate and plasma homocysteine concentrations¹

	Baseline	Week 7	Week 14
All subjects			
Serum folate (nmol/L)	45.7 ± 27.0	13.7 ± 7.0 ²	23.3 ± 12.4 ³
Plasma homocysteine (μmol/L)	9.2 ± 2.8	11.3 ± 3.8 ⁴	10.5 ± 3.2 ⁵
By folate intake			
200 μg/d			
Serum folate (nmol/L)	38.4 ± 20.3	12.1 ± 7.2	14.0 ± 7.6
Plasma homocysteine (μmol/L)	9.4 ± 3.0	11.0 ± 3.0	11.3 ± 3.3
415 μg/d			
Serum folate (nmol/L)	53.6 ± 31.4	15.6 ± 6.5	31.4 ± 9.9 ⁶
Plasma homocysteine (μmol/L)	9.0 ± 2.7	11.6 ± 4.6	9.9 ± 3.0 ⁷

¹ $\bar{x} \pm SD$.

²Decreasing trend from baseline, $P < 0.001$ (ANCOVA with baseline as the covariate).

³Increasing trend from week 7, $P < 0.001$ (ANCOVA with week 7 as the covariate).

⁴Increasing trend from baseline, $P < 0.001$ (ANCOVA with baseline as the covariate).

⁵Decreasing trend from week 7, $P < 0.001$ (ANCOVA with week 7 as the covariate).

^{6,7}Significantly different from the 200-μg/d group (ANCOVA): ⁶ $P = 0.003$, ⁷ $P = 0.009$.

elevations in plasma homocysteine concentrations may be associated with impaired function of the methionine synthase enzymatic conversion of homocysteine to methionine, which is dependent on an adequate supply of folate coenzyme as the one-carbon donor. Because folate metabolism comprises a complex series of one-carbon transfer reactions involving numerous nutrients and regulatory mechanisms, it is not surprising that metabolic changes that occur in response to folate deficiency are also influenced by a multitude of other factors. It is important, therefore, to couple indexes of function such as DNA methylation with standard indicators of blood folate status.

The present study provides evidence that methylation of leukocyte DNA is reduced in response to inadequate folate intake and that DNA methylation status could serve as a functional indicator of folate status. Although the data from this study suggest that the DNA methylation assay is a valid approach to interpreting a functional consequence of marginal folate intake, a quantitative definition of DNA hypomethylation needs to be established. At present, there are no normal ranges established for DNA methylation, therefore, the definition of hypomethylation has not been determined. Before extrapolating changes in leukocyte DNA methylation to whole-body methylation status, it is important to determine whether the methylation status of leukocytes reflects the methylation status of DNA in other body tissues. In addition, the potential influence of the aging process on DNA methylation requires further investigation. Until these standards are established, the definition of genomic DNA hypomethylation remains to be defined.

The influence of the reduction in DNA methylation (in response to moderate folate depletion) on cancer risk is unknown. Previous studies, however, indicate that reductions in genomic DNA methylation are observed early in carcinogenesis and appear to precede the more well-described mutation and deletion events that occur later in the development of cancer (2). Large population studies are needed to establish associations between distinct levels of DNA methylation and disease risk, although the practicality of evaluating DNA methylation status in large populations is currently limited.

In summary, the results of the present study indicate that measurement of the methylation status of genomic DNA can

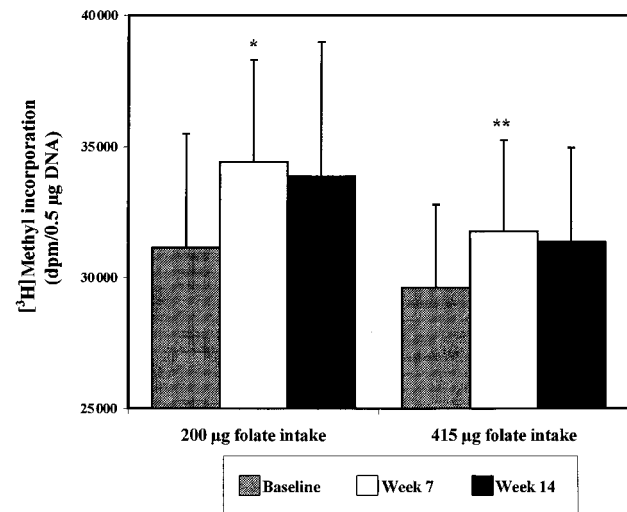



FIGURE 2. Mean (\pm SD) [³H]methyl incorporation into leukocyte DNA of the 200- and 415-μg folate/d groups at baseline, week 7 (postdepletion), and week 14 (postrepletion). **Least-squares mean [³H]methyl incorporation at week 7 significantly greater than at baseline (ANCOVA): *200 μg/d ($P = 0.0012$), **415 μg/d ($P < 0.001$).

be used as a functional indicator of inadequate folate status and may be used in conjunction with other indicators to assess adequacy of folate intake. Further studies need to be conducted to better define normal ranges of DNA methylation and thus provide a standard definition of hypomethylation. The significant reductions observed in genomic DNA methylation status in response to a moderately folate-depleted diet in this group of elderly women emphasizes the need for additional investigations to delineate the role of DNA methylation in the etiology of chronic disease. 

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