

# Folate absorption in women with a history of neural tube defect-affected pregnancy<sup>1-4</sup>

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## ABSTRACT

**Background:** The risk of neural tube defects (NTDs) is significantly reduced by supplemental folic acid. NTD risk may be associated with impaired absorption of polyglutamyl folate, the primary form of naturally occurring food folate, and of folic acid in supplements or fortified food. Stable-isotope methods provide the specificity needed to test this hypothesis.

**Objective:** We determined whether women who had an NTD-affected pregnancy had a reduced ability compared with control women to absorb polyglutamyl folate relative to folic acid.

**Design:** Healthy, nonpregnant women with a history of an NTD-affected pregnancy (cases;  $n = 11$ ) and control women ( $n = 11$ ) were administered an oral dose containing a mixture of [<sup>2</sup>H]pteroylpentaglutamate ([<sup>2</sup>H<sub>2</sub>]PteGlu<sub>5</sub>; 233 nmol) and [<sup>13</sup>C]pteroylmonoglutamate ([<sup>13</sup>C<sub>5</sub>]PteGlu<sub>1</sub>; 567 nmol) after a 30-d saturation protocol (2 mg unlabeled folic acid/d). Relative extents of absorption were evaluated by urinary excretion of <sup>2</sup>H<sub>2</sub>- and <sup>13</sup>C<sub>5</sub>-labeled folates 48 h postdose.

**Results:** During the first 24 h postdose, cases excreted less ( $\bar{x} \pm SD$ ) [<sup>2</sup>H<sub>2</sub>]PteGlu<sub>5</sub> ( $21 \pm 12\%$  compared with  $37 \pm 19\%$ ;  $P = 0.01$ ) and [<sup>13</sup>C<sub>5</sub>]PteGlu<sub>1</sub> ( $17 \pm 8\%$  compared with  $31 \pm 14\%$ ;  $P = 0.007$ ) than did controls. No significant differences between cases and controls were detected in the percentage of [<sup>2</sup>H<sub>2</sub>]PteGlu<sub>5</sub> or [<sup>13</sup>C<sub>5</sub>]PteGlu<sub>1</sub> excreted during the second 24 h postdose or when the data were averaged over 48 h. However, excretion of the [<sup>2</sup>H<sub>2</sub>]folates tended to be lower in cases than in controls over the 48-h period ( $33 \pm 13\%$  compared with  $45 \pm 26\%$ ;  $P = 0.21$ ). A similar trend ( $P = 0.29$ ) for lower excretion of [<sup>13</sup>C<sub>5</sub>]folates in cases was also observed ( $31 \pm 16\%$  compared with  $39 \pm 17\%$ ). The ratio of urinary [<sup>2</sup>H<sub>2</sub>]folates to [<sup>13</sup>C<sub>5</sub>]folates did not differ significantly between cases and controls.

**Conclusion:** These data suggest the need for a larger-scale study using stable-isotope methods to further investigate this hypothesis. *Am J Clin Nutr* 2000;72:154-8.

**KEY WORDS** Folate, neural tube defect, absorption, bio-availability, stable isotopes, women

## INTRODUCTION

Neural tube defects (NTDs) are birth defects occurring in pregnancies of genetically predisposed women in which the fetal neural

tube fails to form properly during early embryonic development (1). NTD risk reduction has definitively been linked to periconceptional consumption of folic acid, a monoglutamyl form of folate used in supplements and fortified foods (1). In contrast with the well-established protective effect of supplemental folic acid, data are insufficient to conclude that increased intake of naturally occurring food folate, which is predominantly in the polyglutamate form, is associated with NTD risk reduction (1-3). The significant reduction in NTD risk in response to supplemental folic acid taken in addition to diet has led to the hypothesis that increased folic acid intake may overcome an unidentified defect in folate utilization (1, 4-9).

The proposed defect investigated in the present study is the malabsorption of food folate, which requires cleavage of the polyglutamyl side chain by jejunal brush border pteroyl-poly- $\gamma$ -glutamate hydrolase (folate conjugase, or  $\gamma$ -Glu-X carboxypeptidase) before active transport of monoglutamyl folate (10). A reduction in conjugase activity, therefore, may reduce the absorption efficiency of food folate without affecting folic acid absorption. In addition, it is proposed that the efficiency of absorption of folic acid may also be reduced in women with NTD-affected pregnancies.

Other investigators have evaluated the hypothesis that absorption of folate is impaired in women with an NTD-affected pregnancy (8, 11-13). Protocols used previously evaluated this question by comparing the serum folate response to food or a folic acid supplement in subjects with NTD-affected pregnancies (cases) relative to that in controls (8, 11-13). Interpretation of these studies is complicated by some of the methodologic approaches used, including differences in the folate status of cases and controls (8, 12, 13), nonphysiologically large test doses (8, 12, 13), and no quantitation of the food folate provided in doses (8, 12, 13).

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In the present study, we used a protocol developed and used repeatedly by our research group (14–19) that was designed to overcome the limitations of previous folate absorption protocols. In an earlier investigation (19), we evaluated the absorption of folic acid alone in both cases and controls and found no significant difference, although there was a trend for lower absorption in cases than in controls. The primary objective of the present study was to determine whether women at risk for an NTD-affected pregnancy have a reduced ability to absorb food folate and folic acid compared with women with normal pregnancy histories. Because the polyglutamyl form of folate is the predominant form that occurs naturally in foods, the inclusion of labeled polyglutamyl folate in this protocol provides new information on potential differences in response to food folate compared with folic acid in women at risk of an NTD-affected pregnancy.

## SUBJECTS AND METHODS

### Subjects

Twenty-two white, nonpregnant women residing in the vicinity of Gainesville, FL, were recruited to participate in the study. Eleven women ( $\bar{x} \pm SD$  age:  $26.5 \pm 5.4$  y) with a history of one NTD-affected pregnancy [spina bifida ( $n = 10$ ) or anencephaly ( $n = 1$ )] were recruited primarily from among mothers of infants who were patients at the Shands Hospital Spina Bifida Clinic in Gainesville to serve as cases. Eleven nonpregnant women (aged  $27.2 \pm 5.0$  y) who had no history of an NTD-affected pregnancy served as controls. The number of previous pregnancies and the period of time since a previous pregnancy did not differ significantly between cases and controls, and subjects were selected on the basis of similarity of socioeconomic backgrounds.

All participants were healthy according to the following criteria: normal serum and red blood cell (RBC) folate concentrations ( $>6.8$  nmol/L and  $>317$  nmol/L, respectively), normal blood chemistry profile, no history of chronic disease or alcohol use, nonsmoking, and not taking medication known to interfere with folate metabolism. In addition, all participants were screened for the following: inadequate vitamin B-12 status (serum vitamin B-12  $<200$  pmol/L and plasma methylmalonic acid concentrations  $>270$  nmol/L), elevated plasma homocysteine concentrations ( $>14$   $\mu$ mol/L), and presence of the C-to-T substitution at nucleotide 677 (C677T) in the methylenetetrahydrofolate reductase gene (*MTHFR*). All participants had normal vitamin B-12 status and plasma homocysteine concentrations and none of the participants were homozygous for the C677T *MTHFR* genotype. No significant differences ( $P \geq 0.05$ ) in dietary intakes for any nutrient were detected between cases and controls according to a 4-d diet record computed by THE FOOD PROCESSOR II (version 7.0; Nutrient Analysis System, ESHA Research, Salem, OR). Participants abstained from all alcohol, medications, and vitamin-mineral supplements during the entire study. The protocol was approved by the University of Florida Institutional Review Board and each subject signed informed consent forms.

### Study protocol

A mixture of folic acid and polyglutamyl folate labeled with 2 different stable isotopes ( $^2\text{H}$  and  $^{13}\text{C}$ ) was given to both cases and controls. The relative absorption of the 2 forms of folate was estimated by comparing the ratio of the 2 labeled folates in urine, and absolute absorption was estimated by calculating the percentage of each labeled folate that was excreted.

Cases and controls underwent a saturation protocol before the absorption test that involved consumption of 2 mg folic acid provided as a 1-mg supplement given twice daily for 30 d. Compliance with the supplementation protocol was confirmed by monitoring serum folate concentrations.

On day 31 at 0800, baseline fasting blood samples were drawn from subjects, who were then given an oral bolus dose containing a mixture of [ $^2\text{H}$ ]pteroylpentaglutamate ([ $^2\text{H}_2$ ]PteGlu $_5$ ; 233 nmol) and [ $^{13}\text{C}$ ]pteroylmonoglutamate ([ $^{13}\text{C}_5$ ]PteGlu $_1$ ; 567 nmol) in 50 mL apple juice. [ $^{13}\text{C}_5$ ]Folic acid was synthesized by coupling pteric acid and [ $^{13}\text{C}_5$ ]glutamic acid (20). To prepare the polyglutamyl folate we coupled [ $^2\text{H}_2$ ]ptericoic acid, prepared as described previously (14), with a  $\gamma$ -linked polyglutamate peptide via solution-phase chemistry (20). The subjects fasted for an additional 2 h after consumption of the test dose to prevent any interference of food with folate absorption. Twenty-four-hour urine collections were obtained for 2 d after the dose (days 31–33). Each 24-h urine sample was collected in amber jugs containing ascorbic acid, was refrigerated, and was then frozen until analyzed for total and labeled folates.

### Sample analysis

Quantitation of urinary [ $^2\text{H}_2$ ]folate and [ $^{13}\text{C}_5$ ]folate was performed after isolation of the labeled compounds by affinity chromatography, chemical cleavage of the C-9–N-10 bond, isolation of the *p*-aminobenzoylglutamate fragment by HPLC, and derivatization before gas chromatography–mass spectrometry (GC-MS) analysis as described previously (21). Absorption was estimated by measuring excretion of [ $^2\text{H}_2$ ]folates and [ $^{13}\text{C}_5$ ]folates derived from [ $^2\text{H}_2$ ]PteGlu $_5$  and [ $^{13}\text{C}_5$ ]PteGlu $_1$ , respectively.

Folate concentrations in urine, serum, and RBCs were measured by the microplate adaptation of the *Lactobacillus casei* microbiological assay (22). Serum vitamin B-12 concentrations were measured by radioligand assay (Quantaphase II; BioRad, Hercules, CA). Total plasma homocysteine concentrations were measured by using a modified HPLC method with fluorescence detection (23). Serum methylmalonic acid concentrations were measured by GC-MS (Metabolite Laboratories, Denver). The presence of the C677T *MTHFR* genotype was determined by using a modification of the method of Frosst et al (24).

### Statistical analysis

The recovery of isotopically labeled folates was expressed as a percentage of the administered dose of labeled folates. The absorption of [ $^2\text{H}_2$ ]folic acid relative to [ $^{13}\text{C}_5$ ]polyglutamyl folate was estimated by determining the ratio of the respective labeled urinary folates. An independent sample *t* test was used to compare pre- and postsupplementation serum folate and RBC folate concentrations and other blood indexes between cases and controls. The difference in the change in serum and RBC folate concentrations after supplementation between cases and controls was also compared by using *t* tests. Additionally, differences in concentrations of excreted labeled compounds and total folate urinary recoveries and isotope ratios between cases and controls were compared by using *t* tests. Analysis of covariance (ANCOVA) was also performed to test for differences in percentage recovery between cases and controls with total urinary folate as the covariate. Statistics were computed by using SAS (version 6.12; SAS Institute Inc, Cary, NC). *P* values  $<0.05$  were considered significant. Results are reported as means  $\pm$  SDs unless noted otherwise.

**TABLE 1**Serum and red blood cell folate concentrations and urinary folate excretion<sup>1</sup>

	Cases (n = 11)	Controls (n = 11)
Serum folate (nmol/L)		
Presupplementation	93 ± 45	95 ± 50
Postsupplementation	168 ± 36 <sup>2</sup>	188 ± 66 <sup>2</sup>
Difference (post - pre)	33 ± 13	42 ± 28
Red blood cell folate (nmol/L)		
Presupplementation	2719 ± 777	2784 ± 886
Postsupplementation	3365 ± 682 <sup>3</sup>	3252 ± 909 <sup>3</sup>
Difference (post - pre)	285 ± 235	202 ± 201

<sup>1</sup> $\bar{x} \pm SD$ . There were no significant differences between cases and controls.

<sup>2,3</sup>Significantly different from presupplementation: <sup>2</sup> $P = 0.0001$ , <sup>3</sup> $P = 0.01$ .

## RESULTS

We found no significant differences between cases and controls in serum or RBC folate concentrations before or after the 30-d supplementation period (**Table 1**). Serum and RBC folate concentrations in both cases and controls were significantly higher after the 30-d supplementation period than before supplementation. There were no significant differences in the change in serum and RBC folate concentrations between cases and controls in response to the 30-d saturation period.

Total urinary folate excretion for the 48-h postdose period was not significantly different in cases (6400 ± 2519 nmol/d) and controls (6967 ± 3324 nmol/d). The large quantity of urinary folate excreted postdose reflects the saturation protocol (2 mg/d) coupled with dietary folate, which included foods fortified with folic acid. The high presupplementation blood and correspondingly high urinary folate concentrations were likely influenced by vitamin supplements taken by subjects before the study.

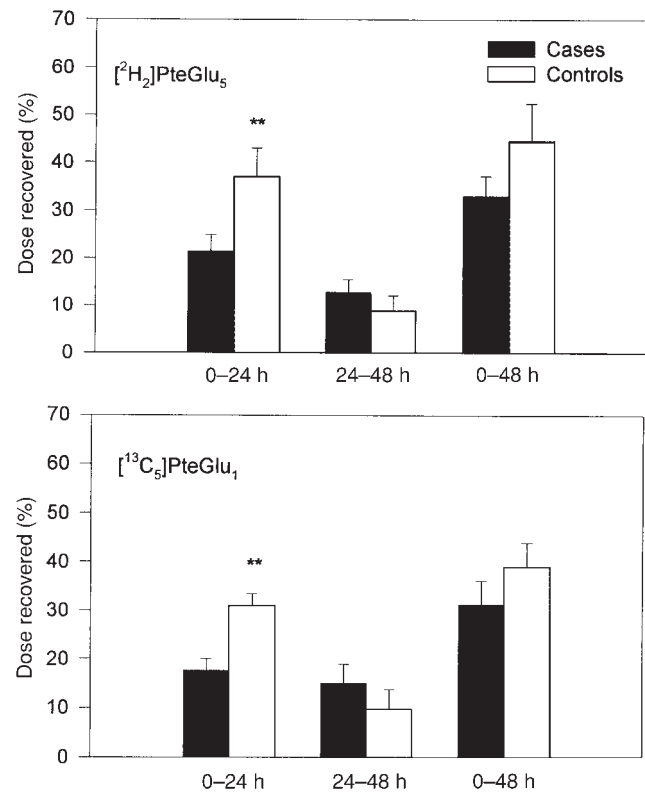
During the first 24 h postdose, cases excreted significantly less [<sup>2</sup>H<sub>2</sub>]folates derived from PteGlu<sub>5</sub> (21 ± 12% compared with 37 ± 19%;  $P = 0.01$ ) and significantly less [<sup>13</sup>C<sub>5</sub>]folates derived from PteGlu<sub>1</sub> (17 ± 8% compared with 31 ± 14%;  $P = 0.007$ ) than did controls (**Figure 1**). No significant difference was detected in the percentage dose excreted of either [<sup>2</sup>H<sub>2</sub>]PteGlu<sub>5</sub> or [<sup>13</sup>C<sub>5</sub>]PteGlu<sub>1</sub> between cases and controls during the second 24-h postdose period or when the data were averaged over 48 h. However, a nonsignificant trend ( $P = 0.21$ ) for lower excretion of the oral dose of [<sup>2</sup>H<sub>2</sub>]PteGlu<sub>5</sub> by the cases than by the controls (33 ± 13% compared with 45 ± 26%, respectively) was observed over the 48-h period. A similar trend ( $P = 0.29$ ) for lower excretion of [<sup>13</sup>C<sub>5</sub>]PteGlu<sub>1</sub> by the cases was also observed (31 ± 16% compared with 39 ± 17%).

The ratio of urinary [<sup>2</sup>H<sub>2</sub>]folates to [<sup>13</sup>C<sub>5</sub>]folates did not differ significantly between cases (1.17 ± 0.32) and controls (1.12 ± 0.21). The comparable bioavailability of polyglutamyl folate relative to the monoglutamyl form agrees with the results of a previous study in which the protocol involved giving the 2 synthetic forms alone in aqueous solution with a folic acid saturation model similar to that used in the present study (16). The percentage of oral dose excreted was not influenced by total folate excretion (ANCOVA). These data indicate no significant difference in the absorption of pteroylpentaglutamate relative to folic acid between cases and

controls. The fact that none of the subjects were homozygous for the C677T *MTHFR* genotype prevented an evaluation of the potential influence of *MTHFR* genotype on folate utilization in this study (5).

## DISCUSSION

The mechanism or mechanisms by which folic acid reduces NTD risk is not known. Increasing folic acid intake, and thus the concentrations of folate derivatives in tissues, might overcome a deficiency in the production of methionine, *S*-adenosylmethionine, or nucleotides near the time of neural tube closure (1). NTD risk reduction has been observed in studies in which supplemental folic acid was taken in addition to a normal dietary intake; therefore, it is hypothesized that supplemental folic acid may overcome a metabolic defect that impairs the absorption of either food folate or folic acid. It is logical to assume that folate from food would be as effective as folic acid after absorption because once polyglutamyl folate has been deconjugated in the jejunal brush border, the monoglutamate produced (predominately 5-methyl-tetrahydrofolate)



**FIGURE 1.** Mean ( $\pm$ SEM) percentage of the dose of [<sup>2</sup>H<sub>2</sub>]pteroylpentaglutamate ([<sup>2</sup>H<sub>2</sub>]PteGlu<sub>5</sub>) and [<sup>13</sup>C<sub>5</sub>]pteroylmonoglutamate ([<sup>13</sup>C<sub>5</sub>]PteGlu<sub>1</sub>) excreted by cases and controls over the 0-24-, 24-48-, and 0-48-h postdose periods. \*\*Significantly different from cases,  $P = 0.01$  ([<sup>2</sup>H<sub>2</sub>]PteGlu<sub>5</sub>) and  $P = 0.007$  ([<sup>13</sup>C<sub>5</sub>]PteGlu<sub>1</sub>). There were no significant differences between cases and controls over the 24-48-h period. For the 0-48-h postdose period, excretion of [<sup>2</sup>H<sub>2</sub>]PteGlu<sub>5</sub> and [<sup>13</sup>C<sub>5</sub>]PteGlu<sub>1</sub> tended to be lower by the cases than by the controls ( $P = 0.21$  and  $P = 0.29$ , respectively). Also, cases excreted  $\approx 25\%$  less [<sup>2</sup>H<sub>2</sub>]PteGlu<sub>5</sub> and  $\approx 20\%$  less [<sup>13</sup>C<sub>5</sub>]PteGlu<sub>1</sub> in the 0-48-h postdose period than did controls.

is the same as that derived from folic acid (10). Once absorbed, the metabolic utilization of folate is identical regardless of whether it was derived from supplements or from naturally occurring food folate. The objective of this study was to determine whether women at risk of an NTD-affected pregnancy had a reduced ability to absorb either polyglutamyl folate or folic acid compared with women with normal pregnancy histories.


The availability of stable-isotope-labeled polyglutamyl folate and folic acid and experience with their use in studies of folate absorption provided our research group with the opportunity to assess the relative absorption of both forms of folates in women with NTD-affected pregnancies. Previous investigations of the hypothesis that folate absorption may be impaired in women at risk of an NTD-affected pregnancy resulted in equivocal results (8, 11–13). The protocol used in our investigation enabled a direct assessment of potential differences in intestinal deconjugation and uptake of mono- and polyglutamyl folate through the use of highly specific isotopic procedures not subject to the ambiguities of previous studies. For example, differences observed by previous investigators (8, 11–13) in serum folate response curves between cases and controls may have resulted from either decreased absorption or increased tissue uptake of folate in the test dose because subjects were not given supplemental folic acid to normalize their folate status before the absorption test. In such studies, the conclusion that the absorption of folate was impaired in the cases compared with that in the controls may have been influenced by differences in folate blood concentrations, reported to be lower in cases than in controls.

In the present study, no significant differences in the absorption of either polyglutamyl folate or folic acid were detected between cases and controls; however, a trend for lower absorption of both forms of folates in cases was observed. These findings suggest that there is a trend for these women to absorb folic acid from supplements or fortified foods and naturally occurring food folate somewhat less efficiently than do women who are not at risk of an NTD-affected pregnancy. We observed a similar trend for a difference in absorption of folic acid in our previous study (18).

Our findings agree with those of Neuhouser et al (13), who estimated the relative absorption of folic acid in supplements and of food folate provided as orange juice in cases and controls. With use of the area under the curve (AUC) as a measure of response to the folate test doses, the controls had a nonsignificantly larger response to the oral challenge with the pteroylpolyglutamic (orange juice) than did the cases (13). The AUC in response to folic acid was significantly higher in controls than in cases (13).

The data from the present study in conjunction with previous findings (13, 18) suggest that a difference in folate absorption between cases and controls may exist; further investigation in a larger-scale study using the stable-isotope method is warranted to confirm these findings. The magnitude of this potential difference appears to be in the range of 20–25%, which may result in a physiologically significant difference in folate status in situations in which folate intake is limited. These findings do not rule out the influence of multiple metabolic abnormalities affecting folate metabolism in at-risk women. Because multiple enzymes and tissue-specific proteins are required for normal folate absorption, transport, and storage, abnormalities in intestinal absorption may coexist with other metabolic defects.

In summary, data from the present investigation suggest that absorption of folate present as naturally occurring food folate and of folic acid in fortified foods, enriched cereal-grain prod-

ucts, or supplements may be somewhat impaired in women at risk of NTD-affected pregnancies. These findings suggest that reduced absorption of both folic acid and naturally occurring food folate may partially explain why some women have a higher risk of NTD-affected pregnancy. The metabolic basis for the dramatic reduction in NTD risk in response to increased folic acid in addition to diet will continue to be the subject of intense investigation. 

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