

Bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid in subjects with different genotypes of the glutamate carboxypeptidase II gene¹⁻³

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ABSTRACT

Background: Before dietary folate is absorbed, polyglutamate folates are deconjugated to monoglutamates by folylpoly- γ -glutamyl carboxypeptidase in the small intestine. The *1561T* allele of the glutamate carboxypeptidase II gene (*GCPII*), which codes for folylpoly- γ -glutamyl carboxypeptidase, may impair intestinal absorption of dietary folates.

Objective: Our aim was to study the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid across *GCPII* 1561 genotypes.

Design: In a randomized study, 180 healthy adults aged 50–75 y received 323 nmol monoglutamyl folic acid/d ($n = 59$), 262 nmol heptaglutamyl folic acid/d ($n = 61$), or placebo ($n = 60$) for 12 wk. Genotypes were assessed after the intervention. The bioavailability of heptaglutamyl folic acid relative to that of monoglutamyl folic acid was calculated by using the changes in serum folate concentration in the treatment groups, after correction for changes in the placebo group and for the administered dose.

Results: No subjects with the *TT* genotype were encountered. At baseline, serum and erythrocyte folate concentrations were higher ($P < 0.05$) in subjects with the *CT* genotype [16.3 nmol/L (geometric \bar{x} ; 95% CI: 13.7, 19.3 nmol/L) and 863 nmol/L (735, 1012 nmol/L), respectively; $n = 19$] than in subjects with the *CC* genotype [13.7 (13.1, 14.3) and 685 (652, 721) nmol/L, respectively; $n = 161$]. Baseline homocysteine concentrations were not significantly different between genotypes. The bioavailability of heptaglutamyl folic acid relative to that of monoglutamyl folic acid was not significantly different between subjects with the *CC* (64%; 52%, 76%) and *CT* genotypes (70%; 49%, 91%).

Conclusions: The *1561T* allele of the *GCPII* gene does not impair the bioavailability of polyglutamyl folic acid. However, the allele is associated with higher folate status. This association may be explained by yet unidentified factors controlling the expression of the *GCPII* gene. *Am J Clin Nutr* 2004;80:700–4.

KEY WORDS Folate bioavailability, polyglutamyl folic acid, serum folate, erythrocyte folate, plasma homocysteine, glutamate carboxypeptidase II gene 1561C→T polymorphism, genetics

INTRODUCTION

The B vitamin folate is essential in the human diet. Folate deficiency or suboptimal folate intake is associated with various pathologies, such as anemia (1), neural tube defects (2–4), hyperhomocysteinemia and cardiovascular disease (5–10), cancer

(11), and neurocognitive dysfunction (12–14). In general, *folic acid* refers to the oxidized forms, which are present in supplements and fortified foods, whereas *folate* refers to the reduced forms, which are present in the diet and in biological tissues.

Folate bioavailability from food may be only 50% (15). *Bioavailability* is defined as the proportion of an ingested nutrient that is absorbed and becomes available for use and storage in the body. Several studies found that impaired folate bioavailability may be caused by conjugation of folate to a polyglutamate chain (16–22), which is the case for about two-thirds of dietary folate (23). Polyglutamate folate requires enzymatic deconjugation to the monoglutamate form before it can be absorbed, whereas monoglutamate folate does not require such deconjugation. Incomplete deconjugation of polyglutamate folate could lead to lower bioavailability.

The enzyme folylpoly- γ -glutamyl carboxypeptidase (FGCP; EC 3.4.19.9), which is encoded by the glutamate carboxypeptidase II gene (*GCPII*) and is present in the brush border of the human small intestine, is responsible for the deconjugation of polyglutamate folate (24). In 2000 a 1561C→T polymorphism in this gene was reported (25). In *in vitro* experiments, FGCP activity in transfected COS-7 cells expressing the H475Y mutagenized *GCPII* complementary DNA, which is comparable to the *1561TT* genotype in humans, was 50% lower than that in transfected cells expressing the wild-type *GCPII* FGCP enzyme. Furthermore, in 75 healthy human subjects, those with the *CT* genotype ($n = 6$) had significantly lower serum folate and significantly higher plasma homocysteine concentrations than did those with the *CC* genotype.

In the present study, we aimed to assess the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic

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acid in subjects with different *GCP11* 1561 genotypes. Our hypothesis was that the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid in subjects with the *CT* genotype would be impaired in comparison with subjects with the *CC* genotype.

SUBJECTS AND METHODS

Subjects

The present study was conducted within a 12-wk, randomized, double-blind, placebo-controlled trial in which we studied the bioavailability of heptaglutamyl folic acid relative to that of monoglutamyl folic acid in 180 healthy subjects. The study protocol was approved by the Medical Ethical Committee of Wageningen University, Wageningen, Netherlands. Details on the recruitment of subjects and the methodologic issues were reported previously (22) and are therefore only briefly summarized here. The subjects were older adults aged 50–75 y. They were screened on the basis of a medical questionnaire and had to have a vitamin B-12 concentration >130 pmol/L, a serum creatinine concentration <125 μ mol/L, and a plasma homocysteine concentration <26 μ mol/L. The subjects had no previous history of cardiovascular diseases and did not suffer from chronic diseases, such as cancer, renal disease, or gastrointestinal disorders. The use of drugs that are known to interfere with folate and homocysteine metabolism, such as antiepileptics, and the use of B vitamin supplements were also reasons for exclusion. All the subjects were informed in writing and orally about the study and gave their written consent.

Study design

After a screening visit, the subjects started with a run-in period of 5 wk in which they took a placebo capsule once per day. This gave the subjects the opportunity to get used to the intervention practice, while in the meantime, their blood samples were analyzed for the screening criteria. If all the inclusion criteria were met, the subjects returned to the research center for baseline measurements. At that time, blood was drawn and anthropometric measurements were made. The subjects were divided into 3 groups that were stratified for plasma homocysteine concentrations at the screening visit. One group received 323 nmol monoglutamyl folic acid/d, the second group received 262 nmol heptaglutamyl folic acid/d, and the third group received placebo capsules. Blood samples were drawn after 12 wk of intervention. All blood collections took place after an overnight fast.

The subjects were asked to refrain from consuming liver and yeast products during the whole study because of their high folate content. Moreover, consumption of liver products was not allowed during 3 d before the blood collections. Habitual folate intake was determined by using a food-frequency questionnaire that was filled out by the participants in the second week of the intervention.

Capsules

Monoglutamyl folic acid (Merck & Co, Inc, Whitehouse Station, NJ) and heptaglutamyl folic acid as the ammonium salt (Schircks; Jona, Switzerland) were obtained (reported chemical purities of >98%). Identical capsules containing monoglutamyl folic acid or polyglutamyl folic acid, with a target amount of 450 nmol/capsule, and placebo capsules were produced manually

(Pharmacy of the Gelderse Vallei Hospital, Ede, Netherlands). The folic acid content of the capsules was determined by using HPLC with ultraviolet detection (26). Polyglutamyl folic acid was hydrolyzed to monoglutamyl folic acid by incubation with rat plasma before injection into the high-pressure liquid chromatograph. Six batches of each type of capsule were analyzed, and each batch consisted of 20 randomly chosen capsules. The folic acid contents expressed as nmol/capsule were as follows: placebo capsules, 0 (range: 0–0); monoglutamyl folic acid capsules, 323 (219–373); and polyglutamyl folic acid capsules, 262 (249–297). Thus, the actual contents of the monoglutamyl folic acid and polyglutamyl folic acid capsules were 71% and 58% of the targeted dose (450 nmol/capsule), respectively. CVs for the folic acid content in the capsule batches did not exceed 6%. Post hoc analysis of the heptaglutamyl folic acid raw material showed that its purity was not 98% as reported, but only \approx 70%, which largely explains the lower content of the polyglutamyl folic acid capsules (22).

Biochemical measurements

Blood samples intended for analysis of plasma homocysteine concentrations were immediately placed on ice and centrifuged at $2600 \times g$ for 10 min within 0.5 h of venipuncture. Homocysteine concentrations in the plasma samples were measured with the use of an HPLC method with fluorescence detection at the laboratory of the Division of Human Nutrition, Wageningen University, Wageningen, Netherlands (intraassay CV: 2%) (27, 28). Separate blood samples were collected in serum separator tubes and centrifuged ($2600 \times g$, 10 min, 4 °C) for determination of serum folate concentrations. A third set of blood samples was used for the analysis of folate concentrations in erythrocytes. For this analysis, after assessment of hematocrit values, whole blood samples were diluted with 4 volumes of ascorbic acid (10 g/L) and, before analysis, were further diluted with Immulite 2000 diluent (Diagnostic Products Company, Los Angeles). Serum and red blood cell folate concentrations were measured with the use of a chemiluminescent immunoassay analyzer (Immulite 2000; Diagnostic Products Company) at the clinical laboratory of the University Medical Centre St Radboud, Nijmegen, Netherlands. The intraassay CVs were <5% for serum folate and <14% for red blood cell folate.

Genotyping

The *GCP11* genotypes were determined in DNA isolated from whole blood samples. The 1561C→T transition creates an *AccI* site, and genotyping was performed with the use of the polymerase chain reaction (PCR) and subsequent restriction enzyme analysis with *AccI*. PCR was performed in a total volume of 50 μ L containing 50 ng of the forward primer 5'-CATTCTGGTAGGAATTTAGCA-3', 50 ng of the reverse primer 5'-AAACACCACCTATGTTTAACA-3', 200 μ mol of each dNTP/L, 10 mmol Tris-HCl/L (pH 8.3), 50 mmol KCl/L, 1 mmol MgCl₂/L, and 1 unit *Taq* polymerase (Life Technologies, Rockville, MD). The PCR conditions were as follows: an initial denaturation step of 3 min at 92 °C; 35 cycles of 92 °C for 30 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 30 s (extension); and a final extension of 7 min at 72 °C. The amplified PCR fragment of 244 base pairs was digested with the restriction enzyme *AccI*, which was followed by gel electrophoretic analysis on a 2% agarose gel. After restriction enzyme

TABLE 1

Serum folate, erythrocyte folate, and plasma homocysteine concentrations at baseline by glutamate carboxypeptidase II gene (*GCP11*) 1561 genotype¹

	CC genotype (n = 161)	CT genotype (n = 19)	P ²
Serum folate (nmol/L)	13.7 (13.1, 14.3)	16.3 (13.7, 19.3)	0.05
Erythrocyte folate (nmol/L)	685 (652, 721)	863 (735, 1012)	0.01
Plasma homocysteine (μmol/L)	10.4 (10.1, 10.7)	10.2 (9.6, 10.7)	0.38

¹ All values are geometric \bar{x} ; 95% CI in parentheses.

² Student's *t* test.

analysis, the *1561TT* genotype results in 2 fragments of 141 and 103 base pairs, respectively, whereas the *1561CC* genotype produces only a fragment of 244 base pairs (25).

Calculations and statistics

Geometric mean folate concentrations in serum and erythrocytes and geometric mean plasma homocysteine concentrations were calculated at baseline for each *GCP11* 1561 genotype. Cross-sectional differences between genotypes were tested by using Student's *t* test. Changes in folate status after 12 wk of intervention were calculated, and differences between groups and genotypes were tested for statistical significance by using two-factor analysis of variance with baseline value as a covariate and Tukey's test to correct for multiple comparisons. The statistical significance level was set at $\alpha = 0.05$. All statistical analyses were performed with SAS version 8.2 (SAS Institute Inc, Cary, NC). The bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid was calculated for each genotype as follows:

$$\text{Relative bioavailability} = (\Delta[C]_{\text{poly}} - \Delta[C]_{\text{placebo}}) / (\Delta[C]_{\text{mono}} - \Delta[C]_{\text{placebo}}) \times (\text{dosage}_{\text{mono}} / \text{dosage}_{\text{poly}}) \quad (1)$$

In this equation, $\Delta[C]$ stands for the change in serum or erythrocyte folate concentration after 12 wk.

RESULTS

At baseline, the 3 study groups did not differ significantly from one another in age, body mass index, serum vitamin B-12 concentrations, serum creatinine concentrations, or plasma homocysteine concentrations, as previously described (22). Habitual folate intake, as determined by using the food-frequency questionnaire, did not differ significantly between the 3 groups. Of the 180 subjects, 161 (89%) had the *GCP11* 1561CC genotype, and 19 (11%) had the CT genotype. No subjects with the homozygous TT genotype were found in this study population. At baseline, serum and erythrocyte folate concentrations in the subjects with the CT genotype were 19% and 26% higher, respectively, than those in the subjects with the CC genotype. Plasma homocysteine concentrations did not differ significantly between genotypes at baseline (Table 1).

Changes in serum folate, erythrocyte folate, and plasma homocysteine concentrations in subjects with the CC and CT genotypes after 12 wk of intervention with either placebo, 323 nmol monoglutamyl folic acid/d, or 262 nmol heptaglutamyl folic

acid/d are shown in Table 2. The interaction between treatment group and genotype was significant only for erythrocyte folate concentrations. For serum folate, the responses to polyglutamyl folic acid were significantly smaller than those to monoglutamyl folic acid. Although there was a significant interaction between treatment group and genotype for erythrocyte folate concentrations, there was no significant difference between the subjects with the CT genotype and those with the CC genotype for a given treatment, probably because of the small numbers.

On the basis of serum folate concentrations, the bioavailability of heptaglutamyl folic acid relative to that of monoglutamyl folic acid was not significantly different between the subjects with the CC genotype and those with the CT genotype [64% (95% CI: 52%, 76%) and 70% (95% CI: 49%, 91%), respectively]. On the basis of erythrocyte folate concentrations, the relative bioavailability of heptaglutamyl folic acid was 72% (95% CI: 42%, 103%) for the subjects with the CC genotype. Because of the significantly different erythrocyte folate concentrations between the 2 treatment groups before the intervention in the subjects with the CT genotype, we did not calculate bioavailability on the basis of erythrocyte folate concentrations among these subjects.

DISCUSSION

We found that the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid is ≈ 65 –70% and does not differ significantly between subjects with the *GCP11* 1561CT genotype and those with the CC genotype. However, baseline and 12-wk folate concentrations in serum and erythrocytes in the subjects with the CT genotype were 20–25% higher than those in subjects with the CC genotype. Plasma homocysteine concentrations did not differ significantly between genotypes. These findings are in accordance with published data from other cross-sectional studies, in which the *1561T* allele of the *GCP11* gene was associated with higher folate concentrations (29–31). One American study (32) did not find such an association, which may be attributed to higher folate intakes due to mandatory food fortification with folic acid.

In our study population, which consisted of 180 healthy subjects, we found that 10% carried the T allele, and no subjects with the homozygous TT genotype were observed. Other researchers also reported prevalences of $\approx 10\%$ for the CT genotype and $\approx 0.5\%$ for the TT genotype (25, 29, 30). Therefore, polymorphism in the *GCP11* gene is rare in comparison with some other polymorphisms, such as the methylene tetrahydrofolate reductase gene 677C→T polymorphism (11% TT, 43% CT, and 46% CC) (10).

After treatment with polyglutamyl folic acid and, unexpectedly, with monoglutamyl folic acid, the responses in erythrocyte folate concentrations in subjects with the CT genotype were smaller than those in subjects with the CC genotype. However, subjects carrying the T allele already had high erythrocyte folate concentrations from the start. Their initial concentrations were comparable with concentrations after 12 wk of supplementation with doses of 200–400 μg folic acid (33). The small changes in these subjects may be attributed to their high initial concentrations. Moreover, erythrocyte folate concentrations in subjects with the CT genotype in the polyglutamyl folic acid group were

TABLE 2

Serum folate, erythrocyte folate, and plasma homocysteine concentrations before and after intervention and changes in concentrations after 12 wk of intervention with placebo, 323 nmol monoglutamyl folic acid/d, or 262 nmol heptaglutamyl folic acid/d by glutamate carboxypeptidase II gene (*GCP11*) 1561 genotype¹

	Placebo		Monoglutamyl folic acid		Polyglutamyl folic acid	
	CC genotype (n = 52)	CT genotype (n = 8)	CC genotype (n = 54)	CT genotype (n = 5)	CC genotype (n = 55)	CT genotype (n = 6)
Serum folate (nmol/L)						
Before intervention ²	14.0 ± 3.7 ³	15.4 ± 5.0	14.9 ± 3.9	17.8 ± 6.1	13.8 ± 5.0	19.3 ± 7.0
After intervention	12.8 ± 3.5	14.1 ± 5.1	25.6 ± 7.3	26.8 ± 9.0	18.8 ± 5.4	23.8 ± 6.0
Change from baseline ⁴	-1.2 (-2.0, -0.5) ⁵	-1.3 (-3.4, 0.8)	10.7 (9.0, 12.3)	9.0 (4.5, 13.4)	5.0 (4.0, 5.8)	4.5 (-0.9, 9.9)
Erythrocyte folate (nmol/L)						
Before intervention ⁶	669 ± 204	784 ± 219	732 ± 216	790 ± 164	756 ± 224	1188 ± 428
After intervention	610 ± 207	801 ± 205	973 ± 257	931 ± 240	873 ± 281	1193 ± 441
Change from baseline ⁷	-59 ^a (-84, -34)	17 ^a (-58, 93)	241 ^c (203, 280)	140 ^c (16, 264)	117 ^b (84, 149)	5 ^{a,b} (-186, 196)
Plasma homocysteine (μmol/L)						
Before intervention ⁸	10.5 ± 1.8	10.1 ± 1.5	10.3 ± 2.2	10.7 ± 1.3	11.1 ± 2.7	10.0 ± 0.6
After intervention	10.6 ± 1.8	10.3 ± 1.9	8.9 ± 1.8	9.6 ± 1.4	9.7 ± 2.1	9.3 ± 0.5
Change from baseline ⁸	0.1 (-0.2, 0.4)	0.2 (-0.3, 0.8)	-1.4 (-1.7, -1.1)	-1.1 (-2.2, 0.01)	-1.3 (-1.8, -0.9)	-0.7 (-1.7, 0.3)

¹ Values with different superscript letters are significantly different, $P < 0.001$ (analysis of covariance and Tukey's test).

² Significant main effect of genotype (borderline, $P = 0.05$) but not of treatment group; no significant interaction between genotype and treatment group.

³ $\bar{x} \pm SD$ (all such values).

⁴ Significant main effect of treatment group ($P < 0.01$) but not of genotype; no significant interaction between genotype and treatment group. The groups given monoglutamyl folic acid were significantly different from those given polyglutamyl folic acid, and those given either form of folic acid were significantly different from those given placebo ($P < 0.001$).

⁵ 95% CI in parentheses.

⁶ Significant main effect of genotype ($P < 0.05$) but not of treatment group; no significant interaction between genotype and treatment group.

⁷ Significant main effect of genotype ($P < 0.05$) but not of treatment group ($P = 0.06$); significant interaction between genotype and treatment group ($P < 0.01$).


⁸ No significant main effects or interaction between genotype and treatment group.

higher than those in subjects with the *CT* genotype in the monoglutamyl folic acid group, although this result had only borderline significance. Therefore, calculation of the relative bioavailability of polyglutamyl folic acid on the basis of changes in erythrocyte folate concentration in these groups was not justifiable.

The similar bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid among the *GCP11* 1561 genotypes suggests that the FGCP activity of the *T* allele-coded enzyme is not reduced in vivo. This is in contrast with our expectations based on experiments in which plasmid complementary DNA of the mutagenized H475Y *GCP11* was brought to expression in transfected COS-7 cells, which had lower FGCP activity than did transfected cells expressing the wild-type *GCP11* FGCP enzyme. This implies that other factors that are yet unidentified may affect the expression of the *GCP11* gene in human subjects.

From a metabolic point of view, our findings on the *GCP11* 1561C→T polymorphism are puzzling. Why would folate status be higher in subjects with the *CT* genotype than in subjects with the *CC* genotype although the bioavailability of folate is similar? The *GCP11* gene is expressed in other body tissues, including the brain (*N*-acetylated alpha-linked acidic dipeptidase) and the prostate (prostate-specific membrane antigen) (25). Folate metabolism may therefore be affected by expression of the *GCP11* 1561C→T polymorphism in these and other body tissues. Further investigation in this area is needed to address these issues to obtain a better understanding of folate metabolism in general.

In conclusion, we found that the bioavailability of polyglutamyl folic acid relative to that of monoglutamyl folic acid does

not differ significantly between *GCP11* 1561 genotypes. At baseline and after 12 wk of supplementation with folic acid, the *1561CT* genotype was associated with 20–25% higher serum and erythrocyte folate concentrations than was the *CC* genotype. This suggests that other, yet unidentified, factors control the expression of the *GCP11* gene. Further research is needed to investigate the mechanism behind these findings. 

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AM-B and PV were involved in designing and conducting the study. KJAL and HJB performed and facilitated the genotyping. AM-B analyzed the data and wrote the manuscript, but all other authors contributed significantly to the interpretation of data and the writing of the manuscript. None of the authors had any financial or personal interests in the sponsoring organization.

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