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Concentrations of unmetabolized folic acid and primary folate forms in plasma after folic acid treatment in older adults

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Abstract

Folate deficiency can cause age-related disease. Folic acid (FA) has been used in studies aiming at disease prevention. Recently, unmetabolized FA in plasma raised public health concerns; but numerous studies used FA for disease prevention. Concentrations of the folate forms FA, 5-methyltetrahydrofolate (5-MTHF), and tetrahydrofolate (THF) were measured before and after 3-week placebo or FA 5 mg, vitamin B6 40 mg, and cyanocobalamin 2 mg per day administrated to 74 older adults (median age, 82 years). Concentrations of 5-MTHF and total homocysteine (tHcy) ($r = -0.392$) and *S*-adenosylmethionine ($r = 0.329$) were correlated at baseline. Twenty-six percent of the elderly subjects had unmetabolized FA in plasma at the start, and concentrations of FA were increased after 3 weeks of FA treatment (median FA = 0.08 nmol/L at baseline and 15.3 nmol/L at the end of the treatment in the vitamin group). Folic acid caused a 10- and a 5-fold increase in 5-MTHF and THF, respectively, and lowered tHcy (median tHcy = 17.2 μmol/L at baseline vs 9.0 μmol/L after treatment). Concentrations of unmetabolized FA were positively related to those of 5-MTHF and THF. People showed wide variations in folate forms at baseline, but these were reduced after FA treatment. Folic acid given to older adults is mostly converted to THF and 5-MTHF and lowered concentrations of tHcy, but caused a substantial increase in unmetabolized FA in the plasma.

1. Introduction

Folate deficiency has been related to numerous disease conditions including anemia, cancer, birth defects, and age-associated diseases [1-4]. 5-Methyltetrahydrofolate (5-MTHF) is the physiologically active folate form whose role is to carry 1-carbon units to the methionine cycle (Fig. 1. Supplemental Data File), thus converting homocysteine (Hcy) to methionine. Methionine synthase and its cofactor methylcobalamin mediate the last reaction. 5-MTHF delivers a labile methyl group as *S*-adenosylmethionine (SAM) for the methylation of DNA, phospholipids, and proteins.

The recommended daily intake of folic acid (FA) is 400 μg for women of childbearing age to prevent neural tube defects (NTDs) [5]. Pregnant and lactating women require up to 800 μg/d. The mandatory fortification with FA provides per 100 g grain 140 μg FA in the United States, 220 μg in Chile, and 150 μg in Canada [6-8]. In addition, over-the-counter multivitamins in many countries contain between 400 and 800 μg FA. Folic acid is reduced by dihydrofolate reductase (DHFR) to 7,8-dihydrofolate and then to 5,6,7,8-tetrahydrofolate (THF) in the liver. The activity of DHFR might be a limiting factor for FA reduction in people consuming greater than the tolerable upper intake level of 1 mg/d of the vitamin [9]. Folate deficiency is common in elderly people and might confer a risk for age-related diseases [10,11]. Numerous trials have investigated the effect of FA supplementation on certain health outcomes in elderly population.

Concern has recently been expressed that increasing FA intake will cause the presence of unmetabolized FA in blood [12-14]. This is supposed to mask cobalamin deficiency and interfere with antifolates [14]. Moreover, concerns about colon cancer risk after FA fortification have been raised [15,16]. Supplemental FA has been investigated in clinical trials as Hcy-lowering agent. Therapeutic doses of FA were used in clinical trials aiming at prevention of recurrent

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The study has been reviewed and approved by the local ethical committee.

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NTDs, dementia, vascular disease, and stroke [17-19]. Supplementation with FA was positively associated with cancer risk in some [20] but not all studies [21,22]. However, a causal link between FA and disease development has not been shown in human studies.

One important issue that remains unclear is how FA is metabolized into active folate forms. The current study aimed at testing the concentrations of primary folate forms (5-MTHF, THF, and FA) in plasma before and after a high dose of FA in elderly people. Because of the role of cobalamin and vitamin B6 for folate recycling, and to ensure that the methylation and the transsulfuration of Hcy are not impaired, we supplemented FA together with vitamin B6 and cobalamin.

2. Materials and methods

2.1. Subjects

The study included 74 older adults (median age = 82 years, 63 women). Study participants were recruited during their stay in the geriatric health center, St Ingbert, Germany, after various illnesses. The inclusion criteria were age > 65 years and glomerular filtration rate greater than 35 mL/min. Elderly people who had acute cancer or those who had coronary or cerebral event or thrombosis in the last 3 months were not eligible for the study. Furthermore, people who had dementia (mini-mental state examination scores <15), those treated with B-vitamins, those with renal insufficiency, and those receiving drugs that affect Hcy metabolism (methotrexate, antiepileptics, L-dopa) were not allowed to participate.

On the day of admission, elderly people were thoroughly examined by the medical personnel; and blood samples for routine blood markers (blood count, lipids, glucose, C-reactive protein [CRP], creatinine) were collected. People who fulfilled our inclusion criteria were informed of the study purpose and were invited to participate (n = 85). Those who agreed to participate and signed an informed consent were included (n = 79). On the third day of admission, blood was collected for the study purposes in fasting conditions; and on the same day, people started taking the vitamins. The study was approved by the local ethical committee.

This study is a 3-week randomized, double-blind, placebo-controlled trial. The randomization was conducted by throwing a coin. Participants were allocated to a placebo or a combination of the oral B-vitamins (5 mg FA, 1 mg cyanocobalamin, and 40 mg vitamin B6) daily, in addition to a 1-mg subcutaneous cyanocobalamin injection per day. The placebo capsule contained 400 mg of mannitol:aerosil (99.5:0.5). Older adults were given the vitamin or the placebo every day by the medical staff, and this was documented. Therefore, adherence to treatment is supposed to be 100%. Of the 79 participants, 3 were hospitalized during the study; and 2 were excluded because of very high blood concentrations of cobalamin and free FA at the start. Seventy-four elderly people completed the study (37 placebo and 37 vitamin). Fasting blood samples were also collected 3 weeks later before discharge. The last FA dose was on the evening before the blood collection (at least 10 hours). Study participants were followed up at homes, and cognitive function was tested 45 days after discharge. The results of cognitive function tests are not shown here.

2.2. Blood sampling and biochemical measurements

Peripheral venous blood samples were obtained from all study participants in fasting conditions (> 8 hours) on the third day of admission and 3 weeks later before discharge. Blood samples were collected in tubes without anticoagulant or those containing K^+-EDTA as an anticoagulant. Samples were placed on ice and centrifuged within 45 minutes for 10 minutes at 2000*g* and +4°C. Serum and EDTA plasma were directly separated. Several aliquots were prepared to avoid freeze-thaw cycles, and those were stored at −70°C until analysis. The EDTA plasma (500 μL) was immediately treated with acetic acid 1 N (50 μL), mixed, and stored at −70°C for *S*-adenosylhomocysteine (SAH) and SAM assays.

Concentrations of total Hcy (tHcy), cystathionine (Cys), and methylmalonic acid (MMA) were measured by gas chromatography–mass spectrometry as described elsewhere [23]. Concentration of total cobalamin in serum was measured by using a chemiluminescence immunoassay (ADVIA Centaur System; Bayer, Leverkusen, Germany). Concentrations of SAM and SAH in EDTA plasma were measured using Ultra Performance Liquid Chromatography Tandem Mass Spectrometry (UPLC-MS/MS) (Waters, Milford, MA) [24]. The coefficients of variation (CVs) for SAH and SAM in plasma were less than 4.5%. The concentration of holotranscobalamin (holoTC) as a marker for cobalamin status was measured at baseline using a specific monoclonal antibody against holoTC, and detection was performed using alkaline phosphatase–labeled antitranscobalamin (AxSYM; Abbott, Wiesbaden, Germany) [25].

Concentrations of betaine, choline, and dimethylglycine (DMG) in EDTA plasma samples were measured by stable-isotope dilution UPLC-MS/MS. The method was modified from Holm et al 2003 [26] using an acetonitrile precipitation step. One in-house prepared pool of EDTA plasma was run each time with the samples to calculate the between-day CVs for the nonautomated methods. The between-day CVs for betaine, choline, and DMG were less than 5%.

Concentrations of primary folate forms were measured in EDTA plasma by UPLC-MS/MS as previously reported [27]. Briefly, plasma samples (250 μL) were incubated with 700 μL ammonium acetate buffer (200 mmol/L, pH 10) containing 10 g/L ascorbic acid. Fifty microliters of internal standard solution mix (1 μmol/L [13C5]-5-MTHF, 0.5 μmol/L [13C5]-FA, and 0.2 μmol/L [13C5]-5-formylTHF) was added. Sample cleanup was performed with Oasis MAX solid-phase extraction columns (Waters) preconditioned with methanol following ammonium acetate buffer.

The samples were loaded, and impurities were removed by washing the columns with 5% aqueous NH_4OH and methanol. The elution of the folates was performed by methanol containing 1% formic acid. Samples were brought to dryness and dissolved in 100 μL H_2O:methanol 60:40 (vol/vol) containing 0.1% formic acid and 1 g/L ascorbic acid. Detectable concentrations of FA in plasma were those greater than the low detection limit of our assay (0.20 nmol/L). Concentration of total folate was calculated by adding the concentrations of the detectable folate forms in the sample.

Data analyses were performed by using SPSS (version 17.0; SPSS, Chicago, IL). Data are presented as median (10th-90th percentiles). Possible differences in medians of continuous variables between the study arms were examined using Mann-Whitney test. χ^2 test was used to test differences in categorical variables. Differences in means of changes in biochemical markers caused by the treatment were investigated by using Student *t* test. Correlations between different variables were examined by Spearman test. All tests were 2-sided, and *P* values < .05 were considered to be statistically significant.

3. Results

The main characteristics and concentration of the routine biochemical markers according to the treatment allocation are shown in Table 1. Older adults in the 2 treatment arms did not differ significantly in their age; cognitive function scores (mini-mental state examination scores); and the incidence of diabetes mellitus, hypertension, coronary disease, or fractures. People who received the vitamins had a higher median concentration of creatinine compared with the placebo group. Other biochemical markers did not differ between the placebo and the vitamin groups (Table 1).

Table 1
Baseline characteristics of study population

Variables	Placebo group	Vitamin group	*P*
Age, y	81 (73-90)	82 (72-88)	.770
Hypertension, n (%)	10 (27%)	8 (21%)	.597[a]
Type 2 diabetes mellitus, n (%)	4 (11%)	8 (21%)	.346[a]
Coronary disease, n (%)	12 (32%)	17 (45%)	.345[a]
Acute fracture, n (%)	17 (46%)	18 (47%)	.999[a]
holoTC, pmol/L	60 (24-104)	55 (28-226)	.797
Cholesterol, mmol/L	5.17 (3.56-6.96)	5.36 (3.07-7.27)	.899
Triglycerides, mmol/L	1.23 (0.77-2.24)	1.29 (0.65-2.28)	.784
HDL cholesterol, mmol/L	1.33 (0.81-1.98)	1.30 (0.85-1.82)	.263
Hemoglobin, g/L	116 (98-128)	111 (88-140)	.999
Mean corpuscular volume, fL	93 (89-99)	91 (87-97)	.055
Creatinine, μmol/L	70.7 (52.0-132.6)	79.6 (61.9-149.4)	.031
Albumin, g/L	36.8 (31.1-40.4)	35.8 (28.3-41.7)	.199
Glucose, mmol/L	5.77 (4.61-8.99)	5.61 (4.27-8.33)	.528

Data are medians (10th-90th percentiles). *P* values are according to Mann-Whitney test. The study included 74 people (37 placebo/37 vitamin). HDL indicates high-density lipoprotein.

[a] According to χ^2 test.

Table 2 shows the concentration of folate cycle biomarkers in the study arms at baseline and at the end of the treatment (3 weeks later). The concentrations of tHcy were significantly lowered in the vitamin group (median tHcy = 17.2 μmol/L at baseline vs 9.0 μmol/L at the end of the treatment). The concentrations of MMA were lower in the vitamin group compared with the placebo group at the end of the treatment (207 vs 318 nmol/L). The concentrations of Cys tended to be lower in the vitamin compared with the placebo group.

At the end of the treatment, the concentrations of plasma betaine were significantly higher in the vitamin group compared with the placebo group (28.2 vs 21.3 μmol/L); and plasma concentrations of choline were not altered by the treatment (Table 2). Furthermore, the concentrations of SAM and SAH were higher in subjects receiving the B-vitamins compared with those receiving the placebo at the end of the treatment.

At baseline, 19 (26%) of the 74 older adults had detectable amounts of unmetabolized FA in plasma (>0.20 nmol/L, the low detection limit of the assay; signal to noise ≥ 5). In the group that received placebo, 14 people (41%) had detectable amounts of FA in plasma at the end of the study. Three weeks after starting the treatment, the placebo group had median concentrations of 5-MTHF (6.5 vs 4.7 nmol/L, *P* = .208) and total folate (11.7 vs 6.5 nmol/L, *P* = .086) that were slightly lower than those at the start. Concentrations of THF were significantly lower in the placebo group at the end of the study compared with values at baseline (5.5 vs 1.5 nmol/L, *P* < .001) (Table 2). In the placebo group, the concentrations of FA at baseline and 3 weeks later were not correlated, whereas the pre- and posttreatment concentrations of THF (Spearman correlation coefficient *r* = 0.365), 5-MTHF (*r* = 0.724), and tHcy (*r* = 0.896) in the placebo group were correlated (all *P* < .05).

In the vitamin group, the concentrations of THF were increased by approximately 5-fold compared with the pretreatment one. In addition, the median concentration of 5-MTHF was almost 10-fold higher in the vitamin group at 3 weeks compared with that at baseline. With only one exception, all of the older adults who received FA had detectable unmetabolized FA in plasma (median 15.3 nmol/L) (Table 2).

Table 3 shows the differences in concentration of folate-related biomarkers according to the treatment arm. In the vitamin group, the concentrations of tHcy were lowered by a median of 8.3 μmol/L (*P* < .001); those of MMA, by a median of 49 nmol/L (*P* = .142). The concentrations of betaine were increased by a median of 6.0 μmol/L in the vitamin group but not in the placebo group (*P* < .001). Plasma concentrations of FA, THF, and 5-MTHF were significantly increased in the treatment group but not in the placebo group (all *P* < .001) (Table 3).

The concentrations of FA, THF, and 5-MTHF showed high interindividual variations ([SD/mean] × 100) at baseline of 316%, 108%, and 107%, respectively (in the total group,

Table 2
Concentration of main metabolites and vitamin forms before and at the end of the study according to the treatment allocation

Variables	Baseline		P	After 3 wk of treatment[a]		P
	Placebo	Vitamin		Placebo	Vitamin	
tHcy, μmol/L	16.4 (8.9-28.6)	17.2 (9.4-29.0)	.289	18.1 (10.3-26.5)	9.0 (6.2-13.5)	<.001
Cys, nmol/L	357 (234-802)	493 (253-1214)	.010	348 (215-741)	297 (167-606)	.109
MMA, nmol/L	249 (160-518)	246 (174-519)	.888	318 (156-708)	207 (133-339)	.001
Betaine, μmol/L	26.5 (15.0-45.3)	21.9 (13.4-36.6)	.158	21.3 (12.8-36.8)	28.2 (19.9-36.8)	.001
Choline, μmol/L	8.0 (5.1-10.9)	7.5 (5.6-11.3)	.372	8.8 (6.2-11.9)	8.9 (5.5-12.3)	.851
DMG, μmol/L	3.1 (2.2-4.3)	3.4 (2.0-5.3)	.189	2.8 (1.9-4.3)	3.0 (2.0-4.6)	.206
Total folate (UPLC), nmol/L	11.7 (4.7-33.1)	11.0 (4.1-34.7)	.469	6.5 (2.7-47.6)	102.9 (49.0-280.9)	<.001
5-MTHF, nmol/L	6.5 (2.5-16.7)	5.6 (2.4-19.1)	.194	4.7 (1.6-25.8)	61.4 (33.2-139.0)	<.001
THF, nmol/L	5.5 (1.0-16.6)	3.4 (1.3-16.0)	.411	1.5 (0.0-5.4)	14.5 (6.0-30.5)	<.001
FA, nmol/L	0.07 (0.0-0.59)	0.08 (0.0-0.85)	.366	0.17 (0.0-4.1)	15.3 (1.02-144.4)	<.001
SAM, nmol/L[b]	180 (116-253)	173 (126-271)	.655	148 (104-266)	181 (129-312)	.041
SAH, nmol/L[b]	27.2 (14.2-59.7)	25.8 (16.7-65.5)	.937	28.5 (13.9-71.4)	31.2 (17.7-85.0)	.116

Data are medians (10th-90th percentiles). *P* values are according to Mann-Whitney test.

[a] Treatment either with placebo or FA plus cobalamin and vitamin B6.

[b] SAM and SAH concentrations were available from 30 placebo and 31 vitamin-treated people at baseline and from 22 placebo and 26 vitamin-treated people posttreatment.

n = 74). This was in contrast to the concentrations of tHcy that showed 44% interindividual variations. The vitamin group showed less interindividual variations at 3 weeks than at baseline: 144% for FA, 58% for THF, 52% for 5-MTHF, and 39% for tHcy. In addition, the increase in FA showed 145% interindividual variations, compared with 99% for THF and 53% for 5-MTHF.

We further tested the hypothesis that high concentrations of unmetabolized FA in plasma might be related to impaired or slower reduction of FA into THF and further metabolism to 5-MTHF. To show this, we divided elderly people who received the B-vitamins (n = 37) by the median posttreatment concentration of FA (15.3 nmol/L) (Table 4).

Table 3
Differences in concentration of biomarkers according to the treatment

Variable	Post- minus pretreatment concentration		P[a]
	Placebo group	Vitamin group	
tHcy, μmol/L	1.1 (−3.4/5.9)	−8.3 (−20.3/−1.9)	<.001
Cys, nmol/L	2.0 (−249/205)	−260 (−695/21)	.721
MMA, nmol/L	15 (−107/395)	−49 (−241/−2.8)	.142
Betaine, μmol/L	−3.91 (−17.8/6.2)	6.0 (−6.4/20.8)	<.001
Choline, μmol/L	0.48 (−2.7/4.3)	1.4 (−2.0/4.8)	.303
DMG, μmol/L	−0.27 (−1.37/0.53)	−0.10 (−1.72/1.49)	.803
Total folate (UPLC), nmol/L	−4.9 (−13.4/11.4)	85.4 (37.8/236.6)	<.001
5-MTHF, nmol/L	−1.27 (−8.0/7.7)	51.8 (29.7/124.9)	<.001
THF, nmol/L	−3.8 (−9.8/0.12)	10.7 (−0.86/27.8)	<.001
FA, nmol/L	0.04 (−0.58/4.07)	14.0 (0.63/125.4)	.002
SAM, nmol/L[b]	−15 (−88/62)	−1 (−75/87)	.319
SAH, nmol/L[b]	−0.7 (−10.3/44.3)	4.4 (−15.5/24.1)	.902

Data are medians (10th-90th percentiles) of the difference between post- and pretreatment concentrations.

[a] Mean differences were compared between the study group using *t* test.

[b] SAM and SAH concentrations were available from 30 placebo and 31 vitamin-treated people at baseline and from 22 placebo and 26 vitamin-treated people posttreatment.

Table 4
Concentration of blood biomarkers after 3 weeks of FA treatment according to the concentration of unmetabolized-FA

Variables	Range of unmetabolized FA		P[a]
	0.1-15.3 nmol/L	15.4-209.3 nmol/L	
Age, y	82 (68/87)	82 (77/92)	.807
Hemoglobin, g/L	12.1 (9.3/14.2)	11.1 (8.6/14.3)	.282
Mean corpuscular volume, fL	91 (87/100)	92 (85/97)	.803
CRP, mg/L	0.5 (0.1/10.4)	0.8 (0.1/3.3)	.580
Creatinine, mmol/L	79.6 (50.4/170.6)	92.8 (44.2/259.0)	.507
tHcy, μmol/L	9.0 (5.9/16.5)	9.0 (6.1/13.3)	.904
tHcy difference, μmol/L	−10.4 (−23.3/−7.0)	−7.5 (−16.9/−2.0)	.989
Cys, nmol/L	299 (160/609)	316 (177/660)	.904
Cys difference, nmol/L	−255 (−927/210)	−299 (−693/19.4)	.190
MMA, nmol/L	189 (112/335)	224 (133/355)	.361
MMA difference, nmol/L	−48 (−232/−4)	−49 (−740/24)	.796
Betaine, μmol/L	29.9 (20.6/38.9)	27.6 (17.8/39.2)	.614
Betaine difference, μmol/L	7.9 (−8.3/25.2)	2.3 (−6.2/17.6)	.368
Choline, μmol/L	9.1 (5.8/12.0)	8.5 (5.6/13.5)	.801
Choline difference, μmol/L	1.8 (−4.9/5.3)	0.5 (−1.4/5.2)	.540
FA, nmol/L	3.8 (0.7/13.5)	48.5 (23.3/183.1)	<.001
FA difference, nmol/L	3.6 (0.4/13.3)	44.8 (22.6/186.4)	<.001
Total folate (UPLC), nmol/L	78.7 (42.8/126.3)	156.2 (81.7/323.2)	<.001
Total folate difference, nmol/L	66.4 (34.9/111.6)	139.8 (61.0/317.5)	<.001
5-MTHF, nmol/L	52.5 (32.1/89.4)	72.8 (33.2/158.3)	.040
5-MTHF difference, nmol/L	46.0 (29.8/83.5)	66.0 (30.4/149.3)	.105
THF, nmol/L	11.2 (2.9/31.1)	19.1 (6.8/35.4)	.196
THF difference, nmol/L	7.6 (−6.1/27.0)	12.7 (−0.1/32.8)	.113

Data are medians (10th-90th percentiles). The concentrations of FA from 37 treated-elderly people were separated by median value (15.3 nmol/L). Differences were post- minus pretreatment concentrations.

[a] *P* values are according to Mann-Whitney test.

The 2 subgroups did not differ in age, blood count, and the concentration of creatinine or CRP. The concentration of tHcy, Cys, MMA, betaine, and choline and their change by treatment (post- minus pretreatment) were not different between people having plasma FA less than or greater than 15.4 nmol/L. Higher concentration of FA in plasma was associated with higher total folate (P <.001) and 5-MTHF (P = .04). However, the concentration of THF and the increase in THF did not differ significantly.

In the total group (n = 74), the baseline concentration of 5-MTHF correlated to that of tHcy (r = −0.392, P = .001) and SAM (r = 0.329, P = .012). In the vitamin-treated elderly people, positive significant correlations were observed between plasma concentrations of FA and those of THF (r = 0.394 pretreatment and r = 0.451 posttreatment) and 5-MTHF (r = 0.417 pretreatment and r = 0.555 posttreatment) (all P values < .05, Fig. 1A and B). In addition, the concentrations of 5-MTHF and THF were positively correlated in the vitamin-treated people (r = 0.511 pretreatment and r = 0.563 posttreatment) (all P values < .05, Fig. 2).

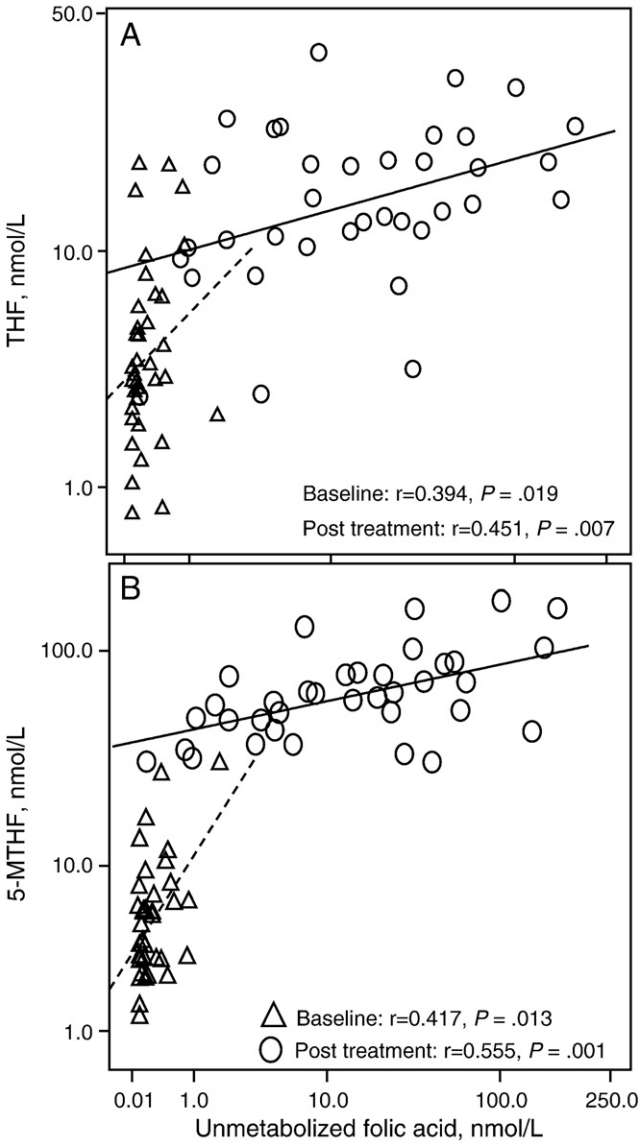

Fig. 1. The correlation between the concentration of unmetabolized FA and that of THF (A) or 5-MTHF (B) in the vitamin-treated elderly people before and after treatment with FA. r is the correlation coefficient according to Spearman test. Log-scales were used to show the results.

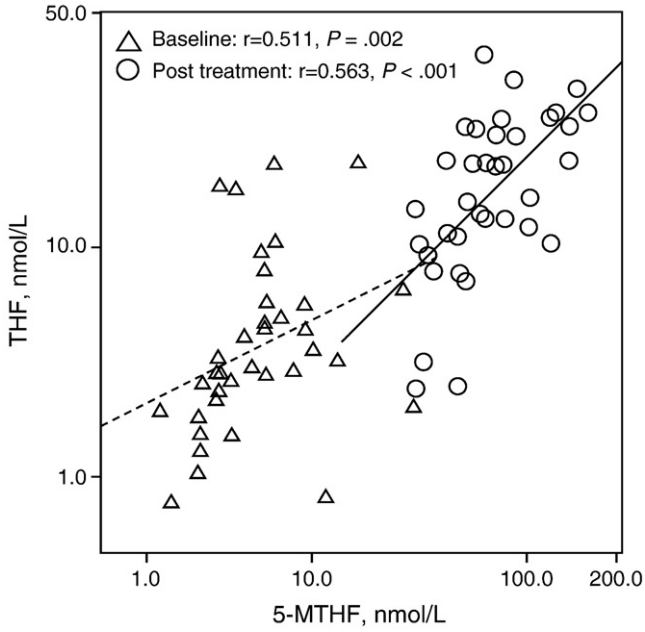

Fig. 2. The correlation between the concentration of THF and that of 5-MTHF in the vitamin-treated elderly people before and after treatment with FA. r is the correlation coefficient according to Spearman test.

4. Discussion

The current study examined the concentration of primary folate forms and related metabolic markers in elderly people receiving therapeutic doses of FA plus cobalamin and vitamin B6. Numerous tHcy-lowering studies applied therapeutic doses of FA (between 1 and 15 mg/d) [10,28-30]. However, only few studies tested the concentration of 5-MTHF in plasma after the treatment [28]. The response of THF and 5-MTHF to FA treatment has not been tested. In addition, the concentrations of unmetabolized FA have been only tested in populations from countries applying mandated fortification with FA. Few studies reported on plasma concentrations of unmetabolized FA from people regularly consuming FA-containing supplements, but those hardly exceeded the upper tolerable limit for FA of 1 mg/d [12,13].

The most important findings in this study are as follows: First, plasma concentrations of 5-MTHF were negatively related to tHcy and positively related to SAM, suggesting that 5-MTHF delivers labile methyl groups as SAM and supporting earlier observations [31]. Second, the concentrations of 5-MTHF were also positively related to FA at

baseline and were increased in the group receiving FA, suggesting that FA is being converted into the active form, 5-MTHF. Third, FA-treated people showed a substantial increase in concentrations of the unmetabolized vitamin in their plasma; but this was related to higher concentrations of THF and 5-MTHF. Finally, the increase in plasma concentrations of 5-MTHF was stronger than that of THF, suggesting that FA is effectively reduced and further converted into its active forms.

We observed a remarkable tendency of concentrations of THF, 5-MTHF, and total folate in the placebo group to be lower at the end of the study compared with baseline concentrations. This might be related to recent changes of folate intake during the stay in the health center. Furthermore, the current study included elderly people from a country not applying a mandated fortification with FA. However, several dietary products in Germany are being fortified with FA, thus probably explaining the detectable unmetabolized FA at baseline in 26% of the study participants who were not receiving FA-containing supplements.

Folic acid, the oxidized form of folate, is reduced in vivo in 2 steps into 7,8-dihydrofolate and THF by means of DHFR. The activity of DHFR in human liver extracts showed significant variations between subjects [9], suggesting wide intersubject differences in folate metabolism. A recent randomized, placebo-controlled intervention has shown that concentrations of unmetabolized FA in milk samples from lactating women did not differ between subjects supplemented with FA or placebo [12]. However, a dose of only 400 μg FA a day was used in this study, which is much lower than the one used in our study and might thus be differentially metabolized by DHFR. Nguyen et al [32] studied the steady state of plasma and red blood cell folate in young women after 5 and 1.1 mg oral FA daily. Our results cannot be directly compared with those of Nguyen et al because no quantification or limit of detection of FA was shown in this study [32]. In our study, the 10th and the 25th percentiles of FA (1.02 and 3.7 nmol/L) in the vitamin-treated subjects are higher than the concentrations reported in fortified populations [13,33]. Bailey et al suggested that the activity of DHFR might be a limiting factor when folate intake exceeds 1 mg/d, which is the case in our current study [9]. This can explain the high concentrations of FA in plasma of vitamin-treated people from the current study.

In the era of FA fortification, vitamin users and nonusers from the Framingham Offspring Study differed significantly in plasma concentration of FA but not in total folate or 5-MTHF [13]. The higher the plasma total folate or 5-MTHF, the higher the proportion of subjects with an increased concentration of FA ($\geq$85%) [13]. Our study confirmed the positive association between FA and 5-MTHF. However, after supplementation, increased plasma concentration of unmetabolized FA was related to higher 5-MTHF but not to differences in THF probably because the turnover rate of THF is much faster than that of 5-MTHF. Our results confirm also earlier observations that FA supplementation can spare betaine as a methyl donor for tHcy methylation [34].

The relatively wide range of interindividual variations in FA (144%), THF (58%), and 5-MTHF (52%) in the vitamin group after the treatment suggest that factors other than folate intake might be responsible. Differences in the absorption, storage, or activities of various folate cycle enzymes may account for such variations. The activity of DHFR has been suggested to vary between individuals. In one study, intakes of FA greater than 500 μg/d caused higher incidence of individuals with high FA in the presence of a 19–base pair deletion polymorphism in DHFR that was supposed to lower the capacity of the enzyme to reduce FA [33]. Age is an additional factor that might account for differences in folate metabolism. Our study included elderly people (median age, 82 years) who might show slowed metabolism of FA. Finally, we also observed that renal function (higher creatinine) was associated with higher concentrations of FA in plasma, suggesting that FA conversion might be limited in renal patients. Nevertheless, because we excluded elderly people with glomerular filtration rate $\leq$35 mL/min, we could not evaluate the effect of impaired renal function on plasma concentration of folate forms after the treatment.

Despite the unequivocal success of the FA in reducing NTDs cases, several studies have questioned the role of unmetabolized FA in blood [13,35-37]. In one study, FA in the circulation greater than 3.0 nmol/L was related to a lower natural killer cell cytotoxicity in women older than 60 years [35]. However, no firm data are available to show the relationship between FA in blood and disease outcomes in humans. In a rat model, long-term supplementation with FA resulted in lower birth weights [38]. In addition, data on the role of FA in tumor development are derived from animal models [39]. However, differences in FA metabolism between humans and rats have been recently demonstrated [9]. The posttreatment plasma concentrations of FA in this study in addition to numerous previous long-term supplementation studies argue against a harmful effect of this vitamin.

In summary, we found that 26% of elderly people from a country not applying mandated fortification with FA had detectable amounts of unmetabolized FA in plasma. Folic acid supplementation caused a marked increase in the concentration of FA, THF, and 5-MTHF and a reduction in tHcy. The increase in the concentration of 5-MTHF after the treatment was stronger than that of THF, suggesting that FA is being effectively reduced and used. The wide interindividual variations in folate forms and their response to treatment might suggest a pharmacogenetic interaction between FA and folate-metabolizing enzymes. This might be important for a health-relevant effect of supplemented FA. Future studies might investigate a dose-response effect of supplemented FA and the effect of 5-MTHF on concentrations of key folate forms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.metabol.2010.06.020.

References

[1] Tamura T, Picciano MF. Folate and human reproduction. Am J Clin Nutr 2006;83:993-1016.
[2] Reynolds E. Vitamin B12, folic acid, and the nervous system. Lancet Neurol 2006;5:949-60.
[3] Duthie SJ, Hawdon A. DNA instability (strand breakage, uracil misincorporation, and defective repair) is increased by folic acid depletion in human lymphocytes in vitro. FASEB J 1998;12:1491-7.
[4] Fioravanti M, Ferrario E, Massaia M, et al. Low folate levels in the cognitive decline of elderly patients and the efficacy of folate as a treatment for improving memory deficits. Arch Gerontol Geriatr 1998;26:1-13.
[5] Institute of Medicine. Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline. 150-195. Washington, DC: National Academy Press; 2000.
[6] Food and Drug Administration. Food Standards. Amendment of standards of identity for enriched grain products to require addition of folic acid. Fed Regist 1996;61(No 44, 21 CFR Parts 136, 137, and 139):8781-807.
[7] Freire WB, Hertrampf E, Cortes F. Effect of folic acid fortification in Chile: preliminary results. Eur J Pediatr Surg 2000;10(Suppl 1):42-3.
[8] Health Canada. Regulations amending the food and drug regulations (1066). Can Gaz I 1997;131(48):3702-37.
[9] Bailey SW, Ayling JE. The extremely slow and variable activity of dihydrofolate reductase in human liver and its implications for high folic acid intake. Proc Natl Acad Sci U S A 2009;106:15424-9.
[10] Ford AH, Flicker L, McCaul K, et al. The B-VITAGE trial: a randomized trial of homocysteine lowering treatment of depression in later life. Trials 2010;11:8.
[11] Obeid R, Kostopoulos P, Knapp JP, et al. Biomarkers of folate and vitamin B12 are related in blood and cerebrospinal fluid. Clin Chem 2007;53:326-33.
[12] Houghton LA, Yang J, O'Connor DL. Unmetabolized folic acid and total folate concentrations in breast milk are unaffected by low-dose folate supplements. Am J Clin Nutr 2009;89:216-20.
[13] Kalmbach RD, Choumenkovitch SF, Troen AM, et al. Circulating folic acid in plasma: relation to folic acid fortification. Am J Clin Nutr 2008;88:763-8.
[14] Smith AD, Kim YI, Refsum H. Is folic acid good for everyone? Am J Clin Nutr 2008;87:517-33.
[15] Hirsch S, Sanchez H, Albala C, et al. Colon cancer in Chile before and after the start of the flour fortification program with folic acid. Eur J Gastroenterol Hepatol 2009;21:436-9.
[16] Mason JB, Dickstein A, Jacques PF, et al. A temporal association between folic acid fortification and an increase in colorectal cancer rates may be illuminating important biological principles: a hypothesis. Cancer Epidemiol Biomarkers Prev 2007;16:1325-9.
[17] Saposnik G, Ray JG, Sheridan P, et al. Homocysteine-lowering therapy and stroke risk, severity, and disability: additional findings from the HOPE 2 trial. Stroke 2009;40:1365-72.
[18] Grosse SD, Collins JS. Folic acid supplementation and neural tube defect recurrence prevention. Birth Defects Res A Clin Mol Teratol 2007;79:737-42.
[19] Durga J, van Boxtel MP, Schouten EG, et al. Effect of 3-year folic acid supplementation on cognitive function in older adults in the FACIT trial: a randomised, double blind, controlled trial. Lancet 2007;369:208-16.
[20] Ebbing M, Bonaa KH, Nygard O, et al. Cancer incidence and mortality after treatment with folic acid and vitamin B12. JAMA 2009;302:2119-26.
[21] Bonaa KH, Njolstad I, Ueland PM, et al. Homocysteine lowering and cardiovascular events after acute myocardial infarction. N Engl J Med 2006;354:1578-88.
[22] Lonn E, Yusuf S, Arnold MJ, et al. Homocysteine lowering with folic acid and B vitamins in vascular disease. N Engl J Med 2006;354:1567-77.
[23] Stabler SP, Marcell PD, Podell ER, et al. Elevation of total homocysteine in the serum of patients with cobalamin or folate deficiency detected by capillary gas chromatography-mass spectrometry. J Clin Invest 1988;81:466-74.
[24] Kirsch SH, Knapp JP, Geisel J, et al. Simultaneous quantification of *S*-adenosyl methionine and *S*-adenosyl homocysteine in human plasma by stable-isotope dilution ultra performance liquid chromatography tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2009;877:3865-70.
[25] Orning L, Rian A, Campbell A, et al. Characterization of a monoclonal antibody with specificity for holo-transcobalamin. Nutr Metab (Lond) 2006;3:3.
[26] Holm PI, Ueland PM, Kvalheim G, et al. Determination of choline, betaine, and dimethylglycine in plasma by a high-throughput method based on normal-phase chromatography–tandem mass spectrometry. Clin Chem 2003;49:286-94.
[27] Kirsch SH, Knapp JP, Herrmann W, et al. Quantification of key folate forms in serum using stable-isotope dilution ultra performance liquid chromatography–tandem mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci 2010;878:68-75.
[28] Duthie SJ, Horgan G, De RB, et al. Blood folate status and expression of proteins involved in immune function, inflammation and coagulation: biochemical and proteomic changes in the plasma of humans in response to long-term synthetic folic acid supplementation. J Proteome Res 2010;9:1941-50.
[29] Zhang SM, Cook NR, Albert CM, et al. Effect of combined folic acid, vitamin B6, and vitamin B12 on cancer risk in women: a randomized trial. JAMA 2008;300:2012-21.
[30] Buccianti G, Raselli S, Baragetti I, et al. 5-Methyltetrahydrofolate restores endothelial function in uraemic patients on convective haemodialysis. Nephrol Dial Transplant 2002;17:857-64.
[31] Loehrer FM, Angst CP, Haefeli WE, et al. Low whole-blood *S*-adenosylmethionine and correlation between 5-methyltetrahydrofolate and homocysteine in coronary artery disease. Arterioscler Thromb Vasc Biol 1996;16:727-33.
[32] Nguyen P, Tam C, O'Connor DL, et al. Steady state folate concentrations achieved with 5 compared with 1.1 mg folic acid supplementation among women of childbearing age. Am J Clin Nutr 2009;89:844-52.
[33] Kalmbach RD, Choumenkovitch SF, Troen AP, et al. A 19–base pair deletion polymorphism in dihydrofolate reductase is associated with increased unmetabolized folic acid in plasma and decreased red blood cell folate. J Nutr 2008;138:2323-7.
[34] Melse-Boonstra A, Holm PI, Ueland PM, et al. Betaine concentration as a determinant of fasting total homocysteine concentrations and the effect of folic acid supplementation on betaine concentrations. Am J Clin Nutr 2005;81:1378-82.
[35] Troen AM, Mitchell B, Sorensen B, et al. Unmetabolized folic acid in plasma is associated with reduced natural killer cell cytotoxicity among postmenopausal women. J Nutr 2006;136:189-94.

[36] Kelly P, McPartlin J, Goggins M, et al. Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. Am J Clin Nutr 1997;65:1790-5.

[37] Wright AJ, Dainty JR, Finglas PM. Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK. Br J Nutr 2007;98:667-75.

[38] Achon M, Reyes L, onso-Aperte E, et al. High dietary folate supplementation affects gestational development and dietary protein utilization in rats. J Nutr 1999;129:1204-8.

[39] Lindzon GM, Medline A, Sohn KJ, et al. Effect of folic acid supplementation on the progression of colorectal aberrant crypt foci. Carcinogenesis 2009;30:1536-43.