

Short communication

Low-dose folic acid supplementation does not influence plasma methionine concentrations in young non-pregnant women

Ingeborg A. Brouwer^{1,2*}, Marijke van Dusseldorp¹, Marinus Duran³, Chris M. G. Thomas^{2,4}, Joseph G. A. J. Hautvast¹, Tom K. A. B. Eskes² and Régine P. M. Steegers-Theunissen^{2,5}

¹Division of Human Nutrition and Epidemiology, Wageningen Agricultural University, Wageningen, The Netherlands

²Departments of Obstetrics & Gynaecology, ⁴Chemical Endocrinology and ⁵Epidemiology, University Hospital Nijmegen St Radboud, Nijmegen, The Netherlands

³Laboratory of Metabolic Diseases, Wilhelmina Children's Hospital, Utrecht, The Netherlands

(Received 1 September 1998 – Revised 8 February 1999 – Accepted 22 March 1999)

An elevated plasma total homocysteine (tHcy) concentration is a risk factor for cardiovascular disease and for having offspring with a neural-tube defect. Folate is a methyl donor in the remethylation of homocysteine into methionine. Although folic acid supplementation decreases tHcy concentrations, effects of folic acid supplementation on plasma methionine concentrations are unclear. There is also concern that folic acid supplementation negatively affects vitamin B₁₂ status. We studied effects of low-dose folic acid supplementation on methionine and vitamin B₁₂ concentrations in plasma. We also investigated whether baseline plasma methionine and tHcy concentrations correlated with the baseline folate and vitamin B₁₂ status. For a period of 4 weeks, 144 young women received either 500 µg folic acid each day, or 500 µg folic acid and placebo tablets on alternate days, or a placebo tablet each day. Plasma methionine, tHcy and plasma vitamin B₁₂ concentrations were measured at start and end of the intervention period. Folic acid supplementation had no effect on plasma methionine or plasma vitamin B₁₂ concentrations although it significantly decreased tHcy concentrations. Plasma methionine concentrations showed no correlation with either tHcy concentrations (Spearman $r_s = -0.01$, $P = 0.89$), or any of the blood vitamin variables at baseline. Baseline tHcy concentrations showed a slight inverse correlation with baseline concentrations of plasma vitamin B₁₂ ($r_s = -0.25$, $P < 0.001$), plasma folate ($r_s = -0.24$, $P < 0.01$) and erythrocyte folate ($r_s = -0.19$, $P < 0.05$). In conclusion, low-dose folic acid supplementation did not influence plasma methionine or plasma vitamin B₁₂ concentrations. Furthermore, no correlation between plasma methionine concentrations and the blood folate and vitamin B₁₂ status was shown.

Folic acid: Methionine: Homocysteine: Vitamin B₁₂

Homocysteine has received a lot of attention because hyperhomocysteinaemia has been shown to be a risk factor for cardiovascular disease and for having offspring with a neural-tube defect (Steegers-Theunissen *et al.* 1994; Boushey *et al.* 1995; Mills *et al.* 1995). Homocysteine is the transmethylation product of the essential amino acid methionine. Methionine can be transmethylated to homocysteine, which in turn can be remethylated to methionine. Thus, in theory, an increase in plasma methionine is expected after a decrease in plasma homocysteine. *N*-5-methyltetrahydrofolate, the predominant form of the B-vitamin folic acid in the

blood, serves as a methyl donor for this remethylation reaction and supplementation with folic acid decreases plasma total homocysteine (tHcy) concentrations (Ward *et al.* 1997; Brouwer *et al.* 1999). Vitamin B₁₂ (methylcobalamin) is a cofactor in this remethylation reaction. Supplementation with folic acid or vitamin B₁₂ increased plasma methionine concentrations in folate- or vitamin B₁₂-deplete subjects (Guttormsen *et al.* 1996).

Since 1947 there has been concern that folic acid supplementation may have deleterious effects on cobalamin deficiency (Heinle & Welch, 1947). We therefore checked in

Abbreviation: tHcy, total homocysteine.

* **Corresponding author:** Dr Ingeborg A. Brouwer, fax +31 317 483342, email ingeborg.brouwer@staff.NutEpi.wau.nl

this study whether low-dose folic acid supplementation influenced plasma vitamin B₁₂ concentrations.

The present study aimed to determine the effects of low-dose folic acid supplementation on plasma methionine and plasma vitamin B₁₂ concentrations in healthy volunteers. We compared these effects with the decreasing effect on plasma tHcy concentrations (Brouwer *et al.* 1999). We also examined whether plasma methionine and tHcy were correlated with the blood folate and vitamin B₁₂ status.

Subjects and methods

The methods of this experiment have previously been described in more detail (Brouwer *et al.* 1999). In short, these are as follows.

Subjects

We recruited healthy, non-pregnant women (18–40 years of age). Exclusion criteria were: smoking, gastrointestinal disorder, use of vitamins, minerals, yeast or seaweed, malaria prophylaxes and anti-convulsants. The final study group comprised 144 women.

The Medical Ethical Committee of the Wageningen Agricultural University approved the study design. All women gave written informed consent.

Methods

After stratification for the use of oral contraceptives, the women were randomized over three intervention groups and received either 500 µg folic acid/d, or 500 µg folic acid and a placebo tablet every second day (on average 250 µg folic acid/d), or a placebo tablet daily (Pharmachemie B.V., Haarlem, The Netherlands) for 4 weeks. The group sizes were chosen to detect an 11% decrease in plasma tHcy concentrations after 4 weeks of intervention (Brouwer *et al.* 1999). The subjects and the laboratory staff were blind to the group assignment. Subjects followed their regular diet except for refraining from the consumption of liver and Marmite, a yeast extract. A 24 h recall was obtained from each subject once during the intervention period to check their intake of macronutrients, vitamin B₆, B₁₂ and folate (Stichting NEVO, 1993; Brants & Hulshof, 1995).

Venous blood samples were collected after overnight fasting at the start (week 0) and at the end of the 4-week intervention period (week 4). We determined plasma methionine concentrations and plasma tHcy, plasma folate, erythrocyte folate and vitamin B₁₂ concentrations in all samples.

Measurements

Samples for determinations in plasma were immediately placed on ice and centrifuged within 60 min at 3000 g for 10 min. Plasma was separated and stored at –20° for methionine, at –35° for folate and vitamin B₁₂ and at –80° for tHcy. For determination of folate concentrations in erythrocytes, whole blood was diluted 5-fold with aqueous sodium ascorbate (10 g/l) and stored at –35°. The

haemolysates were further diluted with IMx Folate RBC Lysis Reagent (Abbott Laboratories, North Chicago, IL, USA). To enable expression of the folate concentration in erythrocytes, packed cell volumes were also measured.

For methionine analysis a quantitative amino acid analysis in plasma was performed on a Biotronik 7000 amino acid analyser (Biotronik, Maintal, Federal Republic of Germany) using S-(aminoethyl)cysteine as internal standard. The separation was achieved by a stepwise gradient of lithium citrate buffers; the detection and quantification was performed with the classical ninhydrin reaction and spectrophotometry at 440 and 570 nm. The method had inter- and intra-assay CV < 5%.

Plasma tHcy was measured by an HPLC technique and fluorimetric detection (intra- and inter-assay CV < 8%) (Araki & Sako, 1987). Folate concentrations in plasma and erythrocytes and vitamin B₁₂ in plasma were determined with the Abbott IMx Vitamin B₁₂ and Folic Acid assays (Abbott Laboratories). The intra-assay CV of the folate assay was < 6%, while the inter-assay CV was < 10%. For vitamin B₁₂, both the intra- and inter-assay CV were lower than 5%.

Statistics

The response to treatment was calculated for each subject as the change in plasma methionine, tHcy and plasma vitamin B₁₂ concentrations between the start (week 0) and the end of the intervention period (week 4). These responses were normally distributed as checked by visual inspection of the normal probability plots (univariate procedure; Statistical Analysis Systems Inc., Cary, NC, USA). To analyse differences in response to intervention Student's *t* tests were used with a significance level of $P < 0.05/3 = 0.017$. Spearman rank correlation coefficients were calculated for the blood variables, because the concentrations (with the exception of plasma methionine) were not normally distributed.

Results

Low-dose folic acid supplementation for 4 weeks had no effect on plasma methionine concentrations, whereas folate concentrations in plasma and erythrocytes increased and plasma tHcy concentrations decreased (Table 1; Brouwer *et al.* 1999). Intervention with folic acid had no effect on plasma vitamin B₁₂ concentrations (Table 1).

The mean change in plasma methionine in the 500 µg group corrected for the change in the placebo group was 0.37 (95% CI –1.6, 2.3) µmol/l; the mean change in plasma methionine in the 250 µg group corrected for the change in the placebo group was –1.2 (95% CI –3.2, 0.7) µmol/l. Fig. 1 shows the individual values of plasma methionine and plasma tHcy before and after intervention.

Baseline plasma tHcy and methionine concentrations of the subjects were not correlated (Spearman $r_s = -0.01$, $P = 0.89$). Baseline plasma tHcy concentrations showed a slight, but significant correlation with baseline concentrations of plasma vitamin B₁₂ ($r_s = 0.25$, $P = 0.003$), plasma folate ($r_s = 0.24$, $P = 0.004$) and erythrocyte folate ($r_s = 0.19$,

Table 1. Plasma folate, erythrocyte folate, plasma vitamin B₁₂, total plasma homocysteine and plasma methionine concentrations before and after 4 weeks of folic acid supplementation (Values are means and standard deviations)

	500 µg folic acid/d group (n 45)		250 µg folic acid/d group (n 50)		Placebo group (n 49)	
	Mean	SD	Mean	SD	Mean	SD
Initial plasma folate (nmol/l)	11	4.0	11	3.4	12	4.2
Final plasma folate (nmol/l)	27	5.1	23	5.3	17	6.1
Initial erythrocyte folate (nmol/l)	400	107	386	85	422	181
Final erythrocyte folate (nmol/l)	508	176	432	126	401	182
Initial plasma vitamin B ₁₂ (nmol/l)	265	117	290	88	286	110
Final plasma vitamin B ₁₂ (pmol/l)	265	110	300	98	287	121
Initial total plasma homocysteine (µmol/l)	11.1	2.6	9.7	2.2	10.4	2.5
Final total plasma homocysteine (µmol/l)	8.5	1.7	8.5	1.7	10.3	2.5
Initial plasma methionine (µmol/l)	22.6	4.8	23.4	4.3	23.4	4.1
Final plasma methionine (µmol/l)	23.7	5.7	22.9	4.3	24.1	4.7

$P=0.03$). However, none of these blood vitamin concentrations was correlated with plasma methionine concentrations.

Users of oral contraceptives ($n=97$) had significantly lower baseline plasma vitamin B₁₂ concentrations (252 (SD 84) pmol/l) than non-users (346 (SD 118) pmol/l). However, no differences were observed for plasma tHcy and folate concentrations between users and non-users of oral contraceptives.

Discussion

This present study shows that 4 weeks of low-dose folic acid supplementation did not influence plasma methionine or plasma vitamin B₁₂ concentrations although it decreased plasma tHcy concentrations and increased plasma and erythrocyte folate concentrations (Brouwer *et al.* 1999). Plasma methionine concentrations were also not correlated with tHcy or plasma or erythrocyte folate concentrations, while tHcy concentrations were inversely correlated with these blood vitamin variables.

In contrast to our study, Guttormsen *et al.* (1996) showed significantly increased fasting plasma methionine concentrations after intervention with folic acid (5 mg/d for 10 d) and/or intramuscular injections with cobalamin (3–5 mg in 2–3 weeks). However, they supplied much higher doses of folic acid and all subjects in that study had a deficient or marginal folate and vitamin B₁₂ status and elevated plasma tHcy concentrations (Guttormsen *et al.* 1996). Our results are supported by those of others who also showed no effects of increased folate intake on plasma methionine concentrations in healthy young men, although plasma tHcy concentrations increased after depletion and decreased after repletion (Jacob *et al.* 1994, 1995; Ubbink *et al.* 1995). All our subjects had plasma methionine concentrations in the normal range (Scriver *et al.* 1985), but they were not in optimal folate status because folic acid supplementation decreased their plasma tHcy concentrations (Brouwer *et al.* 1999). Effects of folic acid supplementation might be different in subjects with either high or low plasma methionine and tHcy concentrations. Repetition of the analysis

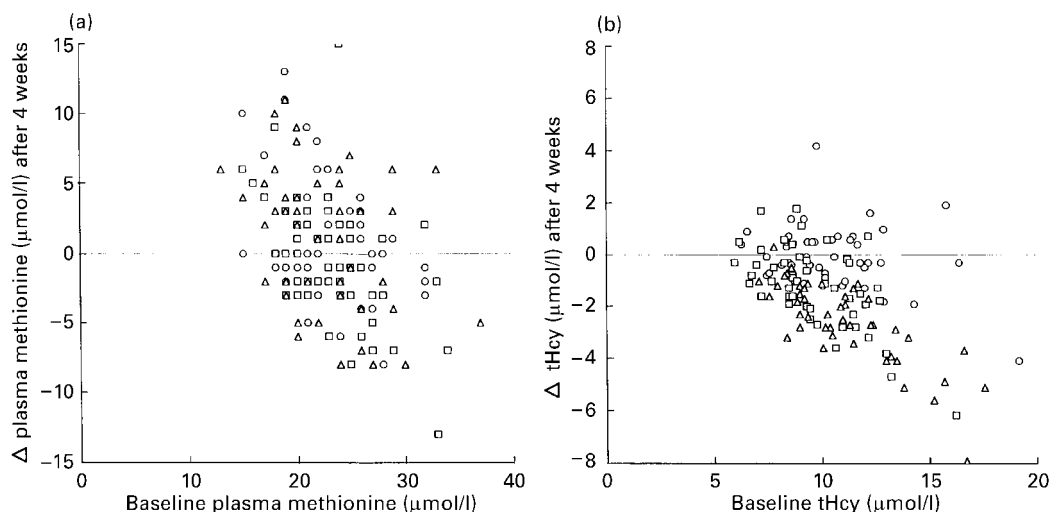


Fig. 1. Changes in (a) plasma methionine and (b) plasma total homocysteine (tHcy) in healthy young women ($n=144$) after four weeks of placebo (○), 250 µg folic acid/d (□) or 500 µg folic acid/d (△).

with exclusion of those subjects with plasma tHcy concentrations above 15 $\mu\text{mol/l}$ did not change the results of the current study.

Potgieter *et al.* (1997) noticed that methionine is very sensitive to oxidation and may even be converted to methionine sulfoxide when samples are stored at -20° until analysis. Thus, in our study spontaneous oxidation of methionine might have occurred and it can not be excluded that this contributed to the lack of effect on plasma methionine.

Differences in methionine intake between the groups is not a very likely explanation for the lack of effect in the present study. Although we did not estimate methionine intake in the subjects, we know from a 24 h recall from all participants during the trial that the total dietary protein intake and the intake of animal protein reflected by the vitamin B₁₂ intake were similar among the groups (Brouwer *et al.* 1999). This suggests comparable methionine intakes between the groups. Besides, it is unlikely that a slight difference in methionine intake would have influenced plasma methionine concentrations: Jacob *et al.* (1995) showed that changes in the intake of dietary methyl groups did not influence plasma methionine concentrations. Moreover, Andersson *et al.* (1990) found that a 3-fold increase in the daily intake of methionine for 13 d had no effect on fasting plasma methionine concentrations on day 14.

On the basis of the actual group sizes (placebo group, n 49; 500 $\mu\text{g/d}$ group, n 45) and the standard deviations for the change in plasma methionine (placebo group, 4.8; 500 $\mu\text{g/d}$ group, 5.1) and a power of 90%, we calculated that a difference of 3.3 $\mu\text{mol/l}$ in plasma methionine between the placebo and the 500 $\mu\text{g/d}$ group would have been statistically significant. Therefore, we can exclude effects of low-dose folic acid supplementation on methionine above this level.

We found no effect of folic acid supplementation on plasma vitamin B₁₂ concentrations. However, users of oral contraceptives had a lower plasma vitamin B₁₂ concentration than non-users at baseline. Plasma tHcy concentrations were not affected by use of oral contraceptives. Green *et al.* (1998) also found lower (33%) plasma vitamin B₁₂ concentrations, but similar plasma tHcy concentrations in adolescent females who used oral contraceptives than those who did not use oral contraceptives. Our study suggests that this finding is not just confined to adolescent females.

In conclusion, fasting plasma methionine levels in healthy women were not related to plasma folate and vitamin B₁₂ concentrations and were not influenced by low-dose folic acid supplementation.

Acknowledgements

The authors wish to thank the volunteers for their participation and Ms C. Schuurman, Ms M. Louwman and Ms M. Laurinen for their assistance during the field work; Dr Robert Passas, Abbott Diagnostics, Maidenhead, Berks, UK, for his support; the laboratories of the division of Human Nutrition and Epidemiology of the Wageningen Agricultural University, of Endocrinology and Reproduction of the University

Hospital Nijmegen St Radboud and of Metabolic Diseases of the Wilhelmina Children's Hospital in Utrecht for their support and expert technical assistance. The work was supported by the Zorg Onderzoek Nederland/Dutch Prevention Fund (28-2559), The Hague, The Netherlands. Tablets (folic acid and placebo) were kindly supplied by Pharmachemie B.V., Haarlem, The Netherlands. Abbott Diagnostics, Maidenhead, Berks, UK, provided the IMx diagnostic testing kits.

References

- Andersson A, Brattström L, Israelsson B, Isaksson A & Hultberg B (1990) The effect of excess daily methionine intake on plasma homocysteine after a methionine loading test in humans. *Clinica Chimica Acta* **192**, 69–76.
- Araki A & Sako Y (1987) Determination of free and total homocysteine in human plasma by high performance liquid chromatography with fluorescence detection. *Journal of Chromatography* **422**, 43–52.
- Boushey CJ, Berford SAA, Omenn GS & Motulsky AG (1995) A quantitative assessment of plasma homocysteine as a risk factor for cardiovascular disease. Probable benefits of increasing folic acid intakes. *Journal of the American Medical Association* **274**, 1049–1057.
- Brants HAM & Hulshof KFAM (1995) *De Ontwikkeling van een Voedingsmiddelentabel met Foliumzuurgehalten (Development of a Food Table with Folic Acid Values)*. Zeist, The Netherlands: TNO-nutrition.
- Brouwer IA, van Dusseldorp M, Thomas GMC, Duran M, Hautvast JGAJ, Eskes TKAB & Steegers-Theunissen RPM (1999) Low-dose folic acid supplementation decreases plasma homocysteine: a randomized trial. *American Journal of Clinical Nutrition* **69**, 99–104.
- Green TJ, Houghton LA, Donovan UM, Gibson RS & O'Connor DL (1998) Oral contraceptives did not affect biochemical folate indexes and homocysteine concentrations in adolescent females. *Journal of the American Dietetic Association* **98**, 49–55.
- Guttormsen AB, Schneede J, Ueland PM & Refsum HM (1996) Kinetics of total plasma homocysteine in subjects with hyperhomocysteinemia due to folate or cobalamin deficiency. *American Journal of Clinical Nutrition* **63**, 194–202.
- Heinle RW & Welch AD (1947) Folic acid in pernicious anemia. Failure to prevent neurologic relapse. *Journal of the American Medical Association* **133**, 739–741.
- Jacob RA, Wu M-M, Henning SM & Swenseid ME (1994) Homocysteine increases as folate decreases in plasma of healthy men during short-term dietary folate and methyl group restriction. *Journal of Nutrition* **124**, 1072–1080.
- Jacob RA, Pianalto FS, Henning SW, Zhang JZ & Swenseid ME (1995) In vivo methylation capacity is not impaired in healthy men during short-term dietary folate and methyl group restriction. *Journal of Nutrition* **125**, 1495–1502.
- Mills JL, McPartlin JM, Kirke PN, Lee YJ, Conley MR, Weir DG & Scott JM (1995) Homocysteine metabolism in pregnancies complicated by neural-tube defects. *Lancet* **345**, 149–151.
- Potgieter H, Ubbink JB, Bissbort S, Bester MJ, Spies JH & Vermaak WJ (1997) Spontaneous oxidation of methionine: effect on the quantification of plasma methionine levels. *Analytical Biochemistry* **248**, 86–93.
- Scriver CR, Gregory DM, Sovetts D & Tissenbaum G (1985) Normal plasma free amino acid values in adults: the influence of some common physiological variables. *Metabolism* **34**, 868–873.
- Steegers-Theunissen RPM, Boers GHJ, Trijbels FJM, Finkelstein JD, Blom HJ, Thomas CGM, Borm GF, Wouters MGAJ &

- Eskes TKAB (1994) Maternal hyperhomocysteinemia: a risk factor for neural-tube defects? *Metabolism* **43**, 1475–1480.
- Stichting NEVO (1993) *Dutch Nutrient Data Base*. The Hague, The Netherlands: Voorlichtingsbureau voor de Voeding.
- Ubbink JB, Vermaak WJH, Delport R, van der Merwe A, Becker PJ & Potgieter H (1995) Effective homocysteine metabolism may protect South African blacks against coronary heart disease. *American Journal of Clinical Nutrition* **62**, 802–808.
- Ward M, McNulty H, McPartlin J, Strain JJ, Weir DG & Scott JM (1997) Plasma homocysteine, a risk factor for cardiovascular disease, is lowered by physiological doses of folic acid. *Quarterly Journal of Medicine* **90**, 519–524.